THE EFFECTS OF DIPHTHERIA TOXIN ON THE CECROPIA SILKWORM*

By A. M. PAPPENHEIMER, JR., AND CARROLL M. WILLIAMS

(From the Department of Microbiology, New York University College of Medicine, New York, and the Biological Laboratories, Harvard University, Cambridge)

(Received for publication, October 17, 1951)

Studies on the metabolism of *Corynebacterium diphtheriae* have furnished evidence that diphtheria toxin is closely related to the protein moiety of diphtherial cytochrome *b* (8). These observations, in turn, have suggested the possibility that diphtheria toxin may exert its injurious action on the tissues of susceptible animals by interfering in some way with the normal functioning of the cytochrome system—perhaps by blocking the synthesis of cytochrome *b* or related components (7).

Efforts to test this hypothesis have unfortunately proved inconclusive. Though changes in succinoxidase activity have been observed after injecting the toxin into pigeons, guinea pigs, and rabbits, the deviations, for the most part, have been relatively small and unconvincing. In the present investigation we have approached the matter from a new direction by a study of the effects of diphtheria toxin on the *Cecropia* silkworm—an insect which, for reasons which we may now consider, seems particularly suited for an analysis of this problem.

Six weeks after hatching from the egg, the mature *Cecropia* caterpillar spins a silken cocoon within which it transforms into a pupa. Morphogenesis then comes to a standstill and the insect persists for 6 to 12 months in a state of dormancy, the pupal diapause. Previous studies (14–20) have demonstrated an endocrine basis for the genesis and termination of diapause. Its onset apparently results from a prolonged delay on the part of the brain in secreting a hormone required for the initiation of adult development. Surgical removal of the pupal brain therefore stabilizes the animal in a state of permanent diapause. But under normal circumstances the pupal brain, activated by the low temperatures of winter, regains its endocrine powers and, by secreting its hormone, functions to terminate diapause the following spring. Adult development then proceeds promptly so that, at a temperature of 25°C., the adult moth emerges 21 days later.

* This study was supported, in part, by grants from the Commonwealth Fund, the American Cancer Society, Inc., and the United States Public Health Service.

For our present purposes these events are of considerable interest because the transitions from larva to pupa to adult are characterized by pronounced alterations in the insect's cytochrome system. Thus the succinoxidase activity of the caterpillar largely disappears at the time of pupation, except for a high concentration which persists in the intersegmental muscles of the pupal abdomen (11). Other pupal tissues continue to respire, apparently by the use of a flavoprotein as terminal oxidase. With the termination of the pupal diapause, however, the initiation and progress of adult development are accompanied throughout the insect by a rapid and progressive synthesis of the cytochrome system (20).

Consequently, through the use of the *Cecropia* silkworm as an experimental animal, it becomes possible to test the action of diphtheria toxin on an organism whose cytochrome system is undergoing large and predictable alterations. If the toxin interferes with the function of the cytochrome system, then a correlation should become evident between its effects on the diapausing pupa, in which the system is essentially absent, and on the caterpillar and developing adult, in which the system is intact and functional.

To this end we have studied the toxicity of purified diphtheria toxin for the *Cecropia* silkworm during successive stages in its life history. For the sake of comparison, analogous studies have been performed using purified tetanus toxin—an agent whose action has no apparent relation to the cytochrome system.

Materials and Methods

1. The Cecropia Silkworm.—Silkworms were reared in large numbers from eggs obtained from fertile females. The larvae used in the toxicity studies were mature fifth instar individuals weighing 12 to 15 gm. In experiments performed on pupae, either unchilled or previously chilled insects were utilized. By the removal of their brains, a considerable number of pupae were stabilized in permanent diapause and stored at 25°C. "Chilled" pupae were stored for 2 to 8 months at 5°C. prior to being used—a treatment which assures the abrupt initiation of adult development a few days after being returned to 25°C. Each pupa was ordinarily provided with a transparent plastic window at the tip of its abdomen; it was therefore possible to look inside the living pupa under the dissecting microscope and directly observe the beating of the heart and the day-to-day progress in adult development.

