THE LYSIS OF GROUP A HEMOLYTIC STREPTOCOCCI BY EXTRACELLULAR ENZYMES OF STREPTOMYCES ALBUS

I. Production and Fractionation of the Lytic Enzymes

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It has been known for many years that certain soil microorganisms have the property of elaborating substances which cause the dissolution of a variety of bacteria (1). According to the classification currently employed, these soil organisms fall in the genus *Streptomyces.*

The lysis of bacteria by *Streptomyces* has been studied in detail by Welsch, using a strain designated as *Streptomyces albus* G, and he has given the name *actinomycetin* to the soluble preparations from this organism which are capable of lysing heat-killed Gram-negative organisms and many species of living Gram-positive organisms. Welsch has prepared an extensive monograph concerned with his investigations (2). Maxted (3) applied the techniques of lysis with *Streptomyces albus* preparations to the practical problem of grouping hemolytic streptococci, and showed that lysates containing the group-specific polysaccharide could be prepared rapidly as a routine laboratory procedure. The value of this procedure has been confirmed in other laboratories (4, 5).

The fact that *Streptomyces albus* elaborates a soluble, extracellular enzyme system which can dissolve hemolytic streptococci suggests that this material may provide a potential tool for the study of cellular components of group A streptococci. The need for gentle, more biological methods of extracting the cellular components of streptococci is emphasized by the nature of the procedures that are now required for obtaining certain of the antigenic constituents in soluble form. The type-specific M protein, for example, is best prepared by heating cells at 100°C. and pH 2 (6). While this procedure has proved useful in the study of M protein, it has no general applicability for the investigation of other cellular constituents. Prior to the use of *Streptomyces albus* enzymes for the release of the group-specific carbohydrate, the application of enzymatic techniques to the extraction of hemolytic streptococci has been limited to the preparation of certain antigens, as exemplified by the release of T antigen from group A cells with the aid of trypsin (7).

The present investigation was undertaken to explore the possibility of using enzyme preparations from *Streptomyces albus* in the disruption of streptococcal cells, with the ultimate goal of studying cellular components of group A streptococci in their native state. The lytic filtrates of *Streptomyces albus*

cultures are known to be strongly proteolytic, and it has been assumed that the proteolytic enzyme is responsible for dissolution of the bacteria. If this assumption were correct, the usefulness of the lytic material for the purposes contemplated would be greatly reduced, since many of the protein components of the cell would necessarily be destroyed in the course of lysis. However, in the case of group A hemolytic streptococci, it is unlikely that proteolytic action could be solely responsible for dissolution of the cells, since a wide variety of other proteolytic enzymes have no such effect even in high concentration. Trypsin, chymotrypsin, pepsin, papain, and ficin have the effect only of removing certain surface constituents, such as the M and T antigens, without bringing about any major disruption of the cells as evidenced either by their microscopic appearance or the release of soluble material. On the basis of these considerations, the initial aim of the present work was to test the hypothesis that some factor (or factors) other than the proteolytic enzyme is responsible for the initiation of lysis by *Streptomyces albus* filtrates. Evidence supporting this hypothesis will be presented.

The numerous problems concerned with optimal growth conditions of *Streptomyces albus* have been studied with a view to obtaining consistent and maximal yields of the lytic material. In addition, a variety of approaches have been used in attempted fractionation of the active filtrates.

Materials and Methods

Strains of Streptomyces albus.—The stock strain of *Streptomyces albus* was obtained from Maxted's laboratory through Dr. Robert Cruikshank. Subcultures of strains also originally from Maxted's laboratory, were obtained from Dr. Floyd W. Denny and Dr. O. T. Avery. In addition, an organism tentatively identified as *Streptomyces albus* on the basis of cultural and biological properties was isolated from garden soil. The four strains showed differences in their ability to grow and produce enzymes on various media despite the fact that three of the strains presumably originated from the same source. The problem of variability of the organism, especially with regard to production of the lytic enzymes, is discussed below.

