NEUROPATHOLOGY IN INSECTS[1]

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Neuropathological pictures resulting from insecticide action have been recorded for the insect nervous system by Krüger (1931), Klinger (1936), Richards (1941), Wigglesworth (1941) and especially by Hartzell and his colleagues (1932–1944). The aim of the present paper is to present the numerous data we have gathered on various compounds, to compare these data with other published data, and to discuss the relation between the various pathological pictures and the cessation of nerve action. The effects of compounds on other tissues is not considered. Analysis of the action of pyrethrum is presented in considerable detail. Other compounds are then treated more briefly, practically as summaries, without specific reference to the hundreds of experimental animals involved. Such a space-saving procedure seems warranted in view of the thesis of the present paper; this thesis being that the visible pathological changes induced in nerves by insecticides are at least largely postmortem and accordingly too complex for analysis at the present time.

MATERIALS AND METHODS

For most of the original data presented in the present paper we used adults or sometimes large nymphs of the American cockroach (*Periplaneta americana*). In some cases mosquito larvæ were employed (*Culex pipiens* and *Aedes ægypti*). The substances tested were introduced in acute dosages (commonly very large doses) either into the tracheæ or hemocœl by means of a syringe, or fed orally or applied to the cuticle. Some volatile

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substances were also used as vapors. Repeated chronic dosing was usually not tried. It has been shown in an earlier paper in this series (Richards & Weygandt, 1945) that organic solvents of low water solubility definitely tend to accumulate in the central nervous system particularly when applied in the tracheæ (Figs. 3, 13, 14). Tracheal injections have several advantages: the distribution of the substance can be checked with fair accuracy on dissection; also the injection may be made at either end of the animal, one end then serving as the experimental end, the other end serving as one kind of control (for some purposes this gives excellent results). The method of tracheal injection was accordingly commonly employed in studying the effects of lipid soluble materials on the nervous system.

The majority of the microscopic observations were made by the polarized light method which is highly sensitive and avoids questions of fixation artifacts (Schmitt & Bear, 1939; Richards, 1944). Quantitative changes in the birefringence of nerve cords can be measured with a high degree of accuracy (see preceding reference for illustrations). It is not possible to repeat the details of this technique here. Suffice it to say that with this method it is possible to analyze the effects of various toxins on both the nerve axoplasm and the surrounding lipo-protein sheath. In normal nerves there is a balance between positive and negative components of the birefringence, the positive elements being slightly stronger (having greater amplitude). Measurements in saline give the amplitude by which the positive components (axis cylinder and sheath proteins) exceed the negative components (sheath lipids). In glycerine (15 per cent in saline) the form birefringence of the proteins is largely masked, and measurements give the amplitude by which the birefringence of the lipid sheath exceeds the protein birefringence in this solution. Theoretically, elimination of *all* the positive (protein) birefringence should show the true negative (lipid) value, and vice versa. It is not possible at present to do this with nerves. But so long as conditions of measuring are kept standard the figures from various specimens can be compared quantitatively. When the measurements in glycerine remain constant (within the normal range), the previous measurements in saline indicate the condi-

tion of the positive or protein components; when both are lowered by the same or different degrees allowance must be made for the fact that we are dealing with a balance; when a nerve is isotropic in saline but not in glycerine then the two opposed components are presumably in balance and the measurement in glycerine gives a measure of the amplitude of each; and only when a nerve is isotropic in both saline and glycerine can we say that there has been an approximately complete decay of the birefringence.

With pyrethrum, valone, aniline, petroleum oils and a number of other materials, serial sections were prepared following routine paraffin procedures. Nerve cords or brains were fixed in 95 per cent ethyl alcohol plus 5 per cent glacial acetic acid, and after sectioning stained with toluidine blue and acid fuchsin. Polarized light analyses are applicable only to nerve fibers; investigation of possible histological effects on the nerve cell bodies must be done by the more usual methods of histopathology.

In many cases pathological effects can be seen even under the ordinary light microscope without fixation or other treatment. This is particularly true of substances which cause opacity, chromatin clumping or the release of droplets in the highly transparent nervous system of mosquito larvæ (Figs. 5, 15).

The condition of the insects was noted after application and at the time of dissection. A further check was made on the functional condition of the nerve cord by applying a direct electrical shock to selected parts of the nerve cords and observing any responses. Platinum electrodes were used either with an ordinary inductorium or an electronic multivibrator (thyratron controlled, condenser discharge circuit) adjustable to give shocks with frequency from 0 to 1000 per second, the duration and interval being separately controlled, and the voltage variable from zero to several hundred volts.

NORMAL STRUCTURE OF THE INSECT NERVOUS SYSTEM

The central nervous system of insects consists of nerve cells and their fibers surrounded by thin lipo-protein sheaths (Richards, 1944) and held together by tracheæ, neuroglia and a tough outer sheath, the neural lamella (Scharrer, 1939). The nerve cells are of typical structure but range from very small to only

moderate in size. The nerve fibers are commonly very small (to less than 1 micron). The nerve sheaths are always thin, and in the case of small fibers are not detectable by ordinary microscopic methods and require polarized light methods for their demonstration.

The central nervous system (Fig. 2) is a solid structure arranged with the major fiber tracts centrally and the cell bodies in a peripheral layer in the segmental ganglia (Hanström, 1928). The interganglionic connectives and peripheral nerves consist of nerve fibers with some tracheæ and neuroglia. All ganglia, connectives and peripheral nerves are surrounded by the neural lamella.

The tracheal network in ganglia is most rich in the boundary between the cell body layer and the central fiber tract region. This is readily seen in favorable preparations (Figs. 1, 2). The distribution of tracheæ at this boundary may weaken it, and also, in cases where toxins are applied via the tracheæ, results in a maximum concentration and effect here. It is not surprising, therefore, that one of the recorded pathological changes in insect ganglia is a tendency for cell body and fiber tract regions to separate (Richards, 1941).

EFFECTS OF PYRETHRUM

For a standard of comparison with a well-known neurotoxic insecticide, two series of experiments were performed with pyrethrum concentrates. In the first series a known pyrethrum concentrate[3] was injected into the first thoracic spiracle of 25 adult cockroaches. Individual records were kept on each specimen: cessation of general movements, cessation of heartbeat, responsiveness of the nerve cord to electrical stimulation at the time of dissection, and the extent of distribution of the pyrethrum

[3] Sample received from McLaughlin, Gormley, King Company, Minneapolis. Assay: 10.80 per cent pyrethrin I, 9.85 per cent pyrethrin II, total 20.65 per cent pyrethrins. In the absence of chemically pure materials there is, of course, no way of evaluating how much of the effect may be due to the other 80 per cent of the material, or whether or not there is any difference between the effects of pyrethrin I and II. This concentrate, however, is the kind used in preparing insecticides.

One should also remember that in tracheal injections we are always dealing with relatively large doses and rapid penetration.

concentrate throughout the tracheal system. Most of these specimens were examined with polarized light but representative specimens were serially sectioned and compared with published figures and descriptions of pyrethrum lesions. In the second series 24 last instar nymphs and adults were injected in the first or second thoracic spiracle with an unassayed pyrethrum concentrate, records kept individually as above, and the nerve cords later removed and examined with polarized light.

Following the tracheal injection there is an immediate initial paralysis that may be in part a reflex immobilization.[4] A few minutes later there is a partial recovery followed by a gradual decline and slower and slower movements of peripheral parts and eventually complete death. The legs, abdomen and heart may continue moving for many hours (up to 52 hours in these experiments). Electrical stimulation to test responsiveness was routinely performed on specimens still moving appendages at the time of dissection; specimens treated ½ to 52 hours prior to dissection showed in no case any response to direct electrical stimulation of the affected nerve cord. The fact that muscular movements may continue for many hours after the nerve cord is irreversibly paralyzed or even highly degenerate is good evidence for the selective nervous action of pyrethrum.

Analyses with polarized light revealed that nerve cords paralyzed with pyrethrum show first an effect on the axoplasmic colloid (axis cylinder) of the nerve, then somewhat later an effect on the lipid component of the nerve sheaths. It seems to be the latter that gives rise to the most prominent pyrethrum lesions. Both the axis cylinder of the nerves and the lipo-protein sheath may lose the ultrastructure responsible for the optical properties prior to the cessation of movement of appendages but not prior to paralysis of the nerve cord. These effects on the ultrastructure responsible for the optical properties are first seen in the region of maximum penetration, but with the passage of time extend to all parts of the nerve cord and to at least the larger peripheral nerves. The experimental technique employed was not sufficiently refined to permit direct demonstration of the

[4] Reflex immobilization from injection of pyrethrum is not a specific action. It may result from any tracheal injection.

status of the optical properties of a nerve at the moment of paralysis, but we can infer that the visible effects are all post-mortem since affected nerves are always paralyzed whereas normal-appearing nerves may or may not be paralyzed. The paralysis prevents both direct stimulation and the passage of impulses through to unaffected regions.

No illustrations of pyrethrum effects are given in the present paper. Adequate illustrations of the more advanced stages of degeneration as seen in serial sections have already been published by Klinger, Hartzell *et al.*, and Wigglesworth. The deterioration of optical properties can be measured with considerable accuracy but there is no point in publishing photographs of the various stages. Such a series of pictures would simply range from those published for normal nerves (Richards, 1944) to a complete absence of contrast (= no picture).

