MORPHOLOGY OF SOUTHERN CORN ROOTWORM (*DIABROTICA UNDECIMPUNCTATA HOWARDI*) REPRODUCTIVE SYSTEMS AND THEIR HISTOCHEMISTRY IN RELATION TO APHOLATE

by

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A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of

The Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject: Entomology

Approved:

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Ames, Iowa

1964

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INTRODUCTION

Apholate or ENT 26,316 is one of the promising chemosterilants being tested on some economically important arthropods. Stoichiometrically, it is very active because of its six functional groups in the molecule. It has never been tried on southern corn rootworm (Diabrotica undecimpunctata howardi Barber) before. The physiological activities of apholate on the reproductive systems of this species are emphasized in this paper.

Since apholate is a derivative of cancer chemotherapeutic agents and phosphatases are predominant in growing tissues such as reproductive and neoplastic tissues, the application of histochemical localization in this study was considered to be a promising approach. Quantitative effects of apholate on the reproductive organs of this species by measuring the intensity of precipitation were not attempted.

This technique for evaluating the effects of a chemosterilant was also conceived because of the relatively long life cycle of southern corn rootworms. Some adults survive for as long as seven months. By the methods used the biochemical effects may be directly evaluated without waiting an indefinite time for egg-laying and hatching. Having a long observation period would expose the experiment to a wide range of factors causing variability.

Furthermore, the effects not visible histologically were thought to be detectable histochemically. Chromosomal breakage and clumping can be observed histologically; but these usually occur after a treatment with a large dose of alkylating agents which is usually very

toxic. The effects on enzymes will not be evident cytologically. The formation of a triester by alkylating compounds with the phosphate groups of DNA is not detectable cytologically unless breakage or clumping occurs. A break in a single strand in the DNA helix is also not likely to be conspicuous.

In connection with histochemistry, the familiarity of the investigator with the morphology and histology of the reproductive system is important. No record could be located concerning the detailed account of the internal reproductive systems of southern corn rootworms. Inasmuch as information about the morphology of the reproductive systems of the male and female southern corn rootworm is practically unavailable, except a very few scanty descriptions of external characteristics, detailed studies on this phase were accomplished.

PART I

MORPHOLOGY AND HISTOLOGY OF THE REPRODUCTIVE SYSTEMS OF *DIABROTICA UNDECIMPUNCTATA HOWARDI* BARBER

REVIEW OF LITERATURE

Sharp and Muir's (1912) meager description of the anatomy of the male tube (phallus) of Diabrotica soror, a synonym of D. undecimpunctata undecimpunctata Barber (1947), is closely analogous to that of D. undecimpunctata howardi. The description is as follows:

> In this Galerucid, the median lobe forms a long, curved tube: the basal foramen extends ventrally from one-third of the length of the tube; at the distal extremity there is a short acumen, and the dorsal face of the tube is membranous for nearly one-third of the length. The tegumen consists of a pair of slender, nearly parallel and nearly contiguous rods, these diverge very abruptly, and then converge again a little so as to partially embrace the median lobe, but they are unconnected by chitin on the dorsal aspect.

The V-shaped sclerite supporting the membrane, the spiculum, was not mentioned in D. undecimpunctata undecimpunctata, but is very distinct in D. undecimpunctata howardi. This as well as the spiculum ventrale are evident in the figures of Araujo Marques (1941), but no reference was given to them.

Araujo Marques (1941) and Barber (1947) figured genitalia of several species of Diabrotica but apparently used only preserved or dry specimens which led to several inaccuracies. They did not describe their figures in detail.

The critical studies of Coleoptera by Jeannel (1949) contributed much towards clarifying some of the disconcerting questions of terminologies. The problem arose because occasionally insect morphologists used some terms loosely on simple or modified genital apparatus whereas their use in a more complex system would be confusing. Singh Pruthi (1924a and b) contributed to this area by delineating the ontogeny of the homologs of male and female genital organs in Tenebrio molitor L.

No histological literature concerning D. undecimpunctata howardi could be found. The histological anatomy of male gonads of some cerambycid beetles (Ehara, 1951) closely resembles that of this species.

MATERIALS AND METHODS

Live, preserved, and macerated specimens of D. undecimpunctata howardi were utilized to verify each structure discussed. The dissection of live specimens was done in insect physiological saline solution under a dissection microscope of 9.9-45X. Measurements of ovaries, oocytes, and sperms were based on living organs. The ovarial ligament was traced using Schiff's and "Light Green SF, yellowish" stains added in saline solution. The ligament stained dark purple, hence it was easily traced. The ovarioles were counted and studied both unstained and stained.

Specimens were fixed with KAAD and were preserved in 70% alcohol. Some were macerated with about 5% KOH. The concentration of KOH could be varied a great deal depending on the worker's availability for keeping close observation of the specimen. They were studied in either water or alcohol under a dissecting microscope. For finer details a high-power phase-contrast microscope was used. Other specimens were mounted in either balsam, glycerine jelly or Hoyer's mounting medium. Basic fuchsin was used to partially stain the structures for clearer contrast.

Another method used was to allow specimens to decompose for less than a day for delicate isolation of the intact female genital chamber linings including a considerable portion of the spermathecal duct. Then they were preserved in 70% ethyl alcohol, and were dissected when needed. In this case mild enzymic reactions and microbial activities substituted for that of KOH. Integuments were cleared of protein and

fatty tissues by this method without decolorizing or bleaching the normal shades. Since proteolytic enzymes from either muscles or microorganisms cause the spoilage of tissues rich in protein and lipases degrade fatty acids with the aid of albuminous protein which tends to emulsify these substrates (cf. electrophoretic fraction I, Patton, 1963), care was taken so that these biological reactions could be practically stopped when the desired texture of the tissues was attained. This was done by preserving the specimens in alcohol. Actual examination of the tissues was found to be the best way to determine the needed time to clean certain parts of the specimen.

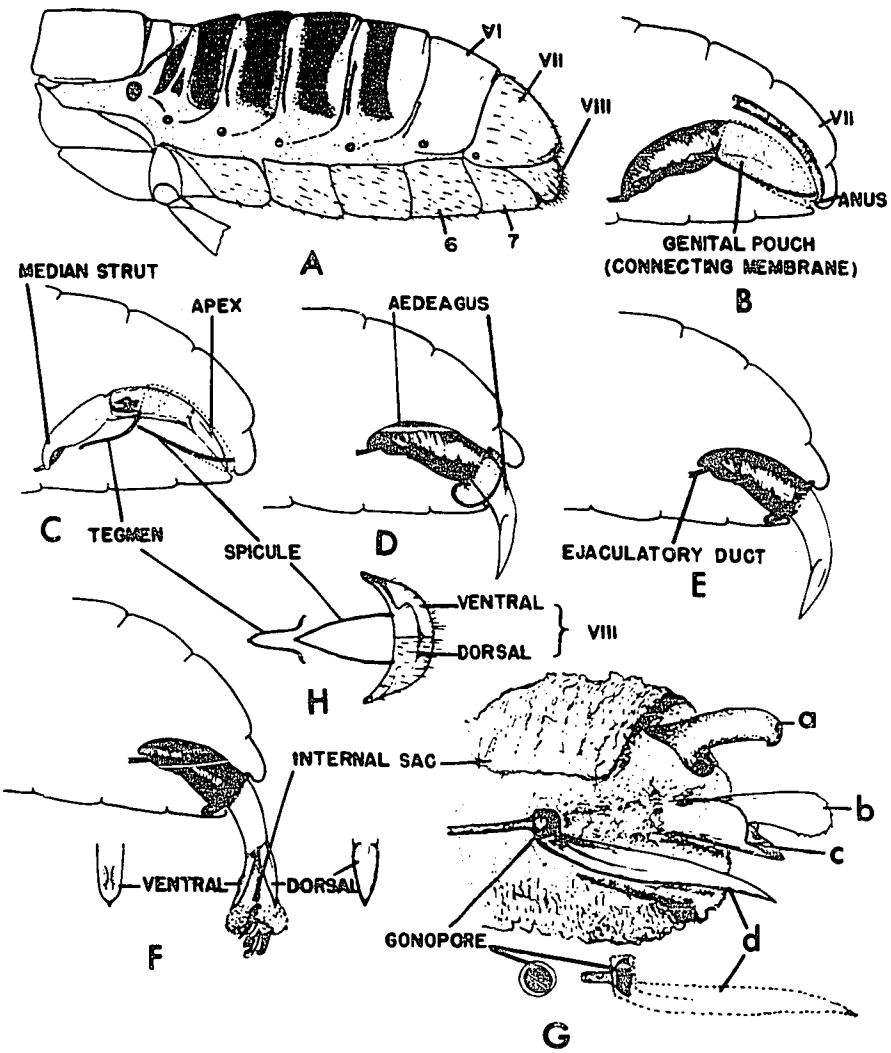
Histological studies were accomplished by using specific methods such as Feulgen's DNA test and "Light Green SF, yellowish" staining for cytoplasm. Tissue sections were obtained by using an automatic rotary microtome. A high-power phase-contrast microscope was also used in these studies. Gomori's technique (Gomori, 1939) with slight modification (see Part II) was followed to test for phosphatase content or activity in acetone-fixed tissues.

DISCUSSION

The terminology used is primarily after Jeannel (1949), Lindroth and Palmén (1956), Lindroth (1957) and Torre-Bueno (1962). Jeannel's French terminology is given in parentheses after the terms used by the other authors. The term phallus is used as Snodgrass (1935) defined it; thus its synonym aedeagus, according to Torre-Bueno and Lindroth, is applied in this paper as only a part of the former.

The abdomen of corn rootworm is flattened dorso-ventrally. There are seven distinct tergal and five sternal sclerites visible at repose, plus the eighth tergite, tergum VIII or supraanal plate (Figure 1A) in the male. The first two sternites are obliterated by the coxal cavity. The sterna are thickly chitinized forming hard segmental plates. On the other hand, the terga are not thickly sclerotized except the seventh. Soft terga are characteristic of most Coleoptera, since the elytra assume the function of protecting the membranous hindwings and abdomen from mechanical injury. Wide dark bands distinctly across terga I-V (Figure 1A and Figure 5A). The dark markings on tergum I are not continuous at the median line, unlike those of II-V. Other narrow dark bands are conspicuous laterally between the wider bands from I-V. These are not usually present in newly emerged imagoes. The first anteriorly is the shortest and forms a wedge-like pattern with the apex pointing towards the midline. All of these narrow, laterally located patterns are not continuous at the midline. The sternites and the seventh tergite possess numerous setae.

Figure 1. The abdominal segments and copulatory apparatus of male D. u. howardi (Roman numeral = tergum and Arabic numeral = sternum) A. External appearance of abdomen. B thru F. Diagrammatic steps in eversion of aedeagus. G. Detail of internal sac. H. Dorsal diagram to show positioning of tegmen and spicule. All drawings about 12X except G which is 80X.


VI
VII
VIII
6
7
A
VII
ANUS
GENITAL POUCH
(CONNECTING MEMBRANE)
B
MEDIAN STRUT
APEX
TEGMEN
SPICULE
C
AEDEAGUS
D
EJACULATORY DUCT
E
VENTRAL
DORSAL
VIII
H
INTERNAL SAC
VENTRAL
DORSAL
F
a
b
c
d
GONOPORE
G

The spiracles on the first tergum are about five times larger than the other six pairs in abdomen. The first six pairs are located on the pleural area which is not distinctly demarked from the terga. The last pair is located laterally on the angle of the seventh tergum.

MORPHOLOGY AND HISTOLOGY OF THE REPRODUCTIVE SYSTEM IN CORN ROOTWORM MALES

External Reproductive System

The abdominal segments of the male are similarly constructed to those of the female except the slight modification of the tergite VII to accommodate the single supraanal plate (Smith and Allen, 1931). This plate is attached on both sides to the dorsal sclerite of the male. It possesses numerous setae posteriorly. Smith and Allen did not provide any illustrations with their description.

When enough pressure is applied on the abdomen, the genital segment protrudes outwardly (Figure 1D, E). The anus is dorsally located in relation to the phallus or male genital tube (Figure 1B). The latter is composed of tough sclerites and membranes arranged around the terminal portion of the ejaculatory duct or ductus ejaculatorius. The ninth segment usually is a part of this apparatus and is therefore called the genital segment. Unlike the female, the rectal portion is not extended out because the perianal tissues are closely anchored on the supraanal plate.

There has been much confusion among insect morphologists concerning which parts represent the aedeagus, penis or phallus. Some used these terms interchangeably which could be erroneous as well as confusing to others. Sharp and Muir (1912) realized that penis is totally fallacious when applied to Insecta. The part in Insecta that most nearly approximates the vertebrate penis is the internal sac. The term should be restricted to a muscular intromittent organ found in other groups of animals than insects. For this particular species of

Diabrotica, presumably all conceivable genital structures are present. The whole genital apparatus is designated phallus.

The phallus consists of aedeagus or aedoeagus (median lobe, Jeannel, 1949) and tegmen (phallobase). The aedeagus is the terminal and tubular part of the phallus as distinct from the phallobase or tegmen. It is highly sclerotized and arcuate. The proximal process is referred to as the median strut (lame basale). The distal part of the aedeagus is the apex. The opening or area through which the internal sac is everted is the ostium. The membranous internal sac or endophallus is everted during copula. The details of this apparatus will be discussed with the internal reproductive system.

The apex is composed of dorsal and ventral V-shaped lobes, at the base of which is the ostium. They partly surround the internal sac during the eversion. The dorsal lobe is convex and beak-like. It is attached to the periphery of the ostium by a narrow, thinly sclerotized bridge at the midline. The ventral lobe is somewhat flattened, and terminates very abruptly to a sharp point giving an outline of an ogee arch. There are bracket-like papillae on the mid-lateral surface. These papillae have minute punctations. Both dorsal and ventral lobes fit snugly with each other.

The dorsal member of the apex is pushed away from the ventral process during copula. The semi-membranous bridge at the base of the dorsal part affords articulation for this beak-like structure. Thus, the internal sac is easily everted.

A very simple tegmen is evident. It consists only of a very slender ventro-lateral basal piece and a median membrane. The tegmen is a

modification of annulate type (type en cavalier). This type has the basal piece forming a complete ring around the aedeagus with which it is loosely connected.

In this species, the basal piece does not form a complete ring around the aedeagus. Its proximal end is attached ventrally on the base of the aedeagus close to the entrance of the ductus ejaculatorius. It runs longitudinally for a very short distance nearly parallel to its pair. Then it bends abruptly upward and forms a semi-annular structure. A thin membranous sheath connects the two basal pieces at the midline. A thin sheath of muscular tissues is attached around the basal pieces forming a bracteate structure at the base of the aedeagus.

The extrusion of the whole phallus is controlled mechanically by the modified pair of spicules, spiculum gastrale or spiculum, wiry sclerites lateral of the phallus (Figure 1H). They support a membranous sheath which is continuous with the membrane associated with the basal pieces. This membrane is folded over the bracteate structure just mentioned during the protrusion of the phallus and prevents extrusion of the basal portion of the phallus. The sclerites articulate on the membrane at the ventral margin of the basal pieces, near the angle of their curvature.

These spicules are the homologs of the rods Lindemann (1875 and 1876) and Hopkins (1911 and 1915) described in their studies of Pissodes and Scolytoidea. Hopkins, however, proposed the ontogeny of this rod as modified ninth sternite as well as other parts of the genitalia without actual experimentation. His theory was rejected by Muir (1915) after studying the ontogeny of the genital tubes in

Coleoptera. Muir considered his statements as contrary to actual observations, and some are quite illogical. Sharp (1918) considered spiculum gastrale to be a part of tegminal layer, from which it diverges, just as the strut of the tegmen is a part of the tegmen.

Where the ninth sternite is well developed there is no spicule, but there is often a pair of projections, or a single projection, from its base (Muir, 1915). According to Muir the latter may be represented by the spicule in such forms as have no ninth sternite. In Hylesinus crenatus Fabricius no chitinized eighth sternite is present, and the spiculum is highly developed and serves in the place of the sternite as part of the tube through which the aedeagus plays (Muir, 1918). This condition is evident in D. undecimpunctata howardi. A V-shaped spiculum was reported by Singh Pruthi (1924a) in Tenebrio molitor L. which is of exactly the same form as in southern corn rootworm males.

