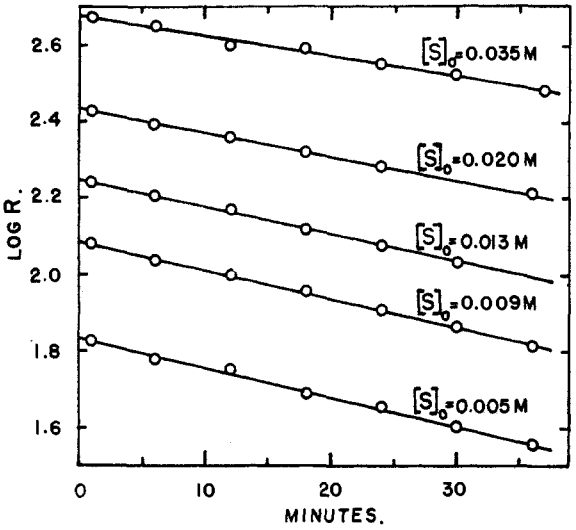

Fig. 10.—Estimation of initial velocities from apparent first-order plots; $[S]_0$ in units of 10^{-3} M acetyl-L-tyrosinhydroxamide; [E] = 0.0294 mg. protein-nitrogen/ml.; R = calorimeter reading; 0.3 M tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer at 25° and pH 7.6.

of the color determined with a Klett–Summerson photoelectric colorimeter equipped with a green filter (filter no. 54—transmission range *ca.* 500–570 mμ). A standard calibration curve was prepared from solutions containing varying amounts of acetyl-L-tyrosinhydroxamide in a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer 0.30 M in respect to the amine component. It was found that for concentrations of acetyl-L-tyrosinhydroxamide up to 0.050 M in the original solution the colorimeter readings were directly proportional to the concentration of the hydroxamide. The proportionality constant was found to be independent of the presence of α-chymotrypsin, acetyl-L-tyrosine, hydroxylamine, acetyl-D-tyrosine ethyl ester, acetyl-D-tyrosinamide and acetyl-D-tyrosinhydrazide, in amounts equal to or greater than the maximum amounts involved in the enzymatic reactions. The same proportionality constant was observed with acetyl-D-tyrosinhydroxamide, and this constant was found to be independent of the hydrogen ion concentration over the pH range from 6.5 to 9.1. The color formed, whether from the addition of aliquots from the enzyme reaction mixtures or from the solutions used for calibrations, was stable over a period of two hours after mixing. All readings were taken within this time interval and were reproducible to within ± 2%.

As the initial portion of the rate curves (20–30% hydolysis) were always observed to be first order, values of $\log_{10}R$ (R = colorimeter reading) were plotted against time (typical curves are given in Fig. 10) and the initial velocity at zero time determined from the apparent initial first-order rate constant and the initial substrate concentration. It should be pointed out that it is only necessary to know that the colorimeter readings are directly proportional to the acetyl-L-tyrosinhydroxamide concentration, since the value of the proportionality constant is not a factor in the determination of the apparent first-order constant.

Apparent Ionization Constants of Acetyl-L-tyrosinhydroxamide.—A 2.00-ml. aliquot of a 0.01002 M aqueous solution of acetyl-L-tyrosinhydroxamide was titrated with a 0.01075 N aqueous sodium hydroxide solution and the titration followed with a Beckman model G pH meter. The data so obtained was treated essentially as described by Simms[30] and from this treatment values of pK'_{A_1} and pK'_{A_2} of 9.0 and 10.2 were obtained. The calculated curve given in Fig. 2 is based upon these latter values. It is believed that the large deviation of the experimental points from this curve at pH values greater than 10.5 is due to the fact that a given error in the pH determination creates a larger and larger error in b' as the pH approaches a value of 12, and that at pH values greater than 10.5 the pH meter becomes inaccurate. A sodium ion correction was made according to the manufacturer's directions but this is clearly only an approximation for pH values above 10.5. Calculated curves based upon the use of pK'_A values that differed from those used for the construction of the curve given in Fig. 2 by ±0.1 of a pK' unit indicated that the pK'_A values given above, *i.e.*, 9.0 and 10.2, can be considered to be accurate to within ± 0.1 of a pK' unit. A duplicate experiment gave data that agreed with that given in Fig. 2 to within ± 0.03 of a pH unit and gave the same pK'_A values to within ± 0.1 of a pK' unit.

(30) H. Simms, THIS JOURNAL, **48**, 1239 (1926).

PASADENA 4, CALIFORNIA

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY AND THE INSTITUTE FOR ENZYME RESEARCH, UNIVERSITY OF WISCONSIN]

Purification and Kinetics of β-D-Galactosidase from *Escherichia coli*, Strain K-12[1,2]

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RECEIVED JULY 7, 1952

β-D-Galactosidase has been purified about 100-fold from an extract of dried *Escherichia coli* cells by fractionation with methanol, $MnSO_4$, $(NH_4)_2SO_4$ and $Ca_3(PO_4)_2$. The effect of pH, substrate concn., type of substrate, temp., competitive and non-competitive inhibitors, and ionic environment on the catalytic activity of the purified enzyme have been investigated.

β-D-Galactosidase, an adaptive enzyme of *Escherichia coli*, strain K-12, has been studied in some detail in the crude state by Lederberg.[4] Cohn and Monod[5] purified a similar enzyme from *E. coli*, strain ML, and investigated many of its properties, especially the effects of ions on the rate of hydrolysis of β-D-galactosides.

It is the purpose of this paper to report a method of purification of β-D-galactosidase from *E. coli*, strain K-12, and to describe some of the catalytic properties of this purified enzyme.

(1) Presented in part before the Division of Biological Chemistry at the 121st Meeting of the American Chemical Society, Milwaukee, Wis., March 30–April 3, 1952.

(2) Supported by grants from the United States Public Health Service and the University Research Committee.

(3) Predoctorate Fellow, National Institutes of Health.

(4) J. Lederberg, *J. Bact.*, **60**, 381 (1950).

(5) M. Cohn and J. Monod, *Biochim. Biophys. Acta*, **7**, 153 (1951).

Experimental

Analytical Methods.—The "chromogenic" substrate, *o*-nitrophenyl β-D-galactoside, introduced by Lederberg,[4] was used for following the purification of the enzyme.

