PROPERTIES OF CERTAIN RAPIDLY ACTING BACTERIAL TOXINS AS ILLUSTRATED BY STREPTOLYSINS O AND S[1, 2]

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The nature of bacterial toxins, their mode of formation and action, and their rôle in the genesis of disease, are problems that have occupied investigators since the early days of medical microbiology. With the recognition that poisonous bacterial products could account for the major manifestations of each of a number of diseases, there developed an active and enduring interest in the qualities of the poisons themselves. In particular, the toxins of the diphtheria, tetanus, and botulinus bacilli have been most intensively investigated.

Apart from the classical exotoxins just mentioned, there exists a rather large group of toxic bacterial products which, on the whole, have been studied less thoroughly. Because of the diversity of their effects, it is difficult to define them, as a group, in a completely satisfactory manner. The toxic bacterial products to be discussed differ from the classical exotoxins in several ways, one of the most striking of which is that their visible effects are not preceded by a latent period of appreciable duration. They act swiftly, and might therefore be termed *rapidly acting* or *acutely acting* toxins. It is noteworthy also that most, if not all, of the rapidly acting toxins are hemolytic, a property which is not shared by diphtheria, botulinus, or tetanus toxin (tetanospasmin). Although none of the rapidly acting toxins has been isolated with certainty as a pure substance, it is clear that the lethal activity of the most potent preparations is of a much lower order than that of the classical exotoxins. Rapidly acting toxins are produced by some strains of staphylococci and streptococci, by pneumococci, by several of the clostridia, and undoubtedly by many other bacterial species.

Among the pathogenic bacteria, a single species may elaborate several distinct toxins of which one or more is of the rapidly acting type. This is true of many strains of *Streptococcus pyogenes*, a fact which has been clarified especially by Todd (23) who differentiated by immunological means, two hemolytic toxins which he designated streptolysin O and streptolysin S. These two substances are, in certain respects, representative of the class of rapidly acting toxins, and in the following discussion they will be considered in some detail. They differ from each other not only immunologically and in the manner in which they are affected by certain organic substances, but also in the conditions governing their formation.

Before discussing the factors involved in formation of streptolysins O and S, it is pertinent to mention some of the conditions necessary for optimal growth of

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the organism. Nutritionally, the pathogenic streptococci are among the most exacting of all bacteria. In addition to salts and an energy source such as glucose, they require an impressive assemblage of amino acids and vitamins (27, 19, 8, 7). However, even when all of the substances known to be required for multiplication are present, growth in terms of bacterial weight per unit volume of medium is likely to be slight compared to that which can, under suitable conditions, be obtained. By increasing the concentration of certain of the nutrients, some of which had been found to be limiting, by reducing the total salt concentration to a minimum, and by neutralizing with alkali the lactic acid formed from glucose, there was evolved a medium of essentially defined composition capable of supporting growth amounting to several grams of streptococci, dry weight, per liter (8, 7).

The mere fact that a toxigenic bacterial species can grow under a particular set of conditions gives no assurance that it will produce toxin under the same circumstances. In fact, there does not seem to be any way of predicting whether the formation of a particular toxin will occur automatically as a consequence of growth. Streptolysin O was found to be produced in the defined medium just as it is in broth, and it appears, therefore, that its formation does not require extra-nutritional factors. The situation in regard to streptolysin S, however, is quite different. Its formation is favored by the presence of serum, either in growing cultures (23) or in contact with resting cells (25, 26), and until recently it was believed that appreciable amounts of streptolysin S are not formed in the absence of serum.