Singh Pruthi (1924a) explained the ontogeny of the spicule. His report is as follows:

> The spiculum arises, while the individual is still in the larval stage, as a pair of ectodermal invaginations of the body-wall just posterior to the ninth sternite; the invaginations lie, one on either side of the genital pocket. The fact that the spiculum is present in the larva, in which the ninth sternite is also distinct, establishes beyond doubt that this organ in the imago is not the modified ninth

sternite, as it was believed to be by numerous investigators, e.g., Hopkins (1911).*

This also rejects the suspicion of Sharp (1918) that the spicule may be a part of the tegminal layer.

The V-shaped spicule has its apex pointing anteriorly. Its base is suspended laterally by muscles on a membrane posteriad and just near the angle of the ventro-lateral protruberance of the supraanal plate (Figure 1H). The membrane attached to the spicule forms a conical pouch around the phallus (Figure 1B). The dorsal base of the cone is attached to the anus which is anchored on the ventral portion of the supraanal plate. Ventrally, it is united with the seventh sternite. The base of the cone, as a whole, is the orifice for the phallus.

Internal Reproductive System

The internal reproductive system of the male southern corn rootworm consists of proximal testes and accessory structures (Figure 2). The two ovoid, sometimes kidney-shaped, testes are normally enveloped by a thin membranous sheath to form a one-lobed structure. Each testis, however, is separated by a thin membrane. It is hollow at the center and is light yellow in young imagoes but changes to deep orange with age.

The enlargements of the paired vasa diferentia known also as seminal vesicles or vesicula seminalis serve as a reservoir for

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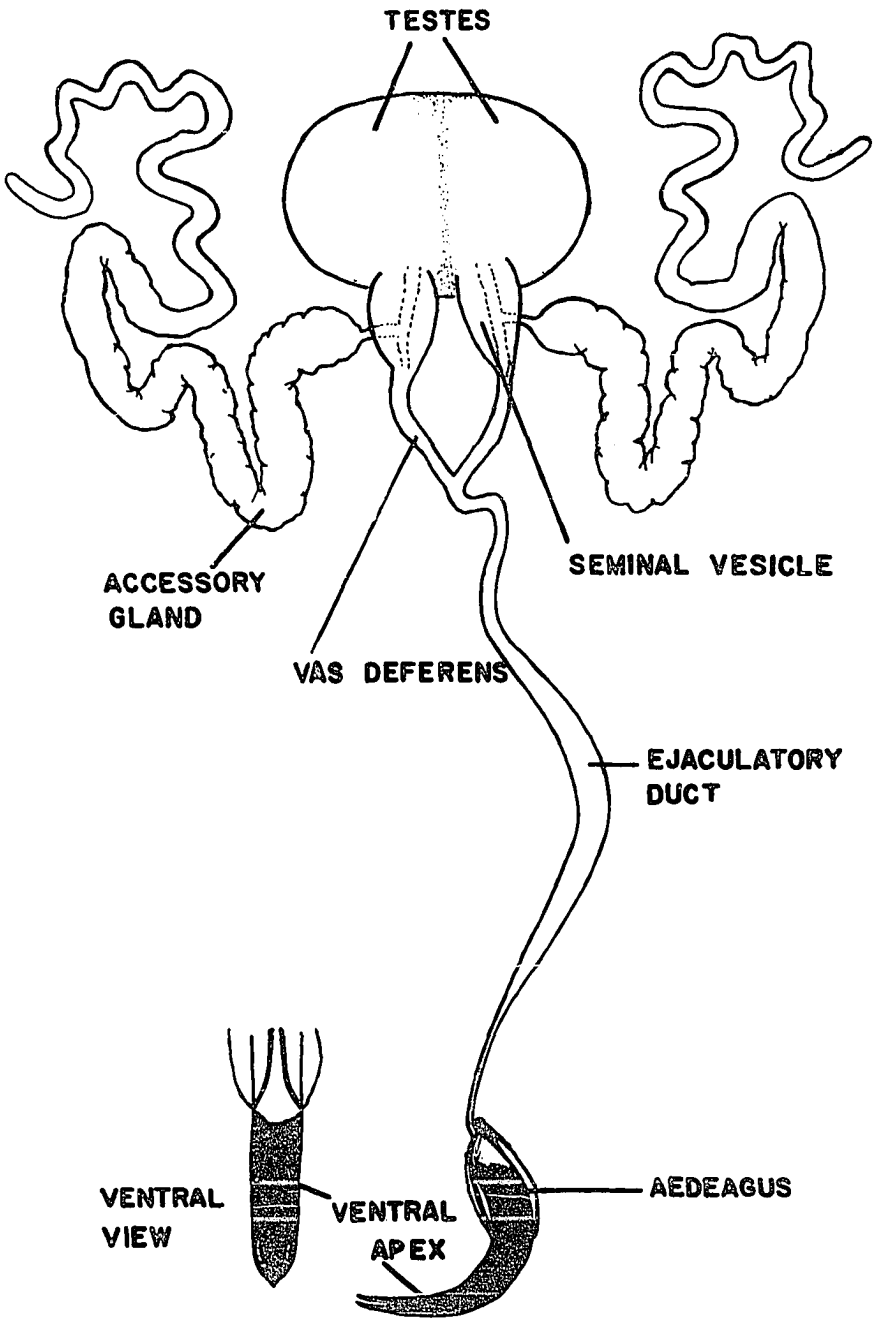
*Actually Hopkins made his "hypothesis" in 1915.

Figure 2. Diagrammatic illustration of the internal reproductive organs of male *D. u. howardi* (about 30X)



REPRODUCTIVE ORGANS OF MALE

DIABROTICA UNDECIMPUNCTATA HOWARDI BARBER

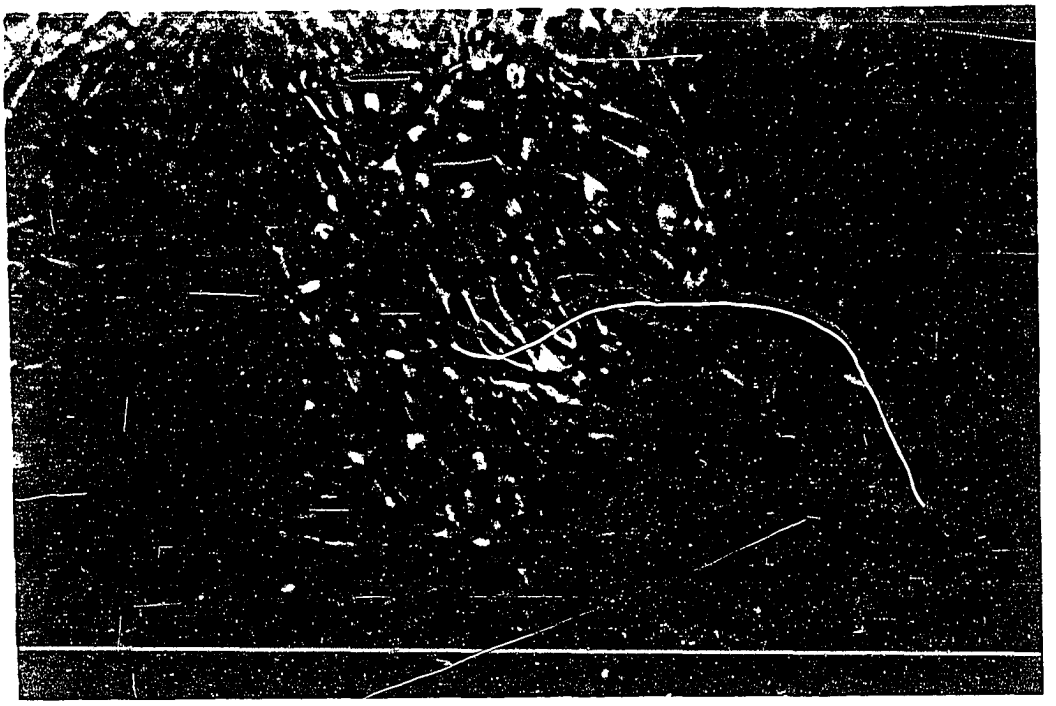
spermatozoa. These are continuous with the testicular cavities and extend distally to the short vasa diferentia or ductus ejaculatorius duplex. These fuse to form a single vas efferens or ductus ejaculatorius.

The paired accessory glands arise laterally near the proximal portion of the seminal vesicle. Each is long, tubular, and coiled about the body cavity and terminates in a blind pouch. It is slender and clear at about the distal half. Proximally, it is much enlarged and opaque with more distinct convolutions in this portion.

The vas efferens is a long tube and gradually enlarges at about the middle portion giving an impression of a slender tubular spindle. It enters into the membranous eversible sac which is known among coleopterists as the internal sac.

The aedeagus is a complicated structure in this species. Besides those strongly chitinized structures described previously, the terminal components of the membranous internal sac give further variation to this apparatus. Four distinct sclerites occur on the terminal portion of the internal sac (Figure 1F, G). A spur-like structure is most ventrally located. This possesses a ring-like base which surrounds the distal orifice of the ejaculatory duct or the gonopore. The gonopore is encircled by a single row of stumpy filaments resembling a sea anemone (Figure 3). Next above it is a scoop-like or petaloid sclerite with a serrate edge. This is more thinly sclerotized than the most ventral structure mentioned above. It resembles a bracket fungus partially enveloping the third dorsal member. The third member gradually broadens at the terminus particularly at one side making a sole-like outline. This is not heavily sclerotized, and has fewer

Figure 3. Photomicrograph and outline of gonopore of male *D. u. howardi* (about 1000X)



serrations at the distal margin. The most dorsal structure, which is akin to a claw, is strongly curved downward at the tip terminating in an abrupt but sharp point. This appears tubular and is as thickly sclerotized as the most ventral structure.

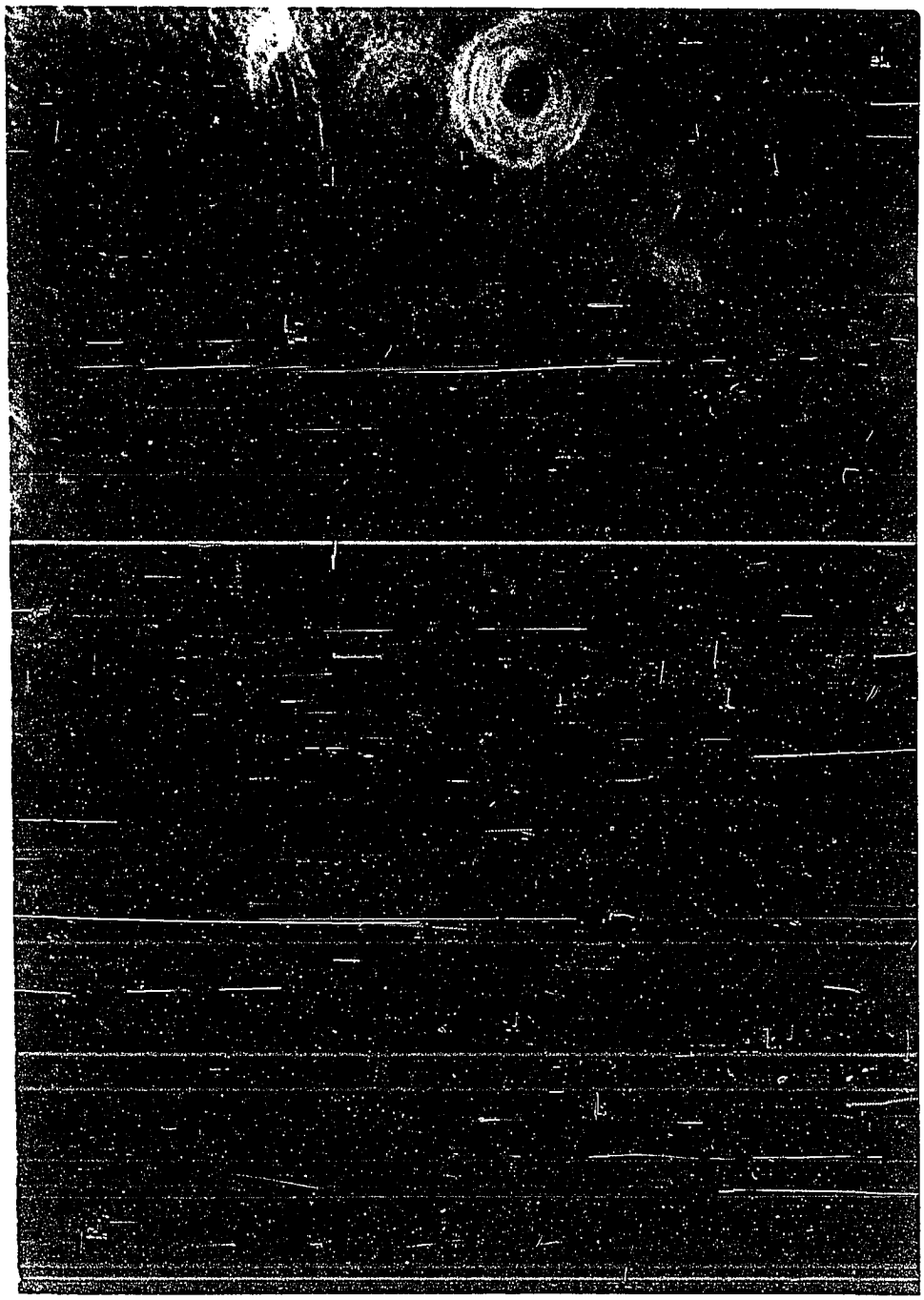
The minute structures mentioned in the preceding paragraph could easily be missed if the internal sac is not examined meticulously. The most ventral and dorsal members presumably are analogous to dent ventrale and dent dorsale of the internal sac of Choleva cisteloides Frol. (Catopidae) (Jeannel, 1922 and 1949).

Histology of the Male Reproductive System

A cross-section of the testis reveals the following layers from outside toward the inside cavity. A very thin membrane sparsely supplied with DNA-rich cells envelops the testis. Underneath it is a band of germarium composed of cells extremely rich in DNA as revealed by the Feulgen reaction. The zones of growth and maturation are indistinguishable although the degree of development can be discerned clearly by the distinct differentiation of the sperm heads. The sperm head is strongly Feulgen positive because it contains mostly nuclear materials.

The spermatozoa are produced in bundles (Figure 4) and are arranged longitudinally with each other. They are inactive within the bundle. Cytoplasmic material is predominant in the tail or flagellum of a sperm. This is demonstrated by "Light Green SF, yellowish" stain which is specific for cytoplasm. Bundles of spermatozoa are contained in the testicular cavity.

Figure 4. Phase-contrast photomicrograph of D. u. howardi sperms. Each circular area is a group of actively swirling sperms and bundle of sperms at the upper left corner (about 600X).



The accessory glands consist of tissues abdundant in glandular cells. Glandular cells are distinctive due to large nucleation and are concentrated in the tubule walls. Secretion is evident and fills the cavity of the gland.

The tissues of the ejaculatory duct have a vigorous squeezing motion under normal conditions. This would appear to be useful in the transport of spermatozoa.

High alkaline phosphatase content or activity was detected in all DNA-rich tissues mentioned above.

Notes on Sperms

The sperms glide as they move in the medium. When they are very active and concentrated in an area, the gliding motion resembles a swirl or an eddy current (Figure 4). The sperms measure about 150 μ with the head about 15 μ in width. This is extremely large as compared to human and herring sperms. The latter has a head nucleus measuring only about 2 μ in diameter (Stacey, et al., 1958).

MORPHOLOGY AND HISTOLOGY OF THE REPRODUCTIVE SYSTEM IN CORN ROOTWORM FEMALES

External Reproductive System

The seventh abdominal tergite fits snugly with the seventh sternite. (Figure 5A). The last abdominal visceral segment tapers narrowly forming a subtriangular pattern. The terga VIII and IX are folded inside this segment. As in Tenebrio molitor L. (Singh Pruthi, 1924b) sternite VIII is present.

The style, stylus or vaginalaster (Figure 5C) is highly sclerotized and dark at the tip. This is attached to a baculiform valvifer which appears like a thick clear membrane. This tendon-like membrane is continuous with the chitinous lining of the vagina and bursa copulatrix. The stylus enlarges at the posterior tip and forms a penpoint-like structure equipped with sensory setae. It has a slit from the tip to about one-third of the total length. The stylus measures about 0.83 mm. It is extended during oviposition.

