# THE NEUROTOXINS OF CLOSTRIDIUM BOTULINUM AND CLOSTRIDIUM TETANI

G. PAYLING WRIGHT

*Department of Pathology, Guy's Hospital Medical School, London, S.E. 1, England*

TABLE OF CONTENTS

I. INTRODUCTION ..... 413
II. BOTULISM ..... 414
General features ..... 414
Immunological types of botulinum toxin ..... 416
Pathogenicity of different types of botulinum toxin for different animal species ..... 417
Resistance of botulinum toxin to heat and other agents ..... 420
Purification of botulinum toxins ..... 421
Potentiation of botulinum toxin by serum and other materials ..... 423
Other "toxins" in *Cl. botulinum* culture filtrates ..... 424
Site of action of botulinum toxin ..... 424
Action of botulinum toxin on enzyme systems ..... 431
Lethality of botulinum toxin for poikilothermic animals ..... 431
Action of anticholinesterase drugs in botulism ..... 432
Fixation and duration of action of botulinum toxin ..... 433
III. TETANUS ..... 434
General features ..... 434
Purification of tetanus toxin ..... 435
Difficulties in the determination of the minimum lethal dose of tetanus toxin ..... 436
Potentiation of the neurotoxins of botulism and tetanus ..... 437
Pathogenicity of tetanus toxin for various animal species ..... 438
Effect of the route of injection on the lethality of tetanus toxin ..... 439
Site of action of tetanus toxin ..... 440
Spread of tetanus toxin from a depot site in the tissues ..... 446
Mode of action of tetanus toxin ..... 453
Tetanus in poikilothermic animals ..... 454
Protection against tetanus toxin by prior injections of tetanus toxoid ..... 456

## I. INTRODUCTION

In his book entitled 'Experimental Science', Claude Bernard (30) began his account of the action of curare with the following remarks. 'Poisons can be employed as means for the destruction of life or as agents for the treatment of the sick, but in addition to these two well recognised uses there is a third of particular interest to the physiologist. For him, the poison becomes an instrument which dissociates and analyses the most delicate phenomena of living structures, and by attending carefully to their mechanism in causing death, he can learn indirectly much about the physiological processes of life. Such is the way in which I have long regarded the actions of toxic substances'. With most of the bacterial toxins there has yet been scarcely any analysis of their mode of action on sensitive tissue elements or of the chemical lesions that they produce, so that for the

most part they are of little assistance to the pharmacologist for the purposes envisaged by Claude Bernard. There are two toxins, however, those formed by *Clostridium botulinum* and *Clostridium tetani*, which are of much greater interest on account of their many unique features. They operate lethally in such minute dosages, with such specific effects and on such wide ranges of animal species that they have long attracted the close and detailed attention of bacterial toxicologists. As a result, so much has become known about their dispersal in the body and their sites of action on susceptible structures, that they are beginning to find places amongst the important group of drugs that can be used to throw light on certain neurophysiological processes. Botulinum toxin with its potent destructive action on peripheral cholinergic mechanisms is of value both in mapping the distribution of this system of nerves in the body and for their selective inactivation in organs and tissues in which their elimination by surgical measures is not feasible. Tetanus toxin, with its apparently similar affinity for cholinergic mechanisms, but those in the central rather than in the peripheral nervous system, may well serve a valuable purpose in the exploration of the neurological mechanisms and transmitter substances involved in excitation and inhibition in the cerebrospinal axis. The present general review of these two neurotoxins has been prepared with the possibility in mind that these exceedingly powerful and now highly purified agents may prove of value in physiological and pharmacological research along the lines to which Claude Bernard directed attention a century ago.

## II. BOTULISM

*General features.* Through the pioneer work of the Belgian bacteriologist van Ermengem between 1895 and 1897 the serious and often fatal form of food poisoning now termed 'botulism' (though formerly often met with under the names 'allantiasis', 'ichthyosism' or 'Wurstvergiftung') is now known to result from the ingestion of a toxin produced by one or other of the five recognised variants of the bacterium *Clostridium botulinum*. From the epidemiological features of early outbreaks, descriptions of which are numerous in German medical literature in the 18th and 19th centuries, the intoxication was originally attributed to the consumption of sausages, preserved fish and other similarly prepared foods of animal origin which had become contaminated with some poison that possessed very distinctive pharmacological properties. (The extensive literature on botulism and the many theories on its etiology have been fully reviewed by van Ermengem (265), Dickson (84) and Meyer (179).) While there is no doubt that such types of food were correctly incriminated as the vehicle for the toxin in almost all these continental epidemics, later outbreaks, especially those in the United States, have much more often followed the consumption of home-preserved vegetables, amongst which string-beans appear to be involved with noteworthy frequency.

It is of much epidemiological significance that a large number of ecological surveys on the natural distribution of *Cl. botulinum* have disclosed that this family of anaerobic, spore-bearing, bacteria are primarily inhabitants of soil and

of stagnant water in which vegetation is decaying. It is noteworthy, too, that in contrast with the other important pathogenic clostridia, *Cl. botulinum* is not a common inhabitant of the intestines of domestic animals or of farmyard manure (89). In a series of papers, Meyer and Dubovsky (180, 181, 182, 183) have recorded the frequent occurrence of botulinum spores in samples of soil taken from very varied kinds of cultivated and uncultivated land in North America, Western Europe and China. A roughly similar prevalence of the organism was found in the soils of central New York State: thirty three positive cultures, mostly of Type A strains, were obtained from 283 samples examined (196). In a survey made in Scotland soon after the Loch Maree outbreak in 1922, toxigenic strains were found in four of 100 samples of soil tested (159); more recently, five similar strains were obtained from 106 samples collected from various southern, mainly agricultural, counties in England (123). It seems worthy of note that in spite of their widespread distribution in soils, botulinum spores—in contrast to those of tetanus and gas gangrene—fail to germinate readily in the tissues (140) and have only rarely been recorded as a complicating infection of wounds (77, 124, 126, 213, 252).

Although botulism was first observed and recorded for man, it became recognised early in the present century that comparable neurological disorders are encountered from time to time as an epizootic in several species of domestic and many species of wild animal. This aspect of botulism has recently been fully reviewed by Verge (267). The distinctive paralytic syndrome, with sudden onset, and high mortality, together with its occurrence in association with typical outbreaks in the families of the owners of the animals, has suggested a more than chance connection between the clinical forms seen in human and veterinary medicine. This suspected kinship was soon firmly established when toxic filtrates from the cultures of the recovered clostridia were prepared and their behaviour and mutual relationships were explored in toxicological and immunological experiments. Amongst the more important of these naturally-occurring intoxications in animals are 'lamziekte' amongst cattle in South Africa (251), and horses, cattle and sheep in Western Australia (29), a form of 'meningo-encephalitis' of horses in several parts of France (136, 158, 211), 'limber neck' in fowls and ducks in many parts of the world (114), and a paralytic syndrome amongst wild ducks on reservations in the western United States (216, 217). In cattle and other herbivora in South Africa and Australia, the intoxication arises from the habit of sarcophagia and osteophagia of carrion which animals grazing on the veldt country develop to satisfy a craving for the phosphorus which is deficient in their ordinary pasturage (251). In France, it seems likely that the horses contract botulism from hay and silage which has become contaminated with the remains of small dead rodents and birds (267). Farmyard birds may become affected through eating the larvae of the 'green-bottle' and other blowflies which are often present on contaminated carcases (27, 28). Wild ducks become intoxicated through the consumption of marsh weeds that are growing in places and under conditions in which oxygen-consuming micro-organisms, notably *Pseudomonas aeruginosa*, flourish and create those anaerobic conditions which make possible

the profuse proliferation of the clostridium and the formation of large quantities of its toxin (217).

Although the syndrome of botulism is highly characteristic and has been frequently described before, the modern idea of the disease as a form of bacterial intoxication was first conceived by van Ermengem (263, 264) in 1897, when he made a very detailed investigation of the Ellezelles outbreak near Ghent two years previously and not only isolated the clostridium for the first time, but also demonstrated its ability to liberate one of the most powerful exotoxins known. Later, Dickson (84), in California, re-examined the whole question from its clinical and experimental aspects and clarified in two admirable pharmacological studies (85, 86) the main features of the mode of action of the toxin. The contributions of these two authors, together with those of Meyer (179), have provided the foundations upon which all subsequent knowledge has been built.

In typical epidemics, a high proportion of the persons who consume the spoiled food develop clinical botulism. About 18 to 24 hours, sometimes longer, after the meal, the affected persons develop a syndrome which is usually ushered in with muscular weakness and ocular disturbances, of which diplopia, lack of power of accommodation and loss of the light reflex are conspicuous features. Troubles with articulation and swallowing soon follow, brought about partly by weakness and incoordination of the pharyngeal muscles (186, 188) and partly by cessation of salivation. Gradually, all the voluntary muscles weaken, and death generally takes place as the result of paralysis of the muscles of respiration. The heart often continues to beat for some time after breathing has ceased. The symptoms and signs of intoxication in several typical small outbreaks have been described by Dickson (84) and by McClasky (169). The case-fatality rates vary widely in different epidemics and localities: in most of those in the United States, in which home-preserved vegetables have been the chief source of the toxin, it has often risen to more than fifty per cent (184), but in the many small, but authenticated, outbreaks that occurred in France during the recent war, where ham and other preserved meats were almost exclusively the vehicle (154), it was much lower. Whether this was due to the greater prevalence of Type B intoxications, or to the fact that the quantities of meat consumed at a meal are usually less than those of vegetables, is not known, but the great difference between the American and the French case-fatality rates is noteworthy. In survivors of epidemics, the paralyses and other clinical features gradually disappear in the course of a few weeks, a point to be noted for the treatment of the disease, because there are now well-developed techniques for continuous artificial respiration over long periods with mechanical insufflators (155, 189).

*Immunological types of botulinum toxin.* Early in the experimental study of botulism it was found that the typical syndrome of the intoxication could be produced by the injection of culture filtrates which, though identical in the way they behaved as poisons, could be separated and distinguished immunologically by the use of specific antiserums (53, 162). On the basis of cross-neutralization tests with antitoxins, five toxins, now termed Types A to E, have been identified, and the variant strains of the Clostridium from which they are obtainable have

been correspondingly named. Though the distinctions between these five toxins have now been established on a fairly firm foundation through the interchange of cultures and other materials between investigators in different parts of the world, the taxonomy of the variant bacteria still needs further clarification; the question of their terminology has been the subject of an interim report to the International Committee on Bacterial Nomenclature (210).

Although the typing of the five botulinum toxins is of great importance as regards both immunological differentiation and relative pathogenicities for different species of animal, there are good reasons for believing that all five produce their toxicological effects through the same kind of injury in the tissues. Further to test this view, Prévot and Brygoo (212) carried out an ingenious series of experiments in which they mixed appropriate fractional doses of two, three or more of the different Types of toxin in such a way that they summated to a single lethal dose. In general, they found that the mice which had received these fractional mixtures died from botulism much as did the control mice which had received one lethal dose of a single Type of toxin: from these results, they concluded that all five Types exhibited mutual summation but not reinforcement of action.

*Pathogenicity of different types of botulinum toxin for different animal species.* Taken collectively, the botulinum toxins are highly pathogenic on parenteral injection for a wide range of both warm and cold blooded animals, in all of which the syndrome of the intoxication follows one common pattern of paralysis. When the Types of toxin are examined individually, however, it becomes apparent that their relative pathogenicities for particular species vary widely. The literature on botulism contains many scattered observations of this comparative kind: while they are sufficient to provide a general impression of the relative susceptibilities of the different species, they are inadequate for any close numerical assessment that may be needed for the purpose of calculating particular lethal doses. Further, many of the earlier observations should be treated with reserve, because changes and amplifications in the nomenclature of the toxin Types have rendered their identification uncertain.

Recently, Prévot and Brygoo (212) have repaired some of these deficiencies by making comparative estimates of the minimum lethal doses of their preparations of Types C, D and E toxin on a number of common laboratory animals. Table 1, which has been taken mainly from their results, but extended by some figures obtained from other sources, gives representative numerical values for the minimum lethal doses on parenteral injection (expressed for all species as per unit of body weight) in terms of those found for mice. Some estimates are also available for the lethal doses for horses (which are highly susceptible animals) of Types A and B (158), for Type B (271) and for Type D (136).

It cannot be emphasized too strongly that such estimates of the relative minimum lethal doses for these species are in the highest degree approximate. Nevertheless, it seems safe to infer from them that mice, guinea-pigs, rabbits and monkeys are notably susceptible to all Types of toxin (except perhaps Type D for monkeys), while cats, dogs, fowls and pigeons are more refractory. It is not

TABLE 1

*Comparative lethal doses (per unit of body weight) of different types of Clostridium botulinum toxin for some of the common laboratory animals*

| Toxin Type | Source | Minimum lethal dose per unit weight of animal | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Mouse | Rat | Guinea-pig | Rabbit | Dog | Cat | Monkey | Fowl | Pigeon |
| A | (a) | 1 | | ½ | ⅓ | | | | 15 | |
| | (b) | 1 | 2½ | | | | | | | |
| | (c) | 1 | | | 1 | | | | | |
| B | (b) | 1 | 1000 | | | | | | | |
| | (d) | 1 | | ⅕ | | | | | | |
| | (e) | 1 | | ⅓ | | | | | | |
| C | (f) | 1 | 6 | 1 | ⅛ | 1000 | 800 | ⅓ | 2000 | 20 |
| D | (f) | 1 | 320 | ¼ | ⅕ | 100,000 | 15,000 | 100 | 100,000 | 2000 |
| E | (f) | 1 | 40 | ½ | 1 | 100 | 400 | 1 | 25 | 25 |

Sources references:

(a) Meyer (179).
(b) Burgen, Dickens and Zatman (52).
(c) Davies, Morgan, Wright and Payling Wright (74).
(d) Walker (271).
(e) Lamanna and Glassman (149).
(f) Prévot and Brygoo (212).

without some interest that this division for species resistance is closely similar to that recorded by van Ermengem (263, 264, 265) as a result of his injecting and feeding animals with extracts of the ham that had been implicated in his famous outbreak at Ellezelles in 1895. But it is abundantly clear that any investigator who contemplates the use of these toxins in pharmacological studies can only use the above figures as very rough guides, and that he must determine for himself the relative toxicities for mice and for any other species of animal that he is using, for the particular preparation of toxin and route of administration that he is employing.

Since naturally occurring botulism, both in man and animals, is primarily a food poisoning, a number of investigators have sought to compare the lethal dosages of toxin preparations when they are given parenterally and by mouth. Again, the figures obtained are very variable, so that taken individually they are of little value. Gunnison, first with Meyer (120) and later with Coleman (119), compared the two for Types B, C and D toxin preparations on mice, guinea-pigs and rabbits, and found that whereas for Type B toxin the two did not differ greatly, for Types C and D toxin, the oral dose was from several hundred to several thousand times larger than that needed for injection. Macacus monkeys resemble the smaller animals in their susceptibility to Type B toxin when it is given by mouth, while goats are correspondingly vulnerable to Type D toxin.

Differences of a similar kind have been found for dogs by Graham and Erikson (113) for a Type A toxin: a preparation which was lethal when injected subcutaneously in a dose of 0.1 ml. failed to kill when given orally in a dose of 100 ml. unless the recipient animal had been starved for two days beforehand. Coleman (58), who gave an unpurified, but nonetheless highly potent, Type A toxin to rats, has recorded perhaps the highest ratio of oral to parenteral lethal doses, for it attained the order of a million. With horses, Legroux and his colleagues (158) found that for both Types A and B toxins, the lethal dose by mouth was about a hundredfold that needed when given subcutaneously. Earlier, Legroux *et al.* (157), had recorded that the lethal dose of a Type B toxin for rabbits was about a thousand times greater when given by stomach tube than when injected: the great difference of this ratio from that found earlier by Gunnison and Meyer merely exemplifies the great variability inherent in experiments of this kind and casts no reflection upon the care exercised by the investigators.

Such ratios as those referred to above illustrate the general finding that though many preparations of the botulinum toxins are highly potent when they are taken in food, they are usually less effective orally than when inoculated directly into the tissues. To what extent the lessened lethality of the toxin when given by mouth results from its partial destruction by digestive juices and other agents within the contents of the alimentary canal and to what extent from the incomplete absorption of the toxic protein molecule through the intestinal mucosa is unknown. The ratio between destruction and unabsorbability almost certainly varies widely from one set of experimental conditions to another.

Exposure of toxic filtrates from cultures of *Cl. botulinum* to conditions simulating those found in the gastric contents appears to have little adverse effect on their potency. Bronfenbrenner and Schlesinger (42) added varying quantities of hydrochloric acid and sodium hydroxide to broth containing Type A toxin and found that between pH 2.3 and pH 5.0, there was little or no change in toxicity even after 24 hours at 37°C.; only when the pH of the solutions was increased did their potencies fall. Nor did the addition of pepsin or trypsin diminish the toxicities of the solutions—results that are in conformity with some of the earlier observations on the destructibility of the toxin. Different results were reached, however, by Coleman (58) when recently he repeated these observations on a highly purified preparation of Type A toxin—a crystalline product that was dissolved in a buffered solution that contained no other organic material. At the end of 8 hours, the solution maintained at pH 1.5 and 37°C. had lost little toxicity while that at pH 6.5 had fallen to roughly one-fifth, but after 72 hours, both solutions had declined to about one-tenth of their initial lethality. He found further that the addition of pepsin and of trypsin and chemotrypsin at pH values of 1.4 and of 6.8 respectively, completely detoxified the solutions by the end of 72 hours. An obvious difference between Bronfenbrenner and Schlesinger's and Coleman's techniques, which might account for the discrepancies in their results, is that the former carried out their digestion experiments in a broth that contained much additional nitrogenous material, while the latter used highly purified preparations of the toxin. The presence of proteins and other organic

materials may have protected the toxin from digestion by the enzyme, a possibility pointed out by Littauer (165) in a discussion of some similar experiments of his own. Clearly, Bronfenbrenner and Schlesinger's experiments reproduce much more closely than those of Coleman the conditions in the alimentary tract under which botulism occurs naturally.

It is evident from the innumerable experiments in which general botulism has been produced by the oral administration of the toxin, that some must gain access to the circulation to bring about an intoxication of such a typically widespread nature. To test this inference, certain observations have been made on the toxicity for mice or other small susceptible animals of samples of blood removed from a large animal during the period of onset of botulism. Jaquet and Prévot (136) gave 100 mg. of a Type D toxin (2000 subcutaneous mouse lethal doses per mg.) in a handful of fodder to a horse that weighed about 250 kg., and subsequently took samples of its blood for assay of their toxicity for mice. Seven hours after the toxin was given, and before the horse showed any signs of intoxication, an appreciable amount of the toxin had entered the circulation, for mice injected with blood taken from its jugular vein died in 60 hours with typical botulism. This detection of toxin in the blood during the prodromal stages of intoxication has a parallel in tetanus, for several instances have been recorded of serum taken from ostensibly healthy horses in diphtheria antitoxin serum stables having caused tetanus subsequently in persons into whom it had been inoculated for the purpose of treatment (see pages 435 and 439).

Although there can be no doubt that botulinum toxin can be readily absorbed through the mucous membrane of the alimentary tract, there is very little evidence to suggest in what portion of the gut the entry takes place. Dack and Gibbard (65) and Dack and Wood (67) introduced Type A toxin into the upper ends of loops of dog's and rabbit's intestines which they perfused with oxygenated blood: they found little indication of absorption. Nor could Haerem, Dack and Dragstedt (122) or Coleman (58) demonstrate the absorption of Type A toxin from the ileum of dogs. Dack and Hoskins (66) found that macacus monkeys were very susceptible to Type A toxin when it was given by mouth, but that similar doses of the toxin failed to produce botulism when it was introduced into surgically isolated loops of their colon. What slender evidence there is available thus suggests that most of the absorption of these toxins must take place in the stomach or in the upper portions of the small intestines.

*Resistance of botulinum toxin to heat and other agents.* The desire to protect the consumers of commercially preserved food from botulism has led to much work on the resistance of both the spores and the toxins of *Cl. botulinum* to heat and other agents. Many of these studies have been undertaken under the auspices of the National Canners' Association in the United States. In 1921, by which date Types A and B toxins had been distinguished, Orr (194) examined the thermal destruction of ten toxin preparations obtained from ten strains of the bacterium: nine of them were inactivated at 80°C. in less than five minutes. The tenth was more resistant to heat, and in the light of present knowledge it may well have been a Type C or D toxin. Bengtson (28) was the first to study the thermal

stability of Type C toxin and to record its greater resistance to heat. Recently, Prévot and Brygoo (212) have re-examined the destructive effects of heat on specimens of all five Types of toxin, and have found that Types A, B and E are relatively thermolabile, in that they are destroyed by exposure to 70°C. for a few minutes, while Types C and D are considerably more resistant. Such estimates of thermal destruction can only be very general, however, for they are dependent upon the pH and other characteristics of the medium in which the toxin is present. For instance, as Schoenholz and Meyer (235) have pointed out, the same Type of toxin often proves more resistant to heat when present in vegetable juices than when in broth; the greater acidity of the former apparently provides some degree of protection.

All the Types of toxin are more stable when dissolved in media with an acid reaction. Even at physiological neutrality, they soon lose much of their potency—especially Types A and B—and when made slightly alkaline they quickly become quite inactive. Nor can this loss of potency be reversed by any subsequent re-acidification of the solution.

