THE ANTIGENIC PROPERTIES OF *CLOSTRIDIUM BOTULINUM* TYPE E TOXOIDS

by

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ABSTRACT

The conditions responsible for the preparation of crude, activated and purified toxins of Clostridium botulinum Type E and the antigenic properties of toxoids prepared from these toxins, are described.

Optimum toxin production and toxin activation are seen to be critically dependent on conditions such as hydrogen-ion concentration, and incubation time and temperature.

Immunization of human volunteers with the formolized toxoids demonstrated that the highest level of immunity occurred in those individuals who received the crude toxoids.

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I. The Problem and Definition of Terms Used.

Clostridium botulinum species, responsible for botulinum food poisoning in man and animals, give rise to five different types of toxin, designated A, B, C, D and E, each of which has been shown to be serologically distinct.

Dolman, in 1956, reported the variation of type E strains into sporolytic, proteolytic and toxigenic variants (designated "O", "T" and "OT" respectively), capable of isolation and propagation in pure cultures.

The further observation by Dolman, that toxin potency could be increased by the addition of proteolytic variants, has led to intensive research on the antigenicity of these "activated" products.

1. The Problem

(a) Statement of the problem. It is the purpose of this investigation to study the antigenic properties of Clostridium botulinum type E toxins and toxoids in animals and man, with the end result being the preparation of a toxoid suitable for human immunization.

(b) Importance of the Investigation. The discovery that a relatively low potency toxin such as type E could be activated to a potency of 300 to 300,000-fold led to the preparation of toxoids from these activated products, the assumption being that antigenicity would increase with potency. The importance of this investigation lay in determining quantitatively the immunity conferred in animals and man by the injection of crude, trypsin-activated, and purified toxoids.

During the course of animal immunization a non-toxic, proteolytic variant (T) was seen to produce a low, but definite, immunity against the

crude and activated toxins. This observation was considered important enough to warrant further investigation, and was included in the programme of human immunization.

2. Organization of the Remainder of the Thesis.

The remainder of the thesis is arranged to cover a review of the literature, experimental studies, discussion and summary. Since there were many aspects of the study, i.e. minimal lethal dose determinations, purification techniques, optimum temperature and hydrogen ion concentration for growth etc., certain of these aspects will be discussed separately for the sake of clarity. The final discussion will be reserved for the more general issue of antigenicity.

II. Introduction.

It is remarkable that, with the exception of the diphtheria bacillus, the organisms forming powerful exotoxins belong almost entirely to the group of anaerobic spore-forming bacilli. Two of these, Clostridium botulinum and Clostridium tetani, give rise to toxins more potent than any other substances with which we are acquainted. Table I lists the diseases, most of them toxaemic, that are associated with some species of Clostridia (1).

Table I.

Organism	Disease
Cl. botulinum, types A-E	Botulism in man and animals
Cl. chauvoei	Blackleg in cattle
Cl. haemolyticum	Bacillary haemoglobinuria (red water) in cattle.
Cl. histolyticum	Sometimes associated with other clostridia in gas gangrene in man.
Cl. oedematiens type A	Gas gangrene in man
" " " " B	Black disease and bradsot of sheep.
" " " " C	Bacillary osteomyelitis of buffaloes.
Cl. septicum	Blackleg and braxy of sheep; gas gangrene in man.
Cl. tetani	Tetanus.
Cl. welchii type A	Gas gangrene in man.
" " " B	Lamb dysentery.
" " " C	"Struck" of sheep.
" " " D	Infectious enterotoxaemia of sheep.
" " " F	Enteritis necroticans in man

Study of the anaerobic spore formers, before the war of 1914-18, was undertaken fitfully and by imperfect methods: much attention had been paid to their pathogenicity, but little to their general biological characteristics. There was confusion in nomenclature, and many organisms with the same name belonged to

different species. The only two about which no doubt existed were those that formed a highly potent toxin, namely Cl. tetani and Cl. botulinum. With the development after the war of new techniques, such as the use of the McIntosh-Fildes anaerobic jar, the obscurity around this group began to disperse.

The earlier work of Fildes (2), Fildes and Knight (3), Pappenheimer (4), and Stickland (5), on the essential nutrients of the clostridia and their methods of utilization has been developed to the point where it is clear that the majority of the pathogenic clostridia are heterotrophs, requiring a large number of amino-acids, vitamins, and carbohydrates for growth in artificial media. The energy-producing mechanisms, especially of those clostridia that depend mainly on amino-acid breakdown for their energy, have been studied in detail by Gale (6), Clifton (7) and Guggenheim (8). Most clostridia grow best at 37°C., but there are exceptions.

Apart from their toxic activity many of the anaerobes, such as Cl. tetani, Cl. welchii and Cl. septicum, produce filterable haemolysins. Kerrin (9), in 1930, stated that atoxic strains of Cl. tetani produce as powerful a haemolysin as do the toxic strains, and that normal rabbit, horse and human serum have a very strong antihaemolytic effect. Fibrinolysins and leucocidins also may be produced by some species, as reported by Reed, Orr and Brown (10).

III. A Historical Review of the Literature

Through the pioneer work of the Belgian bacteriologist van Ermengem in 1895, the serious and often fatal form of food poisoning termed "botulism" (formerly known as allantiasis, ichthyosism, or Wurstvergiftung) is now known to result from the ingestion of a toxin produced by one of the five Types of Clostridium botulinum. The intoxication was originally attributed to the consumption of sausages, preserved fish and other prepared foods of animal origin which had become contaminated with some poison possessing distinct pharmacological properties. Later outbreaks however, especially those in the Western Hemisphere, have followed the consumption of home-canned vegetables as well as the aforementioned vehicles. Van Ermengem (22) conceived the disease to be a form of intoxication when he made an investigation of the Ellezelles outbreak near Ghent. He succeeded in not only isolating the organism for the first time, but also demonstrated its ability to liberate one of the most powerful exotoxins known.

This family of anaerobic spore-forming bacteria are inhabitants of soil, and stagnant water in which vegetation is decaying. In contrast with many other pathogenic clostridia, Clostridium botulinum is not a common inhabitant of the intestine of domestic animals (11). Meyer and Dubovsky (12, 13, 14, 15) have recorded the occurrence of these spores in soil samples taken from various kinds of cultivated and uncultivated land in North America, Western Europe and China.

In spite of this widespread distribution in soils, botulism spores, in contrast with those of tetanus and gas gangrene, fail to germinate readily in the tissues. A few instances of wound complications are mentioned in the literature and will be noted later.

Although botulism was first described in man, comparable neurological disorders were encountered in several species of domestic and wild animals early in the twentieth century. The same syndrome, with rapid onset and high mortality, suggested the occurrence of botulism in animals, and was soon established when toxic filtrates from cultures of clostridia from animals were prepared and studied immunologically. The most common of these naturally-occurring intoxications in animals are:- "lamziekte" amongst cattle in South Africa (16), and horses, cattle and sheep in Western Australia (17); a form of "meningo-encephalitis" of horses in France (18); "limber-neck" in fowl in many parts of the world (19); and a paralytic condition amongst wild ducks on reservations in the Western United States (20). 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 phosphorous deficiency in their ordinary diet (16). In France, botulism among horses is caused by ingestion of hay and silage which has been contaminated with the remains of small rodents and birds (21). Farmyard birds may become infected through eating the larvae of the "green bottle" and other blowflies which are often present on contaminated carcasses. Wild ducks become intoxicated through the consumption of marsh weeds growing in places in which oxygen-consuming micro-organisms, notably Pseudomonas aeruginosa, flourish and create the anaerobic conditions which makes possible the proliferation of the Clostridia (20).

Intoxication

In typical epidemics, a high proportion of the persons infected by consuming the food develop clinical botulism. About 18-24 hours or even longer after the meal, the affected persons develop a syndrome which begins with

muscular weakness and ocular disturbances of which diplopia, lack of power of accommodation, and loss of the light-reflex occur. Trouble in articulating and swallowing soon follow, brought about by weakness and inco-ordination of the pharyngeal muscles and partly by cessation of salivation. Gradually all the voluntary muscles weaken, and death generally takes place as a result of paralysis of the respiratory muscles. The heart often continues to beat after breathing has ceased. These symptoms and signs of intoxication have been described by McClasky (23).

Food Vehicles

Human cases, of which the history is known, result from foods which have been smoked, salted, spiced, or canned, allowed to stand for a period, and consumed without cooking. No cases are known to have been caused by the consumption of fresh foods, cooked or uncooked. In Europe most cases have been due to sausages, ham, spiced meats, game pastes, potted meats, and in Russia to salt fish. In every recorded instance these foods have been eaten without prior reheating. In the Western Hemisphere, on the other hand, most cases have been due to canned vegetables and fruits, such as olives, string beans, corn and peas. A few cases have also been due to cooked meat, cheese and fish (24). In the majority of cases the foods contaminated were noticeably spoiled. Cans often had swollen ends and on opening demonstrated gas bubbles. Meats such as ham were often paler and softer than normal. In some instances, however, foods have been reported as apparently well-preserved. More recently there has been a number of reports on the isolation of Type E botulism from fish, fish products and other marine life (25, 26, 27, 28, 29, 30, 31). This type does not seem peculiar to either the Western or Eastern Hemispheres and isolations have been reported from many points around the world. Food vehicles for Type E toxin will be discussed more fully under the heading "Type E Toxin".

Cell Pathogenicity

Orr (32) reported in 1922 that Clostridium botulinum could be recovered from the intestinal organs of animals which had been fed or injected with toxic cultures and toxin-free spores; and stated that cells of Clostridium botulinum were capable of proliferating and producing toxin in the body of a guinea pig. The presence of the toxin was demonstrated by precipitation as well as direct toxicity tests.

Clostridium botulinum as a complicating factor in wound infections, by growing with other anaerobes and producing toxin, was also reported by Hampson (33) and Mattman (34). These instances are exceedingly rare, but nevertheless should be anticipated.

Introduction to Types

Early in experimental studies of botulism it was found that the typical syndrome of the intoxication could be produced by injection of culture filtrates which, though identical in behaviour, could be separated and distinguished immunologically by the use of specific antisera (35). On the basis of cross-neutralization tests with antitoxin, five toxins, now termed Types A, B, C, D and E, have been identified, and the variant strains from which they are obtainable have been named.

Although the typing of the botulinum toxins is of importance both for immunological differentiation and relative pathogenicities for a wide range of animals, there are good reasons for believing that all five Types produce their toxicological effects through the same kind of tissue injury. Prévot and Brygoo (36) performed experiments in which they mixed appropriate fractional doses of botulinum toxin, using two, three or more different Types, and producing a single lethal dose. 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 Types for Animal Species

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 intoxication follows one common pattern. When these toxin Types are examined individually, however, their relative pathogenicities for species varies widely. Throughout the literature there are many observations concerning animal susceptibility, but changes in nomenclature during the past causes one to treat these findings with caution.

Values obtained by different workers are approximate, but nevertheless one may 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, fowl and pigeons are more refractory (36, 37, 38, 39, 40).

Since naturally-occurring botulism is primarily a food poisoning, a number of investigators have sought to compare the lethal dose of toxins when administered parenterally and by mouth. Figures obtained are quite variable: Gunnison and Meyer (41) and Gunnison with Coleman (42) compared the two for Types B, C and D toxin on mice, guinea pigs and rabbits, and found that for Type B toxin the two did not differ greatly. Using Types C and D toxins, however, the oral dose was from several hundred to several thousand times larger than that needed for injection. Differences of a similar kind were found for dogs by Graham and Erikson (43) for 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 prior to injection. Knowledge as to what extent toxin is destroyed by gastric juices and other agents within the alimentary canal is rather scarce. In general it seems there is wide variation from one set of experimental conditions to another.

Bronfenbrenner and Schlesinger (44) attempted to simulate gastric conditions by adding various quantities of hydrochloric acid and sodium hydroxide to broth containing Type A toxin, and found that between pH 2.3 and 5.0 there was little or no change in toxicity after 24 hours at 37°C. Only when pH values were increased did potencies fall. Nor did the addition of pepsin and trypsin diminish the toxicities of the solutions. Coleman (45), however, obtained different results when he repeated this experiment employing a highly purified preparation of Type A toxin. At the end of eight hours, the toxin at pH 1.5 had lost little toxicity while that at pH 6.5 had fallen to one-fifth, and after seventy-two hours, both preparations had declined to about one-tenth of their original potency; and solutions of pepsin and trypsin at pH values of 1.4 and 6.8 respectively, completely detoxified the solutions by the end of seventy-two hours. Littauer (46) points out the possibility that the presence of proteins and organic materials in the broth employed by Bronfenbrenner and Schlesinger may have protected the toxin from digestion by the enzymes.

Dach and Wood (47) and Dach and Gibbard (48) introduced Type A toxin into the upper ends of loops of dogs' and rabbits' intestines, which they perfused with oxygenated blood, to determine at what point in the alimentary canal the toxin is absorbed. They found little indication of absorption. Haerem, Dach and Drogstedt (49), and Coleman (45) also failed to demonstrate absorption in the ileum of dogs. Dach and Hoskins (50) demonstrated macacus monkeys to be very susceptible to Type A toxin by mouth, but doses of toxin introduced into the loops of the colon failed to produce botulism.

Resistance of Toxin to Destructive Agents

Many studies have been undertaken concerning the resistance of toxin and spores of Cl. botulinum species to heat and other agents. Spores of the organism are as a whole highly resistant, depending on type of culture media, pH, etc. Dolman (51) more recently reported that spores of Type E organisms exhibit very low thermal stability. In general, however, spores of Clostridium botulinum will withstand 100°C. for 3½ to 5 hours, although in 1920, Bigelow and Esty (52) reported strains which would withstand 100°C. for up to 22 hours. As mentioned previously, the pH of the medium has a marked influence on heat sterilization of spores.

Orr (53) 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, considering this in the light of today's knowledge, may have been a Type C or D toxin. Bengston (54) studied the thermal stability of Type C and recorded its greater resistance to heat. In general, Types A, B and E are relatively more thermolabile in that they are destroyed by exposure at 70°C. for a few minutes, while Types C and D are more resistant. Schoenholz and Meyer (55) demonstrated that the same Type of toxin 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.

Mechanism of Toxin Production

Dozier, Wagner and Meyer (56), studying the effect of glucose on biochemical activities, growth and toxin production of Cl. botulinum, reported that its presence stimulated early reproduction, maximum growth, and a greater content of amino acid nitrogen in 96 hours than when it was omitted.

However, the presence of glucose did not exert any effect on the production of toxin, indicating their results to be in harmony with the suggestion that the toxin was an autolytic product. Boroff, Raynaud and Prévot (57), by obtaining a toxin from the particulate bacteria, similar in all respects to that produced from cultures, demonstrated that the soluble toxin may derive from the bacterial cytoplasm. This evidence tends to fix the site for the formation of the exotoxin in the interior or on the surface of the organism. The view of these workers was that the toxic activity is due to relatively simple groupings or to a spatial configuration of these groupings on the molecule of bacterial protein; otherwise ease of detoxification, with substances having affinity for some specific grouping, or by oxidation, would not be possible. The unfolding and splitting of the toxin molecule may occur while under refrigeration, liberating in the process more of its toxic groupings. This may account for the observed augmentation in toxic activity, a phenomenon noted with crude extracts. These workers obtained toxin in colloidal and soluble forms. The soluble toxin (exotoxin) may be split off the colloidal particle by ultrasonic waves in the presence of catalase. They assumed the colloidal toxin to be a precursor of the soluble form. Boroff (58), studying the relation of autolysis to toxin production in Type D, noted that maximum amounts of toxin appear in the culture filtrates after ten days incubation. At this time the culture is past the logarithmic growth phase. The culture filtrate then contains very little, if any, toxin; yet if the cells are removed and cracked, large quantities of toxin are released. This lag period between maximum growth and toxin production, he suggested, was due to a required build-up of autolytic enzymes.

Site of Action of Toxin

The neurological character of the symptoms of botulism directed workers to seek evidence of injuries in the central nervous system. Many of these studies were based on autopsy records of patients who had died in some botulism outbreak. This work was later supplemented with observations on experimentally infected animals. Many complications arose in examination of material due to the slow death of the animal from respiratory failure and progressive anoxaemia. These complications confused the study of initial morphological characteristics of the toxic action. Cowdry and Nicholson (59) recognized many of these difficulties and observed the disorder to be essentially a "biochemical lesion".

