HEMOLYMPH TOXICITY AND FOREGUT BLOATING IN *PERIPLANETA AMERICANA* (L.) CAUSED BY COLD INJURY AND DDT INTOXICATION

by 1264

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B.S., Stephen F. Austin State College, 1965

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Entomology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1970

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INTRODUCTION

Toxins may be liberated into insect hemolymph by several different types of stressors such as intoxication by chlorinated hydrocarbon and organophosphorus insecticides, electrical stimulation, immobilization and induced physical activity (Roan and Hopkins, 1961; Sternburg, 1963). Sternburg and Kearns (1957) first reported a toxic hemolymph factor which was not DDT in DDT-poisoned American cockroaches. Beament (1958) reported that immobilization or constant motion in a revolving powder mill caused paralysis in Periplaneta americana (L.), and that the paralyzing factor was a blood toxin possibly from neural origin. Heslop and Ray (1959) suggest that there is a common reaction of P. americana to insecticidal intoxication that may obscure the specific symptoms of the insecticide and that any type of stressor may cause common symptoms once a certain mechanism is triggered. Patel and Cutkomp (1967) found some similarities and some significant differences in oxygen consumption, heartbeat frequency, and loss of weight between immobilized cockroaches and DDT-treated cockroaches depending on the conditions to which they were subjected. They postulated that secondary effects of DDT were essentially those of stress, but the primary effects were distinctive. Ameel (1965) observed the collection of gas in the foregut of Leucophaea maderae (Fabr.) and P. americana when they were subjected to organophosphorus and chlorinated hydrocarbon insecticides, induced physical exercise and electrical shock. The effects of the various types of stress on the nervous system are not known, but it is possible that the damage results in ion leakage thus producing hyperactivity and nervous tremors. Bloating of the foregut appears to be a secondary symptom after prolonged stress and nervous system break down.

This study was concerned with the effects of cold injury and immobilization on the production of hemolymph toxins, foregut bloating, and comparison of these symptoms to those produced by DDT intoxication in P. americana. The first phase was designed to investigate the syndrome of stress symptoms related to cold injury and how cold injury affects foregut bloating. The second phase investigated the toxicity of the hemolymph at various times after cold injury and the effects of different dosages of toxic hemolymph on normal cockroaches. The third phase was the relationship between induced prostration of cockroaches by topical DDT treatment and hemolymph toxicity. The effects of topical application of DDT to the cerci of dissected nerve cords bathed in saline and production of toxins was also investigated. Insects in each experiment were observed carefully for intoxication symptoms, foregut bloat and whether the method of stress caused toxin to be produced in the hemolymph. In the fourth phase experiments of ligating the esophagus and severing the abdominal tracheal trunks supplying the foregut were to elucidate the origin of the gas causing foregut bloat.

MATERIALS AND METHODS

Experimental Insects

Nymph and adult American cockroaches, Periplaneta americana (L.), were used, and the sex is stated when pertinent to the results of the experiment. Experimental colonies were started from "stock" colonies by transferring approximately 100 cockroaches of various instars into a #2 square galvanized tub. Each tub was equipped with a wooden frame screen top and filled with three to four inches of wood shavings on the bottom. Cockroaches were regularly fed and watered using Purina Laboratory Chow^{R} and a poultry

watering dish. They were not removed from the cultures until enough adults had emerged to guarantee perpetuation of the colony.

Cold Injury and Hemolymph Toxicity

Adults were removed from the culture and placed in covered glass Petri dishes, two to three per dish. The most consistent results were obtained when one sex was used for each experiment, since the male and female cockroaches respond differently to the cold depending on the length of exposure to -20° C. Petri dishes were cooled more uniformly if each was placed individually on the floor of the freezer compartment. At the end of the 12 minute time interval, female cockroaches were removed from the freezer, placed on a table at room temperature (approximately 23° C), and allowed to remain for 24 hours. This was found to be the optimal time period to allow for paralysis, maximal bloating of the foregut, and minimal loss of hemolymph volume.

After the 24 hour period the cockroaches were prepared for hemolymph removal by centrifugation. The cervix was ligated with cotton thread and the legs and antennae were cut off near the body with cuticle scissors. A sagittal incision also was made in the pronotum. The cockroaches were placed head down in a brass screen sieve that fitted the inside of a 50 ml graduated glass centrifuge tube. Cockroaches were centrifuged for 15 minutes at 250 rpm at a temperature of 0° to 4° C in a pre-cooled International Refrigerated Centrifuge (Model PR-2). The hemolymph was removed from the centrifuge tube with a B-D Yale 1/4 cc tuberculin syringe fitted with a two inch #25 B-D Yale hypodermic needle, and 25 µl doses were injected between the sixth and seventh abdominal sterna of normal cockroaches lightly anesthetized with carbon dioxide. A hand operated mechanical micro-applicator was used to make

all the injections of hemolymph. Subsequently the cockroaches were placed in covered Petri dishes for recovery and observed periodically for any abnormal symptoms. No food or water was provided during the observation period.

Control cockroaches received the same treatment as the experimental individuals, except that they were removed from the culture and placed directly in covered Petri dishes at room temperature without being subjected to cold treatment.

The cockroaches that became paralyzed, due to the injection of hemolymph taken from the cold injured cockroaches, were again prepared for collection of their hemolymph by centrifugation. This hemolymph was injected into normal adults using the previously described methods. This series of procedures was continued five times for each experiment to determine if the toxic material in the hemolymph could propagate itself. Those cockroaches that were not centrifuged were checked for bloating of the foregut by examination of the abdomen for expansion, by X-ray techniques, and by dissection.

The anterior part of the ventral surface of the abdomen was examined for bloating to see if there was a translucent area visible just posterior to the metathoracic legs. The size of the translucent area indicated the extent of bloating. An alternate check is to observe whether the light colored intersegemental membranes are visible. Heavy or extreme bloat is usually present when these membranes are visible.

Final conformation of any collection of gas in the foregut was by X-ray. The X-rays were made by taping the cockroaches in rectangular openings in a piece of 1/4 inch plexiglass using transparent tape. The frame and a piece of Kodak Industrial X-ray film were placed in a General Electric Grain

Inspection Unit. After one minute exposure period the film was removed from the X-ray machine and developed in Kodak D-76^{R} developer and Kodak fixer for the recommended time depending on the temperature of the developer and fixer. The developed X-rays were viewed on a ground glass X-ray viewer and each X-ray was assigned a bloated condition on the following basis: (1) no bloat - the foregut is normal with no gas other than occasional small bubbles in the crop, (2) slight bloat - gas content is somewhat greater than a small bubble and usually less than 1/4 inch in diameter, (3) medium bloat - gas caused the crop to fill much of the thorax and into the abdomen but the X-ray lacks density, (4) heavy bloat - gas causes the crop to fill the anterior half of the abdomen with decreased density of the X-ray, (5) extreme bloat - gas is still contained within the crop but enough is present to nearly fill the abdomen with a dark, dense area as shown by the X-ray (Plate I).

After the cockroaches had been X-rayed, they were removed from the X-ray frame. A dorsal sagittal incision was made the entire length of the animal and the flaps were pinned back exposing the foregut. Care was taken while the incision was being made not to puncture the foregut with the point of the scissors. The foregut was examined to determine the extent of gas collection and liquid content.

Three groups of experiments were run to determine when the hemolymph of cold injured cockroaches became toxic enough to produce paralysis and death in normal insects within 16 hours if a 25 µl dose was administered. For each of these experiments 60 adult females were removed from the culture. Thirty-six were used as experimentals and the other 24 as controls. The experimentals were placed in a six inch Petri dish and cold injured as previously described. The controls also were put in a six inch Petri dish but allowed to remain at room temperature. Thirty minutes after their removal from the freezer, three

EXPLANATION OF PLATE I

Fig. 1. No bloating. Control P. americana. (30 hours)

Fig. 2. Slight bloating of foregut of P. americana after cold injury. (18 hours).

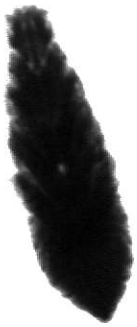
Fig. 3. Medium bloating. P. americana male topically treated with DDT. (24 hours).

Fig. 4. Medium bloating of foregut of P. americana after cold injury. (18 hours).

Fig. 5. Heavy bloating. Cold injured P. americana. (20 hours).

Fig. 6. Extreme bloating. Cold injured male P. americana. (18 hours).

