A Quantitation of the Factors Which Affect the Hydrolase and Transgalactosylase Activities of β-Galactosidase *(E. coli)* on Lactose†

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ABSTRACT: A study was implemented to quantitate the hydrolase and transgalactosylase activities of β-galactosidase *(E. coli)* with lactose as the substrate and to investigate various factors which affect these activities. At low lactose concentrations the rate of galactose production was equal to the rate of glucose production. The rate of galactose production relative to glucose, however, dropped dramatically at lactose concentrations higher than 0.05 M and production of trisaccharides and tetrasaccharides began (galactose/glucose ratios of about 2:1 and 3:1, respectively, were found for these two types of oligosaccharides). At least five different trisaccharides were formed and their patterns of formation showed that they probably utilized both lactose and allolactose as galactosyl acceptors. Allolactose was produced in amounts proportional to glucose at all lactose concentrations (ratios of allolactose/glucose were about 0.88). Analyses of various data, including a reaction analyzed at very early times, showed that the major means of production of allolactose (and the only means initially) was the direct enzymatic transfer of galactose from the 4 position to the 6 position of the glucose moiety of lactose without prior release of glucose from the enzyme. It was shown, however, that allolactose could also be formed in significant quantities by the transfer of galactose to the 6 position of free glucose, and also by hydrolysis of preformed trisaccharide. A mechanism which fits the initial velocity data was proposed in which the steps involving the formation of an enzyme·galactose·glucose complex, the formation and breakage of allolactose on the enzyme, and the release of glucose all seem to be of roughly equal magnitude and rate determining. Various factors affected the amounts of transgalactosylase and hydrolase activities occurring. At high pH values (>7.8) the transgalactosylase/hydrolyase activity ratio increased dramatically while it decreased at low pH values (<6.0). At mid pH values the ratio was essentially constant. The absence of Mg^{2+} caused a large decrease in the transgalactosylase/hydrolase activity ratio while the absence of all but traces of Na^+ or K^+ had no effect. The anomeric configuration of lactose altered the transgalactosylase/hydrolase activity ratios. α-Lactose resulted in a decrease of allolactose production (transgalactosylase activity) relative to hydrolase activities (glucose production) while β-lactose had the opposite effect.

Although lactose [galactosyl-β-D-(1 → 4)-glucopyranose] is known to be the natural substrate of β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) of *Escherichia coli*, only a relatively small number of the many studies carried out with this enzyme have used lactose as the substrate (Cohn and Monod, 1951; Kuby and Lardy, 1953; Reithel and Kim, 1960; Wallenfels et al., 1960a,b; Becker and Evans, 1969; Burstein et al., 1965; Jobe and Bourgeois, 1972). Most of the studies on β-galactosidase have dealt with the hydrolytic action of the enzyme on the synthetic substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG[1]) (Wallenfels and Weil, 1972) and have almost totally ignored the transgalactosylase action which the enzyme possesses. [The exception to this are studies in which simple alcohols were tested as acceptors of galactosyl moieties from synthetic substrates such as ONPG (Shifrin and Hunn, 1969; Wallenfels and Weil, 1972).] Studies quantitating the transgalactosylase reactions of β-galactosidase relative to the hydrolytic reactions have not previously been done. It, therefore, seemed pertinent to carry out a comprehensive quantitative study of the action of β-galactosidase using lactose as the substrate, including the effect of transgalactosylase activity on the overall rate. This seemed especially important since it has been shown that part of the action of β-galactosidase on lactose is the transgalactosylic formation of allolactose [galactosyl-β-D-(1 → 6)-glucopyranose] (Burstein et al., 1965; Jobe and Bourgeois, 1972) and that the allolactose so produced, rather than lactose itself, is the "natural inducer" of the *E. coli lac* operon (Müller-Hill et al., 1964; Jobe and Bourgeois, 1972). The present report describes the quantities and rates at which the different products of the β-galactosidase reaction are formed and broken down over a wide range of incubation conditions. A mechanism by which allolactose can be produced from lactose is also discussed.

Experimental Section

Enzyme. Highly purified β-galactosidase from *E. coli* K-12 was prepared by methods described by Wallenfels et al. (1959a). It was stored in ammonium sulfate at 50% saturation in the crystal form (long storage) or in solution in M/16 phosphate buffer (pH 6.8) (short storage). Only enzyme of high specific activity was used.

Enzyme free of monovalent or bivalent metal ions was prepared as reported previously (Wallenfels and Weil, 1972) and all ionic studies were carried out in carefully washed quartz containers.

Incubation Conditions. The standard incubation mixture consisted of enzyme, substrate, 0.01 M NaCl, and 0.0067 M $MgSO_4$ in 0.04 M imidazole hydrochloride buffer (pH 7.2). The normal enzyme concentration was about 130 μg/ml but this was varied depending on the requirements of the experi-

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[1] Abbreviations used: ONPG, *o*-nitrophenyl-β-D-galactopyranoside; DMF, dimethylformamide.

ment. The incubation temperature was usually 30 °C but some studies (in which it was important to preserve the anomeric configuration as intact as possible) were done at 0 °C (in an ice bath). Temperature did not affect the ratios of products formed but of course much more enzyme was required to obtain a measurable reaction rate at 0 °C (about 1 mg/ml).

The amounts of products formed at very early times were determined with a "quenched flow system". Two Hamilton syringes were joined by thin-diameter (0.15 mm) Tygon tubing leading to a mixing chamber. A delivery tube led from the mixing chamber to the quenching solution. Enzyme was in one syringe and substrate in the other. The quenching solution was the silylating mixture required for volatilizing the sugars for gas liquid chromatography (GLC) analysis (see below) and this solution was vigorously stirred to promote rapid quenching. The reaction times were varied by changing the length of the delivery tube and by varying the rate at which the plungers of the syringe were pressed. Readings as early as 200 ms (at an enzyme concentration of 2 mg/ml and an initial lactose concentration of 0.25 M) could be quantitated.

