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The Enzymatic Synthesis and Disproportionation of Galactosyl Oligosaccharides

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THE ENZYMATIC SYNTHESIS AND DISPROPORTIONATION OF GALACTOSYL OLIGOSACCHARIDES

by

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INTRODUCTION

Transglycosylation has been suggested as the mechanism of synthesis of many naturally occurring oligosaccharides, polysaccharides, plant glycosides, glycolipids and other carbohydrate compounds. Enzymes which catalyze specific reactions in these syntheses have, in many cases, not been isolated, and steps in the reaction mechanisms have not been elucidated. During the hydrolysis of lactose by enzymes prepared from yeasts and other microorganisms, several new galactosyl oligosaccharides are produced, apparently by a transglycosylation type reaction.

Four of these new compounds have been isolated and preliminary structure studies have been reported. Hydrolysis of the compounds and their aldonic acids and identification of the hydrolytic products, paper chromatographic mobilities of the oligosaccharides and analogy with the structures of oligosaccharides synthesized by other transglycosylases indicated the probable structures of the new compounds to be 6-O-β-D-galactopyranosyl-D-glucose, 6-O-β-D-galactopyranosyl-D-galactose, O-β-D-galactopyranosyl-(1-->6)-O-β-D-galactopyranosyl-(1-->4)-D-glucose and O-β-D-galactopyranosyl-(1-->6)-O-β-D-galactopyranosyl-(1-->6)-D-glucose.

A consideration of the structures of the oligosaccharides and independent evidence from isotope experiments point to transgalactosylation as the mechanism of enzyme action.

It was the purpose of this research to obtain unequivocal evidence for the suggested structures of the new oligosaccharides and to obtain evidence in support of the transgalactosylation mechanism of action of the enzyme studied.

LITERATURE SURVEY

In 1944 Pigman (1) observed that emulsins from pancreas, certain fungi (Aspergillus niger and A. oryzae) and bacteria (B. mesentericus), when allowed to react with maltose, produced, in addition to glucose, a small amount of non-fermentable carbohydrate. Stark (2) had made a similar observation using salivary and barley malt diastases. The nature of these non-fermentable products was not ascertained. Later workers, using enzyme preparations from A. niger and A. oryzae determined that the non-fermentable products consisted of a series of glucosyl oligosaccharides (3,4,5,6,7). More recently it has been demonstrated that oligosaccharides are also produced during the hydrolysis, by appropriate enzymes, of sucrose, cellobiose, lactose (8), melibiose and other oligosaccharides (9).

Fischer (10) and Wallenfels (11) in 1951 proposed very similar mechanisms to explain the formation of oligosaccharides during the hydrolysis of disaccharides. According to this mechanism, the enzyme reacts with the disaccharide to form a free monosaccharide and a glycosyl-enzyme complex. This complex may then react with some hydroxyl-bearing compound to yield the free enzyme and an oligosaccharide, glycoside or monosaccharide, depending upon whether the hydroxyl group is on a sugar, alcohol or water molecule. This type of mechanism has since been used to explain many other carbohydrate transfer reactions (7,8,12,13). Since the overall reaction catalyzed by such enzymes is the transfer of a glycosyl group from the substrate to a cosubstrate or acceptor, which may be water, an alcohol or a carbohydrate, these enzymes have been called transglycosylases (14). It is implied in this mechanism that hydrolysis and synthesis of new oligosaccharides are competitive reactions.

The relative amounts of hydrolytic products and oligosaccharides or glycosides formed depend upon the relative concentrations and the affinity of the enzyme for the various acceptor molecules (8). Since enzymatic reactions are of necessity carried out in aqueous solution, the concentration of water is always much greater than that of the other acceptors. However the cosubstrate specificity of different transferases varies greatly. Many of the oligosaccharide-hydrolyzing enzymes have sufficient affinity for sugars as acceptors that new oligosaccharides are produced in appreciable amounts, affording convenient systems for the study of transglycosylation.

The numerous and extensive studies of transglycosylations which have been published since the phenomenon first gained attention have been the subject of reviews (8,14,15) and periodic summaries (16). A A brief review will be included here of the studies which have been made concerning the transgalactosylation reactions observed during the enzymatic hydrolysis of lactose.

Wallenfels (11), in 1951, published the first of a series of papers on the enzymatic synthesis of oligosaccharides from lactose. He reported that paper chromatograms of digests of lactose and an enzyme prepared from Aspergillus species showed the presence of several new oligosaccharides in addition to lactose. Subsequent work (17) showed that two of them were disaccharides. One was composed only of galactose and the other yielded glucose and galactose on acid hydrolysis. A trisaccharide was isolated and shown to be composed of glucose and galactose.

A kinetic study (18) in which enzymes from molds, Helix pomatia, Escherichia coli and calf intestine were used, was interpreted in terms of the following scheme:

Glucose-O-galactose + enzyme..H <=> galactose..enzyme + glucose (1)

Galactose..enzyme + ROH --> galactose-OR + enzyme..H. (2)

R = H, glucosyl, lactosyl, galactosyl, etc.

The enzyme reacts reversibly with lactose in the first step to form glucose and an enzyme-galactose complex. This complex then irreversibly transfers the galactosyl group to an acceptor to form galactose or an oligosaccharide. The trisaccharide is synthesized rapidly at first, since the only acceptor present besides water is lactose. The concentration of trisaccharide then diminishes while that of the disaccharides increases. Then the concentration of all transfer products decreases, as the enzyme action is allowed to proceed for prolonged periods.

Aronson (19), using enzymes from Saccharomyces fragilis and Escherichia coli reported the isolation of four new oligosaccharides from digests with lactose. In addition to the three described by Wallenfels, he reported a compound which yielded, upon acid hydrolysis, glucose and galactose in a mole ration of 1:3. He suggested that it was a tetrasaccharide produced by the addition of a galactosyl group to the trisaccharide, which was formed in the same way from lactose. Formation of oligosaccharides was found to be greater in concentrated lactose solutions. Addition of excess glucose or galactose to the digest caused increased synthesis of the two disaccharides. These results were explained as resulting from the competition between sugar and water molecules for the galactosyl-enzyme complex which was assumed to be an intermediate in the reaction. Xylose and glycerol were also found to act as acceptors of the galactosyl group. Monosaccharides or sugar phosphates did not react with the enzyme.

Roberts and McFarren (20), also using an enzyme prepared from S.

fragilis, reported the detection on paper chromatograms of ten oligosaccharides produced from lactose. They were not characterized except to mention that three of them had chromatographic mobilities between those of lactose and galactose, while the other seven moved more slowly than lactose on the paper. A later paper, by Roberts and Pettinati (21), reported the observation of an additional oligosaccharide moving more slowly than lactose, making a total of eleven transfer products produced in this digest. They found that the optimum conditions for production of the oligosaccharides was at a very high lactose concentration (35%). Under optimum conditions, 44.6% of the lactose was converted to other oligosaccharides.

A lactase prepared from Penicillium chrysogenum (22) was also found to act as a transgalactosylase, its pattern of action being very similar to that of the other lactases which have been discussed.

Information on the structures of the oligosaccharides produced from lactose was gained from studies (23) of the products of partial acid hydrolysis of the compounds and their aldonic acids. From this information and by analogy to the structures of glucosyl oligosaccharides formed by transglucosylation reactions (7) the structures given in the introduction were suggested. This structural information and isotope studies confirmed the transgalactosylation mechanism of enzyme action. Thus inclusion of glucose-C^{14} in the reaction mixture resulted in the synthesis of labeled allolactose (6-O-β-D-galactopyranosyl-D-glucose) and corresponding trisaccharide (O-β-D-galactopyranosyl-(1-->6)-O-β-D-galactopyranosyl-(1-->6)-D-glucose). Inclusion of galactose-C^{14} resulted in the synthesis of radioactive galactobiose (6-O-β-D-galactopyranosyl-D-galactose). These findings are consistent with the two-

step mechanism previously proposed (13) for the enzymatic synthesis of these compounds.

