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THE PHYSIOLOGICAL BASIS OF TOXIGENICITY  
OF CLOSTRIDIUM BOTULINUM TYPES A AND B

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## INTRODUCTION

"Experiment is the interpreter of Nature. Experiments never deceive. It is our judgment which sometimes deceives itself because it expects results which experiment refuses. We must consult experiment, varying the circumstances, until we have deduced the general rules, for experiment alone can furnish reliable rules."

Leonardo Da Vinci

This investigation was initiated as a result of a chance observation. Louis Pasteur once expressed the idea that chance favors only the trained mind. In a few rare instances however, the observations are of such a dramatic nature, they are quite impossible to overlook or ignore.

In an attempt to develop a growth medium suitable for the production of anaerobic spores, it was observed that glucose, above a given level in a complex medium, completely inhibited spore production of Clostridium botulinum type A. This in itself was not very surprising since the formation of clostridial spores has proved to be a very elusive phenomenon. On the other hand autolysis of the cultures after maximum growth had been achieved, was surprising, not because the phenomenon of autolysis is unique among bacterial forms, but because of the rapidity and degree of lysis observed in this case. A priori, there was no reason to assume that a correlation existed between lysis of the vegetative cells of C. botulinum and the quantity of toxin found in the external environment. On the other hand, in view of the uncertain state of the exotoxin and endotoxin concepts, this correlation presented itself

as a possibility distinctly worthy of investigation.

Examination of the literature concerning the *Clostridium* group as a whole and *C. botulinum* specifically, revealed that although autolysis had been accepted as a general characteristic of the entire anaerobic spore-forming group of bacteria, it had not been definitely recognized as being a mechanism whereby the toxin was released into the external environment. The organisms which are classified as producers of "exotoxin" in the classical sense are quite inconsistent in their toxin producing characteristics and the rigid classification imposed upon them seems unwarranted. At one extreme, the Alpha toxin of *Clostridium welchii* is produced and excreted into the medium during the phase of active growth and reaches its highest concentration when the growth of the parent cells has reached its maximum. Conversely, the toxin of *Clostridium tetani*, reaches its highest concentration in the culture filtrate many days after the organism has attained maximum growth. Somewhere between these two extremes, but apparently at no fixed point chronologically, the toxin of *C. botulinum* appears extracellularly.

As originally planned this investigation involved a quantitative study of the growth, lytic and toxin synthesizing processes of accessible strains of *C. botulinum*. This approach introduced many other intriguing possibilities which lend themselves quite readily to the study of protein toxins in general. Unlike problems which are clearly defined before experimental work is initiated, this investigation possessed autocatalytic properties, so to speak, which make speculation and generalization quite difficult to avoid. Realizing that speculation is desirable, it however, must be based on sound experimental observation. With this in mind, the

current investigation was restricted to studies of the growth and lysis of C. botulinum in relation to toxin production; the effect of the physical and chemical environment on these processes; and once synthesized, the possible role of the normal proteolytic enzyme complement of the organism in conferring to the protein toxin molecule its astonishing biological activity.

The results delineated in this thesis represent isolated experimental observations made with a number of strains of one species of organism capable of producing what has been termed a bacterial toxin. Especially since the advent of the Delft school of comparative biochemistry, it has been shown time and time again that amidst apparent diversity a certain amount of unity exists between organisms at opposite ends of the evolutionary scale. It is only the embellishments which tend to obscure the similarities. Therefore, within this one group of bacteria, which are so similar in many other respects, there is no a priori reason to negate the possibility for the existence of a fundamental unity insofar as the toxin producing mechanisms are concerned. It is hoped that the isolated experiments described here, will be useful in the Da Vincian sense, i.e., that they can be incorporated into the body of experimental evidence which will ultimately allow the deduction of the general rules concerning the mechanisms responsible for the toxigenicity of bacterial cells.



## I. HISTORICAL

### General Considerations.

Since van Ermengen's discovery in 1896 of C. botulinum as the causative agent in an outbreak of food intoxication, the protein neurotoxin elaborated by this anaerobic, spore-forming bacterium has been a subject of widespread interest in many areas of scientific research. Food bacteriologists in the canning industries and elsewhere have been interested in the toxin primarily as a potential health hazard and have very successfully coped with the problem as evidenced by the virtual elimination of botulinus food poisoning from commercially prepared materials. Studies of a more theoretical nature, however, have been far more difficult to evaluate and consequently many investigators such as the clinician or pharmacologist, the biochemist, immunologist and cellular physiologist are still faced with many problems concerning this toxin, the answers to which remain relatively obscure.

The idea that harm caused by infectious diseases might be due to microbial poisons was entertained even before the germ theory of disease had been established. Both endotoxins and exotoxins were foreseen in 1713, by Vallisnieri, who suggested in his Riposta that "the little worms of the most atrocious pests are of themselves of a poisonous nature" and that "as often as they enter the humoral mass (they) defile it with their excrement, adulterate it, pollute it." The first practical demonstration of a bacterial toxin was made in 1884, by Loeffler, the discoverer of the diphtheria bacillus. Credit for the discovery of the toxin, however, is generally given to Roux and Yersin (1888) who

demonstrated the toxicity of bacterial filtrates for guinea pigs. This discovery encouraged Roux in the belief that the harmful effects of all pathogenic bacteria were due to poisons; and that by administering gradually increasing doses of these poisons to animals, it would be possible to render the animals refractory, not only to the toxin but even to the microbe itself. Within two years, von Behring and Kitasato (1890) discovered tetanus toxin and announced that "the immunity of rabbits and mice, immunized against tetanus, consists of the power of the cell-free blood fluid to render innocuous the toxic substance which the tetanus bacilli produce." After van Ermengem's discovery of botulinus toxin, considerable stimulus was given to the idea that the harmful effects of all infectious diseases were due to toxins, and medical research was dominated by the conception of antitoxin serum therapy. This led to a tremendous expansion of immunology, then in its infancy, but added little to our knowledge of the exact nature of toxins and the role they play in the pathology of infectious diseases. During the half-century that followed, although many more bacterial toxins were discovered, very little fundamental progress has been made toward an understanding of their mechanisms of action. It is probably more than a coincidence that the three bacterial toxins referred to above, the first ones to be discovered, should be the only ones that are unequivocally responsible for the harmful effects of the diseases in which they are concerned.

The relative importance of toxins in bacterial diseases varies considerably. This is illustrated by anthrax at the one extreme and botulism at the other. In the former, the spores of Bacillus anthracis germinate rapidly and the vegetative cells invade the tissues extensively.

The numbers of bacteria present in the blood as death approaches are so enormous that until very recently, it was thought that death might be caused directly by mechanical blockage of the capillaries. However, Smith and Keppie (1955) have isolated two fractions, one intracellular and the other extracellular, which may be ultimately classified as the bacterial toxins responsible for death in anthrax infections. On the other hand, an animal that dies of botulism is rarely infected by the causative organism, C. botulinum. If the toxin were not effective by mouth the organism would not be considered pathogenic because it very seldom invades the animal host. It was not until 1950 that Hampson reported the isolation of the organism from a naturally infected wound which resulted in botulinus intoxication. Obviously, this is a rare occurrence and botulism is usually results from the ingestion of food in which the organism has grown and produced its highly potent toxin. It can be said therefore, that botulism is a disease which is entirely due to a bacterial toxin.

Exotoxins vs. Endotoxins.

The bacterial toxins are conventionally classified as endotoxins and exotoxins, i.e., according to whether they are found inside or outside the parent organisms. This arbitrary classification is open to many qualifications and the terminology is quite misleading. No less an authority than van Heyningen, as recently as 1950, stated "an exotoxin is excreted during the phase of active growth of the organism and reaches its highest concentration at the time growth reaches its maximum or soon after". In view of experimental evidence obtained during the past decade

this concept of the "true, soluble exotoxin" has undergone a reappraisal. It has been found that the three classical exotoxins, diphtheria, tetanus and botulinus, can be recovered in considerable quantity from washed cells by means of solvents (Raynaud and Second, 1949; Boroff, 1952). On the other hand, exotoxins are assumed to be structural components of the bacterial cells in spite of the fact that they are often found in cell-free autolysates (van Heyningen, 1950). It is apparent, then, that the clear-cut distinction made between the extracellular and intracellular nature of the two classes of bacterial toxins is not valid. Indeed some investigators (Oakley, 1954) are of the opinion that the terminology should be discontinued. Valid differences do exist between the bacterial toxins, but these differences can be attributed primarily to their chemical composition. The exotoxins which have been purified are simple proteins (Lamanna et.al. 1947; Pillemer et.al. 1948; Pappenheimer, 1937) whereas most endotoxins have been found to be polymolecular phospholipid-poly-saccharide-protein complexes (Boivin, 1940/41a; Morgan and Partridge 1942). It is quite natural that the former should be highly antigenic, readily converted to toxiods, heat labile and pharmacologically quite specific in their action. On the other hand, the latter are not generally considered good antigens, are not easily converted to toxiods, are heat stable and the clinical symptoms of the diseases they produce are quite similar, irrespective of the organisms from which they are derived

Nature and Properties of Botulinus Toxin.

Studies of the many toxigenic strains of C. botulinum which have been isolated from various epidemics and epizootics have shown that, in spite of the fact that the clinical symptoms in the affected human

beings or animals presented a single, common, essentially neurological syndrome, their respective toxins presented immunological differences that enabled them to be separated into several distinct types. Five types, A-E, have now received general recognition. In almost all the human outbreaks in which typing has been carried out, either type "A" or type "B" has been incriminated. In epizootics, the other types are generally found.

The biological activity of the toxin produced by C. botulinum is truly astonishing. Even in crude preparations, this bacterial metabolite is unmatched for lethal power by any other poisonous substance known to man. Type A and B toxins have been purified and are chemically distinct. The isolation and crystallization of "A" toxin was accomplished simultaneously by two groups (Lamanna, et.al 1946; Abrams et.al 1946) on the basis of an observation made by Sommer (1937) that the toxin is precipitated at pH levels below 4.0. In 1946 Putnam, Lamanna and Sharp determined the physical characteristics of the toxin and from these data calculated its molecular weight to be approximately 900,000. Kegeles (1946), making a slightly different physico-chemical examination, calculated a slightly higher molecular weight of 1,100,000. The crystalline material appears to be an ordinary globulin-like protein containing no prosthetic groups or any other special characteristics which might account for its astonishing biological activity. When assayed in mice, pure toxin contains more than thirty million minimum lethal doses per mg and this when compared on a weight basis with aconitin, the most toxic drug known, represents fifteen thousand times the toxicity of this drug. A molecular comparison shows that a type A toxin molecule is twenty million times as toxic as aconitin (van Heyningen, 1950).

Lamanna and Glassman (1947) purified type B culture filtrates and again separated the toxin as an electrophoretically homogenous protein having different physical characteristics than those of "A" toxin. It is chemically distinct, is a much smaller molecule and is rather less toxic for mice than type A toxin when compared on a weight basis. Wentzel et.al., (1950) obtained a partially purified preparation of type D toxin and estimate its toxicity to be even more potent than that of purified "A" toxin. If their estimates of the molecular weight and minimal lethal dose of type D toxin are correct, a fatal intoxication of a mouse could be effected with approximately 1000 molecules (Wright, 1955). In the ordinary concepts of pathogenesis, numbers of this order are more commonly associated with the idea of some replicating particle.

A tremendous amount of work has been done in attempting to determine the site(s) and mode of action of botulinus toxins. A large body of evidence has now been brought together to show that botulinus toxin acts widely on all parts of the peripheral nervous system that are cholinergic in character and is without effect on the adrenergic nerves. The evidence suggests that it acts on the nerve endings by interfering with the release and not the synthesis of acetylcholine (Ambache, 1949; Burgen et.al., 1949). In contrast, the cholinergic structures in the central nervous system do not seem to be susceptible to the action of the toxin (Davies et.al., 1953). It should be pointed out that the only significant consideration which seems to implicate the cholinacetylase system as the specific site for attack by this toxin is the indirect evidence that the nerve terminals of the adrenergic system are not vulnerable to the neurotoxin. Attempts to inhibit the cholinacetylase

mechanism in vitro by Payling-Wright (1955) and others, however, have proven unsuccessful.

The literature concerning the effects of the chemical and physical environment on the growth and toxin production of C. botulinum is voluminous. The major portion of the work however was done between 1896 and 1930. By and large, the investigations were of a qualitative rather than a quantitative nature, and many were complicated by the fact that the various strains of C. botulinum isolated from a multitude of sources and being used in these studies had not been adequately described or classified. Consequently, investigators working in this area during the early part of this century frequently found themselves in partial or full disagreement on many aspects of the problem. Probably as a result of the elimination of botulinus intoxication as an important public health problem, by 1930 research in this direction came to a virtual halt. With the possible exceptions of the work at the Hooper Foundation, and Fort Detrick, very little has been done. The former group became involved primarily in the classification of the species, and reports from the latter have been infrequent, due quite likely to security restrictions. During the past few years, interest in the subject has been revived and in view of the fact that earlier research had resulted in many observations which deserved exploitation, the study of botulinus toxin is proving to be a very fruitful field of research.

It is not surprising that the first manuscripts to be published after the twenty year drought, which deal with the origin of the protein neurotoxin synthesized by C. botulinum tend to negate the concept of its extracellular nature. Boroff et.al, (1952) demonstrated that more toxin

could be extracted from the cells of a 48 hr culture of C. botulinum type D than could be found in the medium and concluded that the toxin appeared in the medium only after the death of the organism. In 1955, Boroff definitely correlated the autolysis of C. botulinum type C with the appearance of toxin in the medium. It is interesting to note that some early work had also bared the possibility for the intracellular nature of the toxin. Nelson (1927) made the observation that botulinus toxin was intimately associated with the "bacterial globulin" although not identical with it. Dozier (1924), while trying to determine the effect of salt and sugar on the growth and toxin production of C. botulinum, reported that the decline in the number of viable organisms was related to the appearance of toxin in the medium. Ellberg and Meyer (1939) came to the same conclusion in an investigation concerned primarily with determination of the proteolytic enzyme complement of some organisms in this species. Evidence supporting the extracellular concept, however, is not entirely lacking. Dack et.al., (1928) found that growth and toxin production reached a maximum at the same time and concluded that the toxin was a direct metabolic by-product excreted into the medium during the organism's growth. Raynaud et.al. (1955a,b) also concluded that the toxin was formed principally during the logarithmic phase of growth.

Conflicting reports have also been recently published concerning the possible site of toxin synthesis and the relationship between the cell surface and toxicity of the organism. Kindler et.al. (1956) have found that thoroughly washed cells of Clostridium parobotulinum type A possess a very low level of toxicity for mice. From this evidence they concluded that the "bound" (intracellular) toxin is totally ineffective in exerting



its pharmacological activity and that the surface contributed nothing to toxicity. On the other hand, Boroff (1955) reported that washed cell preparations of C. botulinum, type C, when injected intravenously into mice, killed almost as fast as the soluble toxin. Since death occurred in 30 to 40 minutes, he states "it is not likely that the injected organisms were lysed in the body of the mouse in this short period of time. It is easier to imagine that death is due to a content of the toxic surface substance of the organism".

In the face of the comparatively large number of conflicting reports found in the literature, it became evident that the manifestation of toxicity was not an all or none proposition dependent solely on the synthesis of the toxin by C. botulinum. In effect, it appeared as if other unknown factors were operative, which, depending on the environmental conditions, experimental design, and strain of organism used, resulted in the relative toxicity or non-toxicity of the culture. These alleged unknown factors both consciously and sub-consciously provided the stimulus and directed the thinking employed during the expansion of the following investigation.

## II. MATERIALS AND METHODS

### Organisms.

The source and designation of C. botulinum types A and B used in this investigation are as follows:

- a. C. botulinum 62A; obtained originally from the Hooper Foundation for Medical Research, San Francisco, California.
- b. C. parobotulinum 457A; obtained originally from the stock culture collection of the Bacteriology Department, Indiana University through the courtesy of Dr. McClung.
- c. C. botulinum, type A (JTD-IV) and
- d. C. botulinum, type B (B-201) both sent through the courtesy of Dr. James Duff, Fort Detrick, Frederick, Maryland.
- e. C. botulinum 213B originally obtained from the National Canners Association, Washington, D.C.

### Stock Cultures.

In order to reduce the possibility of spontaneous mutations and to insure uniformity of response, all strains of the organism were grown in a complete medium for 18 hr at 35C. After this incubation period, 2 ml quantities of the cultures were aseptically transferred to sterile 4 ml screw cap vials which were immediately quick frozen at -70C and stored at -20C. All experiments were then initiated from a frozen culture which was thawed at room temperature.

### Media.

It was found that the presence of particulate, proteinaceous matter enhanced the viability of C. botulinum which was subjected to the

freezing and thawing operation outlined above. Consequently, the stock cultures were stored in a beef infusion medium described by Duff (1956) which contained very small particles of meat. In addition, the organisms initially transferred from the frozen state were much more sensitive to the presence of oxygen. A much more effective growth response was obtained if the primary transfers were made into 3% semi-solid thioglycollate (Difco) rather than into a pure broth medium.

The constituents of the complete medium employed for the cultivation of type A strains of C. botulinum after growth in thioglycollate medium had been obtained are as follows:

Tryptic digest of casein, (N-Z Amine, type B)----- 2.0%  
Sheffield Chem. Co.

Autolyzed Yeast extract, (Vico) ----dry solids----- 0.5%

Glucose----- 0.5%

For the cultivation of type B strains, a complete medium with the following composition was employed:

Trypticase (BBL)----- 1.5%

Yeast Extract (Difco)----- 0.5%

Cysteine Hydrochloride (Mathison Co.)----- 0.075%

Glucose----- 0.5%

In some experiments the glucose was replaced by another carbohydrate at the same concentration. In all cases the carbohydrate was added in the form of a sterile 20% (w/v) solution after the medium had been autoclaved at 15 lb pressure for 20 minutes and had been adjusted to pH 7.0 as determined with a Beckman pH meter.

The defined medium used for the cultivation of both types A and B strains of C. botulinum was modified from Mager et.al.(1954).

Defined medium for growth of C. botulinum.

Vitamin-free Casamino acids (Difco)-----	3.0%
Tryptophane (crystalline)-----	100 mg/l
Salts A -----	
-----K <sub>2</sub> HPO <sub>4</sub> -----	0.5 g/l
-----KH <sub>2</sub> PO <sub>4</sub> -----	0.5 g/l
Salts B-----	
-----MgSO <sub>4</sub> .7H <sub>2</sub> O-----	0.2 g/l
-----NaCl-----	0.01 g/l
-----MnSO <sub>4</sub> .3H <sub>2</sub> O-----	0.01 g/l
-----FeSO <sub>4</sub> .7H <sub>2</sub> O-----	0.01 g/l
Sodium Thioglycollate (Difco)-----	500 mg/l
Thiamine-----	4 mg/l
p-Aminobenzoic acid-----	100 µg/l
Nicotinic acid-----	10 mg/l
Pyridoxin-----	5 mg/l
Biotin-----	5 µg/l
Glucose-----	0.5%

The medium was freshly prepared from concentrated stock solutions which were stored at 4 C. After adjustment to pH 6.9, the medium was sterilized by Seitz filtration and dispensed aseptically into screw cap tubes or Erlenmayer flasks as desired. In contrast to other synthetic media described in the literature it was found that the medium described above could be sterilized effectively in toto and did not require separate sterilization of component parts.

The various media employed during the initial survey will be discussed in the appropriate sections under Experimental Results.

General experimental conditions

Adaptation to a given set of environmental conditions was always

carried out before response of the organism was measured. For example, if growth and/or toxin production in a given medium at a given pH were to be assayed, the frozen culture was transferred to thioglycollate medium and then to the test medium which had been adjusted to the desired pH for at least two further transfers. This procedure was used throughout the investigation in order to eliminate any extraneous factors introduced from the initial inoculum and also to eliminate the adaptive mechanisms of the organisms to its previous environment. It was felt that these precautions served as constant experimental controls and thus insured a true response by the organism to those variables under consideration.

Completely **anaerobic** conditions were not necessary for the growth of any strains of C. botulinum used in the study provided comparatively deep cultures were employed. The media were evacuated by immersion in a boiling water bath for 10 minutes, rapidly cooled and immediately inoculated. Growth obtained under these conditions and in a completely anaerobic environment was found to be identical.

Cultures were incubated at 35 C unless otherwise stated. This temperature supported maximum growth and toxin synthesis. It was also found to be quite convenient for experimentation since incubation overnight at this temperature produced cultures near the end of the accelerated growth phase and a sample could be conveniently taken at this critical transition point in the life of the culture. Overnight incubation at higher temperatures usually resulted in cultures already in the stationary phase.

Periodically, microscopic examinations were made to determine

the purity of the cultures and the toxin elaborated was checked by neutralization tests with specific A or B antiserum.

#### Turbidimetry.

Growth curves of C. botulinum were established turbidimetrically with a Klett-Summerson photoelectric colorimeter (Model 800-3) measured at 640 m $\mu$ . The phenomenon of lysis was so dramatic that viable cell counts were found to be unnecessary. As the organisms lysed, the decrease in the optical density was readily measureable. Divergence between the total and viable counts usually encountered with organisms which retain their cellular integrity after multiplication has ceased was not apparent with C. botulinum. Consequently, the turbidimetric method in this case was considered to give reliable indices of viable cell numbers.

#### Preparation of Spore suspensions.

Strain JTD-IV did not sporulate under any conditions, a fact which was confirmed by Duff (1956). Spores of C. botulinum strains 62A, 457 A and B-201 were obtained by growth in 10% casitone (BBL) at 30 C for a period of 7 to 10 days. The spores were harvested by centrifugation and washed thoroughly in sterile distilled water. The final suspensions were adjusted to contain approximately  $10^7$  spores/ml and any vegetative cells which may have been present were inactivated by boiling for 15 minutes.

#### Disintegration of Cells.

Both vegetative and spore forms were disintegrated by sonic

oscillation in a 10KC Raytheon sonic oscillator. To facilitate disintegration of the spores, approximately 1g of Superbrite (Minnesota Mining and Mfg. Co.; grade 10) glass beads per ml of suspension was added before sonic treatment. The addition of glass beads was not necessary for the disruption of the vegetative cells. Examination under the phase microscope revealed very few intact cells left after 30 minutes in the case of the vegetative forms or after 1.0 hour with the spores.

#### Centrifugation.

Harvesting of all cultures was carried out at 4 C. If large volumes of culture fluids had to be clarified for the purpose of obtaining cells, the International Centrifuge at 2500 rpm or the Lourdes Angle-Head centrifuge at 6000 G was used. When cell-free supernatant materials were required, the cultures were sedimented in the Servall SS-1 angle-head centrifuge at 20,000 G. Filtrates obtained by Seitz filtration and the supernatant fluids obtained by high-speed centrifugation were found to give exactly the same toxin titers, thereby showing that both methods were suitable for assaying only the solubilized toxin. Consequently, the terms filtrate and supernatant fluid are used synonymously throughout the investigation.

#### Toxin assay.

Supernatant culture fluids were obtained by high-speed centrifugation or by Seitz filtration at 4 C. If cellular extracts were to be assayed, the cellular debris was removed beforehand in the same manner. Due to the lability of the toxin, certain precautions were taken during manipulation of the samples to be assayed. Dilutions were made in sterile phosphate buffer (pH 6.8) containing 0.5% gelatin. The diluent was dispensed into serological test tubes and cooled in an ice-bath before serial

dilutions of the samples were made. Toxin titers were established by intraperitoneal inoculation of the diluted samples into white mice (20 to 25 g). Four mice were inoculated at each dilution with the inoculum ranging from 0.1 to 0.5 ml. The animals were observed for symptoms of botulinus intoxication over a period of 4 days and toxicity was calculated as minimum lethal doses per ml (MLD/ml) according to the method described by Wadsworth (1947). The procedure was slightly modified so that the number of animals necessary was reduced. The minimum lethal dose is defined as the smallest amount of toxin which when injected intraperitoneally will kill a mouse weighing between 20 and 25 g. The estimation of the true minimum lethal dose or the LD<sub>50</sub> requires the use of large numbers of animals. The minimum lethal dose, also is not usually considered as meaningful as the LD<sub>50</sub>. However, since the number of animals available during the course of the current investigation was limited, toxicity was estimated by determining as closely as economically feasible, the least amount of toxin which proved fatal to the animals. For example, if 0.2 ml of a one to ten thousand dilution of a sample was not fatal and 0.3 ml of the same dilution was fatal to the mice, the number of lethal doses was estimated as  $< 5 \times 10^4$  and  $> 3.3 \times 10^4$ . As can be seen, being more precise than this would involve one or more animal titrations using quantities of sample between 0.2 and 0.3 ml. Since only relative changes in toxicity were important in this investigation, the modification of the method described above was considered justifiable if the procedure as used was standardized. Therefore, although the term minimum lethal dose is used throughout the investigation, it should be kept in mind that this was done solely for



the purpose of expressing toxicity in terms of a definable unit. For the sake of clarity the minimum lethal dose (MLD) was used to express toxicity, but it is well to realize that the limited accuracy of the procedure employed may not fully justify such an absolute expression. Worded in a slightly more precise manner, the minimum lethal dose as used in this investigation represents at least that quantity of toxin which was capable of killing the animal.

