1970

Studies On The Crystalline Protein Toxin From Some Varieties Of Bacillus Thuringiensis

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STUDIES ON THE CRYSTALLINE PROTEIN TOXIN FROM SOME VARIETIES OF *BACILLUS THURINGIENSIS*

by

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Submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

Faculty of Graduate Studies

The University of Western Ontario

London, Canada

August 1969

ACKNOWLEDGMENTS

This program was made possible through the assistance generously provided by John Labatt Limited. The major financial support, the use of Company laboratory facilities and the opportunity to partake in an enlightened employee educational program are indeed most sincerely appreciated. The consideration so kindly given by the Biochemistry Department, University of Western Ontario throughout the course of this protracted study and the facilities provided by the London Research Institute, Canada Department of Agriculture are gratefully acknowledged.

The author wishes to express a deep debt of personal gratitude to Dr. E.Y. Spencer for suggesting the field of study and for guidance and encouragement throughout these studies. The initial introductory assistance provided by Mr. H. Schoenfeld is sincerely acknowledged. The writer also takes this opportunity to thank especially Dr. T.A. Angus and Dr. C.L. Hannay for many helpful comments and suggestions. The technical assistance given by Mrs. M.S. Kendall and Mr. W.G. Layng was gratefully received. A final special debt of gratitude is expressed to my wife, who at times bore more than her fair share of such a program.

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LIST OF ABBREVIATIONS

B.t.	-	*Bacillus thuringiensis* (general species name)
B.t.a.	-	*Bacillus thuringiensis* variety *alesti*
B.t.e.	-	*Bacillus thuringiensis* variety *entomocidus*
B.t.s.	-	*Bacillus thuringiensis* variety *sotto*
B.t.t.	-	*Bacillus thuringiensis* variety *thuringiensis*
Pc	-	Protein concentration (expressed as µgm/ml)
PHB	-	Polymerized beta-hydroxybutyric acid
Tris	-	Trishydroxymethylaminomethane
MW_{min}	-	Minimal Molecular Weight

ABSTRACT

Varieties of the bacterium Bacillus thuringiensis have the ability to produce a protein parasporal inclusion, commonly called a crystal. These intracellular structures are elaborated during the sporulation phase of growth. When cellular lysis occurs both spores and crystals are released into the culture medium. Crystals are characterized by two very unique properties, they may act as toxins on many lepidopterous insects and also their native structure possesses a remarkable degree of structural stability. This latter property was of particular interest during these studies.

Initial efforts were focused on the development of growth conditions that would provide a readily available source of crude material (spores, crystals, cells and cellular debris). Parameters were defined for the production of high crystal yields from three varieties of Bacillus thuringiensis using a liquid semi-synthetic medium. Several physiological characteristics were observed in growing cultures of the bacteria that had not been previously reported, also other properties were noted that were similar to those found in Bacillus cereus. With a ready source of crude material a physical chemical purification technique was developed for the isolation of crystals. This chloroform purification method permitted the recovery of 3,000 crystals for each contaminating spore. Yields, expressed on a dry weight basis, approached 20%.

A review of the amino acid composition of crystals showed that crystals were normal associating globular proteins. Purified crystals were subjected to the effects of many different chemicals and conditions. No single reagent successfully dissociated crystals under conditions judged to be sufficiently mild to avoid possible denaturation. The combined effects of several reagents that apparently disrupted the bound water on the crystal and also ruptured hydrophobic, ionic and hydrogen bonds permitted the complete solubilization of crystals at pH 9.5. Dissociation was achieved through the apparent cleavage of only non-covalent bonds. It is suggested that the combined forces of these different bonds unite to endow the native crystal with its characteristic stability.

I. INTRODUCTION

The idea of controlling noxious insects biologically as suggested by Pasteur (1870) was not quite a century old when Bernal (1958) outlined the concept of a quaternary structure for high molecular weight proteins. These seemingly diverse aspects become intimately related when toxic parasporal inclusions, produced by varieties of the insect pathogen Bacillus thuringiensis, are studied. Initial attention was attracted to this group of bacteria because of their pathogenic effects on desirable insects and early efforts were directed towards protection of the insect and destruction of the bacterium. The obvious converse was readily realized; however little real advance was made in this area until the last decade. This advance was partly stimulated by an increasing apprehension for organic control chemicals, Carson (1962), and by the highly favourable attitude held by food and drug regulation agencies towards the use of B.t. preparations.

Bacillus thuringiensis varieties are members of the 'cereus' group of bacilli. This group is comprised of large cell bacilli; namely Bacillus cereus, Bacillus cereus var mycoides (both ubiquitous soil organisms) and Bacillus anthracis, a mammalian pathogen. They are characterized further by all being aerobic or facultatively anaerobic and producing lecithinase. B.t. varieties are common soil organisms and have been isolated from agricultural environments throughout the world.

In the 1950's two Canadians, C.L. Hannay and T.A. Angus succeeded in unlocking part of the mysterious relationship between bacterium and insect. Hannay (1953) showed that during sporulation certain toxic strains of B.t. invariably produced an intracellular diamond shaped crystal along with a spore. Both these intracellular structures were observed with the light microscope. The crystals varied greatly in size but were always the same shape. They were amazingly stable in various solvents but they could be readily stained with Giemsa, basic fuchsin, crystal violet and Victoria blue. Soaking the crystals in 1N hydrochloric acid for 15 minutes at 60°C failed to alter their size or shape, however they did take up stains more readily. Extreme alkali was necessary for dissociation of the crystal.

Although Hannay suggested a relationship between the crystal and insect pathogenicity, it remained for Angus (1954) to confirm such a correlation. In a further paper Hannay and Fitz-James (1955) showed that crystals were pure protein and assembled in such a way that their surface structure was made up of repeating layers. Since these enlightening studies other investigators have continued to chip away at the secrets of the crystal. However, usually these studies have been concerned more with the end rather than the means to it, hence from the structural aspect comparatively little is known about the proteinaceous crystal.

Interests in the varieties of B.t. have been both of a 'fundamental' and 'applied' nature. Large scale industrial growth systems have been used to produce several varieties of B.t. and field tests have been conducted in France, Czechoslovakia, U.S.S.R. and North America. The world's leading nation in microbial products, Japan, has not become involved in B.t. production because of the disastrous effects

these microbes would have on the silk industry. The high degree of secrecy associated with the fermentation industry, coupled with their willingness to supply 'fundamental' workers with crude starting material, has resulted in relatively little published work on the growth conditions for the varieties of B.t.

In general these organisms grow well on normal laboratory media. However, crystal production is extremely variable and long incubation times are not uncommon. Factors influencing crystal yield seem to be complex, the situation being somewhat similar to that encountered in spore proliferation. Reports of rapid and simple growth conditions suitable for production of hundred gram quantities of crude B.t. preparation are not to be found in the literature.

The extraction of crystals or crystal protein from crude preparations of B.t., i.e. those containing whole cells, spores, crystals and cellular debris, has been complicated greatly by the many common properties shared by crystals and spores. Physical separations based on centrifugal methods have been attempted, as have methods based on induced clumping of one structure and then removal of the other. Degrees of purity have seldom reached as high as one or two spores per 100 crystals. Under conditions of extreme alkalinity crystals are more readily solubilized than spores, however such conditions have been reported to cause inactivation of crystal toxin (Angus, 1956b) and apparent alteration of spores (Doak and Lamanna, 1948). Existing methods for purifying crystals provide only small quantities of pure material after the expenditure of much time and effort. Methods for purifying gram quantities of crystals, necessary for a purely biochemical investigation, are not available in the literature.

Small samples of variously purified crystals have been subjected to limited biochemical investigations. In general these studies were all dependent upon comparatively high alkaline conditions (pH 11.0 - 12.5) for solubilization of the crystals. Such studies have reported protein components with widely varying molecular weights and toxic properties. A common regima has been to go from a protein macromolecule (the crystal) large enough to be seen with a light microscope, down to a molecular weight of about 10,000 $\pm$ 5,000. This final structure usually had some toxic effect on insects. The transition from macromolecule to polypeptide failed to cast any light on the large grey zone between these structures. Methods offering a controlled stepwise dissociation of the crystal to intermediate forms have not been reported.

This present investigation was started with a view of studying:

(1) Growth conditions for crystal forming varieties of Bacillus thuringiensis with the production of hundred gram quantities of crude preparations.

(2) Crystal separation from other biological matter so that gram quantities of highly purified, undamaged crystals would be readily available.

(3) Existing biochemical information that might help to explain the peculiar stability properties of the crystal.

(4) The quaternary structure of the crystal, its bonding forces and other properties that might facilitate a gentle and controlled dissociation of the crystal.

II. HISTORICAL REVIEW

Bacillus thuringiensis and its varieties have been extensively reviewed by many authors from various viewpoints. Included here are the reports of: Krieg (1961), Heimpel (1963)(1967a), Heimpel and Angus (1960)(1963), Martouret and Melair (1963) and Rogoff (1966). This historical setting has been constructed using the information gleaned from the above reviews and other reference papers that offer some insight into crystal elaboration, purification and architecture.

A. Early Investigations

After the work of Pasteur (1870) on diseased silkworms (*Bombyx mori*) and the report of Cheshire and Cheyne (1885) on infected honey bees (*Apis mellifera*), the bacterium was recognized next in Japan. Ishiwata (1901) isolated a spore forming aerobic microbe from diseased silkworms and named the organism *Bacillus sotto*. It was noted that the toxic principle could be separated from the vegetative cell by filtration and that toxicity resided either on the spore or within the spore, i.e. toxicity was associated with a particulate structure. The infective bacilli were isolated from both the blood and gut contents of the diseased insect. A generalized bacterial infection was the apparent cause of death. These results were confirmed by Aoki and Chigasaki (1915, 1916), who also noted that only sporulated preparations infected

the silkworm.

Berliner (1911, 1915) isolated a bacterium from the diseased European flour moths, Anagasta (Ephestria) kuhniella, found in the province of Thuringia, Germany. He named this organism Bacillus thuringiensis. It was noted that during sporulation this bacterium formed two intracellular bodies, one was the normal oval spore, while the other structure was a rhomboidal shaped body which was believed to be part of the nuclear apparatus of the cell. These results were confirmed by Mattes (1927) who reisolated the organism and made it available to investigators on this continent, where it ultimately received considerable attention. It is interesting to note that no one seemed to recognize the significance of the association of disease with a particulate cellular structure and the second intracellular inclusion. For approximately 20 years, from 1930 to 1950, the literature made little note of the crystalliferous bacteria.

B. Recent Investigations

The apparent re-awakening of interest in B.t. varieties was undoubtedly sparked by the work of Steinhaus (1951) and the discoveries of Hannay (1953) and Angus (1954).

1. Taxonomy

Although the B.t. varieties all seem to have certain distinct characteristics, their extremely close biochemical and morphological relationships to Bacillus cereus have left room for a degree of taxonomical doubt (Rogoff, 1968 and Heimpel, 1968). This situation is not too surprising when one considers the complexity associated with the classification of the rest of the genus Bacillus (Breed, Murray and Smith,

1957). Only three of the many crystalliferous isolates were considered in this work, with biochemical studies restricted to only one variety.

Generally it has been accepted that the crystalliferous bacteria were first recognized as such by Berliner (1911, 1915) consequently Bacillus thuringiensis was considered to be the type species (Heimpel and Angus, 1960). The Bacillus sotto recognized by the Japanese workers was found to be closely related to an insect pathogen isolated by Toumanoff and Vago (1951). This latter organism was isolated in the vicinity of Ales, France and because of its close resemblance to Bacillus cereus, it was named Bacillus cereus variety alesti. Steinhaus (1951) isolated two new strains of crystalliferous bacteria that were toxic to silkworms but when compared to B.t.t. they were deficient in certain biochemical characteristics. Heimpel and Angus (1958) created a new species name, Bacillus entomocidus, for these isolates. The history and taxonomy of these and many more B.t. isolates are summarized by de Barjac and Bonnefoi (1968).

With the host of different crystalliferous isolates available, comparative morphological, biochemical and serological techniques have been used extensively. Such tests, together with a consideration of the various toxins produced, form the basis of a taxonomic key proposed by Heimpel (1967b). Although this key has certain points of criticism (Rogoff, 1968) it has been followed during this work, hence the three varieties of immediate interest are designated:

Bacillus thuringiensis var thuringiensis

Bacillus thuringiensis var sotto

Bacillus thuringiensis var entomocidus

2. Growth Conditions

(a) Bacillus Species in General

Conditions for the seemingly optimal growth of many species of bacteria are readily found in the literature, and few complications need be encountered in the production of normal vegetative cells. However, when a species produces an intracellular spore, as encountered within the genus Bacillus, its entire physiology is greatly influenced by the additional metabolic processes imposed by sporulation. Many seemingly contradictory reports and large reviews attest to this fact. With the formation of a second intracellular body, the crystal, an already complex situation is further complicated.

(i) Nutrition

When the bacilli are undergoing vegetative growth sporulation genes as well as other closely linked genes are repressed. These may be activated by the disappearance from the medium of certain essential nutrients (especially glucose) or by metabolic products accumulated in the medium (Halvorson, 1962). In addition to the normal essential nutrients required for vegetative growth, the bacilli also have an absolute requirement for manganese and oxygen if sporulation is to occur.

Manganese appears to be a universal constituent in all media supporting sporogensis. If a medium is completely synthetic, (i.e. simple or defined) then manganese is added directly as a salt. However, if a medium is semi-synthetic or complex then the manganese may be added indirectly as a constituent of another component possessing the required amount of manganese (e.g. bran, yeast extract, corn steep or casein). Halvorson (1962) noted that the maximal effect with manganese was obtained at about 0.00001% (w/v) and suggested that the manganese

might have an essential role in the protease activity associated with sporulation. It is interesting to note that in reports of highly sporogenic media (Kim and Naylor, 1966; Yao and Walker, 1967) magnesium was absent.

In addition to absolute growth requirements, sporulation may be influenced by the presence or absence of a wide variety of other compounds in a growth medium, included here are carbohydrates, amino acids, organic acids, vitamins and inorganic compounds. The influence of the diverse group of chemicals on the integrated sequential biochemical events of sporulation ensures a most involved physiological situation.

(ii) pH

During the initial phases of growth the bacilli secrete as by-products a surplus of organic acids, mainly pyruvic and acetic, formed during carbohydrate metabolism. The effect of these acids is to lower the pH of the medium by about 2.0 $\pm$ 0.5 pH units, depending on the concentrations of carbohydrates and buffering groups present. A growth system at pH 7.6 may readily be changed to pH 5.6 by such acid accumulation (Nakata and Gollakota, 1959). With the depletion of carbohydrate or other energy sources from the medium and the utilization of amino acids, the pH of a growing system will rise to produce a slightly alkaline environment (at least pH 7.5 - 8.0). Murrell (1967) noted that such a pH pattern was very common among bacilli, with no exceptions being cited.

(iii) PHB

During their early stages of growth some bacteria accumulate intracellular granules of polymers of beta-hydroxy-butyric acid. These granules, usually about 0.5μ in diameter, are hydrophobic and possess a

crystalline helical structure (Marchessault, Okamura and Su, 1968). Accumulation of PHB is particularly common among the bacilli (Williamson and Wilkinson, 1958).

Macrae and Wilkinson (1958) showed that the amount of PHB formed within a bacillus cell increased as the glucose in the medium increased. Also when the nitrogen supply in the medium was exhausted PHB synthesis continued if sufficient energy sources (glucose, pyruvic acid or beta-hydroxy-butyric acid) were present. Hence an improperly balanced growth medium could cause an over-production of PHB granules. This work was confirmed by Nakata (1966). In some cases PHB has been shown to constitute up to 50% of the dry weight of the cells (Dawes and Ribbons, 1964). A point of further interest was demonstrated by Slepecky and Law (1961) who showed that when considerable quantities of PHB were accumulated by a growing cell, the material was not utilized for many hours and also sporulation was delayed until a time when PHB was rapidly consumed. These workers, as well as Nakata (1963), showed that PHB production was not a requirement for sporulation. Therefore, it would appear that an imbalanced growth medium (rich in carbohydrate) could cause delayed sporulation (and associated processes) by an over-production of PHB. Macrae and Wilkinson (1958) also showed that in the absence of exogenous energy sources PHB could be metabolized by the bacilli. The ability of PHB granules to act as reserve sources for energy during endogenous metabolism appears to be a property peculiar to the bacteria and suggests a role for PHB similar to that played by starch and glycogen in higher forms of life.

Bacillus cereus has the ability to endogenously metabolize another type of substrate (in addition to PHB) and produce ammonia (Clifton and Sobek, 1961). When this nitrogenous substrate was used

concurrently with PHB, the total ammonia produced by the growing culture was much larger than that produced when only PHB was metabolized. In poorly buffered media a fairly strong alkaline environment (pH 8.0 - 9.0) could be established. Also, during the metabolism of these two substrates, a much greater oxygen demand occurred compared to that required when only PHB was metabolized. The ability to metabolize two endogenous substrates with a requirement for an increased oxygen tension appeared to be a property peculiar to Bacillus cereus (Dawes and Ribbons, 1964).

An observation made by Stokes and Parson (1968) may be worthy of note here. They showed that Sphaerotilus discophorous (a chlamydobacter very much unrelated to the bacilli) accumulated large amounts of PHB. This material acted subsequently as a reserve food supply and allowed the organism to withstand long periods of starvation. When magnesium and oxygen were increased during growth a significant stimulation in PHB utilization occurred. The amount of magnesium added was 0.03% w/v (as $MgSO_4$), a comparatively high level when optimal manganese levels for the bacilli are considered. This work implicated PHB utilization with a requirement for high levels of magnesium and oxygen.

A survey of the magnesium and manganese levels in various bacteriological media where PHB was formed is shown in Table I. It may be noted that all synthetic media contained both manganese and magnesium. However, previous reports have established that magnesium is not required in large amounts in sporulation media.

TABLE I

Levels of Magnesium and Manganese Added to Media Supporting PHB Synthesis

Magnesium (% w/v)	Manganese (% w/v)	Reference
0.002 (1)	0.00006 (5)	Williamson and Wilkinson (1958)
0.010 (2)	0.001 (6)	Macrae and Wilkinson (1958)
0.200 (3)	0.050 (10)	Nakata (1966)
0.080 (3)	0.010 (8)	Hashimoto et al (1960)
0.010 (3)	Nil[a]	Baptist (1962)
Nil[a]	0.005 (6)	Delafield et al (1968)

[a] Semi-synthetic medium was used.

Numbers in brackets indicate the following salt forms:

1. $MgCl_2$
2. $MgCl_2.6H_2O$
3. $MgSO_4$
4. $MgSO_4.7H_2O$
5. $MnCl_2$
6. $MnCl_2.4H_2O$
7. $MnSO_4$
8. $MnSO_4.H_2O$
9. $MnSO_4.4H_2O$
10. $MnSO_4.7H_2O$

(b) Bacillus thuringiensis Varieties

In terms of physiological complexity these varieties represent an extreme as far as the bacteria are concerned. Although the production of crystals was considered to be a stable taxonomic feature of the bacteria (Heimpel, 1967b), it must be acknowledged that the amount of crystal production is quite variable. It would seem that variations in crystal production are more dependent on nutritional changes than on genetic stability. Subcultures of the original isolate of Mattes (1927) are still effective crystal producers (Hannay, 1967). Somewhat analogous to the variation in crystal production is the situation encountered in spore production, where much endeavour has been expended attempting to suppress or induce sporulation. More often than not it is the bacterium that has the final word.

Laboratory methods for the growth of B.t. varieties with subsequent spore and crystal proliferation are somewhat tedious, time consuming and variable. On routine bacteriological solid media (nutrient or Lab Lemco agar) incubation periods of seven to 20 days are common (Pendleton and Morrison, 1966). Steinhaus (1956) described the production of B.t.t. on agar using 200 Povitsky flasks, with a total surface area of 70 sq. ft. After a seven day growth period the surfaces were washed to yield a final total of 140 gms dry weight. Hannay (1967) worked out a semi-synthetic solid media that produced high crystal yields in a comparatively short time (24 - 48 hrs.). The final medium contained 25 constituents that required special aseptic handling and successful use was restricted to certain B.t. varieties. The metal mixture for this medium contained seven different components, magnesium was several fold higher than any other constituent. No carbohydrate was added to this

medium.

Various semi-synthetic and complex media have been reported to support good spore and crystal production for one or more of the B.t. varieties. A comparison of the diverse constituents in these media is highlighted by one common feature. All possessed comparatively large quantities of magnesium (Table 2). The levels of magnesium reported here are high, similar to those found in media supporting the metabolism of PHB (Table 1). As noted before, the bacilli do not require magnesium for sporulation, although trace amounts may be required for growth.

No reports were found in the literature describing PHB metabolism by any B.t. variety. However, several reports exist where reference was made to an intracellular granular stage during the growth of a given B.t. variety (Hashimoto, Black and Gerhardt, 1960; Monro, 1961a; Rogoff, 1966; Singer, Goodman and Rogoff, 1966; and Dubois, 1968). This stage invariably occurred before spore and crystal production. Hashimoto et al (1960) reported that when the granular stage occurred cells of Bacillus cereus were committed to sporulation.

B.t. varieties show many of the growth characteristics associated with the rest of the genus Bacillus. Proom and Knight (1955) conducted extensive studies into the minimal nutritional requirements of some Bacillus species. They showed that the growth requirements for Bacillus cereus and Bacillus thuringiensis were the same. Rogoff (1966) noted that limiting glucose served as an initiator of sporulation and crystal production by B.t. varieties. A nutritional balance between carbohydrate and nitrogen in B.t. growth systems was described by Megna (1963) as being a must in order to obtain high spore and crystal yields. It was essential that the balanced nutrients be depleted at the

TABLE 2

Levels of Magnesium and Manganese Added to Media Supporting Spore and Crystal Proliferation in B.t.

Magnesium (% w/v)		Manganese (% w/v)		Reference
0.020	(4)	Nil[a]		Hannay and Fitz-James (1955)
0.0123	(4)	0.000223	(9)	Grelet (1957)
0.040	(4)	0.00054	(7)	Monro (1961a)
0.005	(3)	0.003	(8)	Cantwell, Heimpel and Thompson (1964)
0.100	(4)	0.015	(8)	Koonz and Greenberg (1966)
0.020	(4)	Nil[a]		Aronson, Bowe and Swafford (1967)
0.098	(4)	0.034	(8)	Hannay (1967)
0.0093	(4)	0.00017	(9)	Cooksey (1968)
0.003	(3)	0.005	(4)	Dubois (1968)
0.025	(4)	0.0006	(8)	Kingan and Ensign (1968)
0.010	(4)	0.001	(8)	Singer and Rogoff (1968)

[a] Semi-synthetic Medium.

Numbers in brackets indicate the following salt forms:

1. $MgCl_2$
2. $MgCl_2.6H_2O$
3. $MgSO_4$
4. $MgSO_4.7H_2O$
5. $MnCl_2$
6. $MnCl_2.4H_2O$
7. $MnSO_4$
8. $MnSO_4.H_2O$
9. $MnSO_4.4H_2O$
10. $MnSO_4.7H_2O$

same time. Mogna also described a typical Bacillus pH profile during growth of B.t.t. The initial pH change in the medium was from 7.4 ± 0.2 to 6.5 ± 0.1, while later in the growth cycle the pH rose to 8.0 ± 0.5 (even though the medium was buffered with 0.1% w/v calcium carbonate). Dubois (1968) reported a similar pH pattern with less dramatic changes, the initial pH of 8.05 fell during the first ten hours of growth and then gradually returned to pH 7.95. This modified pH profile might have been influenced by the high initial pH or the presence of 0.1M dipotassium hydrogen phosphate in the medium.

Under normal conditions spores and crystals are produced together, although Smirnoff (1963a) reported that sub-optimal growth temperatures (12° - 16° C) caused a desynchronization of this togetherness and only crystals were produced. Smirnoff (1963b) also noted that the incorporation of up to 0.95M urea into an agar growth medium prevented cultures from forming crystals, but growth and sporulation occurred. Therefore spore formation and crystal production do not appear to be completely dependent upon each other.

Norris and Watson (1960) observed that crystals were synthesized in close association with the nuclear body of the cell. Aronson, Bowe and Swafford (1966) showed that in normal sporulating cultures of B.t. the crystals were surrounded by ribosomes. Monro (1961b) reported that crystal antigens could not be demonstrated in cells forming crystals and suggested a de novo synthesis of crystal proteins. Young and Fitz-James (1959c) showed that under certain conditions 8-azaguanine could stop crystal production. Such reports tend to implicate the processes of normal protein synthesis in crystal elaboration.

3. Toxins Produced by Bacillus thuringiensis Varieties

Crystalliferous bacteria can produce at least four toxic entities (Heimpel, 1967a). The actual conditions under which each toxin is produced are not well understood. Also different B.t. varieties produce varying amounts of any or maybe all of the toxins, depending on strain characteristics and growth conditions. Initial testing of B.t. preparations produced considerable confusing and conflicting results in the literature due to the presence of an unknown number of toxins, each apparently acting in different ways. Some of the comparative properties of established and suspected toxins are listed in Table 3. In the experiments conducted during this investigation only the crystal toxin has been considered; it is discussed in later sections.

The exotoxins, with one exception, have not been studied in great detail, however it does seem clear that several different forms exist. McConnell and Richards (1959) isolated a factor from B.t. growth systems that was toxic to Diptera which was later called the Fly Factor. Perron and Benz (1968) showed that this heat stable toxin was probably a nucleotide of adenine. The detailed structural studies of Farkas, Sebesta, Horska, Samek, Dolejs and Sorm (1969) have confirmed the work of Perron and Benz (1968). DeBarjac, Burgerjon and Bonnefoi (1966) reported that this factor was produced by many varieties of B.t. and that it was toxic to representatives from several insect groups, including Lepidoptera.

Smirnoff (1964) reported the presence of a substance in commercial preparations of B.t.t. (Thuricide) that was toxic to sawflies, an insect not normally effected by B.t. The active component was shown to be a heat labile peptide (molecular weight about 2,000) and was not

TABLE 3

Properties of Established and Suspected Toxins Produced by Bacillus thuringiensis

Toxins	Heat Stable	Dialysable	Soluble in Water	Toxic to Diptera	Toxic to Lepidoptera	Suggested Structure	Reference
Endotoxin:							
Crystal	No	No	No	No	Yes	Protein macromolecule	Hannay (1953)
Exotoxins:							
Phospholipase C	No	No	Yes			Protein	Toumanoff (1953)
Protease	No	No	Yes			Protein	Bucher (1960)
Nucleotide	Yes	Yes	Yes	Yes	Yes	Nucleotide	McConnell and Richards (1959)
Peptide	No	Yes	Yes	Yes	N.R.	Peptide	Smirnoff and Berlinguet (1966)
Peptide (?)	Yes	N.R.	Yes	N.R.	Yes	Peptide	Cantwell et al (1964)

N.R. - Not Reported

present in all preparations of Thuricide. Later Smirnoff and Berlinguet (1966) showed that the toxin was inactivated at 60°C for ten minutes. Attempts to reproduce quantities of this toxin, presumably by subculturing an isolate from the preparation, failed. Cantwell, Heimpel and Thompson (1964) extracted a water soluble, heat stable exotoxin from dry commercial preparations of B.t.t., which contained a ninhydrin positive fraction possessing an absorbance maximum at 270 - 280 mμ. Burgerjon (1964) also isolated a toxic factor from Thuricide. Propagating an isolate from the original preparation did not produce more of this factor. During normal formation of Thuricide only the particulate matter is used (Megna, 1963), thus little or no soluble toxin would be expected in such preparations. Raun, Sutter and Revelo (1966) reported that when a liquid formulation of B.t.t. was stored it became more toxic, with the potency almost doubling that found in the fresh preparation. The increasing toxic entity was soluble and toxic by injection. In short, at least one toxic low molecular weight peptide, of variable stability, may be isolated from some preparations of B.t.

4. Pathogenicity of Bacillus thuringiensis

Heimpel (1967a) noted that the fly-factor of McConnell and Richards (1959) had a much wider toxicity spectrum than the crystal. Representative insect species from Diptera, Hymenoptera, Orthoptera, Lepidoptera, Coleoptera, Isoptera and Acarina appeared to be susceptible to this factor (Krieg, 1968). The fly-factor, which is produced early in the growth cycle (before sporulation) by certain B.t. varieties, interferred with the metamorphosis in the larvae of Diptera. Sorm (1969) reported that this factor was also toxic to mammals and that it inter-

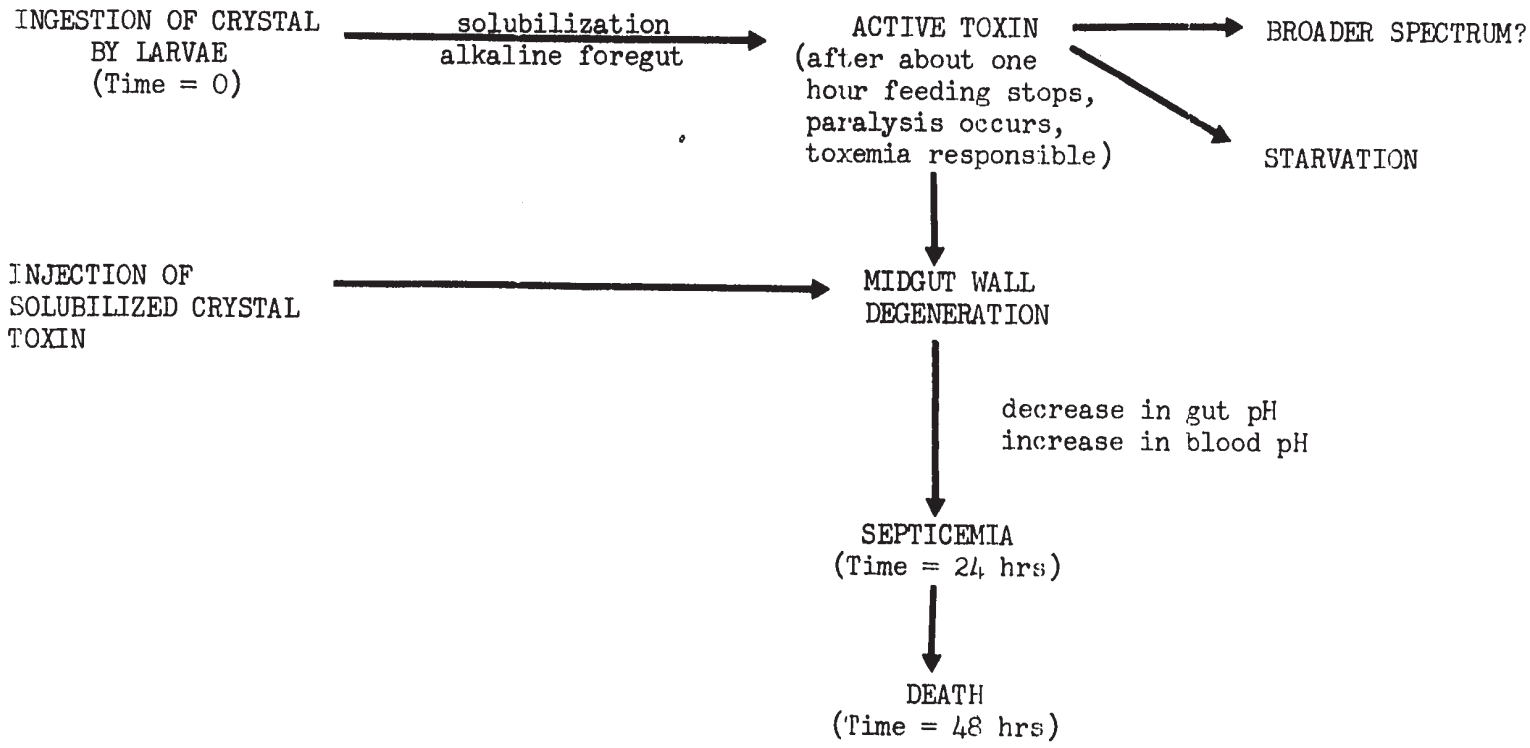
ferred with elongation of ribonucleic acid.