Probably the most extensive studies concerning the action of botulinum toxin were performed by Dickson and Shevky (60, 61) who mapped the field of intoxication in both the viscera and skeletal muscle, and pointed out the similarities between the sites of action of botulinum toxin and acetylcholine. They demonstrated that once botulism had become established in dogs, cats and rabbits, progressive impairment developed in the vagus nerve which induces cardiac inhibition and evokes salivary secretion; in the nervus erigens which causes contraction of the bladder; and in the oculomotor nerve which constricts the pupil. These results led them to conclude that the toxin affects the autonomic nervous system and that impulses along these fibres become blocked at some point at the periphery. They also reported in their second paper the cause of the paralysis that develops in skeletal muscles as the intoxication advances. It was apparent that the weakness, so characteristic of intoxication in man and animals, was not due to loss of contractile power of the muscle fibres themselves, since they reacted as promptly and efficiently as normal

muscles when electrical stimuli were applied. A second observation demonstrated that the musculature paralysis was not due to the inability of the nerve fibres to conduct an electrical impulse. The most characteristic feature was revealed when they observed that when a threshold stimulus was applied the muscle of an intoxicated animal became fatigued very rapidly as compared to the same muscle of a normal animal. From all these observations they 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.

The possibilities offered by Cl. botulinum as an agent in bacterial warfare during the Second World War led to much further research on the mode of action of the toxin, especially at the myoneural junction in skeletal muscles. Earlier observers regarded this paralysis as similar to that which follows the injection of curare. Guyton and MacDonald (62) were among the first to draw attention to the points of difference in the action. By injecting a small amount of acetylcholine intra-arterially they demonstrated that a muscle paralysed by botulinum toxin would contract. This contraction could not be brought about through the action of curare.

The possibility that the release of acetylcholine at the junction might be impaired was examined by Burgen, Dickens and Zatman (40). By a unique apparatus they demonstrated that the amount of acetylcholine released in a paralysed muscle after stimulation is considerably smaller than when a normal muscle is employed. The manner in which botulinum toxin interferes with acetylcholine release at the neuromuscular junction after stimulation of the motor nerve has not as yet been reported.

The early studies on the paralytic action of botulinum toxin on the cholinergic components of the nervous system were made on animals suffering from generalized intoxications, and therefore disclosed chiefly the effect of the toxin on the post-ganglionic fibres. In studies on local tissue intoxication in the region of the ciliary and superior cervical sympathetic ganglia, Ambache (63) has shown that the toxin is also injurious to the pre-ganglionic fibres.

Considerable evidence is now available which shows that botulinum toxin acts very widely on all those portions of the peripheral nervous system that are cholinergic, regardless of whether they form pre- or post-ganglionic components of the autonomic nervous system. There is no experimental information to demonstrate that the toxin exerts any directly injurious effects on the brain and spinal cord. 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; 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 (39).

Immunization

Employing as antigen single cell strains of Cl. botulinum which had been dissolved by alternate freezing and thawing, Dack and Starin (86), in 1924, demonstrated that it was possible to detect the presence of complement-binding bodies in the serum of animals immunized with these same strains. These reactions were specific to A and B Types of Cl. botulinum, and by employing quantitative methods it was possible to demonstrate subgroups

within the Types. This study was not undertaken for immunization purposes, but was developed by the authors for the detection of Cl. botulinum in canned vegetables.

Graham and Thorp in 1929, following the work of Ramon in preparing formolized diphtheria toxins, performed a series of experiments to determine the antigenic value of formalin-treated botulinum toxins A, B and C. The protective character of formolized heat-treated cultures and culture filtrates was demonstrated in horses, mules, rabbits, guinea pigs and chickens (87). They employed formalin to a concentration of 0.5 per cent at 37°C. until detoxification was complete.

In the following ten years studies concerning immunological reactions with Cl. botulinum became rather scarce, and the small number of reports that are available pertain mostly to studying past observations of immunological importance.

Rice et al (88) reported in 1947 the results of a systematic study on the preparation of highly antigenic formolized and alum-precipitated toxoids of Types A and B toxins. Their investigations showed that formalin added to a final concentration of 0.3 to 0.6 per cent produced higher antigenicity than when concentrations below or above this range were employed. Immunization of mice with alum-precipitated toxoids conferred a much higher degree of immunity than when formolized toxoids were employed. They also stated that mice appear to be satisfactory test animals in titrating the toxicity of the filtrates and the immunizing properties of the material, and also in determining the antitoxic titre of sera from immunized guinea pigs. Results obtained in a later experiment (89), comparing the action of alum-precipitated and formolized toxoids of Types A and B on mice and guinea pigs, proved interesting in that

Type B toxoids showed a much lower protective potency than those of Type A.

Investigating the immune response when Types A and B toxoids are combined, Rice (90) indicated that mixing Cl. botulinum Type A toxoid with Type B toxoid improved the immunizing properties of the latter for mice and to a lesser degree for guinea pigs, notwithstanding the fact that the two antigens when used singly induced no cross protection.

In the same year the Camp Detrick workers (91) took the first step in producing toxoids suitable for human use. They developed a medium for the production of toxin by removing those fractions of all constituents of the medium which did not seem to contribute to toxin yield, especially those of large molecular size. During this work these workers observed that the antigenicity of the toxoid was not directly proportional to the toxicity of the culture from which the toxoid was prepared. As a result, higher Lf values were obtained in a medium containing 4 per cent Pepticase than when 2 per cent Pepticase was employed, although the toxicity of the latter was much higher than that of the former. They assumed that although the toxin produced in the former medium was of much lower potency, there was no corresponding reduction in combining power as reflected in its flocculation titre.

In 1954 Boroff and Cabeen (92) reported the induction and formation of antibodies against toxic forms of Type C strains by injection of an atoxic variant of Type C. This phenomenon is mentioned here to substantiate an observation in this laboratory in which it was shown that an atoxic variant of Type E would produce a low but definite immunity to other Type E toxic strains in rabbits and humans.

Immunization experiments with Type E toxoids began recently when Baron and Reed (93) presented a systematic study of methods for the preparation of crude alum-precipitated Type E toxoids employing cultures grown in cellophane bags. These workers reported a similar phenomenon to that described by Rice (90) with Types A and B toxoids, viz. the augmentation of antigenicity of Type E when mixed with Type A and B toxoids.

In 1957 the Camp Detrick workers (94) succeeded in preparing formolized toxoids from crude and 'activated' Type E toxins, and obtained protection of mice approaching that offered by Types A and B toxoids. Their results were similar to those reported by Batty and Glenny (95) for epsilon toxins and toxoids of Clostridium welchii. Both groups of workers found that activation of the respective toxins by trypsin increased the antigenicity in rabbits and guinea pigs, despite a considerable loss in combining power.

In reviewing the literature concerning experimental immunization of man with botulinum toxoids, one finds experience with its use is indeed scarce.

In 1936 Velicanov (96) prepared Types A and B toxoid by treatment of toxin with formalin. These toxoids were used on a small scale experiment to immunize human volunteers. The author concluded that his preparation of toxoid immunized man against the botulinum toxins, and that a single booster injection a year after the initial immunization caused a decided increase in immunity.

Bennetts and Hall (17) used a Type C alum-precipitated botulinum toxoid in performing a field experiment to test the value of immunizing sheep against Type C intoxication. Their experiment proved very successful in that out of 1,249 treated animals 10 died of botulism, an incidence of 0.8 per cent. On the other hand there was an incidence of 6 per cent deaths from botulism among 3,432 non-immunized sheep.

Reames et al (97) immunized human volunteers with Type A and B toxins on a large scale and obtained comparable results with formolized and alum-precipitated toxoids. Within 5 months after starting immunization on a schedule of four bi-weekly injections of fluid toxoid, over 90 per cent of the individuals had protective levels. The importance of the spacing of the injections was illustrated in the experiments with a combined Type A and B alum-precipitated toxoid. A schedule consisting of two doses 8 weeks apart was more effective than one that called for two doses 3 to 4 weeks apart.

Action of Serum and Other Materials on Toxin

Increases in the lethality of botulinum toxins by the addition of serum and other protein-containing materials has been recorded with horse serum (64, 65), rabbit and sheep serum (66), and guinea pig leukocytes (67). Sommer and Sommer (68) reported this phenomenon and evaluated their findings. Using a Type A toxin, they found that exposure to 2 per cent Witte's peptone at 37°C. for several hours could double or triple the potency, while similar treatment with horse serum might raise it even higher. However, with all agents, the period of increase was only for a few hours at the most and was then quickly followed by a rapid decline in toxic activity.

The manner in which these agents cause increases in potency remains obscure. Bronfenbrenner (41) drew attention to the instability of greatly diluted toxins, especially when highly purified, and to the possibility that any added serum might be protective against oxidative or destructive influences.

Other Toxins of Clostridium Botulinum

While studying the properties of highly concentrated toxin of Cl. botulinum Type A, Lamanna (69) 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 (70, 71), 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.

A haemolysin for sheep red cells has been found by Guillaumic and Kreguer (72) in culture filtrates from C and D organisms. Lecithinase activity has been demonstrated in certain culture filtrates of Types A and B organisms (73).

Type A Toxin

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 (74, 75). After sterilizing the cultures in an autoclave, they were able to recover a highly toxic component by repeated precipitations with HCl at pH 3.5 and re-solution in various saline buffers of specified composition. From the last of the concentrates they were able to crystallize a globulin which, on parenteral injection into mice, had the high toxicity of 220×10^6 LD_{50} per mg. of N. On hydrolysis this protein yielded 19 different amino acids, among which microbiological assays disclosed an unusually high proportion of aspartic acid, tyrosine and threonine (76). When examined by electrophoresis, diffusion methods and sedimentation in the ultracentrifuge, the toxin behaved as a homogenous protein whose apparent molecular weight was about 900,000.

Abrams, Kegeles and Hottle (77) also used this highly toxigenic strain of Cl. botulinum in the purification of Type A toxin. Their method of fractionation differed in some respects from that of Lamanna et al. Starting

with a sequence of precipitations with the acid at pH 3.5 and re-solution in phosphate buffer, they completed their purification with partially saturated ammonium sulphate. Their crystalline final product had a toxicity higher than that obtained by Lamanna. The isoelectric point was 5.6, and the molecular weight was about 1,130,000.

Both values for molecular weight were in the range of 1,000,000. Since the toxin is of such a high molecular weight, it is difficult to imagine how this can be absorbed through the intestinal wall. However, Wagman and Batman (78) 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 disruption into much smaller ones whose weight they estimated to be about 70,000. This "dissociated" toxin still possessed the same lethality for mice as the complex toxin, and may prove to be the form in which the toxin is absorbed in naturally-occurring botulism.

Type B Toxin

Lamanna and Glassman (37), again separated the toxin as an electrophoretically homogenous protein. Immunologically, chemically and physically it differed from Type A toxin, although it had a potency of 160×10^6 mouse LD_{50} per mg. of nitrogen, not much below that of the Type A toxin. The most noteworthy difference between the two Types of toxin was that the Type B had a molecular weight of 60,000, a value very close to that of the dissociated form of Type A toxin referred to by Wagman and Batman.

Type C Toxin

No attempt has been made as yet to fractionate toxic filtrates from Type C cultures that can be compared with those undertaken for Types A and B.

Sterne and Wentzel (79) have made a potent toxin preparation by cultivating a toxigenic strain inside a cellophane sac immersed in a large volume of nutrient medium. By this technique they prevented the dispersal throughout the entire culture of the toxin which was formed, thus producing a concentrated product. The greatest potency of their preparations, which they termed "dialysate toxins", was about 3×10^6 mouse M.L.D. per mg. of N.

Type D Toxin

Using the same cellophane sac culture technique for Cl. botulinum Type D as they used for Type C, Sterne and Wentzel (79) produced a highly toxic concentrate which, without further treatment, contained 130×10^6 mouse M.L.D. per mg. of N. Starting with this preparation and employing successive precipitation and re-solution in ammonium sulphate solutions, they obtained an electrophoretically homogenous 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 a toxicity of 4×10^{12} mouse M.L.D. per mg. N (80).

An attempt to concentrate the Type D toxin has also been made at the Pasteur Institute by Boroff, Raynaud and Prévot (57). Their most potent preparation, however, contained only about 14×10^6 mouse LD_{50} per mg. N, a value far different from that obtained by Sterne and Wentzel, but not so very different from that obtained with the Type A and B toxins.

Type E Toxin

Clostridium botulinum Type E was first reported in 1936 by Gunnison, Cummings and Meyer (25), after they isolated the organism from the intestines of sturgeon. They described the morphology, biochemical reactions, thermal resistance of spores, and the toxin production of two cultures sent by Bier

of the Institute at Dniepropetrovsk, in the Russian Ukraine. They showed that the antitoxin of Types A, B, C and D failed to protect guinea pigs against this toxin, and that antitoxin made with these Type E cultures failed to protect against at least 2 to 3 M.L.D. of the toxins of Types A, B, C and D.

One year later, Hazen (26) isolated an organism from a tin of German-canned sprats which had caused fatal botulism in New York State. Hazen noted that the thermo-stability of the spores of this strain (E35396) was very low in comparison with other Types of Clostridium botulinum. In 1938, Hazen (27) isolated a second strain of Type E botulinum from salmon, caught in Labrador and smoked, which had caused a fatal case of botulism in New York State. In this same report the author mentions that Type E strains in the past may have been overlooked due to the low thermo-stability of the spores.

First mention of Type E botulism in Canada came in 1947 when Dolman and Kerr (29) reported the isolation and identification of Type E toxin from home-canned salmon which had caused 3 fatal cases of botulism in Nanaimo, British Columbia. The isolation of another strain from home-pickled herring in Vancouver, British Columbia, by Dolman in 1950 (30), was Canada's second reported incident of Type E botulism.

Meyer and Eddie in 1951 (15) described a small outbreak of botulism among Eskimos in Alaska due to white whale flippers cured in oil. A strain of Type E was later isolated from the infected flippers by Dolman and Chang (31). Outbreaks of Type E botulism have since been reported from Canada (81, 82), Japan (83), France (84) and Greenland (85). These occurrences held one peculiarity in common: they were all isolated from marine foodstuffs, indicating a marked predilection for this type of food.

Data concerning the physical and chemical properties of purified Type E toxin are not available to date. A method of purification has been described by Duff et al. (94) in 1957, in which they obtained a product containing 56×10^3 LD_{50} per mg. N for non-activated toxin and a value of 19×10^6 LD_{50} per mg. N for a trypsin-activated product. In each case, preliminary ultracentrifugation studies indicated that this product was not homogenous, although the value obtained, 19×10^6, is approaching that obtained for purified Types A and B toxins. One interesting observation concerning the purification of Type E toxin is the inability to precipitate the toxin with acid, a procedure which is fundamental in the purification of Types A and B toxins. Instead, precipitation with alcohol, followed by extraction of the toxin with calcium chloride solutions, has proved to be successful.

Variation

The phenomenon of variation in Type strains of Clostridium botulinum was first mentioned in 1928 by Schoenholz (98), while studying colonial growth of various strains on blood agar medium. When 42 stock cultures were streaked on these plates he obtained two to three variants in each strain, and noted five different variants in all. However, it should be noted that in these five variants he succeeded in isolating and propagating in pure culture, no mention was made of a non-toxic variant.

One year later Gunnison and Meyer (99) demonstrated that toxic and non-toxic variants of Cl. botulinum may exist side by side in mass culture from contaminated foods; but the authors stated that it was more likely that these non-toxic organisms were actually a Clostridium sporogenes contaminant rather than non-toxic variants of Clostridium botulinum.

In the same year Townsend (101) reported what would seem to be the first systematic study of non-toxic and toxic variants of botulinum species, although his conclusions that it was not possible to differentiate between toxic and non-toxic strains of Cl. botulinum by cultural, biochemical or serological methods were later demonstrated to be erroneous.

It was not until 1957 that the phenomenon of variation in Clostridium botulinum was fully clarified. At this time, Dolman (51) demonstrated variation in Type E strains and succeeded in differentiating the isolated variants both morphologically and biochemically. These variants consisted of a toxic, non-proteolytic variant ("TOX") which produced a mosaic pattern colony by transmitted light; an atoxic, non-proteolytic, sporolytic variant ("OS"), yielding opaque colonies; and a proteolytic, non-toxic variant ("TP") which produced a flat, transparent colony. The author states that the toxigenic Type E strains tend to degenerate into the sporolytic and less often into the proteolytic variants, consequently losing their toxicity; and secondly, that though both the "TP" and "OS" forms are non-toxic in themselves, or, combined together, they may revert to the toxic phase under the proper conditions. The importance of this observation to the production of toxins of Type E, and in the isolating of Type E strains from infected food material cannot be overestimated.

