

DECOMPOSITION OF THE CAPSULAR POLYSACCHARIDE
OF PNEUMOCOCCUS TYPE III BY A
BACTERIAL ENZYME

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It is now generally recognized that one of the important differences between the two variants of Pneumococcus, the so called R and S forms, is the presence around the latter of a capsule which has been shown to contain a complex polysaccharide; the type specificity of Pneumococcus and the virulence of the S cells are associated with the presence of this capsular polysaccharide which is referred to as the soluble specific substance; the chemical structure of the capsular polysaccharides has been shown to vary from one type of Pneumococcus to another (1); in fact, they are as chemically distinct one from the other as they are serologically specific.

The fact that the capsular polysaccharides of Pneumococcus—and not some impurities carried along with them—are themselves the substances responsible for type specificity, has been shown by the disappearance of their reactivity in the specific antisera after they had been subjected to acid hydrolysis. However, this treatment is a fairly drastic one and may have affected at the same time the hypothetical impurities. It was thought that such an objection would be removed if the polysaccharide could be split by the milder action of an enzyme.

It was interesting also to determine whether the addition of such an enzyme to a medium seeded with the encapsulated pneumococci would effect a dissolution of the capsule. From this point of view the observations of Toenniessen (2) are of special interest. He found that when *Bacillus vulgatus* was seeded together with encapsulated Friedländer bacilli, the latter organisms grew deprived of their capsule. Finally, we had in view experiments to determine whether the injection into the animal body of an enzyme capable of decomposing the cap-

sular polysaccharide would in any way alter the course of experimental infection induced by *Pneumococcus* of the same type as that from which the specific substance had been derived.

A systematic search for specific enzymes of this order has been carried on in this laboratory for several years. A number of enzymes from animal and plant sources, known to be active in the hydrolysis of simpler carbohydrates, were tested, but none of them were found capable of attacking the polysaccharides of pneumococcus origin. In addition, cultures of various molds, yeasts, soil actinomycetes, and bacteria, many of which were known to decompose cellulose and other complex carbohydrates, were tested without success.

Recently, however, a microorganism has been isolated, and an enzyme extracted from it, both of which are capable of decomposing the capsular polysaccharide of Type III *Pneumococcus* (3). The object of this first paper is to describe the technique of isolation, the morphological, cultural, and biological characteristics of the organism; and to define the nature and mode of action of the enzyme derived from the bacterial cells.

EXPERIMENTAL

1. Inoculation Material.—As mentioned previously, many organisms known to decompose actively various polysaccharides were tried and were found unable to decompose the capsular polysaccharides of *Pneumococcus*. Since these specific substances have many of the properties of hemicelluloses, an attempt was made to search in a natural environment for organisms possessing the capacity to split complex substances of this nature. It was thought that those locations where large amounts of organic materials,—especially belonging to the group of “hemicelluloses,”—accumulate and undergo decomposition, were the most likely to harbor the desired organisms. Among the materials tried were leaf mold, composts (of corn cob, rye straw, sphagnum, oak leaves, etc.), farm manure, soils rich in organic matter (different peat soils and soils heavily manured).¹ From a

¹ Many of these materials and especially the peat samples from the cranberry bogs of New Jersey were supplied by Dr. S. A. Waksman and Dr. R. L. Starkey of the Department of Microbiology of the New Jersey Experiment Station to whom we extend our heartiest thanks for their courtesy and cooperation.

sample of soil from the cranberry bogs of New Jersey a microorganism has been isolated which is capable of decomposing the capsular polysaccharide of Pneumococcus Type III.

2. *Medium*.—The mineral medium used was based on one previously described for the isolation of cellulose-decomposing bacteria (4).

Ammonium sulfate (1 gm. per liter) was used as a source of nitrogen, dibasic potassium phosphate (2 gm. per liter) as buffering agent and source of phosphate, tap water supplied traces of the other mineral elements. The reaction of the medium was adjusted to the proper pH with HCl and NaOH. To this mineral solution, the capsular polysaccharide of Type III Pneumococcus was added in final concentrations varying from 0.001 to 0.2 per cent. This substance was the only source of organic carbon in the medium.

The soluble specific substance used in these experiments was prepared from a strain of Type III Pneumococcus by the method previously described (1, 5). This nitrogen-free preparation yields on hydrolysis a mixture of aldobionic acid and glucose. However, the molecule seems to be built up exclusively of aldobionic acid groups, the glucose appearing only as a secondary product of hydrolysis (6).

The use of this simple and specific medium was dictated by the following considerations.