PLATE I



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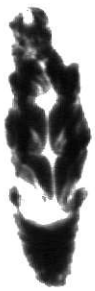

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experimental and two controls were prepared for centrifugation of hemolymph. Three normal individuals were injected with hemolymph from the cold injured ones and two with the hemolymph from the controls. After the injections had been made, they were placed in Petri dishes and observed for symptoms. This procedure was repeated with three cold treated and two control cockroaches every two hours for a period of 24 hours. Each injected group was allowed to remain in the Petri dish for 30 hours before being examined and X-rayed for any collection of gas in the foregut.

DDT Induced Paralysis by Topical Treatment

Adult males were used for these experiments because of a shortage of females. The cockroaches were removed from the culture and were lightly anesthetized with carbon dioxide before being treated with a p,p'DDT acetone solution. Each was treated with four µl of the solution containing 141.2 µg DDT per µl of acetone. One µl of solution was placed on each of the coxal membranes of the prothoracic and mesothoracic legs. The control cockroaches were treated with four µl of acetone. The treating solutions were measured with a hand operated mechanical micro-applicator equipped with a B-D Yale 1/4 cc tuberculin syringe and a two inch #25 blunt B-D Yale hypodermic needle.

After the treatments the cockroaches were placed at room temperature in covered Petri dishes for 24 hours before being put in a constant environment of 15° C $\pm$ 2.5° C for three hours.

Prostration was induced in the p,p'DDT treated group by the three hour exposure to 15° C. At the end of the three hour period the cockroaches were prepared for centrifugation by washing them well with acetone to remove any p,p'DDT residues and the hemolymph centrifuged as previously described.

The cool hemolymph was immediately injected in 25 µl doses into the abdominal cavity of normal adult males. These cockroaches were then placed in covered Petri dishes at room temperature and observed.

The centrifuged cockroaches were removed from the brass screen seive and the ventral nerve cords were dissected leaving the head and the cerci attached. After dissection each nerve cord was placed in oxygenated cold Ringer solution (cockroach heart and blood saline, Yeager) to prolong cell life while other nerve cords were being dissected. When all nerve cords were dissected, they were placed in a glass tissue grinder with 100 µl of Ringer solution for each nerve cord and thoroughly mascerated. After homogenization, the tissue grinder was placed in a padded centrifuge tube shield and centrifuged for 15 minutes at 2000 rpm in the refrigerated centrifuge precooled to 15° C. The supernatant was then injected in dosages ranging from 25 µl to 50 µl into normal adult males. Treated cockroaches were placed in covered Petri dishes and observed. Control cockroaches were treated in the same manner using the homogenized nerve cord supernatant of the acetone treated control cockroaches.

After the saline extract of the homogenized nerve cord was removed from the tissue grinder, an acetone extract was prepared by adding a small amount of acetone to the tissue grinder and thoroughly mixing it with the homogenized nerve cords. The nerve cords then were centrifuged again in the refrigerated centrifuge using the same setting as mentioned above. The supernatant was taken from the tissue grinder and placed in a Syracuse dish and permitted to evaporate to dryness before being reconstituted with 250 µl of Ringer solution per homogenized nerve cord. This material was then injected into normal adult males in dosages ranging from 25 µl to 50 µl per individual. An acetone extract of the control nerve cords also was used to treat the controls; the

dosages also ranged from 25 µl to 50 µl per cockroach. The treated cockroaches were placed in covered Petri dishes, observed and none was given food or water.

DDT Treatment of Dissected Nerve Cords

Adult male cockroaches were removed from the culture and lightly anesthetized with carbon dioxide. The legs, wings, and antennae were removed immediately and the cockroaches were pinned ventral surface down in the bottom of a Petri dish lined with bee's wax. A dorsal sagittal incision was made the entire length of the body and the viscera were removed being careful not to disturb the ventral nerve cord or the cercal nerves. The sterna were then cut along either side of the nerve cord leaving the head and cerci attached. Immediately after dissection each nerve cord was transferred to cold oxygenated Ringer solution and then placed in the refrigerator at 5° C to prolong the life of the nerve cords.

The nerve cords were laid on a glass slide with the cerci overhanging and each cercus was treated with three µl of p,p'DDT solution mentioned earlier. The nerve cords used as controls had the cerci treated with three µl of acetone per cercus. Immediately after treatment each nerve cord was placed in a small trough hollowed out of a block of paraffin. The nerve cords were placed in the trough in such a manner as not to allow the treated cerci to come into contact with the 75 µl of Ringer solution that was put on the abdominal section of the nerve cords. The nerve cords were covered with microscope slides, sealed with petroleum jelly, and placed in the refrigerator at 5° C. The cold temperature enhanced the action of p,p'DDT and also prolonged the life of the dissected nerve cords. After 10 hours the saline was removed from around the nerve cords with a syringe and needle and was

injected in 25 µl doses into normal adult males (these were placed in covered Petri dishes without food or water).

Immobilization Experiments

Immobilization experiments were conducted to see if this type of stress would cause paralysis and the release of toxins into the hemolymph. Third and four instar nymphs were used for these experiments since Beament (1958) had good results using this age cockroach. The immobilizations were done in several different ways: (1) by following the procedure of Beament (1958) and securing the cockroaches to a piece of balsa wood using criss-cross pins, (2) by taping the legs to balsa wood strips, (3) by attaching the cockroaches to a piece of cardboard using modeling clay, and (4) by placing the nymphs in an immobilization block.

The procedure described by Beament (1958) was to anesthetize cockroaches with carbon dioxide and secure them to a 95 mm by 43 mm piece of balsa wood using insect pins. The pins were criss-crossed over the femur and tibia of each leg, and three U-shaped pins were placed over the thorax sufficiently tight to hold the cockroach but not tight enough to restrict respiratory movements. The cockroaches were restrained for 96 hours before being released. The controls and experimentals were provided with water throughout the entire experiment.

The second method used was very similar to the first except that the legs of the CO_2 anesthetized nymphs were secured to the balsa wood rectangles using a piece of tape that was pressed around each leg with forceps. The tape was lightly pressed around each leg. Controls for this experiment also were retained in covered Petri dishes. Both the experimental and controls were provided with water for the 96 hour restraining period.

The third technique used for immobilization was to roll modeling clay into strips and place the strips across the thorax and legs of anesthetized cockroaches. The strips across the thorax were only to restrain any violent body movements that might occur while the cockroach was struggling to free itself. The strips across the legs were used mainly to keep the cockroach from running away and had to be pressed firmly around the femurs of each leg and stuck well to a piece of cardboard. A modified version of this method was used in which U-shaped pins were put over the thorax and modeling clay was pressed across the legs. The modeling clay was removed after the 96 hour immobilization period and cockroaches were placed in covered Petri dishes for observation.

A fourth method of immobilization was to confine the cockroaches in an immobilization block made of balsa wood and plexiglass. The block was made by drilling a 1/2 inch hole through a piece of 4" x 4" x 2" balsa wood. The hole was drilled from side to side so that it was four inches long. The block was split so that a 1/4 inch diameter trough was left in each half of the block. A plexiglass cover was made, two holes were drilled in it and nails put through them to hold the cover in place. A small piece of plexiglass was inserted in the balsa wood across the center of the trough so that two cockroaches could be put in each block. Another small piece of plexiglass was used to keep the cockroaches from backing out of the holes; this was also held in place with two nails. Several small holes were drilled in the cover over the trough so the immobilized cockroaches could be given water and to allow adequate ventilation. The cockroaches were put in the block head first and left there for 96 hours. Each animal was provided with water through holes in the plexiglass top.

Origin of the Gas Causing Foregut Bloat

In an attempt to determine the origin of the gas that collects in the foregut, three different techniques were used. First, the esophagus was ligated; second, the spiracles were plugged; and third, the tracheal trunks were severed.

Adult females were subjected to cold injury as previously described. The adults were held in covered Petri dishes for bloating of the crop to occur, after which a ventral incision was made in the cervix, the esophagus was ligated with thread, and the incision was sealed with hot paraffin. A dorsal sagittal incision was made in the abdomen and the gas was removed from the foregut using a 1/4 cc syringe and a #27 hypodermic needle. Observations were then made for rebloating which usually occurs within 30 minutes to one hour. The viscera were kept moist by occasionally applying a few drops of Ringer saline solution to the incision.

The second technique used was to externally plug the spiracles of the bloated cockroaches with hot paraffin. The paraffin was applied with a pair of forceps to each spiracle and allowed to harden with little or no movement from the anesthetized cockroach. Gas was then removed from the bloated foregut and the occurrence of rebloating noted.