Activation

A marked difference between Type E toxin and the toxins produced by other Types of Clostridium botulinum is the relatively low potency of the former under ordinary laboratory conditions. Potencies exceeding one million M.L.D. per ml. are not uncommon for Types A and B toxins, whereas potencies of 3,000 to 5,000 M.L.D. per ml. are maximum for Type E. Despite this

characteristic, deaths from Type E toxin occur almost as quickly as with those of Types A and B (51). An explanation of this phenomenon has been put forward by Dolman, after observing that high potency Type E toxin can be obtained by the combination of a toxic variant with a non-toxic, proteolytic variant (51). This increase (10 - 100-fold) can be brought about by culturing the two variants together, or by mixing sterile filtrates of these cultures and incubating them for specified periods of time.

Rapid activation of Type E toxin has been reported by Duff et al (102) employing crude and purified trypsin solutions as the activating substances. Activation by this method has also been shown with the epsilon toxin of Clostridium welchii by Turner and Rodwell (100), who suggested the increase in potency was due to the presence in the culture of an inactive toxin or pro-toxin which became active on addition of trypsin, thus allowing a more potent toxin to be produced.

IV. Preliminary Experiments.

The production of botulinum toxins for the purpose of preparing toxoids suitable for human immunization involved much preliminary experimentation. Before these toxoids could be prepared, it was necessary to study the conditions required for optimum toxin production, the minimal lethal dose of the toxin for laboratory animals, the complex phenomenon of toxin activation and the effect of temperature, hydrogen-ion concentration, etc. of the crude and activated toxins.

A. The effect of surface moisture on the isolation of variants.

In the preparation of potent toxins of Clostridium botulinum Type E strains, one must be certain that the toxigenic strain employed has not degenerated into a non-toxic, sporulating state. This point is emphasized when one considers that incubation periods may in some instances be as high as 12 days, as is the case with concentrated dialysate toxins.

It is conceivable that when one sets about to obtain this information by culturing aliquots of the toxic suspension, a false picture concerning the state of the strain may be realized due to inadequate techniques in culturing the sample. For example, the use of a wire loop for streaking a non-toxigenic, proteolytic culture on agar plates is sometimes conducive to the growth of non-toxigenic colonies which resemble the mosaic pattern of the toxic variant when examined by indirect lighting. However, if the inoculum is streaked with the smooth, round end of a glass rod, thus not disturbing the surface of the medium, this phenomenon does not occur.

A second and more important determinant for producing characteristic colonies and for ease of isolation is the amount of surface moisture on the

medium to be inoculated. To obtain some degree of standardization, an experiment was performed to determine the optimum length of time culture plates should be dried before inoculation.

Brain heart infusion plates were poured and allowed to dry at 37°C. for varying lengths of time. After the desired drying time had elapsed, plates were removed and streaked with a toxic, non-proteolytic, non-sporeforming (OT) strain and others with a non-toxic, proteolytic, non-sporeforming (T) strain. The plates were then incubated at 37°C. for 24 hours, in McIntosh-Fildes anaerobic jars. The following table lists the results of this experiment, the criteria for evaluation being the amount of strain variation and ease of isolation. The results shown are an average of those from three separate experiments.

TABLE II.

Drying Time	Culture	Results
0	OT	No isolated colonies.
	T	" " "
5 min.	OT	Colonies fairly well isolated.
	T	No isolated colonies.
10 "	OT	Colonies well isolated.
	T	Few isolated colonies.
20 "	OT	Colonies well isolated, some OT-O variation.
	T	Well isolated colonies.
30 "	OT	Isolated OT colonies, considerable O variants.
	T	Isolated T colonies, considerable variation to the O form. Appearance of some variation to colonies resembling OT in morphology.

Determination of optimum drying-time for plates.

The optimum drying time of the plates is dependent on the variant being cultured. For toxigenic variants this is approximately 5 to 10 minutes at 37°C., whereas for the non-toxigenic, proteolytic variants 10 to 20 minutes would seem to be optimum. If mixed cultures are to be plated the plates should be dried 10 minutes at 37°C. It should also be noted that when the (T) variant is streaked on plates dried for 30 minutes or longer colonies appear which do not conform to those described for (OT) variants (see Appendix A). However, if these colonies are picked and inoculated into glucose-peptone-beef-infusion media (G.P.B.I.), the growth is indicative of normal (T) variants.

B. Production of toxic and non-toxic filtrates.

The production of botulinum toxoids involves the preparation of two types of toxin, designated "crude" and "activated".

Crude toxin is that toxin produced by growing a pure toxigenic variant (OT) of a Type E strain and harvesting the filtrate after the required incubation period. This toxin normally has a potency of 2,000 to 5,000 M.L.D. per ml. of culture filtrate, depending on the medium employed, pH and the condition of the strain itself.

Activated toxin is crude toxin which has an artificially increased potency. This increase may be brought about by (a) culturing together a proteolytic, non-toxic variant (T) with an (OT) variant, (b) by combining sterile filtrates of (OT) and (T) cultures and allowing them to incubate, and (c) by the addition of trypsin to a crude toxin followed by incubation (51). The potency of this activated product may range from values slightly

above that of the crude toxin to as high as 100 million of mouse M.L.D. per ml., again depending on conditions such as incubation time, pH and the state of the crude toxin per se.

1. Production of crude toxin.

For the production of crude toxin, the (OT) variant of strain "Iwanai" was employed. Stock cultures were prepared in G.P.B.I. media and stored at 4°C.

Erlenmeyer flasks containing 4 litres of G.P.B.I. media at pH 7.8 were inoculated with 10 ml. of a 20 hour culture of the (OT) variant grown at 32°C. The flasks were incubated at 32°C. for 7 days followed by refrigeration at 4°C. for 24 hours. At the end of this period, the filtrate was removed and sterilized by filtration through a Seitz filter. Before filtration of the toxin, 100 ml. of sterile nutrient broth at pH 5.2 was passed through the filter pad. The toxin was then titrated by intraperitoneal injections in mice, and normally had a potency of 2,000 to 3,000 mouse M.L.D. per ml.

TABLE III

Days incubation	Potency mouse M.L.D. per ml.	pH
1	2	7.2
2	10-30	6.7
3	50-100	6.1
4	100-300	5.7
5	500-700	5.3
6	2,500-3,000	5.2
7	2,500-3,000	5.2

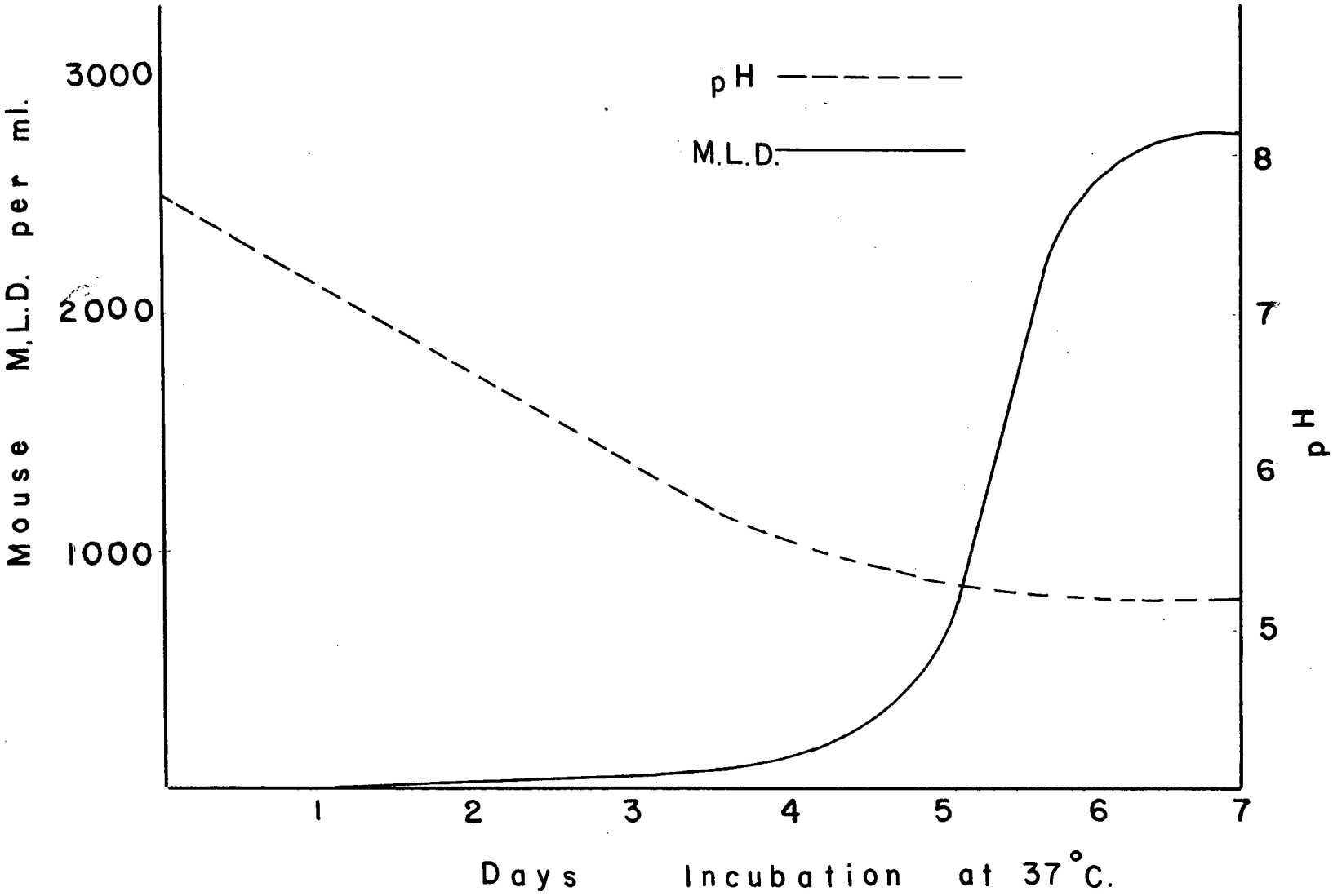

Diagram 1. Production of crude (OT) toxin.

Diagram I indicates the rise in toxin potency and pH decline in the preparation of a crude toxin. This particular toxin had a potency of 2,500 to 3,000 mouse M.L.D. per ml., or 2,272 LD_{50} per ml.

Crude Type E botulinum toxin is very stable at refrigeration temperatures; samples stored at 4°C. for 2 years demonstrated no alteration in potency. As shown in Diagram I, the pH of the culture is of extreme importance from the standpoint of toxin potency and, as will be shown later, for toxin activation.

(a) Effect of pH on production of crude toxin.

The pH of the incubating culture undergoes the same increase in each batch of toxin, and a number of experiments were undertaken to study this action more closely. Diagram II illustrates the effect of an elevated pH on toxin production.

In this experiment the pH of the culture was not allowed to fall below a value of 6.0. This was accomplished by adding N/10 NaOH to the culture beginning on day 3. As seen in this graph, toxin potency did not increase beyond 300-500 mouse M.L.D. per ml. Furthermore, microscopic observation of the culture throughout the incubation period did not demonstrate the characteristic decrease in Gram-positive cells between days 4 and 5, as is noticed with normal cultures.

When the above experiment was repeated and the pH allowed to decline on day 7, toxin potency was seen to again increase to a final titre of 750 - 1,000 mouse M.L.D. per ml. on day 10. Further incubation or decreased pH would not increase this potency. These results are shown in diagram III.

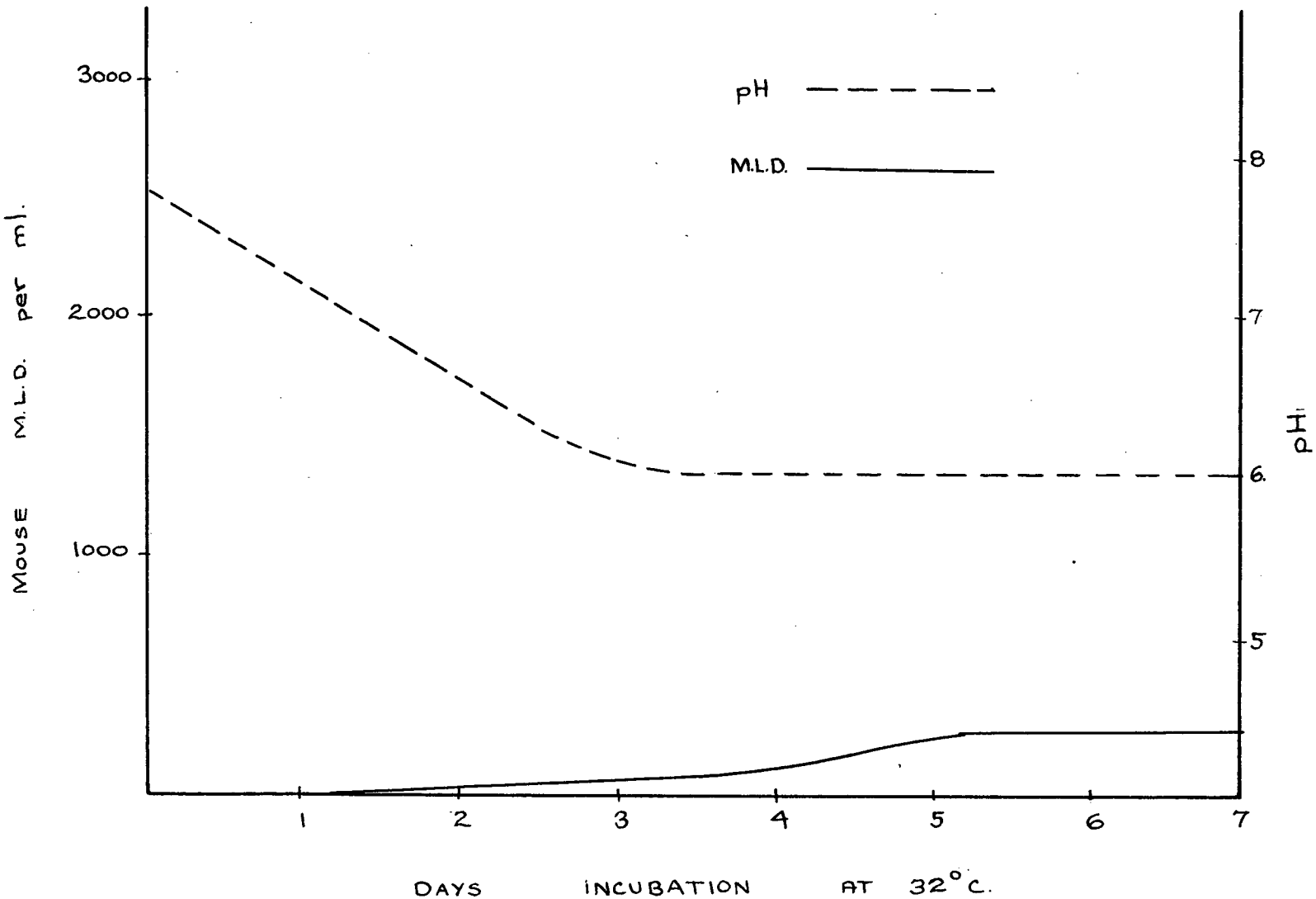

Diagram II.. The effect of an elevated pH on toxin production.

