SYNTHESIS OF TYPE III PNEUMOCOCCAL POLYSACCHARIDE BY SUSPENSIONS OF RESTING CELLS

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In an earlier study (1) it was shown that a bacterial toxin, streptolysin S, is formed by streptococci when the washed organisms are suspended in a solution whose composition permits metabolism, but not growth, to occur. The findings presented in another report (2) indicated that under similar but not identical conditions an extracellular enzyme of streptococci, desoxyribonuclease, is formed also in the absence of growth. It seemed possible that the resting cell technique, used in the studies mentioned, could be applied to pneumococci in order to examine the factors affecting formation of the type-specific polysaccharides. To explore this possibility a strain of type III pneumococcus was selected because the constitution and structure of the polysaccharide characteristic of this type have been intensively investigated (3–5) and because relatively large amounts of the polysaccharide are formed. In addition, it seemed likely that the information to be obtained could provide a background for investigating polysaccharide synthesis by cell-free extracts of pneumococci.

Materials and Methods

Diplococcus pneumoniae, type III, Strain A66, was used.

The enzyme catalyzing the hydrolysis of the type III polysaccharide, termed "SIII enzyme," was prepared according to a method similar to that of Dubos (6) except that the autolysate of the bacilli was centrifuged at high speed instead of being filtered. The supernate of the autolysate was lyophilized without further purification, yielding a powder which assayed (7) at 0.2 to 1 unit of SIII enzyme per mg., depending on the lot.

SIII was estimated photometrically by measuring the absorption caused by antigen-antibody precipitate formed in the presence of excess antibody. To 0.5 ml. of an appropriate dilution of the solution to be assayed was added 0.5 ml. type III antipneumococcal horse serum diluted 1:5. After 60 minutes at 37° the mixture was shaken to disperse the precipitate, and the optical density was read against a reagent blank in a Beckman DU spectrophotometer, using microcells and a wave-length of 650 mμ. The optical density was converted to micrograms of SIII per milliliter by reading from a standard curve prepared by use of a purified preparation of SIII. The primary standard was a specimen of highly purified SIII, obtained from Dr. Michael Heidelberger, and considered to have a purity approaching 100 per cent. The five times diluted antiserum, when mixed with polysaccharide, yielded a precipitate whose optical density was a linear function of SIII when the total quantity of SIII in the system (1 ml.) did not exceed 5 μg. In order to avoid erroneous results, it is imperative that the meas-

urements be made in the region of antibody excess. When the solutions to be assayed contain antigenic materials in addition to SIII, it is necessary either to employ purified SIII-antibody or to apply a correction as explained in the experimental section.

Glucose was estimated by the method of Nelson (8) and lactic acid by the method of Barker and Summerson (9).

EXPERIMENTAL

Formation Anew of Capsular Polysaccharide by Decapsulated Pneumococci.—It was shown by Avery and Dubos (10) that exposure of encapsulated pneumococci to the action of SIII enzyme results in removal of the capsule, and according to Dubos (11) in impairment of type-specific agglutinability. The purpose of the present experiment was to explore with the aid of the agglutination technique the conditions under which capsular resynthesis occurs.

100 ml. of neopeptone infusion broth was inoculated with 0.1 ml. of broth culture of strain A66. After 16 hours at 37°, 12.5 ml. of the culture was added to 50 ml. of broth. At the end of 3 hours at 37°, the optical density (Beckman DU spectrophotometer, 1 cm. cells, wavelength of 650 mμ; broth blank) had reached 0.080. 50 ml. of the 3 hour culture was centrifuged for 15 minutes in an angle centrifuge. The clear supernate was discarded and the sedimented cocci were decapsulated by suspending them in 25 ml. broth containing 15 mg. SIII enzyme. After 20 minutes at 37° the suspended cocci were sedimented in the centrifuge and washed in 25 ml. phosphate-buffered saline. In order to remove remaining traces of SIII enzyme and broth, the cocci were again washed in 25 ml. of buffered saline.