2. Diphtheria Toxin.—The stock solution used in the present experiments contained 2500 Lf per ml. and 40 to 50 guinea pig M.L.D. per Lf. 1 Lf of toxin is defined as the amount of toxin flocculated by 1 Lf of reference standard antitoxin in the flocculation test. Eighty-five per cent of the total nitrogen was specifically precipitable by antitoxin. Dilutions were made in 0.1 M phosphate buffer (pH 7.3) containing 0.002 per cent gelatin. The diluted toxin was injected in a volume of 0.1 ml. into animals anesthetized with carbon dioxide. Larvae were injected by passing a No. 27 gauge hypodermic needle into the thorax, care being taken to avoid puncturing the midgut. Pupae were injected through the lateral side of the second abdominal tergite,

the puncture wound being sealed with melted paraffin. After injection, the animals were stored at a temperature of 25°C. and a relative humidity of 60 per cent; one series of animals was stored at a relative humidity of 100 per cent.

3. Tetanus Toxin.—5900 ml. of crude culture filtrate of *Clostridium tetani* grown in Mueller and Miller's medium (6) was used as starting material.[1] The crude toxin contained 20 Lf per ml., equivalent to approximately 75,000 guinea pig M.L.D. The toxin was precipitated by the addition of 2.5 kg. of ammonium sulfate. After standing 2 days in the cold, the precipitate was collected by filtration through Whatman No. 50 paper and resuspended in 100 ml. of water. The turbid solution was dialyzed overnight in the cold against 20 per cent saturated ammonium sulfate at pH 7.0. After centrifugation, the precipitate was discarded and the supernatant dialyzed against two changes of 60 per cent saturated ammonium sulfate in the cold. The precipitate containing the toxin was collected by centrifugation and stored as a suspension in 60 per cent ammonium sulfate. Each milliliter of the final suspension contained about 2000 Lf (16,000,000 mouse M.L.D.). Since 50 Lf were present per milligram of protein, the toxin was about 10 per cent pure (10).

This partially purified preparation unquestionably contained other active products of the tetanus bacillus. Several months after its preparation, the material was tested for tetanolysin content.[2] 1.6 units of tetanolysin were demonstrated per Lf of tetanus toxin. Following reduction with cysteine the activity increased to 14 units per Lf.

4. Enzyme Studies.—(*a*) *Preparation of heart or intersegmental muscle homogenates.* Chilled pupae were opened longitudinally, immersed in insect Ringer's solution, and dissected. The heart or intersegmental muscles were carefully freed from fat body, washed in insect Ringer's, and homogenized with a small amount of 0.1 M phosphate buffer at pH 7.3, in a glass homogenizer. The 12 to 16 pupae used in each experiment provided 1.5 to 2.0 ml. of heart homogenate and 2.5 to 3.0 ml. of abdominal muscle homogenate. In four experiments each heart contained 0.16 to 0.24 mg. nitrogen; the intersegmental muscles of each pupa contained 0.4 to 0.7 mg. nitrogen.

(*b*) *Succinoxidase activity* was assayed at 30°C. using small Warburg vessels containing 0.1 ml. of 20 per cent KOH in the center well. 0.3 ml. of homogenate was placed in the side arm and, in the vessel itself, 0.5 ml. of 0.16 M sodium succinate, 0.5 ml. of 0.4 M phosphate buffer (pH 7.3), 0.1 ml. of 1.7×10^{-4} M cytochrome *c*, and water to a total volume of 2 ml. The endogenous metabolism was negligible in a control series tested without substrate.

(*c*) *Cytochrome oxidase* activity was measured in 0.1 M phosphate buffer at pH 7.3, using 0.3 ml. of homogenate and 0.016 M phenylenediamine as substrate. 0.4 ml. of 1.2×10^{-4} M cytochrome *c* was added to each vessel and the volume brought to 2 ml.

(*d*) *Succindehydrogenase* activity was measured at pH 7.8. To each vessel was added 0.3 ml. of homogenate, 0.5 ml. of 0.16 M sodium succinate, 0.1 ml. of M/20 KCN, and 0.2 ml. of 0.01 M methylene blue. The total volume was adjusted to 2 ml.

(*e*) *Oxidation of reduced DPN.* Reduced DPN oxidation has been shown by Slater

[1] We are indebted to Dr. J. Stone, of the Massachusetts Antitoxin and Vaccine Laboratory, for supplying this material.