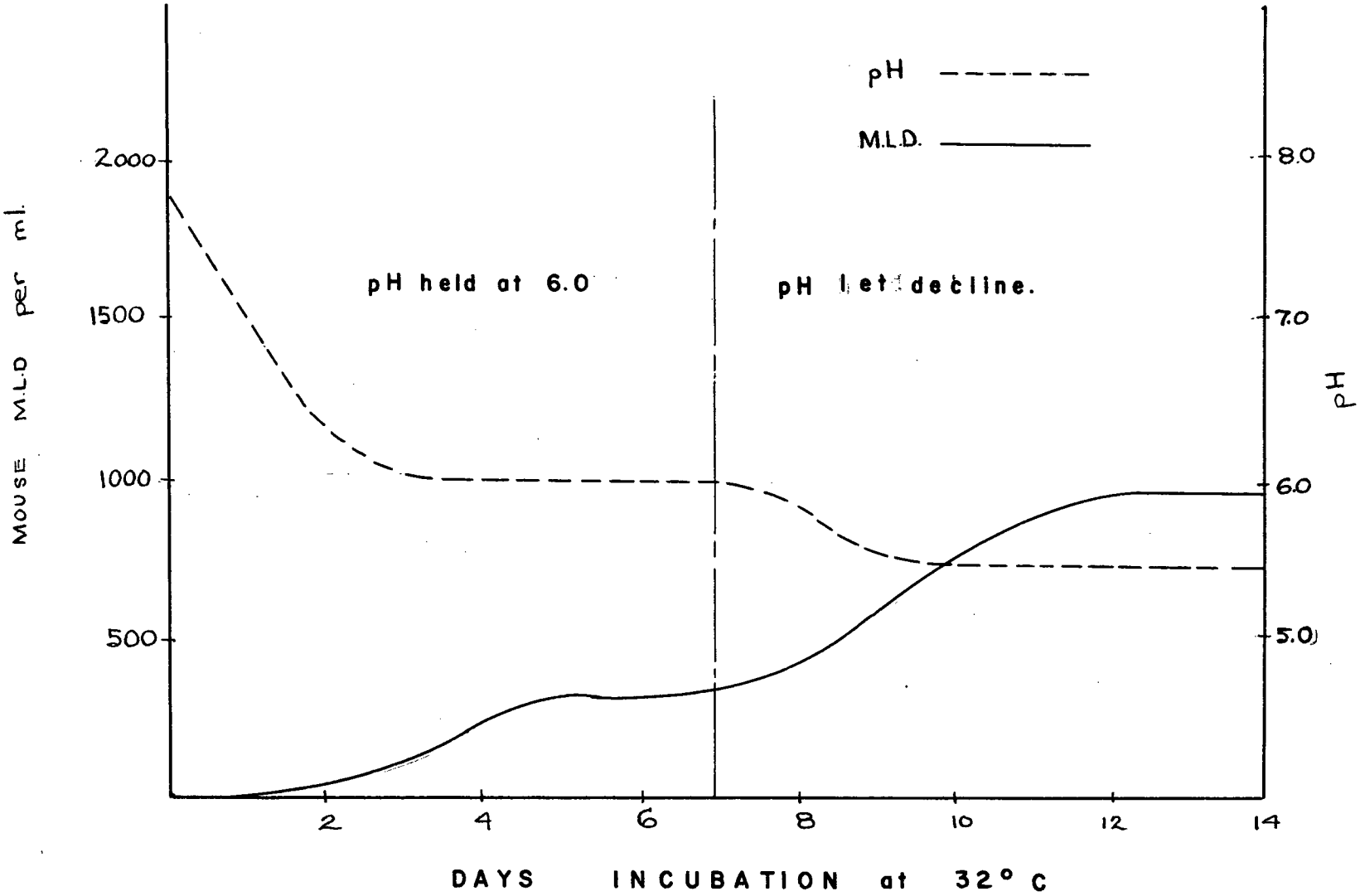

Diagram III. The effect of a delayed drop in pH.

Diagram IV illustrates the effect on toxin potency when the pH is lowered to 5.2, on day 2, by the addition of N/10 HCl.

Toxin production, in this instance, increased normally to a potency of 10 to 30 mouse M.L.D. per ml. on day 2, but failed to increase to any extent during the remaining 5 days of incubation. Adjusting the pH again to 7.0 on day 7 and prolonging the incubation did not cause a potency increase.

From these experiments it was evident that the pH plays an important role in the production of toxin. Not only must the pH reach the required acidity, but it must attain this value at a particular time during the growth of the culture.

I believe these findings also substantiate the theory that the toxin is an exotoxin released from cells on autolysis as described by Boroff (58) for Type D toxin. Boroff's suggestion that the long lag period between maximum growth and toxin production is due to a required build-up of autolytic enzymes might also be applicable here.

The importance of these pH studies is again emphasized under the heading of toxin activation.

2. The production of activated toxin.

The production of activated toxin, as stated earlier, may be accomplished by one of three methods. Each of these has been studied in some detail in an attempt to determine the conditions responsible for toxin activation, and secondly, to determine which method should be employed for the production of an activated product suitable for the preparation of a toxoid.

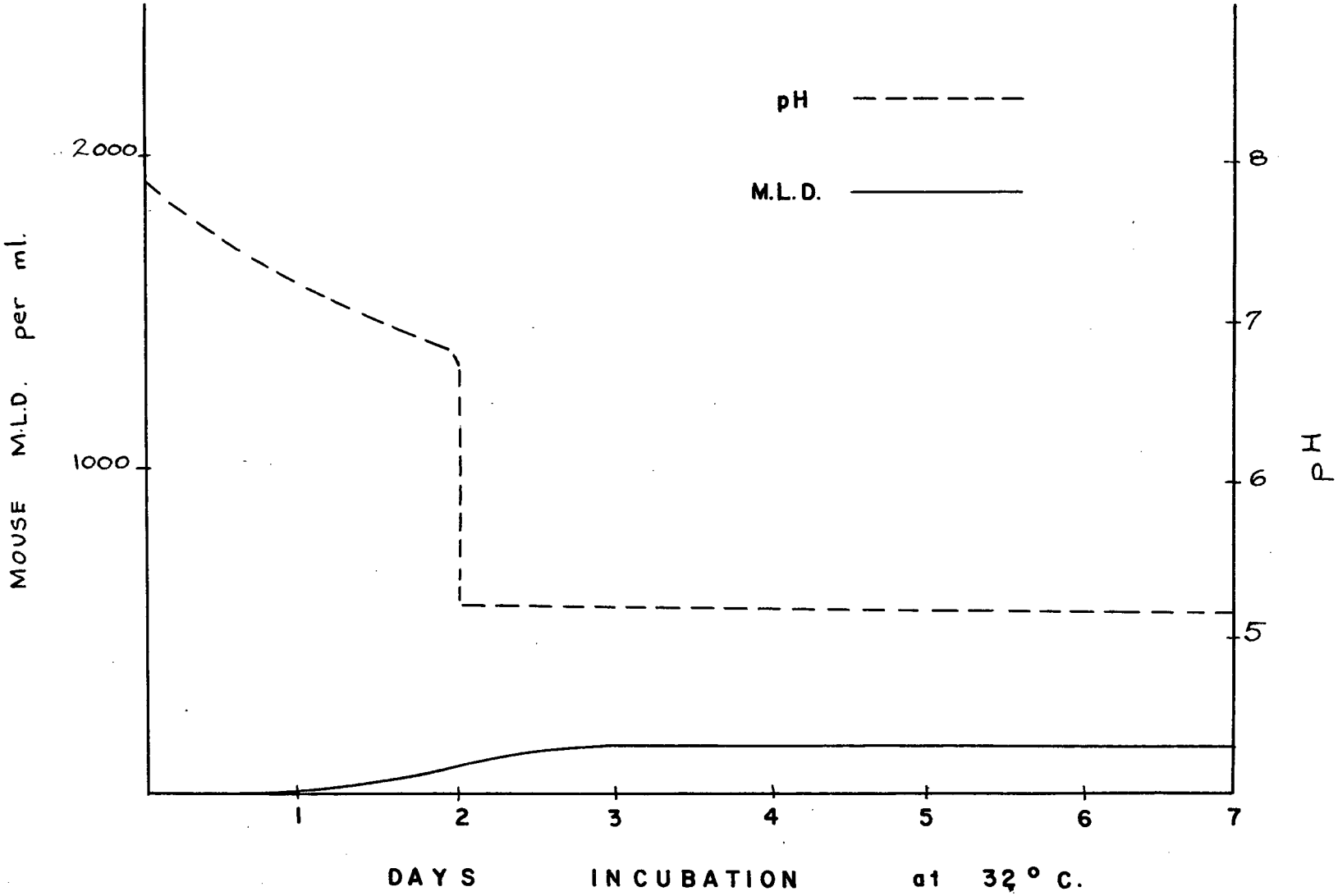

Diagram IV The effect of an early decrease in pH.

(a) Dialysate toxin.

The production of activated toxin by culturing a toxic (OT) variant with a proteolytic, non-toxic (T) variant in dialysis tubes has been employed in this Department for some time (51). A complete description of the apparatus and its mode of operation is contained in Appendix C.

For the preparation of this toxin the apparatus was inoculated with 20 ml. of 16 hour cultures of "Iwanai" (OT) and "VH" (T), grown in G.P.B.I. media at 32°C.

Samples of the toxic filtrate were removed each day throughout the incubation period for the determination of pH and toxin potency. The results of these determinations are listed in Table IV.

TABLE IV

Days incubation at 32°C.	Mouse M.L.D./ml.	pH
0	0	7.8
1	300 - 1,000	7.3
2	10,000 - 30,000	7.0
3	50,000 - 66,000	6.6
4	66,000 - 100,000	6.2
5	100,000 - 150,000	6.0
6	150,000 - 200,000	5.8
7	200,000 - 250,000	5.6
8	1,000,000 - 2,000,000	5.3
9	2,000,000 - 3,000,000	5.3
10	2,000,000 - 3,000,000	5.3
11	2,000,000 - 3,000,000	5.3

Refrigeration of toxin on day 11 for 24 hours caused a further increase in potency to 3 million - 4 million mouse M.L.D. per ml. This latter characteristic has been demonstrated many times with the (T) + (OT) toxin.

The toxin suspension was sterilized by Seitz filtration and stored at 4°C. Activated toxin produced by this method is relatively stable compared with the other types.

Diagram V shows the characteristic curves for pH and potency obtained throughout the incubation period.

(b) Combination of filtrates.

Toxin activation by the combination of sterile filtrates of (OT) and (T) cultures has been reported in a recent publication by Dr. C.E. Dolman of this Department. The potency of activated toxin produced in this manner is much more variable than when the organisms are cultured together. On some occasions a rapid activation to a potency 50 times that of the original toxin will result, whereas on other occasions little or no activity follows combination and incubation. Preliminary experiments indicate that such conditions as time, pH, temperature and the conditions under which the two filtrates themselves are produced are very critical.

The following experiment entails the production of activated toxin by this method.

Two hundred ml. of a sterile filtrate of "Iwanai" (OT) toxin (2,500 - 3,000 mouse M.L.D. per ml.)were mixed with 25 ml. of a sterile "VH" (T) filtrate. The mixture was incubated at 32°C. and gently swirled every few hours to assure a homogenous mixture. At designated intervals, small aliquots were removed and titrated in mice. Table V contains the results of this experiment.

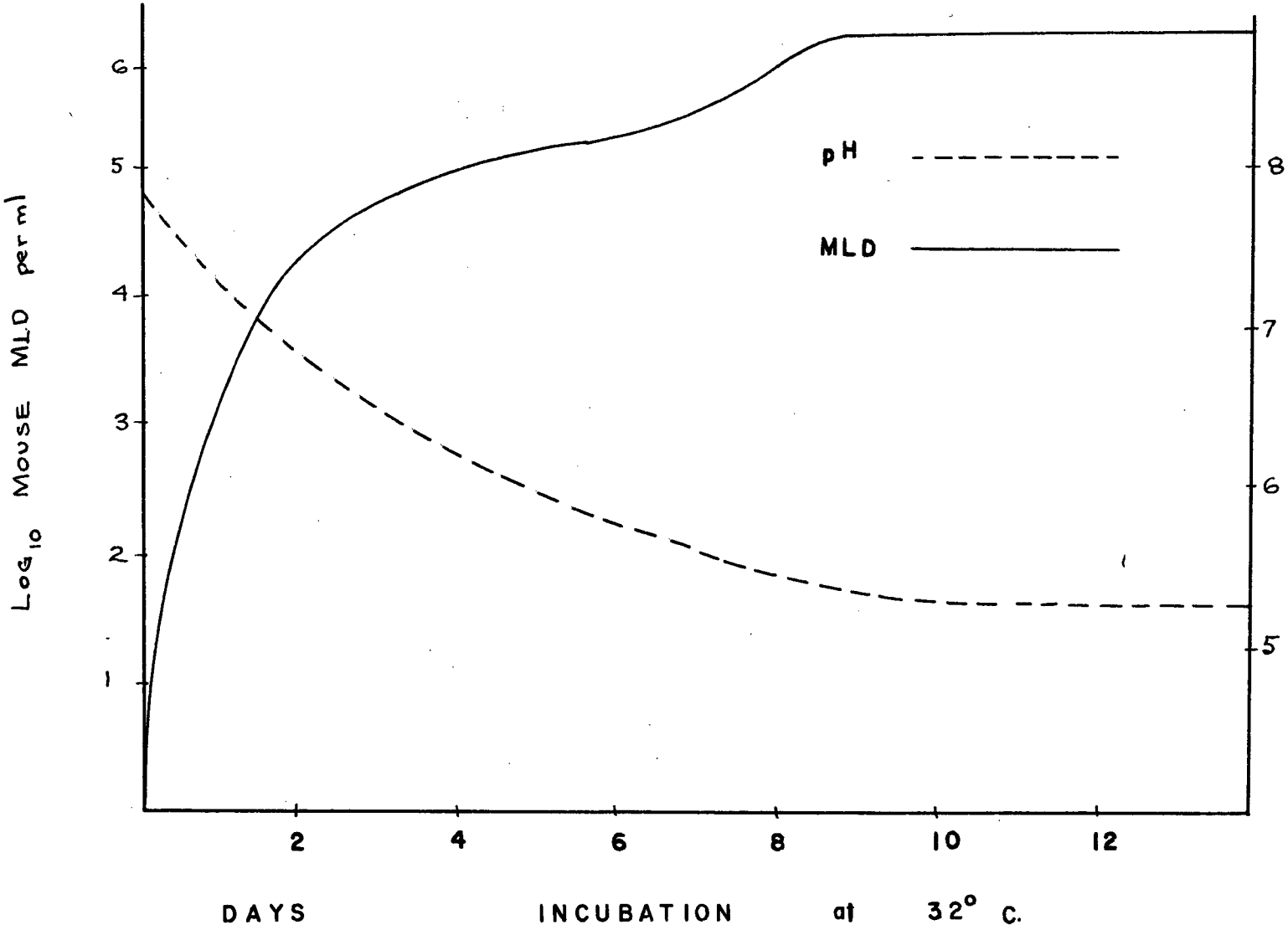

Diagram V Toxin activation by mixed culture.

TABLE V

Time	Mouse M.L.D./ml.
0	2,000 - 3,000
2 hrs.	5,000 - 10,000
4 "	20,000 - 33,000
6 "	33,000 - 50,000
8 "	50,000 - 75,000
10 "	75,000 - 100,000
12 "	100,000 - 125,000
16 "	125,000 - 150,000
20 "	150,000 - 175,000
24 "	175,000 - 200,000
28 "	200,000 - 250,000
32 "	200,000 - 250,000

As stated earlier, it was not always possible to repeat these results. Some experiments gave higher final potencies, while others demonstrated no activation. Consequently, a number of experiments were undertaken to examine more closely the effect of incubation temperature and pH. Titrations of the toxin after maximum potency had been attained indicated a gradual decline in titre both at incubation temperature as well as at 4°C., though the latter was much slower.

(i) Effect of incubation temperature.

Three flasks containing 25 ml. each of "Iwanai" (OT) toxin and "VH" (T) filtrate were incubated at temperatures of 28°C., 32°C. and 37°C. respectively. Titrations of toxin potency were made every 8 hours for 136 hours. Results are tabulated in Table VI.