Samples were assayed as soon as possible but it was found that the toxin titers did not decrease appreciably if the samples were stored in the gelatin-phosphate buffer at 4 C for periods up to 48 hr. Beyond this point, toxin titers were erratic and consequently, samples were never stored longer than 48 hr before assay.

It became evident from the work of this investigation that toxin-free supernatant fluids cannot be obtained even after numerous washings, indicating a constant "leakage" of toxic materials from the cells into the surrounding fluid. The intracellular toxin, therefore, was measured indirectly. An aliquot of the culture was removed and the cell-free supernatant was assayed. Another aliquot was taken at the same time and placed in the sonic oscillator for 30 min, thereby releasing any intracellular toxin into the mother liquid. The cellular debris was removed and the toxicity of the extract represented a summation of both intra and extracellular toxin. Bound intracellular toxin was then calculated by subtracting the free from the total toxin titer.

#### Determination of "Masked" or Protoxin.

The presence of an inactive toxin was demonstrated by treatment of the supernatant culture fluids or cell-free extracts with proteolytic

enzymes. Crystalline trypsin and pepsin were obtained from the Armour Research Foundation. Samples to be treated with the enzymes were clarified by centrifugation. They were adjusted to pH 4.0 if they were to be incubated with pepsin, or pH 6.0 if they were to be incubated with trypsin. Although these pH values are not optimum for the activity of these enzymes, pH 4.0 and pH 6.0 were chosen because of inherent difficulties encountered while working with this system. The optimum pH for pepsin is between 1.0 and 2.0, but it was found that if the pH of the sample was reduced to less than 4.0, the protein was precipitated. Optimum activity with trypsin is obtained at pH 9.0. Since the toxin is inactivated in an alkaline environment, this level of hydrogen ion concentration could not be used. The pH levels used in the experiments represent a compromise between enzyme activity on one hand and stability of toxin on the other.

The concentration of enzyme used was 0.01% and the solutions were sterilized by Seitz filtration. Sterile enzyme solutions were stored at 4 C but were freshly prepared every two weeks to prevent loss of activity. Equal volumes of enzyme and sample were mixed in sterile screw cap tubes (final enzyme concentration of .005%) and incubated in a water bath at 37 C. Prior to mixing, the enzyme and sample were equilibrated to this temperature. Since it is known that the presence of other organisms may result in the inactivation of toxin (Jordan and Dack, 1924), precautions were taken to carry out the entire procedure aseptically. As controls, samples with equal volumes of sterile distilled water were incubated along with the enzyme-sample mixtures. A control tube and a tube of the incubation mixture were removed from the water bath at desired

intervals and the toxicity of each was assayed. Any differences found between the titer of the control tube and the enzyme treated sample were interpreted as being due to the action of the proteolytic enzyme on the protein toxin.

"Resting" cells of C. botulinum.

The term "resting cells", as usually used refers to cells which are deprived of exogenous nitrogen and carbon sources. These cells, although unable to multiply, are able to maintain themselves until their endogenous reserves are exhausted. In this investigation, however, "resting cells" refer to organisms which are unable to proliferate due to the presence of a metabolic inhibitor such as chloramphenicol, penicillin or Versene but are otherwise in an environment which is favorable for the continuation of all synthetic processes; i.e., in a complete medium containing all necessary growth factors.

For preparation of resting cells the procedure employed was to inoculate 1 liter Erlenmayer flasks containing the complete medium with C. botulinum and allowing growth to proceed unimpeded. After 12-16 hr of incubation, while the organisms were still in the accelerated growth phase, they were harvested and washed thoroughly using aseptic technique. To minimize disruption of cells during manipulation, they were centrifuged at comparatively low speeds and washed either in gelatin-phosphate buffer containing 0.05% sodium thioglycollate or in freshly prepared growth medium. The cells were then resuspended in the complete medium to an arbitrarily selected turbidity. The metabolic inhibitors were added to aliquots of the suspension in concentrations high enough to completely inhibit multiplication. The suspensions were then reincubated

at 35 C. Toxin determinations were then carried out after specified time intervals. The possibility of contamination during sampling was avoided by dispensing the original cell suspension equally into sterile screw cap tubes. Stock solutions of antibiotics and metabolic inhibitors were prepared in concentrated form, sterilized by Seitz filtration and stored at 4 C.

Preparation of media for pH experiments.

In the experiments concerning the growth and synthesis of toxin by C. botulinum at different pH levels, special precautions were taken to adjust accurately the initial hydrogen ion concentration. The medium was prepared as usual but rather than adjusting the pH before sterilization, it was placed in the autoclave under flowing steam for 15 minutes. The medium was then cooled and adjusted to the desired pH level. It was next dispensed into tubes or flasks as desired, and sterilized. It was found that the pH of the medium changed considerably upon sterilization, but the initial heating stabilized the medium so that the final sterilization and subsequent addition of glucose and inoculum resulted in no further changes in pH.

Total nitrogen determinations.

The direct Nesslerization method of Johnson (1941) as described by Umbreit et.al. (1949) was used to determine total nitrogen. Cultures were centrifuged at high speed, following which the cells were washed thoroughly in deionized water and resuspended.

For a determination, a 1 ml sample and 1 ml of 2.0 N  $H_2SO_4$  containing 0.02%  $CuSeO_3$  were pipetted into pyrex test tubes. The tubes were placed directly over a heating element and allowed to digest overnight.

Two drops of 30%  $H_2O_2$  insured completion of digestion. After, cooling, the following reagents were added in rapid succession; 4 ml of  $H_2O$ , 2 ml of color reagent and 5 ml of 2.0N KOH. The color was allowed to develop for exactly 15 min and read in the Klett-Summerson colorimeter using a 490 m $\mu$  filter. A solution of ammonium sulphate was used as a standard.

#### Determination of proteolytic activity.

Proteolytic activity of cultures and extracts of C. botulinum was measured using the method described by Northrop et.al. (1948). A 0.01% solution of commercial casein (Borden's) was prepared in distilled water and adjusted to pH 7.4. One ml quantities were allowed to equilibrate in a 37 C water bath. After equilibration, 1 ml of test sample was added to each tube. The mixtures were shaken thoroughly and replaced in the water bath for 2 hr. The non-hydrolyzed protein was then immediately precipitated with 3.0M trichloroacetic acid and the samples were allowed to stand at room temperature for 1 hr before the precipitates were removed by centrifugation. One ml from each of the supernatant fluids was treated with a reagent containing 4%  $Na_2CO_3$ ; 0.02%  $CuSO_4 \cdot 5H_2O$ ; and 0.04%  $Na_2$  tartarate. After standing at room temperature for 45 min, 0.5 ml of 1/3 strength Folin-Ciocalteu reagent was added to each sample. The blue color was allowed to develop for 20 min and read at 660 m $\mu$  in a Klett-Summerson colorimeter. The standard curve was obtained by using a solution of crystalline tyrosine.

#### Protein Determination

The method of Lowry et al. (1951) was used to determine the protein content of cultures of C. botulinum. Rosenbaum (1955) reported that protein determination of Escherichia coli cultures by this colorimetric method gave values approximately 30% less than a dry weight

determination. He found however that this method was quite suitable for detecting relative changes in protein content. Since the current experiments were designed to detect synthesis of protein in the presence of chloramphenicol, absolute quantitation of protein was not necessary and the colorimetric procedure was quite satisfactory.

An aliquot of the culture was precipitated by adding 0.1 volume of 3.0 M trichloroacetic acid followed by cooling to 5 C. The precipitate was solubilized in 0.5 N NaOH. A 0.5 ml volume of the protein solution was then treated with the reagents described above; i.e. the  $\text{Na}_2\text{CO}_3$  and the Folin reagents, and the free tyrosine measured at 660 m $\mu$  as before. Purified bovine albumin was used as the standard.

### III. EXPERIMENTAL RESULTS

#### Growth and lysis of C. botulinum in relation to toxin production.

##### 1. Preliminary observations in a complete medium.

The strain of C. botulinum used initially was 62 A. A complex pork infusion medium (National Canners Association) generally used for the production of anaerobic spores was found to be inadequate for strain 62 A. Consequently, a nutritional survey using dehydrated, commercially prepared media was initiated.

##### a. Growth of C. botulinum 62 A as a function of glucose concentration.

The trypticase broth medium described by Hitzman et al. (1955) modified by the addition of 0.5% yeast extract was used to determine the effect of glucose concentration on growth and spore production. One ml of a 24 hr culture of strain 62 A was inoculated into 100 ml of deoxygenated medium containing glucose at concentrations ranging from 0 to 5.0%. Samples were removed daily for comparison of growth and spore production in the various media. It was found that increasing glucose concentrations gradually decreased sporulation of the vegetative cells and increased the rapidity and degree of autolysis. At glucose concentration of 1.0% and above, lysis proceeded to completion in less than 4 days whereas in the absence of the exogenous energy source or at low

concentrations, lysis was not as evident after the same period of incubation. That lysis was complete in the presence of sufficient glucose was evidenced by the fact that sub-culturing of 4 day old cultures of strain 62 A into fresh medium usually resulted in no further growth. Maximum growth was achieved in less than 24 hr if the glucose concentration was 0.5% or more. It was found, however, that if the concentration of glucose in the medium was raised above 5.0%, growth was inhibited. At concentrations of glucose below 0.5%, the end of the logarithmic growth phase was not reached until approximately 12 hr later than in the case of the cultures grown in the higher glucose concentrations.

As incubation was continued microscopic examination of the cultures revealed that the organisms gradually lost their gram-positive staining characteristic, became granular in appearance and finally lost their cellular integrity. It must be stated, however, that autolysis does not seem to be an all or none phenomenon, nor is it completely dependent on the presence of glucose in the medium. Some lysis was obvious even in the complete absence of the carbohydrate. It is quite likely that the presence of a sufficient quantity of an energy rich carbohydrate such as glucose is essential for maximum synthesis of the autolytic enzymes.



At sub-optimum concentrations of glucose, these enzymes may be synthesized to a limited extent by virtue of the organism's ability to utilize other carbon sources present in the complete medium.

b. pH of culture filtrates.

The fermentative processes of anaerobic organisms may result in an accumulation of organic acids. Consequently, it was conceivable that the lysis of C. botulinum was being caused by a drastic reduction in the pH of the culture fluid. This possibility was excluded, however, by comparing the pH levels of uninoculated medium and the supernatant fluids of growing cultures. It can be seen from table 1 that the hydrogen ion concentration of the medium did not increase sufficiently to cause a physiological disturbance and thus, pH per se was not a factor contributing to the lysis of the vegetative cells.

c. Enzymatic nature of the lytic principle.

The capacity of many "surface active" materials to cause lysis of bacterial cultures is well known. Among such agents are included the detergents of non-biological origin, enzymes such as lysozyme and the polypeptide antibiotics. Waksman (1941) reported the filtrates of cultures of Bacillus mycoides and Bacillus megaterium to be actively lytic against a large number of organisms. These and other antibacterial substances have

Table 1

EFFECT OF GLUCOSE CONCENTRATION ON THE pH OF INOCULATED AND UNINOCULATED COMPLETE MEDIA. (inoculum; C. botulinum 62A)

Glucose concentration	pH Uninoculated media	pH Inoculated culture filtrate	
		24 hr	48 hr
0.1%	7.2	6.9	6.7
0.2%	7.2	6.9	6.7
2.0%	7.2	6.8	6.6
4.0%	7.2	6.8	6.6

two characteristics in common; they are active against a fairly wide spectrum of microorganisms and they do no injury to the microbial strains which produce them. Recently, Greenberg and Halvorson (1954) and Norris et.al. (1957) have reported that certain species of the aerobic, spore-forming bacilli produce a "lytic principle" during growth and subsequent sporulation which can lyse the vegetative cells of the same species. The development of a lytic factor by any of the anaerobic spore forming organisms has never been established but the sequence of events noted with C. botulinum suggested that self-destructive substances, similar to those found with the aerobic organisms, were synthesized.

C. botulinum 62 A was grown in a complete medium and incubated at 35 C until lysis had gone to completion. The cellular debris was removed by high-speed centrifugation and the lysate was tested for its ability to enhance lysis of young cultures. A 12 hr culture of C. botulinum 62 A was harvested by centrifugation and the cells washed in a gelatine-phosphate buffer (pH 6.8) and resuspended in fresh medium. Filtrate from the week old culture of strain 62 A was then added to an aliquot of the young cell suspension and the mixture reincubated at 35 C. An aliquot of the suspension containing filtrate which had been heated at 100 C for 5 min and an aliquot

containing no added filtrate were reincubated simultaneously as controls. Figure 1 shows the kinetics of lysis in the presence of old culture filtrate, heated old culture filtrate and of a normal culture to which no filtrate was added. It can be seen that the presence of the old culture filtrate increased the rate of lysis of young vegetative cells considerably. Boiling the filtrate for 5 min completely eliminated its lytic enhancing ability as evidenced by the fact that the rate of lysis was exactly the same as a normal culture to which no filtrate was added. It can be said then, that the phenomenon of autolysis noted in cultures of C. botulinum 62 A is due to a lytic system which is most likely enzymatic in nature.

d. Quantitation of toxin produced by C. botulinum 62A.

The concept of exotoxin in the past implied that the toxin was excreted into the external environment as a direct product of an organism's metabolic processes. If this were the case, then it would be reasonable to expect that the quantity of toxin present in the culture filtrates would be proportional to the rate of cell multiplication and that maximum toxin titers should be demonstrable in the filtrates soon after the cessation of growth. The conflicting reports found in the literature concerning the rate of toxin production by C. botulinum, prompted an investigation which attempted

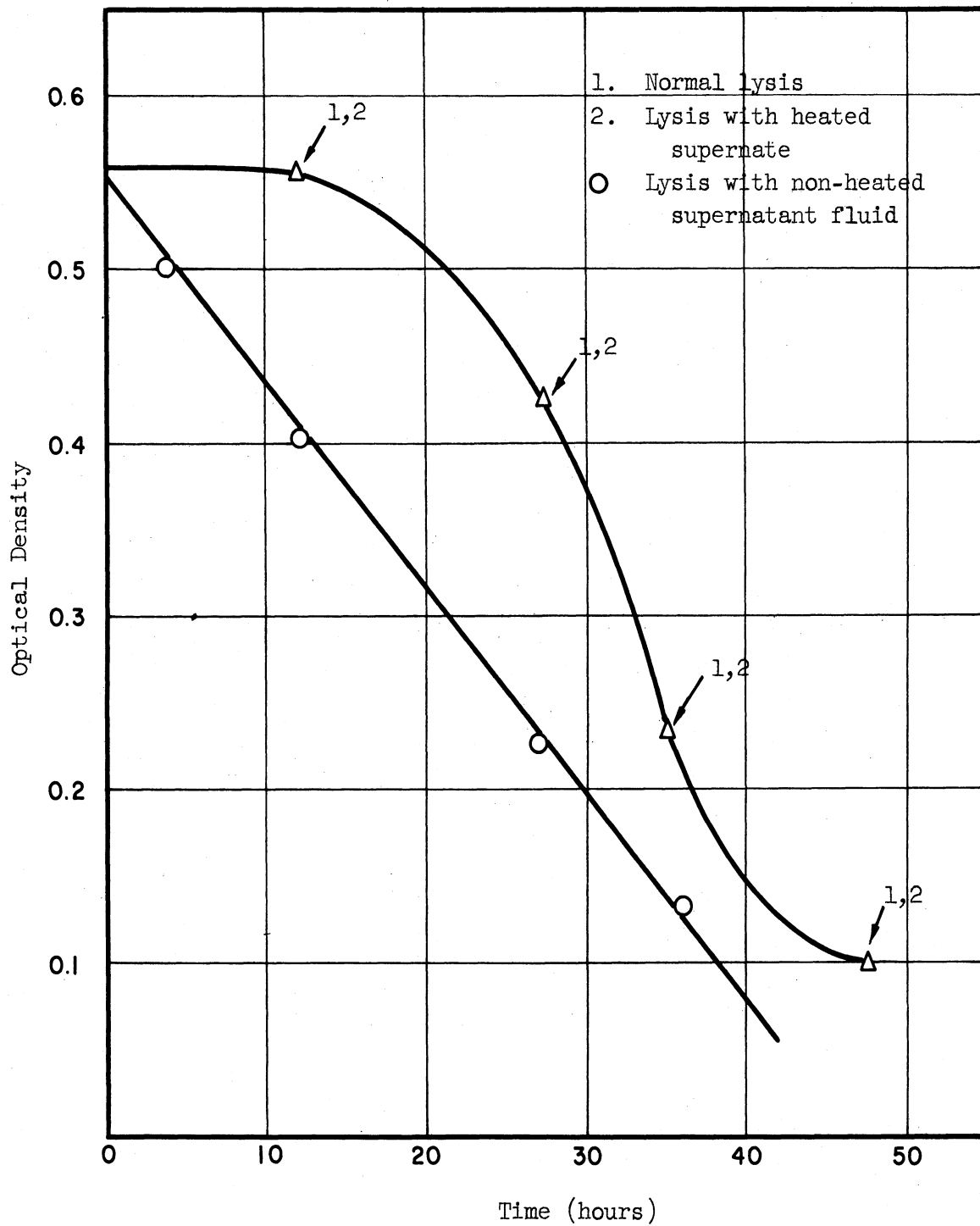


Figure 1. Effect of an old culture filtrate of *C. botulinum* 62A on the kinetics of autolysis of the same organism.

to correlate the kinetics of cell multiplication and subsequent autolysis with the appearance of toxin in the extracellular environment.

Figure 2 gives a typical picture of toxin appearance in the extracellular culture fluid of C. botulinum 62 A grown in a complete medium. After 24 hr of incubation at 35 C, which approximately marked the beginning of the stationary growth phase of the organism, the toxin titer of the filtrate was found to be less than 1000 lethal doses (MLD) per ml. Between 24 and 48 hr when autolysis of the cells become significant, the toxin titer rose sharply and reached a maximum of  $10^5$  MLD/ml after 72 hr. Calculations made from these data showed that the toxicity of 24 hr cultures represented only 1.0% of the toxin found in the filtrates of cultures 48 hr older. The significance of this observation lies in the fact that the 100-fold increase in toxicity took place after cell multiplication had ceased. This evidence suggested that the toxin of C. botulinum is not simply excreted during the period of cellular multiplication but is released upon autolysis.

It had been observed at the initiation of the investigation that the rate of lysis was dependent on the glucose concentration in the growth medium. If autolysis were a mechanism of toxin liberation, it was

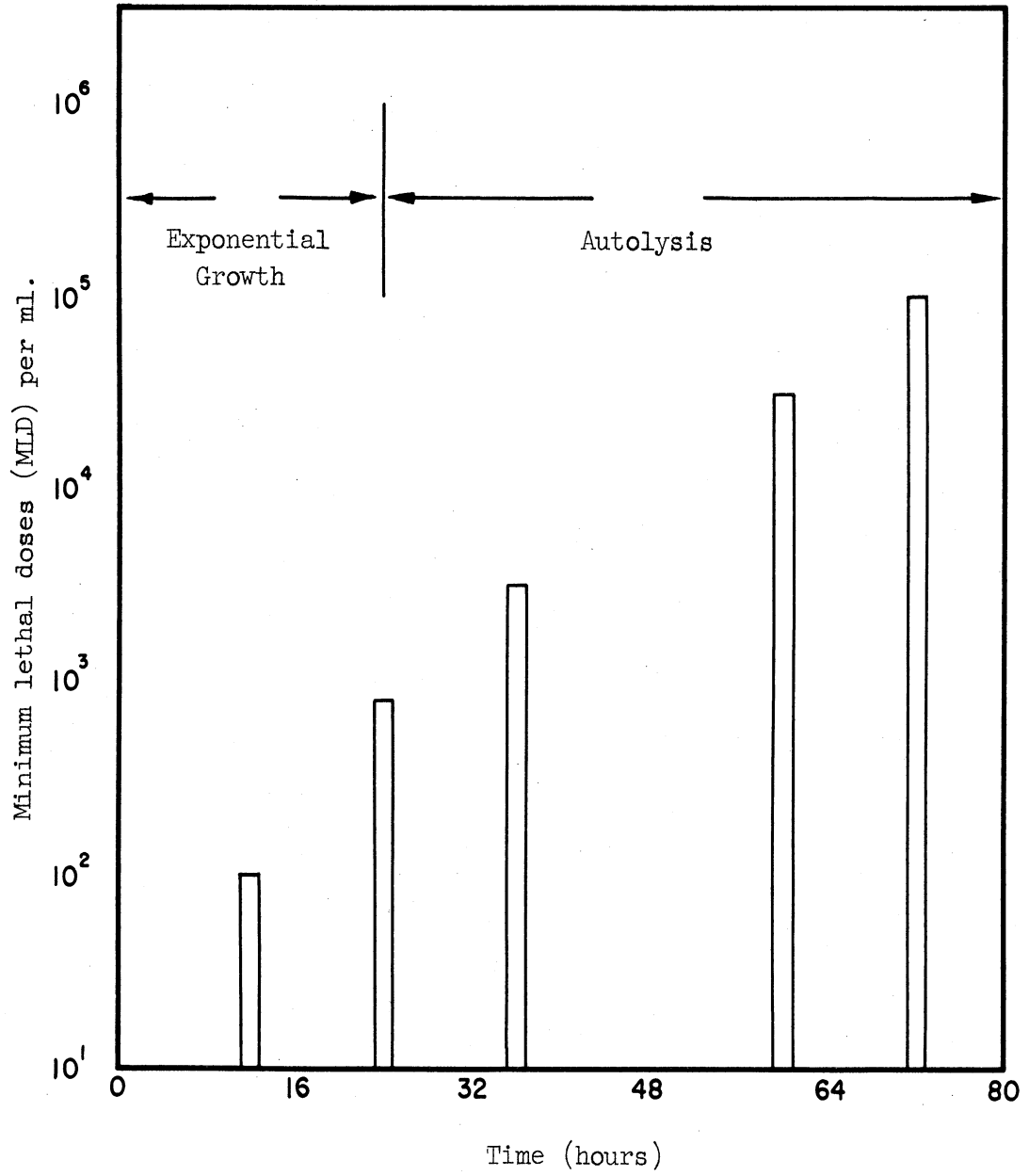


Figure 2. Toxicity of filtrates of C. botulinum 62A grown in a complete medium.

felt that differences in the rate of autolysis, by virtue of changes in the glucose concentration of the growth medium, should be reflected by differences in the rate of toxin appearance in the culture filtrates. Toxin production by C. botulinum 62 A grown in 0.2% and in 2.0% glucose is shown in figure 3. Maximum growth as measured turbidimetrically was attained in 24 hr in the 2.0% glucose medium and 12 hr later at the lower glucose concentration. The maximum cell concentration in both cases, however, was essentially the same. The quantity of toxin present in the filtrates of samples taken at various points during the growth cycle of the organism showed that the toxin titer was proportional not to the growth of the organism, but to the extent of autolysis of the culture at the time the sample was taken. Lysis, as well as growth was much more rapid in the presence of the high concentration of glucose and after 72 hr, a toxin titer of  $10^5$  MLD/ml was demonstrable in the filtrate. After the same incubation period in the medium containing 0.2% glucose, many cells retained their cellular integrity and the toxicity of this filtrate was  $10^4$  MLD/ml. Incubation of the cultures for 144 hr, at which time autolysis had gone to completion in both media, resulted in filtrates of comparable potency.