The third method used required that a dorsal sagittal incision be made the length of the abdomen and the main tracheal trunks to the foregut severed. The flaps were allowed to close and the viscera were kept moist using Ringer saline solution applied with a pipette.

Three groups were similarly treated, exposed to cold treatment and observed for crop bloating. In each case the cockroaches were watched carefully for any signs of rebloat of the foregut, in which case the gas was

again removed when the foregut began to protrude beyond the abdominal cavity. This procedure was continued until no rebloating occurred. The control cockroaches were treated in the same manner except they were not cold injured.

Once each of the techniques had been employed separately and the results recorded, another set of cockroaches was cold treated and a combination of the techniques was tried. On one group the esophagus was ligated and the spiracles were plugged with paraffin. The second group had the esophagus ligated and the tracheal trunks cut using the same techniques as discussed in the preceding section.

OBSERVATIONS AND RESULTS

Cold Injury and Symptoms

Preliminary experiments have shown that cold would produce paralysis and a bloated condition of the foregut in Periplaneta americana (Murdock and Hopkins, unpublished data). American cockroaches subjected to a temperature of -20° C for 12 minutes will become prostrate and bloated within 24 hours. The present series of experiments was performed to determine the incidence and extent of bloating, paralysis and death in the cold treated cockroaches; and a summary of the data are found in Table 1.

Immediately after being placed in the freezer compartment, the cockroaches became hyperactive, running wildly about the Petri dishes, but within one minute they began to become sluggish. By two minutes the cockroaches had stopped movement and showed only periodic twitches of the extremities. All movements ceased within four minutes. At the end of the 12 minutes they were so cold that the extremities had become stiff, but the legs would not

Table 1. Effects of Cold Treatment of Adult Periplaneta americana Resulting in Foregut Bloat, Paralysis, and Death.

Sex	Total number	Foregut bloated at 42 hours		Returned to normal[2/]	Paralyzed and died		Hours until death
		Number and condition	Percent[1/]		Number	Percent	
FEMALE							
Treated	116	32 slight 52 medium 9 heavy 2 extreme	9.4	8	108	93.1	24-48
Control	33	7 slight 1 medium	0	--	0	0	120+
MALE							
Treated	75	16 slight 7 medium 1 heavy	1.3	10	65	86.6	30-52
Control	21	0	0	--	0	0	120+

[1/] Slight and medium bloat were not used to calculate percentages since these may be normal.

[2/] Apparent normal condition when mobility is regained. Mobility is regained between two and four hours after cold treatment.

break off when they were forcibly moved. As the cockroaches returned to room temperature they became flexible and by 30 minutes they periodically had nervous tremors and hyperactive movements of the extremities. One hour after treatment the cockroaches were still prostrate with only occasional muscular spasms. By two hours the muscular spasms were in regular intervals occasionally interrupted by nervous tremors causing the extremities to twitch. After four hours the cold treated cockroaches became hyperactive, nervous tremors were becoming more pronounced, and slight bloat was occurring in several individuals. Cockroaches were able to recover from the initial prostration did so between four and five hours after cold treatment. Those that had not recovered from the cold treatment paralysis by five hours after treatment remained paralyzed until death. At eight hours they possessed heavy bloat and the nervous tremors were still present but none of them would defecate when handled. The maximal number were bloated at ten hours; however, the maximal amount of bloat did not occur until 18 hours after cold treatment. Also at 10 hours it was noticed that the heads had assumed a near prognathus position with necks stretched forward as if the foregut were full of gas. The insects in this condition were the ones with maximal bloat at 18 hours. After 14 hours the cockroaches were generally in the same condition; nervous tremors, occasional muscular spasms, and medium bloat were present in most cases. At 14 hours it was noted also that the cockroaches would readily defecate a liquified material when handled. There was little change in the bloated condition of the prostrate, paralyzed cockroaches from 18 hours after cold treatment until death. Often occasional nervous tremors were present until shortly before death.

Adult males succumb to cold injury more easily than the adult females. The males do not need to be exposed to the -20° C for more than nine minutes

to produce the maximal incident of bloating in the foregut. The females on the other hand, had to be exposed for 12 minutes to obtain the maximal incident of bloating.

Several series of experiments were performed using cold injury on both adult males and females. A total of 116 adult females were cold injured in various experiments and 85% to 95% of these showed some degree of gas accumulation in the foregut when X-rayed. Thirty hours after the cold treatment 32 of the cockroaches showed signs of slight bloat, 48 produced medium bloat, 13 developed heavy bloat, and two demonstrated extreme bloat of the foregut. Four with signs of heavy bloat at 30 hours after cold injury showed no signs of bloat in the foregut at 36 hours but had medium bloat by 42 hours after the cold treatment. Also 13 showed no signs of bloat at 30 hours after cold treatment but were prostrate. These cockroaches did not demonstrate any signs of bloat for the duration of the experiment.

Eight of the original 116 cold injured cockroaches had returned to what appeared to be normal by 30 hours after the treatment. Two of these showed signs of heavy bloat, three showed signs of slight bloat, and three showed no bloating of the foregut. At 42 hours one of the cockroaches that had heavy bloat at 30 and 36 hours had reverted to slight bloat. The bloat appeared to only slightly impair the mobility of the above individuals. All those having heavy bloat were able to move about with little difficulty. Female cockroaches that returned to the apparent normal condition would occasionally lapse into periods of hyperactivity and nervous tremors of the extremities.

Thirty-three female control cockroaches were placed in Petri dishes at the same time that the above experimental cockroaches were placed in the freezer compartment. After 30 hours of confinement in the Petri dishes, the

control cockroaches appeared normal. However, upon closer examination six were slightly bloated and two had medium bloat of the foregut. Forty-two hours after cold treatment one of the medium bloated cockroaches had changed to a slightly bloated condition. These observations were confirmed by X-ray radiographs. The bloated control cockroaches apparently were not impaired by the collection of gas in their foregut.

A total of 75 males were cold-injured in another series of experiments. Twenty-four showed signs of bloat 30 hours after the treatment. Of the 24 that appeared bloated on the radiographs, 15 were slightly bloated, eight had medium bloat, and one was heavily bloated. At forty-two hours 16 cockroaches had slight bloat, seven were medium, and one had heavy bloat of the foregut. Forty-one did not bloat and 10 of them returned to an apparently normal state. None that returned to normal showed any signs of bloating. However, they did occasionally lapse into periods of hyperactivity and nervous tremors of the extremities.

Twenty-one male controls were used in the experiments and one of them became slightly bloated by 36 hours. The male control cockroaches reacted similarly to the female controls except that the incidence of bloat was only .05% in the males and 32% in the females.

The cold-injured cockroaches would readily defecate while being prepared for centrifugation. The fecal material was very liquified in all cases and the cockroaches were usually wet on the exterior surface, as if moisture had diffused through the exoskeleton. The cold-injured cockroaches took from 48 to 72 hours to die after the cold treatment. These cockroaches usually died in abnormal positions and dried up leaving a hard exoskeleton. Cold-injured cockroaches seldom developed a putrid smell when allowed to remain in the Petri dishes after death.

Effects of Hemolymph Extracted from Cold Injured Cockroaches on Normal Cockroaches

Cold injury to cockroaches apparently results in irreversible damage to the nervous system as evidenced by the symptoms previously described including bloating of the foregut, prostration, and eventually death. To determine if naturally produced toxins are released into the hemolymph during the prostration and paralytic stages (as is indicated to be the case in DDT-poisoned insects), hemolymph was tested for toxicity during the post treatment period.

A series of 10 experiments were run using third and fourth instar nymphs as well as adult males and females. The same age and sex cockroaches were used throughout the entire experiment. Ten adults were removed from the cultures and exposed for 12 minutes at -20° C. They were then returned to room temperature for 24 hours. The cockroaches were prepared for centrifugation, the hemolymph was collected, and 25 µl injected into normal nymphs which were placed in Petri dishes until stress symptoms developed. This was determined to be the lowest dosage that would give the most consistent results (Table 2). Eight hours after the injections, the nymphs became sluggish and responded slowly or not at all to various stimuli. After 10 hours they were very sluggish, with only feeble movements when probed. They were completely paralyzed at 12 hours. Although the nymphs did not appear to be bloated, X-ray indicated nine with slight bloat and all 28 nymphs were dead within 15 hours (Table 3). Fourteen control nymphs injected with hemolymph taken from normal cockroaches appeared normal at 12 hours and remained so through 48 hours. X-rays showed none of the controls to have any signs of bloat.

