25 September 1959, Volume 130, Number 3378 SCIENCE

CURRENT PROBLEMS IN RESEARCH

The Most Poisonous Poison

What do we know about the toxin of botulism? What are the problems to be solved?

Carl Lamanna

One of the fundamental contributions of bacteriology to the understanding of disease is the concept that a symptom or specific pathological condition may have its origin in the activity of a single compound elaborated by a pathogenic agent. Historically this principle has been best exemplified by the exotoxins produced by certain bacteria. Among these exotoxins are the neurotoxins produced in vitro by *Clostridium botulinum* under suitable environmental conditions of nutrition, anaerobiosis, and temperature. The botulinal toxins have also been objects of curiosity because they are the most potent poisons known. It is my purpose to outline some of our knowledge of the nature of the botulinal toxins and to record questions of interest that remain to be answered.

Clostridium botulinum is a spore-forming, strictly anaerobic, rod-shaped bacterial species. Under as yet poorly understood conditions of growth it produces intracellularly a neurotoxin which is released into the growth medium after the major increase in the bacterial culture's population has been achieved. What role, if any, the toxin plays in the economy of the parent bacterium is a complete mystery. The organism is generally unable to grow within the body of the warm-blooded animal; hence, from a medical and veterinary standpoint, botulism is a matter of food poisoning. Botulism is a toxemia not involving an infectious process. Only three exceptional clinical cases of probably authentic wound infections by botulism organisms have been reported in man (*1*).

Different strains of *C. botulinum* produce antigenically distinguishable neurotoxins which have been labeled A, B, C, D, and E. The existence of other types or subtypes has not been clearly established. Antitoxin is capable solely of neutralizing the antigenic toxin or toxoid employed for its production, cross-neutralization among the types being unknown. One strain of organism produces exclusively one of the antigenic types of toxin. Mutation in production from one to another type of toxin has never been found, despite the mutability observed with respect to the quantity of a toxin produced under fixed conditions of bacterial growth and the mutability of many other characters of *C. botulinum*.

In man, food poisonings by toxin types A, B, and E have been detected frequently. Only two reported outbreaks of poisoning with type C and one with type D toxins in man are known to us. Poisoning by type E has resulted from consumption of improperly preserved fish foods. Type D is the cause of lamziekte, a toxemia of grazing animals on the South African veldt, and type C has been described most often as causing epizootic outbreaks of botulism in wild birds, such as ducks, that feed on the alkali lakes of western North America. The disparities in the frequencies of occurrence of particular toxin types as causes of natural food poisoning in different animal species are not necessarily due solely to a lack of susceptibility in particular vertebrates to the types of toxin. Rather, these disparities could be due to differences in geographic distribution of the botulinal types and to differences in feeding habits of various animal species.

Type E bacilli are probably marine organisms (*2*). The other types are soil organisms common to virgin soils. By a virgin soil is meant one that has not been intensely cultivated or, if cultivated, has not been subjected to heavy fertilization with manures. The difference in habitat may be responsible for the reputedly greater sensitivity to heat of type E spores than of spores of the other types (*2*). A direct relationship appears to exist between resistance to heat and the temperature limits for growth of bacteria (*3*). Marine species generally possess lower temperature limits for growth and are among the least heat-resistant of the bacteria.

Heavily manured and acid soils seem to favor the growth of species of *Clostridium* that are also found inhabiting the intestinal tract of animals, rather than growth of the botulism organism. We do not know why *C. botulinum* is unable to establish itself in the intestinal tract, as seems to be the case, whereas other clostridial species can do so.

Chemical Nature of Botulinal Toxins

Before 1945 little was known about the chemical nature of the botulinal toxins. While it was suspected that the toxins were proteins, reports existed which challenged this notion. Today, methods for purifying the toxins have been sufficiently advanced to make it certain that the toxins are simple globular proteins (*4*), a simple protein being defined as one composed exclusively of amino acids.

The author is scientific director of the Naval Biological Laboratory at the School of Public Health, University of California, Berkeley.

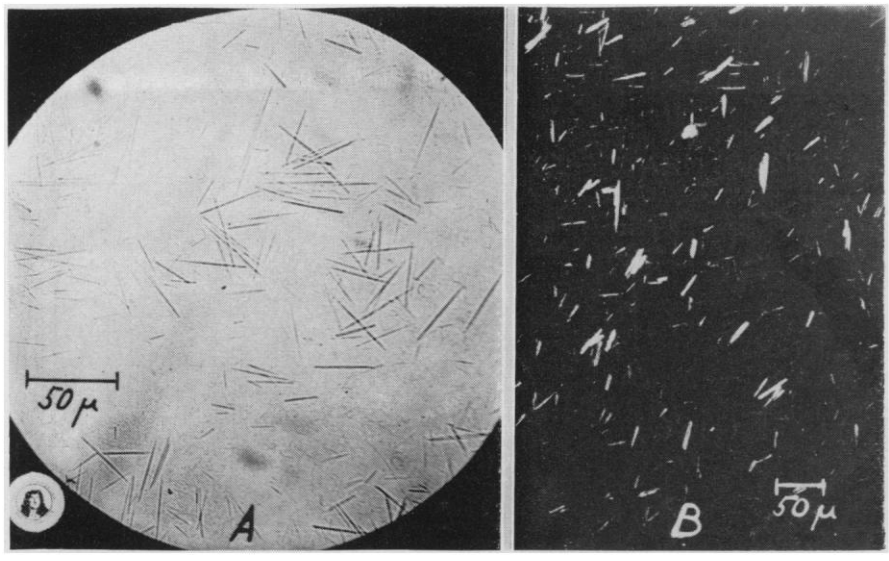

Fig. 1. Crystals of *Clostridium botulinum* type A toxin. (*A*) A direct light photomicrograph; (*B*) a photomicrograph made by dark-field illumination. The size of crystal is dependent upon the rapidity with which the toxin is salted out from a supersaturated solution. The average dimensions were 85 μ (length) by 5 μ (width); the largest crystal was 125 μ by 7 μ.

Only the type A toxin has been purified to a stage where crystallization is possible (Fig. 1). The crystalline type A toxin is a white, odorless protein of high molecular weight and unknown taste. Now, in connection with successful purification of a protein one is always faced with the difficult problem of deciding whether one has actually succeeded in isolating a homogeneous, single, molecular species. Some of the common criteria for molecular homogeneity are met by the type A crystalline material. Separately prepared batches show constancy of biological activity and amino acid composition. These are the same for amorphous and crystalline materials. Recrystallization does not result in increased toxic potency. The toxin molecules all migrate in solution in an electrical field (electrophoresis) at the same rate—a phenomenon that one expects to find when the molecules each possess the same net electrical charge. During analytical ultracentrifugation a single sedimenting boundary appears at various *p*H values on the acid and alkaline side of the isoelectric point, *p*H 5.6. The isoelectric point is that *p*H below which a protein particle bears a net positive electrical charge and above which it bears a net negative charge.

In spite of these indications of homogeneity there do exist disturbing facts about the crystalline material. The phase-rule solubility test for homogeneity cannot be met—that is, for fixed conditions of solution the saturation value is not independent of the quantity of crystalline material exposed to solvent. During ultracentrifugation in the vicinity of the isoelectric region, though only a single sedimenting boundary appears, the boundary spreads at a more rapid rate than can be accounted for solely by diffusion of a homogeneous substance (*5*).