The wall of the genital segments is membranous and highly retractile. Application of pressure on the abdomem causes the extrusion of the bifurcate genital and rectal protrusions (Figure 5C, D). The dorsal branch terminates in the anus and the ventral branch in the vulva. The vulva or genital aperture is located near the base and ventrad of the stylus (Figure 5D).

Internal Reproductive System

The reproductive system consists of two laterally positioned ovarial lobes (Figure 6). The ovaries are profusely provided with

Figure 5. The abdominal segments and genital apparatus of female *D. u. howardi* (Roman numeral = tergum and Arabic numeral = sternum) (about 12X except enlarged style)

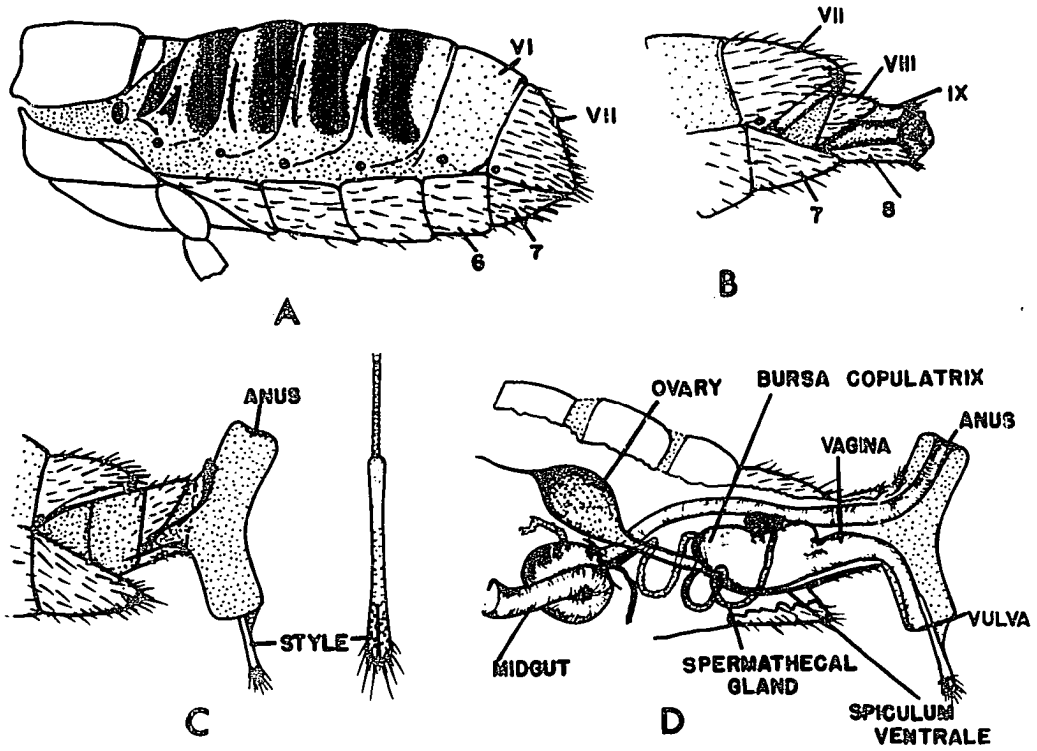
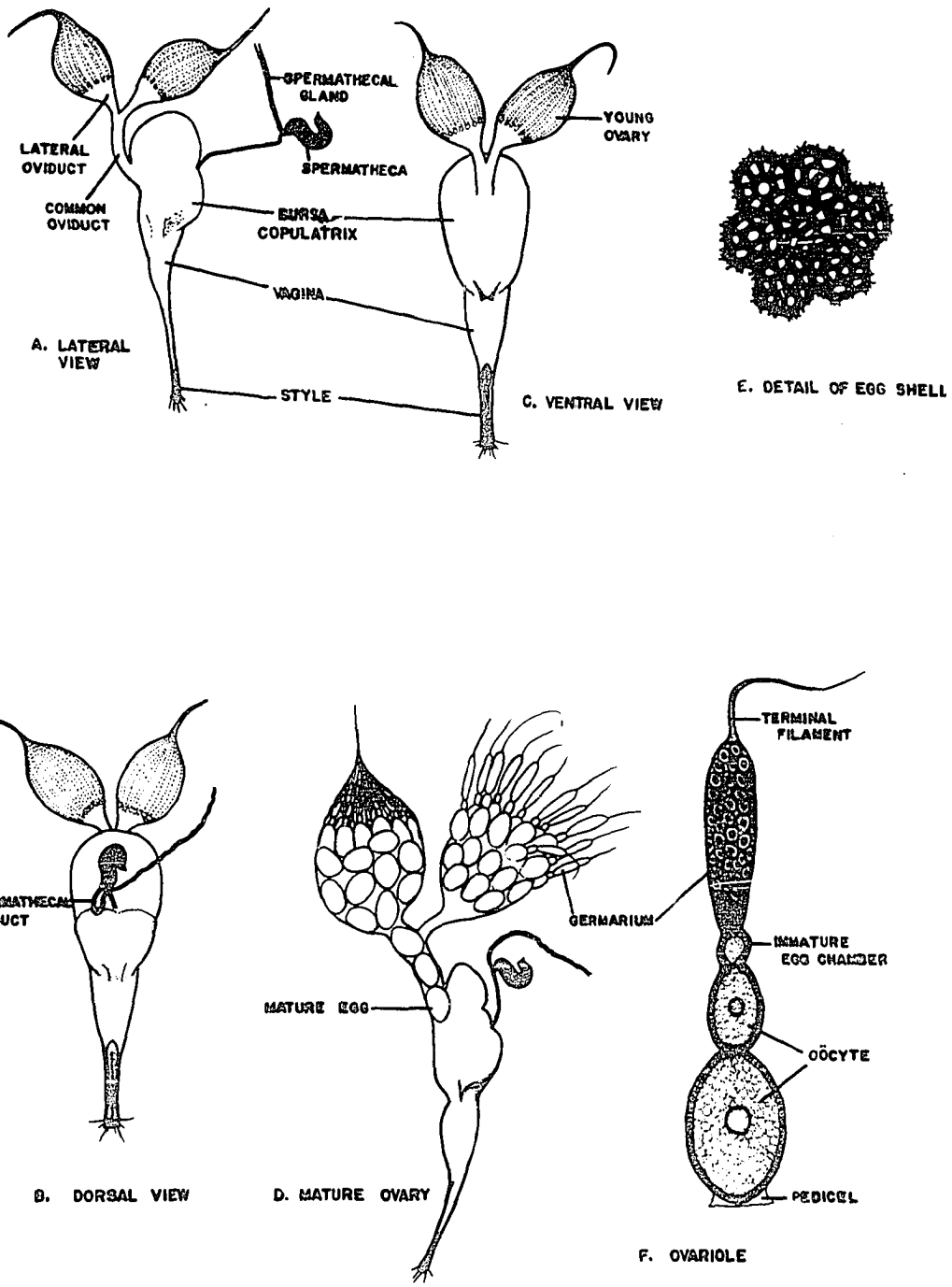

VI
VII
6
7
A
VII
VIII
IX
7
8
B
ANUS
STYLE
C
OVARY
BURSA COPULATRIX
ANUS
VAGINA
VULVA
MIDGUT
SPERMATHECAL GLAND
SPICULUM VENTRALE
D

Figure 6. Diagrammatic illustrations of the internal reproductive organs of female *D. u. howardi* (A, B, C and D, about 18X; E about 320X and F about 30X)



REPRODUCTIVE ORGANS OF FEMALE

DIABROTICA UNDECIMPUNCTATA HOWARDI BARBER

tracheoles which are definitely reinforced with taenidia. Each ovary has about 56 ovarioles, making an average of about 112 ovarioles per female. Five pairs of ovaries were dissected out revealing from 50 to 60 ovarioles per lobe. Each pair does not necessarily have an equal number of ovarioles.

The ovariole is composed of a long terminal filament, a biologically active germarium, a follicle and/or an oocyte depending on the stage of egg development, and a very short stumpy pedicel. The terminal filaments aggregate to a larger ovarial ligament. The ovarial ligament is anchored on the prescutum close to the median notch. This notch is between the two lobes of the anterior phragma. On the other hand, the pedicel connects the ovariole to the surface of the egg calyx. The ovariole may be considered an acrotrophic type of egg tube although no plasmatic strands could be descerned on unstained and stained sections and whole mounts. This assumption is based on the fact that only one oogonium matures at a time; thus its physical relation to the germarium is very close, such that nourishment could be absorbed without the aid of plasmatic strands.

In a few insects, particularly in Hemiptera and some Coleoptera, the cells produced with the oocyte from the oogonia, but destined to become nurse cells, remain in the upper part of the egg tube (Snodgrass, 1935). The oocytes become removed from them as the series of egg cells increase in the vitellarium. The germarium in the acrotrophic type of egg tube, therefore, is also an apical feeding chamber for the oocytes. In this particular species of Diabrotica, usually two to three oocytes are discernible. Those closer to the germarium are very minute as compared to the distal oocyte.

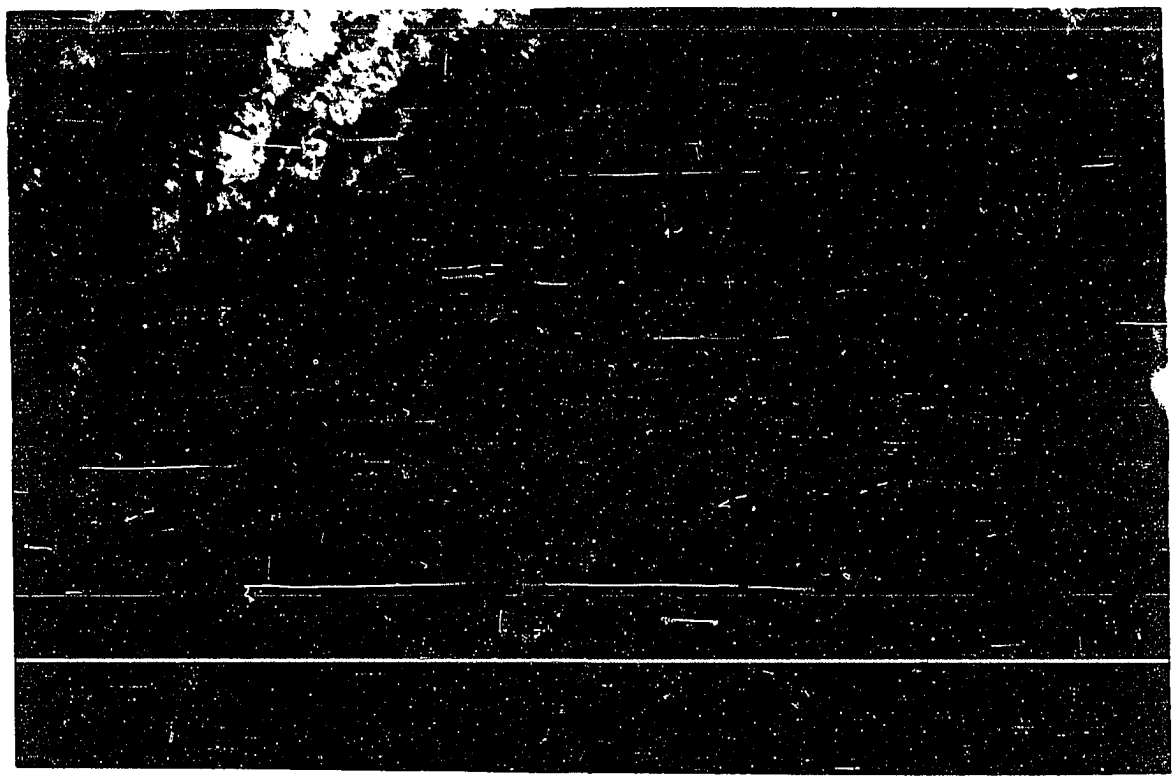
A well-developed egg measures about 0.65 mm in length. It is already enclosed in shell when released in the chalice. Polygonal patterning is conspicuous on the shell or chorion and appears to be an architectural reinforcement of a semi-spherical dome. Each polygon contains about 8 to 11 irregular depressions.

The chalice or egg calyx directly beneath the ovarioles can hold about 50 eggs which would seem to indicate that not all oocytes mature at one time. It has numerous internal folds resembling flaps. The chalice is a continuation of the lateral oviduct.

The two lateral oviducts meet medianly forming a single common oviduct or oviductus communis. The chalice, the oviductus laterales, and the common oviduct are capable of vigorous contraction. The oviducts possess numerous spinous structures lining the inner walls, which apparently prevent the eggs from being pushed back in the chalice during oviposition.

The oviductus communis opens into a much enlarged bursa copulatrix on the ventral side. The bursa copulatrix has a very thick musculature. It extends anteriorly forming a blind pouch. Because of the dorsal muscular fold, it appears as a bilobed structure, the lobes being oriented antero-posteriorly. Internally, the bursa copulatrix is chitinized and studded with teeth- or bump-like sclerotized structures (Figure 7B). These are more profuse towards the junction of the bursa copulatrix and vagina. The chitinization extends to the anterior vagina. Chitinized folds at this junction form valve-like flaps. Numerous convolutions and folds are also evident in the inner walls of the bursa copulatrix.

Figure 7. Portions of bursa copulatrix (A. Internal lining of bursa copulatrix showing the pore at the center in which the spermathecal duct opens, about 680X. B. Section of bursa copulatrix showing the chitinous teeth-like structures, about 2,000X)



A

B

Dorsal to the bursa copulatrix is a strongly chitinized spermatheca or receptaculum seminis. This tubular structure is hook-shaped giving an impression of an almost perfect question mark. Its length from the base to the curvature measures about 0.45 mm. Its diameter is about 0.13 mm which is almost uniform from near the base to the blind end which abruptly terminates in a sharp point directed outwardly from the curvature.

A very long tubular spermathecal gland is attached to the junction of the base of the spermatheca and the spermathecal duct or ductus spermathecae. It is regarded as a diverticulum of the ductus spermathecae and secretes a fluid in which the sperms are discharged.

The spermathecal duct connects the bursa copulatrix and the spermatheca. It enters at the median line of the aforementioned dorsal muscular fold of the bursa copulatrix. Its opening at the bursa copulatrix has two moon-shaped papillae (Figure 7A) which might serve as valves to control the release of sperms. It appears to be lined with thinly sclerotized membrane similar to that of the bursa copulatrix and vagina.

Posterior to the enlarged bursa copulatrix is a chamber called the vagina. It tapers posteriorly. The vaginal area is protected between sclerites of the seventh segment at repose. The vulva constitutes the external opening of the vagina. The base of the stylus is associated dorsally with the vulva.

A very slender sclerotized spiculum ventrale supports the bursa copulatrix and vagina (Figure 5D). It is attached by a muscle ventrally at the junction of the common oviduct and bursa copulatrix. The

posterior end terminates at about the middle of the vagina, and is fused with the eighth sternite.

Histology of the Female Reproductive System

The germarium is very rich is DNA as revealed by the Feulgen test. It consists of numerous cells containing large proportions of DNA. Just below the germarium are the developing oocytes, the distal one being much more advanced in development than those proximal to the germarium.

The follicle or egg-chamber has a single layer of epithelial cells which are rich in DNA. The large nuclei are usually centrally located in the developing oocytes. Cytoplasmic material, as revealed by a "Light Green SF, yellowish" stain, comprises the main bulk of oocytes.

The walls of the egg calyx are also provided with DNA-rich cells. These are located directly beneath the pedicels.

The oviducts and spermathecal gland and duct have a single layer of cells. The spermathecal gland particularly consists of DNA-rich cells.

From the inner to the outer walls of the bursa copulatrix, the tissues evident are 1) a chitinous layer, some areas of which are provided with tooth- or bump-like structures, 2) an almost clear cytoplasmic-rich tissue, 3) a DNA-rich layer, and 4) cytoplasmic-rich striated muscles. Cells with large amounts of nuclear materials are sparsely embedded in the last layer.

Histologically, the vaginal wall is constructed like that of the bursa copulatrix. However, the thick stud-like chitinization is present only in the anterior portion.

