STUDIES ON BACTERIAL ENZYMES.

IV. The Maltase and Lipase of the Botulinus Bacillus.

By JAMES M. NEILL, Ph.D., and WILLIAM L. FLEMING.

(From the Department of Bacteriology and Immunology, Vanderbilt University Medical School, Nashville.)

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INTRODUCTION.

The present paper reports a study of the maltase and lipase derived from *botulinus* bacilli; the activity of the enzyme was demonstrated with sterile solutions of the bacterial cells not only devoid of living bacteria but freed from cell fragments by Berkefeld filtration. The investigation was undertaken as a step toward obtaining further knowledge of the mechanism of metabolism of this toxin-forming species of bacterium. The fact that the *botulinus* bacillus is an anaerobe lends more general interest to a study of its enzymes since relatively little work has been done on the enzymes of anaerobic organisms.

Although maltase and lipase derived from different bacteria have been reported by a number of workers, no previous demonstration of these enzymes from *botulinus* bacilli appear in the literature. Several reports (1) have been made of the proteolytic action of sterile fluids obtained from broth cultures of *botulinus* bacilli; this work has been done with supernatant fluids of broth cultures. The sterile bacterial solutions used in the present study differ from the preparations of previous workers in that they contain the intracellular substances liberated when the bacterial cells are disintegrated in addition to the soluble products of growth.

EXPERIMENTAL.

Methods.

Preparation of Bacterial Extracts, or Enzyme Solutions.—The bacterial extract, or enzyme solution, was prepared from a representative, toxin-producing strain of *botulinus* bacilli (Type A) furnished by Dr. Jacques Bronfenbrenner of The Rockefeller Institute for Medical Research.

Large Florence flasks were filled aseptically with sterile meat infusion broth,

placed in the Arnold sterilizer, and steamed for 1 hour to drive out dissolved air; a heavy layer of sterile vaseline was then poured over the hot broth; the culture medium extended into the neck of the flasks so that only a small surface of the fluid required the vaseline seal for protection from air. This air-free medium was inoculated with a young culture of *botulinus* bacilli and incubated at 35°C. for 15 hours. The broth culture was then distributed into sterile, 250 cc. centrifuge bottles, sealed with vaseline, and centrifuged. The supernatant was removed and the sedimented bacilli from each 250 cc. bottle were suspended in about 5 cc. of the supernatant fluid. (The bacilli were suspended in the supernatant culture fluid because we believe that the presence of certain constituents of the broth either facilitates the subsequent dissolution of the bacteria or renders the liberated bacterial substances more soluble.)

The bacterial suspension was placed in long, narrow Pyrex tubes, sealed with vaseline, and repeatedly frozen and thawed as in the preparation of sterile pneumococcus (2) and meningococcus (3) extracts. The *botulinus* bacilli do not disintegrate easily as do pneumococci and meningococci and it was necessary to subject the suspension to about 100 repeated freezings and thawings. After this treatment, microscopic examination of the *botulinus* suspension indicated that the bacilli were well broken up, since the prepared films consisted almost entirely of bacterial detritus with only an occasional, definitely outlined bacillus. The suspension of disintegrated bacilli was centrifuged at high speed for 1 hour and the supernatant fluid was filtered through a Berkefeld candle. The filtered solution was distributed into long narrow test-tubes, sealed with vaseline, and stored at 2°C.

The sterile filtrate obtained represents an extract of the intracellular substances liberated when *botulinus* bacilli are disintegrated by repeated freezing and thawing. Since the bacilli were originally suspended in small portions of the supernatant culture fluid, these extracts contain broth and the soluble products liberated into the culture fluid during early growth of the culture, in addition to the intracellular substances derived by dissolution of the bacteria. After Berkefeld filtration, the extracts of the *botulinus* bacilli are perfectly clear and are indistinguishable in appearance from uninoculated broth. The presence of a considerable amount of dissolved protein material derived from the disintegrated bacterial cells is indicated by the heavy protein precipitation which occurs when these clear solutions are heated.

These sterile extracts of *botulinus* bacilli should contain both the exocellular and endocellular enzymes of the *botulinus* bacillus, provided that the enzymes are neither destroyed during the preparation processes nor held back by the Berkefeld filter. The term "enzyme solution," when used in the following text, refers to the bacterial extracts described above.