Crystals seem to possess toxicity only for insects in the Lepidoptera group and most studies on B.t. pathogenicity have been primarily interested in the effect of the crystal on these insects. The gut contents of this insect group are characterized by comparatively high pH values (in excess of pH 9.5) plus considerable inorganic ions (Faust, Adams and Heimpel, 1967) and a reducing environment (Day and Waterhouse, 1953); all these are conditions that have been found to enhance crystal dissociation. When one of these factors was absent, the crystals appeared to be ineffective. This was demonstrated by Burges (1964) who noted that when crystals alone were fed to insects with comparatively neutral gut pH no toxic effect occurred.

The pathogenic sequence observed when crystals of B.t.s. were fed to silkworms is outlined in Figure 1. The high alkaline environment along with reducing conditions and proteolytic enzymes found in the insect gut apparently combine to dissociate the crystal into smaller units. It is these steps that convert the crystal from an inactive protoxin to a toxic agent. It will be noted that paralysis and cessation of feeding occur early; while death, which is usually due to septicemia, can take up to 48 hours. For purposes of crop protection the cessation of feeding is the critical point. An interesting observation was made by Krieg (1961) who reported that Mamestra brassicae, normally not susceptible to B.t. could be attacked if preparations were first fed to a sensitive host (Pieris brassicae) and then withdrawn and injected into the non-susceptible insect. Such results suggest that prior activation of the protoxin, i.e. solubilization of the crystal, might increase its toxicity range.

Figure 1

Sequence of Pathogenicity When *Bacillus thuringiensis* var *sotto* is Fed to Silkworms



Although the degeneration of the midgut wall has been followed microscopically, the mechanism by which this is brought about is not clear. Fast and Angus (1965) fed pure crystals of B.t.s. to previously starved silkworms and used labelled sodium carbonate plus glucose to show that although the permeability of the gut wall was disrupted some forces remained to prevent free diffusion. In a later report Angus (1968) showed that a cyclic peptide (Valinomycin) had an effect on silkworms similar to that of B.t.s. It was suggested the toxic effects of both crystals and Valinomycin might be associated with changes in the permeability of the midgut epithelia cells. Such an explanation gains credence when it is considered that crystal toxicity is generally associated with a low molecular weight peptide molecule.

Although the individual responses within the Lepidoptera to B.t. crystals are varied, they have been classified into four general groups by Heimpel (1963). This grouping is as follows:

GROUP I - Response restricted to larvae with high alkaline gut contents (all Lepidoptera).

- General paralysis occurs due to the effect of alkaline gut contents leaking into the hemolymph. Sterile alkaline buffers produce a similar paralysis upon injection into the hemolymph, therefore paralysis is a secondary effect brought about by disruption of gut wall.

GROUP II - The Lepidoptera not included in Group I.

- General paralysis does not occur, (gut contents do not leak into hemolymph) paralysis is restricted to the gut, hence feeding ceases and death is due to starvation.

GROUP III - Few species included here (Anagasta kuhniella and Porthetria dispar, the gypsy moth caterpillar).

- General paralysis does not occur.
- Spores plus crystals and/or the fly factor are responsible for toxicity.

GROUP IV - Resistant Lepidoptera, includes species of Mamestra, Euxoa, Perioroma and Agrotis.

- Although these insects are not attacked by crystals they are affected by the thermostable fly factor, hence B.t. formulations containing both toxins give confusing results when used on this group.

5. Bacteriophage Associated With Bacillus thuringiensis

The general physical and chemical properties of the crystal protein might suggest an association with the structural protein of bacteriophage. Yoder and Nelson (1960) first reported phage active against B.t.t. Later Yoder (1961) showed no apparent relationship between crystal and phage.

Chapman and Norris (1966) reported that the different varieties of B.t. possessed varying degrees of resistance to bacteriophage, with B.t.t. being the most sensitive while maximum resistance was shown by B.t.s. Afrikian (1969) has stated that of all the B.t. varieties, the most phage resistant was Bacillus thuringiensis var cacasicus. Raun et al (1966) noted that phage decreased the rate of B.t. activity, but not the final outcome.

6. Commercial Preparations of Bacillus thuringiensis

Wettable powders and wet stabilized slurries of B.t.t. are available for field use. These preparations contain added diluents, carriers, stickers and stabilizers. No reports are known to exist on the long term effects of these agents on the crystal. The trade names and some information on the different commercial preparations are outlined in Table 4. Final formulations usually contain about 10^9 spores/gm and effective control demands about 10^{12} to 10^{13} spores per acre. It is interesting to note that these preparations are standardized on the basis of spore counts and not on the amount of any toxic entity.

Standardization of toxicity in final products is difficult due to variations in growth conditions, bacterium used and the possible number of toxic factors produced. No accurate in vitro assay exists for any toxic factor of the B.t. varieties. Spore counts are not a direct reflection of crystal protein content as crystal size varies and a cell may produce more than one crystal while only one spore per cell is formed. Different crystal varieties do not have identical effects on a given insect (Angus and Norris, 1968), the converse of this is also true and although in vivo assays possess considerable built-in biological variation they represent the most reliable method of assay. The silkworm has proven to be a suitable insect for bioassay of crystal potency, while the house fly has been used to assay the soluble non-enzymic exotoxins (Heimpel, 1967a).

The advantages and disadvantages offered by commercial crystalliferous preparations of B.t. have been listed by Norris (1965), Heimpel (1967a) and Cameron (1967) as follows:

TABLE 4

Commercial Preparations of Bacillus thuringiensis

(B.t.t. usually used)

Trade Name	Manufacturer	Patent Number		Remarks
Thuricide	Bioferm Corp., California	US 3,073,749	Jan., 1963	Particulate matter only
Biotrol (and Larvatrol?)	Nutrilite Products Inc., California	US 3,086,922	April, 1963	Whole culture medium in final product
Bakthane	Rohm & Haas Co., Pennsylvania	US 3,087,865	April, 1963	
Parasporin	Grain Processing Corp., Iowa	Process not patented		
Agritol	Merck & Co., New Jersey	?		
Biospore	Farbwerke Hoechst AG	French 1,247,677		Final product 10% particulate material and 90% bentonite
Plantibac	French Product?	?		
Entobacterine	USSR Product?	?		
Sporeine	French Product	?		Particulate material and bentonite
?	Pasteur Institut, France	French 1,225,179 Canadian 630,687	1960 1961	Particulate matter only

(a) Advantages

1. Toxic effects are restricted to lower forms of life.
2. Potency is of the same order as Organochlorine and Organophosphorus insecticides.
3. Application is easily accomplished by conventional methods.
4. No build up of toxic residues occurs.
5. Costs appear to be competitive with conventional methods.
6. Insect resistance does not develop.
7. Preparations are stable, with long shelf life.

(b) Disadvantages

1. Repeated applications are necessary.
2. This is not a contact control agent and preparations must be ingested.
3. Preparations are bulky and low volume concentrates are not possible.
4. Spores are undesirable in food products.
5. Specificity is limited to Lepidopterous insects.

At this time it would seem that the inherit safety aspects associated with B.t. crystalliferous formulations could more than offset the above disadvantages.

7. Safety

In 1958 preparations of B.t.t. were the first insecticide to receive official Food and Drug Administration (U.S.A.) blessing when Bioferm Corporation was issued with a temporary exemption from a tolerance. In follow-up orders (Anon, 1966 and 1969) the exemption was extended to cover use on over 25 fruit and vegetable crops. A similar

ruling in Canada allows the use of B.t.t. on 26 different food crops (Anon, 1967).

Fisher and Rosner (1959) outlined a series of toxicological studies using B.t. on many different animals and human volunteers. When Thuricide (apparently a centrifuged preparation) was inhaled and ingested, detailed physical and biochemical tests showed no harmful effects. The human subjects all remained in good health throughout the tests.

8. Crystal Purification

Water soluble entities found in laboratory and industrial crude preparations of B.t. may be readily removed by successive centrifugal washings, however the remaining insoluble material presents a more difficult problem. The ultimate isolation of the crystal is further complicated by the similarity in size and chemical stability of spores and crystals. A comparison of some of the salient properties of spores and crystals is presented in Table 5. When the small differences in size and density are compared to the larger differences in chemical composition it would seem that a purification regima based on physical properties would be less effective than one based on physical chemical properties. Also large variations in crystal overall size (but not morphology) could influence the degree of reproducibility with physical methods.

(a) Physical Methods

The first method describing crystal purification from a crude laboratory preparation was reported by Hannay and Fitz-James (1955). Spores were initially destroyed by disintegration, either with a Mickle

TABLE 5

Comparison of Spores From Bacillus cereus and Crystals From Bacillus thuringiensis

	Spores	Reference	Crystals	Reference
Size	0.5 to 1.2μ by 1.0 to 2.5μ	Bergey et al (1957)	0.6μ by 2.0μ	Mattes (1927)
Density	1.51 to 1.54	Ross and Billing (1957)	1.41	Holmes and Monro (1965)
Total Lipid	0.9% (in wall)	Salton (1960)	nil	
Total Phosphorus	1.2% (in wall)	Salton (1960)	nil	Hannay and Fitz-James (1955)
Total Nitrogen	13.2% (in wall)	Salton (1960)	17.5%	Hannay and Fitz-James (1955)

disintegrator, or by natural lysis. The less dense spore debris was removed from the heavier crystals by differential centrifugation. Fitz-James, Toumanoff and Young (1958) reported that spores and crystals of B.t.a. settled into two distinct layers upon centrifugation. After about 30 separation cycles, involving repeated centrifugings and the removal of the bottom crystal-rich material, a preparation was obtained that was almost free of spores. These authors noted that such a differential precipitation could not be done with B.t.t. or B.t.s.

Robertson and Heimpel (1962) described a method for purification of crystals from commercial preparation of B.t.s. About a 17% (w/v) working suspension was made of the crude preparation in water and this was subjected to a series of washes and subsequent settlings. A final purification ratio (crystal vs spore count) of 49:1 was achieved with an apparent yield of 0.017% (dry weight basis, purified crystals versus starting material). It was noted that repeated washings of the crystals caused them to clump and also that some loss of crystal refractility (refringence), as well as crystal swelling, occurred. The addition of electrolyte (potassium chloride) prevented clumping. The pH of the wash water and the history of the crude preparation were not reported. However, it would seem apparent that the crystals underwent some change.

A refinement in the centrifugal purification approach was worked out by Burges (1965) using a sucrose gradient. In order to be effective it was first necessary to induce germination and lysis of the spores. Final preparations had a purification ratio of 66:1. No yields were reported.

(b) Physical Chemical Methods

The procedures used here generally involved a phase separation based on an apparent difference in the hydrophobicity of spores and crystals. Usually an emulsion was made between an aqueous suspension of crude B.t. and an organic solvent. Trifluorotrichloroethane has been used extensively. When the phases separated most of the spores were in the organic phase while most of the crystals were in the aqueous phase, the actual amount of spores in this latter phase varied with the different methods. The ideal, 100% recovery of pure crystals, became more difficult to reach each time the process was repeated on a given sample.

Angus (1959) outlined the first physical chemical method using 10% trifluorotrichloroethane and 90% of a crude laboratory preparation of crystals and germinated spores. The crude preparation was about 0.15% (w/v) in water. The mixture was shaken rapidly until a milky white emulsion was formed. On standing this emulsion broke down to form two layers. It should be noted that the ability of this fluorocarbon to form an emulsion was not constant with all preparations of B.t., and with some commercial preparations such a system could not be formed (Robertson and Heimpel, 1962). The aqueous upper layer contained largely crystals together with a few viable spores, it was re-treated three more times with fluorocarbon. The purification ratio obtained was 19:1 with ungerminated spores still present. These spores were induced to germinate and a further fluorocarbon treatment produced an ultimate purification ratio of 99:1. This method was reported to be effective for laboratory preparations of B.t.t., B.t.s. and B.t.a.

A modification of the above method was proposed by Bateson (1965) whereby the total time required for purification of a given sample

was reduced to six hours. The final germination step was eliminated and only two fluorocarbon treatments, instead of five, were necessary. A working suspension (5% w/v) of a crude laboratory preparation was made. Tributylcitrate and glass beads were added in order to effect complete separation of spores and crystals (as described by Hannay and Fitz-James, 1955). The shaken suspension was then added to sodium sulphate, more tributylcitrate plus trifluorotrichlorethane and the entire system was mixed in an homogenizer. The homogenate was transferred to a separatory funnel and allowed to separate. The particulate matter from the aqueous upper phase was recovered and the entire process repeated once more. A final purification ratio of 99:1 was obtained with an overall yield of 0.4% (dry weight, purified crystals versus starting material).

Pendleton and Morrison (1966) described a modification of the method of Bateson, (1965) in which an initial cleanup was done by removing some spores via flotation. The starting material was washed initially three times by centrifugation. Then spore flotation was achieved by repeated vigorous shaking of the crude suspension and removal of the froth. This partially purified preparation, with a purification ratio of 4:1, was then subjected to a further cleanup by homogenization with sodium sulphate and carbon tetrachloride. The final crystal-rich aqueous upper phase gave a purification ratio of 99:1 with approximately 35% of the total crystal content being recovered. A purification method was described by Lecadet (1965) using a two phase system brought about by mixing tetrabromoethane with B.t. preparations. The crystal-rich upper phase was removed and washed several times. A purification ratio of 99:1 was achieved with a 6% yield of purified crystals (dry weight, purified crystals versus starting material).

A different two phase system was described by Goodman, Gottfried and Rogoff (1967). The two phases were made of varying amounts of sodium chloride, polyethylene glycol and sodium dextran sulphate. A previously washed suspension of spores and crystals was shaken with the noted reagents. The resulting bottom phase and the interface were crystal-rich. A purification ratio of 1665:1 and a final yield of 2.5% (wet weight basis, pure crystal versus starting material) were reported. A similar purification method has been described by Delafield, Sommerville and Rittenberg (1968).

Assuming that the greater hydrophobicity of the spore could be exploited with flotation techniques, Gingrich (1968) developed a method for isolating crystals from commercial preparations of B.t. containing various carriers. The crystals and spores were initially separated from the other material by a very slow centrifugation (121 x g for 5 minutes). This procedure was repeated 15 times, when no more spores and crystals appeared in the supernatant. After pooling supernatants the spores and crystals were washed by centrifugation. Next the spores were separated from the crystals by flotation. About 23% of the initial crystal total was recovered. A point of particular interest was that it was necessary to wash the original product in order for the spores to show a hydrophobic attraction to the bubbles. No purification ratio or yield were reported.

9. Crystal Structure

(a) Morphological Studies

The first published electron micrographs of a crystal (Hannay and Fitz-James, 1955) indicated that B.t.t. crystals were very pliable, readily bent and under some conditions broken, especially at their tips.

These effects were recorded over a pH range of 5.0 to 8.0. If crystals were bombarded intensely with an electron beam they burst, leaving an oval shaped blob. However, with a lower beam intensity the crystals seemed to be 'fixed' with a surrounding outer layer or coat, suggesting some type of surface denaturation. Young and Fitz-James (1959b) noted that crystal refractility increased during sporulation and that crystal affinity for dyes decreased. At the same time, they suggested that the crystal was undergoing a maturation process. An alternative explanation, that might be worthy of consideration, could also be surface denaturation. An additional observation by Hannay and Fitz-James (1955) of special note, was that the edges of the crystal appeared to be serrated and a repeating surface periodicity was apparent with long rows running parallel to the base of each of the two pyramids forming the crystal (Figure 2a). The rows themselves appeared to contain spherical units, but upon rupture of the crystal with an electron beam, the smallest structure was always a row of spherical units (length not given). When crystals were dispersed in alkali, small globular units were noted with diameters much less than the width of the repeating rows. These authors suggested that different forces might be responsible for holding the crystal together.

The periodicity of the horizontal rows was confirmed by Norris and Watson (1960) using crystals from B.t.a. In addition they reported a row width of 252Å and suggested that the ridging reflected the molecular packing of the subunits within the crystal. These workers also noted that the observed morphology of the crystal suggested an ellipsoidal, rather than a globular, subunit. Labaw (1964) extended the electron microscope work and showed that a periodicity was apparent on the surface

Figure 2

Bacillus thuringiensis crystals. An electron micrograph (160,000 x) and proposed structural models.

Figure 2

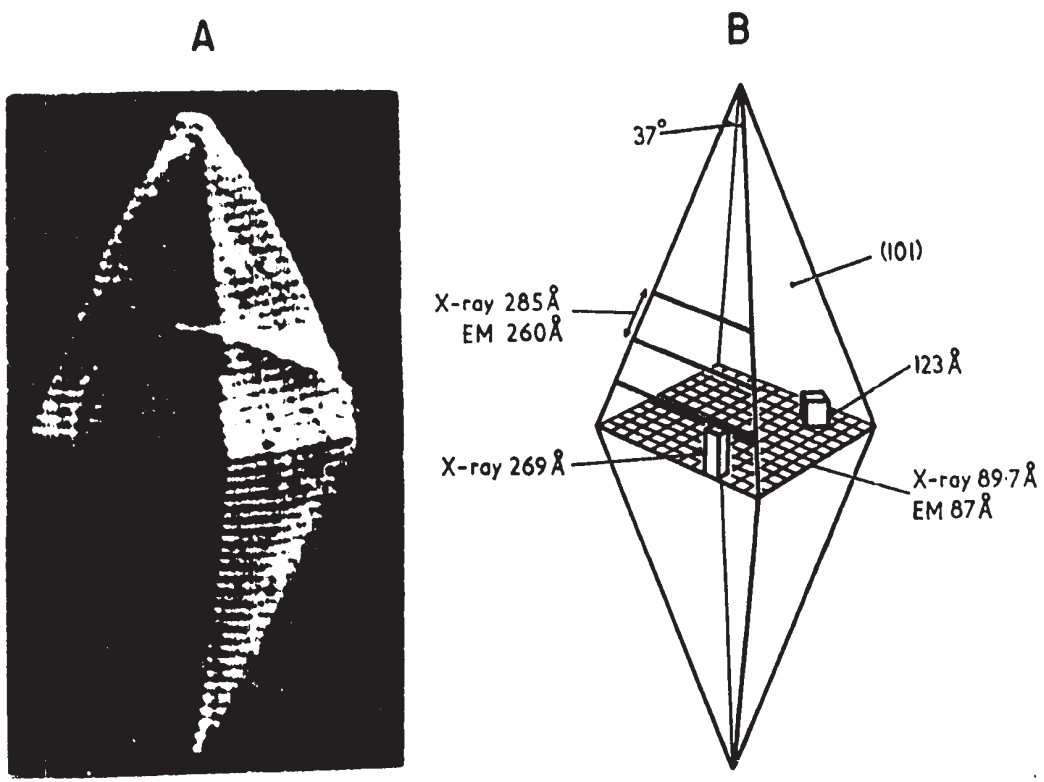
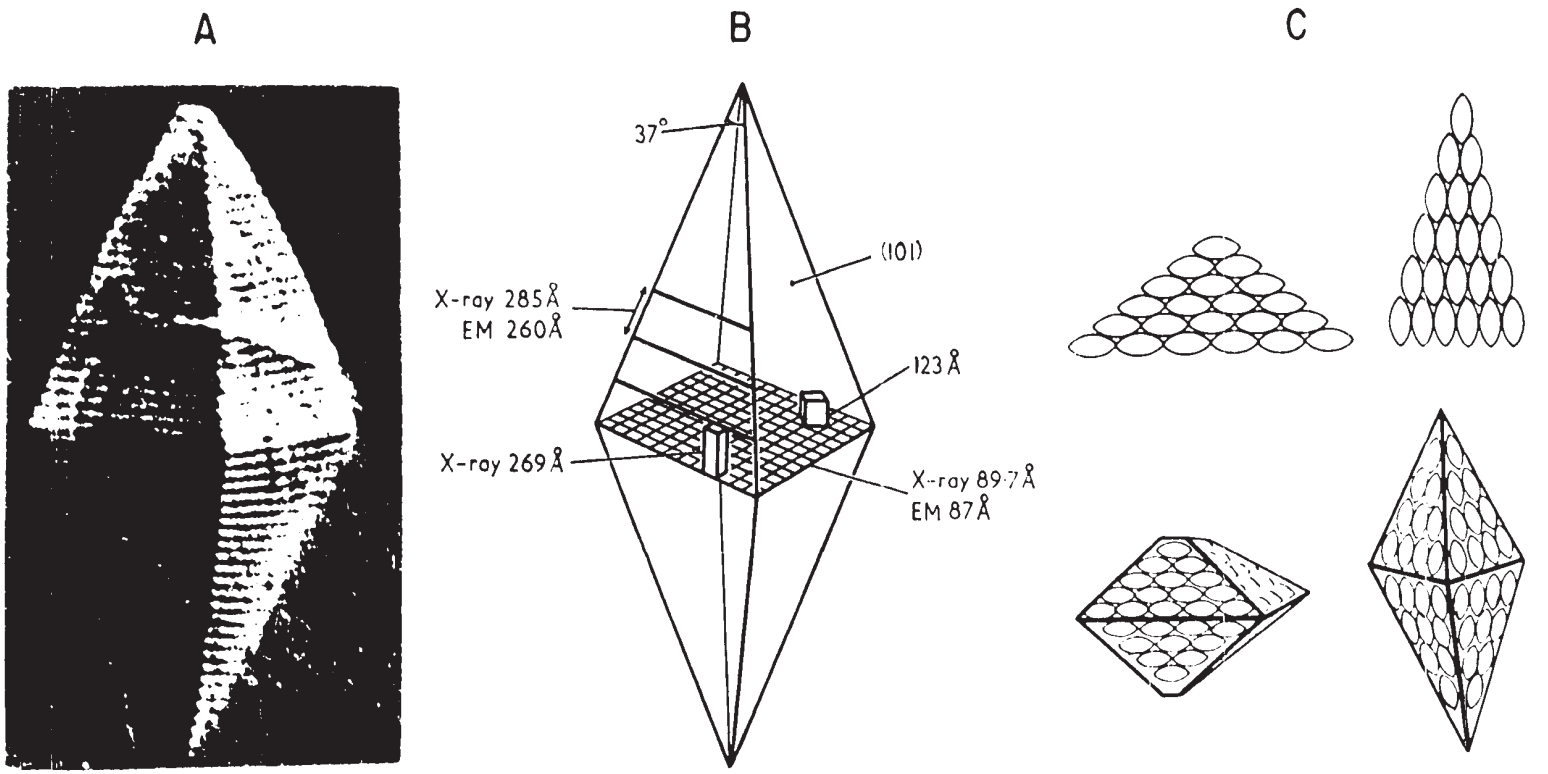


Figure 2



of the crystal in both the horizontal and vertical planes. This latter periodicity ran obliquely away from the crystal base at an angle of 60 degrees to the direction of the horizontal rows. The average width of rows on the crystal surface was 260Å when measured at right angles to the short axis of the crystal. Also each row was marked at intervals of 87Å along its length.

A study employing x-ray crystallography was carried out by Holmes and Monro (1965) using powder diffraction methods because of the small size of the B.t. crystal. They noted that previous electron microscope studies revealed a fine striated surface structure, suggesting a true crystalline nature. Their x-ray data were such as to indicate nearly perfect crystallinity. These data were the same for half grown crystals, mature crystals, and the latter when kept for five years. Only one type of repeating structure was detected, this was an ellipsoidal unit cell, 269Å long and 89Å in diameter with a calculated molecular weight of 230,000. Monro (1959) had previously established that crystals were square in cross section and Holmes and Monro (1965) suggested that the rows seen with the electron microscope were actually made up of the unit cells as detected by x-ray studies. The diagramatic model proposed by these workers is shown in Figure 2b. The slightly smaller dimensions obtained by electron microscopy could be accounted for by shrinkage of the crystals when they were prepared on the grids. These authors further noted that edges frequently seen at the rows on the crystal surface were probably layers in the process of being formed by the fitting into place of the unit cells as they were attracted to the crystal. In short, crystal growth appeared to be a surface phenomenon where building units were attracted and held. Unpublished electron micrographs of Hannay

tend to support this notion.

Grigorova, Kantardgieva and Pashov (1967) studied two different strains of B.t.t., one produced a normal bipyramidal crystal while the other formed cuboidal crystals in addition to normal crystals. Varying shaped crystals, from bipyramidal to cuboidal with intermediate forms, had been reported by Steinhaus and Jerrel (1954) and also by Hannay (1956). From a series of electron micrographs Grigorova et al (1967) showed that both types of crystals possessed a characteristic periodicity of parallel rows. They measured the varying lengths of the rows and the distance between them and from this deduced the size of the basic unit which built the rows in each crystal shape. The measurements showed that differences in lengths of two adjacent rows were constant, as were the differences in distances between the rows. It was shown that the width of the row in the bipyramidal crystal was equal to the difference in the length between the rows in the cuboidal crystal. Conversely, the length difference in the former equalled the width of the rows in the latter. A diagramatic model as shown in Figure 2c, was proposed for the two crystal structures. It will be noted that for both crystal forms the repeating units are ellipsoidal, with the orientation of these units dictating gross morphology of the crystal. At high magnification these workers observed ellipsoidal building units in both forms of crystals. It was further suggested in this work that the greater toxicity associated with cuboid crystals, when compared to bipyramid crystals, might be related to the less stable characteristic of the former. Presumably insect activation of the cuboidal crystal could be more easily accomplished.

(b) Chemical Studies

(i) Effect of Alkaline Conditions

Dissociation of the crystal has always been carried out in an alkaline environment. No reports are known whereby crystals have been solubilized at neutral or acid pH. In some investigations the severity of the alkaline treatment has been lessened by the addition of reducing and/or dispersing agents.

Hannay and Fitz-James (1955) performed a stepwise alkaline extraction on purified crystals of B.t.t. Their findings are summarized in Table 6. It will be noted that although crystal swelling occurred, no protein was solubilized until the last step. A similar extraction process on a spore and crystal preparation showed protein solubilization at each step. Crystals from B.t.a. were reported to swell between pH 10.5 to 11.8 but soluble protein was not released until 11.8 to 12.1 (Young and Fitz-James, 1959b). In an earlier study Doak and Lamanna (1948) showed that an alkali treatment (0.9N KOH) of Bacillus cereus spores apparently altered their serological characteristics. Hence although spores are more stable to alkaline treatment than crystals, it cannot be assumed that no spore protein is removed from this seemingly more stable structure.

After solubilization of purified crystals at pH 12.55 Hannay and Fitz-James (1955) reported that a jelly-like precipitate was formed upon centrifuging at 10,000 x g for 5 minutes and that 84% of the total crystal protein was in this precipitate, i.e., only 16% of the crystal protein was solubilized. The ratio of alkali to protein and the time of reaction were not reported. It was also shown that not all of the solubilized crystal protein was precipitated with 20% trichloroacetic

TABLE 6

Alkaline Extraction of Bacillus thuringiensis var thuringiensis Crystals

pH	Temperature (°C)	Protein Solubilized	Remarks
10.2	Room	Nil	Crystals start to lose refractility
9.2 - 9.5	30 - 37	Nil	Crystals start to lose refractility
10.7	Room	Nil	Crystals increase in size, shape retained
11.8	Room	Nil	Crystal swelling is extreme
12.2 - 12.5	Room	Large amounts	Colorless, viscous mixture is formed

acid, apparently some low molecular weight material was also formed. The different protein fractions possessed slight differences in their ultraviolet absorption spectra. Thus one of the earliest studies on the crystal protein seemed to suggest a heterogeneous crystal structure.

Angus (1956b) solubilized crystals of B.t.s. in 0.05N sodium hydroxide and noted a large decrease in insect toxicity. Only about 7% of the original crystal toxicity remained. This loss was attributed to the action of the strong alkali. When crystals were solubilized with 0.01N sodium hydroxide toxicity losses were less and the preparation retained its toxicity for a longer time. Such preparations were toxic to insects only on ingestion and injection into the hemolymph caused no toxic effect.

The preceeding observations of Angus on B.t.s. were confirmed by Watanabe, Tsutsui and Iwahana (1966) who used the same bacterial variety. In addition it was shown that when crystals were solubilized with alkali at pH 11.5 with 1.0% thioglycollic acid, two major protein fractions (sedimentation coefficients 3.5 and 2.8) and other minor protein components (number not specified) were produced. Toxicity resided in a minor component. Holmes and Monro (1965) also showed that alkali alone (0.1N sodium hydroxide) caused crystals of B.t.t. to swell initially and then to dissociate. Ultracentrifugation studies demonstrated the presence of at least two molecular components of 4.1S and 0.4 - 0.85S, each present in about equal quantities. Alkali solubilized crystal protein could not be induced to re-crystallize. Cooksey (1968) treated spore-crystal mixtures (Pc 2 mg/ml) with 0.04N sodium hydroxide and obtained up to 13 major and minor components on gel electrophoresis.

There is some evidence in the literature suggesting that

crystals from different varieties may possess different degrees of stability. Angus (1956b) reported that B.t.s. crystals were solubilized at pH 10.5 - 11.0, while Hannay and Fitz-James (1955) reported solubilization of B.t.t. at pH 12.2 - 12.5, and Young and Fitz-James (1959b) reported B.t.a. crystal solubilization at pH 11.8 - 12.2. However it must be noted that many variables associated with these reaction systems were not comparable.

Fitz-James et al (1958) studied crystals from B.t.a. and also found a critical pH point for breakdown. When crystals were adjusted to pH 12.2 with sodium hydroxide only 9% of the protein was solubilized and a gel-like pellet was obtained which was highly toxic. When 0.1N sodium hydroxide was used to dissolve the crystals (Pc 1 mg/ml) alkali damage reduced the toxicity several fold. Young and Fitz-James (1959b) reported that if 2.0% thioglycollic acid were added, crystal solubilization was detected at pH 11.5. Thus disulphide bonds were implicated in the crystal structure. The existence of a reducing environment in the gut of many, if not all, susceptible insects indirectly supported the possibility of a stabilizing effect due to disulphide bonds in the crystal.