[2] We are indebted to Dr. A. W. Bernheimer, of New York University College of Medicine, for performing these tests.

(18), using a Keilin-Hartree preparation from beef heart, to by-pass cytochrome *b* and to couple to cytochrome *c* through diaphorase and an unknown factor. This factor apparently controls the rate of oxidation of reduced DPN, provided that an excess of diaphorase is present.

Reduced DPN oxidation was measured at 25°C. in the Beckman spectrophotometer. Each cuvette contained 1 ml. of 0.133 M phosphate buffer. To one cuvette was added 100 gamma $DPNH_2$ in 0.05 ml. of 0.01 N NaOH. 0.05 ml. of 0.01 N NaOH was added to the control cuvette. 0.02 to 0.1 ml. homogenate was then added to each cuvette and the change in absorption at 340 mμ followed as a function of time. This rate was assumed to be proportional to the activity of the Slater factor.

RESULTS

1. Effects on Mature Larvae.—Mature silkworms were injected with graded doses of toxin a few days prior to the onset of spinning or a few days after the completion of the cocoon. As recorded in Table I, the minimal lethal dose varied between 0.025 and 0.25 Lf. The low dose here corresponds to 1 guinea pig M.L.D. or about 0.07 gamma of toxin. The two larvae which died after receiving 0.025 Lf underwent considerable development but failed to accomplish the pupal moult. Caterpillars injected with larger doses (0.25 to 25 Lf) ceased feeding and showed toxic symptoms within 12 to 18 hours. All individuals receiving lethal doses of toxin underwent a gradual and progressive paralysis until, after a minimum of 4 days, they no longer responded to electric shocks delivered from a spark-coil.

Table I also indicates the protection conveyed by prior injection of diphtheria antitoxin. Larvae receiving 100 units of antitoxin just prior to the injection of the largest dose of toxin (25 Lf) spun cocoons and pupated normally.

2. Effects on "Permanent" Larval Abdomens.—If a ligature is applied between the thorax and abdomen of a larva that has just begun to spin, then the isolated abdomen is deprived of the hormonal stimulus for pupation (19). It continues to live for several months thereafter, but undergoes no further development. The state of these larval abdomens may be regarded as somewhat analogous to that of diapausing pupae in terms of the absence of morphogenic changes. However, as indicated in Table I, such "permanent" abdomens were found sensitive to diphtheria toxin although they appeared to be somewhat more resistant than intact caterpillars. Thus two preparations continued to respond to electric shock for nearly 3 weeks following the injection of 0.25 Lf.

3. Effects on Prepupae.—Two to 3 days after spinning the cocoon, the pupa begins to form within the old caterpillar skin. The onset of this prepupal stage is signalled by a retraction of the melanin granules underlying the transparent cornea of the larval ocelli. As recorded in Table I, animals injected at this time showed substantially the same sensitivity to diphtheria toxin as did mature larvae.

4. Effects on Diapausing Pupae.—Pupation is followed by a remarkable decrease in the sensitivity of the insect to diphtheria toxin. Diapausing animals tested 4 to 6 weeks after pupation survived for 3 weeks or longer after the injection of doses as high as 25 Lf—approximately 100 to 1,000 times the minimal lethal dose for the larva. Large doses of toxin caused no apparent effects on the pupae for several days. After this initial period, however, the customary movements of the abdomen in response to electric stimulation became progressively feebler and totally disappeared in most cases after 1 to 3 weeks. In all pupae, regardless of the dose of toxin, the heart continued to function in an entirely normal manner for long periods after all abdominal muscular activity had ceased. For example, among a group of 14 diapausing pupae injected with 5 Lf of toxin, only two individuals remained capable of feeble movements after 2 weeks. At this time, the pupal hearts of all the insects were beating normally. Dissection of the unresponsive pupae revealed that the intersegmental muscles of the abdomen had undergone complete degeneration. The two pupae still able to respond to stimuli showed thin and fragile muscular fasciculi; in histological section apparently normal fibers were found interspersed with others showing pycnotic nuclei, absence of striations, and hyalinization of the sarcoplasm.[3]

Aside from this action on the intersegmental muscles, the toxin caused relatively rapid desiccation of pupae maintained at the customary relative humidity of 60 per cent. This did not occur in a series of treated pupae maintained at a relative humidity of 100 per cent. Recalling that the loss of water vapor from insects occurs principally *via* the spiracles during the process of respiration, we suspect that the toxin caused a degeneration of the minute muscles whose contraction closes the spiracular sphincters. Any interference with this closing mechanism is known to accelerate the rate of water loss (13) and for this reason we believe the desiccation caused by the toxin to be a by-product of its action on muscle.

