Cytolytic Toxins of Bacterial Origin

The nature and properties of cytolytic proteins are discussed with emphasis on staphylococcal α-toxin.

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Bacterial toxins belong to one of four categories: (i) The classical exotoxins that are synthesized by the microorganisms causing diphtheria, tetanus, and botulism. These were the first to be discovered, and they are distinguished by the fact that they continue to be almost the only ones that have been demonstrated to participate in the genesis of human infectious disease. (ii) The endotoxins, which form part of the cell walls of Gram-negative bacteria, are lipo-polysaccharides; they are clearly very different from the classical exotoxins which are proteins. (iii) Cytolytic toxins. (iv) Toxins which do not fall into the foregoing groups, as the leucocidin and enterotoxins of staphylococci, the erythrogenic toxins of streptococci, and the toxins of plague and anthrax bacilli.

We are here concerned exclusively with toxins of the third group. It is composed of staphylococcal α-, β-, and δ-toxins; streptolysin S; streptolysin O; *Clostridium welchii* α- and θ-toxins; *C. septicum* toxin; tetanolysin; pneumolysin; cereolysin; *Streptococcus zymogenes* bacteriocin; and others. Information regarding the occurrence and properties of some of these bacterial products has been reviewed by Guillaumie (*1*). The substances belonging to this group have certain features in common. All of them are found extracellularly. Most of them are proteins, and most give rise to neutralizing antibodies. Streptolysin S, or its active moiety, appears to be a relatively small polypeptide (*2–4*). All of them are lytic for erythrocytes when tested in vitro and some of them are the most potent hemolytic agents known. For example, streptolysin S (*2*), cereolysin (*5*), and staphylococcal β-toxin (*6*) in concentrations of the order of 0.001 microgram per milliliter are lytic for erythrocytes of appropriate species. They are lytic also for a variety of other cell types. Finally, most of them are lethal for laboratory, and presumably for other, animals, the lethal dose being considerably larger than that of the classical exotoxins.

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Staphylococcal α-Toxin

I will discuss in some detail the α-toxin of *Staphylococcus aureus* because it is more or less typical of the group, and for the more cogent reason that my laboratory, among others, has been occupied with its study for several years. This toxin is usually produced during growth of pathogenic strains of staphylococci, but not during growth, or in any other culture phase, of nonpathogenic strains. Although it is synthesized only by staphylococci, these microorganisms form a rather large number of extracellular products of which α-toxin is only one. When a concentrate containing the large molecules that are present in the supernatant fluid of a staphylococcal culture is subjected to electrophoretic separation in starch gel, and the gel is then sliced and stained for protein, at least 13 different proteins are discernible (Fig. 1). Had the more sensitive technique of disc electrophoresis in acrylamide gel been used instead of electrophoresis in starch gel, the number of proteins demonstrable would almost certainly have been appreciably larger.

α-Toxin can be separated from the bulk of the other proteins by fractional precipitation with ammonium sulfate and by continuous-flow electrophoresis (*7*). In this way, partially purified toxin, which we call stage-5 toxin, is obtained in a yield of about 40 percent. It can be further purified by density-gradient electrophoresis (*7*, *8*), by heat-induced aggregation followed by disaggregation in urea (*9*), and in other ways (*10*). Highly purified toxin has ultraviolet absorption characteristics typical of proteins (*7*, *10*), contains less than 0.025 percent phosphorus, and less than 1 percent carbohydrate as glucose (*7*). Its amino acid composition has been determined (*7*, *10*), and it is notable that cystine is absent. It seems to contain only one amino terminal residue, arginine (*10*). It is a basic protein, and the best estimate of the isoelectric *p*H of the major peak, obtained by isoelectric focusing, is 8.6 (*11*). Reports from various laboratories show a sedimentation coefficient in the region of 3*S*. The molecular weight of α-toxin, first estimated by ultracentrifugation (*7*) was found to be about 44,000, and later by gel filtration (*5*) a value of 41,000 was obtained. Coulter (*10*) is of the opinion that 30,000 is closer to the true value. The molecular weights of several lytic agents of bacterial origin are compared in Table 1.

