CHANGES IN THE BIOCHEMISTRY OF THE HEMOLYMPH OF *PLODIA INTERPUNCTELLA* AFTER TREATMENT WITH *BACILLUS THURINGIENSIS*

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SUMMARY: The biochemical changes in the hemolymph of P. interpunctella larvae as a result of treatment with B. thuringiensis showed that the total carbohydrate and protein concentrations were not significantly changed. The electrophoretic patterns of the hemolymph protein showed 10 different bands of which six showed great variation in the control and the treated samples. The change in lipid concentration showed a double fold increase after treatment with B. thuringiensis. The total amino acids, on the other hand, showed no quantitative differences in the hemolymph of normal and treated larvae. Analysis of the hemolymph revealed the presence of 15 amino acids. Serine, proline, glycine, alanine, valine, leucine and lysine exhibited quantitative decrease, while aspartic acid, threonine, isoleucine, tyrosine, phenyl alanine and arginine showed an obvious increase after treatment of the larvae with B. thuringiensis. An increase of sodium, potassium, calcium, magnesium and manganese ions, and a decrease in phosphorous, ferric, zinc and copper ions, were also detected after treatment.

Key words: Hemolymph, plodia, bacillus thuringiensis.

INTRODUCTION

Investigations on insert hemolymph are of particular interest because they provide us with an adequate background to judge the synthetic activity associated with the different processes in developing organisms. Amino acids of the hemolymph play an important role in the osmoregulation in insects and abnormal changes in their concentration through application of microbial insecticides may lead to fatal consequences (9, 19, 20, 29).

The inorganic constituents of insect hemolymph are also of vital importance in view of their role in the neurophysiology of insects and their level inside and outside the nerve membranes have to be maintained for the propagation of impulses.

In the present study, investigations have been carried out to evaluate the qualitative and quantitative changes of proteins, lipids, carbohydrates, amino acids and inorganic ions of *Plodia interpunctella* larvae that was allowed to feed on a diet containing lethal concentration of *B. thuringiensis* endotoxin.

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MATERIAL AND METHODS

Effect of *B. thuringiensis* var. Kurstaki on the hemolymph of *Plodia interpunctella* in order to determine the effect of *B. thuringiensis* on the hemolymph of *Plodia interpunctella*, hemolymph samples were obtained from the last instar larvae in two series of experiments. In the first series of experiments, a group of larvae were fed for seven days on a diet as described by Chippendale (7), containing *B. thuringiensis* var. Kurstaki HD-1 (Dipel 2x) at a concentration of 500 μg/gm of diet. In the second series of experiments, the larvae were fed on untreated diet to be used as a control.

Hemolymph was collected from the larvae of each group. After ligation of the neck of larvae, small incisions were made in the soft cuticle anterior to the first thoracic segment. Capillaries were used to withdraw the exuding hemolymph which was subjected to slow centrifugation at 1200 rpm for 15 mins and then drawn into serological test tubes to be collected with traces of

phenyl-thiourea to prevent darkening. The test tubes were then stored in a deep freezer at -20°C till required.

Investigations were made to determine the total proteins, protein fractions, total amino acids, individual free amino acids, total lipids, total carbohydrates and ion concentrations.

A colony of *P. interpunctella* was raised in the laboratory as previously described (30).

Determination of total protein in the larval hemolymph of *P. interpunctella*

Total proteins can be determined using two methods as follows:

Using the crude hemolymph: The total protein was determined according to the method specified by Lowry *et al.* (16). Protein reacts with Folin ciocalteau reagent to give a blue colored complex due to the reaction of the alkaline copper of the reagents with the protein. The intensity of the color was measured photometrically using a spectrophotometer at 750 nm.

In the control experiments, distilled water replaced the hemolymph while in the standard experiments, the standard protein albumin solution was used. The solutions were mixed well and allowed to stand at room temperature for 15 mins. Then 0.5 ml of the diluted Folin ciocalteau reagent (1:2) with distilled water was added and mixed rapidly. After 45 mins at room temperature, the intensity of the violet color obtained was compared spectrophotometrically against the standard and blank (control) solutions at 750 nm.