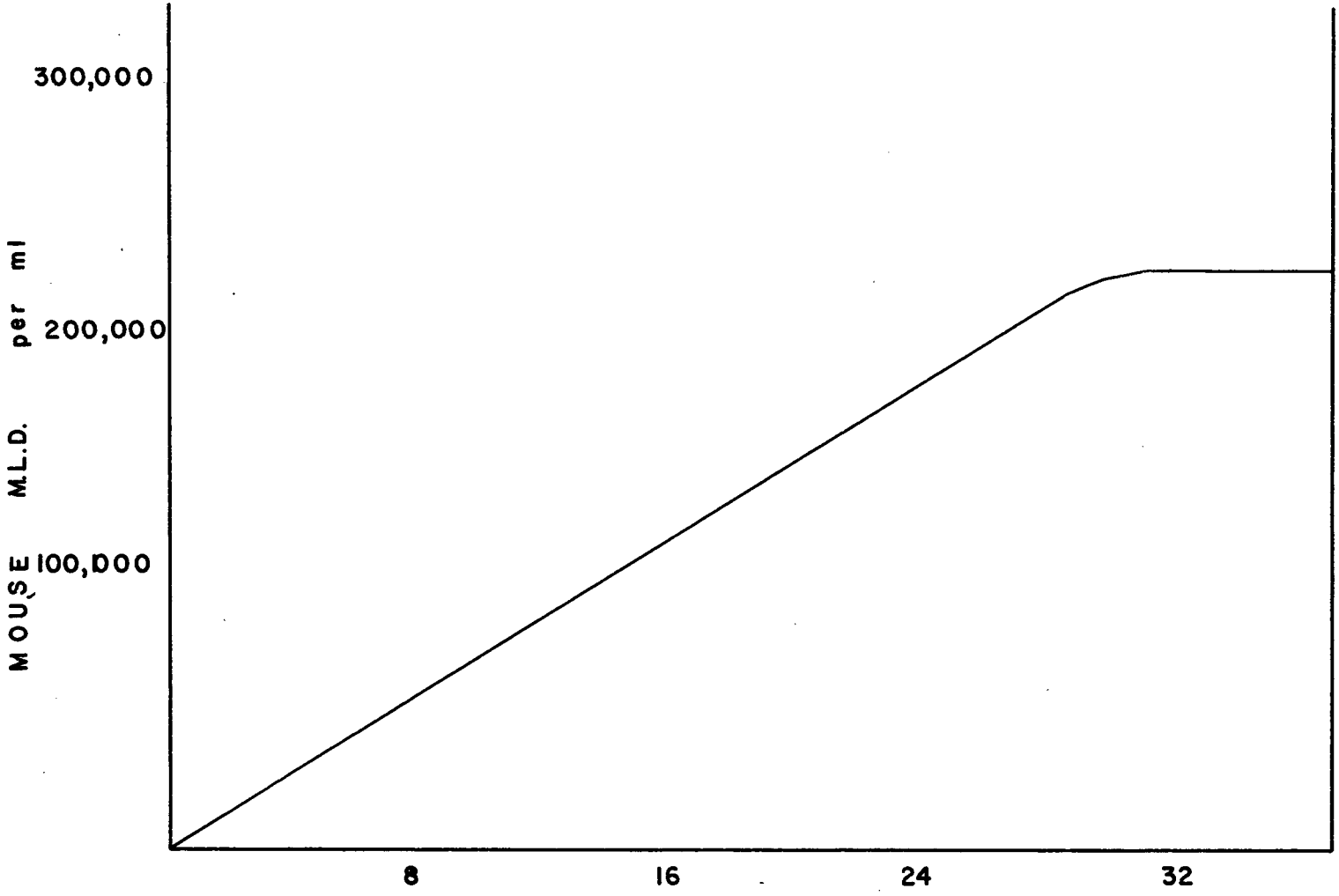

Diagram VI. Toxin activation by sterile proteolytic filtrate.

TABLE VI. Effect of temperature on toxin activation.

Time in hrs.	Mouse M.L.D./ml. 28°C.	32°C.	37°C.
0	1,000 - 2,000	1,000 - 2,000	1,000 - 2,000
8	10,000 - 20,000	33,000 - 50,000	33,000 - 50,000
16	10,000 - 20,000	100,000 - 150,000	100,000 - 150,000
24	20,000 - 30,000	100,000 - 150,000	150,000 - 200,000
32	30,000 - 50,000	150,000 - 200,000	150,000 - 200,000
40	50,000 - 100,000	150,000 - 200,000	50,000 - 100,000
48	50,000 - 100,000	50,000 - 100,000	50,000 - 100,000
56	50,000 - 100,000	50,000 - 100,000	30,000 - 50,000
64	30,000 - 50,000	30,000 - 50,000	10,000 - 30,000
72	10,000 - 20,000	10,000 - 20,000	3,000 - 5,000
80	3,000 - 5,000	3,000 - 5,000	1,000 - 2,000
88	1,000 - 2,000	500 - 1,000	300 - 500
96	500 - 1,000	100 - 300	50 - 100
104	100 - 200	-	-
112	50 - 100	-	-
120	10 - 30	-	-
128	2 - 10	-	-
136	-	-	-

(ii) Effect of pH

Five flasks, each containing 25 ml. of "Iwanai" (OT) toxin and 25 ml. "VH" (T) filtrate, were prepared and adjusted to pH values of 4.8, 5.2, 5.6, 6.0 and 7.0 respectively and incubated in a water-bath at 32°C. At specified intervals, aliquots were removed from each flask and titrated in mice for potency determinations. These results are shown in Table VII.

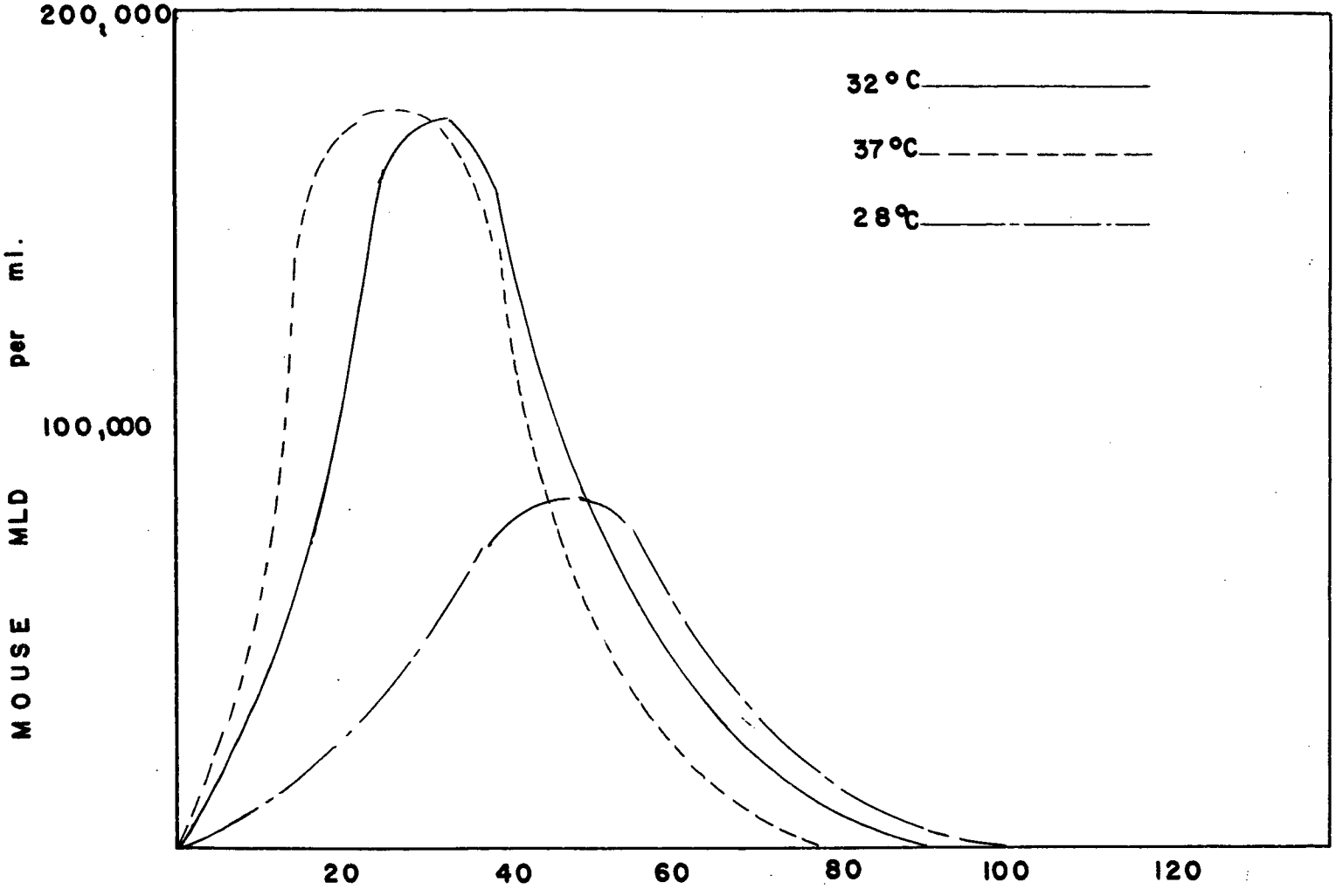

Diagram VII: Effect of temperature on toxin activation.

TABLE VII Effect of pH on toxin activation

Incubation time in hrs.	Mouse M.L.D. per ml.				
	pH 4.8	pH 5.2	pH 5.6	pH 6.0	pH 7.0
0	1,000 - 2,000	1,000 - 2,000	1,000 - 2,000	1,000 - 2,000	1,000 - 2,000
2	1,000 - 2,000	10,000 - 20,000	2,000 - 3,000	1,000 - 2,000	1,000 - 2,000
4	1,000 - 2,000	20,000 - 33,000	3,000 - 5,000	1,000 - 2,000	1,000 - 2,000
8	1,000 - 2,000	33,000 - 50,000	5,000 - 10,000	2,000 - 3,000	1,000 - 2,000
16	500 - 1,000	100,000 - 150,000	20,000 - 30,000	2,000 - 3,000	1,000 - 2,000
24	500 - 1,000	150,000 - 200,000	20,000 - 30,000	3,000 - 5,000	1,000 - 2,000
32	500 - 1,000	200,000 - 250,000	20,000 - 30,000	3,000 - 5,000	1,000 - 2,000
40	500 - 1,000	150,000 - 200,000	10,000 - 20,000	5,000 - 10,000	1,000 - 2,000
48	500 - 1,000	100,000 - 150,000	10,000 - 20,000	5,000 - 10,000	1,000 - 2,000
56	500 - 1,000	50,000 - 100,000	5,000 - 10,000	3,000 - 5,000	1,000 - 2,000

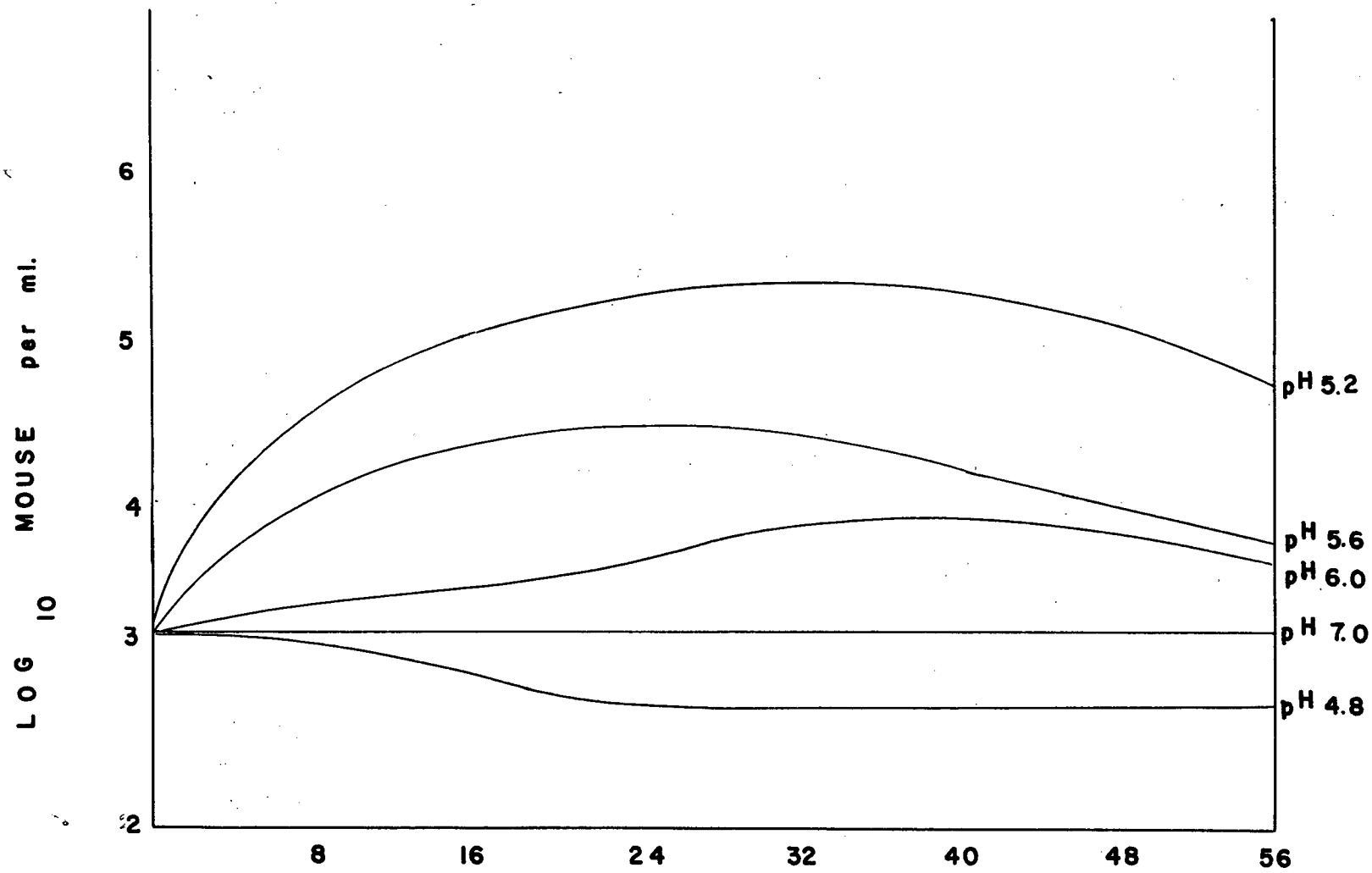

Diagram VIII Effect of pH on toxin activation.

The production of activated toxins by combining filtrates of toxic and proteolytic variants is dependent on the pH of the mixture and the incubation temperature. The pH is seen to be very critical and variations of 0.5 pH units on either side of the optimum (pH 5.2) is detrimental if not inhibitory to toxin activation. The incubation temperature of the reaction mixture is not as critical, activation occurring from temperatures of 32°C. to 37°C. Results are indicative of activation by enzymatic substance or substances contained in the (T) filtrate.

(c) Activation with trypsin.

Duff et al. (102) have recently reported the marked activation of Clostridium botulinum Type E toxin by treatment with trypsin. The authors state that when crude toxins were incubated with 1 per cent trypsin for 45 minutes, potencies could be increased to as high as 50 fold.

Experiments in this Department have confirmed and extended these findings. These studies involved determinations of optimum conditions for activation, inhibition of activation and the stability of the activated products at temperatures required for toxoiding.

(i) Toxin activation.

Fifty ml. of crude "Iwanai" (OT) toxin were incubated at 37°C. with 1 per cent trypsin (Difco 1:250). Aliquots were removed at specified intervals and titrated for potency in mice. The results are shown in Table VIII.

TABLE VIII

Time	Mouse M.L.D./ml.	pH
0	2,000 - 3,000	5.3
30 min.	10,000 - 30,000	5.3
1 hr.	50,000 - 100,000	5.4
2 hrs.	500,000 - 1,000,000	5.4
3 "	30,000,000 - 50,000,000	5.5
4 "	50,000,000 - 100,000,000	5.6
5 "	50,000,000 - 100,000,000	5.6
6 "	30,000,000 - 50,000,000	5.4
8 "	10,000,000 - 30,000,000	5.4
10 "	5,000,000 - 10,000,000	5.3
20 "	1,000,000 - 5,000,000	5.3
40 "	10,000 - 50,000	5.2
50 "	5,000 - 10,000	5.0
60 "	5,000 - 10,000	5.0
120 "	3,000 - 5,000	4.8
240 "	1,000 - 3,000	4.8
480 "	1	4.8

The effect of trypsin on the crude toxin at a pH well below the optimum for this enzyme was very surprising. As seen in the above table, the toxin is very unstable and once the high potency is attained it decreases very rapidly. This detrimental action is retarded somewhat at refrigeration temperatures but still remains comparatively rapid.

The activation itself will not occur with trypsin at a pH of 7.5 (optimum for trypsin) nor with pepsin at pH values of 5.2 or at its optimum pH of 2.5, as demonstrated in Table IX.