When partially purified α-toxin (*7*) is examined in the analytical ultracentrifuge, two components are visible: a larger, slower-moving peak having a sedimentation velocity at 20°C in water of approximately 3, and a smaller, faster-moving peak having a sedimentation velocity of about 12. The 3*S* component is toxic, whereas the 12*S* component is not, and it was at first thought that the 12*S* material was a contaminant unrelated to α-toxin. However, Lominski, Arbuthnott, and Spence (*12*), who independently obtained an ultracentrifugation pattern similar to ours, suggested that the faster-moving component might be a polymerized form of toxin. Recent experiments indicate that the 12*S* component is indeed an aggregate containing toxin molecules because disaggregation by 8*M* urea is accompanied by the appearance of a substantial amount of toxic activity (*9*). Electron-microscopic examination of the 12*S* component reveals that it consists largely of rings having an outside diameter of approximately 100 Å. Each ring appears to be made up of a circular arrangement of subunits measuring 20 to 25 Å in diameter, and application of the image-rotation technique to selected rings indicates that the number of subunits per ring is 6 ± 1 (*9*). The molecular weight of the 12*S* component, estimated from sedimentation velocity and diffusion rate to be in the range of 240,000 to 330,000 (*13*),

Table 1. Estimates of molecular weight of some cytolytic toxins of bacterial origin.

Toxin	Molecular weight	Method of estimation
Staphylococcal α-toxin	44,000	Pseudoequilibrium (*7*); see text
Clostridium welchii α-toxin	31,000	Gel filtration (*5*); see (*62*)
Cereolysin	54,000	Gel filtration (*5*)
Streptolysin S	12,000	Gel filtration (*4*)
Streptolysin O	<10,000	Sedimentation velocity (*63*)
Staphylococcal hemolysin G	~12,000	Sedimentation velocity and gel filtration (*64*)
Staphylococcal δ-toxin	68,230	Ultracentrifugation and amino acid composition (*65*)
Staphylococcal β-toxin	59,000	Ultracentrifugation (*47*)

is consistent with six times 44,000. It is possible, nonetheless, that the 12*S* component does not consist exclusively of toxin. The conditions governing transition of 3*S* toxin to the 12*S* form are not fully understood, but the transition apparently can be brought about by cell membranes and artificial phospholipid spherules (*14*).

In addition to the 12*S* structure, the toxin can exist in still another molecular arrangement. On standing in the cold, solutions of highly purified toxin lose activity, and the loss is accompanied by the formation of a nontoxic, insoluble precipitate (*10*, *15*) which is capable of inducing formation of specific antitoxin. This finding is interpreted as indicating that toxin tends slowly to polymerize to an inactive form which is not 12*S*. A presumably identical polymerization occurs very rapidly when solutions of toxin are brought to 60°C (*9*, *16*). When the precipitate is disaggregated in 8*M* urea, toxin closely similar to, if not identical with, native toxin is regained. In the electron microscope, heat-polymerized toxin does not show the rings characteristic of the 12*S* form. The two states of α-toxin just described can be regarded as two kinds of toxoid—one soluble, the other insoluble, and they clearly represent two different molecular arrangements.

A curious effect of heat on α-toxin was described 60 years ago by Arrhenius (*17*), who found that heating toxin to 60°C resulted in complete loss of hemolytic activity, but subsequent heating to 100°C was accompanied by partial reactivation. The mechanism of the paradoxical Arrhenius effect has been the subject of considerable controversy (*16*, *18*). In light of the above findings it can be explained simply as a polymerization to inactive aggregates induced at 60°C, followed by partial reversal of the reaction at higher temperatures, or perhaps by complete reversal with permanent destruction of some, but not all, of the toxin (*9*).

