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AGROBACTERIUM TUMEFACIENS AFFECTING SURVIVAL, HISTOLOGY AND PROTEIN CONTENT OF *PERIPLANETA AMERICANA* (COCKROACH)

FOUZIA QAMAR, FIRDAUSIA AZAM ALI AND SHAHIDA HASNAIN

Department of Zoology (FQ, FAA), Department of Botany (SH), University of the Punjab, Quaid-e-Azam Campus Lahore-54590, Pakistan

Abstract: *Agrobacterium tumefaciens* was isolated from two sources. One source was the young and freshly cut tumors from trees of *Populus* sp. present along the canal near the vicinity of Quaid-e-Azam Campus, Punjab University, Lahore. The other source was from stink bugs which were always found to be present in the proximity of the tumors of these trees. These insects habitually feed on them. Total of eight isolates were obtained and designated as F_3o, F_3w, F_3y_1, F_3y_2, $F_3y_2(1)$, BS, BSc and BSy. First five were obtained from the tumors and later three were isolated from hemolymph of stink bug. Two of these strains i.e., F_3o and BSy were selected for inoculation studies. These were injected as well as fed in measured dozes to the insects. The effects were observed after 5 hrs, as bacteria-treated insects started showing high rate of mortality after 8 hrs. The insects which received bacteria by means of feeding survived for comparatively longer duration. Blood smear preparations of inoculated insects showed a disturbed hemocyte structure. Histological studies of the gut of bacteria-fed insects revealed a significant decrease in the length of the cells of enteric epithelium. Their total body protein content also showed a significant increase over that of the controls, but no significant increase was noted in two case of inoculated insects.

Key words: Agrobacterium, Periplaneta, insects, enteric epithelium of insects

INTRODUCTION

Agrobacterium is an example of naturally occurring genetic engineering system. *A. tumefaciens* is a ubiquitous plant pathogen, which causes gall tumors in many orders of plants, including dicotyledons, gymnosperms (Mani, 1964; Bevan *et al.*, 1983; Nester, 1983; Schell *et al.*, 1983; Nester *et al.*, 1984) and monocots (Graves and Goldman, 1986; Schafer *et al.*, 1987; Graves *et al.*, 1988). The ability to transform plant cells is correlated with the presence of Ti-plasmid i.e., tumor inducing plasmid in *A. tumefaciens* (Zaenen *et al.*, 1974).

Inspite of the tumorogenecity of *A. tumefaciens* on plants, little attention has been diverted towards it's pathogenecity on animals. It had been observed that generally the bacterial pathogens interfere with the metabolic functions of the host cellular system and cause structural and functional impairing. Earlier workers like Smith (1917) reported the formation of tumors on trout and certain invertebrates. Agrobacterial cultures injected into rabbits caused increased proliferation of the bone marrow elements (Soru and Brauer, 1933). Hamilton and Huisingh (1968) revealed that *A. tumefaciens* and other members of the genus have an acute lethal effect on mice but are noninfectious and lethality is dependent on the presence of Ti-plasmid, which codes for oncogenecity. Curing of plasmid results in the loss of virulence and production of murine toxin (Mitra, 1987). Mitra *et al.*(1988) found that *A. tumefaciens* damages both liver and kidney tissues of mice by disrupting the renal organization as well as by affecting the hepatic infra structure.

A curious observation made by Qazi (Personal communication) is the association of stink bugs and tumors on the plant which is the base of the present work. *A.*

tumefaciens was isolated from tumors and hemolymph of stink bugs and was used to inoculate *Periplaneta americana*, the American small Cockroach. Isolation of this bacterium and effects on this cockroach are being reported here.