(a) It was possible that the materials used for inoculation contained organisms *potentially* capable of decomposing the specific substance but for which other nutrients would act as more readily available sources of energy. Such organisms would attack the specific substance when deprived of any other food but leave it untouched in a complex medium. In fact, the production of an enzyme is often the result of what has been termed a "starvation" phenomenon. For example, Brown and Morris (7) found that the secretion of diastase by germinating barley seeds is inhibited by the presence in the medium of sugars which can be utilized directly by the growing plantlet. In a review on bacterial enzymes, Waksman has cited many similar examples which occur in the microbial world (8). Wortman (9) for instance, found that a certain bacterium had the power of excreting a starch-dissolving enzyme when starch was the only available food, but that no secretion of enzyme occurred if sugar or tartaric acid was offered to the organism along with the starch.

(b) The material used for inoculation was of course a mixture of a

great variety of microbial species. A medium containing the specific substance as sole source of carbon rendered conditions favorable only for these organisms capable of utilizing the specific substance itself or the products of its decomposition. This procedure afforded a means of rapidly eliminating a large number of irrelevant species.

(c) The ultimate object of the work was the preparation of an enzyme specifically directed against the capsular polysaccharide. For study of the action of the enzyme on the growth of *Pneumococcus*, and on the course of pneumococcus infection in experimental animals, it was especially desirable to have a preparation as poor as possible in proteolytic activity. From this point of view, it was expedient to use a mineral source of nitrogen instead of peptone or protein.

3. *Technique of Isolation.*—In order to eliminate as many as possible of the organisms which although unable to attack the specific substance may grow on the products of its decomposition or on the bacterial bodies, transfers were made as soon as growth could be detected in the cultures, in the hope that the first organisms to develop would be the ones attacking the specific substance and that in young cultures they would outnumber the others which could then be eliminated by diluting the inoculum.

4. *Serological Method for Following the Disappearance of the Specific Substance.*—The presence or absence of the specific substance in a culture was tested by the precipitin reaction:

0.5 cc. of fluid to be tested was added to 0.2 cc. of Type III antiserum² and the mixture brought to a volume of 1 cc. by the addition of salt solution. Since the precipitation test gives a positive result with a concentration of specific substance as low as 1:5,000,000, the absence of a positive precipitin reaction was interpreted as evidence of complete decomposition of the specific substance.

The Organism

1. *Isolation.*—The presence in a sample of soil from the cranberry bogs of New Jersey, of an agent capable of decomposing the Type III specific substance, is demonstrated in the following experiment.

² The Type III antipneumococcus serum used in these experiments was furnished through the courtesy of Dr. A. B. Wadsworth, Director of the Division of Laboratories of New York State Department of Health.

Experiment 1. The Effect of Environmental Conditions (pH, Temperature, Aeration) on the Decomposition of the Capsular Polysaccharide by an Agent Present in Peat Soil.—Different lots of basic mineral medium containing 0.002 per cent of Type III specific substance were adjusted to pH 4.5, 5.0, 5.5, 6.2, 6.6, 7.0, 7.8, and 8.5. Each one of these media was divided into three small Erlenmeyer flasks (25 cc. per flask) and into three Noguchi tubes (10 cc. per tube). Each of the tubes and flasks was inoculated with about 0.5 gm. of peat soil (from the cranberry bogs of New Jersey). The Noguchi tubes were incubated under anaerobic conditions (Brown jar) and the Erlenmeyer flasks under aerobic conditions. In each instance one set was kept at room temperature (about 22°C.), a second set at 37.5°C., and a third set at 54°C.

The cultures, in mineral media at different hydrogen ion concentrations and incubated at different temperatures under aerobic and anaerobic conditions, were tested from time to time for the presence of the specific substance. The specific precipitin reaction became negative first in the aerobic flask at pH 7.8, after 24 days incubation at 37.5°C. Within the following 10 days, the test became negative also in all the aerobic cultures at pH 6.2, 6.6, 7.0, and 7.8 at room temperature and 37.5°C. The specific substance did not disappear in the other flasks nor in the anaerobic tubes, even after 2 months incubation.

These results pointed to the existence of an agent capable of decomposing or removing from solution the specific substance between pH 6.2 and 7.8, at room temperature and 37.5°C., under aerobic conditions. That it was a living agent was shown by the fact that it could be transferred in series.

Since we had in view the use of this agent in the animal body, it was advisable to incubate the cultures at 37.5°C. in a medium of approximately neutral reaction; these conditions approach those present in the animal body and had been found in Experiment 1 to be close to the optimum for the activity of the microorganism.

Experiment 2. Attempts to Increase the Activity of the Culture by Repeated Transfers in Specific Medium.—Test tubes containing 10 cc. of the synthetic medium adjusted to pH 7.5 were inoculated with material from the aerobic flask in which the specific substance had first disappeared in Experiment 1. The incubation was carried on at 37.5°C., aerobically.