Attempts by Pazur (12) to separate the hydrolyzing and transferring activities of the enzyme preparation by chromatography on silica columns and electrophoresis on paper strips were unsuccessful. Wallenfels has shown (18) that partial purification of the enzyme from Aspergillus alleaceus by a variety of precipitation procedures failed to produce any separation of the hydrolyzing and synthesizing activities. Enzymes prepared from molds, bacteria, snails or calf intestine all showed the same pattern of action. The effects of pH on the hydrolyzing and transferring activities of the mold enzyme were nearly identical. These results have been interpreted to mean that one enzyme species catalyzes both the hydrolytic and transgalactosylation reactions.

METHODS AND MATERIALS

Paper Chromatography- The method used for qualitative paper chromatography of oligosaccharides was essentially that described by French, Knapp and Pazur (24). The filter paper used was 8 in. by 8.5 in. rectangles of untreated Eaton and Dikeman 613. The substances being tested were applied at intervals of 1 in. along a line ruled 1 in. from one of the shorter edges of the rectangle. The chromatograms were developed by the multiple ascent technique using a solvent composed of 1-butanol, pyridine and water (6:4:3 by volume). For the detection of reducing sugars, the dried chromatograms were sprayed with an alkaline copper reagent (Reagent 60 of Shaffer and Somogyi (25) made up without potassium iodate or potassium iodide), followed by a phosphomolybdic acid reagent (26). Fructose and fructose-containing oligosaccharides were detected using a spray reagent composed of 0.2 g. of phloroglucinol dissolved in 100 ml. of 0.1 N hydrochloric acid.

The procedure used for preparative paper chromatography was very similar except that from 0.1 to 0.2 ml. of the mixture to be separated was applied in a continuous streak along the ruled edge of the paper. After development of the chromatogram, 2 vertical strips 1 cm. wide were cut from each chromatogram 6 cm. from either side. These were sprayed for reducing sugars and used as markers for sectioning the remainder of the chromatograms. The sections of the chromatograms containing a particular compound were combined and extracted with boiling water. The extracts were concentrated to a small volume under reduced pressure. When isolating compounds by this method from complex mixtures it was usually necessary to rechromatograph the concentrated extract to obtain chromatographically pure materials.

Detection and Determination of C^{14}-Labeled Materials- Radioautograms were made for the detection and location of radioactive materials on paper chromatograms. The chromatograms were placed in contact with Kodak No-Screen X-ray film for periods of time varying from one day to several weeks, depending upon the amount of radioactive material present. After development of the films, the intensities of the spots were used as an approximate measure of the amount of radioactive material present. To get a more precise measure of the radioactivity of the materials on the paper, the radioautograms were used as guides to locate the radioactive areas on the paper. The activities were then measured using a Geiger-Mueller counting assembly.

The Enzyme- The enzyme was a partially purified lactase prepared from Saccharomyces fragilis and kindly provided by Dr. W. M. Connors, National Dairy Research Laboratories, Long Island, New York. It was an amorphous yellow powder readily soluble in water. Enzyme solutions were prepared by dissolving a weighed amount of material either in water or in an appropriate buffer. Enzyme activity measurements were made as described below.

Measurement of the hydrolytic and transferring activity of the enzyme preparation was accomplished by measuring the disappearance of lactose-1-C^{14} and the appearance of glucose-1-C^{14}. In the assay procedure 0.05 ml. of a 0.6 M solution of lactose-1-C^{14} (0.36 μC/mg.) were treated with 0.05 ml. of a solution containing 1 mg. of the enzyme preparation in 0.1 M phosphate buffer of pH 6.7. Samples of 5 μl. were placed on chromatograms at the time of mixing and after incubation at room temperature (23° C.) for 1 hour. The reducing compounds were separated on the paper chromatograms and the radioactivity of the

lactose and glucose were then measured directly on the paper, using a G.M. counting assembly. The amount of lactose which disappeared was a measure of the sum of the hydrolytic and transferring activities, while the amount of glucose produced was a measure of the hydrolytic activity. Under the conditions of the assay procedure, of 0.3 millimoles of lactose, 0.065 millimoles were hydrolyzed and 0.026 millimoles were converted to new oligosaccharides by 1 mg. of the enzyme preparation in the 1 hour period. This amount of activity is arbitrarily defined as one unit.

For an approximate determination of the pH optimum of the enzyme, digests were prepared containing 100 mg. of lactose and 10 units of the enzyme dissolved in 1 ml. of 0.02 M phosphate buffer, at pH's of 5.0, 6.0, 7.0, 8.0 and 9.0. The enzyme was inactivated by heating at the end of 4.5 hours. Visual inspection of paper chromatograms of the digests indicated that although the enzyme had appreciable hydrolytic and transferring activity at each pH tested, maximum activities were found at pH 6.0 and 7.0 Since it was found that the phosphate ions interfered with paper chromatography of the sugars, many of the enzymatic experiments were done in unbuffered solutions having pH's of about 6.8.

Since the oligosaccharides produced during the hydrolysis of lactose are also subject to hydrolysis by the enzyme, their concentrations were expected to reach a maximum, then decrease to zero as the reaction progressed. To determine the time at which maximum synthesis had occurred, a digest was prepared containing 100 mg. of lactose and 10 units of enzyme dissolved in 1 ml. of 0.05 M phosphate buffer of pH 6.5. Samples taken at intervals of 1 hour up to 6 hours were chromatographed. Visual inspection of the sprayed chromatograms indicated that maximum

synthesis occurred in about five hours, although one compound reached a maximum in about three hours.

Preparation of the Oligosaccharides- A typical digest for the preparation of the oligosaccharides consisted of a solution of 10 g. of lactose in 50 ml. of water to which was added 2000 units of enzyme dissolved in 50 ml. of water. After standing at room temperature in a closed flask for 4.5 hours, the enzyme was inactivated by heating at 100° C. for 5 minutes. The reducing compounds were then separated by the paper chromatographic method described in an earlier paragraph. The yields of disaccharides were 0.2 g. of compound I; 0.7 g. of compound II; and 0.5 g. of compound III.

Since sucrose, which was present in the enzyme preparation, possesses an Rf value similar to that of compound I, it is difficult to eliminate by chromatography. Hydrolysis of the solution in 0.1 N hydrochloric acid for one hour at 80° C. converted the sucrose to glucose and fructose which were then separated from the oligosaccharides by paper chromatography.

In some instances, a preliminary fractionation of the oligosaccharides was achieved by adsorption and elution from charcoal (27). The digest was concentrated by vacuum distillation and the carbohydrates adsorbed on 100 g. of a 1:1 mixture of charcoal and celite (Celite No. 535, Johns-Manville, New York; Darco G-60 activated carbon, Darco Corporation, New York). The carbohydrates were then eluted by slurrying the celite-charcoal with ethanol-water mixtures containing increasing amounts of ethanol, then filtering with suction. Five hundred ml. filtrates containing 0, 2.5, 5.0, 7.5, 10, 12.5 and 15% ethanol were collected, concentrated to about 10 ml. by vacuum distillation and examined by paper chromatography. Fractions with 0 and 2.5% ethanol were

enriched in the monosaccharide components, fractions with 5.0, 7.5 and 10% ethanol were enriched with the disaccharide components and fractions with 12.5 and 15% ethanol were enriched with the trisaccharides. These fractions were then chromatographed on paper for final purification of the products.