Analyses of Sugars. The reactants and products of the β-galactosidase reaction were identified and quantitated by gas liquid chromatography (GLC), by gel molecular filtration, and by enzyme-linked spectrophotometric assays.

For GLC, samples (10 μl) of the incubation mixture were withdrawn at various time intervals and added to a tube containing 50 μl of dimethylformamide (DMF). This mixture was immediately frozen in liquid nitrogen and a solution consisting of 500 μl of pyridine, 100 μl of 1,1,1,3,3,3-hexamethyldisilazane, and 100 μl of trimethylchlorsilane was added. The content of the tube was thawed and allowed to react at room temperature for 1 h and then centrifuged at 8000*g* for 2 min. The addition of the aliquots from the enzymatic assay incubation to the DMF served to stop the enzymatic reaction, and the DMF plus the cold temperature preserved the anomeric conformation of the sugars (Bentley and Botlock, 1967). The GLC analyses were carried out on μl amounts of the silylated sugar mixture with a Hewlett-Packard (Series 7620A) GLC system. Columns of 0.4 cm diameter (1 m in length) were packed with SE-52 (3% on Chromosorb W, 80–100 mesh) and a helium flow rate of 30 ml/min was used. Temperature programs of 130–260 °C were employed. The quantitation and identification of the silylated sugars were accomplished by means of standards.

In some cases it was desirable to analyze the sugars in the reduced form, especially when the number of structural isomers present were being determined (e.g., for a series of possible trisaccharides the various possible anomers would make analysis very difficult unless the sugars were first reduced). To carry out the reduction, boiled incubation aliquots (1 ml in this case) were reduced overnight with 1 mg/ml of $NaBH_4$ and the borate salts were then converted to acids using a cation exchange column (IR-120 in the H^+ form). Methyl borates were formed by the addition of methanol and these were removed with a rotary evaporator. Large-scale production of lactitol from lactose was also accomplished using this method.

Gel molecular filtration was used to detect oligosaccharide formation (disaccharides and trisaccharides could be detected by the GLC system which was used but higher oligosaccharides could not). β-Galactosidase incubation mixtures which had been placed in boiling water for 2 min to destroy enzyme action were applied to a 3 × 200 cm glass column packed with Bio-Gel P-2 (200–400 mesh). A flow rate of 23 ml/h of quartz-distilled degassed water was used and the eluent reservoir was kept at 55 °C to prevent the uptake of gases in the reservoir. The elution of the sugars was followed with a Winopal Forschung differential refractometer.

When very small quantities of glucose and galactose were to be measured (for initial velocity data), coupled enzyme assays were used. Glucose was measured by the addition of a large excess of hexokinase (10 μg/2.5 ml) and glucose-6-phosphate dehydrogenase (20 μg/2.5 ml) to the incubation mixture described above, which contained 0.167 M ATP and 0.1 M $NADP^+$ in addition to the other components. Galactose was measured with excess galactose dehydrogenase (10 μg/2.5 ml) in the presence of 0.1 M NAD^+, also in addition to the other components mentioned earlier. The production of NADH and NADPH was quantitated with a recording spectrophotometer at 366 nm. In these cases very small amounts of β-galactosidase were added for assay (0.1–0.5 μg/2.5 ml).

The relative amounts of galactose and glucose in trisaccharides and tetrasaccharides were determined by the addition of large quantities of β-galactosidase (1 mg/ml) to incubation mixtures containing these oligosaccharides. The incubations were allowed to proceed at 30 °C for 24 h and the solutions were then analyzed for contents of galactose and glucose by GLC.

The chemical structure of allolactose was verified by paper chromatographic analysis after methylation and hydrolysis by the method of Björndal et al. (1970).

Units of activity were defined as micromoles of product produced per minute at 30 °C with the above incubation conditions.

Results

Products of the β-Galactosidase Reaction. Figure 1 is a reaction substrate and product profile of the reaction of β-galactosidase (130 μg/ml) with 0.5 M lactose. At this high lactose concentration galactose, glucose, allolactose, and oligosaccharides were formed (oligosaccharides will mean trisaccharides or higher saccharides when used throughout). More glucose was formed than galactose and the difference was due to the fact that oligosaccharides were being synthesized. As time progressed the oligosaccharides and allolactose hydrolyzed and the glucose and galactose values eventually became equal. The type of transgalactosylase activity, where the initial aglycone moiety (glucose) is lost before another acceptor (in this case lactose or, as shall be seen later, allolactose) is used to form trisaccharides and eventually tetrasaccharides, shall be referred to as "indirect" transgalactosylase activity.

When 0.05 and 0.01 M lactose were used, identical amounts of glucose and galactose were formed throughout the time period involved and allolactose was formed in significant amounts. Again the allolactose was hydrolyzed as time progressed. If oligosaccharides were formed at these lower lactose concentrations, they could not be detected by GLC or gel filtration.