In this first transfer, the specific substance disappeared in 10 days. The culture was carried in the same medium for several months, transfers being made as soon as the specific substance had been decomposed,

in the hope of increasing the activity of the culture. In fact, after repeated transfers for 6 months, decomposition of 0.002 per cent of specific substance could be obtained regularly in 24 hours provided a young culture was used.

Experiment 3. Attempts to Purify the Mixed Culture by the Dilution Method.—A tube of synthetic medium was inoculated with a loopful of an active culture. 8 hours later stained films showed a fairly abundant growth of many different kinds of bacteria. At this time transfers were made into a series of tubes containing fresh mineral medium, using the following inocula: 0.1, 0.01, 0.001, 0.0001, 0.00001, and 0.000001 cc.

After 18 hours incubation at 37.5°C. stained preparations of these cultures showed that growth had taken place in the first four tubes of the series. Serial transfers were immediately made from the fourth tube (0.0001 cc. inoculum) into fresh medium, using seedings varying from 0.1 to 0.00001 cc. of culture. 11 hours later growth was recognized (by microscopic examination) in the tube which received 0.001 cc. inoculum and from this culture serial transfers were again made.

The technique of purification by the dilution method was continued for 10 days. Each transfer was kept long enough to make sure that the active culture was carried along, as demonstrated by decomposition of the specific substance in 1 to 5 days, the time depending upon the size of the inoculum. Microscopic examination of a young culture of the last of the serial transfers, revealed the presence of only three morphological types of bacilli, a very small Gram-negative rod, a short plump Gram-negative rod, and a large Gram-positive rod. The impure culture thus obtained was plated in the hope of separating these three organisms, but unfortunately one of them was a spreader which overgrew the two other species.

Experiment 4. The Use of Gentian Violet in the Isolation of the Specific Organism from Mixed Cultures.—An active culture was seeded into mineral medium to which had been added 0.001 per cent of gentian violet in the hope that the bacteriostatic action of the dye would inhibit the development of the Gram-positive organism (10). 4 days later, the specific substance could no longer be detected in the culture containing gentian violet and microscopic examination showed the presence of only the two Gram-negative organisms, and a few large spores, apparently belonging to the smaller bacillus.

In the hope that the spore-bearing bacillus was the active form in the mixture, the following experiment was carried out.

Experiment 5. Isolation in Pure Culture of the Specifically Active Spore-Forming Bacillus by Heating the Inoculum at 70°C.—0.2 cc. of the culture just described was inoculated into two tubes of mineral medium containing 0.002 per cent of specific substance; one tube was then heated at 70°C. and the other at boiling temperature for 15 minutes.

After 7 days incubation at 37.5°C. the specific substance had been decomposed in the tube heated at 70°C. but it remained unaffected in the tube which had been boiled. Transfers made from the culture heated at 70°C. grew and decomposed 0.002 per cent of specific substance in 2 days; stained films of this culture showed what appeared to be a pure culture of a small, Gram-negative, spore-bearing rod, exhibiting metachromatic granules.

Young cultures of this organism plated on nutrient agar gave a pure growth of colonies which will be described later, whereas older cultures in the spore stage (5 to 6 days old) failed to grow on agar. Transfers were made from one of these colonies, and the Gram-negative bacillus in pure culture was carried for fifteen generations on blood agar from colony to colony. A colony from the fifteenth transfer was then inoculated into the mineral medium containing 0.002 per cent of Type III capsular polysaccharide. The latter was decomposed in 3 days, the culture showing again a pure growth of Gram-negative rods.

These experiments established the fact that the spore-bearing Gram-negative rod, and not some other agent carried along with it, is responsible for the decomposition of the specific polysaccharide of Type III Pneumococcus. For the sake of simplification, this culture will be referred to as the "S III bacillus."

2. Description of the Culture.—A morphological description of the organism is rendered difficult by its pleomorphism. In the mineral medium containing small amounts of specific substance (0.002 per cent), the organism appears as a minute Gram-negative bacillus, at times smaller than the Pfeiffer bacillus. In this medium also, spore formation takes place within the first 24 hours, and the cells are completely autolyzed by the 3rd day.

The cultural characters of the bacillus are much the same when grown in mineral medium containing larger amounts of specific substance (0.2 per cent); under these conditions the cells, however, are much larger and appear more resistant to autolysis.