The exceptionally high lethality of botulinum toxins has attracted some attention to the possibility of using them as agents in biological warfare (230). They could apparently be used in two ways: firstly, as an aerosol which would lead in unmasked persons to the contamination of the respiratory and alimentary tracts; and secondly, as an agent for the pollution of food and water supplies. It is reassuring to find, therefore, that such Types of the toxin as are known to be pathogenic for man can readily be destroyed under conditions which could be brought into operation even in seriously war-stricken communities. Although the toxins remain potent for many days in cold clean water (49), they are destroyed in a few minutes by exposure to permanganate, chlorine and other oxidizing agents in concentrations now commonly employed for the chemical sterilization of water supplies (50, 138). Moreover, if such polluted water is made slightly alkaline, it can be readily detoxicated by warming to 70°C. for a few minutes.

*Purification of botulinum toxins.* When the toxic cultures obtained by van Ermengem from his Ellezelles strain of *Cl. botulinum* came to be studied experimentally for their effects on animals, it quickly became apparent that a bacterial metabolite was present that was quite unmatched in its lethal power by any other poisonous substance known. Even with the crude filtrates which he prepared, he was able to kill rabbits with subcutaneous injections of a fraction of a cubic millimetre. For many years no serious efforts were made to effect the separation of the toxic components of botulinum culture filtrates by conventional biochemical methods. The progress made in the fractionation of mixed proteins in solution, however, has recently encouraged various investigators to attempt the isolation of these and other potent toxins. At Camp Detrick in the United States, two groups of investigators have had signal success in purifying the neurotoxin present in Type A cultures, and further progress has been made there and elsewhere in concentrating the toxins in Types B and D filtrates.

*Type A toxin.* To obtain the large yields of toxin that they needed for its

chemical characterization, Lamanna and his colleagues cultivated a highly toxigenic strain of *Cl. botulinum* Type A on a simple medium containing casein hydrolysate and corn-steep liquor (148, 151). After sterilizing the cultures in an autoclave, they were able to recover a highly toxic component by the application of a succession of precipitations with HCl at pH 3.5 and re-solution in various saline buffers of specified composition—a technique modelled on that first used by Sommer (246). From the last of the concentrates thus obtained, they were able to crystallise a globulin which on parenteral injection into mice had the remarkably high toxicity of $220 \times 10^6$ $LD_{50}$ per mg. N (214). On hydrolysis, this protein yielded nineteen different amino acid residues, amongst which microbiological assays disclosed an unusually large proportion of aspartic acid, tyrosine and threonine (51). When examined physicochemically by electrophoresis, diffusion methods and sedimentation in the ultracentrifuge, the toxin behaved as a homogeneous protein whose apparent molecular weight was about 900,000 (215). Immunologically, also this crystalline protein appeared to be molecularly homogeneous, both it and its specific antitoxin were fully precipitated in the zone of equivalence (147).

The other Camp Detrick investigators, Abrams, Kegeles and Hottle (6), also used this highly toxigenic strain of *Cl. botulinum* and cultivated it on a similar casein digest-corn steep liquor medium. Their method of fractionation differed, however, in some respects from that of Lamanna *et al.* Starting with a sequence of precipitations with acid at pH 3.5 and re-solution in phosphate buffers, they completed their purification with partially saturated ammonium sulphate. Their crystalline final product had a rather higher toxicity for mice than that of the other investigators ($220 \times 10^6$ m.l.d. per mg. N). It too, behaved as a globulin in solution and likewise possessed an isoelectric point at pH 5.6. One point of difference, and that probably an immaterial one, was the rather higher estimate made by Kegeles (139) for the molecular weight of his purified toxin (1,130,000).

The values for the molecular weights for Type A toxin that were reached in both these studies were each of the order of one million, and are not easy to reconcile with the known absorption of the substance through the mucosa of the alimentary tract. It seems possible, however, that this difficulty may be resolved by some observations of Wagman and Bateman (268, 269), who found that when a solution of Type A toxin is brought to a pH of 7.5, a significant proportion of the large molecules, which are typical of the protein in acid solution, undergo fission into much smaller ones whose molecular weight they estimated to be about 70,000. This 'dissociated toxin' still possesses the same lethality for mice as the 'complex toxin' from which it is apparently derived, and may prove to be the form in which the toxin is absorbed in naturally occurring botulism. As will be seen below, this conception of polymerization of toxin molecules has also been hypothesized for the Type D variety.

*Type B toxin.* In their study on the toxic component of Type B cultures, Lamanna and Glassman (149) again separated the lethal toxin as an electrophoretically homogeneous protein. Immunologically, chemically and physically, however, it differed from the Type A toxin, though in the most concentrated

fraction they obtained it had a potency of $160 \times 10^6$ mouse $LD_{50}$ per mg. N—a value not much below that of their best Type A toxins. Perhaps the most noteworthy biochemical difference between these two Types of toxin was that the Type B one had a molecular weight of only about 60,000—a value not very different from that of the 'dissociated toxin' of Type A cultures referred to by Wagman and Bateman (269).

*Type C toxin.* No account has yet been published of any attempt to fractionate toxic filtrates from Type C cultures that can be compared with those undertaken for Types A and B. A potent toxin preparation has been made, however, by Sterne and Wentzel (248), who cultivated a toxigenic strain of this organism inside a cellophane sac immersed in a much larger volume of nutrient medium. By this device, they prevented the dispersal throughout the entire culture of the toxin which was formed by the multiplying bacteria and in this way they effected what was in essence a primary step in concentration. The greatest potency of any of their preparations, which they termed 'dialysate toxins', was about $3 \times 10^6$ mouse m.l.d. per mg. N.

*Type D toxin.* Using the same cellophane sac culture technique for *Cl. botulinum* Type D as they had used before for Type C, Sterne and Wentzel (248) produced a highly toxic concentrate which without further treatment contained $130 \times 10^6$ mouse m.l.d. per mg. N. Starting with this preparation and employing successive precipitations and re-solutions in ammonium sulphate solutions, they obtained an electrophoretically homogeneous material which from diffusion measurements appeared to have a molecular weight of about one million. When dissolved in a dilute gelatin phosphate buffer solution at pH 6.2, this material had the quite unprecedented toxicity of $4 \times 10^{12}$ mouse m.l.d. per mg. N (275). If the authors' estimate for the molecular weight is correct, it follows that only about a thousand molecules of this toxin are needed to kill a mouse—a level of toxicity that is about 20,000 times greater than that of the crystalline Type A toxin prepared by the two groups of investigators at Camp Detrick.

An attempt to concentrate the Type D toxin has also been made at the Pasteur Institute by Boroff, Raynaud and Prévot (34). Their most potent preparation, however, contained only about $14 \times 10^6$ mouse $LD_{50}$ (about $10^7$ mouse m.l.d.) per mg. N, an order of lethality quite different from that of Wentzel and his colleagues in South Africa, but not so very different from that obtained with the two American Type A toxins. The chief interest of this French study, however, lies less in the level of potency reached by their preparation than in the evidence that it provides for the existence of this toxin in two separable forms, one of which is possibly a polymer of the other. Their inference recalls the recent similar suggestion made by Wagman and Bateman (269) that purified Type A toxin undergoes dissociation when it is brought into a neutral solution.

*Potentiation of botulinum toxin by serum and other materials.* In several investigations of botulinum toxin an increase in the lethality of the preparation has been recorded after the addition of serum and other protein containing materials: horse serum (32, 41, 270), rabbit and sheep serum (137), and guinea-pig leu-

cocytes (57). Sommer and Sommer (245), however, appear to have been the only authors who have attempted to evaluate their findings quantitatively. Using a Type A toxin, they found that exposure to 2 per cent Witte's peptone at 37°C. for several hours could enhance its lethality two or three times, while similar treatment with horse serum might raise it rather more. But with all the agents they used, the period of potentiation was a short one—a few hours at most—and was quickly followed by a marked and progressive decline in the activity of the toxin. The questions raised by the potentiation of the neurotoxins will be discussed more fully below in conjunction with tetanus toxin, for which more information is available (see 437).

*Other 'toxins' in Cl. botulinum culture filtrates.* Although there is no evidence that they participate directly in the neurotoxic phenomena of botulism, attention may be drawn briefly to other biologically active agents present in many *Cl. botulinum* culture filtrates. During his studies on the properties of the highly concentrated toxin of *Cl. botulinum* Type A, Lamanna (146) found that his preparation could agglutinate the red cells of chickens, rabbits, guinea-pigs, sheep and man. Since then this characteristic has received further study (150, 168, 247), from which it has become apparent that the neurotoxic and the haemagglutinative properties of highly purified materials from Types A and D cultures are in many respects independent of one another and may be brought about either by different portions of the same molecule or by different constituent molecules.

A haemolysin for sheep red cells has been found by Guillaumie and Kréguer (116, 117) in culture filtrates from Types C and D organisms. This toxin appears to belong to the well-recognized group of oxygen-labile haemolysins of which streptolysin O, tetanolysin, *Cl. welchii* $\theta$ toxin and *Cl. histolyticum* toxin are the best known examples. Lecithinase activity, as manifested by the digestion of lecithovitellin, has been demonstrated in certain culture filtrates of Types A and B organisms (195) and of Types C and D (48, 117).

*Site of action of botulinum toxin.* The preponderantly neurological character of the symptoms and signs of botulism naturally directed pathologists to seek for evidence of injuries in the central nervous system. This early work has been reviewed by Dickson (84) and Meyer (179). Many of these studies were based on autopsy records of patients who had died in some epidemic; only later were they supplemented by observations on experimental botulism in animals. Much of the human material was only examined several hours after death and consequently at a time when there could no longer be any reasonable expectation of discovering any finer signs of injury to nerve cells. Moreover, the inevitable accompaniments of slow death from respiratory failure—pneumonia and progressive anoxaemia—added complications which inextricably confused the primary and secondary morphological manifestations of the intoxication. Even with animals that are killed in the terminal stages of botulism, such secondary changes have provided many unsuccessfully avoided pitfalls. Cowdry and Nicholson (63) were the first to appreciate the difficulties that confronted any conventional treatment of the problem and to recognise that the disorder was essentially a

'biochemical lesion', an exploration of whose sites and nature would require a more functional approach.

In 1923, three groups of investigators independently published experimental studies which radically altered the previously held conception of botulism by directing attention to various functional disturbances that could be demonstrated in the peripheral nervous system. Although the first of these, Schübel (236, 237), published a preliminary account of his work in 1921, his main study appeared two years later. In some respects, his contribution forms a connecting link between the former idea of botulism as a toxic injury to the central nervous system and the modern concept of the disease as an intoxication of the end-organs of certain peripheral nerves. Schübel carried out most of his work on frogs, but he also showed that on parenteral injection the toxin was lethal for species so widely spaced on the evolutionary scale as earthworms, snails, fish, tadpoles, frogs, birds and mammals. An interesting observation on the sequelae of intoxication in earthworms and snails was the exaggerated secretion of mucus from their cutis. He was chiefly concerned, however, with paralytic phenomena in the skeletal musculature in frogs and attempted to compare the mode of action of botulinum toxin with that of curare by employing Claude Bernard's well known experiment of protecting a limb against the action of a drug by temporary interruption of its circulation. With botulinum toxin, Schübel could demonstrate no such protection, a failure which it now seems safe to attribute to the very different time-scales for the actions of these two poisons. In consequence, he felt forced to infer that the intoxication was not wholly a peripheral one, but that it presented in addition a central component whose effects he believed that he could demonstrate in histological preparations of diseased spinal cords.

By far the most extensive and penetrating of these contemporary studies were those of Dickson and Shevky (85, 86), who carefully mapped the field of intoxication in both the viscera and the skeletal musculature and pointed out the similarities between the sites of action of botulinum toxin and those of acetylcholine, which had been described some years before by Dale (68). In retrospect, it seems regrettable that at the time they were described these toxicological observations should have attracted so little attention from pharmacologists. In many experiments on dogs, cats and rabbits, they showed that once botulism had become established, there developed a progressive impairment in the power: (I) of the vagus nerve for inducing cardiac inhibition; (II) of the chorda tympani for evoking salivary secretion; (III) of the nervus erigens for causing contraction of the bladder and erection of the penis; and (IV) of the oculomotor nerve for effecting constriction of the pupil. This colligation of paralytic manifestations led them to conclude that the toxin affects specifically those portions of the autonomic nervous system included by Gaskell (108) in his 'bulbosacral' and 'prosomatic' outflows, and that impulses conveyed along these fibres become blocked at some point at the periphery. On the other hand, they found no evidence of any comparable paralytic manifestations at the terminations of the fibres (mainly splanchnic) of the 'thoracicolumbar' outflow.

In their second paper, Dickson and Shevky recorded observations on the

course of the paralysis that developed in skeletal muscles as the intoxication advanced. It soon became evident that the progressive weakness, which is so typical a feature of naturally occurring botulism in both man and animals, is not dependent upon any diminution in the contractile power of the muscle fibres themselves, for they react as promptly and forcibly as normal muscles when electrical stimuli are applied to them directly. They also satisfied themselves that the progressive paralysis of the musculature was not due to any deterioration in the ability of the motor nerves to conduct impulses—an observation that was later confirmed by Bishop and Bronfenbrenner (31), Guyton and MacDonald (121) and Ambache (9). The most characteristic feature of the paralysis became apparent when they excited the motor nerve trunk supplying a limb at frequent intervals over a long period; in normal animals the threshold stimulus needed to sustain the maximal degree of contraction underwent little change over a period of several hours, whereas that required for the same purpose in the botulinum animal—though initially the same as that for the normal one—soon rose progressively. Moreover, the promptitude with which this fatigue set in became increasingly apparent as the intoxication advanced. From these observations, they again concluded that the weakness of the voluntary muscles in botulism is dependent upon the intoxication of some end organ and not upon any lesion in the central nervous system.

Edmunds and Long (92), the third of these groups of investigators, used both Types A and B toxin with results that were toxicologically indistinguishable upon frogs, guinea-pigs, rabbits, cats, dogs and fowls. Unlike Schübel, they could demonstrate no effect upon frogs, but in the other species they were able to show that in the voluntary muscles, transmission became impaired at the muscle end organ—an effect which they likened to that of curare. They also noted that in intoxicated fowls the paralysed muscles responded in the same way as normal ones to injections of nicotine; from this they inferred that the nerve ending and not the receptor substance was the element affected by the toxin. As a result of their experiments, Edmunds and Long concluded that the paralyses characteristic of botulism, including that of the vagus nerve in causing bradycardia, could all be accounted for on the supposition that the site of action of the toxin is peripheral rather than central.

Edmund's second paper with Keiper (91) appeared shortly after those of Dickson and Shevky, and while amplifying his earlier observations with Long, it was in the main a confirmation of some of the observations of these other investigators. With frogs, it proved possible, by raising their temperature to 20–22°C. and using a more potent toxin, to repeat Schübel's observation that in advanced botulism the skeletal muscles react normally to direct stimulation but fail to respond indirectly after excitation of their motor nerves. Further, like Dickson and Shevky, they produced paralysis of the chorda tympani, the vagus nerve and the oculomotor nerve in dogs, but failed to find any impairment of the sympathetic innervation of the eye. They recorded the observation, previously noted by Schübel, that with structures innervated by the parasympathetic nerves, the paralysis often appeared to be preceded by a short spell of overactivity.

During the decade before the last war, the importance of acetylcholine as a transmitter agent was becoming widely recognised, and the sites of its action in various neuro-effector systems had been determined by systematic exploration. The resulting clarification in the pharmacology of the autonomic nervous system culminated in the concept, first enunciated by Dale (69, 70), that certain nerves —to which he gave the name 'cholinergic'—operated through the release of acetylcholine at their terminations, while others—the 'adrenergic' nerves—acted through the liberation of adrenalin. This division of the nervous system on a functional chemical basis, rather than the earlier one derived from its anatomical arrangement, has strongly influenced all the subsequent studies on the site of action of botulinum toxin, and has served to emphasize the parallelism between the sites of action of this toxin and those of acetylcholine to which Dickson and Shevky first directed attention.

During the recent war, the possibilities offered by *Cl. botulinum* as an agent in bacterial warfare revived interest in its toxin and led to further published and unpublished studies on its mode of action especially at the myoneural junction in skeletal muscles. This paralysis had been regarded by earlier observers as similar to that which follows the injection of curare: among the first to draw attention to significant points of difference in the nature of the disturbance produced by the two agents were Guyton and MacDonald (121). In both guinea-pigs and rabbits in which local botulism had been produced by the injection of small doses of Type A toxin into the muscles of the lower hind leg, they could evoke an immediate contraction of the paralysed muscles by a close intra-arterial injection of acetylcholine—a response which cannot be brought about by similar injections of this substance in animals rendered paralytic with curare (45). Further, as Edmunds and Keiper had noted previously, the administration of physostigmine to botulinum intoxicated animals had little or none of the beneficial effect that follows its use in the paralysis of curare poisoning (38, 62). The distinction between the modes of action of the toxin and of curare has since been confirmed in other studies (8, 9, 52, 176), in which it was pointed out that the failure of transmission was compatible with some lesion at the myoneural junction which renders it incapable of releasing acetylcholine in quantities sufficient to evoke a contraction of the muscle fibre. Indeed, Guyton and MacDonald rather guardedly described certain morphological changes, disclosed by gold impregnation techniques and visible at moderate magnifications, in the motor end-plates in botulinum intoxicated muscles, but the need for caution in such histological studies of intoxicated myoneural junctions has been emphasized by Denz (80) and by Harris (128). The possibility that the release of acetylcholine at the junction might be impaired was examined by Burgen, Dickens and Zatman (52) on the excised rat's phrenic nerve diaphragm preparation, which can readily be paralysed in less than an hour by the addition of very large doses of Type A toxin to the bath. They found that when the enzymic destruction of the acetylcholine liberated into the fluid of the bath is prevented by the presence of eserine, comparisons of the amounts that can be recovered after identical periods of nerve stimulation were very much smaller with intoxicated than with control preparations.

A reduction in the amount of acetylcholine recoverable from an intoxicated diaphragm might conceivably be brought about in one of two ways. Firstly, the toxin may injure the terminal, largely unmyelinated, portion of the nerve fibril, and in this way interrupt the passage of the impulse to the acetylcholine-release region in apposition to the end-plates. Secondly, the nerve terminals themselves may be damaged in such a way as to lessen their capacity to liberate acetylcholine on the reception of an impulse. Burgen and his colleagues and Guyton and MacDonald are inclined to favour the former hypothesis, basing their belief largely on the observation that anticholinesterase drugs do little to mitigate the paralysis in botulism. For were the second hypothesis correct and the acetylcholine liberated at the intoxicated site merely decreased to some level below the threshold needed to excite the muscle fibre, it might reasonably be expected that the inhibition of the local cholinesterase would permit conduction to be restored in at least a proportion of the blocked junctions and a demonstrable improvement in muscular contraction to follow.

The application of methods for recording muscle end-plate potentials to the investigation of the site of failure of conduction in botulism has so far brought forth only conflicting evidence, though it would seem that eventually the solution of the problem of paralysis must be reached through the employment of such a technique. Stover, Fingerman and Forester (249) observed that in frogs when two impulses are propagated down a motor nerve trunk in rapid succession, two separate end-plate potentials can be recorded from an intoxicated muscle although only the second is followed by a contraction. Brooks (43) carried out a similar study on cats and guinea-pigs which were in various stages of paralysis following an injection of a large dose of botulinum toxin. He was unable to record any end-plate potential which he could attribute to the arrival of the first impulse, though the second impulse often reached the end-plate and gave rise to a muscular response. He concluded that the toxin injured the terminal exposed portions of the nerve filaments close to their site of final branching in such a manner as to lessen their ability to conduct impulses, and that when the first impulse failed to reach the neuromuscular junction, it might none the less facilitate the passage of the second one, provided that the interval between the two were of the order of 200 msec. or less. In short, he regarded the success of the double volley after a single one had failed, to the facilitation induced by the first impulse in the zone of impaired conduction rather than to the summation of two, individually subliminal, liberations of acetylcholine at an injured nerve ending—the suggestion offered by Stover and his colleagues. Brooks was strengthened in his belief that the acetylcholine release mechanism in such an intoxicated and paralysed muscle was not itself damaged, by the observation that when the latter was stimulated directly by current pulses passed through the bath fluid, as much acetylcholine was liberated as there was from a normal muscle which had been excited indirectly by way of its motor nerve. The differing conclusions reached in these two investigations may possibly be attributable, as Brooks has pointed out, to his observations having been made on mammals while those of Stover and his associates were made on

frogs. Until further electrophysiological studies of this kind have been made on intoxicated muscles, the manner in which botulinum toxin interferes with acetylcholine release at the neuromuscular junction after stimulation of the motor nerve will remain obscure.

Many of the symptoms and signs of naturally occurring botulism in man and animals are attributable, as Dickson and Shevky pointed out, to far-reaching paralytic effects of the toxin on certain parts of the autonomic nervous system. In the past few years, several aspects of the intoxication of this system have been re-examined by Ambache, who has established in a series of investigations a close correlation between those parts of this innervation that are vulnerable to the toxin and both those pre- and post-ganglionic elements that are included within the ambit of the cholinergic autonomic system.