Culture Media.—A variety of media were tried for the growth of the *Streptomyces albus*; but for the most part, following preliminary investigation of the effect of various salts, all were comprised of the same salt mixture with differences in the nitrogen and carbon sources employed. The salt mixture used had the following composition per liter: NaCl, 5 gm.; K_2HPO_4, 2 gm.; $MgSO_4 \cdot 7H_2O$, 1 gm.; $CaCl_2$, 0.04 gm.; $FeSO_4 \cdot 7H_2O$, 0.02 gm.; and $ZnSO_4 \cdot 7H_2O$, 0.01 gm.

Measurement of the Lytic Action on Group A Streptococci.—The complexity of the factors involved in the lysis of intact streptococcal cells renders it difficult to devise an accurate quantitative method for measurement of the effect of *Streptomyces albus* filtrates. For example, as will be indicated below, it is apparent that at least two enzymes are involved in the lysis, and changes in the relative concentration of the active components can alter the course of the visible changes occurring during lysis. In addition, the use of whole bacteria as substrate in procedures that involve quantitative measurement by nephelometric or turbidimetric techniques introduces numerous potential errors. Thus, the initial alteration in the cell membrane may proceed without much change in turbidity and the release of cellular contents and visible lysis may be wholly secondary to the limiting enzymatic reaction.

With the various limitations in mind, a turbidimetric technique has been adopted for

the semiquantitative estimation of the lytic activity of the *Streptomyces albus* preparations and has proved useful in following fractionation procedures. Although a wide variety of strains of group A streptococci have been shown to be susceptible to lysis, there are individual variations in sensitivity; and two strains of approximately equal susceptibility were employed for routine tests in the present study: Strain S43, type 6, and strain T28, type 28. The organisms were grown in Todd-Hewitt broth, collected by centrifugation, washed twice in physiological saline, and finally heat-killed at 60°C. for 30 minutes. Fresh suspensions were prepared in this manner at frequent intervals. The use of suspensions of acetone-dried cells, which could be freshly prepared and standardized on a weight basis, was tried extensively but finally abandoned because of an alteration in the sensitivity to lysis on storage of the dried cells. Living streptococci can also be used but are less convenient than killed cells in the routine test.

The turbidity of the suspensions is read in small cuvettes (12 × 75 mm.) in a Coleman Jr. spectrophotometer at 600 mμ. The stock cell suspension is diluted in M/15 phosphate buffer pH 8 so that the turbidity of the reaction mixture, after addition of enzyme, gives an optical density of approximately 0.5. To tubes containing 0.5 cc. of the cell suspension is added 0.5 cc. of enzyme appropriately diluted in the same buffer. The tubes are incubated at 37°C. in a water bath and readings of optical density are made at 5 minute intervals over a period of 30 minutes. Fig. 1 illustrates the type of results obtained with a concentrate of a lytic filtrate and shows the relation of the rate of fall in turbidity to the concentration of active material. It will be noted that there is an apparent initial lag except at the highest concentration, followed by a period in which the decrease in turbidity is essentially linear with time. The slopes of the linear portion of the curves are related to the concentration of active material so that a reasonably accurate comparison of the activity of various preparations can be made. The initial lag varies with the cell suspension and enzyme preparation employed, and at times is more marked than that illustrated in Fig. 1 (see Fig. 3).

It should be pointed out that this system does not provide *optimal* conditions for the action of the lytic substance, since, as shown by Welsch (2), salts have an inhibitory effect. However, phosphate buffer has been used in these experiments to provide a constant and reproducible environment with respect to pH and salt concentration.

Under the conditions described above, one unit of streptolytic activity is defined as that amount of enzyme which causes a linear rate of fall of 0.05 unit per minute in the optical density of the bacterial suspension. Because of the fact that different bacterial suspensions vary in their susceptibility to lysis, this unit has no absolute meaning; and in comparing the activity of various fractions the same streptococcal suspension is used throughout.

Measurement of Proteolytic Activity.—The proteolytic activity of the lytic filtrate and the various fractions was measured by an optical density method similar to that suggested by Kunitz (8) involving the spectrophotometric estimation of the trichloracetic acid–soluble products formed during the digestion of casein. 6 cc. of a 2 per cent solution of casein in M/15 phosphate buffer pH 8 is mixed with 2.0 cc. of the enzyme solution, diluted in the same buffer. 2 cc. samples are withdrawn at 0, 15, and 30 minutes and pipetted into 3.0 cc. of 5 per cent trichloracetic acid. The precipitates are removed by filtration and the optical density of the filtrates is measured in the Beckman quartz spectrophotometer at 280 mμ. One unit of proteolytic activity is defined as that amount of enzyme which results in an increase of one unit in the optical density of the trichloracetic acid filtrate in 30 minutes.