The organism is Gram-negative in the mineral medium but harder to decolorize when grown in plain broth or on agar. At all stages, the cultures exhibit metachromatic granules, and in older cultures these granules appear as chains of minute coccus forms within the empty cell

membrane. The organism grows diffusely in peptone solution. In this medium sedimentation of the growth occurs after several days. On plain nutrient agar, free of dextrose, growth occurs in the form of small whitish colonies, 2 mm. in diameter, circular, slightly raised, umbilicated, with entire edge and fairly smooth surface. When grown in plain broth or in peptone solution, the organism appears as a fairly large bacillus, actively motile by means of peritrichous flagellae, the young cells measuring 2 to 3μ by 0.5μ . Short chains and especially diplo-forms are often observed. Organisms growing in this medium do not autolyze readily; in fact it is difficult to cause disintegration of the cells even by repeated freezing and thawing. Older cultures show the presence of elongated, thread-like involution forms and spores appear in 4 to 5 days. They are polar, oval, and very much larger than the rods. The heat resistance of the spores was measured in the following experiment.

Experiment 6. Heat Resistance of Spores of the S III Bacillus.—Tubes containing 5 cc. of casein peptone broth were inoculated with 0.2 cc. of a 5 day old culture of the bacillus. The inoculated tubes were heated at different temperatures for different lengths of time as indicated in Table I; the presence or absence of growth after 10 days incubation at 37.5°C . is indicated in the same table.

The results of Experiment 6 indicate that the spores of the organism resist heating for 30 minutes at 75°C ., but are killed by boiling for 5 minutes.

The fermentative ability of the specific organism was determined by growth in the basic mineral media to which various sugars, glucosides, and alcohols were added in concentration of 1 per cent. No gas was formed in any of these media and slight production of acid was observed only in the presence of dextrin, galactose, lactose, maltose, salicin, and trehalose.

Of particular interest is the action of glucose on the growth of this organism. It has been found that the addition of this sugar, to an otherwise favorable medium, exerts a decidedly inhibiting action on the development of growth.

In all media thus far tested, the organism is strictly aerobic, no growth occurring under anaerobic conditions.

In media containing, in addition to the capsular polysaccharide, other nutrients such as peptone, the decomposition of the specific

substance is much delayed. The delayed decomposition under these conditions is probably attributable to the so called sparing action of the peptone, which serves as a more readily available source of energy. As an illustration of this fact, it may be mentioned that, in peptone broth and in mineral medium containing a concentration of capsular polysaccharide equivalent to that naturally present in an autolysate of a bouillon culture of Type III Pneumococcus, the rate of decomposition of the specific substance is much slower in both the peptone broth and the autolysate than it is in the mineral solution, although growth develops more abundantly in the peptone-containing media.

TABLE I
Heat Resistance of the Spores of the S III Bacillus

Time of exposure	Growth in casein peptone broth after heating inoculum as indicated						
	70°C.	75°C.	80°C.	85°C.	90°C.	95°C.	100°C.
<i>min.</i>							
5	+	+	+	+	+	+	-
10	+	+	+	-	-	-	-
15	+	+	-	-	-	-	-
20	+	+	-	-	-	-	-
30	+	+	-	-	-	-	-

+ Refers to presence of growth after 10 days incubation.

- Refers to absence of growth after 10 days incubation.

It was of special interest to test the action of the organism on the specific polysaccharides of other types of Pneumococcus and on other polysaccharides of bacterial and plant origin.

Experiment 7. Specificity of the Action of the S III Bacillus.—The specific polysaccharides of Pneumococcus Types I, II, and III, Friedländer bacilli, Types A, B, and C (11), and *Hemophilus influenzae* Type *a* (12), and gum arabic were added to the basic mineral medium at pH 7.5, inoculated with 0.1 cc. of a young culture and incubated aerobically at 37.5°C. Decomposition of the different polysaccharides was tested by the serological method, using in each instance the specific antiserum, except in the case of gum arabic which was tested against Pneumococcus Type III antiserum (13) (see Table II).

The results of Experiment 7 emphasize the extraordinary specificity of the action of the organism on the specific polysaccharide of Type III

Pneumococcus. It is worth noting in particular that the organism does not decompose gum arabic even though this substance reacts in Type III antiserum.

The saprophytic nature of the S III bacillus is shown by the fact that 1 cc. of a young active culture may be injected intraperitoneally into a mouse without affecting the animal.

The Enzyme

The preceding experiments have established the existence of a microorganism which during growth in a synthetic medium breaks

TABLE II
Specificity of the Action of the S III Bacillus

Origin of the polysaccharide	Concentration of polysaccharide	Specific precipitin reaction of cultures after incubation at 37°C. for	
		1 day	30 days
	<i>per cent</i>		
Pneumococcus Type I.....	0.002	+++	+++
Pneumococcus Type II.....	0.002	+++	+++
Pneumococcus Type III.....	0.002	—	—
Friedländer's bacillus Type A.....	0.002	+++	+++
Friedländer's bacillus Type B.....	0.002	+++	+++
Friedländer's bacillus Type C.....	0.002	+++	+++
<i>Hemophilus influenzae</i> Type a.....	0.02	+++	+++
Gum arabic.....	0.01	+++	+++

+++ = marked precipitate formed in the corresponding specific antiserum.

