Bulla, 1971 and Benoit *et al.*, 1990 whereby rapid decrease in reducing sugar was indicated.

Julian and Bulla (1971) indicated that at the end of the logarithmic phase, when glucose is exhausted, the cells undergo a sequential development that results in spore formation. During the transition from the vegetative phase to sporulation, certain metabolic changes occur. This includes elaboration of acetate-oxidizing metabolism. The end-products of glucose catabolism that accumulate during growth are oxidized via the tricarboxylic acid cycle (Bulla, *et al.*, 1990). Also implicated in terminal respiratory activity is the glyoxylic acid cycle (Bulla *et al.*, 1980).

During vegetative growth, energy is provided by oxidation of glucose. Generally, it is believed that glucose is oxidized by simultaneous operation of the Embden-Meyerhof-Parnas and pentose phosphate pathways. For both *B. cereus* and *B. subtilis*, the Embden-Meyerhof-Parnas pathway is the primary mechanism for glucose assimilation, whereas the pentose phosphate pathway aids formation of biosynthetic intermediates rather than functioning as a major respiratory pathway (Goldman and Blumenthal, 1963, 1964 and Wang and Krackov, 1962). This could also be true for *B. thuringiensis* as a related species.

The stationary phase of growth commenced after 8 hours, and continued until the 20th hour. During this phase, a gradual decrease in the amount of reducing sugar was observed for all the species of *B. thuringiensis*. During the transition of vegetative phase to sporulation, organic acids in the medium are oxidized by terminal respiratory reactions to high-energy compounds, and the pH of the culture medium increases (Bulla and Hoch, 1985). This is indicated

in Figure 6 whereby pH increased after the exponential phase for each of the *B. thuringiensis* subspecies. The changes in pH was in correlation with the growth pattern of the bacterial species (Figs. 5 and 6). The increase in pH for Flobac, IPT BT6, IPT BT 15 and IPT BT 16 was observed after 5 hours which corresponded to the end of the logarithmic phase. Meanwhile, IMR BT 8 and IMR BT 16 exhibited an increase in pH after 8 hours which corresponded to the end of the logarithmic phase for these species. The changes in pH are in agreement with those reported by Bulla *et al.* (1971, 1980), Bulla and Hoch (1985), and Benoit (1990).

During the transition from vegetative growth to sporulation, enzymes of the tricarboxylic acid cycle are synthesized (Bulla *et al.*, 1980). Pyruvic and acetic acids that accumulate during vegetative growth are oxidized during the early stages of the sporulation process (Hanson *et al.*, 1963). Obviously systems capable of oxidixing pyruvate and acetate to carbon dioxide are present in *B. thuringiensis*.

Though the general pattern of metabolism for all the *B. thuringiensis* subspecies differed significantly from one another, all subspecies exhibited a similar trend in the change in pH, reducing sugar and biomass content. The lepidopteran-specific, dipteran-specific and lepidopteran- and dipteran-specific subspecies showed a growth pattern which had a correlation to that of any spore forming bacteria particularly the Bacillus family.

5.2 Enzymes synthesized during the various growth phases

Spore and crystal formation in *B. thuringiensis* affords a system for investigation relating to physiological reactions. Recent investigations showed that the genus Bacillus, undergoes metabolic changes, during the course of sporulation, that are related to morphological changes. A number of biochemical events, such as antibiotic and enzyme synthesis particularly with respect to the changes in the tricarboxylic acid cycle enzymes, have been implicated in the early stages of sporulation (Hanson *et al.*, 1963 and Spizizen, 1965).

Protease

The nature of proteolytic enzymes of microbial origin is less well known than that of the proteolytic enzymes of animal or plant origin. In recent years, however, the discovery of uses for some microbial enzymes and their relationship to sporulation in Bacillus have served to stimulate research in this area. In order to determine the role of proteases in sporulation and crystal formation, it is important to determine the activity of these enzymes during various phases of growth. A diverse group of proteolytic enzymes are produced by Bacilli, and several of these are synthesized by the cell during the early stages of sporulation (Doi, 1972; Roitsch and Hageman, 1983).