Aliquots were withdrawn periodically from the reaction mixture and pipetted directly into a Na_2CO_3 solution (final molarity of Na_2CO_3, 0.1 M) to stop the enzymatic hydrolysis and to produce the maximum possible absorption due to the liberated *o*-nitrophenolate ion. *o*-Nitrophenol was determined colorimetrically using the Evelyn colorimeter with a 420 mμ filter. Although Beer's law is followed reasonably well over a fairly wide range of concentration, correc-

tions were made for the small deviation from Beer's law, thus permitting an accuracy of about 1%.

For the *p*-nitrophenyl derivative, *p*-nitrophenol was determined by the absorption at its maximum (400 mμ) in 0.1 *M* Na_2CO_3 using the Beckman DU spectrophotometer ($E^{M}_{1cm.}$ 1.84 × 10^4).

When non-chromogenic β-D-galactosides (except lactose) were used as substrates the liberated galactose was determined by the colorimetric method of Somogyi[6] and Nelson[7] with an accuracy of *ca.* 2–4%.

Lactose was determined by Leloir's[8] modification of the Barfoed test for monoses with *ca.* 10% error.

For protein determinations, the method of Johnson[9] for organic solids was used as an expression of the protein concentration; suitable precautions were taken with the method—*e.g.*, removal of MeOH first, dialysis of various fractions where interference might result, etc. Nitrogen determinations were correlated with the organic solids method where feasible.

Reagents.—*o*-Nitrophenyl-β-D-galactoside was synthesized according to the procedure of Seidman and Link.[10] Other substrates and compounds were kindly supplied as follows: methyl, *n*-butyl, phenyl and *p*-nitrophenyl-β-D-galactosides, *o*-nitrophenyl-α-L-arabinoside and *p*-nitrophenyl-β-D-glucoside by Prof. K. P. Link and Drs. M. Seidman, S. Roseman and J. Snyder; lactositol by Prof. M. L. Wolfrom; methyl-α-D-galactoside and methyl-α-D-manno-D-gala heptoside by Dr. H. G. Fletcher; lactobionate by Dr. E. Dimant. The nitrophenols were Eastman grade recrystallized several times before use. All other reagents were analytical grade (usually Merck or Mallinckrodt). Deionized water twice distilled through an all-Pyrex apparatus was used for all experiments except those involving ionic effects. For the latter, the water was redistilled in an all-quartz apparatus.

Purification of β-D-Galactosidase.—Because of the limited amount of source material available for isolation of the enzyme, and the very small amounts of protein dealt with, the fractionation scheme was so designed as to emphasize both yield and purification.

Escherichia coli, strain K-12, was grown on a lactose–inorganic salts medium,[4] in an 80-gallon steel fermentor with strong aeration and stirring. After 18 to 20 hours the cells were harvested by means of the Sharples centrifuge. The *fresh* cell paste was washed with a minimum amount of distilled water required to form a homogeneous suspension and centrifuged hard in the Spinco preparative ultracentrifuge, model L, head No. 20, at 20,000 r.p.m. for 0.5 hr., to remove the liquid. The sedimented cells were then spread thin over the bottom of petri dishes and dried in evacuated desiccators over P_2O_5 at room temperature, with repeated changes of P_2O_5. After *ca.* 2–3 days, the drying was practically complete, and the cell residue was ground to a fine powder and allowed to dry an additional day. When stored in vacuum desiccators over P_2O_5 in the cold room, the dry cell powder showed only small loss in β-D-galactosidase activity after several months.

Fraction I.—Twenty grams of dried cells and 50 ml. of 0.001 *M* sodium phosphate, *p*H 7.3, were ground in a large porcelain mortar to a homogeneous paste with a heavy porcelain pestle. The paste was then frozen (*ca.* −10°), in the mortar. After freezing, it was thawed somewhat (*ca.* 2°) and ground until the paste assumed the original consistency. The freezing and grinding procedure was repeated five times.

The mortar was then removed to room temperature; 75 ml. of the same buffer added to the mortar and the paste ground to a homogeneous suspension which was diluted to 500 ml. with the buffer. The contents were placed in a one-liter round-bottom flask, immersed in a 30° bath, and stirred (glass) mechanically for 3 hr.

The suspension was then centrifuged ("Spinco" model L, head No. 20) for 0.5 hr. at 15,000 r.p.m. and the supernatant (455 ml., fraction I) decanted. The residue was discarded. The *p*H of the supernatant should be around *p*H 6.0 (5.8–6.2); if not, it should be so adjusted with 0.5 *M* NaH_2PO_4.

Fraction II: Methanol Fractionation.—Fraction I (455 ml.) was transferred to a 2-liter, 3-neck round-bottom flask equipped with a glass stirrer, 250-ml. graduated dropping funnel and thermometer. With the contents at *ca.* 1°, about 1/6th volume of absolute methanol were introduced at *ca.* 40 ml. per min. with efficient mechanical stirring (foaming must be avoided). The flask was then immersed in a −10° bath and allowed to reach temperature equilibrium. Then abs. methanol was added at *ca.* 20 ml./min., with stirring. When a total of 372 ml. had been added, the contents of the flask were allowed to stand 0.5 hr. at −10° and centrifuged at *ca.* −10° in the International refrigerated centrifuge (cat. No. 834 head) at maximum speed for 20 min. The slightly *turbid* supernatant was decanted and retained and the small amount of precipitate discarded.

The supernatant was returned to the flask and allowed to reach −10° if any change in temperature had occurred. Then 386 ml. of methanol (abs.) was added as before. After allowing to stand 0.5 hr. the suspension was centrifuged as before and the *clear* yellow supernatant discarded. The precipitate was resuspended in 100 ml. of 0.001 *M* sodium phosphate, *p*H 7.3, and dialyzed[11] *vs.* 4 liters of the same buffer at *ca.* 2° for 24 hr. The initial suspension which contained some "insoluble" material gradually became clear, with the protein redissolving, until finally at the end of the dialysis the solution (fraction II) was essentially clear and yellow in color. The fractionation was conducted between the levels of 2.45 to 5 ml. of abs. methanol for every 3 ml. of fraction I.

Fraction III: Precipitation of Nucleoproteins.[12]—Fraction II (266 ml.) was made 0.0235 *M* with respect to $MnSO_4$ and allowed to stand at 2° for 38 hr. The precipitate which separated at 15,000 r.p.m. (Spinco, model L, No. 20 head) for 0.5 hr. was discarded; the clear supernatant solution (*ca.* 248 ml.) was retained as fraction III.