The behavior of crystalline toxin in the quantitative toxin-antitoxin precipitin reaction has been particularly instructive (*6*). Upon removal of precipitates by centrifugation, the supernatant solutions from the equivalence zone of a series of mixtures of varying quantities of toxin with a fixed amount of antitoxin are found to be free of both toxin and antitoxin. Such a result is interpreted as indicating the existence of only a single antigen-antibody system. But in other respects the results of mixing toxin and antitoxin are atypical for a pure antigen.

What is surprising is the fact that large discrepancies are found in the amounts of precipitate formed, particularly in the zone of antigen (toxin) excess, when toxin and antitoxin are mixed at different temperatures (Fig. 2). In the usual situation, when equivalently reacting amounts of antigen and antibody are mixed, the amount of precipitate formed will be the same over a wide temperature range and will include all of both reagents. Even more unexpected is the finding that at low temperatures the quantity of botulinal toxin-antiserum precipitate formed is greater than the calculated sum of the amounts of toxin and antitoxin mixed together. Such a result forced consideration of the possibility that serum components other than antitoxin were being precipitated. Therefore, purified albumin and globulin fractions of normal sera were studied, and they were observed to form precipitates in the cold with toxin. In sum, crystalline or any highly concentrated or purified toxin preparation proved to have the unusual ability to form relatively insoluble compounds or complexes with different normal serum proteins. These serum proteins have no toxin neutralizing action and cannot be considered to be of the nature of antibodies or antitoxin.

Assisted by hope and led by intuition, I performed red-cell agglutination tests (hemagglutination) with purified toxin. To our distress and delight, hemagglutination was found to take place in the cold. It was distressful to find that botulinal toxin had another property to worry us but delightful to find that the property was one that could be studied and measured by simple procedures.

Botulinal Hemagglutinin

It is now known that the various neurotoxin types of *Clostridium botulinum* produce hemagglutinating substances. The synthesis of hemagglutinin is coincident with toxin production, and in the purification procedures employed to date, hemagglutinin accompanies the toxin. Since the classification of types of *C. botulinum* is based on the specific serological neutralization of the neurotoxin produced by these organisms, the occurrence of an accompanying hemagglutinin during toxin production poses the question of the type specificity of these substances. The hemagglutinins produced by organisms of types A and B are reciprocally neutralized by commercially available specific A and B antitoxins. Hemagglutinins produced by types C and D are reciprocally neutralized by the specific antisera of these strains. Type E hemagglutinin is neutralized only by specific type E antiserum. The hemagglutinin produced by type A bacilli has been intensively studied, and the remarks that follow derive from these studies (*7*).

The toxin and hemagglutinating activities of toxin preparations might be attributable either to the same molecule or to different molecules. A number of

facts indicate that they are attributable to different molecules. Important evidence for the molecular individualities of toxin and hemagglutinin is the fact that, in laboratory experiments, the hemagglutinin is selectively adsorbed to red cells without concomitant uptake of toxicity from the adsorbed solution. To achieve this result, the correct conditions of acidity, salt concentration, and temperature must be employed. The conditions of adsorption determine the degree to which toxin accompanies the hemagglutinin in the latter's attachment to adsorbents. It is also observed that the diffusion coefficient of toxin becomes larger when hemagglutinin is removed by these methods (*8*). This, of course, is direct proof that toxin freed of hemagglutinin has a smaller molecular size.

In the botulinal culture the toxin does not exist free of hemagglutinin. The presently available methods for isolating and purifying the toxin were developed before the existence of the hemagglutinin was known, and they neither aim at nor inadvertently achieve the separation of hemagglutinin from the toxin. It is obvious that crystalline type A toxin is a complex in which toxin and hemagglutinin exist in an intimate association of unknown nature, and this accounts for the fact that in its behavior in the isoelectric region it simulates a single substance.

The combination of hemagglutinin with toxin is a reflection of the unexpected capacity of hemagglutinin to combine with or be adsorbed to a variety of chemically and biologically unrelated protein materials. Not only does the hemagglutinin form complexes with serum globulin and albumin but it is taken up by homogenates of many tissues other than red blood corpuscles. In other words, the hemagglutinin has the remarkable ability to interact with diverse proteins to form compounds or complexes of reduced solubility relative to that of the reactants. The hemagglutinating property itself is a consequence of the reaction of hemagglutinin with protein structure at the surface of the erythrocyte. This is demonstrated by the fact that proteolytic enzymes and some protein denaturants destroy the capacity of red blood cells to take up the hemagglutinin. The receptor sites for the hemagglutinin do not involve the carbohydrate-type material involved in influenza virus hemagglutination.

Are the red cell sites that act as receptors for the type A-B hemagglutinins also involved in the types C-D and E hemagglutinations? At the present time all we can say is that erythrocytes saturated with type C hemagglutinin are incapable of sorbing type A hemagglutinin. Whether this is due to adsorption of the C hemagglutinin to the same receptor sites that take up A hemagglutinin or simply to the crowding of type C hemagglutinin molecules on the surface of the red cell and the consequent lack of contact between A type hemagglutinin with its specific receptors has not been investigated.

The botulinal hemagglutination phenomenon has the characteristics of a reversible adsorption reaction and does not result in any detectable permanent changes in the surface of the red cell or in the hemagglutinin after repeated cycles of adsorption and elution of the hemagglutinin (*7*). While some hemolysis may result, this appears to be no greater than that attributable to experimental manipulations in the absence of hemagglutinin.

By working in an alkaline environment at low ionic strength, it is possible to adsorb hemagglutinin to erythrocytes and to leave toxin in solution free of hemagglutinin. Employing this scheme, toxin solutions free of hemagglutinin and hemagglutinin solutions with greatly reduced (by more than 99 percent) toxic activity can be prepared. Unfortunately, the procedures for selective adsorption-elution employed to date have not resulted in the isolation of the quantities of pure toxin desired. Losses of toxic activity result, and hence the toxic products freed of hemagglutinin have not been crystallized and contain considerable amounts of biologically inactive