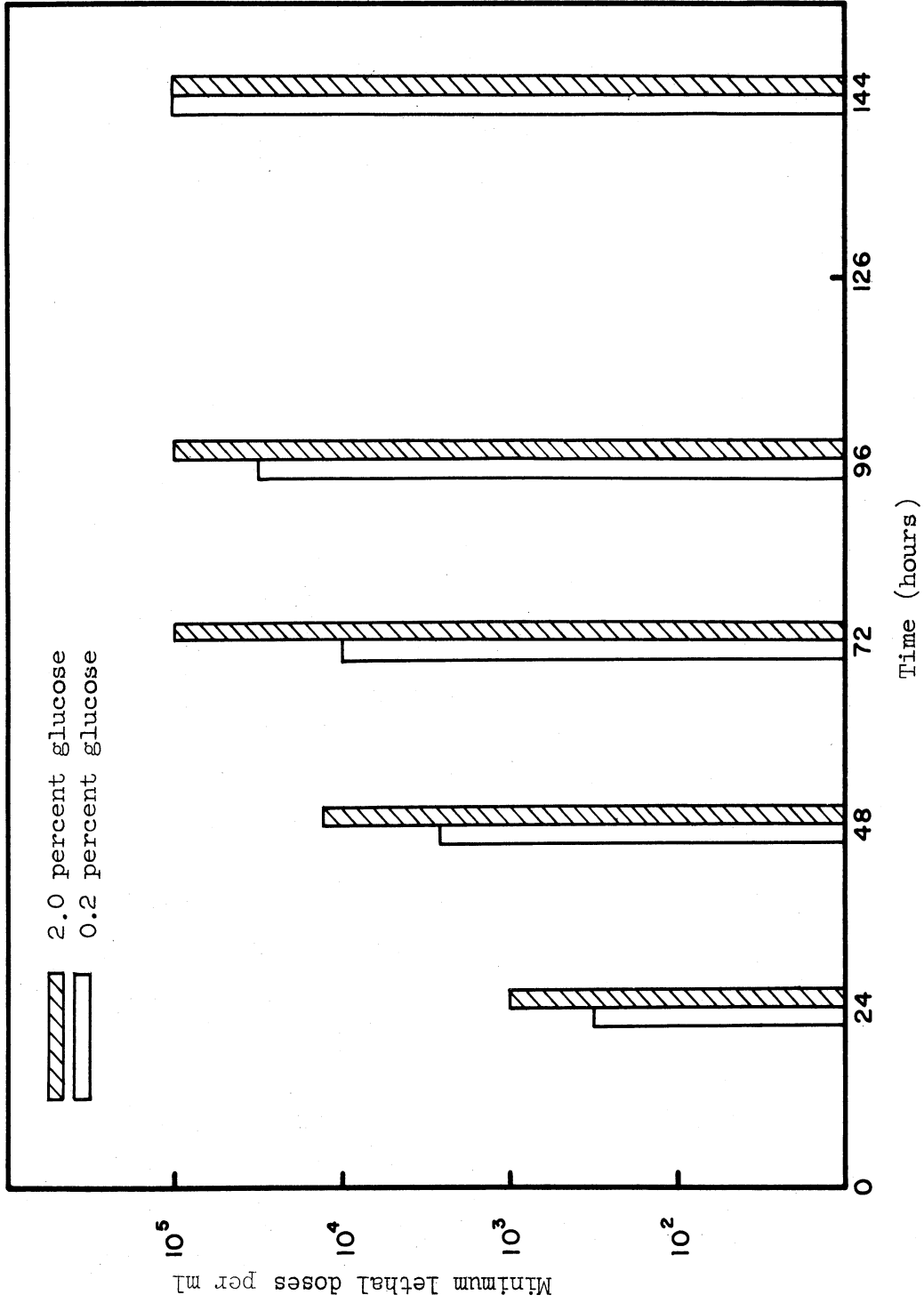


Figure 3. Comparison of extracellular toxin produced by *C. botulinum* 62A in a complete medium containing different concentrations of glucose.

The observations which had been made up to this point, suggested very strongly that at least as far as strain 62 A was concerned, a correlation existed between the degree of autolysis of the vegetative cells and the quantity of toxin found extracellularly. Since it was felt that a generalization of this kind could not be made by studying only one strain of C. botulinum, the investigation was broadened to include other strains of the organism. Before quantitative tests were initiated, however, an attempt was made to standardize the environmental conditions for all strains of C. botulinum to be studied. The most important consideration was to find a growth medium which would support maximum toxin synthesis. A complex medium containing corn steep liquor described by Lewis and Hill (1947) and recommended for high toxin production was found to be inadequate for the strains of the organism used in this investigation. The same can be said for the trypticase medium of Hitzman et al. (1955) and the meat infusion medium usually recommended for storage of stock cultures of C. botulinum. After considerable trial and error, the complete media described in the section "Materials and Methods" were chosen since they supported maximum toxin synthesis and gave the most consistent results. The latter point is significant for two reasons: firstly, the initial nutritional survey conducted showed that

toxin production by C. botulinum on a quantitative level is not a stable biochemical characteristic. Complete loss of the organism's ability to produce toxin is not an uncommon occurrence (Nungester, 1956) and descriptions of non-toxic variants of C. botulinum can be found in the literature (Gunnison and Meyer, 1927). Conversions of this type can most likely be attributed to changes in the genetic constitution of the organisms. The probability of spontaneous mutation occurring was considerably reduced in this investigation by storing the cultures in the frozen state as described previously. Non-toxic mutants excluded, however, toxin synthesis seems to depend on a delicately poised set of environmental conditions, any deviation from which results in a decrease in the amount of toxin which can be demonstrated. Secondly, the actual quantity of toxin synthesized in a given culture is dependent on a complex chain of metabolic events brought about by the interaction of the organism and its environment. The final estimate of the toxin present in this culture fluid, however, is dependent on a complex animal assay system. The basic assumptions of the assay method are: (1) The arbitrary selection of a healthy norm in the test animal and (2) identical response of the animals to a given quantity of the toxic material. The establishment of a healthy norm is an

indeterminate problem and for the most part is never done with any precision or universality. As Miles (1955) has pointed out "one man's normal mouse is another's pox-ridden runt from a back-street dealer, according to their standards of health and rigorous experiment." The response of the animal to the toxic material is dependent on a multiplicity of host resistance factors which are assumed to remain constant from one arbitrarily selected normal animal to the next. Consequently, when by necessity the quantity of toxic material must be measured by the effect it produces on an animal, in this case death of the mouse, it can be seen that the final estimate of lethal doses in a given volume of culture fluid is at best a relative index of the actual amount and in no sense can it be said to represent an absolute quantity.

After the media for the growth of the different strains of C. botulinum had been selected it was found that the phenomenon of rapid autolysis was not restricted to strain 62 A but could be readily demonstrated with all strains of the organism studied; i.e. strains 457A, JTD-IV, 213B and B-201.

2. Intracellular toxin of C. botulinum.

Following the establishment of autolysis as a common characteristic of all strains of C. botulinum, experiments were conducted to quantitate the intracellular toxin. It was felt

that if autolysis was a mechanism by which the toxin was liberated, then intracellular toxin should be demonstrable before spontaneous lysis had occurred.

A comparison of intracellular and extracellular toxin was made by assaying the filtrates of spontaneously lysing and sonically disintegrated cultures. For these experiments, C. botulinum strains JTD-IV, 62 A and B-201 were used. Large pyrex test tubes containing 20 ml of the appropriate complete medium were inoculated with 1 ml of a log phase culture and incubated at 35 C. Two culture tubes were removed after various periods of incubation and treated as follows: One culture was sedimented and the supernatant removed. The other was sonically disintegrated and the cellular debris discarded. Both cell-free samples were then assayed for toxin. Growth curves and toxin titers of strains JTD-IV, 62 A and B-201 are shown in figures 4, 5, and 6 respectively. The overall picture obtained with these three strains of C. botulinum was essentially the same. After multiplication of the organisms had ceased, (approximately 24 hr) autolysis quickly became apparent and after 72 hr of incubation practically no intact cells remained. The toxin titers of the supernatant culture fluids at the end of the exponential growth phase were still comparatively low. In all cases, however, mechanical disruption of the cells before autolysis had taken place resulted in the release of a considerable quantity of toxin. The ratios of intracellular to extracellular toxin

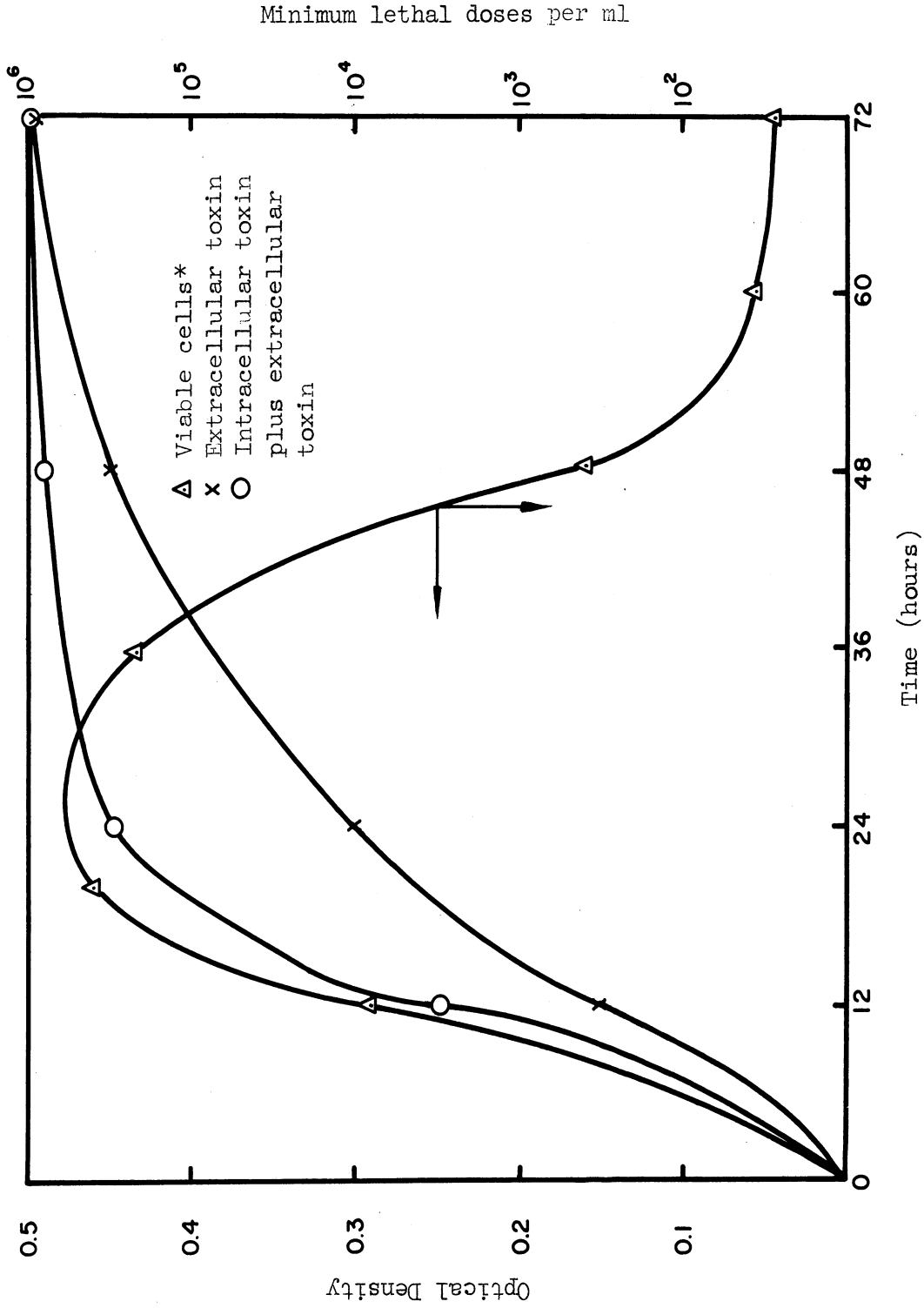


Figure 4. Growth and toxin synthesis by C. botulinum JTD-IV in a complete medium.

\* See Materials and Methods.

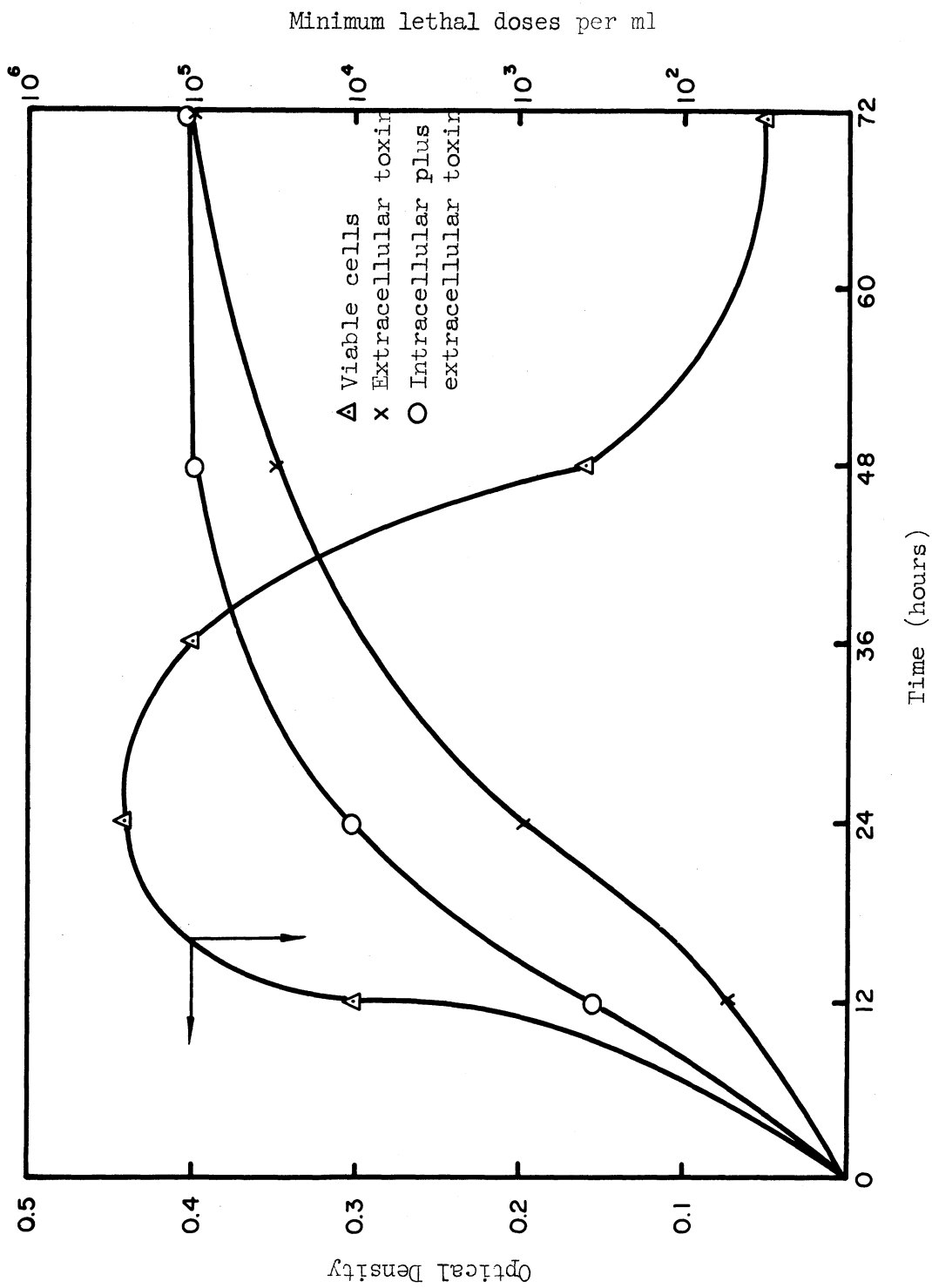


Figure 5. Growth and toxin synthesis by C. botulinum 62A in a complete medium.

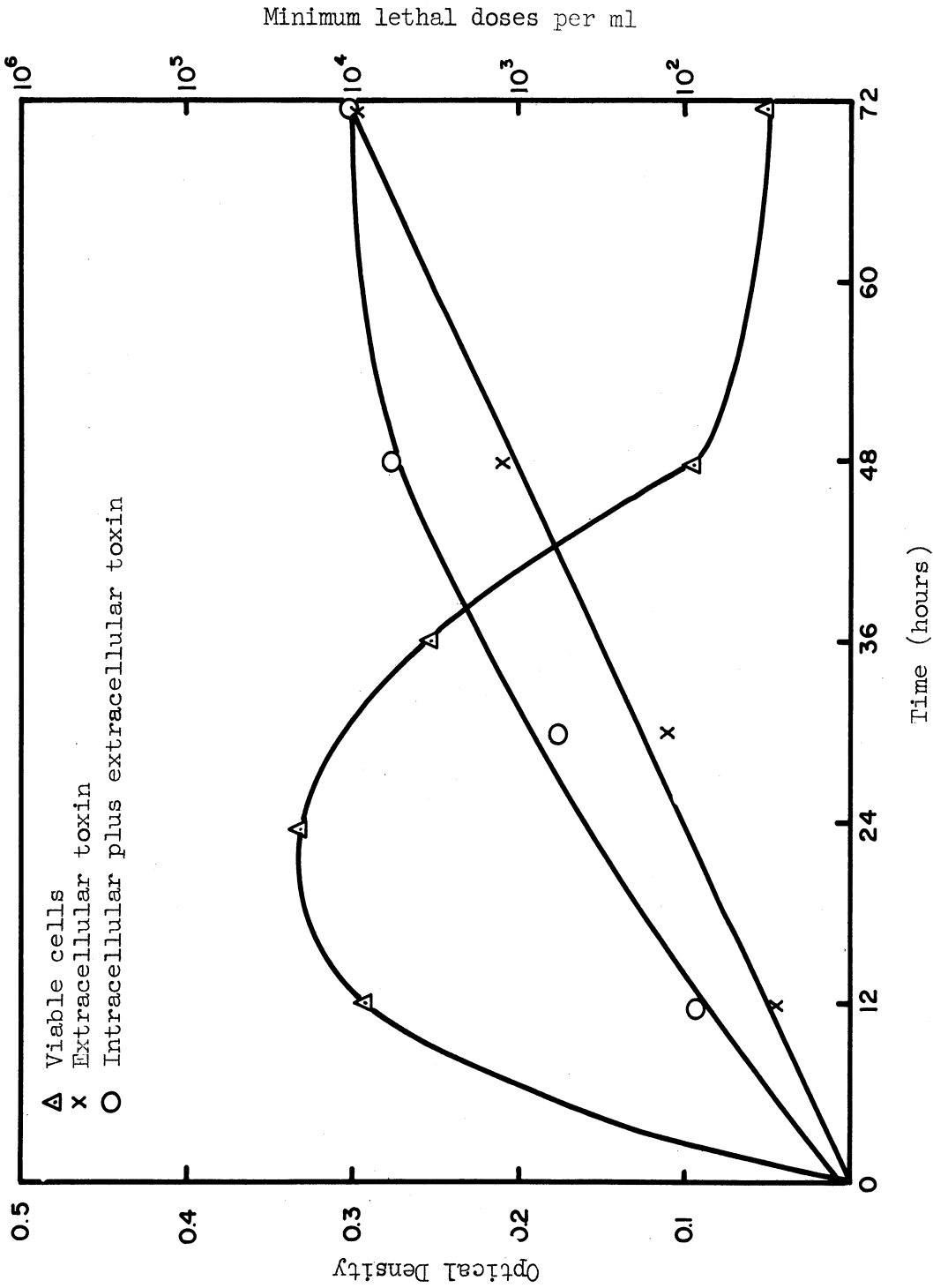


Figure 6. Growth and toxin synthesis by C. botulinum B-201 in a complete medium.



for the 3 strains of C. botulinum during the 72 hr incubation period are given in table 2. It can be seen that before autolysis of the cells took place much more intracellular toxin was present than in the extracellular culture fluids. As lysis proceeded, however, the ratio of intracellular to extracellular toxin decreased until no more intracellular toxin could be demonstrated, owing to the complete autolysis of the vegetative cells.

It was noted that although the foregoing experiments definitely implicated autolysis as a mechanism for the liberation of botulinus toxin, the data indicated as well that autolysis alone was not the sole factor responsible for the ultimate toxicity of the extracellular culture fluids. The appearance of some extracellular toxin in very young cultures could not be accounted for on the basis of loss of cellular integrity with the subsequent release of materials into the external environment. The possibility that lysis was occurring even during the active phase of growth could not be borne out by microscopic examination of young cells. In addition, the inability to obtain toxin-free supernatant fluids after repeated washings indicated that mechanisms other than lysis were operative. Manipulation during the washing procedures did not disrupt the cells as evidenced by the fact that the numbers of viable organisms before and after washing remained approximately the same. While working with C. tetani, Stone (1954) found that the toxin of this organism behaved in the

Table 2

EXTRACELLULAR AND INTRACELLULAR TOXIN OF C. botulinum STRAINS JTD-IV  
62A AND B-201 GROWN IN A COMPLETE MEDIUM.

Length of incubation (hr)	Ratio of intracellular to extracellular toxin		
	JTD-IV	62A	B-201
12	9.0	9.0	2.0
24	14.0	10.0	4.0
36	8.0	7.0	5.0
48	0.8	1.0	5.0
72	-*	-	-

\*- No intracellular toxin demonstrable

same manner. He concluded that although autolysis of the vegetative cells could not be entirely ruled out as a mechanism for the liberation of the toxin, the experimental evidence strongly suggested that the inability to obtain non-toxic wash fluids could be explained on the basis of simple diffusion of toxin from the cell into the extracellular environment. Similarly, the evidence discussed above for botulinus toxin could not exclude simple diffusion as a mechanism of toxin liberation during the early stages of growth. Since the autolytic process appears to be a gradual one, both mechanisms working in conjunction could account for the extracellular toxin found in young cultures of C. botulinum. A priori, it seems logical to assume that the rate of diffusion of toxin is dependent on the extent to which the autolytic enzymes have disrupted the permeability barriers of the cell.

As noted above, sonic disintegration of young cultures of C. botulinum resulted in the release of protein toxin. An experiment was performed in an attempt to establish the strength of binding between the toxin and the cellular debris. A strong bond between toxin and a cellular structure would tend to negate simple diffusion as a mechanism for toxin liberation. Non-adsorption to the cellular debris, on the other hand, would indirectly support simple diffusion of the toxin.

A cellular extract of C. botulinum JTD-IV which had been cleared of debris by centrifugation at 20,000 G for 30 min and an extract which contained the cellular debris in suspension were assayed for toxin. It was found that both samples possessed a toxicity of  $10^6$  MLD/ml. This indicated that the toxin was not bound to any degree to the cell wall or cytoplasmic membrane. While this evidence indirectly supported simple diffusion as a mechanism, it did not exclude other unknown mechanisms which were contributing to the final toxicity of the culture filtrates. That such factors existed can be seen by reexamining the data presented in figures 4, 5 and 6. The toxicity of cell extracts obtained from cultures which had attained maximum growth but had not as yet lysed, although higher than the culture filtrates of the same age, was approximately 10 times lower than the maximum toxicity usually obtained if the cultures were allowed to incubate for 72 hr. If all the toxin were synthesized and was in the molecular form required to exhibit maximum lethality by the time the stationary growth phase was reached, then the sum of the bound intracellular and free extracellular toxin of samples taken from this stage in the growth cycle of the culture should be comparable to the toxin found in the filtrate of a culture which had been allowed to autolyze completely. As the data show, however, this was not the case and consequently it became clear that the final toxicity exhibited by a culture could be explained

solely on the basis of diffusion and autolysis only if the major part of the protein toxin was synthesized during the period of cellular degeneration. Since this phase in the life of most bacterial species is characterized by catabolic rather than anabolic processes, it was felt that de novo synthesis of such large quantities of protein by C. botulinum under extremely adverse conditions would be a rather unique, although possible biological situation. On the other hand, a much more logical possibility existed; namely a pre-synthesized precursor of the active toxin molecule was being enzymatically altered during the terminal stages of the autolytic process thereby allowing the molecule to manifest its full toxic potentiality. Before investigating these and other possible mechanisms, however, a study concerning the effects of the physical and chemical environment on the growth, lytic and toxin synthesizing processes of the cell was carried out. A quantitative investigation of this type was considered to be of interest from both a theoretical and practical point of view. In addition, it could possibly provide information which would be helpful in determining the chain of events which resulted in the tremendously increased toxicity of extracellular filtrates after multiplication of the organism had ceased.

Effect of the chemical and physical environment on growth, lysis and toxin synthesis.

1. Effect of glucose concentration.

Previous experiments showed that the rate of autolysis was

affected by the concentration of glucose in the growth medium. However, at concentrations of glucose as low as 0.1%, maximum toxin synthesis could be obtained if the cultures were incubated long enough to allow lysis to go to completion. The complete medium supported growth of C. botulinum in the absence of glucose but as yet it was not known to what extent toxin synthesis was supported in the absence of this energy rich carbohydrate.

C. botulinum strains JTD-IV and B-201 were grown in the complete medium containing 0.5% glucose and also in the same medium to which no glucose was added. After adaptation of the organisms to growth under these conditions, toxin production was determined. From the data presented in table 3 it can be seen that the absence of glucose had a profound effect on the synthesis of toxin by both strains of C. botulinum. The absence of the carbohydrate resulted in an approximately 1000-fold decrease in the toxicity of the filtrates although growth was unimpeded. Autolysis in the absence of glucose did not go to completion even on prolonged incubation at 35 C. This suggested that the toxin might not have been detected if it were present intracellularly. Sonic disintegration of the cells, however, resulted in only a very slight increase in the toxicity of the filtrates. This indicated that glucose was required, at least in low concentrations, for the synthesis of toxin as well as for the synthesis of the autolytic enzyme system. With B-201, the less toxigenic strain of the organism,

Table 3

TOXIN SYNTHESIS BY C. botulinum STRAINS JTD-IV AND B-201  
IN THE PRESENCE AND IN THE ABSENCE OF GLUCOSE

Age of culture		Toxin Titers (MLD/ml)			
JTD-IV	Culture filtrate		Culture extract		
	with glucose	no glucose	with glucose	no glucose	
20 hr	$5 \times 10^3$	$< 10^2$	$1 \times 10^4$	$1 \times 10^3$	
48 hr	$1 \times 10^5$	$< 10^3$	$5 \times 10^5$	$1 \times 10^3$	
72 hr	$2 \times 10^5$	$< 10^3$	$1 \times 10^6$	$1 \times 10^3$	
<u>B-201</u>					
20 hr	$5 \times 10^2$	neg.	-	-	
48 hr	$1 \times 10^3$	neg.	-	-	
72 hr	$1 \times 10^4$	neg.	-	-	
96 hr	$5 \times 10^4$	neg.	$1 \times 10^5$	$< 10$	

toxin synthesis in the absence of glucose was negligible.