Thioglycollic acid was also used by Lecadet (1966) when dispersing B.t.t., and the alkalinity of the reducing environment was lowered to pH 9.5. This was obtained by using 0.1M carbonate buffer. At least three forces were acting directly to solubilize the crystal. These were: reducing agent, moderate alkalinity and salt ions from the buffer system. It was shown by this treatment that essentially all of the cyst(e)ine sulphur in the crystal was present in the oxidized (disulphide) form. A similar observation was made on bacilli spores

(Vinter, 1961). Lecadet (1966) also analysed the solubilized protein by ultracentrifugation and showed nine different size molecules were present, sedimentation coefficients ranged from 0.8S up to 27.0$S. The longer a crystal preparation remained in the dispersing milieux, the smaller were the sedimentation values for that preparation. An overall reaction equilibrium shifting to the smaller molecules was apparent. Dissociation of different samples of crystals from the same bacterial variety gave variations in molecular size as well as a different percentage composition for each size, however the smallest molecule always had a sedimentation value of 0.83. Such results tend to suggest that the variation in crystal breakdown encountered with different batches of the same crystal stock may be due to slight variations in the storage and/or handling of the different batches, i.e., crystal stability may be influenced by crystal environment.

Few reports may be found in the literature where classical protein dispersants have been used on the crystal. Anderson and Rogoff (1966) reported that crystals could be partially solubilized at pH 9.5 if urea and potassium borohydride were added to the system (concentrations were not specified). Five different fractions were obtained by paper ionophoresis. Lecadet (1967) reported that urea up to 8.0M did not release soluble protein from the crystal but it did cause irreversible modification to the state of aggregation.

The number of protein and peptide fractions obtained upon alkali mediated dissociation of the crystal has been shown to vary greatly. Depending upon individual preference one may pick almost any number between one and thirteen and a report may be found outlining a method for breaking down the crystal to that number of fractions. This situation

was summarized quite adroitly by Rogoff (1966) who stated that "one does not readily rid himself of the gnawing feeling that the methods of solubilization of the crystal are reflected in the components observed".

(ii) Effect of Buffer Salts

Angus (1956a) suspended crude preparations of B.t.s. in gut juice of the silkworm. After reacting, the system was centrifuged and the soluble supernatant was toxic when fed to silkworms. Due to the alkaline nature of the gut juice, solubilization was attempted next using only 0.2M carbonate buffer (pH 10.5). On ingestion the supernatant from this latter system was toxic. Because of the comparatively high pH it was not clear what role, if any, was played by the buffer ions in crystal solubilization. Faust et al (1967) conducted a more detailed investigation into the effect of buffer ions on crystal solubilization. Table 7 summarizes their results. It will be noted from this table that some crystal protein was solubilized by buffer and that additional protein was solubilized by the inclusion of proteolytic enzyme. Such salts and enzymes are common constituents in the gut contents of susceptible insects. In an earlier work Faust and Estes (1966) showed that in addition to carbonate, tris buffer was effective in solubilizing crystal protein. Protein loads during these studies were 1.2 mgm. Such results would tend to implicate ionic bonding in some way in the structure of the native crystal.

(iii) Effect of Proteolytic Enzymes

The involvement of insect peptidases (proteases) in the solubilization and hence activation of the crystal was demonstrated by Angus (1956a). It was later shown by Horie, Tanaka and Ito (1963) that

TABLE 7

Solubilization of Crystals With Buffer Salts and Proteolytic Enzymes

Reagents Used	pH	Protein Solubilized (1)
Bicarbonate Buffer 0.05M	9.2	14.28%
Potassium Carbonate 0.15M (2)	11.2	27.04%
Silkworm midgut contents (undialyzed, heated to 100°C)	8.8	20.99%
Silkworm midgut contents (3) (undialyzed, unheated)	9.0	50.00%
Silkworm midgut contents (dialyzed against distilled water and then supplemented with 0.15M potassium carbonate)	11.2	67.34%
Dialyzable fraction from above (concentrated to original volume)	8.9	21.42%

(1) Pc = 196 µgm/ml (final)

(2) Silkworm gut juice normally contains 0.15M potassium carbonate

(3) Peptidases in silkworm midgut juice have an optimum for activity in the range pH 11.0 - 11.5

both silkworm digestive and midgut juices possessed peptidases capable of acting up to pH 11.5. Peptidase activity in the gut was highest during larval stages of growth, i.e., the stage susceptible to crystals.

Watanabe (1966) demonstrated that silkworm peptidase, in an alkaline environment, activated B.t.s. crystal preparations to a maximum level of toxicity which remained stable on prolonged incubation. However, pepsin, pronase or chymotrypsin caused activation of previously solubilized crystals to a similar maximum level but on prolonged incubation the toxic activity of the preparations decreased. Monro (1961a) showed that native B.t. crystals were not attacked by trypsin until they were first denatured by heating in citrate-phosphate buffer at pH 3.0, for 10 minutes at 100°C.

Lecadet and Martouret (1962) treated crystals with the anterior gut contents of Pieris brassicae. Dialysis of the reaction mixture gave two fractions of different molecular weights, both caused the same toxic effects. In a later study (Lecadet, 1967) it was shown that at least ten fractions were present when crystals were solubilized by these enzyme preparations in 0.1M bicarbonate buffer (pH 10.5). The solubilized material was toxic by ingestion and also by injection. Alkali solubilized crystals were not toxic via this latter route. The degree of activation seemed to be greatest when proteolytic enzymes were present.

(iv) Immunochemical Investigations

Due to its proteinaceous character the crystal has been subjected to many serological studies. The antigen for these studies has been either the native crystal in its intact state or solubilized crystal protein. Pendleton and Morrison (1966) showed that when the antigen was pure native crystals, only one antibody was produced by the host animal. However, if a soluble antigenic preparation were used,

(prepared by using 0.05M sodium hydroxide) then many antibodies were formed, apparently dissolution of the crystal increased the number of antigens. Monro (1961b) demonstrated that whole crystals reacted only weakly to the antiserum against alkali dispersed crystals. It was determined by Murray (1967) that normal rabbit serum had no solubilizing effect on purified crystals of B.t.t. and B.t.s. If a repeating subunit is assumed to build up the final crystal structure, then such observations imply subunit degradation and/or contamination.

The great similarity between crystals of different B.t. varieties was demonstrated by Pendleton and Morrison (1966), who treated 94 alkali solubilized crystal preparations against the antisera from nine different whole crystal preparations. Most isolates reacted with at least one of the nine antisera and some isolates cross reacted with up to four antisera. A similar study, but not as inclusive, was carried out by Krywienczyk and Angus (1965, 1967). They reported a common toxic component in B.t.t., B.t.e. and B.t.s. These workers also reported that old preparations of alkali dissolved crystals gave only a single precipitin band, while fresh preparations showed several antigenic entities. Such results would seem to corroborate the ultra-centrifugal observations of Lecadet (1966).

(v) Miscellaneous Observations

The effect of heat on the crystal was first reported by Angus (1956b), who showed that native crystals could be boiled in water for 30 minutes before a loss of toxicity occurred. Alkali solubilized crystal toxin was inactivated when heated to 50°C for 30 minutes. The native structure of the crystal would appear to endow it with a considerable degree of heat stability. Angus (1956b) also reported that

the alkali solubilized toxin was stable over a wide pH range (3.0 - 12.0), however certain organic solvents (methanol, ethanol, acetone and chloroform) caused a loss of toxicity. Native crystals were not adversely affected by these solvents.

Ultraviolet light has been reported to have an adverse effect on the toxicity of B.t. preparations. Raun et al (1966) showed that it drastically reduced toxicity, and after 72 hours all toxicity disappeared. Cantwell (1967) reported that B.t.s. preparations were more stable to ultraviolet irradiation than those from B.t.t. It is not clear whether such results are due to bacteriocidal action, thereby reducing the possibility of death due to septicemia, or to a photolytic inactivation of the crystal.

A peculiar effect of boric acid on B.t. toxicity has been reported by Doane and Wallis (1964). After checking many organic and inorganic acids it was shown that boric acid (1% w/w) enhanced the toxicity of Thuricide about two fold against gypsy moth larvae. Boric acid alone had no adverse effect on these insects. Faust et al (1967) reported that borate buffer (0.05M at pH 9.0) did not have any solubilizing effect on crystals.

From the preceeding chemical studies it is clear that the stability of the native crystal can be influenced by methods and reagents known to rupture several different types of chemical bonds. It would also seem quite reasonable to assume that the characteristic crystal stability may not be due to a single type of bond but rather to the combined effects of several different bonds.

(c) Molecular Size of Components

Holmes and Monro (1965) subjected purified crystals to x-ray crystallography and found a repeating unit cell that was 89Å in cross section and 269Å in length. This unit cell was believed to be made up of two to four spherical or ellipsoidal subunits. The molecular weight of the unit cell was calculated to be 230,000. The large size of the unit cell probably plays a determining role in the overall size of the crystal aggregate. Labaw (1964) had earlier shown with the electron microscope that the subunit of the crystal was 87Å in diameter. If three subunits are assumed to exist in the unit cell, the subunit molecular weight would be 77,000. The basis for such an assumption is not solid. As pointed out by Holmes and Monro (1965) the subunit molecular weight could be also 115,000 or 57,500.

When crystals from B.t.t. were hydrolysed by gut enzymes from Pieris brassicae at pH 9.5 in 0.2M bicarbonate buffer (Pendleton, 1968) it was shown that only two molecular fractions were obtained by sieving this material on Sephadex. One fraction had a molecular weight of approximately 200,000 and was toxic only on ingestion, the second fraction had a molecular weight of 5,000 - 10,000 (also determined on Sephadex) and was toxic by both ingestion and injection. No molecules of intermediate size were detected. A similar enzymatic solubilization procedure was carried out by Lecadet and Martouret (1967) except that the reaction time was increased from three to six hours. The largest component obtained via the ultracentrifuge had a molecular weight of 50,000 (S = 4.0) while the smallest components had molecular weights of 13,000 (S = 0.99) and 5,000 (S = .41). Watanabe (1966) solubilized B.t.s. crystals with sodium hydroxide at pH 11.5 and 1% thioglycollic acid and

two major molecular fractions (S = 2.8 and 3.5) were obtained, neither was toxic on injection.

Thus it would seem that a unit cell of molecular weight 200,000 - 230,000 might be the repeating building block in the crystal. This structure in turn might be made up of several intermediate molecular forms, with molecular weights around 50,000. Neither of these size ranges are toxic on injection, hence activation is not yet complete. The molecular entity possessing maximum toxicity, appears to be in the molecular weight range of 10,000 $\pm$ 5,000. Pendleton (1968) noted that when crystals were solubilized at least 85% of the crystal protein was recovered as an active toxin with a molecular weight of 5,000 to 10,000.

(d) Amino Acid Composition

The finding that B.t. crystals were essentially protein, as reported first by Hannay and Fitz-James (1955) has been confirmed by other workers using different varieties. The first quantitive report on the amino acid composition (Angus, 1956b) has been supported by the results of Lecadet (1965) as well as Anderson and Rogoff (1966). Spencer (1968) reported on the amino acid composition of five different varieties of B.t., the results for the three varieties of interest in this study are shown in Table 8.

A cursory study of Table 8 shows that the B.t. crystals are made up of amino acids in quantities that would be expected in globular proteins. No single residue exists in a quantity that might explain the unique crystal stability. A more detailed scrutiny of the three sets of values shows that all are high in dicarboxylic residues, a fact that could explain the low isoelectric point of alkali solubilized crystal

TABLE 8

Amino Acid Composition of Parasporal Inclusions From *Bacillus thuringiensis* Varieties

Amino Acid	*sotto*	*thuringiensis*	*entomocidus*
	%	%	%
Glutamic acid	17.0	16.0	16.0
Aspartic acid	12.3	14.8	14.0
Glycine	5.0	4.8	4.8
Alanine	4.9	5.0	4.8
Valine	6.9	7.0	7.8
Isoleucine	6.3	5.6	6.6
Leucine	10.0	9.5	10.5
Serine	5.6	6.0	6.0
Threonine	5.3	7.0	6.8
Cystine	1.2	1.1	1.4
Methionine	0.82	0.85	0.67
Lysine	4.3	4.5	4.4
Arginine	10.2	9.1	9.0
Histidine	3.0	3.0	2.8
Phenylalanine	6.5	6.0	6.7
Tyrosine	6.4	6.5	6.5
Tryptophan	2.4	2.3	2.3
Proline	4.3	4.6	4.4

protein. Also of interest are the low values of cystine, for such a stable structure one might expect higher levels of this residue. A point of further interest is that in all three varieties the limiting amino acid is methionine. The total amino acid content was in the range of 112% - 115%, well within the experimental error for the theoretical yield of 118%.

III MATERIALS AND METHODS

A. Growth Studies

1. Culture Maintenance

Lyophilized strains of B.t.t., B.t.e. and B.t.s. were kindly provided by Dr. T.A. Angus. Each lyophil was slurried in sterile water and then subcultured onto the agar medium of Schaeffer (1961). The constituents of this medium are given in Appendix I. After incubation a microscopic check was made of the colonies and those producing large amounts of free crystals were isolated and subcultured onto a lyophilization medium (Appendix I). Two-step lyophils were prepared from each B.t. variety after it had sporulated. During growth studies a fresh two-step lyophil was opened each week and the entire contents were slurried in sterile water. This slurry was used to streak sufficient slants of Schaeffer's agar to last for one week. Cultures were not stored on agar slants for more than a week.

2. Culture Technique

Cultures of B.t. were propagated under the following conditions:

(a) Shake Flasks

Wide mouth, 300 ml non-baffled shake flasks were obtained from Bellco Glass Inc., Vineland, N.J. Metal closures were used to maintain sterility which was achieved by autoclaving at 121°C for 15 minutes. Unless otherwise noted a liquid volume of 50 ml was used in these flasks. After inoculation the flasks were shaken on a rotary shaker with a two inch throw at 200 revolutions per minute.

(b) Bench Fermentors

The growth systems used here were essentially the same as the 'Bio-cultural Assemblies' produced by Scientific Products, Evanston, Illinois. The fermenting jars had a total capacity of 7.5 liters, however a 5.0 liter working volume was used during these studies. The height to width ratio of the jars was 3:1. After being charged with media and properly assembled the fermenting pot was autoclaved at 121°C for 30 minutes. Systems were stirred at 400 r.p.m. and aerated at a rate of six liters per minute throughout the entire growth period.

(c) Pilot Fermentor

This was designed to complement the bench type fermentor, the total volume was 1,000 liters and a working volume of 600 liters was maintained. The system was sterilized at 121°C for 45 minutes. Agitation was at a rate of 186 revolutions per minute while the rate was 600 liters per minute for the first 24 hours and thereafter 400 liters per minute. Throughout the growth period a back pressure of 15-20 p.s.i. was maintained.

(d) Inocula

An inoculum level of 2.0% (v/v) was used at all stages. Trypticase Soy Broth (Appendix I) was obtained from Baltimore Biological Laboratories, Baltimore, Maryland and used as the inoculum (seed) medium. Growth periods for the inocula were standardized at 11.5 $\pm$ 0.5 hours. Bench fermentors were used to propagate the seed for the pilot fermentor.

(e) Media Constituents

Soytone, an enzymatic hydrolyzate of soybean meal, was obtained from Difco Laboratories, Detroit, Michigan. Totamine, an enzymatic hydrolyzate from casein, was obtained from Delmar Chemicals, Montreal, Quebec. All other chemicals used during growth studies were of reagent grade and obtained from Fisher Scientific Ltd., Toronto, Ontario.

(f) Miscellaneous Parameters

All growth studies were conducted at 28 $\pm$ 1°C. Untreated tap water was used in the make up of production and seed media. Antifoam 'C' (Dow-Corning Silicones Ltd., Toronto, Ontario) was automatically added into the fermentors to suppress foam.

3. Culture Monitoring

The progress of a growth system was followed by aseptically withdrawing culture samples and subjecting them to one or more of the following determinations.

(a) Microscopic Observations

These were done using a Zeiss or a Leitz microscope at a magnification of at least 1,000 x. Samples were negatively stained with an equal volume of 1.0% (w/v) nigrosin.

(b) Turbidity

The changing biomass was followed by absorbance variations at 660 mμ using a Klett-Summerson Colorimeter with filter No. 66.

(c) pH

Changes in the pH of the culture samples were determined using a Radiometer pH meter (model No. 28) with a combined electrode (No. GK2303C). This electrode could also be chemically sterilized with ethanol and formaldehyde and then aseptically fitted into the 7.5 liter fermentors. A continuous pH pattern during growth was then obtained using the above meter and a YSI Recorder (model No. 80). All of the preceding pH equipment was obtained from Canadian Laboratory Supplies Limited, Toronto, Ontario.

(d) Peptidase Assay

The occurrence of extracellular peptidase (protease) in the culture broth was determined by the method of Hagihara, Matsubara, Nakai and Okunuki (1958) using casein as the substrate.

(e) Biomass

The particulate material in a culture sample was precipitated by centrifuging in tared tubes at 10,000 x g for 15 minutes. The supernatant was decanted and the weight of the biomass was determined.

(f) PHB Assay

This was carried out using the alkaline carbonate and hypochlorite treatment of Williamson and Wilkinson (1958). The effects of this procedure on vegetative cells were microscopically followed.

4. Culture Product

At the completion of the growth period particulate matter was recovered from the culture broths by centrifugation. In order to enhance stability this material was lyophilized.

B. Crystal Purification

Variations in size, electrical charge and hydrophobicity appeared to be the major differences between spores and crystals upon which a successful purification method might be based. Centrifugal methods utilizing size differences were not attempted due to the considerable time and effort required for comparatively small amounts of purified crystals. Electrolysis experiments were conducted and it was hoped that negatively charged spores would migrate at a different rate than crystals through an electric field in a liquid bridge. These attempts were fruitless.

The greater hydrophobicity of spores, when compared to crystals, was exploited next. Crude lyophilized preparations of B.t.t., B.t.e. and B.t.s. were used, these possessed few, if any, vegetative cells. To ensure the lysis of any remaining cells a standard working suspension of 0.5% w/v was made with distilled water and stirred gently for two hours at 35°C. As a result of this treatment crude preparations contained essentially spores, crystals and cellular debris. Initial

separation attempts using detergents (Span and Tween series) and organic solvents (benzene and petroleum ether) showed some clumping of spores, but many free spores and crystals remained. Aliphatic solvents offered a higher degree of spore clumping with no concomitant crystal clumping.

From the homologous series, carbon tetrachloride, methylene chloride and chloroform, the latter was finally selected as the best inducer of spore and cell clumping. A purification scheme was worked out where 15 ml of chloroform (reagent grade, Fisher Scientific Ltd., Toronto, Ontario) were added to 10 ml of the previously stirred crude preparation of B.t. This system was contained in a 50 ml screw cap test tube (150 x 25 mm) and subjected to vigorous agitation on a vortex type mixer for 60 seconds. Ten tubes were usually worked on at any one time. A solvent-solid suspension was obtained which was unstable and could be further handled by one of two possible methods.

When the suspensions were allowed to stand on the bench a two phase system settled out after about ten minutes. The upper phase (about 8.5 $\pm$ 0.5 ml) was carefully withdrawn and microscopically observed. Massive aggregates of spores could be seen. Crystals remained essentially free and unclumped, however some crystals could be seen entrapped within the aggregates. The upper phase was withdrawn gently and passed through a filter bed.

In the second method the tubes were subjected to a gentle centrifugation (350 x g for 10 minutes) and a three phase system resulted. The bottom layer was essentially chloroform while the middle layer was a compact mass of aggregated material (including many trapped crystals). The upper layer (about 9.5 $\pm$ 0.5 ml) contained smaller

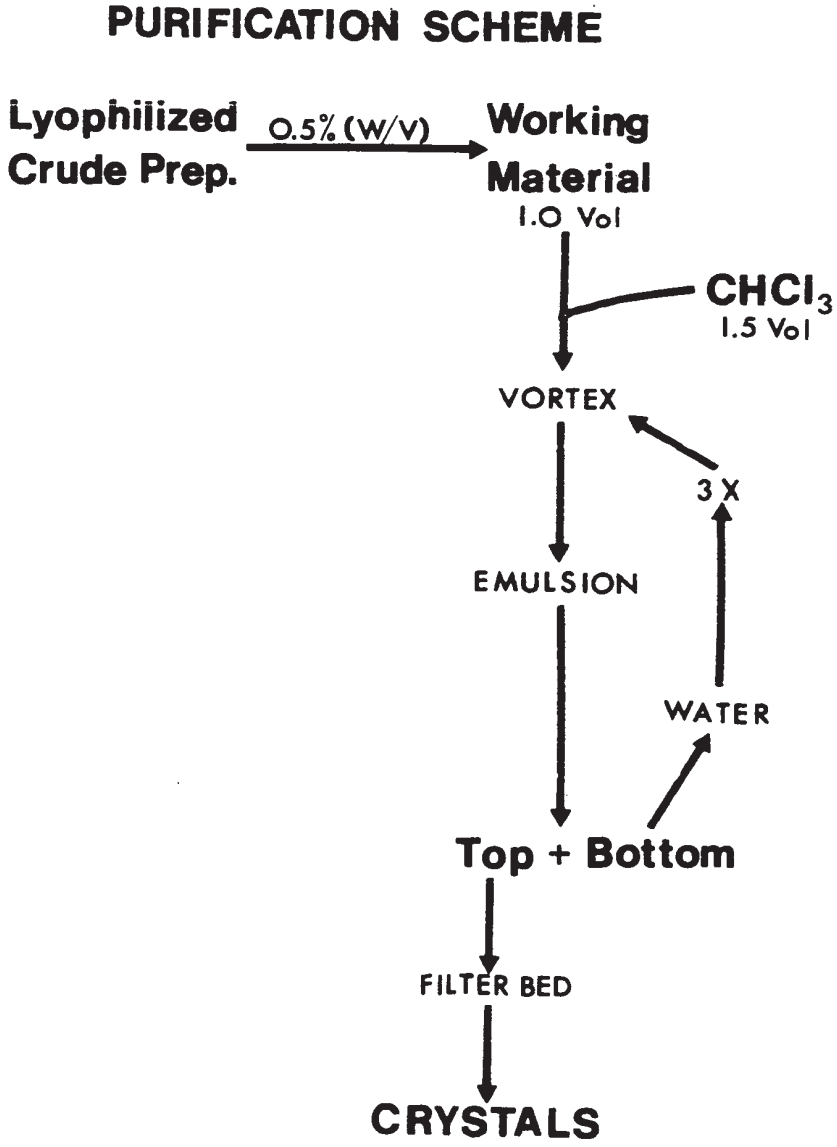
aggregates and more free crystals than the upper phase obtained on standing. This upper layer was also withdrawn and passed through a filter bed.

The purpose of the filter bed was to retain the aggregated material (i.e., spores and vegetative debris) and to permit free crystals to pass through. Various types of filter material were used; thick, loose filter beds proved to be the most effective. These were made by placing different types of filter papers, e.g. Whatman Numbers 4 and 1, together on a Buchner funnel or by using the prefilter pads provided with cellulose acetate microfilters of the Millipore and Gelman type. A glass fiber filter, type E, obtained from the Gelman Instrument Company, Ann Arbor, Michigan was particularly suitable during the latter stages of these studies. However it should be noted that the suitability of a given prefilter was not constant and seemed to vary from supplier to supplier and indeed from batch to batch. Filtration was always done at atmospheric pressure.

The material that remained in the screw cap tubes was again subjected to vortex mixing after a volume of water had been added to replace the upper phase. After phase separation had occurred (either by standing or centrifuging) the crystals were again recovered from the upper layer by filtration. This procedure was carried out a total of three times if just standing were employed and four times if the centrifugal method were followed. Figure 3 schematically outlines this purification method.

The crystal containing filtrates were pooled and washed three times by centrifugation. The final supernatant gave a negative protein value, determined by the method of Lowry, Rosenbrough, Farr and Randall

Figure 3


PURIFICATION SCHEME
Lyophilized Crude Prep.
0.5% (W/V)
Working Material
1.0 Vol
CHCl3
1.5 Vol
VORTEX
3 X
EMULSION
WATER
Top + Bottom
FILTER BED
CRYSTALS

(1951). The washed crystals were suspended in distilled water to a final protein concentration of about 250 ± 50 µgm/ml. Biochemical studies were conducted on these purified, standardized preparations.

C. Amino Acid Calculations on the Minimal Polypeptide

In an attempt to shed some light on any possible relationship between crystal composition and stability the amino acid values (Table 8) for crystals of B.t.t., B.t.e. and B.t.s. were subjected to the following calculations.

1. Minimal Molecular Weight

In each of the three B.t. varieties the limiting amino acid was methionine. Therefore an estimate of the minimal molecular weight was based on the amount of this amino acid using the following formula:

$$M.W._{min} = \frac{\text{M.W. of Methionine x 100}}{\text{\% of Methionine in sample}}$$

2. Number of Residues

Once again these calculations revolved around the limiting amino acid, methionine. The amount of each amino acid, expressed as micromoles per milligram of crystal protein was used to estimate the number of residues. These were expressed to the nearest whole integer.

3. Helical Content

Davies (1964) showed a strong correlation between the helical content of 15 different proteins and their total amount of Val, Ile, Ser, Thr and Cysteine. The graphical relationship found on page 608 of

the original article was used to estimate the helical contents of the minimal polypeptide in each of the three B.t. varieties.

4. Non-Polar Nature

The ratio of polar to non-polar residues in the three polypeptides was estimated by the method of Waugh (1954). The frequency of the non-polar sidechains (N.P.S.) was calculated by using the following equation:

$$N.P.S. = \frac{\text{Residues of Val + Leu + Pro + Phe}}{\text{Total Number of residues in polypeptide}}$$

5. Polarity Ratio

The internal volumes of the polypeptides and also the volume of their external shells were calculated by the method of Bigelow (1967). The polarity ratio, originally defined by Fisher (1964), was calculated as follows:

$$\text{Polarity Ratio} = \frac{\text{Volume of External Shell (Polar Volume)}}{\text{Internal Volume (Non-Polar Volume)}}$$

6. Hydrophobicity

Hydrophobic residues were estimated by the technique of Van Holde (1966) who merely summed the total percentage of Val, Leu, Ile, Pro and Phe present in a polypeptide.

7. Average Hydrophobicity

The hydrophobicities of each amino acid residue, as outlined by Bigelow (1967), were used to calculate the total hydrophobicities of

the three polypeptide chains. The average hydrophobicities were then calculated as follows:

$$\text{Average Hydrophobicity } (H\ \bar{\Phi}\ \text{avg.}) = \frac{\text{Total Hydrophobicity}}{\text{Total Number of Residues}}$$

D. Biochemical Studies

1. General

Only crystals of B.t.s., which had been purified in accordance with the preceding purification method, were used during these studies. Crystals were isolated from lyophilized crude material that had been produced during a single run on the pilot scale fermentor. Immediately after chloroform purification the crystals were at the approximate pH of the culture broth (pH 8.50 $\pm$ 0.30). Crystals were washed with distilled water until a neutral pH environment and a protein free supernatant (Lowry method) were achieved. The size and color of the crystal pellets were noted during washing. All subsequent references to crystals imply purified and washed B.t.s. crystals, unless otherwise stated.

2. Crystal Composition

A readily available supply of highly purified crystals provided the opportunity to confirm the published amino acid composition of B.t.s. (Spencer, 1968) and also to reconfirm the amounts of methionine and cystine. Determination of amino acids was done on a Technicon Amino Acid Analyser using standard methods after hydrolysis of two mg samples under vacuo for 20, 40, 70 and 140 hours at 110°C. A brownish-black humin-like material remained in the hydrolysis tubes even after 140 hours.

A photomicrograph was taken of this material. Cystine and methionine were determined by the method of Moore (1963).

The amount of water bound to crystals was estimated by two methods. Firstly, highly purified and washed crystals were lyophilized or dried over phosphorus pentoxide. The weight of each sample was then determined in a tared vessel. These vessels were opened and the systems were allowed to equilibrate in a humid chamber for 48 hours. The weight of the adsorbed water was determined. In the second method crystals were allowed to air dry and the weight of the material was determined. The dry crystals were resuspended and the total protein content of the sample was assayed according to the Lowry method.

The isoionic point of crystal suspensions was determined after a constant pH was reached by repeated washing of crystals in deionized-glass distilled water. The pH determinations were done using an expanded scale pH meter (Radiometer, model No. 26). The treated water had a conductivity of less than 2×10^{-6} μ mho.

3. Crystal Structure

(a) General

Preliminary experiments were carried out with purified crystals to provide a degree of familiarity when handling this peculiar protein. As a result of these studies certain analytical procedures were selected because of their usefulness and applicability. Microscopic observations on variously treated crystals showed changes in crystal size, shape and refractility as well as crystal clumping. These samples were usually stained with an equal volume of nigrosin (1.0% v/v). Reagents used often reacted with this dye and therefore sometimes unstained preparations

were observed with a green filter placed in the light path of the microscope.

The turbidity of untreated crystal preparations and changes in this turbidity produced by various reagents afforded another analytical approach. Changes in absorbance of a system at 660 mμ reflected changes in crystal size, refractility and the degree of clumping. Absorbance measurements were made using a Klett-Summerson Colorimeter (with a No. 66 filter), a Hitachi Perkin-Elmer Spectrophotometer (model No. 139) or a Beckman Spectrophotometer (model DB-G). Where possible microscopic and absorbance changes were considered together when interpreting the effect of a particular reagent on crystals.

Further analytical tests were carried out when possible by playing on the insoluble nature of the crystal. Protein solubilized during a particular treatment was separated from the remaining crystals and quantitated by the Lowry method. This assay could not always be done because of interference caused by some chemicals, notably guanidine hydrochloride and tris. In some cases the amount of insoluble protein remaining after treatment was determined and then solubilized protein was calculated from the difference.

A summary of preliminary experiments, listing the effects of various reagents on crystals is shown in Table 13. These observations provided a basis from where subsequent biochemical studies could be launched. Unless otherwise noted all reaction volumes were 5 ml in these and the following biochemical studies and glass distilled water, with a conductivity of less than 1×10^{-5} μ mho, was used throughout.