By precipitation of protein by thichloro-acetic acid: To 0.1 ml of diluted hemolymph, 0.1 ml of 20% trichloroacetic acid (TCA) was added and mixed well to make the final concentration of the acid 10%. The mixture was left to cool in a deep freezer for 10 mins, then the solution was centrifuged for 15 mins or more to assure complete precipitation at 5000 rpm. The precipitate was isolated and dissolved in 0.1 ml of 0.1 N NaOH. Therefore, the supernatant and dissolved precipitate were used to measure the protein concentration adopting the same procedure as already mentioned.

Seperation of protein bands by *gel electrophoresis*

The separating gel solution was made (according to Davis, 1964) by mixing 2 ml of a mixture solution (1) containing 18.3 gm (Tris dissolved in 24 ml IN HCL, then 0.11 ml TEMED was added and distilled water to make the volume up to 50 ml), 3.2 ml of another mixture solution (2) containing (15 gm acrylamide dissolved in distilled water, then 0.4 gm Bis acrylamide was added and distilled water up to 50 ml volume), 2.8 ml distilled water and 8 ml of a mixture solution (3) containing (filtrate of 140 gm ammonium persulphate dissolved in 100 ml distilled water). The trapped air bubbles were removed from the final solution by a suction pump. Glass tubes of electrophoresis 87x5 mm were fixed in vertical position and the lower end of each tube was tightly stoppered with a flattened rubber cap (parafilm).

The separating gel was gently added to each tube by means of a syringe to fill the tube almost completely. Then about 0.5 ml of cold distilled water was layered above the upper surface of the gel column to obtain a smooth and horizontal gel surface. Care was taken to avoid mixing of water with gel solution. The gel tubes were then placed in front of a daylight fluorescent lamp at room temperature. Photo-polymerization usually takes place within 1 hr. The above water layer was then drained away. These gel columns were suitable for use within one week when stored at 4°C and were used for separating protein bands as follows:

a) The upper surface of the completely polymerized separating gel column was washed with few drops of a diluted electrode buffer (Tris-glycine buffer pH 8.3), the washing solution was removed by inverting the tubes on an absorbing filter paper.

b) The gel tubes were allowed to stand vertically between the cathodal and anodal buffer solutions after removing the rubber caps from the lower ends of the tubes.

c) Samples of hemolymph were applied in 40% sucrose solution on each gel column (34), the remaining empty part of each gel tube was filled with the diluted electrode buffer solution.

The gel columns were fixed, thereafter, for 1-2 hours in each of the mixture solutions 1, 2 and 3 as mentioned above, respectively; then rinsed in distilled water and stained for 1 hr with Amino black stain. Excess dye was removed by placing the gel columns in several changes of destaining solution (7.5% acetic acid) until the gel between the protein bands became colorless. Areas containing protein bands stained blue. The gel was then stored in 7.5% acetic acid (8). The protein bands were classified according to their mobility resolution.

Gels were scanned directly using a densitiometer (Scanner), Beckman, model R-112, adjusted through fast scan at sensitivity of 80, a slit 4x5 mm and a 550 nm (wavelength), filter gave optimum results.

The protein bands were classified according to their mobility resolution value (Mr.) in relation to the tracking dye which was assigned an Mr. of 100.

Determinations of total lipids

Total lipids were estimated colorimetrically using the method of Knight *et al.* (14). The lipids were extracted as described by Bligh and Dyer (3). To a known volume of diluted hemolymph (one volume of hemolymph: nine volume distilled water) one volume of distilled water and 10 volumes of methanol: chloroform 2:1 (v/v) were added and mixed well. The mixture was centrifuged for about 10 mins; the supernatant was decanted, and the residue was then re- extracted with methanol-chloroform-water 2:1:0.3 (v/v). After centrifugation, the combined supernatant was diluted with chloroform-water 1:1 (v/v) and the phase was allowed to separate in separating funnel. The lower chloroform phase was with drawn and concentrated to dryness in rotary evaporator at room temperature. The residue was dissolved and made up to a convenient volume with chloroform.

Determination of total carbohydrates

The enthrone reaction with carbohydrate depends on the temperature developed by the reaction of the sulphuric acid in the reagent with the aqueous solution (1).

To 1 ml diluted hemolymph samples (one volume of distilled

water), 2 ml of 2% enthrone in 96% sulphuric acid was added and placed in ice bath. The mixture was stirred and heated in boiling water bath for 10 mins. Determination of the optical density at the wavelength of 625 nm was carried out half an hour after cooling. A blank of 1 ml distilled water and standard purified liver glycogen were treated in the same manner for comparison.