2. Growth and toxin synthesis in the presence of various carbohydrate sources.

The previous experiments suggested that the external carbohydrate source in the growth medium was being used not only as an energy source for the synthetic processes of the organism, but was a specific requirement for the synthesis of the protein toxin.

Growth and toxin synthesis by four strains of C. botulinum were followed in the complete medium containing a fermentable carbohydrate source. Strains JTD-IV, 62 A, 457 A and B-201 were first tested for their ability to ferment the carbohydrate sources selected. This was determined in phenol red broth supplemented with the test carbohydrate at a concentration of 0.5%. A loopful of an actively growing culture was inoculated into the medium and the cultures incubated at 35 C for a period of 3 days. Table 4 shows that all of the carbohydrates were fermented with the exception of sorbitol and inositol by strains 457 A and B-201. The four strains of C. botulinum were then tested for their ability to synthesize toxin in a complete medium supplemented with a utilizable carbohydrate. Large pyrex test tubes containing 20 ml of medium were deoxygenated by boiling for 10 min, cooled and inoculated with 1 ml of an 18 hr culture. Transfers were made every 24 hr for 3 days to adapt the organisms to the carbohydrate and to dilute out any contamination by glucose originating from the starting inoculum.



Table 4

FERMENTATION\* OF CARBOHYDRATES BY DIFFERENT STRAINS OF  
C. botulinum

Carbohydrate	Strain			
	JTD-IV	457A	62A	B-201
Galactose	+	+	+	+
Rhamnose	+	+	+	+
Sorbitol	+	-	+	-
Glycerol	+	+	+	+
Maltose	+	+	+	+
Xylose	+	+	+	+
Ribose	+	+	+	+
Lactose	+	+	+	+
Inositol	+	-	+	-
Glucose	+	+	+	+

\*) + growth and acid production in phenol red broth

After the 4th transfer was made, growth was measured turbidimetrically and samples were taken periodically for toxin assay. Table 5 shows the toxicity of the supernatant culture fluids after 48 and 96 hr of incubation. Only glucose and its disaccharide, maltose, supported toxin synthesis fully. Of the other carbohydrates tested, only glycerol, pyruvate and ribose partially supported synthesis of toxin. Toxin produced in the presence of the remaining compounds was no greater than in the control medium which contained no added carbohydrate.

Most anaerobic organisms utilize the glycolytic pathway as the main route of carbohydrate metabolism. The net gain of high-energy phosphate bonds in this metabolic pathway takes place beyond the pyruvate stage and theoretically, the three carbon compounds should be capable of efficiently replacing glucose as an energy source. If a shunt mechanism is operative, then ribose may be converted to three carbon fragments which then can enter the glycolytic pathway. The synthesis of toxin by the four strains of C. botulinum with pyruvate, glycerol or ribose serving as the energy source, however, was considerably repressed. This evidence indicated that glucose was utilized, not primarily as an energy source, but was specifically required for the synthesis of the protein toxin.

Growth and toxin synthesis by strain JTD-IV with the various carbohydrates are presented graphically in figures 7 and 8.

Table 5  
 TOXIN PRODUCTION BY FOUR STRAINS OF *C. botulinum* IN THE PRESENCE OF  
 DIFFERENT CARBOHYDRATES

Carbohydrate	Toxin titer of filtrate (MLD/ml)												
	62A 48hr filtrate		457A 48 hr		96 hr		JTD-IV 48 hr		96 hr		B-201 48 hr		96 hr
Glucose	10 <sup>3</sup>	2 x 10 <sup>4</sup>	2 x 10 <sup>5</sup>	2 x 10 <sup>5</sup>	-	2 x 10 <sup>5</sup>	5 x 10 <sup>5</sup>	2 x 10 <sup>5</sup>	5 x 10 <sup>5</sup>	3 x 10 <sup>3</sup>	5 x 10 <sup>4</sup>	3 x 10 <sup>3</sup>	5 x 10 <sup>4</sup>
Maltose	10 <sup>3</sup>	2 x 10 <sup>4</sup>	2 x 10 <sup>5</sup>	2 x 10 <sup>5</sup>	-	2 x 10 <sup>5</sup>	5 x 10 <sup>5</sup>	2 x 10 <sup>5</sup>	5 x 10 <sup>5</sup>	3 x 10 <sup>3</sup>	5 x 10 <sup>4</sup>	3 x 10 <sup>3</sup>	5 x 10 <sup>4</sup>
Galactose	neg	neg	5 x 10 <sup>3</sup>	5 x 10 <sup>3</sup>	-	5 x 10 <sup>3</sup>	5 x 10 <sup>2</sup>	2 x 10 <sup>2</sup>	5 x 10 <sup>2</sup>	neg	neg	neg	neg
Lactose	neg	neg	10 <sup>3</sup>	10 <sup>3</sup>	-	10 <sup>3</sup>	5 x 10 <sup>2</sup>	2 x 10 <sup>2</sup>	5 x 10 <sup>2</sup>	neg	neg	neg	neg
Xylose	10 <sup>2</sup>	10 <sup>2</sup>	10 <sup>2</sup>	10 <sup>2</sup>	-	10 <sup>2</sup>	5 x 10 <sup>2</sup>	2 x 10 <sup>2</sup>	5 x 10 <sup>2</sup>	neg	neg	neg	neg
Ribose	neg	neg	10 <sup>2</sup>	10 <sup>2</sup>	-	10 <sup>2</sup>	5 x 10 <sup>3</sup>	5 x 10 <sup>3</sup>	5 x 10 <sup>3</sup>	neg	neg	neg	neg
Glycerol	10 <sup>2</sup>	5 x 10 <sup>2</sup>	-	-	-	-	2 x 10 <sup>4</sup>	2 x 10 <sup>4</sup>	2 x 10 <sup>4</sup>	neg	neg	neg	neg
Pyruvate	10 <sup>2</sup>	5 x 10 <sup>2</sup>	-	-	-	-	5 x 10 <sup>3</sup>	5 x 10 <sup>3</sup>	10 <sup>4</sup>	neg	neg	neg	neg
Inositol	neg	neg	-	-	-	-	-	-	-	-	-	-	-
Control	neg	10 <sup>2</sup>	5 x 10 <sup>2</sup>	5 x 10 <sup>2</sup>	-	5 x 10 <sup>2</sup>	10 <sup>3</sup>	5 x 10 <sup>2</sup>	10 <sup>3</sup>	neg	neg	neg	neg

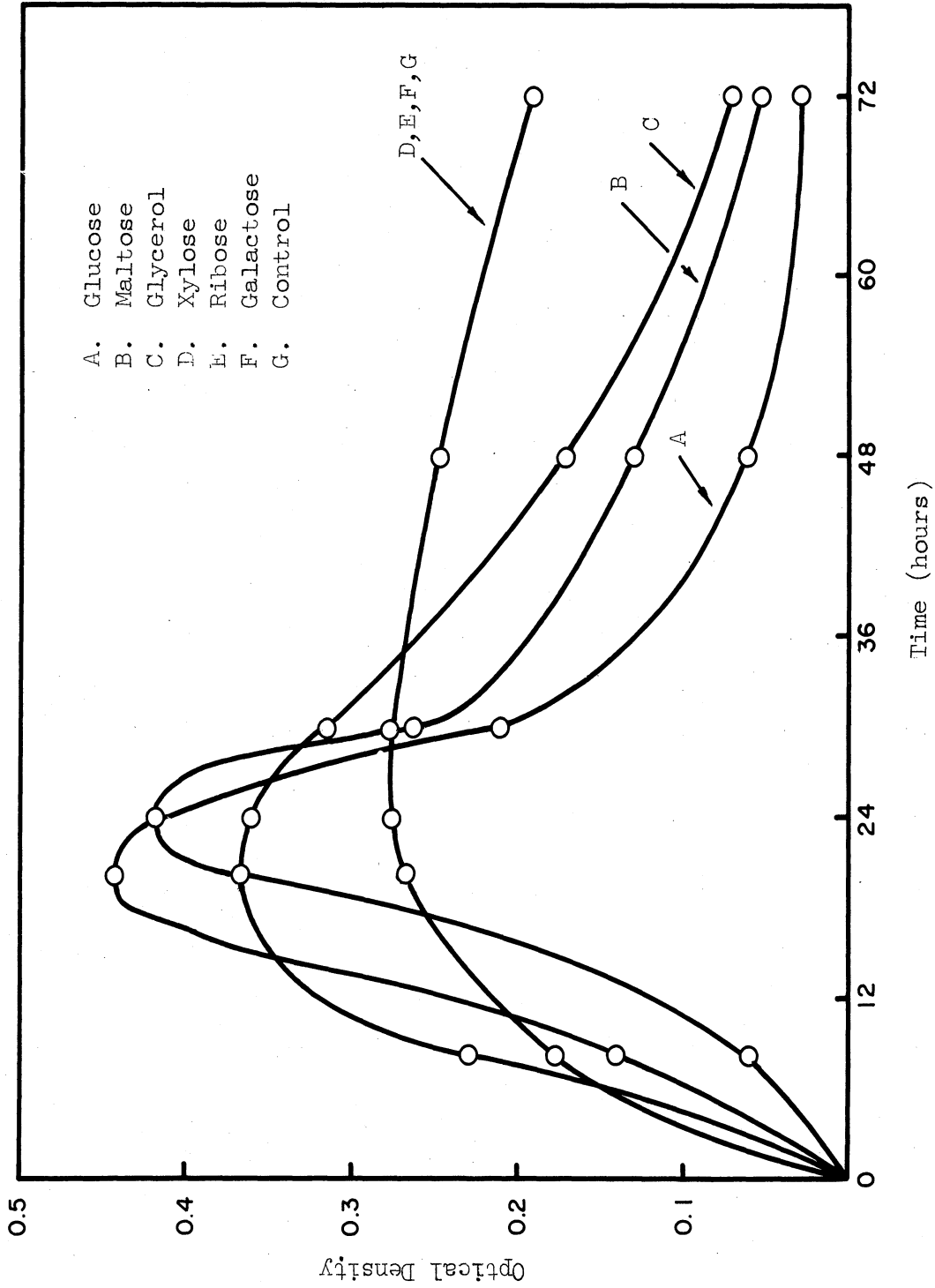


Figure 7. Effect of external carbohydrate source on growth of *C. botulinum* JTD-IV.

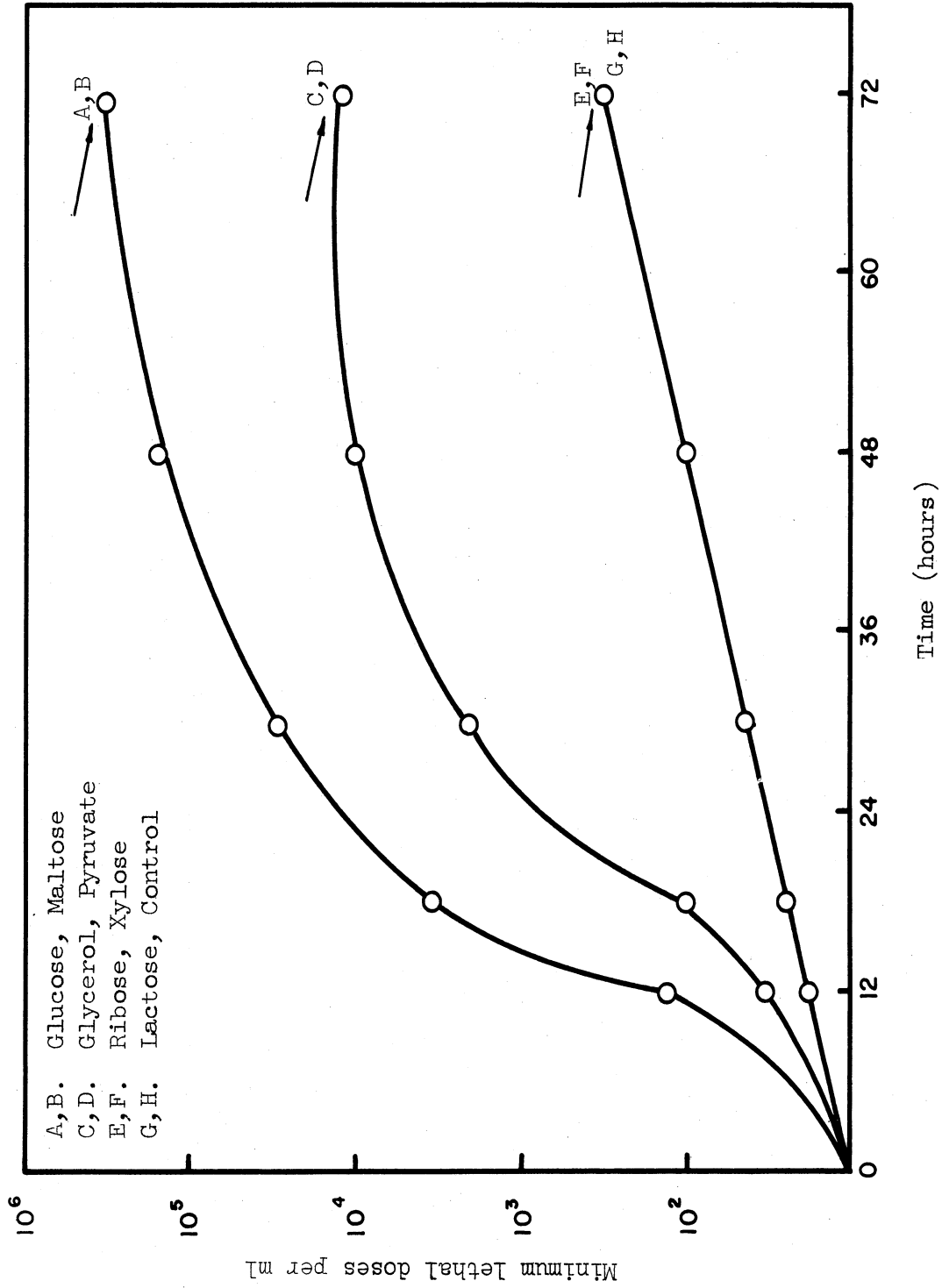


Figure 8. Effect of external carbohydrate source on toxin synthesis by C. botulinum JTD-IV.

Although equal growth rates were observed during the logarithmic phase with all of the carbohydrates, maximum growth was achieved only with glucose and maltose. The degree to which growth was repressed with the other carbon compounds when compared to glucose, however, was not sufficient to account for the extreme differences in toxin production. It was also noted that autolysis of the cultures did not go to completion unless glucose or maltose was included in the growth medium. Sonic disintegration of the intact cells which remained after 72 hr of incubation in the media containing xylose, ribose, glycerol, pyruvate, lactose and galactose did not result in increased toxicity of the supernatant fluids showing that the toxin was not present intracellularly.

The number of carbohydrates tested for their ability to support toxin synthesis by C. botulinum by no means exhausted all the possibilities. The data obtained from this limited survey, however, indicated that glucose was a specific requirement for the synthesis of toxin and that this requirement could not be replaced by other external energy sources tested.

### 3. Effect of pH on toxin synthesis.

The complete medium was freshly prepared and accurately adjusted to pH values between 4.5 and 8.3. The media were dispensed into matched 12/150 tubes in 10 ml quantities so that turbidimetric measurements of growth could be easily made. One tenth ml of an 18 hr culture of JTD-IV was

inoculated into a sufficient number of tubes so that a culture growing at each level of hydrogen ion concentration could be removed after suitable incubation periods and assayed for toxin. This procedure was found to be more advantageous than working with a single culture at each pH level since manipulation during sampling at times resulted in a cessation of growth due to over-oxygenation.

Growth and toxin synthesis by C. botulinum strain JTD-IV are shown in figures 9 and 10. Optimum growth of the organism was obtained between pH 5.5 and 7.5, and sub-maximal growth between 7.5 and 8.3. At pH values of 5.3 and below, the organism was not able to initiate growth. This value is much higher than the minimum pH at which certain strains of C. botulinum have been reported to be capable of growth and toxin production. Gunnison and Meyer (1929) described an outbreak of botulism from home-canned pears, the syrup of which had a pH of 3.86. However, since a lactobacillus was also isolated, it is conceivable that the acids produced by the lactobacillus had increased the acidity of the syrup after the botulinus toxin had been produced. Dozier (1924) and Townsend et al. (1954) reported growth and toxin production by some strains of C. botulinum at pH values slightly under 5.0 but found that the absolute minimum pH values varied considerably from strain to strain and medium to medium. It should be noted that the investigations referred to above were not quantitative in nature. Gas production

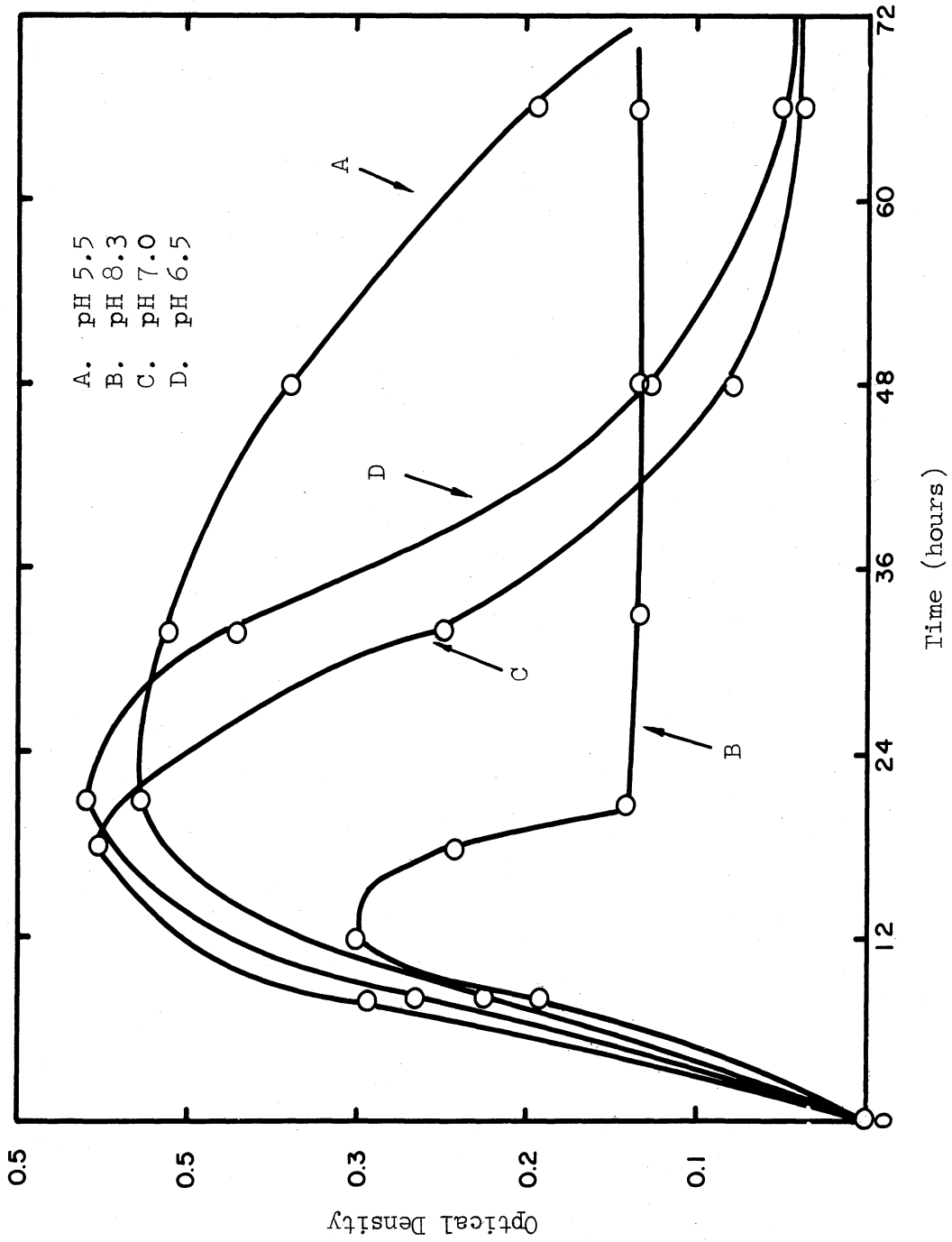


Figure 9. Effect of pH on growth of C. botulinum JTD-IV.



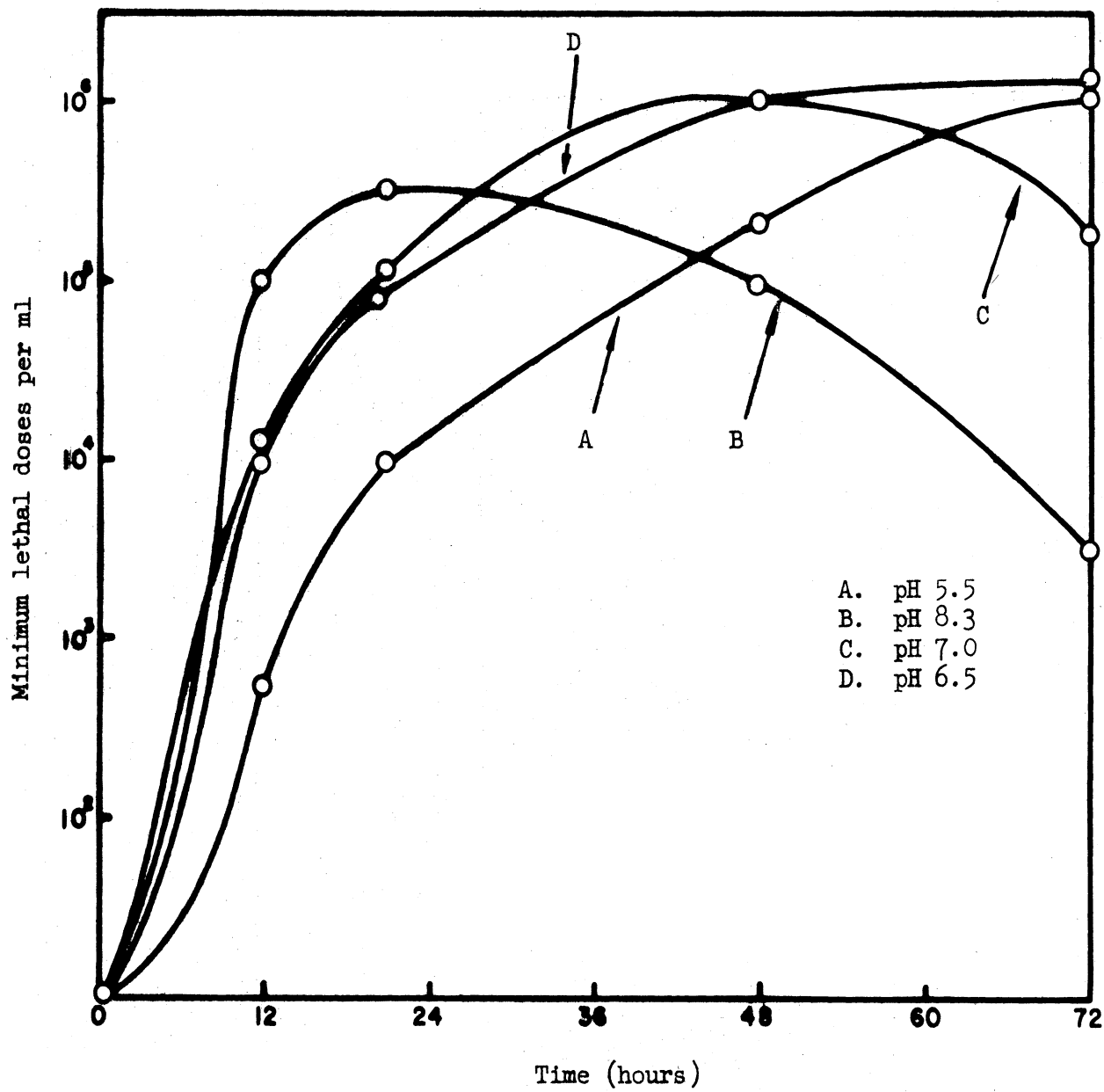


Figure 10. Effect of pH on toxin synthesis by C. botulinum JTD-IV.

was used as the criterion for growth and the presence of toxin was determined by inoculation of the undiluted culture filtrate into mice. Although this method for the detection of toxin has been widely used, it is known that inoculation of a concentrated culture fluid may result in non-specific reactions.

The autolytic process was most rapid between pH 6.5 and 7.5, indicating that the autolytic enzymes have a rather narrow pH range for optimum activity. Maximum toxin titers were obtained between pH 5.5 and 8.0 but it was found that the toxin was much more stable at pH 6.5 and below. Although an alkaline environment resulted in an inactivation of the toxin, it did not seem to affect its actual synthesis. It is interesting to note that the toxin was most stable at 5.5 where the rate of autolysis was comparatively slow. This observation suggested that the stability of the toxin could possibly be increased by virtue of large quantities being bound intracellularly for much longer periods of time than in an environment where autolysis proceeded very rapidly.