MATERIALS AND METHODS

Isolation of Bacteria from Insects and Tumors

Trees along the canal bank were thoroughly surveyed. Trees of *Populus* sp. having young tumors were selected. In the vicinity of the tumors, a large number of stink bugs, i.e., *Halys dentatus (F.)* (Heteroptera: Pentatomidae) could be seen. Bacteria were obtained from the suspension of the young tumors and hemolymph of the bugs using the method of Lelliot and Stead (1987). Purified colonies were used for morphological and biochemical characterization. Growth of bacteria was at 29 $\pm$ 1 °C for 24 - 48 hrs. Two isolates, one from each source, were selected for determining the effects of *A. tumefaciens* inoculation on *Periplaneta americana.*

Morphological and Biochemical Characterization

Morphological studies included both colony and cell morphology. Colony morphology included shape, size, motility, margin, elevation, whereas in cell morphology shape and size was determined. Gram-staining was performed as described by Gerhardt *et al.* (1981). Biochemical characterization was done by performing 3-keto-lactose, oxidation - fermentation, NO_3 reduction, denitrification, oxidase and urease (Gerhardt *et al.*,1981) and catalase tests (Mac Faddin, 1976). 20 biochemical tests were accomplished by using QTS-20 identification strips and reagents supplied by DESTO Laboratories, Karachi. For screening the presence of plasmids, total cell lysate method was followed (Thomas, 1984).

Cockroach Inoculation Studies

Lethal effects of bacterial isolates on cockroaches were tested and for this purpose only males were used in order to eliminate variation. Two bacterial strains F_{30} and BSy, one from each source, were selected. Overnight liquid culture in L.B (Kahn *et al.*, 1979) were used for Inoculation. Following treatments were used for this purpose i) Inoculation of bacterium to the insect by injection; ii) Inoculation of bacterium by means of feeding.

0.1 ml or 100 μl of bacterial inoculum ($\sim 10^7$ cells/ml) was injected in the abdomen of each cockroach with the help of a microsyringe. The insects to be used as a control were injected with 0.1 ml of sterile glass distilled water. 0.5 ml of bacterial liquid culture (10^7 cells/ml) was mixed in equally weighed quantity i.e., 2 gms, of minced meat by means of sterilized glass rods for feeding purposes.

For studying the effects of *A. tumefaciens*, on blood both inoculated and fed insects were punctured from the lateral side of the abdomen as well as from the thoracic region and blood was gently squeezed on the slides. Blood smear

preparations were carried out and staining was done by Giemsa's stain. Cockroaches, that were fed with bacteria, were dissected and midgut region was removed. Histological procedure for section cutting was carried out and sections were cut at 8u. Staining was done in Ehrlich's Hematoxylin and for counterstaining Eosin was used. Total body protein contents were estimated according to Lowry's method (Lowry *et al.*, 1951) modified by Schaettle and Pollack (1973). Folin Ciocaltau's phenol reagent marketed by Merck was used for this purpose.

RESULTS

Total of eight isolates 5 from tumors on trees of *Populus* sp. ($F_{3}o$, $F_{3}w$, $F_{3}y_{1}$, $F_{3}y_{2}$, $F_{3}y_{2}(1)$), and three from hemolymph of stink bug (BS, BSc, BSy) were obtained. All the isolates had circular colonies with entire margins and convex elevations (except for $F_{3}w$ where elevation was raised). Size of the colonies varied from 0.5 - 4.0 mm. All are motile, rod shaped (with length ranged from 1.12 - 4 μm width ranged from 1.16 - 0.5 μm) and Gram-negative bacteria. All of them gave positive results for 3-ketolactose test (specific for *Agrobacterium*), NO_3 reduction, catalase and oxidase tests. They gave negative results for urease and oxidation - fermentation tests. $F_{3}y_{1}$, $F_{3}y_{2}(1)$, $F_{3}y_{2}$ and BS had the ability to denitrify. 20 biochemical tests performed by using QTS-20 strips and reagents, indicated that all the isolates shared many biochemical characters excluding $F_{3}y_{2}$, $F_{3}o$ and $F_{3}y_{2}(1)$, where some differences were observed (Table-1).

Table 1: Results of QTS-20 biochemical tests performed with different strains of *Agrobacterium tumefaciens* isolated from tumors on trees of *Populus* sp. and hemolymph of stink bug.

