RECENT CHEMICAL INVESTIGATIONS OF BACTERIAL TOXINS

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The chemical investigation of bacterial toxins holds much of practical and theoretical significance not only in immunology but also in physiology and chemistry. The ultimate objectives of

these studies are to determine the mode of formation of toxins, their nature, and relationships between their chemical and biological properties. This review will indicate some of the paths by which these objectives have been approached.

True toxins such as those of diphtheria and tetanus may be distinguished from other bacterial poisons by high antigenicity and the production of well-defined physiologic effects when minute amounts are injected into susceptible animals. Although the term toxin has been used to denote any poison of unknown nature, it is used in this paper to mean an antigenic poison. It is difficult to draw the line between "exotoxins" and "endotoxins" because antigenic poisons possess all degrees of toxicity and antigenicity. There are also marked differences in the ease with which toxins are liberated from bacterial cells.

Well-known biologic properties of toxins and immunological phenomena common to toxins and non-poisonous antigens will be discussed in this review only when related to the nature or mode of action of toxins. The reaction between toxin and antitoxin could be discussed in detail more profitably in a review of antigens and antibodies in general, because this reaction probably does not differ essentially from the reaction between any other antigen and the corresponding antibody.

Nothing at all will be said about toxins such as that from *Clostridium welchii*, the toxic substances derived from gram-negative cocci, or the fibrinolysins. In general, what chemical work has been done on these is similar to that on other toxins described in this review.

PART I. THE PRODUCTION OF BACTERIAL TOXINS

1. *The use of media of simplified composition*

Progress in studies on the mode of formation and the nature of toxins will be greatly facilitated when toxins are produced on media of known composition, or, at least, on media much simpler than those at present in use.

Mueller (1922 to 1937) has worked out the cultural requirements of the diphtheria bacillus so that abundant growth of some toxigenic strains may be obtained in a medium consisting of

simple organic and inorganic substances. Pappenheimer and Johnson (1936), in extending the observations of Locke and Main (1931), Scheff and Scheff (1934), and others, have shown that definite low concentrations of iron and copper are essential to toxin production. Previous failure to produce it in Mueller's medium was due to the inhibitory effect of an excess of iron. Pappenheimer, Mueller, and Cohen (1937) have described the production of potent diphtheria toxin in a medium in which the organic constituents are certain amino acids together with pimelic acid, beta-alanine, and nicotinic acid. This medium is not synthetic in the strict sense of the word, because some of the purified constituents were obtained from natural sources and may have contained impurities which stimulate toxin production. In this connection may be mentioned the finding of Pappenheimer and Johnson (1937) that an unknown inorganic constituent of soft glass stimulates the production of toxin in simplified media.

Media consisting of amino acids and small amounts of partially purified but unknown constituents have been developed by Knight, Fildes and their co-workers (1933, 1936) for growth of the staphylococcus and for *Clostridium botulinum.* Knight (1937) has found that the accessory substances necessary for the growth of the staphylococcus are nicotinic acid and vitamin B_1, or closely related substances. However, toxins have not yet been produced in significant amounts in these media. Burrows (1933), using an amino acid medium, was able to produce weak toxin from type A strains of *Cl. botulinum* but not from type B strains. Tryptophane seems to stimulate slightly the production of toxin. Tani (1934) obtained growth and production of toxin by *Cl. botulinum* in a biuret-negative medium made from a sulphuric acid hydrolysate of Witte peptone.

For purposes of subsequent purification of toxins, we may use protein-free media of simplified but unknown composition from which the toxin may be precipitated selectively. The Wadsworth-Wheeler medium (1934) contains no proteins precipitable by acid but does contain proteoses precipitable by ammonium sulphate, alcohol, or acetone. Diphtheria toxoid may be precipitated by acid in a relatively pure state from this medium, but

the toxin is damaged by acid. This medium might be useful for the production and purification of other toxins that remain undamaged by precipitation with acid. A similar medium has been used by Sommer (1937) for botulinus toxin. For the production of diphtheria toxin, Pappenheimer and Johnson (1937) have described a gelatin hydrolysate medium to which certain amino acids and accessory substances have been added. It contains no substance precipitable by ammonium sulphate, and diphtheria toxin produced in it may be considerably purified simply by salting out. Holt (1937) and McLean (1937) have obtained growth and toxin production by *Staphylococcus aureus* in a medium made with the dialysate from nutrient broth. This medium contains no nitrogenous substances insoluble in saturated ammonium sulphate.

2. The possible relationship of oxidation-reduction systems to the formation of toxins

Although direct proof is lacking, a number of observations seem to link the formation of toxins to the activity of respiratory enzymes and other substances concerned in oxidation-reduction processes. In 1931, Coulter and Stone described a complex porphyrin, apparently containing both copper and iron, which is present in the toxic filtrates from cultures of *Corynebacterium diphtheriae* and is not formed by non-toxigenic strains.[1] It may, however, be formed by yeast and certain other organisms. The amount of porphyrin as judged by the intensity of the absorption spectrum is parallel to the amount of toxin formed. These observations were confirmed by Wadsworth, Crowe, and Smith (1935) who also showed that the porphyrin can be adsorbed out of toxic filtrates by charcoal. Wheeler and Crowe (1936) found that removal of porphyrin from, or its addition to, cultures of *C. diphtheriae* does not affect the formation of toxin. Pappenheimer and Johnson (1937) found that the amount of toxin

[1] Since this article has gone to press another paper by C. B. Coulter and F. M. Stone (Proc. Soc. Exp. Biol. Med., 1938, **38**, 423–425) has appeared in which evidence is presented that the pigment in diphtheria culture filtrates is a zinc coproporphyrin compound.

and the amount of porphyrin can be increased or decreased in parallel by varying the concentration of iron in the medium.

These observations and the fact that iron and copper are essential for the production of diphtheria toxin indicate that the porphyrin found in the filtrates may take part in the formation of toxin in the bacillus although it has no effect when free in the culture medium.

Urban and Eaton (1937) have shown that the porphyrins derived from *C. diphtheriae* are reversibly oxidized by oxygen in the presence of reduced cytochrome C and form a redox system with lactoflavin, but, contrary to the observations of Coulter and Stone (1931), these porphyrins are not directly oxidized by potassium ferricyanide.

Levaditi and his co-workers (1934) have called attention to another substance, with an absorption band at the ultraviolet end of the spectrum, which they claim is parallel in amount to the amount of diphtheria toxin formed and is absent from culture filtrates of non-toxigenic strains. This is denied by Ottensooser, Krupski, and Almasy (1935).

Although iron, copper, and cysteine are essential for growth and toxin production by the diphtheria bacillus (and probably other organisms), excess of any one of these substances inhibits the formation of toxin. (Locke and Main, 1931, Pappenheimer and Johnson, 1936). Kligler, Liebowitz, and Berman (1937) report that ascorbic acid added to the culture medium in a concentration of 0.1 mg. per cc. markedly reduces the formation of diphtheria toxin. The reported inhibitive effects of cysteine and ascorbic acid may have been due to the introduction of an excess of iron as an impurity in these substances.

Burky (1933) observed that when toxigenic strains of *Staphylococcus aureus* are grown anaerobically no pigment and no hemotoxin are formed but the concentration of lethal toxin is the same as in filtrates from aerobic cultures. On the other hand, anaerobic cultivation of the streptococcus or pneumococcus does not reduce the production of hemotoxin. The production of diphtheria and Shiga dysentery toxins is inhibited by anaerobic cultivation of the organisms. McBroom (1937) has found a cor-

relation between the ability of strains of staphylococcus to reduce methylene blue and to form hemotoxin. Kodama (1936) has reported that a thermolabile substance extracted in parallel with the cytochrome pigments from muscle stimulates the production of erythrogenic toxin by the hemolytic streptococcus. These observations constitute the fragmentary evidence that oxidation-reduction processes in the bacterial cell may be intimately associated with the formation of at least some of the toxins.

3. Other factors affecting the formation of toxins

Because of the use of complex media made up of substances of indeterminate composition and purity, much of the work on toxin production, although of undoubted practical value, has little significance in the present connection. For this reason an extensive review of the literature on this subject has not been undertaken.