Allolactose was produced at all of the concentrations of lactose tried. Allolactose production shall be referred to as "direct" transgalactosylase activity, because, as shall be shown later, the main method of its production, initially, is direct transfer of galactosyl moieties from the 4 to 6 position of the glucose of lactose without prior release of glucose. In addition to the production of allolactose from lactose, analysis showed that other disaccharides were also produced in small amounts (<3% of the allolactose production) by the action of β-galactosidase on lactose. This was not unexpected as it was shown by Boos et al. (1968) that the synthesis of small amounts of disaccharides with $\beta(1 \rightarrow 3)$, $\beta(1 \rightarrow 2)$, and $\beta(1 \rightarrow 1)$ linkages

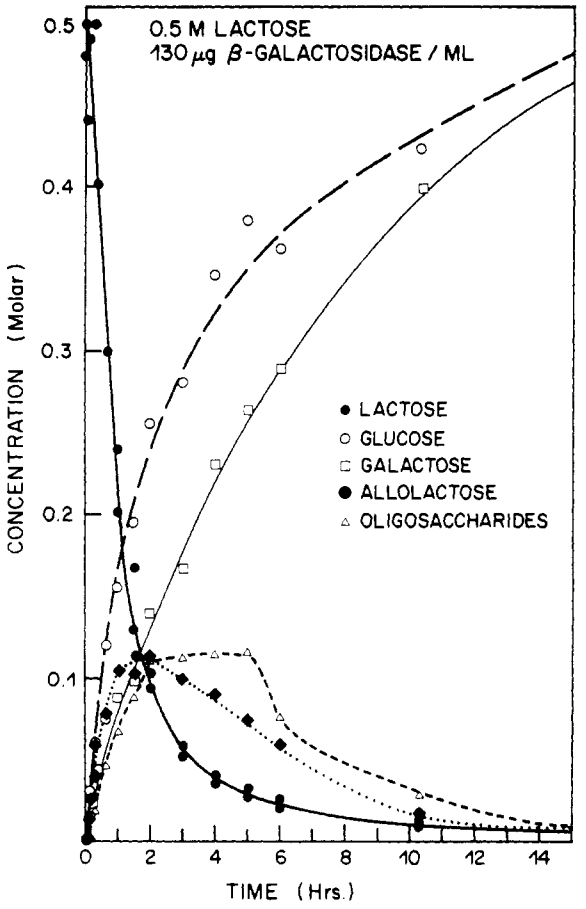


FIGURE 1: Profiles of the rate of utilization of lactose and formation of products with time at 0.5 M lactose. Reactants and products were detected by GLC and the temperature of reaction was 30 °C. The standard incubation mixture was used. Oligosaccharides refer to trisaccharides and tetrasaccharides.

between galactose and glucose could be catalyzed by β-galactosidase.

When the concentration of lactose was 0.5 M, the oligosaccharides produced were trisaccharides and tetrasaccharides as shown by elution from the P-2 column. Penta or higher oligosaccharides were not detected. Ratios of galactose to glucose in the trisaccharides and tetrasaccharide(s) were 1.8:1 and 2.9:1 (galactose:glucose) respectively.

A GLC analysis of the trisaccharides (reduced to eliminate anomeric isomers) present after the action of β-galactosidase (130 μg/ml) on 0.5 M lactose for 0.5 h showed that there were at least five and possibly six main forms of trisaccharide present. Trace amounts of others were also formed. The two trisaccharides which eluted earliest were present in largest amounts. These first two trisaccharides formed rapidly initially and then their rate of production rapidly fell off (by 20 min, the rate of their production was very slow). The other trisaccharides were formed at very slow initial rates followed by rapid rate increases (beginning at about 10 min and continuing to beyond the 30-min time of the experiment). A reasonable explanation for this is that the formation of the trisaccharides which eluted early takes place using lactose as the acceptor while the other trisaccharides were formed using allolactose as the acceptor. This is the logical conclusion since at early times in the reaction only lactose is present while at later times significant concentrations of allolactose accumulate (cf. Figure 1). The allolactose molecules could act as acceptors for the galactosyl moiety being transferred. This would account for the lag before the later trisaccharides are formed. The later peaks did not increase significantly compared with the early peaks and this is further evidence for this idea since the highest allolactose concentration is significantly smaller than the highest concentrations of lactose (see Figure 1).

The fact that allolactose is the acceptor substrate for the formation of some of the oligosaccharides is supported by the appearance of lactose and allolactose as early intermediate breakdown products when β-galactosidase was used to break down trisaccharides isolated by the P-2 column (after reaction to form them by β-galactosidase). No lag in the formation of allolactose was noted and so some, if not most of the allolactose, must have been produced from trisaccharide breakdown rather than from conversion of the lactose.

There are two ways in which allolactose can be generated from trisaccharides by β-galactosidase. The first possibility would occur if the trisaccharide being acted upon by β-galactosidase has the galactose attached by a glycosidic linkage at any hydroxyl position of either the galactose or glucose moiety of allolactose. If this galactose is then removed by the hydrolytic action of β-galactosidase, it will of course regenerate allolactose. Any of the later peaks could be examples of this possibility. (The rate variation with time above indicated that the later peaks are formed from allolactose.) Secondly, if one of the trisaccharides is made up of a galactose attached at the 6-glucose position of lactose, hydrolysis of the galactose present at the 4-glucose position on this trisaccharide would generate allolactose. Either of the first two peaks could be examples of this second possibility. Further studies must be carried out to determine whether one of these two early peaks are examples of the second possibility in order to establish whether or not this possible method of allolactose formation occurs and contributes significantly to total allolactose production.

A final piece of evidence supporting the fact that the early peaks are formed from lactose while the later ones are from allolactose is that, when 0.5 M allolactose was added to 0.05 M lactose with the normal incubation conditions with β-galactosidase, 95% of the trisaccharide product appeared as the later peaks.