	ISOLATES							
Tests	F3w	F3o	F3y1	F3y2	F3y2(I)	BS	BSc	BSy
1. ONPG	-	W+	-	-	W+	-	-	-
2. Sodium citrate	-	-	-	+	-	-	-	-
3. Sodium malonate	-	-	-	+	-	-	-	-
4. Lysine decarboxylase	-	-	-	+	-	-	-	-
5. Arginine dihydrolase	-	-	-	+	-	-	-	-
6. Ornithine decarboxylase	-	-	-	+	-	-	-	
7. H_2S production	-	-	-	-	-	-	-	-
8. Urea hydrolysis	-	-	-	+	-	-	-	-
9. Tryptophane deaminase	-	-	-	-	-	-	-	-
10. Indole	-	-	-	-	-	-	-	-
11. Acetoin (VP)	W+	-	VW+	-	-	-	-	-
12. Gelatin hydrolysis	+	-	W+	+	+	+	W+	W+
13a Acid from glucose	-	-	+	+	-	-	-	-
13b Nitrate reduction	+	-	+	-	-	+	+	+
14. Acid from maltose	-	-	-	-	-	-	-	-
15. Acid from sucrose	-	-	W+	-	-	-	-	-
16. Acid from mannitol	-	-	-	-	-	-	-	-
17. Acid from arabinose	+	+	+	+	+	+	+	+
18. Acid from rhamnose	-	-	-	-	-	-	-	-
19. Acid from sorbitol	-	-	-	-	-	-	-	-
20. Acid from insoitol	-	-	-	-	-	-	-	-

+, positive; -, negative; W+, weak positive; VW+ very weak positive

Total cell lysate of the isolates on agarose gel revealed that each strain harboured a single plasmid.

Results of Inoculation Studies

Cockroaches used for the work had mean body weights 1.08 $\pm$ 0.06 gms. The variability in weights was not significant (< 6%). The bacterial strains designated as F_3o and BSy, showing some differences in morphology but having similar biochemical characters were used (Fig.1a and 1b) for inoculating cockroaches. When experiments were carried out with cockroaches, that were both injected and fed with bacterial liquid culture of *Agrobacterium tumefaciens* it was observed that cockroaches with relatively larger body weights showed low mortality rate, whereas cockroaches with comparatively smaller body weights showed larger mortality rates. Furthermore the cockroaches, that were fed with bacterial cultures survived for longer duration of period than cockroaches which were given the injection of bacterial liquid cultures. The point worthy to be mentioned here is that the mortality among cockroaches, started occuring after 8 hrs of experimental set up.

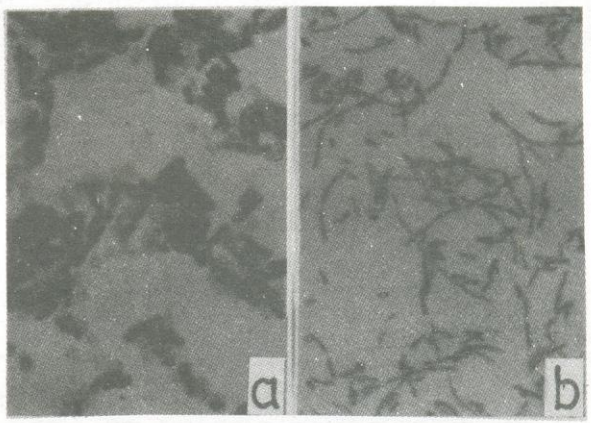


Fig.1. Bacterial cells a) BSy strains from *Halys dentatus;* b) F_3o strains from tumors on *Populus* sp. (700X)

Blood Smear Analysis

For blood smear analysis, blood was obtained from the lateral side as well as from the thoracic region of the insect's body. Smear preparations showed that the cell walls of the hemocytes in the hemolymph obtained from lateral side of the insect's abdomen, injected with bacteria, got badly ruptured, and shape of their nuclei was also distorted (Figs. 2b,c). Only a few bacteria were seen in the hemolymph of the insect fed with bacteria as compared to that of the injected insects (Figs. 3b,c). Studies carried out with blood from the thoracic region of both the injected and fed

cockroaches, indicated that no abnormalities in the hemocyte structure took place in those insects which survived for more than 5 hrs (Figs. 4Ia,4Ib,4IIa,4IIb) of experimental set up. Bacterial presence was indicated in those insects which survived for more than 8 hrs (Figs. 4IIIa,4IIIb). In the insects fed with controlled diet the hemocytes were in a normal state (Fig. 3a). The cell membranes of some of the hemocytes, were found to be ruptured in the insects, that were injected with H_2O (Fig. 2a).