There are certain original and important observations on the formation of streptolysin, made by Okamoto, Itô and their coworkers in Japan that are not well known in this country. In 1939, Okamoto (17) discovered that yeast nucleic acid causes the formation of a very potent hemolysin in cultures of *Streptococcus pyogenes*. The striking effect of yeast nucleic acid can be demonstrated in blood agar plates as well as in broth cultures. The beta hemolytic zones around colonies growing on the surface of blood agar containing sodium nucleate are relatively enormous compared to those of colonies growing on plain blood agar. Although high concentrations of yeast nucleic acid improve the growth of streptococci, it can be shown that the great increase in streptolysin formation seen in plates or in liquid media is not dependent upon increased growth. The yeast nucleic acid effect appears to be specific for beta-hemolytic streptococci, as indicated by the findings of Okamoto (17) who studied several other species of gram positive cocci, and by the results obtained in our laboratory in which a wide variety of gram positive and gram negative organisms were examined. With T. H. Horrigan and H. H. Balch, we have observed the nucleic acid effect not only in strains of Lancefield Group A but also in certain strains belonging to groups D, E, G, H, and L. These findings do not imply that the nucleic acid effect is shown by all strains of these groups nor that it may not be encountered in strains belonging to other Lancefield groups.

The failure of certain agents, such as atmospheric oxygen, sulfhydryl compounds, and cholesterol, to affect the activity of the nucleic acid hemolysin

shows that it cannot be streptolysin O. In all respects studied, however, the nucleic acid hemolysin is identical with streptolysin S. The almost unlimited capacity of bacteria to undergo variation provided a means for testing further the possible identity of the nucleic acid hemolysin with streptolysin S. From a culture of Group A streptococci, it was possible to isolate a mutant which, unlike the parent strain, failed to produce streptolysin S. In broth cultures as well as in plates, nucleic acid, although having its usual effect on the parent strain, failed to stimulate lysin production by the mutant. In addition, examination of a series of strains, some of which produce only streptolysin S, others only streptolysin O, and others both, showed that the nucleic acid effect is demonstrable only in strains having the potentiality for streptolysin S formation. From these results, and the ones already cited, it can be concluded that the nucleic acid hemolysin is streptolysin S (9).

The capacity of ribonucleic acid to induce the formation of streptolysin S is possessed not only by yeast nucleic acid but also by ribonucleic acid from certain other sources, such as mammalian liver, wheat and bacteria. A single preparation of ribonucleic acid from tobacco mosaic virus, tested under the same conditions, failed to cause streptolysin formation—a finding which suggests a difference in structure between the virus ribonucleic acid and that from other sources. Desoxyribonucleic acid, in contrast to ribonucleic acid, appears to be inactive. Negative results were likewise obtained upon testing the products of acid- and alkali-hydrolyzed ribonucleic acid as well as the constituent purine and pyrimidine nucleotides and some of their hydrolysis products.

The streptolysin-forming action of some preparations of ribonucleic acid is markedly and specifically increased by treatment with ribonuclease, the activation, in some instances, being as great as ten-fold. Although different preparations of ribonucleic acid may vary considerably in activity, after they are treated with crystalline ribonuclease they show approximately the same activity, indicating that they possess approximately equal potential capacity to induce streptolysin formation.

On fractionation of the digestion mixture following the action of ribonuclease upon yeast sodium nucleate, it is possible to isolate a polynucleotide which is approximately one hundred times as active as untreated yeast nucleic acid. The active polynucleotide contains a greater proportion of purine nucleotide than is present in the starting material, but its exact composition has not been determined.

The biological significance of desoxyribonucleic acids is clearly established by Avery, MacLeod, and McCarty's demonstration (1) of the ability of desoxyribonucleic acid to direct, in a specific and inheritable manner, the synthesis of pneumococcal polysaccharide. Although ribonucleic acid is commonly believed to perform essential functions in living systems, there are no instances in which its rôle has been clearly defined. Streptococci furnish, so far as we know, the only physiologically specific test for a ribonucleic acid. Further study of what may appear to be but a laboratory curiosity will, perhaps, provide an insight into the function of ribonucleic acid, its structure, or both.

