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STUDIES ON THE LACTASE OF ESCHERICHIA COLI

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A yeast capable of fermenting lactose was first described by Adametz (1889). He found it in his studies on the microorganisms of cheeses and gave it the name *Saccharomyces lactis*. In the same year Beijerinck working with two species of yeast, *Saccharomyces kefir* and *S. tyrocola*, succeeded in demonstrating in the filtrate of his cultures a lactose-hydrolyzing enzyme, which he named "lactase."

Following these investigations lactases were soon detected in many yeasts, molds, bacteria, and in animal tissues. In 1896 Fischer and Niebel voiced the opinion that hydrolysis had always to precede the fermentative decomposition of lactose. From their study of the structure of carbohydrates they concluded that the enzyme concerned must be specific for the alpha-glucose-beta-galactoside linkage of milk sugar. Due to more recent work, however, the validity of these assumptions has become rather questionable.

Lactases are widely distributed in the plant and animal kingdoms. Euler (1922) in reviewing the literature on this subject points out that they are always found in the intestinal tract of young mammals but decrease markedly with age. As to their occurrence in the pancreas there is no agreement among the various authors. More recently Cajori (1935) has reported a lactase from the dog's liver.

Bierry and Ranc (1909) found a lactase in the gastrointestinal tract of the edible snail, *Helix pomatia*, and Wigglesworth (1927) reported it from the midgut of the cockroach, *Periplaneta americana*. It is, however, very doubtful whether these lactases are identical with those of higher animals, and the same holds for the lactases of higher plants, most frequently encountered in the family Rosaceae. The best known example in this group is the enzyme emulsin of bitter almonds, which can hydrolyze lactose as well as beta-glucosides.

Various species of yeasts, molds, and bacteria are capable of fermenting lactose and may contain lactases. Such have been found in *Aspergillus niger* and *A. oryzae* by Hofmann (1934*a*), in *Diplococcus pneumoniae* by Fleming and Neill (1927*a*), in *Clostridium perfringens* by the same authors

(1927*b*), in *Escherichia coli* by Lowenstein, Fleming, and Neill (1929), and in *Escherichia coli mutabile* by Hershey and Bronfenbrenner (1936) and Deere, Dulaney, and Michelson (1936). The presence of lactases in these organisms, however, does not necessarily mean that hydrolysis of the lactose into its constituent sugars has to precede fermentation. The evidence obtained by Willstätter and Oppenheimer (1922) for lactose yeast, by Wright (1936) for *Streptococcus thermophilus*, and more recently by Leibowitz and Hestrin (1939) for maltose yeast points very strongly to the possibility of direct fermentation of lactose and other disaccharides under certain conditions.

Escherichia coli was selected for a general study of its lactase, special emphasis being placed on the kinetics of enzyme action, heat inactivation, and the behavior of the enzyme toward some reducing and oxidizing agents and salts of heavy metals.

EXPERIMENTAL

1. Preparation of the Enzyme Solution

Fleming and Neill (1927) were successful in obtaining cell-free extracts of carbohydrases from pneumococci by subjecting them to repeated freezing and thawing. In this process zymases were destroyed, while the activity of the hydrolytic enzymes was preserved. This method is very tedious and time-consuming and therefore was not investigated further.

Hofmann (1934 *b*, *c*) obtained active lactase preparations from *E. coli* and *B. delbrukii* by treating the bacteria with an alcohol-ether mixture and then drying them at room temperature. This method was found to be unsatisfactory in our hands largely because of the susceptibility of the enzyme itself to the solvents used. The activity of preparations of lactase so obtained was very low and decreased on prolonged contact with alcohol, which sometimes was unavoidable.

To obtain appreciable amounts of enzyme, masses of *E. coli* were grown on standard meat extract agar in which 1.5 per cent of lactose had been incorporated. After 48 hours incubation the organisms were washed off with a physiological salt solution, containing 1 per cent of toluene, and subsequently centrifugated. This procedure was repeated three times in order to reduce to a minimum the concentration of adhering metabolic waste products. The resulting suspension contained 6×10^{11} organisms per ml. It was treated with an additional amount of toluene bringing the total concentration of the latter up to 5 per cent. Toluene serves three purposes: (1) It acts as a preservative, (2) it inactivates the zymase complex without affecting the lactase (Willstätter and Oppenheimer, 1922), and (3) it destroys the semipermeability of the cell walls, bringing about a gradual autolysis of the bacteria.

Several attempts were then made to obtain a cell-free enzyme preparation. As was mentioned above it was found that the lactase apparently was very susceptible to alcohol and ether. It was also completely inactivated on dehydration with acetone. When a toluene-treated cell suspension was incubated overnight at 37°C. a dry gelatinous substance was obtained. This was removed and ground to a powder, the relative activity

of which, as determined by a method to be described later, was found to be 82 per cent of that originally present in the bacterial suspension.

The dried powder, consisting of whole cells and cell fragments, was subjected to more rigorous autolysis. Measured portions were suspended in M/15 phosphate buffer solutions of pH 7.0, 8.0, and 9.0 and incubated overnight at temperatures of 37°C. and 46°C. They were then centrifugated and supernatants and sediments tested for lactase activity. The opaque supernatant fluids were practically inactive, whereas the precipitates still exhibited a marked activity though less than that of the dry powder, probably because of the severity of the treatment to which they had been subjected. A microscopical examination revealed that practically all bacterial cells were disintegrated, and only cell fragments were present. The enzyme, apparently, adhered to these cell fragments.