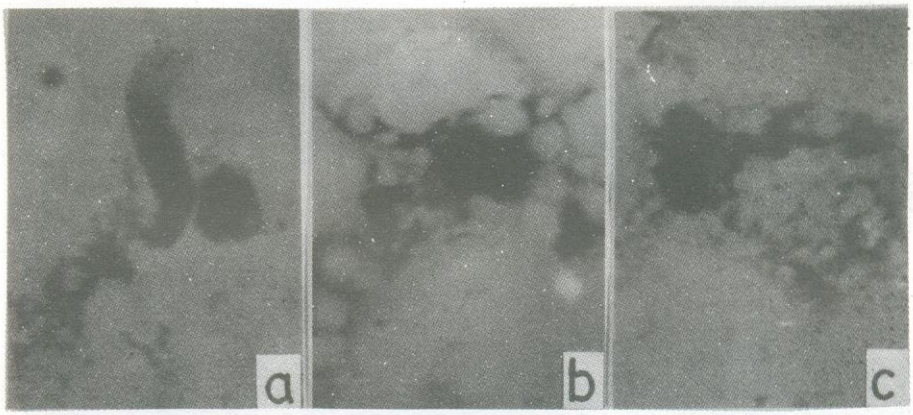


Fig.2. Hemocytes from the lateral region of the abdomen of cockroaches after 5 hours of injection with a) dist. H_2o-control; b) F_3o; c) BSy. (700X)