On one occasion gradient elution chromatography from a charcoal-celite column (28) was employed. A digest was made following the procedure of Roberts and Pettinati (21) in which the lactose concentration was very high. Ten g. of lactose were dissolved in 22 ml. of 0.05 M biphthalate buffer, pH 6.2, and 440 units of enzyme were added. After incubation at 35° C. for five hours, the enzyme was inactivated by heat. A charcoal-celite column 24 cm. long and 34 mm. in diameter was prepared. After washing the column with water, 5 ml. of the digest was added to the top and the column eluted with water until the eluate no longer gave a positive Benedict's test. Ethanol was then added gradually to the solvent by allowing a mixture of ethanol and water (1:4 by volume) to flow through a tube into the bottom of a 4 l. vacuum filtering flask filled with water, which was being stirred mechanically. The solvent overflowed through the sidearm of the flask onto the top of the column and was forced through under 1 to 2 lbs. of air pressure. Fractions of 30 to 40 ml. were collected, concentrated to 1 to 2 ml. by vacuum distillation and examined by paper chromatography. Chromatographically pure allolactose was found in fractions 51 to 61, allolactose and lactose in fractions 62 to 84 and compound I in fractions 92 to 94. Fractions beyond 94 were not collected.

Lactose-C^{14}, glucose-1-C^{14} and galactose-1-C^{14}- Lactose labeled in the glucose and galactose moieties was prepared in this laboratory (29)

from the milk of female guinea pigs which had been injected with glucose-1-C^{14} or galactose-1-C^{14}. Lactose-1-C^{14}, glucose-1-C^{14} and galactose-1-C^{14} were purchased from the National Bureau of Standards.

p-Nitrophenyl-β-D-galactopyranoside- This material was prepared following the procedure described by Seidman and Link (30) for the preparation of o-nitrophenyl-β-D-galactopyranoside. The product after being recrystallized three times from 95% ethanol was a white, crystalline material melting at 140-141° C.

Methyl-α-D-galactopyranoside- Crystalline methyl-α-D-galactopyranoside was kindly provided by Dr. A. S. Perlin, Prairie Regional Laboratory, National Research Council, Saskatoon, Saskatchewan, Canada.

EXPERIMENTAL

Preparation of the phenylosazones- To prepare the phenylosazones of the disaccharides, 0.05 g. of oligosaccharide dissolved in 1 ml. of water was mixed with 1 ml. of 5% acetic acid saturated with sodium acetate, 1 ml. of 50% sodium bisulfite, 0.4 g. of phenylhydrazine hydrochloride and 7 ml. of water. After heating in a boiling water bath for 1 hour, the mixtures were cooled until the phenylosazones precipitated. The osazones were then collected and dried on a filter and finally crystallized from a small amount of 95% ethanol. The melting points of the crystalline phenylosazones obtained are recorded in Table 1 of the appendix. The X-ray diffraction data for the phenylosazones (obtained at the Instrumentation Laboratory of the University of Nebraska) are recorded in Table 2. The first number of each pair is the interplanar spacing in Angstroms, using CuKa radiation; the second number is the relative intensity of the line, estimated visually, on the basis of 100 for the strongest line.

Optical Rotations- The optical rotations of the amorphous but chromatographically pure disaccharides were measured in water solution in two decimeter polarimeter tubes, volume 1.4 ml. The concentrations in grams per 100 ml. of solution were: compound I, 0.9; compound II, 3.3; and compound III, 2.0. The specific rotations are recorded in Table 1.

Lead tetraacetate oxidations of oligosaccharides- Lead tetraacetate oxidations were carried out in a Warburg respirometer at 27° C., following the procedure of Perlin (31). Quadruplicate samples of from 0.27 to 0.56 mg. of the oligosaccharides dissolved in 0.2 ml. of 90% acetic acid were placed in the side arms of the flasks. The vessel

chambers contained 1 ml. of a solution containing 20 mg. of lead tetra-acetate and 10 mg. of potassium acetate in 90% acetic acid. The side arms of the 4 blank vessels contained 0.2 ml. of 90% acetic acid. After equilibration and mixing, the production of carbon dioxide was determined manometrically. At the end of 15 minutes and again after 50 minutes 2 samples and 2 blanks were withdrawn and the oxidation stopped by pouring the reaction mixture into 5 ml. of a "stopping solution" (10 g. of potassium iodide and 50 g. of sodium acetate in 100 ml. of water). Each vessel was then rinsed three times with 1 ml. portions of the stopping solution and the liberated iodine titrated with standard thiosulfate solution.

Disproportionation of II- To study the effect of the enzyme on compound II, a digest was prepared by adding, to 2.5 mg. of II dissolved in 0.03 ml. of water, 0.45 units of enzyme dissolved in 0.03 ml. of water. Samples withdrawn before the enzyme was added and after 1/2, 1, 2, 4 and 8 hours, and an enzyme blank, were chromatographed on paper. The products of the reaction were located by spraying the developed chromatogram with the copper sulfate reagent, followed by the phosphomolybdic acid reagent described in the methods section.

Reaction of II with glucose-1-C^{14}- A digest was prepared by adding 1 unit of enzyme dissolved in 0.05 ml. of 0.1 M phosphate buffer, pH 6.5, to a solution containing 2.5 mg. of II and 2 mg. of glucose-1-C^{14} (2.54 ucurie/mg.) in 0.05 ml. of water. Duplicate 5 ul. samples, taken at the time the enzyme was added and after 2, 4, 8 and 24 hours, were applied to 2 chromatograms. After development of the chromatograms the radioactive materials were located by radioautography and the reducing compounds by spraying the chromatograms with appropriate reagents.

Reaction of V with glucose-1-C^{14}- To 1.2 mg. of V and 2 mg. of glucose-1-C^{14} (1.91 µcurie/mg.) dissolved in 0.03 ml. of water were added 0.9 units of enzyme dissolved in 0.03 ml. of water. Samples were taken before addition of the enzyme and after 1, 2 and 4 hours. The samples were chromatographed by the usual procedure. The radioactive materials were located by radioautography and reducing compounds with spray reagents.

Reaction of galactose and glucose-1-C^{14}- Two digests containing the enzyme and free monosaccharides were prepared, one in which the concentration of the monosaccharides was relatively low, the other having a very high monosaccharide concentration. The first was prepared by dissolving 1 mg. of galactose and 2 mg. of glucose-1-C^{14} (1.91 µcurie/mg.) in 0.03 ml. of water and adding 0.9 units of enzyme dissolved in 0.03 ml. of water. The second digest was prepared by dissolving 12 mg. of galactose, 2 mg. of glucose-1-C^{14} (1.91 µcurie/mg.) and 10 mg. of unlabeled glucose in 0.03 ml. of water and adding 0.9 units of enzyme in 0.03 ml. of water. Samples were taken at the time the enzyme was added and after 1/2, 1, 2 and 4 hours. Reducing and radioactive components in the aliquots were identified by paper chromatography and radioautography.

Disproportionation of III- To bring about the disproportionation of III, a digest was prepared by mixing 1.5 mg. of III dissolved in 0.03 ml. of water with 1.5 units of enzyme in 0.03 ml. of water. Samples were taken and treated as described for the disproportionation of II.

Reaction of III with galactose-1-C^{14}- The reaction of III and galactose-1-C^{14} in the presence of the enzyme was studied by preparing a digest containing 2.5 mg. of III and 2 mg. of galactose-1-C^{14} (2.24 µcurie/mg.) dissolved in 0.05 ml. of water, to which was added 1 unit of

enzyme dissolved in 0.05 ml. of 0.1 M phosphate buffer of pH 6.5. The remainder of the experiment was carried out as described for the disproportionation of II with glucose-1-C^{14}.

Digest of methyl-α-D-galactopyranoside and the enzyme- To 1 mg. of methyl-α-D-galactopyranoside was added 3 units of enzyme dissolved in 0.1 ml. of 0.05 M biphthalate buffer, pH 6.2. Samples were taken at the time the enzyme was added and after 1, 2, 3 and 5.5 hours. The developed chromatogram was sprayed for reducing sugars.

Enzymatic digest of lactose and glucosamine- A solution containing 50 mg. of glucosamine hydrochloride dissolved in 0.5 ml. of water was adjusted to pH 7 with sodium carbonate. Fifty mg. of lactose dissolved in 0.5 ml. of water was added, followed by 20 units of enzyme dissolved in 1 ml. of water. Samples taken at the time the enzyme was added and after 1, 2, 4, 8 and 24 hours were acidified to pH 1-2 with 5 N hydrochloric acid and chromatographed in a solvent composed of 1-butanol, acetic acid and water (4:1:5 by volume). After development the chromatograms were sprayed with ninhydrin to locate the amino compounds or with copper sulfate reagent to locate the reducing compounds.

