Growth and toxin production of Clostridium botulinum type F
by Lillian Haldeman Holdeman

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Bacteriology
Montana State University
© Copyright by Lillian Haldeman Holdeman (1964)

Abstract:
The characteristics of the growth and toxin production of Clostridium botulinum type F have been studied. Arginine, leucine or isoleucine, tryptophan, tyrosine, valine, biotin, and thiamin were found to be essential nutrients, Growth was stimulated by glycine, phenylalanine, methionine, and para-aminobenzoic acid. Five different colony types were separates, The mechanism by which toxin is produced was studied, using cultures of the different colony types.

It was shown that toxin was produced intracellularly A toxin precursor which could be activated by trypsin was present in some cultures.

The precursor was not found in all cultures tested, but was repeatedly demonstrated in cultures with certain colonial characteristics. Like the toxin, the precursor was produced intracellularly. However, precursor also could be found in supernatant fluid from some cultures. The mechanism of toxin production was discussed.

The most highly toxic supernatant fluids were obtained from cultures of a mosaic colony type grown in peptone-glucose medium at 30 C for three days. Such supernatant fluids contained 30,000 to 60,000 mouse IP LD50 doses per ml. The toxicities of synthetic medium cultures usually were about one-tenth those of complex medium cultures.

C. botulinum type F appears to be closely related to the other types of C. botulinum, differing in that its geographical distribution seems to be restricted to a very small area.

GROWTH AND TOXIN PRODUCTION OF CLOSTRIDIUM BOTULINUM TYPE F

by

LILLIAN HALDEMAN HOLDEMAN

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Bacteriology

Approved:

Head, Major Department

Chairman, Examining Committee

Dean, Graduate Division

MONTANA STATE COLLEGE
Bozeman, Montana

June, 1964

ACKNOWLEDGMENTS

The completion of the requirements for this degree was made possible, in large part, by funds made available by Public Law 85-507 and by the Communicable Disease Center. The author wishes to express her appreciation to all those at the Communicable Disease Center who provided facilities, services, and above all, time for the research study. In particular, the author wishes to thank Mrs. Catherine J. Phillips for her competent technical assistance, interest, and encouragement; Mr. Jack Gust for the excellent photographs; and Mrs. Elizabeth Paz for secreterial assistance.

The author feels particularly indebted to Dr. Louis DS. Smith for his advice, encouragement, and guidance. Especially appreciated is the patience he demonstrated in letting her create and resolve her own problems.

And last, but not least, the author wishes to acknowledge the understanding and thoughtful attitude of family and friends.

TABLE OF CONTENTS

List of tables vi

List of figures viii

Abstract ix

Introduction 1

Historical 2

Nutritional requirements 6

Physical and chemical characteristics of the toxin 7

Mechanism of toxin production 11

Cultural studies 18

Procedures and results 21

General procedures 22

Use of the Brewer anaerobic culture jar 22

Media 24

Temperature of incubation 25

Assay of toxin 25

Toxin neutralization tests 25

Confirmation of biochemical and toxic characteristics of the culture to be used 26

Determination of cultural and biochemical properties 31

Determination of optimum pH for obtaining growth from small inocula 34

Effect of different amounts of casitone on toxicity and toxin stability 35

Effect of type of peptone on toxicity 38

Effect of medium constituents, other than peptone, on toxicity 39

Growth of Clostridium botulinum type F in synthetic medium 41

Glassware 44

Growth in synthetic medium 44

Evaluation of technique 46

Nutritional requirements of Clostridium botulinum type F 48

Amino acid requirements 50

Vitamin requirements 69

Growth in a basal synthetic medium 78

Effect of temperature of incubation on toxicity of supernatant fluids 83

Growth of Clostridium botulinum type F in synthetic medium at different temperatures of incubation 89

Site of toxin production 92

Colony variations 100

Demonstration of prototoxin 125

Discussion 143

Summary 153

Appendix 156

Literature cited 165

LIST OF TABLES

1. Cultural and biochemical properties of C. botulinum type F - no. 16 29

2. Toxic properties of C. botulinum type F 30

3. Effect of different concentrations of casitone on toxicity of three-day culture supernates and on toxin stability at various storage temperatures 37