The double innervation of the intraocular musculature with adrenergic and cholinergic fibres renders this organ peculiarly suitable for distinguishing the selective effects of neurotoxins on these two types of nerve. Ambache (8, 9) found that in rabbits the intraocular injection of a dose of botulinum Type A toxin roughly equal to a single intravenous lethal dose for this species was succeeded 24 to 48 hours later by great enlargement of the pupil and a failure of the iris to react to light. No such paralysis occurred if boiled toxin were used. To produce this iridoplegia, the minimum amount of toxin needed was smaller if it was inoculated into the vitreous humour than into the anterior chamber—a difference that is probably attributable to the direction of fluid movements in the eye and to the rapid escape of foreign proteins introduced into the aqueous humour (281). By electrical stimulation of the oculomotor and cervical sympathetic nerves, Ambache could show that the iridoplegia arose from a paralysis of the cholinergic neuromuscular apparatus of the iris and that the adrenergic fibres to the dilator pupillae muscle remained normally effective. Any suggestion that this botulinum paralysis of the cholinergic sphincter pupillae muscle depends upon an atropine-like action of the toxin, however, is no more correct than the earlier belief that its action on striated muscle is curariform, for an injection of acetylcholine into the anterior chamber of such an intoxicated eye brings about an immediate reduction in the size of the pupil. Later, Ambache (10) found that in cats the cholinergic and adrenergic fibres to the intraocular muscles were similarly sensitive and resistant respectively to botulinum toxin. He was also able to show in this species that the infiltration of the nictitating membrane with very large doses of toxin led only to partial paralysis of its retractor muscle when a maximal stimulus was applied to the cervical sympathetic nerve. Since this muscle is largely under the influence of adrenergic fibres (238), these observations provide further support for the belief that this portion of the autonomic nervous system is notably resistant to the action of botulinum toxin.

Ambache (11, 13) supplemented his observations on iridoplegia with experiments on the similarly doubly innervated small intestine. By injecting the toxin locally into the subserosal layer of the wall of the ileum in rabbits, he was able to infiltrate the whole myenteric apparatus. When an intoxicated segment from such a loop of gut was examined in an organ bath, it was found that the contrac-

tile response ordinarily produced by nicotine was suppressed, although the sensitivity of the muscle to acetylcholine was fully maintained. Again, the botulinum paralysis affected only the cholinergic components of the intestinal nerve plexuses, an observation which led him to suggest that the selective unmasking of adrenergic mechanisms by this toxin might prove of value in the analysis of phenomena that involve a mixture of cholinergic and non-cholinergic neurones (13, 15).

A further instance of the botulinum inactivation of a postganglionic cholinergic innervation is provided by the local paralysis of sudomotor nerves. In cats, these fibres occupy an exceptional position in the autonomic nervous system in that, although they arise from that part which is connected with the thoracic outflow, the sweat glands they innervate respond to acetylcholine and not to adrenalin (71). Ambache (10) made unilateral injections of toxin into the hairless pads of the feet of kittens where sweat secretion can readily be elicited by appropriate nerve stimulation. He found that after the lapse of 19 to 72 hours after the injection, stimulation of the brachial plexus or sciatic nerve caused profuse sweating in the control pads, but little or none in those whose subcuticular structures had been intoxicated. Sweating on both sides, however, could be evoked, sometimes copiously, by local injections of acetylcholine and by intravenous or subcutaneous injections of pilocarpine.

The early studies on the paralytic action of botulinum toxin on the cholinergic components of the nervous system were made on animals suffering from a generalized intoxication, and they therefore disclosed chiefly the effect of the toxin on the post-ganglionic fibres. Through studies on local tissue intoxication in the vicinity of the ciliary and superior cervical sympathetic ganglia, Ambache (10, 12) has shown that the toxin is also injurious to the pre-ganglionic fibres of this system. The ciliary ganglion in the orbit contains the synapses between the preganglionic fibres that reach it through the oculomotor nerve and the post-ganglionic fibres that pass to the sphincter pupillae. Feldberg and Gaddum (94) had shown that the pre-ganglionic fibres that enter the superior cervical sympathetic ganglion in cats are cholinergic, and the observations of Macintosh (171) make it likely that this is true also for the ciliary ganglion. By infiltrating the tissues immediately round the ciliary ganglion, Ambache was able to produce paralysis at the synapses between its pre- and post-ganglionic fibres. Similarly, by effecting a localized intoxication of the superior cervical sympathetic ganglion, he was able to abolish the contraction of the dilator pupillae muscle which ordinarily follows the stimulation of pre-ganglionic fibres in the cervical sympathetic nerve.

A large body of evidence has now been collected which without exception shows that botulinum toxin acts very widely on all those portions of the peripheral nervous system that are cholinergic in character irrespective of whether they form pre- or post-ganglionic components of the autonomic nervous system or are somatic nerves supplying skeletal muscles. In spite of many indications that acetylcholine may act as a transmitter substance at certain synapses in the central nervous system, however, there is no suggestion from either clinical or experimental sources that the toxin exerts any directly injurious effects on the

brain and spinal cord. Innumerable clinical accounts of the terminal stages of botulism in man have recorded the full retention of mental faculties when all the typical signs of the disease are fully developed (84, 169). Nor is there any evidence that the toxin is more rapidly lethal or exhibits a higher potency when it is injected intracerebrally than when inoculated parenterally by some other route. The absence of any special vulnerability of the brain stem was described by Davies, Morgan, Wright and Payling Wright (74), who made a comparative study of the lethalities of botulinum and tetanus toxins when they were injected into the medulla oblongata of the rabbit. Whereas tetanus toxin was much more potent by this route, botulinum toxin was less effective than when it was given intravenously. Nor does the exposure of botulinum toxin to finely divided suspensions of brain lessen its toxicity, as happens with tetanus toxin in the well-known Wassermann-Takaki reaction (57, 156). The invulnerability of putatively cholinergic structures in the central nervous system to botulinum toxin thus contrasts very sharply with the widespread susceptibility of the peripheral cholinergic innervation. The difference cannot be attributed to the size of the botulinum toxin molecule or to the relatively impermeable blood-brain barrier, for the resistance to its action is equally manifest after direct inoculations into the brain.

*Action of botulinum toxin on enzyme systems.* Very little is yet known about the biochemical lesions produced by botulinum toxin, nor, in view of the multitude of potentially vulnerable enzyme systems in neurones, does it seem likely that studies on this aspect of the intoxication will prove rewarding until the site of its action has been more closely identified. Burgen and his colleagues (52) found no evidence that the addition of this toxin, even at high concentrations, interfered with the choline acetylase systems present either in fresh minced mouse brain or in the much more potent dried rat brain preparations described by Feldberg and Vogt (95). Some inhibition of this enzyme by the toxin was observed by Torda and Wolff (257), but their experiments were made with relatively crude, unfractionated, Type A and B culture filtrates. In assessing the significance of such experiments, however, it should be borne in mind that neither in clinical nor laboratory botulism is there any indication of involvement of the central nervous system. In view of the functional disturbances typical of botulism, it would seem unlikely that the toxin would exert any inhibition on cholinesterase. This supposition has been confirmed: even at high concentrations, botulinum toxin has no effect *in vitro* on the esterase activities of either the 'true' or the 'pseudo' varieties of this enzyme present in guinea-pig's serum.

*Lethality of botulinum toxin for poikilothermic animals.* While most of the experimental studies on the action of botulinum toxin have been made on birds and mammals, there is ample evidence that many species of cold-blooded animals can be similarly intoxicated, though the dosage required to kill them may proportionately be much larger. Schübel (237) used a wide range of species in his study, and described successful intoxication of snails, earthworms, frogs and fish (barbels). He found that with frogs, the toxin remained innocuous if the animals were kept at a low temperature, but that if it were raised to 20–22°C.,

botulism soon developed. This dependence of the state of intoxication upon temperature was apparently unknown to other investigators who had previously attempted to induce botulism in this species—which was well suited for nerve-muscle studies—though the effects of raised temperatures had long been familiar to students of tetanus in frogs.

Recently, Burgen, Dickens and Zatman (52) have again produced botulism in frogs (*Rana temporaria*); they recorded that whereas a fixed inoculum of toxin (15,000 mouse $LD_{50}$ per 15 g. frog) caused death in 5–8 days at 16–18°C., the same dose was lethal in 16 hours at 30°C. Ambache and Ferreira (14) observed a comparable reduction in the survival time of Brazilian frogs (*Leptodactylus ocellatus*) when their temperatures were raised. With goldfish (*Carassus auratus*) Cartwright and Lauffer (55) found a clear inverse correlation between the lethal dose and the temperature of the tank: at 36°C., the $LD_{50}$ was only about $\frac{1}{80}$th of that at 22°C.

With poikilothermic animals, the extra degree of freedom in experiment introduced by controllable alterations of temperature would seem to offer an additional means for distinguishing the successive stages of the complex phenomena of bacterial intoxication. For botulism, a preliminary attempt at such an analysis has been made by Goldfarb and Peisakhis (109). They first showed that when a medium-sized dose (40 mouse $LD_{50}$) of Type A toxin was injected intramuscularly into frogs, the concentration in the blood reached its peak in about 2 hours. When frogs inoculated with this dose are kept at 16–18°C., except for a single warm spell of four hours at 31°C., at various intervals after injection, the shortening of their survival period was most pronounced in those that were warmed on the second day—that is at a time when most of the toxin had left the circulation. Once the first signs of botulism have appeared, death in intoxicated frogs can be delayed by chilling—those kept at 5°C. survived about twice as long as those at room temperature. From these observations, Goldfarb and Peisakhis concluded that the effect of the raised temperature in botulism is to accelerate the action of the toxin on susceptible structures once it has become fixed, rather than to hasten the initial stages of its absorption and fixation.

An interesting further instance of the action of botulinum toxin on cholinergic innervations is the paralysis of the discharge of the electric eel (*Electrophorus electricus*) (14). The nerve-electroplax system in this discharging organ seems to be analogous with the nerve-endplate structures of voluntary muscle, and is capable of developing an e.m.f. of several hundred volts between the eel's snout and its most caudal segments. These fish, being of tropical distribution, well sustain a temperature of 30–34°C., at which an inoculation of botulinum toxin quickly becomes effective. In two or three hours, the potential between the two extreme ends of the electric organ, which at first were two to three hundred volts, had sunk to a few volts and sometimes to values that were barely recordable. As with skeletal muscle, the paralysis is peripheral, for if the anterior spinal roots in the region intoxicated are stimulated, no response could be elicited from the corresponding segments of the electric organ.

*Action of anticholinesterase drugs in botulism.* The appreciation of the periph-

eral, apparently curariform, nature of the paralysis in experimental botulism led Edmunds and Keiper (91) to administer physostigmine to intoxicated animals in the hope of improving their muscular activity; in none did they observe any favourable result from its use. On several occasions since, the failure of anticholinesterase drugs in botulism has been confirmed. Guyton and MacDonald (121) gave neostigmine to guinea-pigs with a unilateral botulinum intoxication of one hind leg, but failed to alleviate the paralysis. Masland and Gammon (176) also found that physostigmine led to very little improvement in the tension developed in the intoxicated tibialis anticus muscle in cats when it was stimulated indirectly through the sciatic nerve—a result that was quite different from that in animals equally paralysed with curare. Burgen, Dickens and Zatman (52) recorded some slight improvement in the tension produced in the paralysed isolated rat phrenic nerve-diaphragm preparation when eserine (0.1–1.0 μg./ml.), prostigmine (0.01–0.1 μg./ml.) and tetraethylpyrophosphate (0.01–0.1 μg./ml.) were added to the bath. The improvement, however, was no greater than that produced by these drugs in comparable unpoisoned preparations, and much inferior to that displayed by curarised diaphragms.

While there is general agreement that these anticholinesterase drugs are of little value in improving the contractile strength of skeletal muscles on indirect stimulation, hardly any observations have yet been made on their effects on paralyses in structures supplied by the cholinergic autonomic nervous system. It is possible that a systematic survey might disclose a greater effectiveness at such sites.

*Fixation and duration of action of botulinum toxin.* Observations made on rat phrenic nerve-diaphragm preparations in isolated organ baths show that exposure to large doses of toxin leads within a few minutes to its fixation to susceptible structures in amounts sufficient to cause paralysis (52). With such preparations, the subsequent addition of immunologically grossly excessive quantities of specific antitoxin fails to reverse the paralysis or even to arrest its progress. Such observations are in full conformity with the common clinical finding that the administration of antitoxin is only likely to be beneficial if carried out early in the course of the disease. Even then its use is not spectacular, and the main employment of such sera should be directed to prophylaxis amongst persons who may possibly have partaken of the toxin-contaminated food and may therefore be in the incipient stages of intoxication.

Most of the evidence on the persistence of the effect of botulinum toxin has come from the clinical study of patients who have survived epidemics. Generally, in cases that recover, the intoxication reaches its peak in about ten days and improvement then sets in, though convalescence is often extremely slow and tedious. The pharyngeal signs and symptoms are usually the first to diminish. The general muscular weakness may last for several months, and the disturbances of vision even longer. But although the period of recovery is often long, there seems to be no permanent residual disability in any of the structures affected.

So far as the skeletal musculature is concerned, these observations agree with

those of Guyton and MacDonald (121) on the recovery of contractile power in the calf muscles of guinea-pigs in which local botulism has been produced by the regional inoculation of toxin. Immediately after their intoxication, these muscles were virtually unresponsive to indirect stimulation, but after several weeks they had recovered much, though not all, of their former strength. Even after the lapse of many months, they still presented a perceptible disability that could be disclosed by quantitative measurements of their contractile power, though usually it was inappreciable to the ordinary observer of the animals' natural movements. These authors also describe structural changes in the muscle end-plates that can be recognised by the usual histological impregnation techniques.

### III. TETANUS[1]

*General features.* The infectious character of tetanus, although previously suspected, was first incontrovertably demonstrated by Carle and Rattone (54) in 1884 when they succeeded in inducing the disease experimentally in animals (for literature, see Humphreys (131)). They inoculated twelve rabbits with finely divided tissues taken from the primary lesion of a patient who had died of tetanus, and reproduced the classical signs of the disease in all except one of the animals. A few years later, Nicolaïer (192) described the bacillus now known as *Clostridium tetani*, which he recovered from the tissues of animals which he had infected with material obtained from the streets of various German cities, and Kitasato (141, 142) and Tizzoni and Cattani (254) isolated the organism in pure culture. It was from cultures of this anaerobic, spore-bearing, bacillus that Tizzoni and Cattani (255, 256) and Faber (93) in 1890 separated by filtration the highly lethal toxin termed 'tetanospasmin', and showed that its inoculation was followed by all the features typical of naturally occurring tetanus in man and animals. The work of these authors finally established the nosological position of the disease as a bacterial intoxication and its lethal agent as a neurotoxin.

Clinically, the great majority of cases of tetanus met with in man fall into one or other of two forms: first, the 'general', in which, after an ill-defined prodromal stage, the intoxication usually declares itself by an increasing spasticity of the muscles of mastication followed by similar changes in those of the trunk and limbs and still later by generalized convulsions; and second, the 'local', in which the musculature of the region infected, usually a limb, becomes at first painful and later spastic. Experimentally, these two forms can be readily reproduced in laboratory animals, the 'general' by an intravenous and the 'local' by an intramuscular inoculation of the toxin. The fact that the disease, both natural and experimental, can occur in these alternative clinical forms has been responsible for the many controversies on the modes of distribution of the toxin in the body and on the sites of its action, which have rendered the literature on tetanus so polemical.

In naturally occurring tetanus in man, the organisms enter the tissues in the form of spores, often together with contaminating dirt. In the spore state, the

[1] A very comprehensive monograph on tetanus has recently been published by Pelloja (197).

organisms can survive in soil for long periods without changing into the vegetative form, but once they have entered the tissues they usually soon undergo this metamorphosis. The local conditions which render lacerated tissues so conducive to the germination of the spores have been greatly clarified by Fildes (96, 97, 98, 143), who applied the physico-chemical concepts of oxidation-reduction potentials to the problems of infections with anaerobic bacteria. He found that at the usual pH of the tissues, tetanus spores could only germinate when the Eh of their surroundings had fallen to 10 mV or less. At the reaction of traumatized tissues, which usually become acidified through local ischaemia and anoxia, as well as through the metabolism of accompanying proliferating bacteria of other kinds, germination may take place at a much higher oxidation-reduction potential. Hanke and Bailey (127) found that the growth requirements of *Cl. tetani* are not unlike those of *Cl. histolyticum*, and that at pH 6.5, a degree of acidity readily created in traumatized muscle (193, 242), both clostridia will multiply when the Eh is 85 mV or less.

Unfortunately, there are few studies upon the rate at which tetanus toxin is formed at infected foci in tissues, but from a few quantitative experiments made by Francis (101) it would appear that at the time of death a very large depot of toxin is present round the growing bacteria. In dying guinea-pigs, which had been inoculated with tetanus spores under conditions that ensured their germination, he was able to recover more than a thousand guinea-pig lethal doses by simple saline extraction of the infected tissues. From evidence that became available at the enquiry into the tragic outbreak of tetanus at St. Louis amongst seven children who were given diphtheria antitoxin which had been inadvertently obtained from a horse two days before it developed clinical signs of tetanus, Abel and his colleagues (1) calculated that even at this stage in the incubation of the disease, the animal had 280 horse lethal doses of toxin in its circulating blood. Madsen (173) recorded an instance in which a horse at a serum institution had several horse lethal doses of toxin in its blood five days before it presented any clinical sign of tetanus. Even these few observations show how great the accumulated reservoir of toxin may become in the local tissues of an infected person or animal, and the considerations which have led certain surgeons and immunologists to advocate a local as well as a general prophylactic injection of antitoxin.

*Purification of tetanus toxin.* Interest in the biochemical nature of so lethal an agent as tetanus toxin ('tetanospasmin') has led to several attempts to obtain it in a purified form. In the past, most efforts to fractionate toxic culture filtrates have relied chiefly on precipitation of the toxic protein with ammonium sulphate at various degrees of saturation, and even with these simple procedures it has been possible to obtain a high measure of concentration of the toxin. Pickett, Hoeprich and Germain (202), for instance, thus prepared a toxin which had $27 \times 10^6$ mouse m.l.d. per mg. N from a filtrate with a potency of $34 \times 10^4$ m.l.d. per mg. N—an increase of about 80 times. Using adsorption and precipitation techniques with cadmium chloride and ammonium sulphate respectively, they could concentrate the toxin still more—their best preparation with this method contained $43 \times 10^6$ mouse m.l.d. per mg. N.

Pillemer and his colleagues (203, 204, 207, 208) introduced a wholly new method of precipitating the toxic protein with methanol at various concentrations in solutions of specified ionic strength and pH and at particular temperatures. The material they obtained had a potency of $77 \times 10^6$ mouse m.l.d. per mg. N—a value about twice that of the best preparations of Pickett and his colleagues. The toxic protein thus separated could be crystallized from dilute methanol, and proved to be homogeneous as judged by the tests of electrophoresis and ultracentrifugal sedimentation. After acid hydrolysis, thirteen different amino acid residues were identified and their relative proportions determined. In comparison with other simple proteins, the toxin contained exceptionally high percentages of aspartic acid and lysine (88)—features which it is suggested might partly account for its great surface activity and its ability to become adsorbed readily on glass and other materials. It seems of interest too, that the relationships of aspartic acid to glutamic acid and of isoleucine to leucine are similar to those found with Type A botulinum toxin (205) and are the reverse of those typical of most other proteins.

A comparison between the purification methods of Pickett *et al.* and those of Pillemer *et al.* has recently been made by Turpin, Raynaud and Rouyer (261). With the former, the most lethal preparation they obtained had a potency of $8.7 \times 10^6$ mouse m.l.d. per mg. N, while with the latter they attained a value of $57 \times 10^6$ such doses. In this final concentration, the potency of the material was 330 times that of the original parent culture filtrate.

In its most highly purified form, tetanus toxin is unstable, and in the course of a few days undergoes a transformation which appears to be polymerization to a dimer toxoid form (204). While these larger molecules retain their capacity for flocculation with specific antitoxin, they lose their former toxicity; for this reason such highly purified preparations are difficult to use for most toxicological experiments. In its fresh state, however, the highly purified toxin produces typical tetanus with death in 96 hours when given intramuscularly to mice in doses of $0.013 \times 10^{-6}$ mg. N (206). When injected in enormous doses, *e.g.*, 500,000 m.l.d., the inoculated mice show signs of intoxication in about 30 minutes and usually die within an hour. With intermediate doses, the survival times are roughly inversely correlated with the size of the inoculum. There seems to be no reason for not accepting these very rapid deaths as the result of a classical intoxication of extreme severity, for although the clinical features of such animals differ in some respects from those that develop more gradually after an inoculation with smaller doses of the more usual and cruder preparations of this toxin, muscular spasticity and convulsions are again the main manifestations seen.

*Difficulties in the determination of the minimum lethal dose of tetanus toxin.* Accurate estimation of the minimum lethal dose of a highly potent toxin is fraught with considerable difficulty, which becomes greater as the preparation under test increases in purity. Deterioration in the potency of a toxin solution while awaiting assay can occur quite quickly from a variety of causes: *e.g.*, physically, by adsorption on the glass wall of the containing vessel (190) or by

denaturation on any bubbles that may be inadvertently created during the dilution procedures (218, 219, 220), as well as chemically by oxidation. To lessen the risk of such losses, it is now customary to protect the purified toxin during its serial dilution by the addition of some harmless material, such as peptone or gelatin (59, 125, 203). These procedures are closely akin to, and often indistinguishable from, those of toxin potentiation which will be considered below.