A constituent of the medium need not necessarily act directly either by stimulating growth or toxigenicity. It may act by removing an inhibitory factor, by effecting changes in pH or reducing conditions during growth, or by protecting the formed toxin from destruction. Traces of unknown impurities in an "essential" substance may stimulate the formation of toxin. The success of Taylor (1935) in producing very strong diphtheria toxin in a hog-stomach-digest medium is due to factors as yet unknown. The work of Pappenheimer, Mueller, and Cohen (1937) and Pappenheimer and Johnson (1936, 1937) indicates that in a medium containing the organic and inorganic substances required for growth of the diphtheria bacillus, the production of toxin may be brought about by the addition of the proper amounts of inorganic substances. The latter investigators have pointed out that the amount of iron necessary to inhibit toxin formation is considerably less than that found in normal tissues. An excess of iron may also occur in peptones or sugars. Most methods for preparing media for the production of diphtheria toxin involve a step in which phosphates of metal ions are precipitated in alkaline solution. The excess of iron inhibitory to toxin production is carried down and removed in this precipitate.

The manner in which carbon dioxide and soft agar act in stimulating the formation of toxin by the staphylococcus has been the subject of considerable investigation. Bigger (1933) found that good toxin is produced by some strains in a medium containing glycerol and phosphate buffer without carbon dioxide; but McLean (1937) has had less success in substituting other buffers for carbon dioxide. This suggests that the carbonic acid may act as a buffer, or maintain the pH at the optimum level for toxin production. Carbon dioxide as such, and independently of its effect on pH, stimulates the growth of bacteria, and it may in some way affect the metabolic processes concerned in the formation of toxin.

Until recently the use of soft agar for the production of staphylococcus toxin has been considered essential. However, McLean (1937) has found that after adsorption from the medium of an inhibitory substance by kieselguhr, kaolin, filter paper, or cellophane, good toxin may be produced without agar. McLean believes that agar acts in a similar way.

Jordan and Burrows (1935) report that the production of enterotoxic substances by strains of staphylococcus concerned in food poisoning is greatly increased by adding starch to the medium and cultivating the organisms on soft agar under 20 per cent carbon dioxide. After repeated transfer to this medium certain strains of *Streptococcus viridans*, *Bacillus proteus*, and members of the colon-typhoid group also acquire the ability to produce gastro-intestinal poison. It is possible that the enterotoxic substance is a metabolite, not a true toxin, because it is non-antigenic, and, unlike other well-defined toxins, it is soluble in organic solvents (Jordan and Burrows, 1933).

4. Toxins as products of secretion, autolysis, or the action of enzymes on the medium

The production of diphtheria toxin in a medium containing only substances of the degree of complexity of amino acids, and the production of other protein-like toxins in media containing no proteins or proteoses makes it unlikely that these toxins are formed by enzymic degradation of a constituent of the culture

medium. It seems rather that toxins are synthesized in the bacterial cell and then liberated, by diffusion into the medium or by disruption of the cell. Most toxins are intermediate in their properties between two extremes represented, on the one hand, by diphtheria toxin, which appears in the medium even during the first hours of growth and is easily washed out of the bacilli, and, on the other, by the toxic substances in the bodies of the colon-typhoid organisms which are liberated only by prolonged autolysis, tryptic digestion, or extraction with acids.

Nelson (1927) found that intact botulinus bacilli are very toxic and their toxicity is not appreciably diminished by washing. The toxin is apparently combined with a protein of the bacterial cell which can be removed by peptic digestion. More recently Sommer (1937) has reported that botulinus toxin having almost the same activity as the purified toxin separated by him from filtrates can be obtained by dissolution of the dried bacilli in phosphate buffer.

Both the neurotoxin and the enterotoxin of the Shiga dysentery bacillus exist in the bacterial bodies (Boivin and Mesrobeanu, 1937b). The neurotoxin is liberated from the cells by diffusion in an alkaline medium, but the enterotoxin is liberated only by autolysis. Hansen (1936) has obtained strong dysentery toxin by grinding the dried bacilli in water and concentrating the toxin by adsorption and precipitation. Gildermeister and Grillo (1935) obtained increased production of toxin by growing the cultures in broth at pH 8.8 inside a cellophane bag immersed in broth. This was attributed to the escape of metabolic products through the membrane, thus permitting better growth of the bacilli.

In 1934, Weld described a heat-labile hemotoxin of the streptococcus which, after intravenous injection, kills mice and produces extensive intravascular hemolysis. Unlike the pneumococcus hemotoxin which is generally liberated by breakdown of the cells and the staphylococcus hemotoxin which is formed as a soluble substance during growth, the streptococcus hemotoxin is best obtained by treating the organisms with serum. Several successive serum extracts contain hemotoxin of equal potency, and extraction of a larger mass of the cocci with the same amount of serum does not increase the potency. This indicates saturation

of the serum or limitation of the amount of toxin formed by the availability of something in the serum. Schluter and Schmidt (1936) have repeated the work of Weld, and have been unable to obtain hemotoxin by extraction with Tyrode solution or solutions of gelatin, gum arabic, or peptone. With solutions of serum albumin or globulin only weak toxins were obtained. Dialyzed serum failed to extract the toxin but addition of sodium chloride restored its extracting power. Dilution or concentration of the serum also diminished the extracting power. The ability of the streptococci to produce hemotoxin diminishes after 14 hours of growth. Extraction of the organisms with saline, ether, or alcohol also destroys the ability to yield hemotoxin on subsequent treatment with serum. It is possible that this hemotoxin of the streptococcus is not extracted by the serum but is formed by the action of an enzyme of the organism on some constituent of the serum.

PART II. THE PURIFICATION AND CHEMICAL NATURE OF BACTERIAL TOXINS

1. Concentration, partial purification, and separation of toxins

This section will be devoted to the applications of chemistry in the study of toxins which have been concentrated or have been separated from a mixture of toxins but have not in most cases been purified to any great degree.

Procedures such as precipitation with alcohol, acetone, or ammonium sulphate, dialysis or ultrafiltration, and evaporation at low pressure and temperature have long been used to concentrate bacterial toxins. Such methods usually do not effect a great amount of purification except where specially designed and simplified media have been used for production of the toxin. However, concentration may be useful in the study of bacterial products having poorly defined biological properties; and simple chemical procedures may sometimes be used to separate a mixture of two or toxins produced by the same organism.

In considering the destructive effects of a chemical procedure two criteria of the amount of alteration of the toxin should always be observed:

(1) The final yield of toxin (measured in units, such as skin

test dose, minimal lethal dose, or flocculating unit) in the purified or concentrated sample should be a major fraction of the total toxin in the original material.

(2) The toxicity per gram of dry weight in the final preparation should be equal to or greater than that of the original toxin.

a. Toxins of the hemolytic streptococcus, pneumococcus, and staphylococcus. Scarlatinal toxin may be concentrated by fractional precipitation with ammonium sulphate or sodium chloride according to the methods used by Huntoon (1924), Dick and Boor (1935) and others. Acetone has been used as a precipitant by Wadsworth and Quigley (1931). Precipitation with neutral salt has yielded a ten- to twenty-fold purification of the toxin without much loss of toxicity. Simple acetone precipitation has effected a five- to ten-fold purification. These methods undoubtedly involve precipitation of much inactive material from the culture medium.

In 1929 Korschun, Krestownikowa, and Rjachina precipitated scarlatinal toxin with sodium chloride and then with alcohol. The alcohol precipitate was redissolved in acidified water to separate the toxin from an insoluble nucleoprotein. The resulting toxic substance is stated to be a polysaccharide containing nitrogen. A similar substance was obtained by Kodama (1936). Huntoon, and Dick and Boor reported that their scarlatinal toxins were destroyed by tryptic digestion, but Kodama used tryptic digestion as a step in the purification of his scarlatinal toxin. Recently Stock (1937), using methods similar to those of the Japanese and the Russian investigators, has obtained a toxin of considerable activity which gives 60 per cent of reducing sugars on hydrolysis. Stock showed, however, that a similar polysaccharide can be isolated from commercial peptone. Apparently, scarlatinal toxin has not yet been isolated in a degree of purity sufficient to warrant definite conclusions as to its nature.