These observations are contrary to reports by Karström (1930), who obtained cell-free lactase preparations from *E. coli* by suspending the dried organisms in phosphate buffer solution of pH 7.0. They are, however, in agreement with results reported by Hershey and Bronfenbrenner (1936), who were unable to separate the enzyme from the bacterial cell and therefore concluded that it was an intracellular water insoluble enzyme.

In another experiment equivalent amounts of toluene-treated cell suspension were exposed to the action of trypsin and papain. In both instances lactase activity was destroyed.

Finally, 120 ml. of bacterial suspension were ground for 18 hours in a ball mill devised by Krueger (1933). But again lactase was inactivated.

In view of these experiences it was decided to use the original cell suspension in all subsequent experiments, and it will be referred to in this report as "enzyme solution" or "*E. coli* lactase" inasmuch as it was solely employed for hydrolyzing lactose. This preparation was stored in an icebox at 5°C. where its activity decreased only slightly during the course of several months.

2. *Materials and Methods*

Standard sugar solutions: 1 gm. of lactose hydrate and glucose, respectively, were dissolved in 100 ml. of distilled water and a few drops of toluene added.

Throughout the course of the experiments dilutions were prepared from these standard solutions, 1 ml. of which contained 10 mg. of the respective sugar.

The Folin-Wu method (1920) for blood sugar determination was chosen as best fitted for measuring the total amount of sugar present before and after hydrolysis by the enzyme.

Experiments were conducted as follows: The desired dilution of the standard was prepared by the use of M/15 phosphate buffers of measured hydrogen ion concentration. One-tenth ml. portions of enzyme preparation were added to 5 ml. of lactose solution and the tubes shaken in a water bath at 36°C. for a certain length of time. Thereupon, they were centrifugated for 30 minutes and the supernatant liquid used for sugar determination. 2 ml. were pipetted into Folin-Wu sugar tubes, 2 ml. of copper solution added, and the tubes then placed in boiling water for 8 minutes. After cooling 2 ml. of color reagent (phosphomolybdic acid) were added, the tubes made up to a volume of 25 ml. with water, and the resulting color compared with that of a standard.

In preliminary readings, employing glucose and lactose solutions of different concentrations, it was found that 1 mg. of lactose corresponded to 0.504 mg. of glucose. In

all experiments, therefore, the values obtained have been expressed in terms of glucose or total reducing sugar on the basis of the above empirical determination.

For example, if the initial concentration of lactose is 1/40 of that of the standard solution, *i.e.* 2 ml. contain 0.5 mg. of lactose, it will be read as 0.252 mg. of glucose or total reducing sugar, a glucose solution being always used as the standard for comparison.

For each experiment a parallel control had to be set up since most of the chemicals whose effect on the enzyme was to be tested were oxidizing or reducing agents, and the enzyme solution itself slightly reduced copper sulfate. For this purpose corresponding amounts of enzyme and chemical reagent were added to 5 ml. of phosphate buffer solution and the reducing values obtained then subtracted from the total.

Finally, a correction for volume had to be made to an extent dependent upon the amount of enzyme solution and chemical reagent added.

It was impossible to maintain a perfectly uniform rate of hydrolysis for the duration of the experiments. The values fluctuate between 51 and 59 per cent hydrolysis per hour for a 1/40 lactose solution. This circumstance, however, was not regarded as of importance inasmuch as the problem selected concerned merely the comparative study of rates of reaction as affected by hydrogen ion concentration, temperature, and chemicals.

RESULTS

1. The Effect of Hydrogen Ion Concentration

Optimal conditions with regard to hydrogen ion concentration differ for lactases from various sources (Oppenheimer, 1935).

To determine the effect of pH on the activity of *E. coli* lactase, experiments were carried out as follows: M/15 phosphate buffer solutions of different pH were prepared and their hydrogen ion concentration checked by means of a glass electrode. They were then used to make up lactose solution of a concentration of 1/40 with respect to the standard (0.252 mg. of total sugar per 2 ml.).

As described above, 5 ml. were then mixed with 0.1 ml. of enzyme preparation and shaken in a water bath at 36°C. for 1 hour, and the reducing sugar was determined. The results are given in Table I.

The values are plotted in Fig. 1.

The results indicate that the activity of the enzyme is markedly reduced by slight acidity but much less affected by alkalinity of the medium. The optimum pH for the time period and temperature given seems to extend over the range between 7.0 and 7.5. Consequently, all subsequent experiments were carried out at a pH of 7.5.

2. The Mechanism of Enzyme Action

Michaelis and Menten (1913) worked out general rate laws for the action of invertase on sucrose by assuming a chemical combination of the enzyme with its substrate as the governing step in the hydrolysis of the sugar.

The enzyme-substrate equilibrium can be represented by the equation:

$$K_s = \frac{(E)\,(S)}{(ES)}$$

TABLE I

Effect of pH on the Degree of Hydrolysis of Lactose by E. coli Lactase

pH	Amount of total sugar	Hydrolysis	Ratio of activity to that of maximum activity
	mg. per 2 ml.	*per cent*	
5.0	0.254	0.8	0.01
6.0	0.357	41.7	0.71
6.5	0.386	53.2	0.91
7.0	0.399	58.3	0.99
7.5	0.400	58.7	1.00
8.0	0.393	56.0	0.95
9.0	0.381	51.2	0.87