— = negative precipitin reaction, indicating complete decomposition of the polysaccharide.

down the capsular polysaccharide of Type III Pneumococcus. It seemed possible that from cultures of this bacillus a soluble enzyme might be extracted which would decompose the specific substance in the absence of the bacterial cells.

Experiment 8. The Extraction of a Soluble Enzyme Capable of Decomposing the Specific Polysaccharide of Pneumococcus Type III.—The mineral medium (pH 7.5) containing 0.01 per cent of Pneumococcus Type III specific polysaccharide was seeded with a heavy inoculum of the S III bacillus and incubated at 37.5°C.

The specific substance was completely decomposed after 24 hours incubation;

at this time, when microscopic examination showed large numbers of well formed cells with only a few spores, 10 cc. of the culture were removed and passed through an N Berkefeld filter. The filtrate was designated Preparation 3-*a*. After 5 days further incubation, microscopic examination of the original culture showed that all the cells were lysed; the autolyzed culture was then passed through an N Berkefeld filter and the filtrate designated Preparation 3-*b*. Test tubes containing 1 cc. of a 0.001 per cent solution of specific substance (pH 7.5) received varying amounts of Preparations 3-*a* and 3-*b*; the mixtures were made up to a volume of 1.5 cc. and incubated at 37.5°C. for 18 hours in the presence of toluene to prevent any bacterial action. The precipitin test for the presence of specific substance was made after 18 hours incubation. The results of these tests are recorded in Table III.

TABLE III

The Decomposition of Type III Capsular Polysaccharide by Sterile Culture Filtrates of the S III Bacillus before and after Autolysis

Amount of filtrate cc.	Specific precipitin reaction of mixtures of substrate and culture filtrate	
	Before autolysis 3- <i>a</i>	After autolysis 3- <i>b</i>
0.5	+++	-
0.1	+++	+
0 (control)	+++	+++

Plus signs indicate amount of precipitate formed in Type III antiserum.

Minus sign indicates no precipitation in Type III antiserum, showing complete decomposition of the specific polysaccharide.

The results of Experiment 8 show that 1 cc. of a 0.001 per cent solution of specific polysaccharide was completely decomposed by 0.5 cc. of the filtrate of an autolyzed culture (Preparation 3-*b*). On the contrary, 0.5 cc. of the filtrate of a young culture (Preparation 3-*a*) did not affect the specific substance. It is apparent, therefore, that the soluble principle responsible for the decomposition of the specific substance is released only after lysis of the cells. This experiment also seems to justify the assumption that the S III bacillus, when grown on a synthetic medium containing the specific polysaccharide, gives rise to a soluble endocellular enzyme capable of decomposing this substance.

Several enzymes are known to be extremely heat-resistant, most of them, however, are thermolabile and it was interesting to determine

the heat resistance of the active principle responsible for the decomposition of the specific substance in autolytic extracts of the S III bacillus.

Experiment 9. Inactivation of the Specific Enzyme by Heat.—Different lots of Preparation 3-*b* were heated at 50°, 55°, 60°, 65°, 70°, and 75°C. for 10 minutes and added in amounts of 0.1 cc. and 0.5 cc. to 1 cc. of 0.001 per cent solutions of SSS III. The mixtures were incubated for 18 hours at 37.5°C. in the presence of toluene and tested at that time for the presence of the specific polysaccharide by the precipitin reaction with Type III serum (see Table IV).

TABLE IV
Heat Inactivation of the Specific Enzyme

Enzyme heated 10 min. at	Specific precipitin reaction of mixture of enzyme and substrate
°C.	
50	—
55	—
60	+
65	+++
70	+++
75	+++

Plus signs indicate amount of precipitate formed in Type III antiserum.

Minus signs indicate no precipitation in Type III antiserum, showing complete decomposition of the specific polysaccharide.

These results indicate that the soluble principle responsible for the decomposition of the capsular polysaccharide is inactivated after exposure for 10 minutes at 60–65°C.

Experiment 10. Activity of the Enzyme under Aerobic and Anaerobic Conditions.—Two tubes containing 2 cc. of a 0.02 per cent solution of SSS III received 2 cc. of active enzyme Preparation 3-*b*. One of the tubes was incubated at 37.5°C. under aerobic conditions, and the other under anaerobic conditions (Brown jar). After 24 hours incubation, the mixtures containing active enzyme and polysaccharide were tested for the presence of specific substance and it was found that the test had become negative in both tubes.

It has been shown previously that the organism is strictly aerobic and does not decompose the specific polysaccharide under anaerobic conditions. This experiment shows, however, that the soluble prin-

ciple, when extracted from the cells, decomposes the specific substance equally well under both aerobic and anaerobic conditions.

