

Methods of
TESTING
CHEMICALS
on
INSECTS

Volume I

METHODS OF TESTING CHEMICALS ON INSECTS

VOLUME I

by

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CHEMICALS ON INSECTS**

PREFACE

Several years ago the Entomology Subcommittee, Chemical-Biological Coordination Center of the National Research Council, designated the writer as editor of its proposed manual of entomological testing methods. It was expected originally that this manual would be published by the Council. Reorganization of the Coordination Center and finally its termination resulted in all rights and responsibilities being relinquished to the editor, who was to arrange for publication commercially.

This manual is intended to describe methods used in studying any phase of the action of chemicals on insects. However, busy specialists sometimes hesitate to give the time required for preparation of a critical review of the methods available in their fields. It has been decided to publish in this first volume such articles as have accumulated treating of the fundamental phases (effects of chemicals on the physiology of the insect) and relating to general techniques for applying chemicals to insects. A second volume will be devoted largely to special-purpose techniques (wood impregnation, soil treatment, repellents, acaricides, etc.) with chapters added which relate to the factors affecting test results (pre-test, exposure, and post-exposure conditions). If possible, a third volume will include those essential subjects that have not appeared in the earlier volumes, as well as a summary of recent methods to bring up-to-date those topics for which papers were received by the editor early in the project.

Although forms other than insects and their arthropod relatives often are used in studying the relative activities of chemicals as a means of screening insecticides, none of these techniques are included in this manual. The details of insect culture methods are not discussed.

The assistance of numerous collaborators receives the heartfelt thanks of the editor. These well-known authorities in their respective fields have in this manual shared their hard-earned experience with those who look to this book for guidance.

Harold H. Shepard, Editor
March 20, 1958

TABLE OF CONTENTS

	<u>Page</u>
Preface	ii
<u>Chapter</u>	
1 Surface Phenomena in Relation to Insect Cuticle W. M. Hoskins	1
2 Penetration of Insect Cuticle A. G. Richards	17
3 Measurement of Insect Respiration . . . R. Craig	35
4 Electrophysical Preparations in the American Cockroach K. D. Roeder and Elizabeth A. Weiant	45
5 Study of the Circulatory System in Insects R. L. Patton	61
6 Radioactive Tracer Methods A. W. Lindquist	76
7 Resistance Studies W. V. King	83
8 Methods of Topical Application and Injection R. L. Metcalf	92

<u>Chapter</u>		<u>Page</u>
9	Feeding and Drinking Methods F. W. Fisk	114
10	Dipping Methods A. H. McIntosh	130
11	Precision Spraying C. Potter and M. J. Way	154
12	Precision Dusting J. E. Dewey	259
13	Testing Fumigants R. T. Cotton	275
14	Synergism and Antagonism N. Turner	314
	Literature Cited	325
	Index	355

Chapter I

SURFACE PHENOMENA IN RELATION TO INSECT CUTICLE

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In the voluminous literature on the behavior of insecticidal sprays and dusts, relatively little attention has been given to the influence of the integument of insects. This is the more remarkable because it is this integument and, in particular, its outermost surface upon which all contact insecticides exert their first effects. The importance of penetration of the integument is obvious, but this can occur only after the chemical makes intimate contact with the outermost layer. The factors involved in such contact are varied, e.g., wettability is influenced chiefly by the polarity of molecules in the surface and in the adhering liquid, but is modified by such physical matters as roughness, hairiness or recent abrasion. The firmness with which dusts are held upon an insect is affected by these same conditions, with the added influences of moisture content and electrostatic charges.

Aside from its interactions with toxic substances, the outer surface of the integument affects the behavior of insects in many ways. Respiration of mosquito larvae and of other aquatic forms which must come to the surface to breathe depends upon repulsion of the water from the openings of the spiracles. Diving beetles which bear a thin layer of air on some parts of the body can carry on this "plastron respiration" (Thorpe, 1950) only because of a delicate balance between hydrostatic and interfacial forces. Possibility of life in very dry or in very moist environments or among abrasive particles such as the soil, is connected with the ability of the integument to absorb, retain, or lose moisture and to repair injuries. The outer covering of eggs serves much the same purpose during embryonic life

as the integument in later stages. It is obvious that the hatching and the molting periods are times of peril for most insects since at these times they shed their protective outer covering and are relatively susceptible to unfavorable environmental conditions as well as their normal enemies.