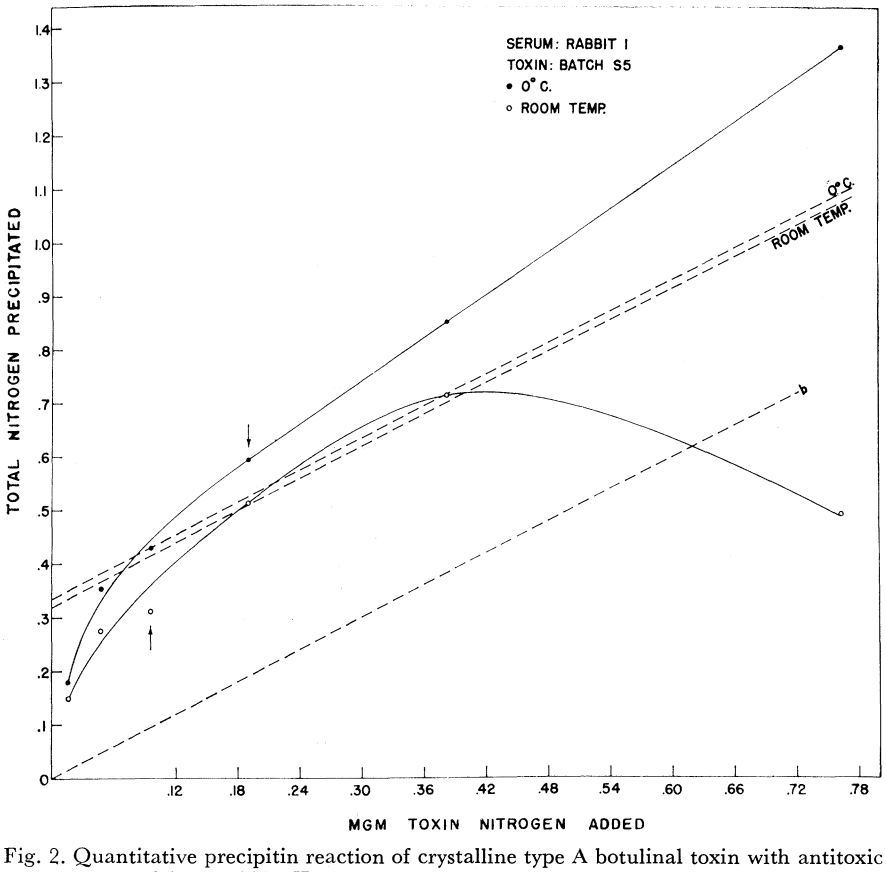

Fig. 2. Quantitative precipitin reaction of crystalline type A botulinal toxin with antitoxic serum prepared in a rabbit. The arrows represent the limits of the equivalence zone—that is, mixtures of toxin and antitoxin in a ratio that should lead to complete precipitation of both the toxin and antitoxin. The broken line (Ob) represents the amount of precipitate that can form if only the toxin itself is precipitated. The other broken lines represent the total precipitate expected at the indicated temperatures if all of the toxin and antitoxin is precipitated. Since the actual amounts of precipitate are greater than amounts obtainable by precipitation of toxin-antitoxin alone, a component other than toxin or antitoxin must be precipitating. As explained in the text, this material proved to be both normal serum albumin and globulin, which can form insoluble products with the hemagglutinins associated with the toxin. [From C. Lamanna and B. W. Doak (*6*), courtesy of Williams and Wilkins Co.]

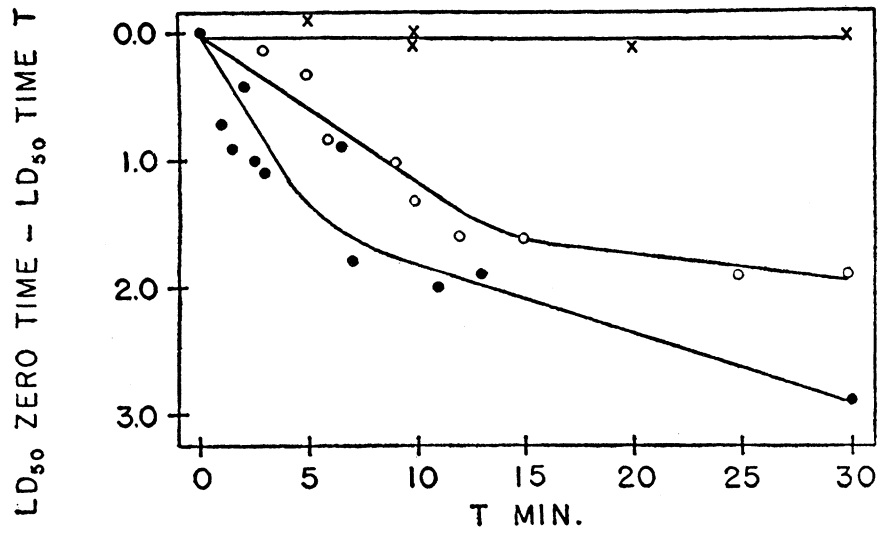

Fig. 3. Thermal inactivation curves for crystalline botulinum A toxin preparation: X, 40°C; O, 50°C; ●, 60°C. LD_{50} indicates logarithm to base 10 of the extent to which a toxin solution must be diluted to give 50-percent mortality when goldfish are inoculated with 0.1 ml to assay toxicity. The ordinates represent differences between logarithms of these dilutions for materials heated zero length of time; the times are indicated on the abscissa. [From T. E. Cartwright and M. A. Lauffer (*10*), courtesy of Society for Experimental Biology and Medicine.]

proteins. Lack of homogeneity in the materials isolated by selective adsorption-elution has been suggested by results of exploratory runs in the analytical ultracentrifuge. It would be a great convenience if a suitable nonprotein adsorbent could be discovered to replace the red cells, particulate denatured chicken egg albumin, and other protein adsorbents employed to date. Nonprotein adsorbents tested have failed in one or more of three ways: They do not adsorb efficiently; they denature toxin; and they do not permit quantitative elution.

Size of Toxic Unit

The promise offered by the successful crystallization of the type A toxin that the ultimate toxic particle might be isolated was followed by the disillusioning discovery that a separable hemagglutinin is present, and this disappointment has been followed by others. The crystalline toxin, with a molecular weight of 900,000, appears to dissociate, when held in solution above *p*H 6.5, into particles of molecular weight in the range of 40,000 to 100,000. It has been claimed that these dissociants are not consequences of chemical changes resulting from detoxification at destructive *p*H values because they retain toxic potency (*9*). These components of low molecular weight have been identified only in the course of ultracentrifugation experiments and have not been isolated in sufficient quantities to permit thorough investigations of their state of purity and of their biological properties.

Detoxification by heating crystalline A toxin is not linear with respect to time (Fig. 3), and ultracentrifugation shows dissociation of heated toxin into two components (*10*). Since similar detoxification results were obtained when toxin was heated both before and after removal of hemagglutinin, these observations cannot be explained by assuming the simultaneous presence of toxin-hemagglutinin complex and free dissociated toxin. This is important to know, since hemagglutinin is less sensitive to heating than toxin. Toxin complexed with hemagglutinin might possibly be more heat-resistant than toxin alone, and this might account for observations of toxin with two differing sensitivities to heat.

The question of the size of the ultimate toxin particle also raises the problem of whether the chemical basis for toxicity is resident in the protein molecule as a whole or in some portion (toxophoric group) of the molecule. Chemical analysis of crystalline toxin has revealed the presence of 19 typical amino acids and has yielded no insight into the nature of the molecular configuration responsible for toxicity (*11*). Alternatively, one might hope to deduce the presence and nature of a toxophore from the conditions and chemical reagents causing detoxification. A variety of unrelated physical and chemical agents for protein denaturation cause detoxification (*12*). The splitting of hydrogen bonds and reactions with free amino and carboxyl groups are among other reactions that probably cause detoxification.

If a specific toxophore exists within a molecule, it might be possible to reveal its presence during hydrolytic splitting of the toxic protein. Proteolysis might proceed as a multistep process during which pieces of the toxin molecule are split off prior to the release or destruction of a specific toxic group. Though comprehensive studies of this possibility are needed, the existence of such a process must be doubted. The various chemical reagents and proteolytic enzymes that cause loss of toxicity attack different kinds of bonds characteristic of simple proteins. Loss of toxicity by proteolysis proceeds without an apparent initial lag in time. The simplest view consistent with these known facts is that maintenance of the structural integrity of a protein molecule as a whole is required for toxicity.

In summary, it is clear that final knowledge of the true size of the ultimate toxic unit remains to be obtained. While it is a useful working hypothesis to consider crystalline toxin to be possibly an "assembly of toxic and inert subunits in association with one or more hemagglutinin units" (*9*), we have reason to guess that the toxic unit itself will prove to be of the dimensions of a protein.