The two series, totalling 49 specimens, were sufficiently consistent so that the data may be combined and analyzed as follows:

In ten specimens that were dissected from ½ to 2½ hours after treatment there was usually a slight but significant decay of the positive component of birefringence but not any clearly demonstrable change in the negative component.[5] This is interpreted as meaning that the proteins of the axis cylinder of the nerves are degenerating but that the nerve sheaths are still normal. The decay of birefringence of the axis cylinder was most evident in the injected regions, the abdominal or posterior abdominal connectives being normal. Another specimen (1½ hrs.) serially sectioned and stained showed seemingly normal nerve tissue. The large nerve cell bodies and the fiber tracts appeared normal; there was a little homogeneous staining in the vicinity of pyrethrum-filled tracheæ but this is of questionable significance, and chromatin clumping was fairly general but not universal in the medium-sized nerve cells, usually absent in the large nerve cells and always absent in the neuroglia cells. The "typical" pyrethrum lesions were not found in this specimen.

[5] In one of the above cases the reading in saline was approximately normal but an unusually high negative reading in glycerine implies an effect on the axis cylinder which was masked by slight stretching of the nerve cord during dissection. This phenomenon can be reproduced experimentally by deliberately stretching pyrethrum-treated nerve cords.

In eleven specimens that were dissected in 3½ to 7 hours after treatment the positive component of birefringence was considerably reduced but the negative component was still strong (nerves isotropic in saline but strongly birefringent in 15 per cent glycerine). This is interpreted as meaning that the optical properties of the proteins of the axis cylinders have degenerated to half or less than half their normal value, but that the lipo-protein sheaths are still normal or nearly so. This decay commonly extended farther posteriorly than the visible distribution of pyrethrum in the tracheæ but was not complete in the posterior abdominal segments. Another specimen (5½ hrs.) serially sectioned and stained showed relatively slight pathological changes, especially in stainability, throughout the area of penetration. Histological effects are thus evident but the changes are not of the extreme type described by Klinger, Hartzell and Wigglesworth.

In six specimens that were dissected from 12 to 14 hours after treatment the positive component of birefringence was low and the negative component was also somewhat reduced (nerves isotropic in saline but moderately birefringent in 15 per cent glycerine). This is interpreted as meaning an advanced state of degeneration of the optical properties of the axis cylinders and a beginning of degeneration of the lipids of the nerve sheaths. The decay is also more general throughout the nerve cord after this time interval. No specimen from this set was sectioned.

In eight specimens that were dissected from 24 to 25 hours after treatment the positive component of birefringence was further reduced or abolished and the negative component was also greatly reduced or even abolished in the regions of greatest penetration (less or not at all affected in regions far removed from the visible distribution of pyrethrum in the tracheæ). This is interpreted as meaning a complete or nearly complete degeneration of the axoplasmic colloid, plus an advanced degree of degeneration of the nerve sheaths. Effects are apparent throughout the nerve cord at 24 hours and are not limited to regions where pyrethrum is visible in the tracheæ. Another specimen (30 hrs.) serially sectioned and stained showed chromatolysis, vacuolization, etc., of the types described as "characteristic"

for pyrethrum by Klinger, Hartzell and Wigglesworth, but commonly the sections illustrated by these authors represent more advanced stages of degeneration than is to be seen in this specimen.

A single specimen that was still moving its metathoracic legs 52 hours after treatment had a nerve cord with seemingly complete decay of all its optical properties (isotropic in both saline and glycerine). As seen without crossed Nicols, the axis cylinder and nerve sheaths were both obviously degenerate.

In nine specimens that appeared to be dead at the time of dissection (29½ to 56 hrs.) there was generally a complete decay of both the positively and negatively birefringent components of the nerve cords. In specimens with shorter exposures (29–30 hrs.) the negative component had usually degenerated completely only in the regions where pyrethrum was visible in the tracheæ; in specimens with the longer exposures (55–56 hrs.) the decay seemed complete throughout the nerve cord although pyrethrum was visible in only the thoracic tracheæ. As seen without crossed Nicols, these nerve cords were obviously degenerate. Another specimen (55½ hrs.) serially sectioned and stained showed extensive degeneration of the "typical" pyrethrum type.

The above data show that pyrethrum does have a selective nervous action as previously reported by others. As seen with polarized light, pyrethrum first causes degeneration of the colloid of the axis cylinder (and likely of the nerve cells). The degeneration of the nerve sheaths occurs later. Degeneration proceeds from the region of application towards and finally to other regions. The death of the animal does not bear any fixed relationship to the degree of degeneration of the central nervous system. All the histological effects seen in our experiments are subsequent to irreversible paralysis and are accordingly to be classed as postmortem pictures.[6] The appearance of vacuoliza-

[6] Hartzell & Scudder (1942) used "moribund" flies four hours after treatment and obtained both slight lesions and chromatin clumping. Differences in technique and in the test animals used prevent strict comparison to these data. However, the use of peripheral movements as an index of life does not prove that the visibly pathological nerve cells were still living. Our "living" cockroaches had "dead" nerve cords. It is possible that some nerve cells may show changes before they die but this would be difficult to prove and has not yet been proven. Chromatin clumping is discussed in a later section entitled Suffocation and Acidity.

tion coincides with the time of breakdown of the lipo-protein sheaths and may be due to sheath products (Richards, 1943). The histological effects develop rather slowly following death of the nerves concerned, and we have found that these effects are remarkably similar to those seen in the autolytic degeneration of nerves in saline. It seems questionable, therefore, whether pyrethrum has any causal relationship to "pyrethrum lesions" other than killing the nerves. It is quite possible that a lethal concentration of pyrethrum develops in the central nervous system so far in advance of that in other tissues that advanced autolysis may develop there before other tissues even die.

EFFECTS OF "THANITE"

This commercial product and its active ingredient, isoborneol thiocyanoacetate (96 per cent) were studied mostly for the effect of the water miscible (soluble ?) fraction in the culture water of mosquito larvæ. Some tracheal injections were also performed with mosquito larvæ. Histological examination was made only by means of stained serial sections. The same results were obtained with "Thanite" as with isoborneol thiocyanoacetate. Selective degeneration of the nervous tissue is not so marked with this material as with pyrethrum but similar lesions (vacuolization) are produced in larvæ still capable of feeble movements (Fig. 7).

EFFECTS OF PETROLEUM OILS

The pathological changes resulting to brains and nerve cords from the application of various petroleum oils in the tracheæ of mosquito larvæ have already been described and illustrated in some detail (Richards, 1941). It was shown that asphyxiation might or might not occur in the experiments depending on the technique employed. It was also shown that the lighter oils containing unsaturated compounds produce a degeneration involving chromatolysis, cell separation and fiber degeneration (stained sections). At that time it was suggested that the histological effects might be postmortem insofar as the cells of the nerve cord were concerned, and that the effect is at least partly intercellular (*i.e.*, on the nerve sheaths). As seen in stained sections there are certain similarities between the histological effects of petro-

leum oils and those of pyrethrum and "Thanite." Cell separation and fiber separation are more distinct with petroleum oils, perhaps partly due to a solvent action of the oils on the sheath lipids.

The saturated, so-called non-toxic, petroleum oils can kill insects. They have not, however, been shown to produce any noticeable change in the nervous system or other tissues prior to death of the insect. When asphyxiation is avoided they do not produce any cytological changes visible in stained sections. Also, saturated petroleum oils do not alter the optical properties of the nerve fibers of cockroaches.

EFFECTS OF COBRA VENOM AND LYSOLECITHIN

A number of neurotoxic snake venoms have been studied by mammalian physiologists. Cobra venom is most commonly used. This venom is a complex mixture of toxins (see Ghosh, 1940; De, 1941; Macht, 1941; etc.) in addition to an enzyme, lecithinase A, which splits the fatty acids radical from lecithin to form the potent hemolytic agent called lysolecithin. In some of the older literature it was assumed that cobra venom acted on mammals by producing lysolecithin *in vivo,* the lysolecithin then causing death (Page, 1937, p. 80). In view of the obvious sheath degeneration caused in insects by some insecticides, this hypothesis was introduced into entomological literature by Richards (1943). Subsequently, however, experiments designed to test this suggestion have not given confirmatory evidence, and it seems advisable to abandon the hypothesis.

Cobra venom[7] is quite toxic to insects. It first paralyzes the nervous system, then later results in the deterioration of the optical properties of the axis cylinders, and only lastly causes a breakdown of the lipids of the nerve sheaths. This makes it seem dubious that lysolecithin formed during sheath breakdown could be an important factor in the action of cobra venom on insects. As a further check, lysolecithin was prepared by the action of crude cobra venom on egg yolks (chicken) following the techniques of King & Dolan (1933) and Levene *et al.* (1923, 1924). The preparation obtained was potent for hemolysis of erythro-

[7] Supplied through the courtesy of Hynson, Westcott & Dunning, Baltimore.

cytes and seemed reasonably pure. Injections of maximal doses of saline suspensions into the hemocœl of cockroaches was without effect. One can question whether the lysolecithin penetrated from the insect's blood into the cells, but certainly the data do not support the idea that lysolecithin is toxic to insects. In vertebrates it seems that the effect of lysolecithin is expressed largely by hemolysis and rupture of capillaries—phenomena that are not found in insects.[8]

Bee venom is known to contain lecithinase A (Feldberg, 1940). Perhaps wasp venom does also. Hartzell (1935) has recorded nerve pathology with vacuolization in the ganglia of cicadas paralyzed by wasp stings. Insect venoms, like snake venoms, are complex mixtures (Beck, 1935). We have not had any venom of *Sphecius speciosus* for study, but certainly with cobra venom the pathological effects on insect nerves are subsequent to the death of the nerve cells.

