How Contact Insecticides Kill
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HOW CONTACT INSECTICIDES KILL.

III. RELATING EVIDENCE, FURTHER, OF CERTAIN PROPERTIES OF CARBON DISULPHIDE, GASOLINE, AND A FEW OTHER FLUIDS, AS WELL AS TEMPERATURE AND SOME DRY POWDERED CONTACT INSECTICIDES, BY MEANS OF WHICH THE INSECTICIDAL ACTION OF THESE AGENTS IS ACCOMPLISHED AFTER THEIR ABSORPTION INTO THE INSECT TISSUES, OR AFTER MERE APPLICATION—INCLUDING ALSO BRIEF SUGGESTIONS FOR POSSIBLE PRACTICE.

BY

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TABLE OF CONTENTS.

HOW CONTACT INSECTICIDES KILL.

III. Relating evidence, further, of certain properties of carbon disulphide, gasoline, and a few other fluids, as well as temperature and some dry powdered contact insecticides, by means of which the insecticidal action of these agents is accomplished after their absorption into the insect tissues, or after mere application;—also brief suggestions for possible practice, by George D. Shafer.

INTRODUCTORY STATEMENTS.

(a) FORMER EVIDENCE.

In a former paper on the subject, "How Contact Insecticides Kill*," it has been shown that many gaseous or volatile contact insecticides mainly become effective after being taken up by the insect tissues. Certain color tests and actual volume measurements demonstrated that gaseous insecticides, (such as H_2S, HCN and NH_4) and the vapor of carbon disulphide, as well as that from many volatile oils (e. g. kerosene, gasoline, benzine, turpentine) are absorbed by the tissues of insects. Much evidence was pointed out which indicated that vapors of gasoline, carbon disulphide and the like, after absorption, accomplish their effect upon insects through some tendency their presence exerts to prevent oxygen assimilation in the tissues.

If the vapors of the insecticides mentioned do interfere with oxygen assimilation, upon what do they act in the tissues to bring about that condition?

At the time the former bulletin was written nothing had been done upon this latter phase of the problem. It is with facts that seem to relate to this phase of the question that the present bulletin has to do. Careful examination was made of the organs of treated insects and many histological studies were made. When an insecticide, such as gasoline, was present in abundance, it was found that after several hours the fatty tissues went more or less into solution. Insects were crushed and an attempt was made to detect changes which carbon disulphide, kerosene, gasoline and the like might have upon the tissue-pulp. It was easy here also to notice the solvent action of gasoline, kerosene and carbon disulphide upon the fats and fat-like substances in the pulp. The action of carbon disulphide upon protein in the pulp could also be seen. The concentrated vapor of carbon disulphide would, in time, coagulate and whiten certain proteins. Egg-albumen readily shows this action of carbon disulphide vapor, after a few hours confinement with air *saturated* at about 68° to 70° F. with that vapor. Studied in this way, however, using only such amounts of the agents in question as were necessary to *kill* insects, there was no visible effect on the tissue pulp which might be held as a sufficient cause for rapid death.

*Parts I and II, Tech. Bulletin No. 11, Mich. Agr. College Exp. Station.

As already pointed out, however, a rise in the respiratory ratio during the time insects were dying, afforded the strongest evidence of an inhibition of oxygen assimilation. It was decided, therefore, to determine the respiratory exchange of fresh insect tissue pulp and to see whether this exchange became influenced by gasoline, for example, in the same manner as had already been found for insects themselves. That the pulp does have a decided respiratory exchange, and that this is influenced by gasoline vapor in the same manner as the latter influences the respiration of insects may be seen by comparing table I-A with table V of part I, Tech. Bull. No. 11.

TABLE I.

A.

Extract pulp of tissues of *Passalus cornutus.*	Treatment.	Temperature.	Respiratory period.	$\frac{CO_2}{O_2}$
5 c. c.	Gasoline	24.2° C.	16 hrs.	$\frac{2.1}{1.2} = 1.7$
5 c. c. same sample	None	Same	Same	$\frac{2.8}{3.4} = 0.82$
5 c. c.	Gasoline	24.2° C.	7½ hrs.	$\frac{0.9}{0.4} = 2.2$
5 c. c. same sample	None	Same	Same	$\frac{0.5}{1.0} = 0.5$
5 c. c.	Gasoline	25.4° C.	16½ hrs.	$\frac{3.1}{1.3} = 2.3$
5 c. c. same sample	None	Same	Same	$\frac{4}{5} = 0.8$

B.