Separation of various substances giving skin reactions and of other toxic products from hemolytic streptococci has been partially successful but much remains to be done. Hooker (1936) has called attention to the multiplicity of toxins or poisons, elaborated by the hemolytic streptococcus, which may play a

part in infections with this organism. In 1934, Hooker and Follensby demonstrated the existence of what they termed A and B toxins of the hemolytic streptococcus. The B toxin is present in the fraction precipitated by 0.60 saturated ammonium sulphate solution, while the A toxin is precipitated at 0.75 saturation. Hooker and Follensby found strains of streptococci which produce mostly A or mostly B toxin. The NY5 strain produces A and B while the Dick strain produces only A. The two toxins differ in their stability to various physical and chemical agents. The B toxin resembles a polypeptide or protein while the A toxin in many respects departs from the properties commonly attributed to a protein. It is interesting to note that the B toxin is digested by trypsin whereas the A toxin resists digestion by pepsin, pancreatin, or trypsin.

From hemolytic streptococci and their culture filtrates, Kodama (1936) has separated several substances which give skin reactions. One of these is a scarlatinal toxin which resists tryptic digestion and which Kodama believes is similar to the A toxin of Hooker. In addition, Kodama describes an alcohol-soluble, acetone-insoluble polypeptide which gives reactions of an allergic nature in certain adults, and a nucleoprotein which also gives allergic skin reactions. The importance of separating toxic from allergic factors in streptococcic filtrates is evident in view of the discussions of these factors in papers such as those by Hooker (1933), Ando, Kurauchi, and Nishimura (1930), and Cooke (1928).

Rane and Wyman (1937a) have demonstrated a definite flocculation of scarlatinal toxin and antitoxin by using toxin concentrated and partially purified by precipitation with ammonium sulphate. O'Meara (1935) and others had previously obtained flocculation by using enormous volumes of unconcentrated toxin relative to the amount of antitoxin, and by incubating for a long time. The concentrated toxin prepared by Rane and Wyman flocculates with a suitable serum in 15 minutes. Although other investigators have failed to demonstrate a relation between the flocculation value and the skin test dose of scarlatinal toxin, Rane and Wyman report relatively constant values of about

60,000 skin test doses (as determined on rabbits) per flocculating unit, using the National Institute of Health unit of antitoxin. In most details the flocculation of scarlatinal toxin closely resembles the Ramon reaction with diphtheria toxin.[2]

The demonstration that hemolytic streptococci and pneumococci form lethal substances has been accomplished by purifying and concentrating these substances. The concentrated streptococcus toxin prepared by Korschun and his collaborators was sometimes fatal to rabbits in doses of 0.02 gram. Rane and Wyman (1937b) report that strong toxins containing 10 to 80 flocculating units per cubic centimeter kill adult rabbits in doses of 5 cc. Young rabbits are more resistant.

In addition to the toxins of hemolytic streptococci just discussed, three streptococcus hemolysins have been described, and two of these are apparently toxic for experimental animals. As was mentioned in the preceding section, the heat-labile substances extracted from streptococci with serum (Weld, 1934) kill rabbits and mice with extensive *in vivo* hemolysis. More recently Czarnetzky, Morgan, and Mudd (1938) have prepared a heat-stable hemolysin by extraction of frozen and dried ("lyophile") streptococci with ether. A crystalline derivative of this hemolysin is also hemolytic and lethal for mice and rabbits in doses of approximately 0.16 mg. per kilogram of body weight. These hemolysins and that described by Weld have not yet been shown to be antigenic. Consequently, they must be provisionally classified as poisons rather than as true toxins. Besides these two hemolysins which are oxygen-stable, Todd (1932) has described an oxygen-labile streptococcus hemolysin which is antigenic and probably similar to pneumococcus hemolysin. This hemolysin may be related to the oxygen-labile antigen of Group A streptococci described by Czarnetzky, Mudd, Pettit, and Lackman (1938).

By concentrating culture filtrates of pneumococcus type III by ultrafiltration, Coca and his associates (1937) have obtained a

[2] Ramon and his collaborators (1937a) report that tetanus anatoxin, concentrated by precipitation with trichloracetic acid, flocculates rapidly with antitoxin.

preparation which kills mice in 1 cc. doses and produces skin reactions in rabbits. Dick and Boor (1937) have obtained a similar substance by precipitation with ammonium sulphate. The fatal dose for mice is approximately 0.05 gram. These preparations also produce skin reactions and fever in human beings. The lethal substances resemble other toxins in that they are antigenic and are neutralized by the corresponding anti-toxin. The pneumococcus toxin is somewhat unusual in being type-specific. However, the large amount necessary to kill experimental animals, and the stability to heat sharply differentiate these substances from toxins such as those of diphtheria and tetanus. The relation between the symptoms produced by the toxin and those resulting from infection with the organism has not been clearly demonstrated. It is possible that insignificant amounts of these substances are produced by pneumococci and streptococci under artificial cultivation but that larger amounts are produced *in vivo* and do play a part in pneumococcus and streptococcus infections. The development of methods for producing, concentrating, and purifying these so-called toxins will lead to a better knowledge of their biological activity.

Like the streptococcus, the staphylococcus probably produces several toxins or poisonous substances which may usefully be investigated by chemical methods. The identity or separability of hemolyzing, necrotizing, and lethal toxins has not definitely been settled. Glenny and Stevens (1935) and Roy (1937) have described α and β staphylococcus toxins which differ in their ability to hemolyze human and rabbit red cells, to produce necrosis in the skin, and to kill susceptible animals. There are corresponding anti-toxins for these α and β toxins. The gastro-intestinal toxin described by Dack and his co-workers (1931) and Dolman (1934) apparently differs both in its chemical and biological properties from the hemolytic and necrotizing toxins of the staphylococcus. This substance is apparently non-antigenic and may not be a true toxin (Jordan and Burrows, 1933).

Some progress has been made in the purification and concentration of staphylococcus toxoid. Holt (1937) prepared toxin in a dialysate medium; and the formalin-toxoid from this toxin

was then precipitated with ammonium sulphate. The resulting product was free of the nitrogenous constituents of the medium but contained a carbohydrate derived from the agar used. Ramon, Boivin, and Richou (1936) have concentrated staphylococcus toxoid by precipitation with trichloracetic acid at pH 4.0.

b. Toxins of the Salmonella, Proteus, colon, and dysentery groups of organisms. Raistrick and Topley (1934) obtained from *Salmonella aertrycke*, by digestion of the acetone-extracted bacilli with trypsin and precipitation with 68 per cent alcohol, an antigenic fraction which produces somatic "O" agglutinins in the serum of immunized animals. As shown by Martin (1934) and Delafield (1934) this fraction is toxic for mice and rabbits. Herter and Rettger (1937) have also described toxic fractions obtained from *S. aertrycke*. Substances of a nature similar to that of Raistrick and Topley have been obtained by Boivin and Mesrobeanu (1937a) from members of the *Salmonella*, *Proteus*, and colon groups by extraction of the bacterial bodies with trichloracetic acid and precipitation of the extracted material with alcohol. These toxic substances are produced by the smooth but not by the rough variants and are independent of the presence of the H antigen. They contain from 3 to 5 per cent of nitrogen, small amounts of sulfur and phosphorus and give 20 to 40 per cent of reducing sugars on hydrolysis. An ether-soluble lipid component is split off by acid hydrolysis. Tests for peptide linkages and tyrosine are positive, but the substances are not precipitated by the ordinary reagents for proteins. This carbohydrate-lipid complex constitutes about 10 per cent of the bacterial bodies, kills mice in doses of 0.1 to 1.0 mg., produces only weak antitoxin, and is stable to heat in a neutral but not in acid or alkaline solutions.

The existence of a second but weaker endotoxin is indicated by the observation of Boivin and Mesrobeanu (1937a) that, after destroying the gluco-lipid complex by heating with dilute acetic acid, residual toxicity remains. This endotoxin, which is destroyed by tryptic digestion, is apparently a polypeptide, and is found in rough variants as well as in smooth.