Toxic Potency

Botulinal toxins are unquestionably the most potent poisons known; less than a 0.1×10^{-3} microgram causes the death of a mouse. Only tetanus and *Shigella* neurotoxins appear to possess potency of the same order. Other protein poisons, such as diphtheria toxin and animal venoms, are hundreds and tens of thousands of times less poisonous. Aconitine, one of the most poisonous nonprotein materials known, is far less toxic than the botulinal toxins (Table 1). The extraordinarily high toxicity recorded in Table 1 for the botulinal D toxin is of questionable validity, since it has been reported once only and has not been repeated in independent investigations.

The absolute measure of toxic potency is the number of molecules required to cause fatalities. While it has been estimated that 20 million molecules of crys-

talline type A toxin are required to kill a mouse, this figure was arrived at before it was known that extraneous hemagglutinin existed in the crystals. Until the botulinal toxins can be separated from the associated hemagglutinin and purified to meet criteria of homogeneity, and until the weights for the physiologically active molecules can be accurately determined, calculations of the number of molecules in a lethal dose will be speculative.

It is of interest to know how different species compare quantitatively in their response to physiologically active substances such as toxins. The favored convention for making such comparisons is to assume that the amount of toxin needed to kill increases with the body weight of an individual animal and to express lethal dose per kilogram of body weight or per kilogram of body weight per unit weight of poison. Experience casts serious doubts on the applicability of this convention to the botulinal toxins. The fact is that in the mouse, the only animal adequately studied, the potencies of botulinal toxins A, B, and C are independent of the weights of the individual animals, whether potency is measured in terms of the time it takes to kill or in terms of the percentage of animals killed by different concentrations of toxin (*13*). The same situation probably exists with respect to the lethal dose for rabbits. In the mouse, the range of body weights studied has included all of the physiological ages from weaning through sexual maturity and adulthood. Whether it is valid to generalize from this experience to include other animal species and other toxins is unknown. A surprising paucity of pertinent experimental data exists. It would appear that the existence of a relationship between body weight and physiological response to toxins has been accepted as a matter of faith, and few students have felt the call to study the question experimentally with adequate numbers of animals.

In nature, there simply is no such thing as a mouse, rat, or guinea pig of 1-kilogram body weight, so expressions of toxicity in terms of 1 kilogram of body weight are extrapolations from findings with actual animals. The conventional extrapolation is not valid for the botulinal toxins, since there is evidence that the assumption that with increases in body weight more toxin is required to yield a given result is untenable. As a matter of fact, in a comparison of findings for male mice weighing 6 to 10 grams with those for male mice weighing 18 to 22 grams, the smaller animals proved somewhat more resistant to type B toxin. Such a finding indicates that scientifically valid expressions of toxicity for various animal species of various body weights are not easily arrived at. What, if any, proportionality exists between body weight and toxic dose must be established empirically for each species and toxin considered. Then, if desirable, dosages can be expressed in units per kilogram of body weight, but only by extrapolation in accordance with experimental findings for each species of animal and toxin. With botulinal toxins, extrapolation for a mouse weight of 1 kilogram is best obtained by simply using the figure for toxicity recorded for the real mice used in titrations, with allowance made for any significant contribution made by the sex of the animals. In resistance to botulism, the female mouse (in line with male suspicions) sometimes proves to be the more hardy member of the species.

That the qualitative estimates of the relative potency of botulinal toxin for different animal species vary in accordance with the method for making comparisons is illustrated in Tables 2 and 3. Until future quantitative studies have been made to define a realistic basis of comparison, it is not possible to state with any degree of confidence what the relative sensitivities of different animal species to the botulinal toxins are. Nor do the available clinical data permit one to make a scientific judgment of the true minimum lethal doses for man. What evidence is at hand points to man as a very susceptible species. Recently it was calculated that ingestion of a dose only 3500 times larger than the dose lethal to mice was responsible for the death of a 104-kilogram man (*14*). This kind of information gives us a notion of the range within which a fatal dose for man falls, but it provides no clue as to what lesser quantity of toxin might prove to be fatal.

Possibly the foregoing discussion justifies my lack of enthusiasm for the task of compiling a list of reported lethal doses of botulinal toxin for susceptible animals. Moreover, to compile such a list might lead to a false impression of the conclusiveness of our knowledge of the relative and absolute difference in sensitivity to the different types of toxin among animal species. The unfortunate fact is that the data obtained by independent investigators often differ widely, and it is injudicious, scientifically speaking, to make choices between the conflicting reports. Let us illustrate. Stevenson *et al.* (*15*) have reported the guinea pig to be 6000 to 8000 times as sensitive as the white mouse to the type B toxin, a conclusion greatly at variance with the data given in Table 2. However, these authors worked with relatively impure toxin derived from a different strain of B type *Clostridium botulinum* than was employed for obtaining the data recorded in Table 2. Nor is information available on possible differences in the genetic backgrounds of the strains of guinea pigs and mice studied. These variables might explain the differences in results, but no inquiries have been made to establish this as a true explanation.

No doubt the lack of dependence of lethal dose on body weight arises from

Table 1. Toxicity with respect to the white mouse of the most purified preparations of the five types of botulinal toxins.

Toxin type	Toxicity* (LD_{50}/mg of N)
A	2.4×10^8
B	2.6×10^8
C	4×10^7
D	4×10^{12}
E	7.7×10^4
Aconitine	3.5×10^3

* It is generally preferred to report toxicity per milligram of nitrogen, since analysis for protein nitrogen is practical and reliable. To convert to toxicity per gram of solid, multiply the figures in column 2 by 1000/6.25. Use of the number 6.25 in the conversion factor is based on the assumption that the nitrogen content of the toxins is 16 percent. The factor for aconitine is 1000/46. Sources for the data on toxicity are as follows: for type A, various authors (*4*); for type B, Duff *et al.* (*4*); for type C, Gordon *et al.* (*4*); for type D, Wetzel *et al.* (*4*); for type E, Cardella *et al.* (*4*).

Table 2. Toxicity of type B toxin for the mouse and guinea pig. If in fact the dose of toxin required to kill is independent of the weights of mice and guinea pigs, then the amount of toxin that kills an actual animal is also the amount required to kill a hypothetical 1-kilogram (or any other weight) individual of the same species. This leads to the conclusion that the mouse is more sensitive to type B toxin than the guinea pig, a conclusion which contradicts that based on the conventional extrapolations of toxic dose for individual animals of different weights.

Lethal dose (mg of N $\times 10^{-9}/LD_{50}$)	Ratio (mouse: guinea pig)
6.2 for 20-g mouse 31.2 for 300-g guinea pig	1:5
310 per 1-kg mouse 103 per 1-kg guinea pig	3:1

the specific nature of the mechanism of poisoning by botulinal toxins. These toxins are known to act strictly at terminal ends of cholinergic autonomic nerves and at the neuromuscular junction. Thus, if the number of these sites of action is fixed at birth and does not change with the growth of the animal to adulthood, then it may be expected that body weight will have no appreciable influence on response to toxin. This thought can be framed in another way. The botulinal toxins are not general cellular poisons. They probably act on biochemical substrates which do not keep pace in quantity with increase in body weight. It would be worth while to learn whether this hypothesis is applicable to other poisons—say, those affecting the nervous system in a quite specific way or in several specific ways.