Progress in the direction of more precise physical and chemical characterization of α-toxin is made difficult by the fact that the toxin in its conventional form (3*S*) is not molecularly homogeneous. Analysis by density-gradient electrophoresis revealed the presence of three or four species of toxin molecules having similar, though not necessarily identical, biological effects (*7*). On the basis of this finding we suggested (*19*) that the toxin may exist in several different states of molecular aggregation, and from these and results of gel diffusion, Coulter (*10*) has postulated that the toxin is an associating system. An observation of Madoff (*20*) also agrees with this concept. Even very highly purified toxin has proved to be molecularly inhomogeneous when examined by ultracentrifugation (*13*) and by isoelectric focusing (*11*). The combined results, derived from a variety of sources, suggest that active toxin could be a mixture of molecules with weights of 21,000 to 55,000, but there is need for better data than now exist.

Table 2. Relative sensitivity of erythrocytes from various animal species to staphylococcal α-toxin. From Cooper, Madoff, and Weinstein (*16*) slightly modified. For additional data see (*19*).

Animal	Sensitivity of erythrocytes compared to those of rabbit (%)
Rabbit	100
Wallaby	20
Hamster	11–27
Dog	10–25
Rat	10
Mouse	9
Cat	8–10
Deer	5
Wood duck	4
Sheep	0.6–1.0
Bear	1
Chicken	0–0.5
Guinea pig	0–0.1
Man	0–0.8
Horse	0–0.06
Monkey	0

Biological Effects and Their Mechanisms

The classical biological effects of α-toxin are hemolysis, death, and dermonecrosis (*21*). A suspension of washed rabbit erythrocytes is lysed by toxin in a concentration of about 0.05 μg/ml. The kinetics of hemolysis have been described repeatedly (*22*, *23*) but with rather different conclusions to the question of whether the primary reaction that leads ultimately to release of hemoglobin is or is not enzymic. There exist striking differences in sensitivity of the red blood cells of different species of animals to α-toxin (Table 2), and even erythrocytes from individuals of the same species, rabbit for example, can vary in sensitivity as much as threefold (*7*, *16*). Unlike *Clostridium welchii* α-toxin which requires calcium ions for hemolysis, unlike staphylococcal β-toxin which requires magnesium ions, and unlike streptolysin O which needs sulfhydryl groups, staphylococcal α-toxin does not require a cofactor to lyse cells.

The toxin is lethal for mice (*19*), rabbits (*24*), frogs (*25*) and other animals. Approximately 1 μg injected intravenously kills mice, but the mechanism of death, even when high lethal doses are used, is obscure. It may involve an action on smooth muscle (*26–28*), but the toxin can also act on skeletal muscle (*29*, *30*) and doubtless on other kinds of tissues as well. A striking, and perhaps unique, effect is the development of paralysis of the hind legs of mice (*29*) and of rabbits (*31*). The toxin is known to liberate histamine from some isolated animal tissues and not from others (*32*). The studies of Brown, Casewell, and Quilliam (*32*) and those of Wurzel, Bernheimer, and Zweifach (*28*) suggest that its spasmogenic action on smooth muscle is not mediated by local liberation of pharmacologically active substances such as histamine, 5-hydroxytryptamine, or acetylcholine, but is due, rather, to a direct action of the toxin on the smooth muscle itself.

Injection of about 1 μg into the skin of a rabbit is followed by development of a necrotic lesion of impressive dimensions. One of several possible mechanisms of the dermonecrotic effect is ischemia resulting from spastic action of toxin on vascular smooth muscle (*27*).

All recent biochemical investigations of α-toxin support the view that its

diverse biological effects are caused by a single kind of protein or by a group of closely similar proteins. Nevertheless, there is evidence suggesting that different strains of *Staphylococcus aureus* may produce α-toxins that do not have identical biological effects (*33*). The investigation of McClatchy and Rosenblum (*34*) indicates that a single mutational event can result in loss of all forms of α-toxin activity. Other mutations give rise to strains that are not hemolytic on rabbit blood agar but which retain dermonecrotic and lethal activities and immunological specificity. Still another mutant exhibited no α-toxicity at all but produced a protein that reacted immunologically with α-antitoxin. The implication of these and other results is that mutations can produce changes in the primary structure of the toxic protein with consequent alterations in biological activity. These important findings provide an approach to identifying the active site (or sites) of the molecule, and ultimately they should help to explain why the protein is toxic.