Determination of amino acids

Colorimetric determination of total amino acids: Amino acids were determined colorimetrically according to the method of Lee and Takahashi (15). To 0.1 ml of previously prepared samples of hemolymph, 1.9 ml of ninhydrin citrate glycerol mixture (0.5 ml of 1% ninhydrin in 0.5 M citrate buffer, pH 5.5; 1.2 ml of glycerol and 0.2 ml of citrate buffer) was added, the final pH of the reaction mixture was adjusted to 6.0. After mixing and shaking well, the test tubes were placed in a boiling water bath for 12 mins and then cooled to a room temperature under running tap water.

The formed color was read spectrophotometrically at 750 nm against a blank experiment in which distilled water replaced the hemolymph sample. For calculations, a sample of known concentration of the standard L-leucine was processed simultaneously with each experiment.

Determination of individual free amino acids: Free amino acids were separated and determined quantitatively by an LKB alpha plus high performance amino acid analyzer, LKB Biochrom LTD England. The hemolymph was deproteinized and then hydrolyzed for analysis of individual free amino acids, thus to 0.1 ml of hemolymph, 5 mg of sulfosalicylic acid were added, mixed and centrifuged for 5 mins at 3500 rpm. The supernatant fluid was used for analysis. A volume containing 50 mg protein was dissolved in 5 ml 5.7 N hydrochloric acid, in a digestive tube that was closed under vacuum. The contents were digested for 24 hrs at 110°C, sample filtered, residue washed with distilled water and filtrate volume completed to 50 ml with double distilled water. Five milliliter of the filtrate were evaporated at room temperature, residue was dissolved in 5 ml loading buffer (0.2 N sodium citrate buffer pH 2.20). The solution was filtered through 0.22 um membrane. Twenty micro-liter of the final filtrate were loaded in the instrument capsule for determination of free amino acids.

Determination of hemolymph ion concentration

The concentration of hemolymph ions was measured using an atomic absorption spectrophotometer.

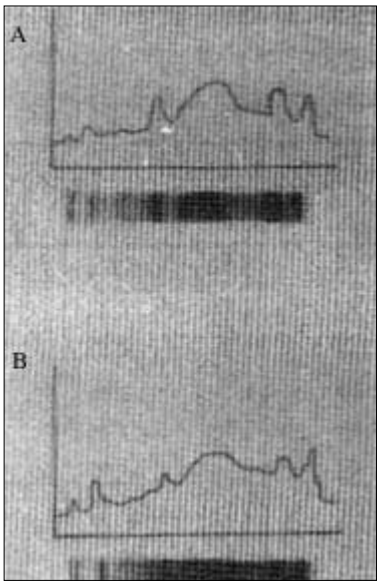

Figure 1: Electropharetic patterns of hemolymph protein in the larvae of *P. interpunctella*. A) Control larvae. B) Treated larvae.

RESULTS

Effects on total hemolymph protein

Proteins are among the substances which are known to be effective in forming complexes with the divalent metals in the hemolymph of insects. The nitrogenous components of hemolymph protein in insects are of vital importance to adequate growth, maintenance and repair of tissues.

From the morphogenetic point of view, investigations of hemolymph proteins of insects as a result of treatment with the biobacterial insecticide are of particular interest because they provide an adequate background to judge the synthetic activity associated with the differentiation processes in the developing organism. Therefore, the quantitative differences of proteins in the normal larvae of *P. interpunctella* as compared with those treated with *B. thuringiensis* have been evaluated.

The data obtained (Table 1) from crude hemolymph samples show insignificant variation in the total proteins between the normal untreated larvae of *P. interpunctella* and those treated with *B. thuringiensis*. In another series of

Table 1: Effects of *B. thuringiensis* on the hemolymph proteins of *P. interpunctella* larvae.

Method used	PROTEIN CONTENT (mg/ml)					
	Control			Treated		
	min	max	mean ± S.E	min	max	mean ± S.E
Crude material	64.0	72.0	68.0 ± 2.31	48.0	64.0	58.67 ± 5.30
Precipitation by TCA	44.4	55.6	50.4 ± 3.25	36.0	55.2	44.80 ± 5.60

Table 2: Changes in the pattern and percentage of the hemolymph protein bands of *P. intepunctella* larvae as affected by *B. thuringiensis* and as shown by electrophoresis.