Fraction IV: Adsorption on $Ca_3(PO_4)_2$ and Elution.—These steps were conducted in a cold room at 2°. To 248 ml. of fraction III, 9.93 ml. of $Ca_3(PO_4)_2$ gel[13] (gel concn., 62.2 mg./ml.; therefore 0.532 mg. gel/mg. protein in fraction III) was quickly added, mixed and centrifuged immediately for *ca.* 4 min. Speed in this operation is essential; therefore this step was usually conducted in the 250-ml. centrifuge bottle. The supernatant was decanted (ppt. discarded) and 55.7 ml. of gel (2.91 mg. gel/mg. protein in fraction III) was added. After stirring for *ca.* 15 min. the suspension was centrifuged for 10 min. and the supernatant was discarded. The precipitate was washed with *ca.* 250 ml. of cold distilled H_2O and collected by centrifugation.

The enzyme was eluted from the gel with 62 ml. of a 25% saturated $(NH_4)_2SO_4$ solution.[14] After stirring for 15 min., the suspension was centrifuged. The eluate was saved and the precipitate re-extracted as above with another 62 ml. of 25% saturated $(NH_4)_2SO_4$. The eluates were combined (fraction IV).

Fraction V–VI: $(NH_4)_2SO_4$ Fractionation. Step 1.—The 122 ml. of IV was brought to 45% saturation by adding 44.3 ml. of saturated $(NH_4)_2SO_4$, *p*H 6.3, slowly with stirring. After allowing to stand in the cold room for 48 hr., the small amount of precipitate was centrifuged off at 15,000 r.p.m. (Spinco, head No. 20) for 30 min. The supernatant was retained for Step 3.

Step 2.—The ppt. was resuspended in 5 ml. of 25% saturated $(NH_4)_2SO_4$ and stirred for *ca.* 30 min. After centrifuging at 20,000 r.p.m. (Spinco, head No. 20) for 30 min. the supernatant was saved and the precipitate was re-extracted in the same manner. The supernatants from step 2 were combined (*ca.* 9.8 ml.—fraction V) and the insoluble residue discarded.

Step 3.—The supernatant from step 1 (*ca.* 160 ml.) was brought to 50% saturation by addition of 15.9 ml. of saturated $(NH_4)_2SO_4$, *p*H 6.3. After 36 hr. at 2° the material was centrifuged at 15,000 r.p.m. (Spinco No. 20 head) for

(6) M. Somogyi, *J. Biol. Chem.*, **160**, 61, 69 (1945).

(7) N. Nelson, *ibid.*, **153**, 375 (1944).

(8) R. Caputto, L. F. Leloir and R. E. Trucco, *Enzymologia*, **12**, 350 (1948).

(9) M. Johnson, *J. Biol. Chem.*, **181**, 707 (1949).

(10) M. Seidman and K. P. Link, THIS JOURNAL, **72**, 4324 (1950).

(11) Dialysis was conducted so that both the bag and the external fluid were stirred; and the external fluid changed *ca.* once every 8 hr.

(12) S. Kaufman, S. Korkes and A. del Campillo, *J. Biol. Chem.*, **192**, 301 (1951).

(13) The gel was prepared by a procedure based upon that of S. Swingle and A. Tiselius, *Biochem. J.*, **48**, 171 (1951).

(14) Obtained by dilution of saturated $(NH_4)_2SO_4$ (A.R.) solution which had been adjusted to *p*H 6.3 (Beckmann glass electrode) with NH_4OH.

TABLE I

FRACTIONATION OF β-D-GALACTOSIDASE

Fraction no.	Total volume, ml.	Units/ml.	Total units	Mg./ml.	Units/mg.	Per cent. recovery from preceding step	Purification over preceding step
I	455	1,635	744,000	16.2	101.0	..	..
II	266	2,285	608,000	8.17	279.5	82	3.61
III	248	2,195	545,000	4.68	469	89.8	1.68
IV	122	3,035	370,500	3.60	843	68.0	1.8
V	9.8	9,250	90,600	2.43	3810	24.5	4.52
V′	19.4	7,555	146,500	2.17	3488	39.6 (from IV)	4.14 (over IV)
V + V′	29.2	8,110	237,100	2.26	3590	64.0 (from IV)	4.26 (over IV)
VI	4.5	40,875	184,000	6.7	6100	77.6	1.70
VII	4.4	40,000	176,000	5.96	6715	95.7	1.10
VIII	3.9	30,000	117,000	3.75	8000	66.6	1.19
IX	2.4	35,350	84,250	3.82	9400	72.5	1.16
X	1.0	56,750	56,750	5.65	10,050	67.0	1.09
						Over-all % recovery from I, 7.63	Over-all purification over I. 99.5

30 min. and the supernatant discarded. The precipitate was eluted with two 10-ml. portions of 25% saturated $(NH_4)_2SO_4$ as described in step 2. The combined supernatants were designated V′.

Step 4.—Fractions V and V′ were combined (fraction V + V′, 29.2 ml.) and brought to 45% saturation by adding 10.6 ml. of saturated $(NH_4)_2SO_4$, *p*H 6.3. After 28 hr. at 2° the ppt. was collected at 20,000 r.p.m. (Spinco, No. 40 head) and was redissolved in 0.001 *M* sodium phosphate, *p*H 6.3 (fraction VI, *ca.* 4.5 ml.).

Fractions VII—X: Recycling.—Fraction VI was brought to 0.0235 *M* $MnSO_4$ and after 21 hr. at 2° the precipitate was collected by centrifugation at 20,000 r.p.m. (Spinco No. 40 head) for 30 min. and discarded. The clear solution (4.4 ml., fraction VII) was then treated with $Ca_3(PO_4)_2$ exactly as above, using the same levels of gel per mg. protein and eluted as before with two 2-ml. portions of 25% saturated $(NH_4)_2SO_4$. The combined eluates were designated fraction VIII (3.9 ml.). This solution was brought to 45% saturation by addition of 1.42 ml. of saturated $(NH_4)_2SO_4$ and allowed to stand at 2° for 24 hr. The precipitate which was collected at 20,000 r.p.m. (Spinco No. 40 head) for 30 min. was resuspended in 1.5 ml. of 25% saturated $(NH_4)_2SO_4$. The very small amount of insoluble residue was removed by centrifugation as above. The supernatant was retained[15] and the residue resuspended in 1.0 ml. of 25% saturated $(NH_4)_2SO_4$ and recentrifuged as above.