The alkaline phosphatase content or activity was detected on the aforementioned tissues rich in DNA.

PART II

HISTOCHEMISTRY OF THE EFFECTS OF APHOLATE ON THE REPRODUCTIVE ORGANS OF *DIABROTICA UNDECIMPUNCTATA HOWARDI* BARBER

REVIEW OF LITERATURE

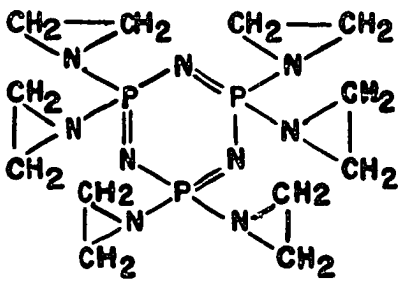
Development of Anticancer Agents and Their Role in Genetics

Analogs of anticancer agents are being intensively evaluated for passible chemosterilant activity on different species of arthropods and mammals. The first to recognize the unique biological features of alkylating compounds, ethylenimine (=aziridinyl) and ethylene oxide, was Paul Ehrlich (1898). Jackson (1959) and Jackson et al. (1961) assessed the antifertility substances, including cytotoxic alkylating agents, on male and female rats. Side effects such as toxicity and risks of carcinogenesis and possible genetic damage were mentioned. The most important outcome of these studies on male rats (Jackson et al., 1961) was the demonstration that selective attack on various stages of the long, complex process of spermatogenesis is practicable. The enhancement of action on spermatozoa with reduction of side effects is encouraging. The most important classes of potential antitumor compounds are alkylating agents, antimetabolites, radiomimetic compounds, and mitotic poisons. This is a superficial classification of biologically active compounds because some can belong to any of them simultaneously.

Apholate and Other Alkylating Agents

Apholate, a relatively new compound, is an ethylenimine (=aziridinyl) derivative (Figure 8). It is also known as APN among textile researchers and ENT. 26,316 among entomologists. This chemical, 2,2,4,4,6,6-hexahydro-2,2,4,4,6,6,-hexakis (1-aziridinyl)-1,3,5,2,4,6-triazatriphosphorine, is synthesized by acting trimeric phosphonitrilic chloride

Figure 8. Apholate chemical reactions (after Hobart et al., 1962) and other alkylating agents

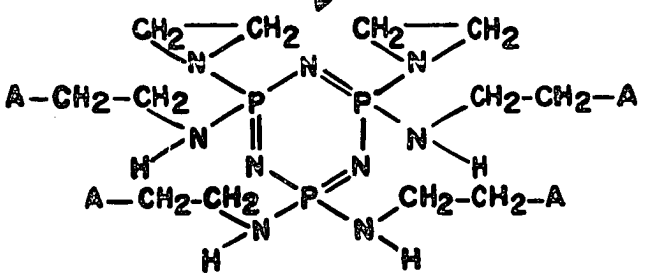


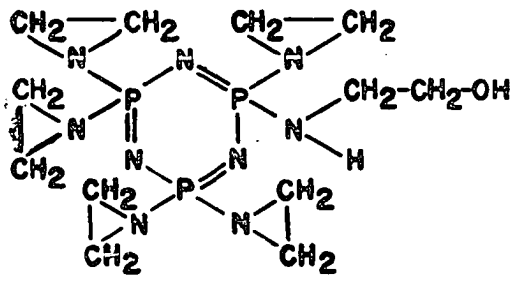
APHOLATE

+

(1) H^+ ACIDIC CATALYSIS

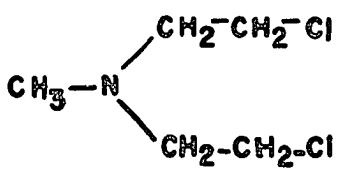
H_2O (2) H^+





A = ANOTHER OR SERIES OF APHOLATE MOLECULES

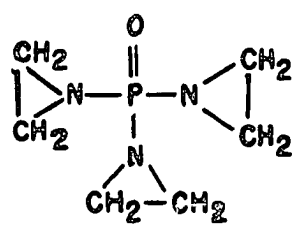
A



HN2, MUSTARGEN

(A NITROGEN MUSTARD)

B



TEPA

C

with ethylenimine in the presence of an acid acceptor such as triethylamine (Hobart, et al., 1962). They also described a procedure for isolation of pure apholate. The product obtained is a white crystalline solid melting sharply at 155° C. It is readily soluble in water and chloroform, but insoluble in ether. Apholate polymorizes slowly on storage at room temperature but under refrigeration there is essentially no polymerization as indicated by water solubility after two months time. Its possible reaction with water is given in Figure 8.

Apholate substantially inhibits carcinoma 755, Dunning leukemia, and Walker 256 test systems (Ross, R. B., 1962). R. B. Ross continued that it has not been afforded extensive clinical trial, since its relative insolubility would be a serious drawback. This discrepancy in solubility might be caused by polymerization of apholate before testing.

Some closely related compounds such as tepa (tris(1-aziridinyl) phosphine oxide) (Figure 8), methiotepa (tris(1-aziridinyl)phosphine sulfide), and tretamine (2,4,6-tris(1-aziridinyl)-s-triazine) are common cancer chemotherapeutic agents. They are trifunctional alkylating compounds while apholate is a hexafunctional. They are known as radiomimetic chemicals because they produce all the biological end-effects observed after treatment with ionizing radiations. Their biological activities are closely related to the study of radiobiology.

Auerbach and Robson (1944) were the first to observe that the chemical warfare agent, mustard gas ($S(CH_2CH_2Cl)_2$), produced mutation. (cf. Figure 10). Koller (1954) observed that this substance causes radiation-like chromosome abnormalities. Koller (1958) and Revell (1958) reported a variety of cytological effects of alkylating agents.

Geneticists such as Fahmy and Fahmy (1956) studied the cytogenetic action of carcinogens and tumor inhibitors in Drosophila melanogaster. Reviews of the mutagenic effects (Auerbach, 1958) and the carcinogenic action (Walpole, 1958) of alkylating agents are of general interest and application to this area. The latter author stated that as yet there is no evidence that any alkylating agent has caused tumors in man, but past experience in the industrial handling of heavy hydrocarbons and aromatic amines indicates that chances should not be taken with alkylating agents.

The fundamental mechanisms of alkylation were discussed by Price (1958) and Warwick (1963). A vast amount of information concerning the biological activities of a number of chemotherapeutic agents was compiled in the Annals of the New York Academy of Science, Volume 68, Article 3 (1958). Ross (1962) wrote a monograph on biological alkylating agents alone. In Part I of his review the chemical reactivity of the compounds is defined with respect to mechanism and likely sites of interaction with cellular material. Part II deals with attempts which have been made to produce agents with selective toxicity towards cancer cells. More recently, a voluminous survey of alkylating agents, including aziridinyl compounds, was issued by the Cancer Chemotherapy National Service Center (Cancer Chemotherapy Reports, No. 26, January 1963). These agents were tested against cancer or described in the literature as prepared for such testing.

Alexander (1952) found that the biologically more active polyfunctional compounds are more effective in reducing the ability of nucleic acid to associate with basic protein than mono-alkylating

agents (Figure 9). It is generally accepted that the phosphate group of nucleic acid is affected, forming *in vitro* an unstable triester which gradually hydrolyzes to a more stable diester (Alexander and Stacey, 1958, and Ross, 1958). This causes a break in the DNA molecule (Figure 10).

Cross-linking of DNA molecules occurs when an alkylating agent with two active groups per molecule attaches to receptors on the DNA chain (Alexander, 1960) (Figure 10). Linking may be between different molecules or between parts of the same molecule. When enough different molecules are joined together, this portion of DNA is not soluble but gel-like.

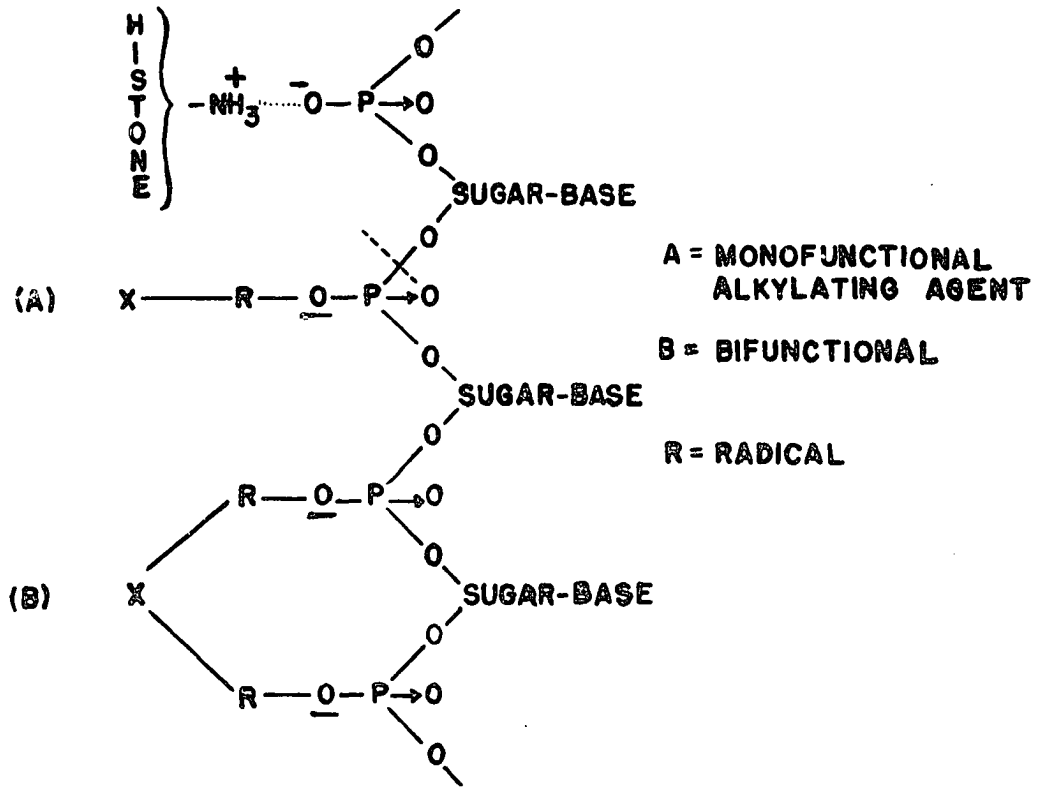
Alkylating agents have a greater affinity for the -SH group as found by Ogston *et al.* (1948) in the mustard gas reactions with compounds bearing this group. The functional groups in protein are shown in Figure 10.

Roitt (1954) reported that after 4 hours incubation of phospho-fructokinase and triosephosphate dehydrogenase with 0.01 M TEM (tretamine or triethylenemelamine) at 38° C showed 75 and 25 per cent inhibition, respectively.

Radiobiology of Enzymes

Ionizing radiation has effects on the enzyme activity *in vivo* which are difficult to analyze. The action of ionizing radiation on enzymes and viruses was discussed by Pollard (1955). Total-body exposure of rats to 500 r of X-irradiation has no effect on the nonmercapto enzymes such as catalase, alkaline phosphatase, esterase, arginase, and rhodanase

Figure 9. Chemical structures of nucleic acid and protein showing their reactive groups affected by alkylating agents (after Alexander, 1952, and Ross, 1958)


HISTONE
SUGAR-BASE
A = MONOFUNCTIONAL ALKYLATING AGENT
B = BIFUNCTIONAL
R = RADICAL
(A)
(B)
NUCLEIC ACID

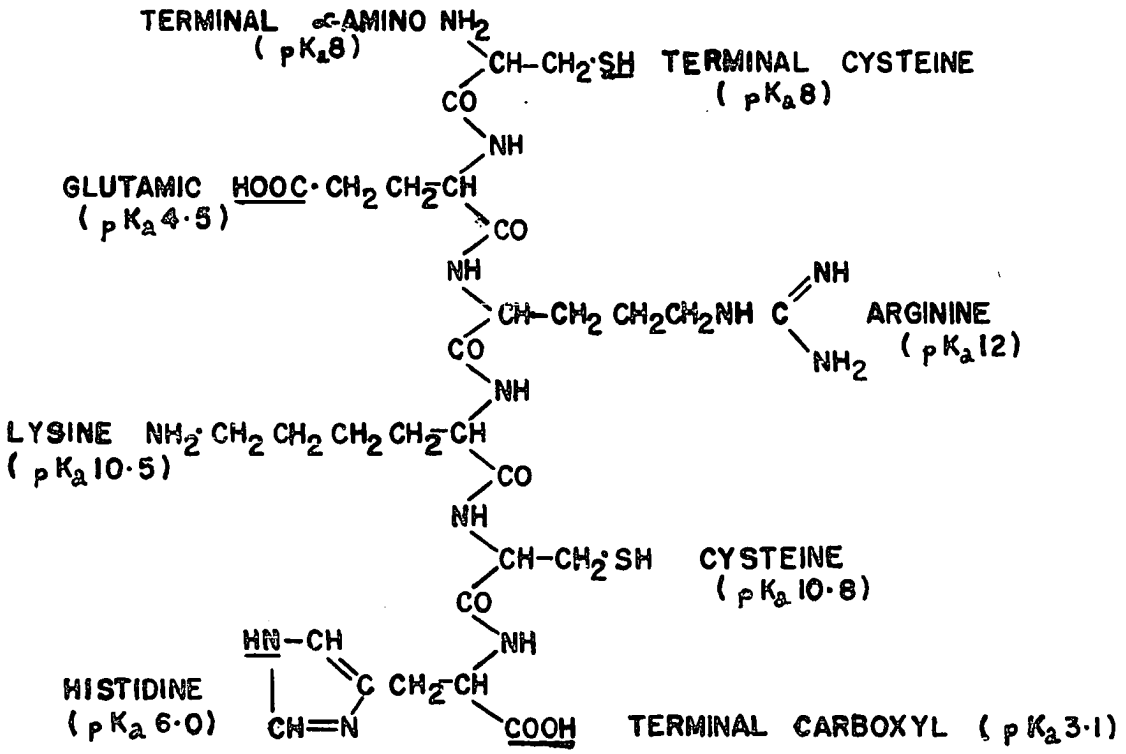
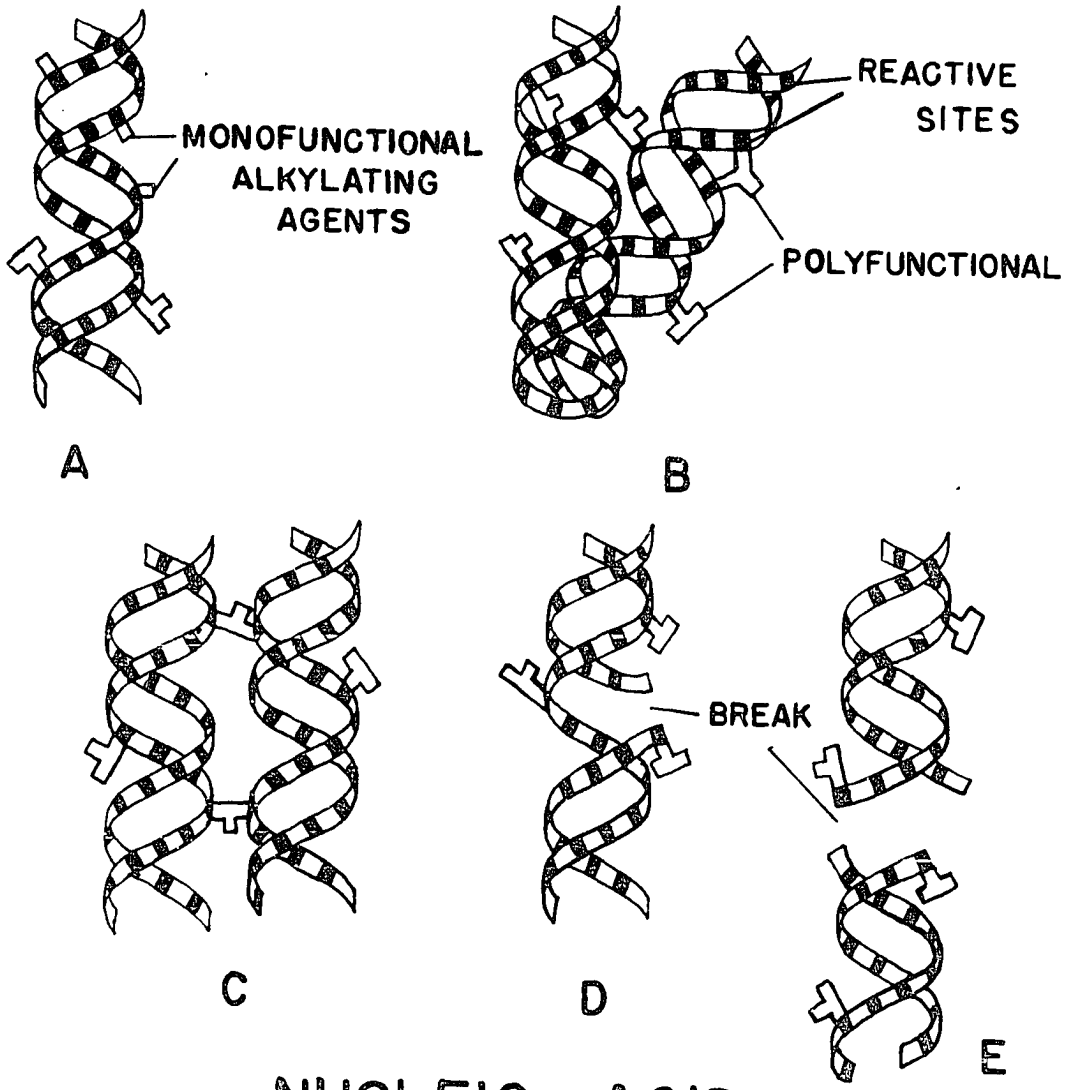

