FORMATION OF A BACTERIAL TOXIN (STREPTOLYSIN S) BY RESTING CELLS*

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It was demonstrated originally by Weld (1, 2) that streptococci produce appreciable amounts of hemolytic and lethal toxin upon shaking the cells, derived from young cultures, in the presence of heat-inactivated horse serum. The observation has been repeatedly confirmed (3–5). The hemolytic toxin, formation of which is favored by serum, was designated streptolysin S by Todd (5) in order to differentiate it from the oxygen labile hemolytic toxin, streptolysin O, formation of which occurs independently of the presence of serum. It was subsequently discovered (6) that yeast nucleic acid causes a very potent hemolysin to appear in broth cultures of streptococci, and this hemolysin seems to be identical with streptolysin S (7). Some of the factors affecting the formation of streptolysin S in cultures containing ribonucleic acid, or a polynucleotide derived therefrom, have been studied (7).

The foregoing facts suggest that it should be possible to form streptolysin S through the interaction of ribonucleic acid and resting streptococci, that is, by employing the system used by Weld but substituting ribonucleic acid for serum. Preliminary experiments, carried out in collaboration with Miss Marcelle Rodbart, showed that streptolysin S did indeed appear in mixtures of commercial yeast nucleic acid and unwashed streptococci from broth cultures. The results obtained from day to day, however, were not consistent, and very frequently no streptolysin at all appeared under conditions apparently similar to those which had yielded appreciable titers. Further work (8) indicated that streptolysin S formation depended upon a multiplicity of factors including the age of the culture employed, the presence of a suitable carbohydrate, whether or not the cocci had been washed free of culture medium, whether commercial yeast nucleic acid or a fraction (AF) of yeast nucleic acid was employed, and others. The purposes of this report are to show that streptolysin S can be formed by resting cocci in a system free of serum or culture medium, to define the conditions necessary for maximal yields of toxin, and to describe some of the proper-

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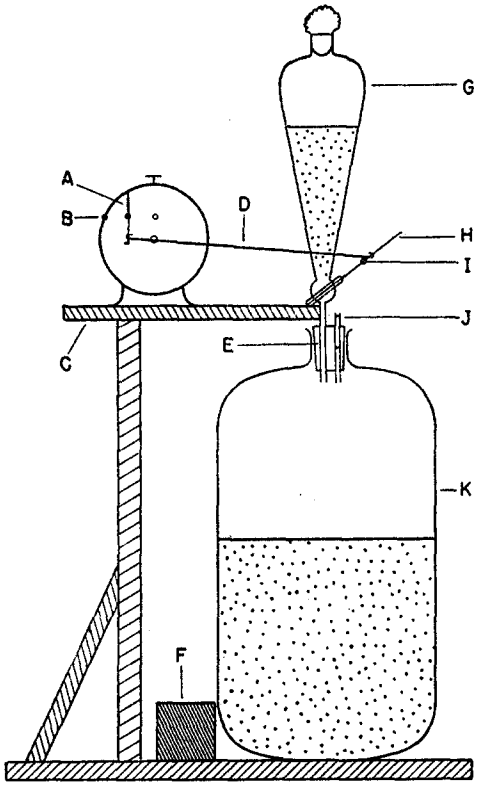

A - lever attached to alarm winder
B - pin stop attached to alarm clock
C - support for clock and funnel stem
D - connecting rod of no. 14 copper wire
E - two-hole rubber stopper
F - block for positioning bottle
G - 500 ml. pyrex separatory funnel containing 300 ml. broth
H - lever wired to stopcock handle
I - adjustable stop
J - cotton plugged air outlet
K - 9 liter pyrex bottle containing 5 liters broth

FIG. 1. Automatic inoculator. Assembly and operation: The separatory funnel containing 300 cc. of broth is autoclaved upright with the previously greased (lubriseal) stopcock in the position shown and with the lower portion of the funnel stem and rubber stopper protected from contamination during subsequent handling. The 9 liter bottle containing 5 liters of broth is plugged with cotton and autoclaved separately. After allowing both vessels to come to 37°, apart, the separatory funnel is inoculated with 0.3 cc. of broth culture and the two vessels are joined as shown. This is usually done at 4 p.m. and at the same time, the alarm clock is set for 3 a.m. and connected by means of rod D to lever H. At 3 a.m. lever D pulls H to the vertical position discharging the 300 cc. seed culture into the 5 liters of broth providing, thereby, a 6 hour growth at 9 a.m.

ties of the toxic product obtained. In addition, experimental results bearing on the mechanism of toxin formation will be presented.

Methods

Strains.—In most experiments, the C203S strain of group A streptococcus was used. In certain instances, a mutant of this strain, designated C203U, was employed. The mutant strain fails to produce streptolysin S but in other respects it resembles the parent strain.

Preparation of Resting Cocci.—Ten cc. of infusion broth, prepared as described in an earlier report (7), and containing 0.01 per cent freshly neutralized thioglycollic acid is inoculated with 0.01 cc. broth culture and incubated at 37°. After 17 hours, 90 cc. of infusion broth containing 0.01 per cent sodium thioglycollate is inoculated with the whole of the 10 cc. culture and incubated in a water bath at 37°. After 5 hours the culture is centrifuged and the sedimented cocci are washed in 50 cc. phosphate-buffered saline (M/15 phosphate at pH 7 and M/12.9 NaCl). The washed cocci are then dispersed in 7 cc. of suspending medium, described in the experimental section, and after placing in a 37° bath for a given length of time, the mixture is chilled and refrigerated until the next day when it is titrated for hemolytic activity. In many experiments, the volumes employed differed from those just stated but the ratio of volume of suspending medium to volume of broth from which the cocci were derived was always the same as described unless otherwise specified. Likewise, the size of the inoculum was always adjusted to the volume of broth to be inoculated so that the ratio of the two was constant.

Automatic Inoculator for 5 Liter Cultures.—For the routine production of streptolysin S on a large scale, it was desired to have available, at the beginning of the working day, 5 to 6 hour broth cultures. This was accomplished for units of 5 liters each by using the contrivance described in Fig. 1.