Table 2. The toxicity of variable dosages of hemolymph from cockroaches paralyzed by cold injury to normal nymphs, Periplaneta americana.

Hemolymph µl	Treated	24 hours after treatment		
		Normal	Paralyzed	Dead
5[1]	20	20	0	0
10[1]	20	19	1	1
15[1]	20	16	4	4
20[1]	20	7	13	13
25[1]	20	0	20	20
30[1]	20	0	20	20

[1] Four controls were used for each dosage; no mortality was observed for 120 hours.

Table 3. Toxicity of 25 µl dosages of hemolymph from cockroaches paralyzed by cold injury to normal nymphs and adult, Periplaneta americana.

Insects	Controls	Treated	Hours until		Foregut bloat
			Paralysis	Death	
Nymphs, third and fourth instar	14[1]	28	12	15	9 slight
Female, adults	12[2]	31	13	16	4 medium
Males, adults[1]	2	8	14	15	0

[1] Controls remained normal for 48 hour observation period.

[2] Two controls were dead at 24 hours and 2 had medium bloat.

To determine if adult cockroaches were affected by the blood toxin, 31 adult females were injected with hemolymph from cold injured adult females. Eight hours after the injections all became sluggish. At 10 hours they were so sluggish that they would make no escape movements when probed. By 13 hours the cockroaches were completely paralyzed in a normal upright position. X-rays showed signs of medium bloat in four individuals, and all the females were dead at 16 hours after the injection. Twelve adult females were injected and used as controls. Two of these were dead 24 hours after treatment, the others remained normal until 48 hours. The two controls that died had medium bloat; the others showed no signs of bloat.

One group of eight adult males was injected with hemolymph from cold treated adult males to determine if both sexes would react to the blood toxin in the same manner. The males started becoming noticeably sluggish at nine hours, were very sluggish at 11 hours, and became paralyzed at 14 hours. These cockroaches became paralyzed in an upright position the same as the nymphs and females, but they died shortly after paralysis with no signs of bloat. The symptoms in the males appeared to be the same as those in the preceding experiments using females with a gradual decline in activity and reaction to stimuli. The paralysis was flaccid and no movement was apparent. However, when a paralyzed cockroach was dissected, parastalic movements of the foregut were present. The two male controls were still normal at 48 hours with no accumulation of gas in the foregut. One of the controls had accumulated a small amount of gas in the foregut by 16 hours after treatment but lost it by 22 hours.

In all cases the paralyzed cockroaches appeared to be in a normal resting position. None of the insects evidenced any signs of hyperactivity or nervous tremors, nor did they release any abnormal fecal material. Those that were

injected with hemolymph extracted from cold injured cockroaches died and turned black in 12 to 18 hours after treatment, were very putrid smelling and would break apart very easily when handled.

Once it had been established that the hemolymph of cold injured cockroaches would cause paralysis and death in a normal cockroach when injected in sufficient quantities, the question arose whether or not the toxic effect would be propagated when injected into a series of normal cockroaches. Normal adult females were paralyzed by cold exposure and their hemolymph was extracted and injected into normal females. After paralysis the hemolymph from these insects was extracted and injected into a third group of adult females. This was continued for two additional groups. Each series of experiments was replicated three times, twice using female cockroaches and once using male cockroaches. Both sexes gave very similar results (Tables 4 and 5).

The symptoms of the cold injured cockroaches were generally the same as those described earlier. The 10 normal adult females that received the hemolymph from the cold injured cockroaches became sluggish 10 hours after injection and were completely paralyzed by 19 hours. No bloat was apparent by either visual or X-ray examination. Death occurred at about 24 hours. The above cockroaches were centrifuged one hour after becoming paralyzed and the hemolymph was injected into 10 normal adult females. They became paralyzed in 15 hours and died in 20 hours as indicated by the one cockroach not centrifuged. One of the experimentals appeared to be slightly bloated on the radiograph. Nine of the paralyzed cockroaches were centrifuged and the hemolymph was collected and injected into eight adult females. These were paralyzed in 14 hours and dead in 20 hours. No bloat occurred in any of these cockroaches before being centrifuged. Their hemolymph was collected

Table 4. Propagation of hemolymph toxins between groups of adult female Periplaneta americana.

Group number	Sex	Controls	Treated	Hours until		Foregut bloat	Remarks
				Paralysis	Death		
I	Female	4[1/]	16	After cold injury	53	4 slight 9 medium 3 heavy	Gp. I was cold injured.
II	Female	4[1/]	10	19	24	0	Gp. II was injected with 25 µl of hemolymph from Gp. I, 24 hours after cold injury.
III	Female	4[1/]	10	15	20	1 slight	Gp. III was injected with 25 µl of hemolymph from Gp. II after paralysis.
IV	Female	3[1/]	8	14	20	0	Gp. IV was injected with 25 µl of hemolymph from Gp. III after paralysis.
V	Female	3[1/]	8	14	19	0	Gp. V was injected with 25 µl of hemolymph from Gp. IV after paralysis.

[1/] Controls remained normal for 96 hour observation period.

Table 5. Propagation of hemolymph toxins between groups of adult male Periplaneta americana.

Group number	Sex	Controls	Treated	Hours until		Foregut bloat	Remarks
				Paralysis	Death		
I	Male	4 1/	8	After cold injury	47	4 slight 4 medium	Gp. I was cold injured.
II	Male	4 1/	8	18	20	0	Gp. II was injected with 25 µl of hemolymph from Gp. I, 24 hours after cold injury.
III	Male	4 1/	7	17	20	0	Gp. III was injected with 25 µl of hemolymph from Gp. II after paralysis.
IV	Male	4 1/	7	15	20	0	Gp. IV was injected with 25 µl of hemolymph from Gp.III after paralysis.
V	Male	3 1/	6	16	19	0	Gp. V was injected with 25 µl of hemolymph from Gp. IV after paralysis.

1/ Controls remained normal for 96 hour observation period.

one hour after paralysis and injected into eight normal adult females. All were paralyzed by 14 hours after injection and died by 19 hours. Once again no bloat was observed nor did any radiographs taken before or after centrifugation demonstrate any bloating of the foregut. A series of control cockroaches was run with the experimentals. They were treated exactly as the experimentals except that the group I control cockroaches were not cold injured. Those that were injected with the hemolymph from the cold injured, paralyzed cockroaches gradually became sluggish and entered a state of complete flaccid paralysis. The paralyzed cockroaches became wet externally, died shortly after paralysis, and rapidly became putrid.

Another experiment was performed using adult males. The procedure for this experiment was the same as that used for the females. Eight normal adult males were cold injured and their hemolymph extracted after 24 hours. This hemolymph was injected into eight normal adult males. These cockroaches were paralyzed within 18 hours, at which time their hemolymph was removed and injected into seven more male cockroaches. Within 17 hours all were in a state of flaccid paralysis. Each time one of the paralyzed cockroaches did not have its hemolymph extracted, it was used as an indicator for when death occurred in those paralyzed. Within 15 hours all seven were paralyzed and death occurred about 20 hours after injection. Extracted hemolymph was injected into six additional male cockroaches that were observed closely. They became paralyzed in 16 hours and died at 19 hours. The experimental males reacted the same as the females with a gradual increase in sluggishness and a constant advance into a state of flaccid paralysis. The other external symptoms previously mentioned for females also developed in the males. Control males also were used and followed the same procedures as the experimentals. Both the female and male controls were observed for 96 hours. None

of the controls showed any signs of bloat, sluggishness, paralysis, nervous tremor, or hyperactivity.

Only in one case was bloat observed in a cockroach injected with toxic hemolymph. This was a case of slight bloat observed in a group III female. In this case, as with the cold injured cockroaches, the individuals that were injected with dark colored hemolymph were the ones that became paralyzed and died more rapidly. It was observed that the foregut became full of a brownish-green watery material after a cockroach was cold injured. To determine if the presence of this material in the hemolymph after centrifugation would cause paralysis and death, 10 cockroaches were cold injured. The contents of the foregut were removed from five and from the rectum of the remaining five. The foregut contents were injected in 25 µl dosages into the abdominal cavity of four normal cockroaches. The rectal contents were injected in the same manner into four more cockroaches. In neither case did any of the cockroaches become bloated, paralyzed, die, or exhibit any other symptoms of the blood toxin. Four control cockroaches were injected with foregut and rectal contents of normal cockroaches. They also showed no abnormal reactions during the 96 hour observation period.