The protease-associated with *B. thuringiensis* has been described in several reports (Berlohr, 1964; Coleman, 1967; Epremyan *et al.*, 1981; Egorov *et al.*, 1983), but no central agreement has been reached regarding protease properties and characteristics. Several reports suggest that the proteases associated with crystal formation are of the serine, metallo, and sulfydryl classes (Bulla *et al.*, 1977; Chestukhina *et al.*, 1978; 1980). Despite the

lack of proof for the role of the proteases in *B. thuringiensis*, their synthesis is interesting because they are produced very early during sporulation, and they interfere with biochemical studies of sporulating bacteria.

Since cell lysis was not observed in any of the *B. thuringiensis* strains during the first 20 hours of growth, protease was apparently excreted from the intact sporulating cells. The presence of proteases inside the cell might constitute one of the modes of regulation for sporulation (Lecadet *et al.*, 1977). These authors further suggested that another regulatory mechanism might be due to the presence of an inhibitor, which could modulate the endocellular activity at different stages of the sporulation process.

Maximum protease activity coincides with glucose depletion in the medium when the cells begin to undergo sporulation. Similar findings have been reported by Berlohr (1964) on *B. licheniformis* and by Coleman (1967) on *B. subtilis*. At the end of the logarithmic phase, there was a general increase in protease activity which reached a maximum in all *B. thuringiensis* subspecies except for Florbac. A possible explanation could be the accumulated intermediates of carbohydrate metabolism may have given rise to catabolite repression during the logarithmic phase, which were removed during the post-logarithmic phase as the repressing substances were further metabolized.

Mandelstam (1961, 1962) conducted detailed studies on catabolite repression and concluded that any compound which an organism can use as a source of carbon and energy can cause catabolite repression of a sensitive system under the right conditions. Especially when growth is prevented by the omission of an essential ingredient from the medium then in the presence of a

carbon source, intermediates can accumulate and conditions will favour the build-up of the repressing catabolite to a value at which it will exert an effect. However, Coleman (1967) rejected this argument and demonstrated that secretion of exoenzymes such as α-amylase, ribonuclease and protease were not subject to catabolite repression.

Although the quantities of protease produced by each of the *B. thuringiensis* subspecies differred significantly, they approximated to a similar trend. Physiologically, the elaboration of protease by *B. thuringiensis* subspecies appears to be the function of a post logarithmic phase metabolism that is committed to or responsible for spore formation. This data, along with the studies by Coleman (1967), Andrews *et al.* (1985) and Lecadet *et al.* (1977) suggests that the protease from *B. thuringiensis* function physiologically in some part of sporulation of the respective cells. This role could involve the degradation of vegetative cellular material for subsequent anabolic or nutritional use for spore release. However, the role of protease in sporulation is yet to be confirmed. Probably the stage of production of protease coincides with that of sporulation because the same process that triggers sporulation also initiates the biosynthesis of the enzyme (Levisohn and Aronson, 1967).

The two peaks observed for IPT BT 15, IMR BT 8 and IMR BT 16 could be due to two different types of proteases produced at different times. The protease enzyme responsible for the first peak probably does not play any role in the sporulation process.

Alkaline phosphatase

It is not certain whether alkaline phosphatase synthesized after the logarithmic phase was identical to the enzyme in the vegetative phase under condi-

tions of phosphate depletion (Hanson *et al.*, 1970). The increase in the specific activity of alkaline phosphatase after 8 hours diminished during the stationary phase (Fig. 9). This suggests that it is dependent on phosphate limitation in the medium. Few studies are available on the occurrence of alkaline phosphatase in *B. thuringiensis*. Thus, further research is required in order to determine whether two enzymes are present or two independent mechanisms control the activity of a single enzyme. Information is also lacking on the function of alkaline phosphatase during sporulation.