Measurement of Hemolytic Activity.—The unit of streptolysin S is that amount which lyses half the erythrocytes (human, 0.35 per cent by volume) contained in 2 cc. of phosphate-buffered saline, pH 7, in 30 minutes at 37°. Details of the method including the preparation of the stock erythrocyte suspension have been published previously (7, 9).

AF.—AF is a polynucleotide which is active in causing the formation of streptolysin S in growing cultures. It is obtained by fractionating yeast nucleic acid according to the method described in an earlier report (7).

EXPERIMENTAL

I. Factors Affecting the Formation of Streptolysin S

Age of Culture.—Although cocci derived from 16 hour cultures sometimes yielded streptolysin when mixed with sodium ribonucleate and peptone, on other occasions they failed to, and it soon became evident that rigorous control of the age of the culture was necessary for reproducible results. In this connection, it may be noted that Weld (1) emphasized the importance of employing young cultures for the formation of streptolysin by serum-cocci mixtures. In the present system, it has been observed that cocci from cultures in the early phase of growth yielded very little streptolysin. This observation is illustrated by Fig. 2 in which cell mass as measured by turbidity, and capacity to form streptolysin, are plotted against the age of culture from which the cocci were derived. It can be seen that over most of the linear part of the growth curve,

that is, when the cell mass was increasing linearly, and maximally, the cocci were capable of yielding only small amounts of streptolysin. However, at the time the growth rate began to decrease, and for several hours thereafter, the cocci uniformly yielded appreciable amounts of streptolysin. It would appear that rapidly growing cocci are in a metabolically different state from that of

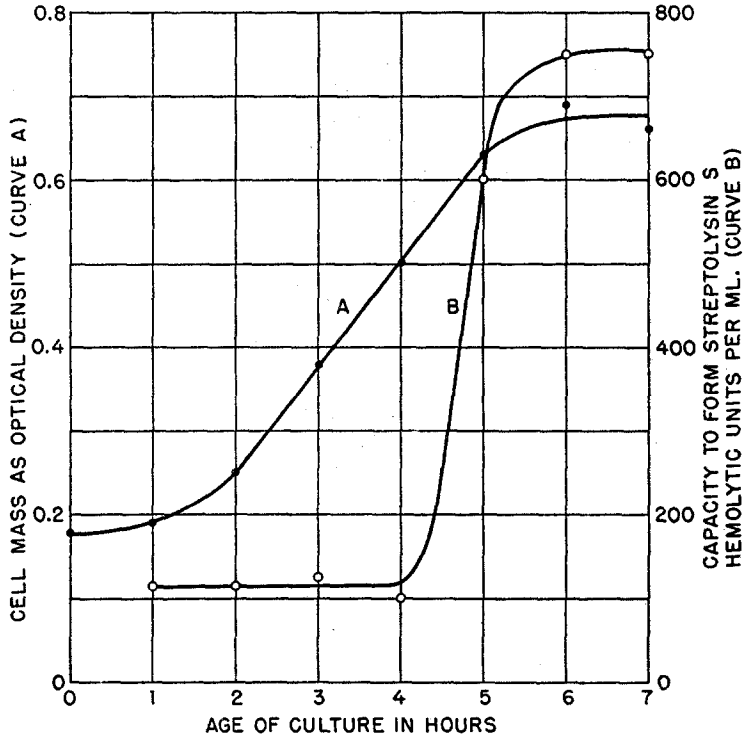

FIG. 2. Variation with age of culture of (A) cell mass, and (B) capacity of cocci to form streptolysin S. At zero hours, 200 cc. sterile broth containing 0.01 per cent sodium thioglycollate was inoculated with 10 cc. of a 16 hour broth culture.

Cell mass expressed as optical density, using 10 mm. cells and wave length of 650 mμ.

Capacity to yield streptolysin measured by mixing unwashed, sedimented cocci from 10 cc. aliquot with 0.5 cc. 1 per cent commercial sodium nucleate and 0.2 cc. 10 per cent Witte's peptone, incubating for 60 minutes at 37°C, and titrating mixture for hemolytic activity. Data from (8).

cocci in a culture which is closely approaching the stationary phase, and that the latter yield much larger amounts of streptolysin than the former. In connection with this observation, it may be noted that certain enzymes of bacteria are known to follow a quantitative variation with cell mass very similar to that described by Fig. 2, and that these are frequently enzymes concerned in catabolic processes (10).

Substances Necessary for the Formation of Streptolysin S.—It has been shown earlier (7) that streptolysin S is readily formed by the C2O3S strain while growing in a medium of essentially defined chemical composition containing, in addition to all necessary nutritional factors, AF and maltose. The purpose of the present experiments was to find the simplest conditions under which washed cocci would consistently yield appreciable quantities of toxin. It was found that significant amounts of streptolysin S appeared in mixtures containing washed cocci, commercial sodium nucleate, and salts, but only when peptone was present. When, however, AF was substituted for commercial sodium nucleate, the peptone was observed to be unnecessary. The simplest suspending medium which was consistently effective was one containing the following factors: (1) AF; (2) a fermentable carbohydrate; (3) sodium, potassium, magnesium, and phosphate ions.

The requirement for these factors is illustrated by the data of Fig. 3 which records the streptolysin titers at various times after mixing washed streptococci with (*a*) the complete suspending medium, and (*b*) the same medium from which one or another of its components had been omitted. It can be seen (Fig. 3 A) that no streptolysin was formed in the absence of AF, and very little (100 units) in the absence of glucosamine (Fig. 3 B). Additional experiments have shown that certain carbohydrates can be substituted for glucosamine, namely, maltose, sucrose, glucose, and cellobiose. On a weight basis, the most active of these substances, in inducing maximum streptolysin formation, were glucosamine and maltose in concentrations of approximately 2 mg. per mg. cocci in a volume of 1 cc. Too great an excess of carbohydrate reduced the yield. Of the disaccharides, maltose was found to be more effective than either sucrose or cellobiose; glucosamine usually yielded somewhat more toxin than glucose. It has been shown earlier (7) that certain substances other than AF have a marked stimulatory effect on streptolysin S production *in cultures*, the most active being maltose and glucosamine. It is notable that in growing cultures, glucose could not replace maltose or glucosamine, but in the resting cell system, glucose, when used in sufficiently large concentration, induced the formation of 50 to 100 per cent as much streptolysin as was obtained when either maltose or glucosamine was used.