Extract pulp of tissues of *Passalus cornutus.*	Treatment.	Temperature.	Respiratory period.	$\frac{CO_2}{O_2}$
5 beetles (uninjured)	None	20.8° C.	1 hr.	$\frac{3.2}{4.49} = 0.71$
Same 5 beetles	Opened and digestive tracts removed.	21° C.	1 hr.	$\frac{0.89}{0.8} = 1.1$
5 beetles	" "	20.4° C.	18 hrs.	$\frac{6.4}{7.9} = 0.81$
5 beetles	" "	20.8° C.	6 hrs.	$\frac{2.7}{2.9} = 0.9$

Gasoline was selected for making this test because of all the volatile insecticides employed, it permitted of the most accurate volumetric determinations of its own vapor, and of the oxygen, carbon dioxide and nitrogen present at the beginning and at the end of the test. The apparatus and the method employed for making the gas determinations has been described in Part I of the former bulletin. The insect-tissue pulp was contained in a small, sterile, open glass stender dish which floated on the mercury in the respiration container. The pulp, or crude tissue-

extract, was prepared as follows: The insects were thoroughly cleaned with absolute alcohol and a cloth. In the case of *Passalus cornutus*, which was mostly used, the wing-covers and wings were first removed. Then the digestive tracts were carefully removed entire, and discarded. The remainder of the tissues—blood, fat, muscles, nervous tissue, etc.—became thoroughly "ground up" in a mortar with a little distilled water.

It will be noticed that the respiratory ratio of the pulp or water extract of insect tissues proved to be rather higher, often, than was the case for healthy, uninjured beetles. A few respiration experiments were run, using insect bodies from which the digestive tracts had been removed. The ratio of carbon dioxide given off to oxygen taken up was in this case, also, a little higher usually than the ratio for normal healthy beetles. (See B, table I). Mechanical injury, through crushing without external mutilation, or through pithing the beetles with a hot needle, however, gave a ratio for the respiratory exchange lower than that of healthy normal beetles. But as has already been pointed out, when air saturated with gasoline vapor was used either with the insects themselves, with the tissue-pulp or with the crude water extract, it regularly caused a decided rise in the respiratory ratio above that given by the same insects, tissue pulp, or crude extract. Thus it began to appear as if whatever bodies were present in the living insect tissues to start and carry forward the respiratory process—these were able to maintain themselves as "respiratory bodies" (with limited activity) for a time, at least, in the insect tissue pulp and in the crude extract of the pulp, where the effects of the insecticides in question might still be studied.

(b) OXIDASES, CATALASES AND REDUCTASES IN ANIMALS AND PLANTS.

In the pulp and in the water extract of various tissues of many vertebrate and other animals and in many plant tissues, workers have described enzymes which are able not only to carry forward oxidation processes in the pulp or extract itself, but also to initiate or accelerate the oxidation of certain other bodies, such as gum guaiac, hydroquinone, tyrosine, etc., when these are added in solution to the extract. Such enzymes have received the general name of oxidases. It is not the purpose here to give a discussion of oxidasic enzymes, their kinds and possible functions in the living tissues in connection with other enzymes or enzyme-like bodies; but a short statement will make clear the import of the experiments which follow in this connection. None of the oxidases have been obtained in a known pure and isolated form, yet much has been learned of their properties, and of their more or less specific action, so that they have generally received names according to the kind of body or bodies upon which they have been found to act—as alcoholases, aldehydases, phenolases, or laccase, tyrosinase, etc. Associated generally, if not always in pulp or tissue-extract with oxidases, various workers have found peroxidases, catalases, and reductase (or reductases). The peroxidases of tissue extracts (when they clearly show themselves present) are substances which activate peroxides—for example they cause hydrogen peroxide to change in such a way that its oxygen may became transferred to guaiac, causing oxidation of the latter to guaiac blue. Peroxidases serve as activators merely; they do not cause the liberation of free oxygen from the peroxides. Indeed an oxidase is now looked upon

by most workers in this field as an organic peroxide, or an oxygenase (i. e. a substance capable of quickly forming an organic peroxide by uniting with oxygen from air) working with a peroxidase. The alpha and beta catalases, as demonstrated by Loew, are enzymes which are capable of breaking down H_2O_2 into molecular oxygen and water, the oxygen coming off in bubbles. Finally, tissue pulp from plants and from animals has been found to possess a more or less strong reducing power. This power has been generally attributed to reducing enzymes called reductases. Knowledge of the functions and properties of the enzymes mentioned above is, as yet, very incomplete. Satisfactory proof has not been obtained in some cases, and there are those who believe that reductases and catalases are not enzymes at all. As experiments and observations accumulate, however, there seems to be a growing number, among those actively engaged in investigation of the subject, who believe that in the oxidases, catalases, and reductases we have to do with a part, at least, of the machinery which accomplishes oxidations and respiration in the protoplasm of living cells. Alexis Bach, for example, has gone so far (in 1913)*, in a discussion of oxidising and reducing enzymes, as to express his belief in their role in the process of respiration rather definitely. E. F. Armstrong in the "Journ. of the Chem. Soc. 104, Part I, p. 543, abstracts Bach's conclusions as follows:

"(1) In order to utilize the oxygen of the air to effect oxidation, the cell produces an enzyme (an oxygenase)—a substance which is readily oxidized, fixing molecular oxygen to form a peroxide. (2) A second enzyme (the peroxydase) accelerates the oxidizing action of the peroxides, acting on them in the same way as ferrous sulphate does toward H_2O_2. (3) The peroxides are readily transformed by hydrolysis into H_2O_2 which is also formed as a primary product during hydrolytic oxidation. Owing to its rapid rate of diffusion, this accumulation of H_2O_2 might damage the cell protoplasm. To guard against this, the cell produces an enzyme—catalase, which rapidly decomposes H_2O_2 into water and inert O_2. Catalase thus acts as a regulator of the respiratory process. (4) To effect hydrolytic oxidation, an enzyme-perhydridase—is present which accelerates both oxidation and reduction just as do the metals of the platinum group. The reductase consists of the enzyme, water and an oxidisable substance which fixes the oxygen derived from the water, leaving the hydrogen free to effect reduction."