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(b) Effect of Succinic Anhydride

The succinylation method of Habeeb (1967) was followed with the exception that pH control was maintained with a pH stat (Radiometer Titrator, model 11 with a model 28 pH meter) instead of a phosphate buffer. A five fold excess of succinic anhydride was used based on an assumed level of 1% free amino groups in the crystal. An 80.0 ml reaction system was set up at 25°C containing a final Pc of 150 µgm/ml. Minimal volumes of sodium hydroxide at various concentrations were used to hold the pH of the system at different levels over the pH range 4.0 - 12.0. The reaction vessel was initially at pH 4.0. After allowing the succinic anhydride - dioxane reagent to react with the protein for 10 minutes the absorbance of the system was determined as was any soluble protein (Lowry method) in 1 ml of supernatant. The pH of the reaction system was gradually raised in a stepwise manner up to pH 12.0 with absorbance and protein determinations being done at each step.

(c) Effect of Detergents

Cationic, neutral and anionic detergents were mixed with crystals and their effects determined over different pH ranges at 25°C. Cetyltrimethylammonium bromide, Triton X-114 and sodium laurylsulphate were each used at a final concentration of 0.75% w/v. The final concentration of crystal protein in each system was 75 µgm/ml. Changes in absorbance and microscopic characteristics were monitored.

(d) Effect of Thiol and Disulphide Reagents

The effect of each of the following reagents was determined on pure crystals; p-chloromercuribenzoic acid, silver nitrate, sodium

thioglycollate, 2 mercaptoethanol and dithiothreitol. All were used in 0.01M carbonate buffer pH 9.5 at a reaction temperature of 35°C. A final Pc of 100 µgm/ml was used. It was assumed that the total cysteine/cystine content of the crystal protein was 2% and a five fold excess of each sulphur reagent was used. A reaction system containing crystals, sulphur reagent and buffer was set up and monitored at a wave length of 660 mµ. The experiment was repeated at pH 10.0.

(e) Effect of Urea

Purified crystals were subjected to increasing quantities of fresh urea at pH 9.5. A final Pc of 150 µgm/ml was set up in systems containing 2.0, 4.0, 6.0 and 8.0 M urea. The systems were incubated at 35°C and changes in absorbance as well as crystal morphology were noted.

(f) Effect of Guanidine Hydrochloride

Increasing concentrations of guanidine hydrochloride were reacted with crystal preparations held at pH 4.5, 7.5 and 9.5. The final Pc was 150 µgm/ml while the reaction temperature was 25°C. During a reaction time of 120 minutes absorbance and microscopic properties of the system were determined.

(g) Effect of Boiling Crystals in Dilute Acids

Crystals at a final Pc of 120 µgm/ml were heated in a boiling water bath in the presence of different dilute acids. These included hydrochloric (0.03N), acetic (0.25M), oxalic (0.25M) and boric (0.002M). Changes in absorbance in each reaction system were monitored. The experiment was repeated using only hydrochloric and oxalic acids and the amount of protein and free amino acids were measured in the supernatants of each system at various time intervals. Total amino acids were

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measured by the ninhydrin method of Moore and Stein (1954) and the absorbance of the reaction mixture was noted at 570 mμ. The supernatant from the hydrochloric acid system was checked for free amino acids by thin layer chromatography.

(h) Effect of Potassium Carbonate

The effect of 0.15M potassium carbonate on purified crystals was determined at different pH levels. The Pc in all cases was 100 μgm/ml, while reaction time and temperature were held constant at 30 minutes and 35°C respectively. The redox potential of each system was determined with a Radiometer pH meter (model No. 28) using a platinum and calomel electrode system. The experiment was repeated at increasing time intervals. The ability of potassium carbonate to solubilize crystals was followed at pH 11.0 where absorbance changes and protein solubilized were monitored.

(i) Characteristics of 'Old' Crystals

Crystals were studied that had been allowed to soak at 4°C in water for five days and in 0.2M tris-maleate buffer (pH 8.0) for five days and for 30 days. Each of the three preparations was subjected to the action of 8.0M urea at pH 8.0 for various times, the final Pc was 100 μgm/ml. Reaction volumes were incubated at 35°C. The experiment was repeated using 0.3M sodium laurylsulphate in place of urea. Absorbance changes were followed in both experiments.

(j) Soaking Crystals in an Alkaline Environment

Purified crystals in water at 25°C were soaked at pH 9.5 for increasing time periods, the initial Pc was 200 μgm/ml. A 20 ml system in a beaker was held at this pH with a Radiometer Titrator (Model TTT-1).

Sodium hydroxide (0.01N) was used as the titrant, volume changes (less than 5%) were considered negligible. A control system consisting of only water was held under identical conditions. The uptake of sodium hydroxide, as well as microscopic and absorbance changes were followed. At the end of the titration the system was centrifuged and a check for soluble protein was done on the supernatant using the Lowry method. The experiment was repeated at pH 10.0 and 10.5.

Crystals that had been soaked at pH 9.5 for four hours were incubated with trypsin (twice crystallized). The final reaction system containing 10 µgm/ml of trypsin and 100 µgm/ml of crystal protein, was adjusted to pH 7.5 ± 0.25 with minimal volumes of sodium hydroxide. The system was incubated at 35°C for various times. A control was established using unsoaked crystals in place of the soaked preparation. Microscopic and absorbance changes as well as protein solubilization were monitored.

Crystals that had been previously soaked at pH 9.5 for four hours were held at that pH and incubated with fresh 3.0M guanidine hydrochloride which had been previously adjusted to pH 9.5. The final Pc was 100 µgm/ml. A similar system was set up using crystals that had not been alkali soaked. Microscopic and absorbance changes, as well as protein solubilized were monitored. The experiment was repeated using 0.15M potassium carbonate and also 2.0M tris, both systems being held at pH 9.5 with minimal volumes of acid.

(k) Effect of Water Breakers

Normal purified crystals, at a final Pc of 100 µgm/ml, were incubated in 3.0M sodium chloride at 35°C for 120 minutes. The pH of the reaction system was maintained at 7.50 ± 0.25. After incubation,

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trypsin (twice crystallized) was added to a final concentration of 10 µgm/ml. Microscopic and absorbance changes were monitored with increasing reaction times. The experiment was repeated using 3.0M guanidine hydrochloride and 3.0M urea. It was realized that this latter reagent is not recognized as a water breaker.

The effect of guanidine hydrochloride and trypsin on crystals was further investigated. Normal crystals at a final Pc of 100 µgm/ml were incubated at 35°C in 3.0M guanidine hydrochloride for 10 minutes at pH 7.5 ± 0.25. After this period trypsin (final concentration 10 µgm/ml) was added to the reaction system. Absorbance changes were monitored. The experiment was repeated at 60, 120, 180 and 1200 minutes of incubation.

(m) Effect of Tris

Crystals were incubated at 25°C and 4°C in 3.0M tris at pH 8.0, the final Pc was 75 µgm/ml. The tris was adjusted to this pH with 6N hydrochloric acid. Absorbance and microscopic changes were observed over a period of 15 days. A similar experiment was set up at pH 10.0 and held at 25°C for 60 minutes, then half of the system was cooled to -5°C. Microscopic and absorbance changes were monitored over a six hour period. This latter experiment was repeated at pH 10.5.

In an additional experiment, crystals at a final Pc of 75 µgm/ml were incubated in increasing concentrations of tris (up to 4.0M) at pH 9.5, 10.0 and 10.5. Two reaction systems were set up, one was incubated at 25°C while the other was at -5°C.

(n) Combined Reagent Effects on Crystals

In an attempt to completely solubilize B.t.s. crystals various conditions known to produce partial solubilization were selected and combined. The following regima was ultimately selected. Crystals at a Pc of 200 µgm/ml were soaked at pH 9.5 for four hours at 25°C. After this treatment the 20 ml sample was added at 25°C to an equal volume of tris (4.4M) so that the resulting Pc was 100 µgm/ml and the tris concentration was 2.2M. The tris was prepared fresh and adjusted to pH 9.5 with 6N hydrochloric acid. The system was then incubated at -5°C for 20 hours. After incubation the mixture was warmed to 35°C and 40.0 ml of 4.0M guanidine hydrochloride were added. The guanidine hydrochloride was prepared immediately before use and adjusted to pH 9.5 with 2N sodium hydroxide. The resulting Pc was 50 µgm/ml. This final preparation was incubated at 35°C for two hours. After incubation the system was centrifuged. The experiment was repeated using 0.3OM potassium carbonate (pH 9.5) in place of the 4.0M guanidine hydrochloride.

4. Miscellaneous

Unless otherwise noted all chemicals were obtained from Fisher Scientific Co., Ltd., Toronto and where possible were 'Fisher Certified Reagent' grade. Guanidine hydrochloride, ultra pure, was obtained from Mann Research Laboratories, New York, N.Y. Dithiothreitol (Cleland's Reagent), 1,1,1,3,3,3-hexafluoro-2-propanol and Triton X-114 were obtained from Calbiochem, Los Angeles; Aldrich Chemicals Co., Inc., Milwaukee, Wisconsin and Rohm & Haas Co., Philadelphia, Pa., respectively. Nutritional Biochemical Co., Cleveland, Ohio supplied sodium thioglycollate and p-chloromercuribenzoic acid. Trypsin (2x crystallized) was obtained from Worthington Biochemical Corp., Freehold, N.J.

IV RESULTS

A. Growth Studies

During preliminary investigations different solid media were used to plate out lyophils of B.t.t., B.t.e. and B.t.s. The best yields of crystals were invariably obtained with the medium of Schaeffer (1961). This medium was made of two basic components, nutrient broth and inorganic ions. It was known that nutrient broth would not support rapid crystal production, hence it was assumed that the success of the Schaeffer medium was at least partly attributed to the influence of inorganic ions. The inorganic mix described by Schaeffer contained $MgSO_4.7H_2O$ - 0.25%, $MnCl_2.4H_2O$ - 0.0002%, KCl - 0.10%, $CaCl_2.2H_2O$ - 0.735% and $FeSO_4$ - 0.0015% (all expressed on the basis of w/v). This formulation was kept constant during initial growth studies and various organic compounds were tested in an attempt to find suitable soluble components for a liquid medium for pilot scale production of crude material.

Early studies in 300 ml shake flasks showed that regardless of the medium being tested crystal yields were greatly influenced by aeration rates. Flasks containing 50 ml of media produced more free crystals than those containing 75 or 100 ml, all other parameters being held constant. Shake flask studies showed that glucose and Soytone or Totamine were promising organic additives. Once this stage was reached experimentation was shifted to bench fermentors (7.5 liters), where

higher rates of oxygenation could be achieved.

One of the best sources for organic nitrogen proved to be Totamine (Amino acid composition given in Appendix II). When glucose and Totamine plus the inorganic ion mix were used as a complete medium a very delicate balance of carbon and nitrogen sources was essential in order to obtain high yields of free crystals. Although the addition of glucose resulted in an increase in biomass (Table 9, line 3) and a less alkaline growth environment (line 7), the degree of cellular lysis was less (line 6). Incomplete lysis was undesirable due to the difficulty encountered in lysing any remaining whole cells prior to crystal purification.

The presence of glucose in the medium also caused an increase in the degree of granulation that could be microscopically observed within growing cells. As glucose concentrations were increased the number of intracellular granules also increased (Table 10). These granules were spherical in shape and varied in size, up to eight could be observed within a single cell. Cells containing these granules were treated with the alkaline hypochlorite reagent of Williamson and Wilkinson (1958). This treatment destroyed evidence of all discrete cellular structures except the granules. When granulation was extensive the culture did not appear to enter into the initial stages of sporulation and no crystals were produced. After prolonged incubation (72 hours) these cells appeared to remain dormant at the pre-sporulant granular stage. However when low levels of granulation were encountered some crystals were subsequently produced. The difficulty encountered in establishing a balanced growth system seemed to be magnified by the addition of glucose. Comparatively low amounts of glucose (0.30% w/v)

TABLE 9

Bacillus thuringiensis Growth Systems in 7.5 Liter Fermentors

	Fermentor Number			
	1	2	3	4
1. Totamine in Medium (% w/v)	0.5	1.0	0.5	1.0
2. Glucose in Medium (% w/v)	nil	nil	1.0	1.0
3. Yield (gm/L)[A]	5.0	11.0	14.0	17.0
4. Crystal Yield %[B]	30	40	20	40
5. Crystal Yield (gm/L)[C]	1.5	4.4	2.8	6.8
6. Degree of Lysis[B]	95%	70%	40%	60%
7. Culture pH (final)	8.75	8.80	7.10	7.90

A Wet weight of all particulate material in culture broth.

B Estimated from microscopic counts.

C Calculated from values in lines 3 and 4.

TABLE 10

Observation of Granular Structures Inside *Bacillus thuringiensis* var *sotto* Cells With Increasing Amounts of Glucose in the Growth Medium

(In addition to organic constituents each medium had inorganic ions as described by Schaeffer (1961).)

Medium	Flask Number					
	1	2	3	4	5	6
Totamine % w/v	1.0	1.0	1.0	1.0	1.0	1.0
Glucose % w/v	1.0	1.0	0.6	0.4	0.2	nil
Granulation*	+4	+3	+1	+1	±	±

* Microscopic observations after 48 hours ranging from very many (+4) to few if any (±).

seemed to aid crystal production, however the degree of variation in crystal yield was still unacceptable. Consequently glucose was deleted from the organic portion of the medium. In subsequent studies it was found that low levels of Soytone had a beneficial effect on crystal yield when used together with Totamine to make up the organic constituents of the medium.

Once the organic portion of the production medium had been optimized and produced encouraging results, attention was then focused on optimizing the inorganic constituents. Growth studies showed that ferrous sulphate could be deleted from the medium. The addition of potassium dihydrogen phosphate had a slightly beneficial effect, perhaps by providing some buffering capacity to the medium. Although the other ions outlined by Schaeffer were necessary additives, their optimal amounts varied slightly.

The final production medium, supporting maximum crystal production and cellular lysis, contained the following constituents:

Totamine	0.80% (w/v)
Soytone	0.20%
$CaCl_2$	0.01%
KH_2PO_4	0.10%
KCl	0.10%
$MgSO_4.7H_2O$	0.06%
$MnCl_2.4H_2O$	0.0005%
Total Solids	1.2705%

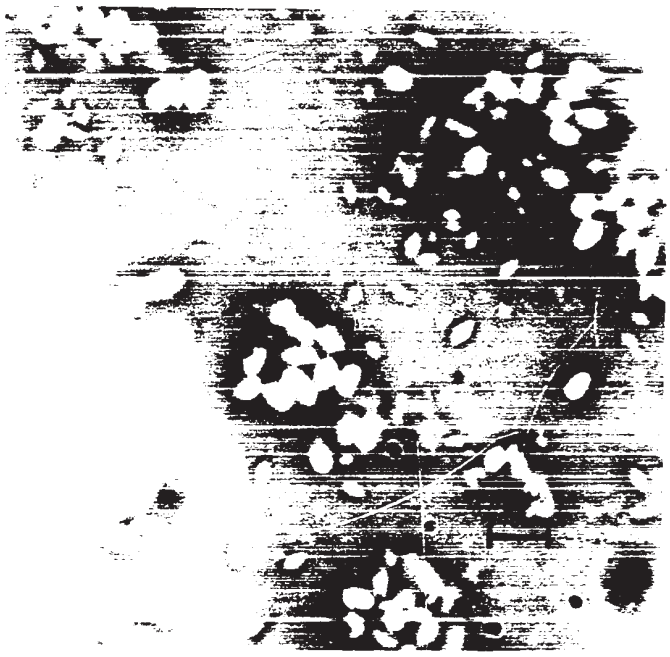
After autoclaving, the medium was at pH 6.20 $\pm$ 0.1. Although all three B.t. varieties produced crystals on this medium, the best yields were obtained with B.t.e. and B.t.s. After a growth period of 36 - 40 hours

the particulate matter was recovered from the culture broth by centrifugation and then lyophilized. Approximately 750 grams of dried crude material were obtained from 600 liters of culture broth.

During the routine monitoring of B.t. growth systems on different production media it was found that the ability to produce many free crystals varied. Such variations were often unexplainable. In short, a complex and somewhat confusing growth picture was encountered with these varieties. Many unsuccessful growth systems were produced, these too added to the overall growth study.

The morphological characteristics observed in growing cells of B.t. seemed to be similar to those of other large cell bacilli. When crystals were produced they appeared initially after about 24 hours as free structures in the culture broth. Maximum numbers of crystals were obtained in good culture media after 36 - 40 hours. When crystals first appeared in the broth they were small, however larger sizes were found towards the end of the growth period (36 - 40 hours). The final crude product contained crystals of varying sizes. The size variation encountered in a preparation of B.t.s. is shown in Figure 4.

Foam produced during aeration of the growing cultures was suppressed by the automatic addition of the polysilicate 'Antifoam-C'. This material was eventually mixed into the culture broth where it formed free microscopic droplets. During routine monitoring of the culture broth these droplets could be seen with many spores adsorbed onto their surface (Figure 5). At no time were crystals ever detected on the surface of these droplets. A difference in the surface adsorptive properties of spores and crystals was therefore suggested.

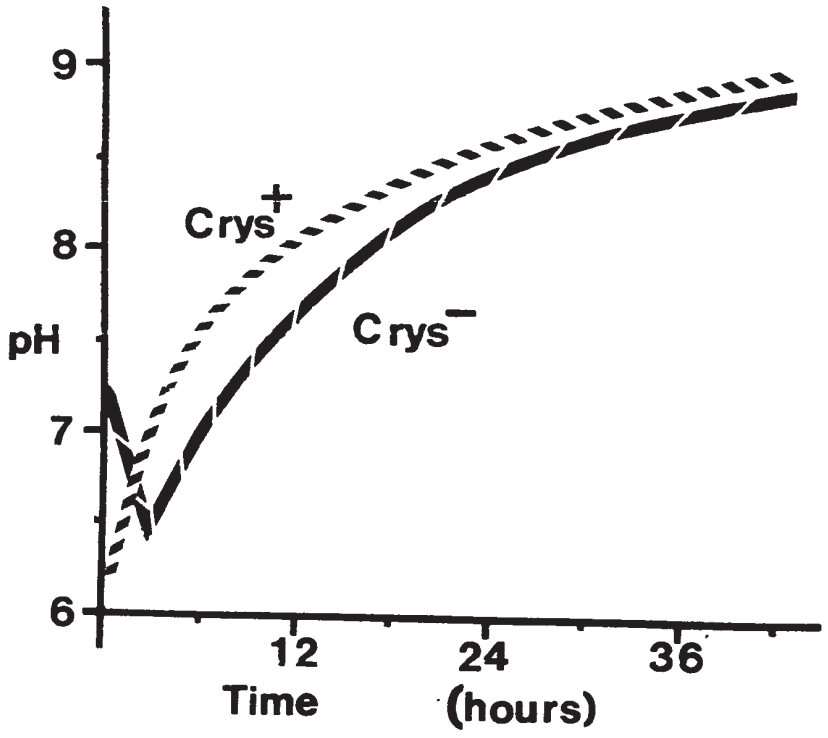


Changes in biomass were followed by monitoring the absorbance of the whole culture broth at various times. Absorbance patterns of a growing B.t. system were similar to those encountered in the growth of other Bacillus species. After a rapid increase in absorbance during the logarithmic phase of growth a plateau occurred which was usually followed by a slight decrease. This decrease was not apparent when cultures remained in the granular stage. The lysis indicated by absorbance decreases was encountered in sporulating systems that produced crystals and those that did not.

The pH profile produced in the culture broth by growing populations of B.t. was initially the same as that characteristic of other members of the genus Bacillus. However, such B.t. cultures produced few, if any, free crystals. When media were finally formulated that supported high levels of crystal production, it was found that atypical pH profiles were produced. The profiles failed to undergo an early acid shift, but instead rose directly towards the more alkaline values. Crystal yield was invariably higher whenever the atypical pH pattern was encountered. These atypical pH profiles were independent of the starting pH of the media. The atypical pH profile was produced by all three B.t. varieties on media low in or devoid of glucose (Figure 6).

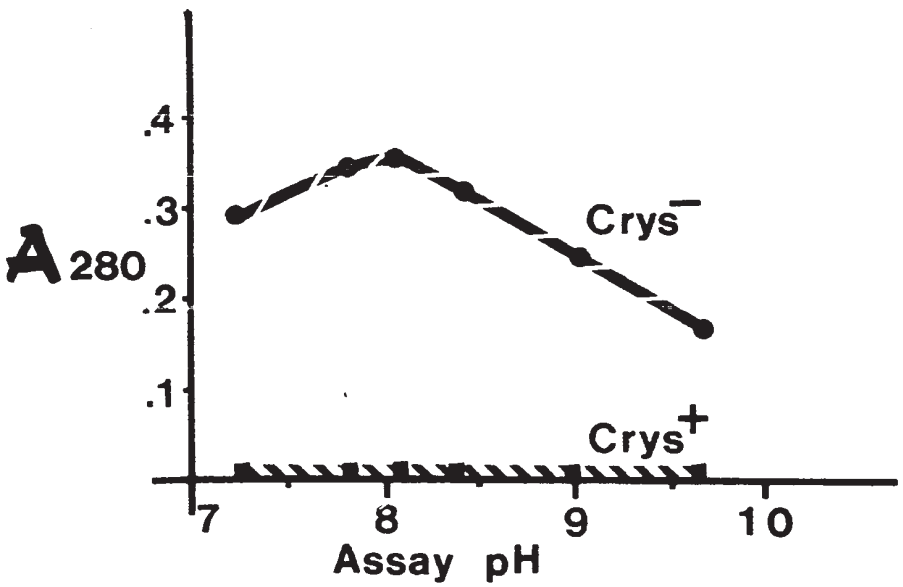
Culture broths from growth systems producing high levels of free crystals did not contain measurable amounts of peptidase (protease). However it was found that the broth from cultures that produced few, if any, crystals contained high amounts of peptidase. It was shown that this peptidase had a slight alkaline pH optimum (Figure 7) and that it was elaborated, apparently as an extracellular enzyme, after 12 hours of growth (Figure 8) during the late logarithmic and early sporulant

Figure 6



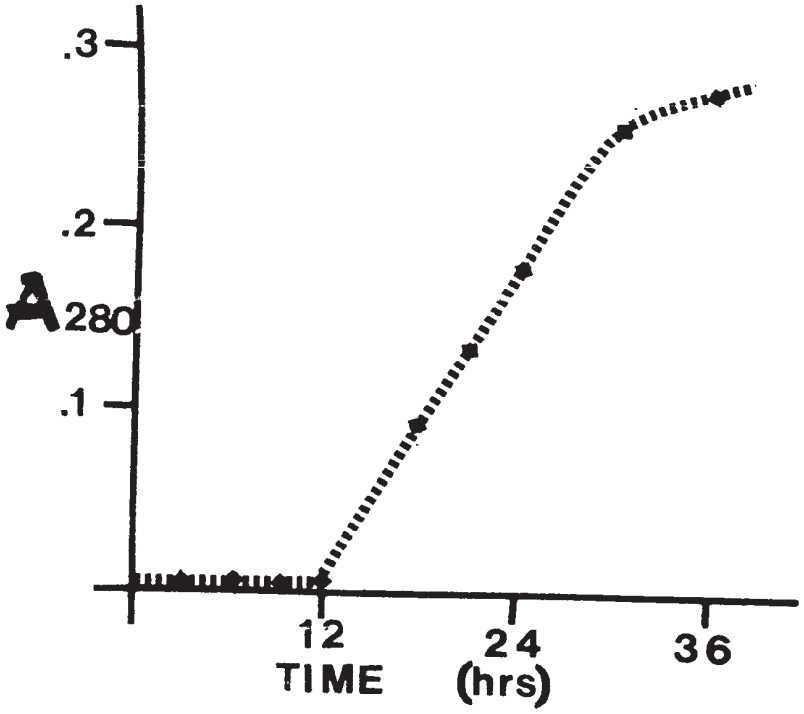
Profiles of broth pH for B.t. cultures failing to produce crystals ($Crys^-$) and other growth systems producing crystals ($Crys^+$).

Figure 7



Peptidase activities in broth of B.t. cultures failing to produce crystals ($Crys^-$) and other growth systems that did produce crystals ($Crys^+$).

Figure 8



Appearance of peptidase activity in culture broths of B.t. growth systems that failed to produce crystals.

phases of growth. When culture filtrates possessing peptidase activity were added to highly purified crystals and the system incubated at 36°C for 24 hours, the crystals lost their characteristic diamond morphology and appeared to be slightly swollen. The remaining structures were unclumped and slightly ellipsoidal.

B. Crystal Purification

The best organic solvent for the induction of massive spore clumping proved to be chloroform. A typical spore aggregate induced in B.t.s. preparation during the purification method is shown in Figure 9. It will be noted that although free crystals surround the clump, additional crystals are entrapped within it. Repetition of the purification method served to remove these latter crystals. The total time that crystals were subjected to the chloroform environment was kept to a minimum.

Each of the two emulsion separation techniques offered certain advantages and disadvantages. The bench settling method required less time but final purification yields were only about 5% (dry weight basis, crystals versus crude material) and purification ratios were in the order of 2000:1 (microscopic count of crystals versus spores). The centrifugal technique required more time but gave a higher yield (about 20%) and a higher purification ratio (3000:1). The increased yield and purification ratio appeared to be related to greater recovery of crystals at each extraction step due to the centrifugal packing of the remaining debris. The bench settling method was used during most of these studies to prepare purified crystals. In a five hour period it was possible to prepare approximately 100 mgs of crystals. Purification of crystals by this method worked well for all three varieties of B.t.

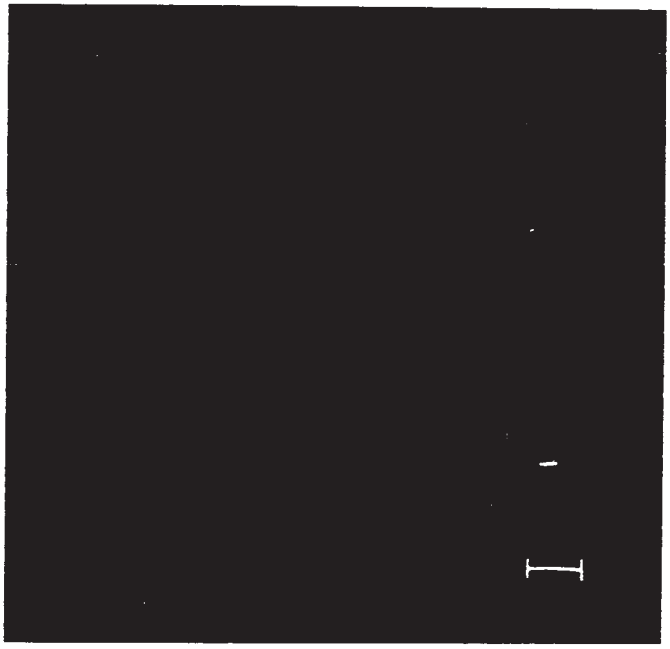
Figure 9

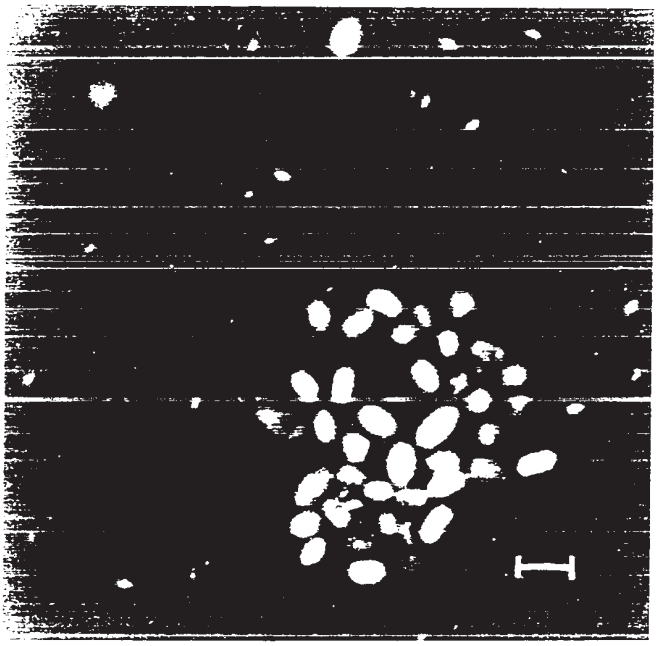
Spore aggregate showing entrapped crystals.

Free crystals may be seen around the spore clump.

Preparation is negatively stained and shown at 3800 x.

Solid line represents two microns.





Purified B.t.s. crystals are shown in Figure 10 while a comparison of B.t.s. and B.t.t. crystals is shown in Figures 11 and 12. It will be noted that the B.t.t. crystals are generally larger than those of B.t.s. All three varieties of crystals purified by this method were toxic to silkworms (Angus, 1965).

C. Amino Acid Calculations on the Minimal Polypeptide

Calculations based on the amino acid composition of crystals from B.t.t., B.t.e. and B.t.s. are summarized in Tables 11 and 12. Differences in any particular parameter did not vary greatly among the three varieties. None of the values was other than what would be expected in a globular associating protein. No peculiarities were found that could account for the remarkable stability of the crystals. Calculations supporting determination of the number of residues, polarity ratios and average hydrophobicities may be found in Appendix III, Tables A, B and C.

D. Biochemical Studies

1. General

Pellets of purified crystals at a pH similar to that of the culture broth were white in color and on centrifuging they packed into a loose and rather large mass. As the crystal environment approached a neutral pH value with washing, the pellets gradually changed color becoming greyish-black in appearance. With washing the crystals had packed together much more and formed a smaller and slightly sticky pellet. Microscopic changes in the crystals could not be detected during the above transitions.

Figure 10

Purified crystals of B.t.s. Preparation is negatively stained and shown at 3800 x. Solid line represents two microns.