The sedimented, decapsulated cocci were now suspended in broth and in other solutions in order to see whether they were capable of re-forming capsules. In each instance, the cocci obtained originally from 5 ml. of broth culture were suspended in 5 ml. of test solution so that the bacterial density was approximately that of the broth culture from which the cocci were derived. The suspensions were placed in a 37° water bath and 0.5 ml. aliquots were removed after various intervals of time. Each aliquot was immediately placed in a 60° water bath for 10 minutes. The agglutinability of the cocci in each aliquot was determined by adding 0.5 ml. anti-type III rabbit serum, diluted 1:10 in buffered saline, and incubating for 2 hours at 37° followed by overnight refrigeration.

As evidenced by loss of type-specific agglutinability, the findings (Table I) indicate that enzymatic treatment destroyed the capsular material. When the decapsulated cocci were placed in broth, they synthesized sufficient capsular polysaccharide in 15 minutes to become agglutinable again in specific antiserum. Sodium iodoacetate (M/10,000) delayed but did not prevent the formation of polysaccharide while fluoride (M/50) and arsenate (M/50) completely prevented capsular resynthesis. The cocci formed polysaccharide when suspended in a solution containing only glucose and salts and failed to do so when glucose was omitted.

Course of SIII Formation by Resting Pneumococci.—The agglutination technique used in the preceding experiments is adequate as a qualitative test for formation of SIII but it provides no information on the amount of polysaccharide formed. Experiments were therefore undertaken to estimate quantitatively the total SIII formed by resting pneumococci.

Decapsulated cocci were prepared as in the preceding experiment except that they were washed twice in 40 ml. instead of 25 ml. of washing fluid, and the latter was modified in this and in subsequent experiments to include glucose in a final concentration of M/1000. The sedimented decapsulated cocci, derived originally from 50 ml. of culture, were suspended in 50 ml. of solution having the following composition: 0.057 per cent $MgSO_4 \cdot 7H_2O$, 0.285 per cent KH_2PO_4 adjusted with alkali to pH 7.0, and M/1000 glucose. The resting cell suspension was placed in an open tube at 37°. After 0, 10, 20, 30, 45, and 60 minutes, two series of aliquots were taken: 2 ml. aliquots which were immediately chilled, and 3 ml. aliquots which were immediately immersed in a 60° water bath and removed after 10 minutes.

TABLE I

Capacity of Decapsulated Cocci to Re-Form SIII in Broth and Other Solutions

Test solution	Type-specific agglutinability of cocci after:					
	0 min.	15 min.	30 min.	45 min.	60 min.	120 min.
Broth	0	+++	++++	++++	++++	+++
Broth containing M/10,000 sodium iodoacetate	0	+	++	+++	+++	+++
Broth containing M/50 sodium fluoride	0	0	0	0	0	0
Broth containing M/50 sodium arsenate	0	0	0	0	0	0
Phosphate-buffered saline, pH 7.0	0	0	0	0	0	0
GMP	0	++	+++	+++	+++	+++
MP	0	0	0	0	0	0

0 indicates either no agglutination or fine particles readily dispersible on shaking.

+, ++, +++, ++++, indicate degrees of agglutination, the clumps in each instance being difficultly dispersible.

GMP, mixture containing M/70 glucose, 0.057 per cent $MgSO_4 \cdot 7H_2O$, and 0.29 per cent KH_2PO_4 adjusted with NaOH to pH 7.0.

MP, same as GMP but glucose omitted.

The 3 ml. heated aliquots were centrifuged and the supernatant fluids were used for estimation of free SIII and lactic acid. The sedimented cocci from the heated aliquots were suspended in 3 ml. phosphate-buffered saline, and the resulting suspension tested for agglutinability. The agglutination tests were carried out in the same way as described in a preceding experiment.

In distinction to free SIII, the SIII which was adherent to cocci was estimated as follows: The 2 ml. chilled aliquots were centrifuged, the supernates discarded, and the sedimented cocci freed as completely as possible from fluid by draining the tubes. The adherent SIII was got into solution by lysing the cocci in 2 ml. of 0.1 per cent sodium desoxycholate solution for 15 minutes at 37°. The lysates were centrifuged and the clear supernates assayed for SIII. It is likely that somatic antigens contributed to the precipitate formed in assaying the lysates for SIII, and therefore a correction had to be applied. The cocci of zero-time aliquots were never found to be agglutinable and hence it is unlikely that they contained a significant amount of SIII. Nevertheless the addition of antiserum to supernates of zero-time lysates resulted in a small amount of turbidity, due presumably to C carbohydrate or to other somatic antigens. The measurements of adherent SIII were therefore corrected by subtracting a constant determined from the turbidity contributed by the supernate of the zero-time lysate.