In view of the importance which physiologists begin to attach to oxidizing and reducing bodies in tissue extracts of animals and plants, and because of the influence which certain of the contact insecticides are now known to have upon the respiratory exchange of insects, it seemed worth while to investigate these bodies in insect tissue extract and to study the effect of gasoline, carbon disulphide, and some other contact insecticides upon the activity of those found to be present.

(c) OXIDIZING AND REDUCING BODIES IN INSECT TISSUE PULP OR EXTRACT.

The method used in preparing insect tisue pulp for study has already been described. In preparing the crude extract the pulp was ground up with a small amount of sterile distilled water—usually about twelve to fifteen cubic centimeters to five or six adult specimens of *Passalus*

*(Arch. Sci. phys. nat., 1913, vol. 35, 240-262).

cornutus. Then the extract was pressed out through Swiss muslin. The crude extract obtained in this way was whitish or creamy in appearance, at first, due to fat and very tiny particles of tissue which came through the muslin. When this extract was filtered through heavy filters under twenty to twenty-five pounds pressure, a clear or slightly straw-colored filtrate was obtained, and (on the filter) a considerable residue. When either the crude extract or the filtrate was allowed to stand for a time, the surface in contact with the air turned dark while the deeper parts of the fluid remained as at first for hours—for more than a day if kept cool or treated with a *little* of some weak antiseptic like chloroform, sodium fluoride solution, toluol or ether. Whenever work with the extract extended over several hours, some such antiseptic was used provided the nature of the experiment would permit. If to about 1 c. c. of the crude extract a few (2 to 5) drops of a saturated solution of alcoholic guaiac were added, the milky mixture would begin to show a tinge of blue within one or two minutes, and would gradually develop a beautiful deep blue coloration. When about 1 c. c. of a standard hydroquinone solution* was added to 3 c. c. of the extract, it slowly began to turn reddish brown; and after several hours, the entire mixture appeared very dark brown or perhaps almost black, giving off a decided odor of quinone. Vernon's alpha-naphthol paraphenylene-diamine mixture† was tried, and this, the crude extract rapidly oxidized to the blue indophenol. Finally, the extract was able to slowly oxidize tyrosine (Merck) until after a few hours one obtained a heavy black or melanic pigment. It was interesting to drop a small pinch of tyrosine crystals into a little extract from *P. cornutus* tissue, and then watch the black coloration develop about it. The extract clearly possessed oxidasic properties. Moreover, the filtrate and the washed residue left from filtering the crude extract under pressure gave the reactions showing the presence of oxidases. That is, oxidases soluble in water and insoluble in water were present. Likewise two catalases, one soluble in water and going with the filtrate, the other insoluble in water and remaining in the washed residue, were found to be present as first described by Loew‡ in the tobacco plant, and both were very active in liberating bubbles of oxygen from hydrogen peroxide.

Some of the fresh extract which had just darkened was confined in the absence of oxygen, and it became rapidly changed back to the appearance it had before it had darkened. If a few drops of a methylene blue solution were added to some of the extract and then the latter were confined from the oxygen of the air, the blue color became entirely reduced. Reducing bodies, therefore, seemed to be present; and besides, the reduction of methylene blue took place whether it was confined with the filtrate of the crude extract or with the washed residue. Thus, oxidases, catalases, and reducing bodies were all found to exist in the pulp and in the crude water extract of *P. cornutus* in two forms, one being soluble and the other insoluble in water. It therefore seemed desirable to make

*This standard solution was made up by dissolving 1.1 grams of hydroquinone in 100 c. c. of distilled water.

(i. e. $\frac{m}{10}$ hydroquinone.)

†Vernon, H. M. The Quantitative estimation of Indophenol Oxidase of Animal Tissues. The Journal of Physiology, Vol. XLII., Nos. 5 and 6, pp. 402-427.

‡Oscar Loew: Report No. 68 Div. of Veg. Phys. and Path., U. S. Dept. of Agr., 1901.

a further study of these oxydasic and reducing bodies of *P. cornutus* and to determine the effect of some of the contact insecticides upon their activity. How, for example, would gasoline vapor or the vapor of carbon disulphide affect the rate at which the crude water extract could oxidize a solution of hydroquinone? When present in concentrated amounts, the rate at which oxygen was absorbed by insects and by crude insect tissue-extract was lowered by these insecticides. Would they reduce the rate at which the oxydases in the extract caused guaiac or hydroquinone to become oxidized?

THE EFFECT OF GASOLINE AND SOME OTHER CONTACT INSECTICIDES UPON THE OXIDASE, CATALASE, AND REDUCTASE ACTIVITY IN EXTRACTS OF THE TISSUES OF *P. CORNUTUS*.