EFFECTS OF TRIORTHOCRESYL PHOSPHATE

This is the causative agent of "ginger paralysis" in humans. It is moderately toxic to mammals but its exact effects are not clear (Lillie & Smith, 1932). Hartzell (1934) has shown that it can be used to produce lesions in the nervous system of insects. We have confirmed this with the polarized light method, but again the histological effects are found only after paralysis (death) of the nerve cells.

EFFECTS OF LIPID SOLVENTS

The selective accumulation of various lipid solvents in the central nervous system of mosquito larvæ has been treated in detail in a preceding paper (Richards & Weygandt, 1945). It was reported therein that organic solvents may diffuse from the tracheæ to become uniformly distributed throughout the fiber tracts (xylol, chloroform, etc., Fig. 14) or they may penetrate the tracheal walls and collect outside the tracheæ as droplets which disperse gradually (essential oils, Figs. 3, 4; various glycol derivatives, Fig. 13, etc.). Stained serial sections may reveal no visible effect in the case of the former, especially those that

[8] Several potent toxins of mammals have little or no effect on insects. In addition to lysolecithin, the list includes histamine and curare.

are common ingredients of fixing fluids, perhaps partly because the same or similar solvents are used in histological techniques. Stained serial sections of brains into which the organic solvent has penetrated as droplets may show a considerable degree of seeming vacuolization, but the so-called vacuoles in these cases may represent the precipitation of tissue components around the periphery of invading droplets which are later dissolved away (Fig. 9).

With the polarized light method we studied the effects of injecting ethyl ether, ethylene dichloride, chloroform, xylol and toluene as fluids into the tracheæ of cockroaches. The effects of ethyl alcohol on extirpated nerve cords were also studied. When these solvents are used as fluids (relatively large amounts) they quickly block metatropic reversal by dissolving the sheath lipids. They do not destroy the optical properties of the axis cylinder, in fact they have good fixing properties except for lipids (see Richards, 1944, Figs. 5–10).

It appears, however, that the detectable solvent effects are not necessarily concerned with the anesthesia or death produced by these substances. When cockroaches are anesthetized or killed with the *vapors* of chloroform or ethylene dichloride there is no detectable change in the optical properties of the nerve cords.

EFFECTS OF INSECT REPELLENTS

Insect repellents are organic compounds, and like most organic compounds have lipid solvent properties. Accordingly, it is not surprising that when introduced into the tracheal system of insects they tend to accumulate in the nervous system, and that large doses of the fluids so introduced abolish (dissolve) the lipid component of nerve birefringence. Of more interest is the fact that they produce no detectable changes other than those which can be ascribed to their solvent properties. As with the preceding materials, the visible effects are subsequent to paralysis and presumably subsequent to death of the cells concerned. Materials tested included dimethyl phthalate, 2-ethyl hexanediol-1,3 ("Rutgers 612"), a,a′-dimethyl-a-carbobutoxydehydro-gamma-pyrone ("Indalone"), and synthetic Oil of Citronella.

When the above repellents are used as *vapors* they can kill cockroaches. It is particularly easy to kill cockroaches with the

vapor (saturated atmosphere at room temperature) of synthetic citronella (12–26 hrs.). Dimethyl phthalate vapors also kill readily. Cockroaches are more resistant to "Indalone" and ethyl hexanediol but are eventually killed. The interesting aspects shown for repellent vapors are: (1) they can kill such a hardy insect as an American cockroach, and (2) they act like the vapors of other lipid solvents in not altering appreciably the optical properties of the nerves they kill.

EFFECTS OF ANILINE

This is an extremely toxic substance for all cells. As such it is not to be called a selective nerve poison; however, its effects on the lipids of the nerve sheaths are so striking that we are treating it separately. When aniline is injected into the tracheæ of mosquito larvæ and the nerve cords dissected out in saline immediately, the whole nerve cord (ganglia and connectives) is diffusely but unusually strongly birefringent (Fig. 17; compare to figures of normal nerve cords in Richards, 1944). If the nerve cord is now transferred to 10 per cent formalin in saline, birefringent particles appear within a few minutes and rapidly increase in size and brilliance (Fig. 18). These irregular particles are found *between* the cells and fibers throughout the ganglia and connectives. They dissolve and disappear within a few seconds in 95 per cent ethyl alcohol. Birefringent particles are not found if the nerve cord is left in saline. Untreated nerve cords in formol-saline retain their normal birefringence. It follows that aniline does not directly produce these particles but that it affects the sheath lipids in such a manner that the particles are produced by the fixation process.[9]

Similar but less striking effects (smaller particles) are obtained by the injection of aniline into the tracheæ of cockroaches and subsequently removing the nerve cords into formol-saline.

Serial sections of mosquito larvæ treated with aniline, and fixed first in formol-saline and then in the alcohol-acetic acid mixture, show many holes ("vacuoles") in positions comparable to those occupied by the birefringent particles (Fig. 10). The presumption is that these holes and also the extensive cell separa-

[9] Nerve cell lipids may be involved as well as the sheath lipids. What can be verified optically is that sheath lipids are involved.

tion result from the production and solution of such particles. Aniline is destructive to nerve cells in other ways too. Figure 10 shows that the nerve cell bodies are somewhat shrunken and stain heavily and nearly homogeneously.

Birefringent particles can be produced in insect nerve cords by several other means. Immersion of normal nerve cords in lower concentrations of ethyl alcohol (30–50 per cent) results in the production of birefringent particles which may appear similar to the above (Richards, 1943). Autolytic degeneration of normal nerve cords in saline gives a gradual diminution and eventually loss of the lipid component of sheath birefringence, but degeneration of normal nerve cords in 15 per cent glycerine in saline gives birefringent particles (Richards, 1944, Fig. 16). Particles that appear somewhat different can be produced by the action of certain essential oils (see next section). Clearly the production of birefringent particles can result from the action of a variety of treatments that seem to have no other obvious common action. So far as is known, all such particles appear postmortem.

EFFECTS OF ESSENTIAL OILS

Chemically, essential oils are diverse compounds, commonly complex mixtures. Included are terpenes, aldehydes, esters, resins, etc. Some are relatively toxic substances, others less so. Some are attractants to certain insects (*e.g.*, eugenol), others are repellents (*e.g.*, citronella). All these tend to accumulate in the insect nervous system (Richards & Weygandt, 1945) where they usually penetrate as clusters of droplets from the tracheæ (Figs. 3, 4); these clusters disperse slowly throughout the nerve cord and eventually lose their identity in mixing with the degenerating nerve substances. Some notes on essential oils have been included in preceding sections.

In terms of histopathological effects the members of this diverse group of substances produce various effects. Observations on gross changes in the nervous system of mosquito larvæ were made during the penetration studies cited above. The list tested included: oil of thyme, natural oil of citronella, citronella "tails," synthetic oil of citronella, eugenol, geraniol, cinnamic

alcohol, "Citrola" and several perfume bases called "Petrodars." As mentioned above these substances first penetrate as droplets clustered around the tracheæ. Serial sections of mosquito larvæ treated with oil of citronella (Ceylon ST) show clear acidophilic nuclei in which no chromatin threads can be seen,[10] and some "vacuoles" around the tracheæ (presumably arising from precipitation of the tissue elements followed by solution of the citronella droplets, Fig. 9). Gross pathological changes were noted only in the cases of eugenol, citronella "tails" and oil of thyme.

Eugenol is striking in that nerve cords fixed in formalin show many rounded particles or globules (Fig. 15) which are weakly birefringent (Fig. 16). It seems most likely that the optically active material in these globules comes from the lipids of the nerve sheaths. The rounded shape and relatively low amplitude of birefringence suggest that they are not identical with the particles resulting from the application of aniline, alcohol or glycerine. These globules in nerve cords treated with eugenol resemble somewhat the "myelin figures" that can be produced by the action of water on preparations of phospholipids. Somewhat similar but less striking results were obtained with the unknown mixture that constitutes the end distillation product of citronella ("citronella tails").

Oil of thyme is a destructive material to various types of cells. The nerve cords of mosquito larvæ treated with this material are opaque and obviously highly pathological. No extensive analysis of the action of oil of thyme was attempted.

EFFECTS OF "VALONE" AND "TERTIARY BUTYL VALONE"

Most of our experiments were performed using "Valone" (2-isovaleryl-1, 3-indandione), and accordingly this compound will be discussed first. Individual experiments were performed on more than 300 adult cockroaches and 200 mosquito larvæ in addition to the accompanying controls. Various types of ap-

[10] Similar dissolution of chromatin has been reported for isobutyl undecylene amide by Hartzell & Scudder (1942) and for piperine by Hartzell & Strong (1944). Several other oils (mustard, croton, cantharidin, colchicine, etc.) are listed by Haas (1941) as primary nuclear poisons in vertebrates.

plications were employed in an attempt to elucidate the erratic results obtained in the use of this compound in our experiments on cockroach control. The variations in effectiveness for practical control programs are not of primary interest to the present paper where only effects on the nervous system are to be considered. Intensive study of these practical variations did, however, lead to the accumulation of an unusually large amount of pathological data.

The methods of application employed included blood and tracheal injections of both oil and alkaline-saline solutions, oral feedings, cuticle applications in sealed wax cells, and *in vitro* studies of the effects on extirpated nerve cords.