*Potentiation of the neurotoxins of botulism and tetanus.* Many years ago, Ricketts and Kirk (228) noted that the lethality of tetanus toxin could be materially raised if goat or rabbit serum was added to it immediately before injection. A similar potentiation of botulinum toxin was later described by Bronfenbrenner (41), and since then the phenomenon has been re-observed on many occasions with these and other agents for both these neurotoxins (for literature, see Traub, Hollander and Friedemann (258)). The extent of such potentiation is usually about ten times, but one of an apparently much higher order has recently been recorded for Type D botulinum toxin by Wentzel, Sterne and Polson (275). They found that the addition of 0.2 per cent gelatine to a solution of this toxin raised its lethality for mice to the unprecedented value of $4 \times 10^{12}$ m.l.d. per mg. N.

The way in which potentiating agents operate is still obscure. Several authors have drawn attention to the instability of greatly diluted toxins, especially when highly purified, and to the possibility that any added serum or peptone might be protective against oxidative or other destructive influences (41, 125, 245). The presence of other neutral colloids might also minimize the loss of the highly surface active toxin through denaturation or attachment to glass surfaces (219).

Zuger, Hollander and Friedemann (286) were the first to try to distinguish between mere protection and true potentiation of tetanus toxin. They soon excluded the possibility that the mixing of toxin and potentiating agent led to the formation of additional toxin, for the same amount of antitoxin sufficed for neutralization both before and after potentiation had occurred. They thus disposed of any applicability to this system of Dernby and Walbum's (83) theory that the effective lethal agent was formed by the action of some bacterial enzyme on some substrate presented to it. Nor did they find evidence of any notably rapid deterioration of their toxin preparation when it was dissolved in saline only, so that they felt compelled to abandon the idea of protection as an explanation for their findings. By the unsatisfactory process of exclusion, they were led to accept the phenomenon as one of true potentiation.

Although the manner in which serum and other kindred agents potentiate these two powerful toxins is still obscure, several of the facts pointed out by Traub and his colleagues must be borne in mind in any attempt to account for the phenomenon: (i) there is no increase in the quantity of toxin present—a finding which excludes the possibility of a conversion of a prototoxin into the toxin, such as has been described for the $\epsilon$-toxin of *Cl. welchii* by Bosworth and Glover (35) and by Turner and Rodwell (259, 260); (ii) it is much more pronounced—sometimes as much as fifty-fold—in some preparations of toxins than in others; (iii) it is more clearly demonstrable on the more susceptible species of animal, mice and guinea-pigs, than on the less susceptible rabbits and cats; and (iv) it

TABLE 2

*Relative minimum lethal doses of tetanus toxin for various species of mammals and birds which have been recorded by various authors*

| Species | Fildes (99) | Abel (151) | Bardelli (23) | Ipsen (133) | Friedemann *et al.* (104) | Smith (243) | Behring (26) | Davies *et al.* (76) | Shumacker *et al.* (240) |
|---|---|---|---|---|---|---|---|---|---|
| Horse | | 1 | | | | | 0.5 | | |
| Guinea-pig | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | 1 |
| Monkey | | 2 | | | | | | | 4 |
| Sheep | | 2 | | | | | | | |
| Mouse | 2 | | 4 | 2–6 | 4–20 | 3–6 | 6 | 6 | |
| Goat | | | | | | | 12 | | |
| Rabbit | 12 | | 4–500 | 24–720 | 50–320 | 3–350 | 900 | | |
| Dog | | 300 | | | | | | | 480 |
| Cat | 1200 | | | | | | | | 960 |
| Goose | | | | | | | 6000 | | |
| Pigeon | | | | | | | 24,000 | 6000 | |
| Hen | | | | | | | 180,000 | | |

does not appear to be due to a toxoid-toxin reversal, though the direct comparative experiments on the relation of lethal to flocculating titres before and after potentiation do not seem to have been made.

Not only are the phenomena of potentiation important for an understanding of the modes of action of these two toxins, but they also have a practical bearing on the methods needed for their quantitative assay. In their valuable study on the distribution and fate of tetanus toxin in the body, Abel, Evans and Hampil (1) pointed out that in any attempt to prepare a balance sheet for the toxin injected and that circulating, fixed and excreted in animals, it was essential to carry out dilution procedures with homologous serum if serious errors from possible potentiation are to be avoided.

*Pathogenicity of tetanus toxin for various animal species.* Although fatal tetanus intoxication can be induced in a wide variety of both warm- and cold-blooded animals, the minimum dose of toxin required varies greatly from one species to another. Some comparative figures, collected from various sources, are set out in Table 2. It must be emphasized at once, however, that such figures can be accepted as broad estimates only, because the particular strains of the clostridium used, the method of preparation of the toxin, the breeds, sexes and ages of the animals employed, as well as many other experimental conditions, differ considerably from one recorded series to another. Moreover, as will be considered more fully below, the particular mode and route of injection adopted affect the lethality of this toxin very greatly—indeed to a much greater extent than with any other known bacterial toxin. Nonetheless, a broad picture emerges: one large group that includes horse, guinea-pig, monkey, sheep, mouse and goat is highly susceptible; cats and dogs are much less vulnerable; while birds are strikingly

resistant. From records of accidental inoculations, it is likely that man is among the more susceptible species, though his position is clearly impossible of close assessment (191). Bolton and Fisch (33) recorded the death of a child aged four years from tetanus after it had received an inoculation with diphtheria antitoxin taken from an infected horse: the dose of serum administered contained 50 guinea-pig lethal doses, which would suggest that on a weight basis human beings and guinea-pigs are about equally susceptible. The exceptional variability in the susceptibility of the rabbit to different preparations of tetanus toxin has been described by several authors (21, 26, 104, 133, 201, 243). Von Behring (26) was the first to comment upon his finding that preparations of toxin that were uniformly and constantly lethal for mice might possess a wide range in potency when tested on rabbits. This variability could be widened if the culture were passed through rabbits before being used for preparing the toxin, and he suggested that the blood vessel endothelium of rabbits possesses a destructive enzyme to which he gave the name "tetanotoxinase". No further work along these lines appears to have been undertaken, though the correctness of von Behring's belief could readily be tested by comparative studies of the variabilities in the lethalities of different toxin preparations for this species when administered intravenously or injected directly into the medulla oblongata itself. There seems little doubt that the variability is a feature of the toxin preparation and not of the breed of rabbit used, for Lamont and his associates (152) employed New Zealand white, Chinchilla and mixed Flemish Giant strains without detecting any significant differences in their susceptibilities. Further, when Ipsen (133) examined seven preparations of toxin, all made from the same strain of the clostridium and on the same medium, but in seven different laboratories in Europe and America, his rabbit-guinea-pig dose ratios only varied from 176 to 518, whereas when he employed six wholly different toxin preparations in similar experiments, the range of ratios broadened to 24 to 720. Ipsen considered that there are differences in the "biological qualities" of the toxins formed by different strains of *Cl. tetani* which manifest themselves most strikingly in lethal tests on rabbits.

*Effect of the route of injection on the lethality of tetanus toxin.* The route by which tetanus toxin is introduced into the body not only determines many of the clinical manifestations of the intoxication but also has a profound effect on the size of the requisite lethal dose. Even in 1890, very soon after the discovery of tetanus toxin, Tizzoni and Catanni (256) demonstrated the greatly enhanced potency of the toxin when it was injected into the sciatic nerve trunk. The results obtained by a number of investigators are summarized in Table 3.

As with estimates of species susceptibilities, those for the lethalities of toxin by different routes of inoculation should be interpreted on broad lines. So many variables enter into such experiments that no precision in such dosage figures can be expected. Yet cumulatively, they leave little room for doubt that irrespective of species, the closer the site of inoculation approaches the vital centers in the brain stem, the more effectively lethal does this toxin become. The single exception to this otherwise uniform finding is with the monkey, but in this species the high ratio of intraspinal to intravenous dosage is largely due to the great

TABLE 3

*The relative minimum lethal doses of tetanus toxin for different species of animal when administered by different routes of injection*

| Species | (source) | Intravenous | Subcutaneous | Intramuscular | Intraneural | Spinal cord | Medulla oblongata | Cerebrum | Intraventricular |
|---|---|---|---|---|---|---|---|---|---|
| *Rabbit* | (a) | 1 | 1 | | | | | 1/10-1/20 | |
| | (b) | | | 1 | | | | | 1/20-1/100 |
| | (c) | | 1 | 1/4 | 1/10 | | | | |
| | (d) | 1 | | | | | 1/1000 | 1/100 | |
| | (e) | | 1 | | | | | 1/25 | |
| *Guinea-pig* | (a) | 1 | 1 | 1 | | | | 1 | |
| | (b) | | | 1 | | | | | 1-1/3 |
| | (c) | 1 | | 1 | | | | | |
| | (e) | | 1 | | | | | 1 | |
| *Hutia* | (g) | | | 1 | | | | 1/7-1/13 | |
| *Cat* | (f) | | | 1 | | 1/300 | | | |
| *Dog* | (f) | | | 1 | | 1/50 | | | |
| *Monkey* | (f) | | | 1 | | 1/4 | | | |
| *Pigeon* | (h) | 1 | | | | | | 1/5 | |

Sources references:
(a) van den Hoven van Genderen (266).
(b) Friedemann and Hollander (104).
(c) Sawamura (233).
(d) Wright (279).
(e) Roux and Borrel (231).
(f) Shumacker, Lamont and Firor (240).
(g) Angulo (20).
(h) Davies, Morgan and Wright (76).

susceptibility of this animal when the toxin is given by this latter route. Indeed, if given directly into the lumbar spinal cord, the minimum lethal doses of tetanus toxin for cats, dogs and monkeys per kg. body weight (3, 5 and one guinea-pig $LD_{50}$ respectively) are not very dissimilar (240).

*Site of action of tetanus toxin.* There is common agreement that in the "general" form of tetanus, in which spasticity is widespread in the skeletal musculature and convulsions occur frequently, the toxin operates at some site within the central nervous system. With "local" tetanus, however, there still remains a sharp difference of opinion as to whether all the manifestations can be wholly accounted for by an intoxication of the regional segments of the central nervous system, or whether the toxin brings about the local spasticity solely through a peripheral action on susceptible structures in the affected muscles themselves. Efforts to settle this question have been numerous, and have led to more ingenious experimentation that is to be found anywhere else in the literature of bacterial toxicol-

ogy. Most observers, perhaps partly actuated by a desire for economy in hypothesis, have inclined to the view that in all forms of tetanus the toxin operates only on elements of the central nervous system, but no crucial experiment has yet been designed that has successfully surmounted all the objections raised by those who oppose this view.

It seems to the reviewer that the source of this disagreement is largely traceable to a failure by some investigators to appreciate adequately that the affected skeletal muscle passes through a succession of separable stages as local tetanus progresses. Although commented upon previously (107, 118, 163), it was very properly emphasized by Ranson and his associates (223, 224, 225) that two characteristic forms of spasticity should be distinguished: an early one, lasting roughly five or six days, in which the contraction of the affected muscle can be terminated promptly by general anaesthesia, curariform drugs or nerve section; and a later one in which the muscle remains firmly contracted for many weeks in spite of isolation from the central nervous system by denervation or drug action. To the former stage, he applied the term "hypertonic contraction" and to the latter, "myostatic contracture". It might at first seem possible that the hypertonic contraction phase of tetanus could be attributable to the action of the toxin on the local somatic innervation and the myostatic contracture to a more slowly progressive intoxication of the muscle itself. But this explanation seems to be excluded by the unanimous finding by many observers that the injection of a previously denervated skeletal muscle is never followed by the development of spasticity. In short, the myostatic contracture of local tetanus is invariably a sequal to the phase of hypertonic contraction, and unquestionably depends upon a wholly different mechanism. From this it is apparent that in any experiment designed to elucidate the pathogenesis of local tetanus, these two phases in the disease must be clearly distinguished. The employment of animals in the later myostatic contracture phase of the intoxication should in general be avoided, for the use of such preparations adds unnecessary difficulties to the analysis of an already complex problem.

1) *Peripheral site of intoxication.* (a) *Myoneural junction.* The pioneer experiments of Vaillard and Vincent (262) on the effect of nerve section upon local tetanus emphasized very clearly the dependence of the clinical manifestations on the integrity of the motor innervation. A number of authors, however, have reached the conclusion that the spasticity originates from some toxic injury to the peripheral endings of these motor neurones and that it is this overactivity at the motor end-plates which maintains the affected muscle in a state of unremitting tension. In recent years, Abel and his associates at Baltimore have been the most influential sponsors of this view (2, 3, 4, 5). They find their support chiefly in three observations recorded by Harvey (129): firstly, that the spasticity persists in intoxicated muscles for several days after their motor nerves have been sectioned and disappears with the degeneration of the nerve endings; secondly, that spasticity can develop in a denervated limb if the toxin is injected directly into the muscle before sufficient time has elapsed for the motor nerve endings to lose their transmitting function; and thirdly, that a single nerve volley

excited by electrical stimulation of the motor nerve trunk—which gives rise to a single action potential in a normal muscle—leads to a repetitive discharge in an intoxicated muscle and, inferentially, to a prolongation in the duration of contraction of its fibres.

Whether Harvey's observations adequately support the interpretation that he has placed upon them may be seriously questioned, for there is a large body of evidence with which they are incompatible. In his denervation experiments, he divided the sciatic nerve high in the thigh, but as several earlier investigators had already made clear, the complete denervation of the hind limb requires in addition the section of the femoral and obturator nerves (60, 200, 209, 233). Further, as Permin (200) has pointed out, in many rabbits there is an additional trunk in the sacral plexus which may escape division unless special precautions are taken. The inadequacy of the denervation methods used by Zupnik (287), in which he observed some residual local tetanus in an ostensibly denervated hind limb, has been pointed out by Sawamura (233), by Permin (200) and by others, who have described in detail the surgical procedures needed to ensure the complete extirpation of the motor nerve supply. When such precautions are taken, no sign of local tetanus follows the inoculation of the toxin into the denervated muscles (132, 233). Pochhammer (209), too, pointed out that incomplete denervation can easily lead to the misconception of a purely muscular tetanus. Nor is there any evidence to corroborate Harvey's statement that tetanus will follow the injection of toxin into a denervated muscle provided that the inoculation is made before the motor nerve endings have had time to degenerate. Sawamura (233) made such experiments on rabbits many years ago, and recently Hutter (132) has repeated them on cats—the same species that Harvey used; both recorded that under these conditions no local tetanus developed. Lastly, Harvey's observations on the electromyograms of intoxicated muscles in which repetitive action potentials followed a single nerve volley, have come under criticism from several later investigators who attempted to repeat his experiment. Göpfert and Schaefer (112), Acheson, Ratnoff and Schoenbach (7), Perdrup (199) and Mackereth and Scott (172) have all been unable to confirm his findings. Göpfert and Schaefer suggested that Harvey's recorded action potentials were artefacts, possibly created by the spread of electrotonus from the stimulating electrodes on the nerve to end-plates in the muscle.

(b) *Proprioceptive sensory nerve endings.* Impressed with the readiness with which convulsive motor activity can be induced reflexly in tetanus, several early investigators sought to determine the extent to which the maintenance of spasticity in local tetanus is dependent on the integrity of the regional sensory nerves. With this in view, they de-afferented portions of the spinal cord—usually the lumbar enlargement—by dividing the posterior nerve roots. Their findings were not unanimous. Courmont and Doyen (61) and Permin (200) recorded that in dogs local tetanus was prevented by this procedure. On the other hand, Gumprecht (118), also using dogs, Brunner (46) and Goldscheider (111) with rabbits, and Fröhlich and Meyer (107), Liljestrand and Magnus (163) and Ranson (224) with cats, found that posterior nerve root section might diminish, but did not

abolish, local tetanus. The significance of some of these experiments is not easily assessed at this distance of time, but in their interpretation, one caveat must be borne in mind. In some species, notably cats and dogs, division of the posterior roots leads to chromatolytic changes in the anterior horn cells (272, 273); such disturbances might well impair the functional activity of the regional motor neurones and consequently their ability to conduct impulses to their respective muscles.

In spite of the inconclusive nature of this early work on deafferentation, some investigators have been reluctant to discard the possibility that during the phase of hypertonic contraction, the overactivity of the affected muscles is brought about by some pathologically intensified influx of afferent impulses into the spinal cord. The manifold similarities between the character and distribution of the spasticity in tetanus and in decerebrate rigidity, to which Sherrington (239) first drew attention, have suggested to various investigators (36, 163, 199, 224, 234) the possibility that the former might depend upon an exaggeration of the proprioceptive reflexes on which normal postural tonus mainly depends; overactivity of such a kind might result, they supposed, from the intoxication of the sensory nerve endings in the muscle from which the reflex originates.

Schaefer (234) supported his belief with experimental observations of two kinds. Firstly, he noted that in guinea-pigs the action potentials that could be recorded from the *distal* divided stump of the motor nerve trunk to one of the heads of the gastrocnemius muscle after a sudden pull had been applied to the tendo Achillis became markedly augmented after tetanus toxin had been injected into the muscle and sufficient time had elapsed for the development of local tetanus. The more numerous action potentials he regarded as indicating the pathological sensitivity of the intoxicated muscle spindles to this mechanical stimulus. Secondly, he found that the electromyograms obtainable after crossed stretch reflexes in control and intoxicated limbs showed greater frequency and amplitude when the tension was applied to the tetanus side and the recordings made from the control one than *vice versa*. Both these observations are susceptible of alternative explanations. The former abnormality probably depends upon changes in the elastic properties of a muscle that is entering, if it is not already in, the phase of myostatic contracture and the augmented stimulation that any sudden stretch would consequently impose on its sensory receptor end organs. The latter can be readily accounted for by the asymmetry of the intoxication in the spinal cord which necessarily follows a unilateral intramuscular injection of the toxin.

An alternative approach to the problem has been made through the use of local injections into affected muscles of procaine (Novocaine)—a drug which in the concentrations used is believed to anaesthetize the sensory receptors without paralysing the myoneural junction (163, 199, 224). Perdrup (199), the most recent worker to use this method, found that the injection of a dilute solution of procaine into a spastic muscle in the early stages of local tetanus abolished its characteristic electromyogram without affecting its normal voluntary or reflex activity. These findings, too, throw no light on the site of action of the toxin,

because they fail to distinguish between an augmentation of the proprioceptive reflex activity of the affected muscles that depends on an increase in the number of centripetal impulses that might originate in the supposedly intoxicated sensory nerve endings, and one in which the number of such afferent impulses remains normal but becomes multiplied through after-discharge at diseased synapses in the spinal cord.

Recently, further evidence from very different types of experiment have concurred in adding to the unlikelihood that proprioceptive reflexes from affected muscles contribute significantly to the initiation and maintenance of their spasticity. The first was provided by Friedemann and his colleagues (105), who repeated some of the earlier studies in which the posterior spinal nerve roots had been divided before toxin was injected into the de-afferented muscles. They used monkeys in this work—a species which is particularly suitable because section of these roots interferes little, if at all, with the vascular supply of the nearby spinal cord. When tetanus toxin was injected into the legs of monkeys, all of whose lumbar and sacral posterior roots had been previously cut, the animals developed local tetanus in the de-afferented limb on the third or fourth day. The second type of evidence comes from an electromyographic study of local tetanus in the hind limbs of rabbits (75). During the early, or hypertonic, contraction stage of the intoxication, the spasticity and its associated continuous electromyographic activity can be promptly suppressed by the intravenous injection of a moderate dose of sodium pentobarbital (Nembutal). At this level of anaesthesia, however, the patella tendon reflex can be elicited just as easily from a limb whose quadriceps extensor muscles have been rendered spastic by a local inoculation of toxin as from one in which this group of muscles is normal. Moreover, the electromyographic record of the tendon jerk possesses the same shape and amplitude after the spasticity has been abolished by the Nembutal as it had before. It seems, therefore, that the monosynaptic reflex arc of Lloyd (166, 167), which is concerned with such tendon jerks, is unaffected by the intoxication, and that the "spontaneous" spinal cord discharges down the motor nerve trunks which are responsible for the initiation and maintenance of the tetanus must have their origin in other neurones.

2) *Central site of the intoxication.* The literature upon experimental tetanus now contains accounts of many observations of very diversified kinds, all of which support the belief that in local tetanus the toxin produces the typical spasticity through its operation on some neural element in the central nervous system. Much of the evidence is bound up with that gained in studying the related problem of the possible intraneural dissemination of the toxin and will be considered below. Some of the earliest, and still some of the most convincing, evidence was brought forward by Meyer and Ransom (185) when they found that typical local tetanus can be induced by the inoculation of very small amounts of toxin intraneurally into the sciatic nerve trunk or the substance of the lumbar spinal cord. In both cases, the muscles of the hind limb developed a characteristic local tetanus, which in the intraneural inoculation experiments was at first ipselateral but which often spread later with reduced severity to the opposite

limb also. These experiments have been repeated frequently during the past half-century with the same results (25, 130, 200, 233, 239, 250). Abel, Hampil and Jonas (5) are the only investigators who have thrown doubt on this method of producing local tetanus, but, as the reviewer has pointed out elsewhere (285), their conclusions are difficult to reconcile with the full published protocols of their experiments. More than half of the 13 dogs whose sciatic nerves they inoculated with scrupulous care with various sublethal doses of tetanus toxin are recorded as having subsequently developed "stiffness of the limb"; moreover, there is a clear correlation between the occurrence of this sign of tetanus and the amount of toxin injected.