Nitrogen Determinations.—Total nitrogen was determined by the direct nesslerization micro-Kjeldahl procedure of Koch and McMeekin (9).

Production of Lytic Material

Importance of Sporulation.—As the result of his extensive investigation, Welsch concluded that the lytic substances formed by *Streptomyces albus*

are elaborated by the cell at the time of sporulation (2). Since sporulation occurs only on aerial mycelium associated with surface growth, the active material is not produced in submerged cultures. The results of the present investigation are fully in accord with this conclusion and somewhat at variance with the findings of Jones, Swallow, and Webb (10). The latter workers obtained filtrates after prolonged incubation of submerged cultures which had lytic activity when tested with heat-killed *Aerobacter aerogenes*.

Welsch employed several techniques in an attempt to demonstrate that the active enzymes do not exist preformed within the vegetative mycelium with subsequent release into the environment upon disruption of the cell at the time of sporulation. He was unable to recover appreciable active material from vegetative mycelium by any of the following meth-

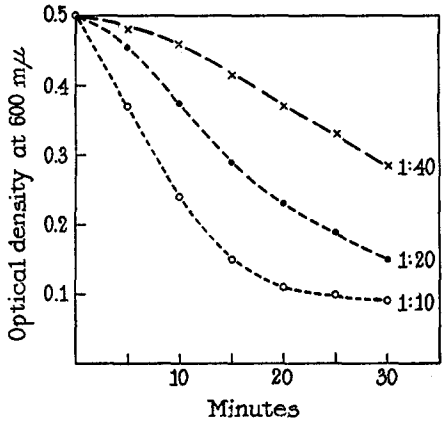

FIG. 1. Lysis of suspension of heat-killed group A streptococci by varying concentrations of enzymes from *Streptomyces albus*.

ods: grinding with sand, autolysis in the presence of chloroform, and freezing and thawing. In the present investigation it has been possible to provide further evidence concerning the absence of active material in the mycelium as a result of the finding that the mycelium of the streptomyces strain used in this laboratory is highly susceptible to lysis by crystalline lysozyme from egg-white. Thus, treatment with lysozyme provides a rapid and gentle procedure for obtaining complete dissolution of the mycelium at various stages in its growth under different conditions. It was not possible to demonstrate any appreciable effect on hemolytic streptococci in lysozyme lysates of vegetative mycelium, even in solutions which represented considerable concentration as compared with the ordinary lytic filtrates. The lysozyme lysates of the mycelium showed a small amount of proteolytic activity on casein. Even when surface growth was subjected to lysozyme lysis at a time when sporulation and release of active material had begun, the activity of the mycelial lysates was small compared to that of the filtered growth medium.

The temporal relationship between sporulation and the appearance of active material is illustrated in Fig. 2. In this experiment, *Streptomyces albus* was grown on the surface of an agar medium in a series of bottles, and filtrates were obtained for testing at daily intervals by freezing and thawing the contents of individual bottles to release fluid from the agar, fol-

lowed by filtration through paper. The mycelial growth appeared to be fully developed after 2 days, but assay at this time revealed the absence of effect on streptococci. On the 3rd day there were small areas of sporulation scattered over the surface and the culture filtrate was weakly active. After incubation for 5 days, sporulation appeared to be reaching completion and was accompanied by a marked increase in lytic activity of the filtrate.