Cockroaches affected with "Valone" become completely paralyzed. The effect from blood and tracheal injections is quite rapid. With acute doses attainment of complete paralysis may require only a few minutes or even a few seconds. With smaller doses the effect is slower. With sublethal doses no effects were noticed. With tracheal injections the immediate paralytic effect can be localized to the region of injection and there does not appear to be any marked stimulation transmitted to the yet unaffected parts of the nervous system. Electrical stimulation of exposed nerve cords shows that "Valone" both paralyzes the affected ganglia and blocks transmission of impulses through to unaffected areas. With oral and cuticle applications the effect is slower (may require days) and highly erratic in that many individuals are never affected. In all cases, irrespective of the mode of application, once animals are affected they show the same paralytic symptoms, and in all of our cases cockroaches once showing paralytic symptoms always died. The variability concerns only the time for the effects to develop and whether or not the effects ever do develop. In part this probably represents unexplained variations in penetration or absorption but it also seems partly due to various uncontrollable degrees of enolization.

Nerve cords of slightly more than 100 cockroaches affected with "Valone" were examined with polarized light. The results were entirely consistent irrespective of the mode of application or the time lapse between application and paralysis. In all cases the nerve cords or the affected regions thereof showed complete

or almost complete loss of the positive component of birefringence. This is interpreted as indicating destruction of the ultrastructure responsible for the optical properties of the axis cylinders of the nerve fibers. Correlated with the above is a great diminution or even loss of the elasticity of the nerve cord, and also a diminution of the photoelastic properties, but we do not as yet sufficiently understand the elastic and photoelastic properties of whole nerve cords to localize or evaluate effects thereon.

It seemed that the effects on the optical properties of the axis cylinder were always subsequent to irreversible paralysis but because of the time lag involved in dissection this was checked by *in vitro* experiments. Extirpated cockroach nerve cords were measured with polarized light in saline, and then while observation was continued a "Valone" solution in alkaline saline was drawn under the cover glass and the beginning and completion of the effect timed. Measurable changes, with only one exception, required 2–4 minutes or longer, and a large effect such as obtained in dissections after treatment required 10–15 minutes or longer. This is much slower than the paralytic action under comparable conditions and so demonstrates that the visible effect is post-paralysis and presumably postmortem.

The effects of "Valone" differ from those of all other substances tested not only in the drastic effect on the axis cylinder but also (and even more strikingly) in the apparent absence of any demonstrable effect whatsoever on the nerve sheaths.

Serial sections of "Valone"-treated cockroach nerve cords showed nearly normal histology. Even the axis cylinders of nerves appeared in reasonably good condition despite the known extensive degeneration of the optical properties. The only abnormal condition found was a moderate amount of chromatin clumping in a fair percentage of the nerve cells. As will be pointed out in the section on Suffocation, this indicates an increased cellular acidity presumably brought about by the "Valone"-injury.

"Tertiary butyl valone" (2-pivalyl-1,3-indandione) was not studied so intensively as "Valone." It was used on 75 cockroaches and 140 mosquito larvæ. The symptoms it produces are comparable to those found for "Valone," and so the effects seem likely to be similar so far as the nervous system is concerned.

EFFECTS OF OTHER DESTRUCTIVE AGENTS

In the course of work on penetration a number of very destructive organic compounds were encountered (Richards & Weygandt, 1945). Octyl alcohol (2-ethyl hexanol) can serve as an example of the more extreme of these. It accumulates in the central nervous system when injected into the tracheæ of mosquito larvæ but since it is somewhat soluble in water (0.1 per cent) it can travel readily in the insect's blood or be used for *in vitro* studies. The results are similar whatever method of application is employed.

The most obvious gross effect of octyl alcohol is to make the normally transparent brain and nerve cord of mosquito larvæ nearly opaque. Commonly the neural lamella becomes separated from the nerve cord by a clear space (Fig. 5). Observation of extirpated nerve cords treated *in vitro* shows that the neural lamella is not detectably altered but that the nervous tissue shrinks away from it. Using high magnifications it is possible to see that considerable cellular dissolution, as well as shrinkage, has occurred in these brains. Without any fixation artifacts being involved, one can see in these whole mounts in saline that octyl alcohol produces opacity, shrinkage and extensive cellular dissolution.

In serial sections of fixed brains extensive destruction is apparent. Commonly the peripheral parts of the brain have lost all appearance of cellular structure and consist of relatively uniform basophilic granular material (Fig. 11). Nuclei of recognizable cells are stained a solid dark blue. The fiber tract regions do not appear to be as badly cytolyzed as with some other agents (Fig. 12). Other tissues are also extensively affected by octyl alcohol, for instance the cytoplasm (but not nuclei) around muscle fibers has nearly or quite disappeared and the muscle fibers themselves show no cross striations.

Obviously the extensive destruction due to octyl alcohol is not selectively on the nervous system, and the dissolution is so great that no analysis of the effects is possible.

More or less similar, but usually less destructive, effects were obtained with a number of other compounds, some of which are used in insecticides. The list includes methyl diethanolamine,

monoisopropanolamine, morpholine, benzyl "cellosolve," cinnamic alcohol, m-cresol acetate, oleic acid, butyl carbitol acetate, butyraldehyde, oil of thyme, trichlorethane, ethylene dichloride, and to a lesser extent a number of other organic substances. The aminated alcohols are especially destructive to tissues.

EFFECTS OF SUFFOCATION AND ACIDITY

In connection with studies on petroleum oils one of us pointed out that asphyxiation causes a reversible clumping of chromatin within nuclei (Fig. 6, see also Richards, 1941). This criterion can, under properly controlled conditions, be used as an index of suffocation. However, the phenomenon can be produced by other agencies (*e.g.*, pressure) and so is no specific result of asphyxia itself. No explanation of the chromatin clumping was offered in the above paper. More recently it has been called to our attention that Nassonov analyzed this phenomenon in a paper published in 1932 (see also Alexandrov, 1932). He presented strong evidence supporting the view that the clumping is due to increased acidity in the nuclei (gut cells of fishes).[11]

Increased cellular acidity can be obtained in a number of ways. Asphyxiation can lower the pH by increasing the CO_2 concentration. It is also rather generally accepted that one of the common effects of injury to cells is increased acidity (Ettisch & Jochims, 1927). Thus one sees references to "the acid of injury" in physiological literature (see, *e.g.*, Heilbrunn, 1943). Several kinds of injury can produce clumping of chromatin, *e.g.*, pressure (Buck & Boche, 1938).

In view of the above, it is not surprising that chromatin clumping has been recently recorded as an effect obtained from the action of an insecticide (Hartzell & Scudder, 1942). We too have found chromatin clumping fairly general but not universal in pyrethrum-treated nerve cells of cockroaches.[11] We have also obtained chromatin clumping in nerve cells from the action of

[11] Chromatin clumping seems to be a general response to asphyxia in cells with large nuclei. It has been observed and studied in gut cells, gland cells and nerve cells. It is, however, not found in the small nuclei of the neuroglia cells of the central nervous system of cockroaches. Since one would expect asphyxia to lower the pH of all cells it would seem that the chromatin of some nuclei is unaffected by this degree of acidification (Fig. 6).

"Thanite" and "Valone." Apparently, this effect can be interpreted as meaning no more than that the affected cells were injured in such a way (manner unknown) that they become somewhat more acid. This, however, is more than one usually learns from a histopathological effect, and it is interesting that some insecticides do this and others do not.[12] When this clumping is produced by asphyxiation it occurs before death and is fully reversible up to the time of death. It would be very interesting (and desirable) to find out whether or not it is premortem and reversible when produced by insecticide action but this has not yet been studied.

TOXINS WITHOUT KNOWN HISTOPATHOLOGICAL EFFECTS

A number of well-known insecticides apparently do not produce any histologically visible effects in insects. Krüger (1931) and Hartzell (1934) both failed to find any effects from the application of rotenone. McIndoo (1916) and Hartzell & Wilcoxon (1933) report no effects from nicotine. The latter workers also found no histopathological effects in nerve cords of insects killed with lead arsenate. In our work we were unable to detect any notable changes in nerve cords treated with formalin, "non-toxic" mineral oils (*e.g.*, Marcol GX), sodium fluoride or "DDT" (2,2-bis-(p-chlorophenyl)-1,1,1-trichloroethane). Possible effects from "DDT" have been studied by us in some detail. The nerve cords of dying cockroaches have normal optical properties and may even still be capable of transmitting impulses set up by electrical stimulation. Stained serial sections prepared from cockroaches dying from the effects of "DDT" showed no clear effects in the nerve cords or other tissues.[13]

It has already been noted in a previous section that lipid solvents used as vapors cause no demonstrable histological effects in insect nerve cords.[14] It seems probable that a rather large num-

[12] This criterion, of course, is not applicable to those agents which cause a dissolution of the chromatin. The work of Haas (1941) is of interest in suggesting possible differences in terms of nuclear versus cytoplasmic action of drugs.

[13] In addition to nerve cords we examined midgut epithelium, malpighian tubules, thoracic muscle, "heart" and nephrocytes.

[14] Shull, Riley & Richardson (1932) concluded, "it is probable that lethal concentrations of most gaseous compounds do not produce marked visible changes in the blood" of cockroaches.

ber of insecticides and other toxins will not give rise to notable histopathological changes in insects.

DISCUSSION

The term "nerve poison" is encountered rather commonly in discussions of insecticide action but seldom defined. Actually, it is a loose term without any very precise meaning. As commonly employed in insecticide literature it is used to categorize the action of compounds that seem to produce symptoms involving the nervous system. Sometimes there is an implication that other systems and tissues are less or not at all involved.