4. Effect of temperature of incubation on toxin synthesis.

The food industries, particularly those preparing canned materials for public consumption have made exhaustive studies to determine the heat resistance of spores of C. botulinum. The vegetative forms have been of no particular consequence to them since vegetative cells are easily destroyed at comparatively low temperatures. The tremendous expansion of

the frozen food industry, and the possibility that irradiation pasteurization of some foods to increase their storage life may be adopted, reestablish botulinus intoxication as a potential health hazard. Since complete sterilization of foods prepared in this manner is not accomplished, the prime consideration is the temperature at which the foods are stored. This situation was considered important enough to warrant an investigation on the effect of temperatures, within the range supporting growth of C. botulinum, on the synthesis of toxin.

The procedure of inoculating the organism into matched test tubes containing 10 ml quantities of a complete medium used in the pH experiments was also adopted for these experiments. The medium was adjusted to a pH of 6.8 and growth and toxin synthesis of cultures incubated between 4 C and 55 C was recorded. The precision of temperature control in the incubators was  $\pm 1$  C. Experiments conducted below room temperature, were carried out by incubation in a cold running water bath which was found to fluctuate between 10 C and 18 C over a one-week period. Since this is the approximate range of temperature at which foods may be stored, it was felt that the information obtained would be of considerable value.

The rates of growth, lysis and toxin synthesis by C. botulinum JTD-IV varied considerably with the temperature of incubation (figures 11 and 12). Between 28 C and 37 C, maximum growth, autolysis and toxin synthesis were obtained

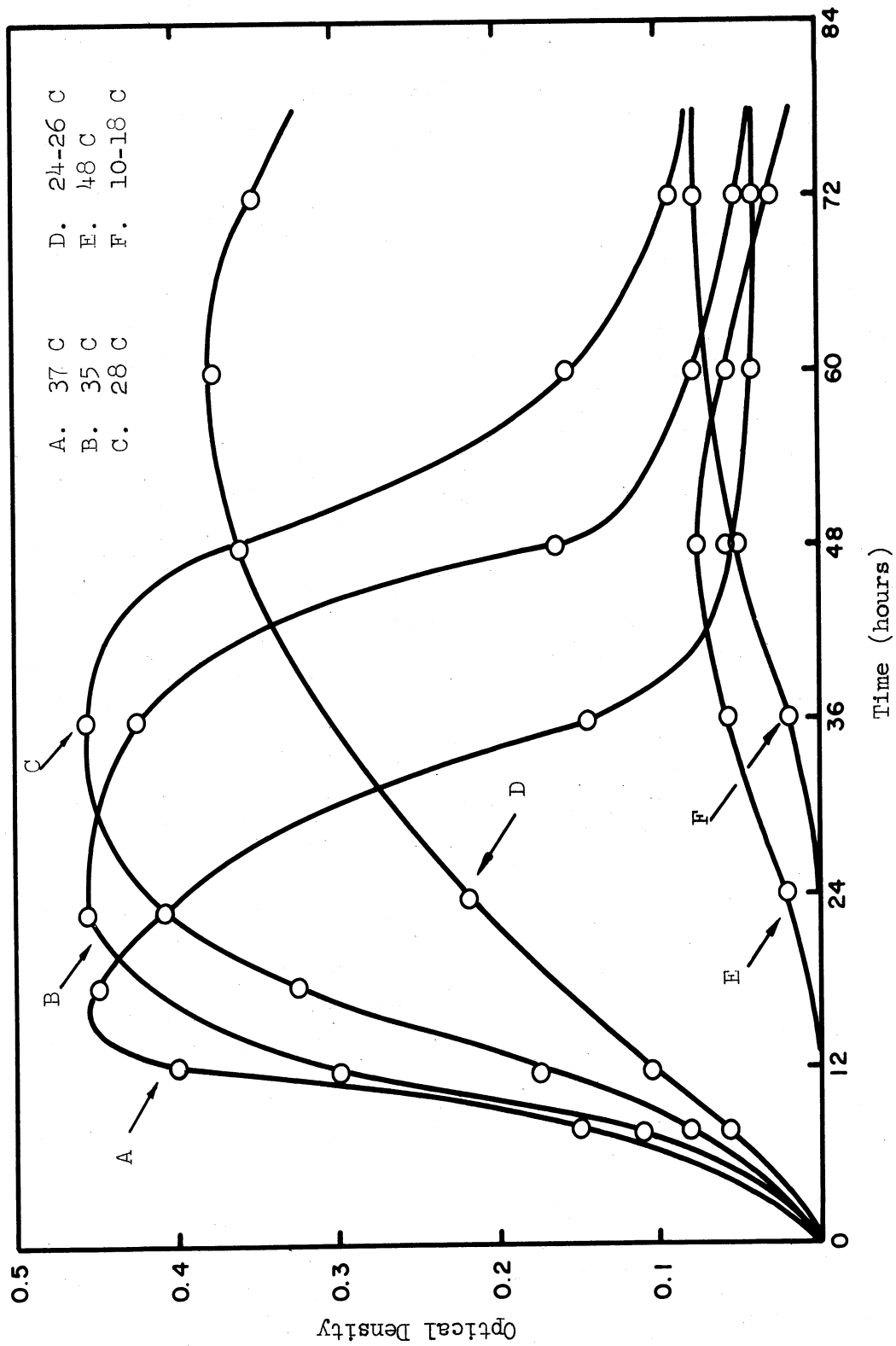


Figure 11. Effect of incubation temperature on growth of *C. botulinum* JTD-IV.

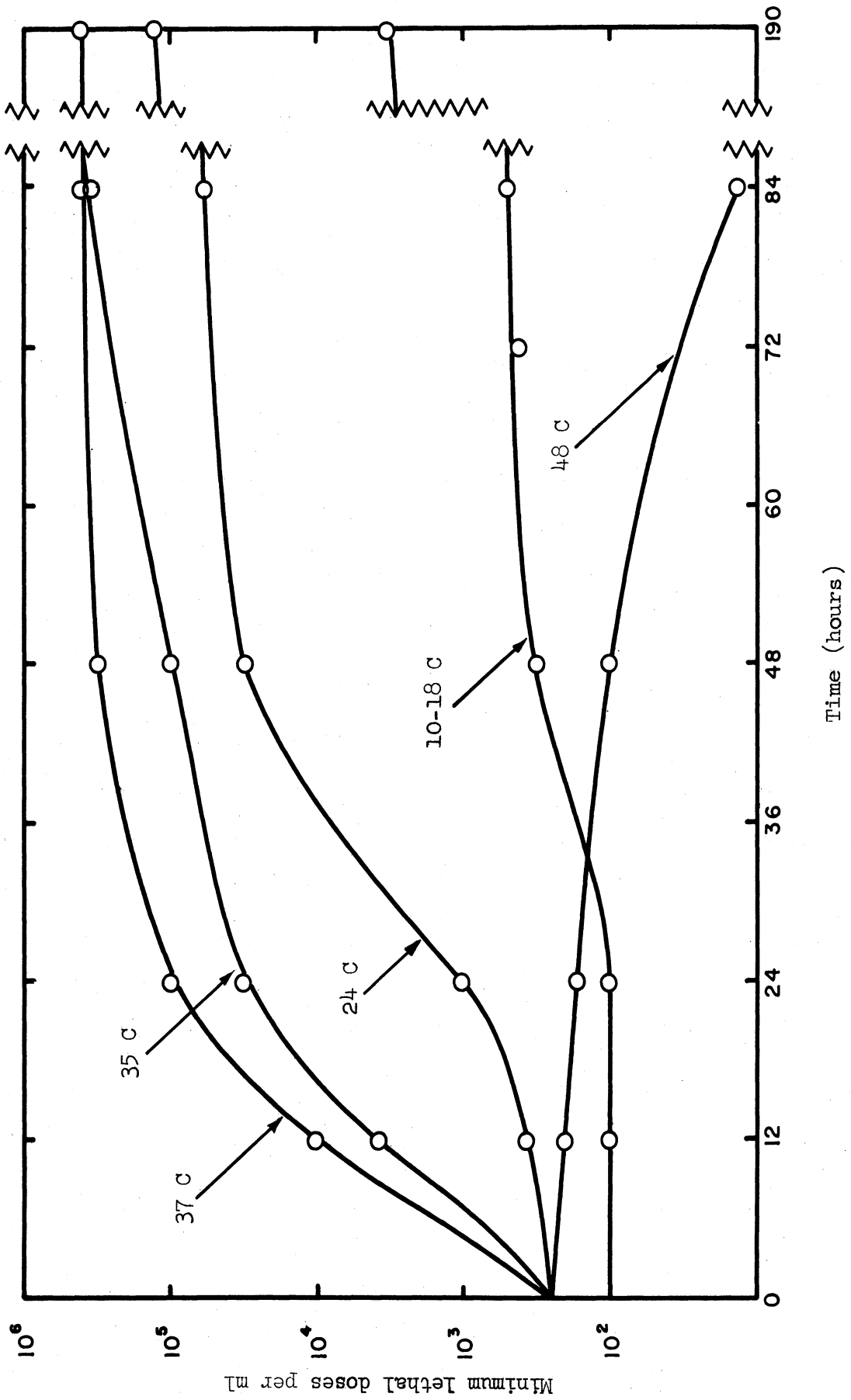


Figure 12. Effect of incubation temperature on toxin synthesis by *C. botulinum* JTD-IV.

although the rate at which these processes occurred increased with increasing temperature. In all cases, maximum toxicity of the culture filtrates became apparent only after autolysis was complete. Temperatures between 20 C and 26 C supported growth adequately but not optimally. At these temperatures, lysis was retarded and unlike cultures which had completely autolyzed, prolonged incubation between 20 C and 26 C resulted in a gradual increase in the quantity of toxin found extracellularly. The maximum temperature which supported growth was 48 C. At this temperature, however, although some growth could be measured turbidimetrically, no increase in the toxicity of the filtrates could be detected. Furthermore, the toxicity conferred on the cultures by the initial inoculum was abolished during the incubation period at 48 C.

Since the organism was able to multiply at this elevated temperature, it is quite unlikely that toxin synthesis was completely inhibited. The evidence suggests that any toxin which was elaborated was inactivated by gradual denaturation.

No growth or toxin synthesis was demonstrable at temperatures between 4 C and 10 C. However, when cultures of C. botulinum JTD-IV were incubated in the water bath which fluctuated between 10 C and 18 C, growth and toxin synthesis were noted. At these temperatures, the toxin is fairly stable and consequently, prolonged incubation resulted in a gradual increase in toxicity. After 190 hr of incubation, the culture

filtrates contained 5000 MLD/ml. These results suggest that the hazard of botulinus intoxication resulting from irradiation pasteurized or frozen foods is a possibility only if the storage temperatures are not adequately controlled.

De novo synthesis of protein toxin vs activation of "protoxin."

It was noted before that autolysis and simple diffusion of toxin could not account for the total toxicity of the cultures after lysis had proceeded to completion. Therefore, at this point, efforts were directed toward obtaining experimental evidence which would support de novo synthesis of protein during the period of cellular degeneration and/or the activation of a precursor of the toxin by physical or enzymatic agents.

1. Toxin production by "resting" cells (non-multiplying) of C. botulinum.

Kindler et al. (1956) reported that a strain of Clostridium parobotulinum was able to synthesize toxin actively in a complete medium containing high concentrations of penicillin. Their interpretation of de novo synthesis of protein was based on the fact that the filtrates of the cultures increased in toxicity in the absence of cell multiplication. At the same time this information was presented in the literature by Kindler et al., experiments of a similar nature were being conducted in this investigation in an attempt to separate completely the growth and toxin synthesizing processes.

Two strains of C. botulinum were grown in complete medium and the cells harvested at the end of the exponential growth phase. The organisms were then washed 5 times in freshly prepared medium and resuspended in complete medium to approximately twice the original cell concentration. Penicillin was then added so that the final concentration of the antibiotic was 200 units/ml. At this level, penicillin completely inhibited cell multiplication. The resting cells were reincubated and samples were subsequently taken at intervals for both extracellular and intracellular toxin assay. Table 6 shows the increase in toxicity noted with resting cells of C. botulinum strains JTD-IV and 457A. Microscopic examination of the cells during the incubation with penicillin revealed a loss of cellular integrity at a much faster rate than observed in a normally autolyzing culture. Penicillin, at the concentration utilized, abolished the gram-positive staining characteristic of both strains of C. botulinum in approximately 2 hr. Maximum toxicity of the filtrates was not attained until the cells had been reincubated with the antibiotic for 48 hr. If the time required for obtaining maximum growth of the organisms is taken into consideration, the total incubation time required for development of maximum toxicity in the filtrates i.e. incubation before and after the addition of penicillin, was 72 hr. A normally autolyzing culture of C. botulinum, as noted before, required the same length of time for maximum toxicity to manifest itself although the stationary growth phase was reached in 24 hr. Therefore, although the addition



Table 6

TOXIN PRODUCTION BY TWO STRAINS OF C. botulinum  
IN A COMPLETE MEDIUM CONTAINING PENICILLIN.

Hours of incubation with penicillin	Toxin titer (MLD/ml)			
	JTD-IV		457A	
	Filtrate*	Extract"	Filtrate	Extract
0	$5 \times 10^3$	$1 \times 10^4$	$3 \times 10^3$	$1 \times 10^4$
6	$1 \times 10^5$	$2 \times 10^5$	$4 \times 10^4$	-
18	-	-	$1 \times 10^5$	$2 \times 10^5$
24	$5 \times 10^5$	$1 \times 10^6$	$2 \times 10^5$	$3 \times 10^5$
40	-	-	$3 \times 10^5$	$5 \times 10^5$
50	$1 \times 10^6$	$2 \times 10^6$	$3 \times 10^5$	$5 \times 10^5$

\* Filtrate - extracellular toxin

" Extract - extracellular + intracellular toxin

of penicillin to log phase cells of C. botulinum insured the cessation of cell multiplication, the greater than 100-fold increase in toxicity obtained after 48 hr in the filtrates of resting cells could not be definitely interpreted as de novo synthesis of protein as suggested by Kindler et al. (1956). While such an interpretation of the data remained a possibility, experimental evidence concerning the synthetic capabilities of C. botulinum during the period of cellular degeneration was necessary before de novo synthesis of toxin could be considered as the mechanism responsible for the increased toxicity of the non-proliferating cultures. Such evidence was not presented by Kindler et al. (1956). In addition, de novo synthesis of protein in such large quantities is not usually considered a "normal" metabolic process during the terminal stages of the growth cycle. On the other hand, the evidence suggested the possibility of a toxin precursor being gradually converted to active toxin.

2. Toxin production by "resting" cells of C. botulinum in the absence of glucose.

It had been found that adaptation of C. botulinum to growth in the absence of an exogenous supply of glucose resulted in a marked inhibition of toxin synthesis. Consequently, in an attempt to distinguish between de novo synthesis of protein and activation of a toxin precursor, a comparison was made of toxin production by non-multiplying cultures of C. botulinum JTD-IV incubated with and without an exogenous source of glucose.

The organism was grown in a complete medium containing 0.5 glucose and the cells harvested at the end of the logarithmic growth phase. The cells were washed thoroughly to remove any contaminating glucose and resuspended in the complete medium to which no glucose had been added. Penicillin was added and the resting cell suspension divided into 2 equal parts. After 0.5% glucose had been added to one part, both portions of the original cell suspension were reincubated at 35 C.

The increase in toxicity of the filtrates of both resting cell suspensions during the 48 hr incubation period with penicillin was of the same order of magnitude. This indicated that the initial 24 hr growth period in the presence of glucose had satisfied the organism's requirements for maximum toxin synthesis and consequently, the removal of the carbohydrate during the remainder of the incubation period had no effect on the ultimate toxicity of the non-multiplying culture filtrates. When the above experiment was repeated utilizing cells of strain JTD-IV which initially were adapted to growth without glucose in the medium, it was found that further incubation of the resting cells in the absence of the external energy source resulted in only a slight increase in the toxicity of the filtrate. The results of these experiments, presented graphically in figure 13, would not appear to support the concept of de novo synthesis of protein toxin

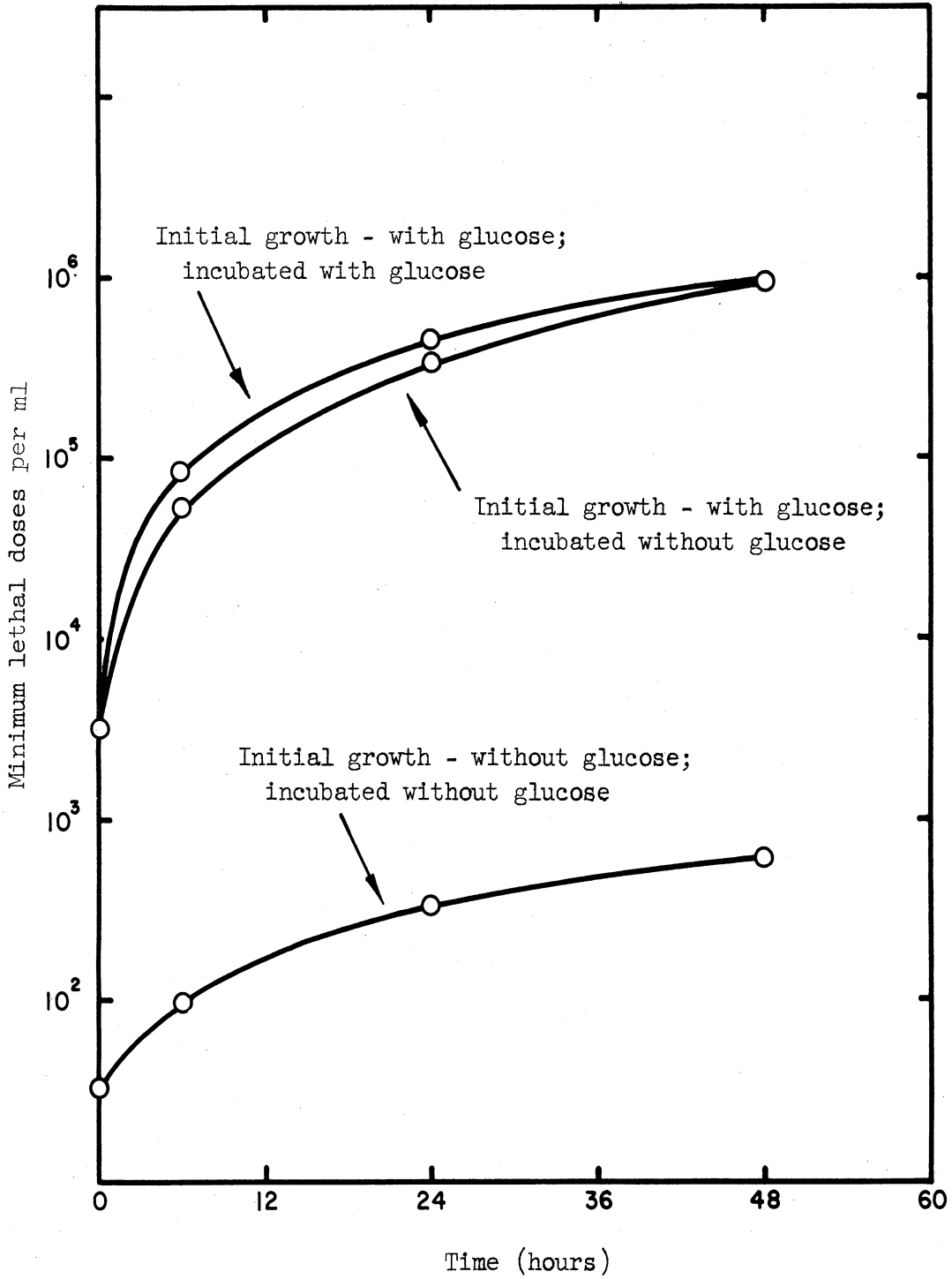


Figure 13. Toxin production by washed cells of *C. botulinum* JTD-IV suspended in a complete medium containing high concentrations of penicillin.

during the 48 hr incubation period with penicillin. The fact that glucose was essential for toxin synthesis in actively growing cultures of C. botulinum, and that removal of the sugar after maximum growth was attained had no effect on the final toxicity would seem to indicate that the actual synthesis of protein takes place during the initial 24 hr although maximum toxicity is not demonstrable by the animal assay system employed until 48 hr later.

3. Growth and toxin synthesis in a chemically defined medium.

The use of a chemically defined medium at this point in the investigation was deemed necessary for a number of reasons; (a) a chemically defined medium allows the control of constituents which can not be controlled in a complex medium; (b) in order to reject or sustain the hypothesis of de novo synthesis of toxin during the period of cellular degeneration, it was considered desirable that total protein determinations be carried out. Protein determinations made in a complex medium would be meaningless since they do not distinguish between the precipitable proteins in the medium and the protein synthesized by the cells; (c) a defined medium would be more suitable than a complex medium in obtaining evidence for establishing the presence of an inactive precursor of the toxin molecule.

The defined medium previously described under "Materials and Methods" initially was used without the addition of tryptophane. With the exception of C. botulinum strain B-201, this

medium supported the growth of the remaining strains for only three transfers, indicating that an essential growth factor was missing. Strain B-201 continued to grow poorly in the tryptophane deficient medium until the sixth transfer when growth became much more luxuriant. An assay of the supernatant culture fluid showed that no toxin had been synthesized in the defined medium. The fact that this strain alone continued to grow after the third transfer in the defined medium, introduced the possibility that the organism being carried was a contaminant. However, no morphological differences from the original strain of B-201 could be detected by microscopic examination. The biochemical reactions of this strain are not described in the literature and toxin production is the only basis on which it can be satisfactorily differentiated from other spore-forming, anaerobic; gram-positive rods. On the strength of the non-toxic organism's morphological identity with the parent strain of B-201 and the fact that toxigenic strains of C. botulinum revert to non-toxic forms quite readily, the investigation was continued on the assumption that the non-toxic organism was not a contaminant. Growth of the organism through 7 transfers in a complete medium did not result in restoration of toxigenicity. This was taken as evidence that the tryptophane deficient medium had either selected out a non-toxic variant of the original B-201 strain present in a heterogeneous population or had induced a non-toxic mutant which eventually replaced the parent population.

Bacterial toxins, in some cases may be excreted in an inactive form during the early stages of growth and become active later. Turner and Rodwell (1943) found that the Epsilon toxin of Clostridium welchii is excreted as a non-toxic antigenic precursor which is slowly converted into active toxin by proteolytic enzymes produced by the organism. Ross et al. (1949) showed that the Iota toxin of C. welchii is activated in the same manner. A phenomenon very similar to this was reported by Cohen et al. (1942) for still another organism. Pneumococcal cells which were allowed to undergo "maturation" at 5-10 C for a period of 15 hr, increased in total hemolysin content by at least 4-fold. More recently, Seki et al. (1954) although they did not demonstrate the phenomenon conclusively, postulated that the toxin of C. tetani may also be synthesized as a non-toxic precursor. On the strength of these precedents, a concerted effort was made to demonstrate an activation phenomenon of C. botulinum toxin. If a comparatively inactive precursor to the toxic molecule was shown to exist, it would serve to reconcile the conflicting reports on toxin production by C. botulinum found in the literature. In addition, the tremendous increase in toxicity noted during the period of active autolysis could be reconciled on an entirely different basis than the de novo protein synthesis concept advanced by Kindler et al. (1956).

Preliminary experiments were carried out with the non-toxic strain of C. botulinum B-201. All attempts to induce an active

from an inactive form of the toxin by treatment of the culture filtrates with trypsin, pepsin, and trypsin plus chymotrypsin were unsuccessful. Another approach was used by incubating mixtures of B-201 toxic extracts, obtained by growth of the parent strain in a complex medium with the non-toxic extracts of the variant. The rationale employed was that the enzymes or other factors necessary for activation of the toxin might be present in the toxic extracts and they could conceivably act to unmask the inactive form in the non-toxic extracts.

For these experiments 48 hr cultures of the two strains were sonically disintegrated and the resulting extracts mixed in equal proportions. The mixture was incubated for 6 hr at 35 C and assayed for toxin. A comparison of the extract toxin titers, singly and in combination is shown in table 7. The results were rather surprising since a partial inactivation, rather than an increase in toxicity was noted. Variation of the proportions in which the extracts were mixed or varying the length of the incubation period resulted in no further inactivation of the toxin.

No attempts were made to characterize the substance in the non-toxic extracts responsible for the partial inactivation of the protein toxin. An animal experiment in which 10 mice were used, however, indicated that the detoxifying ability of the substance was not effective in vivo. Injection of non-toxic extracts, prior to or in conjunction with active toxin was totally ineffective in protecting the mice.