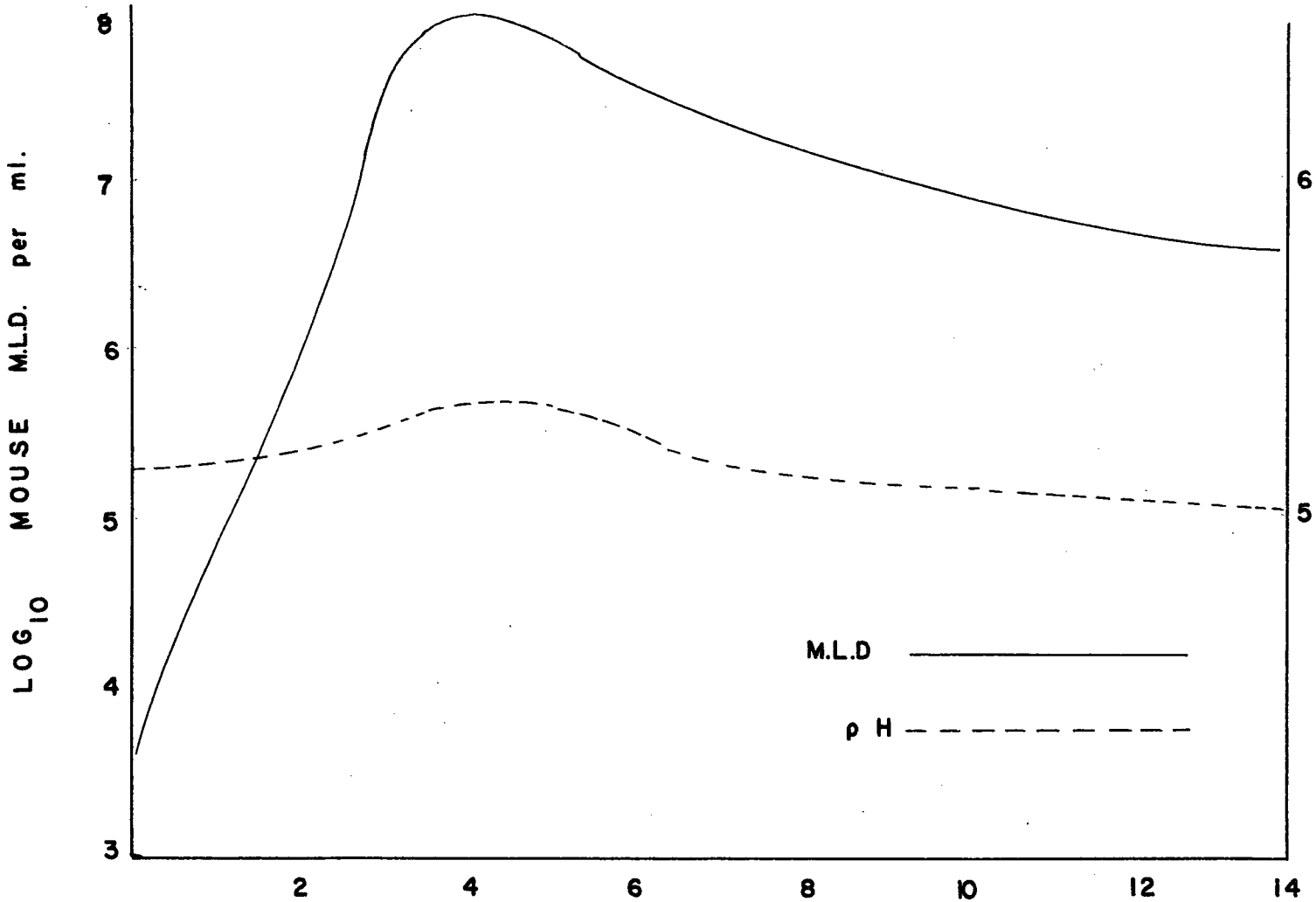

Diagram IX: Toxin activation by trypsin.

TABLE IX Activation of toxin with crude trypsin and pepsin

Time in hrs.	1% Trypsin		1% Pepsin	
	pH 5.2	pH 7.5	pH 5.2	pH 2.5
0	2,000 - 3,000	2,000 - 3,000	2,000 - 3,000	2,000 - 3,000
1	30,000 - 50,000	2,000 - 3,000	2,000 - 3,000	1,000 - 2,000
2	100,000 - 300,000	1,000 - 2,000	2,000 - 3,000	1,000 - 2,000
3	1,000,000 - 3,000,000	1,000 - 2,000	2,000 - 3,000	500 - 1,000
4	10,000,000 - 30,000,000	500 - 1,000	2,000 - 3,000	300 - 500
8	10,000,000 - 30,000,000	300 - 500	2,000 - 3,000	100 - 200
16	1,000,000 - 3,000,000	50 - 100	2,000 - 3,000	10 - 30
24	300,000 - 500,000	10 - 30	2,000 - 3,000	1

(ii) Inhibition of the activating phenomenon.

The apparent instability of this activated toxin, as shown by its rapid decrease in toxin potency, does not allow for its use as such in the preparation of toxoids.

Duff, in a recent publication (102), employed purified trypsin as the activating substance and succeeded in inhibiting toxin activation by adding purified soya inhibitor. Consequently, experiments were undertaken in this laboratory to determine if destruction of the activated toxin could be inhibited by treating the product with soya inhibitor. Preparation of the solutions employed may be found in Appendix B.

Twenty ml. of a 0.02 per cent purified trypsin solution were added to an equal volume of crude "Iwanai" (OT) toxin and the mixture incubated at 37°C. At times 1, 2, 4, 8 and 16 hours, 4 ml. of the mixture were removed and added to 4 ml. of a 0.02 per cent soya inhibitor solution. The aliquots were re-incubated and titrated in mice for toxin potency after 16 hours and after 3 days further incubation.

The results shown in Table X demonstrated that the inhibitor protected activated toxin from decreasing in potency after maximum activation for a minimum of 16 hours, and allowed only a slight decrease after 3 days at 37°C. Meanwhile, activated toxin without added inhibitor had diminished to a potency of 1 million - 3 million in 16 hours of further incubation and to as low a potency as 3,000 - 5,000 after 3 days.

TABLE X Effect of purified soya inhibitor on activation of toxin with trypsin.

Time inhibitor added	Activated toxin (no inhibitor)	Activated toxin (at 16 hr. incubation) + Inhibitor (at 3 days)	
1 hour	100,000 - 300,000	100,000 - 300,000	100,000 - 300,000
2 "	1,000,000 - 3,000,000	1,000,000 - 3,000,000	300,000 - 1,000,000
4 "	100,000,000 - 300,000,000	50,000,000 - 100,000,000	50,000,000 - 100,000,000
8 "	10,000,000 - 30,000,000	10,000,000 - 30,000,000	10,000,000 - 30,000,000
16 "	1,000,000 - 3,000,000	1,000,000 - 3,000,000	300,000 - 1,000,000
3 days	3,000 - 5,000	-	-

The activated toxins, with and without added inhibitor, were refrigerated at 4°C. for a period of one month and re-titrated. In this time toxin potency had only slightly diminished in the samples with added trypsin inhibitor, while those without the inhibitor had become non-toxic.

3. Production of non-toxic filtrates.

For the production of non-toxic, proteolytic filtrates the (T) variant of the "VH" strain was employed. Early in the investigational studies a number of media were tested to determine which one supported the best growth and enzyme production for this "VH" (T) strain. Evaluation of media was based on the ability of the proteolytic filtrate produced to activate crude toxin. Media tested were as follows:-

#1 Brewer's thioglycollate anaerobic medium.

#2 Beef-heart infusion medium with added thioglycollate.

#3 G.P.B.I. medium.

#4 Yeast extract medium.

Preparation of the above media is discussed in Appendix B.

One hundred ml. aliquots of each medium were prepared in Erlenmeyer flasks and inoculated with 0.3 ml. of a 20-hour culture of "VH" (T). The flasks were incubated at 32°C. for 48 hours. Following incubation the proteolytic filtrates were removed from the flasks containing media #1, #3 and #4. (Medium #2, beef-heart infusion with added thioglycollate, would not support growth of the "VH" strain). The proteolytic filtrates were sterilized by Seitz filtration and 10 ml. added to 50 ml. quantities of crude "Iwanai" (OT) toxin. Mouse titrations for toxin activation were performed at intervals of 8, 16, 32, 64 and 128 hours. Table XI contains the results of the experiment.

TABLE XI

Media	Incubation time with crude toxin (values in thousand mouse M.L.D. per ml.)				
	8	16	32	64	128
# 1	3 - 10	10 - 30	30 - 50	30 - 50	30 - 50
# 3	30 - 50	50 - 75	100 - 125	100 - 125	100 - 125
# 4	50 - 75	50 - 75	100 - 125	100 - 125	50 - 75

Yeast extract and G.P.B.I. media both supported good growth and proteolytic activity of the "VH" (T) strain, as indicated by toxin activation. Brewer's thioglycollate medium, under the conditions stated, did not produce an active proteolytic filtrate comparable to the two aforementioned media.

Both types of media were employed in later experiments, depending on the desired function of the (T) filtrate.

C. Purification of Type E toxin.

Two procedures were employed for the preparation of Type E toxin for subsequent toxoiding. The first method was that employed by Prevot and Raynaud of the Pasteur Institute, and involved essentially the precipitation of the toxin by purified sodium metaphosphate at a pH of 3.5 and a temperature of -15°C. The precipitate was re-dissolved in an acetate buffer and final precipitation was effected by addition of concentrated phosphate buffer.

Reasonable results were obtained by this method, but the final concentrations of the various added components were found to vary with the toxin preparation being purified. The final concentration of precipitating agents required to purify one batch of toxin could not always be applied to a

second lot of toxin and, as a result, a number of trial experiments were undertaken throughout the procedure.

The second method employed for the purification of toxin was that reported by Duff et al. (103), which involved the precipitation of toxin by the addition of 95 per cent ethanol. The extraction of toxin from the precipitate was effected with a calcium chloride solution and phosphate buffer.

1. Purification with phosphates.

(a) Procedure

Six litres of crude "Iwanai" (OT) toxin (potency of 2,000 to 3,000 mouse M.L.D. per ml.) were employed for the experiment.

(1) Sodium chloride was edded to the toxin to a final concentration of 30 per cent and the temperature lowered to -15°C.

(2) A 10 per cent solution of sodium metaphosphate (Difco purified Na_3PO_4), in distilled water, was added to make a final concentration of 1 per cent.

(3) Normal sulphuric acid was added until the pH of the mixture was lowered to 3.5, and the mixture was refrigerated for 24 hours at -15°C.

(4) The precipitate was collected by centrifugation for 30 minutes at 2,000 r.p.m. at 0°C., and dissolved in 0.015 M sodium acetate solution at pH 6.0 to one-tenth the original culture volume. (Designated NaAc extract).

To this point the process followed that reported by Prévot and Raynaud. However, when 3.5 M K_2HPO_4-KH_2PO_4 buffer was added to a concentration of 30 per cent, precipitation would not occur. Aliquots of the NaAc extract were

then removed and precipitation was attempted using varying concentrations of phosphate buffer. The results of this experiment are contained in Table XII(a)

TABLE XII (a)

Tube Number	Percent 3.5 M Phosphate Buffer (ml.)	Distilled Water (ml.)	Toxic Complex (ml.)
1	5	8.5	1
2	10	8.0	1
3	15	7.5	1
4	20	7.0	1
5	25	6.5	1
6	30	6.0	1
7	35	5.5	1
8	40	5.0	1
9	45	4.5	1
10	50	4.0	1
11	55	3.5	1
12	60	3.0	1
13	65	2.5	1
14	70	2.0	1
15	75	1.5	1
16	80	1.0	1

A control series was prepared substituting distilled water for the toxic complex.

Flocculation began in tube #9 of the test series, i.e. 45 per cent concentration of phosphate buffer. No flocculation occurred in the control series.

Each tube in the series was then filtered through a #1 filter pad to leave the precipitate, if any, on the pad. The filter paper was then washed

three times with the same sample of sodium acetate at -10°C. The supernatant and resuspended precipitate were then titrated in mice for M.L.D. determinations. Results of these titrations are shown in Table XII(b)

TABLE XII(b)

Tube Number	Toxicity in M.L.D. per ml. (in thousands) Supernatant	Precipitate
1	15 - 20	-
2	15 - 20	-
3	15 - 20	-
4	15 - 20	-
5	15 - 20	-
6	15 - 20	-
7	15 - 20	-
8	15 - 20	-
9	3 - 5	10 - 15
10	1 - 3	10 - 15
11	1 - 3	15 - 20
12	1 - 3	15 - 20
13	-	15 - 20
14	-	15 - 20
15	-	15 - 20
16	-	15 - 20

Titrations indicated that the phosphate buffer if added to the toxin-complex to a concentration of 55 per cent yielded a precipitate containing a high percentage of toxin

(5) 3.5 M phosphate buffer was added to the toxic complex to a concentration of 55 per cent, and the mixture was refrigerated at -10°C.

(6) The precipitate was collected by centrifugation and re-dissolved in 1 N NaCl to one-tenth the original culture volume. (Designated NaCl extract).

(7) Extraneous protein was then removed by partial precipitation at -10°C. with phosphate buffer to a concentration of 26 per cent.

(8) The mixture was then centrifuged and the precipitate discarded. (NaCl-2 extract).

(9) Phosphate buffer was added to the supernatant to a concentration of 28 per cent to precipitate the toxin, and the mixture allowed to remain at -10°C. for 24 hours.

(10) The precipitate was collected by centrifugation at 0°C. and re-dissolved in 50 ml. of NaCl.

It should be noted that the concentrations of phosphate buffer employed in steps 7 and 9 were determined by experiments similar to that shown in step 5.

(b) Results.

Nitrogen determinations on the various extracts were obtained by direct nesslerization technique (104). The results indicated in Table XIII are an average of two separate trials.

TABLE XIII

Sample	Fold Conc. From Culture	Mouse M.L.D./ml. (in 1,000)	Mouse M.L.D./mg. N (in 1,000)	Per cent Recovery	Fold Purif.
Crude	undiluted	3	5	-	-
NaAc	10	22	40	70	8
NaCl-1	10	15	125	50	25
NaCl-2	10	12	350	38	70
Final	120	40	375	11	75

2. Purification by alcohol precipitation.

(a) Procedure.

Five litres of crude "Iwanai" (OT) toxin were employed for this experiment. The potency of this toxin was 2,000 - 3,000 mouse M.L.D. per ml.

(1) Ninety-five per cent ethanol was added to the toxin to a concentration of 25 per cent. Micron bentonite (Volcay Bentonite, BC grit free, American Colloid Company), a highly refined diatomaceous earth, was added to a concentration of 1 gram per litre of culture to aid settling of the toxin precipitate. The mixture was allowed to stand 48 hours at $-7^{\circ}C$.

(2) The supernatant was siphoned off and the precipitate collected by centrifugation for 30 minutes at 4,000 r.p.m. at $-7^{\circ}C$.

(3) The precipitate was diluted with distilled water to one-sixth the culture volume and stirred for 1 hour at room temperature. (Designated first alcohol fraction).

(4) The alcohol-precipitated toxin was then diluted to one-quarter the culture volume with distilled water, and 1.0 M $CaCl_2$ solution to a final concentration of 0.075 M $CaCl_2$. The pH was adjusted to 6.0,

and the suspension stirred for 2 hours at room temperature, and then clarified by centrifugation at 20°C. for 30 minutes at 4,000 r.p.m. (The supernatant is referred to as $CaCl_2$ fraction).

(5) After adding 95% ethanol to a final concentration of 25 per cent, at -7°C., the $CaCl_2$ fraction was allowed to settle overnight.

(6) The precipitate was again collected by centrifugation at -7°C. and 4,000 r.p.m. for 30 minutes, and dissolved in 0.08 M phosphate buffer, pH 6.0. The mixture was then clarified by centrifugation at 20°C. for 30 minutes, at a speed of 4,000 r.p.m. (Designated second alcohol fraction).

(7) Ninety-five per cent ethanol was again added to a final concentration of 25 per cent at -7°C., the suspension was allowed to stand overnight and the precipitate collected by centrifugation at -7°C. for 30 minutes and a speed of 4,000 r.p.m.

(8) The precipitate was dissolved in 0.2 M succinate buffer at pH 5.5 to a volume one-half that of the second alcohol fraction.

(b) Results.

The average results of three separate purification attempts by this method are shown in Table XIV. Nitrogen determinations were made by direct nesslerization techniques, and potency determinations were performed in mice.

TABLE XIV

Fraction	Fold Conc. From Culture	Mouse M.L.D./ml. (in 1,000's)	Mouse M.L.D./mg. N (in 1,000's)	Per cent Recovery	Fold Purif.
Whole culture	undiluted	3	4	-	-
First alc. fraction	6	15	40	83	10
$CaCl_2$	4	8	90	66	22
Second alc. fraction	16	28	325	58	81
Third alc. fraction	32	32	450	33	113

Purification of toxin by the alcohol-precipitation technique was found to give a purer product and a higher recovery of toxin in comparison with the phosphate precipitation method. This method was easily executed and the results obtained were easily reproducible.

Further experiments to determine the degree of purity obtained were not undertaken. Preliminary ultracentrifugation studies by Duff et al. on the third alcohol fraction indicated that it was not homogeneous.