Enzymatic digest of lactose and sucrose- Two units of enzyme dissolved in 0.1 ml. of water were added to 40 mg. of lactose and 10 mg. of sucrose dissolved in 0.2 ml. of water. Samples taken at the time the enzyme was added and after 4 hours were chromatographed. The chromatograms were sprayed with phloroglucinol reagent (0.1% solution of phloroglucinol in .1 N hydrochloric acid) to locate the fructose-containing oligosaccharides or with copper sulfate reagent to locate the reducing compounds.

Enzymatic digest of lactose and fructose- The effect of adding

fructose to lactose digests was studied by dissolving 400 mg. of lactose and 400 mg. of fructose in 2 ml. of water and adding 40 units of enzyme dissolved in 2 ml. of 0.1 M phosphate buffer, pH 6.5. Samples taken at the time the enzyme was added and after 1, 2, 3, 4, 5 and 6 hours were chromatographed in the usual manner. The developed chromatograms were sprayed with phloroglucinol reagent for locating fructose containing oligosaccharides.

Enzymatic digest of lactose and planteose- Forty mg. of lactose, 15 mg. of planteose and 2 units of enzyme were dissolved in 0.3 ml. of water. In a control digest, 15 mg. of planteose and 2 units of enzyme were dissolved in 0.3 ml. of water. Samples were withdrawn at the time of mixing and after 4 hours. The developed chromatograms were sprayed with phloroglucinol reagent.

Enzymatic digest of lactose and raffinose- A digest designed to determine the effect of adding raffinose to a lactose digest was prepared by dissolving 40 mg. of lactose and 15 mg. of raffinose hydrate in 0.2 ml. of water, then adding 2 units of enzyme dissolved in 0.1 ml. of water. A control digest contained 15 mg. of raffinose and 2 units of enzyme in 0.3 ml. of water. Samples were taken at the time of mixing and after 5 hours. The developed chromatograms were sprayed with phloroglucinol.

Enzymatic digests using p-nitrophenyl-β-D-galactopyranoside as substrate- Digests prepared to determine whether or not p-nitrophenyl-β-D-galactopyranoside (NPG) is a substrate and whether or not maltose, melibiose and cellobiose are cosubstrates for the enzyme were prepared by dissolving, in 0.1 ml. of 0.05 M biphthalate buffer, pH 6.2, the following materials: (1) 10 mg. of NPG and 2 units of enzyme, (2) 10 mg.

of NPG, 20 mg. of maltose and 2 units of enzyme, (3) 10 mg. of NPG, 20 mg. of melibiose and 2 units of enzyme, (4) 10 mg. of NPG, 20 mg. of cellobiose and 2 units of enzyme, (5) 20 mg. of maltose and 2 units of enzyme, (6) 20 mg. of melibiose and 2 units of enzyme, (7) 20 mg. of cellobiose and 2 units of enzyme and (8) 10 mg. of NPG. Samples withdrawn from each digest at the time the enzyme was added and after 1, 2, 3 and 5.5 hours were chromatographed and the chromatograms sprayed for reducing sugars. The unreacted NPG was located by viewing the dried chromatograms under an ultraviolet lamp.

RESULTS AND DISCUSSION

In figure 1 of the Appendix is reproduced a photograph of a chromatogram of a digest of lactose with the lactase prepared from Saccharomyces fragilis. Also on the chromatogram are each of the major components of the digest in chromatographically pure form. In addition to lactose and the hydrolytic products, five new oligosaccharides may be seen in the digest. These have been numbered in order of decreasing paper chromatographic mobility. Compounds II, III, IV and V have previously been isolated by Pazur (13, 23). The tentative structures assigned to them were given in the introduction. Apparently II, III and IV correspond to compounds studied by Wallenfels (11, 17, 18) and by Aronson (19).

Compound I probably corresponds to one of the three new oligosaccharides detected chromatographically by Roberts et al. (20, 21) which have chromatographic mobilities between those of lactose and galactose. Since compound I has not been previously isolated in pure form structural information on this compound has not been available. Unpublished work in this laboratory has shown that I is a disaccharide composed of glucose and galactose, the glucose being the reducing moiety. Its chromatographic mobility is typical of a 1,3 linked disaccharide (32).

Of the various procedures available for determining the positions of glycosidic bonds in oligosaccharides, lead tetraacetate oxidation as described by Perlin (31,33) seemed to be the most advantageous. By this procedure one can make an unequivocal distinction between various possible linkages using samples of less than a milligram.

The action of lead tetraacetate upon carbohydrates resembles that of periodic acid in that it oxidizes glycols to dialdehydes. However,

Perlin has demonstrated that the oxidation of reducing sugars by lead tetraacetate begins with the α-hydroxyhemiacetal group and proceeds stepwise down the carbon chain (34). Ordinary glycols are attacked at a much lower rate. The initial product in the oxidation of a typical disaccharide having a 1,4 linkage is a 3-O-glycosyl-4-O-formyl pentose. The pentose immediately cyclizes and is again oxidized to a 2-O-glycosyl-3,4-di-O-formyl tetrose. The initial rapid oxidation stops here and the slower oxidation of the glycol groups of the non-reducing moiety becomes apparent. Oxidation of the non-reducing moiety of the disaccharide and, to a lesser extent, of the ordinary glycols of the reducing moiety, results in the production of formic acid, which in turn is oxidized to carbon dioxide. The amount of lead tetraacetate consumed by the oxidation of formic acid is estimated by measuring in a Warburg respirometer the amount of carbon dioxide produced.

From inspection of the structural formulas of 1,3-, 1,4- and 1,6-linked reducing disaccharides it can be seen that the rapid initial oxidation of the reducing moieties of these compounds should consume 1, 2 and 3 moles of oxidant, respectively. It is necessary to subtract, from the total amount of lead tetraacetate reduced by a disaccharide, the amount consumed under the same conditions by a glycoside corresponding to the non-reducing moiety of the disaccharide. The difference in these two quantities is the amount of oxidant consumed by the reducing moiety of the disaccharide, from which the position of the glycosidic linkage is deduced.

In Table 3 of the Appendix are recorded the data pertaining to each oxidation which was performed. In Table 4 the values for duplicate oxidations have been averaged and the amount of oxidant consumed by the

galactosyl group subtracted from the total amount consumed by each disaccharide. The results of the lead tetraacetate oxidations clearly indicate that compound I has a 1,3 linkage, while compounds II and III have 1,6 linkages. As would be expected lactose and melibiose, containing a 1,4 and a 1,6 linkage respectively, required 2 and 3 moles of the oxidant.

The non-reducing moieties of the new oligosaccharides are assumed to be in the pyranose ring form because qualitative observations of the rates of acid hydrolysis of these compounds have not indicated that any of them possess the high degree of lability toward acid hydrolysis that is typical of furanosides.

The stereochemical configuration of the glycosidic linkage in II has been shown to be β, because the compound has been shown to be structurally identical in every other way to melibiose, which has an α-1,6 linkage. The physical properties of the two materials and their derivatives are sufficiently different to leave no doubt they are different compounds. In view of the stereospecificity of the transferring enzymes (8,16), the other new compounds should also have β-linkages. Further evidence for this assignment comes from the fact that the enzyme used hydrolyzes all of the new compounds, lactose and p-nitrophenyl-β-D-galactopyranoside, but is inactive toward methyl-α-D-galactopyranoside, melibiose, cellobiose and maltose.

On the basis of the evidence presented, the structures of compounds I, II and III appear to be 3-O-β-D-galactopyranosyl-D-glucose, 6-O-β-D-galactopyranosyl-D-glucose and 6-O-β-D-galactopyranosyl-D-galactose, respectively. The specific rotations of the compounds and the melting points of the crystalline osazones are recorded in Table 1 of the Appendix.