Table 7

TOXICITY OF CELLULAR EXTRACTS OF C. botulinum B-201  
GROWN IN COMPLEX AND DEFINED MEDIA

	Toxin titer (MLD/ml)
Cell extract - 48 hr complex medium	$5 \times 10^4$
Cell extract - 48 hr defined medium	0
Mixture of ex- tracts; 1:1 incubated 6 hr	$5 \times 10^3$

a. Fortification of the defined medium with tryptophane

The nitrogen source in the chemically defined medium was vitamin free casamino acids, prepared by acid hydrolysis of casein. This process results in virtually a complete destruction of tryptophane. This well established fact had been overlooked in the initial experiments and led to the isolation of the non-toxic variant of C. botulinum B-201 referred to above. The defined medium, when fortified with 100 µg/ml of tryptophane, adequately supported the growth of all strains of C. botulinum. After adaptation to growth in this medium, the culture filtrates of strains JTD-IV and B-201 were assayed for toxin. Table 8 gives a comparison of toxin production by the organisms when grown in a complex and in the defined medium. The latter supported toxin synthesis to a lesser degree than the complex medium. It was also found that autolysis was very rapid and the toxin quite unstable in the defined medium. Since stability of the toxin was considered mandatory in an intelligent search for an inactive "protoxin", attention was shifted once again to spores and vegetative cells grown in a complex medium.

4. Activation of botulinus toxin by proteolytic enzymes.

The first successful attempts to activate botulinus toxin were very recently reported by Duff et al. (1956). They found that the toxicity of culture filtrates of C. botulinum, type E

Table 8

SYNTHESIS OF TOXIN BY C. botulinum, STRAINS JTD-IV AND B-201 IN A CHEMICALLY DEFINED MEDIUM AND A COMPLEX MEDIUM

Age of culture	Toxin titer (MLD/ml)	
	Complex medium	Defined medium
JTD-IV		
24 hr	$1 \times 10^4$	$5 \times 10^4$
48 hr	$2 \times 10^5$	$1 \times 10^4$
B-201		
24 hr	$5 \times 10^2$	neg
48 hr	$5 \times 10^3$	$5 \times 10^2$

could be increased considerably by treatment with trypsin and postulated the existence of an inactive, type E "protoxin". These investigators also pointed out that the activation of types A and B toxin had still not been demonstrated. Type E strains of C. botulinum are generally regarded as only slightly proteolytic while types A and B usually possess a full complement of proteolytic enzymes (Elberg and Meyer, 1939; Maschman, 1939). On the basis of this information, it was reasoned that if "protoxins" of types A and B were in fact synthesized, the manifestation of their potential toxicity was dependent upon an enzymatic conversion by the normal proteolytic enzyme complement of the organisms. The consequence of such an hypothesis would be to suggest that type E toxin can be activated by trypsin or other enzymes any time after synthesis of "protoxin" has occurred since type E strains do not themselves produce the enzymes required for activation. It is not surprising then, that the activation of type E toxin by Duff et al. (1956) was accomplished with cultures 120 hr old. On the other hand, types A and B which produce many proteolytic enzymes, can activate their respective "protoxins" gradually as the inactive precursors and enzymes are synthesized. Activation, in this case, can only be demonstrated before the phenomenon occurs naturally.

The inability to observe an activation phenomenon with cultures of types A and B strains of C. botulinum of the same

age as the type E cultures used by Duff et al. (1956), and a successful demonstration of activation by the addition of trypsin to much younger cultures of strains A and B, was considered an adequate test for the presence of "A" and "B" toxin precursors. C. botulinum strains JTD-IV and B-201 were inoculated into a complete medium and allowed to incubate at 35 C for 12 hr. The filtrates of these young cultures were then incubated with a sterile solution of crystalline trypsin (0.01%) at 37 C for 2 hr. Filtrates to which no enzyme had been added were also incubated at 37 C as controls. The toxicity of the culture filtrates is shown in table 9. It can be seen that the toxicity of both types A and B filtrates was increased 15-fold by the 2 hr incubation with the proteolytic enzyme. Ninety-six hr culture filtrates, treated with trypsin in exactly the same manner as the 12 hr filtrates, did not increase in toxicity but rather showed a slight decrease. This then represented the first successful demonstration of activation of the toxin molecules of C. botulinum types A and B and strengthened the hypothesis that a conversion of "protoxin" to active toxin occurs spontaneously during the complete life cycles of these organisms, through the mediation of their own proteolytic enzymes.

In an attempt to increase the degree of activation of the toxin, culture filtrates of still younger cells were employed. In addition, the ability of pepsin, as well as

Table 9

EFFECT OF TRYPSIN ON THE TOXICITY OF C. botulinum TYPES  
A AND B CULTURE FILTRATES

Sample*	Toxin Titer (MLD/ml)	
	JTD-IV	B-201
12 hr filtrate	$1 \times 10^4$	$2 \times 10^2$
12 hr filtrate + trypsin	$> 2 \times 10^5$	$1 \times 10^4$
96 hr filtrate	$2 \times 10^6$	-
96 hr filtrate + trypsin	$1 \times 10^6$	-

\* All samples incubated at 37 C for 2 hr.

trypsin, to activate the toxin was tested. C. botulinum JTD-IV was grown in a complete medium for 8 hr, the cells harvested and the supernatant fluid incubated with the proteolytic enzymes. Aliquots of the filtrate were added to equal volumes of trypsin solution (pH 6.0) and the mixtures incubated in a water bath held at 37 C. The same procedure was carried out using a 0.01% solution of crystalline pepsin of porcine origin (pH 4.0). Samples of the enzyme-filtrate mixtures were taken at different time intervals and assayed for toxin. The toxicity of the 8 hr culture filtrates which had been treated with the proteolytic enzymes is shown in table 10. As can be readily seen, the degree of activation was much greater with 8 hr culture filtrates than that obtained with the 12 hr culture filtrates. Activation of the toxin by proteolytic enzymes seems to be not only a function of the age of the cultures when subjected to this treatment, but also of the incubation period with the enzyme. Maximum increases in toxicity were noted after 2 hr with both pepsin and trypsin treated filtrates. Prolonged incubation resulted in a subsequent decrease in toxicity. In the case of the trypsin treated filtrates, the toxicity had declined to its original level of  $10^3$  MLD/ml after 12 hr. On the other hand, after 12 hr, the pepsin treated filtrates had lost only 60% of the activation obtained during the initial 2 hr incubation period.

Table 10

EFFECT OF PROTEOLYTIC ENZYMES ON THE TOXICITY OF AN EIGHT  
 HOUR CULTURE FILTRATE OF C. botulinum TYPE A.\*

Length of incu- bation with enzyme	Toxin titer (MLD/ml)	
	Pepsin treated filtrate pH 4.0	Trypsin treated filtrate pH 6.0
0	$1 \times 10^3$	$1 \times 10^3$
30 min	-	$2 \times 10^4$
75 min	-	$2 \times 10^4$
2 hr	$1 \times 10^5$	$5 \times 10^4$
12 hr	$4 \times 10^4$	$1 \times 10^3$

\* C. botulinum JTD-IV; 8 hr filtrates incubated with  
 enzyme at 37 C.



Since the ultimate unit of toxicity is not known, the **manner** in which the proteolytic enzymes activate the protein toxin is a matter of conjecture. It is possible that the protein as originally synthesized is a very large molecule whose toxicity is masked owing to chemical or physical binding of specific toxic units. The action of proteinases is one of hydrolyzing protein between specific amino acid linkages and consequently it is possible that the protein toxin molecule is degraded into smaller fragments which results in the freeing of the chemical grouping(s) responsible for the toxin's biological activity. The ease with which botulinus toxin can be inactivated by simple oxidizing agents or mild heat treatment suggests that simple chemical groupings, which are free to react, in conjunction with molecular configuration and amino acid sequence, are essential for the biological activity of the protein toxin.

It was conceivable that the proteolytic enzymes act not on the toxin molecule directly but on an inhibitor of the toxin present in the growth medium. In order to eliminate such a possibility it would be necessary to demonstrate an activation of purified toxin by the enzymes. Since all attempts to purify Type A toxin by the method of Lamanna et al. (1947) were unsuccessful, this experiment was not performed. However, the activation of type E toxin was accomplished by Duff et al. (1956) with purified toxin. If it is assumed that the proteolytic enzymes act similarly on types A and B toxin, then

it can be said that the enzymes act directly on the toxin molecule and not on a constituent of the medium.

5. Effect of heat-shock and sonic oscillation on the toxicity of *C. botulinum* culture filtrates.

The investigations of Kaplan (1955) have shown that a few yeast enzyme systems are inactive under normal environmental conditions. The enzymes, if heat-shocked are activated. Similarly, Church and Halvorson (1955) demonstrated this phenomenon with enzyme systems of aerobic bacterial spores. On the basis of the work of these investigators, an activation of botulinus toxin by other than enzymatic means was attempted.

Eight hr culture filtrates of *C. botulinum* JTD-IV were subjected to heat-shock at 80 C for a period of 30 seconds, 1 min and 2 min. The toxicity of non-heated and heat-shocked filtrates was then compared. No activation of the toxin was demonstrable in the filtrate heated at 80 C for 30 seconds. The longer heat treatments inactivated the toxin. Similarly, the subjection of young culture filtrates to sonic oscillation for 30 min neither decreased nor increased their toxicity. These experiments, suggest that the activation of botulinus toxin can not be accomplished by non-specific physical agents but can be brought about only by specific enzyme mediated reactions.

6. Toxicity of *C. botulinum* spores and spore extracts.

Spores of *C. botulinum* are considered to be relatively innocuous for animals susceptible to botulinus intoxication.

Other than the report by Orr (1922) that feeding of massive numbers ( $5 \times 10^9$ ) of spores to guinea pigs at times resulted in death of the animals, no reference could be found which established the relative toxicity of C. botulinum spores. Therefore an experiment was performed to ascertain the toxicity, if any, of intact spores and spore extracts. The ability of proteolytic enzymes to increase the toxicity of spore extracts was also tested.

Spore suspensions of 3 type A strains of C botulinum were prepared as previously described under "Materials and Methods". The suspensions were placed in a boiling water bath for 15 min to insure inactivation of any free toxin which may have been present due to vegetative forms. Aliquots of 0.5 ml were taken from the heated spore suspensions for toxin assay and the remainder sonically disintegrated. The spore debris was removed by centrifugation and 0.5 ml aliquots of the spore extracts also taken for assay. In addition, the spore extracts were incubated with equal volumes of a 0.01% solution of pepsin or trypsin to test for the presence of a "protoxin". Table 11 shows that 0.5 ml quantities of the heated spore suspensions containing approximately  $10^7$  spores/ml were non-toxic. Spore extracts, however, contained 10 MLD/ml indicating that a small quantity of intracellular toxin had been liberated. Neutralization by specific type A antitoxin established an immunological identity of the lethal factor present in the spores with type A botulinus toxin. The inability of either pepsin or

Table 11

TOXICITY OF SPORES AND SPORE EXTRACTS OF 3 STRAINS OF  
C. botulinum TYPE A.

	Toxin titer (MLD/ml)		
Heated spores (5 x 10 <sup>6</sup> )	neg	neg	neg
Spore extracts (0.5 ml)	10	10	10
Spore extract- antitoxin mixture	neg	neg	neg
Extract-trypsin mixture (pH 6.0) 90 min incubation	10	10	10
Enzyme-pepsin mixture (pH 4.0) 90 min incubation	neg	neg	-

trypsin to activate the lethal factor further, indicated that the spore extracts contained no "protoxin". This evidence, however, is not sufficient to state that an inactive form of the toxin is completely absent in the spores of C. botulinum. It may be that the unique chemical make-up of the spore in some way protects the "protoxin" by making it inaccessible to the action of the proteolytic enzymes, or inhibits the enzymes themselves.

7. Effect of metabolic inhibitors on toxin production.

Metabolic inhibitors were used as tools in attempting to establish conclusively the toxin activation phenomenon by the constitutive enzyme systems of the bacterial cells and the contribution of de novo synthesis of protein to the increased toxicity shown by the culture filtrates after the cessation of cell multiplication.

A procedure similar to that employed in the preparation of resting cells of C. botulinum by the addition of penicillin was employed. The information obtained with the penicillin resting cells, although significant, does not allow for an intelligent choice between the possible mechanisms which can account for the ultimate toxicity of the filtrates. The data can be interpreted on the basis of any one or all of the existing possibilities; i.e. autolysis, diffusion, activation of "protoxin" and de novo synthesis of protein. This situation is due primarily to the fact that the exact mode of action of penicillin is still unknown. The only statement which

can be positively made concerning the metabolic activities of C. botulinum cultures containing a sufficiently high concentration of penicillin, is that cell multiplication has been completely inhibited. The untoward effects of the antibiotic can be seen after a given period of time by microscopic examination of the cells but the specific metabolic reactions and enzyme systems inhibited can not be ascertained with any degree of certainty. Recently, some evidence has been obtained by Park and Strominger (1957) which suggests that penicillin inhibits ribose nucleic acid (RNA) synthesis. If this proves to be the case, then it is entirely possible that protein synthesis is indirectly inhibited by penicillin since Landman and Spiegelman (1955) have presented convincing evidence that protein synthesis does not take place in the absence of RNA.

The modes of action of a few antibiotics and metabolic inhibitors other than penicillin, however, are sufficiently well established so that they lend themselves more readily to studies of this nature. For example, it is known that 2-4 dinitrophenol (DNP) uncouples oxidative phosphorylation and consequently inhibits the formation of high-energy phosphate bonds, Versene is a strong chelating agent capable of binding divalent cations which are necessary as cofactors in many enzymatic reactions, and chloramphenicol completely inhibits protein synthesis while nucleic acid synthesis is

unaffected (Gale and Folkes, 1953; Wisseman et al.(1954).

It was considered possible that these metabolic poisons could be exploited by studying quantitatively their effects on the toxicity of C. botulinum cultures and then interpreting these data in the light of the reliable information available concerning the specific metabolic systems which had been poisoned.

A culture of C. botulinum JTD-IV, grown in a complete medium for 16 hr was harvested and the cells washed thoroughly in gelatin-phosphate buffer (pH 6.8). The cells were then resuspended in the complete medium containing 0.5% glucose and aseptically transferred in 9 ml quantities to sterile screw-cap test tubes. Sterile solutions of the metabolic inhibitors, prepared in concentrated form, were added to the cell suspension so that the final volume in each tube was 10 ml. The final concentration of the antibiotics used were: **Versene** (0.001 M), 2-4-dinitrophenol (0.0005 M), chloramphenicol (200 µg/ml), penicillin (1000 units/ml). Cell suspensions to which no inhibitor was added were included in the experiment as controls. Growth was measured turbidimetrically (figure 14) and samples were taken periodically for toxin assay (figure 15). At the concentrations used, penicillin, chloramphenicol and **Versene**, completely inhibited cell multiplication. Growth of strain JTD-IV in the presence of DNP was comparable to growth obtained with the control. An approximate 200 fold increase in the toxicity of the filtrates was noted during

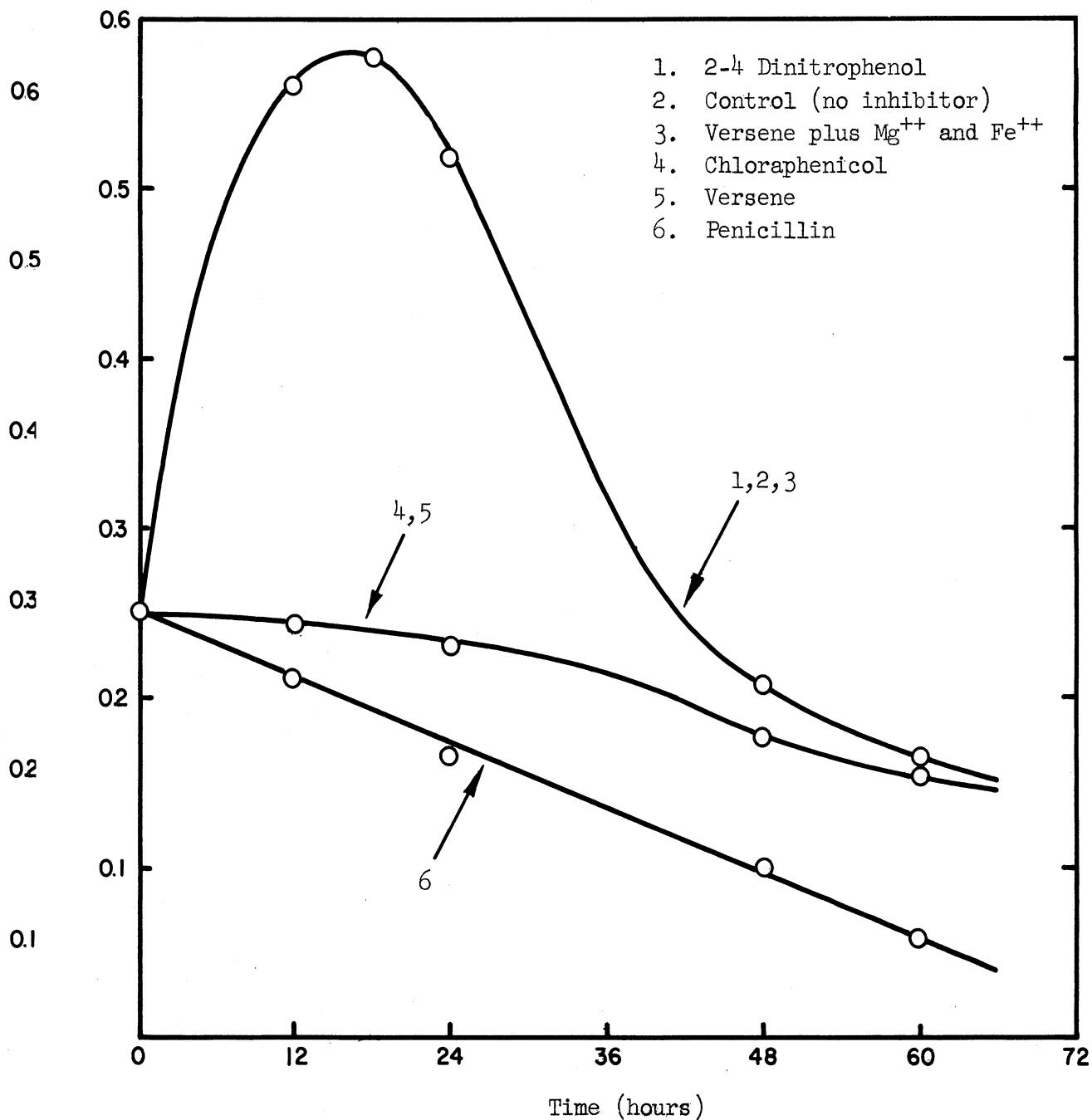


Figure 14. Effect of metabolic inhibitors on the growth of 16 hour washed cells of C. botulinum JTD-IV suspended in a complete medium.



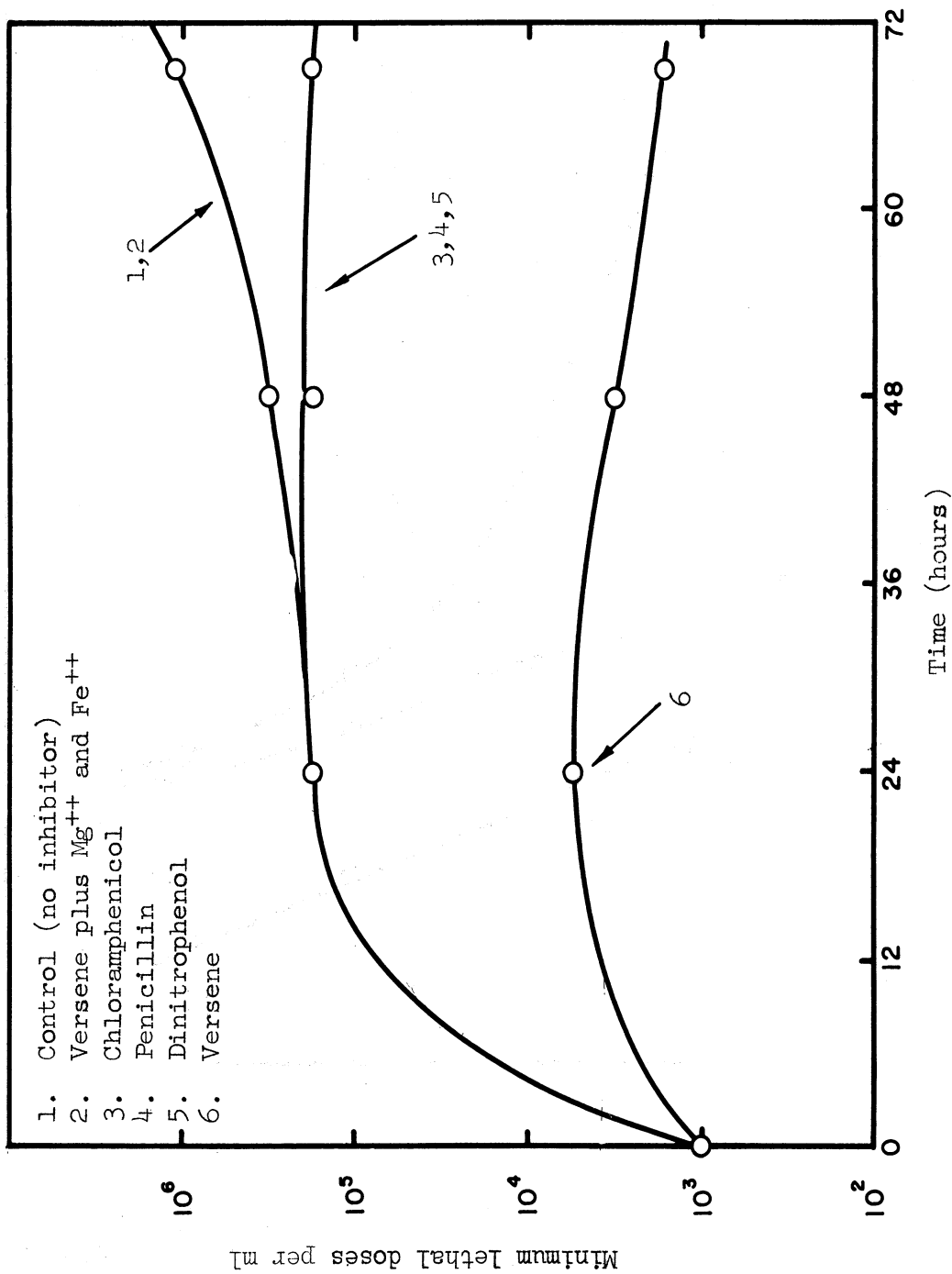


Figure 15. Effect of metabolic inhibitors on toxicity of 16 hour cells of C. botulinum JTD-IV suspended in a complete medium.

the incubation period with penicillin and chloramphenicol. Versene, on the other hand, completely inhibited an increase in toxicity. This inhibition was found to be completely eliminated, however, by the addition of sufficient magnesium and iron salts to the cell suspensions exposed to the chelating agent. The control, which continued to increase in cell number, was 2000 times as toxic as the original cell suspension after 72 hr. Although DNP did not inhibit cell multiplication, only a 200 fold increase in toxicity was noted.

These results, when interpreted on the basis of the modes of action of the metabolic inhibitors employed, strongly support activation of presynthesized "protoxin" and tend to negate the possibility of de novo synthesis of protein. Considering the metabolic poisons individually, the data can be interpreted as follows: (1) Control - The original cell suspension increased in optical density from 0.25 to a maximum of 0.57 before autolysis of the culture became apparent. During the 72 hr incubation period, the toxicity of the filtrates increased in toxicity from  $4 \times 10^3$  MLD/ml to  $2 \times 10^6$  MLD/ml. The maximum toxin titer can be assumed to represent that amount of toxin which a culture of strain JTD-IV is capable of synthesizing and activating in a favorable environment. Under no conditions, were toxin titers higher than this ever attained. In addition, filtrates possessing this maximum toxicity could not be activated further

by proteolytic enzymes. (2) DNP - Although growth in the presence of this metabolic poison was comparable to that obtained in the control, the toxicity of the filtrates did not reach the level of the control filtrates. Toxicity was increased from  $4 \times 10^3$  to  $2 \times 10^5$  MLD/ml. This emphasizes the mutually exclusive nature of the growth and toxin synthesizing processes. It is difficult to attribute the inhibition of toxin appearance by DNP to inhibition of protein synthesis since cell multiplication was unimpeded. It is much more likely that DNP inhibits the formation of adequate high energy bonds needed in the enzymatic reactions responsible for activation of the toxin. (3) Chloramphenicol - If, on the strength of what is known concerning the mode of action of chloramphenicol, it is assumed that protein synthesis was inhibited, then the increase in toxicity of the culture filtrates from  $4 \times 10^3$  MLD/ml to  $2 \times 10^5$  MLD/ml after 72 hr of incubation with the antibiotic cannot be attributed to de novo synthesis of protein. Since chloramphenicol has no effect on pre-synthesized protein, the data suggested that an inactive form of the toxin was gradually converted to the active form by the bacterial enzyme systems synthesized during the initial 16 hr growth period in the absence of any metabolic inhibitor. (4) Penicillin - The data obtained with penicillin-treated cultures were comparable to those obtained with chloramphenicol; growth was completely inhibited and the toxicity of the

filtrates increased to  $2 \times 10^5$  MLD/ml after 72 hr. If penicillin inhibits protein synthesis indirectly by virtue of its effect on RNA synthesis, then the data indicate that penicillin, like chloramphenicol, had no effect on the activation of "protoxin" synthesized before the addition of the antibiotic. (5) Versene - The chelating agent not only inhibited cell multiplication but also suppressed any increase in the toxicity of the filtrates. The necessity for divalent cations as cofactors by many enzyme systems made it appear that Versene, unlike penicillin and chloramphenicol, inhibited the enzymes responsible for activation of the toxin precursor.