The main criticism levelled by Abel at those investigations in which the inoculation of the sciatic nerve trunk had been followed by local hind-limb tetanus was that it is difficult—perhaps impossible—to make such intraneural injections without the retrograde leakage of some of the toxin solution along the track of the needle when the latter is withdrawn. This technical defect appeared to them to vitiate the positive results and interpretations recorded by previous investigators. As long as the available evidence on the results of intraneural inoculations was derived wholly from studies made on the great nerve trunks of the limbs as they pass between the muscles they innervate, it might not be unreasonable to suppose that subsequent spasticity could have resulted from an accidental unnoticed escape of some of the injection fluid. There are, however, several pieces of evidence which greatly diminish the weight of Abel's apprehensions.

When small quantities of tetanus toxin are injected into the sciatic or femoral nerve trunks of rabbits not far from their origins in the lumbar and sciatic plexuses, individual electromyograms taken from the main muscle groups in the limb show that two distinctive patterns of local tetanus emerge (75). With the femoral nerve, the quadriceps extensors of the thigh alone are involved. With the sciatic nerve, not only are the hamstring muscles of the thigh affected, but typical tetanus appears at the same time in both the anterior tibial and the calf group of muscles in the lower leg. Since, as Romanes (229) has shown for the cat—and the same arrangement is probably also true for the rabbit—the motor nucleus for the quadriceps muscles is situated in the spinal cord one to two segments cephalad to the three closely placed nuclei from which the other muscle groups derive their innervation, the pattern of these two forms of local tetanus seems more readily explicable on the basis of the spatial arrangement of the respective nuclei in the spinal cord than on the assumption of any hypothetical contamination by toxin of muscles in the lower leg which are far removed from the site of operation. Similar evidence for a central site of intoxication comes from the observation of Mackereth and Scott (172) that local tetanus develops in the diaphragm after the inoculation of muscles which are anatomically remote from it but which possess a common segmental innervation.

More difficult to reconcile with Abel's contention that local tetanus develops as the result of regional tissue contamination are some observations made by Meyer and Ransom (185), Ambache and Lippold (16) and Wright, Morgan and Payling Wright (282) on the effects of injections of tetanus toxin into the vagus nerve

and other motor nerves of the brain stem. The last-named authors inoculated toxin solution into each of three main cranial motor nerves and thus transferred the region of experimentation from the lumbar spinal cord, with its relatively simple group of motor nuclei for limb skeletal muscles, to the brain stem with its very diversified motor nuclei ranging from that controlling the external rectus muscle in the orbit by way of the abducent nerve to that of the vagus with its many peripheral ramifications in the thoracic and abdominal viscera. For when the toxin is injected into the facial, vagus or hypoglossal nerve trunks, it is inconceivable that any local leakage at the site of inoculation could bring about all the diversified motor responses typical of this form of bulbar tetanus—the strabismus, torticollis, salivation and bradycardia—through any contamination of the local structures of the kind which Abel visualised. Clearly, with such a widely radiating system of emergent motor nerves, any leakage of toxin from a single site of inoculation in one of these cranial nerve trunks could not contaminate more than a small fraction of the effector organs that are actually thrown into activity, and which would all be potentially at risk, if the intoxication were a central one. Since the whole syndrome of bulbar tetanus is the same irrespective of whether the toxin is injected into the facial, vagus or hypoglossal nerve, the simplest and most comprehensible explanation for these findings would seem to be that all these signs result from the central action of the toxin on these closely spaced brain stem nuclei whose neurones provide the final common pathway for the activation of these diverse effector organs.

The belief that the site of action of tetanus toxin is central rather than peripheral receives further support from some observations on the suppression of spasticity in the early phase of the intoxication by barbiturate anaesthetics (284). In rabbits, the length of the thigh makes it possible to isolate temporarily much of the hind limb from the general circulation by the inflation of a suitably placed sphygmomanometer cuff. In local tetanus, the closure of the limb arteries for a period of a minute has no effect on the character of the electromyogram. But if Nembutal is injected into the general circulation at a time when its access to the leg is prevented by such vascular occlusion, all traces of both spasticity and exaggerated electromyographic activity disappear completely within 15 seconds. The termination of the signs of local tetanus by the Nembutal, whose entry into the limb is prevented by pressure from the inflated cuff, demonstrates the dependence of the spasticity on the functional mediation of some centrally-situated neural structures to which the anaesthetic continues to have unimpeded access. This finding seems irreconcilable with Harvey's belief that spasticity arises from a peripheral intoxication at the myoneural junctions.

*Spread of tetanus toxin from a depot site in the tissues.* The pathogenesis of the two distinctive forms of tetanus—the "ascending" and the "descending"—which can be reproduced experimentally by intramuscular and intravenous injections of the toxin respectively, has provoked prolonged controversy as to the possible routes by which this substance can reach the central nervous system. The mass of evidence obtained from intramuscular injections, a short summary of which will be given below, now seems overwhelmingly to favour the belief that in

"ascending" tetanus the toxin is carried centripetally along the regional motor nerve trunks from its depot in the tissues to the spinal cord or brain stem. Much more obscure and open to debate is the route by which toxin circulating in the blood stream reaches the vulnerable structures in the central nervous system to produce "descending" tetanus.

*"Ascending" tetanus.* 1) *Spread of toxin in peripheral nerve trunks.* Very soon after the discovery of tetanus toxin, Bruschettini (47) found that mice died from tetanus if portions of the main motor nerve trunks supplying tetanus intoxicated muscles in rabbits were implanted in their tissues. This and similar experiments have since been repeated with varying results by several investigators (25, 87, 209, 233); the measure of their success in demonstrating the presence of toxin appears to depend upon how much of it was initially injected into the muscles peripherally and the care employed in extracting it from the excised regional nerve trunks. But though the recognition of toxin in proximal portions of such motor nerves is compatible with their serving as conduits for its passage to the central nervous system, positive findings do not in themselves suffice to prove that these trunks participate in this way in the pathogenesis of local tetanus. For it is now known that the larger nerve trunks possess numerous lymphatics in their epineurium whose centripetally-flowing contents pass not to the spinal cord and theca as was formerly assumed, but to the chain of lymph nodes that lie anteriorly along the vertebral column (78), and the extraction methods employed provide no means of distinguishing such toxin as may be present in the "Lymphräume" or tissue spaces inside the trunk from any that may be present in the externally-placed lymphatics proper.

Among the many experiments undertaken to throw light on the pathogenesis of "ascending" tetanus, those designed to effect some impediment to the centripetal movement of toxin along the large nerve trunks have figured prominently. The obstacles set up to lessen or prevent any such movement have assumed three main forms: an immunological barrier created by a proximal injection of specific antitoxin; the transection of the nerve trunk at some site between the point of injection of toxin and the spinal cord; and lastly, the structural disorganisation of the tissue spaces in the nerve trunk by the intraneural injection of some sclerosing solution several weeks before the toxin is inoculated.

Meyer and Ransom (185) were the first to show that the local tetanus that ordinarily follows a suitable inoculum of toxin into a limb muscle can be prevented by the simultaneous injection of specific antitoxin into the regional nerve trunk at some proximal site. This observation has since been confirmed by others (200, 233, 250). It may be questioned, however, whether the usually accepted interpretation of this experimental finding—that at the site of its injection the depot of antitoxin neutralises any centripetally-borne toxin—can be regarded as compatible with the belief in the movement of fluid in nerve trunks. A better explanation of these findings would seem to be that the antitoxin, like the toxin, is carried upwards along the nerve to enter the tissue fluids of the spinal cord and it is there that it exerts its specific protective power. Moreover, this upward spread of the antitoxin would account for Abel, Firor and Chalian's (3) lack of success

when they repeated these earlier experiments with amounts of antitoxin that immunologically were very small.

The objection that the barrier placed in the possible pathway of the toxin may itself have moved from the site at which it was first laid down, cannot apply to experiments in which the obstruction is created by a zone of sclerosis in the substance of the nerve trunk itself. Teale and Embleton (250), who were the first to employ this method, injected small quantities of tincture of iodine into the sciatic nerves of rabbits before the distal inoculation of the toxin, and found that by so doing they could postpone or avert the onset of spasticity. Their observations were severely—and probably correctly—criticised by Abel and his colleagues (3) on the grounds that tincture of iodine or 90 per cent alcohol introduced into a nerve trunk in this way causes such severe injury to its fibres that the procedure "is the equivalent of nerve section". The more recent introduction into surgery of much milder sclerosing agents, such as ethanolamine oleate, has made it possible to create zones of sclerosis in nerve trunks without causing injury to their constituent axons (25, 283). In rabbits whose sciatic nerve trunk had been sclerosed in the mid-femoral region in this way, no local tetanus followed the inoculation into the calf muscles of an amount of toxin much in excess of that needed to produce full local tetanus in the leg of a normal rabbit (25). That this failure to develop tetanus was not due to any serious incapacitation of the neuromuscular structures of the limb by the sclerosing agent was clearly shown by the finding that in those sclerosed rabbits which had failed to develop spasticity after a distal intramuscular inoculation of toxin, typical local tetanus promptly followed the inoculation of a small dose into the sciatic nerve trunk above the site of sclerosis and close to the lumbar spinal cord. In such experiments, therefore, there can be no question of the sclerosis being the "equivalent of nerve section".

The belief that in fatal "ascending" tetanus, the toxin passes centripetally to vulnerable centres in the brain stem is supported by an experiment made by D'Antona (72, 73) in which he administered toxin and antitoxin to guinea-pigs in various combinations of route and amount. His main findings are summarized in Table 4. From this table it can be seen that intravenous doses of antitoxin which are capable of preventing fatal tetanus when the toxin is given directly into the blood stream are incapable of doing so when the inoculation is made intramuscularly into a limb. Yet this latter mode of administering the toxin fails to kill the animals if the regional motor nerves to the injected muscles have previously been divided.

Consistent with, and complementary to, D'Antona's findings are some observations of Friedemann, Hollander and Tarlov (105) to which they have applied the term "route phenomenon". They observed that if 10 to 20 minimum lethal doses of tetanus toxin are injected into guinea-pigs, in one group intramuscularly, in a second also intramuscularly but after a regional motor denervation and in a third intravenously, an intravenous injection of antitoxin some 20 to 80 times greater is required to protect the first group than either of the others (106). Such a difference is compatible with the view that toxin injected into

TABLE 4

*The effect of the route of injection of tetanus toxin on the survival of guinea-pigs that had received antitoxin intravenously*

| Toxin | | Prior treatment | Intravenous antitoxin | Result |
|---|---|---|---|---|
| Dose | Route | | | |
| *mg.* | | | *i.u.* | |
| 17.5 | intravenous | none | 25 | survived: no tetanus |
| 17.5 | intravenous | none | 50 | survived: no tetanus |
| 17.5 | intramuscular | none | 25 | death after 6 days |
| 17.5 | intramuscular | none | 50 | death after 10 days |
| 17.5 | intramuscular | nerve resected | 25 | survived: no tetanus |
| 17.5 | intramuscular | nerve resected | 50 | survived: no tetanus |
| 7.5 | intravenous | none | none | death after 2 days |
| 7.5 | intravenous | none | none | death after 2 days |

muscles which still possess their normal innervation is carried to the vital centres in the medulla oblongata by some route along which it is sheltered from the neutralising action of the circulating antitoxin. Like Meyer and Ransom, Friedemann and his colleagues believe that the first portion of this route is the motor nerve trunk that supplies the region inoculated, for, as they point out, with so much unbound antitoxin in the blood, it would be impossible for any unneutralized toxin to reach the brain by way of the circulation.

Even if the centripetal movement of the toxin along regional motor nerves is accepted as prerequisite for the pathogenesis both of local tetanus and of the first stage in "ascending" tetanus, there would still remain uncertainty as to which component of the nerve trunk forms the necessary conduit for its passage. Meyer and Ransom supposed—and many others since have rather uncritically accepted their supposition—that the toxin passes upwards in the interior of the motor axons themselves. In the light of De Rényi's microdissection studies (81, 82) on the structure and consistence of both vertebrate and invertebrate nerve fibres, such a belief seems no longer tenable. Diffusion alone would be wholly incapable of accounting for the ascent of a protein at a rate of many millimetres an hour (39, 40), and in such a viscous filament of protoplasm as a nerve fibre it is difficult to conceive that it would be carried by any centripetal mass streaming of the axoplasm. An explanation that seems more in accordance with the known internal structure of nerve trunks and their high tissue fluid contents (56) is that the toxin is borne in a central direction along the highly oriented tubular clefts that lie between the individual nerve fibres, and that the force needed for its propulsion is provided by the great increase in the pressure of the tissue fluid in the belly of a muscle when it undergoes contraction (22, 25, 274). Furthermore, the prevention of local tetanus by a degree of sclerosis of the nerve trunk which fails to impair the conductivity of its fibres for nervous impulses, seems better

explicable on the basis of a compression and obliteration of the intraneural tissue clefts in a motor nerve than on the supposition of an axonal carriage of the toxin (285).

2) *Spread of toxin in the cerebrospinal axis.* There are strong grounds for the belief that once the toxin has reached the spinal cord it ascends within its substance to the vulnerable centres lying in the medulla oblongata. The first, and still the most convincing, evidence in favour of this cephalad spread of the toxin came from experiments in which the spinal cord had been transected at some level in its lower thoracic or upper lumbar regions. This procedure was employed by Meyer and Ransom (185), who showed that a dose of toxin which when injected into the sciatic nerve of a normal kitten was capable of producing firstly local tetanus, later advancing tetanus of the back, forelimbs and neck, and ultimately death on the 5th day, brought about tetanus in the hindquarters only in a similar animal whose spinal cord had previously been divided in the upper lumbar region. On the 13th day, this latter kitten was quite active although the hindlimbs were in almost unremitting convulsions; the animal died from exhaustion on the 21st day. Experiments revealing closely similar results have been repeated on dogs (100), monkeys (105) and rabbits (24). Indeed, were it not for the non-specific complications which, even with careful attention to nursing are difficult to avoid in paraplegia, there is little doubt that such animals could be maintained free from generalised "ascending" tetanus indefinitely.

The belief engendered by these transection experiments that toxin gaining access to the cerebrospinal axis even at its lumbar end can spread upwards with rapidity (39, 40) receives support from studies on the spread of other materials in the spinal cord. Permin (200) found that the injection of specific antitoxin into the lumbar enlargement prevented the subsequent development of local forequarter tetanus when the toxin was injected intramuscularly into a forelimb—a result that is probably attributable to the centripetal movement of the antitoxin up the spinal cord. Recently, the introduction of radioactive isotopes into pathology has much facilitated studies on the spread of labelled materials in the tissues, and both $^{32}P$ containing sodium phosphate (37) and $^{131}I$ marked homologous serum proteins (283) have been shown to pass quickly upwards along the spinal cord to the brain stem in rabbits.

That the quantity of toxin ascending in this way that is required to kill an animal need only be a small fraction of that entering the lower portion of the spinal cord is shown by the very small size of the minimum lethal dose and its rapid fixation to susceptible structures when the inoculation is made directly into the medulla oblongata (100, 279, 280). The extraordinarily high lethality of this toxin when injected at this site is evident from Table 5, which summarises some of the observations made by Firor, Lamont and Shumacker (100) on dogs.

*"Descending" tetanus.* In man, the most common initiating symptom of tetanus is a sensation of stiffness in the masseter muscle and difficulty in mastication. This trismus is soon followed by the other signs of "descending" tetanus—stiffness in the neck and back, difficulty in swallowing, progressive spasticity of the limbs and lastly generalised convulsions (for details, see Linder (164)). Though the

TABLE 5

*The effect of various doses of tetanus toxin injected directly into the medulla oblongata on the time of onset of symptoms and duration of survival*

| Number of dogs used | Dose toxin injected | Symptoms | | Duration of survival | |
|---|---|---|---|---|---|
| | | Nature | Onset | Mean | Range |
| | *m.l.d.* | | *hours* | *hours* | *hours* |
| 2 | 1/4500 | | | | |
| 2 | 1/3000 | pharyngeal spasm | 48–188 | 192 | 144–240 |
| 3 | 1/1000 | pharyngeal spasm | 132 | 184 | 144–216 |
| 2 | 1/500 | pharyngeal spasm | 24–48 | 144 | 72–216 |
| 3 | 1/40 | pharyngeal spasm | 18–24 | 43 | 27–60 |
| 2 | 1/20 | pharyngeal spasm | 5 | 23 | 22–24 |
| 5 | 1/15 | pharyngeal spasm | 7 | 22 | 18–27 |
| 7 | 1/10 | pharyngeal spasm | 6 | 21 | 17–26 |
| 2 | 1/8 | pharyngeal spasm | not seen | 18 | 17–19 |
| 2 | 2.5 | pharyngeal spasm | 2 | 11 | 10–12 |
| 2 | 3.0 | pharyngeal spasm | 2 | 10 | 9–11 |

modal incubation period lies between 7 and 10 days, typical signs may appear within a day or two of infection or may be delayed for 3 or 4 weeks. As might be expected, there is a close correlation between the brevity of the incubation period and the case fatality rate. In animals, too, the first evidence of spasticity appears in the muscles of the head and neck and gradually extends thence to those of the back and limbs. In horses, which are highly susceptible, the earliest sign is often the drawing of the nictitating membrane across the eye especially when the head is raised (46).

The clinical features of "descending" tetanus in laboratory animals can be faithfully reproduced by an intravenous injection of the toxin. After an incubation period which depends on the size of the dose, but which is briefer than that which follows an infection, the typical prodromata appear and pursue the same course as is seen in the natural disease. Such a correspondence strongly suggests that the "descending" form of tetanus results from the carriage of the toxin from the tissue depot to the central nervous system by the circulating blood in contrast to the neural transport found in the local and "ascending" forms of the disease. This inference is borne out by the often repeated identification of toxin in the blood of animals that have acquired a natural infection.

Apart from some experiments of McClintock and Hutchings (170), no attempt has yet been made to determine the time of appearance and the progressive rise in titre of tetanus toxin in infected as contrasted with intoxicated animals. Most observers have sought to study the progressive fall in concentration of toxin, which has been injected intravenously in a single inoculum, as it becomes distributed in the various tissue fluid compartments of the body. Using sheep and dogs, because their large blood volumes allowed frequent sampling for toxin assay without serious exsanguination, Abel and his colleagues (1) undertook a notably careful quantitative study on the rate of the progressive decline. As can be seen from Table 6, in sheep, the toxin disappears from the blood at a rate

not very dissimilar from that observed for the loss of labelled bovine serum albumin—a protein of comparable molecular size—from the circulation of rabbits after its intravenous injection (144, 153). In dogs, the fall was more precipitate. (Some earlier and less carefully conducted studies on the loss of toxin from the blood are discussed by Abel in the same paper.) When the toxin is injected intravenously, much of it soon escapes into the lymph and tissue fluids with which it soon reaches an equilibrium (221, 222). If the injection is made subcutaneously, the concentration of the toxin in the blood seems to rise slowly, in much the same manner as has been found with similarly injected labelled foreign plasma albumin (232), and again falls as it becomes redistributed in the various body fluid compartments. The quantitative analysis of the movement of such an agent as tetanus toxin between a 'source' in the tissues and a 'sink' in the central nervous system presents a problem of the greatest complexity. Even with much simpler systems, the difficulties are considerable (244).

The route taken by the toxin in its passage from the blood to susceptible elements in the central nervous system is still obscure, and is closely connected with the physiological and pathological behavior of the postulated haematoencephalic barrier (102, 103). Although the capillaries of the central nervous system in general are known to be notably impermeable to certain constituents of the plasma (145), those in certain sites, particularly the *areae postremae* in the floor of the fourth ventricle permit the escape of substances from the blood with noteworthy ease (277). It may be that this local capillary permeability in the floor of the fourth ventricle may, by permitting the early intoxication of the nuclei of the lower cranial motor nerves, be responsible for the prompt involvement of the muscles of the jaw and neck in general tetanus. How small a quantity of toxin would suffice to bring about their intoxication becomes apparent from experiments on the induction of medullary tetanus (100, 279).

The time interval that elapses between the intravenous injection of a lethal dose of tetanus toxin is inversely related to its size, though the correlation is far from linear for the four species, guinea-pig (60), dog (100), rabbit (134, 279) and

TABLE 6

*Percentage blood concentrations of intravenously injected tetanus toxin (sheep) and labelled bovine serum albumin (rabbits) after various intervals*

| Time after injection | Percentage remaining of initial inoculum | | |
|---|---|---|---|
| | Tetanus toxin | Bovine serum albumin | |
| | | (Knox *et al.*) | (Laws *et al.*) |
| *hours* | | | |
| 0 | 100 | 100 | 100 |
| 5 | | | 65 |
| 7 | 62 | | |
| 10 | | 52 | |
| 24 | 60 | 41 | 40 |
| 48 | 52 | 31 | 28 |
| 72 | 43 | 28 | |

mouse (135), for which such observations have been recorded. Much the most careful study of these 'deathtime-dosage' relationships (which can serve usefully for the potency-titration of unknown toxin solutions) has been made by Ipsen (135); he found that his observations were well fitted with a curve of the following formula

$$\left(\frac{D}{d} - 1\right)^{0.5} \times \left(\frac{T}{8} - 1\right) = 10.3$$

where D is the dosage used, d, the minimum lethal dose and T, the deathtime. This relationship, which is close within the range of doses which Ipsen used, fails completely when extrapolated to the enormous doses of their highly purified toxin that were used by Pillemer and Wartman (206)—500,000 m.l.d. with a deathtime of about one hour.