5.2.2 Endoenzymes

The tricarboxylic acid cycle in Bacillus has been studied by several authors (Fortnagel and Freese, 1968; Diesterhaft and Freese, 1973; Fortnagel, 1970; Bulla *et al.*, 1980). Most investigations concern *Bacillus cereus*, *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus larvae* (Bernlohr, 1964; Baillie and Norris, 1963; Hanson *et al.*, 1963; Hanson and Cox, 1967). Little is known about the relationship between the tricarboxylic acid cycle and sporulation particularly in *B. thuringiensis*. Also of interest is whether these reactions are related to crystal formation, as well as if the control mechanisms regulating sporulation and crystal production are related. In our study three endonzymes were evaluated, namely, pyruvate carboxylase, isocitrate dehydrogenase and citrate synthase. This was with a view to determine trends related to metabolic changes which occurred in vegetative and early sporulation phases of growth and if any correlation existed between the metabolic pattern and the toxicity spectrum.

Carbohydrates are the major source of energy and carbon for microorganisms, and serve a dual function namely in anabolism and in the formation of acetylCoA or ATP in the catabolic process resulting in carbon dioxide, water, organic acids, or alcohols. This is achieved through glycolysis, the pentose phosphate pathway and the tricarboxylic acid cycle (Fig. 15).

Carbon dioxide is fixed primarily through the constitutive enzyme pyruvate carboxylase, which is strongly activated by acetylCoA. This enzyme is necessary for microbial growth on glucose but is not required for sporulation (Diesterhaft and Freese, 1973).

Pyruvate carboxylase forms an inducible pyruvate shunt which may supply oxaloacetate for gluconeogenesis, aspartate production and metabolism via the tricarboxylic acid cycle. However, this shunt apparently consumes too much energy to be the only way of converting malate to oxaloacetate; malate dehydrogenase is a necessary alternative, as mutants deficient in this activity grow extremely slowly on its substrate. In the shunt one ATP is used and thus more energy is required than in the direct conversion of malate to oxaloacetate.

Nevertheless, malic dehydrogenase does not produce oxaloacetate at the rate necessary for optimal growth, indicated by the slow growth of a pyruvate carboxylase mutant on malate (Diesterhaft and Freese 1973). The equilibrium constant of malic dehydrogenase and the stabilizing effect of NADH favour the production of malate. Thus, Diesterhaft and Freese (1973) concluded that the inducible pyruvate shunt is used during microbial growth on malate mainly for the production of substantial amounts of oxaloacetate, while malic

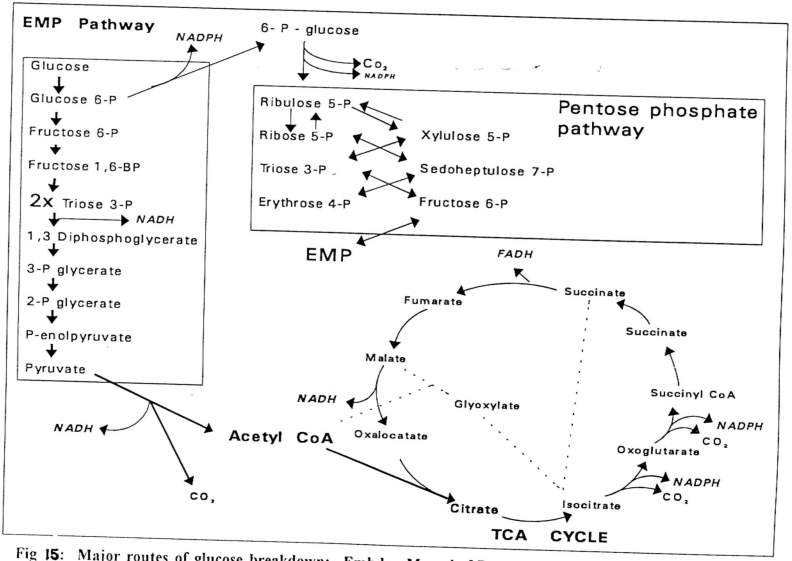

Fig 15: Major routes of glucose breakdown: Embden-Meyerhof-Parnas (EMP) pathway, pentose phosphate patway, TCA cycle, glyoxylate shunt (dashed lines) and interconnecting reactions. (Source: Neidhardt *et al.*, 1990)

dehydrogenase functions primarily to provide energy via the tricarboxylic acid cycle.