Relative Initial Rates of Formation of Various Products. It can be seen from the profile above (Figure 1) and in other studies (Huber et al., 1975) that allolactose and the oligosaccharides are not only formed in significant quantities by the action of β-galactosidase but that the enzyme also hydrolyzes these compounds. To determine accurately the proportion of each product produced by β-galactosidase from lactose, it is, therefore, necessary to obtain measurements of the products at very early times (i.e., before hydrolysis of allolactose and the oligosaccharides has begun). It would also be advantageous to use as little enzyme as possible so that there is very little chance of hydrolysis of even the small amounts of allolactose and the oligosaccharides produced. These conditions are easily achieved for the measurement of the rates of galactose and glucose produced by the use of the very sensitive enzyme-coupled spectroscopic assays for glucose and galactose. Very little enzyme is required (0.1–0.5 μg/2.5 ml). Trisaccharide and tetrasaccharide titer is also simple since it can be determined by the excess of glucose over galactose formed. In the case of allolactose, however, the only feasible and relatively easy analytical technique to use is GLC, and the GLC analysis of allolactose lacks the high sensitivity of the spectrophotometric assays of glucose and galactose. Relatively accurate determinations of initial allolactose formation can be made, however, if high concentrations of lactose are used (so that lactose would tend to inhibit allolactose hydrolysis) and if the

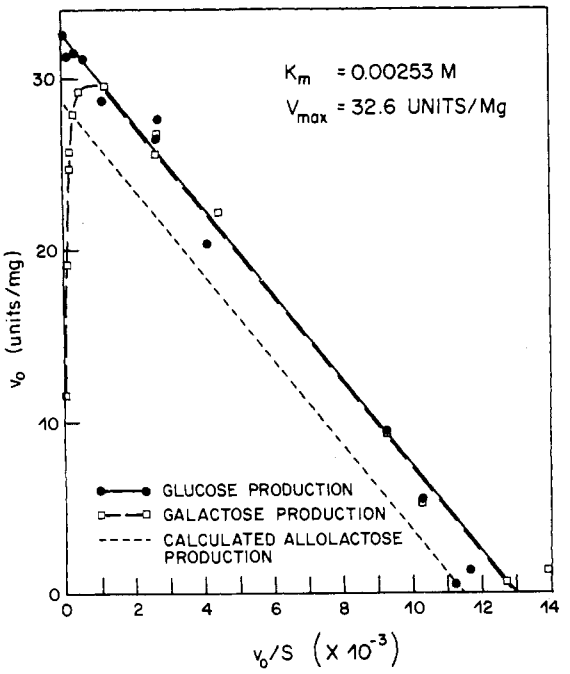


FIGURE 2: Hofstee (1959) plot of the initial rates of production of glucose and galactose by the enzyme. Glucose and galactose were detected by spectroscopic assays as presented in the text. A calculated line for allolactose production, based upon the fact that allolactose production is independent of initial lactose concentration, is shown.

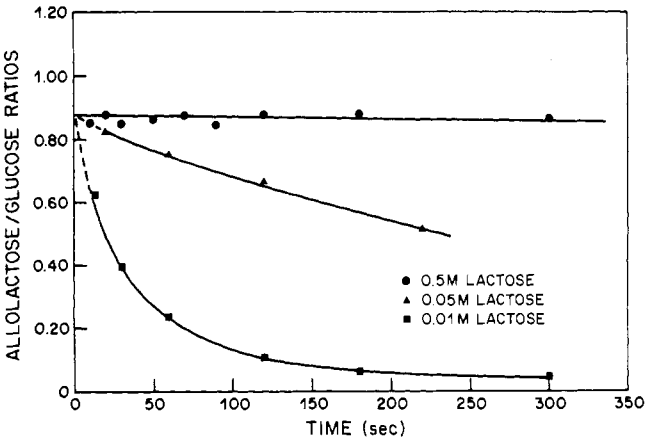


FIGURE 3: The ratio of allolactose to glucose production as a function of time at three lactose concentrations and 3 μg/ml of β-galactosidase. Allolactose and glucose were analyzed by GLC.

ratios of allolactose to glucose formation obtained are extrapolated back to zero time at as low concentrations of β-galactosidase as possible.

Figure 2 is a plot of the initial velocities of the glucose and galactose produced (enzyme-linked spectroscopic assays) from lactose plotted according to the method of Hofstee (1959). Note that at low lactose concentrations the initial velocities of the production of both glucose and galactose are similar. This is expected when oligosaccharides are not formed. When the lactose concentration is over 0.05 M, however, the rate at which galactose is produced drops dramatically. At 0.5 M lactose the initial galactose production rate relative to the initial rate of glucose production is less than one-half. The difference between glucose and galactose produced can be attributed to trisaccharide and tetrasaccharide production. Since these oligosaccharides are not produced until the lactose concentration is greater than 0.05 M, the binding of lactose as an acceptor substrate must not occur readily until more than 0.05 M lactose is present (since these are initial velocities, the use of allolactose as an acceptor would not be significant). The rate of glucose production results in a rectilinear response even above this point, indicating that the step(s) leading to glucose production are rate limiting. This shall be discussed in more detail later. The K_m and V_{max} values from the glucose line are 0.00253 M and 32.6 units/mg, respectively. Values for K_m and V_{max} for glucose production have been published previously and the values we obtained compare favorably with values in the literature (Wallenfels and Weil, 1972) if differences in assay temperatures and pH are taken into account. The point at which galactose production dips and trisaccharide production begins has not previously been determined.