High doses of toxin were found to affect dormant pupae in two additional respects. Thus it was commonly observed that the epithelium (hypodermis) gradually lost its normal intimate attachment to the overlying pupal cuticle and underwent a process of retraction throughout the insect, accompanied, in certain instances, by degeneratory changes in the region of the legs and antennae. In addition to this effect, the toxin also blocked wound healing at sites where transparent plastic windows had been placed. The customary development of connective tissue and outgrowth of hypodermis failed to occur and the surgical defect in the integument remained unrepaired.

5. Recovery of the Toxin from Diapausing Pupae.—The great decrease in sensitivity following pupation suggested the possibility that the pupa might

[3] We are indebted to Dr. Sigmund Wilens, of New York University College of Medicine, for the preparation and examination of these sections.

TABLE I

Effects of Graded Doses of Diphtheria Toxin on Larvae, Prepupae, Pupae, and Developing Adults of the Cecropia Silkworm

Dose (Lf)	No. of animals	Result	Remarks
Mature larvae (1 to 2 days before spinning)			
25	9	D3 to 6	
2.5	2	D7, D7	
0.25	2	D10, D10	Spun outer capsule of cocoons within 24 hrs.; no inner capsule
0.025	2	D14, D20	Spun complete cocoons; died in process of pupal molt
0.0125	2	Survived	Normal pupae
25 Lf + 100 units antitoxin	2	Survived	Normal pupae
Larvae (after spinning but before retraction of eye pigment)			
25	1	D7	
2.5	1	D11	
0.25	1	D16	
0.025	1	Survived	Normal pupa
25 Lf + 100 units antitoxin	1	Survived	Normal pupa
"Permanent" larval abdomens			
25	1	D7	
2.5	1	D8, D20	
0.25	2	D20, D20	
0.025	2	Survived	
Prepupae			
25	2	D5, D5	
2.5	2	D6, D6	
0.25	2	D6, D8	
0.025	2	Survived	Normal pupae
Brainless pupae			
25	13	D19 to 30	Flaccid after 8 to 19 days
10	21	D19 to 100	Flaccid after 8 to 60 days
2.5	3	D30, D38, D45	Flaccid after 18 days
1	5	D30, D34, D35, >29, >35	Flaccid after 18 to 32 days
0.25	3	D44, D44, D55	Flaccid after 30 to 35 days
0.1	2	Survived	Survived longer than 3 mos.
0.025	2	Survived	Survived longer than 3 mos.

TABLE I—*Concluded*

Dose (Lf)	No. of animals	Result	Remarks
		Chilled (potentially developing) pupae	
25	12	D18 to 44	No development; flaccid after 8 to 26 days
10	19	D14 to 41	No development; flaccid after 9 to 34 days
2.5	5	D30, D35, D50, >21, >35	No development; flaccid after 12 to 22 days
1	4	D30, D35, D40, D54, >30	No development; flaccid after 12 to 25 days
0.25	5	D18, D26, D39, D53, D57	No development; flaccid after 11 to 40 days
0.1	2	Survived	Normal adults
0.025	5	D67, >50	Three normal adults
		Pupae showing early development	
10	2	D6, D13	No further development
2.5	4	D12, D12, D30, D30	No further development
0.25	4	D8, D30, D30, >30	No further development
0.025	6	D30, D30, D35, D35	All show extensive development; two normal adults
		Pupae showing advanced development	
2.5	8	D5, D7, D9, D9, D9, D9, D9, >9	No further development
0.25	8	D9, D11, D11, D12, D12, D12, D12, D12	All show slight progress
0.025	9	Survived	Normal adults
230 Lf toxoid	2	Survived	Normal adults

D records the day of death; > signifies that the heart was found beating on the day the animal was sacrificed and dissected.