The correlation between sporulation and the production of lytic enzymes suggests that these enzymes may be involved in the mechanism of sporulation or release of spores from the vegetative cell. However, it has been found that extensive sporulation is not invariably accompanied by a high yield of enzymes active in dissolving streptococci, and under certain adverse cultural

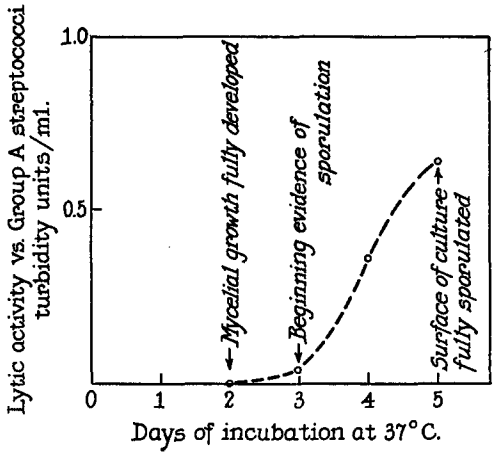

FIG. 2. Relationship between sporulation of culture and release of lytic enzymes by *Streptomyces albus*.

conditions feebly lytic filtrates have been obtained from heavily sporulated cultures.

Cultural Conditions for Production of Active Filtrates.—Considerable difficulty has been experienced in obtaining consistently good yields of active filtrate, and this problem has apparently been encountered by other investigators. A wide variety of culture media and growth conditions have been used in the present investigation in an attempt to define reproducible conditions for the production of the lytic enzymes. In the preparation of material to be used for the release of group-specific polysaccharides from hemolytic streptococci, Maxted (3) grew the *Streptomyces albus* on agar medium and recovered a fluid preparation by freezing and thawing the agar after maximal growth. Other workers applying Maxted's method for grouping streptococci have used the agar method. This procedure has certain advantages for the consistent production of surface growth and was employed for part of the present work.

However, in obtaining material for purification and fractionation of the enzymes it was found that the agar contributed a troublesome water-soluble polysaccharide fraction which interfered with purification.

The disadvantages of the agar polysaccharide were partly offset in early work by the fact that good yields of active material were obtained on a simple agar medium containing 0.5 per cent ammonium phosphate and 0.5 per cent glucose in addition to the salt mixture described under Methods. The activity of the best preparations obtained with this medium was comparable to that obtained with more complex media containing peptones, and information concerning the relationship of the proteolytic enzymes and other enzymes of the filtrate to lysis of streptococci resulted from fractionation of this type of material. However, it was necessary to abandon this medium because variable and unpredictable results were obtained, despite the fact that the effect of a large number of variables (temperature, humidity, agar concentration and preparation, effect of various added substances, etc.) was studied in an attempt to obtain consistent results.

The organism grows well on media containing commercial peptones of many different types. However, most peptones contain inhibitory substances and optimal conditions for rapid growth are more readily obtained with simple media. The medium selected as providing the most consistent results consists of 0.5 per cent casamino acids[1] and 0.5 per cent glucose with the salt mixture as previously described. On this medium maximal sporulating growth is obtained in 48 to 72 hours at 37°C.

Growth in Fluid Medium.—Since the elaboration of lytic enzymes appears to be dependent on the formation of aerial mycelium and sporulation, the production of active material on fluid medium requires conditions that promote surface growth. This can be achieved with certain media, such as the casamino acid medium described above, by the inoculation of a shallow layer of the medium with a suspension of spores containing little vegetative mycelial growth. Under these conditions the lyophobic spores float on the surface and germinate, leading to a continuous sheet of surface mycelial growth which is well formed within 24 hours and heavily sporulated within 48 hours. There may be little or no submerged vegetative growth. The development of this type of surface growth appears to be dependent both on the characteristics of the strain of *Streptomyces albus* and the nature of the culture medium. Two of the strains employed in this study give excellent surface growth on the casamino acid medium, and the yield of active material is roughly proportional to the luxuriance of the sporulated growth. Undiluted fluid from a culture of this type is capable of lysing a suspension of group A streptococci (approximately 1 mg. per cc.) in 10 minutes or less. The use of fluid media has obvious

[1] Bacto-casamino acids, purified; Difco Laboratories.

advantages over agar-containing media for large scale production of the lytic enzymes.