As shown in Fig. 3 C, a considerably greater amount of streptolysin was formed in the presence of $MgSO_4$ than in its absence. The effect of $MgSO_4$ was duplicated when $MgCl_2$ was used in the same molar concentration, but not when $CaCl_2$ or calcium acetate was employed at the same concentration and pH. In some experiments the effect of omitting Mg^{++} was smaller than that shown in Fig. 3 C. The reason for this variation is not clear.

In addition to magnesium ion, both potassium and phosphate ions are required (Fig. 3 D). The KH_2PO_4-NaOH mixture could be replaced neither by pure mono- and disodium phosphate at pH 7.0 nor by KCl or K_2SO_4. Pre-

sumably, phosphate is needed for synthetic processes, and it is unlikely that it functions through its buffering action since as much lysin was formed in a system in which the initial pH was set at 6.5 as was formed when the initial pH was 7.0.

Although the results described by Fig. 3 indicate that AF, carbohydrate, and salts function in the streptolysin-forming system, there is an alternative inter-

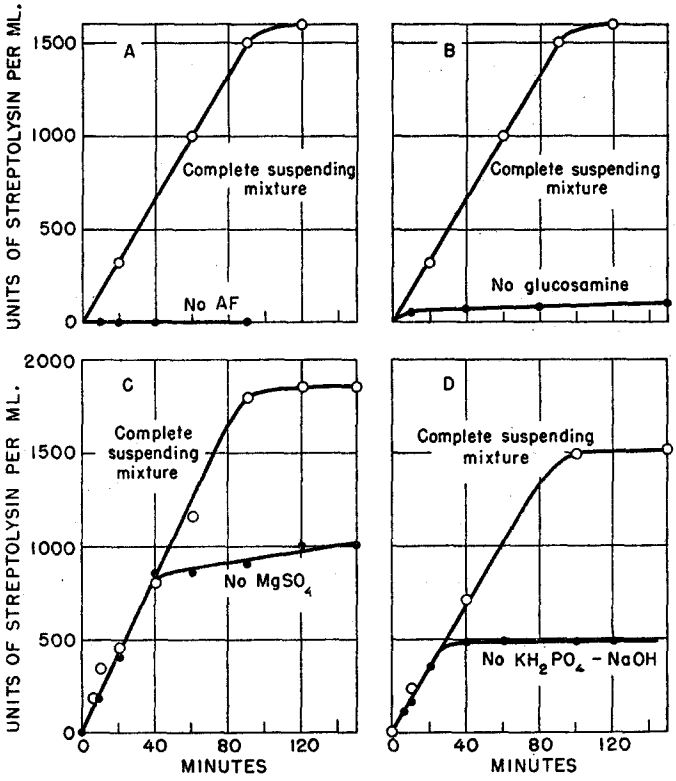

FIG. 3. Effect of omitting various components of suspending mixture. Complete suspending mixture for washed cocci derived from 50 cc. broth contained: 1 cc. water containing 0.41 mg. AF, 1 cc. M/20 glucosamine hydrochloride, 0.5 cc. 2 per cent KH_2PO_4 brought to pH 7 with NaOH, 1 cc. 0.2 per cent $MgSO_4 \cdot 7H_2O$.

pretation, namely that these substances function instead in the hemolytic or test system. In order to distinguish between the two possibilities, portions of washed cocci were mixed with suspending media deficient in each of the four components, and then incubated 90 minutes at 37°. Immediately prior to titrating for hemolytic activity, the missing components were added to the mixtures. In no instance did addition of previously missing components result in formation or activation of streptolysin. It is therefore clear that AF, carbo-

hydrate, and salts function in the streptolysin-forming system rather than in the test system. The observation that carbohydrate is an essential component of the streptolysin-forming system suggests that energy is necessary for the synthesis or liberation of streptolysin. In order to study more fully the changes occurring in the resting cell system the disappearance of glucosamine was measured in the following experiment:

Approximately 16 mg. (dry weight) of cocci, derived from 200 cc. of a 5 hour broth culture, was washed in 100 cc. saline phosphate and suspended in 14 cc. of the following mixture: 2 cc. M/20 glucosamine hydrochloride, 1.64 mg. AF, 0.4 cc. 2 per cent $MgSO_4 \cdot 7H_2O$, 0.2 cc. 20 per cent KH_2PO_4 previously neutralized with NaOH, and 11.4 cc. water. After placing the suspension in a 37° bath, 2 cc. aliquots were removed and chilled at 30, 50, 70, and 90 minutes. The aliquots were centrifuged in the cold, and the clear supernates were assayed for glucosamine as reducing sugar using the method of Nelson (11) and for lactic acid (12). The hemolytic activity and pH were also measured.

The results, illustrated in Fig. 4, indicate that in 90 minutes, 70 per cent of the glucosamine disappeared and 65 per cent of that which disappeared could be accounted for as lactic acid. The results show that the cocci are actively metabolizing during the time streptolysin appears in the mixture. In similar experiments in which glucose was substituted for glucosamine, all the glucose was utilized in 60 minutes.

Effect of Temperature on Rate of Streptolysin Formation.—The effect of temperature on the formation of streptolysin by resting cells was studied in the range of 5 to 45°C. At temperatures below 15°C., no detectable streptolysin was formed in 80 minutes. Similarly, none appeared at 46°C. Between 20 and 37°C., however, sufficient toxin was produced to permit rate measurements. Fig. 5 describes the rate of appearance of streptolysin at 14.7, 21.8, 29.9, and 37.0°C. It is notable that at suitable temperatures the cocci begin to form streptolysin without appreciable delay and that the production of streptolysin follows a linear course for the first 30 minutes or longer. Calculation of the Arrhenius constant using the linear rates of formation of streptolysin at 21.8 and 29.9°C., yielded, in two successive experiments, values of 37,000 and 34,500 calories per mole, respectively.