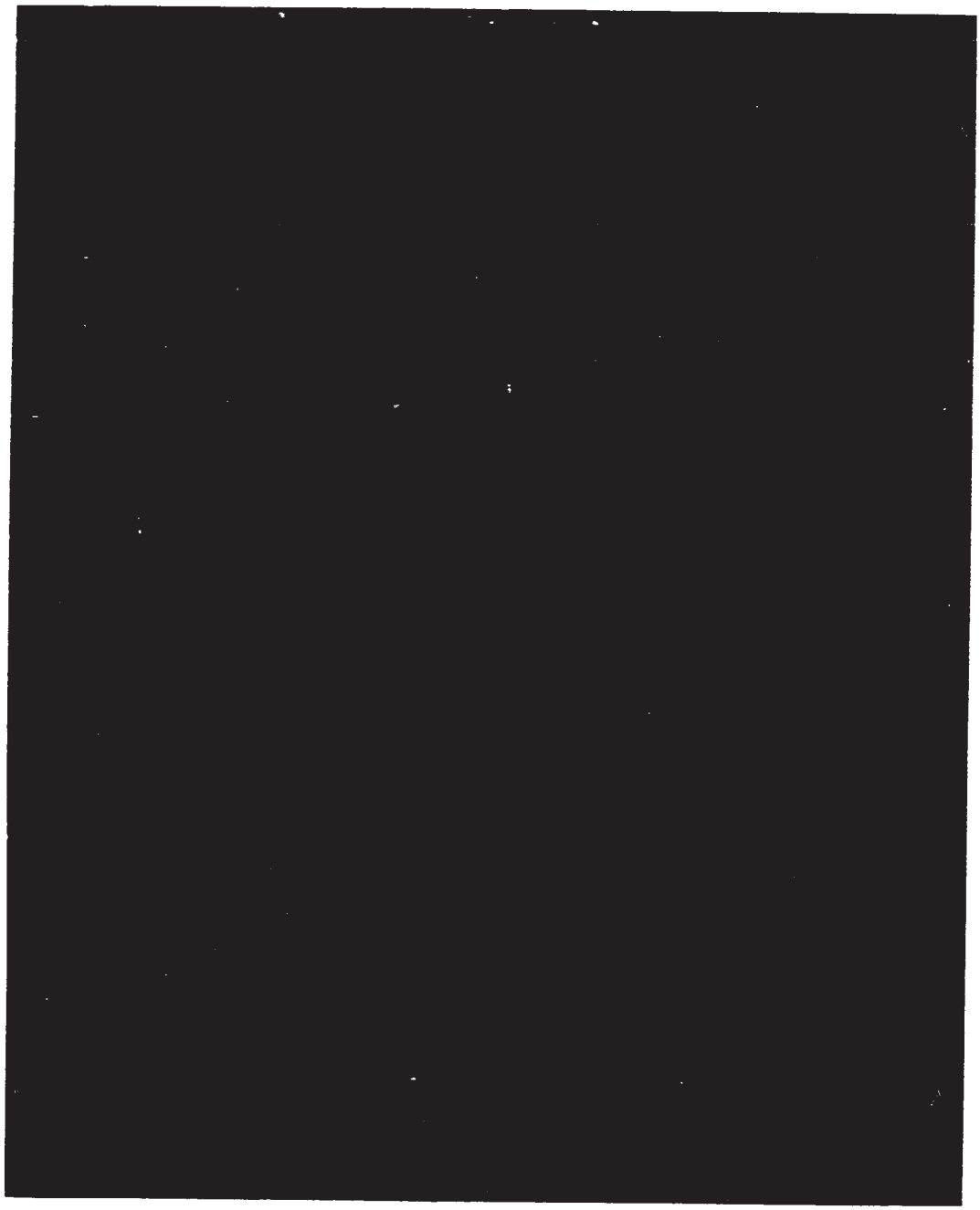
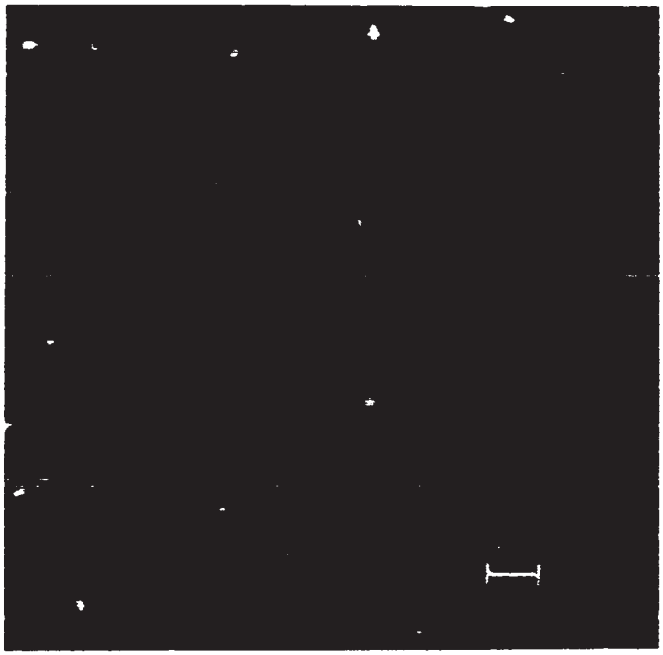


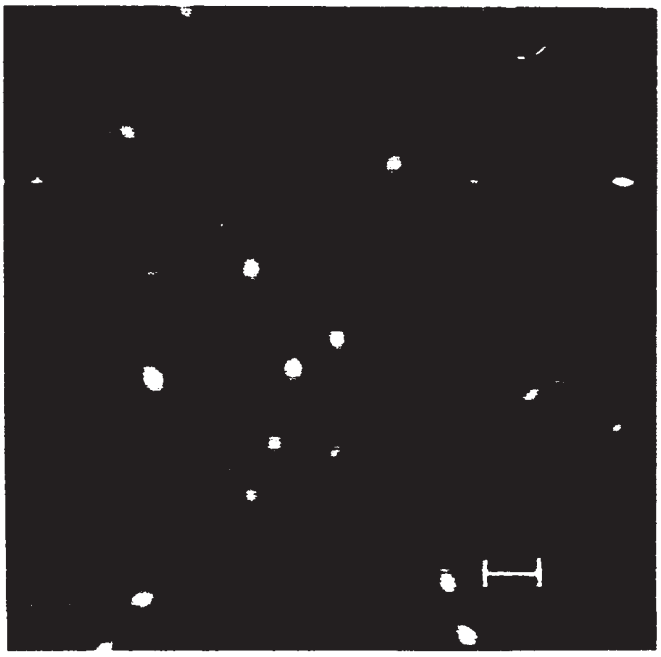
Figure 11

Purified crystals of B.t.s., details as for Figure 10.

Figure 12

Purified crystals of B.t.t., details as for Figure 10.





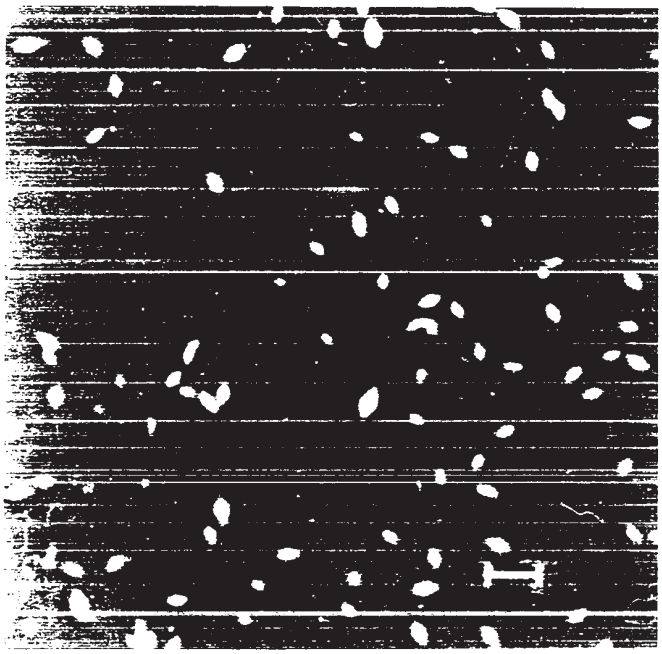


TABLE 11

Number of Residues in the Minimal Polypeptide of *Bacillus thuringiensis* Varieties

Polar Residues	B.t.t.	B.t.e.	B.t.s.
Glx	19	24	21
Asx	20	23	17
Ser	10	13	10
Thr	10	13	8
Arg	9	12	11
Lys	5	7	5
His	3	4	4
Tyr	6	8	6
Nonpolar Residues	**B.t.t.**	**B.t.e.**	**B.t.s.**
Trp	2	3	2
Phe	6	9	7
Pro	7	9	7
Gly	11	14	12
Ala	10	12	10
Val	11	15	11
Ile	9	13	9
Leu	15	21	14
Met	1	1	1
Cysteine	3	5	4

TABLE 12

Summary of Amino Acid Calculations on the Minimal Polypeptide of *Bacillus thuringiensis* Varieties

A	B	C	D	E	F	G	H
Crystalliferous Variety	Min M.W.	Number of Residues	Helical Content	N.P.S.	Polarity Ratio	Hydrophobicity	Average Hydrophobicity (K calories/ residue)
B.t.t.	17,500	157	41%	.31	1.105	32.7%	1.076
B.t.e.	22,200	206	42%	.33	1.020	36.0%	1.136
B.t.s.	18,200	147	52%	.33	1.105	34.0%	1.164

2. Crystal Composition

Results obtained for the amino acid composition of B.t.s. crystals agreed well with the published values. These results are shown in Appendix V. Methionine was the limiting amino acid. No unexpected peaks were found on the aminograms. The photomicrograph (Figure 14) of the residual matter after acid hydrolysis clearly showed some crystals. However many of these had undergone damage as their apices were removed. A few B.t.s. crystals apparently withstood the effect of 6N hydrochloric acid at 110° for 140 hours, possibly through the protective effect provided by massive crystal clumping initially induced by the acid.

When 88.48 mg of lyophilized crystals were allowed to equilibrate in a humid environment it was found that 47.41 mg of water were taken up, i.e. weight increase of 33.5%. If the crystals were dried over phosphorus pentoxide *in vacuo*, it was found that upon equilibration 84.98 mg of material took up 50.91 mg of water, a weight increase of 37.4%. When 2.140 mg of air dried crystals were assayed for protein content, 1.290 mg of protein were present and it was therefore assumed that 0.850 mg of water was bound in some way to the crystal protein. This represented a total water content of 39.5%. The average content of bound water in B.t.s. crystals was estimated to be about 37%.

The isoionic point of crystals in deionized-distilled water was 6.450. This determination was carried out on preparations that were exposed to the atmosphere.

3. Crystal Structure

(a) General

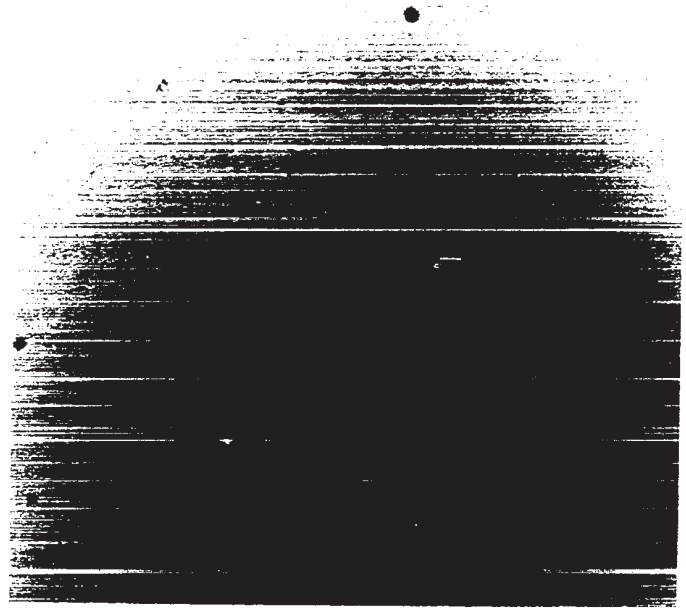
The typical crystal clumping induced by many reagents in purified crystal preparations is shown in Figure 13. It will be noted that the

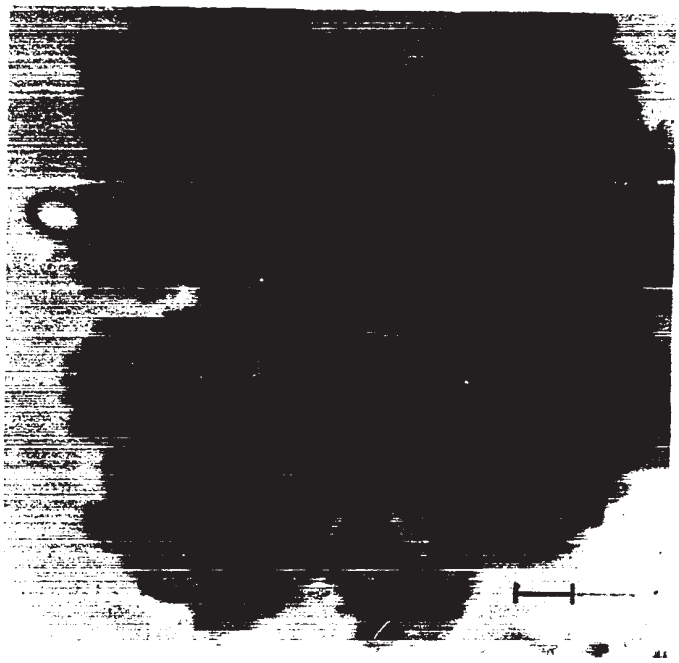
Figure 13

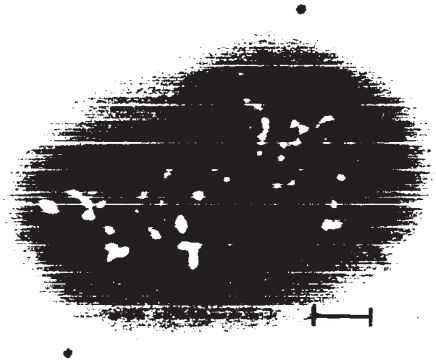
Massive crystal clump induced by many reagents, no free crystals remain. Reagent used here was guanidine hydrochloride. Preparation is negatively stained and shown 3800 x. Solid line represents two microns.

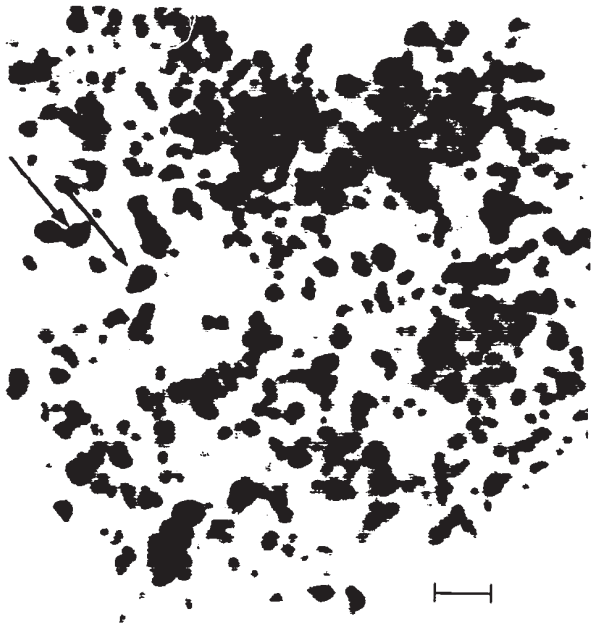
Figure 14

Humin-like material remaining after hydrolysis in 6N hydrochloric acid at 110°C for 140 hours. Preparation was not stained, structures were refractile. Magnification is 3800 x and the solid line represents two microns.









crystals are still refractile and that no swelling has occurred. Turbidity changes in crystal systems usually involved a decrease, however in a few cases increases were encountered. Decreases in turbidity indicated crystal clumping, loss of refractility or swelling with loss of refractility or a combination of any of these effects. Table 13 shows the effects of various reagents on crystals. It may be noted that few treatments caused anything greater than crystal clumping. In all cases where swelling or loss of refractility occurred the amount of reagent, when compared to the total protein load, were judged excessively high.

(b) Effect of Succinic Anhydride

This reagent was partially successful in dispersing crystals (Figure 15) however little protein solubilization was encountered below pH 11.0 and only about 35% of the total protein load was solubilized. The remaining protein was present in large amorphous masses.

(c) Effect of Detergents

Crystals treated with cetyltrimethylammonium bromide showed only slight clumping in the weakly acidic range (pH 6.0 - 7.0). Triton X-114 caused an increase in absorbance with no clumping nor loss of refractility. Crystal dispersion was achieved at pH 9.5 with prolonged stirring (120 minutes) in sodium laurylsulphate. The ratio of detergent to protein in this latter case was about 100:1, expressed on a weight basis. These effects are shown in Figure 16.

TABLE 13

Effects of Various Reagents on Crystals of Bacillus thuringiensis var sotto

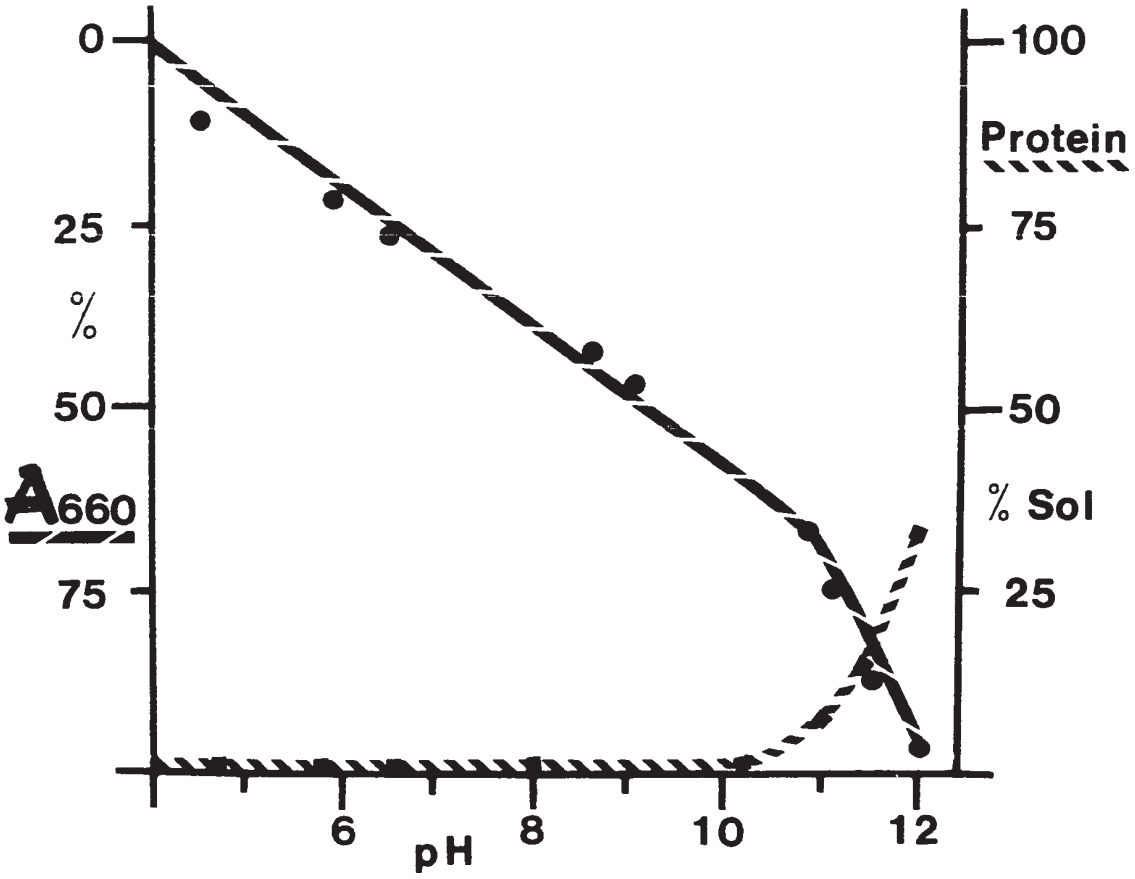
(Reaction times were < 120 minutes, unless otherwise stated. Final Pc was < 300 µgm/ml in each system)

Reagent	Concentration	pH	Temp. °C	Crystal Clumping	Crystal Swelling	Refractility Loss
Hydrochloric Acid		1.1	25	+4	-	-
Acetic Acid		1.6	25	+6	+2	±
Water (1)		6.5	4	+3	-	-
Water		6.5	Boiling	-	-	-
Water		6.5	-20	+6	-	-
Ethylene Diamine Tetraacetic Acid (2)	0.1M	8.0	25	+4	-	-
Cetyltrimethyl Ammonium Bromide	0.75%	6.2	25	+1	-	-
Triton X-114	0.75%	8.0	25	-	+2	-
Sodium Lauryl Sulphate	0.75%	9.0	25	+4	+5	+3
Dimethyl Formamide	1.3M	9.5	4 or 35	-	-	-
Dimethyl Sulphoxide	1.3M	9.5	4 or 35	-	-	-
Hexafluoro Propanol	3.8M	6.5	35	+6	-	-
Potassium Chloride	1.0M	9.5	25	±	-	-
Potassium Bromide	1.0M	6.2	25	+4	±	-
Potassium Carbonate	0.15M	10.5	35	+4	±	-
Lithium Sulphate	1.0M	7.6	25	-	-	-
Sodium Acetate	3.0M	9.3	25	+4	-	-
Urea	6.0M	9.5	35	+4	+4	+3
Guanidine Hydrochloride	4.0M	9.5	35	+5	+4	+3
Tris	3.0M	10.5	4	-	+4	+4
Methanol	4.0M	6.9	-5	-	-	-
Ethanol	4.0M	7.1	-5	-	-	-
Iso-Propanol	4.0M	7.1	-5	-	-	-
Sucrose	22%	9.8	-5	±	-	-
Glycerol	22%	10.2	-5	±	-	-
Formaldehyde	36%	6.0	4	+3	-	-

(1) After 60 days
(2) After 48 hours

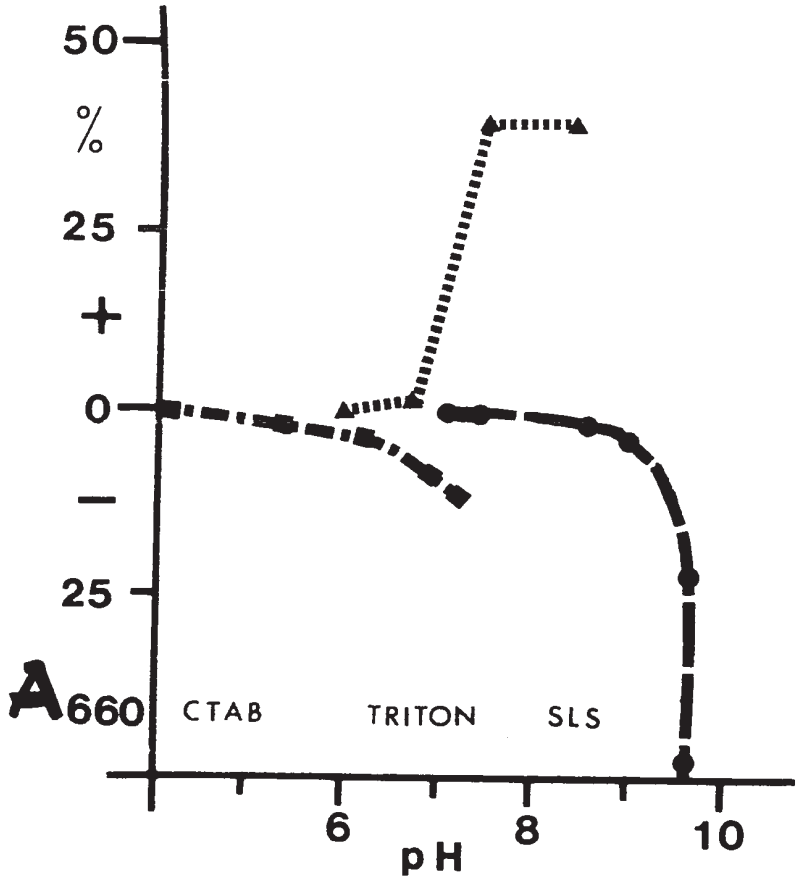
Rating Scale: +6 Massive Change, +5 Very Large, +4 Large, +3 Moderate, +2 Slight, +1 Very Slight, ± Doubtful, - No Change

Figure 15



Effect of a five fold excess of succinic anhydride on crystals. Reaction temperature was 25°C and the final protein concentration was 150 µgm/ml.

Figure 16



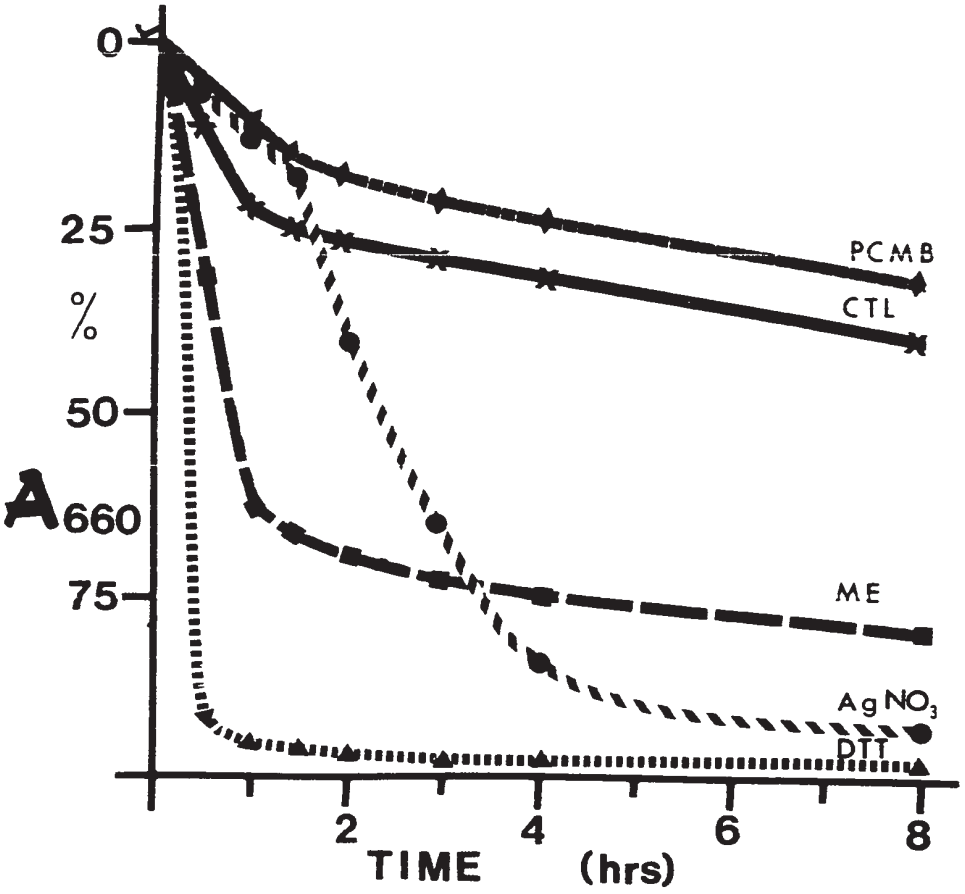
Effects of detergents on crystals at 25°C. All detergent concentrations were 0.75% w/v, while the final protein concentration was 75 µgm/ml.

(d) Effect of Thiol and Disulphide Reagents

When crystals were incubated in carbonate buffer an initial decrease in absorbance was encountered (Figure 17). This decrease appeared to be primarily due to crystal clumping. If p-chloromercuri-benzoic acid were added to the system, absorbance changes were essentially the same as that for the control. Sodium thioglycollate provided a similar effect. However when 2-mercapoethanol or dithiothreitol were used a rapid decrease in absorbance occurred. This decrease was due to crystal solubilization. Dithiothreitol was more effective than 2-mercaptoethanol in dispersing crystals, however this difference could be eliminated by using a ten fold excess of the latter reagent. The effect of silver nitrate was different in that no change from the control systems was noted until after approximately 90 minutes incubation, then a sudden decrease in absorbance was recorded. No parameter was changed throughout the course of this eight hour experiment. These effects were all pH dependent and at pH 9.5 none of the changes outlined in Figure 17 was produced.

(e) Effect of Urea

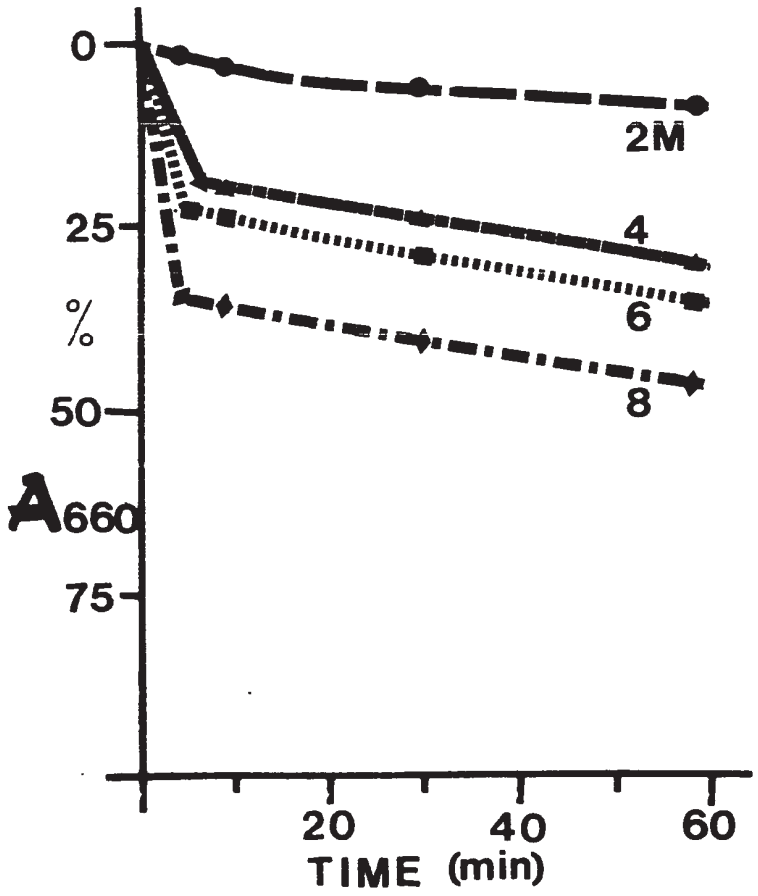
At high concentrations this classical protein dispersant caused initial clumping of crystals which occurred within the first ten minutes of reaction (Figure 18). This effect was followed by a second and much slower reaction that was associated with loss of refractility and swelling (Figure 19). Crystal swelling occurred in at least two planes resulting in the formation of enlarged crystals possessing their normal shape. The urea effects were pH dependent, however only the effects at pH 9.5 are reported.



The influence of various thiol and disulphide reagents at 35°C on crystals at pH 10.0. The final Pc in each system was 100 µgm/ml. A control system (CTL) of crystals in 0.01M potassium carbonate buffer was used.

PCMB - p-chloromercuribenzoic Acid
ME - 2-mercaptoethanol
$AgNO_3$ - Silver nitrate
DTT - Dithiothreitol

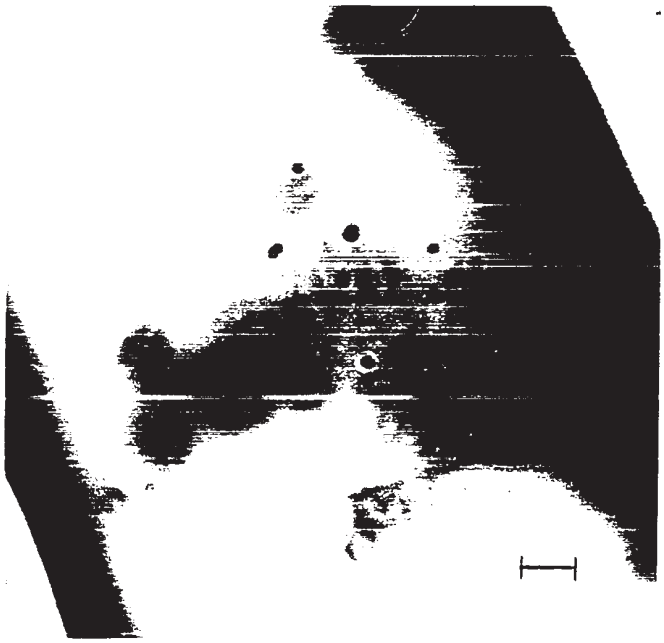
Figure 12



The effect of increasing concentrations of urea on crystals at pH 9.5. The final Pc in each system was 150 μgm/ml and the reaction temperature was 35°C.