Variability of Streptomyces albus.—Waksman (11) stresses the high degree of variability of *Streptomyces* under continuous cultivation on artificial media as indicated by changes in such characteristics as pigment formation, growth pattern, etc. Variability of this sort has been observed with the *Streptomyces albus* strains used in this study, and in addition to the properties described by Waksman, the variation has been found to involve the capacity for formation of the lytic enzyme system. Mutations involving this property provide a partial explanation for the inconsistency in the results with respect to enzyme

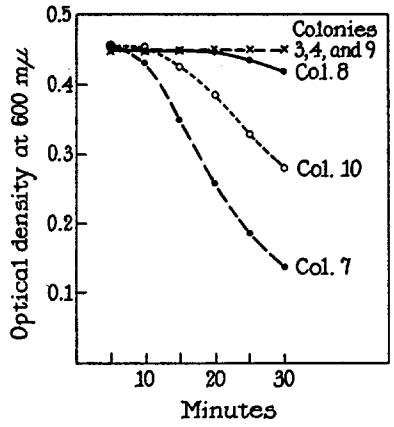

FIG. 3. Streptolytic activity of six substrains derived by single colony isolations from one strain of *Streptomyces albus*.

production on the same medium at different times. Evidence for this variability is indicated by the following experiment:—

A strain of *Streptomyces albus* which had been producing a good yield of lytic enzyme on the simple agar medium containing ammonium phosphate as nitrogen source gradually gave increasingly poor results. A dilute suspension of spores of this culture was plated out to provide isolated colonies rather than confluent growth. After 5 days incubation, the colonies were examined with the aid of a low-power microscope and several colonies which appeared to differ from one another in minor details of surface configuration were transferred to fresh medium. Upon testing these substrains for their capacity to produce streptolytic enzyme under identical conditions, results were obtained which are presented graphically in Fig. 3.

Three of the substrains produced little or no enzyme, one produced a small amount, one a moderate amount, and one produced amounts comparable to the best obtained. The same pattern was maintained for three subsequent subcultures, but on continued subculture substrain 7 again began to show diminished activity.

In consideration of these findings it is important to avoid serial subculture of the strains used for production of the enzyme. In practice, a large number of agar slants are inoculated with a spore suspension from a culture of known activity, and after incubation for 5 days at 37°C. the tubes are stored at 4°C. to serve as a source of inoculum in subsequent experiments.

Fractionation of Lytic Filtrates

Fractionation Procedure.—The most satisfactory material for fractionation is obtained by growth in a simple liquid media. The procedure currently employed is as follows:—

Casamino acid medium (0.5 per cent) is distributed in 500 cc. amounts in 5-liter pyrex bottles. Sterilization is carried out by autoclaving. Each bottle is inoculated with the spores washed from one agar slant culture of *Streptomyces albus* with 5 cc. distilled water. The bottles are incubated at 37°C. in a horizontal position to provide a relatively shallow layer of medium. Growth on this medium is rapid, and a continuous sheet of surface growth, well covered with a white sporulated surface, is obtained in 48 to 72 hours. The maximum yield of streptolytic enzymes is achieved at this time, and it is important not to continue incubation beyond this point since the activity of the filtrates decreases after reaching the maximum. The amber culture medium is removed from beneath the sheet of surface growth by suction and pooled for fractionation.

The pooled culture fluid is brought to 0.7 saturation with $(NH_4)_2SO_4$ by the addition of 472 gm. of solid salt per liter of fluid. One gram per liter of filter cel is added, and the precipitate recovered by suction filtration. The filter cake containing the precipitate and filter cel is suspended in M/15 phosphate buffer pH 8 (about 10 cc. for each liter of original culture fluid) and stirred to bring the precipitate completely into solution. The solution is filtered with suction to remove the filter cel and the filter pad is washed with several portions of buffer until the filtrate is no longer colored.

The solution of the precipitate is dark brown or brownish green in color and contains 75 per cent of the original activity. The solution is dialyzed first against distilled water and finally 0.0001 N HCl, and a dark brown precipitate forms which contains most of the enzyme responsible for the initiation of lysis of hemolytic streptococci. The pale yellow supernate contains most of the proteolytic activity. Resolution of the precipitate in phosphate buffer yields a highly colored solution. The colored material apparently represents pigments arising from the organisms and can be removed by adsorption on calcium phosphate. This is accomplished by the following procedure: 0.1 volume of 10 per cent CaCl is added to the solution in phosphate buffer. The heavy precipitate which forms is thrown down by centrifugation and carries with it most of the pigment, leaving a pale yellow or greenish yellow supernate. The precipitate is washed with water and the washings combined with the original supernate. Two-thirds to three-quarters of the active material is recovered in the decolorized fraction. This material is concentrated by reprecipitation at 0.7 saturation with $(NH_4)_2SO_4$ and finally dialyzed against phosphate buffer.