In addition to ribonucleic acid, or a fraction thereof, at least one other factor is essential for the formation of appreciable amounts of streptolysin S in cultures (9). This factor is present both in peptone and meat infusion, but is absent from the defined medium supplemented with polynucleotide. Study of its properties has shown that it can be replaced by minute amounts of maltose or by somewhat larger amounts of glucosamine. With the exception of trehalose, which is about one-fifth as active as maltose, other disaccharides, including the beta-glucosidic isomer, cellobiose, possess less than 10 per cent of the streptolysin-forming activity of maltose. As little maltose as M/64,000 can cause significant streptolysin formation in the presence of about 180 times as many glucose molecules. Essentially nothing is known of the mechanism whereby such relatively minute amounts of maltose exert their effect. Although observations like this appear to take us rather far afield, they may eventually contribute to the solution of other problems such as the mechanisms of formation and action of the toxin.

The chemical nature of the streptolysins has been the subject of a number of investigations. Both products are very labile substances of high molecular weight. Highly purified preparations of streptolysin O, obtained by Smythe and Harris (21), Herbert and Todd (12), and ourselves (4), exhibit the properties and elementary composition of proteins. Streptolysin O contains a relatively large amount of sulfur which occurs either in disulfide or sulfhydryl form, and it is activated by substances which reduce disulfide links to sulfhydryl groups. It is destroyed by proteolytic enzymes, and there is little reason to doubt that streptolysin O is a protein. Evidence of close chemical and biological similarity between streptolysin O and pneumolysin is indicated by the work of various investigators (15, 16, 20, 11 *et al.*). That streptolysin O is immunologically related to pneumolysin, tetanolysin and *Clostridium welchii* theta toxin, has been shown by Todd (22, 24).

The chemical nature of streptolysin S is more obscure. Herbert and Todd (13) fractionated filtrates of serum-broth cultures, and obtained a product which suggested that streptolysin S is, or is associated with, a lipoprotein. Okamoto and co-workers (18) fractionated filtrates of nucleic acid broth cultures, and obtained preparations of great potency, of which the most active was largely or exclusively polynucleotide in nature, and said to be free from protein. Finally, we (4) have fractionated filtrates of defined medium cultures containing maltose and a fraction of yeast nucleic acid, and have obtained a very potent product whose properties suggest that streptolysin S may be a protein or a nucleoprotein. Further work is needed.

Having considered in some detail the nature of the streptolysins and the circumstances attending their formation we may now proceed to some of their biological properties of which by far the best known is the capacity to lyse blood cells *in vitro*.

Study of hemolysis caused by rapidly acting toxins, including streptolysins O and S as well as lytic agents from other sources, has revealed rather striking similarities and differences in the kinetics of the hemolytic reaction—findings which must in turn reflect similarities and differences in the mechanisms of cellular injury (3). For example, rate of hemolysis, under a particular set of experimental

conditions, is directly proportional to concentration of lytic agent when the latter is streptolysin S or any one of several other rapidly acting toxins—a result that would be expected if these substances function catalytically, that is, as enzymes. Results entirely different from these are seen when the lytic agent is saponin, taurocholate, or tyrocidine, and still other results when it is streptolysin O. Other studies concerned with the effect of temperature on the kinetics of hemolysis have provided an independent means of classifying rapidly acting toxins, and have shown in addition, that these substances possess activation energies most of which fall within the range of those of enzymes.

Although it is self-evident that the substances under discussion act upon erythrocytes in ways that lead to the liberation of the cell contents, the nature of the initial reaction and that of the sequence of events that follow are relatively obscure. A partial but important exception to this statement is found in the discovery of MacFarlane and Knight (14) that the alpha toxin of *Clostridium welchii* specifically catalyzes the hydrolysis of lecithin. This toxin is hemolytic and generally cytotoxic, presumably because it destroys the lecithin of cell membranes.

Analysis of the *Clostridium septicum* hemolytic system (2, 3) indicates that lysis is preceded by two phases, the first of which is an irreversible alteration of erythrocytes caused by the toxin. This phase is specifically inhibited by antibody. In the second phase, the altered cells undergo swelling until they burst. The second phase, which is inhibited by sucrose but not by antibody, can be explained as resulting from loss of selective permeability, which leads to a kind of osmotic hemolysis. The hemolytic action of streptolysin S, although not studied as intensively, shows many of the features of the *Clostridium septicum* system. That of streptolysin O, however, appears to be fundamentally different; the stage of swelling is either lacking or lasts for such a short time that it escapes observation.