4. Effect of different peptones on toxicity of three-day culture supernates 38

5. Effect of medium constituents, other than peptone, on toxicity of three-day culture supernates 40

6. Composition of chemically defined medium 42

7. Stock solutions used in the preparation of the chemically defined medium 43

8. Effect of various test conditions on obtaining growth from small inocula in synthetic medium 47

9. Growth of C. botulinum type F in amino acid test media 55-66

10. Growth of C. botulinum type F in methionine-deficient medium after "back-passage" through complete medium. 67-68

11. Growth of C. botulinum type F in vitamin test media. 70-77

12. Growth of C. botulinum type F in a basal synthetic medium . . . 79-80

13. Growth of C. botulinum type F in basal synthetic medium with added amino acids 81

14. Growth of C. botulinum type F in basal synthetic medium with added vitamins 82

15. Toxicity of supernatant fluids from cultures incubated at different temperatures and sampled at different times after inoculation 84

16. Effect of sonic treatment on toxicity of synthetic medium cultures incubated at 30 and 37 C 88

17. Toxicity of a 17-hour culture of C. botulinum type F 96

18. Toxicity of a 22-hour culture of *C. botulinum* type F 98

19. Toxicity of TOX and TP phase cultures 109

20. Characteristics of *C. botulinum* type F colony types 114

21. Toxicities of culture supernatant fluids from different colony types 115

22. Effect of 1% crude trypsin on toxicity of three day culture supernates 126

23. Toxicity of cultures of *C. botulinum* type F after treatment with sonic vibration and with trypsin 130

24. Release of toxin and toxin precursor from synthetic medium culture OGB-34 by treatment with sonic vibration . . 132

25. Demonstration of endocellular toxin and precursor in synthetic medium culture of OPY-26 133

26. Ability of various enzyme preparations to activate *C. botulinum* type F toxin precursor 135

27. Toxicity of TOX and TP cultures of *C. botulinum* type F after treatment with sonic vibration 137

28. Gelatinase activity of supernatant fluids from 24-hour synthetic medium cultures 140

29. Toxicity and gelatinase activity of type F cultures 141

LIST OF FIGURES

I. Growth of *C. botulinum* type F at different temperatures 86

II. Growth of *C. botulinum* type F at 30 C 91

III. Growth of *C. botulinum* type F 94

IV. Growth of *C. botulinum* type F. Top - spreading edge of TP growth. Bottom - TOX colonies 116

V. Type F colony types. TOX (mosaic) and TP phases 117

VI. TP phase microcolonies of *C. botulinum* type F. 118

VII. TP phase growth of *C. botulinum* type F. Top - microcolonies. Bottom - heavy part of streak 119

VIII. TOX phase microcolonies of *C. botulinum* type F 120

IX. TOX phase microcolonies of *C. botulinum* type F 121

X. TOX phase microcolonies of *C. botulinum* type F 122

XI. Granular colony types. Top - opaque, patterned colony. Bottom - opaque, granular colonies 123

XII. Colony types of *C. botulinum* type F 124

Abstract

The characteristics of the growth and toxin production of *Clostridium botulinum* type F have been studied. Arginine, leucine or isoleucine, tryptophan, tyrosine, valine, biotin, and thiamin were found to be essential nutrients. Growth was stimulated by glycine, phenylalanine, methionine, and para-aminobenzoic acid. Five different colony types were separated. The mechanism by which toxin is produced was studied, using cultures of the different colony types.

It was shown that toxin was produced intracellularly. A toxin precursor which could be activated by trypsin was present in some cultures. The precursor was not found in all cultures tested, but was repeatedly demonstrated in cultures with certain colonial characteristics. Like the toxin, the precursor was produced intracellularly. However, precursor also could be found in supernatant fluid from some cultures. The mechanism of toxin production was discussed.

The most highly toxic supernatant fluids were obtained from cultures of a mosaic colony type grown in peptone-glucose medium at 30 C for three days. Such supernatant fluids contained 30,000 to 60,000 mouse IP LD_{50} doses per ml. The toxicities of synthetic medium cultures usually were about one-tenth those of complex medium cultures.

C. botulinum type F appears to be closely related to the other types of *C. botulinum*, differing in that its geographical distribution seems to be restricted to a very small area.

INTRODUCTION

Historical 2

Nutritional requirements 6

Physical and chemical characteristics of the toxin 7

Mechanism of toxin production 11

Cultural studies 18

"Let us hope that intellectual challenge alone will bring support for activities that will lead to increased understanding of the botulinal toxins." Lamanna, 1959.