D. Immunization of rabbits with Type E toxoids.

During the preliminary experiments on toxin activation and purification, an experiment was undertaken to study the immunization of rabbits with a number of Type E toxoids which had been prepared up to that time. Information obtained from this experiment regarding toxoiding-time, animal response and duration of immunization aided considerably in the preparation of toxoids for human immunization.

1. Toxoids.

Four toxoids were prepared by the formolization of different toxins. The toxoids, designated A, B, C and D, were prepared as shown below.

Type A - derived from an activated toxin produced by culturing together, in dialysis sacs, strains "VH" (T) and "Iwanai" (OT). The potency of this preparation was 1-3 million mouse M.L.D. per ml. Detoxification of the toxin was effected by the addition of formalin to 0.4 per cent final concentration, and incubation at 30°C. The time required for complete detoxification, as indicated by mice injections, was 25 days. Complete loss in potency was indicated by the failure of five mice to develop symptoms of intoxication and loss in weight over a period of 2 weeks after receiving 0.5 ml. of the toxoid intraperitoneally. Toxoids were stored at 4°C.

Type B - derived from an activated toxin produced by the combination of sterile filtrates of "VH" (T) and "Iwanai" (OT) cultures. The final potency of the preparation was 200,000 to 250,000 mouse M.L.D. per ml. Detoxification was effected by addition of formalin to 0.3 per cent and incubation at 30°C. Detoxification time was 20 days.

Type C - derived from a crude toxin produced by the cultivation of strain "Iwanai" (OT) in G.P.B.I. medium as outlined under the heading of "Toxin production". Potency of the crude toxin was 2,500 - 3,000 mouse M.L.D. per ml. The toxin was detoxified by the addition of formalin to a final concentration of 0.4 per cent and incubation at 30°C. for 19 days. Criteria for complete detoxification were the same as for Types A and B.

Type D - derived from a non-toxic "VH" (T) culture as outlined under production of non-toxic filtrates. Formolization of this filtrate was, of course, not required.

Sterility tests were conducted on all four toxoids before animal injections. Five ml. of each toxoid were inoculated into tubes of G.P.B.I. medium and tryptose-phosphate broth, and were incubated aerobically and anaerobically for two weeks at 37°C. Macroscopic and microscopic observations of each tube showed the toxoids to be sterile.

2. Immunization schedule.

Each toxoid was injected intravenously into 2 rabbits, designated #1 and #2. Animal bleeds and injections were performed in accordance with the schedule shown in Table XV.

TABLE XV

Rabbit immunization schedule.

Day	Toxoids							
	A		B		C		D	
	#1	#2	#1	#2	#1	#2	#1	#2
1	bleed	bleed	bleed	bleed	bleed	bleed	bleed	bleed
1	0.25 ml.	0.25 ml.	0.25 ml.	0.25 ml.	0.25 ml.	0.25 ml.	0.25 ml.	0.25 ml.
4	0.75 "	0.75 "	0.75 "	0.75 "	0.75 "	0.75 "	0.75 "	0.75 "
10	1.00 "	1.00 "	1.00 "	1.00 "	1.00 "	1.00 "	1.00 "	1.00 "
20	bleed	bleed	bleed	bleed	bleed	bleed	bleed	bleed
50	2.00 ml.	2.00 ml.	2.00 ml.	2.00 ml.	2.00 ml.	2.00 ml.	2.00 ml.	2.00 ml.
60	bleed	bleed	bleed	bleed*	bleed	bleed	bleed	bleed
90	"	"	"	-	"	"	"	"
120	"	"	"	-	"	"	"	"
150	" *	"	"	-	"	"	"	"
200	-	"	"	-	"	"	"	"
300	-	"	"	-	"	"	"	"

* Death of animal

3. Results.

Rabbit serum was titrated for antitoxin by toxin-neutralization tests in mice; the titres designated are the average values of anti-mouse M.L.D. per ml. of rabbit sera. Table XVI and Diagram X illustrate the results of these titrations.

TABLE XVI

Day	Anti-mouse M.L.D. per ml. (in 1,000's)			
	A	B	C	D
0	1	1	1	1
20	0.02	0.2	0.15	0.005
60	1.15	15.0	10.0	0.15
90	1.0	15.0	10.0	0.10
120	0.75	7.5	5.0	0.02
150	0.4	4.0	2.0	0.02
200	0.4	4.0	2.0	0.02
300	0.15	2.0	2.0	0.015

Over the period of the first three injections, all 4 toxoids elicited a very slow immune response. The anemnestic response was well indicated by a marked increase in serum anti-M.L.D. following the administration of the booster injection. The level of immunity then decreased slowly and could still be demonstrated eight months after the final injection.

Activated toxoid (B) produced the highest level of circulating antibody. Animals immunized with the crude toxoid (C) demonstrated the second highest titre. Activated toxoid (A) did not stimulate antibody production comparable to toxoids B or C.

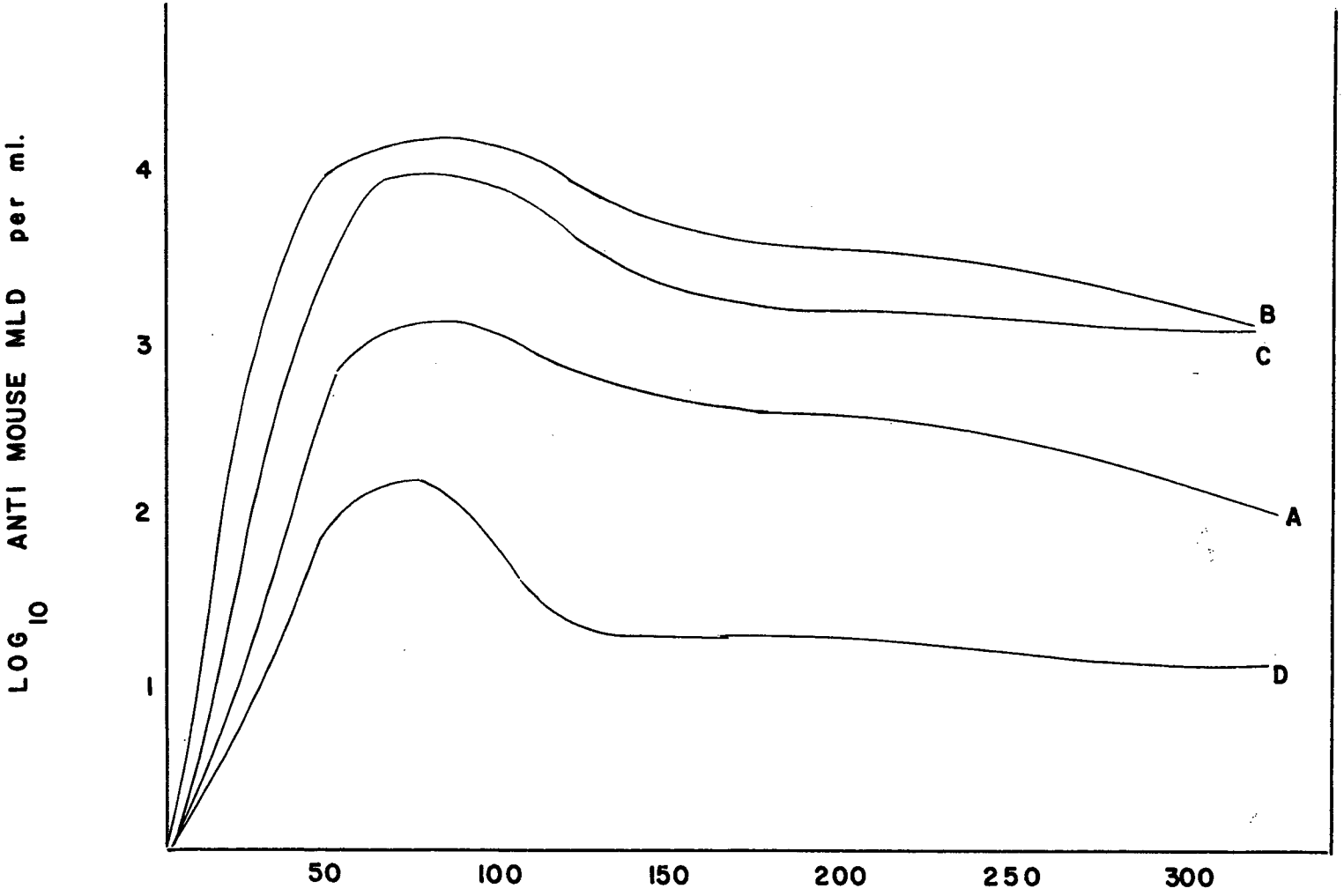

Diagram X Rabbit immunization.

A rather unexpected result was obtained with toxoid D, prepared from a non-toxic culture. Both animals receiving this toxin developed a low but definite immunity to crude Type E toxin.

When the four types of antisera were titrated against their homologous toxins, i.e. Type A antisera titrated against the activated toxin employed for the production of toxoid A, they demonstrated ranges not unlike those already shown for the titrations against the crude toxin. Although all these values are not listed here, it should be mentioned that the crude toxoid (C) and the activated toxoid (B) produced almost identical antitoxin titres when titrated against all three toxins. These values are shown in Table XVII.

TABLE XVII

	Anti-mouse M.L.D. per ml. (in 1,000's)		
	Toxin A	Toxin B	Toxin C
Antiserum B	10	15	15*
Antiserum C	10	15	10*

*Reported in Table XVI

V. Human Immunization.

Five toxoids were employed for the immunization of human volunteers against Clostridium botulinum Type E toxin.

The high titres obtained in rabbits with toxoids B and C in the previous experiment, as well as the development of a low immunity in these animals when injected with a non-toxic filtrate (D), led to the use of these 3 toxoids in the human immunization programme.

A toxoid prepared from a purified toxin, and a second, prepared from a trypsin-activated toxin, were also used.

1. Preparation of toxoids.

Toxoid #1 was derived from an activated toxin produced by combining sterile filtrates of "Iwanai" (OT) and "VH" (T) and allowing the mixture to incubate 30 hours at 30°C. The potency of the activated product was 200,000 to 250,000 mouse M.L.D. per ml. One hundred per cent formalin*was added to the toxin to a final concentration of 0.3 per cent. Complete detoxification required 23 days at 30°C. and was demonstrated by the failure of five mice to develop botulinum intoxication symptoms, or loss in weight over a period of two weeks.

Toxoid #2 was derived from a trypsin-activated "Iwanai" (OT) toxin to which had been added soya-inhibitor to prevent a rapid loss in toxicity.

The conditions required for optimum toxin activation were determined in the preliminary experiments and applied to the production of the toxin for the preparation of this toxoid. The potency of the activated product was 50 million to 100 million mouse M.L.D. per ml. Detoxification was brought about

*37.4 per cent HCOOH

by the addition of formalin to a final concentration of 0.3 per cent. Time required for complete detoxification, as indicated in mice, was only six days.

Toxoid #3 was derived from a purified "Iwanai" (OT) toxin. For the preparation of this toxoid, the third alcohol-fraction, made up in 0.2 M succinate buffer at pH 5.5, of the alcohol precipitation purification method was employed. This toxin suspension had a potency of 30,000 to 40,000 mouse M.L.D. per ml., or 350,000 to 400,000 mouse M.L.D. per mg. of nitrogen. Detoxification was effected by the addition of formalin to a final concentration of 0.3 per cent. Detoxification time was four days, again surprisingly short.

Toxoid #4 was derived from a crude "Iwanai" (OT) toxin filtrate. Potency of this toxin was 2,000 to 3,000 mouse M.L.D. per ml. Detoxification was effected by the addition of formalin to a final concentration of 0.3 per cent. Detoxification time for this crude filtrate was 23 days at 30°C.

Toxoid #5 was derived from a non-toxic "VH" (T) filtrate. Formolization of this filtrate was not required.

The five toxoids were dispensed in sterile 100 ml. serum bottles and stored at 4°C.

2. Sterility tests.

Three ml. of each toxoid were inoculated into each of 6 tubes of G.P.B.I. medium and 6 tubes of tryptose-phosphate medium. Three tubes of each medium were then incubated aerobically and three anaerobically, and observed for contamination every second day for a period of two weeks.

Sterility checks on the toxoids were also conducted in guinea pigs.

Five ml. of each toxoid were injected intraperitoneally into each of two guinea pigs, and the animals observed for a period of three weeks. None of the animals showed symptoms of botulinum intoxication or loss in weight during the three-week period.

3. Immunization schedule.

Twenty volunteers were available for the testing of the five toxoids. These were divided into 5 groups (A, B, C, D and E) each group containing four subjects.

Group A received toxoid #1

Group B received toxoid #2

Group C received toxoid #3

Group D received toxoid #4

Group E received toxoid #5

Toxoids were injected into the deltoid muscle and blood removed from a vein of the arm. The immunization programme is contained in Table XVIII.

TABLE XVIII

Day 0	10 ml. of blood removed from each volunteer.
" 0	0.1 ml. toxoid injected.
" 7	0.2 ml. toxoid injected.
" 21	0.5 ml. toxoid injected.
" 35	10 ml. blood removed from each volunteer.
" 115	1.0 ml. toxoid injected.
" 125	10 ml. blood removed from each volunteer.

4. Results.

The level of circulating antibody was measured by toxin-neutralization tests in mice, the titre being expressed in anti-mouse M.L.D. per ml. of serum.

Blood titrations of the volunteers prior to injection of toxoid indicated the absence of circulating antibody against Type E toxin in all subjects.

The results of the human immunization programme are contained in Table XIX.

TABLE XIX

Group	Subject	Anti-mouse M.L.D. per ml. of serum	
		Day 35	Day 125
A	1	6 - 10	300 - 350
	2	6	150 - 200
	3	6 - 10	350 - 450
	4	10 - 15	350 - 400
B	1	6	50 - 75
	2	6	40 - 50
	3	6	40 - 50
	4	6	-*
C	1	6 - 10	50 - 75
	2	6 - 10	30 - 40
	3	10 - 15	30 - 40
	4	6 - 10	40 - 50
D	1	20 - 30	-*
	2	30 - 40	1,000 - 2,000
	3	30 - 40	2,000 - 3,000
	4	15 - 20	800 - 1,000
E	1	6	6 - 10
	2	6	10 - 15
	3	6	6 - 10
	4	6	15 - 20

* Volunteers no longer available.

Group D, receiving toxoid #4, prepared from crude "Iwanai" (OT) toxin, demonstrated the highest level of antibody production.

Group A, receiving the toxoid prepared from combined filtrates of toxic and proteolytic variants, displayed the next best antibody level.

Groups B and C, receiving the toxoids prepared from trypsin-activated and purified toxins respectively, fell far below Groups A and D in antibody levels.

Group E, receiving the non-toxic filtrate, developed very little immunity to Type E toxin. However, the values in themselves are significant and will be discussed later.

Time did not permit further observations concerning the duration and rate of decline of immunity in the five groups.

VI. Discussion.

Clostridium botulinum Type E toxin, produced by the cultivation of a crude (OT) variant, is a stable toxin of relatively low potency. Preliminary experiments would suggest that the toxin is either released from the cells on autolysis, or the toxin is excreted outside the cells during life in a protoxin form, the protoxin then becoming active by the action of the intracellular enzymes released on autolysis. This latter explanation has been forwarded by Boroff (57) after he noticed that the addition of a 10 day culture to a young culture resulted in an immediate increase in toxicity. Maximum cellular autolysis, and consequently toxin production, is dependent both on the incubation period and the hydrogen-ion concentration; this pH (5.2 - 5.5) is not normally attained until 5 - 7 days of incubation at 32°C., long after the culture is past the logarithmic growth phase for this organism (58).

The characteristic rise in toxin potency which occurs when the culture is refrigerated for 24 hours following incubation may be the result of (a) a "shock" to the whole cells in the culture which have not yet lysed, causing their dissolution, or (b) a folding or splitting of toxin molecules already present, thus liberating more of the toxic groupings (57).

Activated toxins are relatively unstable and of extremely high potency. As with the crude toxin, incubation time, temperature and pH are seen to be very critical.

Several similarities are apparent when toxin is activated by the following methods:-

(i) the combination of sterile filtrates of toxic and proteolytic variants,

(ii) the growth of toxic and proteolytic variants in mixed culture,

(iii) the addition of trypsin.

In method (iii) activation of toxin is extremely rapid and the activated product very unstable, rapidly declining in potency after attaining maximum activity. In contrast to this, activation by methods (i) and (ii) is relatively slow and the activated product is relatively stable.