TERMINAL α-AMINO NH2 (pKa 8)
TERMINAL CYSTEINE (pKa 8)
GLUTAMIC (pKa 4·5)
ARGININE (pKa 12)
LYSINE (pKa 10·5)
CYSTEINE (pKa 10·8)
HISTIDINE (pKa 6·0)
TERMINAL CARBOXYL (pKa 3·1)
PROTEIN

Figure 10. Diagrammatic structures of nucleic acid and protein showing the binding sites of alkylating agents


MONOFUNCTIONAL
ALKYLATING
AGENTS
REACTIVE
SITES
POLYFUNCTIONAL
A
B
BREAK
C
D
E
NUCLEIC ACID
PROTEIN

in liver (Ludewig and Chanutin, 1950). The result of irradiation on liver enzymes are, however, not consistent. In a study of the effects of a whole-body exposure of mice with 600 r, Hori (1956) found a strong decrease in activity of catalase and oxidative phosphorylation and an increase in alkaline phosphatase, succinodehydrogenase, choline oxidase, cytochrome oxidase, and deoxyribonuclease. No changes were found in ribonuclease, arginase, esterase, and tyrosinase.

In rat spleen, acid DNase activity increases from 6 to 24 hr after 500 r; it drops back to normal at the forty-eighth hour. Meanwhile, the neutral activity (cf. Privat de Garilhe and Laskowski, 1955, and Novikoff et al, 1962), normally rather weak, progressively decreases (Douglass et al, 1954, and Fellas et al., 1954).

After a local exposure of rat bone to relatively high doses (2000 r) of X-rays there is a reduced uptake of Ca^{45} in the irradiated leg (Cohn and Gong, 1953). The alkaline phosphatase activity is sharply depressed, the depression closely parallel to the impairment of bone growth. The decreased uptake of P^{32} was also observed in the knee joint and tibia of six-week-old mice (Wilson, 1957). This effect is maintained 8 to 10 weeks after the exposure, when slow recovery begins.

Bacq and Alexander (1961) theorized that increased activity may be due "a) to the destruction of an inhibitor; b) to the release of an activator; c) to an increased synthesis of enzyme; d) in certain experimental conditions, to the release of bond (inactive) enzyme from its links with intracellular structures such as nucleus, mitochondria, microsomes and membrane". The release of hydrolytic enzymes due to break-down of lyzosomes ("suicide bags", Beaufay and De Duve, 1959,

and De Duve, 1963) after irradiation is a very tempting mechanism, but there is no evidence to support this view (Bacq and Alexander, 1961). The latter authors reported that lyzosomes in isolation require more than 10,000 r before a leakage of enzymes can be detected. The possibility remains that the lyzosomes are more sensitive within the cell.

A decreased enzyme activity may be due not only to the destruction of the enzyme but also to the failure of its synthesis, to the release of an inhibitor or to the disappearance of an activator (Bacq and Alexander, 1961). Enzymes, like DNases for instance, have inhibitors in plasma or urine.

Pure enzymes in vitro in dilute acqueous solution are much more radiosensitive than the same molecules "in vivo", or even in homogenates or extracts (Bacq and Alexander, 1961). Enzymes on isolated structures (mitochondria) are in vitro more resistant than in vivo. Addition of 1 per cent liver extract completely prevents the inhibition of a solution of pure glutamic acid dehydrogenase by 5 kr (Maass and Schubert, 1959). The only alteration observed after irradiation in vitro of pure enzymes is inactivation. Several authors have proposed possible ways in which the primary chemical effects of ionizing radiations can be reduced (Alexander and Charlesby, 1955; Hollaender, 1960; and Thompson, 1962).

A decrease in "pseudo" cholinesterase activity in rat, hydroquinone oxidase in the ova of Melanoplus differentialis, and catalase activity in the liver of mice after exposure to radiation were reported in the review of Bacq and Alexander (1961). However, many workers observed an increase activity of some enzymes. Goutier (1961) emphasized that

activation of DNases in mammalian cells is not an early phenomenon. It occurs rather late after important lesions of nucleotide phosphorylation and DNA synthesis have already been detected. There was an inhibition of DNA synthesis at relatively high doses of X-rays. It may be attributed to a) a decrease in phosphorylation of nucleotides leading to nucleotide triphosphates and b) an inhibition of the specific polymerase.

Presumably, the susceptibility of the different tissues to radiations influences that of the enzymes. Clemedson and Nelson (1960) discussed the radiobiology of the different tissues, cells, organs, and systems of adult organism. They classified the organs according to their diminishing radiosensitivity:

1. Differentiating tissues: lymphatic nodes, spleen, thymus, lymphatic tissue in other organs, bone marrow.

2. Rapidly dividing tissues: testes, ovaries, mucous membranes of intestinal tract, skin, hair follicles.

3. Slowly dividing tissues: cartilage, growing bone tissue, liver, adrenals, kidney, pancreas, lung, nervous system, muscles, connective tissues, and bone tissue.

Alkaline Phosphatase in Cancer and Neoplastic Tissues

That the term "alkaline phosphatase" describes a series of enzymes of similar but not identical properties has been recognized for many years (cf. Folley and Kay, 1936, and Moog, 1961). The physiological significance of phosphomonoesterases and alkaline phosphatases was reviewed by Moog (1946 and 1962). Phosphatases are commonly found in the cytoplasm of growing cells, regenerating, and secreting cells in which

protein synthesis is being carried on. There appears to be a correlation in such cells between the content of nucleic acid and of phosphatases. The review of Novikoff et al. (1962) relates the function of specific phosphatases to transport mechanism across membranes. Phosphatases are undoubtedly present in considerable amounts and/or activities in neoplasms which are hypothetically analogous to reproductive tissues.

Burstone (1962) gave an excellent account of phosphatases in relation to neoplasms. Numerous investigators studied the importance of enzymes in tumors and cancers. The histochemistry of alkaline and acid phosphatases and other enzymes in spontaneous mammary carcinoma of mouse was investigated by Murata et al. (1963). They found that alkaline phosphatase activity was in the luminal surface of the tubular structure but not in the periacinar region. Activity of alkaline phosphatase was variable in different portions of the same specimen. Richardson and Pearson (1954) reported that alkaline phosphatase activity was intensified by the estrogenic treatment as shown histochemically on the mammary cancer in stilbestrol-treated mice. No basic difference in intensity, however, was observed during the various stages of tumor formation. Alkaline phosphatase activities were demonstrated in brain tumor (Feigen and Wolf, 1959). Mori et al. (1962) showed histochemically a marked alkaline phosphatase activity in the hamster pouch stromal elements, e.g., inflammatory cells, vascular components, and proliferating connective tissue fibers, whereas it was absent in both the neoplastic and homologous non-neoplastic epithelia. They cited several authors who found increased alkaline phosphatase activity in human neoplasia, in experimental carcinogenesis, and in inflammatory gingiva of periodontal

diseases. Normal hamster pouch epithelium showed no alkaline phosphatase, whereas it appeared on the surfaces of neoplastic epithelia with severe inflammation. Murata et al. (1963) assumed that the appearance of this enzyme in neoplastic epithelia during experimental carcinogenesis is mostly related to inflammation, but also to some extent to the reproduction of the neoplastic epithelium as a result of cell injury from the topical application of the carcinogen.

Alkaline Phosphatase in Reproductive Organs

Moog (1944) localized alkaline and acid phosphatases in the different tissues of the early embryogenesis of the chick using sodium glycerophosphate substrate. Alkaline phosphatases are localized over the Feulgen-positive structures, in nucleoli, and in the cytoplasm of young oocytes in the gonads of some isopod crustaceans (Asellus aquaticus, Meinertia parallela, and Anilocra physodes) using sodium-β-glycerophosphate, RNA and DNA substrates (de Nicola, 1949). She indicated that they are involved in the metabolism of nucleic acids because of the close coincidence of their localization with that of the latter.

The cytochemical studies on the embryonic development of Drosophila melanogaster by Yao (1950a) showed alkaline phosphatase, using sodium glycerophosphate, in the ventral ectoderm near the future thorax during or shortly after the contraction of the germ band. The enzyme activity then spreads to the whole embryo following definite patterns, until finally the whole embryo becomes active. No alkaline phosphatase was demonstrated in drosophila ovaries taken from 1-day-old females.

However, the epithelium of the oviducts gives a moderate to strong reaction. The localization of alkaline phosphatase during the post-embryonic development of D. melanogaster was also described by Yao (1950b). Larval and prepupal gonads react very weakly. A very definite increase of phosphatase activity is noticeable in the 32-hour pupae, when gonads are found to be already joined by the genital ducts. Moderately strong alkaline phosphatase was observed in sperm heads, follicular cells, nurse cells and oocytes.

Kugler et al., (1952) detected no alkaline phosphatase in the follicular epithelium of ovaries in Periplaneta americana (L.). The tall columnar cells of the spermatheca, walls of colleterial glands, and the cytoplasm of the glandular epithelial cells of the right collaterial gland, known to secrete aquinoid tanning agent, showed a pronounced alkaline phosphatase activity. They dismissed, however, their negative findings in follicular epithelium of the ovary as due mainly to the mechanics of the technique because the same method was followed by Kugler (1953) to demonstrate high alkaline phosphatase in the same structure of all growth stages of oocytes of blue gill, Lepomis machochirus (Rafinesque). Nevertheless, the species difference was not considered in this assumption.

Sections of adult human testicles and of testicles from dog, rabbit, guinea pig, rat, and mouse were studied for nonspecific alkaline phosphatase and for 5'-nucleotidase (Wachstein and Meisel, 1954). The most outstanding feature in the testicles of all species examined is the presence of 5'-nucleotidase predominantly located in the nuclei of the spermatogenic elements. Alkaline phosphatase activity increased

in the cytoplasm during the maturing stage of oocytes of Notopterus notopterus (Pallas), a fresh water fish, and thereafter it decreased (Venugopalan, 1961). The nucleoli consistently exhibited high alkaline phosphatase activity.

Krugelis (1942) found alkaline phosphatase activity in the mitotically active chromosomes of mouse testes but not in spermatids or sperm. Alkaline phosphatase activity was demonstrated in the banded structures of larval salivary chromosomes of D. melanogaster (Krugelis, 1945).

Apholate and other Chemosterilants in Entomological Research

The effects of anti-mitotic agents such as aminopterin, nitrogen mustard, colchicine, and podophyllotoxin in skim milk fed to the house fly, Musca domestica (L.) were studied by Mitlin et al. (1957). They found the first three to inhibit oviposition and also ovarian growth. Folic acid and thymidine did not reverse the effect of aminopterin on fly ovaries.

Many more cancer chemotherapeutic agents are currently being tested. Up to the time of the writing, no compounds are legally certified as insecticides for commercial distribution. Apholate, tepa, methiotepa, metepa, and tretamine show promising effects as arthropod chemosterilants.

Numerous publications and research on chemosterilants are currently saturating almost all areas of entomology. Borkovec (1962) and Knipling (1962) gave inclusive reports on the possibility of this group of chemicals for eradication of harmful insects. Smith et al. (1964) surveyed the literature pertaining to chemosterilants up to March, 1963. They

tabulated individual chemicals and the corresponding test species on which they had been used.

The aziridinyl derivatives adversely affect the physiology of reproduction of male and female insects. Apholate is among the most active chemosterilants currently under investigation (Knipling, 1962). The general effects are inhibition of ovarian growth, egg production, and hatchability on house flies, Musca domestica L. (LaBrecque, 1961); stable flies, Stomoxys calcitrans (L.) (Borkovec, 1962); screw-worm flies, Cochliomyia hominivorax (Coquerel) (Crystal and LaChance, 1963); citrus red mites, Panonychus citri (McGregor) (Cressman, 1963); and boll weevils, Anthonomus grandis Boheman (Hedin et al., 1964). Morgan and LaBrecque (1962) observed that apholate in feed inhibited but did not eliminate ovarian development. The germarium was also inhibited. Apholate caused complete sterility of screw-worms, applied on ground-beef mixture (Chamberlain, 1962) and to house flies when mixed with skim milk (Painter and Kilgore, 1964). The latter considered this compound as a "permanent" sterilant.

The above phenomena parallel the findings of Walker and Brindley (1963) on X-irradiated European corn borer Ostrinia nubilalis (Hubner). There was a decrease in egg production and hatchability as the dose increased. Similar observations on different species of insects studied by various investigators were tabulated in their paper.

Each group of investigators used different methods of applying chemosterilants. Some topically applied these compounds in solution while others mixed them with skim milk, manufactured house fly food or raw meat. The disadvantage to these methods is that the protein

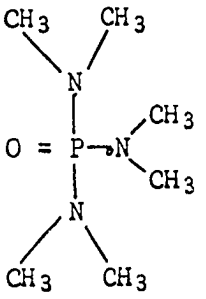
in the media could degrade a considerable amount of chemosterilant before the test species could pick it up. Consequently, this will give experimental errors. Murvosh et al. (1964) proposed a method to measure the effect on house flies of metepa, apholate, and tepa mixed with diet by plotting valid concentration/sterility regression lines.

Many of these chemosterilants showed toxicity on different insect species at various condition. To cite an example, apholate at higher concentrations is lethal to most insects studied.

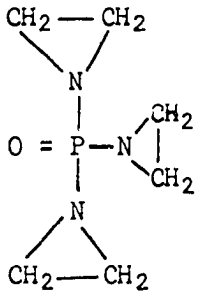
More recently, two insect chemosterilants with low mammalian toxicity but lacking alkylating properties are under investigation (Chang et al., 1964). They are hexamethylphosphoramide (HMPA) and hexamethylmelamine (HMM) (Figure 11). The former is closely related to tepa and the latter, to tretamine. They are found to be effective in sterilizing male house flies.