The present account is concerned with experimental methods designed to test the role of interfacial phenomena in the functioning of the integument. Attention is focused therefore chiefly upon the behavior of the outermost layers when liquids or solids are brought into contact with the insect body. The process of penetration as such is not covered except as certain aspects throw light upon the nature and functioning of the outer layers.

PROCEDURES DEPENDING UPON APPEARANCE UNDER VARIOUS CONDITIONS

Even casual inspection of some species of aphids or scales shows sheets or threads of wax on their bodies and the American roach appears to be covered by a film of grease. By examination of vertical sections through the body wall, especially after staining, it is possible to determine the details of structure of the cuticle. In the epicuticle alone, i.e., that part containing no chitin, two to four more or less distinct layers may be recognized. The general stain for lipoids, Sudan black B, indicates an outer layer from 0.1 to about 0.5μ thick which extends over the whole body including sensory cells, at least in the case of the bee antenna (Richards, 1952). It doubtless is of complex nature for X-ray diffraction shows paraffin type molecules, whereas the infrared spectrum indicates fatty acid esters in the case of Calliphora erythrocephala larvae (Dennell and Malek, 1953) and in other cases sterols are present as shown by the Liebermann-Burchardt test. The argentaffin reaction - reduction of silver from ammoniacal silver nitrate - probably is due to polyphenols invading the lipid layer from underlying tissues in the case of Sarcophaga falculata larvae (Dennell, 1946). Despite its thinness, the

lipoid layer may be made visible to the unaided eye in the following way. If a nymph of Rhodnius prolixus is rubbed with a sharp dust such as alumina and kept in moist air for a few days, the rubbed area develops a milky bloom which is easily seen and may be stained deeply with Sudan black B (Wigglesworth, 1945).

If a piece of cuticle is left for a considerable time in cold, concentrated hydrochloric acid, all parts are dissolved except the thin lipoid layer. Persistence of at least part of it after treatment with hot alkali is consistent with the behavior of either paraffin hydrocarbons or unsaponifiable sterol esters. While details differ from one species to another among the few so far studied, a fairly consistent pattern has emerged, i. e., a mixture of lipoids of varying reactivity and melting points makes up the bulk of the layer, but polyphenols and proteins are usually present in smaller amounts.

Lipoids are not entirely confined to the outer part of the epicuticle for if the underlying portion, called the cuticulin by Wigglesworth, the lipoprotein by Richards or the impregnated protein epicuticle by Dennell and Malek (1953), is heated with nitric acid-potassium chlorate solution or in strong alkali yellow droplets of an oily appearance are formed. However, such lipoids as this test reveals have no important part in the behavior of the surface layer except as they may cause orientation of it, as will be discussed later.

In a number of species it is possible to demonstrate a thin layer of proteinaceous material lying over at least parts of the lipoid layer. Thus a piece of Rhodnius prolixus cuticle placed in concentrated sulfuric acid swells and peels off an outer layer which is soluble in warm concentrated hydrochloric acid or warm alkali and gives other reactions compatible with the theory that it is a somewhat tanned protein. Among several species of the ticks only the Argasid, Ornithodoros moubata, possesses a similar layer which is easily seen as a separated structure in paraffin sections cleared by xylol (Lees, 1947). It has been called the cement layer (Wigglesworth, 1948), the tectocuticle

(Richards, 1951) and by various other names by older writers. A useful method for obtaining the layer free from all other tissues was used by Way (1950) who built a strong layer of collodion upon the surface of a tomato moth larva. Upon pulling the collodion away the cement layer came too and could be put upon a slide by dissolving away the collodion in acetone. When the integument is deeply folded or puckered as in case of the tomato moth larva, Diataraxia oleracea, this outermost layer is discontinuous, lying chiefly in the depressions (Way, 1950). Similarly, spines, hairs and other projections doubtless are free from it.

PROCEDURES INVOLVING DETERMINATION OF DEGREE OF WETTING

Irrespective of the particular process concerned, the general principle holds that the available energy of a system must decrease in any spontaneous change and conversely that a process resulting in an increase of available energy can occur only with expenditure of energy from outside the system. One of the best known examples is the rule that a liquid will spread spontaneously over a surface if the initial surface energy of the solid exceeds the sum of the interfacial energy and the liquid surface energy of the resulting film. The numerically equivalent surface tensions may be used to give the familiar inequality for spreading: $\sqrt{s} > \sqrt{i} + \sqrt{l}$. From this the spreading coefficient may be defined as $s.c. = \sqrt{s} - \sqrt{i} - \sqrt{l}$.