(a) EFFECT UPON OXIDASIC ACTIVITY.

It has been pointed out that both soluble and insoluble oxidases were found to be present in the extract of *P. cornutus*. The insoluble oxidase (oxydone*) remained in the mixed residue on the pressure filter after filtering the crude extract, while the soluble form went through in the clear or straw-colored filtrate. When the latter was treated with alcohol or saturated with ammonium sulphate until the proteins were thrown down, the oxidase separated with them and could be filtered out. The oxidasic activity of this alcoholic or ammonium sulphate precipitate could be preserved for hours or even days, if the precipitate were kept moist and cool; but drying, even at room temperature, almost if not entirely destroyed the activity of the oxidase in a few hours. On the other hand, when the washed residue containing the oxydone was moistened after having been dried at room temperature for 36 hours or even longer, it exhibited oxidasic action toward alcoholic guaiac almost undiminished. Moreover, in the dry condition, the oxydone was very resistant to heat. It could then be kept at 90° to 100° C. for an hour without destroying all of its activity. When moist, as in the crude extract, however, both the oxidase and the oxydone were destroyed if kept at a boiling heat for 10 minutes; and nearly all oxydasic activity was destroyed if a crude extract were kept at 80° to 81° C. for 15 minutes. When kept at 68° to 69° C. for one hour, such an extract was 5 to 6 times longer in oxidizing alcoholic guaiac than a check kept at room temperature for the same time. In making this comparative test with alcoholic guaiac, the same amount of extract was used for the check as for the heated portion, and at the end of the test period a certain number of drops of the guaiac solution were added to each portion. Then the time required to oxidize the guaiac in the treated and untreated extracts to the same depth of blue coloration was noted. This was a method by which only well marked differences might be compared, but by its use one could easily see also that solutions of hydrochloric acid, borax, sodium fluoride, nicotine (to-bak-ine) and ammonia all exerted a more or less harmful

*Insoluble oxydases in animal tissues are referred to as "oxydones" by Batelli, and (Mlle) Lina Stern, Biochem. Zeitsch. 1913, 52, p. 226. Rev. in J. of Chem. Soc. vols. 103-104 pt. i, p. 929.

The work of these investigators did not come to the notice of the author until early in 1914, when this phase of his own work was nearly finished—having been begun in 1912.

influence upon the ability of the crude extract to oxidize guaiac (exposed to the air) when they were added in small amounts. Both the oxidase and the oxydone were affected. The influence of carbon disulphide, gasoline, and hydrocyanic acid gas were studied in the same manner. In the case of carbon disulphide and gasoline, the influence of both the vapor and the liquid of each fluid was studied—using the vapors to saturation in air, and the liquids at the rate of three drops to 2 c. c. of extract thoroughly mixed to an emulsion.

Carbon disulphide deterred the oxidation of guaiac by the extract greatly;—sometimes the blue coloration did not begin to develop until after two hours, when the extract had been treated with the concentrated vapor or with the liquid for a few hours before testing. The influence of carbon disulphide was most marked when the extract was rich in fat-emulsion.

The influence of concentrated gasoline vapor on the oxidasic properties of an extract, as studied by the aid of alcoholic guaiac, was never very decided. However, the liquid gasoline first used appeared quite destructive to the oxidase, but that sample of gasoline was later found to be slightly acid in reaction; and when this acidity was rendered just neutral, the liquid gasoline then acted scarcely more harmfully than the concentrated vapor. In fact if either the vapor or the liquid gasoline were used with perfectly fresh milky extract (rich in fat) it seemed to hasten the bluing of guaiac—or to hasten the darkening of the extract itself on standing alone in air.

In the case of hydrocyanic acid gas, it was found that if an extract were tested immediately after being removed from the treatment, the guaiac blued very much more slowly than in the check. If, however, the treated extract were allowed to stand in air for an hour or so until it became free from the odor of hydrocyanic acid, the guaiac blued almost as rapidly upon being added as in the check extract.

Borax (3 grams in 100 c. c. distilled water) was very destructive to both the oxidase and the oxydone, when a little of the solution was shaken up with the extract and allowed to stand for a few minutes.

Sodium fluoride, in saturated solution, was used. It retarded the development of guaiac-blue when added in considerable amounts to an extract, but even 1 c. c. of the solution mixed with 1 c. c. of the extract did not prevent the development of the blue coloration after an hour or two. When added in only small amounts to a fresh extract, sodium fluoride solution seemed to hasten the bluing of guaiac. Likewise chloroform and ether did not seem to injure the oxidasic activity of the tissue extract toward alcoholic guaiac, except very slightly when they were used in large amounts and thoroughly shaken up with the extract.