These results also indicate that the soluble principle is not of the nature of an oxidative enzyme, since there was no hydrogen acceptor in the mixture incubated under anaerobic conditions. Preliminary results, which indicate the presence of reducing sugars following the decomposition of the specific substance by the soluble principle, suggest that the action is one of hydrolysis.

It has been shown previously (Experiment 7) that of the polysaccharides thus far tested the specific substance of Type III Pneumococcus

TABLE V
Specificity of the Action of the Enzyme Derived from the S III Bacillus

Origin of the polysaccharide	Concentration of the polysaccharide	Specific precipitin reaction of enzyme substrate mixture incubated at 37°C.	
		Aerobically	Anaerobically
	<i>per cent</i>		
Pneumococcus Type I.....	0.002	+++	+++
Pneumococcus Type II.....	0.002	+++	+++
Pneumococcus Type III.....	0.002	—	—
Friedländer's bacillus Type A.....	0.002	+++	+++
Friedländer's bacillus Type B.....	0.002	+++	+++
Friedländer's bacillus Type C.....	0.002	+++	+++
<i>Hemophilus influenzae</i> Type <i>a</i>	0.02	+++	+++
Gum arabic.....	0.01	+++	+++

Plus signs indicate amount of precipitate formed in Type III antiserum.

Minus signs indicate no precipitation in Type III antiserum, showing complete decomposition of the specific polysaccharide.

is the only one to be decomposed by the S III bacillus. The following experiment demonstrates that the same extraordinary specificity applies to the activity of the enzyme in cell-free filtrates of autolyzed cultures.

Experiment 11. Specificity of the Action of Enzyme Extracted from the S III Bacillus.—To different portions of enzyme Preparation 3-*b* were added the specific polysaccharides of Pneumococcus Types I, II, and III, Friedländer bacilli Types A, B, and C (11), *Hemophilus influenzae* Type *a* (12), and also gum arabic (13). The mixtures were incubated aerobically and anaerobically and tested for the presence of the polysaccharide after 1 month incubation at 37.5°C. (see Table V).

The results of Experiment 11 indicate that the soluble principle extracted from the bacterial cells is as specific in its action as is the living organism itself, decomposing only the capsular polysaccharide of Type III Pneumococcus.

Since it was proposed to test the effect of this specific enzyme on the course of pneumococcus infection in experimental animals, it was of interest to establish the influence of normal serum on the rate of decomposition of the capsular polysaccharide by the soluble principle.

Experiment 12. The Influence of Normal Serum on the Rate of Decomposition of the Capsular Polysaccharide by the Specific Enzyme.—To each of three tubes

TABLE VI

Influence of Normal Serum on the Rate of Decomposition of the Capsular Polysaccharide by the Bacterial Enzyme

Incubation period <i>hrs.</i>	Bacterial extract + specific substance +		
	Salt solution	Rabbit serum	Beef serum
0	++++	++++	++++
1	+++	+++	+++
2	+	++	+
3	±	+	±
4	—	±	—
5	—	—	—

Plus signs indicate amount of precipitate formed in Type III antiserum.

Minus signs indicate no precipitation in Type III antiserum, showing complete decomposition of the specific polysaccharide.

containing 3 cc. of enzyme Preparation 3-*b* and 3 cc. of 0.002 per cent solution of SSS III, were added respectively 2 cc. of normal rabbit serum, 2 cc. of normal beef serum, and 2 cc. of physiological salt solution, the last serving as control. The tubes were incubated at 37.5°C. and samples of the mixtures were removed from time to time to follow the progress of the decomposition of SSS III, as measured by the specific precipitin reaction (see Table VI).

The results of this experiment indicate that the rate of decomposition of the Type III polysaccharide by the specific enzyme is not affected by the presence of normal beef or rabbit serum in the mixture.

It was of interest to devise a method for measuring the concentra-

tion or at least the comparative activities of different enzyme preparations. One possible method was to compare the rate of decomposition of a given concentration of specific substance by equal amounts of different preparations. A second method was to determine whether the minimum amount of bacterial enzyme capable of decomposing a given amount of specific substance—independently of time—would vary from one preparation to another, in other words, whether there existed a definite quantitative relationship between total amount of substrate decomposed and amount of active enzyme used. The following experiment was planned to compare the rate of decomposi-

TABLE VII

The Rate of Decomposition of Specific Substrate by Two Different Enzyme Preparations

Incubation period <i>hrs.</i>	Specific precipitin reaction of enzyme-substrate mixture	
	Prep. 3- <i>b</i>	Prep. 4- <i>a</i>
1	++++	++
2	+++	++
3	++	—
4	+	—
5	—	—

Plus signs indicate amount of precipitate formed in Type III antiserum.