Figure 19

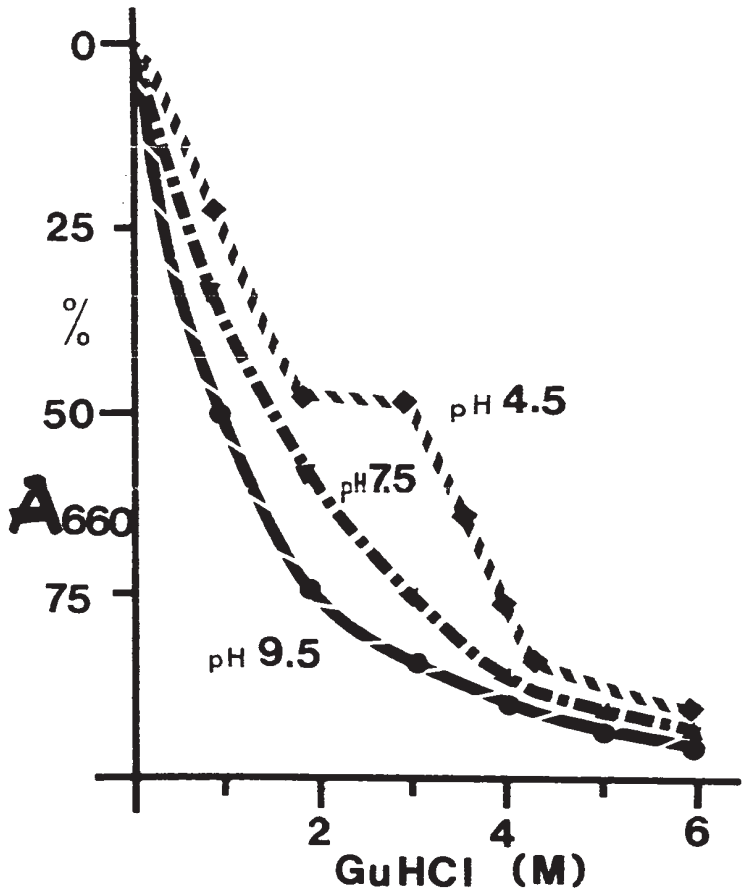
Crystals in urea solution at pH 9.5 showing swelling and loss of refractility. Preparation is negatively stained and shown at 3800 x. Solid line represents two microns.



(f) Effect of Guanidine Hydrochloride

The influence of guanidine hydrochloride on crystals was concentration and pH dependent. Massive crystal clumping occurred initially at all pH levels. At pH 4.5 an initial rapid drop in absorbance was indicative of this clumping. Changes in absorbance were slight as the concentration of reagent was raised from 2.0M to 2.0M. At concentrations higher than this latter level a rapid decline in absorbance continued. Complete clearing was never reached, and on centrifuging an insoluble precipitate remained. The absorbance decreases at pH 7.5 and 9.5 were less concentration-dependent and did not show a stepwise change (Figure 20). The effect of guanidine hydrochloride on crystals could be divided into at least three distinct phases. The early clumping effect, as seen in Figure 13, was quickly replaced by a condition where long needle like crystals could be seen (Figure 21). These crystals had only begun to lose their refractility and reaction with the nigrosin dye was moderate. Swelling in the needle shaped crystals appeared to be only in the longitudinal plane. The third phase was characterized by highly swollen, non-refractile crystals that readily reacted with the nigrosin (Figure 22). Crystal swelling was now in both planes. Signs of crystal disintegration could be detected at the tips of the crystals. The distinct phases observed when crystals and guanidine hydrochloride were incubated depended upon the combined effect of various parameters. These included reactant concentrations, pH, time and temperature. The three different reaction rates shown at pH 4.5 in Figure 20 correspond roughly to the observed phases.

Figure 2.



Effect of increasing concentrations of Guanidine Hydrochloride on crystals at different pH values. Incubation was at 25°C for 120 minutes, the final Pc was 150 µgm/ml.

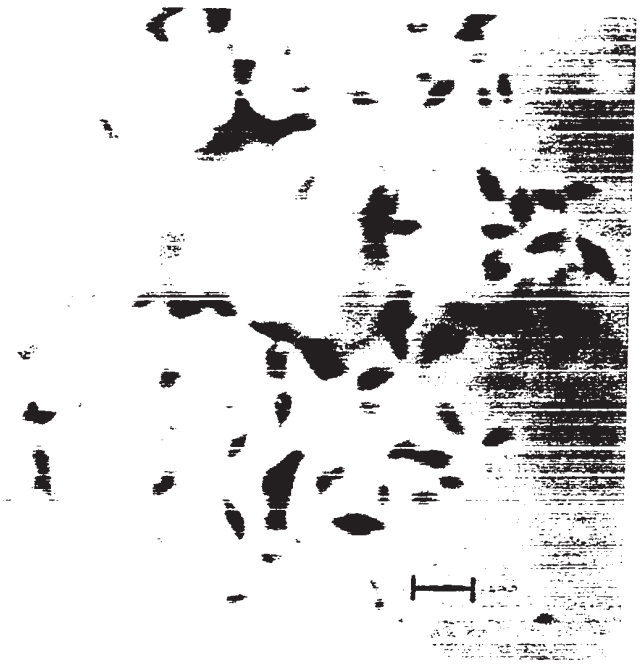
Figure 21

Crystals incubated in guanidine hydrochloride showing long needle-like shapes, the second phase of crystal-dispersant reaction. Preparation is negatively stained and shown at 3800 x. Solid line represents two microns.

Figure 22

Highly swollen, non-refractile crystals in guanidine hydrochloride, the third phase in crystal-dispersant reaction. Preparation is negatively stained and shown at 3800 x. Solid line represents two microns.

100



(g) Effects of Boiling Crystals in Dilute Acids

Hydrochloric (0.03N) and oxalic (0.25M) acids were effective in solubilizing crystals, while acetic acid (0.25M) was only partially effective and boric acid (0.002M) merely caused a slow clumping reaction (Figure 23). The first two acids readily solubilized the crystal protein and also liberated considerable free amino acids (Figure 24). A check of the soluble material in a hydrochloric reaction system by thin layer chromatography indicated aspartic acid was present.

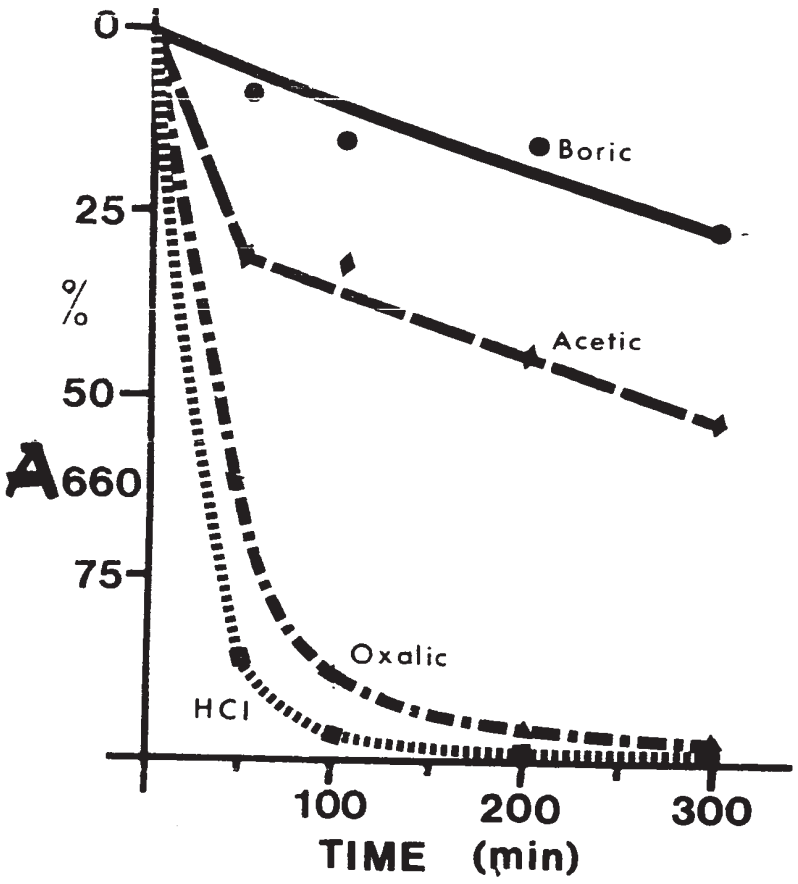
(h) Effect of Potassium Carbonate

The ability of potassium carbonate to solubilize crystals was pH dependent when all other factors were held constant (Figure 25). The redox potential in these systems decreased as the pH moved towards the more alkaline values. The dependence of solubilization on pH was further demonstrated in Figure 26, where initial clumping only accounted for a portion of the large absorbance decrease at the higher pH values. The ability of 0.15M potassium carbonate to solubilize crystals at pH 11.0 was shown in Figure 27. After the initial clumping reaction considerable protein (> 75%) was present in the supernatant.

(i) Characteristics of 'Old' Crystals

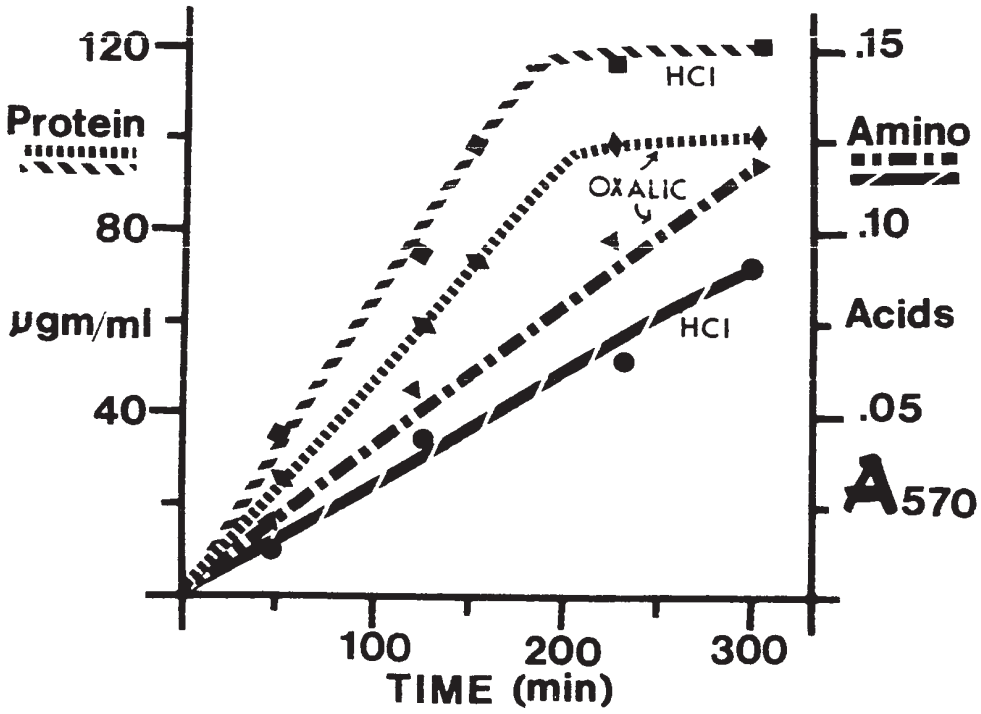
Crystals that had been stored at 4°C under varying conditions showed different degrees of stability towards urea and sodium lauryl-sulphate (Figures 28 and 29). The most unstable crystal preparation being the one held for 30 days at pH 8.0 in 0.2M tris-maleate buffer.

Figure 23



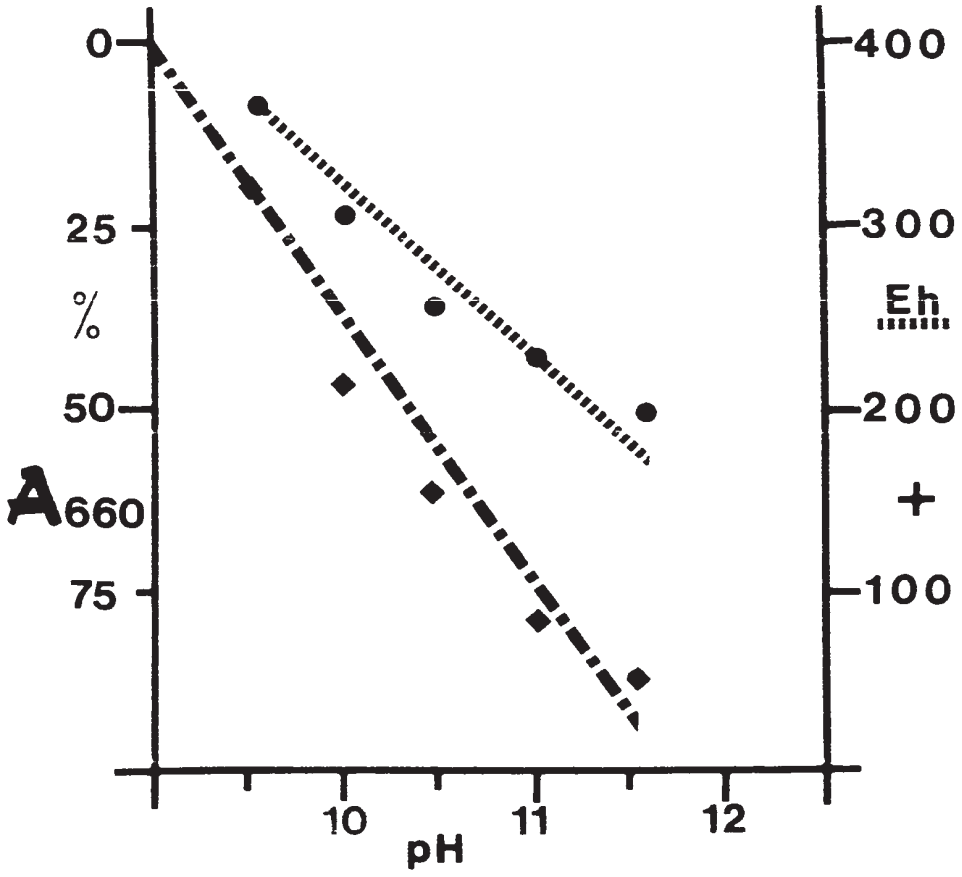
Effect of boiling crystals (Pc 120 µgm/ml) in dilute boric, acetic, oxalic and hydrochloric acids for increasing time intervals.

Figure 24



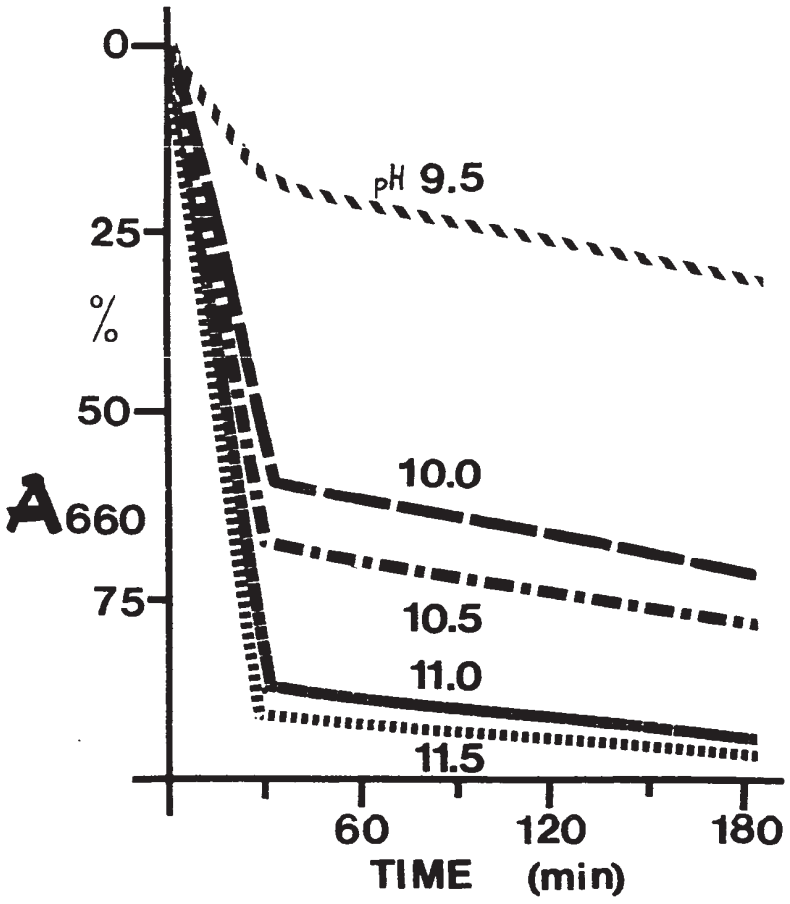
Solubilization of protein and release of amino acids when crystals were boiled in dilute hydrochloric acid and oxalic acid.

Figure 25



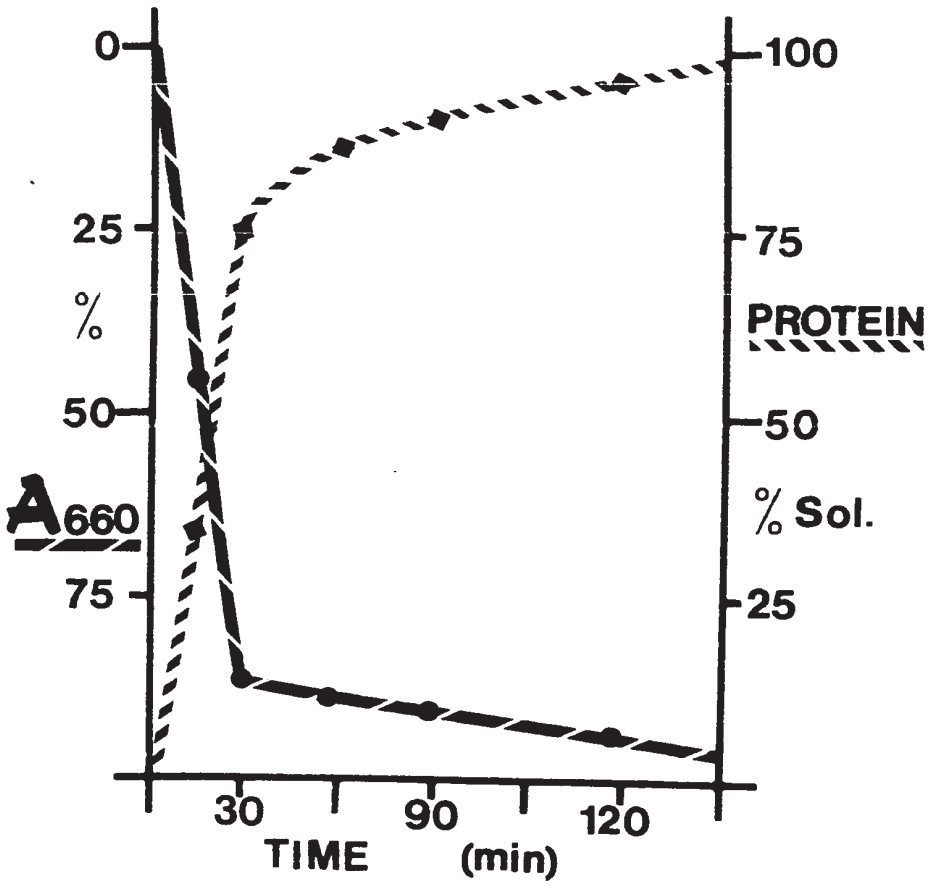
Incubation of crystals (Pc 100 µgm/ml) in 0.15M potassium carbonate at increasing pH levels for 30 minutes at 35°C. The redox potential (Eh) of the reaction systems was measured in millivolts.

Figure 26



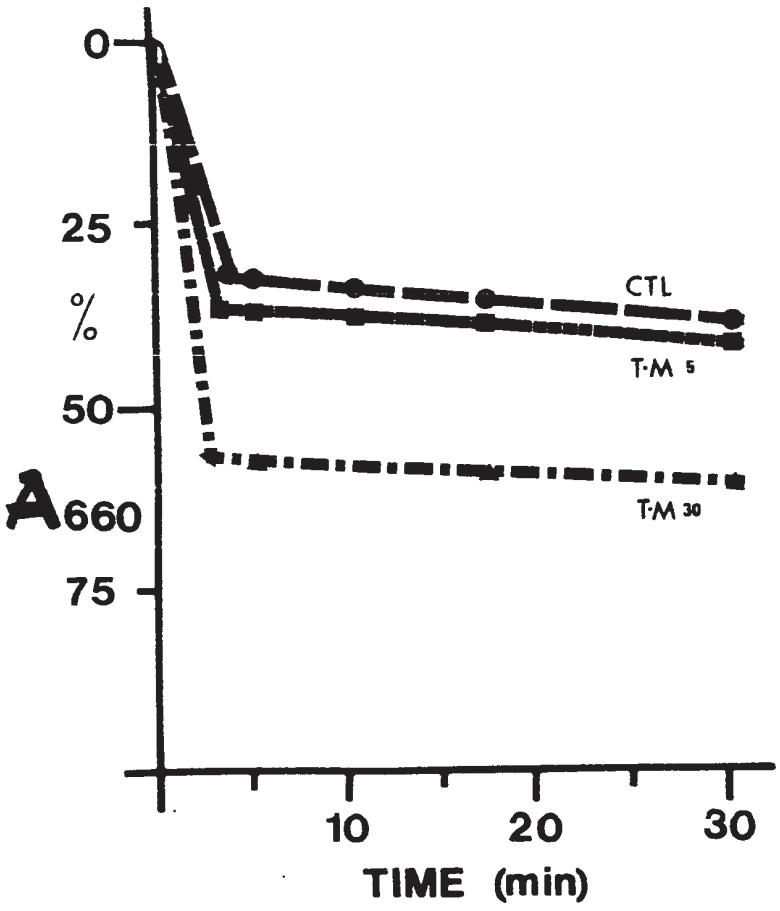
Effect of increasing time and pH when crystals were incubated in 0.15M potassium carbonate at 35°C. The final Pc was 100 µgm/ml. Data used to construct this Figure are shown in Appendix IV, Table A.

Figure 27



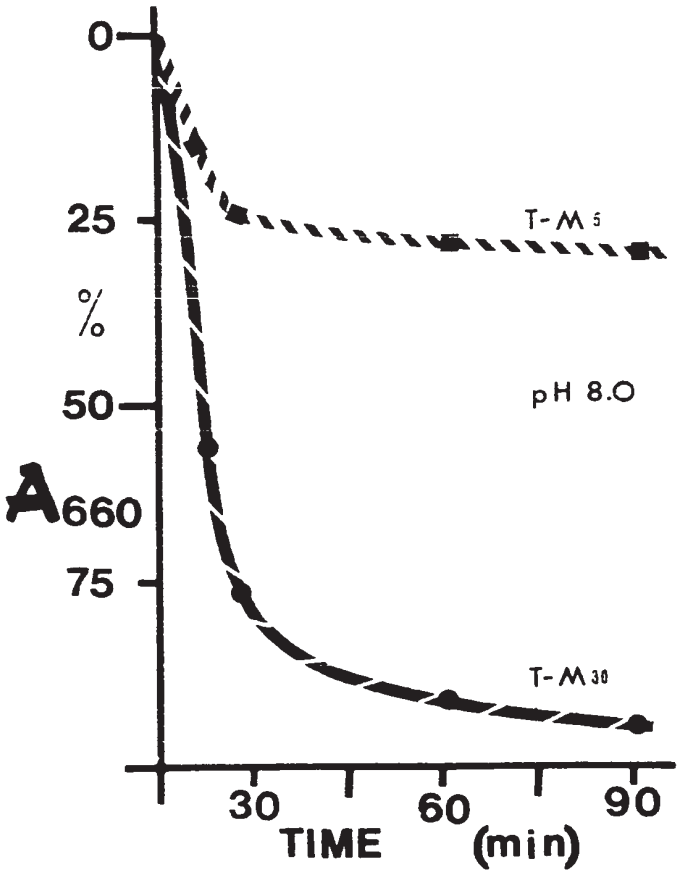
Absorbance decrease and protein solubilized when crystals were incubated at 35°C in 0.15M potassium carbonate (pH 11.0). Final Pc was 100 μgm/ml.

Figure 28



Effect of 8M Urea on 'old' crystals. The control crystals (CTL) had been held in water for five days before urea treatment. The two tris-maleate samples (T·M 5 and 30) had been incubated for 5 and 30 days respectively before urea addition.

Figure 29



Effect of 1.3M sodium laurylsulphate on 'old' crystals. The T-M samples had been incubated in tris-maleate buffer for 5 and 30 days prior to the detergent treatment.

(j) Soaking Crystals in an Alkaline Environment

The uptake of sodium hydroxide by crystals was approximately twice that of a water blank (Figure 30). The sodium hydroxide appeared to be neutralized in some way by the crystal protein. Soaking crystals in sodium hydroxide at pH 9.5 caused no swelling or refractility loss and no protein solubilization (Table 14). These effects were similar when crystals were soaked at pH 10.0 and 10.5 with the exception that in both cases the crystal pellet became sticky and difficult to work with.

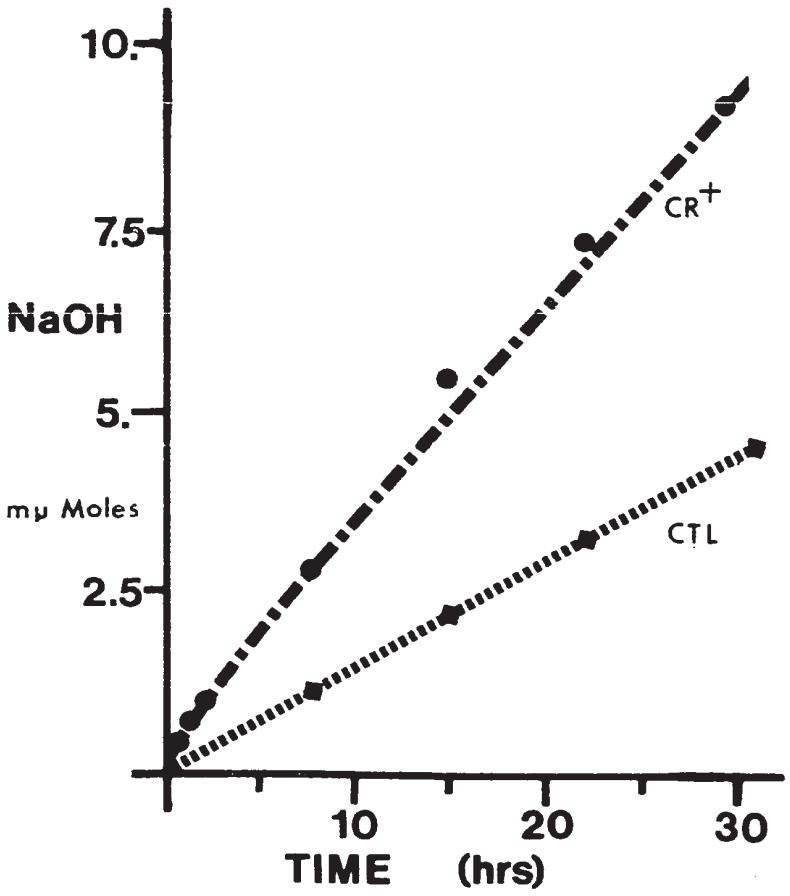
When normal crystals were reacted with trypsin no significant microscopic or absorbance changes were detected. However when trypsin was incubated with crystals previously soaked at pH 9.5 massive clumping was observed (Table 14). This effect was maximal after four hours of soaking. Attempts to measure solubilized protein were not conclusive due to the presence of soluble trypsin protein and the low protein concentrations being evaluated.

Soaking crystals in sodium hydroxide failed to alter significantly their reactive characteristics to potassium carbonate. When tris was incubated with soaked crystals a slight increase was determined only in the amount of crystal clumping. The effects of guanidine hydrochloride on soaked crystals were such that absorbance changes were 25% less but an additional 20% of the total protein load was solubilized. Crystal clumping was the same in both cases (Table 14).

(k) Effect of Water Breakers

The effect of trypsin on crystals pre-treated with sodium chloride, guanidine hydrochloride and urea is shown in Figure 31. It may be noted that absorbance changes caused by the addition of trypsin to crystals soaked in urea are essentially the same as

Figure 30



Neutralization of sodium hydroxide by crystals (Pc 200 μgm/ml) when soaked at pH 9.5. The control system was merely a water blank under identical conditions. Both systems were incubated at 25°C.

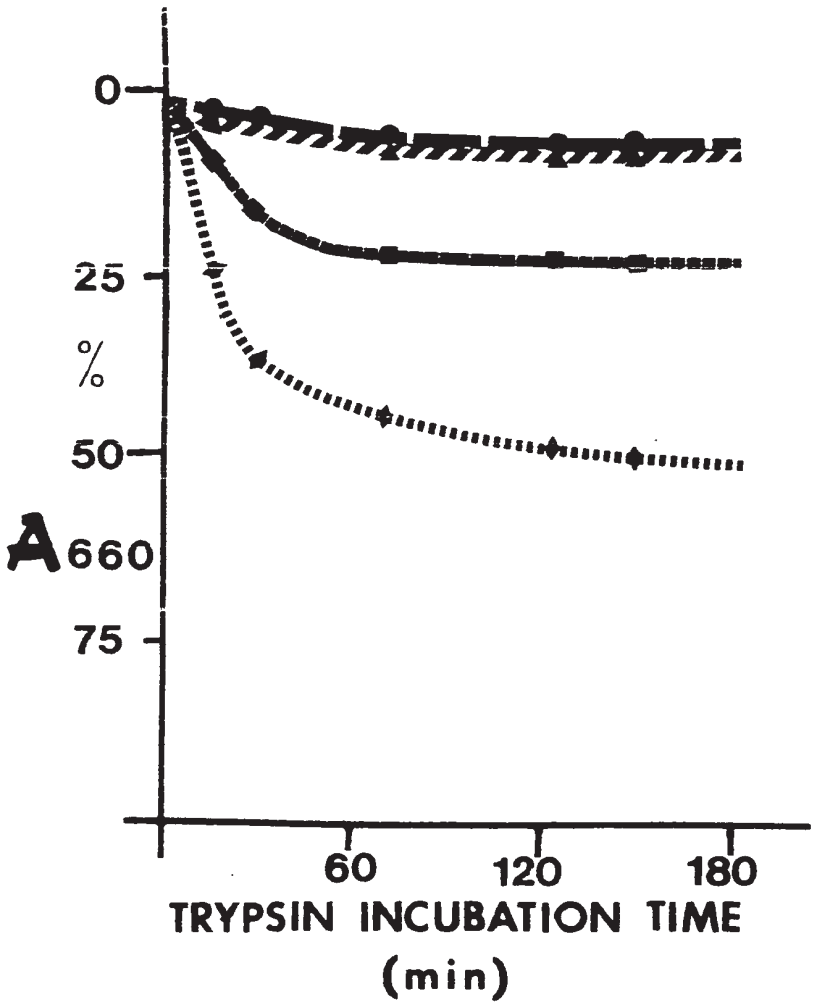
TABLE 14

Effect of Various Reagents on Crystals Soaked in Sodium Hydroxide at pH 9.5 for Four Hours

Reagents at pH 9.5	Amount of Clumping		Absorbance Decreases		Protein Solubilized	
	Crystals Not Soaked	Crystals Soaked	Crystals Not Soaked	Crystals Soaked	Crystals Not Soaked	Crystals Soaked
Control (water only)	-	-	-	-	-	-
Trypsin	-	+4	-	50%	±	±
Potassium Carbonate (0.15M)	+2	+2	25%	25%	29%	32%
Tris (2.0M)	-	+2	36%	40%	49%	54%
Guanidine Hydrochloride (3.0M)	+4	+4	75%	49%	20%	39%

Numerical rating as outlined in Table 13

Figure 31



Effect of trypsin (10 µgm/ml) on crystals preincubated for 120 minutes in sodium chloride, guanidine hydrochloride and urea. All incubations were at 35°C and pH 7.5 ± 0.25, the final Pc was 100 µgm/ml in each system.