No. of Protein fraction	Control sample		Treated sample	
	mg/ml	Relative %	mg/ml	Relative %
1	0.35	0.52	0.41	0.70
2	1.96	2.88	2.25	3.83
3	1.29	1.89	0.82	1.39
4	1.16	1.71	1.64	2.79
5	1.16	1.71	2.04	3.48
6	7.59	11.16	5.32	9.06
7	34.46	50.67	27.59	47.04
8	4.67	6.87	4.50	7.67
9	11.10	16.32	7.97	13.59
10	4.26	6.27	6.13	10.45

Table 3: Effects of *B. thuringiensis* on the hemolymph proteins of *P. interpunchtella* larvae.

Test	Lipid content (mg/ml)			Carbohydrate content (mg/ml)		
	min	max	mean±S.E.	min	max	mean±S.E.
Control	17.50	28.80	22.80±3.41	1.88	3.41	2.48±0.22
Treated	30.00	58.75	47.92±9.02	1.76	2.05	1.80±0.05

experiments, the proteins were precipitated by trichloroacetic acid (TCA).

The data obtained also showed insignificant differences between the normal (50.40±3.25 mg/ml) and treated individuals (44.80±5.60 mg/ml).

The hemolymph protein of *P. interpunctella* larvae was electrophoretically separated into 10 different bands by using Amido black stain (0.1% Amido black in 30% ethyl alcohol and 0.7% acetic acid) (Table 2) and (Figures 1A and 1B). The concentration of each hemolymph protein band is expressed in terms of mg/ml of the determined total protein and also, in terms of a relative percentage.

Bands No. 8 and 10 formed 6.87% (4.67 mg/ml), and 6.27% (4.26 mg/ml) of the total protein determined in the control samples, respectively, being increased in the treated samples to 7.67% (4.50 mg/ml), and 10.45% (6.13 mg/ml), respectively. Band No. 10 which lies at the application point to the gel is primarily an oxidized melanin (32).

On the other hand, bands No. 2-5 are small bands, those of 2, 4 and 5 being increased in the treated samples to 3.83% (2.25 mg/ml), 2.79% (1.64 mg/ml) and 3.48% (2.04 mg/ml) of the total protein, respectively, as compared to 2.88% (1.98 mg/ml), 1.71% (1.16 mg/ml) and 1.71% (1.16 mg/ml) in the control samples, respectively. While that of band No. 3 being slightly decreased from 1.89% (1.29 mg/ml) of the total protein in the control to 1.39% (0.82 mg/ml) of the total protein in the treated sample.

However, band No. 1 was the smallest band, representing 0.52% (0.35 mg/ml) in the control sample, being increased to 0.70% (0.41 mg/ml) of the total protein determined in the treated sample.

Table 4: Pattern of amino acids in the hemolymph of *P. interpunctella* as affected by treatment with *B. thuringiensis.*

No. Amino acid	Control		Treated	
	Conc.	Relative %	Conc.	Relative %
	(gm/ 16 gm N)		(gm/ 16 gm N)	
Aspartic acid	2.02	9.90	1.52	10.87
Threonine	0.46	2.24	0.83	5.99
Serine	2.22	10.86	0.65	4.64
Proline	1.52	7.44	0.70	5.04
Glycine	0.96	4.70	0.45	3.21
Alanine	0.96	4.68	0.52	3.74
Valine	1.44	7.03	0.60	4.34
Methionine	0.13	0.610	0	
Isoleucine	1.53	7.49	1.20	8.62
Leucine	1.49	7.29	0.89	6.36
Tyrosine	0.34	1.69	0.33	2.34
Phenyl alanine	0.78	3.84	0.56	4.04
Histidine	0.88	4.33	0.61	4.40
Lysine	1.08	5.29	0.54	3.88
Arginine	4.62	22.61	4.53	32.53

Effects on total hemolymph lipids and carbohydrates

Data given in Table 3 show the quantitative changes in the total lipid content in the hemolymph of *P. interpunctella* as a result of treatment with *B. thuringiensis*. It appeared that the lipid content of healthy larvae was 22.08±3.41 (17.50-28.80) mg/ml compared to 47.92±9.02 (30.00-58.75) mg/ml with 2.17 fold increase in the treated larvae.

Data given in Table 3 show that the total carbohydrate contents in the healthy larvae was 2.48±0.22 (1.88-3.41) mg/ml and that of the treated larvae was 1.80±0.05 (1.76-2.06) mg/ml, this did not show any significant effect of the bacterial infection on the total carbohydrates content.