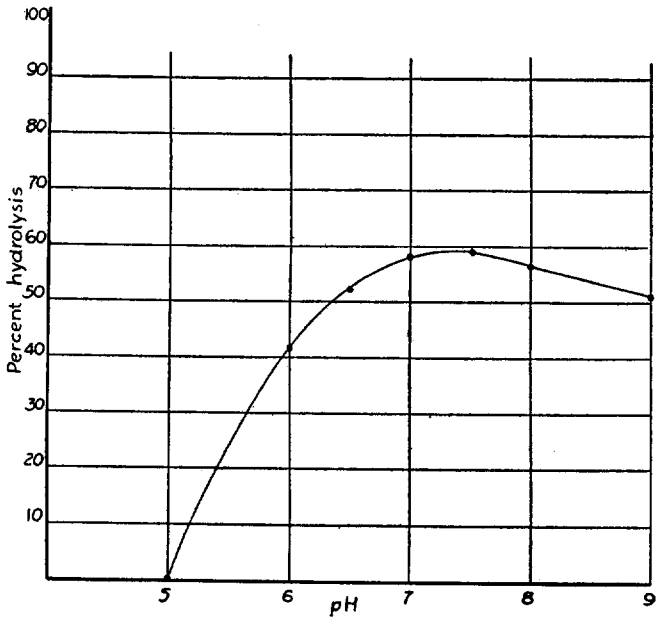

FIG. 1. Effect of pH on the rate of hydrolysis of lactose by *E. coli* lactase.

where (E) and (ES) refer to the concentration of free and combined enzyme respectively and (S) to the concentration of the substrate.

The constant k_s could be determined by simple mathematical calculation, leading to the equation

$$\frac{v}{V_m} = \frac{(S)}{k_s + (S)} \text{ or } k_s = (S)\left(\frac{V_m}{v} - 1\right)$$

in which v represents the initial velocity at the substrate concentration (S), V_m the maximum velocity, k_s, therefore, being equivalent to the substrate concentration at which half the limiting velocity is reached.

Lineweaver and Burk (1934) developed graphic methods for determining dissociation constants of enzyme-substrate compounds. Since in some cases one molecule of enzyme reacts with several molecules of substrate they modified the Michaelis-Menten equation accordingly:

$$k_s = \frac{(E)\,(S)^n}{(ES_n)}$$

and

$$\frac{v}{V_m} = \frac{(S)^n}{(S)^n + k_s}$$

The latter equation can then be written

$$\frac{1}{v} = \frac{k_s}{V_m(S)^n} + \frac{1}{V_m},$$

in which V_m, the maximum velocity, and k_s are constant.

A plot of $\frac{1}{v}$ against $\frac{1}{S^n}$ must therefore give a straight line for some integral value of n. The intercept of this line on the $\frac{1}{v}$ axis is $\frac{1}{V_m}$ and its slope $\frac{k_s}{V_m}$. In this fashion, then, the constants are easily determined.

When the above equation is multiplied by $(S)^n$ it assumes the form $\frac{(S)^n}{v} = \frac{k_s}{V_m} + \frac{(S)^n}{V_m}$. By plotting $\frac{(S)^n}{v}$ against $(S)^n$ a straight line is again obtained. The intercept on the $\frac{(S)^n}{v}$ axis is $\frac{K_s}{V_m}$ and the slope is $\frac{1}{V_m}$.

The latter plot is not only of importance in checking the values obtained by the former but also in discovering any departure from a straight line due to substrate inhibition. In such a case plots of $\frac{(S)^n}{v}$ against $(S)^n$ give curves that rise concavely with increasing substrate concentration.

The following solutions were prepared:

(*a*) A 1/10 dilution of the standard (2 mg. of lactose per 2 ml. = 29.3×10^{-4}M)

(*b*) A 1/20 dilution of the standard (1 mg. of lactose per 2 ml. = 14.6×10^{-4}M)

(*c*) A 1/40 dilution of the standard (0.5 mg. of lactose per 2 ml. = 7.3×10^{-4}M)

(*d*) A 1/60 dilution of the standard (0.33 mg. of lactose per 2 ml. = 4.9×10^{-4}M)

The results of hydrolysis after 30 and 60 minutes are given in Table II.

Upon plotting $1/v$ against $1/S$ and S/v against S practically straight lines were obtained. (See Figs. 2 and 3.) Consequently, it can be concluded that one molecule of enzyme combines with one molecule of lactose as is the case with all the other carbohydrases so far investigated.

TABLE II

Rate of Hydrolysis of Varying Concentrations of the Substrate

	Amount of total sugar	Velocity	1/v (av.)	1/S	S/v
	mg. per 2 ml.	*per min.*			
1. Substrate A					
30 min.	1.164	0.0053	192	0.5	384
60 "	1.312	0.0051			
2. Substrate B					
30 min.	0.628	0.0041	260	1.0	260
60 "	0.722	0.0036			
3. Substrate C					
30 min.	0.332	0.0027	392	2.0	
60 "	0.394	0.0024			196
4. Substrate D					
30 min.	0.233	0.0022	500	3.0	167
60 "	0.273	0.0018			

The intercept on the $1/v$ axis is at 138, hence $V_m = 1/138 = 0.0072$ mg. per 2 ml. per minute.

$K_s = V_m \times$ slope $= 0.0072 \times 132 = 0.95$ mg. per 2 ml. $= 13.9 \times 10^{-4}$M or 0.00139

V_m and k_s, as evaluated from the second plot, are somewhat higher.

The intercept on the S/v axis is at $132 = k_s/V_m$. $1/V_m = 130$, hence $V_m = 0.0077$ and $k_s = 1.02 = 14.9 \times 10^{-4}$M, or 0.00149.

From these plots it may be inferred that the substrate has no inhibiting effect on the rate of hydrolysis by the enzyme.