possess some mechanism for destroying the toxin. To test this possibility a pupa weighing 6 gm. was injected with 25 Lf (*ca.* 1,000 guinea pig M.L.D.). Ten days later the animal was sacrificed and 1 ml. of its blood was collected and diluted with 4 ml. of insect Ringer's solution. The latter contained 0.01 M KCN in order to inhibit the blood's tyrosinase and prevent its melanization. A 250 gm. guinea pig injected subcutaneously with 0.5 ml. of this solution diluted 1:5 died within 24 hours. A second guinea pig which received 0.5 ml. of a 1:50 dilution died of typical diphtherial intoxication on the 4th day. A control guinea pig receiving 0.5 ml. of a 1:5 dilution of toxin-free insect blood in cyanide-Ringer's solution showed no symptoms. It thus appears that

almost all the toxin was still present in the living insect 10 days after injection.

6. Effects on Previously Chilled Pupae.—As previously described, pupae stored at 5°C. for 2 months or longer initiate adult development a few days after being returned to 25°C. Such potentially developing animals, injected with diphtheria toxin, behaved as indicated in Table I.

As far as survival is concerned, the same low sensitivity to the toxin was encountered as in the case of brainless pupae. However, it will be observed in Table I that the normal onset of adult development was blocked by doses as low as 0.25 Lf. Though still lower doses permitted the onset and progress of adult development, the latter required for its completion approximately 4 to 5 weeks instead of the customary 3. These findings indicate that the biochemical processes underlying growth have a lower threshold to diphtheria toxin than do the processes responsible for maintaining life in the diapausing condition.

7. Effects on Pupae Showing Early Adult Development.—The selective effect of the toxin on the growth process is even more evident in this group of animals injected immediately after the onset of adult development. Though 2.5 Lf was not inconsistent with survival for 3 weeks or longer, progress in development was promptly and completely blocked by one-tenth this dose. The still lower dose of 0.025 Lf permitted considerable progress in all cases, but the rate of development was diminished by one-third to one-half.

8. Effects on Pupae Showing Advanced Adult Development.—This series of experiments, recorded in Table I, demonstrates that a high sensitivity to diphtheria toxin persists during the final week of adult development. No animal survived as long as 2 weeks following the injection of 0.25 Lf. As in the preceding series, the toxin blocked the process of development before killing the animal. It is noteworthy, however, that definite progress in development occurred after injection of 0.25 Lf, thus signalling a less prompt action of the toxin than in the case of animals showing early development.

9. Effects of Toxoid.—In contrast to the effects of the toxin, diphtheria toxoid[4] produced no demonstrable reaction even when as much as 230 Lf was injected into developing pupae (Table I).

10. Neutralization of Diphtheria Toxin with Antitoxin.—Mention was made in section 1 of the protection afforded by prior injection of antitoxin into the larval insect. In order to establish this important point more firmly, a toxin-antitoxin titration was performed as summarized in Table II. Increasing amounts of antitoxin were added to a constant amount of toxin and 0.1 ml. of the resulting mixtures injected immediately into pupae showing advanced adult development. In column 3 of Table II, the amount of "free" toxin in

[4] We are indebted to Dr. Leo Levine, of the Massachusetts Antitoxin and Vaccine Laboratory, for this sample of highly purified diphtheria toxoid.

each mixture has been calculated on the assumption that 1 unit of antitoxin neutralizes 1.5 Lf of toxin (9).

The results establish conclusively that the observed effects on the insect are caused by the toxin itself and not by some impurity present in the preparation. Free toxin in concentrations greater than the minimal lethal dose at this stage (0.25 Lf) was lethal, while lower concentrations permitted the completion of adult development.

11. Effects of Partially Purified Tetanus Toxin.—In order to provide further evidence for the specificity of the action of diphtheria toxin, a number of pupae were injected with purified tetanus toxin. As seen from Table III there

TABLE II

Neutralization of Diphtheria Toxin in Pupae in Late Development by Specific Antitoxin*

Antitoxin‡ injected	Toxin injected	Free toxin§	Results‖
units	*Lf*	*Lf*	
0.2	1.25	0.95	Development blocked: D5, D6
0.4	1.25	0.65	Development blocked: D7, D11
0.8	1.25	0.05	Normal adults emerged 5th and 11th days
1.25	1.25	None	Normal adults emerged 4th and 11th days
2.5	1.25	None	Normal adults emerged 6th and 8th days
0	1.25	1.25	Development blocked: D5, D5
0	0.63	0.63	Development blocked: D7, D11
0	0.31	0.31	Fully developed 11th day but did not emerge spontaneously ¶

* Pupae injected on 10 to 16th days of development. Each dose tested on two animals.