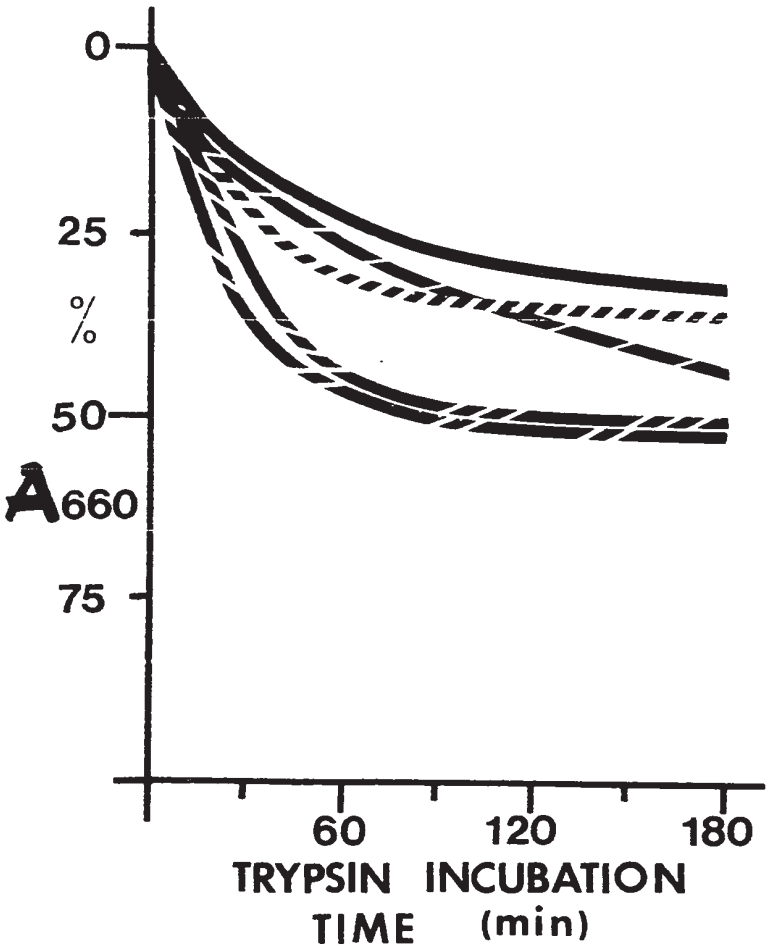
Control (water)
NaCl (3.0M)
GuHCl (3.0M)
Urea (3.0M)

those found in the control system. However changes induced by the addition of trypsin to sodium chloride or guanidine hydrochloride systems indicated increased clumping. When crystals and 3.0M guanidine hydrochloride were incubated for various time intervals and then treated with trypsin it was found that an incubation period of 120 minutes (for crystals and guanidine hydrochloride) was sufficient for maximum absorbance decreases (Figure 32). Incubation periods in excess of 120 minutes appeared to cause a diminished trypsin effect.

(m) Effect of Tris on Crystals

When crystals were incubated in 3.0M tris at pH 8.0 the change in absorbance with increasing time was much more pronounced at 4°C than at 25°C (Figure 33). Reaction of tris and crystals was shown to be especially dependent on pH and temperature (Figure 34). Microscopic observations showed no massive crystal clumping when tris was incubated with crystals. At the lower temperature the crystals showed a tendency to form very straight chains with about 25 to 50 crystals in each. This was particularly pronounced at -5°C and pH 9.5, it suggested inter-crystal bonding occurred at the apices of the short axes. The individual crystal morphology appeared to be changing from the typical bipyramidal structure to a more cube-like shape. These crystal forms could not be photographed due to the adverse reaction of tris with nigrosin and also the optical interference of tris in unstained preparations. The straight chain artifacts are represented diagramatically in Figure 35. After about 24 hours at -5°C the crystals had assumed a cuboidal shape with a length to width ratio of approximately 1:1. This transitory phase was gradually replaced by swelling in all planes giving a crystal of normal morphology but about 2-3 times larger.

Figure 32



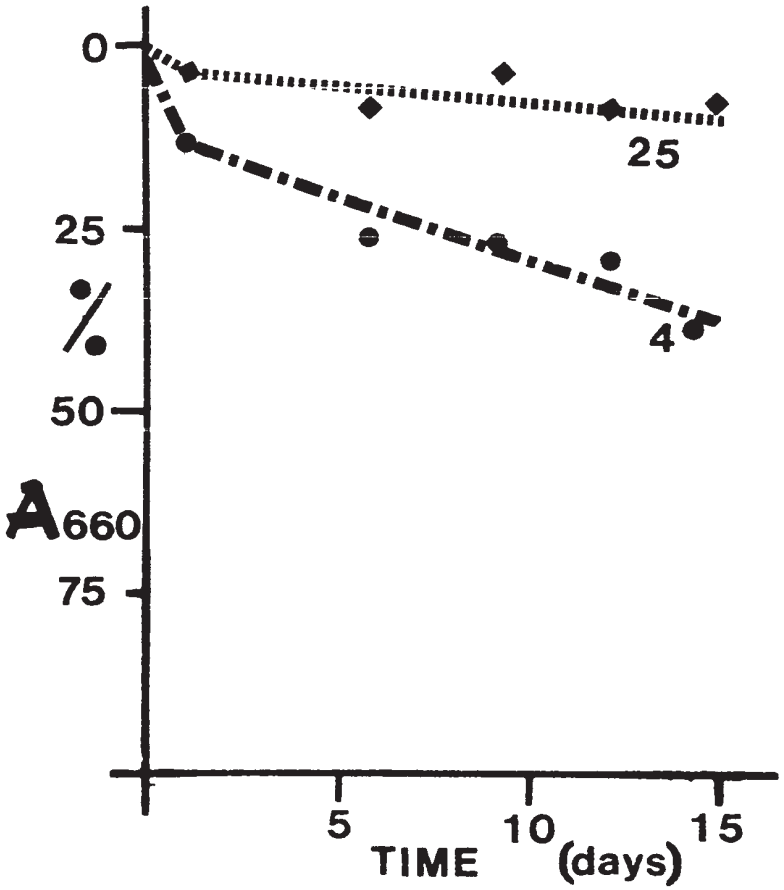
Incubation of crystals in 3.0M guanidine hydrochloride for increasing time intervals, followed by further incubation in trypsin (10 µgm/ml). All reactions were at 35°C and pH 7.5 ± 0.25, final Pc was 100 µgm/ml.

Guanidine Hydrochloride Incubation Time

10 minutes
60 minutes
120 minutes
180 minutes
1200 minutes

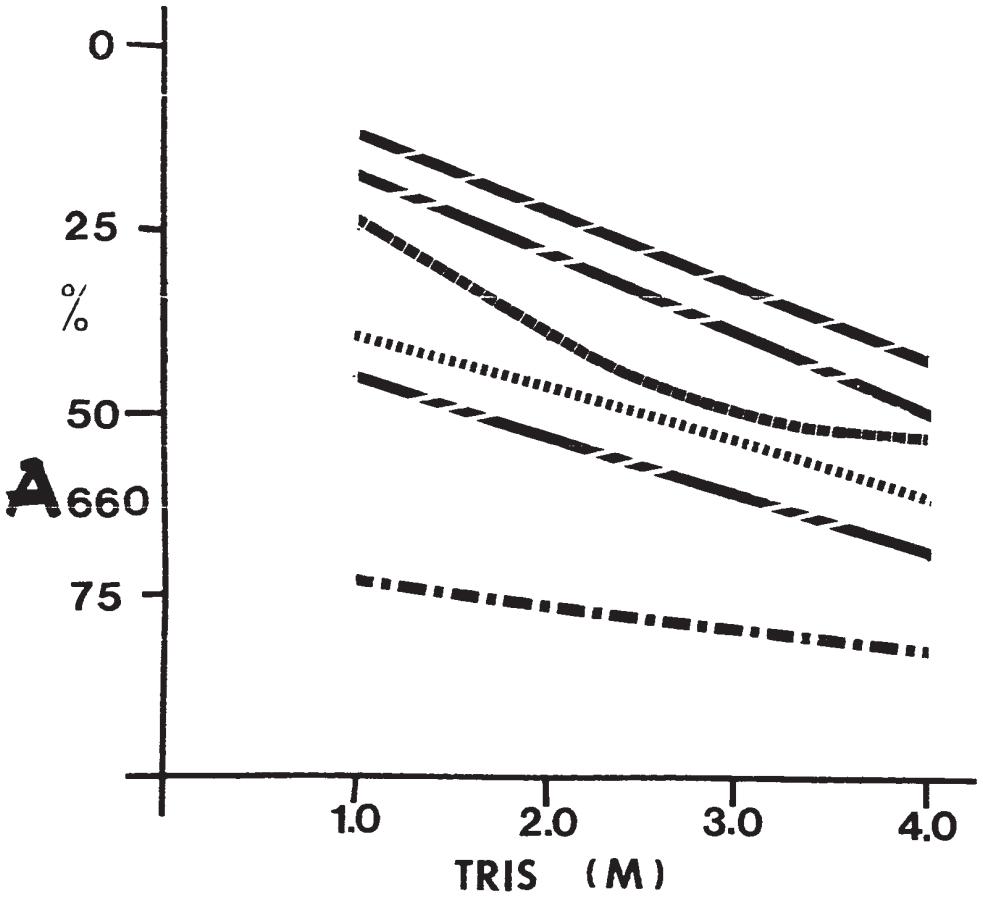
Data used to construct this Figure are shown in Appendix IV, Table B.

Figure 33



Incubation of crystals (Pc 75 µgm/ml) in 3.0M tris (pH 8.0) for increasing periods of time.

Figure 34

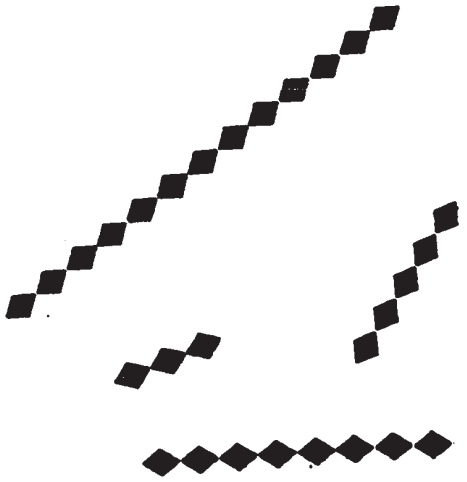


Effect of tris on crystals at different temperatures and pH levels. Incubation was for 120 minutes at a final Pc of 75 µgm/ml.

25°C	pH	-5°C
▰▰▰	9.5	▬▬▬
▰▰▰	10.0	▪▪▪
▰▰▰	10.5	▬▪▬▪

Data used to construct this Figure are shown in Appendix IV, Table C.

Figure 35



Diagramatic effect of 3.0M tris (pH 9.5) on crystals. Incubation temperature was -5°C.

Systems of crystals and tris at pH 10.0 and 10.5 showed a considerable decrease in absorbance when the reaction temperature was changed from 25°C to -5°C (Figure 36), demonstrating clearly that the ability of tris to react with crystals was more effective at reduced temperatures.

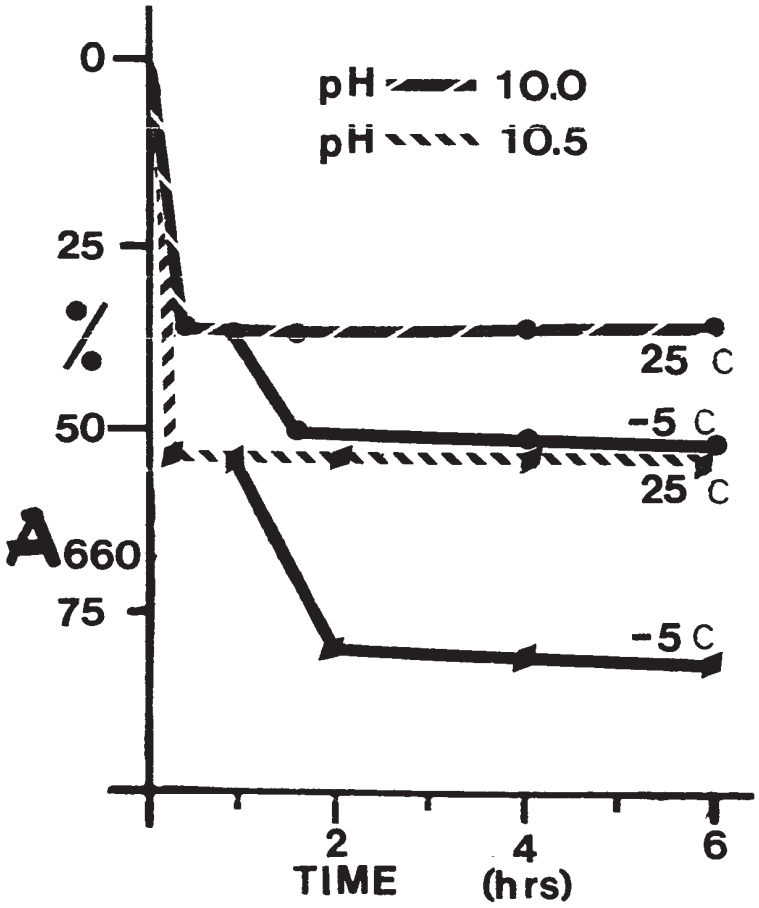
(n) Combined Reagent Effects on Crystals

The combined action of sodium hydroxide soaking followed by tris and guanidine hydrochloride all acting on the crystals at pH 9.5 at varying temperatures completely solubilized crystal protein. This procedure is schematically outlined in Figure 37. After centrifuging no particulate matter could be found in the bottom of the centrifuge tubes. A less effective solubilization was achieved by replacing the guanidine hydrochloride with potassium carbonate (0.15M final concentration), with approximately 50% of the total protein load being solubilized. The effectiveness of this scheme depended upon the stepwise attack on crystals by each of the reagents in the exact sequence outlined.

E. Experimental Reproducibility

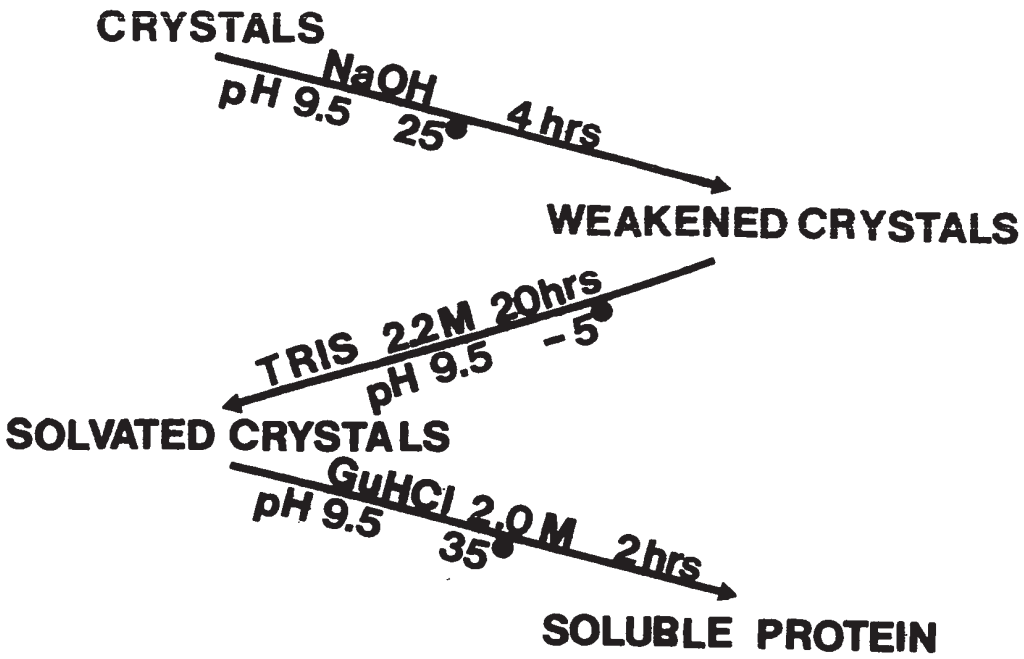
The atypical biological characteristics encountered in almost all aspects of the crystals, together with the possibility of complex chemical and physical interactions in the various reaction systems necessitated that results be of a clear, definite nature and many effects are supported with photomicrographs. All experiments reported in this dissertation were carried out at least twice. Positive results upon which subsequent experimentation was based were repeated many more times. Data presented are either the average of several trials or the most complete set of typical data for a particular effect.

Figure 36



Effect of a downward temperature shift on crystals incubated in 3.0M tris at pH 10.0 and 10.5.

Figure 37



Schematic outline of combined crystal solubilization method. Initial Pc was 200 μgm/ml.

DISCUSSION

A. Growth Studies

Although the ability to produce an intracellular crystal is regarded as a stable genetic property of the B.t. varieties it must be acknowledged that the actual production of these structures is extremely variable. This suggests the need for the maintenance of a delicate metabolic balance in order to realize high crystal yields. A rough indication of this was encountered with the balancing of the carbon and nitrogen sources in the final production medium. An even more delicate balance was necessary with the levels of manganese and magnesium. When B.t.t., B.t.e. and B.t.s. were cultured on the final production medium, crystals were produced in 36 to 40 hours. The crystals were initially small in size but by the end of the growth period crystals of varying sizes (Figure 4) were present. If crystal size is dependent upon the crystallizing out of protein subunits, as suggested by Anderson and Rogoff (1966), then the larger crystals could be the first crystals formed. Pulse labelling studies with amino acids could possibly answer this question. Another explanation might be that only small crystals are produced but those first produced swell in the slightly alkaline growth medium. As the medium contained only seven comparatively simple nutrients and required no special handling it was suitable for the production of crystals on the pilot scale. However it should be noted that when this

medium was used some variation in crystal yield was encountered which could not be attributed to any particular variable. Refinement of the medium to produce even higher crystal yields should be possible.

Many morphological and physiological relationships exist between Bacillus cereus and Bacillus thuringiensis. A few of the physiological properties have been slightly extended by these studies. The well documented requirement of Bacillus cereus for additional oxygen during sporulation (Halvorson, 1962) also appears to exist in growing B.t. populations. Growth systems in both the bench and pilot scale fermentors required extra high levels of oxygen well past the logarithmic growth phase.

These two species of bacilli also seem to be related in their ability to produce PHB. There can be little doubt that the 'granular stage' so often observed in growing populations of B.t. was in reality the intracellular accumulation of PHB granules. The formation of these structures with increased glucose in the medium, the large number of granules per cell, the insolubility of the granule in the Williamson and Wilkinson reagent and the apparent cessation of cell differentiation upon accumulation of large numbers of granules, all give overwhelming support to this conclusion. Although it has been noted that PHB accumulation is not a requirement for sporulation, and that accumulation of large amounts of PHB delayed sporulation, it has been reported by Fitz-James (1965) that if PHB is present at the onset of sporulation it disappears during the intermediate steps of sporulation. Hence it would seem that the low levels of PHB recognized in some B.t. growth systems may be acceptable. Therefore the total elimination of glucose from the production medium may not be necessary.

The essential role of magnesium in B.t. growth systems may be tied in with PHB metabolism. If it is agreed that the intracellular PHB must be consumed in order to attain high levels of sporulation, and hence crystal production in the case of the B.t. varieties, then magnesium could be involved in the degradation of the PHB. This role for magnesium is known with Sphaerotilus species (Stokes and Parsons, 1968). Also some metabolic similarities between Bacillus and Sphaerotilus species have been suggested by Armbruster (1969). Additional indirect support for a key role by magnesium in crystal production is acquired from the fact that almost all media supporting PHB and crystal production contained fairly high levels of this ion (Tables 1 and 2). Media supporting high levels of sporulation with no concomitant crystal production generally did not contain these high levels of magnesium. This suggests that crystal production may be closely related to PHB metabolism.

Another common physiological relationship between Bacillus cereus and Bacillus thuringiensis appears to be their ability to endogenously metabolize two substrates. Clifton and Sobek (1961) reported that Bacillus cereus could simultaneously metabolize both PHB and a nitrogenous substrate. At the end of their sporulation phase B.t. cultures reached an abnormally high pH level (8.5 - 9.0) and a very strong ammonia odour suggested deamination. The metabolism of two endogenous substrates and the requirement for increased oxygen tension peculiar to Bacillus cereus (Dawes and Ribbons, 1964) also seemed to be characteristic of B.t. varieties. The increased oxygen demand, high final pH and ammonia production occurred in all B.t. populations that sporulated regardless of their degree of crystal production.

A further observation on the metabolism of growing B.t.

cultures concerned an atypical pH pattern in the culture broth. The normal pH profile observed during growth of most, if not all bacilli, failed to manifest itself when B.t. cultures produced high crystal yields. Cultures that produced few if any crystals showed a normal pH curve. Cultures of B.t.t., B.t.e. and B.t.s. grown in both sizes of fermentors were most consistent with regard to broth pH pattern. If the culture started to become more acidic during the first two or three hours of growth then the system invariably produced little or no crystals. If however the pH started to rise during this time then the experiment was always successful and high yields of crystals were realized. During these studies no intermediate situations were encountered. Reports in the literature (Megna, 1963 and Dubois, 1968) showed that crystals are obtained with an initial acid shift however it is not clear if such growth systems produced intermediate yields of crystals. The acidic pH shift and PHB accumulation, both adverse conditions for high crystal yields, are probably related.

The appearance of an alkaline peptidase (protease) in culture broths during sporulation by the bacilli is a well documented phenomenon (Schaeffer, 1967; Levisohn and Aronson, 1967). The role of the peptidase at this stage of growth is not clear, however it is known to be linked closely to the sporulation genes. Sporulating cultures of B.t.s. that produced high crystal yields failed to elaborate any detectable amount of extracellular peptidase. However when cultures of B.t.s. did not produce crystals, large amounts of peptidase were detected in the culture broth. Although the enzyme was not tested for esterase activity its late appearance and slight alkaline pH optimum suggest that it might be the peptidase associated with sporulation. The ability of this peptidase to

cause surface deformation of native crystals implied that the crystal protein might act as a substrate. It seems improbable that the peptidase protein could be adsorbed onto the crystal surface in sufficient quantity to cause the observed change in morphology. Rogoff (1966) has noted that high peptidase production can cause dissociation of B.t. crystals as they are formed. The presence of peptidase or crystals, but not both, suggests the possibility of a crystalline pro-enzyme that is active in the dissociated state. Srinivasan and Craig (1968) speculate that such may be the case for a similar situation found in the insect pathogen Bacillus popilliae. These authors also state that the late peptidase of Bacillus popilliae is different from the alkaline peptidases associated with sporulation. Rogolsky (1968) has shown that an asporulant, peptidase-deficient strain of Bacillus subtilis could form an insoluble intracellular protein structure. This was hydrolysed by the sporulation peptidase produced by the parent strain. It was suggested that the insoluble protein was an intracellular substrate which upon degradation provided the necessary amino acids for sporulation. The relationship between B.t.s. crystals and this extracellular peptidase remains unclear. Pfister, Lundgren and Remsen (1969) have stated that PHB granules have an enzymatically active surface layer responsible for storage and degradation of this polymer. If the insoluble protein reported by Rogolsky (1968) is a storage protein, could a hydrolytic enzyme be similarly associated with it and with B.t. crystals? The occurrence of an atypical pH pattern in growth systems that produce crystals but not peptidase invites speculation. Could the atypical pH influence protein solubility?

B. Crystal Purification

The presence of lipids in the spore wall and a lack of any lipid material in crystals coupled with the known hydrophobicity of spores provided the basis for initial attempts at a physical chemical purification technique. The observation that polysilicate globules attracted many spores but no crystals (Figure 5) added to the strength of this working hypothesis. Crystals held within the spore aggregates were readily washed out suggesting merely a physical entrapment. Crystals were subjected to a chloroform environment for a minimum time due to the denaturing effects this solvent has on some proteins. However no adverse effect could be attributed to the action of chloroform on the native crystals.

In order that the purification method might be effective with a high degree of reproducibility, the following factors are emphasized. Starting material must be in a dried, preferably lyophilized, state as there were some indications that the amount of water bound to spores could influence the success of the method. The ratios of weights and volumes followed in this method must be held within the limits outlined. An efficient, but gentle mixing is necessary for the solvent to react with the spores and cellular debris. Rapid mixing on a vortex type mixer proved to be the most suitable. Any filter system may be used provided it has the ability to trap clumped material and allow the free crystals to pass through.

The final purification ratio (crystal to spore count) of 2,000:1 to 3,000:1 with a crystal yield of 5-20% (dry weight basis, purified crystal versus starting material) exceeded those reported in the literature. Earlier methods generally achieved purification ratios

in the order of 99:1 with yields not greater than about 5%. An exception was a somewhat more involved method outlined by Goodman et al (1967) who reported a purification ratio of 1665:1 with a final yield of 2.5%. Because of the simplicity and comparatively short time required by the chloroform method it was possible to prepare gram quantities of highly purified crystals for biochemical studies.

C. Amino Acid Calculations on the Minimal Polypeptide

A cursory examination of the amino acid composition of crystals showed that despite similarities within the B.t. varieties (Table 8) no single amino acid was present in sufficient quantity to account for the overall stability of the crystal. Excesses of amino acids as found in wool, silk and gelatin were not present in parasporal proteins. It is well documented that crystals from different B.t. varieties do not have the same toxic effect on a given insect. For example B.t.s. crystals are about 150 times more toxic to silkworms than those from B.t.t. Hence the close similarity in amino acid composition of the different B.t. crystals suggests that toxicity variations may be due to differences at the primary or quaternary level. The former involving sequence changes and therefore alterations in the structure of the toxic entities, while the latter could involve changes in stabilizing forces influencing the degree of solubilization achieved by the gut contents of the host insect.

A further examination of the amino acid composition of crystals suggested a strong correlation between stabilizing forces and the content of nonpolar amino acids. The minimal molecular weight of the limiting polypeptide was about 19,000 (Table 12) and the number of residues in each chain showed many similarities (Table 11). An estimation of the

helical content produced values not unusual for globular proteins. Calculation of non-polarity by the N.P.S. ratio of Waugh (1954) and of polarity by the method of Fisher (1964), both suggested a higher than normal content of nonpolar residues. The total content of hydrophobic residues, estimated according to Van Holde (1966), clearly showed an excess of nonpolar residues. An even greater indication of a strong stabilizing role played by nonpolar residues was found in average hydrophobicity values calculated by the method of Bigelow (1967). Here all values were in excess of 1050 calories per residue, suggesting the hydrophobic bond was a significant stabilizing force in B.t. crystals.

It is interesting to note that crystal proteins bear many similarities to the structural proteins of membranes (Green, Haard, Lenaz and Silman, 1968) and of connective tissue (Timpl, Wolff and Weiser, 1968). All are characterized by similar amino acid compositions and all require fairly harsh solubilization procedures which usually demand the presence of several dissociating forces and/or extreme chemical or physical conditions. Sund and Weber (1966) noted that the stability of quaternary proteins varied widely from one protein to another and that dissociation may be brought about by disruption of covalent or non-covalent bonds due to:

1. Changes in pH .
2. Temperature changes.
3. Solvent modifications by the addition of organic solvents, urea, guanidine hydrochloride and other salts.
4. Addition of detergents, heavy metals and chelators.
5. Chemical modifications and substitutions in functional groups.

Joly (1965) has reviewed the physical and chemical agents involved in

protein denaturation, the differences between this phenomenon and crystal solubilization appear to be rather subtle.

Timpl et al (1968) used 0.2M sodium hydroxide in order to extract non-collagenous structural proteins from various connective tissues. These proteins were insoluble in 8.0M urea (pH 7.0) and only partially soluble in 6.0M guanidine hydrochloride. The amino acids found in the alkali solubilized proteins lacked hydroxyproline and hydroxylysine, in many ways the amino acid composition of these structures resembled crystal protein and also those proteins in the class proposed by Green et al (1968). These latter workers observed that protein fractions, similar to structural protein of beef heart mitochondria, have been isolated from membranes of microsomes, chloroplasts, nuclei, sarcoplasmic reticulum, muscle sarcolemma and red blood cell ghosts. Also they showed that core protein, a mitochondrial noncatalytic structure, represented a group of proteins not grossly dissimilar to structural proteins.

Structural proteins, core proteins, and the predominant protein species in repeating units of microbial, plant and animal membranes were remarkably similar in amino acid composition, peptide maps, degree of hydrophobicity and overall solubility. On this latter point Green et al (1968) noted that solubilization regimas for structural and core proteins exhibited a bewildering array of solubility characteristics. Under some extraction conditions high concentrations of urea and guanidine hydrochloride failed to solubilize these proteins and they remained resistant to proteolytic enzymes, while under other conditions the converse was true. In almost all cases complete solubilization required the combined effects of several dispersing reagents. Although Green et al (1968) did not include virus coat proteins in their class of structural proteins,

many common characteristics appear to be shared by these two groups. Criddle (1969) has considered the similarities of these two protein types in a review of structural proteins.