Sterility Controls.—After filtration, the sterility of the enzyme solution itself was proved by cultures in glucose broth. No antiseptics were added to any of the hydrolysis mixtures, since the sterility of each mixture was controlled by cultural methods.

Demonstration of the Hydrolysis of Maltose by Botulinus Enzymes.

The carbohydrate-fermenting proclivities of *botulinus* bacilli are not pronounced. While glucose is commonly fermented, there is considerable dispute in the literature concerning their fermentation of the common disaccharides, due largely to the fact that different strains of *botulinus* vary in their disaccharide fermentation. Living cultures of the strain used in this investigation fermented maltose rather slowly, and seemed completely devoid of the property of producing either acid or gas from lactose or sucrose. These fermentation properties, while not agreeing with all reports in the literature, are possessed by perhaps the majority of strains of *botulinus* (Type A), since the order of frequency of fermentation of these sugars by different strains seems to be maltose, sucrose, and lactose (4).

Since the carbohydrate-splitting enzymes of *botulinus* bacilli had never been demonstrated, it seemed of interest to determine whether or not the sterile extract prepared from the intracellular substances of the bacilli possessed the property of hydrolyzing maltose. Tests were included with sucrose and lactose as representing disaccharides not fermented by the living cultures of this strain, as well as with maltose which is slowly fermented by the living culture.

Hydrolysis Mixtures.—Sterile solutions of maltose, sucrose, and lactose were prepared as follows: 30 per cent solutions of each of the sugars 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 of the sugar. The final solutions were distributed into sterile Pyrex tubes.

The hydrolysis test mixtures were prepared by adding 2.0 cc. of the *botulinus* enzyme solution to one tube of each of the three sugar solutions. (This relatively large amount of enzyme was used because preliminary experiments had shown that the maltase action of the *botulinus* extract was weak and difficult to demonstrate.) One portion of the enzyme solution was boiled for 15 minutes to inactivate the enzymes, and a series of controls of heat-inactivated enzyme was prepared for each of the substrates.

The mixtures of enzyme solution plus the sugar substrate were incubated for 72 hours and then tested for the presence of hexoses.

Detection of the Hexose Products of the Enzyme Action.—The hydrolysis of the disaccharides was tested by the biological method described in detail in preceding papers (3, 5). The method is based upon the acid fermentation of the hexoses by bacteria which do not attack the disaccharide from which the hexoses are derived. 1 cc. of each of the test and control hydrolysis mixtures was put into a sterile Pyrex test-tube; 1 cc. of a suspension of bacteria which produce acid from hexoses but which do not attack the test disaccharide was then added to the different tubes. (An atypical strain of colon bacilli which does not attack maltose was added to the

maltose tests; Shiga dysentery bacilli which do not attack maltose, lactose, or sucrose were used for the lactose and sucrose tests was well as for the maltose series.)

These "fermentation mixtures" (equal portions of hydrolysis test mixture plus the bacterial fermenting agent) were shaken, incubated in the water bath at 38°C. for 1 hour, and then centrifuged at high speed to remove the bacteria. The production of acid in the "fermentation mixtures" was detected by colorimetric estimations of the pH of the clear supernatant fluids of each of the test mixtures. The series of controls consisting of heat-inactivated enzyme plus the test disaccharide serve to limit all changes in reaction of the fermentation mixtures to acid produced by the fermentation of hexoses previously formed by the active *botulinus* enzyme.

The protocol of the experiment is summarized in Table I.

TABLE I.

Action of Botulinus Enzyme Solution upon Maltose.

	Hydrolysis mixture	pH after action of bacteria used as fermenting agent	Change in pH due to bacterial fermentation of the hexose previously formed by *botulinus* enzyme
		pH	ΔpH[1]
Maltose	Active *botulinus* enzyme solution	7.0	0.6
	Heat-inactivated *botulinus* enzyme solution	7.6	0
Sucrose	Active *botulinus* enzyme solution	7.6	0
	Heat-inactivated *botulinus* enzyme solution	7.6	0
Lactose	Active *botulinus* enzyme solution	7.6	0
	Heat-inactivated *botulinus* enzyme solution	7.6	0

[1] ΔpH when used in Tables I to IV indicates change (decrease) in pH.