The X-ray diffraction data for the osazones are recorded in Table 2.

Figure 2 shows a chromatogram of a digest of II with the enzyme. The reference compounds (R) are glucose (GL), galactose (GAL), II or allolactose (A) and the trisaccharide V (T). The sample taken at zero time contained only II. In succeeding samples, II gradually disappeared, while the concentrations of glucose and galactose increased. In the 1/2, 1 and 2 hour samples, a reducing compound having the chromatographic mobility of V may be seen. Synthesis of this compound from II supports the tentative structure which has been previously assigned to it, O-β-D-galactopyranosyl-(1-->6)-O-β-D-galactopyranosyl-(1-->6)-D-glucose (23).

The probable mechanism by which V is synthesized from II may be represented by the following equations:

$$\text{Gal(1-6)Gl} + \text{E} \rightleftharpoons \text{Gal.E} + \text{Gl} \qquad (1)$$

$$\text{Gal.E} + \text{Gal(1-6)Gl} \rightleftharpoons \text{Gal(1-6)Gal(1-6)Gl} + \text{E} \qquad (2)$$

Gal(1-6)Gl represents II, E the enzyme, Gal.E a galactosyl-enzyme complex, Gl free glucose and Gal(1-6)Gal(1-6)Gl compound V. In the first step, a molecule of II reacts reversibly with the enzyme to yield free glucose and a galactosyl-enzyme complex. In the second step, the galactosyl enzyme complex reacts with another molecule of II to form a molecule of V and the free enzyme. The reversibility of each step of this mechanism has been demonstrated by the use of radioactive isotopes.

To demonstrate the reversibility of step (1), the following reaction was employed:

$$\text{II} + \text{glucose-1-C}^{14} \rightleftharpoons \text{II-C}^{14} + \text{glucose} \qquad (3)$$

A digest of II with glucose-1-C^{14} and the enzyme was found to result in the rapid incorporation of radioactive glucose into II. The activities of the products of this reaction as measured on the paper chromatograms

are shown in Table 5. Smaller amounts of radioactivity were found in spots having relative Rf values corresponding to I, lactose and V.

The reaction used to demonstrate the reversibility of step (2) was:

$$V + \text{glucose-1-C}^{14} \rightleftharpoons II + \text{II-C}^{14} \qquad (4)$$

Figure 3 shows (on the left) a chromatogram of a digest of V with glucose-1-C^{14} and the enzyme and (on the right) a radioautogram made from the chromatogram. The reference compounds were glucose (GL), galactose (GAL), II or allolactose (A), III or galactobiose (G) and V (T). The sample taken before the enzyme was added contained only V and glucose-1-C^{14}. As the reaction proceeded, galactose and II could easily be detected and a small amount of III was formed. The radioautogram shows that there was a rapid incorporation of radioactive glucose into II by the transfer of galactosyl groups to glucose-1-C^{14} in the solution. A quantitative measure of the extent to which glucose-1-C^{14} was incorporated into II is given in Table 6, in which the relative activities of the products of the reaction are recorded.

To determine whether or not oligosaccharides could be synthesized by the enzyme from free monosaccharides, digests were made containing galactose, glucose-1-C^{14} and the enzyme. One digest contained the monosaccharides at about the same concentrations as the oligosaccharides used in other experiments. The other digest contained the monosaccharides at very high concentrations (20% glucose and 20% galactose, w/v.). At the low monosaccharide concentration, radioautograms showed the presence of a small amount of material corresponding in chromatographic mobility to II. The relative activities of the glucose-1-C^{14} and II found on the chromatogram are recorded in Table 7. The radioautogram

of the digest containing monosaccharides at a high concentration did not show the presence of any radioactive oligosaccharides. It has been demonstrated before that lactases are inhibited by galactose (13). The relatively small amount of synthesis of II-C^{14} from free monosaccharides as compared to that from glucose-1-C^{14} and V supports the view that the synthesis of oligosaccharides in the system occurs by glycosidic exchange reactions in which small changes in free energy are involved rather than by reversion type reactions of monosaccharides.

Further evidence for the reversible transgalactosylation mechanism of action of the enzyme was obtained from the disproportionation of III. This disaccharide was also hydrolyzed by the enzyme with the simultaneous formation of a trisaccharide. The trisaccharide, which was not isolated, had a slightly lower paper chromatographic mobility than V. To demonstrate the reversible nature of the enzyme's action on III, a digest was prepared containing III and galactose-1-C^{14}. The radioactive monosaccharide was quickly incorporated into the disaccharide, as may be seen from the relative activities of the compounds listed in Table 8.

An effort was made to determine what steric requirements must be satisfied in order that a compound may act as a cosubstrate or acceptor for the enzyme. It has been demonstrated that the enzyme is capable of transferring galactosyl groups to glucose, galactose and to oligosaccharides having β-galactosyl groups as the non-reducing end groups. A number of other sugars were tested as acceptors by adding them to digests of the enzyme with lactose or p-nitrophenyl-β-D-galactopyranoside. When glucosamine, fructose or fructose-containing oligosaccharides were tested as cosubstrates, lactose could be used as the substrate, because specific color tests are available which can distinguish between the

oligosaccharides formed from lactose alone and those which might be formed from the new cosubstrate. When other compounds were used as cosubstrates, p-nitrophenyl-β-D-galactopyranoside was used as the substrate because the resulting reaction mixture was more readily analyzed.

Glucosamine was tested as a cosubstrate because of its structural relationship to glucose and because of the possible relationship of the product of the reaction to the glucosamine-containing oligosaccharides which have been isolated from human milk (35). In addition, such a reaction would provide a means of synthesizing reference oligosaccharides for use in studying the structures of such materials as immunopolysaccharides and bacterial cell walls. When glucosamine was added to a digest of lactose and the enzyme, a compound which reacted with ninhydrin and had the chromatographic mobility of a disaccharide was detected on paper chromatograms of the digest. In figure 4, a photograph of a chromatogram of such a digest, it can be seen that the concentration of the compound passed through a maximum and the compound was apparently hydrolyzed by the enzyme. In this figure, glucosamine is labeled GL-NH_2 and the new compound NC. In view of the mode of action of the enzyme in its reactions with glucose and galactose as cosubstrates, the probable structure of this compound is 6-O-β-D-galactopyranosyl-D-glucosamine.

When sucrose was used as a cosubstrate, two compounds reactive with phloroglucinol and having lower chromatographic mobilities than sucrose were detected. Addition of the enzyme to sucrose solutions in the absence of lactose did not result in synthesis of new compounds. Since new compounds were not produced from fructose and lactose, it is likely that the transfers to the sucrose molecule occurred at the

glucosyl moiety. Structural studies on the new compounds were not undertaken.

The fructosyl oligosaccharides raffinose and planteose, which are O-α-D-galactopyranosyl-(1-->6)-α-D-glucopyranosyl-β-D-fructofuranoside (9) and O-α-D-galactopyranosyl-(1-->6)-β-D-fructofuranosyl-α-D-glucopyranoside (36) were tested as substrates and cosubstrates for the enzyme. Neither raffinose nor planteose functioned as substrates for the enzyme. Since a new fructosyl oligosaccharide was synthesized from planteose and lactose, the planteose did function as an acceptor of galactosyl units. Whether the transfer occurred to the glucose or galactose moieties of the trisaccharide is not known at present. A small amount of a fructose-containing oligosaccharide was synthesized from lactose and raffinose indicating that raffinose was a very weak cosubstrate of the enzyme.

When p-nitrophenyl-β-D-galactopyranoside was treated with the enzyme, it was hydrolyzed at a rate comparable to the rate at which lactose is hydrolyzed, with the simultaneous formation of a reducing compound having the same chromatographic mobility as compound III. When maltose, melibiose or cellobiose were added to such digests, no new reducing compounds could be detected, indicating transfer to these materials did not occur, although the substrate was rapidly hydrolyzed. Maltose, melibiose and cellobiose were unaffected by the enzyme.