Effects of Cold Injury on the Appearance of Toxic Factors in the Hemolymph with Time

Fifty adult females were removed from the culture and placed in eight inch Petri dishes. One hour after cold injury three of the treated cockroaches were removed and prepared for extraction of their hemolymph by centrifugation. Twenty-five µl of the collected hemolymph was injected into six normal adult females, which were held for observation. Each time that cold injured cockroaches were centrifuged, two controls also were centrifuged and their hemolymph

injected into a normal adult female control cockroach. Hemolymph was collected from similarly treated cold injured cockroaches the following hour and every subsequent two hours for 24 hours (Table 6). The experiment was replicated three times.

The injected cockroaches appeared be normal for several hours after the treatment. The first definite signs of sluggish movement appeared 14 hours after the injections were made. The hemolymph extracted from cockroaches one hour after cold injury did not cause paralysis or death within the 96 hour observation period. Hemolymph extracted from cockroaches two hours after injury caused one cockroach to become paralyzed in 18 hours and die within 27 hours after injection; the other five treated cockroaches remained normal. Injections of hemolymph extracted four, six, and eight hours after cold treatment caused no paralysis or death in any of the 18 treated cockroaches. Hemolymph extracted at 10 hours caused paralysis in two specimens by 20 hours after treatment and death by 24 hours. The four remaining cockroaches treated at 10 hours showed no abnormal symptoms. The hemolymph collected at 12 hours also demonstrated no effect when injected into the normal cockroaches. The only effects observed in those treated at 14 hours were that two became slightly bloated and paralyzed by 16 hours after the injection and died about 24 hours after the treatment. The remaining four appeared normal for the duration of the experiment. The extracted hemolymph started becoming toxic in a high percentage of the treated 16 hours after cold treatment. Hemolymph collected at 16 hours paralyzed six cockroaches within 20 hours and caused their death by 26 hours after injection. All of the cockroaches treated with hemolymph collected from 18 hours to 24 hours after cold injury became paralyzed and later died. The time interval between injection and paralysis and between paralysis and death became shorter as the time interval between cold injury and

Table 6. Effects of cold injury of adult female Periplaneta americana on the appearance of toxic factors in the hemolymph with time.

Hours after cold injury	Cold injured	Numbers of insects receiving hemolymph injections						Remarks
		Treated	Foregut bloat	Paralyzed	Died	Hours until		
						Paralyzed	Death	
1	9	6	0	0	0	0	0	
2	9	6	0	1	1	18	27	Eight treated remained normal.
4	9	6	0	0	0	0	0	
6	9	6	0	0	0	0	0	
8	9	6	1 medium	0	0	0	0	
10	9	6	0	2	2	20	24	Four treated remained normal.
12	9	6	0	0	0	0	0	
14	9	6	2 slight	2	2	16	24	Four treated remained normal.
16	9	6	0	6	6	16	26	
18	9	6	0	6	6	20	24	
20	9	6	0	6	6	24	28	
22	9	6	2 slight	6	6	14	20	
24	9	6	1 slight	6	6	12	18	
Controls	78	36	3 temporary	0	0	0	0	

treatment increased from 18 to 24 hours. Those cockroaches treated 18 hours after cold injury became paralyzed by 20 hours after treatment and were dead by 24 hours; those treated at 20 hours became paralyzed at 24 hours and died by 28 hours; those treated at 22 hours became paralyzed by 14 hours and died by 20 hours after injection, two of which showed signs of slight bloat; and those treated with hemolymph extracted 24 hours after cold injury became paralyzed in 12 hours and died within 18 hours. One of the latter was slightly bloated. The controls in each of the above cases were injected with hemolymph extracted from normal cockroaches that were placed in Petri dishes at room temperature when the experimental cockroaches were cold injured. All of the controls remained normal for 96 hours except for some temporary bloat in three of the specimens injected at 18 hours. Medium bloat appeared two hours after treatment but it disappeared within the next hour.

None of the those injected with the hemolymph from cold injured cockroaches showed any signs of hyperactivity or nervous tremors. They gradually became sluggish until they entered a state of complete paralysis. The symptoms were generally the same as those described in the previous section for cold injury. In each case the cockroaches injected with hemolymph that was a brownish-yellow color were the ones that became paralyzed and later died. The darker the hemolymph, the quicker paralysis and death occurred. Death occurred very shortly after complete paralysis, and the cockroaches became putrid and deteriorated rapidly, probably the result of proteolytic activity and necrosis of the cells. This is quite different from the chain of events following death due to cold injury where the cockroaches may remain in a paralyzed state 48 to 72 hours prior to death and then become dry leaving a hard exoskeleton.

Topical Treatment with DDT

Topical treatment with DDT was performed as described by Shankland and Kearns (1959). The DDT treated cockroaches became hyperactive but not prostrate by 24 hours, but exposure to 15° C caused them to become prostrate within 35 minutes. However, it was discovered that if they were centrifuged at this time, the extracted hemolymph caused no abnormal symptoms when injected into normal cockroaches even if the dosage was as high as 75 µl per cockroach. Three hours exposure to 15° C caused prostration, paralysis, and death when the hemolymph was injected into normal cockroaches in 25 µl dosages. Three hours of exposure to the 15° C temperature appeared to have no different external effects on these DDT treated cockroaches than a 30 minute exposure. In both instances the cockroaches became more hyperactive for the first 10 minutes. As the hyperactivity increased, they often fell on their backs and gradually became incapable of righting themselves. Within 30 minutes all were prostrate and had continual nervous tremors and muscular spasms of their extremities.

The extracted hemolymph was injected first in 25 µl doses; this resulted in paralysis in all cases by 18 hours and death by 27 hours (Table 7). It also was observed that the hemolymph extracted from the DDT treated cockroaches initiated a considerable amount of bloat. Of the 15 cockroaches treated, 13 became bloated: four slight, one medium, six heavy, and two extreme. The controls remained normal and showed no signs of bloat. Those that were injected with the hemolymph followed the same syndrome of symptoms as those that had been injected with the hemolymph extracted from cold injured cockroaches in earlier experiments.

Table 7. Toxic effects of hemolymph from DDT prostrate cockroaches when injected into normal Periplaneta americana.

Treatment	Sex	Treated	Foregut bloat	Normal	Paralyzed and died	Hours until	
						Paralyzed	Death
Injected with 25 µl of hemolymph extracted after topical treatment	Male	15	13	0	15	18	27
Injected with 25-75 µl of saline extract of homogenized nerve cords after topical treatment	Male	10[1/]	0	10	0	--	--
Injected with 25-50 µl of acetone extract of homogenized nerve cords after topical treatment	Male	10	1	10	0	--	--
Controls	Male	13	0	13	0	--	--

[1/] One treated died of unknown causes.

A second group of cockroaches was injected with a saline extract from the homogenized nerve cords of the above centrifuged cockroaches. Ten adults were treated with injections varying from 25 µl to 75 µl per individual. None of the cockroaches demonstrated any signs of paralysis irrespective of the amount of injection. However, one of them died at 30 hours from unknown causes. The dead cockroach was neither bloated nor did it exhibit the previously observed symptoms that accompany paralysis and death due to toxic hemolymph. The nine other experimentals as well as the controls appeared normal for the 96 hours that they were observed. Two of the controls had slight bloat at 53 hours after treatment, but this was not regarded as abnormal. A third group of cockroaches was treated with dosages of an acetone extract from the homogenized nerve cords. Ten were used as experimentals and three were used as controls. Injections, ranging from 25 µl to 50 µl, caused none of the cockroaches to show any symptoms of paralysis or prostration. One of the experimentals that had been injected with 30 µl demonstrated medium bloat at 24 hours, but no impairment was observed. None of the controls became bloated or showed any other signs of abnormality for the 96 hour observation period.

The major difference between the symptoms produced by the hemolymph extracted from topically treated cockroaches and the hemolymph from cold injured cockroaches was that significantly less bloat was produced by hemolymph taken from the cold injured cockroaches. Bloat in cockroaches injected with hemolymph extracted from DDT treated cockroaches may have been caused by small amounts of DDT remaining in the hemolymph. Otherwise, the stress symptoms produced by the hemolymph in no way reflected the type of stressor the donor cockroach had received.

The 15 experimental cockroaches, those given the injections of hemolymph extracted from the DDT treated cockroaches, were centrifuged for extraction of hemolymph. The hemolymph was given in 25 μl doses to normal adult females to determine if the same symptoms could be produced as those that resulted when the hemolymph from cold injured cockroaches was injected into normal adults. The 15 hemolymph injected cockroaches became paralyzed and prostrate within 13 hours. The symptoms produced were the same as those that result when normal cockroaches were treated with hemolymph from cold injured cockroaches: a gradual onset of sluggishness, lack of response to stimuli, flaccid paralysis, and death within 20 hours after treatment. These cockroaches also did not show any signs of bloat when X-rayed.