Notes on Enzyme Phosphatase Localization

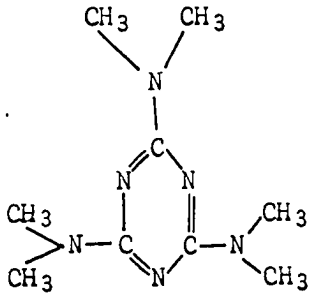
The calcium phosphate precipitation method of localizing enzyme phosphatase in tissues was first done by Robison (1923). It was developed by Gomori (1939) and Takamatsu (1939) as a standard histochemical procedure in enzymology (Figure 12). Danielli (1946) and Gomori and Benditt (1953) proved in their critical studies that sites and reactions are quantitatively determined. More recently, Danielli (1958) discussed in detail the theory of the method, instrumentation, and general techniques employed in calcium precipitation method for alkaline phosphatase. Gomori (1952), McManus and Mowry (1960), and



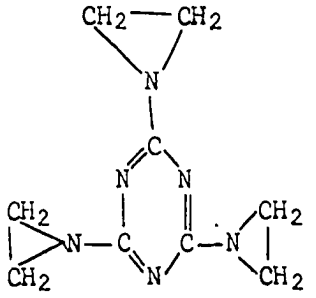
HMPA



TEPA



HMM



TRETAMINE

Figure 11. Two new compounds HMPA (hexamethylphosphoramide) and HMM (hexamethylmelamine) with low mammalian toxicity and showing chemosterilant properties

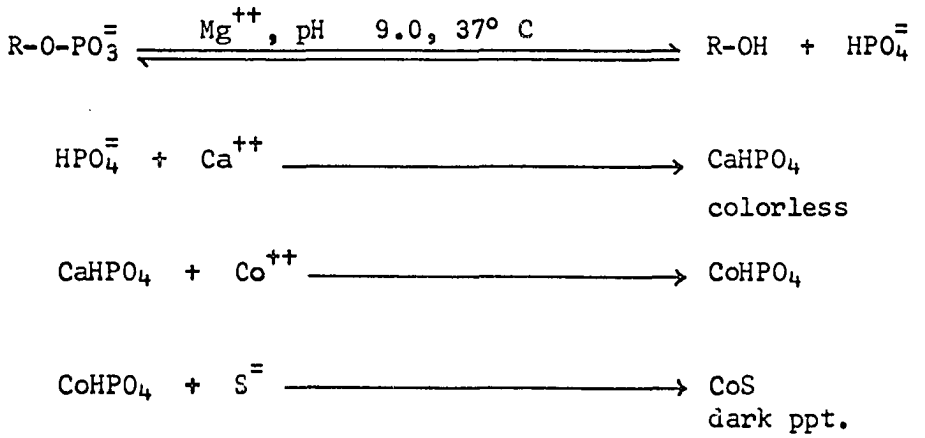


Figure 12. Calcium phosphate precipitation method

Burstone (1962) pres ent histological methods and preparations valuable to localization of enzymes in tissues.

Alkylating Agents and Their Reactions with Protein

Alexander (1954) summarized that the reactions of carboxyl groups of proteins near their isoelectric point with the cytotoxic alkylating agents is anomalous. From chemical consideration, supported by experiments with model substance and other macromolecules, reaction with the carboxyl groups and its reaction with amino groups are to be expected. Mustard gas comes closest to these predictions; but the ready reaction of amino groups with nitrogen mustards, epoxides, and possibly also with ethylenimine and mesyloxy compounds is completely contrary to expectations. This anomaly may be due to the fact that groups are hidden within the protein and sterically inaccessible to alkylating agents, but this does not seem likely.

Alexander (1954) and Davis and Ross (1952) have suggested that the unexpectedly high reactivity of amino groups in some proteins is due to the salt linkage with a carboxyl group having the structure

$$—NH_2...HOOC—$$

rather than

$$—\overset{+}{N}H_3...\overset{-}{O}OC—.$$

The first form is uncharged and therefore reactive.

MATERIALS AND METHODS

General Procedures

A diagrammatic representation of a general experimental procedure is shown in Figure 13. The in vivo treatment was subdivided into different volumes from 2 µl to 10 µl of 0.5 to 2% (w/v) apholate in 0.05 M glycine-NaOH buffer solution at pH 7.4. These are represented by Arabic numerals in Figure 13. The tissue sections for histochemical tests are represented by capital letters in the same figure.

There were two types of controls used. One was the control for apholate treatment in vivo and the second control for histochemical techniques in vitro. The former received nothing but the buffer solution. The histochemical control consisted of sections of reproductive tissues from in vivo treated and control individuals. These sections were placed into the incubating solutions which contained no phosphatase substrates.

The choice of buffer for pH 7.4 is not ideal; however, this condition is more stable than that of distilled water alone. Since glycine has a simple chemical structure, has known chemical reactions, has no cancer chemotherapeutic importance (Lettre, 1953, and Stock, 1953), and most of all has no adverse effect on southern corn rootworm, it was used as a buffer solution in this experiment. Acid catalysis of apholate with itself was prevented under this condition.

The pH range of hemolymph for almost all insects is 6.0 to 8.0 (Patton, 1963). The pH was maintained within this limit by using glycine-NaOH buffer solution. It was assumed that any descrepancy in apholate reactivity due to the presence of glycine could be accounted for, unlike

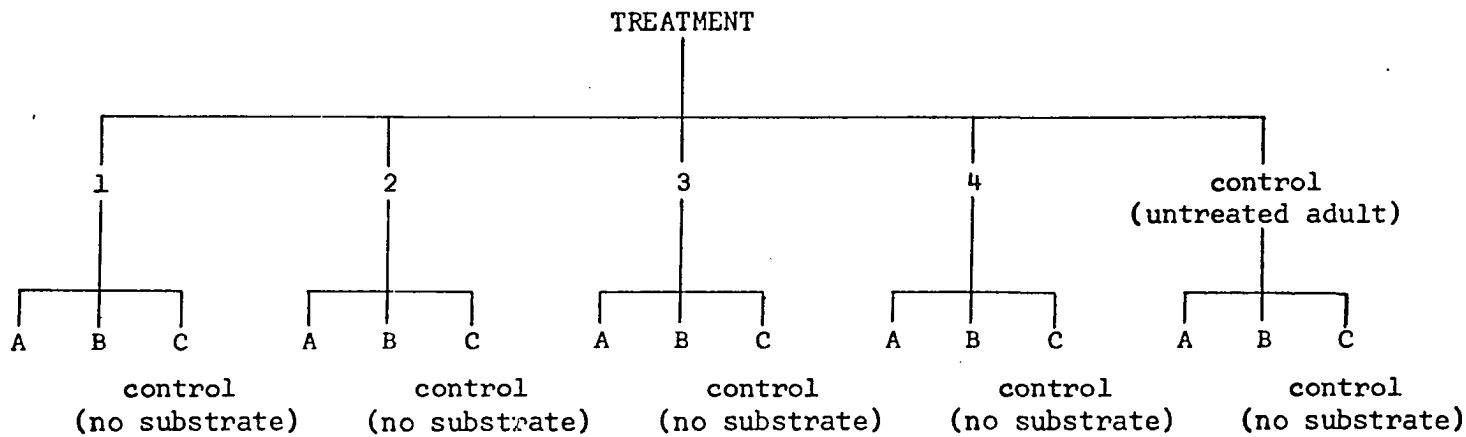


Figure 13. Diagrammatic representation of experimental procedures

that due to the formation of a network of crosslinks under acid conditions.

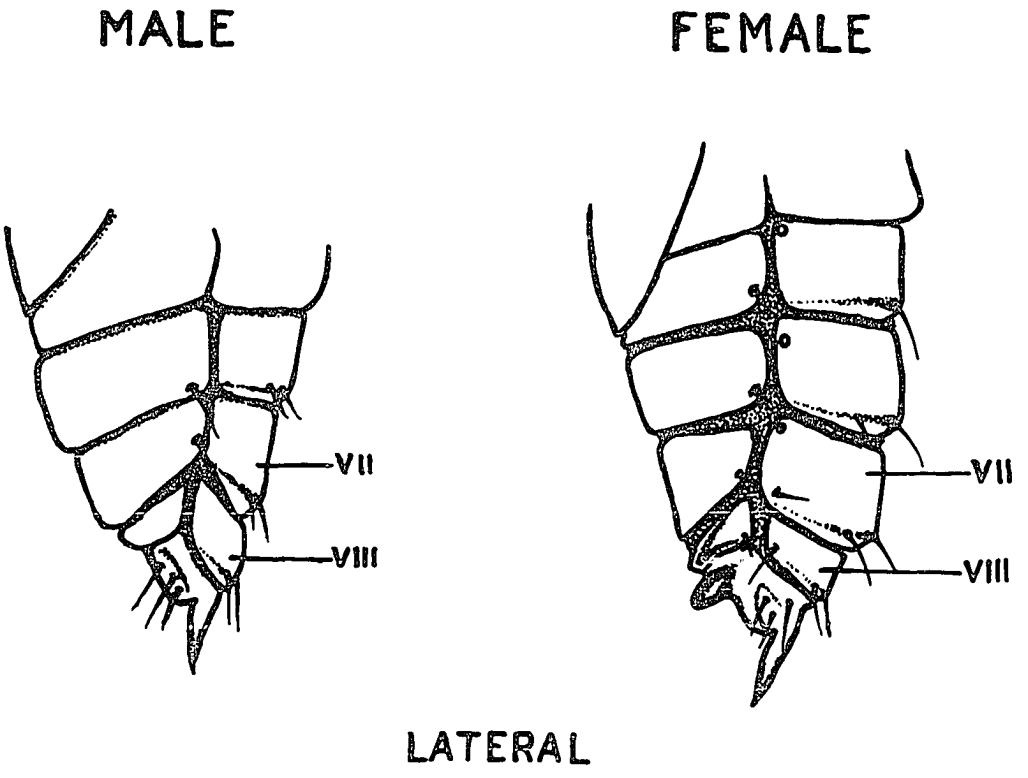
Apholate solution was injected through the pleural suture of adult southern corn rootworms with a 50-µl Hamilton microsyringe having a gage 31 needle with Kel-F hub. Only virgin adults were used. Mating was prevented by separating the sexes during the pupal stage. The female pupa has stubby papillae anterior to the anal pore (Figure 14). These are lacking in male pupae.

Apholate was also added to the adult artificial diet. The concentration used was 0.5 g apholate to 100 g diet. The adults were allowed to feed for 4 to 10 days on the diet with the following composition:

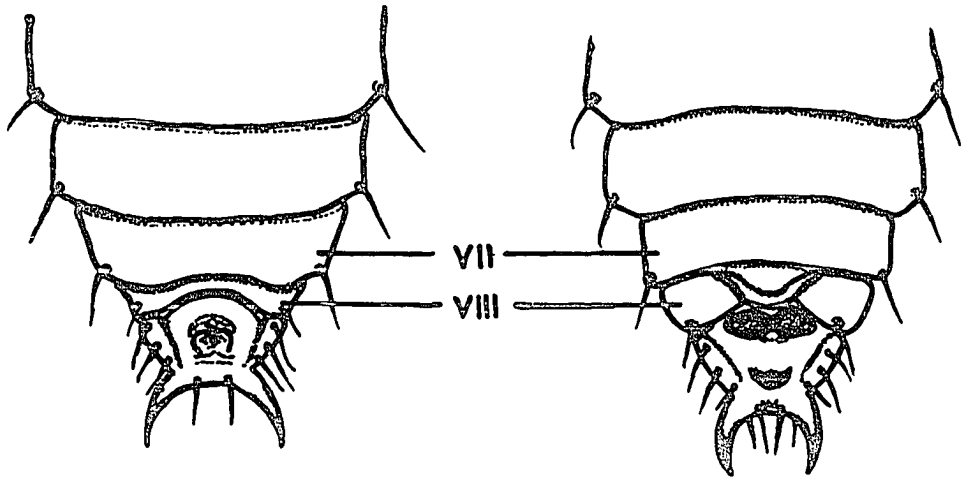
Water	80.0 ml
Agar	2.8 g
Cellulose	5.5 g
Glucose	17.4 g
Casein (vitamin-free)	17.4 g
Cholesterol	1.4 g
Corn Oil (plus 1% α-tocopherol)	1.4 ml
Salt mixture	2.1 g
Choline chloride	0.4 g
Brewers' yeast	13.9 g
Ground corn leaves	13.9 g
Riboflavin	0.5 g

This mixture is essentially the conventional European corn borer diet. Riboflavin was added because, for an unknown reason, the beetles seemed to feed readily when this was mixed with the diet. The feeding period was limited on this diet to prevent any adverse effect on the adults due to the dietary factors. After 4 to 10 days in this medium, the adults were fed lettuce or corn silks.

Figure 14. Abdominal portion of *Diabrotica undecimpunctata howardi* pupae showing sexual dimorphism (about 25X)



LATERAL



VENTRAL

Live adults were dissected in saline solution under a dissecting microscope. Five- and ten-day histochemical observations were made. The reproductive organs were taken out and freed of fat bodies. These were then transferred immediately into cold acetone. Testes were observed for sperm activity by squashing the testes in a small drop of insect-physiological saline solution between a microscope slide and a glass cover slip.

The alkaline phosphatase activities in the reproductive tissues were studied before and after apholate treatment. To test the interaction of alkaline phosphatase and apholate, normal reproductive tissue sections were incubated in solutions with DNA, with DNA plus 1 ml of 1% apholate solution, and without DNA or apholate. The tissues incubated in the first solution were a positive control and the last, a negative control. The latter is a test for the detection of preformed $CaHPO_4$ in the tissue. Those sections in the second incubating solution were a test for the involvement of alkaline phosphatase in causing the precipitation of DNA phosphate groups.

Observation of alkaline phosphatase activity with DNA is one of the bases for the determination of apholate effects on the reproductive tissues of southern corn rootworms. Some phosphomonoesterases belong to the alkaline phosphatase group. Phosphomonoesterase I, nucleotide phosphatases, 5'-nucleotidase, and presumably nucleosidephosphatase are but a few of the members of this group. Many of these are very specific in terms of substrate and physical factors while others have a broad spectra of reactivity. Acid phosphatase is another subdivision

of the phosphatases, and possible activities of these were also tested.

Histochemical Procedures

Gomori's technique (1930), with minor modifications which are described later, was used to determine the phosphatases in the reproductive tissues. The male and female reproductive organs were fixed with cold acetone for two to six hours. Then they were transferred into cold acetone for 15 to 30 minutes followed with cold xylene for three to five minutes. From xylene, the tissues were transferred into 57° C melted paraffin. The paraffin was not allowed to heat beyond 60° C. Impregnation with and embedding in paraffin of tissues were subsequently done within 30 minutes at 56 to 60° C. An improvised vacuum pump was used to impregnate tissues with paraffin.

Sectioning of the tissues was done within less than a day from embedding. The microtome was set at the 8-μ mark which was thick enough to obtain decent sections and thin enough to allow adequate observation of the product of chemical reactions. By using a cold microtome blade better sections were obtained. Not less than 6 sections were randomly affixed on a microscope slide and at least two microscope slides were used for replication.

Deceration was done from three to five minutes and hydration from 7 to 10 minutes. From absolute alcohol, the slides were dipped momentarily into 0.5% collodion to protect against the loss of enzymes during the hydration procedure. A series of diminishing concentrations of ethyl alcohol was used to hydrate the tissue sections.

The tissues were incubated at 37° C for 12 hours in an incubating veronal-buffer solution containing substrates. The pH was maintained at 9.0 to 9.5 for sodium β-glycerophosphate (0.1 and 0.01 M), RNA (50 mg in 50 ml solution), AMP (0.05 and 0.005 M), dCMP (0.005 M), and DNA, not highly polymerized, (50 mg in 50 ml solution) substrates[1]. The dissolution of $CaHPO_4$ precipitate at lower pH levels was avoided. For 5'-nucleotidase detection, however, the solution with AMP was kept at pH 8.7 although its maximum activity is at about pH 8.3. Similarly, from this pH up to 8.8 the dissolution of $CaHPO_4$ was prevented without losing the enzyme activity. To compare the substrate-enzyme reactions, the incubating solutions with AMP and with DNA substrates were kept at about pH 8.7 and 8.8, respectively. The descrepancy in pH was considered not significant because phosphatase activity occurs over a wider pH range.

After 12 hours, a predetermined adequate incubation period, the slides with tissues were rinsed momentarily with distilled water and were immersed in 1% $Co(CH_3COO)_2 \cdot 4H_2O$ solution for five minutes. Then, they were rinsed thoroughly with water, and were transferred into dilute yellow ammonium sulfide (0.4 ml:50 ml) for three minutes. The slides were rinsed again before mounting the tissues in glycerin jelly.

For the acid phosphatase localization, the same technique was employed but at pH 4.8. Incubation periods from 30 minutes to 12 hours in length were tried.

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[1]Biochemicals purchased from Nutritional Biochemical Corporation, Cleveland, Ohio.