Effect of Gaseous Environment and of Agitation on Streptolysin Formation.—The system under study is very sensitive to physical and chemical factors of many kinds, and principally for this reason, extensive kinetic studies have not been attempted. During the time streptolysin is being formed the suspended cocci partially settle, thereby causing the system to become inhomogeneous. Agitating the cocci, either with a mechanical contrivance or by bubbling with oxygen or nitrogen, markedly affected the rate at which streptolysin was formed, and in some instances, the total amount of streptolysin formed. These effects are illustrated in Fig. 6 in which replicate suspensions of resting cocci were (*a*) not agitated except at times of sampling, (*b*) agitated by bubbling

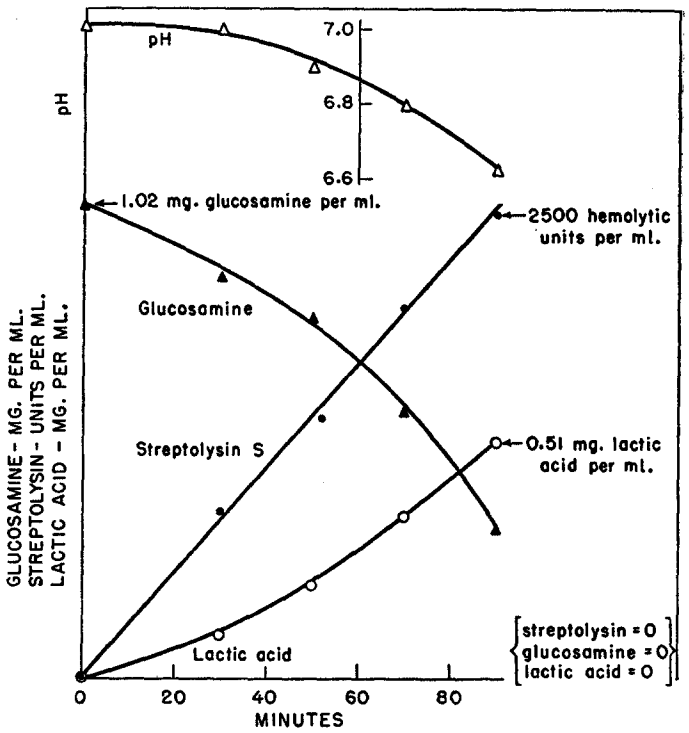

FIG. 4. Disappearance of glucosamine, formation of lactic acid, and change in pH during course of streptolysin formation.

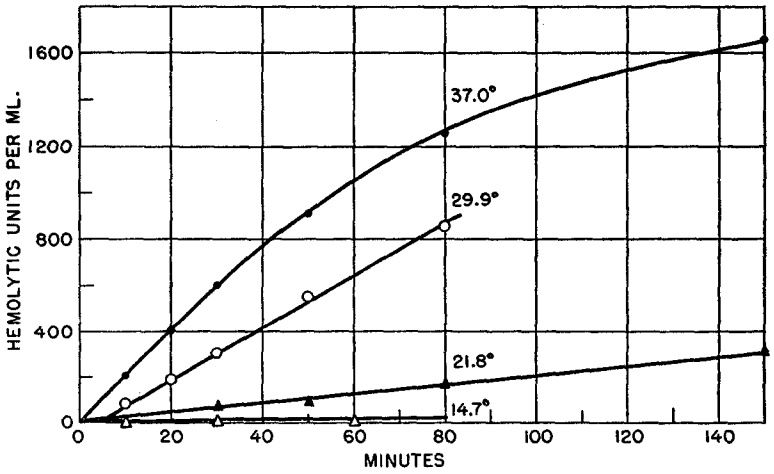

FIG. 5. Rate of appearance of streptolysin S at various temperatures. Complete suspending mixture as indicated in Fig. 3. Three-tenth cc. aliquots were removed after various intervals of time, chilled, and titrated for hemolytic acitivity.

with nitrogen, (*c*) agitated by a glass plunger reciprocating at one to two cycles per second. It can be seen that bubbling with nitrogen markedly decreased the rate of streptolysin formation, and that agitation by means of the moving glass plunger caused the appearance of streptolysin to cease after approximately 20 minutes.

The mechanism of these effects has not been fully explored but it has been found that streptolysin formation proceeds anaerobically in Thunberg tubes at the same rate as in tubes exposed to the air. Other experiments have shown that

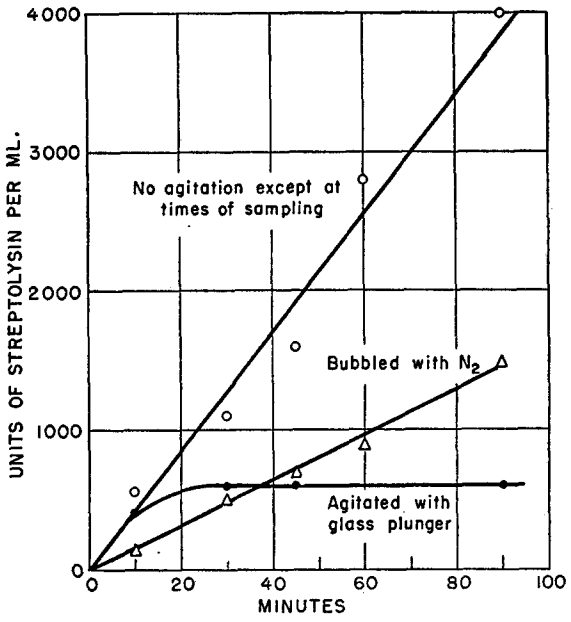

FIG. 6. Effect of gaseous environment and of agitation on the rate of appearance of streptolysin S. Complete suspending mixture as indicated in Fig. 3. Temperature 37°C.

cysteine in a final concentration of 0.1 per cent abolishes completely the inhibition caused by mechanical agitation. These observations indicate that streptolysin S is formed only when the milieu is sufficiently reducing.

Effect of Enzyme Poisons on Streptolysin Formation.—The foregoing results leave little doubt that the appearance of streptolysin S in suspensions of resting cocci is associated with metabolic processes. Further evidence of the dependence of toxin production on cellular metabolism was adduced in experiments in which the effects of a series of enzyme poisons were studied. The substances tested were incorporated into resting cell suspensions similar to those previously described. The resting cell suspensions were titrated for streptolysin after incubating for 90 minutes at 37°C., and the difference in streptolysin concentration in

the presence and absence of enzyme poison was expressed as per cent inhibition. In most instances the effects of five-fold differences in concentration of enzyme poison were measured; in some cases more closely spaced concentrations were used. In addition all the substances tested were examined for possible effects in the test hemolytic system.