Toxin as an Oral Poison

A puzzling fact about the botulinal toxins is that they are oral poisons. The fact that the toxins chemically are typical proteins which apparently survive passage through the alimentary tract raises challenging questions. How can such proteins survive running the gamut of digestive juices, and how can molecules of the large dimensions of protein molecules pass through the alimentary barrier into the general circulation? While the older literature reports that botulinal toxins are resistant to detoxification by proteolytic enzymes, recent work done with quantitative techniques, adequate numbers of experimental animals, and type A toxin of varying degrees of purity has shown detoxification capabilities for trypsin and chymotrypsin, enzymes of the small intestine. Results with pepsin have been conflicting (*16, 17*). With type E toxin, trypsin has been shown to act both as an activator and as a detoxifying agent. In vitro contact of this toxin with trypsin leads to an initial increase in the number of lethal parenteral doses, followed by decreases (*18*).

Since detoxification can result from exposure to trypsin and chymotrypsin, botulinal toxin might be thought of as acting as an oral poison because it is absorbed before it enters the small intestine. The attraction of this hypothesis is diminished by findings which make it doubtful that absorption occurs in any significant degree from the stomach, where toxin would be exposed only to pepsin (*19*). No critical studies delineating the possibility that the oropharynx and esophagus act as major avenues of systemic absorption have been reported. Such a demonstration would help to explain reports that botulism has occurred in human beings after the mere tasting of contaminated foods. It is known that toxin can be absorbed from the lower reaches of the respiratory tract, which possesses only a thin layer of epithelial cells, and through abrasions and burns of the skin and mucous membranes. Thus, mouth wounds, and possibly even dental caries, may permit absorption of toxin before contact with the proteolytic enzymes of the alimentary tract occurs. These considerations, interesting though they may be as conjecture, have little merit unless they provoke future research. On the basis of published evidence, the small intestine must be regarded as the major site of systemic absorption in natural cases of poisoning by ingested toxin. The stomach and colon are minor absorption sites (*19*). We conclude that ingested toxin appears to have every opportunity for contact with proteolytic enzymes.

Actually, what may be significant is the persistence of residual amounts of toxicity for long periods of time in the face of destruction of toxin by proteolytic enzymes. In vitro, the rate of detoxification decreases with time, and reduction to zero toxicity tends to be approached asymptotically. We may surmise that these relationships are repeated in vivo. Relative to a protein such as casein, proteolytic enzymes act more slowly when type A toxin is the substrate. These observations imply that toxin need not be resistant to enzymatic proteolysis in an absolute sense in order to act as an oral poison. It is needful only that the rate of digestion of the toxin before complete destruction be such that a lethal dose can pass the barriers of the alimentary tract. Such a situation would be in keeping with the oft-repeated observation that much larger quantities of toxin—larger by as much as 100,000-fold—are required for a lethal oral than for a lethal parenteral dose. It is also possible that the known difference in potency of oral doses of various types of toxin in a given animal species, or of oral doses of one type of toxin in different species (and these differences are considerable) might reflect differences in the resistance of the various types of toxin to the digestive juices of animals of different species. A case in point is the nonsusceptibility of the *Rhesus* monkey to C and D toxins administered orally (*20*).

For a complete picture it would be necessary to know whether protein from extraneous sources, such as foods, can slow down or prevent proteolytic destruction of toxin, and whether ingested toxin can seriously interfere with the secretion of enzymes into the alimentary

Table 3. Comparison of the susceptibility of mouse, ferret, and mink to botulinal toxin types A and B administered by the intraperitoneal (i.p.) and oral routes. [From Moll and Brandley (*21*), except for data in column 8. Courtesy of the American Veterinary Association]

Route	Type of toxin	Animal	Average wt. (g)	Minimum lethal dose (mg)	Minimum lethal dose/g	Resistance per gram wt. (based on minimum lethal dose in mouse)	Resistance (irrespective of wt. differences based on minimum lethal dose in mouse)
I.p.	A	Mouse	20	0.000001	0.00000005	1	1
I.p.	A	Ferret	1620	0.010000	0.00000060	12	10,000
I.p.	A	Mink	840	0.010000	0.00000120	24	10,000
I.p.	B	Mouse	20	0.000001	0.00000005	1	1
I.p.	B	Ferret	1520	1.000000	0.00060000	1200	1,000,000
I.p.	B	Mink	1000	1.000000	0.00010000	2000	1,000,000
Per os	A	Mouse	20	0.010000	0.00050000	1	1
Per os	A	Ferret		Variable			
Per os	A	Mink	1000	60–80	0.06–0.08000000	120–160	6,000–8,000
Per os	B	Mouse	20	0.010000	0.00050000	1	1
Per os	B	Ferret		Variable			
Per os	B	Mink	980	20.000000	0.02000000	40	20,000

tract—possibilities which have not been seriously explored in any animal species. A remaining and likewise untested hypothesis is that quantitative differences in animals of different species with respect to oral poisoning result from large differences in permeability of tissue to protein. A resolution of the various possibilities could be achieved through comparative quantitative balance studies on the disappearance of toxin from the alimentary tracts in various animal species. The experimental data needed are the precentages of recovery, in time, of toxin taken from the digestive system and from the blood stream and lymph. In such studies, nonrecoverable toxin would represent loss of toxin by destruction in the alimentary tract.

In Table 3 are listed lethal doses determined by one group of workers in a comparative study of the white mouse, ferret and mink (*21*). The results are presented to illustrate the differences often found between data for intraperitoneal and data for oral exposure to toxin, and the fact that the relative sensitivities of species to different types of toxin introduced parenterally is not a reliable index of sensitivities to these toxins when they are ingested.

Some toxicity can be detected in intestinal contents or feces for several days after ingestion of toxin. Since botulinal toxin does not characteristically cause the direct irritation of intestinal surfaces that is found in other types of bacterial food poisoning, such as salmonellosis and staphylococcus enterotoxin poisoning, diarrhea or vomiting do not necessarily follow ingestion of botulinal toxin. These processes, which might be considered self-cleansing mechanisms, limiting the period of absorption of toxin, thus do not regularly occur in the animal in botulism. Constipation is not infrequently observed in botulism. Consequently, death is potentially the outcome, even when rates of absorption are slow. These observations suggest that in clinical treatment of botulism every effort should be made to promptly clear the alimentary tract *in toto*. Physicians have not always used high enemas in addition to the stomach pump.

How botulinal toxins pass across the membranes of the alimentary tract is not known. While there has been reluctance to believe that the toxin is absorbed systemically in the form of a whole protein particle, there is no present evidence in support of any other conclusion. Absorbed toxin may not have the molecular weight of crystalline toxin (900,000) (*5*), but the molecule is large enough to be nondialyzable (*19*). The sojourn in the intestinal tract does not lead to fragmentation of the toxin to absorbable dialyzable toxic molecules. During enzymatic digestion observed in vitro, toxin does not appear in the small molecular-weight fractions, which become soluble in cold 0.25-percent trichloroacetic acid (*17*). Nor does the toxin exposed to pepsin, trypsin, or chymotrypsin in vitro become able to pass through the wall of laboratory dialysis tubing.