This study with alcoholic guaiac was instructive, but a method was sought which would show by *volume measurements* of the oxygen taken up just what influence carbon disulphide and gasoline (for example) had upon the rate at which oxygen was absorbed by insect-tissue-extract plus some other substrate than guaiac. Hydroquinone solution was selected as the substrate to be oxidized because it was easy to keep a comparatively constant, clear, standard solution for some time, and because as the oxidation to quinone proceeded, the color of the solution changed progressively from a reddish to a darker and darker coloration. Thus, a color check was afforded, upon the volume of oxygen used in the case

of treated (with insecticide) and untreated extract samples. (See Fig. 1, Plate I.) So many factors were involved that it was necessary to use extreme precaution in order to be at all sure that a difference in volume of the oxygen absorbed by hydroquinone-extract-mixture in air and hydroquinone-extract in gasoline-vapor-air was due alone to the influence of gasoline vapor. It was necessary always to thoroughly mix a fairly concentrated extract and then to remove two equally measured samples for the experiment at as nearly as possible the same time. Furthermore, exactly equal measures of a standard hydroquinone solution must be added to the two samples at the same time, and then the tests with the two equally prepared mixtures must necessarily be run (the one in air and the other in gasoline-air) under like conditions of temperature for the same period of time. It would not do to run the tests for equal periods, merely. They must cover the same period as nearly as that might be made possible. The reason for these precautions, if not apparent here, will be noticed later in the discussion. Another difficulty presented itself. Oxygen was absorbed rather slowly in the tests, and so it seemed advisable to find some method of measuring directly the entire amount of carbon dioxide that might be given off, as well as the entire amount of oxygen taken up, rather than to depend upon percentage estimations from samples. No method was available for removing and accurately estimating carbon disulphide vapor from air used in a test, and so it seemed necessary to be able to measure the oxygen taken up and the carbon dioxide given off *in the presence* of the insecticide employed during the test. Finally, an apparatus was devised which complied very well with the requirements just outlined. The essential features of the apparatus are represented in Figure 1, and a brief description follows here.

The apparatus (Fig. 1), consisted of two systems which were almost exact duplicates, each made up of a gas-burette "a" connected with a mercury-mug "b"; a gas-container "c"; a potash absorption flask "d"; a mercury manometer "e"; a burette "f" (graduated to O. 1 c. c.) for the hydroquinone solution; and a circulating pump "g". The gas container was of about 300 c. c. capacity, with two two-way cocks above and a wide mouth (2½ inches) beneath. The wide rubber-stopper for this container was connected with a mercury-mug "h", and a hooked U-tube also passed from the stopper to connect with the hydroquinone burette "f". Mercury could thus be used not only to seal the mouth of the gas-container, but to float the stender dish up under the hooked end of the U-tube. The gas-burette of each system was connected by tubes (with airtight sealed joints) through the corresponding gas-container, absorption-flask, mercury manometer and circulating pump of its system, as represented in the figure. Between the "pump-gas-container connecting tube" and the "absorption-flask-pump connecting tube" was a cross tube with a stopcock "St." Fig. 1. This cock was kept closed when the pumps were running. It was opened as soon as the pumps were stopped, and always, before the manometers were adjusted, the circulating wheel of each pump was given a quarter turn backward. Thus, any air that might be compressed in a pocket was released into the upper air-chamber, leaving the pockets filled with mercury. The circulating pump of each system was actuated by belts from the same double pulley of a reducing

gear "j", which was run by a small motor not shown in the figure. It was thus possible to start and stop the pumps (which were duplicates) at the same time and to run them at the same speed. There was not found, on the market, a small pump in which the gas to be circulated did not come in contact with the boxing of the pulley-journal of the