From the results of this experiment (Table I), it is evident that the *botulinus* enzyme solution contains an active maltase, which can be rendered inactive by heat. The maltose-splitting activity of the solution of the intracellular substances of the *botulinus* bacilli is relatively weak in comparison to the activity of the same type of bacterial solution prepared from either meningococci (3) or pneumococci (5). This relatively weak action of the sterile solution of the *botulinus* bacilli is paralleled by the slow fermentation of maltose by living cultures of the strain of *botulinus* from which the enzyme solution itself was prepared.

It is also evident in Table I that no acid is formed by the hexose-fermenting bacteria when added to previously incubated mixtures of the *botulinus* enzyme and lactose or sucrose solution. This fact may be accepted as proof of the absence of lactase or sucrase in the sterile extract of the *botulinus* bacilli. The failure of the sterile extract of the *botulinus* bacilli to hydrolyze either lactose or sucrose is in agreement with the inability of living cultures of the same strain to produce either acid or gas from the same disaccharides. Apparently,

TABLE II.

Splitting of Tributyrin by Sterile Extracts of Botulinus Bacilli.

Test mixture	Amount of bacterial extract	Reaction of test mixtures after 48 hrs. at 38°C.	Change in reaction due to splitting of tributyrin
	cc.	*pH*	*ΔpH*
Tributyrin plus active bacterial extract	0.8	6.3	1.2
	0.2	6.8	0.8
Tributyrin plus heat-inactivated bacterial extract	0.8	7.5	0
	0.2	7.6	0
Phosphate solution plus active bacterial extract	0.8	7.5	0
	0.2	7.6	0
Phosphate solution plus heat-inactivated bacterial extract	0.8	7.6	0
	0.2	7.7	0
Tributyrin solution plus broth	0.8	7.5	0
	0.2	7.6	0

the general rule (5) that the acid fermentation of disaccharides depends upon their preliminary hydrolysis to hexoses holds true for the anaerobic *botulinus* bacillus.

Demonstration of Lipase Action of Botulinus Enzyme Solutions.

The lipase action of the *botulinus* enzyme solution was demonstrated by tests of the production of acid from the splitting of tributyrin.

A 2 per cent solution of tributyrin prepared in 0.1 M phosphate (pH 7.8) was sterilized at 120°C. and 5.0 cc. portions of the sterile solution distributed into sterile tubes; 0.8 cc. and 0.2 cc. of the enzyme solution were then added to separate

tubes of the tributyrin. Three series of controls were included to limit the production of acid to that formed through the action of the enzyme on the tributyrin; (1) phosphate solution plus unheated enzyme; (2) phosphate solution plus heated enzyme; (3) tributyrin solution plus broth. The first two series served as controls on the possibility of changes in reaction due to changes in the enzyme solution itself; the third series controlled the possibility of a spontaneous hydrolysis of the tributyrin itself during the incubation period allowed for enzyme action. A fourth control series consisting of heat-inactivated enzyme plus tributyrin solution was included to prove that the lipase was a heat-labile substance.

The hydrolysis test mixtures and the series of controls were incubated at 38°C. for 48 hours. Tests for the splitting of the tributyrin were made by determining the changes in reaction of the various mixtures. The results are given in Table II.

The results presented in Table II furnish evidence of the action of lipase derived from *botulinus* bacilli. There is a direct relation between the extent of the hydrolysis and the concentration of the sterile bacterial extract.

Resistance of Botulinus Lipase to Exposure to Air.

The lipase of Pneumococcus is susceptible to oxidation and can be inactivated by treatment with an oxidizing agent, such as hydrogen peroxide (6), but it is considerably more resistant to oxidizing agents than are the carbohydrate-splitting enzymes of the same bacteria. Since the *botulinus* bacillus is an anaerobic organism, it seemed of particular interest to determine the effect of exposure to air upon the activity of its lipase enzyme.

The question of the effect of exposure to air was investigated from two points of view: (1) the effect of exposing the bacterial extract to air before its addition to the tributyrin solution; (2) the effect of free exposure of the mixture of tributyrin and bacterial extract during the incubation period allowed for the enzyme action.