Hanson *et al.* (1963) postulated that the tricarboxylic acid cycle is indispensable to sporulation. Energy formed during the oxidation of acetate is stored or used to convert acetate to intermediates that are later oxidized. Perphaps an electron acceptor other than oxygen is utilized during this oxidation. The results displayed in this study were consistent with this view as the activity of pyruvate carboxylase was absent in sporulating cells but present in vegetative cells. These enzymes of the tricarboxylic acid cycle are formed during the transition from vegetative growth to sporulation, and that their formation is required before sporulation occurs.

That isocitrate dehydrogenase was repressed by glucose in the medium (Fortnagel, 1970 and Nickerson *et al.*, 1974) was supported by the findings in this study, where the activity of isocitrate dehydrogenase was inversely proportional to the amount of reducing sugar (Fig. 7). The very high activity of isocitrate dehydeogenase after 8 hours of growth was found to be very unique for IPT Bt 6. This was reconfirmed by another assay done on Florbac and IPT Bt 6 (Fig. 11b) at the end of this study. Thus, it could be concluded that isocitrate dehydrogenase could act as a marker for IPT Bt 6. This could be an useful tool to identify IPT Bt 6.

Although the mechanism of citratic synthase activity is not clearly defined Carls and Hanson (1971) and Freese and Marks (1973) reported that an intact tricarboxylic acid cycle was required. However, Yousten and Hanson (1972) provided the first evidence to the contrary whereby the tricarboxylic

acid cycle may not be an absolute requirement for sporulation. They showed that mutants of *B. subtilis* with lesions in the tricarboxylic acid cycle could be made to sporulate by appropriate manipulation of the culture media. Later, Nickerson *et al.* (1974) provided direct evidence that a fully operational tricarboxylic acid cycle is not required for sporulation. Therefore, it is difficult to describe the function of the tricarboxylic acid cycle in relation to the growth and sporulation of *B. thuringiensis* and offer an explanation of the nature and significance of the observed phenomena in terms of biochemical properties of the cells at different stages of the life cycle.

As observed with the other endoenzymes, citrate synthase too exhibited the highest specific activity after 8 hours. This is true for all the *B. thuringiensis* subspecies except for IPT Bt 16 and IMR Bt 8 where the highest specific activity was after 5 hours and 16 hours, respectively (Figure 13). The high specific activity of citrate synthase at 16 hours for IMR Bt 8 could be due to the substantial amount of reducing sugar still left in the culture broth compared to that in IPT Bt 16. This finding is consistent with the view that glucose represses enzymes of tricarboxylic acid cycle (Nickerson *et al.*, 1974). It is possible that tricarboxylic acid cycle enzymes are specifically repressed, inactivated or destroyed during sporulation. Alternatively, all these enzymes may be synthesized at a reduced rate, or not at all, at later stages of sporulation.

5.3 Purification of crystals from spore-crystal complex

Problems have been encountered in separating the spores and crystals of *B. thuringiensis* as they are similar in size and surface characteristics. However, a preliminary separation is absolutely essential in order to charac-

terize the parasporal crystals. Studies of parasporal crystals and spores of *B. thuringiensis* require that they be efficiently and completely separated. The earliest attempts to obtain purified crystals relied on spontaneous germination and autolysis of the spores, followed by repeated differential centrifugation of the crystals (Cooksey, 1971 and Lecadet, 1970). These techniques were followed by several biphasic systems in which organic solvent emulsions were formed; while the crystals remained in the aqueous phase. Among the solvents employed were trifluorotrichloroethane (Angus, 1959), tetrabromomethane (Lecadet, 1970), carbon tetrachloride (Pendleton and Morrison, 1966), and chloroform (Murray and Spencer, 1966). Unfortunately, the extractions had to be repeated several times to achieve acceptable purity, yields were low, and the danger of crystal modification by the organic solvents was imminent.

