STUDIES ON BACTERIAL ENZYMES.

I. MENINGOCOCCUS MALTASE.

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INTRODUCTION.

The fermentation of maltose is one of the most important characters employed in the differentiation of meningococci from some of the saprophytic cocci found in the respiratory tract. It is of importance, therefore, to determine whether maltose hydrolysis can be effected by enzymes liberated from the bacterial cells. In the present paper this question has been answered by an investigation of the maltose-splitting capacity of sterile filtered solutions of the intracellular substances of meningococci.

Measurement of the action of maltase involves an estimation of glucose in the presence of maltose. Since both are reducing sugars, glucose in such mixtures cannot be estimated by the usual, simple or direct copper reduction methods. Polarimetric determination of glucose requires special apparatus not always at hand in the bacteriological laboratory. Barfoed's method is applicable to the detection of glucose in the presence of maltose, but requires rigid controls especially when the amount of maltose greatly exceeds the glucose. Preliminary tests by this method on mixtures of the bacterial enzyme and maltose did not prove satisfactory. Precise readings were difficult to make because of the voluminous precipitation of the phosphate in the test solutions by the acetic acid in Barfoed's reagent.

Since only qualitative estimations were desired, a biological method was employed, which is based upon the acid fermentation of glucose by bacteria which have little or no action upon maltose.

EXPERIMENTAL.

Methods.

Preparation of Meningococcus Enzyme Solutions.—The enzyme solution was prepared from a strain of normal meningococci (Type II Gordon) furnished by Dr. Elliott S. Robinson of the Massachusetts Antitoxin and Vaccine Laboratory.

Meat infusion agar enriched by the addition of Avery's (1) yeast extract was inoculated heavily with young meningococci suspended in infusion broth. Luxuriant growth was always obtained within 12 to 18 hours; the meningococcus cells were then scraped off the surface of the agar and suspended in sterile 0.1 M phosphate (pH 7.5). Since the medium in which the bacteria were suspended included the broth introduced with the original inoculum, the final solution in which the bacteria were extracted contained about an equal volume of infusion broth and phosphate solution. The meningococcus suspension was placed in long narrow Pyrex tubes, sealed with vaseline, and repeatedly frozen and thawed as in the preparation of sterile pneumococcus extracts (2).

The suspension of disintegrated bacteria was centrifuged at high speed for 1 hour; the supernatant was then pipetted off from the bacterial residue. To eliminate any possibility of the presence of intact meningococcus cells in the enzyme solutions, the supernatant was finally filtered through a Berkefeld candle.

Preparation of Maltose Solutions.—To avoid the hydrolysis which frequently accompanies the sterilization of dilute solutions of maltose, the sugar was heated in concentrated solutions. 30 per cent solutions of maltose were boiled for 15 minutes and then added aseptically to sterile 0.1 M phosphate (pH 6.9) in amount sufficient to give a final concentration of 3.0 per cent maltose. The final maltose solution was distributed into sterile Pyrex tubes.

The Detection of Glucose by Bacterial Acid Fermentation.—The procedure used to detect glucose by bacterial acid fermentation consisted in the following steps: (1) the preparation of hydrolysis mixtures (maltose plus meningococcus enzymes); (2) the incubation of the hydrolysis mixture to allow time for the action of the enzyme upon maltose; (3) the addition of certain glucose-fermenting bacteria to samples of the hydrolysis mixtures and measurement of the acid produced by the bacterial fermentation of glucose.

1. "Hydrolysis Mixtures."—The hydrolysis mixtures consisted of maltose solution plus unheated, active meningococcus enzyme solution and control mixtures of maltose solution plus heat-inactivated enzyme solution. These mixtures were incubated for 48 or 72 hours to allow time for the enzyme action.

2. "Fermentation Mixtures." (*a*) *Bacterial Fermenting Agents Employed.*—The bacteria used to detect the presence of glucose consisted of a strain of Shiga dysentery bacillus and an atypical strain of colon bacillus. (The strain of Shiga dysentery bacillus possessed the usual fermentation characteristics of these bacteria. The atypical strain of colon bacillus was isolated from stools by Mr. William L. Fleming. This strain, at least when first isolated, fails to ferment maltose al-

though actively attacking both glucose and lactose with prompt acid and gas formation, which is a comparatively rare combination of fermenting properties among the known intestinal bacteria.) Ideal fermenting agents for the detection of glucose in mixtures of maltose and glucose should possess the property of fermenting the hexose with acid production but should be without action upon maltose. Although it was found that concentrated suspensions of the strains used in our experiments do form small amounts of acid in maltose solutions, it simplifies the description of the method to define them as "non-maltose-fermenting bacteria." Any error from the slight fermentation of maltose by the "non-maltose-fermenting" bacteria was eliminated by the rigid controls described below.