As a result of these experiments, it is apparent that the enzyme does not transfer galactosyl groups at random to any sugar in the solution. The fact that the enzyme does exhibit cosubstrate specificity indicates that a rather intimate association of enzyme and cosubstrate must take place before the transfer reaction can occur. This may be explained

if one visualizes, as has been proposed, that the reaction of the enzyme and substrate produces a galactosyl-enzyme complex, with the reducing moiety of the substrate then diffusing away from the enzyme as a free sugar molecule. Its place on the enzyme surface may then be occupied by water, if hydrolysis is to occur, or if transfer is to occur, by a sugar molecule having a configuration which allows it to fit onto the active site of the enzyme. Further work is necessary to establish the precise structural features necessary in a compound in order that the compound may function as a cosubstrate in the transgalactosylation reaction of the lactase from S. fragilis.

SUMMARY

Five of the galactosyl oligosaccharides produced during the hydrolysis of lactose by an enzyme prepared from Saccharomyces fragilis have been isolated in chromatographically pure form. The three having the highest chromatographic mobilities have been shown to be 3-O-β-D-galactopyranosyl-D-glucose, 6-O-β-D-galactopyranosyl-D-glucose and 6-O-β-D-galactopyranosyl-D-galactose. The positions of the glycosidic linkages in these compounds were determined by use of the lead tetraacetate oxidation procedure. These three disaccharides have been characterized by measurement of their optical rotations and by preparation of the crystalline phenylosazones.

Evidence has been obtained in support of the following transgalactosylation mechanism of synthesis of oligosaccharides from lactose:

Lactose + enzyme ⇌ galactosyl-enzyme + glucose (1)

Galactosyl-enzyme + acceptor ⇌ oligosaccharide + enzyme (2)

Both steps of this mechanism have been shown to be readily reversible.

The cosubstrate or acceptor specificity of the enzyme was investigated. Glucosamine, sucrose, planteose and raffinose, in addition to the sugars normally found in digests of lactose and the enzyme, were found to be cosubstrates. Fructose, maltose, cellobiose and melibiose were not cosubstrates.

APPENDIX

TABLE 1

PHYSICAL CONSTANTS OF DISACCHARIDES AND THEIR PHENYLOSAZONES

COMPOUND	SPECIFIC ROTATION		MELTING POINT OF PHENYLOSAZONE	
	Observed	Literature	Observed	Literature
I	+28°	------	176-183°C	------
II	+25°	+25°(37);+30.7(38)	186-188°C	176°C(37);188-189°C(38)
III	+31°	+34° (39,40)	188-190°C	207°C(39); 189°C (40)

TABLE 2

X-RAY DIFFRACTION DATA FOR DISACCHARIDE PHENYLOSAZONES

Compound I: 8.83 - 50; 8.10 - 40; 6.60 - 20;
5.60 - 50; 5.24 - 50; 4.92 - 40; 4.74 -100; 4.45 - 70;
4.26 - 40; 4.09 - 40; 3.89 - 40; 3.72 - 80; 3.49 - 90;
3.23 - 60; 3.07 - 10; 2.93 - 10; 2.73 - 20; 2.52 - 10;
2.38 - 5.

Compound II: 12.60 - 30; 10.26 - 20; 9.20 - 20;
6.31 - 40; 5.56 - 40; 5.30 - 40; 4.87 - 60; 4.61 - 60;
4.30 - 80; 4.11 - 80; 3.79 -100; 3.54 - 20; 3.32 - 40;
3.15 -100; 2.96 - 30; 2.81 - 30; 2.62 - 70; 2.46 - 20;
2.25 - 10; 2.20 - 10; 2.15 - 10; 2.12 - 10; 2.07 - 20;
1.96 - 30; 1.85 - 50; 1.81 - 30; 1.68 - 20; 1.62 - 10;
1.52 - 30.

Compound III: 10.89 - 50; 8.57 - 20; 7.18 - 30;
5.63 - 30; 4.69 -100; 4.35 - 80; 3.98 - 80; 3.58 - 60;
3.32 - 10; 3.10 - 5; 2.93 - 5.

TABLE 3

LEAD TETRAACETATE OXIDATIONS

Compound	Sample (μmoles)	Time Min.	$Pb(OAc)_4$ consumed, total*	CO_2 Produced**	$Pb(OAc)_4$ Consumed, Corrected***
Methyl-α-D-galactopyranoside	1.44	15	1.6	0.6	1.0
		50	2.2	0.6	1.6
p-nitrophenyl-β-D-galactopyranoside	1.47	15	1.5	0.3	1.2
		50	2.4	0.6	1.8
I	1.64	15	2.7	0.5	2.2
		50	3.5	0.7	2.8
Lactose	1.11	15	3.6	0.5	3.1
		50	3.9	0.5	3.4
Lactose	1.11	15	3.5	0.4	3.1
		50	4.7	0.6	4.1
II	0.91	15	5.4	1.8	3.6
		50	7.3	2.7	4.6
II	0.91	15	5.5	1.6	3.9
		50	7.6	2.7	4.9
III	0.81	15	5.8	1.9	3.8
		50	7.3	2.5	4.8
III	0.81	15	5.3	1.7	3.6
		50	7.7	2.5	5.2
Melibiose	0.79	15	5.7	1.7	4.0
		50	8.0	3.1	4.9

* Moles of $Pb(OAc)_4$ reduced per mole of compound in the sample.
** Moles of carbon dioxide produced per mole of compound in the sample.
*** Corrected for the amount of $Pb(OAc)_4$ reduced in the production of carbon dioxide.

TABLE 4

CONSUMPTION OF LEAD TETRAACETATE BY DISACCHARIDES

Compound	Total Moles of Lead Tetraacetate consumed Per Mole of Compound*		Lead Tetraacetate Consumed by Reducing Moieties of Disaccharides**		Theoretical***
	15 Min.	50 Min.	15 Min.	50 Min.	
I	2.2	2.8	1.1	1.1	1
Lactose	3.1	3.8	2.0	2.1	2
II	3.8	4.8	2.7	3.1	3
III	3.7	5.0	2.6	3.3	3
Melibiose	4.0	4.9	2.9	3.2	3

* Corrected for carbon dioxide produced.

** Determined by subtraction of values of 1.1 and 1.7 from the 15 minute and 50 minute values, respectively. The 1.1 and 1.7 were the moles of lead tetraacetate consumed per mole of methyl-α-D-galactopyranoside or p-nitrophenyl-β-D-galactopyranoside.

*** Calculated for a 1,3-, 1,4- and 1,6-linkage in the compounds.

TABLE 5

RELATIVE ACTIVITIES OF THE PRODUCTS OF THE REACTION OF II AND GLUCOSE-1-C^{14} IN THE PRESENCE OF LACTASE FROM S. FRAGILIS

Compound	Time (hours) from the addition of the enzyme				
	0	2	4	8	24
Glucose	24500 cpm	23100 cpm	22700 cpm	22300 cpm	21800 cpm
I	120	400	440	510	540
Lactose	170	350	390	470	530
II	170	1050	1350	1590	1940
V	60	80	100	110	160

TABLE 6

RELATIVE ACTIVITIES OF THE PRODUCTS OF THE REACTION OF V AND GLUCOSE-1-C^{14} IN THE PRESENCE OF LACTASE FROM S. FRAGILIS

Compound	Time (hours) from the addition of the enzyme			
	0	1	2	4
Glucose	49800 cpm	48600 cpm	48600 cpm	48900 cpm
II	200	1400	1400	1100

TABLE 7

RELATIVE ACTIVITIES OF THE PRODUCTS OF THE REACTION OF GALACTOSE AND GLUCOSE-1-C^{14} IN THE PRESENCE OF LACTASE FROM S. FRAGILIS

Compound	Time (hours) from the addition of the enzyme			
	0	1	2	4
Glucose	49900 cpm	49800 cpm	49800 cpm	49600 cpm
II	100	200	200	400