To determine the effect of different concentrations of *E. coli* lactase on the rate of hydrolysis of lactose an experiment was set up in the ordinary way, using a 1/40 lactose solution but adding varying amounts of enzyme preparation. The results are recorded in Table III.

Plotting the figures of the third column against those of the first gives practically a straight line (Fig. 4). This may be also expressed in a mathe-

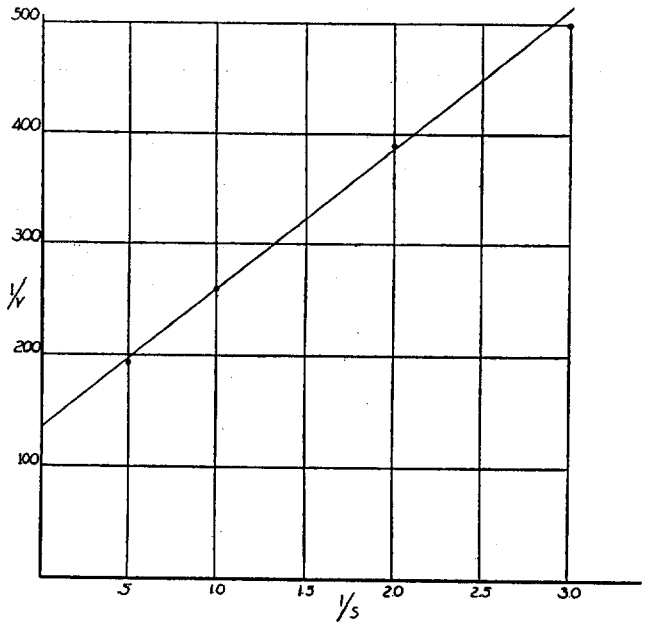

FIG. 2. Nature of the enzyme-substrate intermediate of *E. coli* lactase with lactose.

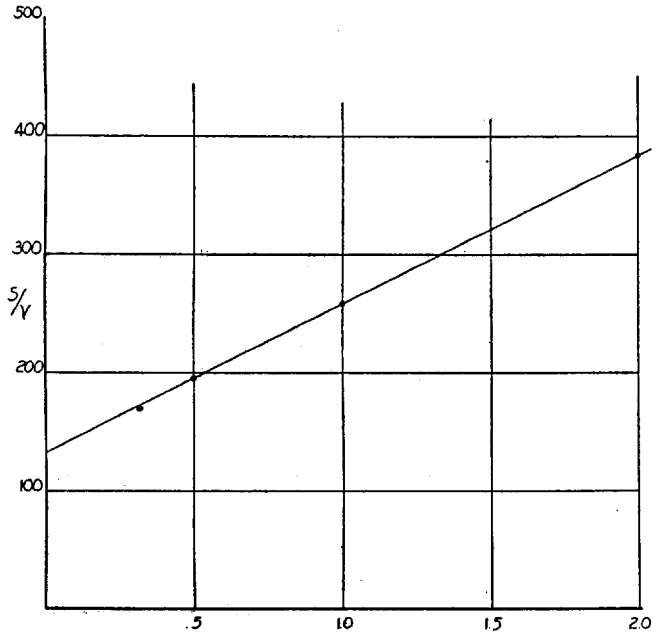

FIG. 3. Test of enzyme-lactose intermediate.

matical form by the equation: $K = \frac{x}{Et}$, where x represents the amount hydrolyzed, E the enzyme concentration, and t the time, which in the above experiment was constant; *viz.*, 1 hour. It is at once evident that

TABLE III

Hydrolysis by Varying Enzyme Concentrations

Amount of enzyme	Amount of total sugar	Amount hydrolyzed (x)	$K = \frac{x}{Et}$	Schütz constant $K_1 = \frac{x}{\sqrt{Et}}$
ml.	*mg. per 2 ml.*			
0.05	0.319	0.067	1.34	0.30
0.10	0.383	0.131	1.31	0.41
0.15	0.452	0.200	1.33	0.52
0.20	0.499	0.247	1.24	0.53

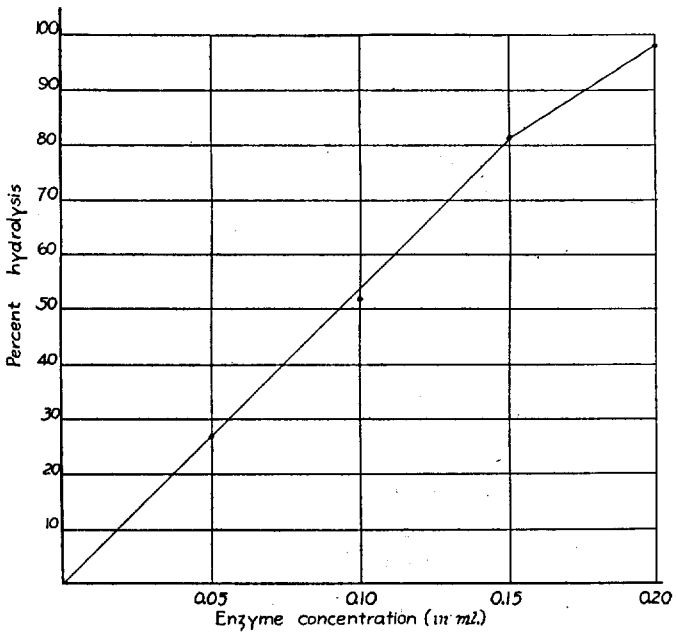

FIG. 4. Relation between enzyme concentration and the rate of hydrolysis.

the values for K fit the above data far better than those for K_1, the so called Schütz constant (1885). Analogous results with yeast lactase were reported by Willstätter and Oppenheimer (1922). In view of the fact that, as has been shown previously, a definite equilibrium between enzyme and substrate is established the products of reaction apparently do not decrease the rate of hydrolysis.