We do not propose to review completely the literature on the effects of cytolytic bacterial toxins on diverse kinds of tissues, cells and subcellular structures. References to a number of papers can be found in (*19*), (*35*), and (*36*). Some of this information is presented in concise form as Table 3, the data of which are an oversimplification and require qualification. The cytotoxic agents listed are not equally efficient in lysing rabbit erythrocytes, and had erythrocytes of horse rather than rabbit been chosen, the differences would be so great as to lead to the conclusion that some of the agents are not hemolytic at all, or at best only feebly so. Also, not all kinds of cells in tissue culture undergo cytopathic changes in response

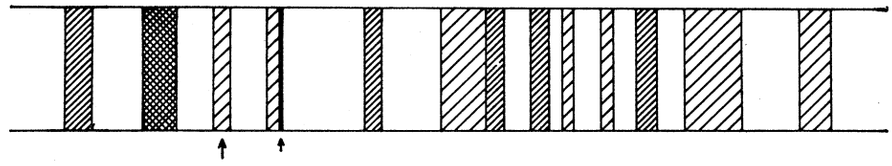

Fig. 1. Representation of starch-gel electrophoretic pattern of extracellular proteins produced by a coagulase-producing staphylococcus. Small arrow indicates the position at which the sample was applied; large arrow indicates band identifiable as α-toxin. Cathode is to left of origin; anode is to right [modified slightly from (*19*)].

to all the agents. Finally, some of the data were obtained in tests of toxins that were not of the highest purity, and therefore not all of the results can be regarded as definitive. Although the cytolytic toxins are capable of affecting a remarkably wide variety of cells and subcellular elements, they are by no means indiscriminate (see Table 2, for example). They do not appear to have any effect on whole bacterial cells or on ciliated protozoans (*37*).

The mechanisms by which cytolytic bacterial proteins damage cells have been studied most extensively with erythrocytes. Before the era of molecular biology, when much attention was focused on energy metabolism, it could be explained that interference by toxins with the generation of energy needed for maintenance of normal cell permeability could easily lead to hemolysis. This facile explanation, having no basis in fact so far as cytolytic toxins are concerned, has given way to the concept that cytolytic toxins directly produce chemical alterations in the cell membrane, destroying its semipermeable character (impermeability to cations), and inducing lysis which in at least some, and possibly in all, instances is osmotic in nature. There is much circumstantial evidence supporting this theory, not the least important of which is the fact that isolated membrane-bound, osmotically sensitive, subcellular elements—lysosomes—are lysed by all toxins that are hemolytic (*38*).

The latter concept, derived in part from the work of Wilbrandt (*39*), was developed in a model hemolytic system based upon the action of toxin of *Clostridium septicum* (*40*). Two phases were clearly demonstrated: one occurring before lysis in which the erythrocyte membrane was altered by direct action of the toxin, and one which was independent of the toxin, a temporally secondary phase involving cell swelling and escape of hemoglobin. The two phases could be inhibited independently of each other. A closely similar sequence of events has been elucidated for staphylococcal α-toxin (*23*).

The fact that cytolytic toxins act on external cell membranes does not preclude the possibility that some of their damaging effects could also be due to an action on intracellular membranes. Polymorphonuclear leukocytes contain cytoplasmic granules, lysosomes, which disappear when the cells are exposed either to streptolysin O or to streptolysin S, and this happens before most other morphological signs of damage are evident. It has been suggested (*41*) that in these systems lysosomal enzymes may be discharged directly into the cytoplasm with consequent destruction of intracellular structure. However, the findings of others (*42*) have not lent much support to this idea.

Table 3. Sensitivity (+) and insensitivity (0) of various biological structures to some cytolytic bacterial proteins.