From the alimentary tract, toxin is passed into lymph rather than directly to blood and is not dialyzable as recovered in the lymph (*19*). The explanation of the permeability of the alimentary tract with respect to toxin may rest on some unexpected capacity for proteins in general to escape in small amounts by a lymphatic route, a possibility currently under investigation at the Naval Biological Laboratory. The alimentary tract may not be such an absolute and impenetrable barrier to systemic absorption of whole unmodified protein as common sense would suggest. In reality, only extremely small quantities of a protein such as a toxin need penetrate the intestine for a fatal result to ensue. The potency of ingested botulinal toxin may be attributable to its extraordinary toxicity alone and not to any unusual ability to escape destructive intestinal processes or to possession of toxophores of small molecular weight released from a larger carrier particle by intestinal mechanisms. Parenthetically, it is worthy of remark that botulinal toxins could prove to be unique tools for the study of small breaks in permeability barriers to the free diffusion of proteins in vivo.

Mechanism of Poisoning

In botulism the immediate cause of death is usually a paralysis of the skeletal musculature, which results in interference with breathing and in terminal asphyxia. Observation for visible gross and microscopic pathological changes in botulism is unrewarding. When histological changes are found, they prove to be secondary to the primary mechanism of poisoning and are accentuated when death is postponed. The greater the dose of toxin, the more rapidly death ensues, and the less likely it is that histopathological changes will be observed.

Botulism is a paralysis of the efferent autonomic nervous system—that is, of the portion of the nervous system that stimulates the self-acting (involuntary) muscles of the body. The central nervous system is not affected. Intracranial injection of toxin results in the same symptoms of poisoning and times of death that are observed when injection is made at other sites. Sensory nerves are not harmed. The actual site of poisoning is at the synapses of efferent parasympathetic nerves and somatic motor nerves (the end plate or myoneural junction). The reaction at the site of action is a subtle one, which leaves no visible mark as yet recognized by the human observer. The productive techniques for studying the mechanism of poisoning in botulism have been, therefore, those of the neurophysiologist. The neuroanatomist still has an opportunity to discover ways to make his contributions.

Transmission across synapses is performed chemically. An impulse in a nerve releases a chemical substance at the terminal fibers. The released substance then acts to initiate an impulse in the recipient postjunctional structure, whether this be the dendrites of another nerve cell or a muscle end plate. The system for synaptic transmission is composed of two subsystems, one (the adrenergic system) depending on an adrenaline-like substance, sympathin, as a chemical transmitter and the other (the cholinergic system), on acetylcholine. Only the cholinergic system is affected by botulinal toxins.

The mechanism of botulism poisoning is rigidly specific; hence, these toxins could be employed as tools to map the cholinergic system, though historically this has not been the means for such mapping. It is probable, then, that only those species of animals that possess an organized cholinergic nervous system are affected by botulinal toxins. The mere appearance of acetylcholine in a living system is not a sufficient condition for the action of botulinal toxin. An organized nervous system must exist. Thus, a microorganism such as the bacterium *Lactobacillus plantarum,* which synthesizes acetylcholine and secretes it into the growth medium, is not affected by botulinal toxin, either in its capacity to produce and secrete acetylcholine nor in any of its other life functions (*22*). Tissue cultures of mammalian cells are also unaffected. Work with tissue cultures must be pursued cautiously, since any effects noted upon the addition of toxin may be traceable to extraneous impurity and not to the neurotoxin proper. Nor will the prevention through

contact with antitoxin of any observed effects of a toxin preparation on tissue culture constitute of itself proof of toxin activity. Antitoxins as generally prepared contain antibody against impurities and will most certainly be found to contain antihemagglutinin.

Though quantitative differences in the toxic dose for animals exist, all but one of the vertebrates tested have been poisoned; exception is claimed for the American turkey vulture (*Cathartes aura septentrionalis*) (*23*). The vulture was tested, since these birds under observation in the natural state did not show botulism when feeding on carcasses of other birds dead of the toxemia. Kalmbach attributed this phenomenon to a neutralizing property of vulture serum which may have arisen as an evolutionary adaptation to the natural risk of feeding on the carrion of animals dead of botulism. The laboratory work, done with only four birds, is worthy of repetition. Studies with botulinal toxins in vultures might have the added value of revealing the existence of unsuspected peculiarities in the avian cholinergic nervous system.

Demonstration of the specificity of the site of action of botulinal toxin provides a tool hitherto ignored for physiological studies requiring long-term immobilization of a muscle or muscle group. The perfecting of techniques for employing toxin for this purpose in a safe manner might lead to successful application in therapeutic situations. Recovery from nonfatal doses of toxin is protracted. Through localizing the distribution of toxin and neutralizing, by means of antitoxin, any toxin escaping from a localized area into the circulatory system, it is feasible to render a muscle inactive without interrupting unrelated vital activity. The potentialities of such techniques have been demonstrated through single local injections of toxin into the eye; paralysis of cholinergic nerves alone for more than six months ensues without manifestations of any interference with the transmission of impulses by neighboring adrenergic nerves (*24*).

By the use of isolated muscle-nerve preparations it has been possible to demonstrate unequivocally that there is a period of latency between the time of exposure to toxin and paralysis. For as long as a half hour after introduction of the toxin, such muscle-nerve preparations may respond to nerve stimulation. Yet shortly after toxin is introduced—often in a matter of a few minutes—it becomes impossible to prevent by any known treatment, including the use of antitoxin, the paralysis that follows the period of latency. In essence the reaction of toxin with an unknown receptor is rapid and irreversible and the consequences of this reaction are delayed in time. The event or events leading to paralysis of the mechanism of synaptic transmission are thought to be chemical in nature. The chief evidence for this lies in the high temperature coefficients demonstrated by the great reduction in the length of the period of latency that accompanies increase in temperature of preparations exposed to toxin.

For botulism poisoning to occur, the terminal endings of sensitive nerves must be exposed to toxin solutions. Experimental bathing of the axon in toxin is without effect. If the muscle paralyzed by toxin is stimulated directly, it will respond. If acetylcholine is added to a muscle-nerve preparation which has been paralyzed by being bathed in a solution of toxin, the muscle will respond.

In normal tissue an enzyme, cholinesterase, is produced which acts to destroy acetylcholine. As a result, the acetylcholine released at the synapse by any one nerve impulse can act only once and only for a moment. This is a necessary phenomenon if muscle is to be capable of an individual response to each nerve stimulus it receives by way of the chemical transmitter and able to respond to each stimulus in a rapid succession of stimuli. In botulism, paralysis is not reversed by cholinesterase inhibitors. This distinguishes botulism from curare poisoning, which in the past was thought to be possibly an analogous phenomenon. These as well as other available facts all point to the same conclusions—namely, that the basis of paralysis in botulism is specific interference with the release of acetylcholine from the terminal ends of nerves and does not involve events in muscle-nerve physiology that follow upon the release of acetylcholine (*25*). Stevenson (*25*) has presented a detailed review of what is known of the physiological basis for poisoning in botulism.

The pressing challenging problem in the physiology of botulism is the need to determine the precise site and nature of the reactions leading to the failure of poisoned nerves to release acetylcholine. The elucidation of the reactions would represent a double victory—one in our understanding of botulism and another, a more intimate knowledge of the biochemistry of nerve terminals. An extra dividend might be the development of methods for treating botulism, based on such understanding. Such therapy is greatly needed, since all current medical treatment is simply supportive. The irreversible nature of toxin uptake and the short period of latency renders the use of antitoxin no more than a psychotherapeutic measure in the patient in whom symptoms have already developed, except for possible neutralization of toxin still in the process of escaping from the alimentary tract at the time antitoxin is injected.