The combined supernatants (fraction IX, 2.4 ml.) were brought to 35% saturation by adding 0.37 ml. of saturated $(NH_4)_2SO_4$, *p*H 6.3. After 24 hr. at 2° the precipitate was collected by centrifugation at 20,000 r.p.m. (Spinco head No. 40) for 30 min. and redissolved in 0.082 *M* Na^+ phosphate buffer (*p*H 6.3) (fraction X, 1 ml.). It was then dialyzed against the same buffer at 2°. This preparation is stable for several months if stored just above 0°.

With some batches of cells, where the initial specific activity of I was unusually low the specific activity of all subsequent fractions was also low. In these cases X was brought to 33% saturation with $(NH_4)_2SO_4$. The precipitate contained about ⅔ of the total units and the specific activity was raised to the normal range.

Table I summarizes a typical fractionation in terms of percentage recovery and purification for each individual step in the scheme. An over-all purification (from I) of about 100-fold was achieved with about 8% recovery of the enzyme. The specific activity of fraction X varied from 7,000 to 10,500 units per mg. in various experiments. Further fractionation by the use of solvents (methanol or acetone), salts ($MgSO_4$, Na_2SO_4, NaH_2PO_4), or adsorbents (alumina C_γ, starch, Dowex 2 anion exchanger) have not yielded higher specific activities.

Fraction X shows one component by micro electrophoresis at *p*H 6.3, but two by sedimentation. Because of the very small amounts of protein isolated, detailed and critical analyses of its homogeneity have not as yet been undertaken.

(15) For these very small volumes, the liquid phase was removed quantitatively by means of a capillary.

Determination of Enzymatic Activity.—A unit of enzyme was defined as that amount of enzyme which will catalyze the hydrolysis of 0.001 *M* *o*-nitrophenyl β-D-galactoside, at *p*H 7.25 and 30° in the presence of 0.14 *M* Na^+ (phosphate), at the rate of 0.012 μmole/ml. of reaction mixture/min., providing the reaction is zero order with respect to the substrate. This amounted to less than 0.1 μg. of protein of the purest preparations.

The reaction tubes containing 3.5 ml. of 0.2 *N* Na^+ (phosphate, *p*H 7.25) and 0.5 ml. of 0.01 *M* *o*-nitrophenyl β-D-galactoside were equilibrated in a 30° bath; 1 ml. of the enzyme was added at the required dilution[16] and a zero time aliquot was removed and pipetted directly into an Evelyn tube containing Na_2CO_3 (final molarity 0.1, total vol. 10 ml.). Periodically, aliquots were removed from the reaction mixture, pipetted into Evelyn tubes, containing Na_2CO_3, and the *o*-nitrophenol liberated was colorimetrically determined as described above.

Kinetic Studies

For the kinetic studies, several preparations of the enzyme were combined giving a protein concentration of 7.0 mg./ml. (organic solids value[9]) and a specific activity of 8125 units/mg. This preparation was dialyzed *vs.* 0.1 ionic strength (Na^+ phosphate buffer), *p*H 6.3 and stored at 2°, A standard concentration of enzyme, for purposes of comparison, was arbitrarily selected as a 1:25,000 dilution of the preparation, representing 0.28 microgram of protein/ml. of reaction mixture.

Figure 1 shows that the enzymatic hydrolysis of 0.001 *M* *o*-nitrophenyl β-D-galactoside displays zero order kinetics up to *ca.* 60% hydrolysis. The reaction proceeds to essentially quantitative hydrolysis of the substrate. For each mole of *o*-nitrophenol liberated, 1 mole of reducing sugar, *i.e.*, galactose, is liberated. The reaction is strictly a hydrolytic cleavage of the β-D-galactoside bond, and phosphate plays no part in the reaction, since the velocity is identical in $NaHCO_3$ and Na^+ phosphate, at the same *p*H (7.25) and Na^+ concentration (0.14 *M*). The velocity is directly proportional to the first power of the enzyme concentration.

With *o*-nitrophenyl β-D-galactoside as substrate maximal enzyme activity was obtained between *p*H 7.2–7.3, at constant Na^+ concentration (0.14 *M*) (Fig. 2).[17]

(16) Glass redistilled water, equilibrated to 30° was used; dilution was made immediately before use.

(17) It will be shown later that Na^+ is the activating ion in the system. To maintain constant Na^+ concentration throughout the *p*H range, appropriate volumes of 0.10 *M* Na_2HPO_4 and 0.20 *M* NaH_2PO_4 were mixed, yielding a 0.20 *M* Na^+ buffer, final dilution in the reaction mixture resulting in 0.14 *M* Na^+ concentration.

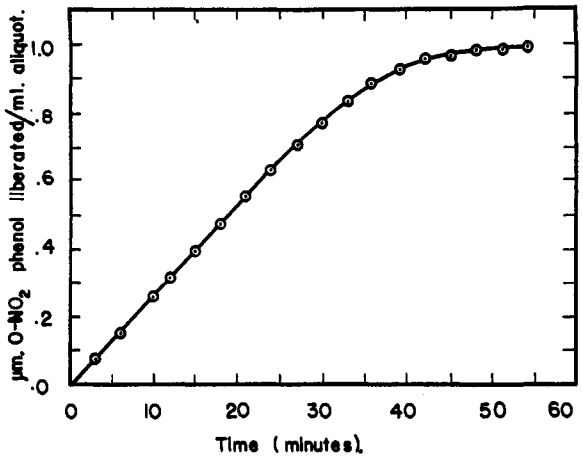

Fig. 1.—Enzymatic hydrolysis of *o*-nitrophenyl β-D-galactoside: initial substrate concentration, 0.001 *M*; *p*H 7.25; [Na^+] = 0.14 *M* (phosphate); 30°.

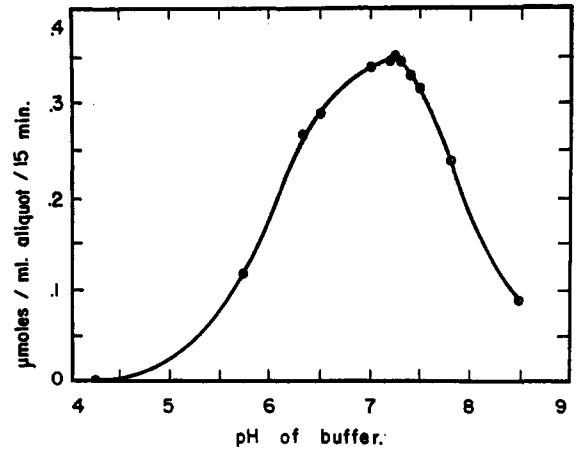

Fig. 2.—Effect of *p*H on enzymatic activity: initial substrate concentration, 0.001 *M* *o*-nitrophenyl β-D-galactoside; [Na^+] = 0.14 *M* (phosphate); 30°.