HISTORICAL

The concept that bacterial toxins, separated from parent organisms, could produce harmful effects in living tissues was confirmed near the end of the nineteenth century. Loeffler, who discovered the diphtheria bacillus in 1884, observed that diphtheria organisms could not be isolated from organs that apparently were affected by the disease and concluded that a "poison produced at the seat of inoculation must have circulated in the blood". In 1888 Roux and Yersin demonstrated that sterile filtrates from cultures of the diphtheria bacillus would kill guinea pigs and that the lesions were identical with those observed after inoculation of living organisms. They also concluded that the poison was a kind of enzyme.

Two years later (1890) von Behring and Kitasato described tetanus toxin and the phenomena which later were called active and passive immunity. Almost simultaneously von Behring announced that the immune response also could be demonstrated with diphtheria toxin.

The most potent of the biological toxins, that of Clostridium botulinum, was identified soon thereafter, in 1896, by van Ermengem. After a meeting of a music club in Ellezelles, Belgium, three persons died and 23 became ill. All had eaten a portion of raw ham. The ham was softer and paler than normal and had a rancid odor. From the ham and

from the intestine and spleen of one victim von Ermengem isolated an anaerobic organism which produced an exceedingly potent toxin.

During the following years a number of outbreaks of botulism were investigated. Leuchs (1910) produced antitoxins in horses against two strains of C. botulinum, the Ellezelles strain and the Darmstadt strain. The Darmstadt culture had been isolated by Landmann in 1904 from bean salad implicated in an outbreak of botulism. On the basis of antitoxin protection tests, Leuchs demonstrated the existence of two toxigenic types. Burke (1919) separated the twelve strains with which she was working into two types, which she designated A and B. By this time the Ellezelles and Darmstadt cultures had been lost, but it is generally believed (Meyer and Gunnison, 1929) that the Ellezelles strain was a non-proteolytic type B and the Darmstadt strain a type A.

Within the next decade Bengston (1922a, 1923), Seddon (1922) and Theiler et al. (1927) described three additional possible types. The Bengston strain, isolated from fly larvae implicated in a paralytic disease of chickens, was later designated type C_a. The Seddon strain, isolated from a beef carcass, produced a toxin immunologically similar to the Bengston strain and has been designated C_b. The strain which Theiler et al. isolated from animal carcasses produced another immunologically distinct toxin and was called type D. Type E was described by Gunnison, Cummings, and Meyer (1936) on the basis of studies of two strains isolated from canned fish in Russia.

Møller and Scheibel (1960) reported an outbreak of human botulism in 1958 on the Danish island of Langeland. Of the five persons who consumed

the incriminated liver paste, one died, three developed severe symptoms, and one was unaffected. The organism isolated from the liver paste was similar to C. botulinum in saccharolytic and proteolytic properties, but the toxin was not neutralized by antitoxin to types A, B, C, or D. The toxin was neutralized by excessive amounts of type E antitoxin, but apparently was not identical with type E, for it was not activated by trypsin. The culture was more extensively studied by Dolman and Murakami (1961) and designated type F.

C. botulinum generally is associated with food poisoning. Nevertheless, there are three reports of possible botulism due to wound infections: Hampson, 1951; Davis, Mattman, and Wiley, 1951; and Thomas, Keleher, and McKee, 1951. However, the presence of C. botulinum in wounds does not always lead to the development of botulism, for Hall (1945) found this organism in wounds of patients in which no symptoms compatible with botulism developed.

Botulism caused by types A and B has been reported in man, mink, and birds. Type C botulism has been reported in ruminants, birds, and mink. Type C is responsible for sporadic epidemics of alkali disease, or western duck disease, in wild ducks. Type D botulism is found primarily in ruminants. The disease in ruminants, known as "lamsiekte" or "loin disease", is associated with a diet deficient in phosphorus and the resultant ingestion of toxic carrion. Raw or improperly preserved fish or marine mammals have almost always been the vehicles for type E toxin. Although botulism caused by type E has been confined almost exclusively to man,

Skulberg (1961) has reported an outbreak of type E botulism in mink.