Figure 3 is a graph of the ratio of the amount of allolactose formed relative to the amount of glucose produced (both determined by GLC) as a function of time at three concentrations of lactose and at a low enzyme level (3 μg/ml). At all concentrations the value extrapolated at zero time is between 0.8 and 0.9, indicating that the ratio of allolactose to glucose produced is independent of lactose concentration. The value at 0.5 M lactose should be the most accurate since the high level of lactose relative to the low amount of enzyme (3 μg/ml) present would mean that the error occurring as a result of allolactose hydrolysis would be small even if not extrapolated to zero time since it is reasonable to assume that high lactose would tend to competitively inhibit the hydrolysis of allolactose. That this is actually the case is seen by the horizontal line obtained for the ratio at this lactose concentration. The extrapolated value at 0.5 M lactose and zero time is 0.88 or, in other words, about 47% of all the lactose molecules present in an anomeric equilibrium mixture are converted to allolactose ("direct" transgalactosylase activity), while the other 53% are converted either to the glucose and galactose hydrolytic products or to glucose and the oligosaccharides ("indirect" transgalactosylase activity), depending on the initial lactose concentration. This is the first report quantitating the amounts of allolactose and oligosaccharides formed by the action of β-galactosidase on lactose and indicates that previous values for V_{max} of β-galactosidase with lactose are somewhat misleading since 47% of the activity of β-galactosidase on lactose is unaccounted for by simply measuring glucose or galactose released. Since the ratio of allolactose to glucose produced is independent of lactose concentration and since a value of 0.88 is obtained for the ratio of allolactose to glucose, a calculated line of allolactose produced can be determined and such a line is shown on Figure 2. A V_{max} of 28.7 units/mg for the amount of allolactose formed is calculated from this line. Addition of the two V_{max} values gives a total V_{max} value of 61.3 units/mg of lactose utilized.

Since allolactose forms in a fixed ratio to glucose at extrapolated zero times, its initial formation must be independent of the prior formation of free glucose or of trisaccharides as also shown by Jobe and Bourgeois (1972). It may, however, be argued that the time span is too large to be extrapolated back to zero time accurately and that at very early times much different results would obtain. Studies at very early times were, therefore, carried out.

Rapid Reaction Studies. The results of the rapid analysis of reaction products of β-galactosidase in the presence of 0.25 M lactose by the quenched flow technique indicated that even at very short times (as low as 200 ms) the formation of all products has begun and their production is essentially linear throughout (except for the production of trisaccharide which levels out—possibly because tetrasaccharides are being

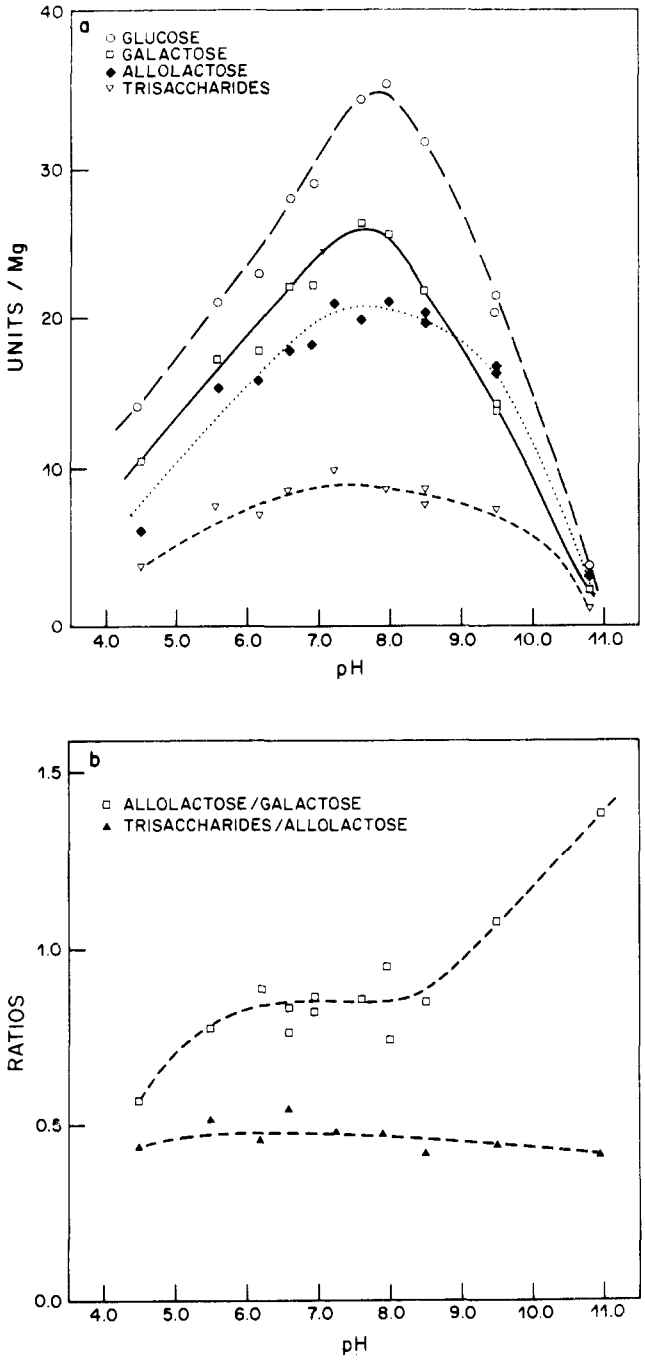


FIGURE 4: (a) Rate of production of various products as a function of pH (0.5 M lactose); (b) ratios of allolactose/galactose production and trisaccharide/allolactose production as a function of pH. Products were analyzed by GLC.

formed). There were no indications of lags in any of the lines. This implies that each of the reactions occurring is independent of the others at very early times and that none of the products are precursors of any of the others. An initial formation of free glucose and/or galactose does not seem to be necessary for the formation of allolactose. The formation of allolactose is also not a necessary early step for the formation of glucose and galactose as they are also formed independently. In addition to this the formation of trisaccharides and tetrasaccharides is not required for allolactose production. Initially, therefore, allolactose production occurs by "direct" transgalactosylase activity. This form of allolactose production has been reported previously (Jobe and Bourgeois, 1972) but was not substantiated for this short a time span.