The effect of *p*H on the stability of the enzyme was determined by a prior incubation at 30° for 36 min., in the absence of substrate, in sodium phosphate buffers at various *p*H values and the activity was then determined under standard conditions.

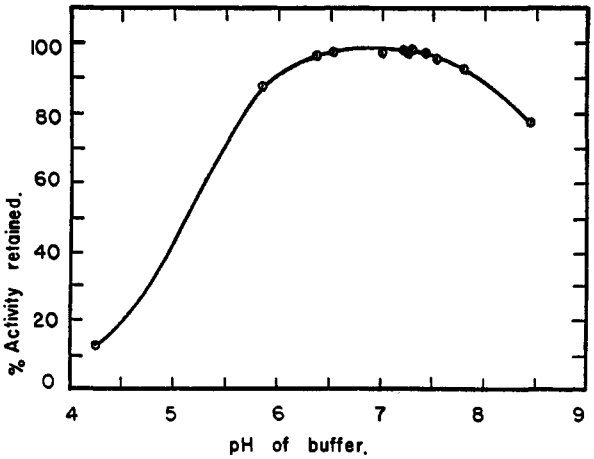

Fig. 3.—Effect of *p*H on stability of enzyme; sodium phosphate buffer.

The enzyme was fairly stable between *p*H 6 and 8 (Fig. 3).

Effect of Substrate Concentration, Determination of k_s and v_{Max} for Various Substrates.—The substrate concentrations were varied over a reasonably wide range, usually 10-fold and in some cases 25-fold, and the simple Lineweaver–Burk[18] plots have been found to hold (Fig. 4). In all cases

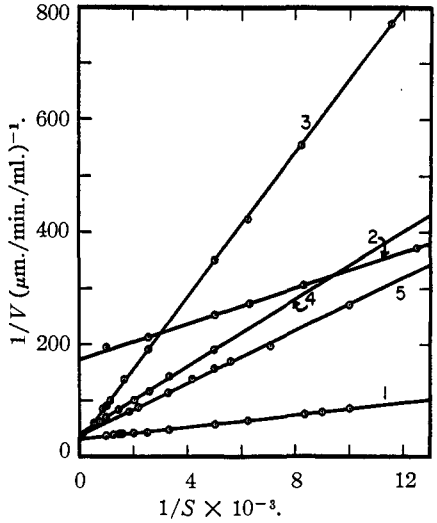

Fig. 4.—Lineweaver–Burk plots for several substrates: 1, *o*-nitrophenyl β-D-galactoside; 2, *p*-nitrophenyl β-D-galactoside; 3, *o*-nitrophenyl α-L-arabinoside, $1/V \times 5 \times 10^{-2}$ *vs.* $1/S \times 10^{-3}$; 4, *n*-butyl β-D-galactoside, $1/V \times 10^{-1}$ *vs.* $1/S \times 10^{-3}$; 5, methyl β-D-galactoside, $1/V \times 10^{-1}$ *vs.* $1/S \times 10^{-2}$; [Na^+] = 0.14 *M* (phosphate buffer), *p*H 7.25, 30°; *V*, the initial zero order velocity expressed in μm./ml./min./std. concn. of enzyme; *S*, the initial substrate concn. expressed in moles/liter.

the initial velocity (zero-order kinetics) has been used for the plotting. Table II summarizes the data obtained for the various substrates in terms of the Michaelis constants (K_s) and maximal velocities (V_{Max}). They illustrate the enormous effect of the aglycone group on the rate of reaction and K_s. For example, K_s ranges from 0.93×10^{-4} *M* for *p*-nitrophenyl β-D-galactoside to 0.04 for lacto-

TABLE II

MICHAELIS CONSTANTS AND MAXIMAL VELOCITIES FOR SEVERAL SUBSTRATES

Compound	K_s[b]	V_{max}[a]
o-Nitrophenyl β-D-galactoside	1.8×10^{-4}	32×10^{-3}
p-Nitrophenyl β-D-galactoside	0.93×10^{-4}	5.8×10^{-3}
o-Nitrophenyl α-L-arabinoside	2.6×10^{-3}	2.2×10^{-3}
Phenyl β-D-galactoside	7.3×10^{-4}	5.0×10^{-3}
Methyl β-D-galactoside	6.9×10^{-3}	2.9×10^{-3}
n-Butyl β D-galactoside	6.9×10^{-4}	2.3×10^{-3}
Lactose	$\sim 10^{-3}$	$\sim 3 \times 10^{-3}$
Lactositol	~0.03	$\sim 2.5 \times 10^{-4}$
Lactobionate	~0.04	$\sim 1.0 \times 10^{-4}$

[a] V_{max} expressed in μm./ml./min./standard concentration of enzyme. [b] K_s in moles/liter.

(18) H. Lineweaver and D. Burk, THIS JOURNAL, 56, 658 (1934).

bionate, about a 400-fold difference. V_{max} ranges from 32×10^{-3} for *o*-nitrophenyl β-D-galactoside to 10^{-4} for lactobionate, about a 320-fold range.

It is evident that the nitrophenyl group decreases the K_s; the *p*-nitrophenyl derivative displayed the lowest K_s, followed by the *o*-nitrophenyl derivative. Removal of the 6th carbon of galactose, as in *o*-nitrophenyl α-L-arabinoside (which has the same configuration as the first 5 carbons of galactose in *o*-nitrophenyl β-D-galactoside), increases K_s by *ca.* 15-fold and decreases V_{max} by a factor of *ca.* 15. Substitution of the nitrophenyl group by methyl or *n*-butyl increases K_s, *n*-butyl having $^1/_{10}$ K_s of the methyl derivative. Substitution of the nitrophenyl group by glucose (as in lactose), by sorbitol (as in lactositol) or by gluconate (as in lactobionate), progressively increased the K_s values.

The *o*-nitrophenyl derivative displays the highest V_{max} followed by the *p*-nitrophenyl and phenyl derivative in that order. Lactobionate and lactositol are hydrolyzed extremely slowly.