Prophylaxis

The incidence of botulism in human beings is low (*26*). Consequently, no special incentive has existed for immunizing large numbers of people against botulism. It is useful as a precautionary measure nonetheless, to have an immunizing product available and to use it to immunize laboratory personnel. Because of their potency, it has been suggested that the botulinal toxins might be used as agents of warfare. As a result, responsible governmental agencies have sponsored research aimed at mastering techniques for large-scale manufacture of effective immunizing preparations for defensive use in human populations. There also exist special situations when it is useful to be able to immunize domestic animals. Immunization of mink, fox, and other species raised on fur farms is a sound economic policy, since inadvertently these animals are not infrequently fed poisoned spoiled meats.

Botulinal toxins are highly antigenic proteins. Small quantities are capable of stimulating an animal to produce specific neutralizing antibody. After passive immunization of rabbits with antitoxin, small amounts of active toxin can be given in a series of injections, with a high but not absolute degree of safety, which will subsequently result in a self-engendered immunity (active antitoxin synthesis). Through such a procedure, injection of small quantities of crystalline type A toxin (2 mg and less of toxin nitrogen) will stimulate rabbits to produce maximal quantities of antitoxin.

Simpler and still safer methods can be applied in medical and veterinary practice. Fortunately it is not a difficult matter to detoxify the botulinal toxins by

chemical means without destroying their antigenic properties. The addition of formaldehyde to toxin solution under controlled conditions of reagent concentrations, of acidity, and of temperature, is the favored method. The resulting preparations (toxoids) are nontoxic, strongly immunogenic, and quite stable. Much has been published on the production of botulinal toxoids (*27*). For use in human beings a preparation of highly purified toxoid of great efficacy is now available (*28*). It is polyvalent, consisting of a mixture of toxoids of types A, B, C, D, and E. While the individual toxoids in this preparation differ in degree of purity, a satisfactory response is engendered by each of the five components.

In the manufacture of toxoids, the presence of hemagglutinins has been ignored. Since hemagglutinin is inactivated more slowly by formaldehyde than is the neurotoxin (*7*), we have not been surprised to find that the toxoids as currently prepared possess considerable hemagglutinating activity. As an example we may cite a purified type A product with 11 flocculating units of toxoid per milliliter, whose hemagglutinating activity was not reduced below detectable macroscopic levels until the preparation had been diluted more than 73,500 times. Two forms of this toxoid—an unmodified form and a special preparation from which the hemagglutinin had been removed by adsorption to erythrocytes—were injected separately into different groups of mice for the purpose of detecting any histopathological changes the toxoid might induce. A comparison was made with untreated control mice held under the same laboratory conditions (*29*). Since no differences were observed among the mice receiving the several treatments, the hemagglutinin may be considered to be harmless. But more intensive work must be done before such a conclusion can be regarded as definitive.

To do away with the hemagglutinating activity of toxoid, higher temperatures, greater concentrations of formalin, and more extended toxoiding periods can be employed in manufacture of the toxoid. However, these modifications of manufacture are not without a drawback since they would inevitably tend to reduce the immunogenic potency of toxoid. Immunogenicity can be preserved in toxoid formation by reaction with formaldehyde only up to a point. Extension of the action of toxoiding agents results in an antigenically inferior product. The best practice is to terminate toxoiding processes just beyond the point of complete detoxification.

Even if it could be conclusively shown that hemagglutinin is harmless from a physiological standpoint, and even if the hemagglutinin could be inactivated, its presence in toxoid in active or inactive form could not be accepted with equanimity. The ideal is to strive for a preparation that places no unnecessary burdens on the body and does not require it to dispose of foreign protein. It will, therefore, be necessary in the future to consider ways to remove the hemagglutinin after toxoiding or to prepare toxoids from purified toxin free of hemagglutinin. For either of these purposes human type O red blood cells can be recommended as adsorbents for the removal of hemagglutinin. Until possibly more satisfactory methods of freeing toxoid from hemagglutinin can be developed, such cells can be employed with the satisfying knowledge that any contaminating substance that these erythrocytes add to toxoid will be substances natural to the chemistry of human blood.

Conclusion

In a survey of the work done on the subject of botulism, one is struck by the fact that efforts have been largely concentrated on studies of the type A toxin. This probably arises from two circumstances, the predominance of this type as a cause of botulism in human beings, particularly in North America, and the existence in culture collections of a number of strains of type A bacilli which produce large quantities of toxin in laboratory media (on the order of a million lethal doses in mice per milliliter). A task for the future is to bring the level of information about the other types to that of information available on type A toxin. Yet, to gain equal knowledge about the different types of toxin is only one goal.

In truth, the research of the past has raised scientifically important and challenging questions which are unanswered. The significant question from the bacteriological standpoint is that of the role of the toxin in the economy of the producing organisms. From the physiological standpoint, the important questions are these: How do the toxins escape from the alimentary tract and what is the exact nature of the mechanism of synaptic poisoning? With respect to chemistry, the problem is to discover what it is about the molecular structure of these most potent of poisons that accounts for their specificity and extraordinary toxicity. The search for answers to each of these questions promises to provide new insights into basic problems in each of these three fields of knowledge. The quest for new information offers the further possibility of discovering uses for the botulinal toxins as agents in neurophysiological research—an exciting prospect, since biological knowledge always leaps ahead when a new tool is available.

The most pressing task is that of isolating the toxins from all identifiable extraneous protein. This must be done to make it worth while to undertake investigations of the chemical structures of the toxins by means of the new and elegant methods which, in recent times, have become available to the protein chemist.

The lack of success that has so far attended efforts to isolate truly pure toxins stems from an ignorance that does not appear to have been dissipated at a rate commensurate with the really remarkable progress made in our knowledge of proteins and in methodologies for isolating proteins. The history of research in botulism is a history of efforts of fluctuating intensity, correlated with periods of excited interest in the solution of practical problems. The first period of great activity was coincident with the realization that the canning industry must solve the problem of packaging foods without the danger of harboring living *Clostridium botulinum* and its toxin. This was the period in which the increase in our knowledge of the characteristics of the organism of botulism was most rapid and in which the existence of toxin types was discovered. Practical canning methods for killing the spores of *C. botulinum* were invented and adopted. Botulism ceased to be a threat to the economics of the food-packaging industry and to the health of individuals in an urbanized society. Interest in botulism then waned until World War II, when the threat of the use of botulinal toxins as chemical warfare agents revived interest. A new surge of effort was generated, rapid progress was made toward purification of the toxins; effective, though imperfect, toxoids for use in human beings were developed, and practical large-scale manu-

facturing methods were designed. And now we are once again in a period of declining interest. It is to be hoped that no new threat will be needed to generate a renewed wave of investigations. Let us hope that the intellectual challenge alone will bring support for activities that will lead to increased understanding of the botulinal toxins (*30*).