The last obstacle was overcome by the use of biphasic systems containing dextran sulfate 500 and polyethylene glycol 6000 (Delafield *et al.*, 1968 and Goodman *et al.*, 1967). Fast (1972) later described a method of crystal purification employing isopycnic density gradient centrifugation in caesium chloride (CsCl), whereas, Sharpe *et al.* (1975) recommended the use of Renografin gradients. Both these methods are convenient and give crystal preparations of high purity. However, the biochemical characterization of the crystals requires large quantities of purified crystal than that are usually available from density gradient centrifugation in a swinging bucket rotor. This could be overcome in zonal gradient centrifugation (Anderson, 1962). Zonal centrifugation employs a hollow bowl instead of tubes. Thus, the entire 360 rotor radius is available for the density gradient, and the consequent gradient volume allows a larger sample capacity. In this study the methods of Goodman *et al.* (1967) and Pendleton and Morrison (1966) were employed.

5.3.1 First method

The method of Goodman *et al.* (1967) involved a two-phase aqueous system consisting of polyethylene glycol 6000 and sodium dextran sulfate 500. The spore-crystal suspension was added to the lower phase. Fresh upper phase was added and when the funnel was shaken vigorously. Both the phases which appeared were found to contain the same material. It was difficult to differentiate between the spores and the crystals. Presumably, both the top and the bottom phase contained both spores and also crystals. Purification carried out in this manner failed to give pure crystals. Most of the spores and crystals were suspended in the bottom phase due to the gravitational force. There was no interface as described by the authors. Thus, this method was not employed for the extraction of crystals from the spore-crystal complex, as it was only suitable for certain subspecies of *B. thuringiensis*.

5.3.2 Second method

This method consisted the use of a cheap organic solvent; carbon tetrachloride, rather than the trifluorotrichloroethane used by Angus (1959). The success of this method depends on the removal of most of the spores in the aqueous phase by flotation before mixing this phase with the organic solvent.

The crystals of Florbac were different in shape compared to IPT Bt 15. The spores of Florbac were eliminated by removing the froth by the flotation process. IPT Bt 6 produced a thick froth which enabled the successful removal of spores before the organic solvent was used.

Yields of pure crystals prepared by this method varied with the starting preparation. Typically 80mg of crystals was isolated from 1L culture broth, with approximately 98% of crystals and less than 1% of spores in the final product. This is a qualitative value observed under the microscope. An ideal method would recover 100% crystals completely free of spores. Generally the greater the degree of crystal purity required, the lower is the yield.

5.4 Molecular weight of crystal subunits

Much of the evidence in the literature indicates that several distinct polypeptide components make up the parent glycoprotein crystal although their exact number and properties are not well defined. Herbert *et al.* (1971) electrophoretically separated several polypeptide fractions from the crystals of *B. thuringiensis* that had been solubilized at pH 10.5. Major products generated by this treatment had apparent molecular weights of 55 000 and 120 000. These investigators attributed toxicity to the smaller component but they did not report the function of any of the other components. By using 1N NaOH to solubilize the crystals, Prasad and Shethna (1974) isolated an insecticidal and antitumour protein with a molecular weight of 130 000. Sayles *et al.* (1970) employed 8M urea (pH 6.0), 0.5% dithiothreitol, and gel filtration chromatography to dissociate the crystals into several polypeptides with molecular weights of approximately 1000. These results have been ascribed to differences in the strains analyzed and in the methods of solubilization, as well as to differences in the methods of molecular weight determination.