Toxin	Erythrocytes (rabbit)	Polymorphonuclear leukocytes (rabbit)	Platelets (rabbit)	Tissue culture cells (various)	Mitochondria (rabbit)	Lysosomes (rabbit)	Whole bacteria	*Staphylococcus* protoplasts	*Escherichia coli* spheroplasts	*Mycoplasma neurolyticum* A	Ciliated protozoa
Staphylococcal α-toxin	+ (*7*)	+ (*66*)	+ (*55*)	+ (*67*)		+ (*38*)	0 (*68*)	+ (*50*)	+ (*50*)	+ (*51*)	0 (*69*)
Streptolysin S	+ (*48*)	+ (*41*)	+ (*55*)	+ (*36*)	+ (*70*)	+ (*71*)	0 (*68*)	+ (*50*)	+ (*50*)	+ (*51*)	0 (*72*)
Streptolysin O	+ (*48*)	+ (*41*)	+ (*55*)	+ (*73*)	+ (*70*)	+ (*71*)	0 (*68*)	0 (*50*)	+ (*50*)	+ (*51*)	0 (*72*)
Staphylococcal β-toxin	+ (*35*)	0 (*66*)	+ (*55*)	+ (*74*)		+ (*38*)					0 (*69*)
Staphylococcal δ-toxin	+ (*75*)	+ (*76*)	+ (*55*)	+ (*35*)		+ (*38*)					
C. welchii α-toxin	+ (*77*)		+ (*55*)		+ (*78*)	+ (*38*)					

Substrates and Possible Substrates

Accepting that cell membranes are the site of action, the most important questions remain to be asked: Are the cytolitic toxins enzymes or are they proteins other than enzymes, whose topography includes strongly hydrophobic and hydrophilic regions? And, in either case, what is the identity of the membrane constituents with which they react? Studies of the kinetics of hemolysis have not provided an unequivocal answer to the first question. The α-toxin of *Clostridium welchii* has been known for more than 25 years to be a phospholipase C (*43*) having a substrate preference for phosphatidyl choline. But as MacFarlane (*44*) has pointed out, the fact that one bacterial toxin is an enzyme does not necessarily mean that all are. Recently a second cytolytic toxin, staphylococcal β-toxin, has been demonstrated also to be a phospholipase C but with a substrate preference for sphingomyelin and lysophosphatidyl choline (*45*, *46*). It is curious that this toxin also has glycosidase activity (*47*).

The enzymatic or nonenzymatic nature of the remaining members of the group is mainly a matter of conjecture. They possess the general properties of enzymes, and some information is at hand concerning the identity of the membrane constituents that probably participate in their toxic action (Table 4). In each instance, lipid either is involved or appears to be.

Streptolysin O (*48*) is a cytolytic toxin of some practical importance because it is an essential reagent in the measurement of a specific antibody, antistreptolysin. Increase in the amount of antistreptolysin in serum following streptococcal infection of man is used in the differential diagnosis of streptococcosis and rheumatic fever. Streptolysin O is the prototype of a group of closely similar "oxygen-labile" toxins that are produced by diverse Gram-positive bacteria, other examples of which are pneumolysin, tetanolysin, cereolysin, and the θ-toxin of *C. welchii*. The lytic action of streptolysin O, and probably that of all other members of this group, is specifically inhibited by cholesterol in low concentration. Certain other sterols that are structurally closely related to cholesterol also inhibit lysis (*49*). All cells that are sensitive to streptolysin O contain cholesterol as a constituent of their cell membranes, and all cells so far examined that lack membrane cholesterol are insensitive to its lytic action (*50*, *51*). The combined evidence is therefore very strong that membrane cholesterol is involved in the lytic action.

Table 4. Probable natural substrates or receptors for cytolytic toxins.

Toxin	Substrate or receptor
Clostridium welchii α-toxin	Phosphatidyl choline (*43*)
Staphylococcal β-toxin	Sphingomyelin* (*45*)
Streptolysin O and congeners	Cholesterol (see text)
Streptolysin S	Phosphatides (*56–58*)
Staphylococcal α-toxin	Phosphatides ?
Staphylococcal δ-toxin	Phosphatides ?