The yields and activities obtained in one preparation of this type are given in Table I. Most of the material is recovered by the initial step of ammonium sulfate precipitation, and it is apparent that dialysis against 0.0001 N HCl effects considerable separation of the proteolytic enzyme from the component or components active against the streptococcus. The degree of purification of

the two types of active substances is indicated by the increase in activity per mg. N.

Relation of the Proteolytic Enzyme to Enzymatic Lysis.—Fractionation experiments of the type described in the previous section make it clear that the enzyme which hydrolyzes casein is incapable of dissolving streptococci. By

TABLE I

Distribution of Proteolytic and Streptolytic Activity in Fractions of S. albus Culture Filtrate

Fraction	Proteolytic activity (casein)		Streptolytic activity	
	Total units	Units/mg. N	Total units	Units/mg. N
1. Original culture filtrate (9 liters)	2070		1840	
2. 0.7 saturated $(NH_4)_2SO_4$ precipitate	1650		1650	
3a. Supernate after dialysis *vs.* 10^{-4} N HCl	1480	27.0	<10	<2
3b. Precipitate after dialysis *vs.* 10^{-4} N HCl	137	7.3	1600*	85
4. Final material: fraction 3b after removal of pigment on calcium phosphate	101	22.6	1260*	350

* Activity measured in presence of 0.025 mg./cc. crystalline trypsin.

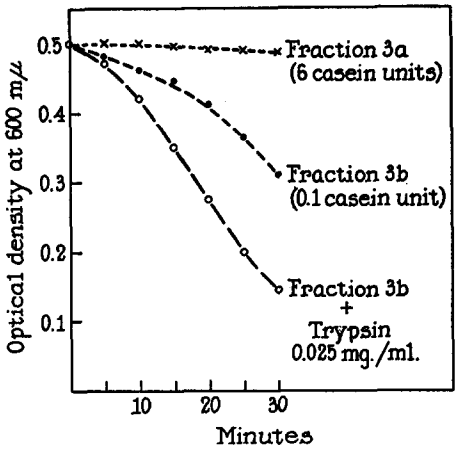

FIG. 4. Comparison of the effect of fractions of *Streptomyces albus* enzyme preparation on group A streptococci.

several different procedures, preparations of the proteolytic enzyme have been obtained which even in high concentration have no observable effect on the turbidity of streptococcal suspensions. Furthermore, these preparations cause no alteration in the morphology or staining characteristics of the cells. However, it has been found that proteolytic activity plays a role in the complete clearing of the suspension once dissolution has been initiated. The proteolytic enzyme of *Streptomyces albus* has no special properties in this regard,

and it can be replaced by other proteolytic enzymes such as trypsin. These relationships are illustrated by data obtained with the fractions described above (Table I). In Fig. 4 it is shown that fraction 3b has a marked effect on streptococci at dilutions possessing weak activity against casein, but that this action is considerably enhanced by the addition of trypsin. A similar effect is obtained by the addition of the proteolytic fraction, 3a. Fraction 3a by itself, at concentrations containing 60-fold larger amounts of proteolytic enzyme, has no appreciable action on the streptococcal cell. Thus, it is clear that the complete clearing of suspensions of streptococcal cells is a complex phenomenon involving the action of more than one enzyme, and that the initial steps in lysis involve an enzyme which appears to be non-proteolytic in character.

The interrelationship of the various enzymes of the filtrates has practical significance in the measurement of streptolytic activity. Since the method employed is turbidimetric and is dependent on complete lysis of the cells, fractions which are low in proteolytic activity may give misleading results. To avoid having the concentration of proteolytic enzyme become the limiting factor in analysis of fractions of this type, the tests are carried out in the presence of 0.025 mg. per cc. trypsin. This was done, for example, in the case of fractions 3b and 4 included in Table I. In the absence of added trypsin, the apparent activity of fraction 3b was approximately 40 per cent lower than the figure recorded in the table.