Effects on total and free hemolymph amino acids

In the present study, investigations were carried out to determine the quantitative differences of amino acids in the hemolymph of larvae of *P. interpunctella* as affected by treatment with *B. thuringiensis*.

The data obtained showed that in healthy larvae, the

Table 5: Effect of *B. thuringiensis* on hemolymph ion concentration of *P. interpunctella*.

	Hemolymph ion concentration (mg/ml)					
	Control			Treated		
	min	max	mean ± S.E	min	max	mean ± S.E
Phosphorous	4.08	32.40	14.14000± 9.15	1.92	31.20	12.1800±0.58
Potassium	2.21	3.22	2.65000±0.30	2.74	3.64	3.2900±0.28
Calcium	0.05	1.40	0.52000±0.44	0.20	21.10	7.9000±6.63
Magnesium	0.67	2.30	1.50000±0.47	1.46	2.30	1.8900±0.24
Sodium	1.24	6.47	3.09000±1.69	1.49	15.40	7.8400±4.06
Ferric	0.0023	0.0304	0.02000±0.0089	0.0054	0.0124	0.0097±0.0022
Manganese	0.00107	0.00157	0.00140±0.00015	0.0011	0.0042	0.0022±0.001
Zinc	0.0179	0.0468	0.03100±0.0085	0.0232	0.0397	0.0297±0.0051
Copper	0.0077	0.10133	0.03931±0.031	0.0028	0.0089	0.0050±0.0018

amount of amino acids was 19.67±0.17 (19.50-20.00) mg/ml compared to an average of 20.33±1.09 (19.00-22.50) mg/ml in treated larvae.

Analysis of hemolymph of *P. interpunctella* reveal the presence of 14 amino acids (Table 4). Comparison of the concentrations (expressed as gm/16 gm N_2) of these amino acids in both normal and treated hemolymph samples showed a wide range of variations. It appears that the pattern of some amino acids showed an obvious decrease in the hemolymph of the larvae after treatment with *B. thuringiensis*; these amino acids were serine, proline, glycine, alanine, valine, leucine and lysine. On the other hand, some amino acids showed an obvious increase after treatment with *B. thuringiensis*, e.g. aspartic acid, threonine, isoleucine, tyrosine, phenyl alanine and arginine. Methionine was completely lost and histidine showed no obvious quantitative changes in the hemolymph of the larvae after treatment with *B. thuringiensis*. A special characteristic of the resulting data was the high titre of the total amino acids in both control and treated Indian meal moth larvae, respectively.

Effects on hemolymph ion concentrations

Breakdown of the cells of the midgut by *B. thuringiensis* causes leakage of the gut contents in the hemolymph, as consequence of this leakage hemolymph components among which ions are changed abruptly and its pH rises sharply and causes general paralysis after this was firstly occurred in the gut.

Data given in Table 5 showed that treatment of *P. interpunctella* with *B. thuringiensis* resulted in an increase of sodium, potassium, calcium, magnesium and manganese ion concentrations in hemolymph with 2.6, 1.2, 15, 1.26 and 1.5 fold increase compared with those determined for the control.

On the other hand, a marked decrease in phosphorus, ferric, zinc and copper ions in hemolymph was observed as compared with those of normal larvae of *P. interpunctella*, but in varying degrees (Table 5).

DISCUSSION

Bacillus thuringiensis causes biochemical changes in the hemolymph of *P. interpunctella* larvae when treated with it. Total proteins showed insignificant differences between the larvae treated with *B. thuringiensis* as compared to the control. Govindarajan *et al.* (12) reported similar findings with the larvae of *Achoea Janata* L. after treatment with *B. thuringiensis*. Salama *et al.* (29), on the other hand, showed marked decrease in the total proteins concentration of *S. littoralis* larvae after their treatment with *B. thuringiensis*. Rupp and Spence (28) showed that the treatment of Manduca sexta larvae with microbial agents caused changes in the protein profile in the midgut contents and in the hemolymph. In this concern, however, hypoproteinemic effects was reported in the blood plasma of the 6th instar larvae of variegated cutworm *Peridroma saucia* during the infection of nuclear polyhedroses virus (17,18). Electrophoretic patterns of the hemolymph protein showed 10 different bands but six out of them showed great variations in the control and the treated samples.