The early work with C. botulinum was concerned primarily with the investigation of outbreaks and the characterization of growth and toxin production as related to conditions that might produce toxic foods. According to Meyer (1956) "the spectacular nature and high case-fatality rate in the USA gave botulism a place out of proportion to its frequency as a cause of death". The development of the commercial canning industry and the legal and economic consequences of toxic foods stimulated research in better methods of preservation.

Interest in botulism declined between 1930 and 1946. Sporadic outbreaks occurred, but were primarily caused by improperly home-preserved foods. Since 1946, interest in the increasing occurrence (or recognition) of botulism caused by type E, the isolation and purification of crystalline toxin, and the possible use of botulism toxin in biological warfare have directed trends in investigative inquiry.

For the sake of historical continuity and in deference to readers from other disciplines, perhaps some time and space should be devoted to terminology as related to toxins. The toxins of Clostridium botulinum, Clostridium tetani, and Corynebacterium diphtheriae were first called "exotoxins", probably because they were observed in cell-free filtrates. The assumption followed that the toxins were "secreted into the medium", a process that might involve some mechanism, probably energy requiring, that would enable these high molecular weight compounds to pass through the cell wall. Also, such molecules would be expected to be at maximum

concentration in the surrounding fluid at the end of the logarithmic phase of growth.

Even the very early investigators recognized that "aged" cultures were the most toxic and correlated cell autolysis with toxic activity. However, the idea that toxins of the gram-positive organisms are "extracellular and secreted" during their growth became well entrenched and, in spite of numerous publications to the contrary, is still perpetuated in the literature, especially in reviews and textbooks. In other instances it is difficult to ascertain whether the apparent misuse of the term "exotoxin" is due to definition or theory. "Toxin production" is a misleading term in itself; nevertheless, the term has to be used. In this paper we will refer to toxin production as the final result of the numerous steps required to convert nutrient material to toxic substances. "Intracellular" and "extracellular" as used in this paper will refer to sites of various activities with respect to the intact cell wall.

NUTRITIONAL REQUIREMENTS

Interest in the metabolism of the organism and the search for a medium in which potent toxins could be produced with a minimum of non-essential protein material stimulated investigations of the nutritional requirements of C. botulinum. Wagner, Meyer, and Dozier (1925) attempted without success to grow C. botulinum in media composed largely of pure amino acids. The subsequent reports of Burrows (1933), Fildes (1935) and Elberg and Meyer (1939a) did not clearly define all the factors necessary for growth. The elusive "sporogenes vitamin" of Knight and Fildes (1933),

necessary for growth of C. botulinum as well as for C. sporogenes, was not a single chemical entity. It could be replaced by a mixture of several vitamins of the B group (Shull and Peterson, 1948; Lamanna and Lewis, 1946; Roessler and Brewer, 1946).

Since the observations of Roessler and Brewer (1946) were not entirely in agreement with those of Lamanna and Lewis (1946), the nutritional requirements of C. botulinum type A were reexamined by Mager, Kindler, and Grossowicz (1954). These authors later reported on a similar investigation of C. sporogenes and C. botulinum types A through E (Kindler, Mager, and Grossowicz, 1956a). They found that these organisms could be classed in two nutritional groups. C. sporogenes and proteolytic C. botulinum types A and B gave rapid and heavy growth in a chemically defined medium with some variations in vitamin requirements. C. botulinum types C, D, and two strains of E failed to grow in the defined medium or in a partially defined medium containing acid hydrolysate of casein supplemented with all known B vitamins. Three type E strains grew in the partially defined medium. These results obtained with C. botulinum types A and B and with C. sporogenes were confirmed by those of Campbell and Frank (1956).

PHYSICAL AND CHEMICAL CHARACTERISTICS OF THE TOXIN

Type A toxin was purified and crystallized in 1946 independently by two groups (Lamanna, McElroy, and Eklund; Abrams, Kegeles, and Hottle) based on the observation by Sommer (1937) that partial purification could be accomplished by acid precipitation and subsequent dialysis. This was the first bacterial toxin to be isolated in crystalline form. The

crystalline toxin - a white, odorless protein - contained 2.4×10^8 mouse LD_{50} per milligram nitrogen (Lamanna et al., 1946) and had a molecular weight of about 1,000,000. Electrophoretic analysis showed a single component. Abrams et al. (1946) reported that the globulin had an isoelectric point of pH 5.6 and a nitrogen content of 14.1 per cent.