*Mode of action of tetanus toxin.* Scarcely anything is known about the nature of the injury inflicted by tetanus toxin on neural elements in the central nervous system. At the beginning of the century, when Nissl's techniques for displaying chromatolysis were much in use, several investigators recorded the finding of morphological abnormalities in neurones in the affected region of the spinal cord (174). Later, with more experience of the method and better controls, such observations have not been substantiated (198, 253). In the light of present ideas on the location of action of the toxin in the cord, it is possible that in these studies too much attention was focused on the large anterior horn cells. Were more directed instead to the smaller internuncial neurones, as is suggested by recent work on the abnormalities in reflex activity that develop in local spinal cord intoxication (44, 75), some morphological change might come to light.

Exactly a hundred years ago, Sir James Simpton, while reviewing a long series of cases of puerperal tetanus, likened the symptomatology of the disease to that of strychnine poisoning (241). Later authors have made the same comparison, but Sherrington (239), during his study of the mutual interactions of reflexes, was the first to suggest that the common element in the modes of action of the two poisons was their conversion of synaptic inhibition into excitation. Recently, two further parallels between the two have been described. First, it has been found that in tetanus, as in moderate grades of strychnine poisoning, the monosynaptic tendon jerk elicitable from the quadriceps extensor muscle in rabbits is hardly affected by the intoxication (75). Second, Brooks, Curtis and Eccles (44, 90), while studying in cats the effects of suitably timed impulses evoked by single maximum volleys in the large stretch afferents from the quadriceps muscles on the monosynaptic tendon reflexes from the biceps-semitendinosus muscle, have found that strychnine and tetanus toxin both lessen and eventually abolish the power of the former to exert its typical inhibitory action on the latter. To account for their finding, they advance two possible explanations for the action of tetanus toxin: first, that, like botulinum toxin, it may prevent the release of the transmitter substance (see below); or second, that, like strychnine, it may become attached to the subsynaptic inhibitory areas on the motoneuronal membrane, preventing the action thereon of the inhibitory transmitter. They suggest, too,

that in generalised tetanus, the convulsive and other manifestations may be similarly explicable by a widespread suppression of synaptic inhibitory action as the toxin spreads throughout the nervous system.

There is little information, too, about the biochemical lesions that occur during tetanus intoxication. Such as there is has come from studies on the effect of sub-lethal doses of toxin injected into the anterior chamber of the rabbit's eye on the intrinsic neuromuscular apparatus of the iris (17, 79). Within 24–48 hours of such an injection, the pupil becomes widely dilated, the light reflex is lost and the sphincter pupillae muscle ceases to respond to stimulation of the third cranial nerve. Throughout such intoxications, however, the dilator pupillae muscle contracts promptly on stimulation of the cervical sympathetic nerve. This selective paralysis, which was later also found to follow comparable intraocular injections of botulinum toxin (9), thus affects only the cholinergic and spares the adrenergic mechanism in the iris. Its progressive development in intraocular injections is also accompanied by a marked reduction in the concentration of acetylcholine in both the iris and the aqueous humour (18). It would seem, therefore, that tetanus toxin behaves less as a general neural poison than as a specific impediment to acetylcholine formation and release—a property which it shares with botulinum toxin. Knowledge of the humoral transmitters in the central nervous system is still too fragmentary to allow such observations to find their place in any explanation of the pathogenesis of the spasticities and convulsions of fully established tetanus. Nonetheless, the double action of tetanus toxin in interfering with acetylcholine release and in impairing reflex inhibition in the spinal cord (see above) seems worthy of note as a possible starting point for further work.

In his discussion on the possible pathogenesis of local tetanus in cats, Harvey (129) suggested that in addition to some disturbance affecting acetylcholine metabolism at skeletal motor nerve endings, the toxin might bring about some reduction in cholinesterase activity at the myoneural junction. No such reduction, however, was detected by Martini, Torda and Zironi (175) in the gastrocnemius or tibialis anticus of rats with tetanus, or by Ambache, Morgan and Payling Wright (18) in the enzyme in either its 'true' or 'pseudo' forms in the iris muscles of rabbits or the quadriceps femoris or rectus abdominis muscles of guinea-pigs. What little evidence there is that this enzyme is inhibited by tetanus toxin has been derived from observations made *in vitro*, most of which were carried out before the distinction between the 'true' and 'pseudo' cholinesterases had been recognized. Schaefer (234) described a slight reduction in cholinesterase activity in muscles that had been incubated with tetanus toxin for several days, and similar findings have been recorded by Werle and Stüttgen (276) and by Ammon (19). In most of these studies, very large concentrations of the toxin were used, and the slight reductions that were found do nothing to disturb the general consensus of opinion that in the dosages ordinarily used to produce experimental tetanus the toxin has little or no detectable effect upon this enzyme. They give no support, therefore, to Harvey's suggestion as to the pathogenesis of the spasticity in this intoxication.

*Tetanus in poikilothermic animals.* In 1892, Courmont and Doyon (60) described the production of fatal tetanus intoxication in frogs, though for the weight

TABLE 7

*Tetanus in frogs: the relationship between temperature and deathtime*

| Numbers of frogs used | Temperature | Median deathtime |
|---|---|---|
| | (°C.) | *days* |
| 16 | 28 | 3 |
| 12 | 27.5 | 4 |
| 10 | 23 | 7.5 |
| 6 | 22 | 10.5 |
| 12 | 19.5 | 9 |
| 18 | 18 | over 21 |

of the animal the dose required was much greater than that needed for small mammals. Their most significant observation, that the temperature to which the animal is exposed is of paramount importance in pathogenesis, was soon confirmed by others (177, 178, 187): below about 18°C., no tetanus developed even though the toxin could be detected in large amounts in the circulating body fluids. Although aware that modifications in temperature provided the observer with a valuable additional degree of freedom in any experimental analysis of the mode of action of the toxin, Courmont and Doyon went no further than to contrast the striking influence of temperature on tetanus intoxication with its relative lack of effect in strychnine poisoning.

Recently, observations on tetanus in frogs (*Rana temporaria*) have been extended by Rowson (personal communication), who has examined the effect of temperature on various stages in the process of intoxication. The relationship which he found between temperature and median deathtime in groups of frogs is shown in Table 7. Toxin assays on mice of samples of blood taken from frogs at various intervals after inoculation showed that the prolongation of survival times at the lower temperatures does not result from any significant retardation in the escape of the toxin from the dorsal lymph sac—its site of injection—into the circulation. But although the blood toxin levels following inoculation pursue closely similar courses in frogs at 15°C. and 27°C., a lethal dose becomes fixed to susceptible structures much more rapidly at the latter temperature. For, whereas an immunological excess of antitoxin given to the warmer frogs 48 hours after the inoculation of the toxin fails to protect them, the cooler ones can be saved in this way even after 7 days. Not only does cooling delay fixation, but it also retards the rate at which the toxin injures vulnerable tissues after it has become fixed. When a group of frogs that have been inoculated with toxin and kept warm at 26°C. for 48 hours to ensure the fixation of a lethal dose, is divided into subgroups, one of which is retained at that temperature and others cooled to much lower temperatures for various periods and then re-warmed, the prolongation of survival times (see Table 8) are almost the same as the duration of the period of cooling. From Rowson's analysis, it seems that the protective effect of cooling is not due to any decreased rate of absorption of the toxin from the lymph sac, but to both a retardation in its rate of fixation to, and an inhibition of its action upon, the susceptible elements in the nervous system.

Although most of the studies on tetanus in poikilothermic animals have been

TABLE 8

*The effect of cooling on the survival times of frogs which have already fixed a lethal dose of tetanus toxin*

| Numbers of frogs used | Temperatures | | Duration of cooling | Median deathtime | Survival Time Increase |
|---|---|---|---|---|---|
| | Warm | Cool | | | |
| | °C. | °C. | *days* | *days* | *days* |
| 7 | 26 | 18 | 0 | 5.5 | |
| 7 | 26 | 18 | 3 | 8.5 | 3 |
| 6 | 26 | 18 | 6 | 10.5 | 5 |
| 6 | 26 | 18 | 9 | 13 | 7.5 |
| 6 | 26 | 5 | 0 | 7 | |
| 6 | 26 | 5 | 6 | 14 | 7 |
| 6 | 26 | 5 | 9 | 16.5 | 9.5 |

made on frogs, many species have been shown to be susceptible to the toxin provided that they are maintained at sufficiently elevated temperatures. As with frogs, the quantity of toxin required is relatively larger than with mammals. Grasset and Zoutendyk (115), in an extensive study of the actions of various toxins on cold blooded animals, found that a number of South African reptiles of the orders *Crocodilia* and *Chelonia* and of the suborders *Ophidia* and *Lacertilia* can be fatally intoxicated with tetanus. Similarly, Cowles and Nelson (64) and Wright, Morgan and Payling Wright (personal observation) have respectively observed that the American crested lizard (*Dipsosaurus dorsalis dorsalis*) and the Madeira lizard (*Lacerta dugesi*) can be killed readily with tetanus toxin when kept warm.

*Protection against tetanus toxin by prior injections of tetanus toxoid.* Lemétayer and his colleagues (160, 226) have made the interesting observation that if both mice and guinea-pigs are given a large inoculum of tetanus toxoid of very high immunological potency 24 hours before the injection of one m.l.d. of tetanus toxin, death is delayed or even prevented. This phenomenon, which they have termed "specific precocious protection", has since been studied in greater detail (110, 161, 227). Raynaud and Wright (227) made clear two points: first, that the phenomenon is specific, for it succeeded with tetanus but failed with diphtheria toxoid; and second, that it is independent of any accelerated specific active immunization—an explanation to which Lemétayer and his associates at first inclined—because if the intervals between the injections of toxoid and toxin are progressively spaced out by days from one to six days, the measure of protection steadily falls to rise again later when classical antitoxin immunity eventually develops. They suggested as an alternative and more satisfactory explanation, that the toxoid molecules (of which several million were injected for every one toxin molecule) exert either a prior blocking of the hypothetical receptor sites in the central nervous system, similar to an interference phenomenon, or inhibit in some competitive manner the action of the toxin. Some observations that bear further on this point have been made by Wolters and Fischoeder (278). They

found that a brei made from mouse brain could take up tetanus toxin readily from a solution in which the two were present (the "Wassermann-Takaki phenomenon"), but that if the brei were exposed to tetanus toxoid before the toxin, its uptake of the latter was materially reduced. It seemed as though susceptible sites in the nervous elements had been occupied in some irreversible way by the toxoid molecules.

How significant these toxoid protection experiments are for an understanding of the mechanism of action of the toxin is not yet clear. It seems inherently unlikely that the conversion by formaldehyde of the latter into the former can be a single stage transformation. More probably, the change is steplike, so that when such an enormous excess of toxoid is present, some incompletely detoxicated molecules still persist which retain the capacity to unite with susceptible structures but have lost the power to inflict injury upon nervous elements at a rate faster than that at which it can be made good by their inherent capacity for repair.

One observation, however, made by Lemétayer and his colleagues in the course of their study of specific precocious protection, has in addition a bearing on the contentious question (see page 446) of the possible centripetal movement of the toxin in local tetanus from a site of intramuscular inoculation to the central nervous system. They found that if, in guinea-pigs, the prior intramuscular injection of the toxoid was made into the same leg as the subsequent inoculum of toxin, no local rigidity developed, but that if the two were injected into the opposite hind legs, spasticity appeared in the toxin-inoculated limb though rather later in its onset than in unprotected control animals. These toxoid experiments, therefore, provide further support for the belief that intramuscularly injected toxoid and toxin both ascend to the regional segments of the spinal cord along pathways provided by their motor nerve trunks.

## REFERENCES


1. Abel, J. J., Evans, E. A. Jr. and Hampil, B.: Researches on tetanus. V. Distribution and fate of tetanus toxin in the body. Bull. Johns Hopkins Hosp., 59: 307–391, 1936.
2. Abel, J. J., Evans, E. A. Jr., Hampil, B. and Lee, F. C.: Researches on tetanus. II. The toxin of bacillus tetani is not transported to the central nervous system by any component of the peripheral nerve trunk. Bull. Johns Hopkins Hosp., 56: 84–114, 1935.
3. Abel, J. J., Firor, W. M. and Chalian, W.: Researches on tetanus. IX. Further evidence to show that tetanus toxin is not carried to the central neurones by way of the axis cylinders of motor nerves. Bull. Johns Hopkins Hosp., 63: 373–403, 1938.
4. Abel, J. J. and Hampil, B.: Researches on tetanus. IV. Some historical notes on tetanus and commentaries thereon. Bull. Johns Hopkins Hosp., 57: 343–372, 1935.
5. Abel, J. J., Hampil, B. and Jonas, A. F. Jr.: Researches on tetanus. III. Further experiments to prove that tetanus toxin is not carried in peripheral nerves to the central nervous system. Bull. Johns Hopkins Hosp., 56: 317–336, 1935.
6. Abrams, A., Kegeles, G. and Hottle, G. A.: The purification of toxin from *Clostridium botulinum* Type A. J. Biol. Chem., 164: 63–79, 1946.
7. Acheson, G. H., Ratnoff, O. D. and Schoenbach, E. B.: The localised action on the spinal cord of intramuscularly injected tetanus toxin. J. Exper. Med., 75: 465–480, 1942.
8. Ambache, N.: Peripheral action of botulinum toxin. Nature, London, 161: 482–483, 1948.
9. Ambache, N.: The peripheral action of botulinum toxin. J. Physiol., 108: 127–141, 1949.
10. Ambache, N.: A further study of the action of *Clostridium botulinum* toxin upon different types of autonomic nerve fibre. J. Physiol., 113: 1–17, 1951.
11. Ambache, N.: Unmasking, after cholinergic paralysis by botulinum toxin, of a reversed action of nicotine on the mammalian intestine, revealing the probable presence of local inhibitory ganglion cells in the enteric plexuses. Brit. J. Pharmacol., 6: 51–67, 1951.

12. AMBACHE, N.: Effect of botulinum toxin upon the superior cervical ganglion. J. Physiol., 116: 9P, 1952.
13. AMBACHE, N.: Autonomic ganglion stimulants. Arch. internat. pharmacodyn., 97: 427–446, 1954.
14. AMBACHE, N. AND FERREIRA, H. M.: Paralysis of the discharge in *Electrophorus electricus* produced by botulinum toxin. An. Acad. Brasil. Ciencias, 24: 225–239, 1952.
15. AMBACHE, N. AND LESSIN, A. W.: *In vitro* effects of botulinum toxin (Type D). J. Physiol., 122: 63P–64P, 1953.
16. AMBACHE, N. AND LIPPOLD, O. C. J.: Bradycardia of central origin produced by injections of tetanus toxin into the vagus nerve. J. Physiol., 108: 186–196, 1949.
17. AMBACHE, N., MORGAN, R. S. AND WRIGHT, G. PAYLING: The action of tetanus toxin on the rabbit's iris. J. Physiol., 107: 45–53, 1948.
18. AMBACHE, N., MORGAN, R. S. AND WRIGHT, G. PAYLING: The action of tetanus toxin on the acetylcholine and cholinesterase contents of the rabbit's iris. Brit. J. Exper. Path., 29: 408–418, 1948.
19. AMMON, R.: Substances which inhibit cholinesterase action. Ergebn. Enzymol., 9: 35–69, 1943.
20. ANGULO, J. J.: Efectos de la toxina tetanica, introducida per diferentes vias en la *Jutia conga*. Vida nueva, 51: 185–194, 1943.
21. ARISTOVSKI, V. M., KAMBOROVA, O. T. AND FIRSOVA: On the pathogenesis of tetanus. IV. On the sensitivity of different species of experimental animals to tetanus toxin. Arch. Sc. Biol., Moscou, 51: 32–33, 1938.
22. BARCROFT, H. AND DORNHORST, A. C.: Demonstration of the "muscle pump" in the human leg. J. Physiol., 108: 39P, 1949.
23. BARDELLI, P. C.: Sulle varietà del bacillo del tetano. Ann. Igiene, 37: 386–390, 1927.
24. BAYLIS, J. H., JOSEPH, J., MACKINTOSH, J., MORGAN, R. S. AND WRIGHT, G. PAYLING: The effect of transection on the ascent of tetanus toxin in the rabbit's spinal cord. J. Path. & Bact., 64: 47–52, 1952.
25. BAYLIS, J. H., MACKINTOSH, J., MORGAN, R. S. AND WRIGHT, G. PAYLING: The effect of sclerosis of the nerve trunk on the ascent of tetanus toxin in the sciatic nerve of rabbits and on the development of local tetanus. J. Path. & Bact., 64: 33–45, 1952.
26. BEHRING, E. VON: Einführung in die Lehre von der Bekämpfung der Infektionskrankheiten. August Hirschwald, Berlin 1912, p. 228 *et seq.*
27. BENGTSON, I. A.: Preliminary note on a toxin-producing anaerobe isolated from the larvae of *Lucilia caesar*. U.S. Treasury, Pub. Health Rep., 37: 164–170, 1922.
28. BENGTSON, I. A.: Studies on organisms concerned as causative factors in botulism. U.S. Treasury, Hyg. Lab. Bull. No. 136, p. 36, 1924.
29. BENNETTS, H. W. AND HALL, H. T. B.: Botulism in sheep and cattle in Western Australia. Australian Vet. J., 14: 105–118, 1938.
30. BERNARD, C.: La science expérimentale. Baillière, Paris 1875, p. 237.
31. BISHOP, G. H. AND BRONFENBRENNER, J. J.: The site of action of botulinum toxin. Am. J. Physiol., 117: 393–404, 1936.
32. BITTER, L.: Der Botulismus. Ergebn. allg. Path. u. path. Anat., 19 (ii): 733–799, 1921.
33. BOLTON, B. M. AND FISCH, C.: An estimate of the amount of toxin in the blood of horses infected with tetanus. Tr. Am. Physicians, 17: 463–467, 1902.
34. BOROFF, D. A., RAYNAUD, M. AND PRÉVOT, A. R.: Studies on the toxin of *Clostridium botulinum* Type D. J. Immunol., 68: 503–511, 1952.
35. BOSWORTH, T. J. AND GLOVER, R. E.: The effects on *Clostridium welchii* toxins of a substance present in the normal intestine. Rep. Inst. Animal Path., Univ. Cambridge, 4: 79–93, 1934–35.
36. BREMER, F., TITECA, J. AND VAN DER HEIREN, L.: Sur la sensibilité au curare de la rigidité tétanique locale. Compt. rend. Soc. biol., 97: 895–898, 1927.
37. BRIERLEY, J. B. AND FIELD, E. J.: The fate of an intraneural injection as demonstrated by the use of radioactive phosphorus. J. Neurol., Neurosurg. & Psychiat., 12: 86–99, 1949.
38. BRISCOE, G.: Changes in muscle contraction curves produced by drugs of the eserine and curarine groups. J. Physiol., 93: 194–205, 1938.
39. BROMEIS, H.: Die Wanderungsgeschwindigkeit des Tetanusgift im Tierkörper. Deutsche Ztschr. Chir., 250: 402–410, 1938.
40. BROMEIS, H.: Weitere Versuche über die Verteilung des Tetanusgiftes bei seiner Wanderung im Tierkörper. Deutsche Ztschr. Chir., 252: 285–293, 1939.
41. BRONFENBRENNER, J. J.: Further studies on potency in botulinus toxin. Proc. Soc. Exper. Biol. & Med., 21: 318, 1923–24.
42. BRONFENBRENNER, J. J. AND SCHLESINGER, M. J.: The effect of digestive juices on the potency of botulinum toxin. J. Exper. Med., 39: 509–516, 1924.
43. BROOKS, V. B.: The action of botulinum toxin on motor nerve filaments. J. Physiol., 123: 501–515, 1954.
44. BROOKS, V. B., CURTIS, D. R. AND ECCLES, J. C.: Mode of action of tetanus toxin. Nature, London, 175: 120–121, 1955.
45. BROWN, G. L., DALE, H. H. AND FELDBERG, W.: Reactions of the normal mammalian muscle to acetylcholine and to eserine. J. Physiol., 87: 394–424, 1936.
46. BRUNNER, C.: Experimentelle und klinische Studien über den Kopftetanus. Beitr. klin. Chir., 12: 523–588, 1894.
47. BRUSCHETTINI, A.: Sulla diffusione del veleno del tetano nell'organismo. Riforma med., 8: 256–259 and 270–273, 1892.
48. BRYGOO, E. R.: Recherches sur l'hémolysine de *Clostridium botulinum* D. Ann. Inst. Pasteur, 78: 795–798, 1950.
49. BRYGOO, E. R.: Résistance des toxines botuliques en dilution dans l'eau. Ann. Inst. Pasteur, 84: 1039–1040, 1953.
50. BRYGOO, E. R.: Action des agents épurateurs sur les toxines botuliques en dilution dans l'eau. Ann. Inst. Pasteur, 84: 1040–1041, 1953.