Extending their observations, Boivin and Mesrobeanu (1937

b, c) demonstrated the existence of endotoxins in the bodies of Shiga and Flexner dysentery bacilli. These gluco-lipid complexes produce acute gastro-intestinal symptoms in experimental animals and are similar to the substances obtained from other gram-negative bacilli. Morgan (1936) has isolated and analyzed a specific nitrogenous polysaccharide from the Shiga bacillus. Other toxic fractions from dysentery organisms have been described by Olitski, Reibowitz, and Berman (1937).

Boivin and Mesrobeanu (1937c) have carried out a chemical separation of the endotoxin from the exotoxin in filtrates of cultures of Shiga dysentery bacilli. The exotoxin, which is formed by both R and S variants, is precipitated by trichloracetic acid at pH 3.5. After complete precipitation of the exotoxin, the endotoxin remains in solution and is recovered by precipitation with alcohol. In contrast to the endotoxin, the exotoxin is destroyed by tryptic digestion and by heat, has the general properties of a protein, and acts on the central nervous system but not on the gastro-intestinal tract.

This work on the Shiga dysentery toxin has been confirmed by Haas (1937a) who separated a heat-labile neurotropic exotoxin from a heat-stable endotoxin using the methods of Raistrick and Topley and of Boivin and Mesrobeanu. The endotoxin prepared by Haas is biuret-negative, gives a strongly positive Molisch test, and contains only 2.25 percent of nitrogen. It is slightly more active and may, therefore, be purer than the substances obtained by other investigators. Haas points out that since nutrient broth gives a protein precipitate with trichloracetic acid, the protein nature of the exotoxin has not been conclusively demonstrated.

2. *Purification of bacterial toxins*

The isolation of bacterial toxins in a pure state must depend upon the simplification of culture media and the perfection of chemical methods for separating the toxins from the constituents of the culture media and from proteins and other non-toxic products of the bacteria.

Although the plant toxin, ricin, was isolated as a protein by

Osborne, Mendel, and Harris in 1905 by fractionation with ammonium sulphate, the technical difficulties of separating bacterial toxins in an unaltered condition from the mixture of proteins, proteoses, and peptones with which they are associated in crude filtrates have not been so easily overcome. The simplest and generally most successful method has been precipitation of the toxin-containing protein fraction with acid, as applied to diphtheria toxin by Watson and Wallace (1924) and Locke and Main (1928), to botulinus and tetanus toxins by Snipe and Sommer (1928) and Sommer (1937), and to diphtheria, tetanus, and staphylococcus toxins and anatoxins by Boivin and Izard (1937). It is evident, however, from recent work that diphtheria toxin and possibly other toxins are damaged by precipitation with acid. Diphtheria toxin may be purified without alteration by adsorption on aluminum hydroxide and elution with phosphate buffer (Lindstrøm-Lang and Schmidt, 1930, and others), but some proteins, proteoses, and other constituents of the culture medium are adsorbed and eluted under the same conditions as the toxin. More extensive references to the literature on the purification of diphtheria toxin will be found in the paper by Eaton (1936a).

Using a new method which consists in precipitating the toxic fraction with the salts of aluminum and cadmium under carefully controlled conditions, Eaton (1936a) succeeded in obtaining highly purified, unaltered diphtheria toxin. It was produced in the proteose peptone medium of Wadsworth and Wheeler (1934). Pappenheimer (1937) obtained one of similar purity by simple ammonium sulphate fractionation of toxin produced in a medium made from hydrolyzed gelatin, amino acids, and accessory substances from liver extract.

Partial separation of toxin from bacterial proteins has been accomplished by fractionation with ammonium sulphate and fractional adsorption of the bacterial proteins on the colloidal hydroxides of magnesium or aluminum (Eaton, 1936a, Pappenheimer, 1937). One of the bacterial proteins is precipitated by 0.33 saturated ammonium sulphate solution at pH 7.0, and it is thus easily separated from the toxin which is precipitated be-

tween 0.4 and 0.6 saturation. A second bacterial protein precipitated at the same concentration of ammonium sulphate as the toxin may be separated by precipitating the toxin at pH 5.4 in a 0.33 saturated ammonium sulphate solution (Eaton, 1937b). Although the toxin is slightly damaged by this procedure it is the only successful method yet devised. The character of the

TABLE 1

Diphtheria toxin and anatoxin

INVESTIGATOR	M.L.D. OF BODY WEIGHT (GUINEA PIG)*	NITROGEN PER Lf UNIT*
	grams per kgm.	*mgm.*
Locke and Main (1928)	Not given	0.0006–0.0008
Eaton (1936a)	4.0×10^{-7}	0.00046–0.00055
Pappenheimer (1937)	4.0×10^{-7}	0.00046
Theorell and Norlin (1937)	Anatoxin	0.00088
Boivin and Izard (1937)	Anatoxin	0.00045

Other toxins

INVESTIGATOR	TOXIN	TEST ANIMAL	M.L.D. OF BODY WEIGHT*
			grams per kgm.
Osborne (1905)	Ricin†	Rabbit	5.0×10^{-7}
Sommer (1937)	Botulinus	Mouse	2.0×10^{-7}
Sommer (1937)	Tetanus	Mouse	1.0×10^{-6}
Eaton (unpublished)	Tetanus	Mouse	4.0×10^{-7}
Eaton (1936c)	Tetanus	Guinea pig	1.5×10^{-7}

* Calculated in some cases from figures given in other terms by the authors in order to make the results comparable.

† Not a bacterial toxin. Cited for comparison.

bacterial protein separated by differential adsorption on metallic hydroxides is not known at present.

Bacterial proteins in purified diphtheria toxin have been detected by precipitin tests with anti-sera against these proteins. Eaton used anti-serum prepared by injecting rabbits with washed whole diphtheria bacilli. Pappenheimer prepared anti-serum against the bacterial protein precipitated by one-third saturated ammonium sulphate; however, it seems likely that this antiserum might fail to detect the bacterial protein which is pre-

cipitated at the same concentration of ammonium sulphate as the toxin (at 0.4 to 0.6 saturation). Estimation by the precipitin test of the amount of bacterial protein in purified toxin was made by titrating the antibacterial serum first against known amounts of bacterial protein free of toxin, and then against the preparation of purified toxin (Eaton, 1937b). The results indicate that in preparations containing the minimal amount of total protein per Lf unit, 10 to 15 per cent of the protein is bacterial precipitinogen and the rest toxin. The amount of bacterial protein may be reduced to 1 or 2 per cent by fractionation with acid as previously described, but not by simple fractionation with ammonium sulphate.

A summary of the more successful results of purification is presented in table 1. In all of these experiments the bacterial toxins have been separated from over 99 per cent of the nitrogenous impurities.

In two cases botulinus and tetanus toxins more active than the purest diphtheria toxin have been prepared. This is not surprising in view of the fact that these two toxins in the crude state are more active than diphtheria toxin. Diphtheria toxin and ricin are the only two toxins for which convincing evidence of purity has been advanced. Both are apparently proteins coagulable by heat.

3. *Criteria of Purity*

Since it has not been possible as yet to crystallize toxins, we must use criteria of purity which are not generally applied in organic or inorganic chemistry but which are of considerable value when applied to proteins with well-defined biological properties.

The attainment of a constant ratio of weight or nitrogen content to biological activity has been applied as a criterion of purity to diphtheria toxin and ricin. Karrer and his associates (1924) were unsuccessful in an attempt to separate from ricin, prepared by the method of Osborne, Mendel, and Harris, more active fractions by methods of adsorption or precipitation. Fractions showing less activity were sometimes obtained but these were probably

toxin altered by the chemical treatment. The nitrogen per Lf unit of diphtheria toxin has not been reduced below 0.00045 milligram by repeated precipitation with a variety of reagents, by fractional adsorption on metallic hydroxides or kaolin, or by repeated fractionation with ammonium sulphate (Eaton, 1936a, 1937b, Pappenheimer, 1937). It is possible that repeated chemical fractionation of a toxin may cause an amount of destruction that equals or slightly exceeds the further purification attained. In the separation of diphtheria toxin from bacterial protein by fractionation with acid, it can be demonstrated by the the precipitin test that impurities have been removed but the nitrogen per Lf is not reduced and may be, in certain instances, slightly increased because of partial destruction of the flocculating properties (Eaton, 1937b).