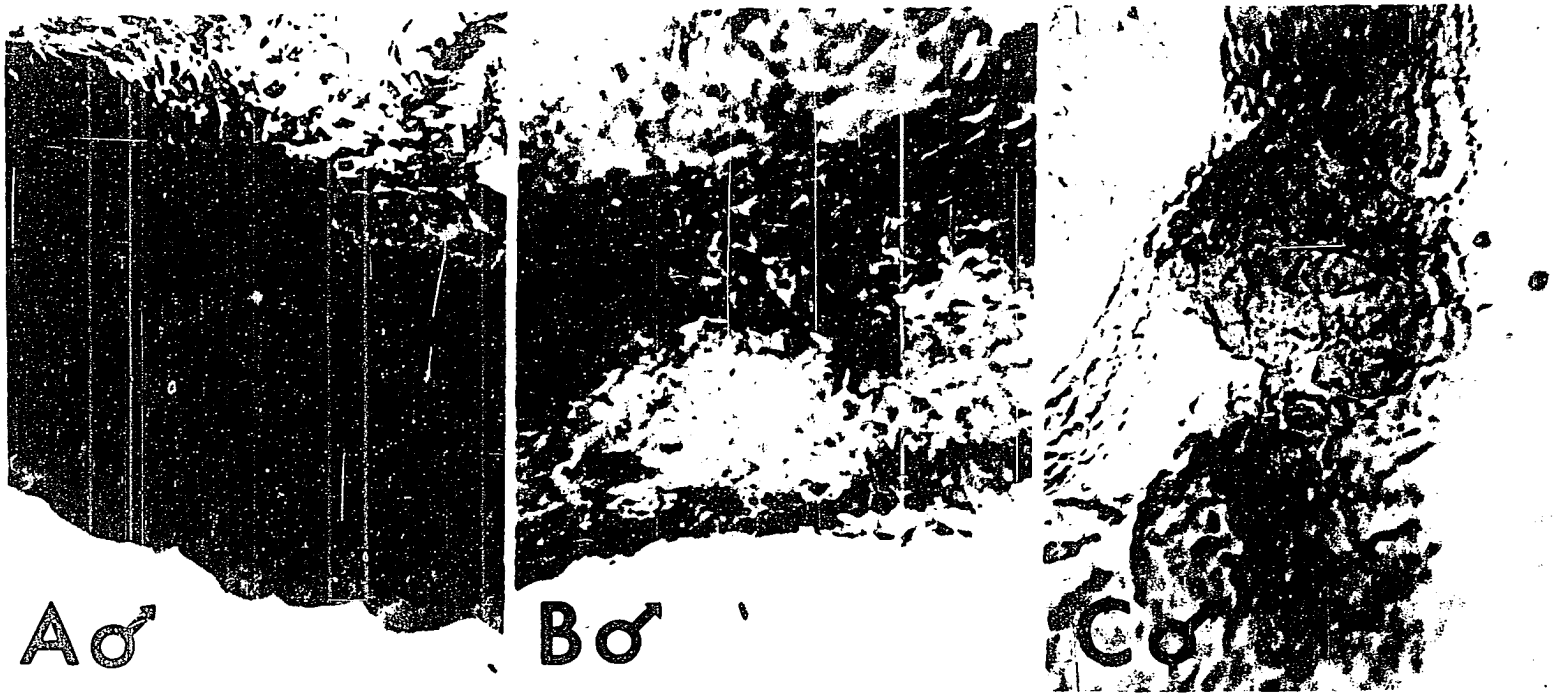
RESULTS

Of the various compounds for which specific tests were attempted, only alkaline phosphatase was readily demonstrable. Acid phosphatase with the activity below pH 7, frequently between pH 3.8 and 6.0, was not detected in the tissues probably because it may not be present in considerable amounts and thus its reaction product is not visible with the phase-contrast microscope. Moreover, acid phosphatase is not as widely distributed in animal tissues as its alkaline counterparts (Moog, 1962). Negative phosphatase activities were obtained from sodium-β-glycerophosphate, dCMP, and RNA. No reliable and precise results were observed from AMP-treated tissues for 5'-nucleotidase test. A positive deposition of precipitate was observed only on tissues incubated in DNA-Gomori's stock solution at about 9.4.

Roles of Alkaline Phosphatase and Apholate in the Calcium Precipitation

The normal tissues incubated in solution with DNA substrate and 1 ml 1% apholate showed a characteristic clearing (Figure 15) indicating the failure to obtain calcium phosphate precipitation. This may be due to direct inactivation of enzyme and alkylation of DNA substrate; both of which will prevent precipitation of calcium phosphates. Alkylation can change the reactivity of the enzyme. The effect of apholate on southern corn rootworm will be elucidated further in succeeding paragraphs.

Figure 15. Photomicrographs of D. u. howardi testicular tissues showing effect of apholate on the deposition of calcium phosphate precipitate (see center picture) (about 900X)



DNA

DNA +1ml 1% apho-late

no DNA

UNTREATED TISSUES

Intense precipitation of calcium phosphates was observed in tissues incubated in DNA-Gomori's solution. Very slight precipitation was evident in tissues incubated in Gomori's solution, indicating the negligible role of the preformed calcium phosphates in the experiments.

Effects on the Sperms

Injection with 2 µl of 1% apholate gave no marked adverse effort on the sperms; most males exhibiting many very active sperms as shown by characteristic swirling (cf. Figure 4), much the same as the untreated individuals. Increasing the volume of 1% apholate to 5 µl weakened and inactivated sperms and at this rate the characteristic swirling of the sperm aggregates was generally not detected. At 2 µl of 2% apholate, likewise, sperms appeared weak or inactive, and swirling was not observed in any case. Increasing the injected volume of 2% apholate solution resulted in more lethal effects on sperms.

Cantwell and Henneberry (1963) reported that after the eighth day from irradiation or apholate treatment time there were abnormal changes in the testes of D. melanogaster and a cessation in the production of sperm as the test progressed. They delivered approximately 320 r/min or fed 0.25 or 1% apholate in sugar-yeast bait for 24 hours to Drosophila adults. Few sperm packets were visible in the tube after 10 days. The activity of sperms was not observed, however.

The sperm viability of the boll weevil, A. grandis, was decreased by allowing the weevils to feed on a diet containing from 0.001 to 0.020% of apholate (Hedin et al., 1964). Similar results were observed when the weevils were fed plants sprayed with 0.5 and 2.5% solution of apholate.

Actual examination of the motility of sperms was not performed. Their assumption was based on hatchability of eggs laid by untreated females mated with treated males.

Effects of Apholate on Alkaline Phosphatase in the Male Reproductive Organs

The effect of apholate on alkaline phosphatase is shown histochemically (Figures 16 and 17). As de Nicola (1949) observed, the site of alkaline phosphatase activity coincides with the Feulgen-positive areas (cf. Part I). Intense precipitation was noticeable on male reproductive tissues from untreated individuals. These are the germaria and zones of growth and maturation which are not distinctly demarked.

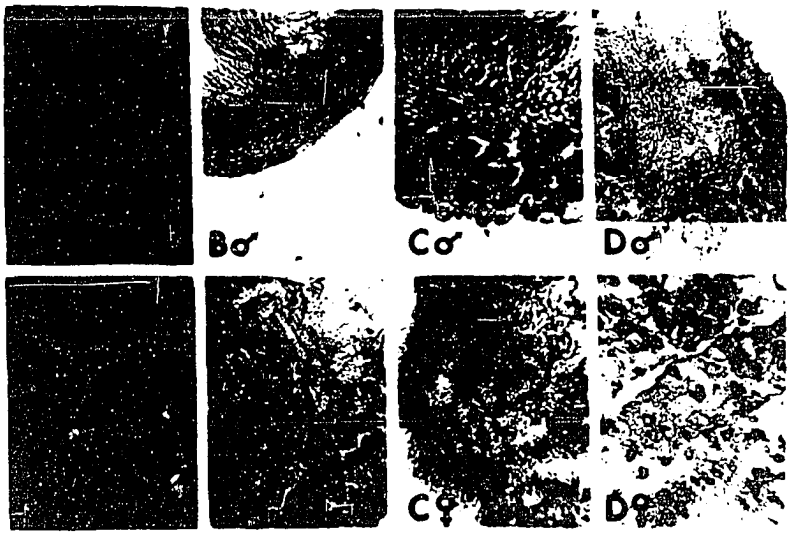
The reproductive tissues from the apholate-treated males showed a very marked clearing or failure of the calcium phosphate to precipitate (Figure 16B, C, and D males). This was due to the inactivation of alkaline phosphatase on the male reproductive tissues of southern corn rootworm.

Effects of Apholate on Alkaline Phosphatase in the Female Reproductive Organs

As was observed in the male reproductive tissues, the germaria of ovarioles of untreated females showed intense alkaline phosphatase activity (Figures 16 and 17). Likewise, heavy precipitation of calcium phosphate was evident on the epithelia of the egg chambers. The cytoplasm of the young oocytes was also positive for alkaline

Figure 16. D. u. howardi male testicular and female germarium tissues showing the effect of apholate treatment (about 330X)

Figure 17. D. u. howardi female genital chamber and germarium tissues showing the effect of apholate treatment (about 330X)



control 2 µl 3 µl 4 µl
2% apholate

MALE TESTES & FEMALE GERMARIUM TISSUES

A. control B. 2 µl, C. 3 µl, D. 4 µl of 2% apholate
E, F, & G. histochemical control. (♀ genital chamber tissues except G = germarium)

phosphatase activity but not the mature ones. Similar findings were reported by Venugopalan (1961) in the maturing stage of oocytes of N. notopterus, a fresh water fish.

The reproductive tissue sections from apholate treated females similarly exhibited diminution of alkaline phosphatase activity. This effect was evident on the germarium, young oocytes, follicular epithelium, and the Feulgen-positive layer of tissues of the genital chamber.

Effect of Increasing Concentration of Apholate on Alkaline Phosphatase

The minimum effective amount evident histochemically was 2 µl of 1% apholate. Observations were made five days after injection. The effect was evident within the female ovaries. There seems to be no marked effects on testes. Five microliters of 1% and 2 µl of 2% showed a very distinct effect on both testes and ovaries. A very noticeable inactivation of phosphatase was manifested by treatments of higher concentrations.

Notes of Mortality of Adults

The summary of the tests involving injection of apholate solutions into the adult southern corn rootworms is shown in Appendix A, Table 3. Mortality records are also included.

Injection of 50 µg of apholate into adult southern corn rootworms resulted in about 50% mortality during the 5-day observation, Table 1. Increasing dosages up to 100 µg augmented the mortality up to 80%.

Percentage morality during the 10-day period after the injection of apholate into adult southern corn rootworms is shown in Table 2. Mortality ranged from 68 to 100%. As was mentioned before, apholate is

Table 1. Percentage mortality of injected Diabrotica undecimpunctata howardi Barber adults during the five-day period after injection

	1% apholate					2% apholate				Glycine control					Non-injected control
	2 µl	3 µl	4 µl	5 µl	10 µl	2 µl	3 µl	4 µl	5 µl	2 µl	3 µl	4 µl	5 µl	10 µl	
Male	25	0	12	52	0	50	61	67	80	36	7	15	54	0	13
Female	22	-	-	42	34	37	73	67	60	43	0	12	44	33	7

Table 2. Percentage mortality of injected Diabrotica undecimpunctata howardi Barber adults during the 10-day period after injection

	1% apholate					2% apholate				Glycine control				
	2 µl	3 µl	4 µl	5 µl	10 µl	2 µl	3 µl	4 µl	5 µl	2 µl	3 µl	4 µl	5 µl	10 µl
Male	100	100	75	76	100	87	87	100	80	36	14	31	62	0
Female	87	-	-	68	100	82	90	66	100	57	0	12	48	33

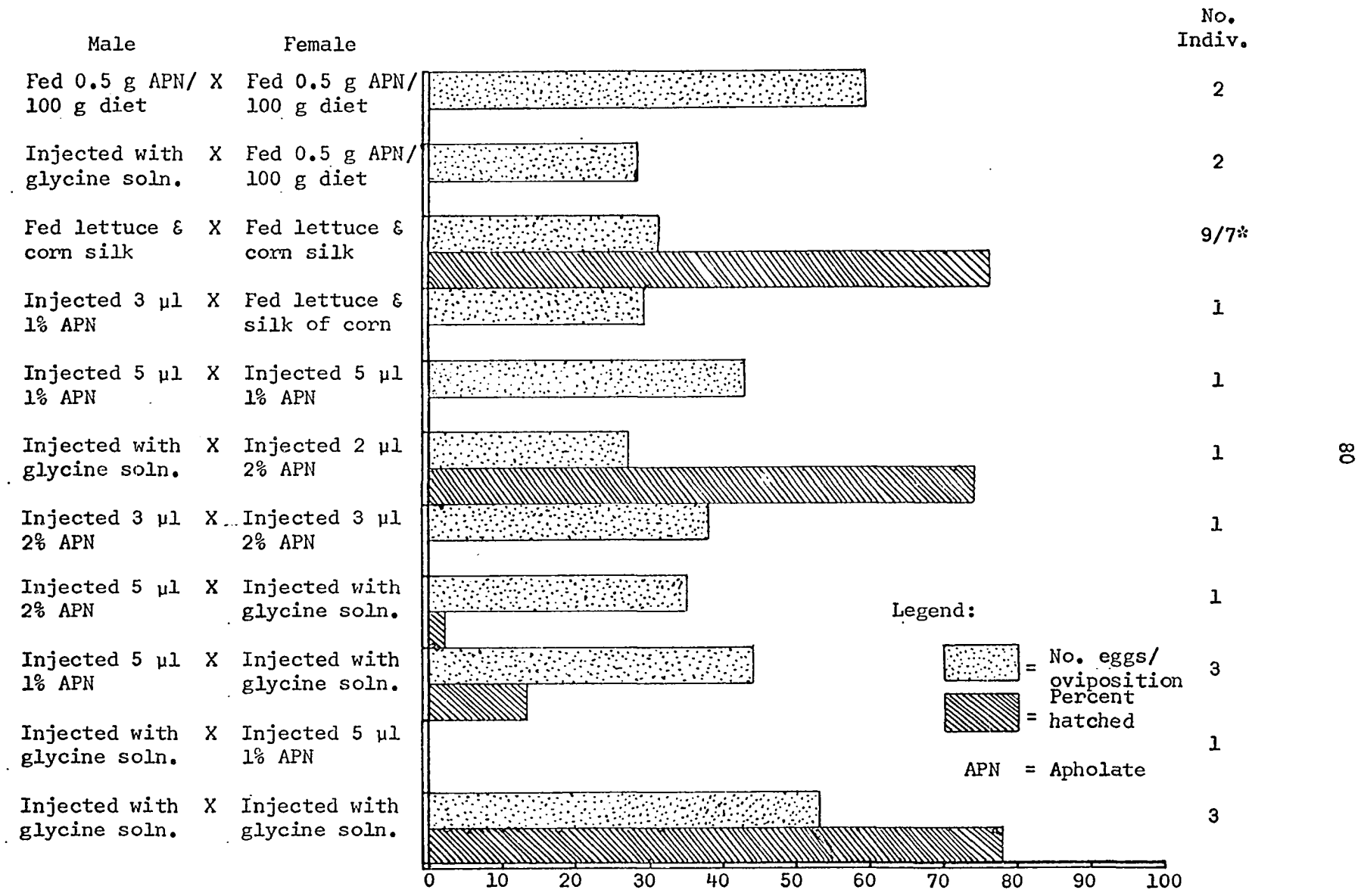
highly toxic to insects, and evidently adult southern corn rootworms will be killed by dosages just above those necessary for sterilization.

Effects of Apholate on Egg Production and Hatchability

Figure 18 indicates that the egg production was not inhibited by the highest concentration of apholate applied either by feeding or by injection. Apparently, male virility was adversely affected as shown by very low hatchability to complete non-hatching of the eggs of the untreated females when mated with treated males (cf. Hedin et al., 1964). Hatching of eggs was observed in no case where both males and females were treated. A high hatching percentage was observed on eggs laid by a female injected with 2 µl 2% apholate and mated with the control male. The very small volume of this treatment might have brought mechanical error during injection. Contrary to the rest of the observations, the female injected with 5 µl of 1% apholate did not lay any eggs although it lived for about 4 months.