These experiments, then, strongly suggested that the potential toxicity of a culture of C. botulinum at the terminal stages of the exponential growth phase was potentially much greater than the toxicity demonstrable by animal assay of the filtrates. If chloramphenicol completely blocked de novo synthesis of protein in this system, then it must be assumed that the original suspension of log-phase cells which contained a comparatively small quantity of biologically active toxin possessed at least enough synthesized inactive precursor to the toxin to account for the 200-fold increase in the toxicity noted during the incubation period with chloramphenicol.

In order to test further the validity of this hypothesis, experiments were conducted to compare the toxicity of 16 hr

culture extracts treated with proteolytic enzymes and the toxicity of cultures of the same age exposed to the metabolic inhibitors.

C. botulinum JTD-IV was inoculated into 1 liter of complete medium and incubated at 35 C for 16 hr. The cells were harvested, washed thoroughly and resuspended in the complete medium to give a heavy suspension with an optical density of 0.55. Aliquots of this cell suspension were then subjected to the treatments described in table 12a and assayed for toxin.

It can be seen that the toxicity of the suspension composed of thoroughly washed log-phase cells was increased more than 100-fold by sonic disintegration in the mother liquid and subsequent incubation of the resulting extract with trypsin for 30 min. Prolonged incubation with trypsin did not activate the toxin any further. The increase in toxicity was accomplished in approximately 1 hr after the cell suspension had been prepared; 30 min for sonic disruption of the cells and 30 min incubation with the enzyme. It is quite unlikely that synthesis of protein during this 1 hr period contributed in any way to the increase in toxicity. The temperature during sonic oscillation was maintained at 10 C by means of cold water circulated through coils in the oscillator. In addition, incubation of the cell-free extract in a complete medium to which no trypsin was added did not increase in toxicity after 24 hr. It can be said then, that

Table 12a

TOXICITY OF 16 HOUR WASHED CELLS OF C. botulinum JTD-IV  
SUSPENDED IN A COMPLETE MEDIUM AND SUBSEQUENTLY SUBJECTED  
TO SONIC DISINTEGRATION AND TREATMENT WITH TRYPSIN.

Treatment of aliquots of original cell suspension	Toxin titer (MLD/ml)
1. Washed, intact cell suspension; cells removed and filtrate assayed at 0 time.	$2 \times 10^3$
2. Extract obtained by sonic disruption of the cell suspension. Assay represents sum of intracellular and extracellular active toxin.	$2 \times 10^4$
3. Cell extract incubated with trypsin (pH 6.0) at 37 C for 30 min.	$> 2 \times 10^5$
4. Same as # 3-incubated with trypsin for 1 hr.	$> 2 \times 10^5$
5. Same as # 3-incubated with trypsin for 3 hr.	$5 \times 10^4$

the original cell suspension was potentially at least 100 times more toxic than could be demonstrated by assay of the filtrates before freeing the intracellular toxin and activation by trypsin.

Aliquots of the cell suspension used in the above experiments were also incubated with metabolic inhibitors so that the increase in toxicity obtained in this case could be compared with the degree of toxin activation observed by treatment of the extracts with trypsin. The metabolic inhibitors were added to aliquots of the cell suspension which were reincubated at 35 C. Table 12b shows the growth of the cells and the increase in the toxicity of the filtrates in the presence of penicillin (1000 units/ml), chloramphenicol (200 µg/ml), and chloramphenicol (200 µg/ml) plus versene (0.01M).

The increase in toxicity of the culture filtrates obtained after 48 hr of incubation with chloramphenicol and penicillin was slightly greater, although in the same range, as the increase noted after treatment of the cell extracts with trypsin. Stated in other terms, the toxicity of the filtrates after prolonged incubation with the antibiotics was only slightly higher than the potential toxicity of the original cell suspension. The controls increased to the maximum toxicity of  $2 \times 10^6$  MLD/ml. Incubation of the cells with chloramphenicol plus **Versene** almost completely inhibited any increase in the toxicity of the filtrates. These experiments presented fairly

Table 12b

CHANGES IN THE OPTICAL DENSITY AND TOXICITY OF 16 HOUR WASHED CELLS OF C. botulinum JTD-IV SUSPENDED IN A COMPLETE MEDIUM AND INCUBATED WITH METABOLIC INHIBITORS

Treatment of aliquots of cell suspension	Time (hr)	Optical Density	Toxin (MLD/ml) titer
1. Cell suspension control, (no inhibitor)	0	0.55	$2 \times 10^3$
	24	0.44	$5 \times 10^5$
	48	0.13	$10^6$
	72	0.11	$2 \times 10^6$
2. Cell suspension plus penicillin	0	0.55	$2 \times 10^3$
	24	0.27	$2 \times 10^5$
	48	0.16	$4 \times 10^5$
	72	0.11	$4 \times 10^5$
3. Cell suspension plus chloramphenicol	0	0.55	$2 \times 10^3$
	24	0.55	$2 \times 10^5$
	48	0.13	$4 \times 10^5$
	72	0.13	$5 \times 10^5$
4. Cell suspension plus chloramphenicol and versene	0	0.55	$2 \times 10^3$
	24	0.54	$5 \times 10^3$
	48	0.51	$5 \times 10^3$
	72	0.50	$5 \times 10^3$



convincing evidence that the toxin of C. botulinum is synthesized as a comparatively non-toxic molecule during the period of cell proliferation and that the period of cellular degeneration which follows is characterized by autolysis and activation of the toxin precursor by the normal proteolytic enzymes of the bacterial cells.

It is interesting to note that a combination of chloramphenicol and Versene, in addition to inhibiting the manifestation of the cell suspension's potential toxicity, also resulted in a dramatic stabilization of cellular integrity. From table 12b it can be seen that the optical density of the cell suspension did not change during the 72 hr incubation period with the metabolic inhibitors. Microscopic examination of the cells revealed typical, gram-positive organisms, very similar to those seen in a normal, log-phase culture of C. botulinum. This virtual abolishment of the autolytic process indicates that Versene inhibits the activity of the autolytic enzymes as well as the enzymes required for activation of the "protoxin".

In order to validate conclusively the chain of events described above as being responsible for the ultimate toxicity of culture filtrates of C. botulinum, type A, it was considered necessary to determine (a) if the strains of the organism used in the investigation synthesized enzymes capable of hydrolyzing protein; (b) if Versene acted as an inhibitor of these enzymes;

(c) if the period of cellular degeneration was characterized by comparatively little protein synthesis; (d) and if chloramphenicol was capable of effectively stopping protein synthesis by C. botulinum.

8. Proteolytic activity of C. botulinum.

A qualitative determination of proteolytic activity by the different strains of C. botulinum was carried out by observing growth in sodium caseinate agar. The constituents of the medium were: sodium caseinate (Difco), 1.5%; yeast extract, 0.5%; glucose, 0.2%; and agar agar (Difco), 1.5%. Proteolytic activity of an organism can be easily ascertained in this opalescent medium by the development of a clear zone surrounding the bacterial colony due to the digestion of the casein.

Prickett tubes containing 25 ml of the medium, were inoculated with the test organism while the medium was still in the liquid state. Rapid solidification was accomplished by immersion of the tubes into an ice bath after which the tubes were incubated at 35 C for 48 hr. C. botulinum strains JFD-IV, 62 A, 213 B, and B-201, when tested by this non-quantitative method were found to digest actively the casein substrate.

It was also considered desirable to determine the proteolytic activity of the organisms quantitatively so that the degree to which Versene affected these enzyme systems could be measured. Twelve hr cultures of the 4 strains of

C. botulinum were harvested, washed and suspended in gelatin-phosphate buffer containing 0.05% sodium thioglycollate. One ml aliquots of the suspensions were incubated with equal volumes of a 1.0% commercial casein solution at 37 C for 2 hr. The cell suspensions and 0.01 M versene (final concentration) were also incubated with the casein solution. In addition, 1 ml of a 0.01% solution of crystalline trypsin was allowed to digest the casein solution for the same period of time as the cells so that a comparison of proteolytic activity by the cells and a purified enzyme could be made. After precipitation of the unhydrolyzed protein by 3.0 M trichloroacetic acid, the supernatant fluids were analyzed for free tryosine as previously described under "Materials and Methods".

The proteolytic activity of the organisms and the degree of inhibition by Versene are shown in table 13. It can be seen that all of the strains of C. botulinum hydrolyzed the casein to approximately the same extent and that this degree of hydrolysis represented approximately 50% of the activity of a highly purified protease. Versene was found to inhibit proteolytic enzyme activity of the organisms by more than 50%. These results show that the organisms used in this investigation synthesize the proteolytic enzymes which theoretically are capable of activating the toxin precursor. The inhibition by Versene of proteolytic activity as well as toxin appearance, also fits in very well with the hypothesis

Table 13

PROTEOLYTIC ACTIVITY OF C. botulinum, TYPES A AND B AS MEASURED  
BY TYROSINE LIBERATION FROM A CASEIN SUBSTRATE

Strain	µg tyrosine/ml		$\frac{\text{(activity of cells)}}{\text{(activity of trypsin)}}$	inhibition by versene (0.01 M)
	cells	cells plus versene		
JTD-IV	62	33	0.43	0.47
B-201	62	28	0.43	0.55
62 A	71	32	0.49	0.55
213 B	65	28	0.49	0.57
.01% trypsin	145	--	--	--

of activation. It should be pointed out, however, that there seems to be no quantitative correlation between proteolytic activity and the comparative toxigenicity of the strain of C. botulinum. For example, strain JTD-IV which is much more toxigenic than strain B-201 does not seem to possess greater proteolytic activity than B-201.

9. Effect of chloramphenicol on total protein content of cultures of C. botulinum JTD-IV incubated in a defined medium.

In order to establish whether or not chloramphenicol effectively inhibited protein synthesis by C. botulinum, total protein determinations of cultures incubated with chloramphenicol were made. Strain JTD-IV was grown in a complete medium for 12 hr and the cells harvested and washed thoroughly in gelatin-phosphate-thioglycollate buffer (pH 6.8). The cells were resuspended in the chemically defined medium and chloramphenicol added in concentrations of 200 µg/ml. The cultures were reincubated at 35 C and protein determinations by the method of Lowry et al. (1951) were carried out periodically. Table 14 shows growth, as measured turbidimetrically. Total protein is also indicated in terms of free tyrosine found in aliquots of the cultures incubated in the presence and absence of chloramphenicol. It can be seen that the antibiotic completely inhibited growth and increases in total protein of the cultures during the 50 hr incubation period. This evidence shows that the 200-fold increase in toxicity obtained with cultures of

Table 14

EFFECT OF CHLORAMPHENICOL ON TOTAL PROTEIN CONTENT OF CULTURES  
OF C. botulinum JTD-IV SUSPENDED IN A DEFINED MEDIUM.

Sample*	Optical Density	Total tyrosine per ml of culture	% increase
Control, no antibiotic			
0 hr	0.152	140 µg	-
18 hr		190 µg	36
30 hr	0.340	184 µg	34
50 hr	0.210	200 µg	43
Chloramphenicol, (200 µg/ml)			
0 hr	0.144	140 µg	-
18 hr		125 µg	0
30 hr	0.130	140 µg	0
50 hr	0.110	140 µg	0

\* Original cell suspension made from 12 hr culture grown in complete medium, washed and suspended in defined medium.

C. botulinum incubated with chloramphenicol (table 12b)

takes place in the absence of any increase in total protein.

10. Changes in total protein of C. botulinum JTD-IV cultures during the normal growth cycle.

A knowledge of the relative changes in protein content of a culture of C. botulinum, especially after cell multiplication ceases, was considered important so that the changes in the quantities of total protein and active toxin could be compared chronologically. Strain JTD-IV was adapted to growth in the defined medium and subsequently growth and total protein of aliquots of a culture were measured during a 72 hr incubation period. Table 15 shows that after cessation of growth, the protein content of the culture decreased slightly. This evidence indicated that little or no protein synthesis occurred during the final 48 hr of incubation. Consequently, it was considered unlikely that de novo synthesis of protein toxin was responsible for the tremendous rise in toxicity of culture of C. botulinum usually observed during this period.

Table 15

CHANGES IN TOTAL PROTEIN OF CULTURES OF C. botulinum JTD-IV  
GROWN IN A DEFINED MEDIUM.

Age (hr)	Optical Density	µg tyrosine/ml of culture
0	0	-
4	0.03	392
24	0.136	530
42	0.108	510
72	0.110	430



## DISCUSSION

The experimental evidence compiled has borne out the original assumption that the toxicity of cultures of C. botulinum is dependent, not merely on the synthesis and excretion of the toxin molecule, but on several inter-related environmental and organismic factors. The investigation, by implication, has also served to narrow considerably the gap existing between the concepts of exotoxins and endotoxins of bacterial origin. For the sake of clarity, the environmental and organismic factors will be discussed as separate entities and then an attempt will be made to discuss the total picture of toxin production in the light of the information already found in the literature and the results of this investigation.

The toxin of C. botulinum, due to the striking toxicological activity, is unique and this uniqueness has at times had the tendency to mask the fact that the toxin is a protein molecule synthesized by a functioning organism. Within the limits imposed by currently available knowledge of protein structure and physical properties, and the limited number of techniques available for studies of this nature, it can be said that crystalline botulinus toxin is an ordinary protein molecule which requires no complexing with lipid, polysaccharide or nucleic acid for activity. Consequently, if for the time being, its toxicological properties are ignored, then it becomes clear that the toxin molecule must be considered on exactly the same basis as would any enzyme or structural protein of the cell. No specific metabolic function as far as the bacterium is concerned can be assigned to the

toxin. The synthesis of this "nonsense" protein molecule, if one wishes to consider it as such, is fundamentally subject to the same laws which govern the synthesis of any cellular protein. If the experimental results dealing with the effects of the physical and chemical environment on production of botulinus toxin are viewed in this light, then the molecule loses its unique character and behaves very much like its vulnerable counterparts.

The pH range in which C. botulinum is able to initiate growth and synthesize toxin in a complete medium was found to lie between 5.3 and 8.5. Generally speaking, these are the approximate limits of hydrogen ion concentration within which the synthetic processes of most heterotrophic organisms will occur. The rate at which toxin was synthesized did not vary markedly with pH. However, the rate limiting factors of the quantity of toxin found extracellularly appeared to be the degree to which the culture had autolyzed and the stability of the toxin in its environment. The autolytic enzymes were found to be most active between pH 6.5 and 7.5. Consequently, cultures grown at these hydrogen ion concentrations liberated the greater part of their toxin shortly after maximum growth was achieved. Since the toxin is very unstable in an alkaline environment, however, a considerable amount was inactivated above pH 6.8. Thus, if the toxicity of the extracellular fluids were determined at only one point in the growth cycle of C. botulinum, after 48 hr of incubation for example, one could interpret the results by stating that more toxin is synthesized between pH 6.5 and 6.8 than at any other level of hydrogen ion concentration. An interpretation of this kind may in fact be one of the contributing factors

for the conflicting reports found in the literature. The results of these experiments strongly indicate that pH does not affect the synthesis of the toxin but indirectly affects the degree of toxicity of the filtrates by virtue of the narrow pH optimum for the autolytic enzymes and the sensitivity of the toxin molecule to an alkaline environment.

Similarly, it was found that the synthesis of toxin was not "abnormal" when C. botulinum was incubated at different temperatures. The growth, autolytic and toxin synthesizing processes were maximally supported within the temperature range expected for any mesophilic organism. Placed in an environment containing all the essential components, C. botulinum carried out the metabolic processes, as one would expect, at increasing rates with increases in temperature between 28 C and 40 C. Extending in either direction above or below these temperatures, the overall metabolic pattern of the organism was increasingly affected and, consequently, the inhibition of growth, autolysis and toxin synthesis all contributed to the lessened toxicity of the extracellular fluids. The non-toxicity of the cultures incubated at 48 C, the maximum temperature at which growth of the organism occurred, is evidence that the toxin molecule is as sensitive to heat denaturation as most enzymes. At temperatures of 10 C or below, all metabolic processes apparently were completely inhibited. The observation of growth and toxin production at incubation temperatures fluctuating between 10 C and 18 C is of practical significance since it emphasizes the potential hazard of botulinus intoxication from non-sterilized foods which may be improperly stored.

The complex nutritional requirements of C. botulinum for growth and toxin synthesis also denote conformity rather than individuality with the fundamental nature of fastidious organisms. Although the specific nitrogen and vitamin requirements for growth and toxin synthesis were not ascertained, it is evident from the futile attempts to obtain a defined culture medium which would support optimum growth and toxin synthesis, that these requirements are very complex. The requirement by C. botulinum for tryptophane and 8 other essential amino acids in comparatively large quantities, in addition to biotin and the B<sub>6</sub> vitamins, is well documented (Mager et al. (1954)). Maximum growth and toxin synthesis by the strains of C. botulinum studied in this investigation were attained only in a complete medium containing the full complement of amino acids, yeast extract and an energy rich carbohydrate. The experiments involving the effect of the external carbohydrate on growth and toxin synthesis in the complete medium pointed out that the two processes were not necessarily proportional to each other. In fact, that they may be entirely independent of each other was shown by the 1000-fold decrease in toxicity of cultures adapted to growth in the absence of the external carbohydrate. The results also showed that glucose, or a disaccharide of this sugar, is a specific requirement for toxin synthesis which could not be replaced by a number of other carbohydrates tested. Since the other compounds tested included pyruvate and glycerol, it is quite likely that glucose is necessary not primarily as an energy source but in the formation of a satisfactory enzyme balance for toxin synthesis. Glucose was also necessary for the complete autolysis of cultures. Incomplete autolysis

of the cultures grown in the presence of carbohydrates other than glucose, however, was not responsible for the decreased toxicity of the filtrates since artificial disruption of the unautolyzed cells resulted in the liberation of very little toxin.

The discussion up to now has dealt with some environmental factors influencing the growth and toxin synthesizing mechanisms of C. botulinum and no mention has been made of the organismic factors. This distinction is a purely arbitrary one since, admittedly, the organism is for the most part dependent on its environment for support of the metabolic activities which contribute to the final toxicity of the culture filtrates. When attention is shifted from the effects of the environment on the organism to the organism's inherent properties, which potentially are capable of bringing about the formation of the extremely potent neurotoxin, C. botulinum assumes its distinctive character and seems to occupy a niche, perhaps not exclusively for itself, but at least restricted to a very small number of toxin producing organisms.

The phenomenon of autolysis has been observed with members of the genus Clostridium time and time again, but it was not until very recently that it was associated with toxin production (Seki et al.(1954); Stone,(1954); Boroff, (1955); Kindler et al.(1955)). The initial experimentation carried out in this investigation concerned itself primarily with the relationship between autolysis and toxicity. The results obtained definitely implicated autolysis as a mechanism for the liberation of botulinus toxin. Without exception, it was found that growth and appearance of toxin in the filtrates of the different strains of

C. botulinum were not parallel processes. At the end of the logarithmic growth phase, much smaller quantities of toxin could be demonstrated extracellularly than intracellularly, and only after autolysis of the cultures had proceeded to an advanced stage was maximum toxicity of culture filtrates obtained. The autolytic process was not due to extreme changes in the physical environment but appeared to be brought about by cellular components, most likely enzymatic in nature. This conclusion was based on the observations that the pH of a culture of C. botulinum did not change appreciably during growth, that filtrates taken from a completely lysed culture hastened autolysis of a young culture considerably, that heating the filtrate at 100 C for 5 min abolished this activity, and that the pH and temperature optima for autolytic activity were quite narrow.

Although autolysis of the cells was found to be correlated with the quantity of toxin found extracellularly, it could not account for the extracellular toxin present in very young culture filtrates. An approximate calculation showed that under the most favorable environmental conditions, using the most toxigenic strain of C. botulinum available, i.e. strain JTD-IV, at least 1000 cells were required for every minimum lethal dose of toxin ultimately present in 1 ml of culture filtrate. If it were assumed that autolysis is an all or none phenomenon, then the toxicity of 1000 MLD/ml (the approximate toxin titer of young culture filtrates) would represent  $10^6$  autolyzed organisms per ml of culture fluid. Debris resulting from the autolysis of this number of cells should be visible by microscopic examination. Since autolysis in very young cultures was not apparent, it became obvious that toxin

was liberated probably by a process of simple diffusion by organisms which, for all intents and purposes, still retained their cellular integrity. If the process of autolysis is now visualized as a gradual one, operative during the complete life of a culture of C. botulinum, then it follows that the autolytic enzymes are continually being synthesized, and their destruction of cell wall begins during the first few hours of growth. Since the process is a gradual one, diffusion of intracellular materials most likely takes place at a very low level during the initial growth phase and accelerates as the autolytic enzymes accumulate. The first microscopic sign of this degenerative process is the loss of the gram-positive staining characteristic. This visible manifestation of the autolytic process, however, is good evidence that extensive disintegration of the cell wall has already occurred and can account for the extracellular toxin found in "pre-lysing" cultures.

Further calculations made from the data indicated that mechanisms other than autolysis and diffusion were responsible for the ultimate toxicity of autolyzed cultures. The sum of intracellular and extracellular toxin demonstrable immediately after cessation of cell multiplication ( $\approx$  24 hr) was approximately one tenth that of culture filtrates assayed 48 hr later. This situation presented two intriguing possibilities; either C. botulinum was able to synthesize 90% of its toxin during the period of active autolysis or the period of cell multiplication was characterized by the synthesis of a "protoxin" which was converted to active toxin during the final 48 hr of incubation. Since both possibilities were considered reasonable, experiments were carried out to determine directly and indirectly, the

contributions of de novo synthesis of protein and activation of a toxin precursor to the final toxicity of culture filtrates of C. botulinum.

The experiments using cell suspensions whose multiplication was prevented by penicillin showed that their toxicity increased at least 200-fold during the incubation period with the antibiotic. Kindler et al. (1956) interpreted a similar observation using a strain of C. parobotulinum on the basis of active toxin synthesis. Since the exact mode of action of penicillin is not known, this interpretation was not considered fully justified, since the synthetic capabilities of the organism in the presence of the metabolic inhibitor had not been investigated. In addition, the fact that glucose was required for toxin synthesis in actively growing cultures of C. botulinum, and that the absence of the carbohydrate from the incubation medium after maximum growth was attained had no inhibitory effect on the final toxicity of the resting cell filtrates, were taken as evidence that the actual synthesis of a toxin precursor takes place while the cells are actively multiplying with the final toxicity becoming apparent only after the period of cellular autolysis.

Precedent for the activation of bacterial toxins is now fairly well established (Turner and Rodwell, (1943); Duff et al. (1956)). In the present study, attempts to demonstrate an activation of the toxins of C. botulinum types A and B with proteolytic enzymes were successful only with filtrates of young cultures. Incubation of filtrates of autolyzed cultures (> 72 hr) with pepsin and tyrpsin resulted in no further increases in toxicity. These observations strongly indicated



that the toxins of C. botulinum types A and B were initially synthesized as comparatively inactive molecules which slowly were converted to the active forms by the normal proteolytic enzymes of the cells. It should be emphasized that the activation of culture filtrates by pepsin or trypsin does not in itself constitute proof that such a phenomenon occurs normally. Indirectly, however, the evidence suggests this as a very likely possibility for two reasons: the results show first that such an activation by the action of proteolytic enzymes is possible; and second that the molecule is altered in some manner on prolonged incubation after cessation of cell multiplication, through the mediation of the organism's metabolism, so that it can no longer be activated artificially. The artificiality of the activation phenomenon by purified proteolytic enzymes is emphasized by the fact that prolonged incubation in their presence resulted in a subsequent decrease in toxicity after the initial increase noted during the first 2 hr of incubation. The differences in the degree of inactivation of the toxin in the filtrates observed after the first 2 hr of incubation with trypsin and pepsin may be explainable on the basis of differences in the activity of these enzymes on proteins in general. After the initial 100-fold increase in toxicity had been obtained with trypsin and pepsin, the toxicity was found to gradually decrease on further incubation. This decrease was much greater with trypsin than with pepsin and may be due to the fact that trypsin hydrolyzes protein down to its constituent amino acids whereas pepsin hydrolyzes them only to the level of proteoses and long chain polypeptides. The process of activation of

"protoxin" through the mediation of the proteolytic enzymes of C. botulinum, however, apparently is a much more efficient process since the subsequent decrease after maximum toxicity is attained does not occur.