Disappearance of glucose and accumulation of lactic acid were followed by making measurements on the supernates of the 3 ml. heated aliquots.

The results are shown in Fig. 1 in which the curve designated "total SIII" is a plot of the sum of the "free SIII" and the "adherent SIII." It can be seen that the total SIII formed by the washed pneumococci is appreciable, amounting in 60 minutes to 15.5 μg. per ml., or slightly more than one-quarter the dry

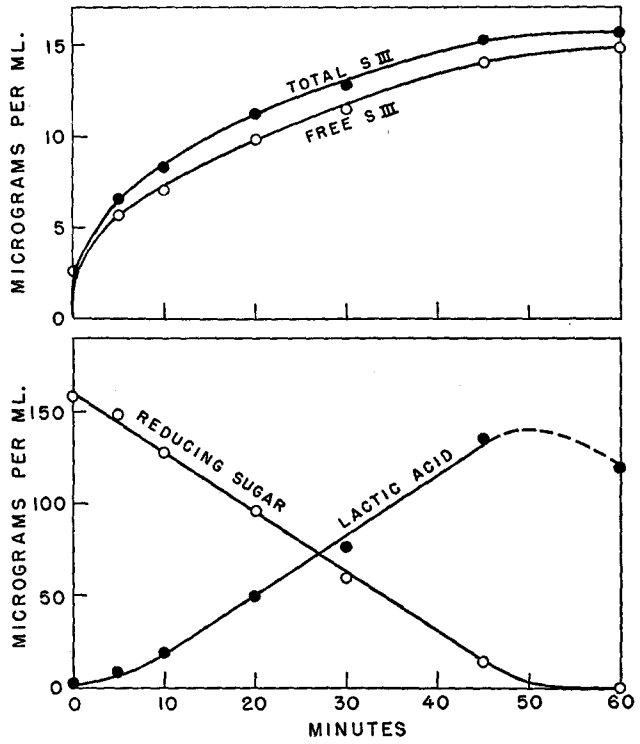

FIG. 1. Course of appearance of free SIII, total SIII, and lactic acid, and disappearance of reducing sugar as glucose. Aerobic, 37°.

weight of the cocci. In this and similar experiments the rate of SIII appearance was greatest during the first 15 minutes or longer, after which the rate slowly decreased. In some experiments, the total SIII curve suggested that the rate of polysaccharide production was constant during the first 20 to 30 minutes. In no instance was a lag period observed, indicating that the cocci were capable of synthesizing SIII promptly upon placing them in a suitable environment.

Adherent, or capsular SIII, appeared within 5 minutes. In some instances, adherent SIII persisted as such for the duration of the experiment; in others, it apparently diffused away from the cocci faster than new SIII was synthesized.

The cocci were found not to be agglutinable by type-specific antiserum at zero minutes, but in general, they became agglutinable within 10 minutes, and remained so thereafter.

The curves of Fig. 1 show that the cocci were actively glycolyzing during the time SIII was being synthesized and that glucose was utilized at an approximately constant rate during most of the experiment. In this and similar experiments virtually all the glucose disappeared in 60 minutes, and lactate accounted for 50 per cent or more of the glucose taken up.

SIII Formation by Resting Pneumococci Incubated Anaerobically.—In order to know whether SIII is synthesized by resting cocci in the absence of molecular oxygen, decapsulated cocci were suspended in glucose-salt mixture having the same composition as that used in the preceding experiment. Instead of using open tubes, however, the resting cell suspension was distributed in 5 ml. amounts among a series of Thunberg tubes which were promptly evacuated at

TABLE II

Quantity of SIII Synthesized Anaerobically and Aerobically

	(*a*) Total SIII found after 60 min. at 37° anaerobically	(*b*) Total SIII found after 60 min. at 37° aerobically	$a/b \times 100$
	μg. per ml.	*μg. per ml.*	*per cent*
Trial I	6.7	15.6	43
Trial II	7.2	19.5	37
Trial III	7.4	17.9	41

20°. The evacuated tubes were incubated at 37° for various lengths of time after which the contents of each were divided into 2 ml. and 3 ml. portions, and these were assayed for SIII, glucose, and lactic acid as in the preceding experiment.