The bacteria actually used as fermenting agents were taken from young agar cultures and were suspended in equal parts of meat extract broth and salt solution.

(*b*) *Preparation of the "Fermentation Mixtures."*—The "fermentation mixtures" were prepared as follows: An aliquot of each hydrolysis mixture, usually 0.5 cc. or 0.7 cc., was put into a series of small sterile Pyrex test-tubes. Then an equal volume of a suspension of the bacterial fermenting agent was added to each of the samples under test for the presence of glucose.

Although the reaction of the hydrolysis mixture was pH 6.9, it was our practice to adjust the initial reaction of the fermentation mixture to approximately pH 7.5. Not only is this pH nearer the optimum for acid fermentation, but with the initial reaction at pH 7.5 the production of small amounts of acid causes larger changes in pH than at pH 6.9 where the "buffer value" of the phosphate is at its maximum. A further advantage of having the more alkaline initial reaction is that smaller decreases in pH can be accurately estimated between pH 7.5 and pH 6.9 by the use of phenol red (3), than can be detected with brom-cresol purple in a zone slightly below pH 6.9. The reaction of the bacterial suspensions, therefore, was adjusted to approximately pH 8.5 which trial tests showed was sufficiently alkaline to yield a pH of 7.5 in a mixture of equal parts of the bacterial suspension and pH 6.9 (0.1 M) phosphate.

(*c*) *Incubation of "Fermentation Mixture" for Acid Fermentation of the Glucose.*—The "fermentation mixtures" (test solution plus glucose-fermenting bacteria) were shaken and then incubated in the water bath at 38°C. for 45 minutes. The factors to be considered in choice of the length of the incubation period will be considered below (Fig. 1).

(*d*) *Estimation of Acid Produced in the "Fermentation Mixture."*—0.5 cc. portions of properly diluted solutions of phenol red or of brom-cresol purple were placed in a series of small selected tubes of equal bore. Colorimetric standards were prepared by adding 0.5 cc. of buffer mixture to the appropriate indicators. 0.5 cc. portions of the centrifuged supernatants of the fermentation mixtures were then added to tubes containing the indicators. These supernatants were themselves practically water-clear and were diluted with an equal volume of the indicator solution so that accurate readings could be obtained by direct comparison with the buffer standards.

Controls on Acid Production in the "Fermentation Mixtures."—The following controls were used to limit the changes in reaction of the fermentation mixture to

the acid formed by the fermentation of glucose previously hydrolyzed by the active meningococcus enzymes.

1. Errors due to any hydrolysis of the maltose by some other agency than the active meningococcus enzyme were eliminated by a control series of heat-inactivated enzyme plus maltose. This series was incubated under the same conditions as the corresponding mixtures of active enzyme plus maltose.

2. In some of the experiments, slight differences existed in the initial pH of the different mixtures of enzyme plus maltose. This was controlled by preparing a duplicate series of "fermentation mixtures" in which heat-killed bacteria were used instead of active bacteria.

Slow Fermentation of Maltose by Shiga Dysentery Bacilli.

In the preceding description of the detection of glucose in the presence of maltose by acid fermentation, it was convenient to speak of the Shiga dysentery and atypical colon bacilli used for fermenting agents as "non-maltose-fermenting bacteria." Cultures of these strains made in maltose broth with the usual sizes of inoculum showed no reddening of Andrade indicator after 48 hours incubation. Although this would be sufficient to classify the strains as "non-maltose fermenters" in a routine study of intestinal bacilli, a gradual fermentation of maltose even under the routine conditions was indicated by a faint reddening of the indicator after 1 or 2 weeks storage of cultures at room temperature. In such cases, however, the question of maltose-fermenting capacity is complicated to some extent by the possibility of a gradual hydrolysis of maltose in dilute (1 per cent) solutions.

The question of the extent of maltose fermentation by Shiga dysentery bacilli assumed importance in determining the period of time that should be allowed for the incubation of the final fermentation mixtures of maltose and glucose with the fermenting agents. The mixture, obviously, should be incubated long enough to insure the complete fermentation of the glucose, but if maltose is slowly fermented the incubation period should not be extended too long or the acid formed from the larger amount of maltose will obscure the acid formation from the hexose. Even a slight capacity to produce acid from maltose, which, with the fewer number of cells in a routine broth culture, would result in only traces of acid, may result in a

very significant amount of acid in tests with the concentrated suspensions of bacilli used in our experiments.