3. *Kinetics of Lactose Hydrolysis by E. coli Lactase*

The hydrolysis of lactose by *E. coli* lactase follows a course between a zero and first order reaction which is quite common for hydrolytic enzymes. Michaelis and Menten (1913), confronted with such difficulty in the

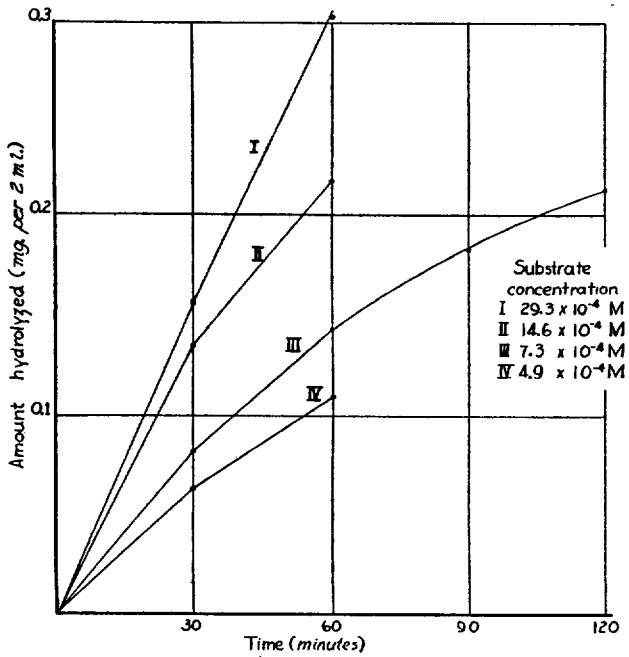

FIG. 5. Relation between substrate concentration and the rate of hydrolysis.

case of invertase, showed that its action could be expressed by a formula that is actually a combination of zero and first order equations.

$$\text{Zero order: } k = k_0 t$$

$$\text{First order: } \ln \frac{a}{a - x} = k_1 t,$$

$$\text{Michaelis-Menten equation: } V_m t = x + k_s \ln \frac{a}{a - x}$$

Essentially the same formula was derived by Van Slyke and Cullen (1914) for the action of urease on urea. Barendrecht (1913) showed that it also held for lactase prepared from yeast.

As suggested in the original paper of Michaelis and Menten, the data of Table II have been presented graphically in two ways. In Fig. 5 the amount hydrolyzed (x) is plotted against time (t). It is readily seen that

for the highest concentration a straight line is obtained indicating that the zero order reaction holds in this case which is in agreement with Michaelis and Menten's observations.

In Fig. 6, $x + 2.3\ k_s \log \frac{a}{a-x}$ is plotted against time, and practically straight lines result, at least for the 1st hour. Only in the case of the 1/40 lactose solution were additional data for the 2nd hour available ($x = 0.185$

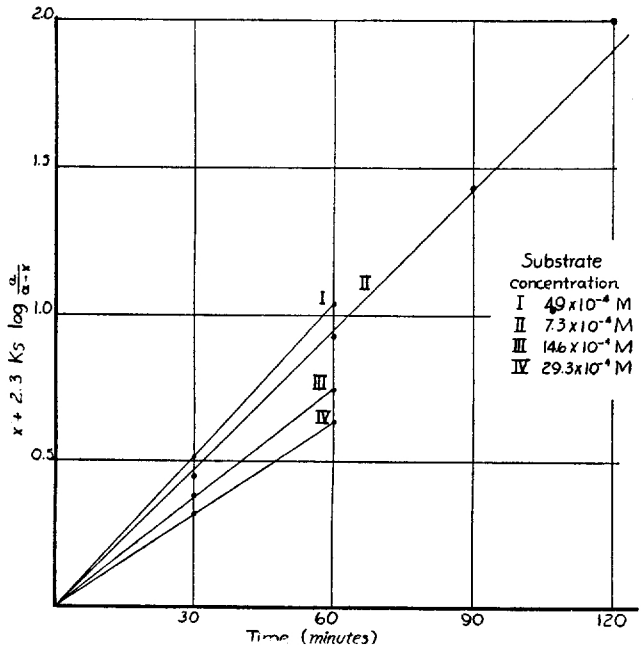

FIG. 6. Kinetics of the hydrolysis of lactose by *E. coli* lactase.

after 1.5 hours and $x = 0.214$ after 2 hours), and these, too, follow practically a straight line. Hence, one can draw the conclusion that hydrolysis of lactose by *E. coli* lactase approximates the reaction course of the integrated Michaelis-Menten equation.

4. The Effect of Temperature

5 ml. of a 1/40 lactose solution were incubated with 0.1 ml. of enzyme preparation for 30 minutes at 26°C., 36°C., 46°C., and 56°C. after preheating the enzyme for 5 minutes at the respective temperature. Table IV shows the results obtained.

The recorded drop of lactase activity between 36°C. and 56°C. may be attributed most probably to a more rapid heat inactivation of the enzyme at the higher temperatures.

To elucidate this point further a few experiments were set up designed to determine the rate of enzyme destruction at different temperatures. Test tubes containing measured amounts of enzyme solution were immersed in a water bath at the desired temperature which was closely controlled.