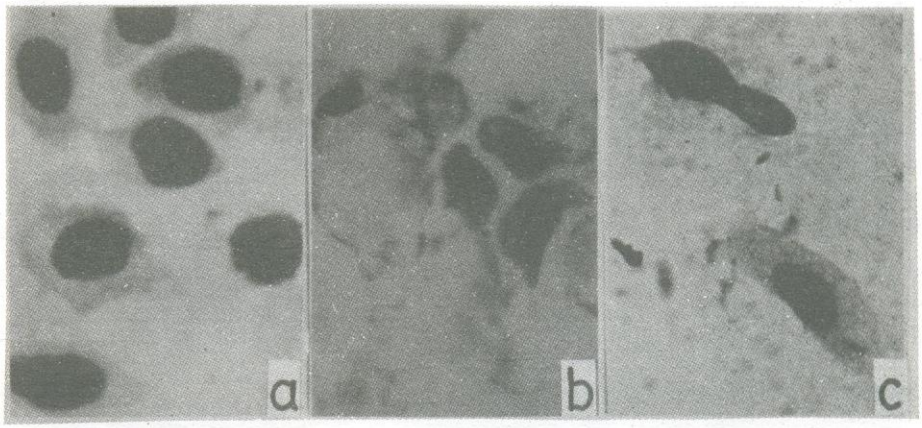
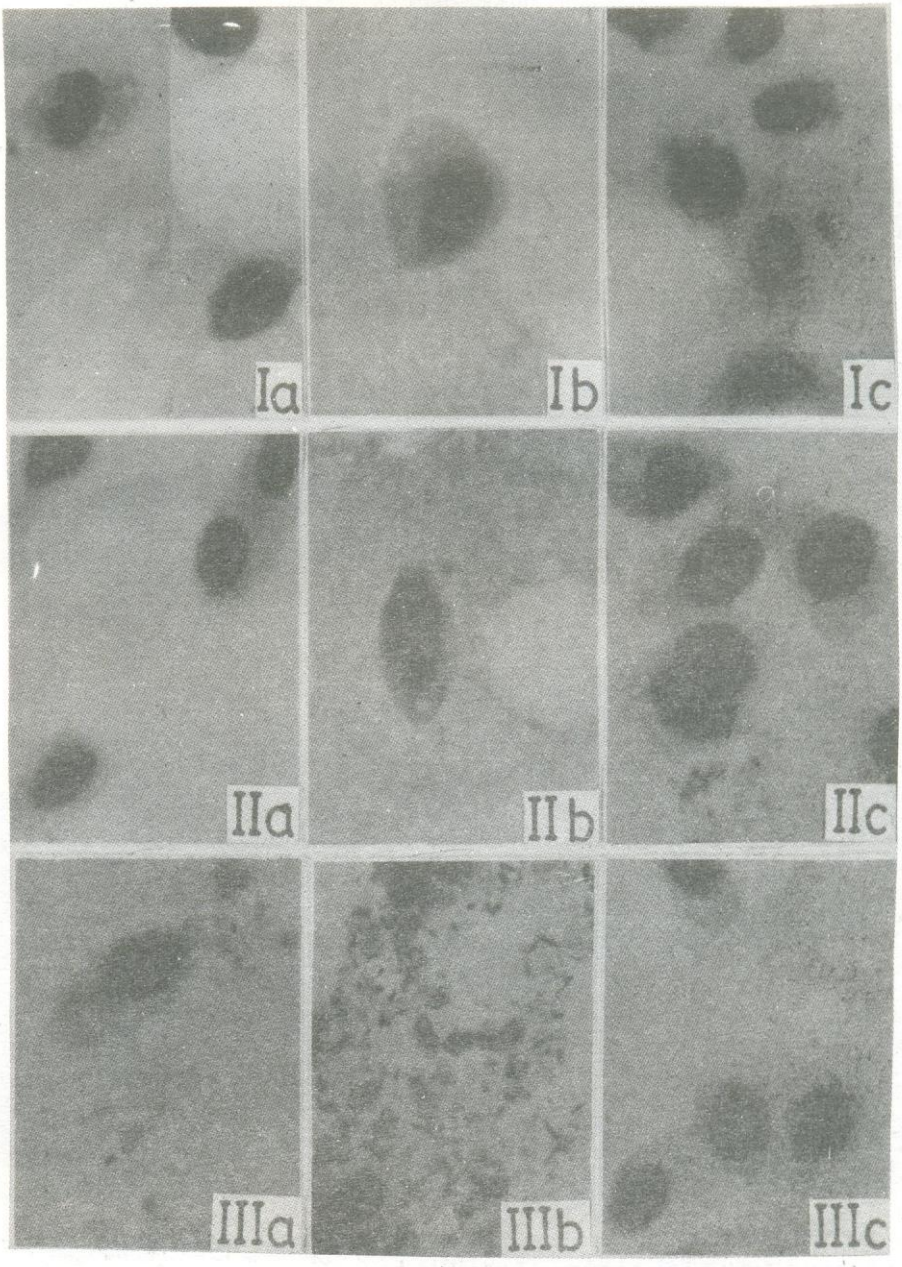


Fig. 3. Hemocytes from the lateral region of the abdomen of cockroaches after 5 hours of feeding with a) control diet; b) BSy; C) F_3o. (700X)


Ia
Ib
Ic
IIa
IIb
IIc
IIIa
IIIb
IIIc

Histological Studies

These studies showed that the length of the nuclei of columnar epithelial cells of the midgut wall in the control ones were greater as compared to the treated ones. Due to the bacterial treatment the nuclei of columnar cells had also shrunk in length (Table-2a) (Figs. 5a,b,c). No significant change in width of nuclei was observed (Table-2b).

Table-2: a) Results of length of epithelial nuclei of columnar epithelial cells in the mid gut region. (Means of three replicates).

Strains	N	Length of Nuclei (μm)	t value
Control	8	17.25 ± 0.125	
BSy	8	15.50 ± 0.125	9.94***
F_{30}	8	14.42 ± 0.260	9.82***

b) Result of width of epithelial nuclei of columnar epithelial cells in the mid gut region (Means of three replicates).