The elemental amino acid composition of crystalline type A toxin was studied by Buehler, Schantz, and Lamanna (1947). Nineteen amino acids were found. The phosphorus content of different lots was variable. No nucleic acids were detected. From their results they could offer no explanation for the unusual toxicity of the substance. The molecular weight was 900,000. From the data on the analysis of cysteine and cystine content, they estimated a minimum molecular weight of 45,000. Kegeles (1946) also studied the physical characteristics of the toxins. These data showed the toxin to be an ordinary globulin-like protein containing no "prosthetic" groups or any other special characteristics which might account for its astonishing biological activity.

The other botulinum toxins, except that of type F, have now been partially purified, but none crystallized. Type B has been partially purified by Lamanna and Glassman (1947) and Duff et al. (1957); type C by Boroff, Raynard, and Prevot (1952) and Cardella et al. (1958); type D by Wentzel, Sterne, and Polson (1950) and Cardella et al. (1960), and type E by Gordon et al. (1957) and Gerwing, Dolman, and Arnott (1961, 1962).

Sterne and Wentzel (1950) described a method for producing "hightitered toxin" by using intussuscepted cellophane tubing. They found

that the protein nitrogen per mouse MLD of toxin produced by this method was about 1% of that obtained in the best crude extracts. Wentzel et al. (1950) obtained an extremely potent type D toxin with the cellophane bag method. This toxin had a molecular weight of 1,000,000 and 4×10^{12} mouse MLD per milligram nitrogen. This high toxicity for type D (about 20,000 times as toxic as crystalline type A) has been reported only once and, according to Lamanna (1959), has not been encountered on independent investigations. If these estimates of Wentzel et al. (1950) of the molecular weight and minimal lethal dose were correct, approximately 1000 molecules would cause fatal intoxication of a mouse (Wright, 1955).

Even though the type A toxin has been crystallized, there is still some question as to the homogeneity of the substance. Several characteristics of the crystalline toxin suggest that it is homogenous. Electrophoretic analysis showed a single component (Abrams et al., 1946; Lamanna et al., 1946; Putnam, Lamanna, and Sharp, 1948). Recrystallization did not result in decreased potency (Abrams et al., 1946). Crystalline toxins prepared by several different methods possessed identical serological properties (Putnam et al., 1948).

There also are data to suggest the heterogeneity of the product. Putnam et al. (1948), reporting on ultracentrifugation experiments, noted that in the isoelectric region the single sedimenting boundary spread at a more rapid rate than could be accounted for solely by diffusion. They suggested that this may indicate heterogeneity that can be attributed to the lability of the protein.

Wagman and Bateman (1951) found that crystalline type A toxin dissociated on ultracentrifugation at pH 7.5. The majority of the toxin formed a rapidly sedimenting boundary while about 14-20% moved more slowly. This slowly sedimenting component was studied further (Wagman and Bateman, 1953; Wagman, 1954). At ionic strength 0.13 and above, slowly sedimenting particles of molecular weight 40,000 to 100,000 were split off. At pH 7.5 in ionic strength 1.0 the faster sedimenting particles were precipitated almost intact and had a specific toxicity two to three times that of the original sample. The hypothetical structure suggested consists of 12 loosely bound segments, one-third of them being toxic. At higher ionic strengths the heavier fragments and inert materials precipitate out and the solution contains predominately toxic fragments. At an acid pH, i.e., 3.8, the inert portions act as a "cement", permitting reaggregation. It is further postulated that the hemagglutins are small toxic units loosely attached to the parent molecule, but not to the smaller fragments. Raynaud, Turpin, and Bizzini (1960) have presented evidence suggesting that tetanus toxin also exists in various states of aggregation and that depolymerization can be effected by rupture of disulfide bonds.

Cartwright and Lauffer (1958) studied the thermal inactivation of crystalline toxin and found the inactivation curves characteristic of a two component system. One component, constituting about 5% of the toxic preparation, was inactivated slowly. The other 95% was inactivated rapidly. Neither component was the hemagglutin. Also, sedimentation diagrams taken before and after heating were interpreted to suggest that the toxin might be dissociating to form two particles with different properties.