It is evident that the *initial* actions of streptolysins O and S differ fundamentally from each other and from those of certain other lytic agents. The rapidly acting toxins can be visualized as destroying specifically one or another linkage of the lipids, proteins or complexes thereof, which comprise the surface of the erythrocyte. Knowledge of the chemical nature of the initial change, that is, of the particular linkages attacked, should help elucidate the molecular architecture of the red cell membrane.

Because of the ease with which their presence can be detected using erythrocytes, the rapidly acting toxins just discussed are commonly designated as hemolysins. This term, however, is not altogether desirable because it implies that the action of the substances under consideration is limited to blood cells. As will now be shown, this is not the case.

Injection of partially purified preparations of streptolysin O into mice causes a fatal toxemia. Since the injected mice do not die of intravascular hemolysis, but rather of cardiovascular and perhaps other injuries, it is clear that cells in addition to those of the blood are attacked. Another indication that the action of streptolysin O is not limited to erythrocytes is shown by unpublished work of A. Brittis in our laboratory, who has found that streptolysin O, and indeed also other rapidly acting toxins, cause *in vitro* the dissolution of lymphocytes prepared

from mesenteric lymph nodes. The findings suggest that many or most substances that are erythrocytolytic are also lymphocytolytic.

A different approach to the action of streptolysin O has been provided by studying its effects on the isolated heart of the frog, washed free of blood, and filled with Ringer's solution. A heart prepared in this way will continue to beat approximately 35 times each minute for a great many hours provided it is supplied with oxygen. It has been shown (5, 10) that a single administration of partially purified, and considerably diluted, streptolysin O has little or no apparent effect. When, however, the first dose of streptolysin O is removed, and the heart washed twice with Ringer's solution and then treated with a *second* dose of streptolysin O identical with the first, the heart quickly stops beating. Application in a single dose of the total amount of streptolysin O given in two doses does not cause cardiac standstill. Two doses must be used, the first dose *sensitizing* the heart to the second. Sensitization occurs because the first dose releases from the heart tissue (and thereby deprives it of) a substance which inhibits the toxic action of streptolysin O. The protective substance derived from the tissue is removed in the perfusate when the heart is washed with Ringer's solution. This substance inhibits not only the systolic contracture-producing action of streptolysin O but also the lethal effect for mice. The inhibitory effects of the protective substance appear to be specifically directed against streptolysin O and closely related toxins. It would be of great interest to know what this inhibitor is but study of its chemical nature has been seriously hampered by the very minute quantities that can be conveniently obtained.

Whether the mechanism observed to operate in the frog's heart functions also in the mammalian heart is not certain, but there are observations which suggest that it does (6). Injection into mice of a just sublethal dose of streptolysin O causes the mice to become refractory to the effect of a subsequently administered lethal dose of the same substance. The refractoriness, which is not due to antibody, develops within six hours and lasts less than forty; it may well depend upon the liberation into the bloodstream of a toxin inhibitor similar to that released by the isolated frog's heart. In any event, it is notable that there exists in the mouse, and presumably in other mammals, an immune mechanism which is distinct from that underlying classical antitoxic immunity.

In conclusion, we have attempted to review some of the distinguishing features of streptolysins O and S, bearing in mind that they represent a class of biologically active substances distinct from the better known exotoxins. While these bacterial products do not constitute a field of study that is new, it is nevertheless true that many of the most basic questions concerning them have been only partially answered. Many of the other members of this class have been very lightly explored, but enough is known to indicate that the investigator who studies them will not be disappointed in what he sees, for they are almost as varied in their effects as bacteria themselves.[3]

[3] Many persons have contributed to the work which has been discussed. The author is particularly indebted, for collaborative experimentation, to Dr. G. L. Cantoni, Long Island College of Medicine, and in less tangible ways to his associates at New York University College of Medicine.

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