TABLE IV

Effect of Temperature on the Degree of Hydrolysis of Lactose by E. coli Lactase

Temperature	Amount of total sugar	Hydrolysis	Ratio of activity
°C.	*mg. per 2 ml.*	*per cent*	
26	0.295	17.1	1.81
36	0.330	31.0	1.63
46	0.379	50.4	0
56	0.249	0	

TABLE V

Rates of Heat Inactivation of E. coli Lactase at Different Temperatures

Temperature of preheating	Time of preheating	Amount of total sugar	Amount hydrolyzed	First order constant
°C.		*mg. per 2 ml.*		
45	0	0.379	0.127	
	15	0.354	0.102	0.0146
	20	0.345	0.093	0.0156
	30	0.332	0.080	0.0154
53	0	0.379	0.127	
	3	0.336	0.084	0.138
	5	0.319	0.067	0.128
	7	0.300	0.048	0.139

After heating for varying times the tubes were placed in ice water to check as quickly as possible further destruction of enzyme. The residual lactase activity was then determined in the ordinary way of mixing 0.1 ml. of enzyme preparation with 5 ml. of a 1/40 lactose solution and shaking it in a water bath of 36°C. for 1 hour. The results of experiments carried out at temperatures of 45°C. and 53°C. are given in Table V.

It was found that thermal inactivation of *E. coli* lactase followed the equation of a simple first order reaction:

$$2.3 \log A_0/A = kt,$$

where A_0 is the activity of the unheated enzyme solution, in other words, the amount hydrolyzed under ordinary conditions, A the activity of the enzyme heated for the time t, and k the constant of heat inactivation.

The average values for k are thus 0.0152 at 45°C., and 0.135 at 53°C. They can be determined also by plotting log A against t as has been done in Fig. 7.

It is at once evident that the rate constant for heat inactivation changes

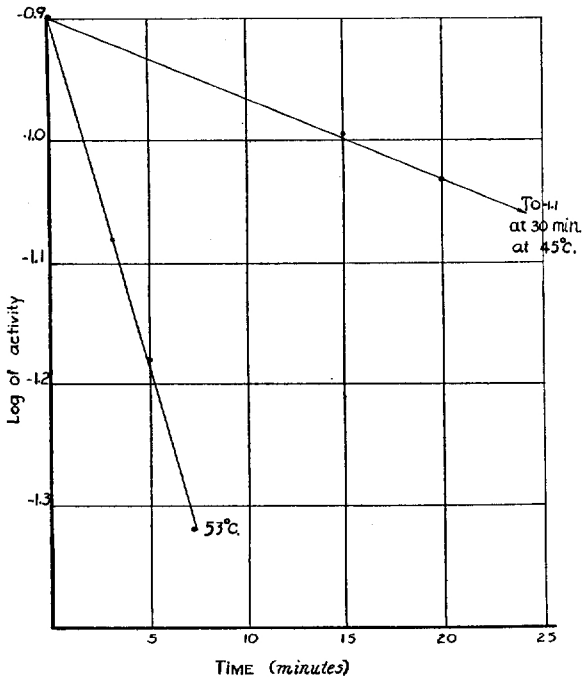

FIG. 7. Rate of heat inactivation of *E. coli* lactase.

very considerably with a relatively small change in temperature. Similar observations have been made with all the enzymes so far investigated, and they are in close agreement with those reported with regard to the denaturation of proteins.

Destruction rates are best considered in their relation to the corresponding "heats of enzyme inactivation." These latter values, known as "critical thermal increments," can be calculated with aid of the van't Hoff-Arrhenius equation

$$\frac{d \ln k}{dt} = \frac{\Delta H}{RT^2}$$

in which k is the reaction velocity constant, T the absolute temperature, R the gas constant, and ΔH the "critical thermal increment."

Integrated between the limits T_2 and T_1, the above equation assumes the following form:

$$\ln \frac{k_2}{k_1} = \frac{\Delta H}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

Since $k_1 = 0.0152$, $k_2 = 0.135$, $T_1 = 318°$, $T_2 = 326°$, and $R = 1.99$ calories, the value of ΔH is calculated as 56,400 calories per mol.

This thermal increment is of the same order of magnitude as those measured in protein denaturation and hence indicates the possibility that *E. coli* lactase may be a protein. Besides, previously cited experiments on destruction of lactase activity through the agency of trypsin and papain constitute another strong evidence for the protein nature of the enzyme.

5. Activation and Inhibition by Chemicals

Inhibition phenomena have been most thoroughly investigated with respect to the action of yeast invertase on cane sugar.

Among others, Euler and Svanberg (1920) and Myrbäck (1926) have studied in great detail the inactivation of this enzyme by various chemical reagents. From the results of his experiments Myrbäck has derived some tentative conclusions as to the nature of the groups that enable the enzyme to decompose its substrate. According to him, an acidic, a basic amino, and an aldehydic group are parts of the invertase molecule concerned with the hydrolysis of cane sugar. He found no evidence that sulfhydryl was an essential group.

Only recently, however, Manchester (1939) noted an acceleration of invertase activity due to the addition of potassium cyanide which is in close agreement with the results obtained for *E. coli* lactase. As will be discussed later this may point to the presence of SH groups in the enzyme molecule.

Activation and inhibition of *E. coli* lactase is produced by a variety of chemical agents (see Tables VI–XIII). All experiments were carried out at 36°C. and at a pH of 7.5 for a period of 1 hour. The substrate, as usual, was 5 ml. of a 1/40 lactose solution. One-tenth ml. of enzyme preparation was at first treated with the chemical agent whose action on the rate of hydrolysis was to be determined, by incubation for about 15 minutes and then added to the substrate.