51. Buehler, H. J., Schantz, E. J. and Lamanna, C.: The elemental and amino acid composition of crystalline *Clostridium botulinum* Type A toxin. J. Biol. Chem., 169: 295-302, 1947.
52. Burgen, A. S. V., Dickens, F. and Zatman, L. J.: The action of botulinum toxin on the neuro-muscular junction. J. Physiol., 109: 10-24, 1948.
53. Burke, G. S.: Notes on bacillus botulinus. J. Bact., 4: 555-565, 1919.
54. Carle, A. and Rattone, G.: Studio sperimentale sull'eziologia del tetano. Gior. Roy. Acad. Med., Torino, 32: 174-180, 1884.
55. Cartwright, T. E. and Lauffer, M. A.: Assay of botulinum A toxin with goldfish. Proc. Soc. Exper. Biol. & Med., 81: 508-511, 1952.
56. Caubet, G.: The effect of pressure on nerve fibres. J. Anat., 82: 262-270, 1948.
57. Coleman, G. E.: Action of leucocytes and brain tissue on toxin of bacillus botulinum. J. Infect. Dis., 34: 614-620, 1924.
58. Coleman, I. W.: Studies in the oral toxicity of *Clostridium botulinum* toxin Type A. Canad. J. Biochem. Physiol., 32: 27-34, 1954.
59. Condrea, P. and Pohnaru, H.: Recherches sur la nature de la toxine tétanique. Influence de la peptone sur la toxicité de la toxine tétanique. Compt. rend. Soc. biol., 112: 1484-1487, 1933.
60. Courmont, J. and Doyon, M.: Le tétanos. Baillière, Paris 1899, p. 53.
61. Courmont, J. and Doyon, M.: Quelques points particuliers de la pathogénie des contractures du tétanos. Arch. Physiol., 25: 64-74, 1893.
62. Cowan, S. L.: The action of eserine-like and curare-like substances on the responses of frog's nerve-muscle preparations to repetitive stimulation. J. Physiol., 93: 215-262, 1938.
63. Cowdry, E. V. and Nicholson, F. M.: A histological study of the central nervous system in experimental botulinus poisoning. J. Exper. Med., 39: 827-836, 1924.
64. Cowles, R. B. and Nelson, N. B.: Studies on thermal sedation in suppression of symptoms of tetanus toxin. Proc. Soc. Exper. Biol. & Med., 64: 220-224, 1947.
65. Dack, G. M. and Gibbard, J.: Permeability of the small intestine of rabbits and hogs to botulinum toxin. J. Infect. Dis., 39: 181-185, 1926.
66. Dack, G. M. and Hoskins, D.: Absorption of botulinum toxin from the colon of *Macaca mulatta*. J. Infect. Dis., 71: 260-263, 1942.
67. Dack, G. M. and Wood, W. L.: Impermeability of the small intestine of rabbits to botulinum toxin. J. Infect. Dis., 40: 585-587, 1927.
68. Dale, H. H.: The action of certain esters and ethers of choline and their relation to muscarine. J. Pharmacol. & Exper. Therap., 6: 147-190, 1914-15.
69. Dale, H. H.: Nomenclature of fibres in the autonomic nervous system. J. Physiol., 80: 10P-11P, 1933.
70. Dale, H. H.: Walter Ernest Dixon Memorial Lecture: Pharmacology and nerve endings. Proc. Roy. Soc. Med., 28: 319-332, 1935.
71. Dale, H. H. and Feldberg, W.: The chemical transmission of secretory impulses to the sweat glands of the cat. J. Physiol., 82: 121-128, 1934.
72. D'Antona, D.: La propagation de la toxine tétanique par la voie nerveuse démontrée par la résection des nerfs chez les animaux en état d'immunité. Rev. immunol., 13: 128-141, 1949.
73. D'Antona, D.: Le tétanos: Synthèse théorique et pratique de nos principales connaissances. Rev. immunol., 15: 93-157, 1951.
74. Davies, J. R., Morgan, R. S., Wright, E. A. and Wright, G. Payling: The results of direct injections of botulinum toxin into the central nervous system of rabbits. J. Physiol., 126: 618-623, 1954.
75. Davies, J. R., Morgan, R. S., Wright, E. A. and Wright, G. Payling: The effect of local tetanus intoxication on the hind limb reflexes of the rabbit. Arch. internat. physiol., 62: 248-263, 1954.
76. Davies, J. R., Morgan, R. S. and Wright, E. A.: The susceptibility of pigeons to tetanus toxin. J. Path. & Bact., 1955 (in press).
77. Davis, J. B., Mattman, L. H. and Wiley, M.: *Clostridium botulinum* in a fatal wound infection. J. A. M. A., 146: 646-648, 1951.
78. Defrise, A.: Ricerche anatomo-typographiche e microscopiche sui vasi linfatici del nervi. Arch. Gen. Neurol. Psichiat., 11: 103-117, 1930.
79. De Lavergne, V., Helluy, J.-R., Faivre, G. and Henry, C.: Réproduction expérimentale du tétanos céphalique. Ann. Inst. Pasteur, 75: 259-271, 1945.
80. Denz, F. A.: Myoneural junctions and toxic agents. J. Path. & Bact., 63: 235-247, 1951.
81. De Rényi, G. S.: The structure of cells in tissues as revealed by microdissection. II. The physical properties of the living axis cylinders in the myelinated nerve fibre of the frog. J. Comp. Neurol., 47: 405-425, 1929.
82. De Rényi, G. S.: Architecture of the nerve cell as revealed by microdissection. In: Cowdry: Special Cytology. 2nd ed., Vol. 3, p. 1370. Hoeber, New York 1932.
83. Dernby, K. G. and Walbum, L. E.: Studien über die Bildung des Diphtherietoxins. Biochem. Ztschr., 138: 505-560, 1923.
84. Dickson, E. C.: Botulism: a clinical and experimental study. Rockefeller Inst. Med. Res., Monograph 8, New York 1918.
85. Dickson, E. C. and Shevky, E.: Botulism: studies on the manner in which the toxin of *Clostridium botulinum* acts on the body. I. The effect on the autonomic nervous system. J. Exper. Med., 37: 711-731, 1923.
86. Dickson, E. C. and Shevky, E.: Botulism: studies on the manner in which the toxin of *Clostridium botulinum* acts on the body. II. The effect on the voluntary nervous system. J. Exper. Med., 38: 337-346, 1923.

87. Doerr, R., Seidenberg, S. and Magrassi, F.: Kritische und experimentelle Studien zur Frage des Nachweises von Tetanustoxin in peripheren Nerven. Ztschr. Hyg. InfektKr., 118: 92–116, 1936.
88. Dunn, M. S., Camien, M. N. and Pillemer, L.: The amino acid composition of tetanal toxin. Arch. Biochem., 22: 374–376, 1949.
89. Easton, E. J. and Meyer, K. F.: Occurrence of bacillus botulinus in human and animal excreta. J. Infect. Dis., 35: 207–212, 1924.
90. Eccles, J. C., Fatt, P. and Landgren, S.: The 'direct' inhibitory pathway in the spinal cord. Australian J. Sc., 16: 130–134, 1954.
91. Edmunds, C. W. and Keiper, G. F.: Further studies on the action of botulinus toxin. J. A. M. A., 83: 495–501, 1924.
92. Edmunds, C. W. and Long, P. H.: Contribution to the pathologic physiology of botulism. J. A. M. A., 81: 542–547, 1923.
93. Faber, K.: Die Pathogenese des Tetanus. Berl. klin. Wchnschr., 27: 717–720, 1890.
94. Feldberg, W. and Gaddum, J. H.: The chemical transmitters at synapses in a sympathetic ganglion. J. Physiol., 81: 305–319, 1934.
95. Feldberg, W. and Vogt, M.: Acetylcholine synthesis in different regions of the central nervous system. J. Physiol., 107: 372–381, 1948.
96. Fildes, P.: Tetanus. VI. Conditions under which tetanus spores germinate *in vivo*. Brit. J. Exper. Path., 8: 387–393, 1927.
97. Fildes, P.: Tetanus. VIII. The positive limits of oxidation reduction potential required for the germination of spores of *B. tetani in vitro*. Brit. J. Exper. Path., 10: 151–175, 1929.
98. Fildes, P.: Tetanus. IX. The oxidation reduction potential of subcutaneous tissue fluids of the guinea-pig: its effect on infection. Brit. J. Exper. Path., 10: 197–204, 1929.
99. Fildes, P.: Medical Research Council: A system of bacteriology in relation to medicine. H.M. Stationary Office, London 1929, vol. 3, p. 298.
100. Fiber, W. M., Lamont, A. and Shumacker, H. B. Jr.: Studies on the cause of death in tetanus. Ann. Surg., 111: 246–274, 1940.
101. Francis, E.: Laboratory studies on tetanus. U.S. Treasury, Hyg. Lab. Bull., No. 95, p. 72, 1914.
102. Friedemann, U.: Blood-brain barrier. Physiol. Rev., 22: 125–145, 1942.
103. Friedemann, U.: Dynamics and mechanism of immunity reactions *in vivo*. Bact. Rev., 11: 275–300, 1947.
104. Friedemann, U. and Hollander, A.: Studies on tetanal toxin. I. Qualitative differences among various toxins revealed by bioassays in different species and by different routes of injection. J. Immunol., 47: 23–28, 1943.
105. Friedemann, U., Hollander, A. and Tarlov, I. M.: Investigations on the pathogenesis of tetanus. J. Immunol., 40: 325–364, 1941.
106. Friedemann, U., Zuger, B. and Hollander, A.: Investigations into the pathogenesis of tetanus. II. The influence of section of the nerve on the neutralisation of intramuscularly injected tetanal toxin by circulating antitoxin. J. Immunol., 36: 485–488, 1939.
107. Fröhlich, A. and Meyer, H. H.: Untersuchungen über den Tetanus. Arch. exper. Path. u. Pharmakol., 79: 55–92, 1915.
108. Gaskell, W. H.: The involuntary nervous system. Longmans Green, London 1916.
109. Goldfarb, D. M. and Peisakhis, L. A.: The mechanism of action of bacterial toxins on the organism in sensitive and resistant types of animal. Uchen. Zap. Vtoroi Mosk. Gosud. Med. Inst. Imenis, 1: 217–222, 1951.
110. Goldman, L., Turner, T. B. and Stafford, E. S.: Protective action of tetanus toxoid unrelated to active immunisation in mice. Proc. Soc. Exper. Biol. & Med., 86: 545–548, 1954.
111. Goldscheider, A.: Wie wirkt das Tetanusgift auf das Nervensystem? Ztschr. klin. Med., 26: 175–189, 1894.
112. Göpfert, H. and Schaefer, H.: Ueber die Mechanik des Wundstarrkrampfes. Arch. exper. Path. u. Pharmakol., 197: 93–122, 1940.
113. Graham, R. and Eriksen, S.: Experimental botulism in dogs. J. Infect. Dis., 31: 402–406, 1922.
114. Graham, R. and Schwarze, H.: Avian botulism (Type A) or limberneck. J. Infect. Dis., 28: 317–322, 1921.
115. Grasset, E. and Zutendyk, A.: Immunological studies in reptiles and their relation to aspects of immunity in higher animals. Publ. S. Afr. Inst. med. Res., No. 29, 4: 377–460, 1931.
116. Guillaumie, M. and Kréguer, A.: Nouvelles recherches sur les hémolysines oxydables. Ann. Inst. Pasteur, 78: 467–480, 1950.
117. Guillaumie, M. and Kréguer, A.: Contribution à l'étude des hémolysines bactériennes: propriétés des toxines botuliniques des Types C et D. Compt. rend. Soc. biol., 145: 179–182, 1951.
118. Gumprecht, F.: Versuche über die physiologischen Wirkungen des Tetanusgiftes im Organismus Ar.ch. ges. Physiol., 59: 105–152, 1894.
119. Gunnison, J. B. and Coleman, G. E.: *Clostridium botulinum*, Type C, associated with Western Duck Disease. J. Infect. Dis., 51: 542–551, 1932.
120. Gunnison, J. B. and Meyer, K. F.: Susceptibility of monkeys, goats and small animals to oral administration of botulinum toxin, Types B, C and D. J. Infect. Dis., 46: 335–340, 1930.
121. Gutton, A. C. and MacDonald, M. A.: Physiology of botulinus toxin. Arch. Neurol. & Psychiat., 57: 578–592, 1947.
122. Haerem, S., Dack, G. M., and Dragstedt, L.: Acute intestinal obstruction. II. The permeability of obstructed bowel segments of dogs to *Clostridium botulinum* toxin. Surgery, 3: 339–350, 1938.
123. Haines, R. B.: The occurrence of toxigenic anaerobes, especially *Clostridium botulinum*, in some English soils. J. Hyg., 42: 323–327, 1942.

124. HALL, I. C.: The occurrence of bacillus botulinus, Types A and B, in accidental wounds. J. Bact., 50: 213–217, 1945.
125. HALTER, K.: Untersuchungen über die Auswertung und die Wirkungsweise des Tetanus toxins. Ztschr. Hyg. InfektKr., 118: 245–262, 1936.
126. HAMPSON, C. R.: Note: a case of probable botulism due to wound infection. J. Bact., 61: 647, 1951.
127. HANKE, M. E. AND BAILEY, J. H.: Oxidation-reduction potential requirements of *Clostridium welchii* and other clostridia. Proc. Soc. Exper. Biol. & Med., 59: 163–166, 1945.
128. HARRIS, C.: The morphology of the myoneural junction as influenced by neurotoxic drugs. Am. J. Path., 30: 501–519, 1954.
129. HARVEY, A. M.: The peripheral action of tetanus toxin. J. Physiol., 96: 348–365, 1939.
130. HORSTER, H. AND WHITMAN, L.: Die Methode der intraneuralen Injektion. Ztschr. Hyg. InfektKr., 113: 113–124, 1931–32.
131. HUMPHREYS, F. B.: Anaerobic bacteria: Tetanus. In: Gay, F. P.: Agents of disease and host resistance, p. 864. Thomas, New York 1935.
132. HUTTER, O. F.: Effect of denervation on development of local tetanus. J. Physiol., 112: 1P, 1951.
133. IPSEN, J.: Comparison of tetanus test toxins prepared by seven institutes from the same strain and by the same method. Bull. Hlth Org., L.o.N., 9: 453–475, 1940–41.
134. IPSEN, J.: Die Auswertung des direkten Giftwertes des Tetanusgiftes als Beispiel der biomathematischen Ausnutzung der Absterbedauer. Arch. exper. Path. u. Pharmakol., 197: 536–549, 1941.
135. IPSEN, J.: Contributions to the theory of biological standardisation. Munksgaard, Copenhagen 1941, part III.
136. JAQUET, J. AND PRÉVOT, A. R.: Recherches sur le botulisme équin expérimental. Ann. Inst. Pasteur, 81: 334–336, 1951.
137. JENSEN, L. B.: Some influences of antitoxic and other serums on botulinum intoxication. J. Infect. Dis., 39: 413–423, 1926.
138. JUDE, A., GIRARD, P. AND CARRAT, P.: Action destructrice, *in vivo*, de certains agents chimiques sur les toxines botulique et tétanique. Compt. rend. Soc. biol., 143: 318–319, 1949.
139. KEGELES, G.: The molecular size and shape of botulinus toxin. J. Am. Chem. Soc., 68: 1670, 1946.
140. KEPPIE, J.: The pathogenicity of the spores of *Clostridium botulinum*. J. Hyg., 49: 36–45, 1951.
141. KITASATO, S.: Ueber den Tetanusbacillus. Ztschr. Hyg. InfektKr., 7: 225–233, 1889.
142. KITASATO, S.: Experimentelle Untersuchungen über das Tetanusgift. Ztschr. Hyg. InfektKr., 10: 267–305, 1891.
143. KNIGHT, B. C. J. G. AND FILDES, P.: Oxidation reduction studies in relation to bacterial growth. III. The positive limits of oxidation-reduction potential required for the germination of *Bacillus tetani* spores *in vitro*. Biochem. J., 24: 1496–1502, 1930.
144. KNOX, W. C. AND ENDICOTT, F. C.: $I^{131}$ as an antigen label in the circulating serum on non-immune rabbits. J. Immunol., 65: 523–528, 1950.
145. KROGH, A.: Croonian Lecture: The active and passive exchanges of inorganic ions through the surfaces of living cells and through living membranes generally. Proc. Roy. Soc. London, s.B., 133: 140–200, 1946.
146. LAMANNA, C.: Hemagglutination by botulinal toxin. Proc. Soc. Exper. Biol. & Med., 69: 332–336, 1948.
147. LAMANNA, C. AND DOAK, F. W.: The antigen-antibody reaction with Type A botulinal toxin. J. Immunol., 59: 231–247, 1948.
148. LAMANNA, C., EKLUND, H. W. AND MCELROY, O. E.: Botulinum toxin (Type A); including a study of shaking with chloroform as a step in the isolation procedure. J. Bact., 52: 1–13, 1946.
149. LAMANNA, C. AND GLASSMAN, H. N.: The isolation of Type B botulinum toxin. J. Bact., 54: 575–584, 1947.
150. LAMANNA, C. AND LOWENTHAL, J. P.: The lack of identity between hemagglutinin and the toxin of Type A botulinal organism. J. Bact., 61: 751–752, 1951.
151. LAMANNA, C., MCELROY, O. E. AND EKLUND, H. W.: The purification and crystallization of *Clostridium botulinum* Type A toxin. Science, 103: 613–614, 1946.
152. LAMONT, A., FIBOR, W. M. AND SHUMACKER, H. B. JR.: The "lethal" dose of toxin in experimental tetanus. Bull. Johns Hopkins Hosp., 67: 25–40, 1940.
153. LAWS, J. O. AND WRIGHT, G. PAYLING: The disposal and organ distribution of radioiodinated bovine serum protein in control and specifically sensitised rabbits. Brit. J. Exper. Path., 33: 343–353, 1952.
154. LEGROUX, R., JÉRAMEC, C. AND LEVADITI, J.-C.: Statistique du botulisme de l'occupation, 1940–1944. Bull. Acad. Méd., 129: 643–645, 1945.
155. LEGROUX, R., JÉRAMEC, C. AND LEVADITI, J.-C.: Traitement du botulisme aigu expérimental au moyen de la sérothérapie et du 'poumon acier'. Bull. Mém. Soc. Méd. Hôp. Paris, 60: 432–433, 1944.
156. LEGROUX, R. AND LEVADITI, J.-C.: Degré d'inhibition du pouvoir pathogéne des toxines botuliques après contact avec la substance nerveuse. Ann. Inst. Pasteur, 72: 480–484, 1946.
157. LEGROUX, R., LEVADITI, J.-C. AND JÉRAMEC, C.: Influence des voies d'introduction de la toxine sur le botulisme expérimental du lapin. Ann. Inst. Pasteur, 71: 490–493, 1945.
158. LEGROUX, R., LEVADITI, J.-C. AND LAMY, R.: Le botulisme expérimental du cheval et la question du botulisme naturel. Ann. Inst. Pasteur, 72: 545–552, 1946.
159. LEIGHTON, G. AND BUXTON, J. B.: The distribution of *Bacillus botulinus* in Scottish soils. J. Hyg., 28: 79–82, 1928–29.
160. LEMÉTAYER, E., NICOL, L., GIRARD, O., CORVAZIER, R. AND CHEYROUX, M.: Recherches expérimentales sur les interactions du sérum, de l'anatoxine, de la toxine spécifique et des tissus dans le traitement préventif et curatif de l'intoxication tétanique. Ann. Inst. Pasteur, 79: 672–710, 1950.