Separation of the Enzymes by Adsorption on Cellulose.—Because chemical fractionation has not provided material completely free of proteolytic activity, it has not been possible to use this material to define the effect of the non-proteolytic component on intact streptococcal cells. More complete fractionation of the components has been obtained by the use of adsorption techniques, since it has been found that the proteolytic enzyme is much less readily retained by various adsorbents than the non-proteolytic enzyme. However, elution of the latter enzyme has proved to be highly variable in different experiments and at best only partial elution has been achieved. For this reason, adsorption techniques have had limited usefulness in large scale fractionation and the experiments will not be described in detail. The general procedure is outlined briefly because of the bearing of the results on the problem of the role played by the various components in the lysis of hemolytic streptococci.

A column 1 x 30 cm. is prepared from a slurry of equal parts of powdered cellulose[2] and celite 503 in 1 M $(NH_4)_2SO_4$. 2 cc. of a concentrate of the streptomyces enzymes is placed on the column in the same solvent. On continued elution with 1 M $(NH_4)_2SO_4$, practically all of the caseolytic activity appears in the effluent with the column volume, but even after 18 hours none of the fractions recovered possess lytic activity when tested with streptococcal suspensions. When the eluting fluid is changed to M/15 phosphate buffer pH 8, a fraction is

[2] Solka-floc, Brown Co., New York.

obtained which contains a portion of the lytic activity but no more than traces of proteolytic enzymes. In some experiments only a small fraction of the lytic activity is recovered and the greatest recovery, regardless of solvents used, is approximately 50 per cent.

Samples from the adsorption column were used to study the effect of the non-proteolytic enzyme on group A streptococci. Although the turbidity of the suspension of streptococci progressively decreases after the addition of this material, the rapid and complete clearing characteristic of the complete lytic system is not obtained. However, microscopic examination reveals that the cells become Gram-negative within a few minutes and that they appear indistinct and irregular in outline. The addition of trypsin to a suspension of these altered cells accelerates the clearing of the suspension, and the solution develops an increasing viscosity which can be shown to be due to the presence of desoxyribonucleate. It would appear, therefore, that the non-proteolytic enzyme brings about a marked structural alteration in the streptococcal cells, but leaves material which does not readily pass into solution without the intervention of a proteolytic enzyme.

None of the various fractionation experiments have provided evidence suggesting that more than one non-proteolytic enzyme is involved in lysis of streptococci. Thus, the total effect on the streptococcus may be attributable to the action of two enzymes, one of which can be replaced by a variety of known proteolytic enzymes.

Significance of Other Enzymes Present in Streptomyces Filtrates.—Tai and van Heyningen (12) in a study of the lysis of heat-killed *Escherichia coli* by filtrates of a *Streptomyces* sp. have described the presence of two proteolytic enzymes, one which digests a casein and another which digests proteins present in *E. coli*. This second proteolytic enzyme does not appear to be involved in the lysis of group A streptococci. Heat-killed *E. coli* are rapidly lysed by fractions containing the caseolytic enzyme (*e.g.*, fraction 3a in Table I) even though this material is without effect on streptococcal suspensions. On the other hand, fractions containing high streptolytic activity in relation to caseolytic activity (fraction 3b, Table I) are only weakly active in causing lysis of *E. coli*, and the activity is roughly proportional to the concentration of proteolytic enzyme. Similarly, the peptidase which Webb *et al.* (10, 13) have described as a component of the extracellular bacteriolytic system plays no role in the lysis of group A streptococci. The preparations described in the present paper contain only small amounts of peptidase when tested by the formol titration method, and all of this occurs in the fractions possessing caseolytic activity which are ineffective in lysing streptococci.

Two other enzymes, ribonuclease and lysozyme, which are present in the enzyme complex from *Streptomyces albus* require consideration as possible factors in the lysis of streptococci. The effect of ribonuclease in rendering Gram-positive cells Gram-negative has been extensively studied (see review by

Bartholomew and Mittwer (14)). Muggleton and Webb (13) have found this enzyme to be present in culture filtrates of their strain of soil actinomyces and are of the opinion that the lytic activity against Gram-positive cells is dependent on the presence of an enzyme of the ribonuclease type. However, the concentrated lytic preparations obtained by the methods described above possess only traces of ribonuclease activity. Furthermore, the addition of crystalline pancreatic ribonuclease in high concentration to crude concentrates or to the various fractions has no effect on the lytic activity against group A streptococci. It is reasonable to conclude, therefore, that ribonuclease is not an important factor in the lysis of group A streptococci under the conditions of the present experiments.