As shown in Table I all ten substances tested inhibited streptolysin formation although some were very much more potent inhibitors than others. For example the concentrations of malonate and mercuric chloride required to produce 50 per cent inhibition were in the ratio of 10,000 to one. The most potent inhibitors were mercuric chloride (3×10^{-6}M), arsenite (3×10^{-5}M), iodoacetate (4×10^{-5}M), and dinitrophenol (5×10^{-5}M). It is notable that the first three are sulfhydryl inhibitors. Since streptolysin formation occurs anaerobically and

TABLE I

Effect of Enzyme Poisons on Formation of Streptolysin S

	Molar concentration producing approximately 50 per cent inhibition of streptolysin S formation
Sodium malonate	3×10^{-2}
Potassium cyanide	2×10^{-2}
Sodium fluoride	1.5×10^{-2}
Sodium azide	5×10^{-3}
Sodium arsenate	2×10^{-3}
Sodium selenite	5×10^{-4}
Dinitrophenol	5×10^{-5}
Sodium iodoacetate	4×10^{-5}
Sodium arsenite	3×10^{-5}
Mercuric chloride	3×10^{-6}

since streptolysin formation was found to be prevented by inhibitors of aerobic metabolism, namely malonate, cyanide, and azide, it would appear that these substances affect metabolic processes in addition to those involved in respiration. This conclusion was further supported by the observation that approximately the same concentration of malonate, cyanide, and azide inhibited streptolysin formation in tubes from which oxygen had been excluded as in tubes exposed to the air.

None of the enzyme poisons in the concentrations listed in Table I was found to inhibit hemolysis induced by preformed streptolysin S. However, this finding does not imply that higher concentrations than those tested may not affect the hemolytic system.

Estimation of Preformed Streptolysin in Cocci.—Weld (1) and Herbert and Todd (13) interpreted their findings as indicating that appreciable quantities of streptolysin S exist in or on the surface of streptococci and that in serum-cocci

mixtures preformed streptolysin S is extracted from the cocci into the serum. In order to estimate the quantity of preformed streptolysin present, washed cocci which had not been in contact with AF or with serum were disrupted by exposure to sonic vibration and the resulting mixture was assayed for streptolysin activity:

The cocci (approximately 18 mg. dry weight) from 400 cc. of a 5 hour broth culture were centrifuged and washed in 100 cc. saline-phosphate buffer. Half of the cocci were suspended in 14 cc. of saline-phosphate buffer and exposed for 30 minutes to the vibration of a 9000 cycle oscillator (Raytheon Corporation, Waltham, Massachusetts). The resulting mixture was found to contain 5 hemolytic units per cc. The remaining half of the cocci were caused to form streptolysin S by suspending them in 14 cc. of solution containing AF, glucosamine, and salts. After 45 minutes at 37°, the mixture was centrifuged, and the supernatant fluid was found to contain 750 hemolytic units per cc. The cocci were washed twice in 50 cc. saline-phosphate and exposed to sonic vibration for 30 minutes. The disrupted cells were found to contain 1.5 hemolytic units per cc. Control experiments in which preformed streptolysin S was treated in the sonic vibrator in the presence of washed cocci established that streptolysin S is not destroyed by the procedure used.

The results show that only traces of preformed streptolysin exist in or upon the streptococcal cells and that this is true of cocci taken directly from a broth culture as well as of cocci taken from a system in which the cells are actively participating in the formation of streptolysin.

II. Preparation and Properties of High Potency Streptolysin S

The foregoing experiments show that streptolysin S can be produced in a very simple system and under well defined conditions. Employing the information disclosed by these experiments a method has been developed for routine preparation of toxin of high potency using the resting cell system.

Method of Preparation of Streptolysin S.—Five liters of a 6 hour growth of C203S streptococci is prepared with the aid of the apparatus described in Fig. 1. The turbidity of the culture is sufficient to give an optical density of 0.95 ± 0.20 when read against a culture supernate blank in a spectrophotometer carrying 10 mm. cells and employing light of 650 mμ. The cocci in the 5 liter culture are sedimented in bucket centrifuges and washed twice, using for each washing, 400 cc. phosphate-buffered saline, pH 6.8 to 7.0. The washed cocci, having a total dry weight of approximately 350 mg., are suspended in 70 cc. of the following mixture: 3 cc. AF solution containing 16.8 mg. AF per cc., 675 mg. maltose, 6 cc. 20 per cent KH_2PO_4 previously brought to pH 7 with NaOH, 12 cc. 2 per cent $MgSO_4 \cdot 7H_2O$, and 63 cc. distilled water.

The resting cell suspension is placed in a 37° water bath and is stirred for a moment at the end of the 1st and 2nd hours. After a total of 3 hours, the cell suspension is centrifuged at high speed, the supernate is decanted and chilled, and the sedimented cocci are discarded. The clear supernate contains approximately 6000 units of streptolysin S per cc. or a total of about 400,000 units. The supernate is dialyzed in the cold, in 23/32 cellulose casing, against 4 changes of distilled water, 2 to 3 liters each, over a period of 45 hours. During dialysis, the distilled water is stirred continuously and the contents of the cellulose sac are mixed occasionally.

The dialyzed supernate is frozen and lyophilized, and a white or grayish white product is obtained. Average yield, 16 mg. The product contains 20,000 to 30,000 units of streptolysin S per mg.—a hemolytic potency considerably greater than that of any other hemolytic agent of which we are aware. For purposes of discussion, the streptolysin-containing product will be designated "C203S product." The findings of a typical experiment of the kind just described can be summarized by the following statement:

$$350 \text{ mg. cocci} + 42 \text{ mg. AF} \xrightarrow[\text{salts}]{\text{maltose}} 16 \text{ mg. soluble non-dialyzable product.}$$

C2O3U Product.—A product has been prepared in an identical manner as the C2O3S product except that the C2O3U strain has been employed in lieu of C2O3S. The yield of this product is approximately equal in weight to that obtained when C2O3S is used. It contains no active streptolysin S and it will be designated "C2O3U product."