With each series of anaerobic tubes, one tube was incubated aerobically. The quantity of SIII synthesized in it was found consistently to be greater than in the anaerobic tubes. As shown in Table II, approximately 40 per cent as much SIII is formed anaerobically as aerobically.

Further details concerning the rate of SIII formation, glucose disappearance, and lactic acid accumulation are shown in Fig. 2. It is notable (*a*) that SIII is formed at a considerably slower rate anaerobically than aerobically, and (*b*) that anaerobic synthesis of SIII ceases altogether after 20 or 30 minutes (*cf.* curves of Figs. 1 and 2). Also, glucose apparently disappears at an appreciable rate only during the first 20 minutes and this is the same period of time in which nearly all the SIII makes its appearance. Although the total quantity of lactic acid formed in 60 minutes is approximately equivalent to the glucose which disappeared in that period of time, the same is not true during the first

20 minutes. The reducing sugar and lactic acid curves of Fig. 2 suggest that an intermediate of unknown identity accumulates during the time SIII is being formed and that this substance is later converted to lactate.

It was observed, in a series of experiments, that oxygenation of the resting cell suspension resulted in increased SIII formation. The following pairs of figures are the quantities of SIII, in micrograms per milliliter, formed in 60

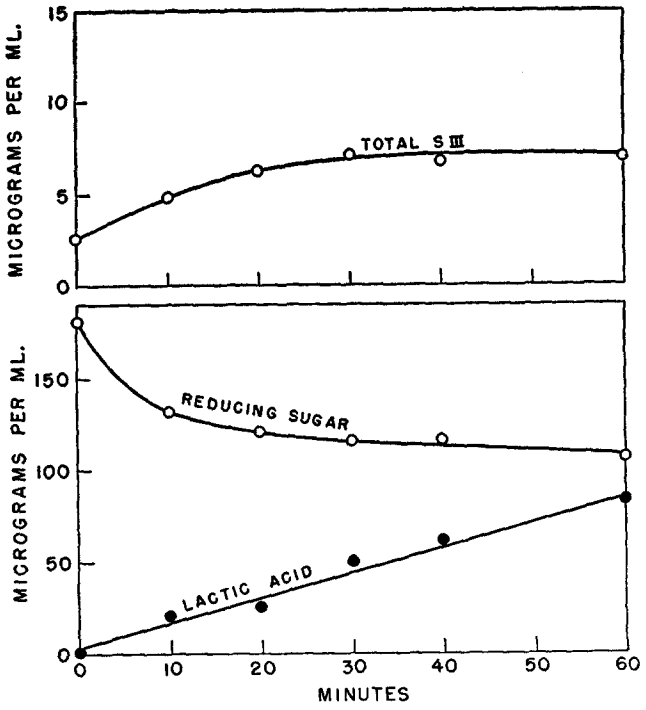

FIG. 2. Course of appearance of SIII and lactic acid, and disappearance of reducing sugar as glucose. Anaerobic, 37°.

minutes at 37° (*a*) in open tubes, and (*b*) in open tubes through which oxygen was bubbled: 17.5 and 31; 15.4 and 30; 11.2 and 24.5; 14.7 and 25.2; 17.5 and 26; 19 and 33.6 (mean ratio equals 0.55). Comparing these figures with the average of Table II, it is evident that only about one-fourth as much SIII is formed anaerobically as in suspensions which had been oxygenated.

Influence of Composition of Suspending Medium on Quantity of SIII Formed.— The suspending medium used in previous experiments was modified by omitting one or another of its components. Decapsulated cocci were suspended in solutions whose compositions are given in Table III. The total SIII formed was measured after oxygenating the suspensions for 60 minutes at 37°. The results

show that little or no SIII was synthesized in the absence of glucose and that a larger quantity of SIII was formed in the presence of Mg^{++}, K^+, and phosphate than when any one of these ions was missing. It is evident that the effect of phosphate cannot be due exclusively to buffering action because substitution of a bicarbonate buffer system for phosphate resulted in a reduced yield of polysaccharide. Experiments in which the concentrations of glucose and salts were altered from those used in this and previous experiments did not result in increased yields of SIII.