It is also evident that a decrease in K_s does not necessarily parallel an increase in V_{max}; *e.g.*, compare the *o*-nitrophenyl and *p*-nitrophenyl derivatives, and the *n*-butyl and methyl derivatives. This is not unexpected, for K_s is a function of at least three independently varying velocity constants, whereas V_{max} is a function of only one.

Effect of Temperature on the Rate of Hydrolysis.—The various substrates were employed at sufficiently high concentrations so that their zero-order velocity constants approached their respective limiting maximal velocities. Figure 5 shows the result of plotting in the conventional Arrhenius manner $-\log k_0$ *vs.* $1/T$ (k_0 = zero order velocity constant, T = absolute temperature).

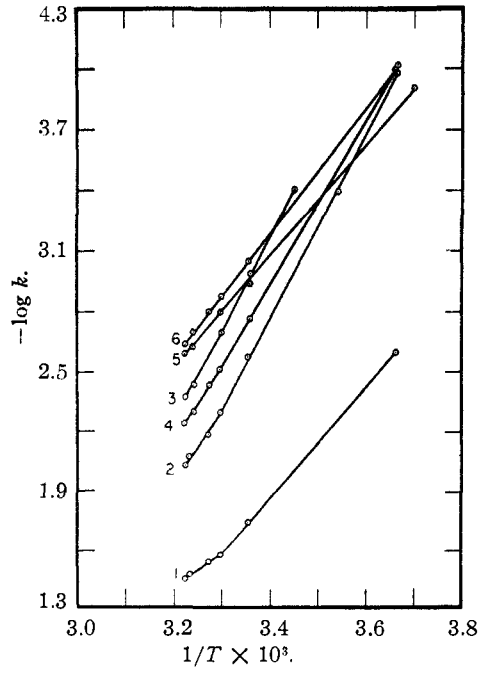

Fig. 5.—Arrhenius plots for several substrates: 1, *o*-nitrophenyl β-D-galactoside (init. concn. 1.00×10^{-3} *M*); 2, *p*-nitrophenyl β-D-galactoside (init. concn. 1.00×10^{-3} *M*); 3, *o*-nitrophenyl α-L-arabinoside (init. concn. 6.00×10^{-3} *M*); 4, phenyl β-D-galactoside (init. concn. 2.60×10^{-3} *M*); 5, methyl β-D-galactoside (init. concn. 4.80×10^{-3} *M*); 6, *n*-butyl β-D-galactoside (init. concn. 5.00×10^{-3} *M*); $[Na^+]$ = 0.14 *M* (phosphate buffer), *p*H 7.25; k_0, the initial zero order velocity constant (μm./ml./min./std. concn. of enzyme); T, absolute temperature.

Only in the case of the methyl derivatives and for all practical purposes in the case of the *n*-butyl derivative are the plots linear over the entire temperature range studied (0° to *ca.* 37°); the others deviated from linearity, the greatest deviation is observed in the case of the *o*-nitrophenyl derivative. For purposes of calculating average ΔH^* values we have fitted the curves to two straight lines.[19]

Table III summarizes the data in terms of the average Arrhenius energies of activation (ΔH^*) and log $PZ_{average}$ values (calculated from $k = PZe^{-\Delta H^*/RT}$ for the designated temperature ranges). An increase in temperature is associated with a decrease in ΔH^* and a parallel decrease in log PZ; thus apparently both ΔH^* and ΔS^* vary concomitantly with temperature with ΔF^* remaining constant. *o*-Nitrophenyl β-D-galactoside has a very low K_s, the highest V_{max}, and shows the lowest ΔH^*. In the case of the α-L-arabinoside, the increase in K_s and decrease in V_{max} is reflected in an increased ΔH^* over *o*-nitrophenyl β-D-galactoside. However, there is no consistent correlation between ΔH^*, K_s and V_{max}; witness the methyl galactoside with ΔH^* = 13 kcal./mole and compare it with the *p*-nitrophenyl galactoside which had a greater ΔH^* but a much lower K and higher V_{max}.

TABLE III

AVERAGE ENERGIES OF ACTIVATION AND LOG PZ FACTORS FOR SEVERAL SUBSTRATES

Substrate	Temp. range, °C.	$\Delta H^*_{av.}$ kcal./moles	log $PZ_{av.}$[a]
o-Nitrophenyl β-D-galactoside	30–37	7.3	3.7
	0–30	13.1	7.7
p-Nitrophenyl β-D-galactoside	30–37	15.5	8.9
	0–30	21.0	12.8
o-Nitrophenyl α-L-arabinoside	30–37	19.3	11.2
	16–30	21.6	12.9
Phenyl β-D-galactoside	30–37	16.9	10.7
	0–30	19.6	12.6
Methyl β-D-galactoside	0–37	12.6	7.3
n-Butyl β-D-galactoside	25–37	13.4	6.8
	0–25	14.3	7.5

[a] Calcd. from $k_0 = PZ\ e^{-\Delta H^*/RT}$ where k_0 expressed in μm./ml./min./std. concn. of enzyme.

Effect of Inhibitors. Determinations of K_I, $\bar{K}_I$ and ΔF^0. A. Competitive Inhibitors.—Lactose (Fig. 6) and *n*-butyl-β-D-galactoside competitively inhibit the rate of hydrolysis of *o*-nitrophenyl β-D-galactoside—competitive inhibition of one substrate by another. Assuming the following Michaelis mechanism for two substrates (S_1, S_2) interacting with one enzyme (E):

$$E + S_1 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES_1 \xrightarrow{k_3} E + \text{Products}_1$$

$$E + S_2 \underset{k_2'}{\overset{k_1'}{\rightleftharpoons}} ES_2 \xrightarrow{k_3'} E + \text{Products}_2$$

(19) G. B. Kistiakowsky and R. Lumry (THIS JOURNAL, 71, 2006 (1949)) consider a sharp change in slope unlikely.

one may derive the kinetic expression

$$\frac{1}{V_{1\text{ init}}} = \frac{1}{V_{1\max}}\left(K_{s_1} + \frac{K_{s_1} S_2}{K_{s_2}}\right)\frac{1}{S_1} + \frac{1}{V_{1\max}}$$

where $K_{s_1} = (k_2 + k_3)/k_1$, $K_{s_2} = (k_2' + k_3'/)k_1'$ and V_1, $V_{1\max}$ refer to the initial velocity and maximal velocity with respect to substrate S_1. This takes the same form as the Lineweaver–Burk[18] expression for competitive inhibition

$$\frac{1}{V_{\text{init}}} = \frac{1}{V_{\max}}\left(K_s + \frac{K_s I}{K_I}\right)\frac{1}{S} + \frac{1}{V_{\max}}$$

where I is the initial inhibitor concentration and K_I is the inhibitor dissociation constant (a ratio of 2 velocity constants).