References and Notes

1. J. B. Davis, L. H. Mattman, M. Wiley, *J. Am. Med. Assoc.* 146, 646 (1951); C. R. Hampson, *J. Bacteriol.* 61, 647 (1951); C. G. Thomas and M. F. Kelcher, *A.M.A. Arch. Pathol.* 51, 623 (1951).
2. C. E. Dolman, *Japan. J. Med. Sci. & Biol.* 10, 383 (1957).
3. C. Lamanna, *J. Bacteriol.* 44, 29 (1942).
4. A. Abrams, G. Kegeles, G. A. Hottle, *J. Biol. Chem.* 164, 63 (1946); C. Lamanna, O. E. McElroy, H. W. Eklund, *Science* 103, 613 (1946); C. Lamanna, H. W. Eklund, O. E. McElroy, *J. Bacteriol.* 52, 1 (1946); C. Lamanna, H. N. Glassman, *ibid.* 54, 575 (1947); J. T. Duff, G. G. Wright, J. Klerer, D. E. Moore, R. H. Bibler, *ibid.* 73, 42 (1957); J. T. Duff, J. Klerer, R. H. Bibler, D. E. Moore, C. Gottfried, G. E. Wright, *ibid.* 73, 597 (1957); M. Gordon, M. A. Fiock, A. Yarinsky, J. T. Duff, *ibid.* 74, 533 (1957); M. A. Cardella, J. T. Duff, C. Gottfried, J. B. Begel, *ibid.* 75, 360 (1958); L. M. Wetzel, M. Sterne, A. Polson, *Nature* 166, 739 (1950).
5. F. W. Putnam, C. Lamanna, D. G. Sharp, *J. Biol. Chem.* 176, 401 (1948).
6. C. Lamanna and B. W. Doak, *J. Immunol.* 59, 231 (1948).
7. C. Lamanna, *Proc. Soc. Exptl. Biol. Med.* 69, 332 (1948); C. Lamanna, J. P. Lowenthal, *J. Bacteriol.* 61, 751 (1951); J. P. Lowenthal and C. Lamanna, *Am. J. Hyg.* 54, 342 (1951); ———, *ibid.* 57, 46 (1953).
8. E. A Meyer and C. Lamanna, *J. Bacteriol.* in press.
9. J. Wagman and J. B. Bateman, *Arch. Biochem. Biophys.* 31, 424 (1951); J. Wagman, *ibid.* 50, 104 (1954).
10. T. E. Cartwright and M. A. Lauffer, *Proc. Soc. Exptl. Biol. Med.* 98, 327 (1958).
11. H. J. Buehler, E. J. Schantz, C. Lamanna, *J. Biol. Chem.* 169, 295 (1947).
12. E. J. Schantz and L. Spero, *J. Am. Chem. Soc.* 79, 1623 (1957); L. Spero and E. J. Schantz, *ibid.* 79, 1625 (1957); L. Weil, T. S. Seibles, L. Spero, E. J. Schantz, *Arch. Biochem. Biophys.* 68, 308 (1957); L. Spero, *ibid.* 73, 484 (1958).
13. C. Lamanna, W. I. Jensen, I. D. Bross, *Am. J. Hyg.* 62, 21 (1955).
14. K. F. Meyer and B. Eddie, *Z. Hyg.* 133, 255 (1951).
15. J. W. Stevenson, V. A. Helson, G. B. Reed, *Can. J. Research* 25E, 14 (1957).
16. I. W. Coleman, *Can. J. Biochem. and Physiol.* 32, 27 (1954).
17. G. Halliwell, *Biochem. J.* 58, 4 (1954).
18. G. Sakaguchi and Y. Tohyama, *Japan. J. Med. Sci. & Biol.* 8, 255 (1955); J. T. Duff, G. G. Wright, A. Yarinsky, *J. Bacteriol.* 72, 455 (1956).
19. A. J. May and B. C. Whaler, *Brit. J. Exptl. Pathol.* 39, 307 (1958).
20. J. B. Gunnison and K. F. Meyer, *Proc. Soc. Exptl. Biol. Med.* 26, 89 (1928).
21. T. Moll and C. A. Brandly, *Am. J. Vet. Research* 12, 355 (1951).
22. J. W. Stevenson and G. T. Girvin, *Atti congr. intern. microbiol. 6th Congr. Rome* (1953), p. 133.
23. E. R. Kalmbach, *J. Am. Vet. Med. Assoc.* 94, 187 (1939).
24. N. Ambache, *J. Physiol.* 108, 127 (1949); ———, *ibid.* 113, 1 (1951); C. Kupfer, *Proc. Soc. Exptl. Biol. Med.* 99, 474 (1958).
25. E. C. Dickson, R. Shevky, *J. Exptl. Med.* 38, 327 (1923); A. C. Guyton and M. A. MacDonald, *A.M.A. Arch. Neurol. Psychiat.* 57, 578 (1947); A. S. V. Burgen, F. Dickens, L. J. Zatman, *J. Physiol.* 109, 10 (1949); V. B. Brooks, *J. Physiol.* 123, 501 (1954); J. W. Stevenson, *Am. J. Med. Sci.* 235, 317 (1958).
26. K. F. Meyer, *Bull. World Health Organization* 15, 281 (1956).
27. G. A. Hottle and A. Abrams, *J. Immunol.* 55, 183 (1947); G. A. Hottle, C. Nigg, J. A. Lichty, *ibid.* 55, 255 (1947); A. R. Prévot and E. R. Brygoo, *Ann. inst. Pasteur* 79, 1 (1950); M. Sterne and L. M. Wentzel, *J. Immunol.* 65, 175 (1950); G. S. Appleton and P. G. White, *Am. J. Vet. Research* 20, 170 (1959).
28. M. A. Cardella, M. A. Fiock, G. G. Wright, *Bacteriol. Proc.* (*Soc. Am. Bacteriologists*) 58, 78 (1958).
29. R. C. Millonig and C. Lamanna, unpublished data.

30. This work was supported by the Office of Naval Research under a contract with the University of California. Opinions contained in this report are not to be construed as reflecting the views of the Naval Service.

Self-Absorption Correction for Carbon-14

A new treatment yields a correction factor that is linearly related to the thickness of the sample.

Richard W. Hendler

Carbon-14 is undoubtedly the most widely used isotope in biochemical research. However, because of its weak emission, beta particles of maximum energy 0.156 Mev, there is an appreciable absorption and scattering of radioactivity within the sample itself, as a function of the weight of the sample. It is of the utmost importance to be able to correct accurately for the loss of observable radioactivity due to self-absorption (*1*). In the past the problem has been dealt with by (i) counting at "infinite thinness"; (ii) counting at constant thickness; (iii) counting at "infinite thickness"; (iv) correcting by use of an empirically determined curve; (v) or correcting by use of a theoretical equation with an empirically determined constant.

The thinner the sample, the less the self-absorption. Therefore, if all samples are counted at extremely low weights (infinite thinness), the correction will be negligible. This requires always having material of sufficiently high specific radioactivity to permit accurate determinations on very small quantities. The criterion is frequently difficult to meet in biochemical work.

If the samples counted are always of the same thickness, the correction factor will always be the same, and the results will all be comparable. It is very tedious to weigh all samples to exactly the same weight and this method is rarely used.

The observable count rate of a material of given specific activity will increase as the thickness of the sample is increased. The lowest layer contributes less radioactivity to the total observed, as more material is added, since its radiation is progressively absorbed. There is a weight of material over a given area which will be sufficient to absorb virtually all of the radiation from this lowest layer. Adding more radioactive material after this does not increase the total observed radioactivity, since the effective thickness of radioactive material contributing to the total observable radiation is essentially not altered. This is known as infinite or saturation thickness, and the maximum count rate observed from a sample whose thickness is greater than the saturation thickness is directly proportional to the specific activity of the material. This technique is quite reliable but requires relatively large amounts of material and therefore is not always convenient in biological systems.

The most general technique involves the use of a correction factor so that the

The author is on the staff of the Laboratory of Cellular Physiology and Metabolism, National Heart Institute, National Institutes of Health, Bethesda, Md.