161. Lemétayer, E., Raynaud, M., Nicol, L. and Turpin, A.: Action préventive spécifique précoce de l'anatoxine tétanique employée à doses massives. Ann. Inst. Pasteur, 87: 1–24, 1954.
162. Leuchs, J.: Beiträge zur Kenntnis des Toxins und Antitoxins des *Bacillus botulinus*. Ztschr. Hyg. InfektKr., 65: 55–84, 1910.
163. Liljestrand, G. and Magnus, R.: Über die Wirkung des Novokains auf den normalen und den tetanusstarren Skeletmuskel und über die Entstehung der lokalen Muskelstarre beim Wundstarrkrampf. Arch. ges. Physiol., 176: 168–208, 1919.
164. Linder, F.: Tetanus. In: Handbuch der inneren Medisin, ed. by v. Bergman, Frey and Schwiegk, p. 243. Springer, Berlin, 4th Ed., 1952, vol. 2, pt. 1.
165. Littauer, U.: Observations on the Type A toxin of *Clostridium botulinum*. Nature, London, 167: 994–995, 1951.
166. Lloyd, D. P. C.: Neuron patterns controlling transmission of ipselateral hind limb reflexes in cat. J. Neurophysiol., 6: 293–315, 1943.
167 Lloyd, D. P. C.: Conduction and synaptic transmission of the reflex response to stretch in spinal cats. J. Neurophysiol., 6: 317–326, 1943.
168. Lowenthal, J. P. and Lamanna, C.: Factors affecting the botulinal hemagglutination reaction and the relationship between hemagglutinating activity and toxicity of toxin preparations. Am. J. Hyg., 54: 342–353, 1951.
169. McClaskey, G. W : *Bacillus botulinus* poisoning: with a report of seven cases, four of which proved fatal. Am. J. Med. Sc., 158: 57–67, 1919.
170. McClintock, C. T. and Hutchings, W. H.: The treatment of tetanus. J. Infect. Dis., 13: 309–330, 1913.
171. MacIntosh, F. C.: The distribution of acetylcholine in the peripheral and the central nervous system. J. Physiol., 99: 436–442, 1941.
172. Mackereth, M. B. and Scott, D. J.: Local tetanus in the rat diaphragm. Proc. Univ. Otago Med. Sch., 32: 13–14, 1954.
173. Madsen, T.: Tetanusgift im Serum eines diphtherieimmunisierten Pferdes 5 Tage vor dem Ausbruch von Tetanus. Zentralbl. Bakt. (Orig.), 46: 276–278, 1908.
174. Marinesco, G.: La cellule nerveuse. Doin, Paris 1909, vol. 2, p. 323 *et seq.*
175. Martini, E., Torda, C. and Zironi, A.: The effect of tetanus toxin on the cholinesterase activity of the muscles of rats. J. Physiol., 96: 163–171, 1939.
176. Masland, R. L. and Gammon, G. D.: The effect of botulinus toxin on the electrogram. J. Pharmacol. & Exper. Therap., 97: 499–506, 1949.
177. Metchnikoff, E.: Recherches sur l'influence de l'organisme sur les toxines. Ann. Inst. Pasteur, 11: 801–809, 1897.
178. Metchnikoff, E.: Recherches sur l'influence de l'organisme sur les toxines. Influence du systeme nerveux sur la toxine tétanique. Ann. Inst. Pasteur, 12: 81–90, 1898.
179. Meyer, K. F.: Botulismus. In: Handbuch der pathogenen Mikroorganismen, ed. by Kolle, W. and Wassermann, A. v., vol. IV, pp. 1309–1364. Fischer, Jena; Urban & Schwarzenberg, Berlin, 3rd ed., 1928.
180. Meyer, K. F. and Dubovsky, B. J.: The distribution of spores of *Bacillus botulinus* in the United States. J. Infect. Dis., 31: 559–594, 1922.
181. Meyer, K. F. and Dubovsky, B. J.: The distribution of spores of *Bacillus botulinus* in the Territory of Alaska and the Dominion of Canada. J. Infect. Dis., 31: 595–599, 1922.
182. Meyer, K. F. and Dubovsky, B. J.: The occurrence of spores of *Bacillus botulinus* in Belgium, Denmark, England, the Netherlands and Switzerland. J. Infect. Dis., 31: 600–609, 1922.
183. Meyer, K. F. and Dubovsky, B. J.: The occurrence of spores of *Bacillus botulinus* in the Hawaiian Islands and China. J. Infect. Dis., 31: 610–613, 1922.
184. Meyer, K. F. and Eddie, B.: Fifty years of botulism in the United States and Canada. George Willams Hooper Foundation, University of California Medical Centre, California 1950.
185. Meyer, H. and Ransom, F.: Untersuchungen über den Tetanus. Arch. exper. Path. u. Pharmakol., 49: 369–416, 1903.
186. Michon, P., Heully, F., Lesure, J. and Delestre, J.: A propos du botulisme. Étude de quelques troubles humoraux et de la dysphagie. Bull. Mém. Soc. Méd. Hôp. Paris, 62: 377–380, 1946.
187. Morgenroth, J.: Tetanus des Frosches. Arch. internat. pharmacodyn., 7: 265–282, 1900.
188. Mounier-Kuhn, P., Monnet, P., Moinecourt, J. and Favre-Gilly, J.: Les troubles de la déglutition au cours du botulisme. Dysphagie simple et paralysie de l'oesophage. J. Méd. Lyon, 27: 629–632, 1946.
189. Mouquin, M., Bastin, M. and Lefevre, M.: Un cas de guérison de botulisme aigu grave traité par la respiration artificielle (poumon acier). Bull. Mém. Soc. Méd. Hôp. Paris, 60: 430–433, 1944.
190. Muttermilch, S., Belin, M. and Salomon, E.: Technique permettant de déterminer la toxicité réelle de la toxine tétanique. Compt. rend. Soc. biol., 114: 1005–1008, 1933.
191. Nicholas, J.: Sur un cas de tétanos chez l'homme par inoculation accidentelle des produits soluble du Bacillus de Nicolaier. Compt. rend. Soc. biol., 5: 844–846, 1893.
192. Nicolaïer, A.: Ueber infectiösen Tetanus. Deutsche Med. Wchnschr., 10: 842–844, 1884.
193. Oakley, C. L.: Gas gangrene. Brit. Med. Bull., 10: 52–58, 1954.
194. Orr, P. F.: Studies on bacillus botulinus. I. Destruction of botulinus toxin by heat. J. Med. Res., 42: 127–136, 1920–21.
195. Owen, C. R., Langohr, J. L. and Blakely, E.: Adaptations of the Macfarlane lecitho-vitellin test. J. Path. & Bact., 59: 261–266, 1947.
196. Parry, E. W.: Prevalence of *Clostridium botulinum* in soils in central New York State. Food Res., 11: 203–209, 1946.
197. Pelloja, M.: Le tétanos expérimental par la toxine tétanique. Masson, Paris 1951.

198. Penfield, W. G.: Alterations of the Golgi apparatus in nerve cells. Brain, 43: 290-305, 1920.
199. Perdrup, A.: Electromyographic investigations on the mode of action of tetanus toxin. Acta pharmacol. et toxicol. 2: 121-137, 1946.
200. Permin, C.: Experimentelle und klinische Untersuchungen über die Pathogenese und Therapie des Starrkrampfes. Mitt. Grensgeb. Med. Chir., 27: 1-21, 1913-14.
201. Petrie, G. F.: Observations on the variable interactions of tetanus toxins and antitoxins. Bull. Hlth Org., L.o.N., 10: 113-143, 1942-43.
202. Pickett, M. J., Hoeprich, P. K. and Germain, R. O.: Purification of high titre tetanus toxin. J. Bact., 49: 515-518, 1945.
203. Pillemer, L.: The immunochemistry of toxins and toxoids. I. The solubility and precipitation of tetanal toxin and toxoid in methanol-water mixtures under controlled conditions of pH, ionic strength and temperature. J. Immunol., 53: 237-250, 1946.
204. Pillemer, L. and Moore, D. H.: The spontaneous conversion of crystalline tetanal toxin into a flocculating atoxic dimer. J. Biol. Chem., 173: 427-428, 1948.
205. Pillemer, L. and Robbins, K. C.: Chemistry of toxins. Ann. Rev. Microbiol., 3: 265-288, 1949.
206. Pillemer, L. and Wartman, W. B.: The clinical behaviour, incubation period, and pathology of tetanus induced in white swiss mice by injections of crystalline tetanal toxin. J. Immunol., 55: 277-281, 1947.
207. Pillemer, L., Wittler, R. G., Burrell, J. I. and Groesberg, D. B.: The immunochemistry of toxins and toxoids. VI. The crystallisation and characterisation of tetanal toxin. J. Exper. Med., 88: 205-221, 1948.
208. Pillemer, L., Wittler, R. G. and Groesberg, D. B.: The isolation and crystallisation of tetanal toxin. Science, 103: 615-616, 1946.
209. Pochhammer, C.: Experimentelle Untersuchungen über die Entstehung des Starrkrampfes und die Wirkung des Tetanustoxins im menschlichen und tierischen Organismus. Samml. Klin. Vortr., No. 520, Leipsig 1909.
210. Prévot, A. R.: Rapport d'introduction du président du sous-committé *Clostridium* pour l'unification de la nomenclature des Types toxigènes de *Clostridium botulinum*. Internat. Bull. Bact. Nomen. Taxon., 3: 120-123, 1953.
211. Prévot, A. R. and Beygoo, E. R.: Étude de la première souche française de *Clostridium botulinum* D. Ann. Inst. Pasteur, 78: 274-276, 1950.
212. Prévot, A. R. and Beygoo, E. R.: Nouvelles recherches sur le botulisme et ses cinq types toxiniques. Ann. Inst. Pasteur, 85: 544-575, 1953.
213. Pulaski, E. J., Melenet, F. L. and Spaeth, W. L. C.: Bacterial flora of acute traumatic wounds. Surg., Gynec. & Obst., 72: 983-988, 1941.
214. Putnam, F. W., Lamanna, C. and Sharp, D. G.: Molecular weight and homogeneity of crystalline botulinus toxin, Type A. J. Biol. Chem., 165: 735-736, 1946.
215. Putnam, F. W., Lamanna, C. and Sharp, D. G.: Physicochemical properties of crystalline *Clostridium botulinum* Type A toxin. J. Biol. Chem., 176: 401-412, 1948.
216. Quortrup, E. R. and Holt, A. L.: Detection of potential botulinus-toxin producing areas in western duck marshes with suggestions for control. J. Bact., 41: 363-372, 1941.
217. Quortrup, E. R. and Sudheimer, R. L.: Some ecological relations of *Pseudomonas aeruginosa* to *Clostridium botulinum* Type C. J. Bact., 45: 551-554, 1943.
218. Ramon, G., Lemétayer, E. and Nicol, L.: De l'instabilité des dilutions de toxine tétanique. L'influence de l'agitation sur les propriétés du poison tétanique en solution dans l'eau physiologique. Compt. rend. Soc. biol., 127: 1060-1062, 1938.
219. Ramon, G., Lemétayer, E., Richou, R. and Nicol, L.: Des causes d'erreur dans la détermination, chez l'animal d'expérience, de l'activité de la toxine tétanique. Compt. rend. Soc. biol., 127: 1157-1160, 1938.
220. Ramon, G. and Richou, R.: Sur le titrage des toxines, des anatoxines, et des antitoxines diphtérique, tétanique et staphylococcique. Rev. Immunol., 14: 161-194, 1950.
221. Ransom, F.: Die Lymphe nach intravenöser Injektion von Tetanustoxin und Tetanusantitoxin. Ztschr. physiol. Chem., 29: 349-373, 1900.
222. Ransom, F.: Weiteres über die Lymphe nach Injektion von Tetanusgift. Ztschr. physiol. Chem., 29: 553-567, 1900.
223. Ranson, S. and Ranson, S. W.: Recovery from myostatic contracture caused by tetanus toxin. Arch. Path., 7: 949-954, 1929.
224. Ranson, S. W.: Local tetanus: study of muscle tonus and contracture. Arch. Neurol. & Psychiat., 20: 663-701, 1928.
225. Ranson, S. W. and Morris, A. W.: Studies on muscle tonus. V. Tetanus contracture. J. Comp. Neurol., 42: 99-111, 1926.
226. Raynaud, M., Lemétayer, E., Turpin, A., Nicol, L. and Rouyer, M.: Installation rapide d'un état de résistance à l'intoxication tétanique expérimentale par l'influence de doses massives d'anatoxine tétanique. Compt. rend. Acad. Sc., 233: 586-588, 1951.
227. Raynaud, M. and Wright, E. A.: Rapid specific preventive action of tetanus toxoid. Nature, London, 171: 797, 1953.
228. Ricketts, H. T. and Kirk, E. J.: The adjuvant action of serum, egg albumin and broth on tetanus intoxication. J. Infect. Dis., 3: 116-127, 1906.
229. Romanes, G. J.: The motor cell columns of the lumbo-sacral spinal cord of the cat. J. Comp. Neurol., 94: 313-363, 1951.
230. Rosebury, T. and Kabat, E. A.: Bacterial warfare: A critical analysis of the available agents, their possible military applications, and the means for protection against them. J. Immunol., 56: 7-96, 1947.

231. Roux, E. and Borrel, A.: Tétanos cérébral et immunité contre le tétanos. Ann. Inst. Pasteur, 12: 225-239, 1898.
232. Rowson, K. E. K. and Morgan, R. S.: The absorption of bovine albumin in rabbits after its intramuscular and subcutaneous injection. J. Path. & Bact., 68: 623-626, 1954.
233. Sawamura, S.: Experimentelle Studien zur Pathogenese und Serumtherapie des Tetanus. Arb. Inst. InfektKr., Bern, 4: 1-103, 1909.
234. Schaefer, H.: Weitere Untersuchungen zum Mechanismus und zur Therapie des Wundstarrkrampfes. Arch. exper. Path. u. Pharmakol., 203: 50-84, 1944.
235. Schoenholz, P. and Meyer, K. F.: Effect of direct sunlight, diffuse daylight and heat on potency of botulinum toxin in culture mediums and vegetable products. J. Infect. Dis., 35: 361-389, 1924.
236. Schübel, K.: Über das Botulinustoxin. Deutsche med. Wchnschr., 47: 1047, 1921.
237. Schübel, K.: Über das Botulinustoxin. Arch. exper. Path. u. Pharmakol., 96: 193-259, 1923.
238. Secker, J.: The chemical agent in the sympathetic control of retraction of the nictitating membrane of the cat. J. Physiol., 89: 296-308, 1937.
239. Sherrington, C. S.: The integrative action of the nervous system. Yale Univ. Press, New York 1906, pp. 112 and 303.
240. Shumacker, H. B. Jr., Lamont, A. and Fibor, W. M.: The reaction of "tetanus sensitive" and "tetanus resistant" animals to the injection of tetanal toxin into the spinal cord. J. Immunol., 37: 425-433, 1939.
241. Simpson, J. Y.: An account of 26 cases of obstetrical and puerperal tetanus. Month. J. Med. Sc., 18: 97-119, 1854.
242. Smith, L. D.: Clostridia in gas gangrene. Bact. Rev., 13: 233-254, 1949.
243. Smith, M. L.: Note on the complexity of tetanus toxin. Bull. Hlth Org., L.o.N., 10: 104-112, 1942-43.
244. Solomon, A. K.: Equations for tracer experiments. J. Clin. Invest., 28: 1297-1307, 1949.
245. Sommer, E. W. and Sommer, H.: Studies on botulinus toxin: 5. Influence of nitrogenous and lipoid compounds on the potency of botulinum toxin. J. Infect. Dis., 43: 496-506, 1928.
246. Sommer, H.: Purification of botulinus toxin. Proc. Soc. Exper. Biol. & Med., 35: 520-521, 1936-37.
247. Sterne, M.: Hemagglutination by *Clostridium botulinum* Type D. Science, 119: 440-441, 1954.
248. Sterne, M. and Wentzel, L. M.: A new method for the large-scale production of high-titre botulinum formol toxoid Types C and D. J. Immunol., 65: 175-183, 1950.
249. Stover, J. H. Jr., Fingerman, M. and Forester, R. H.: Botulinum toxin and the motor end-plate. Proc. Soc. Exper. Biol. & Med., 84: 146-147, 1953.
250. Teale, F. H. and Embleton, D.: Studies in infection. II. The paths of spread of bacterial exotoxins with special reference to tetanus toxin. J. Path. & Bact., 23: 50-68, 1919-1920.
251. Theiler, A., Viljoen, P. R., Green, H. H., Dutoit, P. J., Meier, H. and Robinson, E. M.: Lamsiekte (parabotulism) in cattle in South Africa. 11th and 12th Rep., Vet. Res. S. Africa, part ii, 819-1361, 1927.
252. Thomas, C. G. Jr., Kelleher, M. F. and McKee, A. P.: Botulism, a complication of *Clostridium botulinum* wound infection. Arch. Path., 51: 623-628, 1951.
253. Thomas, G. A. and Wright, G. Payling: Personal observations.
254. Tizzoni, G. and Cattani, G.: Ricerche batteriologiche sul tetano. Riforma med., 5: 512-513, 1889.
255. Tizzoni, G. and Cattani, G.: Über das Tetanusgift. Zentralbl. Bakt., 8: 69-73, 1890.
256. Tizzoni, G. and Cattani, G.: Untersuchungen über das Tetanusgift. Arch. exper. Path. u. Pharmakol., 27: 432-450, 1890.
257. Torda, C. and Wolff, H. G.: On the mechanism of paralyses resulting from toxin of *Clostridium botulinum*. J. Pharmacol. & Exper. Therap., 89: 320-324, 1947.
258. Traub, F. B., Hollander, A. and Friedemann, U.: The potentiation of tetanus toxin by broth and serum. J. Bact., 52: 169-177, 1946.
259. Turner, A. W. and Rodwell, A. W.: The epsilon-toxin of *Clostridium welchii* Type D. I. Proteolytic conversion of epsilon-prototoxin into epsilon-toxin by trypsin and other proteases. Austral. J. Exp. Biol. Med. Sci., 21: 17-25, 1943.
260. Turner, A. W. and Rodwell, A. W.: The epsilon-toxin of *Clostridium welchii* Type D. II. Mechanism of its development in cultures through the action of extracellular proteinases on epsilon-prototoxin. Australian J. Exper. Biol. & Med. Sc., 21: 27-36, 1943.
261. Turpin, A., Raynaud, M. and Rouyer, M.: Purification de la toxine et de l'anatoxine tétanique. Ann. Inst. Pasteur, 82: 299-309, 1952.
262. Vaillard, L. and Vincent, H.: Contributions à l'étude du tétanos. Ann. Inst. Pasteur, 5: 1-39, 1891.
263. Van Ermengem, E.: Über einen neuen anaëroben Bacillus und seine Beziehungen zum Botulismus. Ztschr. Hyg. InfektKr., 26: 1-56, 1897.
264. Van Ermengem, E.: Contributions à l'étude des intoxications alimentaires. Arch. internat. pharmacodyn., 3: 213-350 and 499-601, 1897.
265. Van Ermengem, E.: Der Bacillus botulinus und der Botulismus. In: Handbuch der pathogenen Mikroorganismen, ed. by Kolle, W. & Wassermann, A. v., vol. IV, p. 909. Fischer, Berlin, 2nd. ed., 1912.
266. Van den Hoven van Genderen, A. J.: Die Empfindlichkeit von Meerschweinchen und Kaninchen für intravenös und intraserebral injisiertes Diphtherie- und Tetanustoxin. Zentralbl. Bakt. (Orig.), 128: 129-138, 1933.
267. Verge, J.: Toxi-infections animales et alimentation. Le botulisme. Rec. Méd. Vét. École Alfort, 127: 767-828, 1951.
268. Wagman, J.: Isolation and sedimentation study of the low molecular weight forms of Type A botulinus toxin. Arch. Biochem., 50: 104-112, 1954.

269. WAGMAN, J. AND BATEMAN, J. B.: Botulinum Type A toxin: properties of a toxic dissociation product. Arch. Biochem., 45: 375–383, 1953.
270. WAGNER, E., MEYER, K. F. AND DOZIER, C. C.: Studies on the metabolism of *Bacillus botulinus* in various media. XXVI. J. Bact., 10: 321–412, 1925.
271. WALKER, A. B.: The relation between "Grass Disease" of horses and botulism. Brit. J. Exper. Path., 10: 352–360, 1929.
272. WARRINGTON, W. B.: On the structural alterations observed in nerve cells. J. Physiol., 23: 112–129, 1898–99.
273. WARRINGTON, W. B.: Note on the ultimate fate of ventral cornual cells after section of a number of posterior roots. J. Physiol., 30: 503–506, 1904.
274. WELLS, H. S., YOUMANS, J. B. AND MILLER, D. G. JR.: Tissue pressure (intracutaneous, subcutaneous and intramuscular) as related to venous pressure, capillary filtration and other factors. J. Clin. Invest., 17: 489–499, 1938.
275. WENTZEL, L. M., STERNE, M. AND POLSON, A.: High toxicity of pure botulinum Type D toxin. Nature, London, 166: 739–740, 1950.
276. WERLE, E. AND STÜTTGEN, G.: Zur Kenntnis der Cholinesterase des Blutserums. Klin. Wchnschr., 21: 821–822, 1942.
277. WIELOCKI, G. B. AND PUTNAM, T. J.: Note on the anatomy of the *areae postremae*. Anat. Rec., 19: 281–285, 1920.
278. WOLTERS, K. L. AND FISCHOEDER, E.: Über die Bindung von Tetanustoxin an Hirnsubstanz ohne und nach Vorbehandlung mit Tetanustoxoid. Ztschr. Hyg. InfektKr., 139: 541–544, 1954.
279. WRIGHT, E. A.: The effect of the injection of tetanus toxin into the central nervous system in rabbits. J. Immunol., 71: 41–44, 1953.
280. WRIGHT, E. A.: The fixation of tetanus toxin to the brain stem of guinea-pigs. J. Path. & Bact., 68: 131–135, 1954.
281. WRIGHT, E. A., MORGAN, R. S. AND WRIGHT, G. PAYLING: The rate of escape of proteins following their injection into the anterior chamber of the rabbit's eye. J. Path. & Bact., 64: 461–465, 1952.
282. WRIGHT, E. A., MORGAN, R. S. AND WRIGHT, G. PAYLING: Tetanus intoxication of the brain stem in rabbits. J. Path. & Bact., 62: 569–583, 1950.
283. WRIGHT, E. A., MORGAN, R. S. AND WRIGHT, G. PAYLING: The movements of toxin in the nervous system in experimental tetanus in rabbits. Brit. J. Exper. Path., 32: 169–182, 1951.
284. WRIGHT, E. A., MORGAN, R. S. AND WRIGHT, G. PAYLING: The site of action of the toxin in local tetanus. Lancet 263: 316–319, 1952.
285. WRIGHT, G. PAYLING: Nerve trunks as pathways in infection. Proc. Roy. Soc. Med., 46: 19–30, 1953.
286. ZUGER, B., HOLLANDER, A. AND FRIEDEMANN, U.: Potentiation of tetanus toxin by tissues and chemicals. J. Infect. Dis., 65: 86–91, 1939.
287. ZUPNIK, L.: Die Pathogenese des Tetanus. Deutsche med. Wchnschr., 31: 1999–2004, 1905.