Preliminary tests were made to determine the most desirable period of incubation to allow the maximum acid formation from glucose with the minimum acid production from the maltose. A typical experiment is outlined below.

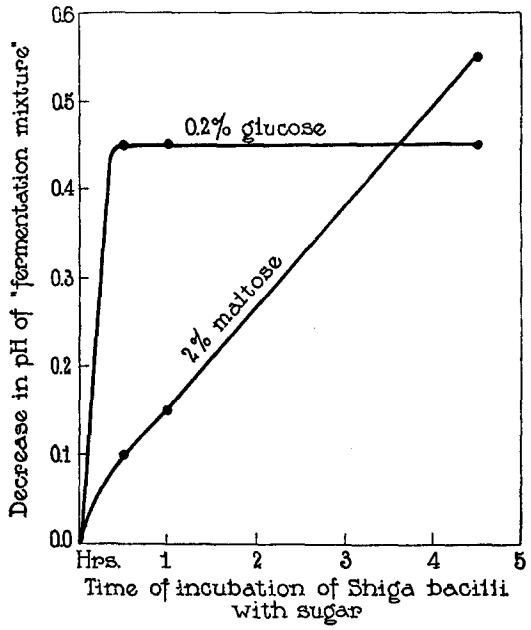

FIG. 1. "Slow fermentation" of maltose by Shiga dysentery bacilli. Relative rates of acid production by action of concentrated suspensions of the bacilli upon glucose and maltose.

Three tubes were prepared: (1) 5 cc. pH 7.6 phosphate solution plus 1.0 cc. sterile H_2O; (2) 5 cc. phosphate plus 1.0 cc. 20 per cent maltose solution; (3) 5 cc. phosphate plus 1.0 cc. 2 per cent glucose. To each of these tubes 3 cc. of concentrated suspension of Shiga dysentery bacilli were added. The final mixtures, therefore, represented "fermentation mixtures" of the same initial pH and the same buffer value containing respectively: (1) no sugar, (2) 2 per cent maltose, (3) 0.2 per cent glucose.

The mixtures were shaken and placed in the water bath. Samples were removed and pH measurements made after centrifugation at the end of 30 minutes, 1 hour, and 4 hours. The results are given in Fig. 1.

Fig. 1 illustrates the differences in rates of fermentation of glucose and maltose by our strain of Shiga bacilli. It is seen that glucose is fermented extremely rapidly, since the maximum acidity was reached within 30 minutes. Maltose, on the other hand, was fermented slowly as only a slight increase in acidity (0.1 pH) was evident at the time the maximum amount acid had been formed from the glucose. The continued, although gradual, increase in acidity in the 2 per cent maltose mixture (Fig. 1) emphasizes the necessity of avoiding prolonged incubation periods since it is obvious that the acid formed from traces of glucose would in time be obscured if an excess of maltose is present in the system.

From the relations revealed in this and other preliminary experiments it was concluded to adopt 45 minutes as the period of incubation of the test "fermentation mixtures." This period was greater than that required for the complete fermentation of the glucose with concentrated suspensions of the fermenting agents. In all cases, therefore, it was necessary to control the slight amount of acid produced from the maltose by the methods previously described.

All that is desired here is to establish the conditions for detection of glucose in the presence of maltose. Whether the "slow" fermentation of maltose is due to the presence of a small proportion of maltose-fermenting variants, or to a very slight maltose-fermenting capacity inherent in all dysentery bacilli, is of relatively little importance. A slight acid formation by Shiga bacilli in maltose solutions has been ascribed by some investigators to traces of glucose resulting from the heating of maltose during sterilization, or from a gradual hydrolysis in media which have been stored for a long time before use. Fig. 1, however, shows clearly that this explanation does not hold for the gradual fermentation of maltose by our strain. The differences in the form of the curves of acid production in the glucose and maltose can be due only to differences in rates of fermentation of the two sugars, since the curves would be similar in nature if the acid production in the "maltose solution" were due to traces of glucose. The above results, therefore, are also of importance as evidence that the production of small amounts of acid in maltose solutions by at least some strains of Shiga dysentery bacilli is a definite property which certainly cannot be explained by the presence of traces of glucose in the sterilized maltose.

Activity of Meningococcus Maltase and the Influence of Concentration of the Enzyme Solution upon Glucose Formation.

In the following experiment, the maltose-splitting activity of sterile, filtered solutions of meningococci is tested by the biological method just described. The relation of the degree of maltose hydrolysis to the concentration of the bacterial enzyme is demonstrated by tests made upon mixtures containing different concentrations of the meningococcus cell solution. The protocol of the experiment is summarized in Table I.