Bulla *et al.* (1980) suggested that the crystal is composed of a single subunit that is converted to smaller components by the usual conditions of dissolutions. Compelling evidence for this hypothesis has been presented by Chestukhina *et al.* (1977) and Bulla *et al.* (1977). Bulla *et al* (1977) have demonstrated that the parasporal crystal of *B. thuringiensis* is composed of a single glycoprotein subunit with an apparent molecular weight of about 90 000 to 130 000. Huber *et al.* (1981) reported that when crystals of *B. thuringiensis* variety *israelensis* was dissolved in 0.1M NaOH and alkylated, gave one major band with a high mobility, corresponding to a molecular weight of 24000, and diffused minor bands with molecular weight ranging from 50 000 to 90 000.

A study conducted by Herbert and Gould (1973) showed that the crystal protein of *B. thuringiensis* var. *tolworthy*, a highly potent toxin for lepidopteran larvae consists of two polypeptide components of molecular weights 55 000 (component A) and 120 000 (component B) in a molar ratio of 2:1. They further demonstrated that the smaller component A retains the full toxicity of the intact crystal, whereas the larger component B is nontoxic. The results obtained from this study strongly favours the findings of these investigators, whereby, Florbac, IPT Bt 6 and IPT Bt 15 showed the presence of similar subunits with the molecular weights of 120 000 and 56 000. This is also strongly supported by the work done by Widner and Whiteley (1989). These investigators found that an open reading frame designated *orf2* encodes for a protein corresponding to a molecular weight of approximately 50 000 in *B. thuringiensis* var. *kurstaki* HD-1.

Holmes and Monro (1965) and Nagamatsu *et al.* (1978) suggested that the molecular weight of the intact crystal is 230 000. Whereas the molecular weight of the dissolved protein in denaturing agents ranges from 80 000 to 150 000, depending on the methods used. Similar findings have been reported by Huber *et al.* (1981) where *B. thuringiensis* var. *thuringiensis, finitimus, kurstaki, sotto, galleriae, entomocidus, aizawai, morrisoni,* and *tolworthy* gave a molecular weight of approximately 230 000.

Electrophoretic profile of all 4 subspecies (Florbac, IPT Bt 6, IPT Bt 16 and IPT Bt 16) revealed protein with a similar molecular weight of 39 000. In particular, 56 000 dalton polypeptides were present in both lepidoptera and lepidoptera- and diptera- toxic crystals. The absence 120 000 molecular weight subunit in IPT Bt 16 could be due to problems created by contaminating proteases (Nickerson, 1980). This is in agreement with the earlier studies of Bulla *et al.* (1977) and Chestukhina *et al.* (1978) who showed that the parasporal crystal has the capacity for proteolysis possibly involving a sulfhydryl or serine or metallic proteases that are activated under alkaline conditions. Chestukhina *et al.* (1980) has indicated the presence of protease in the crystals and their solutions. The difference in the polypeptides produced in *B. thuringiensis* subspecies could be attributed to the different types of proteases present in the crystals.

The appearance of the polypeptides with molecular weights of 39 000 and 19 000 in IPT Bt 16 could be due to the denaturing of the major subunit (molecular weight of 56000). This is further supported by Chestukhina *et al.* (1982) where *B. thuringiensis* toxic to lepidoptera was reported to produce crystals with molecular weights of 130 000 to 145 000. These proteins con-

tained an N-terminal domain (molecular weight of 65 000 - 85 000) resistant to proteolysis whereas their C-terminal moieties (molecular weight of 65 000) undergo an extensive degradation by trypsin that leads to stepwise cleavage of the fragments with molecular weight of 15 000 - 25 000. Studies by Yamamoto *et al.* (1983) also strongly favour this hypothesis, whereby a 92 000 protein had a peptide profile exactly the same as that of 120 000. Furthermore, these authors showed that a group of 25 000, 38 000 and 42 000 proteins showed no significant differences in their peptide patterns.