An observation made by Nelson (1927) seems to be quite pertinent to the phenomenon of toxin activation by proteolytic enzymes although at the time he did not recognize it as such. He found that the toxin of C. botulinum could be released from the cells by alternate freezing and thawing and concluded that the toxin was elaborated within the cell in close association with the "bacterial globulin" (this last term is interpreted as being synonymous with structural proteins of the cell). In an attempt to separate these two components, he treated the mixture with pepsin and stated that the "bacterial globulin" was hydrolyzed while the toxin was not since there was even more toxicity than present in the original sample. Nelson interpreted this increase in toxicity as being due to the release of the toxin from the intracellular masses. However, in the light of more recent work and the results of this investigation, his observations can be interpreted as an activation of the toxin by pepsin. It is quite interesting to note that the retention of toxicity was used by Nelson as the criterion for the resistance of the toxin molecule to the action of pepsin and this reasoning has very likely prevailed for many years since statements to the effect that the toxin of C. botulinum is not sensitive to proteolytic enzymes can be found quite readily in the literature. One of the reasons why the toxin is so effective when it is ingested orally is that the toxicity is not destroyed by the proteolytic enzymes

present in the intestinal tract of the susceptible animal. On the strength of the demonstration of activation of the toxin, it is conceivable that the enzymes of the gut in some cases may partially hydrolyze the toxin, releasing toxic sites and consequently enhance rather than minimize the lethality of the toxin. Pappenheimer (1948) has pointed out that it is difficult to picture a substance with a molecular weight of close to one million being absorbed from the intestinal tract and suggested that the molecule may in fact be broken down to toxic fragments by the proteolytic enzymes of the gut. This is a reasonable suggestion since it is very difficult to visualize why botulinus toxin, which appears to be no different from any other protein molecule, should be so resistant to digestion. The assumption that the toxin is not broken down is based on the retention of toxicity following treatment with these enzymes but toxicity is not necessarily a criterion of intactness and no one has ever shown that the molecule is not broken down. In this investigation activation of a purified "protoxin" was not demonstrated. Consequently, the activation of the toxin in the culture filtrates cannot be considered definite proof of the toxin molecule's enzyme susceptibility. These results, however, strongly indicate that the toxin is in fact broken down into more toxin fragments by pepsin and trypsin. This concept of toxic fragments of the molecule is a very nebulous one, since knowledge of the fundamental units of toxicity is negligible. Protein chemistry has not as yet advanced sufficiently to explain toxicity on a molecular level, i.e. to ascertain the functioning portions of the molecule. This statement holds true not only for the action of bacterial toxins but for enzymatic and antigen-antibody reactions as well. It is known that botulinus toxin

in some manner interfere with the release of acetylcholine at the cholinergic nerve endings but this knowledge sheds no light on what constitutes the toxic moiety of the molecule. The meager evidence at hand, however, indicates that toxicity is due to simple chemical groupings, not only because of the ease with which it is inactivated by oxidizing agents and mild heat treatment but also because of the strange behavior of the protein. Wagman and Bateman (1953) observed that when they precipitated a culture of C. botulinum type A at different pH levels, they obtained 2 separate dissociation products of the toxin which were as toxic as the original supernatant fluid. More surprising, they found that after precipitation, the supernatant had not diminished in toxicity. Dack and Wagenaar (1956) obtained 2 toxic components of purified toxin by ultracentrifugation. All these observations indicate that fragmentation of the molecule, up to a point, results in cumulative toxicity by virtue of more toxic sites being available for activity. Until such time as the toxin molecule is adequately characterized, however, the manner in which proteolytic enzymes activate the toxin remains a matter of conjecture.

The use of metabolic inhibitors, whose mode of action is known, served to determine indirectly whether or not de novo synthesis of protein during the period of cell degeneration contributed to the ultimate toxicity of culture filtrates. It was found that the toxicity of cell suspensions, incubated in the presence of chloramphenicol, a known inhibitor of protein synthesis, increased 200-fold. The same cell suspension could be increased in toxicity to approximately the same extent by simply disrupting the cells and treating the resultant extract with

trypsin. This showed that the potential toxicity of the original culture was far greater than initially apparent. The inhibition of toxin appearance by Versene and the reversal of this inhibition by magnesium and iron indicated that the chelating agent inhibited the proteolytic enzymes of the organism responsible for activation of the toxin. Further experimental evidence showing that the strains of C. botulinum used in this investigation synthesized proteolytic enzymes whose activity was greatly diminished by Versene, the evidence showing that chloramphenicol effectively inhibited increases in total protein of cultures of C. botulinum and finally the observation that increases in total protein were negligible during the period of time in which the greater part of the active toxin appeared, i.e. after cell multiplication ceased, all pointed to activation of a "protoxin" rather than de novo synthesis of protein as the mechanism responsible for the increased toxicity of the culture filtrates. It should be emphasized that this conclusion is based for the most part on indirect rather than on direct evidence. The interpretation of the data is considered to be justified but until such time as a "protoxin" is isolated and can be shown to be capable of activation by the specific enzyme systems responsible for the phenomenon, no definite proof for activation of types "A" and "B" toxin can be claimed. The characterization of both the autolytic and toxin-activating enzyme systems would appear to be a very rewarding area for future research. Since Versene was found to inhibit autolysis as well as activation of the toxin it is quite likely that these two enzyme systems are closely related to each other and may in fact be identical.

De novo synthesis of toxin in autolyzing cultures as a contributing mechanism was eliminated also on the basis of indirect evidence i.e. the observations of a 200-fold increase in toxicity which occurred in the presence of an effective inhibitor of protein synthesis and little or no increases in total protein by normally growing cultures of C. botulinum after cessation of cell multiplication. Possibly the discipline of immunology could be exploited to study this phase of the problem further. Quantitative precipitin determinations could be used to correlate the relative changes in toxicity of cultures. Another approach which seems worth while is the addition of radioactive nitrogen sources to cultures of different ages and the subsequent quantitative measurement of the radioactivity of purified toxin fractions after suitable incubation periods in the presence of the isotopes.

On the basis of the experimental evidence compiled in the investigation, an hypothesis describing the chain of events contributing to the final toxicity of culture filtrates of C. botulinum types A and B can be schematically represented as follows:

0-----> Period of active----->24 hr syntheses	-----> Period of greatly-----> 96 hr reduced syntheses
a. cell multiplication	a. little or no <u>de novo</u> protein synthesis
b. synthesis of "protoxin"	b. cell degeneration; autolysis of cells and liberation of toxin
c. accumulation of auto- lytic and proteolytic enzymes	c. activation of "protoxin"
d. no visible autolysis and little "protoxin" activation	

Admittedly, the burden of proof for the validity of this chain of events rests upon a great deal more future research. Yet it is

tempting to end this discussion by speculating still further on future development of knowledge of the bacterial toxins of Clostridial origin. Evidence is slowly accumulating which makes the following suggestion more than mere conjecture. It may be that some time in the future, all of the protein toxins elaborated by the Clostridium group will be found to be synthesized as toxin precursors which are then acted upon by still other metabolic products of the organisms thereby resulting in the biologically active molecules. Secondly, since the phenomenon of autolysis is so widespread among the toxigenic organisms in this group, and since toxigenicity has been associated with lysogenicity in the case of Corynebacterium diphtheriae, it is also conceivable that the toxin producing Clostridia will be found to be restricted to those organisms in the lysogenic state.

## SUMMARY

The conflicting evidence found in the literature concerning the metabolic events leading to the ultimate toxicity of cultures of C. botulinum types A and B prompted an investigation which sought, not only to reconcile some of these apparent contradictions, but also to establish on a physiological level, the mechanisms responsible for the toxigenicity of a number of type A and B strains of the organism. The investigation was restricted primarily to quantitative estimations of growth and toxin synthesis in relation to the autolytic processes of the organisms, the effect of certain physical and chemical environmental factors on these processes, and the possible function of the organisms' constitutive enzyme complement in converting postulated comparatively non-toxic "protoxins" into the active forms which possess such astonishing biological activity.

Using conventional turbidimetric methods for estimation of growth and autolysis, and mouse assay for quantitation of the toxin, the following results were obtained and conclusions reached:

1. The normal growth cycle of all strains of C. botulinum studied was found to be characterized by a period of active cell multiplication ( $\cong$  18 to 24 hr) immediately followed by active autolysis of the cells ( $\cong$  48 to 72 hr), which continued until such time as few or no viable organisms remained.
2. Autolysis was established as an important mechanism for the liberation of the toxin by the observations that the toxicity of culture filtrates was proportional, not to the



number of intact organisms, but rather to the degree of autolysis of the cultures after cell multiplication ceased; and that artificial disruption of cultures grown in a complete medium at the end of the logarithmic growth phase resulted in the liberation of large quantities of intracellular toxin ( $\approx$  10-fold increase in toxicity of the filtrates).

3. The inability to obtain non-toxic wash fluids from intact cells and the low level of toxicity observed in young culture filtrates were attributed to simple diffusion of the toxin. The rate of diffusion presumably gradually increases as the permeability barriers are broken down by the autolytic enzymes.

4. Glucose was found to be utilized, not primarily as an energy source but rather as a specific requirement for toxin synthesis. A number of carbohydrates, including glycerol, pyruvate, xylose and ribose were unable to substitute efficiently for glucose. It was concluded that glucose created the proper enzymatic balance for optimal toxin synthesis.

5. Within the limits which supported growth, the pH of the culture media did not affect the synthesis of botulinus toxin. The hydrogen ion concentration indirectly affected the toxicity of culture filtrates by virtue of the narrow pH optimum for the autolytic enzymes and the instability of the toxin in an alkaline environment.

6. The maximum temperature at which growth of the organism occurred was 48 C, at which temperature the toxin was gradually inactivated. Between 28 and 40 C, the growth, autolytic

and toxin synthesizing processes were supported optimally. At 10 C or below, metabolic activity was not detectable. Temperatures fluctuating between 10 and 18 C partially supported growth and toxin synthesis.

7. The tremendous increase in toxicity of the culture filtrates during the period of cellular degeneration could not be attributed to de novo synthesis of protein since this increase was apparent in the presence of chloramphenicol, a known inhibitor of protein synthesis. Furthermore, no increases in total protein could be demonstrated during the period of active autolysis.

8. The potential toxicity of cultures in the exponential growth phase was found to be much greater than their apparent toxicity. This was demonstrated by the 100-fold increase in toxicity after artificial disruption of the cells and treatment of the cell-extracts with proteolytic enzymes.

9. The activation of toxin by trypsin or pepsin could be accomplished only with young culture filtrates. It was concluded that the toxins of autolyzed cultures (72 to 96 hr) of C. botulinum types A and B had been activated by the proteolytic enzymes of the organisms and consequently could not be activated any further.

10. The activation phenomenon could not be demonstrated by other than enzymatic means. Heat-shock and sonic oscillation had no effect on the toxicity of the filtrates.

11. Versene which inhibited increases in toxicity of cell suspensions was also found to inhibit the proteolytic and

autolytic enzymes of the organism. Chloramphenicol inhibited any increases in total protein of cultures of C. botulinum.

On the basis of the experimental evidenced described above it was concluded that the biologically active toxins of C. botulinum types A and B are initially synthesized as large molecules which exhibit comparatively little toxicity. These toxin precursors must be partially degraded, probably by the proteolytic enzymes of the organisms, before manifesting their full toxic potentialities.

## APPENDIX

Two manuscripts, published in 1955 (Boroff; and Kindler et al.) expressed diametrically opposite viewpoints concerning the relative toxicity of washed intact cells of C. botulinum. The former concluded that the washed cell surface was as toxic as the solubilized toxin and the latter contended that washed cells were practically innocuous. This contradictory situation emphasized the fact that the site of toxin synthesis was not known or more specifically, if the synthesis of toxin was associated with a cell structure such as the cell wall or cytoplasmic membrane. Since toxin synthesis by cell free extracts of C. botulinum could not be demonstrated, it was considered that a suitable method for the preparation of protoplasts of C. botulinum would be valuable. A study of the toxin-synthesizing capabilities of protoplasts, which can be considered sub-cellular units, could give some insight as to the site of toxin synthesis, involvement of the cell wall and whether or not complete cellular integrity is necessary.

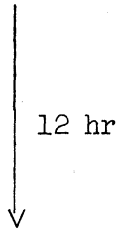
The classical methods for preparation of protoplasts of B. megaterium and E. coli were not successful with C. botulinum. Although the organism was sensitive to lysozyme in phosphate buffer, the cell wall was not stripped off completely. A method using lysozyme and Versene in Tris buffer (pH 8.0) yielded a very small number of protoplasts but conditions could not be defined to increase the yield or stabilize the structures. A method which took advantage of the spontaneous autolysis of C. botulinum was used with some success for the preparation of protoplasts.

Strain JTD-IV was grown in a complete medium for 12 hr at which time sterile sucrose was added so that the final concentration of the sugar was 0.5 M. The filtrate from a completely autolyzed culture of the organism was also added so that the rate of lysis would be enhanced. The mixture was reincubated at 35 C and examination of the cells was made under the phase contrast microscope periodically to determine if protoplasts were formed as the organisms autolyzed. This procedure yielded approximately 10% protoplasts in 12 hr and approximately 40% after 24 hr. That the structures formed were protoplasts was verified by the fact that they burst when suspended in a hypotonic solution. Incubation beyond 24 hr did not increase the yield of protoplasts. Attempts to separate the protoplasts from the vegetative cells by differential centrifugation were not unsuccessful.

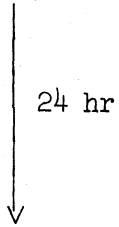
This method is not nearly as efficient as the methods described for the preparation of protoplasts of the aerobic spore forming organisms. In the latter case, over 90% protoplasts are formed in a matter of minutes. In the case of C. botulinum, it is very difficult to explain why only a part of the bacterial population should form protoplasts. It has been found that the age of the organisms is important and that young cells only are able to form the sub-cellular structures. Since the method described above requires incubation for over 24 hr, it may be that the age of the cells is the limiting factor in their formation. However, if a suitable technique can be devised to separate the protoplasts from the cells without destroying the former, this method conceivably could be used to study the synthesizing capabilities of a sub-cellular particle of C. botulinum.

The following diagram describes the method for the preparation of C. botulinum protoplasts.

1% inoculum of 18 hr culture of strain JTD-IV  
in 100 ml of the complete medium.



Add 100 ml of 1.0 M sterile sucrose solution,  
and 50 ml of lysate from a 96 hr culture of  
strain JTD-IV



40% protoplasts

## BIBLIOGRAPHY

1. Abrams, C., Kegeles, G., and Hottle, G. A. 1946, The purification of toxin from Cl. botulinum type A. Jour. Biol. Chem. 164: 63-79.
2. Ambache, N. 1949, The peripheral action of Cl. botulinum toxin. J. Physiol. 108: 127-141.
3. Boivin, A. 1940/41a Les deux toxines du bacille de Shiga et leur place dans la classification generale des toxines bacteriennes. Rev. d'Immunol. 6: 86-115.
4. Boroff, D. A. 1955, Study of toxins of Cl. botulinum. III. Relation of autolysis to toxin production. J. Bact. 70: 363-367.
5. Boroff, D. A., Raynaud, M. and Prevot, A. R. 1952, Studies of toxin of Cl. botulinum type D. J. Imm. 68: 503-511.
6. Burgen, A. S. V., Dickens, F. and Zatman, L. J. 1949. The action of botulinum toxin on the neuromuscular junction. J. Physiol. 109: 10-24.
7. Church, B. and Halvorson, H. 1955, Glucose metabolism by resting spores of aerobic bacilli. Bact. Proc. p. 41.
8. Cohen P., Halbert, S. P. and Perkins, M. E. 1942, Pneumococcal hemolysin. The preparation of concentrates and their action on red cells. J. Bact. 43: 607-627.
9. Dack, G. M. 1926, The influence of some anaerobic species on the toxin of Cl. botulinum with special reference to Cl. sporogenes. J. Inf. Dis. 38: 165-173.
10. Dack, G. M. and Wagenaar, R. O. 1956 Effect of irradiation of Cl. botulinum toxin subjected to ultracentrifugation. Unpublished.
11. Dack, G. M., Wood, W. and Dehler, S. 1928. Toxin production and proteolytic activity of Clostridium botulinum in peptone and peptone egg white medium. J. Inf. Dis. 42: 176-178.
12. Davies, J. R., Morgan, R. S., Wright, E. A. and Wright, G. Payling 1953. The results of direct injections of botulinum toxin into the central nervous system of rabbits. J. Physio. 120: 618-623.
13. Dozier, C. C. 1924, Optimum and limiting hydrogen ion concentration for Bacillus botulinus and quantitative estimation of its growth. J. Inf. Dis. 35: 105-133.

14. Dozier, C. C. 1924, Inhibitive influence of sugars and salt on the viability and growth and toxin production of Bacillus botulinum XVII. J. Inf. Dis. 35: 134.
15. Duff, J. T. 1956, Personal communication.
16. Duff, J. T., Wright, G. G., and Yarinsky, A. 1956 Activation of Clostridium botulinum type E toxin by trypsin. J. Bact. 72: 455-460.
17. Elberg, S. and Meyer, K. F. 1939. The extracellular proteolytic system of Cl. parobotulinum. J. Bact. 37:541.
18. Gale, E. F. and Folkes, J. P. 1953, Assimilation of amino acid by bacteria. 15. Actions of antibiotics on nucleic acid and protein synthesis in Staph. aureus. Biochem. J. 53: 493.
19. Greenberg, R. A. and Halvorson, H. O. 1954 Studies on an autolytic substance produced by an aerobic spore-forming bacterium. J. Bact. 69: 45.
20. Gunnison, J. B. and Meyer, K. F. 1929 The occurrence of non-toxic strains of Cl. parobotulinum. J. Inf. Dis. 45: 79.
21. Gunnison, J. B. and Meyer, K. F. 1929 Botulism due to home-canned pears. J. Inf. Dis. 45: 135-147.
22. Hampson, C. R. 1951 A case of probable botulism due to wound infection. J. Bact. 61: 647.
23. Hitzman, D. Zoha, S. M. S., and Halvorson, H. O. 1955 The requirements for germination of spores of some anaerobic bacteria. Bact. Proc. p. 40.
24. Johnson, M. J. 1941 Isolation and properties of a pure yeast polypeptidase. J. Biol. Chem. 137: 575.
25. Kaplan, J. G. 1955 The alteration of intracellular enzymes. I. Yeast catalase and the Euler effect. Exptl. Cell Res. 8: 305.
26. Kaplan, J. G. 1955 The alteration of intracellular enzymes. II. The relation between the surface and the biological activities of altering agents. J. Gen: Physiol. 38: 197.
27. Kegeles, G. 1946 The molecular size and shape of botulinum toxin. J. Amer. Chem. Soc. 68: 1670.
28. Kindler, S. H., Mager, J. and Grossowicz, N. 1956 Toxin production by Cl. parobotulinum. J. Gen. Microbiol. 15: 394.
29. Lamanna, C. and Glassman, H. N. 1947 The isolation of type B botulinum toxin. J. Bact. 54: 575-584.



30. Lamanna, C., McElroy, O. E. and Eklund, H. W. 1946 The purification of toxin and crystallization of Cl. botulinum type A. *Science* 103: 613.
31. Landman, O. E. and Spiegelman, S. 1955 Enzyme formation in protoplasts of B. megaterium. *Proc. Nat'l Acad. Sci.* 41: 698-704.
32. Lewis, K. H. and Hill, E. V. 1947 Practical media and control for producing highly toxic cultures of Cl. botulinum type A. *Bact.* 53: 213-230.
33. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951 Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265.
34. Mager, J., Kindler, S. H., and Grossowicz, N. 1954 Nutritional studies with Cl. parobotulinum type A. *J. Gen. Microbiol.* 10: 130.
35. Maschman, E. 1939 Zurkenntnis der Aminopeptidase. *Naturwissenschaften* 27: 819-820.
36. Miles, 1955 Mechanisms of Microbial Pathogenicity Univ. Press, Cambridge, England.
37. Morgan, W. T. J. and Partridge, S. M. 1942 An examination of the O antigenic complex of Bact. tyhosum. *Brit. J. Exp. Path.* 23: 151-165.
38. National Canners Association Bulletin. Preparation of spore suspensions, N.C.A. method. *Nat'l. Canners Assoc. Washington, D.C.*
39. Nelson, C. I. 1927 The relationship between the intracellular globulin and the toxin of Cl. botulinum. *J. Inf. Dis.* 41: 9-12.
40. Norris, J. R. 1957 A bacteriolytic principle associated with cultures of B. cereus. *J. Gen Micro.* 16:1.
41. Northrup, J. H., Kunitz, M. and Herriott, R. N. 1948 Crystalline Enzymes. 2nd ed. Burgess Publishing Co. Minneapolis, Minn.
42. Nungester, W. J. 1956 Personal Communication.
43. Orr, P. F. 1922 The pathogenicity of Bacillus botulinus. *J. Inf. Dis.* 35: 134.
44. Pappenheimer, A. M. 1937 Diphtheria toxin. I. Isolation and characterization of a toxic protein from Corynebacterium diphtheriae filtrate. *J. Biol. Chem.* 120: 543-553.
45. Pappenheimer, A. M. 1948 Proteins of pathogenic bacteria. *Adv. Prot. Chem.* 4: 132-153.

46. Park, J. T. and Strominger, J. L. 1957, Mode of action of penicillin. Science, 125: 99-101.
47. Pillemer, L., Wittler, R. G. and Grossberg, D. B. 1946 The isolation and crystallization of tetanal toxin. Science 103: 615-616.
48. Putnam, F. W., Lamanna, C. and Sharp, D. G. 1946 Molecular weight and homogeneity of crystalline botulinus A toxin. J. Biol. Chem. 165: 63 735-736.
49. Raynaud, M. and Second, L. 1949 Extraction des toxines botuliniques a partir des corps microbiens. Ann. Inst. Past. 77: 316-321.
50. Raynaud, M., Turpin, A., Mangalo, R. and Bizini, R. 1954 Croissance et toxigenise. Ann. Inst. Past. 87: 599.
51. Raynaud, M. 1954 Croissance et toxigenise. Ann. Inst. Past. 88: 24.
52. Rosebaum, M. 1955 The Role of Protein Synthesis in the Early Stages of Bacterial Virus Infection. PhD Thesis, University of Michigan, Ann Arbor, Michigan.
53. Ross, E. H., Warren, E. M. and Barnes, J. M. 1949 Clostridium welchii iota toxin: its activation by trypsin. J. Gen. Microbiol. 3: 148.
54. Roux, E. and Yersin, A. 1888 Contribution a 'etude de la diphterie. Ann. Inst. Past. 2: 629.
55. Seki, T., Takaki, M., Hirabayashi, H. and Yamaguchi, H. 1954 Toxin production by autolysis of Cl. tetani. Med. Jour. of Osaka Univ. 5: 271-289.
56. Smith, H. and Keppie, J. 1955 Mechanisms of Microbial Pathogenicity. Pathogenicity of Bacillus anthracis. Univ. Press, Cambridge, England.
57. Sommer, H. 1937 Purification of botulinus toxin. Proc. Soc. Exp. Biol. and Med. 35: 520.
58. Stone, I. L. 1954 On the mode of release of tetanus toxin from the bacterial cell. J. Bact. 67: 110.
59. Strange, R. E. and Dark, F. A. 1957 A cell wall lytic enzyme associated with spores of Bacillus species. J. Gen. Microbiol. 16: 236-249.
60. Townsend, C. T., Yee, L. and Mercer, W. A. 1954 Inhibition of the growth of Cl. botulinum by acidification. Food Res. 19: 536.

61. Turner, A. W. and Rodwell, A. W. 1943a The epsilon toxin of Cl. welchii type D. 1. Proteolytic conversion of epsilon protoxin into epsilon toxin by trypsin and other proteosases. Australian J. Exp. Biol. and Med. Sci. 21: 17-25.
62. Turner, A. W. 1943b The epsilon toxin of Cl. welchii type D. 2. Mechanism of its development in cultures through the action of extracellular proteinases upon epsilon protoxin. Australian J. Exp. Biol. and Med. Sci. 21: 27-36.
63. Umbreit, W. W., Burris, R. H. and Stauffer, J. F. 1949 Manometric Techniques and Tissue Metabolism. 2nd ed. Burgess Publishing Co. Minneapolis, Minn.
64. van Ermengem, E 1896 Recherches sur des cas d'accidents alimentaires. Rev. Hyg. Pol. Sanit. 18: 761.
65. van Heyningen, W. E. 1950 Bacterial toxins. 1st ed. Chas. C. Thomas, Publisher, Springfield, Ill.
66. von Behring, E. and Kitasato, S. 1890. Uber das Zustandekommen Diphtherieimmunitat und die Tetanusimmunitat bei Tieren Dtsch. Med. Wschr. 16: 1113.
67. Wadsworth, A. B. 1947 Standard Methods of the Division of Laboratories and Research of the New York State Department of Health. 3rd. ed. The Williams and Wilkins Company, Baltimore, Maryland.
68. Wagman, J. and Bateman, J. B. 1953 Botulinum type A toxin; properties of a toxin dissociation product. Arch. Biochem. and Biophys. 45: 375.
69. Waksman, S. 1941 Antagonistic relations of micro-organisms. Bact. Revs. 5: 231-291.
70. Wentzel, L. M., Sterne, M. and Polson, A. 1950 High toxicity of pure botulinum type D toxin. Nature, 166: 739-40.
71. Wisseman, C. L., Smadel, J. E., Hahn, F. E., and Hopps, H. 1954 Mode of action of chloramphenicol. I. Action of chloramphenicol on assimilation of ammonia and on synthesis of proteins and nucleic acids in Escherichia coli. J. Bact. 67: 662.
72. Wright, G. Payling, 1955 Mechanisms of Microbial Pathogenicity. Botulinum and tetanus toxins. Univ. Press, Cambridge, England.

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