DDT Treatment of Dissected Nerve Cords

The ventral nerve cords were removed and the cerci treated with DDT on thirty individuals. They were covered with saline and then exposed to 5° C for 10 hours. The nerve cords did not appear to be harmed and retained their normal color and appearance. Approximately 25 μl of saline, which becomes turbid, was withdrawn from around each of the treated nerve cords. Twenty normal adult male cockroaches became noticeably sluggish eight hours after their injections. The degree of sluggishness gradually increased until a state of flaccid paralysis existed 14 hours after the injections. The paralyzed cockroaches showed the same symptoms as those that had previously been injected with hemolymph from cold injured cockroaches. None of the 20 treated cockroaches at any time showed signs of hyperactivity, muscular contractions, or nervous tremors. X-rays taken two hours after paralysis also demonstrated no bloat in any of the cockroaches. The control consisted of five nerve cords which had the cerci treated with acetone. The saline was

collected from around these nerve cords and injected into five normal adult males. Observations were made for 96 hours before being X-rayed for bloat. One had a small bubble of gas in the foregut but not enough to constitute a condition of bloat. None of the controls had any signs of paralysis or other symptoms of toxic factors in the hemolymph.

The above treated and the controls were centrifuged 16 hours after treatment and the collected hemolymph injected into 20 normal cockroaches. Sixteen of these were treated with hemolymph from the centrifuged prostrate cockroaches and four were used as controls. The hemolymph collected from the paralyzed cockroaches was a dark brownish-yellow, a color which seems to be characteristic of hemolymph that is toxic when injected in sufficient quantities into normal cockroaches. Once again this hemolymph proved to be toxic, causing gradually increasing sluggishness until a state of flaccid paralysis existed and resulting in death to the individual. The 16 injected cockroaches were given 15 µl dose which caused paralysis in 18 hours and death in approximately 22 hours. The four controls had no signs of bloat, hyperactivity, muscular contractions, or nervous tremors. At 19 hours the paralyzed and control cockroaches were centrifuged and the hemolymph collected and injected in 15 µl doses into 12 additional normal adult males. This time they also became paralyzed by 16 hours and died at approximately 20 hours after the injections. Symptoms were the same as those observed in the preceding group. The three controls also did not show any abnormal symptoms (Table 8).

In each of the experiments a gradual onset of paralysis, prostration, and death occurred in approximately the same duration of time. In no case was bloat or hyperactivity observed in any of the injected cockroaches. The cockroaches did not have any abnormal movements of the mouth parts or defecation of abnormal fecal material.

Table 8. DDT treatment of the cerci of dissected nerve cords of adult males and the effects of injections of the bathing saline on normal adult Periplaneta americana.

Group number	Treatment	Treated	Paralysis and death	Hours until	
				Paralysis	Death
1	Injected with 25 µl of saline after 10 hours	20	20	14	18-22
	Controls	5	0	--	-
2	Injected with 15 µl of hemolymph from group one paralyzed cockroaches after 16 hours	16	16	18	20-24
	Controls	4	0	--	-
3	Injected with 15 µl of hemolymph from group two paralyzed cockroaches after 19 hours	12	12	16	18-22
	Controls	3	0	--	-

Immobilization

The immobilization experiments did not produce any stress symptoms even though several methods were used. A total of 40 third and fourth instar nymphs were immobilized in an effort to produce stressing symptoms and bloat when the cockroaches were immobilized for 96 hours. Nineteen cockroaches were secured to balsa wood rectangles using criss-crossed pins, but none showed any abnormal symptoms for 72 hours after being released. Eight were placed at 15° C for two hours immediately after removal from the balsa wood blocks to try to induce bloating and prostration but the cockroaches were only slowed down by the cold temperature. The cockroaches that were pinned or taped down struggled to free themselves more than any of the other immobilized cockroaches. Four of the pinned cockroaches struggled so violently that they completely dismembered themselves; two cockroaches lost three legs; one lost two legs; and three lost one leg. The remaining nine did not lose any legs while they were immobilized. Two cockroaches that were taped down lost two legs each. None of the remaining immobilized cockroaches lost any legs. The six cockroaches that were placed in the immobilization block did not struggle to free themselves. They appeared to be content and very seldom moved during the 96 hours of confinement.

The procedure of using modeling clay to restrain the cockroaches also proved to be a poor method. The first method in which strips of modeling clay were placed over the thorax and legs proved to be fatal to the insects, possibly because of the oils present in the clay. The second method of putting pins over the thorax and clay across the legs also caused the cockroaches to die.

Origin of Gas in the Foregut

One of the factors causing bloat of the foregut may be the swallowing of air due to over-activity of the mouth parts during stress or insecticidal poisoning. Other possibilities are that the gas enters through the trachea that supply the foregut plentifully, or that the permeability of the foregut is affected either by: (1) a biologically active compound, (2) the induced stress, or (3) both. This allows the gas to enter the foregut but not to leave it, thus resulting in a bloated condition.

Fifty adult females were cold injured, and 33 of the injured cockroaches showed substantial bloat after 30 hours. Sixteen of these were ligated externally around the cervix; and the other seventeen had an incision made in the ventral side of the cervix and the esophagus was ligated with a piece of thread (care was taken not to distrub any of the trachea or the ventral nerve cord). Sixteen controls were placed in Petri dishes and observed from the beginning of the experiment. None of the 16 externally ligated cockroaches showed a recollection of gas in the foregut once the gas producing the initial bloat was removed. One of the 17 cockroaches that had only the esophagus ligated had a small bubble form in the foregut two hours after the initial bloat was removed; however, this gas may have come from a bubble trapped in the anterior part of the foregut. No controls for the preceding experiments of this section were used since rapid rebloating of the foregut was known to occur in stressed (Hopkins and Ameel, unpublished data) and cold injured cockroaches.

A second group of ligation experiments very similar to the preceding were performed. In this group 14 adult females were ligated externally around the cervix and another 14 had only the esophagus ligated. Twenty

(ten of each group) were cold injured and observed for 48 hours. The injured cockroaches had no abnormal defecation, but they did become wet as has been stated earlier. None of the injured cockroaches showed any signs of bloat visually or by X-ray. When the hemolymph from the injured was injected into normal individuals in 25 µl doses, all cockroaches died. While ligating the esophagi small bubbles were observed passing through. The mouth parts were moving in a chewing manner but not rapidly. When the esophagus was ligated the bubbles would collect in front of the thread and cause the esophagus to bloat and protrude through the incision made in the cervix.

Another 35 adult female cockroaches were cold injured at -20° C for 12 minutes. Twenty-six of the cockroaches had some collection of gases in the foregut varying after 30 hours from two extreme bloats to four that were only slightly bloated. At this time their spiracles were plugged with paraffin and the gas in their foreguts was withdrawn with a syringe. Sixteen of the 26 bloated cockroaches became rebloated, at least as much as the initial bloat, within 30 minutes after the gas was removed. Of the remaining ten, three gained a small bubble and the other seven did not rebloat. Eleven of the cockroaches that became bloated a second time, bloated a third time to a state equal to the first bloat. It took 25 to 35 minutes for complete rebloat the third time. The three that collected a small bubble of gas and the five that bloated a second time did rebloat. Three of the 11 that rebloated three times collected a small bubble of gas in the foregut 67 minutes after the gas from the third bloat had been removed (Table 9). Eight controls were placed in Petri dishes for 30 hours before being partly anesthetized with CO_2 and having their spiracles plugged with paraffin. None of these controls bloated although one did collect a small bubble of gas in

Table 9. Origin of gas accumulation in the foregut of adult female Periplaneta americana due to cold injury.

Treatment	Treated	Cold injury bloat	Reaccumulation of gas in foregut		
			Second	Third	Fourth
Cervix ligated 30 hours after cold injury	16	16	0	0	0
Esophagus ligated 30 hours after cold injury	17	17	1[1/]	0	0
Cervix ligated before cold injury	14	0	0	0	0
Esophagus ligated before cold injury	14	0	0	0	0
Spiracles plugged 30 hours after cold injury	26	26	16	11	3[1/]
Trachea severed after cold injury	25	25	18	15	1
Trachea severed before cold injury	20	15	8	5	0

[1/] A small bubble was observed in each insect.

the foregut. Thus, it appears that air probably enters the foregut via some other way than the spiracles since plugging the spiracles did not affect the amount or rapidity with which rebloating occurred.