‡ Toxin-antitoxin mixtures injected in volume of 0.1 ml. immediately after mixing.

§ Calculated assuming 1 unit antitoxin neutralizes 1.5 Lf toxin (9).

‖ D indicates that blood had darkened and animals could no longer be stimulated by electrical shock. The number following indicates the day on which death presumably occurred.

¶ Only 1 pupa tested.

was no demonstrable effect of tetanus toxin on either developing or brainless diapausing pupae until doses of 32.5 Lf equivalent to 260,000 mouse M.L.D. were injected. After these very high doses, developing pupae showed definite progress for several days; the blood then commenced to darken and the pupae became soft and electrically inexcitable after 10 to 13 days. It is clear that the delayed death caused by this large dose was not due to tetanus toxin, itself, since specific antitoxin provided no protection. As previously noted, the toxin preparation was heavily contaminated with tetanolysin. It is doubtful, however, whether tetanolysin was responsible for the delayed death of pupae following large doses of the tetanus toxin preparation. For, though reduction with cysteine increased the lytic activity for erythrocytes ninefold, it failed to increase the toxicity of the preparation for the pupae.

DISCUSSION

The present experiments demonstrate that diphtheria toxin is a lethal agent, not only for the mammal, but also for the *Cecropia* silkworm. This result apparently represents the second instance in which an invertebrate has been found susceptible to the injection of a bacterial toxin. The only previous affirmative result was Chorine's (1) finding that relatively small doses of diphtheria toxin caused the death of larvae of the wax moth *Galleria*. According to

TABLE III

Effects of Partially Purified Tetanus Toxin on Pupae during Diapause and Early Development

Dose injected*		Stage of development	Results
Lf units	Mouse minimal lethal dose		
2	16,000	4–5th day	Normal adults after 16 days
8	64,000	4–5	Normal adults after 17 and 21‡ days
32.5	260,000	4–5	Progress to pink hair stage after 9 days; D10, D13
32.5 Lf + 35 units antitoxin		4–5	Progress to white hair stage after 4 days; D5, D6
0 + 35 units antitoxin		2	Normal adults after 19 and 20 days
2	16,000	Brainless diapausing	No effect
8	64,000	Brainless diapausing	No effect
32.5	260,000	Brainless diapausing	Some darkening of blood but otherwise no effect§
32.5 Lf + 35 units antitoxin		Brainless diapausing	D6, D7
0 + 35 units antitoxin		Brainless diapausing	No effect

* Each dose tested on two animals.

‡ Did not emerge spontaneously.

§ In a previous experiment this dose caused delayed death.

Chorine, *Galleria* larvae were unaffected for several days following injection, but then gradually became covered with small black lesions. Metamorphosis was blocked and death occurred 5 to 30 days following injection. Interestingly enough, Chorine also demonstrated that *Galleria* larvae were resistant to the injection of relatively large doses of tetanus toxin.

These observations together with those of the present investigation therefore stand in sharp contrast to the results of several previous investigations of great historical interest. Thus in the early studies of Metchnikoff (5) and others, protozoa, invertebrates, and plants (including yeasts and other fungi)

(3) were found wholly insensitive to diphtheria or tetanus toxins. Indeed, these toxins affected cold-blooded vertebrates such as amphibia and reptiles only when their body temperatures were raised to 25 or 30°C. (2, 4, 5).

Of considerable importance to the analysis of the action of diphtheria toxin is the observation that the sensitivity of the *Cecropia* silkworm is not constant but, on the contrary, undergoes sudden alterations in synchrony with the progress of metamorphosis. Though mature caterpillars and developing adults are less sensitive per unit mass than guinea pigs, they nevertheless are killed 1 to 2 weeks after the injection of as little as 0.07 to 0.7 gamma of toxin (1 to 10 guinea pig M.L.D.). The diapausing pupa, by contrast, is resistant to 70 gamma of toxin and may survive even this enormous dose for over 4 weeks. It seems necessary to conclude that the toxin finds a less extensive biochemical target within the diapausing pupa than within the caterpillar or developing adult.