TABLE I.

Influence of Concentration of Enzyme upon Glucose Formation by Meningococcus Maltase.

Hydrolysis mixture — Amount of enzyme solution added to maltose solution		Hydrolysis mixture treated with the Fleming strain of colon bacilli	Hydrolysis mixture treated with Shiga dysentery bacilli	Change in pH due to fermentation of the glucose previously formed by meningococcus maltase enzyme (determined by fermentation with)	
		Unheated bacilli	Unheated bacilli	Fleming strain of colon bacilli	Shiga dysentery bacilli
cc.		*pH*	*pH*	*pH*	*pH*
2.0	Active enzyme	6.3	6.2	0.9	1.0
	Heat-inactivated enzyme	7.2	7.2		
1.0	Active enzyme	6.4	6.3	0.7	0.8
	Heat-inactivated enzyme	7.1	7.1		
0.5	Active enzyme	6.8	6.8	0.3	0.3
	Heat-inactivated enzyme	7.1	7.1		
0.2	Active enzyme	7.0	7.0	0.1	0.1
	Heat-inactivated enzyme	7.1	7.1		
0.1	Active enzyme	7.1	7.1	0.0	0.0
	Heat-inactivated enzyme	7.1	7.1		
0.0	(Control)	7.1	7.1	0.0	0.0

The results presented in Table I demonstrate that meningococci possess an enzyme which hydrolyzes maltose. The glucose formed

by the splitting of the maltose was detected by the formation of acid by glucose-fermenting bacteria. Too many factors are involved for one to attempt to show a precise quantitative relation between the different pH increments and the actual amount of glucose formed. However, the fact that the largest amounts of acid were formed in the mixtures in which the enzyme concentration was greatest, indicates that there existed a direct relation between the concentration of the meningococcus maltase and glucose formation.

TABLE II.

Comparison of Action of Meningococcus Enzyme Solution upon Sucrose, Raffinose, and Maltose as Indicated by Changes in Reactions.

Hydrolysis mixture		Change in pH due to fermentation of the hexose previously formed by meningococcus enzyme
		pH
Maltose	Active meningococcus enzyme solution	0.9
	Heat-inactivated meningococcus enzyme solution	0
Sucrose	Active meningococcus enzyme solution	0
	Heat-inactivated meningococcus enzyme solution	0
Raffinose	Active meningococcus enzyme solution	0
	Heat-inactivated meningococcus enzyme solution	0

Action of Meningococcus Enzyme Solution upon Sucrose and Raffinose.

The preceding experiment has demonstrated that meningococci possess a maltase enzyme. Since the method of demonstration of the maltase was indirect, it seemed important to apply the same biological method to solutions of sucrose and raffinose, neither of which is fermented by meningococci.

Solutions of sucrose and raffinose were prepared like the maltose solution. 2 cc. of active enzyme were added to 3 cc. of each of the sugar solutions; a second series was prepared with the same amount of heat-inactivated enzyme. The hydrolysis mixtures were incubated at 37°C. for 72 hours. The protocol of the experiment is summarized in Table II.

From Table II, it is seen that no acid is formed by hexose-fermenting Shiga bacilli when added to previously incubated mixtures of meningococcus enzymes and sucrose or raffinose solutions. This fact proves that the meningococcus cell solution hydrolyzes neither sucrose nor raffinose. Since it is commonly assumed that the inability of bacteria to ferment certain disaccharides or trisaccharides is due to their inability to hydrolyze the sugar, the above demon-

TABLE III.

Heat Lability of Meningococcus Maltase.

Hydrolysis mixture — Maltose solution plus meningococcus enzyme solution		Hydrolysis mixture treated with Shiga dysentery bacilli	Change in pH due to the fermentation of the glucose previously formed by action of the meningococcus maltase	
Treatment of enzyme solution	Amount of enzyme solution		2.0 cc. enzyme	1.0 cc. enzyme
	cc.	*pH*	*pH*	*pH*
Unheated	2.0	6.2	0.9	0.6
	1.0	6.4		
Heated 10 min. at 45°C.	2.0	6.6	0.5	0.3
	1.0	6.7		
Heated 10 min. at 50°C.	2.0	6.8	0.3	0.1
	1.0	6.9		
Heated 10 min. at 55°C.	2.0	7.1	0.0	0.0
	1.0	7.0		
Heated 10 min. at 60°C., 65°C.; 70°C.; 80°C.; 100°C.	2.0	7.1	0.0	0.0
	1.0	7.0		

strated absence of sucrase and raffinase is to be expected. However, the fact that significant increases in acidity occur only in the maltose solution treated with enzyme is important as evidence of the validity of the described biological method as a means of demonstrating a bacterial maltase enzyme.