Measurements of physical and chemical properties such as optical rotation, molecular weight, precipitability by protein reagents, iso-electric point, and content of nitrogen and various amino acids have been used to characterize diphtheria toxin and ricin as proteins. The demonstration of the absence of impurities such as pigments, carbohydrate, proteose, peptone, and compounds containing phosphorus, from purified preparations of toxic protein constitutes evidence that the protein is the toxin. However, none of these tests proves that it is not mixed with another, atoxic, protein having otherwise similar properties.

Pappenheimer and Robinson (1937) have shown that the nitrogen per Lf unit of diphtheria toxin specifically precipitated by antitoxin corresponds to the nitrogen per Lf unit of the purest preparations obtained by chemical methods. Unless it proves possible to split off part of the flocculating protein without affecting the toxic properties, this is evidence that the purified preparations are almost pure toxin. Under the conditions of the experiment, bacterial protein detectable by anti-bacterial serum remained in the supernatant. The identity with the purified toxic protein of the substance precipitated by antitoxin from the crude preparation was not proved except by the close correspondence of nitrogen per Lf unit. Full proof would require that the experiment be performed with purified antitoxin.

Theorell and Norlin (1937) purified diphtheria anatoxin by cataphoresis in the Theorell apparatus and obtained a preparation containing about one and a half times as much nitrogen per Lf unit as diphtheria toxin purified by other methods. The authors conclude that their preparation is a pure protein because it travels as a unit in the electric field, but purer anatoxin has been prepared by purification of crude anatoxin and by detoxification of purified toxin with formaldehyde. Uniformity of motion in an electric field is not, therefore, a valid criterion of purity in this case.

Titrations of the equivalent weight of ricin done by Karrer and his associates (1924) indicated that their preparation consisted of higher and lower molecular fractions although it was not possible to separate these by any of the methods used. Even crystallization does not always guarantee the uniformity of a protein as shown by Hewitt (1936) for serum albumin. Thus far, apparently no studies of purified toxins or attempts to purify these substances by the ultracentrifugation method have yet been made. Pappenheimer (1937) gives the minimum molecular weight of diphtheria toxin as about 18,000.

4. Hydrolysis, digestion, and analysis of toxins

Up to the present time, chemical analyses of purified ricin and diphtheria toxin have yielded no clue as to the nature of the toxic structures. Karrer was unable to find any unusual grouping in ricin by analyzing the products resulting from tryptic digestion and hydrolysis with acid, although two kilograms of the purified protein were available for this study. Ricin contains cysteine. Eaton (1936a) found that diphtheria toxin gives negative nitroprusside and lead acetate tests for cysteine sulfur. Pappenheimer (1937) finds that his preparations of diphtheria toxin contain 0.75 per cent sulfur but give a negative nitroprusside reaction. It is probable, therefore, that the sulfur does not exist in the usual sulfhydryl configuration. Neither diphtheria toxin nor ricin contains phosphorus; they both contain about 16 per cent nitrogen. The high content of arginine and glutamic acid in ricin indicates that this protein has many free basic and acidic groups.

Diphtheria toxin apparently also contains much arginine as indicated by the strong Sakaguchi test. Tryptophane and tyrosine are present in both diphtheria toxin and ricin. Eaton (1936a) obtained weak qualitative reactions for tryptophane in 0.1 per cent solutions of diphtheria toxin, and Pappenheimer finds that a 1 per cent solution gives strong reactions for tryptophane. Diphtheria toxin contains 1.14 per cent of this amino acid as compared with 0.4 per cent in ricin.

With the possible exception of botulinus toxin, the endotoxins of the *Salmonella*, colon, and dysentery groups and certain of the toxic products of the hemolytic streptococcus, bacterial toxins are destroyed by digestion with proteolytic enzymes. This signifies that peptide linkages similar to those found in proteins are essential to the activity of the toxin. It does not necessarily mean that the chemical group or groups in toxins which poison living cells are polypeptide chains. The numerous examples of enzymic destruction of toxins will not be reviewed in detail; some are referred to in other sections of this paper, but the resistance of certain toxins to digestion will be discussed more specifically here.

The resistance of botulinus toxin to digestion has been demonstrated by Bronfenbrenner and Schlessinger (1924), Schubel (1923), Tani (1934), and others. Nelson (1927) found the toxin to be intimately associated with a globulin from the bacterial cell. Digestion with pepsin removed this globulin without destroying the toxin. Botulinus toxin may not be, however, absolutely refractory to digestion. The fact that the minimal lethal dose by mouth is about a hundred times as great as by injection indicates either that much of the toxin is destroyed in the gastrointestinal tract or only a small part is absorbed. In at least one instance partial destruction by pepsin has been observed (Tani, 1934). Karrer (1924) found that ricin is digested very slowly by trypsin. Of a 50 gm. lot of the toxin only two-thirds, as determined by parallel measurements of amino nitrogen and toxicity, was digested by 40 cc. of pancreatic juice in five months.

Resistance to tryptic digestion in the case of the endotoxins of the *Salmonella*, colon, and dysentery groups of organisms is

attributable to the fact that these substances are complex compounds of carbohydrate and lipid. This may also be true of one of the scarlatinal toxins.

PART III. THE CHANGES PRODUCED BY THE ACTION OF VARIOUS PHYSICAL AND CHEMICAL AGENTS ON TOXINS

The demonstration that a given chemical or physical treatment detoxifies a toxin is of little significance unless the nature of the changes produced in the toxin can be determined or deduced from the experiment. We shall consider here only those studies in which effects on other properties besides toxicity have been examined.

1. Denaturation

Most toxins are destroyed by the action of heat, strong acid, or alkali. From observations on crude unconcentrated toxins, the nature of the changes induced is not clear. It has been assumed that the lability of some toxins to heat is analogous to the heat-lability of many proteins. The demonstration that purified and concentrated diphtheria toxin is coagulated by heat and denatured by acid and alkali lends support to the view that the destruction of toxins by these agents is actually a denaturation of protein.

Although little is known about the chemical changes which occur when proteins are denatured, it is possible to correlate alterations in biological properties with denaturation. Partial denaturation, without loss of solubility at pH 7.0, apparently affects first those properties of diphtheria toxin designated by Ehrlich as the "haptophore groups." If the denaturation is carried farther, the protein is coagulated and all of the biological properties of the toxin are destroyed. According to the degree of denaturation, diphtheria toxin loses in various degrees its ability to combine and flocculate with antitoxin, its antigenicity, and its toxicity, as shown by Eaton (1936b). An increase in the time required to flocculate with antitoxin is probably the most sensi-

tive indicator of denaturation, and increased Kf[3] is apparent before changes in solubility or other gross evidence of denaturation. The length of time toxin is exposed to denaturing agents affects in a corresponding degree its Kf and antigenicity. Denatured toxin or toxoid of low antigenicity may be separated from unaltered toxin or toxoid of higher antigenicity by chemical treatment of partially denatured preparations. Papers bearing on this subject have been reviewed previously (Eaton, 1936b, 1937b).

The loss of toxicity which accompanies denaturation may be considered either as a decomposition of the toxic groups or as an impairment of the ability to combine with or attack susceptible cells. Possibly heat and high concentrations of hydrogen or hydroxyl ions destroy the toxic groups and at the same time denature the protein and destroy its antigenic properties. It appears, however, that at lower temperatures in neutral or slightly alkaline solutions the rate of toxoid formation or modification of the toxicity is more rapid than the rate of denaturation, while at higher temperatures, and especially in pH ranges above 8.0 and below 5.0, denaturation is more rapid than formation of toxoid. The range of pH for maximum stability varies, of course, for different toxins. Diphtheria toxin is most stable in neutral solution, while botulinus toxin is apparently most stable in acid solution and relatively unstable in an alkaline solution.