The kinetics of detoxification were examined by Wright et al. (1960). The formalin detoxification was carried out at pH 5.5, since Hottle, Nigg and Lichty (1947) had determined this to be the optimum pH for obtaining toxoids with the highest antigenicity. Wright et al. (1960) report that detoxification of types A and B deviated markedly from a first order reaction. They suggested that the slower detoxification reaction represented the inactivation of a second, more stable toxin, which was responsible for only a small portion of the toxicity of the original toxic material.

It can be seen, then, that the homogeneity of the crystalline toxin and the size of the toxic entity have not been definitely determined. Perhaps when these have been resolved we will be better able to understand the mechanism by which toxin passes from the alimentary tract into the circulatory system.

MECHANISM OF TOXIN PRODUCTION

Since toxins first were recognized in extracellular material, it was natural that they were regarded as possible cellular secretions or metabolic byproducts. Wagner et al. (1925) were among the first to investigate the mechanism of toxin production. They suggested that toxin might be formed by one of three processes: (1) excretion from the cell, (2) formation of some abnormal product in the presence of certain nutrients, or (3) release through autolysis.

The concept that toxin was formed in the presence of certain nutrients was based on the "potentiation phenomenon": when bacteria-free toxic filtrates were mixed with certain substances such as normal serum or milk the

toxicity was sometimes increased as much as 30-fold. The phenomenon has been reported for botulinum toxin by Bronfenbrenner (1924); Jensen (1926); Stark, Sherman, and Stark (1928a, b); and for tetanus toxin by Traub, Hollander, and Friedemann (1946). Bronfenbrenner (1924) thought that the serum acted as a "sparing" agent, preventing spontaneous deterioration of the toxin. Stark et al. (1928a, b) believed that the increase in toxicity was caused by extracellular production of toxin by C. botulinum enzymes acting on certain protein substrates. Traub et al. (1946) felt that a true potentiation, not sparing action, was involved, but suggested no mechanism. This phenomenon has never been satisfactorily explained.

Dozier (1924a, b) was among the first to correlate cell autolysis and the appearance of toxin in the surrounding medium. Subsequent investigators suggested that toxin was produced intracellularly during growth of the organism, being released by cell disintegration, and that toxin production was intimately associated with the enzymatic activity of the culture (Wagner et al., 1925; Nelson, 1927; Dack and Wood, 1928; Stevenson, Helson, and Reed, 1947). However, Elberg and Meyer (1939b) were unsuccessful in their attempts to correlate extracellular proteolytic enzyme production with toxin production.

The intracellular nature of toxin production has been demonstrated by using hypertonic salt solutions to extract toxin from young, washed cells (Raynaud, 1949; Raynaud and Second, 1949; Boroff et al., 1952; Raynaud et al., 1955). Raynaud and Second (1949) reported that the amount of toxin which could be extracted varied inversely with the age of the culture. In a quantitative study of intracellular and extracellular toxin in relation

to growth, Raynaud et al. (1955) determined that the intracellular concentration of toxin was much higher during the exponential phase of growth. The presence of some extracellular toxin during logarithmic growth was attributed to diffusion through cell walls.

The relation between cell wall disintegration and the appearance of toxin in the surrounding environment has been studied by Boroff (1955); Kindler, Mager and Grossowicz (1955, 1956b); Bonventre and Kempe (1960a, b); Prévot and Sillioc (1962). Boroff (1955) studied spontaneously lysing cultures and found that the increase in toxicity of the supernatant fluid was proportional to the increase in amounts of protein nitrogen in the culture supernate. Furthermore, the addition of a ten-day culture filtrate to a young culture increased the rate of autolysis, which was accompanied by an increase in toxin in the supernatant fluid.

Proteolytic enzymes produced by other bacteria also are able to attack cell walls and release toxin, as reported by Prévot and Sillioc (1962). They observed that toxin could be released from sedimented cells of C. botulinum types A, B, C, and E; C. sordelli; and C. tetani by a culture filtrate of B. licheniformis. Similar results were not obtained with trypsin.

Kindler et al.(1955, 1956b); and Bonventre and Kempe (1960a) artificially disintegrated intact cells by treatment with sonic vibrations to determine relative amounts of intracellular and extracellular toxin. They agreed that toxin appeared within the cell before it could be demonstrated in the surrounding environment. Bonventre and Kempe, like Raynaud, attributed the low toxicity of young culture filtrates to simple diffusion,

since they were unable to obtain non-toxic wash fluids from intact cells.