TABLE III

Requirement for Glucose, Magnesium, Potassium, and Phosphate Ions

Suspending medium	Total SIII
	μg. per ml.
Complete*	48
Glucose omitted	6
$MgSO_4 \cdot 7H_2O$ omitted	28
$MgSO_4 \cdot 7H_2O$ replaced by equimolar Na_2SO_4	31
KH_2PO_4–NaOH replaced by equimolar $KHCO_3$–HCl‡	20
KH_2PO_4–NaOH replaced by equimolar $KHCO_3$–HCl + M/70 NaCl‡	20
KH_2PO_4–NaOH replaced by equimolar KH_2PO_4–KOH	46
KH_2PO_4–NaOH replaced by equimolar NaH_2PO_4–NaOH	21

* Complete suspending medium:
0.7 ml. M/10 glucose
2.0 ml. 0.2 per cent $MgSO_4 \cdot 7H_2O$
1.0 ml. 2 per cent KH_2PO_4–NaOH buffer, pH 7.0
3.3 ml. H_2O.

‡ Suspensions bubbled with O_2 containing 5 per cent CO_2; others bubbled with O_2.

Effect of Various Sugars and Related Compounds on SIII Formation.—Decapsulated cocci were suspended in solutions of the composition shown in the footnote to Table III except that various carbon compounds were substituted for glucose. The suspensions were incubated in open tubes for 60 minutes at 37°. The results (Table IV) show that none of the compounds yielded as much SIII as glucose and that the compounds which yielded appreciable amounts of polysaccharide are those known to be fermented by pneumococcus. The only exception to this rule is glycogen which apparently was not split under the conditions of the experiment.

Effect of pH on SIII Formation.—Decapsulated cocci were suspended in solutions with the composition shown in the footnote to Table III except that phosphate buffer was prepared to give pH values varying by half-unit increments between 5.0 and 8.0. The concentration of phosphate was constant (M/50) and the cation was K^+. The free and total SIII formed in 60 minutes at 37° are plotted against pH in Fig. 3. It is evident that an appreciable quantity

of SIII was formed at all pH values used including the relatively unphysiological extremes of 5.0 and 8.0. However, SIII synthesis was greatest in the region of

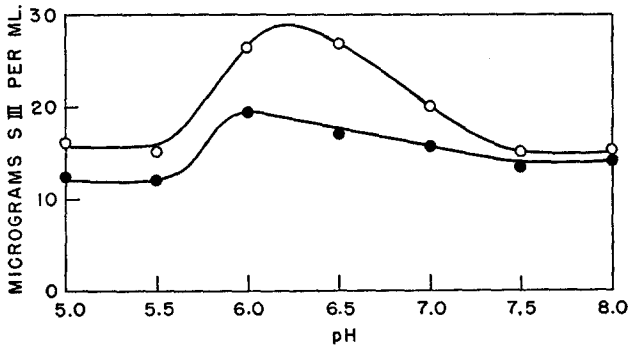

FIG. 3. SIII synthesis as a function of pH. Upper curve, total SIII. Lower curve, free SIII. Both aerobic, 37°.

TABLE IV

SIII Synthesis in the Presence of Various Carbon Compounds

Substance	SIII-forming activity* (glucose = 100)
Fructose (M/100)	72
Galactose (M/100)	50
Maltose (M/200)	63
Sucrose (M/200)	38
Lactose (M/200)	65
Cellobiose (M/200)	0
Glucosamine (M/100)	47
Glucose-1-phosphate, dipotassium (M/100)	11
Glycerol (M/100)	3
Potassium 3-phosphoglycerate (M/100)	19
Sodium glucuronate (M/100)	3
Sodium cellobiuronate (M/200)	0
Menthol glucuronide (M/100)	0
Glycogen (1.8 mg. per ml.)	13

$$* \; 100 \times \frac{\text{(SIII formed in presence of C compound) minus (SIII formed in absence of C compound)}}{\text{(SIII formed in presence of glucose) minus (SIII formed in absence of C compound)}}$$

pH 6.0 to 6.7. The curves of Fig. 3 also indicate that the tendency of polysaccharide to diffuse away from the organisms is a function of pH and is minimal in the region of pH 6.3.