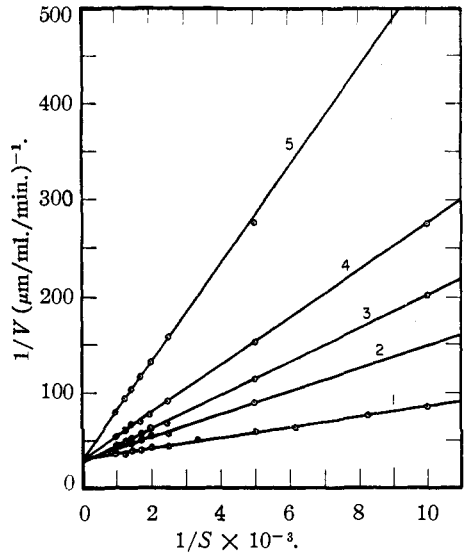

Fig. 6.—"Substrate competitive inhibition" by lactose: 1, init. lactose concn., 0.00 *M*; 2, init. lactose concn., 1.38×10^{-3} *M*; 3, init. lactose concn., 2.56×10^{-3} *M*; 4, init. lactose concn., 4.00×10^{-3} *M*; 5, init. lactose concn., 1.00×10^{-2} *M*; *S*, the initial concn. of *o*-nitrophenyl β-D-galactoside, in moles/liter; *V*, the initial zero order velocity, in μm/ml./min./std. concn. of enzyme; $[Na^+] = 0.14$ *M*, *p*H 7.25 (phosphate buffer), 30°.

Since the rate of disappearance of one substrate could be specifically determined in the presence of the other, K_{s_2} could be indirectly determined from a knowledge of K_{s_1}, S_1, S_2 and $V_{1\text{ init}}$. (K_{s_2} is denoted as $\bar{K}_I$ to indicate its indirect determination by means of competitive inhibition.) The $\bar{K}_I$ values for lactose and *n*-butyl β-D-galactoside agreed, within experimental error, with the direct determination of their K_s values (*cf.* Tables III and IV).

Galactose, the α-galactosides and methyl α-D-*manno*-D-*gala*-heptoside, which are not attacked by the enzyme, also act as competitive inhibitors.

B. Non-competitive Inhibitors.—Glucose and sucrose (a glucoside) apparently act as non-competitive inhibitors at relatively high concentrations; below 0.01 *M* their effect is extremely small.

The data obtained for the inhibitors are summarized in Table IV, together with the standard free energy changes (ΔF°), calculated for the reaction E + I ⇄ EI, *i.e.*, in the direction of formation of the complex. For the *competitive substrate inhibitors*, the assumption was made that $\bar{K}_I \cong k_2'/k_1'$ (see above), this leads to relatively high $-\Delta F^\circ$ values (*ca.* 4 kcal./mole) for lactose and *n*-butyl β-D-galactoside. Interestingly, the α-methyl heptoside shows a fair degree of "affinity" for the enzyme.

TABLE IV

ENZYME–INHIBITOR DISSOCIATION CONSTANTS

Inhibitor	K_I[a]	$-\Delta F^{\circ}$[b]
Melibiose	2.0×10^{-2}	2.4
Methyl α-D-galactoside	2.2×10^{-2}	2.3
Methyl α-D-*manno*-D-*gala*-heptoside	3.3×10^{-3}	3.5
Galactose	0.94×10^{-2}	2.8
Lactose	1.1×10^{-3}	4.1
n-Butyl β-D-galactoside	7.3×10^{-4}	4.4
Sucrose	~0.04	~1.9
Glucose	~0.07	~1.6

[a] For lactose and *n*-butyl β-D-galactoside, $\bar{K}_I$ values (see text) are listed. All other compounds, except sucrose and glucose, are considered to be competitive inhibitors and their K_I's calculated accordingly, using $K_s = 1.76 \times 10^{-4}$ for *o*-nitrophenyl β-D-galactoside. K_I's for sucrose and glucose were calculated as non-competitive inhibitors, assuming 1 molecule of inhibitor per molecule of enzyme. [b] (For E + I ⇄ EI); ΔF° expressed in kcal./mole for 30°.

Effect of Temperature on $\bar{K}_I$ for Lactose and K_s for *o*-Nitrophenyl-β-D-galactoside.—The effect of lactose, acting as a *substrate competitive inhibitor* for *o*-nitrophenyl β-D-galactoside was determined as a function of temperature; Fig. 7 shows the result of plotting $-\log \bar{K}_I$ for lactose and $-\log K_s$ for *o*-nitrophenyl β-D-galactoside. One may conclude from the non-linearity of the van't Hoff plots that either the enthalpy change for formation of the ES complex is a function of the temperature (as is true for all chemical reactions) or that K_s does not approximate a thermodynamic dissociation constant.

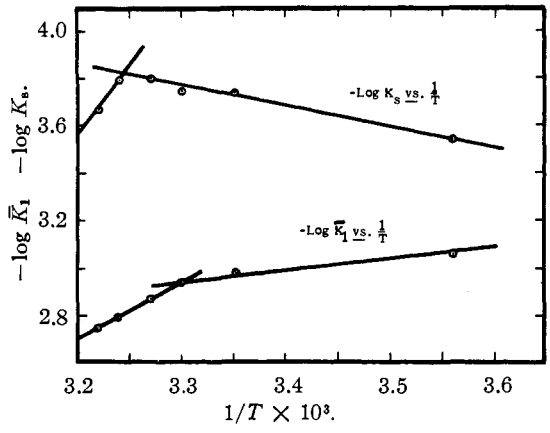

Fig. 7.—Effect of temperature on K_s of *o*-nitrophenyl β-D-galactoside and $\bar{K}_I$ of lactose.