Minus signs indicate no precipitation in Type III antiserum, showing complete decomposition of the specific polysaccharide.

tion of the same amount of specific substance by two different enzyme preparations.

Experiment 13. Comparison of the Activity of Different Enzyme Preparations as Measured by the Rate of Decomposition of Capsular Polysaccharide.—Two preparations (3-*b* and 4-*a*) were compared. The former was the filtrate obtained from an autolyzed culture grown in a mineral medium containing 0.01 per cent of SSS III; 4-*a* was the filtrate of a culture grown in 0.2 per cent SSS III. These two preparations were added in amounts of 5 cc. to 5 cc. of a 0.002 per cent SSS III in buffer pH 7.5. The mixtures were incubated at 37°C. with toluene, and precipitin tests were made from time to time to follow the disappearance of the SSS III (see Table VII).

Table VII shows that when the time required for the decomposition of 1 cc. of a 0.002 per cent solution of specific polysaccharide by 1 cc.

TABLE VIII

Quantitative Relationship between Total Amount of Specific Polysaccharide Decomposed and Amount of Enzyme Used

Enzyme preparation		Specific precipitin reaction of enzyme-substrate mixture incubated for			
No.	Amount	1 hr.	6 hrs.	12 hrs.	24 hrs.
1	cc.				
	2.0	+++	+	-	-
	1.0	+++	++	-	-
	0.5	++++	++++	++	++
	0.2	++++	++++	+++	+++
	0.1	++++	++++	++++	++++
	0.05	++++	++++	++++	++++
	0.02	++++	++++	++++	++++
	0.01	++++	++++	++++	++++
3-b	2.0	++	-	-	-
	1.0	+++	+	-	-
	0.5	++++	++	-	-
	0.2	++++	++	+	+
	0.1	++++	++++	++++	++
	0.05	++++	++++	++++	++++
	0.02	++++	++++	++++	++++
	0.01	++++	++++	++++	++++
4-a	2.0	+	-	-	-
	1.0	+++	-	-	-
	0.5	+++	-	-	-
	0.2	++++	++	-	-
	0.1	++++	++	-	-
	0.05	++++	++++	+	+
	0.02	++++	++++	++	++
	0.01	++++	++++	+++	++++

Plus signs indicate amount of precipitate formed in Type III antiserum.

Minus signs indicate no precipitation in Type III antiserum, showing complete decomposition of the specific polysaccharide.

of the bacterial extract is used as a measure of the activity, enzyme Preparation 4-a is much more active than Preparation 3-b. This

method of titration makes it possible to compare the relative activity of different enzyme preparations.

The following experiment was planned to compare the total amount of specific substance decomposed by given amounts of different enzyme preparations after different incubation periods.

Experiment 14. Titration of Activity of Different Enzyme Preparations as Measured by the Total Amount of Specific Substrate Decomposed.—Three preparations were compared. Preparation 1 was the filtrated autolysate of a culture grown in mineral medium containing 0.001 per cent SSS III; Preparations 3-*b* and 4-*a* were the same as described in the preceding experiment.

The enzyme preparations were added in amounts of 1 cc., 0.5, 0.2, 0.1, 0.05, 0.02, 0.01 cc. to 2 cc. of a 0.001 per cent solution of SSS III; the mixtures were made up to a volume of 3 cc. and were incubated with toluene at 37.5°C. 0.5 cc. samples were taken out from time to time to test for the disappearance of SSS III as determined by the precipitin reaction in Type III antipneumococcus serum.

The results recorded in Table VIII indicate that with a given amount of enzyme the maximum amount of decomposition of specific polysaccharide is reached after 12 hours incubation at 37.5°C. and that no further decomposition takes place thereafter. It appears in this particular instance that it required more than 1 cc. of Preparation 1 to decompose 2 cc. of the standard solution of Type III polysaccharide, whereas the same result was obtained with little more than 0.2 cc. of Preparation 3-*b* and 0.05 cc. of Preparation 4-*a*.

These results indicate that after a definite incubation period, the total amount of specific substrate decomposed bears a quantitative relationship to the concentration and activity of the enzyme preparation used. As a result of these experiments, it has been found that a convenient method of titrating the activity of an enzyme preparation is to determine the minimum amount that would decompose 1 cc. of a 0.001 per cent solution of specific capsular polysaccharide in 18 hours at 37.5°C.

DISCUSSION

The technique of isolation, and the biological characteristics of the bacillus described in this paper illustrate the great possibilities of the "starvation" method for stimulating certain potential properties of microorganisms. Although limited in its ability to utilize carbohy-

drate other than the capsular polysaccharide of Type III Pneumococcus, this organism grows rapidly and abundantly on ordinary media, plain broth, peptone solution, and casein hydrolysate. When grown on these media, however, the presence of other more readily available nutrients exerts a sparing action on the capsular polysaccharide.