Quantitative changes in the total lipid content in the hemolymph of *P. interpunctella* larvae occurred after treatment with *B. thuringiensis*. The lipid content showed double fold increase after treatment with *B. thuringiensis*. These findings were in contrast with those reported early by Boctor and Salama (6) and by Bennett and Shotwell (2) who showed a decrease in the total lipid content in the hemolymph of *S. littoralis* and *Popilliane japonica* larvae after their treatment with *B. thuringiensis* and *B. popilliane*, respectively. They explained this process by the fact that the infested larvae may produce enzymes that utilize lipids in an effort to remove the invading organism. In the pres-

ent study, however, it is suggested that the increase of lipid may be due to the conversion of some proteins to fats (24, 27) during the course of starvation. Total carbohydrate content in the hemolymph of *P. interpunctella* larvae showed in significant difference in the larvae treated with *B. thuringiensis* as well as the control ones.

No quantitative differences of amino acids were detected in the hemolymph of normal larvae and after their treatment with *B. thuringiensis*. Other workers, however, found that the quantity of amino acids present in diseased caterpillar was higher than that present in healthy caterpillar (19). Similar reports have been made by Drilhon *et al.* (9) and Ramakrishnan and Pant (26) in the case of *Bombyx mori* and *Earias fabia*, respectively.

Analysis of hemolymph of *P. interpunctella* revealed the presence of 15 amino acids, the concentration of which in normal and treated samples showed a wide range of variation. Some amino acids showed an obvious decrease in the hemolymph of the larvae after treatment such as serine, proline, glycine, alanine, valine, leucine and lysine, while some others showed an obvious increase after treatment.

The disappearance of glutamic acid may be attributed to its oxidation into glutamine and that of cysteine may be attributed to its loss during hydrolysis (21, 22). The disappearance of methionine as an essential amino acid may be due to its consumption in the methylating intermediary pathways (4). The increase of tyrosine in the treated larvae seems to be a result of its accumulation since in the normal larvae it is used in the formation of polyphenols and quinones that are concerned with hardening process of the cuticle of insects (13). The fact that arginine increased after treatment was related to its proposed function in the metabolic process in the healthy larvae or to its accumulation as guanidine derivative (22). Naralyanan *et al.* (20) reported an obvious increase in the amino acids in the larvae of Plutella maculipennis curt. treated with *B. thuringiensis*. Boctor (5) found that most of the amino acids in the hemolymph of the larvae of *S. littoralis* infected with nuclear polyhydrosis virus decreased, while few others showed an increase than those in the normal larvae.

Treatment of *P. interpunctella* with *B. thuringiensis* resulted in an increase of sodium, potassium, calcium, magnesium and manganese. A marked decrease in phosphorus, ferric, zinc and copper ions was detected, on the other hand, after treatment. The increase in potassium after treatment coincides with the results of Ramakrishnan (25), Pendleton (23), Fast and Morrison (10), Smirnoff and Valero (31) on the occurrence of hyper-potassemia during *B. thuringiensis* infection of Choristoneura fumiferana.

The present study showed a marked increase of potassium after treatment of *P. interpunctella* with *B. thuringiensis*. Smirnoff and Valero (31), however, reported a marked decrease in the level of calcium after treatment of *C. fumiferana* and *Malacosoma disstria* with *B. thuringiensis*; this decrease was tripled during *B. thuringiensis* infection.

Calcium and potassium levels in the organism may be of value in biochemical activities. In additional to influencing glycerol and protein synthesis, potassium affects membrane polarization, nervous conductivity, transmission of signals to the synaptic and neuromuscular functions and muscular excitability and muscular movements (muscular contraction depends on Ca^{++} interaction with regulating proteins) and affects the action of many organs (31).

The infection by *B. thuringiensis* thus, provoked a disturbance in the regulation mechanisms of K^+ and Ca^{++}, a hyperpotassemia together with hypercalcemia were noted in the hemolymph of *P. interpunctella* larvae.

The ratio of Na/K in *P. interpunctella* was significantly increased by a factor of 2.4 after *B. thuringiensis* treatment. In this concern, Tiwari and Mehrotra (33) reported that the changes observed in sodium and potassium ion concentrations and Na^+/K^+ ratio in *Achoea janata* L. and S. litura F. treated with *B. thuringiensis* may not be important as far as toxicity is concerned. Florkin and Jeniaux (11), on the other hand, found that the absolute concentration of various cations in the hemolymph is not as important as the ratio of sodium and potassium.

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