Lysozyme must be considered in connection with the present studies, since it is clear that its action is not limited to the few species of microorganisms that are readily lysed by this enzyme. For example, Webb (15) has shown that a variety of Gram-positive organisms are rendered susceptible to lysis by trypsin when suspensions of heat-killed cells are treated with lysozyme in relatively high concentrations. However, group A streptococci appear to provide an exception to this behavior of Gram-positive cells. In experiments carried out in this laboratory with several streptococcal strains, this lysozyme effect was not obtained although control strains of *Staphylococcus aureus* were dissolved by the technique described by Webb.

A lysozyme-like enzyme occurs in the *Streptomyces albus* filtrates, but it differs in certain respects from crystalline egg-white lysozyme. It possesses only weak activity as measured by lysis of *Micrococcus lysodeikticus*, but has relatively high viscosity-reducing action on lysozyme substrate prepared from this organism by the method of Meyer and Hahnel (16). Caseolytic fractions of *Streptomyces albus* which are ineffective in lysing streptococci contain this lysozyme-like enzyme, and it is thus apparent that it cannot be the factor responsible for initiation of lysis.

DISCUSSION

Because the primary aim of the present investigation was to gain further information concerning group A streptococci, study of the lytic filtrates of *Streptomyces albus* has been limited for the most part to their action on this group of organisms. With regard to the general problem of bacterial lysis by the streptomyces enzyme system, it is clear that the same mechanism is not involved in the case of all species of microorganisms. This is illustrated by the differences between Gram-negative and Gram-positive organisms, since the lysis of heat-killed Gram-negative cells is dependent upon the proteolytic activity of the filtrate while the dissolution of Gram-positive cells requires the action of other enzymes. In addition, the nature of the enzyme involved in the initiation of lysis of Gram-positive organisms may not be the same for all

species. Thus, the lysozyme-like enzyme present in the filtrates, although it does not appear to be directly involved in the lysis of group A streptococci, may be important in the dissolution of certain other Gram-positive organisms.

In view of the foregoing facts, it is impossible at the present time to generalize concerning the nature of bacterial lysis by the extracellular enzyme system of *Streptomyces albus*. The experiments recorded in the present paper show that the proteolytic enzyme produced by *Streptomyces albus* is unable to dissolve group A streptococci, and that the primary action of the lytic system resides in a second enzyme which does not attack ordinary protein substrates. The results of the various fractionation procedures indicate that this second enzyme is probably a single entity and not a mixture of two or more unknown enzymes. However, the data presented provide no clue concerning the identity of this enzyme. Indirect evidence on this point is offered in the following paper which deals with an attempt to localize the site of action of the enzyme on the streptococcal cell.

From the point of view of the proposed use of the *Streptomyces albus* enzymes for the investigation of the cellular constituents of group A streptococci, it is encouraging that the primary effect is not proteolytic and that a non-proteolytic enzyme appears to cause a profound alteration in the structure of streptococcal cells. Thus, it is likely that increased extractability of streptococci can ultimately be achieved without concomitant destruction of protein constituents. However, at the present time the separation of the enzymes is not sufficiently quantitative to provide material for this type of study.

SUMMARY

The lysis of group A hemolytic streptococci by extracellular enzymes of *Streptomyces albus* has been studied. The most favorable material for fractionation of the lytic enzymes was obtained by surface growth on shallow layers of liquid medium containing an acid hydrolysate of casein, glucose, and salts. The results of fractionation experiments show that the potent proetolytic enzyme of *Streptomyces albus* is not able to lyse group A streptococci, and that the initiation of lysis is dependent upon the action of a second, non-proteolytic enzyme. The nature of the non-proteolytic enzyme has not been determined. It does not appear to be a ribonuclease or a lysozyme-like enzyme.

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