Logically at least four degrees or categories of effects on the nervous system can be recognized: 1) A substance may affect all cells (octyl alcohol, aniline, fixing fluids, etc.), and may cause either degeneration or fixation of nerve cells non-selectively. These compounds are not referred to as nerve poisons. 2) A substance may have a significantly lower threshold for its action on nerves. At somewhat higher levels it may affect other tissues. 3) A substance may accumulate in the central nervous system and so reach toxic levels there sooner than in other tissues. This is rather general for lipid soluble substances. Since the concentration of a substance in a bathing solution is not necessarily an index of the concentration within cells or tissues it is commonly very difficult to separate truly lower thresholds from accumulation phenomena. In most cases no attempt is made to do so. Whether or not one is to call substances nerve poisons or selective nerve poisons when they affect other tissues at slightly higher concentrations or slightly longer times seems to be a matter of definition (and accordingly will vary with the purpose for which the term is used). 4) Lastly, there are some substances such as atropine which are thought to act specifically on nerve systems. One could add drugs such as eserine (physostigmine) to this last category, but eserine not only poisons the specific cholinesterase in the nervous system but also poisons other esterases from other tissues. An animal poisoned with eserine apparently dies because of the anti-cholinesterase action on the nervous system but less vital esterases of other tissues are also poisoned. With so many possibilities and gradations the term "nerve poison" cannot have any specific meaning. By the time the action of a substance is

sufficiently understood to state what is meant physiologically by calling it a nerve poison, a more precise term or statement would seem possible. Despite all its vagueness, or perhaps because of its vagueness, the term "nerve poison" is useful at times to express an action involving the nervous system.

Pyrethrum is not only one of the most standard of insecticides but also the classical example of an insecticide that is termed a "nerve poison." With the above preamble in mind, we would like to discuss the present state of our knowledge concerning the neurotoxic action of pyrethrum. The literature is voluminous and the following citations make no pretense of completeness. In 1924, Juillet, d'Everlange & Ancelin first suggested that pyrethrum was a neuromuscular poison because of the paralysis produced. Saling (1928) added cogent evidence since he could obtain what he thought were effects on the nervous system but could detect no effects on the respiratory system or blood. A considerable series of papers treat or mention the nervous symptoms of stimulation, narcosis, paralysis and death from pyrethrum, notably Buchmann (1929), Krüger (1931), Hartzell & Wilcoxon (1932), O'Kane *et al.* (1933), Gösswald (1934), Wigglesworth (1941), Eagleson (1942) and especially Hutzel (1942). Although the skeletal muscles seem to be stimulated extensively (Krüger, 1931; Klinger, 1936; Hutzel, 1942), the heart is only slowly affected (Krüger, 1931; Belleuvre, 1938). A number of authors have recorded and figured extensive histopathological changes in the central nervous system (Krüger, 1931; Hartzell *et al.*, 1932–1942; Klinger, 1936; Wigglesworth, 1941; and the present paper). Krüger and Hartzell also both record histological changes in other tissues, especially muscles. The recent paper by Sweetman & Gyrisko (1944) sounds as though they can obtain in firebrats a progressive localized narcosis involving various tissues but their data do not yet permit any real analysis of the situation (they also get the "typical" pyrethrum symptoms). Hurst (1943) considered the question of cuticle penetration of pyrethrum and other substances from a physico-chemical viewpoint, and Richards & Weygandt (1945) showed that pyrethrum, like other lipid solvents or lipid soluble materials, selectively penetrates into and accumulates in the nervous system. The present paper deals

rather extensively with the time relationships between loss of responsiveness of nerve cords to electrical stimulation, blocking of nerve transmission, paralysis and the appearance of histopathological changes. Preliminary oscillographic studies of the effect of pyrethrum in inducing spontaneous outbursts of impulses and modifying normal impulses have been presented by Lowenstein (1942) and Ellis, Thienes & Wiersma (1942).[15] The recent papers by Roy *et al.* (1943, 1944) seem to us not convincing and not significant; they will not be considered in the following discussion. The latest review of the action of pyrethrum seems to be that of Campbell (1942); older discussions are given by Shepard (1939) and Hoskins (1940). A number of papers have appeared on the effects following injection of pyrethrum into mammals; the most recent of these is by Leonard (1942) who agreed with previous authors in reporting nervous stimulation leading to convulsions and respiratory paralysis but a depression of muscle contractions in isolated pieces of intestine and an absence of neuropathology.

These lines of evidence for a neurotoxic action of pyrethrum may be grouped under five headings: 1) stimulating effect leading to paralysis and death, 2) histopathological changes, 3) accumulation and threshold concentrations, 4) early loss of responsiveness of nerves to electrical stimulation, and 5) effects on the action potential.

The nervous symptoms and diagnostic effects are thoroughly discussed by Hutzel (1942) and reviewed by Campbell (1942). The sequence of activation, convulsions and paralysis, as they point out, suggest stimulation of peripheral sense organs or sensory nerves, stimulation of the central nervous system and then paralysis. These data are quite suggestive and seem correctly interpreted, but they leave unanswered the question of possible effects on other tissues and the question of why the heart is affected so slowly.

The histopathological changes seem to us the poorest line of evidence. Unlike certain other tissues, a functioning nerve undergoes no visible changes.[16] All one can study is chemical processes

[15] This paper deals with the peripheral nerves of crayfish (crustacea) but the data are nevertheless of entomological interest.

[16] With tissues in which one can follow the functioning cytologically, a stronger case can be made for histopathology (note for instance how little

and electrical phenomena. Using direct electrical stimulation of nerves and ganglia as a means of verifying the functional status of the nerve cord, we were unable to produce visible changes with pyrethrum (or any other insecticide) until after the nerves concerned were dead.[17] The degenerative effects and lesions therefore seem postmortem and accordingly incapable of being analyzed at present. Also there is no fixed relation between the degree of degeneration of the central nervous system and the death of the experimental insect. More serious is the fact that, except for possible differences in the time factor, the degeneration of pyrethrum-killed nerves follows a course similar to that of nerves degenerating in saline (present paper) or in the body after suffocation (Richards, 1941). Pathological changes have also been recorded for muscles (Krüger, etc.). In view of the fact that the central nervous system degenerates more rapidly than other insect tissues (Richards, 1941), it seems at least possible that the degeneration seen in pyrethrum-killed insect nerve cords might be due to autolysis.[18] If this is true, then the recorded histopathology from pyrethrum could be interpreted as indicating no more than death of the central nervous system prior to that of other tissues.

The chromatin clumping in nerve nuclei (Hartzell & Scudder, 1942) is a good criterion but seemingly indicates only that the cells have become somewhat more acid (Fig. 6). It seems likely that this increased acidity may be due to the pyrethrum but it

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reference is made to nerves in Ludford's review). However, although histopathology is of great use for diagnostic purposes in medicine, it is not viewed with favor by cellular physiologists or biochemists. It may in some cases give clues for study but it does not seem likely to explain much of the physiology of toxic action.

[17] Data from vertebrates are probably not strictly comparable but we can note that Schmitt, Bear & Palmer (1941) were unable to affect nerve sheath structure *in vitro* with detergents, autolysis, calcium or potassium prior to the death of the nerves, and Leonard (1942) found no pathology in the brains of rabbits and mice in convulsions from pyrethrum.

[18] The difficulty in this connection is to decide what is the "normal" course of autolysis. Some method must be used to kill the cells. This automatically complicates the analysis. We have found that physical methods such as heat and cold introduce invalidating errors. Any chemical is suspect. The two methods recorded here seem to us best although it cannot be claimed that either is "normal."

would be difficult to prove that an "acid of injury" is really involved in this case. It is not yet known whether this is premortem (cell viewpoint) when produced by insecticide action.

Very few data are available on cytopathology of insect nerves from the action of pyrethrum or any other insecticide. The data from optical analyses given in the present paper are cytological (and even based on submicroscopic structure) but cover only the optical properties of the nerve fibers. The degeneration we found in these properties was postmortem for the specific cells concerned.[17] Except for the phenomenon of chromatin clumping discussed above, other possible cytological changes in insect nerve cells (Nissl patterns, mitochondria, Golgi apparatus, etc.) have been scarcely or not at all studied (see especially review by Ludford, 1942).

Nothing is known about the threshold for the action of pyrethrum. MLD determinations can be made accurately but they give no indication of the quantitative distribution within the insect. Until actual thresholds for different tissues are determined (or a specific chemical action to nerves alone demonstrated), it does not seem possible to state that pyrethrum has a *specific effect* on nerves since the material has been shown to penetrate selectively into and accumulate in the nervous system (Richards & Weygandt, 1945). In any experimental insect one has almost certainly a greater concentration in the nervous system than in other tissues. It is conceivable, but not necessarily true, that the apparent selective action is a result of the differences in distribution in the insect. Some distribution phenomenon such as this may possibly account also for the slow effect on the insect heart and its intrinsic nerves.

It was found in our work that pyrethrum paralyzed nerves so that they would not respond to direct electrical stimulation, and also blocked the transmission of impulses through affected ganglia. Yet peripheral movements might proceed for many hours. This is a direct proof of nerve paralysis. It substantiates the conclusions already drawn by others from the general symptoms and general paralysis. The paralysis, however, is still open to the same questions mentioned above; namely, that other tissues may be affected and the quicker effect on nerves may merely reflect the distribution of the pyrethrum.

Preliminary data on action potentials also corroborate the stimulatory and paralytic effects of pyrethrum. This insecticide can alter normal action potentials in cockroach nerve cords (Lowenstein, 1942) and induce spontaneous discharges in crayfish peripheral nerves (Ellis, Thienes & Wiersma, 1942).