Effect of Ionic Environment on the Reaction Velocity.—The enzyme is activated by Na^+ and to a lesser extent by K^+ with *o*-nitrophenyl β-D-galactoside as the substrate (Fig. 8). The lower plots in Fig. 8 enable one to calculate the dissociation constants associated with the reaction: E. cation ⇄ E + cation. For Na^+, $K = 3 \times 10^{-4}$; for K^+, $K = 1.6 \times 10^{-3}$; the ratio of the $V_{\max}$'s are $Na^+/K^+ = 1.6/1.0$. Also K_s for *o*-nitrophenyl β-D-galactoside in $K^+ = 3.2 \times 10^{-4}$ as compared with *ca.* 1.8×10^{-4} in Na^+ at 0.14 *M* cation concentra-

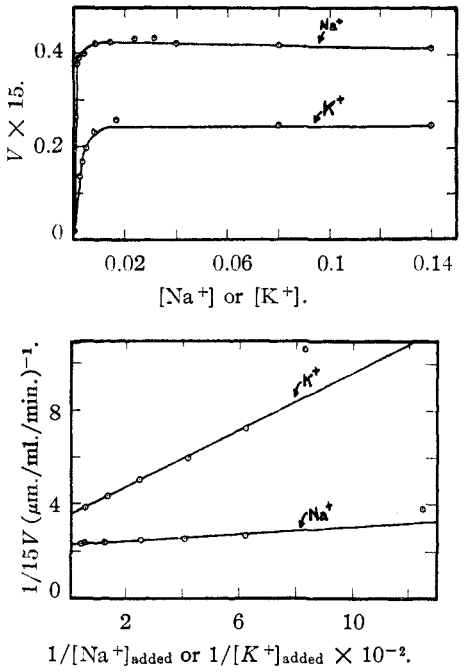

Fig. 8.—Effect of Na^+ and K^+ on the reaction velocity. $[Na^+]$ and $[K^+]$ were varied individually by varying their respective phosphate buffers in the reaction mixture. Deionized, Pyrex distilled water was redistilled through quartz; reaction vessels of Pyrex; temperature, 30°; V, the initial zero order velocity in μm./ml./min./std. concn. of enzyme; $[Na^+]$ or $[K^+]$ in moles/liter.

tion (with phosphate present as the anion). That this effect by Na^+ is not simply a Brönsted salt effect is evidenced by the fact that a whole range of effects may be obtained with monovalent and polyvalent anions and cations (see also Monod, *et al.*,[5] and Lederberg[4]). These effects have not as yet been fully investigated, but *preliminary* evidence points to the following activation series by univalent cations (with *o*-nitrophenyl β-D-galactoside as the substrate)

$$Na^+ > \begin{Bmatrix} K^+ \\ Cs^+ \end{Bmatrix} \geqslant \begin{Bmatrix} Rb^+ \\ NH_4^+ \end{Bmatrix} \geqslant Li^+ \geqslant R{-}NH_3^+$$

where the substituted ammonium ion is definitely inhibitory, *e.g.*, tris-(hydroxymethyl)-aminomethane as the phosphate, 0.14 *M* with respect to tris ion results in essentially complete loss of activity in the absence of Na^+. 0.072 *M* tris buffer in presence of 0.068 *M* Na^+ (both as phosphate) results in a 49% inhibition, even though the Na^+ concentration is at an optimum level for activation. As for the divalent ions, Ca^{++} appears to be inert, Cu^{++} causes a 90% inhibition at a level of 0.006 *M*; Mg^{++} and Mn^{++} activate slightly in a system fully activated by Na^+.

It was observed that certain of the anions can cause inhibition in a fully activated Na^+ system. Preliminary evidence points to the following series for inhibition with *o*-nitrophenyl β-D-galactoside as the substrate

$$SO_3^= \geqslant Cl^- \geqslant NO_3^- \geqslant \begin{Bmatrix} NO_2^- \\ Br^- \end{Bmatrix} \geqslant \begin{Bmatrix} F^- \\ I^- \\ SO_4^= \end{Bmatrix} \geqslant \begin{Bmatrix} \text{Phosphate} \\ HCO_3^- \end{Bmatrix}$$

where phosphate and HCO_3^- were taken as standards; identical rates are obtained in HCO_3^- and in phosphate at *p*H 7.25 and 0.14 *M* Na^+. This activation effect by cations and inhibition by anions makes it very difficult to ascertain their individual effects quantitatively.

A few miscellaneous effects might be mentioned. Methanol and ethanol (at 0.002 *M*) produce a slight activation; cysteine appears to be inert; *o*-nitrophenol up to 5×10^{-4} *M* produces no effect within experimental error (this concentration corresponds to that produced by 50% hydrolysis of 0.001 *M* *o*-nitrophenyl β-D-galactoside); α-D-galactosides, α- or β-D-glucosides, α-D-mannosides and β-D-fructosides are not hydrolyzed enzymatically.

Cohn and Monod[5] found that the adaptive lactase of *E. coli*, strain ML, hydrolyzes lactose more rapidly in the presence of K^+ than Na^+ but the converse was true with *o*-nitrophenyl-β-D-galactoside as substrate. This phenomenon was not exhibited by the enzyme we have obtained from *E. coli*, strain K-12. This behavior, together with the fact that triethanolammonium phosphate inhibits our enzyme but not that of Cohn and Monod, leads to the conclusion that the two enzymes are subtly different.

The results of Snyder and Link[20] on the non-enzymatic acid and base hydrolysis of the nitrophenyl, glycosides reveal some interesting contrasts with the enzymatically catalyzed hydrolysis. The above workers found that the nitrophenyl glycosides could be arranged in the following order of decreasing rate of hydrolysis in either 0.1 *N* HCl or 0.1 *N* NaOH: *o*-nitrophenyl α-L-arabinoside, *o*-nitrophenyl-β-D-galactoside, *p*-nitrophenyl β-D-galactoside; in alkali this corresponded to the order of increasing energy of activation. However, in the case of enzymatic catalysis, the order of decreasing reaction velocity or increasing energy of activation is: *o*-nitrophenyl β-D-galactoside, *p*-nitrophenyl β-D-galactoside, *o*-nitrophenyl α-L-arabinoside. Of the three compounds, the arabinoside is hydrolyzed most rapidly by H_3O^+ and OH^- but least rapidly by the enzyme.

Acknowledgment.—We are much indebted to Prof. J. Lederberg for valuable suggestions and for assisting in the culture of the organism.

MADISON, WISCONSIN

(20) J. A. Snyder and K. P. Link, THIS JOURNAL, **74**, 1883 (1952).