The stabilizing forces found in globular proteins possessing a quaternary structure appear to vary greatly. Consequently the gross stability of the native structure may be such that mere dilution can cause dissociation, as is the case with beef heart glutamic dehydrogenase, or more harsh conditions and the combined effects of several dispersing forces may be required to effect solubilization. Many intermediate situations may be found in the literature, some of these are considered by Sund and Weber (1966). An additional complication when studying associating proteins is their low solubility in normal buffers and a natural tendency to re-associate after dissociation has been achieved. It is clear that successful solubilization of these more stable proteins require a somewhat empirical approach carried out under rigidly defined and highly reproducible conditions.

D. Biochemical Studies

Although B.t. crystals are extremely stable structures being able to withstand the effects of high heat and various concentrations of acid, it is apparent that their surfaces are capable of considerable reactivity. Surface reactive properties of crystals have been shown by Hannay (1953) and Robertson and Heimpel (1962). Changes in packing and color of purified B.t.s. crystals at different pH levels also suggested some type of surface reactivity. The clumping of crystals induced by many reagents (Table 13) further suggested a surface reactivity. If the crystal is assumed to be a homogeneous protein made up of repeating units then the solubilization of this structure may be linked with

accessibility of different reagents to the inner depths of the crystal.

The water content of B.t.s. crystals was approximately 60% on a weight basis. This value is at the high end of the range for water bound to globular proteins (Mathews, 1968). However it does not seem unreasonably high if one considers the number of polar groups in B.t. crystals and the relationship between polar residues and water binding capacity (Bull and Breese, 1968). If this large amount of bound liquid in crystals is present in interconnecting channels, as is normal with globular proteins (Wyckoff et al, 1967), then small molecules might have access to the interior of the crystal. The large amount of water bound to B.t.s. crystals could be important in the overall crystal stability.

At near neutral pH, succinic anhydride was not effective in dispersing crystals (Figure 15), hence the disruptive force usually brought about by this concentration of reagent was not sufficient to overcome other stabilizing forces within the crystal. Cationic and neutral detergents had no dispersing action on crystals (Figure 16) over a period of several hours. Neutral detergents did cause an increase in absorbance of crystal-detergent systems, suggesting some type of reactivity, but no changes in crystal morphology were detected. These latter detergents are added to some commercial preparations of B.t., thus if their reactivity with crystals over a period of many months could cause partial crystal dispersion then this might explain the sometimes noted increase in toxicity of these preparations on prolonged storage. The anionic detergent, sodium lauryl sulphate, gradually solubilized crystals at pH 9.5 (Figure 16), however the ratio of detergent to protein on a weight basis was 100:1. This high ratio together with the ability of proteins to bind up to 1.4 times their weight of sodium lauryl sulphate (Pitt-

Rivers and Impiombato, 1968) discouraged further use of the detergent in selectively dissociating the crystal.

Crystals in carbonate buffer at pH 10.0 were treated with different thiol and disulphide reagents (Figure 17). Para-chloromercuribenzoic acid failed to disperse the crystal. This was not surprising as crystals appear to contain little or no free cysteine (Lecadet, 1966). The negligible effect of sodium thioglycollate could be explained by the low weight ratio of reagent to protein (1:10). The ability of mercaptoethanol or dithiothreitol to rupture disulphide bonds seemed to explain the rapid dispersing effect of these reagents on the crystals. However it must be remembered that crystals contain only 1.20 $\pm$ 0.20% cystine, an amount that can hardly account for the gross stability of the crystal. The effect of silver nitrate on crystals was somewhat surprising. After an initial delay of about 90 minutes, crystals soaked in this reagent began to undergo absorbance changes and after four hours they were 90% dispersed. Although it is generally agreed that silver nitrate will not rupture disulphide bonds it appears that above pH 9.0 cleavage of these bonds may occur (Cecil, 1963). It should be noted that all of the above reagents showed no effect on the crystals at pH 9.5, hence it is doubtful that the sulphur reagents were the only disruptive forces acting on the crystals.

If the minimal polypeptide with a molecular weight of about 19,000 does contain four cysteine residues (Table 11), it would be possible to have both interchain and intrachain disulphide bonds formed with a resulting molecular weight of at least 38,000 or 57,000 i.e. the approximate size of the intermediate structure in the crystal unit cell. Because interchain disulphide bonds are generally more labile than intra-

chain bonds the dispersion of crystals by disulphide rupture might be brought about under comparatively mild conditions by cleavage of the weaker bonds.

The delayed effect of silver nitrate on crystals is of further interest. If disulphide bonds are cleaved, why the delay? The situation found here may be similar to that encountered with potato virus by Reichmann and Hatt (1961). They showed that when bulky radicles like para-chloromercuribenzoic acid were tagged to free thiol groups, the virus structure was dissociated. However when smaller groups (iodoacetate or silver nitrate) were reacted with the thiol groups no dispersion occurred. Although the free thiol groups were not involved in stabilizing the quaternary structure of this virus the dispersion was explained on the basis of steric stress induced by the larger bound radicles. It is possible that similar steric stresses play a role in dispersing B.t.s. crystals once disulphide bonds have been ruptured. Greater stress could be brought about with mercaptoethanol and dithiothreitol while the silver nitrate effect, although less, could be potentiated by alkaline pH and buffer salts acting on a weakened crystal. Hence the delayed effect of silver nitrate.

Studies continued on the effects of rupture of noncovalent bonds. Crystal reactivity with urea (Figure 18) and guanidine hydrochloride (Figure 20) was shown to depend upon protein to dispersant ratios, pH and time. The latter reagent seemed to have a stepwise effect on crystals and initial crystal clumping (Figure 13) was followed by swelling in the longitudinal plane only (Figure 21). This was then followed by swelling in all planes (Figure 22). Crystal swelling induced by urea at 35°C was not stepwise and appeared to be in all planes (Figure 19).

When crystals were subjected to high concentrations of urea or guanidine hydrochloride at an alkaline pH, total solubilization was never accomplished. Some disintegration could be observed microscopically at crystal tips but flocculent masses of protein and reddish-brown, gel-like precipitates invariably formed.

Boiling crystals in dilute acid caused rapid dispersion (Figures 23 and 24) and the appearance of free aspartic acid. The selective effect of dilute acids on proteins has been reported by other workers (Partridge and Davis, 1950; Leach, Rogers and Filshie, 1964 and Tsung and Fraenkel-Conrat, 1965). This behaviour suggested that aspartic acid might be involved in the crystal bonding forces through the establishment of ionic bonds. Subsequent studies showed that alkaline potassium carbonate could solubilize some crystal protein (Figures 25, 26 and 27). The optimum concentration of this reagent was essentially that found in the gut contents of silkworms. It is generally accepted that carbonates have some disruptive effects on proteins through ionic interaction. Other investigators have shown potassium carbonate to have a dissociating effect on membrane protein (Neville, 1967) and wool (Mellet, 1968).

The effect of various reagents on 'old' crystals merely represented tests on crystals that had accumulated in cold storage. These tests were done at a time when it was becoming increasingly difficult to duplicate prior work. All tests suggested that as crystals were stored in a liquid environment for one or more weeks there was a change in their reactive properties. The different effects of urea (Figure 28) and sodium lauryl sulphate (Figure 29) on fresh and 'old' crystals confirmed these suspicions. However they did not establish

whether crystal weakening was due to alkaline soakings at pH 8.0 to 10.0 or to long term reaction with buffer ions or both.

When crystals were soaked at room temperature in an alkaline environment (pH 9.5) with no buffer ions present, hydroxyl groups were taken up (Figure 30) and the crystal structure appeared to be weakened (Table 14). It is suggested that these changes involved the disruption of water bound to the surface of the crystal. Hence the reason for trypsin reacting with soaked crystals to produce massive clumping while no similar effect was observed with unsoaked crystals. The inability of trypsin to attack many proteins, including B.t. crystals, has been clearly established. It is believed that many proteolytic enzymes fail to attack native proteins because of the protective effect afforded the substrate by an outer layer of bound water. Sodium chloride and guanidine hydrochloride, both shown to be effective water breakers by Katz (1968), also caused crystals to become sensitive to trypsin (Figures 31 and 32). Urea did not demonstrate this effect. Tanford (1957) has reported that acid soaking of ferrihemoglobin caused an uptake of protons and a weakening of the molecule.

When crystals were soaked in cold tris it was found that they were dispersed to a limited extent (Figures 33 and 34). The effect of the same concentrations of urea at reduced temperatures was similar but not as extensive. Dispersion at reduced temperatures is a characteristic of proteins presumed to be hydrophobically bonded (Kauzmann, 1959). The hydrophobic values of B.t. crystals (Table 12) are such that this type of bonding could account for considerable stabilization. The effect of tris, and to a lesser extent urea, suggested that these compounds enhanced the destabilization of hydrophobic bonds at reduced temperatures. Many

reports in the literature attest to the dispersing effect of tris on proteins. Aconitase has been separated into two subcomponents after incubation at 0°C for six days apparently in 0.05M tris at pH 7.4 (Peters and Shorthouse, 1969). The solubilizing effect of EDTA and lysozyme on bacterial cell walls has been enhanced by tris (Cox and Eagon, 1968). Tris has also been shown to dissociate tryptophanase (Gopinathan and DeMoss, 1968), release compounds from human dermal tissue (Einbinder, Walzer and Mandl, 1966) and inhibit xylanase (Walker, 1967).

Crystals incubated in tris showed some interesting morphological changes. Although final crystal swelling was always in all planes and a grossly swollen structure of normal shape resulted, early changes showed a tendency for crystals to bind together forming very straight chains (Figure 35). These then changed to cube shaped crystals. On occasion a transitory cube shape was also observed in urea-crystal systems. Crystals in guanidine hydrochloride showed a similar final morphology, i.e. grossly swollen structures with normal diamond shape. However the transitory phase produced long needle-like crystals (Figure 21). Cuboidal shaped crystals were never observed in guanidine hydrochloride systems. This effect suggested swelling in the longitudinal plane only, while the tris influence suggested swelling in a different plane. A possible explanation of these two transitional shapes is that the reagents weakened different stabilizing bonds thereby providing a weakening in different planes. The final grossly swollen crystal of normal shape could result from rearrangement of the remaining stabilizing forces in an attempt to hold the structure together. It was noted by Grigorova et al (1967) that cuboidal shaped parasporal inclusions were

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less stable than the usual diamond shaped structures.

Findings at this time suggest that several different forces combine to stabilize the crystal. These are formed as a result of high levels of polar and nonpolar residues, a high content of bound water and a comparatively low but still significant disulphide level. Reagents known or believed to react with each of these properties gave partial crystal dispersion. Although the effectiveness of these reagents was usually dependent upon concentrations, temperature, time and pH, the chemical characteristics of each reagent were sufficiently unique to suggest a specific effect.

The solubilizing effect of potassium carbonate on crystals was presumably due to a rupture of ionic bonds or possibly to an interaction with amide groups (Robinson and Jencks, 1965a). Although this reagent gave nearly complete solubilization at pH 11.0 (Figures 26 and 27) the pH level was judged to be too high for a useful in vitro dispersion. At the lower and more acceptable level of pH 9.5 some crystal reactivity remained but the overall effect was less (Figures 25 and 26).

Under certain conditions guanidine hydrochloride at high concentrations could solubilize crystal protein (Figure 20) however much the resulting protein was in an apparently denaturated state. At lower concentrations (2.0 to 3.0M) this reagent still possessed some reactivity towards crystals as it caused considerable crystal swelling (Figure 22) and therefore an apparent loosening of the quaternary structure.

The dispersing effect of tris and to a lesser extent urea, both acting at reduced temperatures, implicated hydrophobic bonds as stabilizing forces within the crystal. The effect of tris was pH dependent (Figure 34)

and once again only partial solubilization occurred at pH 9.5.

The peculiar, but similar, reactivity with trypsin of crystals soaked at pH 9.5 or with water breakers suggested a disruption of water bound to the crystal and a subsequent weakening of the structure. Although such treatments produced no soluble protein, they seemed to provide some way to weaken the crystal and thereby make it more susceptible to different reagents (Table 14). The question now of paramount importance was, would the disruptive influence of two or more of the preceding reactions, acting together at the acceptable pH level, cause a competitive, additive or synergistic effect?

Combinations of different treatments ultimately showed that it was possible to completely disperse crystals at pH 9.5 (Figure 37), however the sequence of crystal reactivity with each reagent proved to be critical and a sequential attack was more effective than the simultaneous addition of all reagents. To achieve solubilization it was necessary to first soak the crystal at pH 9.5. This presumably weakened the bound water on the crystal surface thus weakening the protein structure and making it more accessible to other reagents. After this treatment it was essential that crystals be treated next with cold tris before a maximum beneficial effect could be obtained by subsequent treatment with guanidine hydrochloride or potassium carbonate. If it is accepted that the tris treatment attacks hydrophobic bonds then it would appear that these bonds must be ruptured immediately after water disruption but prior to ionic and hydrogen bond attack. With the weakening of bound water and hydrophobic bonds the crystal was then more susceptible to the effects of guanidine hydrochloride and potassium carbonate. Potassium carbonate, when used instead of guanidine hydrochloride in the final

step of the solubilization procedure, was not as efficient.

In order to be effective, the stepwise dissociation of crystal protein described here must be conducted in the sequence outlined. This suggests an interdependence between stabilizing forces, where it is necessary to weaken one force before another is accessible or sensitive to attack. The use of a low concentration of protein throughout these studies was done in an attempt to ensure that sufficient dispersing reagent was available to react effectively with the crystal protein. The complete solubilization of the crystal at pH 9.5 with moderate concentrations of reagents was brought about by the apparent rupture of only noncovalent bonds. This implies a secondary structural role for the disulphide bridges in the crystal. Perhaps they are involved in a smaller structure than that produced by the described solubilization scheme.

The suggestion that the peculiar stability of native B.t. crystals is due to the combined stabilizing effects of different non-covalent bonding forces, rather than a predominance of only one type of stabilizing force, appears to offer a plausible explanation for the gross stability of these structures. However it must be asked, how reliable are the assumptions upon which this suggestion is based? If each proposed stabilizing force is considered in sequence, then the bound water of hydration must be considered first. Is this water weakened or disrupted by the alkaline soaking of the crystals at pH 9.5? If a water lattice structure did form around the crystal subunits, and consequently around the crystal, it would appear to be a logical first barrier. Such an effect would be in agreement with the suggestion of Klotz (1965) that hydrophobic areas on the surface of proteins are

surrounded by structural water and that disruption of this water is necessary for destabilization of hydrophobic forces. The hydrophobic content of the crystals is such that not all of these forces can be readily buried inside the protein subunit.

It has been shown that B.t. crystals can bind reasonably high levels of water. Also, it has been established that alkaline soaking must be carried out before any other step in the solubilization scheme. Katz (1968) has presented data on the effects of various salts, including sodium chloride and guanidine hydrochloride, on bovine serum albumin. In this report small changes in the partial molar volume of the protein were measured and related to the water-structure distorting ability of these salts. Both sodium chloride and guanidine hydrochloride possessed the ability to modify the structural water of bovine serum albumin. Both of these salts, at concentrations similar to those used by Katz (1968) also caused some changes within or on the B.t. crystal. These changes were demonstrated best by the effect of trypsin on treated and untreated crystals. Crystals not treated with either salt failed to undergo any microscopic or absorbance changes when treated with trypsin. However crystals that had been soaked in these salts underwent massive clumping when the enzyme was added. Alkali soaked crystals reacted in the same microscopic manner to trypsin as did salt soaked crystals. Although this evidence is of an indirect nature, the similar effect of trypsin on crystals soaked in salts and in alkali suggests that at the quaternary level the structural water of the crystals may have been weakened.

The next stabilizing force to be considered in the crystal solubilization scheme is believed to be the hydrophobic bond since the low temperature treatment was only effective when used as the second step. The increased instability of the hydrophobic bond with decreasing

temperatures (Kauzmann, 1959) has been supported by many original reports. Some of these were reviewed by Nemethy (1967); there can be no doubt about the instability of the hydrophobic bond at reduced temperatures. However, an additional mechanism for protein solubilization at reduced temperatures has been proposed by Robinson and Jencks (1965b). These workers suggested that a 'non-hydrophobic' effect may occur when urea and guanidine hydrochloride act on peptide models and proteins. The 'non-hydrophobic' effect was believed to arise from the specific formation of bifunctional hydrogen bonds between these compounds and the amide bonds of the peptide groups. An increasing degree of alkylation in either of these two compounds reduced their solubilizing effectiveness, suggesting that the presence of free amino groups was necessary for the 'non-hydrophobic' effect to occur.

Incubation of B.t. crystals in tris at reduced temperatures clearly weakened these structures. Although this effect was mediated best by tris, similar concentrations of urea produced a comparable, but not as extensive, effect. When the solubilization scheme was changed and guanidine hydrochloride was used at the second step, i.e. at reduced temperatures, no such weakening could be observed.

As several mechanisms are possible for the interaction of the above compounds with proteins and few hard and fast generalizations may be made safely concerning such interactions, let us consider the specific conditions encountered in the B.t. solubilization scheme. Firstly, there are few reports in the literature on the ability of tris to act as a protein dispersant. It is commonly used as an emulsifying agent, in addition to its role as a buffer. It possesses three hydroxyl groups plus one amino group per mole. Secondly the reduced temperature

weakening of the crystals was most effectively brought about by tris (one amino group per mole), followed then by the lesser effect of urea (two amino groups per mole). Guanidine hydrochloride with two amino groups per mole, failed to show any effect at this step. The effective relationship of these three compounds is in the reverse order of what would be expected if the 'non-hydrophobic' effect of Robinson and Jencks (1965b) were operative. An additional point to be considered here is that in order to achieve the observed degrees of crystal swelling at least part of the tris or urea must be acting at the quaternary level, i.e. some intermolecular forces must be destabilized. In keeping with the generally accepted properties of quaternary protein structure significant amounts of these forces will be hydrophobic interactions, (Sund and Weber, 1966; Nemethy, 1967). No reports are known where the 'non-hydrophobic' effect has been implicated at the quaternary level of globular proteins. It should be remembered that at this point in the solubilization scheme the crystal is grossly swollen but still possesses its characteristic morphology, i.e. protein changes at the secondary or tertiary level would seem remote. Although the possibility of a 'non-hydrobic' effect cannot be completely ruled out at this time it would seem more probable that a hydrophobic interaction has occurred.

The final step in the solubilization scheme employed guanidine hydrochloride to effect the solubilization of the grossly distended and weakened crystal. This compound would seem well suited for this task as it has been shown to disrupt bound water (Katz, 1968), hydrogen bonds and 'non-hydrophobic' forces (Robinson and Jencks, 1965b) and also hydrophobic bonds (Wetlaufer, Malik, Stoller and Coffin, 1964).

No experimentation was undertaken to characterize the soluble

material obtained from this procedure. The presence of several stabilizing forces within the crystal evidently explains the failure of large amounts of single reagents to solubilize this protein. It suggested that the combined strengths of hydrogen, hydrophobic and ionic bonds endow the parasporal proteins with their characteristic stability. With a procedure now available for the dissociation of crystals via non-covalent attack it is hoped that stable structural intermediates may now be isolated and characterized.

VI SUMMARY

1. Growth parameters have been established for the production of high yields of parasporal bodies (crystals) from three different varieties of Bacillus thuringiensis. A liquid semi-synthetic growth medium was formulated that was suitable for production on the pilot scale thus providing a readily available supply of impure crystals from a single batch. Crystal production was characterized by an atypical pH profile and the absence of extracellular peptidase (protease) in the culture broth during growth. Several metabolic characteristics observed in growing cultures of B.t. were shown to be similar to some already known in Bacillus cereus.

2. A physical chemical method has been described for the purification of crystals from the crude material produced via submerged culture. The method uses a chloroform emulsification step and is based on the apparent differences in the hydrophobic properties of crystals and spores. Yields of highly purified, undamaged crystals may be as high as 20% with the presence of only one spore per 3,000 crystals in the final product. The simplicity and efficiency of this method permitted the preparation of comparatively large amounts of purified crystalf for biochemical studies.

3. Studies on the published amino acid composition of B.t. crystals showed that they did not contain a predominance of any particular

residue and that they possessed the properties of normal associating globular proteins.

4. Purified crystals of B.t.s. were subjected to the individual effects of different reagents known to react with proteins in various ways. No single reagent successfully dissociated the crystals under conditions judged to be sufficiently moderate to warrant their individual use. However crystal weakening or partial solubilization was achieved with several reagents, each known to react differently with crystals. Consequently it was suggested that the gross stability of crystals was due to the combined effect of several non-covalent bonding forces acting in such a way that their overall additive effect imparted a high degree of stability to the native structure.

5. The combined utilization of techniques that apparently disrupted bound water on the crystal and also ruptured hydrophobic, ionic and hydrogen bonds permitted the complete solubilization of crystals at a moderate pH level. Further evidence of the interrelationship of the stabilizing forces was suggested in the inflexible sequence by which these methods had to be employed in order to be effective.

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APPENDIX I

Media Constituents

1. Schaeffers Medium

Nutrient Broth	0.80 % w/v
$MgSO_4.7H_2O$	0.025
$MnCl_2.4H_2O$	0.0002
KCl	0.10
Agar	1.70

Water - Distilled

Adjust pH to 7.0 before autoclaving

After autoclaving aseptically add:

$CaCl_2$	5×10^{-4} M (final concentration)
$FeSO_4$	1×10^{-6} M (final concentration)

2. Lyophilization Medium

Powdered Milk	0.75 % w/v
Nutrient Broth	0.20
Glucose	1.00

Water - Distilled

Dispense 50 ml per 100 ml flask and autoclave at 10 pounds per square inch for 10 minutes. Incubate for 48 hours before use in order to check sterility.

3. Trypticase Soy Broth

Trypticase (a pancreatic digest of casein)	1.70 % w/v
Phytone	0.30
NaCl	0.50
K_2HPO_4	0.25
Glucose	0.25

Water - Cold Tap

Autoclave at 121°C for 15 minutes, final pH should be 7.2 ± 0.1.

4. Production Medium (used during these studies to culture B.t.t., B.t.e. and B.t.s.)

Totamine	0.80 % w/v
Soytone	0.20
$CaCl_2$	0.01
KH_2PO_4	0.10
KCl	0.10
$MgSO_4.7H_2O$	0.06
$MnCl_2.4H_2O$	0.0005

pH 6.20 ± 0.1 (after autoclaving)

APPENDIX II

Free Amino Acids Present in Totamine

(Expressed as mg of amino acid per gm of Totamine)

Glu	25.20
Asp	4.55
Ser	8.80
Thr	9.57
Arg	22.50
Lys	48.50
His	10.50
Tyr	4.37
Asn	19.60
Trp	11.00
Phe	29.30
Pro	3.55
Gly	2.21
Ala	8.41
Val	21.20
Ile	16.00
Leu	54.90
Met	14.90
Cysteine	7.93

APPENDIX III

TABLE A

		B.t.t.			B.t.e.			B.t.s.		
(1)	(2)	(3a)	(3b)	(3c)	(4a)	(4b)	(4c)	(5a)	(5b)	(5c)
Amino Acid	M.W.	%	μmoles/mg	Residues	%	μmoles/mg	Residues	%	μmoles/mg	Residues
Glx	147.13	16.0	1.088	19	16.0	1.088	24	17.0	1.155	21
Asx	133.10	14.8	1.112	20	14.0	1.052	23	12.3	.924	17
Ser	105.09	6.0	.571	10	6.0	.571	13	5.6	.533	10
Thr	119.12	7.0	.588	10	6.8	.571	13	5.3	.445	8
Arg	174.21	9.1	.522	9	9.0	.517	12	10.2	.586	11
Lys	146.19	4.5	.308	5	4.4	.301	7	4.3	.294	5
His	155.16	3.0	.193	3	2.8	.181	4	3.0	.193	4
Tyr	181.19	6.5	.358	6	6.5	.358	8	6.4	.353	6
Trp	204.22	2.3	.113	2	2.3	.113	3	2.4	.118	2
Phe	165.19	6.0	.363	6	6.7	.406	9	6.5	.394	7
Pro	115.13	4.6	.400	7	4.4	.382	9	4.3	.374	7
Gly	75.07	4.8	.640	11	4.8	.640	14	5.0	.666	12
Ala	89.09	5.0	.561	10	4.8	.539	12	4.9	.550	10
Val	117.15	7.0	.598	11	7.8	.666	15	6.9	.589	11
Ile	113.17	5.6	.495	9	6.6	.583	13	6.3	.480	9
Leu	113.17	9.5	.839	15	10.5	.928	21	10.0	.762	14
Met	149.21	0.85	.0570	1	0.67	.0450	1	0.82	.0549	1
Cysteine	121.21	2.20	.182	3	2.8	.231	5	2.40	.198	4
		114.75		157	116.87		206	116.87		147

APPENDIX III

TABLE B

Amino Acid	Volume A^3	B.t.t. Residues	B.t.t.	B.t.e. Residues	B.t.e.	B.t.s. Residues	B.t.s.
Glx	84.7	19	1609.3	24	2032.8	21	1778.7
Asx	68.4	20	1368.0	23	1573.2	17	1162.8
Ser	54.9	10	549.0	13	713.7	10	549.0
Thr	71.2	10	712.0	13	925.6	8	569.6
Arg	109.1	9	981.9	12	1309.2	11	1200.1
Lys	105.1	5	525.5	7	735.7	5	525.5
His	91.6	3	274.8	4	366.4	4	366.4
Tyr	116.2	6	697.2	8	929.6	6	697.2
(Polar Volumes)			6717.7		8586.4		6849.3
Trp	135.4	2	270.8	3	406.2	2	270.8
Phe	113.9	6	683.4	9	1025.1	7	797.3
Pro	73.6	7	515.2	9	662.4	7	515.2
Gly	36.3	11	399.3	14	508.2	12	435.6
Ala	52.6	10	526.0	12	631.2	10	526.0
Val	85.1	11	936.1	15	1276.5	11	936.1
Ile	102.0	9	918.0	13	1326.0	9	913.0
Leu	102.0	15	1530.0	21	2142.0	14	1423.0
Met	97.7	1	97.7	1	97.7	1	97.7
Cysteine	68.3	3	204.9	5	341.5	4	273.2
(Non Polar Volumes)			6081.4		8416.8		6197.9

Polarity Ratios: $\frac{6717.7}{6081.4} = 1.1046$ $\frac{8586.4}{8416.8} = 1.020$ $\frac{6849.3}{6197.9} = 1.105$

APPENDIX III

TABLE C

Amino Acid	Hydrophobicity*	B.t.t. Residues	B.t.t. H Φ	B.t.e. Residues	B.t.e. H Φ	B.t.s. Residues	B.t.s. H Φ
Trp	3.00	2	6.00	3	9.00	2	6.00
Ile	2.95	9	26.55	13	38.35	9	26.55
Tyr	2.85	6	17.10	8	22.80	6	17.10
Phe	2.65	6	15.90	9	23.85	7	18.55
Pro	2.60	7	18.20	9	23.40	7	18.20
Leu	2.40	15	36.00	21	50.40	14	33.00
Val	1.70	11	18.70	15	25.50	11	18.70
Lys	1.50	5	7.50	7	10.50	5	7.50
Met	1.30	1	1.30	1	1.30	1	1.30
Cysteine	1.00	3	3.00	5	5.00	4	4.00
Ala	0.75	10	7.50	12	9.00	10	7.50
Arg	0.75	9	6.75	12	9.00	11	8.47
Thr	0.45	10	4.50	13	5.85	8	3.60
Gly		11		14		12	
Ser		10		13		10	
His		3		4		4	
Asp		20		24		21	
Glu		19		23		17	
Total		157	169.00	206	233.95	147	171.07

* K cal/residue

Average Hydrophobicities (K cal/residue): $\frac{169.00}{157} = 1.076$ $\frac{233.95}{206} = 1.136$ $\frac{171.07}{147} = 1.164$

APPENDIX IV

TABLE A

Data for Figure 26 showing percent decrease in absorbance at 660 mμ with increasing time

Incubation pH	Time (minutes) 15	30	60	120	180
9.5	6.5	20.8	24.5	28.6	32.5
10.0	39.5	60.2	62.3	66.5	69.0
10.5	38.2	67.5	70.1	74.7	77.5
11.0	63.7	83.1	87.5	92.0	94.5
11.5	69.8	87.8	91.5	96.0	98.5

TABLE B

Data for Figure 32 showing percent decrease in absorbance at 660 mμ for crystals previously soaked in guanidine hydrochloride and then incubated with trypsin

Guanidine Hydrochloride Incubation Time (minutes)	Trypsin Incubation Time (minutes) 10	30	60	120	150
10	6.3	17.1	24.5	30.8	31.7
60	9.1	18.5	27.6	38.2	39.5
120	27.2	36.3	42.9	49.5	49.8
180	16.8	29.8	43.4	49.5	50.2
1200	10.0	19.5	29.7	33.8	34.0

TABLE C

Data for Figure 34 showing percent decrease in absorbance at 660 mμ when crystals were incubated in tris

Incubation pH	Incubated at -5°C: Tris Concentration 1.0M	2.0M	3.0M	4.0M	Incubated at 25°C: Tris Concentration 1.0M	2.0M	3.0M	4.0M
9.5	24.8	45.6	52.8	56.0	14.0	32.1	28.2	46.5
10.0	39.2	52.8	55.2	62.3	20.0	37.5	44.4	51.2
10.5	71.2	80.0	77.6	78.4	44.5	55.6	56.5	68.8

APPENDIX V

Amino Acid Composition (%) of *Bacillus thuringiensis* var *sotto* Crystals

Amino Acid	Values on Highly Purified Crystals (Para D.2. - Page 61) Hydrolysis Time (hours) 20	40	70	140	Value at Zero Time	Values From Table 8 (page 49)
Glutamic Acid	15.0	10.6	13.8	9.1	16.0	16.0
Aspartic Acid	14.3	11.0	13.7	8.9	15.1	14.0
Glycine	4.4	3.5	4.4	2.9	4.7	4.8
Alanine	4.4	3.5	4.3	2.8	4.7	4.8
Valine	6.0	5.0	6.2	4.1	6.3	7.8
Isoleucine	5.7	4.8	6.1	4.0	6.0	6.6
Leucine	9.1	7.2	9.0	6.0	9.7	10.5
Serine	5.8	4.2	4.7	2.5	6.4	6.0
Threonine	6.2	4.6	5.4	3.3	6.7	6.8
Cystine					1.8	1.4
Methionine					0.6	0.67
Lysine	3.9	3.0	3.8	2.5	4.1	4.4
Arginine	8.8	6.9	8.6	5.7	9.0	9.0
Histidine	2.3	2.2	2.2	1.6	2.4	2.8
Phenylalanine	6.1	4.9	6.2	4.0	6.5	6.7
Tyrosine	6.2	4.2	5.9	3.5	6.6	6.5
Tryptophan					-	2.3
Proline	4.2	3.4	4.6	2.9	4.5	4.4
Ammonia	1.9	1.1	1.2	0.9	2.0	-