Diptera-specific *B. thuringiensis* subspecies (IMR Bt 8 and IMR Bt 16) have a common subunit with a molecular weight of 80 000. Chungjatuparnchai *et al.* (1988) demonstrated that IMR Bt 8 which is highly toxic to larvae of *Aedes aegypti* produced crystals with a molecular weight of 130 000. Proteolytic cleavage of this protoxin gave rise to toxic fragments of 60 000 to 78 000. Thus, the crystal subunit of IMR Bt 8 and IMR Bt 16 (molecular weight of 80 000) could be the toxin produced from the cleavage of the native crystal. Haider *et al.* (1986) reported that a 130 000 dalton protoxin in the native crystal was converted to a 52 000 dalton dipteran toxin by enzymes produced by *Aedes aegypti*. This could explain the appearance of the 45 000 dalton polypeptide in IMR Bt 16 which could be another product from the cleavage of a major polypeptide.

The study conducted by Hofte and Whiteley (1989) also explains the 80 000 dalton subunit present in IMR Bt 8 and IMR Bt 16. These authors reported that Dipteran-specific crystals are made up of proteins with predicted molecular weights of 135 000, 128 000, 78 000, 72 000 and 27 000. These proteins assemble together to form an ovoid crystal complex. The 80 000

dalton subunit present in IMR Bt 8 and IMR Bt 16 more or less correspond to the 78 000 dalton protein mentioned by Hofte and Whiteley (1989). Orduz *et al.* (1994) reported that *B. thuringiensis* serovar. *medellin* (strain 163-131) which is very toxic to mosquito larvae showed polypeptides at 100 kDa, multiple bands at 80, 75, 70, 67 and 65 kDa, and two duplets at 40-41 and 28-30 kDa. Yamamoto *et al.* (1983) concluded that the crystals of *B. thuringiensis* var. *israelensis* which is mosquitocidal are composed of proteins with molecular weights of 17 000, 28 000, 42 500, 55 000, and 120 000. Later, Nicolas *et al.* (1993) discovered that the 42,000 dalton protein inclusions alone were found to be toxic to *Culex pipiens* larvae. Both insect toxicity and hemolytic activity have been attributed to the 28 000 protein (Thomas and Ellar, 1983).

The lepidopteran-specific subspecies (Florbac and IPT Bt 6) clearly possess a common subunits with the molecular weight of 120 000 and 56 000 whereas the lepidopteran- and dipteran-specific subspecies (IPT Bt 15 and IPT Bt 16) share one common subunit (56 000). The diptera-specific subspecies (IMR Bt 8 and IMR Bt 16) share no common subunits with the lepidopteran-specific or the lepidopteran- and dipteran-specific subspecies. This indicates the presence of a new class of a delta-endotoxin of *B. thuringiensis*. The completely different host spectrum could be due to minute differences in the amino acid make up of the polypeptides. This was shown by Widner and Whiteley (1989) where crystals of different host range were compared. Despite the fact that these two proteins (combined dipteran- and lepidopteran-specific and lepidopteran-specific) display 87% identity in amino acid sequence, they exhibit different specificities with respect to the toxins.

Although identical values of molecular weight of the crystal proteins formed by various subspecies do not provide sufficient evidence as a definite proof of their identity, considering the similarities in the amino acid composition of the crystals, it may be considered that these proteins are largely similar from the structural point of view. It does not imply, of course, that all the proteins synthesized by the various strains are identical. On the contrary, the differences found in the entomocidal activity indicate variations in the chemical structure of the proteins, e.g. in their amino acid sequences. We can only assume that each subspecies synthesizes primarily one protein of a structural type common to all the strains. Before or during the formation of the crystal this protein may be exposed to secondary modification by limited hydrolysis.

Nevertheless it is impossible to exclude the possibility that the appearance of the same polypeptides in the crystal (Florbac, IPT Bt 6, IPT Bt 15 and IPT Bt 16) might depend on the action of two closely related structural genes.

5.5 Bioassay

Total dependence on a single insecticide for the control of *Plutella xylostella* in the past, led to problems related to the build-up of resistance. The search for biological control agents yielded several agents of which IPT Bt 6 is currently the most promising.