TABLE II

Relationship between Yield of Streptolysin S and Quantity of AF Converted to Dialyzable Form

AF in suspendng medium	Cocci per mg. AF	AF converted to dialyzable form	Yield of streptolysin S
mg.	*mg.*	*mg.*	*hemolytic units*
21	28	14.0	290,000
21	19	14.9	307,000
21	17	13.2	210,000
42	10.2	20.2	440,000
42	8.6	18.4	508,000
42	7.1	27.3	600,000
63	6.5	30.9	620,000

Fate of AF during Streptolysin Formation.—Although the conditions described above have been used as a routine for the preparation of high potency streptolysin S, on a number of occasions the amounts of AF and of cocci used have been deliberately varied. The quantity of AF converted to dialyzable form can be calculated by subtracting the total 256 mμ absorption of the C2O3S product from that of the original suspending medium. In making this calculation, it is assumed that the 256 mμ-absorbing material in the C2O3S product is residual AF, and it is further assumed that little or no AF-purine or -pyrimidine enters the bacterial cells. The latter assumption appears to be justified because the total 256 mμ absorption of the 3 hour supernate, before dialysis, has never been found to be less than that of the AF initially present in the suspending medium. Moreover, from half to two-thirds of the 256 mμ-absorbing capacity of the 3 hour supernate has been found to leave the cellulose sac during dialysis.

The data of Table II show considerable parallelism between the yield of toxin and the quantity of AF hydrolyzed during the course of streptolysin formation, and they suggest that a direct proportionality exists between the two. It is evi-

dent, also, that in the range studied the yield of toxin is a function of the quantity of AF used.

In studying the mutant strain, which fails to form streptolysin S, it was found that this strain also hydrolyzed AF, and approximately to the same extent as C2O3S. It is clear, therefore, that the failure of C2O3U to form toxin is not due to a deficiency of AF-hydrolyzing enzymes on the part of the mutant strain.

Chemical Nature of C2O3S and C2O3U Products.—Samples of both products as well as of AF were dried to constant weight in a vacuum desiccator at room temperature. Solutions of the dried preparations containing approximately 1 mg. per cc. were analyzed for total phosphorous by the method of Lohmann and Jendrassick (14), for nitrogen by a modified micro Kjeldahl procedure, and for pentose by the orcinol reaction (15). Reducing sugar was measured by the method of Nelson (11) after refluxing 30 minutes in 2 N HCl; hexosamine was

TABLE III

Analyses of C203S Product, C203U Product, and AF

	C203S product	C203U product	AF
Extinction coefficient at 256 mμ, pH 7	15.6	15.5	27.5
Total P, *per cent*	5.39	5.35	8.47
Total N, *per cent*	10.3	10.0	15.8
Reducing sugar as glucose, *per cent*	20.9	21.4	16.1
Pentose, *per cent*	20.4	20.0	28.0
Hexosamine, *per cent*	8.8	5.1	0.0

estimated by a modification of the method of Elson and Morgan (16). The results are shown in Table III.

In addition, absorption curves of the three preparations were determined between 220 and 300 mμ, at neutrality, in N/100 NaOH and in N/100 HCl. The absorption characteristics of the three preparations were found to be essentially identical, with maxima at or near 258 mμ, although the extinction coefficient of AF was observed to be considerably greater than that of the C2O3S and C2O3U products (see Table III).

With the exception of the figures for hexosamine, the analytical data reveal no differences in the composition of the C2O3S and C2O3U products. Comparison of the composition of the two products with that of AF, however, suggests that both products contain 60 per cent residual AF and 20 or 30 per cent carbohydrate. Evidence for the presence of a small amount of protein in the C2O3S product is presented later.

Solutions of the C2O3S product containing 1 mg. per cc. were examined for streptokinase by the method of Christensen (17), for proteinase by employing the copper phenol reagent (18, 19) and using denatured fibrinogen and casein as substrates, and for hyaluronidase by the acid serum turbidity method (20).

These enzymes were found to be absent. On the other hand, assay of the C203S product for desoxyribonuclease, using the method of McCarty (21), indicated the presence of approximately 1000 units of enzyme per mg. of product. A similar amount of desoxyribonuclease was found to be present in the C2O3U product.

Lability of Streptolysin S.—(*a*) *In Solution:* The pH stability of the C2O3S product was examined by following the hemolytic activity of solutions containing 0.05 mg. per cc. at 22° and at 37°. The results are set out in Table IV. It can be seen that in 60 minutes at 37° there was appreciable loss of activity in all solutions tested, and at times longer than 60 minutes a still larger loss occurred in most instances. At 22°, however, inactivation proceeded more slowly, occurring to the least extent in the region of pH 5 to 6, and to the greatest extent at

TABLE IV

Lability of Streptolysin S

Activity at zero time: 750 hemolytic units per cc. Temperature: 22°					Activity at zero time: 700 hemolytic units per cc. Temperature: 37°				
pH	Buffer	Hemolytic units per cc. after			pH	Buffer	Hemolytic units per cc. after		
		60 min.	120 min.	240 min.			60 min.	120 min.	240 min.
3.0	M/10 acetate	400	250	150	4.0	M/10 acetate	300	300	125
4.0	" "	600	550	330	4.5	" "	250	160	80
5.0	" "	600	550	500	5.0	" "	400	330	250
6.0	" "	725	670	670	5.5	" "	400	330	225
7.0	" "	670	525	330	6.0	" "	80	<50	<50
8.0	" "	670	500	330	6.5	" "	200	<50	<50
7.0	M/15 phosphate	685	500	330					

pH 3. In contrast to these results it has been observed that solutions containing 1 mg. per cc., pH 6.8 to 7.0, maintain their activity for at least a week when they are kept at 3°.

Additional experiments in which dilute solutions of C2O3S product were shaken at room temperature in the presence, as well as in the absence, of 0.01 per cent gelatin indicated that the instability of the toxin is not due to surface denaturation.

(*b*) *In Dry Form:* Preparations of C2O3S product, dried by the lyophile process and stored at room temperature, either *in vacuo* or at atmospheric pressure, have been observed to undergo no loss of activity in 6 months.