Figure 18. Effects of different treatments and mating combinations on production and hatchability of eggs

*Number of eggs per oviposition period was based on 9 individuals and hatching percentage was based on 7


Male
Female
No. Indiv.
Fed 0.5 g APN/ 100 g diet X Fed 0.5 g APN/ 100 g diet
2
Injected with glycine soln. X Fed 0.5 g APN/ 100 g diet
2
Fed lettuce & corn silk X Fed lettuce & corn silk
9/7*
Injected 3 µl 1% APN X Fed lettuce & silk of corn
1
Injected 5 µl 1% APN X Injected 5 µl 1% APN
1
Injected with glycine soln. X Injected 2 µl 2% APN
1
Injected 3 µl 2% APN X Injected 3 µl 2% APN
1
Injected 5 µl 2% APN X Injected with glycine soln.
1
Injected 5 µl 1% APN X Injected with glycine soln.
3
Injected with glycine soln. X Injected 5 µl 1% APN
1
Injected with glycine soln. X Injected with glycine soln.
3
Legend:
= No. eggs/ oviposition
= Percent hatched
APN = Apholate
0 10 20 30 40 50 60 70 80 90 100

DISCUSSION

Gomori (1939) stated that if the tissue is well dehydrated, exposure to paraffin at 56 to 60° C for two hours will do no harm. Similarly, I found no histochemical alteration among tissues impregnated at this temperature range. However, impregnation with and embedding in paraffin of tissues were subsequently done within 30 minutes at the same temperature range.

Histochemical control showed that the preformed calcium phosphate is negligible. The detection of alkaline but not acid phosphatase may be explained by Roche (1950) and Burstone (1960) findings, that tissues endowed with an intense alkaline phosphatase activity are practically devoid of acid phosphatase.

The specificity of this group of alkaline phosphatases to DNA is quite remarkable. Calcium precipitate can only be obtained histochemically upon the cleavage of phosphate groups from a substrate molecule. With DNA as substrates, there are at least two possibilities that might explain this phenonemon. First, the enzyme may be very specific to DNA phosphate end-groups. These phosphate groups are expected to predominant in the medium because the DNA used was not highly polymerized. Oligonucleotides and mononucleotides can be expected in this preparation. On the other hand, the precipitation observed may be due to a combination of enzyme systems, each component is reactive at alkaline pH and is specific to DNA molecules. Longer protions of DNA chain are known to be cleaved off by DNase I (alkaline DNase). In addition, the presence of "neutral" DNase (cf. Privat de Garilhe and Laskowski, 1955) can

further depolymerize shorter polynucleotides. This degradation of DNA will cause an increase of phosphate end-groups that will be exposed to the action of alkaline phosphatase. Thus, the calcium precipitation is subsequently intensified.

The involvement of an enzyme phosphatase was very evident in the preceding results of the tests. At this junction it is worthwhile to note a report of Grushina (1957), on the effect tepa on the activity of acid and alkaline phosphatases in transplanted Sarcoma "45". Under the influence of tepa a diminution in the activity of acid and alkaline phosphatases during the process of tumor regression in transplanted rat Sarcoma "45" was observed. In a number of degenerating tumor cells, however, phosphomonoesterase activity continued at a high level in the karyoplasm of the pycnotic nuclei and nucleoli. The diminution of alkaline phosphatase levels in the reproductive tissues after the injection of southern corn rootworm adults with apholate is further substantiated by the review of Wheeler (1962) which indicated a decrease in this enzyme activity of rat kidney and small intestine by mannitol mustard.

The decrease of alkaline phosphatase in reproductive organs obtained when southern corn rootworm adults were treated with apholate may somehow elucidate the mode of action of apholate or alkylating agents. Borkovec (1962) presented his ideas as follows:

> The most important, and still unresolved, question concerns the identity of this biologically significant molecule. The mode of action of some antimetabolites (for example, 5-fluorouracil) has been elucidated to a considerable extent (Timmis, 1961), and it appears that

> with these compounds the biologically significant molecule is an enzyme which is normally required for the *in vivo* synthesis of nucleic acids. There seems to be no reason to believe that the same mechanism is not applicable to an insect organism. ...A recent suggestion by Timmis (1961) is of interest in this connection. He speculates on the possibility that the action of alkylating agents is essentially similar to that of the antimetabolites although the blocking of the important enzyme may not be accomplished by the alkylating agent itself but rather by an antimetabolite which the agent forms *in situ* with an available metabolite. A second alkylating function would be an added advantage because the newly formed antimetabolite would be able to react irreversibly with the enzyme and its efficiency would be thus increased. It is interesting to note that this hypothesis not only would explain the apparently higher activity of the *bis*-aziridines as compared with monoaziridines but would account also for the lack of difference between *bis*-, *tris*-, and other oligoaziridines.

Philips (1950) and Ross (1962) reviewed the 'enzyme inactivation' theory by alkylating agents. On the other hand, Hendry *et al.* (1951) postulated structures for the reactive polymer formed *in situ* from bis-ethyleneimine and a nitrogen mustard (Figures 19 and 20). The possibility of the formation of a polymer-cell-component complex is

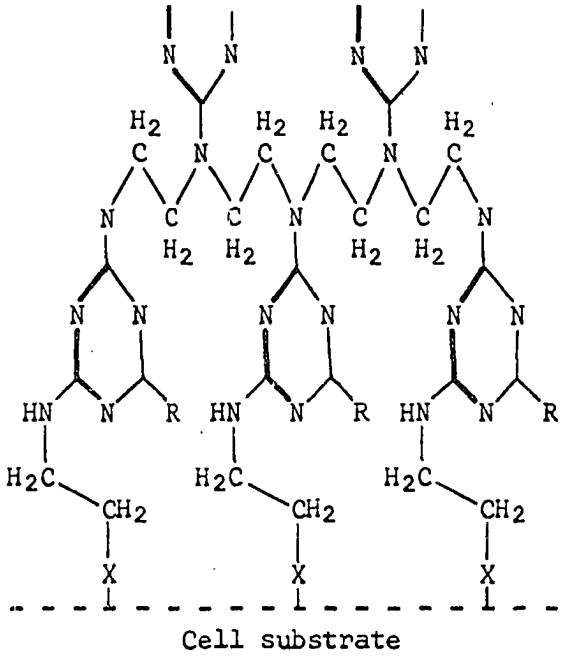


Figure 19. Structure postulated for the reactive polymer formed in situ from ethyleneimine (Hendry et al., 1951)

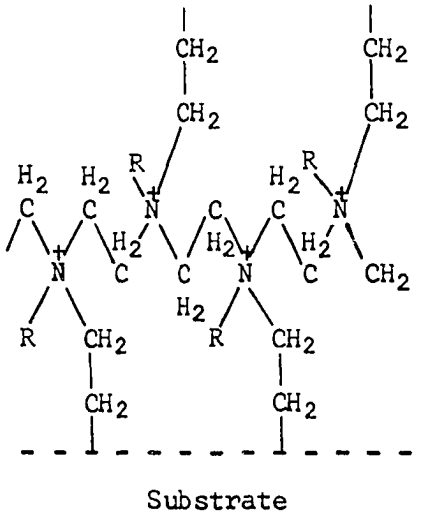
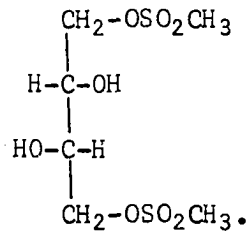


Figure 20. Structure postulated for the reactive polymer formed in situ from a nitrogen mustard (Hendry et al., 1951)

applicable to ethyleneimines (=aziridinyls). Hendry et al. continued that it is not inconceivable that these compounds diffuse in monomeric form into cells. There, one imine radical becomes involved in the formation of a polyethyleneimine chain, while the second (and, if present, a third such radical) combines, for example, with protein or nucleoprotein. Alternatively, chemical union of the monomer with the cell-component may first occur, followed later by polymer formation through the intermolecular condensation of the remaining pendent imine groups. This polymer-cell-component complex could also be an explanation of the lack of difference in activity between bis-, tris-, and other oligoaziridines. Reactive radicals are blocked through polymerization in situ. Moreover, the stereochemistry of a molecule can also be very important as exhibited by a member of the methanesulfonate compounds, namely the 1,4-bis(methanesulfonate) of L-threitol (Ross, R., 1962):



Its D-form is inactive and its meso-form, about half inactive of the L-form when used against the Dunning rat IRC-741 leukemia system.

Likewise, Borkovec (1962) summarized the effects of apholate on adult stable fly. Firstly, the compounds affect both males and females. Secondly, mating of treated males with untreated females yielded a normal number of eggs which had a low viability. Thirdly, mating of

treated females with untreated males yielded an abnormally low number of eggs which had a normal viability. Lastly, only mating of treated males with treated females, however, yielded a low number of eggs none of which hatched.

His first result parallels to the effects of apholate on both sexes of southern corn rootworm adults. The second one can be explained by the lethal and debilitating effect on the sperms based on the observation on southern corn rootworm sperms. Moreover, the inactivation of alkaline phosphatase could possibly cause a diminution of sperm production. Weak sperm is likely to be produced also. The third phenomenon was not observed in southern corn rootworm females treated with apholate. Comparable numbers of eggs per oviposition were obtained from control and treated female rootworms. However, egg hatch ability was greatly diminished, in most cases to zero. Probably, the concentrations used were enough to affect hatching but not the total egg production. In only two cases, females injected with 40 and 50 µg apholate were observed by dissection to have produced 11 and 19 eggs. These figures are definitely small as compared to about 100 eggs usually observed in the chalice at a time before oviposition commenced. The potential for egg production was probably recovered in southern corn rootworm although inviability of eggs persisted. Obviously, the last consequence on stable flies may be deduced as due to the combination of the second and third effects.

SUMMARY

Part I is primarily a morphological dissertation of the external and internal reproductive systems of southern corn rootworms (Diabrotica undecimpunctata howardi Barber). Detailed description of the morphological characteristics of the internal reproductive organs are presented. Likewise, genital accessories are described and illustrated. Sclerites associated with the internal sac, les dents du sac interne (Jeannel, 1922 and 1949), seldom mentioned in the literature, are delineated as well as illustrated. A photomicrograph of the male gonopore is presented. Likewise, chitinous linings of the female genital tract and the pore leading into the spermathecal duct are included in the discussion.

Part II gives a comprehensive review of the literature concerning the development and use of alkylating and some cancer chemotherapeutic agents in the chemosterilzation of arthropods. It particularly emphasizes the role of apholate in the chemosterilization research in entomology. The chemical reactions of this compound are illustrated.

Literature about alkaline phosphatase in relation to cancer, neoplastic, and reproductive tissues was reviewed. Similarly, the role of alkylating agents with regards to the enzyme systems aids in understanding this histochemical study of southern corn rootworm.

Apholate markedly inhibited the reactivity of alkaline phosphatase. This was determined by the calcium phosphate precipitation method of Gomori (1939) and Takamatsu (1939). This method is based on the principle that phosphatases cleave off the phosphate group from nucleotides or phosphate-bearing sugars used as substrates. The freed phosphates react with the

ionized calcium in the incubating solution. The calcium phosphate precipitates are converted into visible compounds by substitution reactions with cobalt acetate and finally with dilute yellow ammonium sulfide solution.

The alkaline phosphatase activity in the reproductive tissues of male and female southern corn rootworms was adversely affected by the apholate treatments of 2 µl of 2% apholate and higher dosages. Actual dissection of the testes revealed inactivation of the sperms. In two cases, treated females dissected out before oviposition showed few mature eggs in the chalices or egg calycis. However, total egg production did not show great decrease. Apparently, male virility was adversely affected as shown by very low hatchability to complete non-hatching of eggs of the untreated females when mated with the treated males.

The preceding findings were congruous with those that other investigators observed. Discussion of this aspect was attempted to verify the biological actions of apholate and related compounds pertaining to insect chemosterilization.

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ACKNOWLEDGEMENTS

I sincerely acknowledge Dr. Don C. Peters for his technical advice and constructive criticism during the course of experimentation and preparation of this manuscript. Likewise I deeply appreciate his interest in the pursuance of this study.

Special thanks is due to Miss Lynda L. Richards for her commendable assistance in the laboratory.

I wish to express my appreciation to Dr. Edwin T. Hibbs and Dr. Earl S. Raun (Department of Zoology and Entomology, Iowa State University, Ames, Iowa) for letting me use their special equipment; and to Mr. Douglas L. Dahlman (Department of Zoology and Entomology, Iowa State University, Ames, Iowa) for his assistance in one way or another during the experimentation.

Dr. Stanley A. Hall (Entomology Research Division, United States Department of Agriculture, Beltsville, Maryland) is also acknowledged for supplying the chemosterilants. Thanks are extended to Dr. Frank M. Schabel, Jr. (Southern Research Institute, Birmingham, Alabama) and Dr. Howard Bond (Cancer Chemotherapy National Service Center, Silver Spring, Maryland) for their communications concerning the cancer chemotherapeutic status of apholate and to Dr. Robert Scott Allen (Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa) and Dr. Helen W. Deane (Albert Einstein College of Medicine, Yeshiva University, New York, New York) for their technical suggestions concerning biochemistry and histochemistry.

Drs. Tom A. Brindley, David R. Griffith, Jack Horowitz, Oscar E. Tauber, and Martin J. Ulmer are gratefully acknowledged for their generosity in sharing their valuable time and/or for the critical reading of the manuscript.

APPENDIX A

Table 3. Survival of adult southern corn rootworms (D. u. howardi) injected with apholate in glycine-NaOH buffer[a]

1% Apholate					
Vol. injected	Sex	No. treated	No. dissected	No. dead within 5 days	No. dead within 10 days
2 µl	Male	10	4	2 (3)[b]	6 (5)
	Female	10	2	2 (2)	7 (6.5)
3 µl	Male	6	0	0 (0)	6 (9)
	Female	-[c]	-	-	-
4 µl	Male	8	0	1 (4)	6 (8.5)
	Female	-	-	-	-
5 µl	Male	56	15	23 (4)	31 (4.1)
	Female	35	6	13 (3)	21 (5.4)
10 µl	Male	6	0	4 (3)	6 (4)
	Female	3	0	1 (4)	3 (6)

[a]Other remaining individuals dissected out after 10 days from date of treatment. Some were observed for hatching of their eggs.

[b]Figure in parenthesis is an average number of days individuals died during 5- and 10-day observation periods.

[c]Dash means none was treated.

Table 3. (Continued)

2% Apholate					
Vol. injected	Sex	No. treated	No. dissected	No. dead within 5 days	10 days
2 µl	Male	21	5	9 (4.5)	15 (5)
	Female	20	4	7 (4)	14 (5)
3 µl	Male	22	7	11 (4)	13 (4)
	Female	13	3	8 (3.2)	9 (3.4)
4 µl	Male	17	3	10 (3.5)	14 (4.5)
	Female	10	3	6 (4)	6 (4)
5 µl	Male	5	0	4 (4)	4 (4)
	Female	5	0	3 (4)	5 (5)

Table 3. (Continued)

Control*					
Vol. injected	Sex	No. treated	No. dissected	No. dead within 5 days	10 days
2 µl	Male	12	1	4 (4)	4 (4)
	Female	7	0	3 (4)	4 (5)
3 µl	Male	15	2	1 (4)	2 (7)
	Female	12	1	0 (0)	0 (0)
4 µl	Male	13	0	2 (2.5)	4 (8.5)
	Female	8	0	1 (5)	1 (5)
5 µl	Male	28	4	13 (3.3)	17 (3.6)
	Female	26	1	11 (3.3)	12 (3.7)
10 µl	Male	3	0	0 (0)	0 (0)
	Female	3	0	0 (0)	1 (3)

*Eleven more males and 18 more females injected were dissected.

APPENDIX B

Addendum

The Information Circular on RADIATION TECHNIQUES AND THEIR APPLICATION TO INSECT PESTS published by International Atomic Energy Agency, Vienna, Austria beginning March 1963 has come to my attention since completing the Review of Literature. It is trying to compile summaries of recently published or unpublished works on other areas of studies relevant to sterile male technique such as the following:

a. Insect tagging techniques

b. Tracer application in ecology, e.g. flight range determination

c. Insect population dynamics

d. Development of mass rearing techniques

e. Effects of radiation

f. Radiation sterilization applications

g. Irradiation of commodities and materials to control insects

h. Chemosterilants.

The aim of this circular is to provide a rapid means for the dissemination of research information to research workers in this field and to stimulate further research.