B. thuringiensis is compatible with a wide range of chemical pesticides. Thus it was envisaged that the effectiveness of one or both could be enhanced if used in combination. Furthermore, this may accomplish economic use of both *B. thuriengiensis* and chemical at lower rates. The results obtained from the study give some very useful information.

IPT Bt6 was chosen for the bioassay based on the studies done by Singh (1994) which showed IPT Bt6 to be superior to the other strains of Bt in his early preliminary tests.

The combination of IPT Bt 6 with Agrimec increased the efficacy of the latter at a lower concentration. Chemical insecticides are known to act as stressors promoting contraction or activation of infectious diseases which make insects more susceptible to the action of microbial toxins (Benz, 1971). The treatment of insects with certain insecticides reduced the number of haemocytes. Since phagocytosis is an important defence mechanism, such reduction can increase susceptibility of insects to microorganisms. In some synergistic combinations, microorganisms seem to be the synergist of the insecticide especially when the microorganism slows down the detoxification of the insecticide.

Though, the combination of Agrimec with IPT Bt 6 (0.25g/L) enhanced the effectiveness of IPT Bt 6, the mortality level obtained from IPT Bt 6 (1.0g/L) showed that the combination was unneccesary. Whether exposing the insects to two different insecticides simultaneously may cause resistance to develop faster to both the insecticides is unknown. Thus, it would not be prudent to recommend the lower dosage of IPT Bt 6 in combination with Agrimec at this time.

There was a significant difference in mortality between Florbac (1.0g/L) and IPT Bt 6 (1.0g/L) on the larvae of *P. xylostella*. Mortality was only 23% for Florbac on the 6th day compared to 100% for IPT Bt 6. This was attributed to resistance developed by the insects towards the former, due

to continued use of Florbac on cabbage in Cameron Highlands. Thus, a substitute for Florbac would be appropriate and the prospect of IPT Bt 6 appear to be promising.

A five fold dilution of IPT Bt 6 (0.25g/L) performed much better than Florbac at 1.0g/L. This indicated clearly the existence of resistance within insects. Florbac at 0.25g/L did not differ significantly in terms of the mortality from the control (Agrimec). Combined with Agrimec (0.1ml/L), there was 53% mortality on the 5th day which was still much lower than the combined effect of IPT Bt 6 (0.25g/L) and Agrimec (0.1ml/L).

The mixture of IPT Bt 6 (0.25g/L) and Agrimec (0.1ml/L) increased the effectiveness of both leading to synergy and compatibility. Thus, a reduced amount of the chemical may be used for the crops. However, it would be premature to draw conclusions before full scale field trials are carried out.

The combination of IPT Bt 6 (0.11g/L) and Agrimec (0.1ml/L) was the most effective. Similar finding was obtained on the Kampung Raja population. Thus, the combination could form a very effective formulation against *P. xylostella*, although IPT Bt 6 by itself could be an effective insecticide. The mortality rate for the combination of Florbac (0.03g/L) and IPT Bt 6 (0.11g/L) was 73% on the 6th day, which was much higher than that of Florbac (0.03g/L) alone. Thus, IPT Bt 6 enhanced the efficacy of Florbac as well.

Agrimec (0.1 ml/L) improved the efficacy of Florbac (0.03g/L). Total mortality was achieved on the 5th day. This is necessary for an insect host which has build up resistance. Although much is yet to be learned about the

use of combinations of insecticides it is apparent that IPT Bt 6 and Agrimec showed a synergistic effect which improved the effectiveness by an enhanced action which may ensure a more appropriate application.

More research should be devoted to the development of synergistic mixtures which should include different dosage levels of Agrimec, Florbac and IPT Bt 6. The increasing emphasis on integrated pest control should centre on studies related to compatibility, between chemical and microbial insecticides. It would also be of interest to determine whether *P. xylostella* could be controlled effectively under simultaneous or sequential treatments consisting of Agrimec and IPT Bt 6 each with its own mode of action.