The effect of previous aeration of the bacterial extract upon its subsequent lipase activity was studied as follows: 5.0 cc. of the sterile bacterial extract were placed in a shallow layer in a 300 cc. Erlenmeyer flask; another portion of the same bacterial extract was placed in a long, narrow test-tube, and carefully sealed with vaseline. Both the aerated and the sealed bacterial extract were held at 38°C. for 24 hours. Equal portions of the aerated and sealed extract were then tested for their lipase activity as described in the preceding experiment.

The effect of exposure of the hydrolysis mixture to air during the incubation period allowed for enzyme action was tested as follows: 4.0 cc. of tributyrin solution were placed in each of a series of narrow (11 mm.) test-tubes; the same amount of tributyrin solution was placed in a series of wide (50 mm.) tubes. 1.0 cc. and 0.3 cc. of the sterile bacterial extract (not previously exposed to air) were then added to separate narrow tubes of tributyrin solution; these tubes were then carefully sealed from air by a heavy layer of sterile vaseline. The same amounts of the same enzyme solution were added to the wide tubes of tributyrin; this series was not sealed with vaseline. The two series then represented hydrolysis mixtures, one of which was protected from air by a high column of fluid overlaid with vaseline, while the other was freely exposed to air in a shallow layer. Controls with heat-inactivated enzyme solution were included for both the sealed and aerated series.

TABLE III.

Effect of Exposure to Air upon the Lipase Activity of Sterile Extracts of Botulinus Bacilli.

Amount of bacterial extract	Effect of previous aeration upon subsequent lipase activity (enzyme solution aerated in absence of substrate)		Effect of aeration of the hydrolysis mixture during incubation period allowed for enzyme action (enzyme solution aerated in presence of substrate)	
	Change in reaction due to hydrolysis of tributyrin		Change in reaction due to hydrolysis of tributyrin	
	Bacterial extract previously exposed to air for 24 hrs.	Bacterial extract not previously exposed to air	Aerated hydrolysis mixture	Sealed hydrolysis mixture
cc.	ΔpH	ΔpH	ΔpH	ΔpH
1.0	1.5	1.5	1.4	1.4
0.3	0.8	0.8	0.8	0.8

After 60 hours incubation at 38°C., the degree of hydrolysis of the tributyrin was estimated by comparisons of the changes in reaction in the different test mixtures. The results are summarized in Table III.

As shown in Table III, previous aeration of the extract of the bacterial substances was without effect upon the subsequent activity of the lipase, and likewise the presence of air during the incubation of the enzyme-substrate mixture did not influence the action of the enzyme upon tributyrin. The resistance of the lipase to aeration is in striking contrast to the susceptibility of the living anaerobic cells from which the enzyme was derived.

Exocellular or Endocellular Nature of the Maltase and Lipase of the Botulinus Bacillus.

Experiments were carried out to determine whether the maltase and lipase of *botulinus* bacilli are endocellular or exocellular. These experiments consisted in comparisons of the activities of the supernatant fluid of a young unautolyzed broth culture with the activities of a sterile extract prepared by disrupture of the centrifuged bacterial cells of the same culture.

The preceding demonstrations of the lipase and maltase of *botulinus* were made with sterile extracts of the bacilli suspended in the supernatant fluids of broth cultures. Obviously, these enzyme solutions contained both the exocellular sub-

TABLE IV.

Exocellular or Endocellular Nature of the Maltase and Lipase of the Botulinus Bacillus.

Hydrolysis test mixture		
Source of enzymes	Substrate	
	Maltose	Tributyrin
Sterile bacterial preparation containing	Change in pH due to fermentation of glucose formed by *botulinus* maltase	Change in pH due to splitting of tributyrin by *botulinus* lipase
	ΔpH	ΔpH
Only exocellular products of *botulinus* bacilli	0.0	1.0
Both exocellular and endocellular products of *botulinus* bacilli	0.5	1.0

stances liberated during the growth of the culture and the intracellular substances set free by the disintegration of the suspended bacteria. For the following experiment, it was necessary to obtain a sterile solution containing only the exocellular products which are liberated into the culture fluid during the period of active growth and before bacterial dissolution had progressed.