The so-called reversible detoxification of diphtheria toxin by acid, as reported by earlier investigators, is probably the result of a combined precipitation and coagulation. Later investigations have shown that the acid-detoxification is only partially reversed by bringing the pH of the solution back to neutrality. It is also possible that the acidified toxin solution precipitates proteins at the site of injection, thus hindering the spread of the toxin.

2. Agents which produce destruction, denaturation, or modification

Many reports dealing with the action of chemicals on bacterial toxins have failed to state what effects were produced on the

[3] Kf is the time required for flocculation of a mixture of toxin and antitoxin in optimal or equivalent proportions. Since this depends on the concentration of toxin and antitoxin in the equivalence mixture, the Kf of different preparations of toxin must be compared by using solutions with the same number of Lf units per cc.

antigenicity or combining power for antitoxin. A review of the earlier work has been published by Bacher (1927). More recently Schmidt (1932) has carried out an extensive investigation of the effects of many aliphatic, aromatic, and alicyclic compounds on the toxicity and Lf value of diphtheria toxin. Only formaldehyde, acetaldehyde, glyoxal, glucose, furfural, hexamethylene tetramine, and certain halogenated hydrocarbons reduced the toxicity without a corresponding diminution in the Lf values. All the other compounds studied either were inert or diminished the Lf in parallel with the toxicity. Judging from the results of Schmidt and other investigators, it seems that the effect of most chemical compounds on bacterial toxins is very similar to that of heat. Many of the substances which destroy toxins also denature proteins.

Von Groer, Altenberg, and Lille (1935) have reported that diphtheria toxin is destroyed by the ortho- and para-dihydroxyphenols but not by the meta-compound. The authors imply that there may be a stereochemical relationship involved, but the o- and p-compounds are much more reactive with a variety of substances than is the m-compound. Schmidt (1932) found that most of the aromatic phenols rapidly destroy diphtheria toxin.

Many of the toxins from anaerobes are quite readily destroyed by oxidation. Diphtheria toxin is not particularly sensitive to mild oxidation or reduction (Hewitt, 1930). Scarlatinal toxin may be in part modified to toxoid by oxidation. Cowles (1936) has shown that cysteine catalyzes an oxidative modification of tetanus toxin. Atmospheric oxygen is used up in the process. The detoxified preparations are antigenic and retain 20 to 50 per cent of their combining power for antitoxin. The work of Halter (1936) indicates that tetanus toxin, when it is diluted in sodium chloride solution, may be destroyed by oxidation. The recent observation of Jungeblut (1937) that about 2 M.L.D. of tetanus toxin may be inactivated by ascorbic acid in amounts between 0.5 and 10 mg. is possibly also connected with an oxidative change. Lippert (1935) finds that methylene blue in the presence of light brings about the destruction of tetanus toxin. He believes that the reaction is an oxidation.

Studies of the photodynamic action of methylene blue on other toxins have been reported by Lin (1936) and Li (1936). Lin states that diphtheria toxin modified by the photodynamic action of methylene blue is apparently more antigenic than formol-toxoid. However, the detoxification was not complete, and, since eight injections were used for immunization, a considerable immunity could have been produced by the residual toxin. No reports of the effect on flocculation were given. Li was able to remove completely the hemolytic, dermo-necrotizing, and lethal properties of staphylococcus toxin by the combined action of methylene blue and light. The toxoid thus produced was equal in antigenicity to formol-toxoid and alum precipitated formol-toxoid.

3. *The action of formaldehyde*

Formaldehyde apparently acts directly on the toxic groups without affecting other parts of the toxin molecule which are concerned with antigenicity and combining activity. The nature of the chemical reaction or series of reactions that occur may be successfully studied only with highly purified toxin because many other substances in crude toxin also react with formaldehyde.

Diphtheria toxin seems best suited for studies on the process of toxoid formation, because it is easy to measure the amount of antigenic material by the flocculation test and because the toxin may be obtained in a relatively pure state. Bunney (1931) reported that diphtheria toxin purified by acid precipitation could not be detoxified by formaldehyde without destroying the antigenic properties. Others have not confirmed Bunney's results. Eaton (1937c) observed that an excess of formaldehyde in an alkaline solution impairs the flocculating, combining, and immunizing properties of purified toxin during modification to toxoid. The presence of small amounts of nitrogenous impurities in partially purified toxin will markedly affect the action of formaldehyde and prevent destruction.

For detoxification in a solution at pH 6.0 there is required a concentration of formaldehyde one hundred times as great as that sufficient to modify purified toxin to toxoid in a solution at

pH 8.6. Follensby and Hooker (1936), using diphtheria toxin partially purified by acid precipitation, showed that the reaction between formaldehyde and toxin to form toxoid has the characteristics of a unimolecular reaction. The velocity constant of the reaction is stated to be directly proportional to the concentrations of hydroxyl ions and formaldehyde.

Hewitt (1930) has pointed out that the reaction between formaldehyde and toxin to form toxoid is slow and irreversible while that between formaldehyde and the free amino groups of proteins, polypeptides, or amino acids is rapid and reversible. With both crude and purified toxin the amount of formaldehyde required for detoxification in a reasonable time is not greater than the theoretical quantity necessary to combine all of the free amino nitrogen (Eaton, 1937c). In the reaction which occurs in the Sørensen titration a large excess of formaldehyde (about 60 times the theoretical amount) must be added to combine with all of the amino groups. From these facts it is obvious that the reaction which occurs in toxoid formation is not the ordinary reaction between formaldehyde and amino groups.

Compounds of formaldehyde or acetaldehyde and ammonia form toxoid more slowly than do the free aldehydes. Aldehyde bisulphites modify diphtheria toxin to toxoid slowly and incompletely (Eaton, 1937a). Wadsworth, Quigley, and Sickles (1937) have observed that the addition of histidine in a quantity sufficient to combine all of the formaldehyde in a mixture with toxin prevents the formation of toxoid. Apparently the affinity of the toxic group for formaldehyde is greater than that of ammonia or amino groups but less than that of the bisulphite ion or the imidazole group of histidine.

Purified diphtheria toxoid contains about two-thirds of the free amino nitrogen found in purified toxin, and the bound amino nitrogen in toxoid is not liberated by removal of the free formaldehyde (Eaton, 1937c). Wadsworth, Quigley, and Sickles (1937) were able to detect only minute amounts of amino nitrogen, or none at all, in diphtheria toxin partially purified by ultra-filtration. Pappenheimer (1937) found about 1.2 per cent, and Eaton found over 2.0 per cent of free amino nitrogen in their purified

preparations. In the experiments of Wadsworth, Quigley, and Sickles about one-tenth of the formaldehyde from a 0.22 per cent solution disappeared during the complete conversion of toxin to toxoid over a period of 20 days. This quantity of formaldehyde appears to be greater than the equivalent of all the amino groups in both toxin and impurities.

The combination of formaldehyde and amino groups is, of course, not necessarily the reaction concerned in detoxification. Present data are not accurate enough to indicate whether all of the formaldehyde used up during detoxification combines with amino groups, but measurements of this sort are not impossible with highly purified and concentrated toxin. Follensby and Hooker (1936) suggest that the formation of toxoid from toxin may be a reaction catalyzed by formaldehyde and hydroxyl ions. If this is true, the disappearance of formaldehyde to combine with amino nitrogen and other groups is only an incidental reaction. Recently Goldie (1937) has studied the action on crude and partially purified diphtheria toxin of ketene, an acetylating agent that combines directly with free amino groups. In the various samples of crude and of purified toxin 30 to 50 per cent of the amino groups were combined after 10 to 25 minutes' action of the ketene, and the toxicity had been reduced to 1/6 to 1/300 of the M.L.D. in the original sample. At this stage of the acetylation the toxin flocculated with antitoxin, indicating that a partial modification of toxin to toxoid had occurred. With more prolonged action of the ketene, further combination occurred and both flocculating ability and toxicity were destroyed.

Formaldehyde-toxoid is more stable to denaturation than toxin (Eaton, 1937c). It is not at present known whether the acquirement of increased stability is directly connected with loss of the toxic properties. Other proteins also become less subject to denaturation, as judged by decrease in solubility, after the action of formaldehyde. The changes which occur are not understood as yet. The fact that purified toxin and toxoid have the same optical rotation suggests that the optically active atoms adjacent to the peptide linkages may not be affected in the change to toxoid.