TABLE 8

RELATIVE ACTIVITIES OF THE PRODUCTS OF THE REACTION OF III AND GALACTOSE-1-C^{14} IN THE PRESENCE OF LACTASE FROM S. FRAGILIS

Compound	Time (hours) from the addition of the enzyme			
Galactose	14850 cpm	14720 cpm	14690 cpm	14660 cpm
III	150	280	310	340

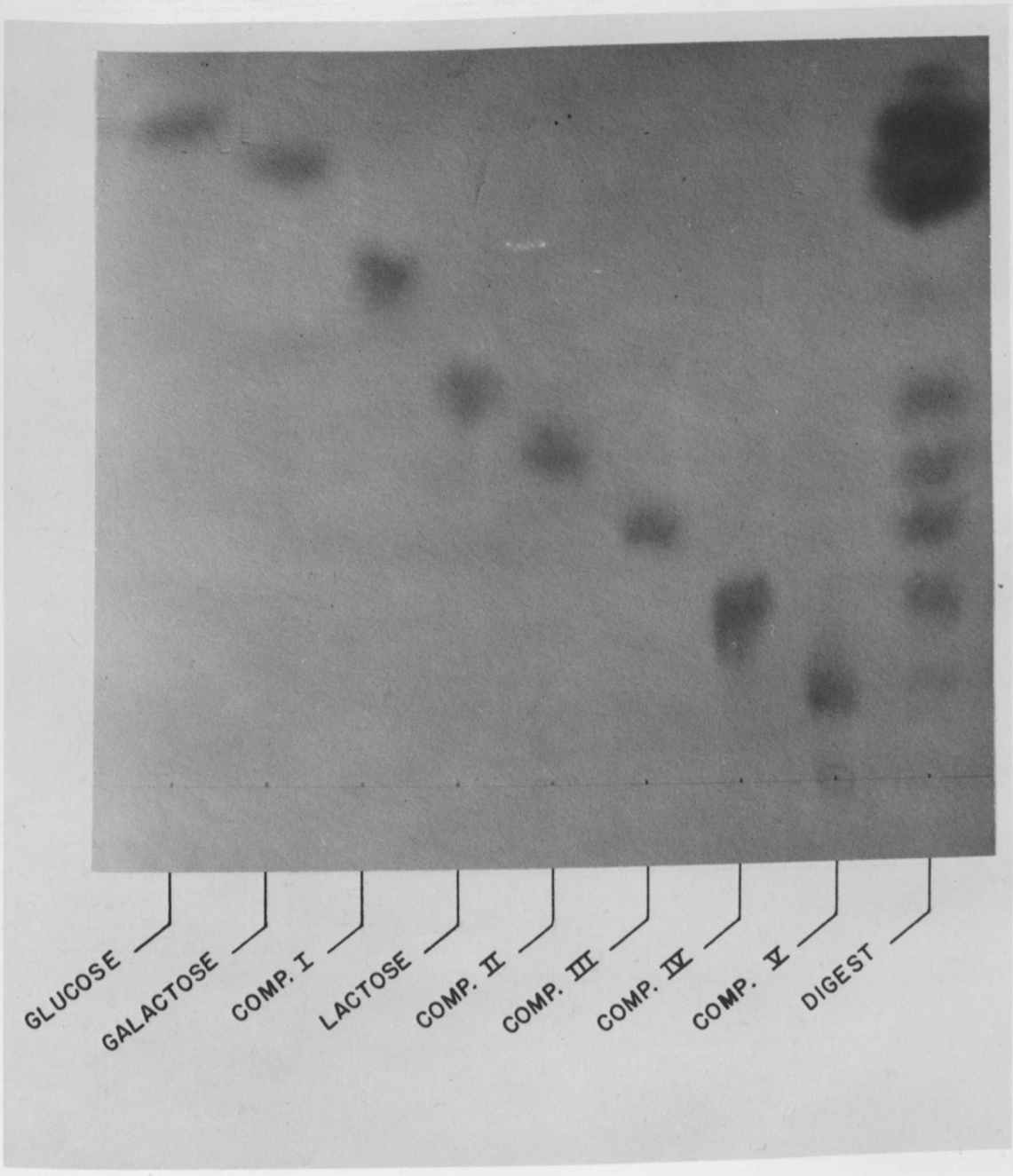