Effect of Proteolytic and Other Enzymes on Streptolysin S.—Previous investigations of streptolysin S have not provided convincing evidence concerning its chemical nature. Okamoto and coworkers (22) fractionated nucleic acid-broth filtrates and obtained potent preparations which were largely polynucleotide

TABLE V

Effect of Various Enzymes on Streptolysin S

100 γ streptolysin S (C203S product) contained in 1 cc. plus:	Loss of hemolytic activity after 60 min. at 20–25°
	per cent
1 cc. M/15 phosphate buffer, pH 7	0
100 γ crystalline ribonuclease*	0
100 " desoxyribonuclease‡ in M/1000 $MgCl_2$	6
100 " crystalline lysozyme§	0
100 " crude testicular hyaluronidase	0
100 " crystalline trypsin‖	0
100 " " chymotrypsin‖	71
10 " " "	74
1 " " "	9
0.1 " " "	0
10 " crystalline chymotrpysin previously heated for 60 min. at 100°	0
100 " ficin¶	92
10 " "	0
1 " "	0
100 " papain**	0
10 " "	0
1 " "	0
100 " papain in 0.1 per cent cysteine	>90
10 " " " 0.01 per cent cysteine	85
1 " " " 0.001 per cent cysteine	17
1 cc. M/20 acetate buffer, pH 5.5	3
1 " cathepsin solution‡‡ 1:5 in M/20 acetate buffer, pH 5.5	37
1 " " " 1:10 " " " " " " "	6
1 " " " 1:20 " " " " " " "	0
1 " " " 1:5 " " " " " " " containing 0.1 per cent cysteine	94
1 cc. cathepsin solution 1/10 in M/20 acetate buffer, pH 5.5 containing 0.1 per cent cysteine	94
1 cc. cathepsin solution 1/20 in M/20 acetate buffer, pH 5.5 containing 0.1 per cent cysteine	90
1 cc. 0.1 per cent cysteine	0

* Prepared according to Kunitz (23).
‡ Kindly supplied by Dr. M. McCarty.
§ Armour.
‖ Plaut Research Laboratory.
¶ Merck.
** Purified according to Grassman (24) and kindly supplied by Dr. J. S. Fruton.
‡‡ Prepared and kindly supplied by Dr. E. Racker.

and which gave negative tests for protein. The authors considered their streptolysin to be a polynucleotide, possibly a highly polymerized form of ribonucleic acid. Herbert and Todd (13) fractionated filtrates of serum-broth cultures and

suggested that streptolysin S is, or is associated with, lipoprotein. Analyses (Table III) of the best preparations we have obtained indicate that polynucleotide and carbohydrate are present in major amount, but provide no clue as to which, if either, is responsible for the toxic activity. Moreover, as has already been noted the composition of the C2O3S product resembles very closely that of the C2O3U product, the latter being non-toxic and presumably containing no streptolysin. It seemed likely that the use of appropriate enzymes would throw some light on the problem.

Various enzymes were tested for their effect on the C203S product, and in each case 1 cc. of enzyme solution was mixed with 1 cc. of 0.01 per cent C203S product in M/15 phosphate buffer, pH 6.8 to 7 0, or, in some instances, in M/20 acetate buffer, pH 5.5. After standing for 1 hour at 20 to 25°, the solutions were chilled to stop enzyme action, and then titrated for hemolytic activity. The hemolysin titers were compared with those of control solutions containing no enzyme, and the results, shown in Table V, expressed as per cent loss of activity in the presence of enzyme. In order to simplify the presentation of the data a considerable number of controls, including tests for hemolytic activity of enzyme alone, have been omitted from the table.

The results show that, under the conditions used, ribonuclease, desoxyribonuclease, lysozyme, trypsin, and crude hyaluronidase failed to cause a significant degree of inactivation of the toxin. In contrast to these negative results, chymotrypsin, ficin in sufficiently high concentration, papain, and a mixture of cathepsins each effectively inactivated the streptolysin. It is notable that inactivation of toxin by papain occurred only when cysteine was present, and that the cathepsin solution was activable by cysteine although not by ascorbate (the latter not shown in the table). In summary, of a variety of enzymes tested, none of the non-proteolytic enzymes inactivated streptolysin S while all the proteinases, with the exception of trypsin, proved effective as inactivators.

The results suggest that the active constituent of C2O3S product is a protein or polypeptide but they do not preclude the possibility that the polynucleotide present may also be essential for toxic activity. A method of testing this possibility was provided by phosphatase derived from calf intestinal mucosa (25), preparations of which have been shown (25, 26) to be effective in dephosphorylating polynucleotides of the ribose type.

One-half mg. C2O3S product was mixed with 0.5 mg. phosphatase in 5 cc. 1 per cent $NaHCO_3$. After 1 hour at room temperature, the mixture, as well as control solutions containing toxin plus heated phosphatase, and toxin alone, was titrated for hemolytic activity and analyzed for inorganic P. It was found that the active enzyme had converted 40 per cent of the total P of the C2O3S product to inorganic P and that the toxin had undergone no loss of activity. Since a substantial portion of the polynucleotide was hydrolyzed without concomitant loss of toxic activity, it would appear that at least some of the polynucleotide is not essential for toxicity. It would be of interest to know the effect

on streptolysin of complete dephosphorylation, but owing to the lability of streptolysin in the absence of added enzymes it has not been possible to carry out such an experiment.

DISCUSSION

While this work was in progress the manuscript of a paper by Ito, Okami, and Yoshimura (27), on the production of streptolysin by resting streptococci, was received from Doctor H. Okamoto. These authors found that streptolysin was formed by washed cocci of strain "s" suspended in Ringer-phosphate solution containing the sodium salt of commercial nucleic acid. The results of Ito, Okami, and Yoshimura and those reported here are similar in that they lead to the general conclusion that the interaction of washed cocci and ribonucleic acid (or a fraction thereof) results in the formation of streptolysin but they differ in certain other respects. Thus, Ito, Okami, and Yoshimura found that appreciable quantities of streptolysin are formed in the absence of an added carbohydrate while the observations reported here indicate that relatively little streptolysin is formed unless a fermentable carbohydrate is present. In addition, the system which we have employed makes use of a fraction of ribonucleic acid, AF, in place of commercial nucleic acid.