Both groups of workers observed that the total amount of toxin (intracellular and extracellular) which was apparent at the end of the exponential growth phase was less than that detected in older cultures, although they did not agree as to the mechanism by which this increase in toxicity occurred.

Kindler et al. (1955, 1956b) employed a technique using non-proliferating "resting cells". Actively growing cells were washed and resuspended to a concentration three to four times that of the original culture in a nutrient medium containing penicillin. Omission from the medium of glucose, phosphates, or a single amino acid necessary for growth resulted in a drastic reduction in toxin yield. They interpreted their results to mean that most of the toxin is produced in the phase of decline instead of in the logarithmic phase of growth. It should be emphasized, however, that their results demonstrate that toxin synthesis can take place in the absence of cell multiplication, not that it can take place in the absence of growth. There is considerable evidence that penicillin can inhibit cell wall formation and cell division while allowing protein synthesis to continue.

Bonventre and Kempe (1960a, b), studying cultures maintained in a normal environment, observed that the potential toxicities of cultures were much greater than the apparent toxicities as determined by artificial disintegration of cells at the end of the exponential phase of growth. They previously had observed that toxicity of young culture filtrates could be enhanced by treatment with trypsin (Bonventre and Kempe, 1959a). No

increase in toxicity was noted, however, when filtrates from older cultures were treated with trypsin. They suggested (1960b) that the toxin might be formed during active growth as a precursor which normally was spontaneously activated by cellular enzymes. Inukai (1963) also demonstrated that a protoxin was present in young cell extracts of C. botulinum type A. He showed that protoxin could be activated by a factor found in culture filtrates and that substances which inhibited the proteolytic activity of the culture filtrates reduced their activating capacity.

The concept of a toxin precursor was not entirely new. The presence of toxin units of various molecular weights in crystalline type A toxin had been taken to mean that a large precursor molecule might be composed of smaller toxic units. A series of studies by Dolman et al. in Canada, Sakaguchi et al. in Japan, and Duff et al. in the United States had demonstrated in vitro enhancement of type E toxin in culture supernatant fluids and in cellular extracts.

The high case fatality rate of type E botulism and the high toxicity of food extracts were not consistent with the low toxic potencies found in pure cultures of C. botulinum type E (Sakaguchi et al., 1954; Dolman, 1957). These investigators, apparently independently, found that toxin of higher potency was obtained from mixed culture growth of the non-proteolytic, toxic type E and a proteolytic, non-toxic organism (Dolman, 1953; Sakaguchi and Tohyama, 1955a, b). The proteolytic organism described by Dolman was a non-toxic mutant of C. botulinum type E. Sakaguchi and Tohyama did not identify their "contaminant", other than to determine that

it probably was a species of Clostridium.

Sakaguchi and Tohyama (1955b) suggested that the activating principle was protein in nature, probably enzymatic in activity, and that the precursor was produced intracellularly. Within a year Duff, Wright and Yarinsky (1956), and then Gordon et al. (1957) demonstrated that trypsin treatment of type E toxin at pH 5.5 to 6.5 increased the toxicity 12 to 47-fold when tested by intraperitoneal or subcutaneous routes. The toxicity as determined by oral administration remained unchanged. Both culture filtrates and partially purified toxins could be activated, and both treated and untreated preparations were neutralized by type E antitoxin. It was suggested that the precursor was elaborated from the cell and that activation took place extracellularly.

Now that the existence of a toxin precursor which could be enzymatically activated had been fairly well established, attention was turned to the nature of the precursor, the mechanism of the activation, and the site of the activation with respect to cellular location. Sakaguchi and Sakaguchi (1959, 1961); Gerwing et al. (1961, 1962); and Sakaguchi, Sakaguchi, and Imai (1964) have studied these problems with partially purified toxins prepared in different ways.

The toxins of Sakaguchi and Sakaguchi (1959, 1961) were prepared by extracting washed cells with acetate buffer, fractionating with ammonium sulfate, and dialyzing against distilled water. The toxin was not soluble in distilled water but, after activation, had a potency of 3.2 to 9.8 x 10^7 mouse LD_{50} per milligram of nitrogen, which compares favorably with the activity of crystalline type A toxin. Gerwing et al. (1961, 1962)