With certain proteins, such as casein, formalin produces polymerization, and some investigators have taken the view that toxoid is a polymerization product of toxin. However, in the case of diphtheria toxin, one Lf unit of toxin is modified by the action of formaldehyde to one Lf unit of toxoid, and there is no appreciable change in the nitrogen per flocculating unit. Consequently, polymerization of two or more molecules of diphtheria toxin to form toxoid could only be possible if all the groups which combine with antitoxin remained free. In the case of staphylococcus toxin, the formation of toxoid by the action of formaldehyde is accompanied by a reduction in the combining power for antitoxin to approximately one-half. An indication of a difference in the combining powers of diphtheria toxin and toxoid is found in the work of Madsen, Jensen, and Ipsen (1937) who studied the combination *in vivo* of injected toxin or toxoid with antitoxin in the blood of actively and passively immunized animals. Their results indicate that diphtheria toxin binds twice as much antitoxin *in vivo* as *in vitro*, while toxoid binds the same amount of antitoxin *in vitro* as *in vivo*.

Although denatured toxin of less combining power and toxicity may be separated by chemical means from a mixture with unaltered fully active toxin, the separability of toxin from toxoid has never been demonstrated. The progressive formation of toxoid from toxin may be a process affecting step-wise all the molecules of toxin at once so that there is not at any time an equilibrium between "completely toxic" toxin and "completely atoxic" toxoid. The fact that the complete reaction is irreversible points against the existence of such an equilibrium. There may however, be a reversible equilibrium between molecules modified to different degrees before complete detoxification. This is indicated by some observations of Wadsworth, Quigley, and Sickles (1937) who found that, following the removal of formaldehyde by ultrafiltration from partially modified toxin, the toxicity increased when the preparation was incubated but not when it was kept in the cold room. This implies a partial reversal of the reaction at an intermediate stage.

4. The action of soaps, lipids, and sterols on bacterial toxins

Vincent (1926) observed that 0.2 to 1.0 per cent solutions of bile and soaps neutralize several hundred M.L.D. of tetanus and other toxins. This author termed the effect a masking of toxicity rather than an inactivation because the reaction was partly reversible. Precipitation of the palmitic acid of the soap with hydrochloric acid liberated enough tetanus toxin to kill guinea pigs, but the killing doses were considerably larger than the M.L.D. of the original toxin.

Larson and Nelson (1924) attributed the detoxifying effects of sodium ricinoleate on diphtheria and tetanus toxins to the property of this substance of forming colloidal aggregates capable of adsorbing other colloids. Larson and Halvorson (1925) observed that the toxin-soap mixture was dissociable. Dilution caused it to become toxic and the firmness of combination increased with time. Bayliss (1936) found that sodium ricinoleate and sodium chaulmoograte are the most effective detoxifying soaps, a 1 per cent solution neutralizing about 35 M.L.D. of diphtheria toxin. Other soaps of chemical composition similar to the ricinoleates and chaulmoogrates were less effective. Unsaturated soaps were generally more active than salts of the saturated fatty acids; bile salts were least effective.

A somewhat different effect of the salts of fatty acids on diphtheria toxin has been observed by Schmidt (1932). The lower members of the series are inactive but, beginning with the fatty acid containing eight carbon atoms in the chain, a destruction of toxicity, flocculating, and immunizing properties is produced by solutions as dilute as hundredth normal acting over a period of several weeks. In these experiments the effect of pH was controlled. Schmidt also observed that a large excess of ricinoleate was necessary to detoxify diphtheria toxin in 24 hours. Contrary to the results of Larson and his collaborators who claimed that their preparations were good antigens, Schmidt states that ricinoleate-toxin has only weak antigenic properties which may be due to traces of free toxin.

Fixation of toxin at the site of injection as a result of previous

adsorption on colloidal particles has been advanced by Ramon and his collaborators (1937b) as the explanation for the neutralizing effects of lanolin on diphtheria and tetanus toxins. By emulsification with 3 or 4 grams of lanolin, 40 M.L.D. of diphtheria toxin or 200 M.L.D. of tetanus toxin were rendered innocuous. Addition of cholesterol diminished the neutralizing effect of lanolin on tetanus toxin. Removal of the lanolin by extraction with acetone, toluol, or chloroform liberated part of the toxin. Mixtures harmless to guinea pigs were found to be toxic for rabbits. Local fixation of the toxin injected with lanolin was demonstrated by studies on the rate of distribution of the toxin in the body of the animal. One or two injections of the toxin-lanolin emulsion are said to produce better immunity than the injection of an equivalent amount of anatoxin. Ramon's results have been confirmed by Eisler and Gottdenker (1937). Using a solution of cholesterol in olive oil, these investigators found that the degree of detoxification of diphtheria toxin depends on the relative volumes of toxin and oil and the length of time these are shaken together. The results apparently depend on surface effects in the droplets of oil. Aqueous emulsions of cholesterol do not affect diphtheria toxin. According to Ramon cholesterol is less effective than lanolin as a detoxifying agent. Schwartz (1936) has reported that the hemolytic, necrotizing, and lethal properties of staphylococcus toxin are markedly diminished by shaking with olive oil.

The colloidal phenomena just described differ in several ways from the strictly chemical effect of formaldehyde on toxin. Relatively enormous amounts of the soap or lipid (in the proportion of about 100,000 parts to 1 of toxin) are required to detoxify. The effects on the antigenic and combining properties of the toxin are not at present clear. Soaps apparently affect the properties of the toxin in a way which is similar in part to denaturation and in part to combination with antitoxin. Possibly partial denaturation of toxin is produced by surface effects in emulsions just as shaking with air denatures purified and concentrated diphtheria toxin (Pappenheimer, 1937), and proteins generally. On the other hand, some of the effects are at least

partially reversible. The high antigenicity of lanolin-toxin mixtures seem to preclude denaturation. Probably some of the agents act by delaying absorption of the toxin at the site of injection. Others, such as soaps, may act by binding those parts of the molecule which attach the toxin to susceptible cells, antibody-producing cells, or antitoxin. True modification of toxin to toxoid probably does not occur in any case.

5. The effects of various chemicals on toxins and other substances that act on red blood cells

Substances that hemolyze or agglutinate red cells are of interest in connection with the chemistry of toxins, because factors affecting the combination of active substance and susceptible cells may be studied. Staphylococcus toxin and plant toxins such as ricin also produce toxic effects *in vivo*. Others acting only *in vitro* are pneumococcus hemolysin, tetanolysin, and the crystalline protein concanavalin A (Sumner and Howell, 1936), which agglutinates red cells and precipitates a variety of carbohydrates and lipids.

Hypertonic salt solutions inhibit hemolysis by immune serum and complement and also by bacterial hemotoxins. Rigdon (1937) finds that the combination of staphylococcus hemotoxin with rabbit red cells is prevented by 6 per cent sodium chloride solution. Avery, Rigdon, and Johlin (1937) report that magnesium sulphate and several salts of sodium and potassium inhibit hemolysis by staphylococcus toxin. The production of skin necrosis by this toxin is also inhibited by hypertonic solutions of sodium chloride, magnesium sulphate, and lithium chloride. The latter salt, however, does not prevent hemolysis. Smith (1937) has found that glycerol, ethylene glycol, sucrose, and glucose, diminish the necrotizing and lethal properties of staphylococcus toxin but have no effect on the titration of toxin by hemolysis of red cells. Weinstein (1937) reports that the hemolysis produced by colonies of streptococci and staphylococci growing on blood agar is inhibited by adding lecithin to the medium. Cholesterol prevents this inhibition of hemolysis. This recalls antagonistic action of cholesterol on the neutralization of tetanus

toxin by lecithin as observed by Ramon. Salts, lecithin, and polyhydroxy-alcohols may act directly on the hemotoxin, or they may alter the surface of the cells so as to prevent attachment of the toxin.