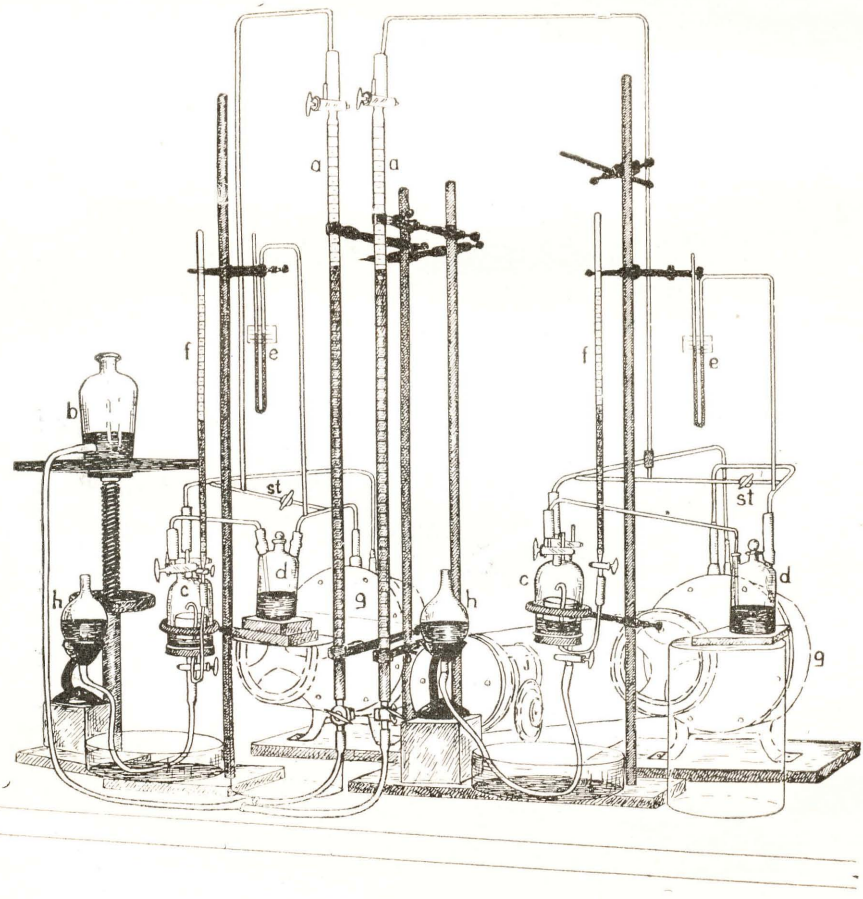

Fig. 1. Apparatus used in determining the influence of gasoline and carbon disulphide upon the absorption activity for oxygen of insect tissue-extract plus hydroquinone solution. (Duplicate systems) a = gas burettes; b = mercury mugs; c = gas-containers; d = potash absorption flasks; e = mercury manometers; f = burettes for hydroquinone solution; g = circulating pumps; h = mercury mugs; j = reducing gear.

pump. In these experiments, it was necessary to *know* that no leak or gas exchange could take place at that point. The two circulating pumps represented in Fig. 1 were, therefore, designed to meet the necessity. Each pump was filled with mercury to a certain level as shown by line

"a-c", Fig. II. The circulating wheel "w" pointed out in the same figure was made to revolve in the mercury in the direction of the large arrow. Each pocket in the wheel thus carried gas from the air-chamber in the upper part of the pump down into the mercury to the position "p", when the air emptied toward the center (due to its tendency to rise in the mercury) and came out into the outlet tube "o" as shown in Fig. II B. The pump was therefore able to circulate air against a pressure equal to the mercury-height "s-t", and the confined gas in the pump could not come in contact with the pulley journal "j" under any circumstances, since the lowest level "s", to which the mercury could be brought, was higher than the pulley-journal. As a precaution, a small

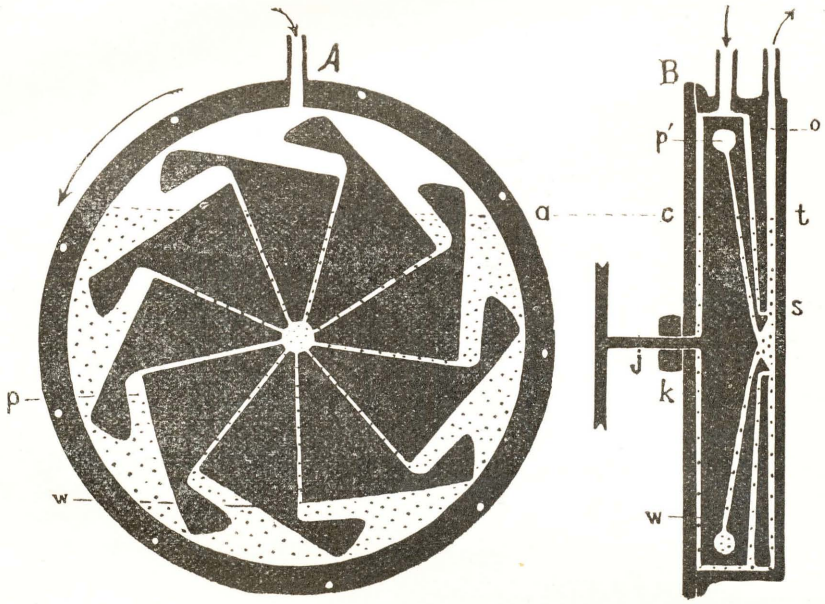

Fig. II. Diagrammatic projections of the working parts in gas-circulating-pump (see "g" Fig. 1). A = Side-projection; B = Edge-projection; a-c = level of mercury; w= circulating wheel; p = position of air pocket when it empties toward the center; p' = air pocket; o= outlet tube; s = bearing around the common central outlet for the air pockets in the circulating wheel; j = pulley journal.

cup was set beside each pump (when in use) just beneath the boxing "k". If a mercury leak should have occurred from this point, the volume of the leak could have been known at once from the mercury caught in the cup. When the apparatus was set up, much trouble was experienced in getting all connections sealed gas-tight. Tests were not begun with the extract, however, until the apparatus was able to stand, for several hours, under a pressure greater than any that might occur in the regular experiments, without showing a measurable leak. Moreover, test experiments were run with samples of the same extract in air in both gas-containers of the apparatus until comparatively uniform results