It should be mentioned here, however, that activation will continue after the toxin produced by the growth of mixed cultures has apparently reached its maximum activity, and has been stored at refrigeration temperatures. This slow activation continues until a maximum is reached and then, as with the trypsin-activated toxin, a slow destruction of toxin follows. The entire process may require a number of years. This has been demonstrated by the periodical titrations of stored toxin activated by this method.

It has also been shown that stored toxin which had been activated in this manner and had not attained maximum potency, could be rapidly activated by the addition of trypsin with the consequent rapid decline in potency after maximum activity was attained.

The different processes of activation, then, would seem to be basically the same, differing only in rate of reaction and possibly the height of maximum potency. In this process, purified trypsin is seen to act faster than crude trypsin, which in turn acts faster than a proteolytic filtrate.

The experiments on activation of toxin by trypsin indicated a definite rise in pH during the period of toxin activation. Since trypsin acts specifically to break peptide bonds adjacent to either of the two basic amino

acids, arginine or lysine, it is suggested that this activating process may be:-

(a) a result of the removal of contaminating protein from the surface of the toxin molecule, thus revealing an increased number of toxic endings, or

(b) a cleavage of the toxin molecule by the breakage of the specific peptide bonds adjacent to arginine and lysine residues, thus producing a large number of smaller toxic residues, and at the same time an increased number of basic amino acid endings, resulting in an increase in pH. The ionization of acidic amino acid endings, which would also have been produced would be suppressed by the acid pH of the filtrate.

Should this latter concept of the cleavage of the toxin molecule be the cause of activation, one would expect a decrease in weight of the toxin molecule on activation. There is no information of this type available for Type E toxin as yet. However, Wagman and Bateman (78), studying the absorption of Type A botulinum toxin, reported a decrease in the molecular weight of Type A toxin from 900,000 to 60,000 after this toxin had been adjusted to a pH of 7.5 with trypsin, simulating the conditions of the upper small intestine.

The experiments in animal and human immunization indicated that activated toxin had little, if any, advantage over crude toxin in the preparation of toxoids. If a comparison is made of the crude and activated toxins, on the basis of which one will produce the best toxoid, the following points are noted.

(1) Crude botulinum toxin can be prepared with little difficulty, and providing the same strains and medium are employed each time, cultures of uniform potency are obtained. Activated toxin, on the other hand, requires rigid control of such conditions as pH, temperature and duration of incubation, for its production.

(2) The crude toxin is very stable, undergoing no detectable change in potency for over two years when stored at refrigeration temperatures. Activated toxin is not stable and even at refrigeration temperatures will fluctuate in potency, regardless of the means of activation. The amount of variation in potency is seen to depend on the method employed for the production of the activated toxin. Each time this activated toxin is employed for the titration of an immune serum, it must be first tested for potency. In the case of highly activated toxins this test for potency should be conducted at the same time as the titration of the serum.

The addition of trypsin-inhibitor to these activated toxins moderates the potency decrease to a great extent; nevertheless the detrimental action will still occur.

(3) Finally, toxoids prepared from crude toxins were seen to produce a higher immunity in man than those prepared from activated toxins. This may have been brought about by the presence of extraneous protein surrounding the toxin molecule similar in antigenic structure to the toxin molecule per se. Supporting evidence, concerning the property of a toxoid prepared from a low-potency toxin producing a higher antigenic response in animals than a toxoid prepared from a high potency toxin, is given by the work of Nigg et al. (91). These workers noted

that the antigenicity of Types A and B toxoids were not proportional to the toxicity of the culture from which the toxoids were prepared. That is, antigenicity does not necessarily increase with potency.

Higher antibody levels than were obtained were expected for the toxoids prepared from highly activated and purified toxins. In view of the short periods of detoxification with 0.3 per cent formalin shown for these toxins, it might be suggested that the concentration of formalin employed was too high. Since these toxins would be in a more purified, and consequently more vulnerable state than the crude toxin, the formalin may have acted to cause considerable antigenic destruction of the toxin molecule. It is therefore suggested that lower concentrations of formalin should be employed for the detoxification of such activated and purified products.

The production of antibodies against Type E botulinum toxin by the injection into animals and humans of a non-toxic filtrate, produced from the atoxic "VH" (T) variant, would suggest a close similarity in molecular structure between the toxin of the (OT) filtrate, and the active substance in the proteolytic filtrate (T). It may be argued that the (T) filtrate actually contains a very minute and undetectable concentration of toxin which would account for the production of antibody. However, should this be the case, one would expect that this low concentration of toxin would be detectable in mice upon trypsin-activation of the (T) filtrate. This, in fact, was attempted but still failed to demonstrate the presence of one mouse M.L.D.

Immunization of animals with a non-toxic variant was also demonstrated by Boroff and Cabeen (92) with Clostridium botulinum Type C.

VII SUMMARY.

1. Crude Type E toxin prepared from a pure toxic variant (OT) is very stable and of relatively low potency.

2. The activation of Type E toxin is enzymatic in nature, and is therefore dependent on temperature, pH, enzyme concentration, and the condition of the substrate (toxin) per se.

3. Activation of Type E toxin may be accomplished by one of three methods:-

   (a) Culturing together proteolytic and toxic variants of "VH" (T) and "Iwanai" (OT) respectively.

   (b) By combining sterile filtrates of proteolytic (T) and toxic (OT) variants followed by incubation at 32°C.

   (c) The addition of crude and purified trypsin to a toxic filtrate, followed by the required incubation period.

4. Trypsin activation of toxin and destruction of activated toxin was shown to be arrested by the addition of purified soya-inhibitor.

5. Type E toxin was partially purified by two methods, one involving the precipitation of the toxin by concentrated salt solutions, and the other involving toxin precipitation by ethyl alcohol. The latter method was more easily executed and yielded a purer product, as shown by nitrogen determinations on both products.

6. Type E toxoids, suitable for human and animal immunization, were produced from crude, activated and purified toxins.

7. Formolized toxoid, produced from a crude toxic filtrate, was seen to produce the highest level of circulating antibody in man.

8. A low but definite immunity was also produced in human volunteers and rabbits by the injection of filtrate from a non-toxic proteolytic Type E variant, indicating a similar antigenic relationship and structure between the toxic and proteolytic molecules.

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IX. Appendices

Appendix A.

Two strains of Clostridium botulinum Type E were employed.

For the production of proteolytic filtrates the (T) or non-toxic proteolytic variant of the "Vancouver Herring" ("VH") strain was used. Type E toxin was produced by the cultivation of the toxic, non-proteolytic variant (OT) of the "Iwanai" strain.

The terms "T" and "OT", referring to the non-gas producing, proteolytic, non-toxic variant and the toxic, non-proteolytic, gas-producing variant respectively, have frequently been employed throughout this manuscript. As mentioned in the "Historical Review" this designation of variants was first employed by Dr. C.E. Dolman, and in a recent publication (51) the colonial morphology and biochemical characteristics of these variants are described. In his publication, however, the author altered the terms "T", "OT" and "O" to the more descriptive designation "TP", "TOX" and "OS" respectively. To demonstrate these characteristics more clearly, a picture of these two variants growing in G.P.B.I. medium is given (Plate I). Examination of tubes 1 and 3 in this picture clearly differentiate the gas-producing, non-proteolytic, toxic variant (OT) in tube 1, from the non-gas producing, proteolytic, non-toxic variant (T) in tube 3.

1 2 3

PLATE I.

Tube 1 Non-proteolytic, gas-producing, toxic variant (OT)

Tube 2 Control

Tube 3 Proteolytic, non-gas-producing, non-toxic variant (T).

Appendix B.

1. Media employed.

All the following media were sterilized by autoclaving at 15 pounds pressure, the time being dependent on the volume. Glass tubes containing 75 ml. of medium and Erlenmeyer flasks containing 4 litres of medium were sterilized for 20 minutes and 45 minutes respectively. Medium which was not used on the day it was prepared was boiled 5 minutes and cooled before inoculation.

(a) Glucose peptone beef infusion medium, with added meat (G.P.B.I.)

(Ref. James Morton, B.A. Thesis, 1944).

Infuse one pound of fat-free beef (minced) in one litre of tap-water overnight in the refrigerator. Infuse at 65°C. for 45 minutes. Strain first through one thickness and then two thicknesses of cheese-cloth. Filter through #1 Whatman paper. Take the volume and add the following:-

NaCl	0.5 per cent.
Difco peptone	1.0 per cent.
$Na_2HPO_4.12H_2O$	0.2 per cent.

Boil for 3 minutes and filter through #1 Whatman filter paper. Adjust to pH 7.8.

Dispense in large tubes with about one inch of meat in the bottom and approximately 25 ml. of broth. Sterilize. A sterile 50 per cent glucose solution is prepared. This is added to the tubes aseptically, before inoculating to give a final concentration of 2.0 per cent.

(b) Brain heart infusion broth (Difco).

Dissolve 37 grams of Bacto-Heart Infusion and 10 grams of sodium thioglycollate in 1,000 ml. of distilled water. Dispense and sterilize. The pH is 7.4. Fifty per cent sterile glucose is added before inoculation to a final concentration of 2.0 per cent.

(c) Brain heart infusion agar.

Preparation is the same as (b) with the addition of 1.5 per cent Bacto-Agar.

(d) Yeast extract broth (Difco).

(Ref. Duff et al. J. Bacteriol., 1957, 72, 455).

Dissolve 20 grams of Difco Yeast Extract and 20 grams of Bacto-Peptone in 1,000 ml. of distilled water. Dispense and sterilize. The pH is 7.2. Fifty per cent sterile glucose solution is added before inoculation to a final concentration of 2.0 per cent.

(e) Brewer's thioglycollate anaerobic broth.

Dissolve 40.5 grams of Brewer's medium in 1,000 ml. of distilled water. Sterilize and dispense. The pH is 7.2.

2. Activation of toxin with crude trypsin.

Add trypsin (Difco 1:200) to the toxin filtrate to a final concentration of one per cent weight to volume, and swirl the flasks gently until all the trypsin is dissolved.

3. Activation of toxin with purified trypsin.

(Ref. Duff et al. J. Bacteriol., 1957, 72, 453.)

Dissolve 0.005 gms. of Crystalline Trypsin (Difco) in 25 ml. of

distilled water, giving a final concentration of 0.02 per cent. Adjust to pH 6.0 with N/200 NaOH. For activation of toxin mix equal volumes of toxic filtrate and trypsin solution.

4. Purified soya inhibitor solution.

(Ref. Duff et al. J. Bacteriol., 1957, 72, 453.)

Dissolve 0.005 grams of purified Difco Soya-Inhibitor in 25 ml. of a 0.0025 M HCl solution giving a final concentration of 0.02 per cent. Adjust to pH 6.0 with N/200 NaOH. For inhibition of the purified trypsin, add an equal volume of the purified soya inhibitor solution.

5. Activation of toxin with crude pepsin.

Add Pepsin (Difco 1:250) to the toxin filtrate to a final concentration of 1 per cent weight to volume and swirl gently until all the pepsin is dissolved.

Appendix C.

The dialysis apparatus for the preparation of toxin is assembled as shown in Plate II and Diagram XI. The apparatus is sterilized by autoclaving for 2 hours at 15 pounds pressure. It is then cooled and assembled in the presence of an ultra-violet lamp, to reduce the chance of possible contamination, in an incubator at 32°C.

Flask (A) (see Diagram XI) contains 6 litres of G.P.B.I. medium and flask (B) 1,500 ml. of 0.85 per cent NaCl solution. After assembly, as shown in the diagram, clamps #3 and #4 are closed and clamp #1 is opened. Media in flask (A) is placed under negative pressure, with a suction pump attached to the air tube of flask (C) until the media begins to flow into the discard flask. At this time clamp #1 is closed allowing the media to flow into the dialysis tube (E) surrounding the dialysis sac (D).

When the dialysis tube (E) is approximately four-fifths filled, clamp #2 is closed. Clamp #4 is now opened, allowing the saline to flow into the dialysis sac (D) until the volume of saline is at an equal level to the media in the surrounding dialysis tube. Clamp #4 is then closed.

The apparatus is then allowed to remain 24 hours as a sterility check. If, at the end of this period, there are no visible signs of contamination the apparatus is inoculated through the inlet tube.

The medium is removed every 24 hours by opening clamp #1 and allowing the expended medium to run into the discard flask (C). Clamp #1 is then closed and clamp #2 opened, refilling the dialysis tube.

This procedure is repeated every 24 hours for the entire incubation period. Samples of toxin may be removed by opening clamp #3 at the base

of the apparatus and collecting the toxin in a sterile tube.

At the end of the incubation period the toxin culture is removed by opening clamp #3. The suspension is refrigerated for 24 hours and then clarified and sterilized by filtration.

PLATE II Dialysis apparatus.

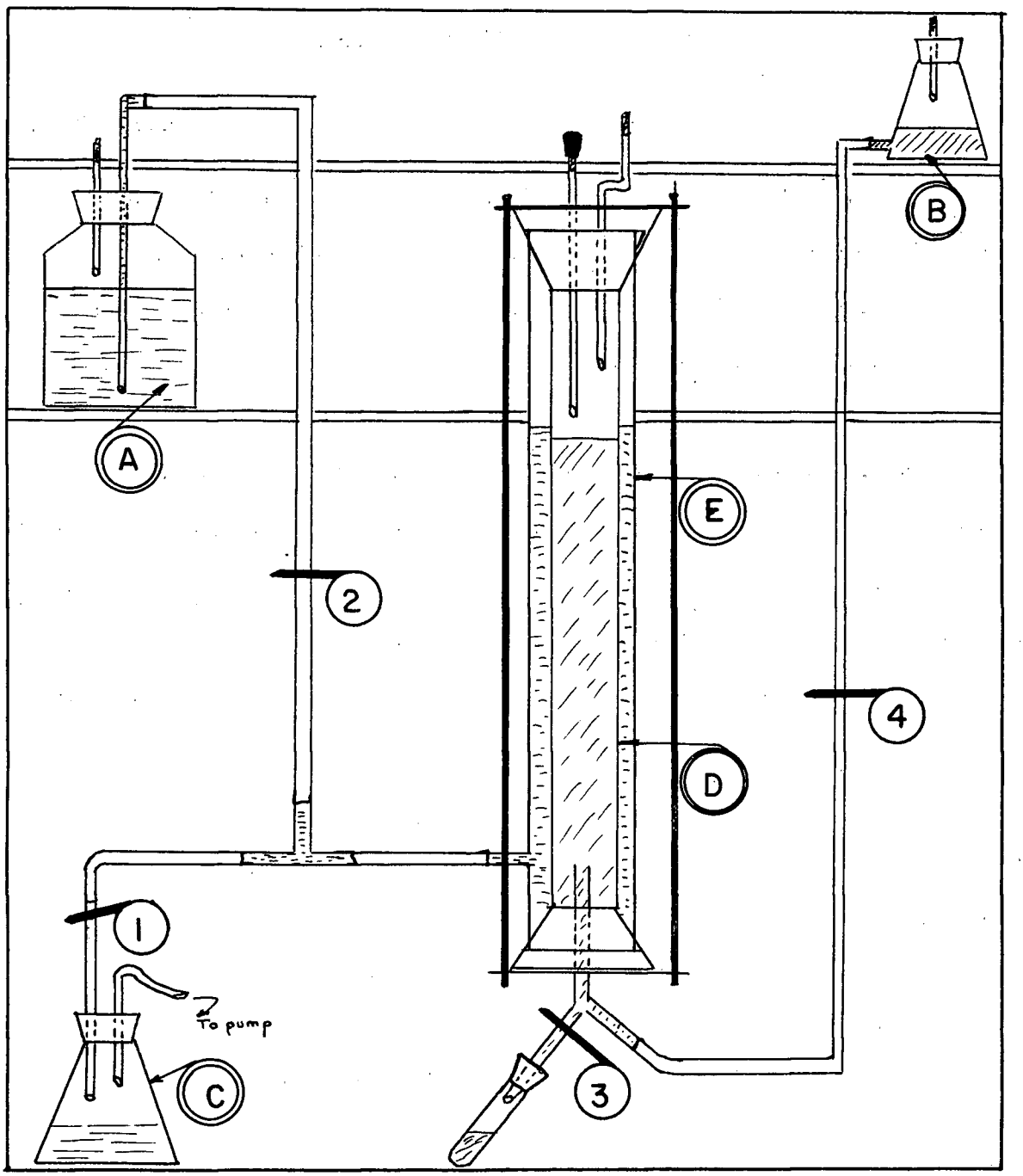

Diagram XI Dialysis apparatus for toxin production. *

(* Vinet, G., and Prodette, V., "Apperatus for the culture of bacteria in cellophane tubes", Science, 1951, 114, 549.)