6. *Attempted Reactivation*

Von Euler and Svanberg (1920) succeeded in reactivating, by means of hydrogen sulfide and sodium cyanide, yeast invertase poisoned by heavy metal salts such as silver nitrate and mercuric chloride. A similar reactivation of the protease papain is well known. It was demonstrated first by Vines (1902) and Mendel and Blood (1910) and later by many other investigators.

Only recently, however, were Hellerman, Perkins, and Clark (1933)

TABLE VI

Activation by Potassium Cyanide

Amount of KCN added	Amount of total sugar	Hydrolysis	Ratio of activity to that of untreated enzyme
	mg. per 2 ml.	*per cent*	
None	0.391	55.2	1.00
0.1 ml. 10^{-4}M	0.388	54.0	0.98
0.1 ml. 10^{-3}M	0.418	65.8	1.19
0.1 ml. 10^{-2}M	0.433	72.2	1.31
0.1 ml. 10^{-1}M	0.381	51.2	0.93

TABLE VII

Activation by Sodium Sulfide

Amount of Na_2S added	Amount of total sugar	Hydrolysis	Ratio of activity to that of untreated enzyme
	mg. per 2 ml.	*per cent*	
None	0.380	51.2	1.00
0.1 ml. 10^{-4}M	0.382	51.6	1.01
0.1 ml. 10^{-3}M	0.422	67.4	1.32
0.1 ml. 10^{-2}M	0.412	63.5	1.25

and Hellerman and Perkins (1934) successful in restoring to normal the activity of urease and papain by suitable reducing agents after a preceding inactivation by salts of heavy metals and oxidants.

On the basis of these findings analogous experiments were set up with *E. coli* lactase. They were carried out as usual, the enzyme, however, being treated first with the inhibiting, then with the reducing agent, each for about 15 minutes.

The results are given in Table XIV.

E. coli lactase was irreversibly inactivated by most of the enzymic poisons used, in contrast to yeast invertase, urease, and papain. (See Table XIV.)

TABLE VIII

*Activation by Cysteine**

Amount of cysteine added	Amount of total sugar	Hydrolysis	Ratio of activity to that of untreated enzyme
	mg. per 2 ml.	*per cent*	
None	0.389	54.4	1.00
0.1 ml. 1.8×10^{-3} M	0.388	54.0	0.99
0.1 ml. 4.5×10^{-3} M	0.416	65.1	1.20
0.1 ml. 9.0×10^{-3} M	0.413	64.2	1.18

* Dilutions were prepared from Pfanstiehl's cysteine hydrochloride adjusted to pH 7.0.

TABLE IX

Inhibition by Mercuric Chloride

Amount of $HgCl_2$ added	Amount of total sugar	Hydrolysis	Ratio of activity to that of untreated enzyme
	mg. per 2 ml.	*per cent*	
None	0.395	56.7	1.00
0.1 ml. 10^{-4} M	0.395	56.7	1.00
0.1 ml. 2.0×10^{-4} M	0.353	40.0	0.71
0.1 ml. 3.3×10^{-4} M	0.318	26.0	0.46
0.1 ml. 5.0×10^{-4} M	0.282	11.9	0.21
0.1 ml. 10^{-3} M	0.250	0	0

TABLE X

Inhibition by Silver Nitrate

Amount of $AgNO_3$ added	Amount of total sugar	Hydrolysis	Ratio of activity to that of untreated enzyme
	mg. per 2 ml.	*per cent*	
None	0.398	57.9	1.00
0.1 ml. 10^{-4} M	0.398	57.9	1.00
0.1 ml. 3.3×10^{-4} M	0.311	23.4	0.43
0.1 ml. 5.0×10^{-4} M	0.281	11.5	0.20
0.1 ml. 10^{-3} M	0.252 to 0.260	0 to 3.0	0 to 0.05

TABLE XI

Inhibition by Copper Sulfate

Amount of $CuSO_4$ added	Amount of total sugar	Hydrolysis	Ratio of activity to that of untreated enzyme
	mg. per 2 ml.	*per cent*	
None	0.382	51.6	1.00
0.1 ml. 10^{-4} M	0.382	51.6	1.00
0.1 ml. 2.0×10^{-4} M	0.351	39.4	0.76
0.1 ml. 3.3×10^{-4} M	0.333	32.1	0.62
0.1 ml. 10^{-3} M	0.262 to 0.270	4.0 to 7.1	0.08 to 0.14

TABLE XII

Inhibition by Iodine (Aqueous Solution in Potassium Iodide)

Amount of I_2 added	Amount of total sugar	Hydrolysis	Ratio of activity to that of untreated enzyme
	mg. per 2 ml.	*per cent*	
None	0.392	55.6	1.00
0.1 ml. 10^{-4} M	0.392	55.6	1.00
0.1 ml. 10^{-3} M	0.324	28.6	0.53
0.1 ml. 10^{-2} M	0.255	1.2	0.02

TABLE XIII

*Inhibition by Hydrogen Peroxide**

Amount of H_2O_2 added	Amount of total sugar	Hydrolysis	Ratio of activity to that of untreated enzyme
	mg. per 2 ml.	*per cent*	
None	0.382	51.6	1.00
0.1 ml. 2.0×10^{-3} M	0.383	52.0	1.01
0.1 ml. 10^{-2} M	0.364	44.3	0.86
0.1 ml. 10^{-1} M	0.361	43.3	0.84

* Dilutions were made from 30 per cent hydrogen peroxide (Merck's Superoxol).