The identification of at least one chemical group associated with the activity of pneumococcus hemotoxin has been accomplished by Cohen and Shwachman (1936), and Shwachman, Hellerman, and Cohen (1934). Pneumococcus hemolysin is inactivated by oxidation and reactivated by reagents that can restore free thiol groups; therefore the lytic activity is associated with the presence of sulfhydryl groups in the preparation. Reversible oxidation is produced by a variety of agents, and zones of reduction potential in which the hemolysin is active or inactive have been defined. The iodoacetate ion which inactivates certain enzymes also inactivates the hemolysin, but does so reversibly.

Cohen, Shwachman, and Perkins (1937) have examined the effects of various sterols on pneumococcus hemolysin. Irreversible inactivation is produced by cholesterol and coprostenol which contain double bonds and are precipitable by digitonin. The saturated sterol, coprostanol, is less active and various sterols not precipitated by digitonin are practically without effect. Binding of the hydroxyl group by esterification as in cholesteryl acetate also removes the inhibitory action. These observations are similar to previous ones on tetanolysin and saponin. Diphtheria toxin differs from these substances in being neutralized both by sterols and their esters. This indicates that a different mechanism is involved (Eisler and Gottdenker, 1937).

Active pneumococcus hemolysin combines rapidly with red cells and with cholesterol. Inactive hemolysin does not combine with red cells nor, apparently, with cholesterol, because after treatment with the latter the hemolysin may be reactivated as usual. It remains to be seen whether or not this indicates that the active hemolysin combines with cholesterol in the red cells. Should it do so, then the attachment could not occur through the sulfhydryl groups because these apparently remain free

(positive nitroprusside reaction) in the hemolysin after inactivation with cholesterol. At any rate, the state of oxidation of the sulfhydryl groups apparently conditions the affinity of other, unknown groups in the lysin for sterols and for red cells. The actual lysis of the red cells may be due to enzymic properties of the hemolysin. The loss of hemolytic power is apparently not accompanied by a loss of the ability to combine with antibody. Hull (1936) found no change in the combining capacity for antibody of hemolytic extracts of the pneumococcus after storage in the ice-box for a length of time sufficient to cause a marked decrease in the hemolytic property.

6. *The effect of vitamin C (ascorbic acid) on diphtheria toxin in vitro and in vivo*

The effect of vitamin C on poisoning with diphtheria toxin has been studied extensively since Greenwald and Harde (1935), and Jungeblut and Zwemer (1935) observed that feeding vitamin C to guinea pigs increased their resistance to 1 or 2 lethal doses of toxin. The effect of vitamin C on toxin *in vitro* is of doubtful significance. The investigators just cited reported that up to 10 M.L.D. of toxin were inactivated by 10 mg. of ascorbic acid per M.L.D. (The M.L.D. of pure diphtheria toxin is 0.0001 mg. or less.) Grooten and Bezssonoff (1936) state that 100 mg. partly detoxify 4 M.L.D. Hanzlik and Terada (1936) observed that neutralization of toxin was absent or irregular in alkaline solutions of vitamin C; and they found no protection of pigeons by vitamin C against diphtheria toxin. Torrance (1937b) reports that heated and unheated crude toxin catalyzes the oxidation of vitamin C in lemon juice, an effect that may be due to porphyrins in the crude filtrate. Lemon juice has no effect on the toxicity or Lf value of the toxin. Sigal and King (1937b) find that properly neutralized and buffered solutions of vitamin C do not inactivate diphtheria toxin when oxidation is inhibited by diethylthiocarbamate. The effects of vitamin C on diphtheria toxin *in vitro* may be attributed to oxidation and acidity.

The injection of sublethal doses of diphtheria toxin in guinea pigs causes a depletion of vitamin C from the suprarenals, pan-

creas, and kidneys (Lyman and King, 1936; Torrance, 1937a; Haas, 1937b). Lyman and King observed an increase or a decrease of vitamin C in the liver, depending upon the dose of toxin and the amount of vitamin given daily. Torrance reports that injection of small amounts of toxin causes a mobilization of vitamin C in the suprarenal gland, but Haas was unable to confirm this. Sigal and King (1937a) have studied the mode of action of vitamin C in diphtheria intoxication. Injection of sublethal doses of toxin into animals on a diet deficient in vitamin C produced a degeneration of the islets of Langerhans, a hyperglycemia, and a low glucose tolerance. The effect was less marked in animals on an adequate diet, but the amount of vitamin necessary for a maximum effect on the intoxication was much larger than that necessary to protect against scurvy and maintain the normal growth rate.

CONCLUSION

The last five years have witnessed definite advances in our knowledge of the mode of production and the nature of bacterial toxins. The development of media containing relatively simple nitrogenous substances of known composition, such as those used for the cultivation of *Corynebacterium diphtheriae* and *Staphylococcus aureus*, will undoubtedly make possible more exact chemical studies of toxins. The complex conditions under which toxins are formed can be worked out most easily when the constituents of the media are known. Among the important factors affecting the formation of toxins are conditions related to oxidation-reduction systems. Many other factors have not yet been clearly defined in their relationship to the mechanism of toxin formation.

The ease with which various toxins are liberated from the bacterial cells differs greatly. Some toxins such as that of *C. diphtheriae* appear to be secreted in a readily soluble form by the bacteria. Others are liberated only by death and autolysis of the cells. The view that toxins are formed by enzymic degradation of proteins or peptones in the culture media is becoming less tenable as more toxins are produced in media containing

substances not much more complex than the amino acids. It is possible, however, that some of the bacterial poisons, such as the enterotoxin of the staphylococcus and the hemolysin of the streptococcus, may be metabolites or products of the action of bacterial enzymes.

At the present time, the only bacterial toxin which has been isolated in a state approaching purity is diphtheria toxin. The concentration and partial purification of others by various chemical procedures has assisted in the study of bacterial products with poorly defined biological properties. Chemical studies of the toxic products of the hemolytic streptococcus have led to the discovery that this organism forms a host of substances, each having some of the characteristics of a toxin. In the separation of the endotoxin from the exotoxin of the Shiga dysentery bacillus it was shown that the gastro-enteric endotoxin is a carbohydrate-lipid complex, while the neurotropic exotoxin is a protein. This is an advance which may eventually enable us to differentiate endotoxins from exotoxins on a chemical basis.

Attempts to demonstrate the existence of a toxic group in toxins by chemical analysis have so far been unsuccessful because of the difficulty of obtaining toxins in a pure state and in sufficient quantity. Both ricin (a plant toxin) and diphtheria toxin appear to be proteins which cannot be further degraded without destroying their biological activity. There is at present no evidence that these are conjugated proteins containing a prosthetic group which is responsible for their physiologic activity. However, it is possible that other toxins, such as those of *Streptococcus hemolyticus, Clostridium botulinum,* and the endotoxins of the *Salmonella,* colon, and dysentery groups, may prove to be non-protein substances with some unique chemical configuration which gives them their toxic properties.

Studies on the action of various physical and chemical agents on toxins have begun to yield suggestive data. Toxins are apparently affected in three different ways: (1) by agents which produce denaturation or coagulation, (2) by agents (soaps and lipids), which reversibly mask the toxic and biologic properties, and (3) by agents (certain aldehydes, halogen compounds, and oxidizing

agents) which produce modification to toxoid. Further investigations with purified toxins and chemical reagents classified into these three groups will doubtless yield important information on the nature of toxins.

Many toxins produce pharmacologic effects resembling those of the alkaloids. Toxins differ, however, from ordinary poisons in their tremendous activity and in the properties connected with antigenicity and the ability to combine with antitoxins and with susceptible cells. The association of antigenicity and great toxicity may be more than incidental. It is not unlikely that the same property or chemical grouping which causes an immunologic response when the toxin is injected may also bring about a selective combination with susceptible cells, just as an enzyme combines selectively with certain substrates. The pharmacologic effect would then follow by the action of other parts of the toxin molecule. From this it would appear that the nature of toxins as proteins or protein-like substances may hold the secret of their most characteristic biological properties.

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[4]In preparing this review, the author has not attempted to cover every paper having a bearing on the subject. Enough references are given to illustrate each point but there are many other papers on similar work which have not been cited. Generally the most recent papers have been reviewed without regard to historical development or priority. The papers cited usually contain adequate references to earlier work.

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