Hexokinase and Glycerol Addition Studies. In order to confirm further that the major initial mechanism for the formation of allolactose from lactose was by direct transgalactosylase activity without prior release of glucose from the enzyme, as indicated by the zero time allolactose/glucose ratios and by the rapid reaction studies, work was carried out in the presence of hexokinase and of glycerol to eliminate completely the possibilities of allolactose forming from free glucose or from trisaccharides. The work showed, however, that free glucose, at higher concentrations, can substantially contribute to the production of allolactose. The experiments were carried out in 0.05 M lactose, a concentration at which trisaccharide and tetrasaccharide formation was not detected. Relatively low amounts of enzyme (13 μg/ml, only $^1/_{10}$ of that used in Figure 1) were used so that the reaction proceeded slowly and hexokinase and glycerol would be sure to act. The results are compared under four conditions: (1) control; (2) addition of 400 μg of hexokinase/ml and 0.167 M ATP to the incubation mix in order to convert any free glucose formed to glucose 6-phosphate and thus render the free glucose unavailable for allolactose formation; (3) addition of 0.5 M glycerol to eliminate the slight possibility that small amounts of trisaccharides and tetrasaccharides would form as precursors (no dissociation of β-galactosidase to subunits was detected at this concentration of glycerol and the pH and buffer conditions used); (4) addition of both hexokinase and glycerol.

At short times (up to 20 min) the elimination of the possibility of free glucose acting as an acceptor and/or the elimination of trisaccharide and tetrasaccharide formation had no effect on the amount of allolactose produced. This is consistent with the conclusions drawn from both the zero time allolactose/glucose ratios and the rapid reaction experiments; that is, the initial formations of all products are independent of each other. At later times in the study (>20 min) there were, however, significant decreases in the amounts of allolactose formed relative to the control both in the hexokinase and the glycerol experiments (up to 45% decrease). This can be accounted for by the fact that free glucose is either not available for allolactose formation (hexokinase experiments) or is competitively inhibited from reacting as an acceptor molecule by the presence of a high concentration of glycerol (glycerol experiments). It thus seems that free glucose is significant as an acceptor in the formation of allolactose when it is present in high enough amounts, although at initial times, the major reaction is a direct transgalactosylis of lactose to allolactose. The data suggested that, when the free glucose concentration was greater than 0.01 M, it began to act as an acceptor.

Transgalactosylase Activity with Glucose as Acceptor. Studies were carried out adding 0.5 M glucose to a spectrum of lactose concentrations in the presence of 130 μg/ml of β-galactosidase under the standard reaction conditions. At low initial lactose concentrations there was a significant increase in allolactose production over the case when no glucose was present (up to 100% increase depending on the initial lactose concentration). At high initial lactose concentrations (>0.10 M), the allolactose production did not increase over normal. This indicates that free glucose can substitute for bound glucose until the concentration of bound glucose (from lactose which is being acted on by β-galactosidase) becomes high. It is thus conceivable that the formation of allolactose using free glucose as an acceptor may be a physiological process.

pH Effects of the Reactions of β-Galactosidase on Lactose. Figure 4a shows the rates at which the various products of β-galactosidase action on lactose are produced at different pH

values. (Similar results were obtained when piperazine hydrochloride was substituted as the buffer in place of imidazole hydrochloride.) Each of the products was formed optimally between pH 7.7 and 7.9. The curves representing transgalactosylase activity (allolactose and trisaccharide formation) are, however, significantly skewed toward higher pH values as compared with the curves for galactose and glucose production. This is more readily noted in Figure 4b where the ratios of allolactose to galactose and of allolactose to trisaccharides are plotted. If the allolactose produced represents "direct" transgalactosylase activity, galactose production represents hydrolysis, and trisaccharide production represents "indirect" transgalactosylase activity; it can be noted that there is little change in the ratio of "direct" to "indirect" transgalactosylase activity with pH whereas the ratio of "direct" or "indirect" transgalactosylase to hydrolase activity changes somewhat at low pH values and dramatically at high pH values. At intermediate pH values the ratios are constant with pH. This indicates that, although there is not a fundamental difference between "direct" and "indirect" transgalactosylase activity with respect to pH, there is a large difference when both types of transgalactosylase activities are compared with hydrolysis at various pH values. The changes at the high pH values are interesting since they begin at the point where the hydrolytic activity of the enzyme begins to drop (at about pH 7.8). This is not the case for the drop in the ratio at low pH values since that decrease begins at the point of acid denaturation of the protein (Wallenfels et al., 1959a) which is at a pH significantly lower than the point at which activity begins to decrease. The profiles for the production of glucose and galactose from both lactose and allolactose are almost the same (Huber et al., 1975). The increase in the allolactose/galactose ratio at the high pH values must, therefore, represent an increase in the formation rate of allolactose relative to lactose hydrolysis rather than a decrease in the breakdown rate of allolactose. These studies must be pursued further but they suggest that at high pH values there are groups on the enzyme which are involved in either activating or binding the acceptor molecules which cause the saccharide acceptors to be relatively more active than H_2O. Preliminary studies using ONPG as the β-galactosidase substrate and glycerol or glucose as the acceptor, compared with water, give results which are very analogous to the above at the high pH values.

Effects of Metals. The monovalent cations K^+ and Na^+ did not seem to affect the capacity of β-galactosidase to form allolactose as compared with the hydrolytic products (ratios of allolactose/glucose production remained at about 0.88 no matter which of these two ions was present and also in the absence of all but traces of these ions) but the absence of all but traces of the Mg^{2+} dramatically affected the ratio of the products produced (allolactose/glucose ratio decreased from 0.88 to 0.29). Further studies will be required to elucidate the precise action of Mg^{2+} in this system. Preliminary studies with ONPG and glycerol (or glucose) as acceptor gave results similar to the above in the absence of Mg^{2+}.