A clue as to the identity of this target is afforded by the correlation between sensitivity to the toxin and the changes which occur in the cytochrome system during the course of metamorphosis. Thus the high succinoxidase activities of the larva and developing adult are accompanied by sensitivity to the toxin. Similarly, the low succinoxidase activity of diapausing and chilled pupae is associated with resistance to the toxin.

This correlation is further revealed by studies of the toxin's effect within the individual insect. Though diapausing pupae survive relatively large doses of diphtheria toxin for considerable periods of time, the toxin is not completely without effect at this stage. It has already been mentioned that the intersegmental muscles of the abdomen are affected within a few days following the injection of toxin, although the heart continues to beat and the pupae remain alive for several weeks thereafter. Potassium cyanide likewise acts selectively on the abdominal muscles of the pupa without affecting the heart; indeed the isolated heart continues to beat for several hours when placed in insect Ringer's solution containing 0.001 M KCN. In the case of cyanide, however, the paralysis of the abdominal muscles is prompt rather than delayed, and temporary rather than persistent; recovery ensues after a few days, presumably because the cyanide is lost as HCN *via* the tracheal system.

In view of these differences in the sensitivity of the abdominal and heart muscles to diphtheria toxin and cyanide, it is not surprising to find that the two types of muscle show striking differences in cytochrome content. As may be observed in Table IV, heart muscle shows only a single faint absorption band when dissected from pupae and examined under the Zeiss microspectroscope in the presence of dithionite.[5] Except for its low concentration, the characteristics of this band are identical with those described for the cytochrome

[5] A previous report that the pupal heart contains a normal cytochrome system was erroneous, due to contamination of the heart with intersegmental muscle (11).

x(e?) of the midgut of the larval silkworm (11). No bands of cytochromes b, c, or $a + a_3$ were evident even when a considerable number of hearts were pooled to provide a longer light path through the tissue. The low activities of several components of the heart's cytochrome system are also revealed by manometric determinations of cytochrome oxidase and succinoxidase and spectrophotometric determinations of Slater's factor (Table IV). By contrast, the abdominal muscles of the pupa show intense bands of cytochromes b, c, and $a + a_3$; moreover, high activities were demonstrated for the several components of the succinoxidase system (Table IV).

The most striking effect of the toxin is that observed in animals at the outset of adult development. Within a few hours or less the toxin causes cessation of development and enforces a state of artificial diapause. However, no obvious

TABLE IV

Succinoxidase Activity of Heart and Intersegmental Muscles of Diapausing Pupae at 30°C.

	Heart muscle*	Intersegmental muscle
Position of cytochrome α-bands, $m\mu$	556 (very faint)	603, 562, 550 (all prominent)
Cytochrome oxidase‡ (Q_{O_2} per mg. N)	9–29	150–190
Complete succinoxidase system (Q_{O_2} per mg. N)	7–18	75–120
Succindehydrogenase (Q_{O_2} per mg. N)	10–12	60–100
$DPNH_2$ oxidation§ (units per mg. N)	*ca.* 30	166

* In all cases the activities of the heart preparations were very low and therefore subject to considerable error.

‡ Q_{O_2} values represent the range encountered in four separate experiments using muscle pools dissected from 12 to 16 pupae for each experiment.

§ 1 unit is defined as a change of 0.001 division in the Beckman spectrophotometer per minute.

damage to the tissues takes place for 3 days or longer and death is delayed for up to 4 weeks. We feel that these facts lend support to the theory that diphtheria toxin blocks the *synthesis* of one or more components of the cytochrome system. In the case of animals at the outset of adult development, during the period when growth is apparently dependent on progressive cytochrome synthesis, the effect of the toxin becomes evident immediately. On the other hand, in tissues such as the intersegmental muscles of the pupa or in animals showing advanced adult development, the toxin acts more slowly because cytochrome synthesis is here required only to maintain the integrity of a system that has already been established.[6]

[6] In this connection it is worth noting that diphtheria toxin has no effect on the cytochrome system *in vitro*. The oxygen uptake by succinoxidase preparations from normal tissues of susceptible mammals remains undiminished even when as much as 1000 Lf/ml. (50,000 M.L.D./ml.) of purified toxin is added.