Strains	N	Width of Nulcei (μm)	t value
Control	8	8.33± 0.260	
BSy	8	7.83±0.0721	0.85
F_{30}	8	7.58±0.191	2.32

Fig. 4. I- Hemocytes from the thoracic region of cockroaches after 5 hours of injection with a) F_{30}; b) BSy; c) control.

II- Hemocytes from the thoracic region of cockroaches after 5 hours of feeding with a) F_{30}; b) BSy; c) control diet.

III- Hemocytes from the thoracic region of cockroaches after 8 hours of feeding with a) F_{30}; b) BSy; c) control

(7ooX)

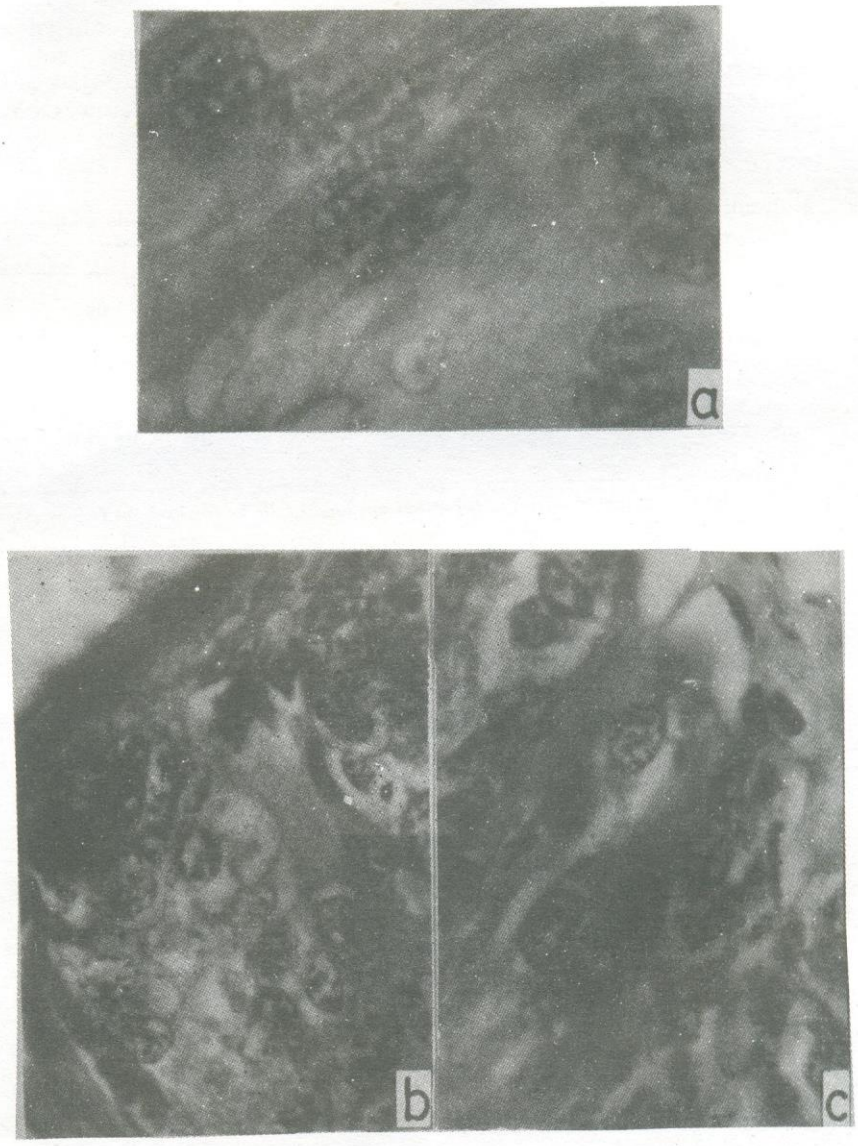


Fig. 5. Transverse section through midgut region of cockroaches after 8 hours of feeding with a) control diet; b) F_3o; c) BSy. (700X)

Protein Content Analysis

Protein content estimation revealed a significant increase in the total body protein contents of insects fed with F_{3O} and BSy strains over the controlled ones. In case of the injected insects, though there was increase in total body protein contents over that of the controls, but it was not significant (Table-3).