1. Solution Containing Only Exocellular Substances.—The fluid used as the solution of exocellular substances consisted of the supernatant fluid obtained by centrifugation of an 18 hour broth culture.

2. Solution Containing Both Exocellular and Endocellular Substances.—The bacterial solution was prepared from the sedimented bacterial cells of the same culture as that used for Solution 1. The bacilli were suspended in a small amount of the supernatant fluid as described under Methods for the preparation of bacterial extracts, and subjected to the freezing and thawing process.

Both the supernatant fluid and the bacterial solution were finally filtered

through a Berkefeld filter. The comparison of the maltase and lipase activities 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) show a difference in the nature of the maltase and lipase of *botulinus*. The maltase must be endocellular in nature, since maltose is hydrolyzed only by the sterile preparation which contains the intracellular substances of the bacilli and not by the sterile filtrate which contains only the *botulinus* products of exocellular origin. The lipase, on the other hand, seems to represent an extracellular rather than an intracellular substance, since tributyrin is split as actively by the sterile *botulinus* filtrate which contains only exocellular products as by the extract of the bacterial substances. The agreement in the extent of glyceride hydrolysis by the two *botulinus* preparations indicates that no lipase is contained within the *botulinus* bacilli themselves. Thus, while the endocellular maltase can be accepted as an actual constituent of the *botulinus* bacterial cell, the glyceride-hydrolyzing enzyme must be considered a substance of different nature which the bacillus liberates into the culture fluid during the period of active growth before bacterial autolysis occurs.

DISCUSSION.

This investigation presents experimental evidence of the maltase and lipase of the *botulinus* bacillus and thus demonstrates the enzymatic nature of two biochemical activities of this anaerobic organism. The proof of the maltase not only shows that *botulinus* bacilli possess an enzyme capable of hydrolyzing maltose in the absence of the bacteria themselves, but furnishes evidence that the capacity of these anaerobic bacilli to ferment different disaccharides depends upon their possession of a specific enzyme to hydrolyze the disaccharide to its component hexoses. The sterile bacterial extract prepared from a strain of *botulinus* which ferments maltose, but not lactose and sucrose, contains an active maltase but no lactase or sucrase.

The maltase enzyme, like the carbohydrate-splitting enzymes of certain aerobic bacteria, as pneumococci (5–7) and meningococci (3), is endocellular in nature, and hence is an actual constituent of the *botulinus* bacterial cell. The lipase, on the other hand, is an extracellular substance, and is extruded into the culture fluid during the period of growth of the bacteria. The lipase of *botulinus* differs in this respect from the endocellular one of Pneumococcus (6, 7) but agrees with those from a number of other aerobic bacteria (8) which also are exocellular in nature.

The results of the experiments presented show that the lipase of *botulinus* is resistant to exposure to air. It was found that 24 hours

aeration of the bacterial extract at 38°C. had no detectable effect upon the subsequent activity of this enzyme; similarly, free exposure to air of the enzyme-substrate mixtures during the incubation period allowed for enzyme action did not seem to affect its activity. The indifference to air which is shown by the heat-labile lipase of *botulinus* is in striking contrast to the susceptibility of the living bacillus. If oxygen be considered toxic to anaerobes, the resistance to oxidation shown by the lipase of these anaerobic bacilli is analogous in some respects to other known examples of the relative resistance of individual cell products to deleterious influences which are toxic to the living cell as a whole. Frequent examples are known of enzymes of aerobic bacteria which withstand heating treatment which kills the cell itself; similarly, concentrations of disinfectants which promptly end the life of the bacterial cell as a whole, often have but a slight inhibitory effect upon many of the enzymes derived from the same bacteria. Nevertheless, in spite of the frequency with which somewhat similar phenomena are encountered, it is of considerable interest to observe that the fat-hydrolyzing enzyme of this anaerobic bacillus, after its elaboration, is apparently indifferent to conditions of oxygen tension which render impossible the growth of the bacilli from which the enzyme is derived.

SUMMARY.

Botulinus bacilli yield a maltase and a lipase, which retain their hydrolytic activity independent of the presence of the formed bacterial cell. The maltase is an endocellular substance, while the lipase is extracellular. The lipase, when separated from the anaerobic bacillus, does not seem to be affected by exposure to air.

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