In the usual situation when the amount of liquid is limited, the degree of spreading is intermediate between nil and complete and is indicated by the angle which the spreading liquid makes with the solid. Thus spreading will continue until $\sqrt{s} = \sqrt{i} + \sqrt{l} \cos \theta$, in which θ is the angle of contact. In this equation, \sqrt{s} and \sqrt{i} are in general very difficult to determine even approximately and hence much effort has been expended in measuring \sqrt{l} and θ . The former is of course a property of the liquid alone, but θ is influenced also by the nature of the underlying solid and hence

offers a means for studying the surface properties of insects at various stages as well as those of plants or any other object.

In the application of sprays in the form of a mist of very small droplets, as with fly sprays or concentrated outdoor sprays formed by the so-called fog sprayers, the point of primary importance is that the individual droplets should spread to the optimum extent for control of the particular pest concerned. Unfortunately there is very little exact information regarding the optimum distribution on a micro scale of any insecticide when used against any one pest and not even a start has been made in determining the proper distribution of the numerous available materials against the host of pests.

On the other hand, in the application of large volumes of dilute spray by conventional high pressure sprayers, the surfaces of insects and plants are completely covered during the process but afterward the liquid withdraws and runs off to an extent governed by the wetting power. It is important to notice that \sqrt{i} may be changed by solutes such as emulsifiers which in some cases alter the nature of the solid surface. Thus if an insect is sprayed or held beneath a liquid long enough for an emulsifying agent to enter into the surface structure, the latter may be changed drastically so that removal of the adhering liquid layer does not restore the original solid surface. Richards (1951, page 140) has given a timely warning on this point and has expressed the hope that methods may be devised for determining the normalcy of insect surfaces after they have been used in wettability experiments. Perhaps the best approach is to determine the rate at which the angle of contact changes and the amount of washing, e.g., with water, required to restore the surface so the original angle is again obtained.

The usual methods for measuring angle of contact have been used with insects, with only such modifications as the small size of the available surface made necessary. Thus Stellwaag (1924) calculated the angle from measurements of the

diameter and thickness of drops of water or various sprays on an aphid and a psyllid, finding the latter much more readily wet. Measurement of a photograph or a projection of the profile of a drop resting on a surface was employed by O'Kane et al. (1932) in studies with water and solutions of various wetting agents on fifteen species from several orders. Pal (1950) by the same technique studied the effect of time of contact upon the advancing angle using distilled water, odorless distillate and methyl naphthalene on a beeswax plate. This was done in recognition of the fact emphasized by Ben-Amotz and Hoskins (1938) that the degree of wetting secured during the application of sprays is determined by the "dynamic" angle of contact prevailing at the time and not by the "static" angle of contact which is affected greatly by surface tension-reducing substances in the spray. Photography of individual drops as they strike a surface, rebound and spread upon it gives a more dramatic demonstration of the effect of wetting agents (Upholt and Hoskins, 1940), but apparently has not been applied to insect surfaces.

Beament (1945a) has attempted to secure "dynamic" conditions by letting liquid into a drop through one micropipette while withdrawing it at the same rate through another. This doubtless ensures that the surface of the liquid drop is renewed rapidly but it does not prevent accumulation of wetting agents at the solid-liquid interface with consequent reduction in the interfacial tension, \sqrt{i} . In another paper Beament (1945b) used the renewable drop method and the older tilting plane procedure with lipoids from six species spread on a sheet of glass. This simplifies the operations greatly but departs from natural conditions in at least two ways: a) in the natural epicuticle the lipid molecules are laid down upon a protein substrate whose arrangement of areas of different polarity, e.g., hydrocarbon radicals versus oxygen- or nitrogen-containing groups, gives rise to a characteristic orientation of the lipid molecules which cannot be duplicated on a substrate of different nature; b) the surface of insects is often thrown into sharp folds and tubercles which make it well-nigh impossible to establish a baseline from which the angle of contact is to be measured. When a

cement layer is present over part of the lipid layer a mosaic results which in a strict sense can have no single interfacial energy or angle of contact as a whole.

In an analysis of the factors involved in the respiration of certain diving beetles which use a thin film of air, a plastron, on part of the body as an artificial lung, Crisp and Thorpe (1948) have shown that the wetting of the hairs which overlie the plastron is expressed by the equation: $\Delta p \cdot a \cdot dx + \sqrt{\cos \theta} \cdot n \cdot ds = 0$, in which ds is the element of area wetted on each of n hairs when an area a of free water surface moves through a distance dx under the pressure Δp . Since the pressure may be varied by depth of water or imposed air pressure and invasion of the plastron may be detected by a change in the sheen, with a few reasonable assumptions it is possible to estimate the angle of contact upon the hairs which support the plastron. They are wet with about as much difficulty as a paraffin surface but Crisp and Thorpe were not able to demonstrate the presence of a lipid layer on the hairs.