Table-3. Results of effect of Agrobacterial inoculation on the total protein contents (mg/gm) of *Periplaneta americana* (Means of four replicates)

	Protein Content (mg/gm)			
Type Of Inoculation	Control	BSy	F_{3O}	L.S.D (P = 0.05)
Fed	4.717 ± .33	8.22 ± 1.09	7.042 ± .48	2.22
Injected	6.418 ± .88	9.714 ± 1.25	8.805 ± 1.57	4.078

DISCUSSION

The main aim of the work carried out here, was to study the toxic effect of *Agrobacterium tumefaciens* on *Periplaneta americana*. Bacterial strains isolated from tumors and bugs were similar, having the same colony morphology and they shared many biochemical characters, which explained the associiation of bugs with the tumors.

A survey of the effects of *Agrobacterium tumefaciens* on mammalian system has indicated that *Agrobacterium tumefaciens* has an acute lethal effect on mice, but is not infectious (Hamilton and Huisingh, 1968). Similar type of phenomenon was observed in the current work. The cockroaches showed no infections but the strains of this crown gall producing organisms were lethal for the cockroaches after 8 hrs of inoculation. Hamilton and Huisingh (1968) attributed these effects to toxicity of *Agrobacterium tumefaciens* in mice. They assumed that the lethality in mice was the result of a toxic agent and they found no evidence of infection in the histopathological studies. In cockroaches, agrobacterial toxic effect was apparent as the hemocytes got destroyed and cytoplasmic contents and fragments of cell membranes of hemocytes got scattered in the plasma (Figs. 2 b,c). Phygocytosis defense mechanism, which is generally effective against microorganisms might have become ineffective against the toxins secreted by *Agrobacterium tumefaciens* which resulted in distortion of nuclei and breakage of cell membranes. The cell membrane and the polysaccharide endotoxin component of bacteria seem to represent a special tartget for defense mechanism since this chemical's uniqueness offers an Achille's heel for destruction substances (Arnold, 1974). The toxicity produced by *Agrobacterium tumefaciens* was shown to be dependent on the presence of Ti-plasmid in bacterium, which codes for oncogenicity and virulence (Mitra *et al*, 1988). Strains described here have this plasmid, therefore, destruction of hemocytes might be ascribed to the toxin produced by Ti-plasmid. The toxin production is actually stimulated by the enzyme lysing the bacterial cell. Antibodies in the blood might have been released against the bacterial antigen. As the antibodies are proteins, they might have contributed to a significant increase in body protein level in the case of bacteria-fed insects. The supposition that

antibodies might have been released against bacterial toxin provide an evidence for the phenomenon which was observed during the cockroach inoculation studies. The cockroaches fed with bacterial cultures survived for longer duration of time than cockroaches which were given the injection of bacterial liquid cultures. It means that bacteria fed cockroaches which survived for longer duration, were able to produce antibodies against bacterial toxin as reflected by the increase in protein content, whereas in injected cockroaches non-significant increase in protein level suggests the formation of some antibodies but it may not be enough to counteract bacterial toxins. The difference in the presumptive antibodies (proteins) as well as survival of injected and fed cockroaches might be due to differences in the number of bacteria, being introduced in their bodies.

Histological examination of midgut sections of fed cockroaches have shown that no histopathological anomalies were noticed in the midgut layers, apart from nuclear length of columnar epithelial cells in the treated ones which decreased to a significant degree. No sign of bacterial presence could be revealed. Hamilton and Huishingh (1968) reported that after injection into the mice, murine toxin is released from the bacterial cells as a result of enzymatic lysis. This might explain the absence of bacterial cells from the midgut cavity of the cockroaches.

The exact nature of the toxin produced by *Agrobacterium tumefaciens* is not clear but previous reports suggest a relationship between a toxin produced by *Agrobacterium* in animal system and the tumor inducing principle in plants, the Ti-plasmid. Whether Ti-plasmid is involved in this toxicity, remained to be determined and further work at molecular level will reveal this aspect.

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