were given. The working difference which might occur between the two systems of the apparatus was about 0.2 c. c. When a test to obtain the influence of gasoline-vapor or carbon disulphide vapor upon the hydroquinone-oxidase activity of insect tissue extract was to be run, equal samples from the same tissue-extract were placed in each of two sterile stender dishes of 36 m. m. diameter, and the dishes were introduced into the two gas-containers "c." "c.", Fig. I. Mercury seal was made at the mouth of each gas-container, and the U-tubes were connected with the hydroquinone burettes. Then, by means of the mercury-mug and the free opening of one of the two-way cocks of one gas-container, as much insecticide vapor as was wanted could be introduced from a separate stock-container of concentrated insecticide vapor. After that, the cocks of the gas-containers were opened to the outside an instant until both mercury manometers read level. The temperature was recorded. Then the cocks were all properly set and the motor started, so that in the two systems the gas or air was made to circulate until it was uniformly mixed. The motor was stopped and started in this way several times (pressure adjustments being made between times, if necessary) until the manometers stood level after each short interval. The cocks of the gas-containers were then quickly opened to the outside, and hydroquinone solution was carefully let down from the burettes until it reached the openings of the hooked U-tubes above the stender dishes in the two containers "c." "c." A measured amount, usually 2 c. c. of hydroquinone solution to 5 c. c. of the tissue extract, was measured into each extract stender dish. Test was again made to see that the pressure manometers stood level and then both cocks of each gas-container were closed. The temperature was again noted. The barometer reading and the readings of the two gas-burettes were recorded. The apparatus then stood during the desired period of the test in a room where the temperature varied but little—and only very slowly—and one could be sure that both systems of the apparatus were at the same temperature when readings of the gas-burettes were made. At the end of the test period, the cocks at the top of the gas-containers were turned so as to connect each container with it's own system. The mercury in each gas-burette was quickly manipulated so as to bring the mercury manometers level, and the readings of the two gas-burettes were taken. Then the cocks "st" and "st'" were closed and the pumps were started. The gas or air in each system was thus made to circulate through the absorption flasks until trial showed that the readings of the gas-burettes (after short runs of the pumps) were constant, when the two manometers "e" and "e" stood at zero. The reading of each gas-burette was then again recorded. Thus there were three gas-volume readings for the system containing the insecticide-treated extract and three for the check. The first reading in each set showed the constant volume of the gas under certain recorded conditions of temperature and barometric pressure; the second showed the volume after oxygen absorption (i. e. by the extract containing hydroquinone solution), before any carbon dioxide had been removed; and the third reading gave the volume after the carbon dioxide produced during the experiment period had been circulated through the hydroxide flask and absorbed. The readings were all reduced to the

volume which would be given at 0°C. and 760 m. m. mercury pressure so that they might be safely compared. The second reading, then, minus the third (in each set of reduced readings) showed the volume of carbon dioxide produced during the period; and since no carbon dioxide was present at the beginning, the first reading minus the third gave the volume of oxygen that had been absorbed during the test. The volume of oxygen absorbed in the case of the insecticide-treated "extract plus hydroquinone" and the volume of oxygen absorbed by the untreated "extract plus hydroquinone" could thus be compared directly; and it is believed that any difference must be attributed to the influence of the insecticide present, since other conditions were kept as nearly as possible the same.

Checks tried separately had shown that gasoline and carbon disulphide vapors were not appreciably affected by the potassium hydroxide solution used, and that they did not interfere with its absorption of carbon dioxide.

The records of a test are given here as they were kept for a regular experiment:

Exp. 7. Used 5 c. c. of an extract (made from 4 large "white grubs" in 20 c. c. distilled water, plus 3 drops of Toluol) in each of the two containers—right and left.

Ran pump at intervals until readings became constant. Added 2 c. c. or $\frac{N}{100}$ hydroquinone solution to each. Ran pumps short interval—readings were constant.

Started 17 Aug. 1912, 4:45 P. M.
Ended 18 Aug. 1912, 8:00 A. M.

At start —Bar. =740 m.m.; Temp. =19.3° C.
At end — " =734 m.m.; " =20.4° C.

Ext. =Left-hand system (CS_2—treated).	Ext. =Right-hand system (no treatment).
Gas-burette reading	Gas-burette reading
27.5 c.c.	20.5 c.c.
30.5	23.5
27.5	20.5

Readings reduced to 760 m.m. mercury pressure and 0°C:—
Left-hand system (CS_2—treated).

430 c.c. =Volume of left-hand system, exclusive of gas-burette.
27.5 c.c. =Volume of gas in left-hand gas-burette.

457.5 c.c. =Total volume of left-hand system at start.

$$\frac{457\ (740-16.655)273}{760(273+19.3)} = 406.6 \text{ c.c. vol. at start (reduced).}$$

430.0 c.c.
30.5

460.5 =Total vol. at end of test; before CO_2 had been absorbed.

$$\frac{460.5\ (734-17.826)273}{760(273+20.4)} = 403.7 \text{ c.c. Vol. before } CO_2 \text{ had been absorbed (reduced).}$$

430.0
27.5

457.5 =Total vol. at end of test (after CO_2 was absorbed).

$$\frac{457.5(734-17.826)273}{760(273+20.4)} = 401.1 \text{ Vol. at end of the test (reduced).}$$

Then 406.6
401.1

5.5 c.c. O_2 absorbed during test by the "extract plus hydroquinone" in the presence of CS_2 — vapor in air.

and 403.7
401.1

2.6 c.c. CO_2 given off during test in the CS_2 —treated gas-container.