The one function that a nerve has (transmit an impulse) is affected by pyrethrum as shown by general body reactions, spontaneous discharges, altered discharges, and loss of responsiveness. Without question, then, pyrethrum has a definite and strong effect on the insect nervous system, and it seems safe to conclude that its normal action on an intact insect is to stimulate and then paralyze. It remains for further work to show how much other tissues are affected, and in how far the selective nervous action is due to selective penetration and accumulation in the nervous system. It seems superfluous to add that as yet we have no idea as to what specific effect pyrethrum has on nerves or other tissues (in terms of cellular physiology or biochemistry).

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It may be convenient to some to have a summary of the types of pathological effects recorded in the present paper.[19] The use of optical analyses of treated nerve fibers has a number of advantages over the usual routine histological procedures (Schmitt & Bear, 1939; Richards, 1944). Outstanding among the advantages, the changes in optical properties can be measured accurately and expressed quantitatively. At least with those substances studied by us intensively, the method either reveals changes not detectable by ordinary sectioning methods (since based on submicroscopic structures and organization) or is at least more delicate (shows small measurable changes sooner). Fixation and sectioning, with their attending artifacts, can be avoided. A clear distinction between effects on the axis cylinder and effects on the nerve sheaths is usually obtained as a routine result of the several measurements in different media. The optical method, however, is applicable only to nerve fibers; it cannot be used to study the nerve cell bodies.

[19] Of the many treatises on vertebrate, especially human, neuropathology, we might recommend Spielmeyer (1928), Weil (1933), Speransky (1935), Page (1937), Young (1942), and Ludford (1942) as particularly useful references.

Nerves may become granular in internal appearance. This is the first visible change in normal nerves viewed in ordinary light as they die and begin to degenerate in saline. The granularity is preceded by a loss of the optical properties of the axis cylinder, which in turn is preceded by death of the nerve. This granularity is best seen in intact nerves viewed in saline; it is commonly not detectable in stained sections.

Various kinds of larger particles may occur with some materials. These may be either inside or outside the cells and fibers. Birefringent particles which are soluble in lipid solvents and occur *outside* the fibers most probably originate from the release of the optically active lipids of the nerve sheaths although one can not exclude the possibility that some of the lipids might have been drawn out of the interior of the nerve fibers (Figs. 15–18). Isotropic particles and globules are of more uncertain origin.

Vacuoles, like particles, may occur either within or between the cells and fibers, but the largest ones are found *outside* of the cells (Figs. 7–10). The holes called "vacuoles" in insect histopathology do not or at least do not necessarily represent vacuoles in the usual cytological sense. They represent the precipitation of tissue constituents around some particle or droplet which is subsequently dissolved during preparation of the section. The nature of those that are found *inside* cells is unknown, and it does not seem possible to attribute their presence directly to the action of the killing agent since they might represent autolytic phenomena (see Ludford's review). The holes that are found *outside* of the cells and fibers can sometimes be identified with fair certainty. The possibilities are that they represent breakdown products of the nerve sheaths produced either by the action of the toxin or by autolysis, or that they represent actual droplets of the toxin, or that they represent material withdrawn from or extruded by the cells. The last possibility is difficult to exclude but examination of unfixed specimens in saline helps in certain cases. In attempting to determine from what the holes originate it is desirable to examine specimens in saline because in several cases (*e.g.*, aniline) we have been able to demonstrate that the particles and hence "vacuoles" are fixation phenomena. In stained sections the "vacuoles" appear simply as holes bearing no label as to their previous contents.

There are three other phenomena that seem best studied in unfixed nerve cords in saline. These are shrinkage, opacity and chromatin clumping. So much shrinkage is involved in making sections that this effect should be studied in saline, preferably the toxin applied after measuring and during observation. Shrinkage most likely indicates a water loss, but in insecticide studies the reasons for such a loss do not seem easy to analyze. Opacity must be examined in unfixed material since fixation produces opacity, and clearing in mounting media masks it. Opacity may be used as an index of abnormality, but interpretation of it would seem difficult. Chromatin clumping can be readily observed either in transparent tissues in saline (or even in intact transparent animals) or in stained sections. As a reversible physiological phenomenon it should be experimented with either *in vivo* or *in vitro* but as a product of a particular treatment it can be just as well seen in stained sections. Since it appears to be caused by changes in the cellular pH, care must be taken to be sure that the effect is really due to the action of the insecticide. Its possible reversibility when produced by insecticide action merits investigation.

Other forms of degeneration are best or sometimes only seen in sections.[19] Chromatolysis or various forms of staining and nonstaining that differ from controls (not necessarily "normals") is usually studied in stained sections (Figs. 10 & 12). With certain dyes this is possible both *in vivo* and *in vitro* but such techniques have not yet been applied to insect nerve pathology. An alteration in staining capacity is the least radical of changes recorded in previously published literature. Extensive changes in the staining properties of insect nerve cells and fibers treated with insecticides are, in our experience, subsequent to irreversible paralysis of the nerves and so presumably postmortem. It is quite possible that some changes in stainability and in fine cytological structure may occur in nerve cells prior to the death of the cells concerned but this is not easy to study or prove and has not yet been done.

More extreme forms of degeneration are numerous. One could apply many terms and describe long series of stages. In general, two types of extreme degeneration can be distinguished although

they commonly occur together: cell and fiber separation, and cell and fiber degeneration. Separation is produced by agents which injure or destroy the nerve sheaths, and may be due directly to the toxin (Fig. 10) or to autolysis following death caused by the toxin (Fig. 7). Separation is greatly affected by fixation since the cells and fibers can shrink independently instead of as a unit. Separation is always accompanied by more or less degeneration of the nerves but may be extreme at a time when the nerve cells and fibers still appear fairly typical. The separation is commonly most noticeable at the boundary between the central fiber tract area and the peripheral layer of nerve cell bodies. Probably several factors are involved: this seems to be the weakest part of the tissue and also the layer in which most of the tracheæ occur (Fig. 2) and in which "vacuoles" may be particularly prevalent (Richards, 1941). The destructive agents which cause radical degeneration, lytic or otherwise, are recordable but beyond analysis by the methods used in this paper.

As mentioned previously, the data in this and other papers deal with the effects of single acute doses. No localized action on particular centers of the insect central nervous system has been reported. In our work we have not noticed any such local effects from insecticide action. It seems that the substances studied affect all nerve cells indiscriminately when applied in the doses we used. At least one reservation must be made. To date no one seems to have studied seriously the possible occurrence of localized effects (more susceptible nerve centers) resulting from prolonged, repeated chronic doses.

In conclusion on the types of histopathological changes found in nerves following insecticide action, we can say that a fairly large number of varieties have already been recorded in the present and other papers. There is no reason to think that others cannot be found. A number of insecticides produce effects which parallel and so presumably represent autolysis phenomena (pyrethrum, "Thanite" & petroleum oils); certain other insecticides produce effects more or less distinct from autolysis ("Valone," lipid solvents, certain essential oils, etc.). It seems that one could go on indefinitely performing such experiments and describing in detail the histological and cytological pictures obtained. The

value of such a mass of data seems to us questionable. At least in the best analyzed case (acute doses of pyrethrum) there is no fixed relation between the degree of degeneration and death of the insect. And in no case have we found detectable pathology from an insecticide prior to the irreversible paralysis (and presumably death) of the cells concerned. Chromatin clumping is a possible exception since when it is produced by suffocation it is premortem and then reversible. Chromatin clumping is a phenomenon that seems to us profitable for further study.

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Cytologically the insect nervous system is similar to that of vertebrates, especially to the non-myelinated fibers such as are found in the vertebrate autonomic system. The cells are smaller, the sheaths thinner, and the connective tissue less, but the basic structure of the cells and fibers so far as has been determined is the same (Richards, 1944; Scharrer & Scharrer, 1944–1945). The chemical or at least lipid components are comparable (Patterson, Dumm & Richards, 1945). Also insect nerves are cholinergic, *i.e.*, have an acetylcholine-cholinesterase mechanism, but there are at least qualitative differences in the cholinesterases of insect and vertebrate nerves (see Richards & Cutkomp, 1945).

Accordingly, we may conclude that the insect nervous system is similar in many ways to the vertebrate nervous system, particularly to the vertebrate autonomic system (Jordan, 1928; the Roeders, 1939; the Scharrers, 1944–1945), but that some differences seem to exist (cholinesterase). A more intensive study of the insect nervous system and its relation to insecticide action is needed. It seems to the present authors that histopathology has little if anything to offer this further analysis. The possible use of cytopathology of insect nerves has not been sufficiently studied for evaluation but the slight amount of data available is not encouraging. Studies of the reactions of insects to drugs (including some insecticides), of nerve enzymes and biochemical processes, and of electrical phenomena are the methods that seem promising for studying the action of neurotoxic insecticides.

SUMMARY

1. Data are presented on the histopathological effects caused by acute doses of various materials including pyrethrum, "Tha-

nite,'' petroleum oils, venoms, triorthocresyl phosphate, lipid solvents, insect repellents, aniline, essential oils, ''Valone,'' acidity, and a number of highly destructive compounds of which octyl alcohol is taken as an example. Cockroaches and mosquito larvæ were used as test animals. No visible effects were obtained with ''DDT'' and certain other compounds. Optical analyses, routine stained sections and *in vitro* analyses were used; electrical stimulation of the nerve cords was employed to determine the physiological state of the nerves being studied.

2. The various types of pathological pictures can be described by the use of terms such as decrease or loss of one or more of the components of the optical properties, granularity, chromatin clumping in the nuclei, and various stages of cell dissolution ranging from chromatolysis to particle production, ''vacuolization,'' opacity, shrinkage, and extensive cell and fiber separation and disintegration.