Effects of Anomeric Conformation. As the proportion of β-lactose in the β-galactosidase incubation decreased relative to the level of α-lactose, the overall reaction rate increased (that is the rate of decrease of lactose concentration). In addition to this, it is noted that the steady-state level of allolactose increased as the β-lactose proportion increased. In Figure 5 it is seen that the extrapolated allolactose/glucose ratio at zero time with 100% α-lactose is 0.25. This ratio increased with increasing β-lactose concentration to a high ratio (well over 2.0) at 79% β-lactose. Thus the anomeric conformation is important

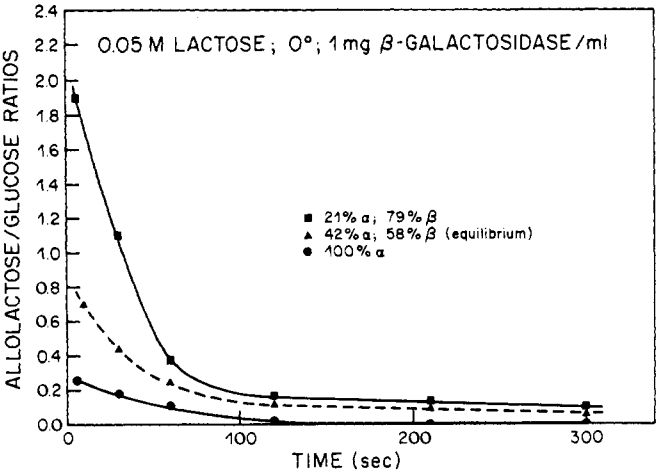


FIGURE 5: The allolactose/glucose production ratios at the three anomeric compositions as a function of time (0 °C).

in directing the catalytic action of the enzyme toward either hydrolase or transgalactosylase activity. High proportions of β-lactose result in greater "direct" transgalactosylase activity (production of allolactose).

α-Lactose was converted to only α-allolactose and α-glucose while β-lactose seemed to be converted to only β-allolactose and β-glucose. The catalysis, therefore, does not result in a change at the anomeric lactose carbon.

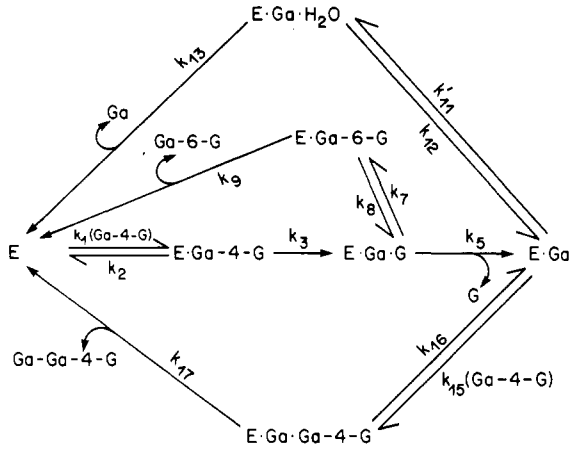
These studies were done at 0 °C. It should be noted that mutarotation is very slow at 0 °C and did not interfere significantly with the above results.

When lactitol (reduced lactose) was the substrate (0.5 M), the reaction went very slowly with no detectable formation of allolactitol.

Discussion

The main products of the action of β-galactosidase (*E. coli*) on lactose are glucose, galactose, and allolactose at low initial concentrations of lactose. At higher initial levels at least five different trisaccharides and some tetrasaccharides are formed in addition to the above products. Although it has been known for some time (Wallenfels and Weil, 1972) that trisaccharides are formed by the action of β-galactosidase on lactose, this is the first report of tetrasaccharides being formed and it is the first report quantitating the number of trisaccharides formed and showing the determination of their sugar composition. Oligosaccharides larger than tetrasaccharides were not detected at the concentrations and times used. At lactose concentrations less than 0.05 M, oligosaccharides are not formed in detectable amounts while with increasing concentrations above 0.05 M the amount formed increased significantly (see Figure 2). Studies quantitating the substrate concentration at which oligosaccharides begin to form have not previously been reported. It is difficult to speculate on whether or not this oligosaccharide formation is a physiologically important process in *E. coli* since the physiological concentration of lactose in the vicinity of the enzyme is really not known.

The initial proportion of allolactose formed in comparison to glucose was independent of the concentration of lactose. At an equilibrium anomeric composition of lactose about 47% of the initial reaction product was allolactose ("direct" transgalactosylic product) while 53% was either hydrolytic product or "indirect" transgalactosylic product (depending on the initial lactose concentration). This is the first study in which the proportion of allolactose produced from lactose by the



Ga-4-G	≡LACTOSE
Ga-6-G	≡ALLOLACTOSE
Ga	≡GALACTOSE
G	≡GLUCOSE
Ga-Ga-4-G	≡TRISACCHARIDE
E·	≡ENZYME COMPLEX
k'_{11}	$\equiv k_{11}(H_2O)$

FIGURE 6: Postulated mechanism for the action of β-galactosidase on lactose.

β-galactosidase (*E. coli*) reaction has been quantitated.

A probable mechanism for the initial reaction of β-galactosidase with lactose which takes into account the above findings is shown in Figure 6. The mechanism is for initial velocities without added glucose. In the presence of glucose, the step where glucose is released (k_5) would be reversible. The mechanism fits the data very well if certain assumptions regarding the rate-determining step (or steps) are made. There are several possible rate-determining conditions which, when the appropriate k_{cat} and K_m values are derived, result in good fits. The most likely is that all of the central steps (i.e. k_3, k_5, k_7, and k_8) on Figure 6 are of roughly equal magnitude and are rate determining. For this case the k_{cat} and K_m values are as follows.

$$k_{cat(glucose)} = \frac{k_3 k_5}{k_3 + k_5 + k_7}$$

$$k_{cat(allolactose)} = \frac{k_3 k_7}{k_3 + k_5 + k_7}$$

$$k_{cat(galactose)} = \frac{k_3 k_5 k_{11}' k_{13}(k_{16} + k_{17})}{(k_3 + k_5 + k_7)[k_{11}' k_{13}(k_{16} + k_{17}) + k_{15}(S)k_{17}(k_{12} + k_{13})]}$$

$$k_{cat(trisacch)} = \frac{k_3 k_5 k_{15}(S) k_{17}(k_{12} + k_{13})}{(k_3 + k_5 + k_7)[k_{11}' k_{13}(k_{16} + k_{17}) + k_{15}(S)k_{17}(k_{12} + k_{13})]}$$

$$K_m = \frac{k_2}{k_1} \frac{k_5 + k_7}{k_3 + k_5 + k_7}$$

(k_{cat} expressions which contain concentrations of substrates are not in fact constants).