Without attempting to evaluate the fragmentary data on wetting of insects it may be said that there is a direct correlation between the hardness of the surface lipid and resistance to wetting. Thus Beament (1945b) was able to arrange several species in the same order according to melting point of their epicuticular lipoids and the angle of contact of water upon films placed on glass. He even was able to separate the lipoids from *Pieris brassicae* pupae and show that the two fractions fell into the series. During the process of molting, the old exoskeleton is cast off before the new cuticle is fully formed. Consequently, some sharp changes in wettability may occur within a brief period. Wigglesworth (1947) has given a chronological log for fifth stage nymphs of *Rhodnius prolixus* as follows: 11:45, water drops roll off surface freely; 12:15, slight adherence of water drops; 12:30, angle of contact now about 90° ; 12:45, spreading occurs in some areas; 1:15, spreading in all areas tanning process started as indicated by slight darkening; 1:45, darkening stronger, no noticeable change in wettability; 5:00,

fully darkened, surface now resists wetting once more.

Insects which live in very moist conditions or in water present a difficulty as yet largely unresolved. Thus Sarcophaga larvae which during the feeding period, live submerged to their rear spiracles in flesh and liquefied products, have no appreciable layer of lipid and the inner portion of the epicuticle is free of it (Dennell, 1946). Larvae of the petroleum fly, Psilopa petrolei, live in pools of petroleum (Thorpe, 1930). Their surface and especially the posterior spiracular openings are not wet by the oil and hence are strongly hydrophilic and cannot have lipid molecules in the outer layer. Pryor (1940) has suggested that the epicuticle of this larva contains proteins whose reactive groups have reacted but slightly with hydroxyphenols, i.e., the normal tanning process does not occur.

Mosquito larvae are freely wettable except around the spiracular openings, but their ability to maintain a steady osmotic pressure of the body fluid in hypo- or hypertonic solutions indicates some mechanism for controlling penetration or exit of water from the body. A proteinaceous cement layer could ensure wettability but apparently this had not been demonstrated. An interesting example of how the relatively easy wetting of parts of the body enables a vital function to operate has been pointed out by Manzelli (1941). Mosquito pupae are top heavy and would turn over and be unable to breathe if it were not for the water-repellent nature of the integument about the respiratory trumpets which literally pull these organs out of the water. Addition of surface tension-reducing solutes prevents this action and drowns the pupae.

PROCEDURES INVOLVING DETERMINATION OF PERMEABILITY OF THE EPICUTICLE

It may be expected that the various layers of the cuticle will interfere with the passage of water into or out of insects and that, because of its hydrophobic nature, the

lipoid layer of the epicuticle will be most effective in this respect. To a lesser extent the cement layer when present may affect movement of water or other liquids. Since the work of Kühnelt (1928) this subject has received steadily increasing attention because of its importance in the life processes of insects, in their adaptation to various environments and in the effects of insecticides. The experimental procedures may be divided grossly into those using intact insects, dead or alive, those using detached portions of integument and those using artificial membranes impregnated or overlaid with lipoids from insect epicuticles.

While it has been known for a very long time that dusts of apparently innocuous nature often cause high mortality of insects to which they are applied, the first clear statement that dehydration of the body is concerned appears to be due to Zacher and Kunike (1930) who thought that the dusts absorbed body moisture through the integument. They weighed the treated insects at intervals after applying powdered silica, magnesium oxide, charcoal and other substances. Essentially this same method has been used by nearly all experimenters since. The suggestion that the lipoid material of the cuticle is important in water conservation was made by Ramsay (1935) on the basis of the observation that a droplet of water placed upon the back of an American roach evaporates much more slowly than a similar drop on a glass surface so long as the temperature is kept below about 30 °C. If this is exceeded there is a sudden increase in the rate. Ramsay thought that a film of the roach grease surrounded the droplet and interfered with water vapor formation and that above the critical temperature the film became unstable.

The general applicability of this theory to insects was shown by Wigglesworth's (1945) proof that for each of numerous species there is a characteristic critical temperature at which loss of moisture begins to rise drastically. This varies widely, e. g., from about 15 °C in the case of Tipula larvae to 32 ° for Blatella nymphs and Nematus larvae, 55 ° for Rhodnius nymphs and 68 ° for eggs of the fruit tree red