After completion of the experiments described in this report, there appeared a paper by Hosoya, Hayashi, Mori, Homma, Egami, Shimamura, Yagi, and Suzuki (28) on the formation and properties of streptolysin produced under conditions similar to those we have used. In agreement with Ito and coworkers (27) and in disagreement with our observations, Hosoya and coworkers found that appreciable amounts of streptolysin were formed in the apparent absence of fermentable carbohydrate. It is of interest to note, also, that Hosoya and coworkers consider that the mechanism of streptolysin formation does not involve an extraction, and they suggest that the toxin may arise "by the action of ferments in the living hemolytic streptococci on the nucleic acid." In regard to the effects of enzymes on streptolysin S, our findings concerning ribonuclease and chymotrypsin confirm those of Hosoya and coworkers. The results of the two investigations are at variance, however, concerning the effect of intestinal phosphatase on streptolysin, and it would appear that further study is necessary in order to settle this point.

Attention may be called to the fact that all the results in the present paper are based upon experiments conducted with a single streptococcal strain, or its mutant, and that the optimal conditions for the formation of streptolysin S may be different when other strains are used. For example, using the Blackmore strain in lieu of C2O3S but otherwise employing conditions identical with those described, appreciable amounts of streptolysin S failed to appear. When, however, cocci from 17 instead of 5 hour cultures of the Blackmore strain were used, relatively large amounts of streptolysin S were formed. Similar results were

obtained in experiments in which the cocci were suspended in normal horse serum in place of the mixture of AF, glucosamine, and salts. The dependence of streptolysin formation upon the "age" of the culture from which the cocci are taken is not due primarily to differences in the number of cocci present but to some other factor—presumably the same one as underlies the effect described by Fig. 2. At present, it does not seem possible to offer a more satisfactory explanation than to suggest that the cocci can form streptolysin S only when they are in a suitable metabolic state. It may be noted that similar observations have been made in connection with the formation of a number of bacterial enzymes (10).

The evidence presented shows that under appropriate conditions washed cocci are capable of forming appreciable quantities of streptolysin S and that the system studied can be utilized for the preparation of toxin of high potency. The criticism can be made that the appearance of toxin may simply depend upon autolysis of the rather thick suspension of cocci used. That the presence of streptolysin S in the suspending medium does not depend upon autolysis is indicated by several facts. First, there is no decrease in the optical density of the bacterial suspension while streptolysin is being formed. Second, streptolysin develops as a specific response to very low concentrations of AF. Third, the total quantity of non-dialyzable substance liberated by the cocci is very small, amounting probably to less than 1 per cent of their dry weight.

Although it is clear that streptolysin S does not have its origin in autolysis, the precise manner in which the toxin does arise is obscure. The commonly expressed belief that the appearance of streptolysin S in systems containing washed cocci involves simply a physical extraction of preformed streptolysin from the cocci into the suspending solution seems, likewise, to be incompatible with many of the findings reported here. The necessity of an energy source, the effect of temperature on the formation of streptolysin, and the inhibition of toxin formation by a variety of enzyme poisons, demonstrate that streptolysin S is formed only when the cocci are actively metabolizing. The available information concerning the kinetics of toxin formation, as illustrated in Fig. 3, also suggests that the rate of appearance of streptolysin S depends upon rate of synthesis. In agreement with this is the observation that only traces of streptolysin S can be found in sonically disrupted cocci, and this fact seems to us to refute conclusively the extraction hypothesis. The results are best interpreted as indicating that streptolysin S is synthesized in the resting cell system rather than in the culture from which the cocci are derived, that the rate of appearance of streptolysin S is limited by the rate at which a toxin precursor is synthesized, and that the latter rate in turn is limited by energy-yielding or other metabolic processes of the cocci.

The findings presented in Part II of this paper are disappointing in that they fail to establish decisively the chemical nature of streptolysin S. The observa-

tion that the toxin is inactivated in the presence of chymotrypsin, ficin, papain or cathepsins, and not by the other enzymes tested, strongly suggests that protein or polypeptide is essential for activity. In contrast to this inference the analytical data of Table III indicate that of the best preparations obtained thus far, approximately 60 per cent is polynucleotide, and of the remaining 40 per cent a substantial portion is carbohydrate. This leaves room for only a small amount of protein. There is no direct evidence that either the polynucleotide or the carbohydrate is essential for toxic activity, and there is some reason to think that they may not be, for both are present in the non-toxic C2O3U product. Moreover, it has been shown that a significant portion of the polynucleotide of the C2O3S product can be dephosphorylated without loss of activity. Although all the observations presented, and in addition, independent findings concerning the kinetics of hemolysis (29), can be explained by assuming that the toxic activity is due to a small amount of protein in the C2O3S product, the evidence as it now stands does not seem to us to warrant the conclusion that streptolysin S activity is due to protein alone. It appears likely that the chemical nature of the substance responsible for streptolysin S activity can be conclusively established by further purification of the high potency material now available.

SUMMARY

The interaction of washed cocci, prepared under specified conditions, and a polynucleotide (AF) results in the formation of streptolysin S provided a fermentable carbohydrate is present. Maximum toxin formation requires, in addition, the presence of magnesium, potassium, and phosphate ions. Streptolysin S production proceeds anaerobically as well as aerobically but under the latter condition, apparently only if the system is sufficiently reducing. Temperature has a marked effect on the rate of appearance of toxin, the critical thermal increment having a value of approximately 36,000. The formation of streptolysin S is inhibited by mercuric ion, arsenite, iodoacetate, dinitrophenol, azide, and other enzyme poisons. The development of streptolysin S in resting cell systems depends neither upon autolysis nor upon physical extraction of preformed toxin but upon toxin synthesis.

From the supernatant fluid of the resting cell system, a product containing 20,000 to 30,000 units of streptolysin S per mg. dry weight can be isolated. Information concerning the pH stability of the product is presented. The product is free of streptokinase, hyaluronidase, and proteinase, but possesses appreciable desoxyribonuclease activity. Chemical analyses and other findings indicate that polynucleotide and carbohydrate are present in major amount, and that a small but undetermined quantity of protein is present. Inactivation of streptolysin S by chymotrypsin, ficin, papain, or cathepsin, and not by a variety of other enzymes, indicates that protein is essential for activity, but the precise chemical composition of the toxin remains to be established.

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