Though *Cecropia* pupae are resistant to the action of a large number of drugs and poisons, a few low molecular weight substances have been found which mimic to varying degrees the effects of diphtheria toxin. It is of particular interest and significance that *all* the simple compounds known to block adult development selectively are also inhibitors of certain components of the succinoxidase and cytochrome systems. These inhibitors include carbon monoxide under pressure (20) which blocks the succinoxidase system by combining with the reduced form of cytochrome oxidase; potassium cyanide which prevents the reduction of cytochrome oxidase; certain imidazoles which inhibit several succinoxidase components to a varying degree (21); and deuterohemin which inhibits succindehydrogenase (cytochrome *b*?) and Slater's factor (21).

In summary, the several lines of evidence derived from the study of the effects of diphtheria toxin on the *Cecropia* silkworm support the view that the toxin acts by blocking the synthesis of one or more components in the cytochrome system.

SUMMARY

1. The metamorphosis of the *Cecropia* silkworm is accompanied by large and systematic changes in the insect's sensitivity to diphtheria toxin.

2. Injection of less than 1 gamma of toxin into mature caterpillars, prepupae, or developing adults causes cessation of development followed by delayed death 1 to 5 weeks later.

3. Dormant pupae, on the contrary, are resistant to 70 gamma of toxin and may survive even this enormous dose for over 4 weeks. One-hundredth of this dose, however, prevents pupae from initiating adult development.

4. Tetanus toxin, to which the insect is insensitive, failed to duplicate any of these effects.

5. Maximal sensitivity to diphtheria toxin is characteristic of those stages in the life history which depend on the presence and function of the cytochrome system. Resistance to the toxin, as in the case of the diapausing pupa, is correlated with the existence and utilization of metabolic pathways other than the usual cytochrome system.

6. This correlation persists within the individual insect. Thus, within the diapausing pupa, the toxin fails to affect the heart in which a normal cytochrome system is absent, but, within the same insect, causes a degeneration of the intersegmental muscles in which an intact cytochrome system is present.

7. These several lines of evidence are interpreted in support of the conclusion that diphtheria toxin acts by blocking the synthesis of one or more components in the cytochrome system.

REFERENCES

1. Chorine, V., *Bull. biol. France et Belgique*, 1931, **65**, 292.
2. Courmont, J., Doyon, M., and Paviot, *Arch. physiol. norm. et path.*, 1896, **8**, 321.

3. Gengou, O., *Ann. Inst. Pasteur*, 1898, **12,** 465.
4. Grasset, E., and Zonderdyk, A., *Pub. S. African Inst. Med. Research*, 1931, **4,** 377.
5. Metchnikoff, E., Immunity in Infective Diseases, Cambridge University Press, 1905.
6. Mueller, J. H., and Miller, P., *J. Immunol.*, 1943, **47,** 15.
7. Pappenheimer, A. M., Jr., *Fed. Proc.*, 1947, **6,** 479.
8. Pappenheimer, A. M., Jr., and Hendee, E. D., *J. Biol. Chem.*, 1947, **171,** 701.
9. Pappenheimer, A. M., Jr., and Robinson, E. S., *J. Immunol.*, 1937, **32,** 291.
10. Pillemer, L., Whittler, R. G., and Grossberg, D. B., *Science*, 1946, **103,** 615.
11. Sanborn, R. C., and Williams, C. M., *J. Gen. Physiol.*, 1950, **33,** 579.
12. Slater, E. C., *Biochem. J.*, 1950, **46,** 484.
13. Wigglesworth, V. B., Principles of Insect Physiology, New York, Dutton and Company, 4th edition, 1950.
14. Williams, C. M., *Biol. Bull.*, 1946, **90,** 234.
15. Williams, C. M., *Biol. Bull.*, 1947, **93,** 89.
16. Williams, C. M., *Biol. Bull.*, 1948, **94,** 60.
17. Williams, C. M., *Growth*, 1948, **12,** 61.
18. Williams, C. M., *Biol. Bull.*, 1949, **97,** 111.
19. Williams, C. M., *Scient. Am.*, 1950, **182,** 24.
20. Williams, C. M., *Fed. Proc.*, 1951, **10,** 546.
21. Williams, C. M., and Pappenheimer, A. M., Jr., 1952, data to be published.