When first isolated, the organism required 10 days to decompose the specific substance in concentration of 0.002 per cent; in its present state of activity, it decomposes a 0.01 per cent concentration of the polysaccharide in 24 hours. By repeated transfers in the specific medium this potential property has been greatly enhanced. It seems likely that this increase in activity is associated with an increased elaboration of the specific enzyme. Further data concerning this point will be presented later.

The fact that the growth of this organism is at least partly inhibited by the presence of glucose in the medium is unexplained but not a completely new phenomenon. There are on record at least two cellulose-decomposing species of bacteria the growth of which is inhibited by reducing sugars and especially glucose. It may be mentioned in passing that these organisms (*Sp. cytophaga* Hutchinson and Clayton (14), and "Y" bacillus Dubos (4)) are also extremely specific in their activities since cellulose is the only material on which it has been possible to grow them.

A comparison of the heat resistance of the spores and of the active enzyme extracted from the bacterial cells brings out the interesting fact that whereas the former resist heating at 95°C. for 5 minutes the latter is inactivated by 10 minutes exposure to 60°C.

It is also worth noting that although the organism is so strictly aerobic, the isolated enzyme responsible for the decomposition of the specific substance is equally active under anaerobic and aerobic conditions. It is likely that this soluble principle belongs to the group of hydrolytic enzymes.

Further work is in progress concerning the cultural conditions affecting the elaboration and activity of the enzyme. A fact of practical importance for experimentation is the possibility of titrating the activity of the enzyme *in vitro* by taking advantage of the quantitative relationship which exists between the total quantity of substrate decomposed and the amount of enzyme used.

Finally it may be mentioned that the first question which fostered this inquiry has been answered. The decomposition of the capsular polysaccharide of one of the specific types of *Pneumococcus* by a mild enzymatic action results in the loss of specific precipitability of this substance in antipneumococcus serum of the homologous type; that the polysaccharide, and not some impurity carried along with it, is responsible for type specificity, is once more proved, and probably beyond doubt.

The specificity of the types of *Pneumococcus* is illustrated also by the remarkably specific action of this enzyme which attacks only the capsular polysaccharide of Type III *Pneumococcus*; in fact this enzyme appears as specific as an antibody.

The answer to the two other questions mentioned at the beginning of this paper, namely the influence of the enzyme on the growth of Type III *Pneumococcus in vitro* and on the course of pneumococcus infection in experimental animals will be considered in subsequent papers.

SUMMARY

1. An organism has been isolated from peat soil which decomposes the specific capsular polysaccharide of Type III *Pneumococcus*.

2. The isolation has been made possible by the use of a synthetic mineral medium containing the specific polysaccharide as sole source of carbon. By repeated transfers in this medium the potential capacity of the organism to decompose the specific substance has been progressively increased.

3. The organism is a pleomorphic bacillus, motile and spore-bearing, exhibiting metachromatic granules; its reaction to the Gram stain varies according to the medium on which it is grown. It is strictly aerobic and grows well in plain broth and peptone solutions; it does not produce gas in any media and it forms small amounts of acid only on dextrin, galactose, lactose, salicin, and trehalose; its growth is inhibited by glucose.

4. The organism decomposes the capsular polysaccharide of Type III *Pneumococcus* aerobically, between pH 6.2 and 7.8, at room temperature and at 37.5°C., but not at 54°C. The decomposition of the specific substance is inhibited by the presence in the medium of other

nutrients, such as peptones, which act as a more readily available source of energy. The action of the organism is specific; it does not attack the soluble specific substance of Type I or Type II Pneumococcus, nor any of the other bacterial polysaccharides thus far tested.

5. The organism possesses an endocellular enzyme. This enzyme has been extracted by autolysis of the bacterial cells; in sterile solution it exhibits the same specific action as do the organisms from which it is derived, decomposing only the capsular polysaccharide of Type III Pneumococcus.

6. This enzyme decomposes the Type III specific polysaccharide under anaerobic as well as under aerobic conditions; it is inactivated at 60–65°C.; the rate of decomposition of the specific substance is not affected by the presence of normal serum.

7. There exists a quantitative relationship between the total amount of specific substance decomposed and the amount of enzyme preparation used; the existence of this relation makes it possible to express the activity of a given enzyme preparation in terms of the minimal amount required for the complete decomposition of a given amount of specific substance.

8. The specific decomposition of the capsular polysaccharide of Type III Pneumococcus, by the organism as well as by the enzyme it produces, illustrates once more the specificity of the types of Pneumococcus and confirms the fact that the capsular polysaccharides, and not some impurities carried along with them, are responsible for type specificity.

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