In the next group of experiments the spiracles were plugged before the cockroaches were cold injured. This method did not prove to be successful since in all cases the specimens managed to break the paraffin plugs loose while struggling during the cold injury treatment.

The next method used was that of dissecting the tracheal trunks to the foregut. The plugging of spiracles gave some idea of what was happening, but some of the gases may have entered around a loose paraffin plug or some other way. To eliminate this possibility the tracheal trunks were severed on 25 cockroaches after they had become bloated due to cold injury. Eighteen of these rebloated in 30 minutes and the other seven did not rebloat. When the gas was removed from the 18 bloated cockroaches, 15 of them rebloated in 40 minutes (only one of these collected any gas a fourth time and this was only enough to be about comparable to slight bloat). Each of the cockroaches that rebloated did so to the same approximate extent as the original bloat. The 10 control cockroaches had the tracheal trunks to their foregut cut 30 hours after they were put in Petri dishes. One of them had medium bloat 15 minutes after the trunks were severed; it did not die but the bloat remained for 96 hours.

In the second part of this experiment the tracheal trunks were cut on 20 adult females before cold injury. Thirty hours later 15 of the specimens had collected enough gas in their foreguts to be visibly bloated. Four had slight bloat, nine medium bloat and two had heavy bloat. The gases were removed from the foreguts of the 11 cockroaches having heavy and medium bloat. The two that had heavy bloat, rebloated to medium bloat twice each in

23 minutes. Of the nine that had medium bloat, six rebloated to medium bloat in 35 minutes. Three of these rebloated again to slight bloat in 37 minutes. None of them bloated a fourth time.

The last group of experiments conducted involved the severing of the tracheal trunks to the foregut and the ligation of the exposed esophagus. This experiment also was run two different ways. The first method involved exposure to cold injury of the adult females thereby causing bloat and then to cut the trachea and ligate the esophagus. The second method was to cut the trachea and ligate the esophagus before cold injuring the cockroaches. Fifteen were treated each way and three of the cockroaches that were cold injured prior to cutting the tracheal trunks and ligating the esophagus showed slight bloat when X-rayed 30 hours after removal from the refrigerator. The 15 specimens that were cold injured after the tracheal trunks were severed and esophagus ligated did not show any bloat. Reaccumulation of gas in the foregut will rapidly occur two or three times after the gas is removed if the esophagus is left unobstructed. Ligation of the cervix or the esophagus completely eliminates reaccumulation of gas in the foregut. Plugging of the spiracles or severing of the trachea apparently does not affect the rapidity or extent of rebloat.

DISCUSSION AND CONCLUSIONS

Abnormal accumulations of gas in the alimentary tract and bloating of the foregut can be caused by both physical and chemical stressors and provides more evidence of a "stress syndrome" in insects. The American cockroach exhibits unusual susceptibility to bloating when exposed to several types of stress. The symptoms caused by organic insecticide intoxication and cold

injury are both very similar. The cockroaches, especially the females when cold injured, become hyperactive and display nervous tremors, occasional muscular spasms, prostration, and bloat in many cases. Cold injury apparently causes irreversible damage to the central nervous system (possibly in the nerve membranes resulting in ion leakage). This study indicates that the toxin produced by cold injury is different than Sternburg's DDT toxin which is a neurotoxic agent. The toxin produced by cold injury exhibits characteristics that are possibly due to protelytic enzymes from lysosomes or from bacterial septicema.

The sex of the cockroaches affected their reaction to cold injury. The males required less exposure time and not as low a temperature to cause paralysis, but they did not display as high a percentage of bloat as the females. Age was not controlled carefully enough to make any statements about its effect.

As paralysis progressed, the frequency and regularity of the nervous tremors increased until death occurred. However, the tremors never became continuous. There seemed to be no correlation between the severity of the tremors and the amounts of bloat. The bloated condition of the foregut may develop and be lost, then develop again and remain until after death. Once a cockroach has bloated, it will usually remain bloated at death regardless of the number of times the bloat is lost during the paralytic stage. A few adults appear to recover from the cold injury but they periodically lapse into periods of nervous tremors. This may indicate that the nervous system suffered less damage during treatment. Prostration without the occurrence of foregut bloat may be due to the rapid progression of symptoms and the by-passing of the extended prostration phase or the actual losing of the gas shortly after its appearance.

The cold injured individuals appear to lose some control of their osmoregulatory system of the alimentary tract, especially that of the rectum. Cockroaches that were centrifuged 14 hours after cold injury contained very little hemolymph. Jochum (1956) found this same condition to exist in larvae of Bombyx mori and Dendrolimus pini that had been subjected to parathion poisoning. Beament (1958) also reports that after 10 to 15 days of paralysis, the hemolymph within the American cockroach is reduced and the hindgut contained abnormally large quantities of water. Cockroaches that had died from cold injury always had moisture under the wings possibly indicating that water is readily being lost through the exoskeleton or abdominal intersegmental membranes. Another example of the loss of physiological and nervous control is the abnormal accumulations of gas in the foregut.

The toxin produced by cold injury becomes more toxic if it is extracted between 14 and 24 hours after cold injury. Past this time the quantity of hemolymph in the cold injured cockroaches is so small that its collection is impractical. Those injected with hemolymph from cold injured cockroaches did not become hyperactive as had the cold injured cockroaches. Instead they gradually became sluggish until entering a state of complete paralysis. In all cases the paralyzed individuals appeared to be in normal resting positions. The cold injury toxin caused death and deterioration rapidly after complete paralysis. The deterioration probably is due to proteolytic activity and necrosis of the cells. Either of these hypotheses would agree with work reported by Krchma (1967) that the toxicity of the toxin produced by cold injury was destroyed by heat and was non-dialyzable. Colhoun (1960) reported prostration in P. americana treated with DDT, TEPP, Dieldrin, and KCN. Necrosis, darkening of the cuticle and autolysis of internal organs was observed when the prostrate cockroaches were joined by parabiosis to untreated cockroaches. The joined untreated cockroaches did not show any

symptoms for 48 hours after parabiosis. These symptoms were similar to those of the toxic hemolymph causing cockroaches to become dark, fragile and putrid. Patel and Cutkomp (1961) found that the proteolytic enzyme fraction I and III of a water extract of American foulbrood scale residue was very toxic and proved lethal when fed to house flies (Musca domestica L.), milkweed bugs (Oncopeltus fasciatus (Dallas)), hide beetles (Dermestes maculatus DeGeer), and cockroaches (Periplaneta americana (L.) and Blatella germanica (L.)). The affected insects were immobilized slowly, without excitation, and dissection of the moribund insects showed extensive lysis of tissue. However, as reported by Patel and Cutkomp (1961), this does not exclude the fact that the toxic effect may be due to proteolytic enzymes of nonbacterial origin, since crystalline beef trypsin caused the same effect and certain other proteinases were not toxic.

After cold injury, the hyperactivity of the treated cockroaches increases until about five or six hours which may indicate further deterioration of the nervous system membranes. The toxin may not be released for some time after the cold injury or it may be increasing in quantity as is indicated by a substantial number of cockroaches not being affected by hemolymph injections taken from cold injured cockroaches unless they were extracted at least 12 hours after cold injury. No toxic effects are produced by the hemolymph extracted from cold injured cockroaches until a dose of at least 15 µl is injected. Injections of 25 µl of hemolymph containing the toxin causes death and paralysis in approximately the same length of time in third and fourth instar nymphs, females, and males. However, in a few cases, the nymphs did become slightly bloated, but this condition is difficult to distinguish from normal foregut gas accumulation. Hemolymph toxicity can be

transferred by injections of toxic hemolymph between groups of cockroaches 12 hours after the injection. The same toxic hemolymph symptoms are produced each time the hemolymph is transferred from a paralyzed cockroach to a normal one. Hemolymph extracted from those that have been treated with hemolymph toxins, whether produced by cold injury or transferred injections, was found to be a dark yellowish brown color; this color may be caused by melanization as a result of the increasing tissue lysis and the release of diphenols and phenylase enzymes. The dark colored toxic hemolymph caused paralysis and death more rapidly than lighter colored toxic hemolymph. As the color of the hemolymph approached normality, the toxicity decreased. Toxic hemolymph caused almost 100% paralysis and death within a few hours after injection, but it rarely caused any abnormal collection of gas in the foregut.