Heat Lability of Meningococcus Maltase.

The following experiment was made to determine the relative degree of heat lability of meningococcus maltase.

Tubes of enzyme solution, sealed with vaseline to prevent oxidation at the higher temperatures, were immersed in a water bath that was constantly stirred, and heated for 10 minutes at the following temperatures: 45°, 50°, 55°, 60°, 65°, 70°, 80°, and 100°C. Samples of 2.0 cc. and 1.0 cc. of each of the heated enzyme solutions were added to separate tubes containing 3 cc. of 3 per cent maltose. The hydrolysis mixtures were incubated for 72 hours at 37°C. and were then tested for glucose by the fermentation method used in the preceding experiments. The protocol is summarized in Table III.

It is evident from the results of this experiment (Table III) that meningococcus maltase is entirely inactivated by 10 minutes exposure to 55°C. Since Table I has shown that the concentration of active enzyme is related to the amount of acid produced by the glucose-fermenting bacteria, differences in the final acidity offer a rough index of the relative amount of enzyme remaining active after the various heatings. When analyzed from this standpoint, the above results show that the enzyme is destroyed to some extent when heated to temperatures lower than 55°C. A significant loss in activity occurs during 10 minutes exposure at 45°C., and only a small proportion of the maltase remains active after 10 minutes at 50°C. Thus, this enzyme, like the carbohydrate-hydrolyzing enzymes of pneumococci (4) is an extremely heat-labile substance.

Endocellular Nature of Meningococcus Maltase.

The method of preparation of the meningococcus derivatives which we have studied indicates that the maltase is an endocellular substance freed when the bacterial cell is disrupted. However, to obtain actual proof of its endocellular nature the following comparison was made of the maltase activity of the supernatant fluid of a broth culture with the activity of a solution prepared from the centrifuged cells of the same culture.

1 liter of a 24 hour yeast extract broth culture of meningococci was centrifuged, and the supernatant removed from the bacterial sediment.

The meningococcus cells were collected and subjected to the freezing and thawing process. Both the supernatant fluid and the cell solution were finally filtered through a Berkefeld filter. The comparison of the maltase activity of the two preparations was made by the procedure used in the preceding experiments.

The results are summarized in Table IV.

The results of this experiment (Table IV) prove that meningococcus maltase is an endocellular substance, since maltase activity is exhibited by sterile solutions prepared from the bacterial cells and not by the supernatant or filtrate of the unautolyzed broth

TABLE IV.

Endocellular Nature of Meningococcus Maltase.

Hydrolysis test mixture	Change in pH due to fermentation of glucose formed by meningococcus maltase	Maltase activity
Maltose plus filtered solution of meningococcus cells	0.8	+
Maltose plus filtrate of broth culture of meningococcus	0.0	−

culture. Needless to say, the endocellular nature of the enzyme does not preclude the possibility of a demonstration of maltase activity by filtrates of broth cultures in which considerable cell autolysis has occurred (5).

DISCUSSION.

Studies on the enzymes of pathogenic bacteria are of more than academic interest. The life and growth of the bacteria, either in artificial culture or in the infected host, are dependent upon their metabolic functions, most of which in turn depend upon the action of enzymes. Knowledge concerning the enzymes leads to a better understanding of the mechanism of the biochemical processes exhibited in the test-tube and of the changes accompanying the growth of the bacteria in the body and the factors concerned in the satisfaction of their nutritional needs. The endocellular nature of many of the enzymes introduces a further point of interest since these enzymes must be considered as actual constituents of the bacterial cell which, in the case of pathogenic microorganisms, would be liberated into the tissues of the host if lysis of the bacteria occurs at any stage of the infection.

The results of the experiments presented in this paper demonstrate the maltose-hydrolyzing activity of filtered solutions of the intracel-

lular substances of meningococci. Hence, the splitting of maltose, the first step in the acid fermentation of this sugar by living meningococci, can be referred to a maltase enzyme the activity of which is independent of the presence of intact bacterial cells. The amount of maltose hydrolysis effected by the meningococcus maltase bears a direct relationship to the concentration of the bacterial enzyme. The maltase of meningococci, like the carbohydrate-splitting enzymes of pneumococci, is an extremely heat-labile substance. It is inactivated by prolonged exposure to 45°C., and is entirely destroyed by a short exposure to 55°C.

SUMMARY.

Meningococci possess an endocellular maltase enzyme. The splitting of maltose by this enzyme has been demonstrated by a biological method based upon the acid fermentation of glucose by bacteria which have little or no action upon maltose.

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