These categories are not sharply defined or mutually exclusive. A particular toxin may produce a more or less characteristic picture under a particular set of conditions but at least with pyrethrum there is no fixed relationship between the death of the insect and the degree of degeneration of the nervous system.

3. The physiological and histological effects of pyrethrum are considered in some detail. It is concluded that previous workers are correct in calling this insecticide a ''nerve poison'' but that the histological (and what is known for cytological) changes are similar to those produced by autolysis and may not be directly caused by the pyrethrum. Pyrethrum penetrates selectively into and accumulates in the nervous system of insects. Its threshold for nerves, its possible thresholds for and effects on other tissues, the relation between these thresholds, how it kills and whether or not it has a *specific* effect on nerves, are points not yet covered by the existing literature.

4. Lipid solvents used as fluids in considerable quantity remove the lipid component of the sheath birefringence. Used as vapors, however, they kill without producing any visible effect. Accordingly the visible effects that can be produced cannot be the cause of death from these substances.

5. Insect repellents have a visible effect on nerves comparable

to that of lipid solvents. Used as fluids they abolish the lipid component of birefringence but used as vapors they kill without visibly altering the structure of the central nervous system.

6. In all cases studied, nerves were paralyzed and presumably dead *prior* to the appearance of any abnormalities or lesions with the possible exception of chromatin clumping. Accordingly all histopathological pictures recorded for insect nerves, with the possible exception of chromatin clumping, are to be classed as "postmortem," and their further analysis is of questionable value. The same statement may be made for the little that is known about insect nerve cytopathology. It seems to us that histopathology of insect nerves may at times give some slight help in localizing the action of certain insecticides but that it is at best a crude and likely to be misleading measure of physiological effects in insects. The reservation should be repeated that existing data refer to the effects of acute doses; the possibility of obtaining more localized effects on particular centers by prolonged chronic dosing has not been sufficiently investigated as yet.

7. The term "nerve poison" is a rather vague concept. It is a convenient term but not a specific one. It can mean either that the substance has a lower threshold in nerves, or accumulates there more rapidly or in greater amounts, or it may mean a truly specific action. However, the analysis of insecticide action on nerves requires more specific techniques than histopathology and the demonstration of paralysis.

8. The suggestion (Richards, 1943) that lysolecithin formed by the breakdown of nerve sheath lipids may be concerned in insect paralysis, is discredited. Cockroaches are not visibly affected by the injection of maximal quantities of lysolecithin, and the nerve sheaths are not detectably affected by cobra venom prior to paralysis. Certain other substances highly toxic to vertebrates have little or no effect on cockroaches (histamine, curare).

SUPPLEMENTARY NOTE

While this paper was in press Hartzell (1945) published a paper on the histopathological effects of several compounds used in insecticides, including "DDT." He stresses the selective action of certain substances on nuclei, nuclear membranes, nerve cell cytoplasm, nerve fibers or intercellular spaces, and notes that

these differences connote something different in the way of action of the substances concerned. He further suggests that the synergistic effect of certain substances may be due to the activator attacking one cellular component, the insecticide another. The paper is subject to the same criticisms pointed out in the above text, and it is difficult to attempt interpretation on the basis of such data.

Hartzell records "relatively slight" pathological effects from "DDT." He has no real knowledge of whether or not the nerves concerned were living or dead but since the time interval was short (and "DDT" effects are relatively slow) they may well have been still functional. However, at least in cockroaches, more variation is seen in long series of controls (or normals) than Hartzell shows for the differences between normal and DDT-killed houseflies. Possibly Hartzell did obtain a slight effect (assuming that histology of the central nervous system is less variable in houseflies than in cockroaches) but the slight effects recorded agree with the usual autolysis picture. We can only repeat that in our "DDT" experiments dying cockroaches which had responsive nerve cords showed no effects that could not be ascribed to normal variation and matched by control preparations.

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PLATE IV

Figure 1. Whole mount of supra- and sub-œsophageal ganglia of mosquito larva showing the distribution of tracheæ. The tracheæ are filled with Eugenol saturated with Black Sudan B. Magnification 55 ×.

Figure 2. Obliquely tangential section of prothoracic ganglion of an adult cockroach showing the distribution of tracheæ principally in the boundary region between the fiber-tracts and cell-bodies. One half is cut tangentially through this boundary and shows several tracheæ in longitudinal section. Magnification 45 ×.

Figure 3. Whole mount of supra- and sub-œsophageal ganglia of mosquito larva showing spotty penetration of stained oil of citronella from the filled tracheæ. Magnification too low to show the droplet-type of penetration (see figure 4). Magnification 45 ×.

Figure 4. Portion of whole mount of supraœsophageal ganglion of mosquito larva showing the droplet-type of penetration. Tracheæ incompletely filled with stained "Citrola." Magnification 220 ×.

Figure 5. Whole mount of supraœsophageal ganglion of mosquito larva. Tracheal injection of octyl alcohol has caused the neural lamella to separate from the brain (shrinkage of the nervous tissue). Magnification 55 ×.

Figure 6. Portion of section of a thoracic ganglion from a suffocated cockroach. Shows extreme chromatin clumping in most but not all of the nerve cells, whereas the nuclei of the neuroglia cells (vertical row on right side) are normal. Magnification 470 ×.

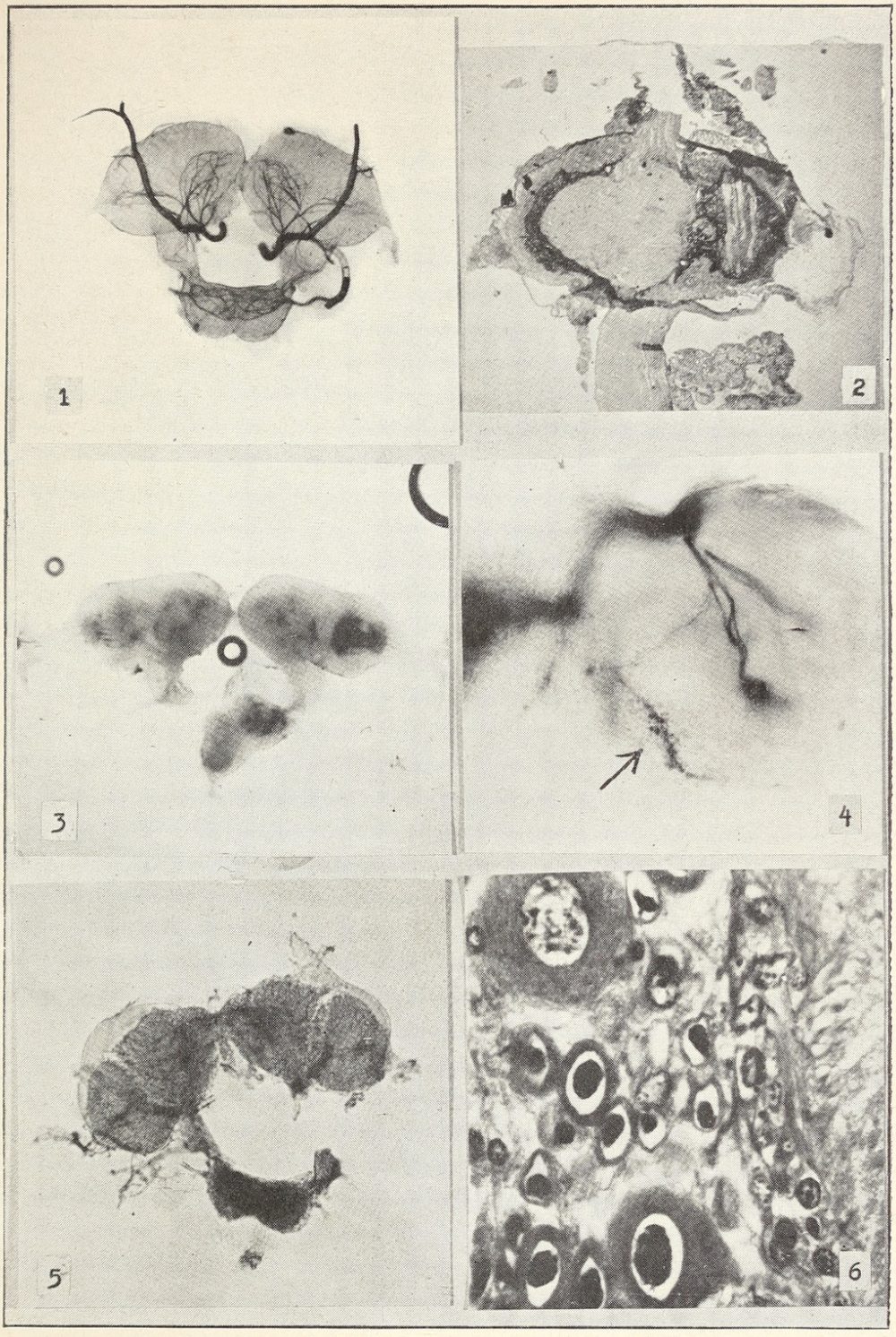
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PLATE V

Figure 7. Longitudinal section of fourth abdominal ganglion of mosquito larva dying from the effects of a tracheal injection of isoborneol thiocyanoacetate (active principal of "Thanite"). Note "vacuolated" fiber tract region. Compare figure 8. Magnification 470 ×.

Figure 8. Longitudinal section (slightly oblique) of fourth abdominal ganglion of mosquito larva with tracheal injection of a "nontoxic" mineral oil ("Marcol GX"). Control for figure 7. Magnification 470 ×.