* Possibly also polysaccharides (*47*).

Streptolysin S (*36*) is of current interest because it is a mitogenic agent (*52*), it is capable in minute doses of inducing experimental arthritis (*53*), and it has been suspect as an agent that may be of importance in the pathogenesis of rheumatic disease (*54*, *55*). Its lytic action is inhibited by small amounts of phosphatidyl choline (*56*) and by other phosphatides (*57*). Moreover, it disrupts artificial lipid spherules made up of phosphatidyl choline, cholesterol, and dicetyl phosphate (*58*). It seems probable therefore that phosphatidyl choline, or other membrane phosphatides, or both, are specifically involved in its action.

Although staphylococcal α-toxin has been intensively studied there is no definitive information on the chemistry of its action. Despite a claim to the contrary (*59*), it does not have proteolytic activity (*7*, *10*), nor is there evidence that it is a phospholipase (*60*). However, its spectrum of action is somewhat similar to that of streptolysin S, and this suggests, rather tenuously, the participation of phospholipids. Like streptolysin S, it interacts with artificial lipid spherules (*61*), but it is not entirely clear to what extent the mechanisms underlying the behavior of this model system are identical with those operating in biological entities bound by naturally occurring membranes.

Implications

The cytolytic bacterial toxins comprise a group of proteins that have both practical and theoretical implications for a variety of biological and medical problems. As products of pathogenic microorganisms some of them contribute to the genesis of disease, but the degree to which each functions in this way is largely unsettled. Quite apart from this, their specificity suggests they may prove to be useful reagents for the general study of cell membranes. The mechanisms by which these relatively large molecules escape from the bacterial cell are poorly understood, but elucidation of them may have relatively broad biological significance. Equally intriguing, and equally a matter of speculation, is the question of why these substances are synthesized at all, which is to ask how they contribute to the economy of the cells that produce them. Application of the powerful techniques of modern protein chemistry should lead to the solution of some of these problems.

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Standardized Ability Tests and Testing

Major issues and the validity of current criticisms of tests are discussed.

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Standardized ability tests have been a source of considerable controversy in recent years. Growing competition for jobs and for all educational opportunities has intensified the search for better ways to evaluate individual abilities and aptitudes and to identify intellectual potential at progressively earlier ages. Standardized tests of various types increasingly are used to identify applicants throughout the educational system, as well as by the military, the civil service, and business and industry (*1–3*).

This reliance on results of standardized tests has caused questions to be raised about the validity of the tests used, as well as their effects on those who take them and on the society that uses them to differentiate among its members. Thus far, there have been very few, if any, attempts to bring together all of the criticisms that have been leveled against tests, and to place them in an analytical framework that would permit a systematic evaluation of their validity. In this paper the validity of standardized tests is discussed, and major criticisms of tests are summarized within such a framework.

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Three Variables of Criticism

Criticisms of testing relate to three variables: the type of test, how it is used, and assumptions regarding its validity. First, the type of test being used must be considered. Ability tests may be divided into tests that attempt to measure inherent capabilities, potentials, or abilities acquired over a long time, and tests designed to measure specific achievements.

Intelligence and aptitude tests are implicitly assumed to measure a relatively deep and enduring quality. This quality may be viewed as changeable; however, startling changes are assumed to be rare except under specific conditions, as when extreme cultural deprivation is ameliorated. Intelligence and aptitude tests therefore generate anxiety in people tested. The high cultural value placed on intellectual abilities in our society also makes any instrument which purports to measure general intellectual abilities a source of fascination. For these reasons, such tests have been a major source of controversy and debate.

Although less often perceived as unfair, since they measure skills acquired in a particular area over a short time, achievement tests potentially exert a considerable influence on subject matter and teaching methods, as well as on what skills appear desirable. Among all tests, they are distinctive in that it is