GLUCOSE
GALACTOSE
COMP. I
LACTOSE
COMP. II
COMP. III
COMP. IV
COMP. V
DIGEST

FIGURE 1

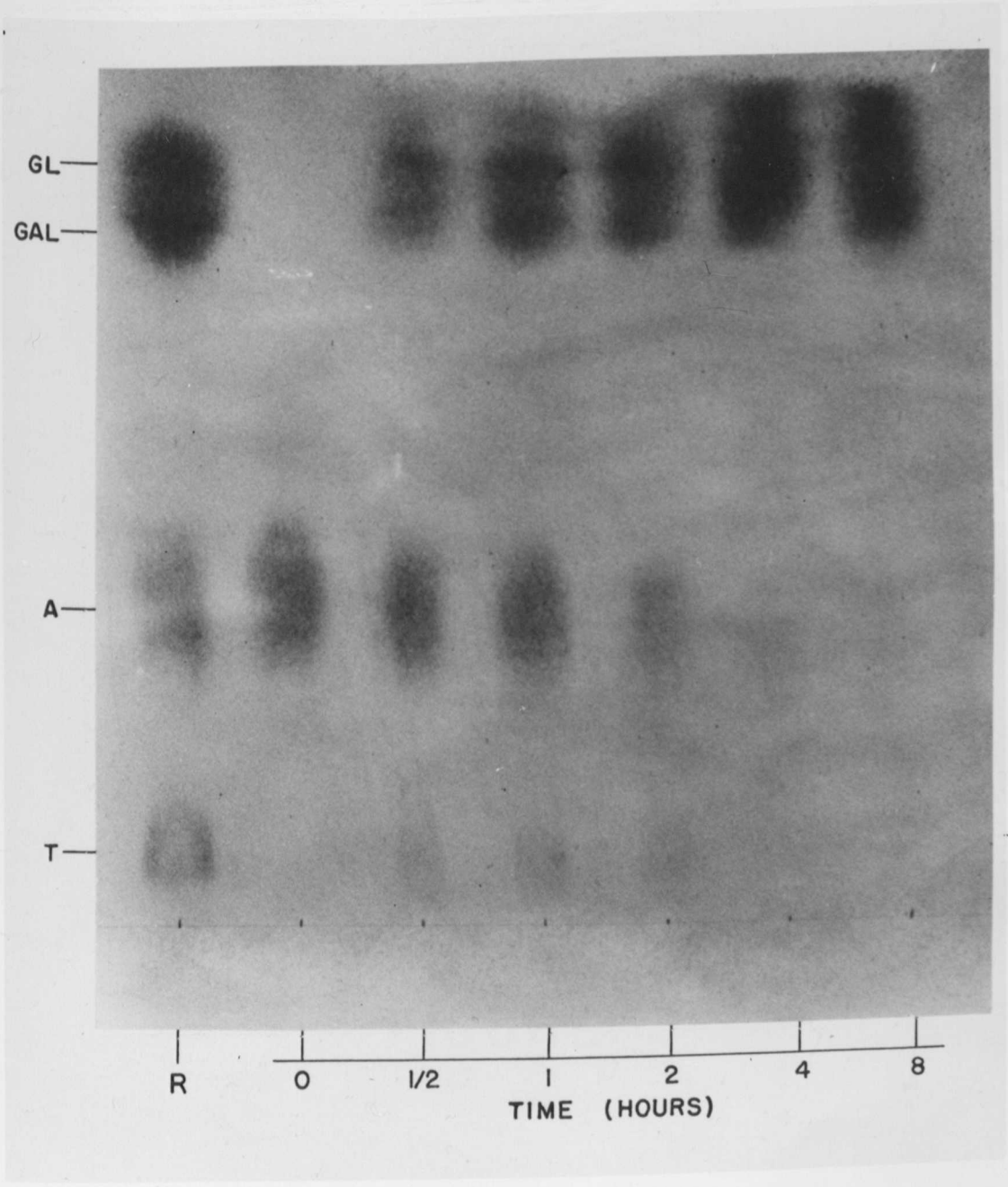

FIGURE 2

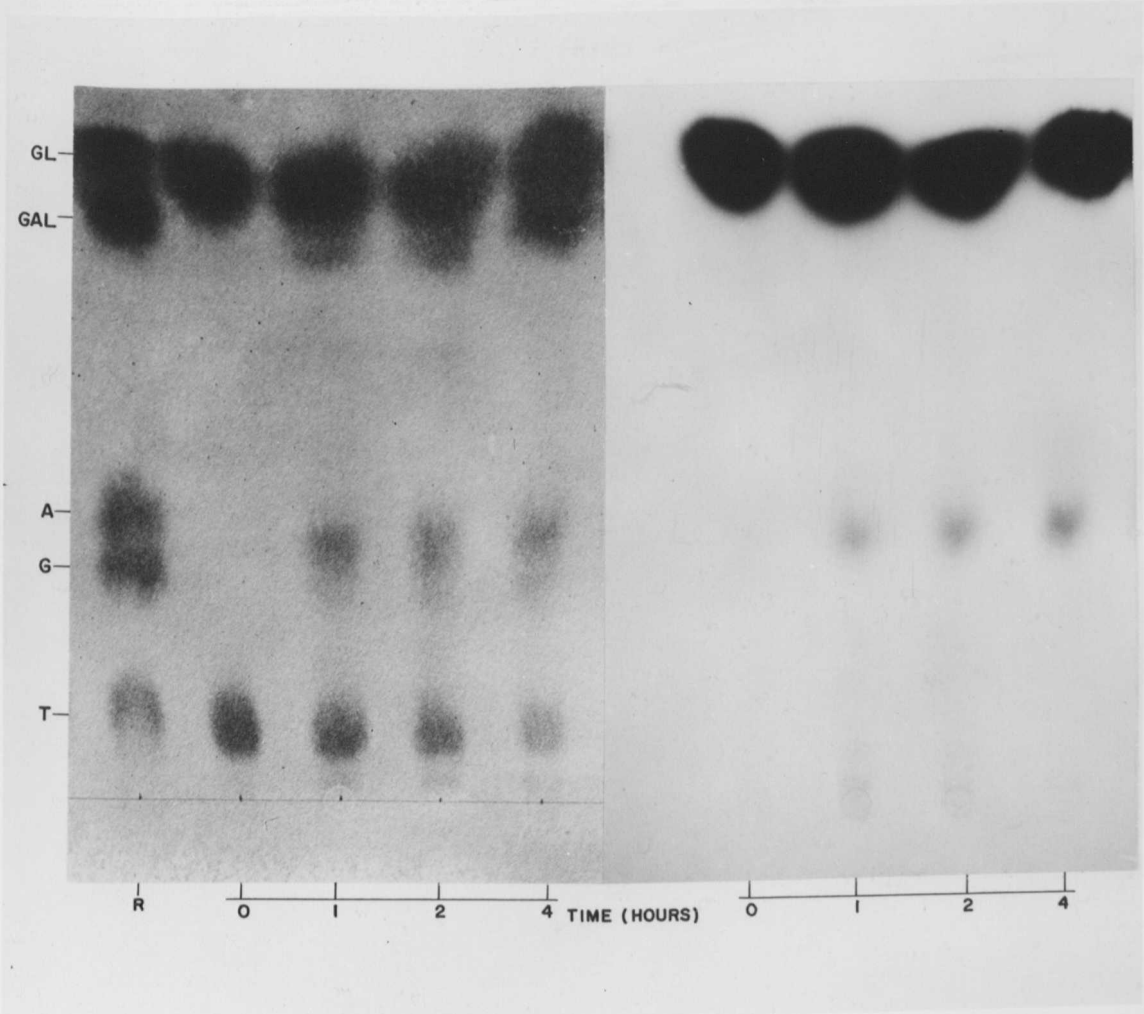

FIGURE 3

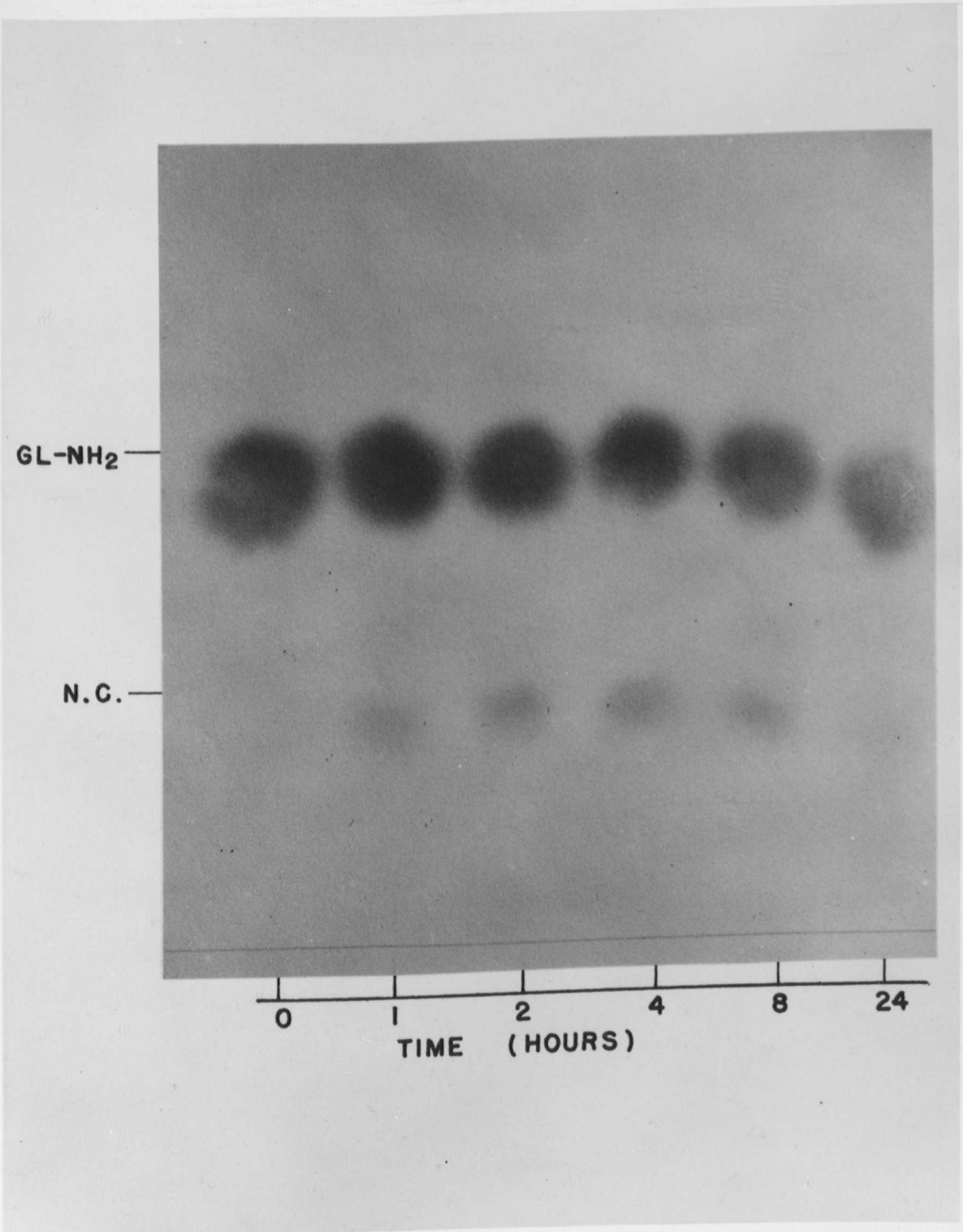

FIGURE 4

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