Figure 9. Section of supraœsophageal ganglion of mosquito larva. Tracheæ filled with stained oil of citronella. The arrow points to a cross-section of a trachea. The holes adjacent to this trachea presumably represent droplets of citronella. Magnification 470 ×.

Figure 10. Section of subœsophageal ganglion of a mosquito larva killed by a tracheal injection of aniline. Magnification 435 ×.

Figure 11. Portion of a section through the cell-body region of supraœsophageal ganglion of a mosquito larva killed by a tracheal injection of octyl alcohol. Note indistinctness of cells in central part and disintegration to granular layer in peripheral part. Magnification 400 ×.

Figure 12. Section of subœsophageal ganglion of a mosquito larva killed by a tracheal injection of octyl alcohol. Magnification 470 ×.

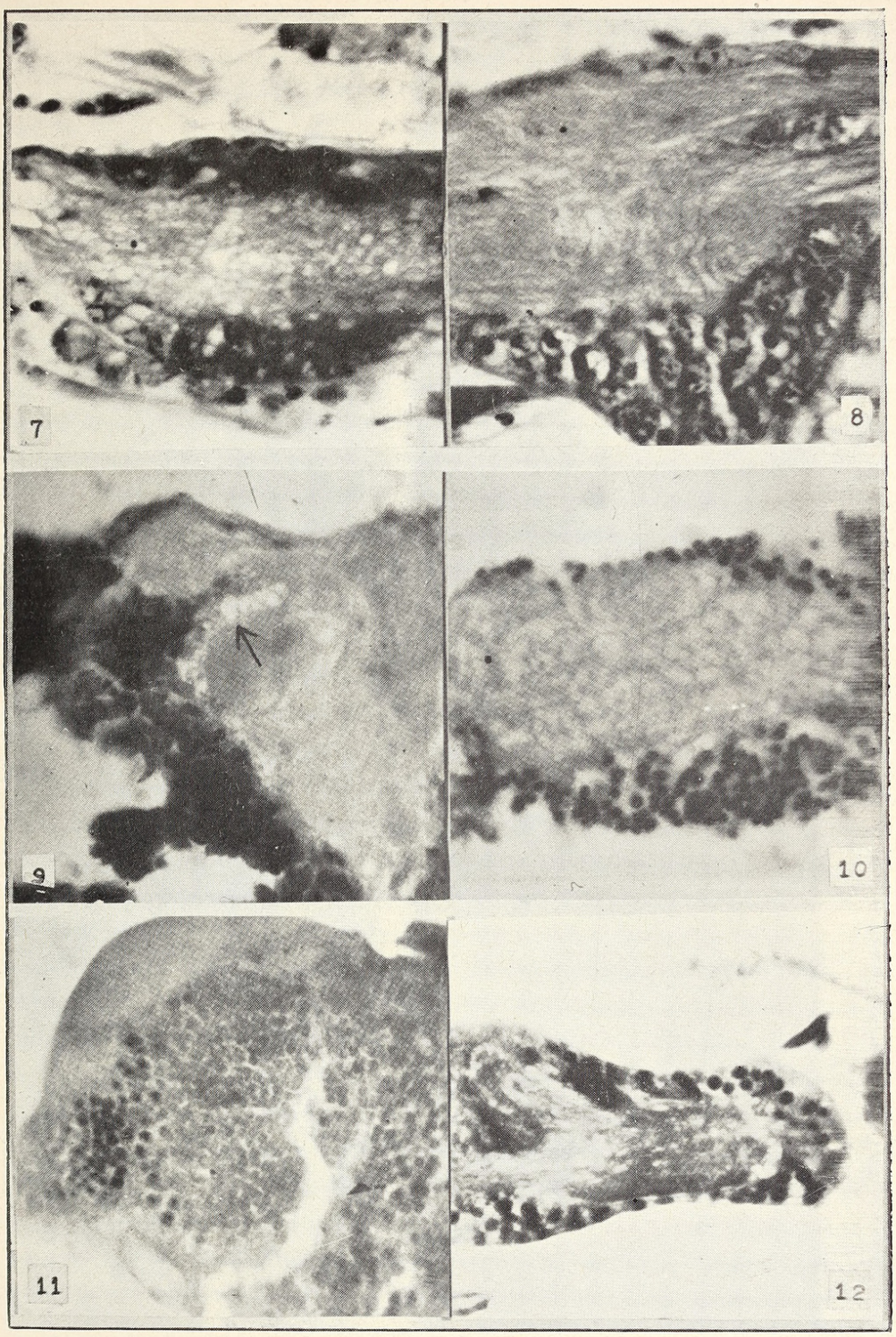
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PLATE VI

Figure 13. Portion of a whole mount of supraœsophageal ganglion of mosquito larva showing penetration of stained droplets of propylene glycol monolaurate from injected tracheæ. Magnification 470 ×.

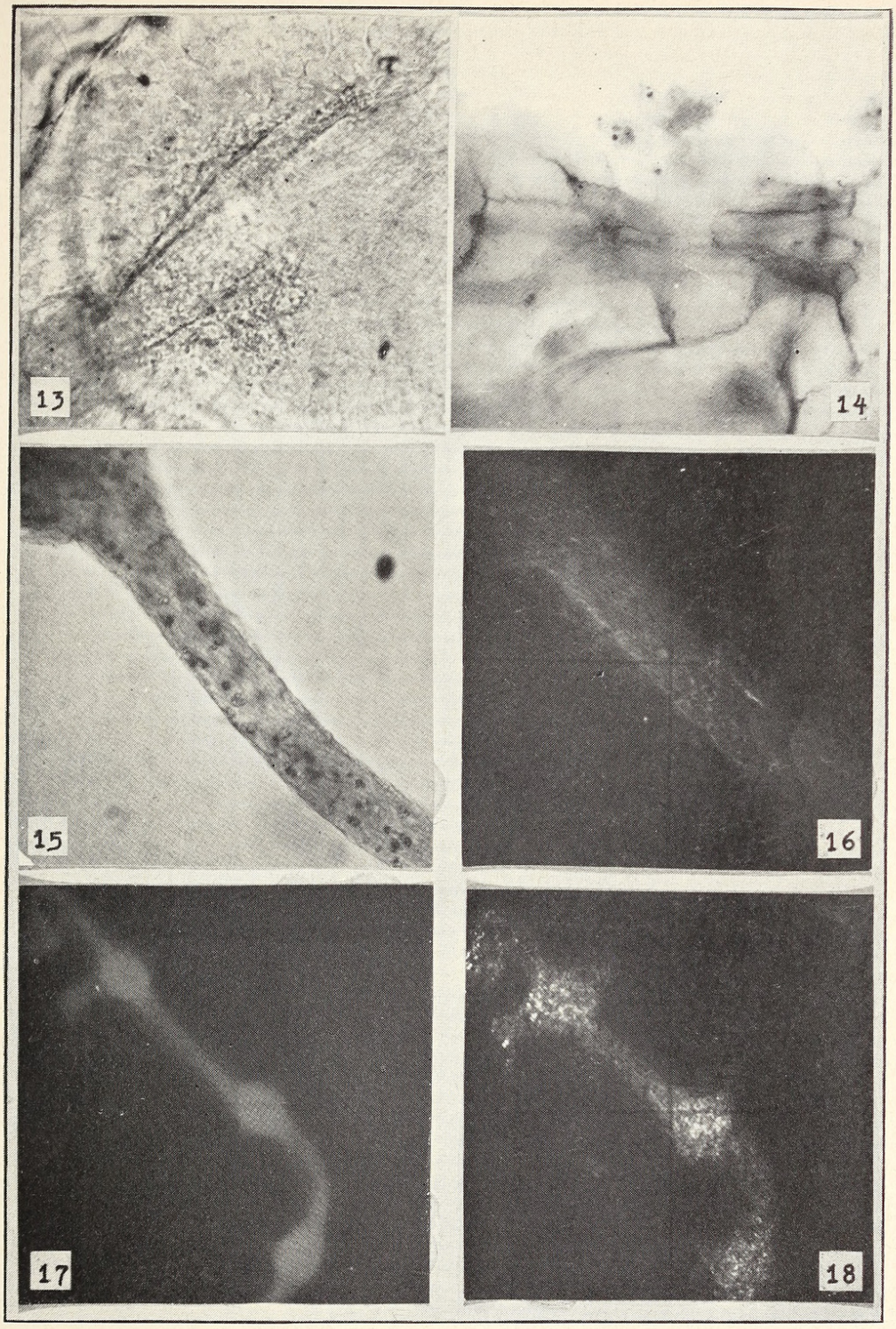
Figure 14. Whole mount of two thoracic ganglia of a mosquito larva showing the diffuse penetration of stained chloroform. This is an early stage picked to show gradient from the fluid-filled tracheæ into the nervous tissue. Magnification 220 ×.

Figure 15. Whole mount of abdominal connective of a mosquito larva killed by a tracheal injection of Eugenol. Photograph in ordinary light to show rounded particles. Magnification 300 ×.

Figure 16. Same as seen in polarized light between crossed Nicols. The rounded particles are very faintly birefringent. Magnification 300 ×.

Figure 17. Whole mount in saline of abdominal nerve cord of a mosquito larva following a tracheal injection of aniline. Photographed in polarized light between crossed Nicols. Compare the relatively strong, diffuse birefringence with normal nerve cord figured by Richards (1944). Magnification 50 ×.

Figure 18. Same after fixation for 50 minutes in formol-saline. The strongly birefringent particles are of irregular shapes. Magnification 50 ×.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