The major difference in the symptoms produced by hemolymph extracted from DDT topically treated individuals and hemolymph from those cold injured was that significantly less bloat was produced by hemolymph taken from the latter. Ameel (1965) reported a high incidence of foregut bloat in this species when topically treated with DDT. The additional bloat caused by hemolymph extracted from the DDT treated cockroaches may have been a result of small amounts of DDT remaining in the hemolymph. Davey and Treherne (1963) reported the foregut system of this species is normally able to maintain a relatively constant volume in the crop that would most probably involve the action of stretch receptors. The cold injury or DDT poisoning probably causes damage to the nervous system resulting in the loss of response to the foregut stretch receptors, thus allowing foregut bloat. Foregut bloat is probably a secondary result of stress since it does not appear unless a prolonged stressor is used.

If the ventral nerve cord is dissected intact with the cercal nerves and cerci and the cerci are treated topically with DDT, the saline bathing the nerve cord preparation will gradually accumulate the toxic agent and become dark colored. If this toxic saline is injected in 25 µl doses into normal cockroaches, their response is the same as if they had been given an injection of hemolymph from cold injured cockroaches. Thus the source of the toxicant is apparently the central nervous system itself during periods of excessive nervous activity initiated by direct action of DDT (Sternburg, 1959) or by cold injury.

Immobilization experiments did not produce any observable hemolymph toxic symptoms or any paralysis or death when the extracted hemolymph of immobilized cockroaches was injected into normal ones. This may have been due to not immobilizing the cockroaches long enough or because they did not struggle to free themselves. Beament (1958) found that mechanically immobilized cockroaches were affected by a form of paralysis, and that a similar physiological state could be produced by mechanical stimulation.

The problem of the source of gas present in the crop has not been solved complete, but more light has been shown on the problem. Salkeld (1951), Wiesman and Kocher (1952), and Jochum (1956), suggested that it may have been swallowed during the prolonged prostration. The fact that all the ligated specimens had none or occasionally only slight bloat and the non-ligated cockroaches became bloated, suggests that swallowing is an important factor in bloating of the foregut.

Even though the foregut is well supplied with trachea, there is little evidence to indicate that this is the means by which the foregut becomes bloated. When the tracheal trunks were cut or plugged and the esophagus was not obstructed the cockroaches would become bloated when stressed or would

rebloat when the gas was removed from the foregut. The ligation experiments of the cervix and those of only the esophagus suggest that the brain is not needed for the release of the toxin into the hemolymph. Hemolymph extracted from cold injured cockroaches with the cervix ligated produced the same toxic symptoms as that from cold injured cockroaches. When ligations of the exposed esophagus were being made, bubbles of gas were noticed passing down the esophagus. There was little noticeable movement of the mouth parts but the passage of bubbles was constant. Once the esophagus was ligated, the gas would collect in front of the ligation and often cause the esophagus to protrude through the incision. This is further evidence indicating that the main source of the gas is from swallowing.

This study reports that symptoms of DDT poisoning and cold injury are initially very similar, except that DDT poisoning produces a much higher incidence of bloat. However, the secondary symptoms more closely parallel those caused by other types of mechanical stress. The secondary symptom of foregut bloat appears to be a result of gas bubbles entering through the esophagus.

SUMMARY

1. This study was concerned with the effects of cold injury and immobilization on the production of hemolymph toxins, foregut bloating, and comparison of these symptoms to those produced by DDT intoxication in Periplaneta americana. Other experiments were designed to determine the source of the gas causing foregut bloat in cockroaches that had received prolonged stress.

2. Bloating was detected through the use of X-ray techniques. Females were cold injured by exposing them to -20° C for 12 minutes. Acetone

solutions of DDT used were applied topically with a microdrop applicator. Physical stress was applied with various means of immobilization.

3. The toxicity of the hemolymph of cold injured cockroaches is greatly affected by various time intervals after cold injury and by the quantity of the injections given to normal cockroaches. Twelve to 14 hours after cold injury a 25 µl dose of hemolymph will cause rapid paralysis, death, and deterioration of the body cells. The rapid deterioration may be caused by proteolytic enzymes from lysosomes or from bacterial septicemia.

4. The primary symptoms of cold injury and DDT intoxication caused the cockroaches to become hyperactive and display nervous tremors, occasional muscular spasms, and prostration. Hemolymph extracted from those cold injured or from cockroaches treated with DDT and injected into normal ones caused the same response.

5. The cerci of dissected cockroach ventral nerve cords were treated topically with DDT and allowed to remain in the bathing saline for 10 hours after treatment. The bathing saline was tested for toxicity by injecting 25 µl doses into normal cockroaches. The injections caused a gradual increase in sluggishness until a state of flaccid paralysis existed and subsequent death.

6. Several different types of ligation experiments combined with severing the tracheal trunks to the foregut were used to determine the entry route of gas causing foregut bloat. Those stressed would bloat and often rebloat if the esophagus was not obstructed. Gas bubbles were observed passing through the esophagus and entering the foregut causing it to become bloated. If the esophagus were obstructed, the passage of bubbles would cease and the foregut would not bloat.

ACKNOWLEDGMENTS

I express thanks to Dr. T. L. Hopkins, my major professor, for his patience, suggestions, and assistance contributed toward the research and writing of this thesis.

I also thank Professor Donald A. Wilbur and Dr. Robert B. Mills for the use of the X-ray and photographic materials in their laboratories.

Appreciation is extended to Larry L. Murdock and Patrick A. Walters for their suggestions and help as well as to Kermit O. Bell for his assistance with the X-ray machine.

Special gratitude is extended to my wife, Donna, for her encouragement and assistance in typing.

Funds for this research were provided by a National Institutes of Health Grant number AI-04643, Stress Effects on Insect Alimentary System Physiology.

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HEMOLYMPH TOXICITY AND FOREGUT BLOATING IN *PERIPLANETA AMERICANA* (L.) CAUSED BY COLD INJURY AND DDT INTOXICATION

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B.S., Stephen F. Austin State College, 1965

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

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1970

The effects of cold injury and immobilization on the production of hemolymph toxins and foregut bloating in the American cockroach, Periplaneta americana (L.) were compared with the symptoms produced by DDT intoxication. The syndrome of stress symptoms caused by cold injury was also investigated.

The cockroaches were cold injured by placing them in a freezer for 12 minutes at -20° C. The symptoms produced were very similar to those caused by DDT intoxication, both of which caused hyperactivity, nervous tremors and foregut bloat. A major difference was that cold injury produced a significantly lower incidence of foregut bloat.

Cold injury produced hemolymph toxins which when injected into normal cockroaches caused flaccid paralysis and rapid death. Deterioration of the body followed, probably due to lysis of the cells by lysosomal enzymes or by bacterial septicemia. A 25 µl dose of hemolymph injected into normal cockroaches 12 to 14 hours after cold injury resulted in death by 18 hours after treatment. The treated cockroaches were always putrid and very fragile after death.

The toxicity of the cold injury hemolymph varied with time. Hemolymph from cold injured cockroaches produced no toxic symptoms when injected into normal cockroaches until 10 or 12 hours after cold injury. The maximum toxicity of a 25 µl dose was reached at 22-24 hours. Toxic hemolymph was brownish-yellow in color.

Cockroaches treated with topical applications of DDT showed the typical DDT symptoms of prostration, foregut bloat, paralysis, and death. Twenty-four hours after DDT treatment the symptoms were accelerated if the cockroaches were exposed to 15° C for three hours. Hemolymph extracted from these prostrate cockroaches caused normal cockroaches to gradually enter a state of flaccid

paralysis. The symptoms were the same as those produced by injections of hemolymph from cold injured cockroaches, except that more bloat was produced.

Observations were made on symptoms associated with topical application of DDT to the cerci of dissected ventral nerve cords bathed in saline for 10 hours. The saline bathing the dissected nerve cords gradually accumulated a toxic agent that produced the same symptoms as cold injury toxic hemolymph when injected into normal American cockroaches.

Experiments were performed using several different methods of immobilization, including criss-crossed pins, immobilization blocks, tape strips, and clay strips. In all cases none of the cockroaches showed any symptoms of stress. Often they did not struggle to free themselves and those that did usually broke off one or more legs.

Ligation experiments and severing of the tracheal trunks supplying the foregut were used to elucidate the source of the gas causing foregut bloat. The presence of gas was detected by X-ray techniques and a scale of none, slight, medium, heavy, and extreme was established on the basis of X-ray density and gas volume. Bloated cockroaches were observed to rebloat if the gas was removed from the foregut with a syringe as long as the esophagus was not obstructed. Bubbles of gas were observed passing through the esophagus prior to ligation indicating that the gas causing foregut bloat is swallowed.