These equations describe the reaction and the data fit the expected curves. Since $k_{cat(glucose)}$ and $k_{cat(allolactose)}$ are constants in both cases, they should give rectilinear responses when plotted by the Hofstee (1959) method. Figure 2 confirms this. In addition the equations predict that the value of $k_{cat(galactose)}$ is equal to $k_{cat(glucose)}$ at low concentrations of substrate but tends to approach zero as high substrate values are reached. This corresponds precisely with the data as seen in Figure 2 where the rate of galactose production is equal to the rate of glucose production at low substrate concentration but then falls off rapidly as the lactose concentration is increased. The value $k_{cat(trisacch)}$ would be expected to be very low at low lactose concentrations and then increase rapidly with lactose concentration, reaching a limiting value of $k_{cat(glucose)}$ at very high substrate concentrations (note that there is a hyperbolic relationship). Indeed, Figure 2 shows that galactose production is approaching zero at high substrate concentrations and the difference between glucose and galactose production rates is, therefore, approaching the V_{max} for glucose. This difference is the trisaccharide (and tetrasaccharide) production rate.

It is possible that, in addition to the above step, k_{13} and k_{17} may also be rate determining. This would result in the same type of equations (not the same ones) and the conclusions about k_7 and k_5 (below) would also be the same.

An interesting aspect of the equations above is that the proportion of allolactose compared with glucose formed depends on the values of k_5 and k_7 relative to each other. If k_7 is greater than k_5, more allolactose than glucose is formed. If k_5 is greater than k_7, more glucose than allolactose is formed. From the relative proportions of allolactose and glucose formed, the value of k_7 must be 0.88 times the value of k_5 at pH 7.6, with Mg^{2+} and with an equilibrium mixture of α- and β-lactose.

Although we showed that at very early initial times (200 ms) allolactose is formed directly on the enzyme ("direct" transfer) without previous release of either free glucose or trisaccharide, it was also noted that allolactose can be formed in significant amounts using free glucose as the acceptor and can be formed from trisaccharide hydrolysis. The only initial mechanism of allolactose formation in the absence of added glucose or other acceptor is "direct" transgalactosylis but other mechanisms are possible at later times after trisaccharides are formed or if free glucose is added in large amounts in the presence of low concentrations of lactose.

There are several factors which dramatically affect the proportion of the various products of the β-galactosidase reaction on lactose. The pH of the incubation mixture is very important in terms of the proportion of hydrolysis and of transgalactosylase activity taking place. The effect is most dramatic at high pH values where the ratio of transgalactosylase (direct or indirect) to hydrolase activity increases between pH 7.8 and 10 and this increase begins at the point at which the decrease of the bell-shaped optimum curve for glucose and galactose on the high pH side begins. It is not clear at the moment whether binding of acceptor (glucose or lactose relative to water), activation of acceptor to form the final product, or both are involved. In addition to the pH effect it was also found that at very low Mg^{2+} concentration the ratio of allolactose/glucose is low. Thus Mg^{2+} activation of β-galactosidase (Reithel and Kim, 1960; Tenu et al., 1972) seems to be related in some way to the hydrolysis and transgalactosylase mechanisms. Again it is not clear how Mg^{2+} acts but it must affect either the binding of the acceptor (glucose or lactose as compared with water) or its activation to form final product. Shifrin and Hunn (1969) also observed a lowering of transgalactosylase relative to hydrolase activity in the absence of Mg^{2+} for β-galactosidase from *E. coli*. It is interesting that pH and Mg^{2+} affect the transferolysis/hydrolysis ratio since at least two studies have previously implicated an interaction of their effects on the catalytic mechanism of β-galactosidase

(Reithel and Kim, 1960; Tenu et al., 1971).

The anomeric conformation of lactose is another factor which seems to direct the type of product formed. If the conformation is α, the glucose, once severed from the galactose prefers to leave the enzyme surface rather than be converted to allolactose (k_5 may have a relatively greater value than k_7). If the conformation is β there is a distinct preference for allolactose formation. Either the β conformer directs the glucose into the proper juxtaposition with the enzyme for allolactose formation (k_7 may change for β compared with α and be relatively greater than k_5) or the β-glucose remains bound somewhat longer than the α (k_5 may be relatively smaller for β as compared with α than k_7) and thus a larger proportion will have a chance to become allolactose simply because there is more time to act. (It is also, of course, possible that both k_7 and k_5 are changing in opposite directions.) It is not clear whether this effect of anomeric conformation has any biological significance. It is obviously possible that the transport process of *E. coli* for lactose is specific for one or the other anomer or that only one anomer of allolactose can bring about induction of the *lac* operon but data of this sort are not yet available. When the glucose moiety of lactose was reduced to glucitol, the enzyme acted only slowly and detectable levels of allolactitol were not formed. The active site of β-galactosidase is not, therefore, well equipped to handle a long straight chain aglycone moiety which does not have an α–β structure and "direct" transgalactosylase activity seems to be ruled out in the case of the reduced lactose.

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