TABLE XIV

Attempted Reactivation of E. coli Lactase

Nature and amount of inhibitor	Nature and amount of reducing agent	Amount of total sugar	Hydrolysis	Ratio of activity to that of untreated enzyme
		mg. per 2 ml.	*per cent*	
None	None	0.382	51.6	1.00
0.1 ml. 10^{-3} M $HgCl_2$	None	0.250	0	0
Same	0.1 ml. 10^{-2} M KCN	0.253	0	0
Same	0.1 ml. 10^{-2} M Na_2S	0.248	0	0
0.1 ml. 10^{-3} M $AgNO_3$	None	0.252 to 0.260	0 to 3.0	0 to 0.06
Same	0.1 ml. 10^{-2} M KCN	0.256	1.6	0.03
Same	0.1 ml. 10^{-2} M Na_2S	0.251	0	0
0.1 ml. 3.3×10^{-4} M $AgNO_3$	None	0.311	23.4	0.43
Same	0.1 ml. 10^{-2} M Na_2S	0.311	23.4	0.43
0.1 ml. 10^{-3} M $CuSO_4$	None	0.262	4.0	0.08
Same	0.1 ml. 10^{-2} M KCN	0.331	31.4	0.61
Same	0.1 ml. 10^{-2} M Na_2S	0.305	21.0	0.41
0.1 ml. 10^{-2} M I_2	None	0.255	1.2	0.02
Same	0.1 ml. 10^{-2} M Na_2S	0.258	2.4	0.05
Same	0.1 ml. 9×10^{-3} M cysteine	0.262	4.0	0.08
0.1 ml. 10^{-3} M I_2	None	0.324	28.6	0.53
Same	0.1 ml. 10^{-2} M Na_2S	0.324	28.6	0.53
0.1 ml. 10^{-2} M H_2O_2	None	0.364	44.3	0.86
Same	0.1 ml. 10^{-2} M Na_2S	0.377	49.9	0.97
Same	0.1 ml. 2×10^{-2} M KCN	0.388	54.0	1.05

Similar observations with heavy metal salts have been reported recently by Winnick, Davis, and Greenberg (1940) with regard to asclepain, a protease from the latex of the milkweed *Asclepias speciosa*. This enzyme is even inhibited by the practically insoluble sulfides of silver and mercury.

In this respect *E. coli* lactase behaves differently. Equal amounts of 10^{-3}M solutions of mercuric chloride and silver nitrate and of a 10^{-2}M solution of sodium sulfide were mixed and then added to the enzyme.

Table XV gives the results obtained.

TABLE XV

Effect of Heavy Metal Sulfides on E. coli Lactase

Nature and amount of chemicals added	Amount of total sugar	Hydrolysis	Ratio of activity to that of untreated enzyme
	mg. per 2 ml.	*per cent*	
None	0.387	53.6	1.00
0.1 ml. 10^{-3} M $AgNO_3$ +0.1 ml. 10^{-2} M Na_2S	0.402	59.5	1.10
0.1 ml. 10^{-3} M $HgCl_2$ +0.1 ml. 10^{-2} M Na_2S	0.386	53.2	0.99

DISCUSSION

In its reaction course *E. coli* lactase obviously follows the general pattern of carbohydrases as best exemplified by yeast invertase.

Studies of heat inactivation and destruction by proteases indicate its protein nature, but beyond this little can be said about the active groups of the enzyme molecule responsible for the decomposition of the substrate.

It is doubtful how far Hellerman, Perkins, and Clark's theory (1933) concerning the oxidation-reduction state of urease may be applicable to *E. coli* lactase. The above investigators postulated sulfhydryl groups to be part of the active enzyme molecule. They contended that oxidation of SH groups to the dithio-stage or the formation of mercaptides with heavy metal ions led to an inhibition of the enzyme studied.

There seems to exist some analogy to their findings in the case of *E. coli* lactase. Its slight activation by reducing agents such as sulfide and cysteine and by cyanide and its readily reversible inactivation by hydrogen peroxide point to easily oxidizible and reducible radicals such as sulfhydryl groups. The same holds for yeast invertase. But if they play any rôle in this connection it is apparently of minor importance. Maybe they are protected in the lactase and invertase molecules or not as actively functional as in proteolytic enzymes. Groups other than sulfhydryl seem to be essen-

tial for enzyme action. Through them the enzyme molecule apparently forms insoluble complexes with mercury and silver ions, whereas cupric ions are bound in a looser combination.

As for the action of iodine one might speculate on the formation of addition compounds such as have been suggested by Herriott (1936) in the case of pepsin. He was able to isolate diiodo-tyrosine from pepsin that had been inactivated previously by treatment with iodine. On the other hand, he noticed no appreciable oxidation of the enzyme by iodine.

SUMMARY

A "lactase solution" was prepared from *Escherichia coli*. The mechanism of its action has been studied and changes in the rate of hydrolysis under various conditions investigated.

The hydrolysis of lactose by the enzyme approximates the course of reaction of the integrated Michaelis-Menten equation. One molecule of enzyme combines with one molecule of substrate.

E. coli lactase is readily inactivated at pH 5.0, and its optimal activity at 36°C. is reached between pH 7.0 and pH 7.5.

The optimal temperature for its action was found to be 46°C. when determinations were carried out after an incubation period of 30 minutes.

Its inactivation by heat follows the course of a first order reaction, and the critical thermal increment between the temperatures of 45°C. and 53°C. was calculated to be 56,400 calories per mol.

The enzyme is activated by potassium cyanide, sodium sulfide, and cysteine, and irreversibly inactivated by mercuric chloride, silver nitrate, and iodine.

After inactivation with copper sulfate partial reactivation is possible, while the slight inhibition brought about by hydrogen peroxide is completely reversible.

The possible structure of the active groups of *E. coli* lactase as compared with other enzymes has been discussed.

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