Effect of Enzyme Poisons on SIII Formation.—Decapsulated cocci were suspended in solutions of the composition shown in the footnote to Table III

except that they contained various enzyme poisons. In most instances, the effects of twofold differences in concentration of poison were measured. Mercuric chloride (2×10^{-6} M), iodoacetate (5×10^{-4} M), and dinitrophenol (5×10^{-3} M) reduced the quantity of SIII formed in 60 minutes by one-half. Cyanide and fluoride likewise caused 50 per cent inhibition when used in concentrations of 5×10^{-2} M and 2×10^{-2} M, respectively, while azide, arsenite, and malonate did not inhibit.

DISCUSSION

The foregoing experiments show that it is possible to study the factors affecting synthesis of SIII under conditions far simpler than those prevailing in a growing culture. It may be noted that the absence of organic nitrogen from the medium in which the washed cocci are suspended precludes the occurrence of growth in the sense of increased mass of protoplasm. Under optimal conditions the washed decapsulated cocci synthesize in 60 minutes approximately half their weight of capsular polysaccharide when provided only with glucose, salts, and oxygen.

The findings show that four or five times as much SIII is synthesized when the suspension of cocci is oxygenated as compared with anaerobic conditions. It is possible that oxygen promotes the formation of carboxyl groups present in the glucuronic acid moiety of the polysaccharide, although this is only one of many mechanisms which can be postulated to explain the oxygen effect. It would be of interest to know whether formation of the specific polysaccharides of other pneumococcal types is similarly favored by oxygen. The observation has been made (12) that oxygen is required for the synthesis of cellulose by resting cells of *Acetobacter xylinum*.

It is reasonable to suppose that glucose, or part of it, serves not only as a source of energy for synthesis but also as a structural precursor of polysaccharide. Hence when conditions are arranged, as by addition of an enzyme poison, to interfere with the utilization of glucose, and no SIII is formed, it is not clear whether the failure of synthesis is due to a deficiency in utilizable energy or to a deficiency in formation of a structural precursor. It is conceivable that clues to the nature of the intermediates involved in SIII synthesis could be obtained by providing the cells with a combination of two types of substrates—one, such as pyruvate or lactate whose oxidation to acetate might be expected to yield energy for synthesis, and another, such as cellobiose or glucuronic acid, which might serve as a precursor of SIII. Experiments of this kind were not fruitful although the possibilities tried were not exhaustive.

A considerable number of attempts were made to obtain synthesis of SIII in the absence of living cells. The general procedure used was to remove the capsules with SIII enzyme and to mix either a sonic extract, or an autolysate of the decapsulated cocci, with a solution containing salts, adenosinetriphos-

phate, and glucose or other substrate. The mixture was incubated and then assayed for SIII using absorbed antiserum. The substrates tested included glucosamine, glucose-1-phosphate, glucuronic acid, α-glucuronic acid-1-phosphate, cellobiose, cellobiuronic acid, and the dialyzable fraction of an enzymic digest of SIII. In no instance was serologically detectable SIII formed. Evidently the methods employed result in the destruction or inhibition of one or more enzymes or cofactors essential for the synthesis of SIII, and it appears likely that further progress will depend upon the discovery of methods for obtaining the synthesizing enzymes in active form.

SUMMARY

The capsular polysaccharide (SIII) of type III pneumococci was removed enzymatically, and the cells thus deprived of preformed SIII were washed and examined for capacity to synthesize SIII anew. The washed, decapsulated cocci lost their capacity to be agglutinated in type-specific antiserum but again became agglutinable and formed readily measurable amounts of SIII, after suspension in a solution containing only glucose and salts.

Maximal SIII synthesis required the presence of glucose, magnesium, potassium and phosphate ions, and oxygen. Other fermentable sugars could be substituted for glucose but then the yield of SIII was reduced. Synthesis of SIII occurred anaerobically but was increased four- to fivefold by oxygenation of the suspension. The effects of pH and of enzyme poisons on the capacity of the cocci to form SIII are described.

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