Right-hand System (check).
420.0 c.c. =Vol. of right-hand system, exclusive of gas-burette (at start.)
20.5 =Vol. of right-hand system gas burette.

440.5

$$\frac{440.5(740-16.655)273}{760(273+19.3)} = 394.7 \text{ c.c. Vol. at start (reduced).}$$

420.0 c.c.
23.5

443.5 c.c. =Total vol. at end of test (before CO_2 had been absorbed.)

$$\frac{443.5(734-17.826)273}{760(273+20.4)} = 389.3 \text{ c.c. above vol. (reduced).}$$

420.0 c.c.
20.5

440.5 c.c. =Total vol. at end after CO_2 had been absorbed.

$$\frac{440.5(734-17.826)273}{760(273+20.4)} = 386.6 \text{ c.c. above volume (reduced).}$$

Then 394.7
386.6

8.1 c.c. O_2 absorbed during test by the check.

and 389.3
386.6

2.7 c.c. CO_2 given off during test by the check.

8.1 −5.5 =2.6 c.c. more O_2 abosrbed in case of the check (i. e. not treated with CS_2 —vapor).

Note was kept of twenty-one experiments carried out in adjusting and in learning to manipulate and use the apparatus to obtain trustworthy results. It was found that the greatest care must be taken to be sure that the two systems of the apparatus were at the same temperature. A door, opened for a short time to admit air of a different temperature from one side of the room, could cause a variation in the volume of the gas in the two systems which would entirely vitiate results if readings were made at that time. It was discovered also that a variation in the influence of the insecticide-vapor occurred, depending on whether the extract were used immediately after it had been prepared, or not until a few hours later—this seemed to be especially true when gasoline was used. This fact served to emphasize the necessity of taking, from the prepared extract, the sample to be treated with the insecticide and the check sample at the same time, and of carrying out the tests with the two during the same period—although the reason for the variation mentioned above was not appreciated until later in the investigation. If the insecticide vapor in the air used in an experiment was near saturation, then it was necessary not to allow the room temperature to fall below that at which the experiment was started. Otherwise some of the insecticide-vapor would be condensed and the vapor tension of the gas-mixture changed. Correction for change in vapor-tension of water vapor due to a change in temperature could be readily made, but no method was at hand for making a similar correction in case of gasoline or carbon-disulphide vapor when the apparatus illustrated by Fig. I was being used. Furthermore, it was found that high concentrations of the insecticide

vapor were necessary to show a marked influence, especially if the water content of the extract were high.

After the preliminary tests, written record was kept of more than thirty experiments, including cautionary check experiments, which were made to get the influence of gasoline and carbon disulphide vapors upon the oxygen absorption activity of "insect extract plus hydroquinone solution". Some of these experiments were performed during as many as three successive periods. In all of these tests unless otherwise stated, the stender dish of each gas-container held 5 c. c. of insect tissue extract from the same stock extract mixed with 2 c. c. of a standard hydroquinone solution (1.1 grams of hydroquinone in 100 c. c. of distilled water.) Representative results of these experiments are given in table II.

The first ten results in the table relate directly to the influence of carbon disulphide or of gasoline vapor upon the activity of "insect tissue extract plus hydroquinone solution" for oxygen absorption.

A study of the table will show that the higher concentrations of insecticide vapors caused a marked reduction in oxygen avidity under that shown by the corresponding checks. Weak concentrations of the insecticides, on the other hand, seemed to accelerate, very slightly, rather than to retard oxygen absorption. One may appreciate the influence of the insecticide, perhaps, better by comparing the total sum of the oxygen absorptions in the insecticide chamber with the total corresponding absorptions in the check gas-chamber.

In case of carbon disulphide vapor the ratio is 6.7 c. c. to 19.1 c. c. In other words, only about 7/20 as much oxygen was absorbed during the series of tests in the carbon disulphide chamber as was absorbed in the check gas-chamber with air alone present. For gasoline-vapor the ratio is 10.2 c. c. of oxygen absorbed in the gasoline chamber to 13.9 c. c. in the pure air chamber (i. e. about 7/10); and if those tests in which the vapor was in high concentration are considered alone, then barely over one-half as much oxygen was absorbed in the gasoline-vapor chamber. The ratio of carbon dioxide given off to the oxygen absorbed by the mixture of "insect tissue-extract and hydroquinone solution" as expressed by the totals for the insecticide-treated samples is 0.8+, and the similar ratio as expressed by the totals for the corresponding checks is 0.4. That is, the respiratory ratio of the "tissue-extract plus hydroquinone solution" proved to be decidedly higher in the presence of strong gasoline or carbon disulphide vapors than was found to be the case in pure air. Now, as has already been mentioned, an oxidase in the insect tissue-extract is able, it is believed, to accelerate the oxidation of hydroquinone to quinone and water—the quinone showing its presence by its odor and by a change in color of the extract-solution. If, then, an oxidase in the extract does cause that kind of an oxidation of hydroquinone, it would naturally follow that an extract to which the latter had been added might take up more oxygen in a given period than it otherwise would, and that the added hydroquinone would therefore decrease the respiratory ratio $\left(\frac{CO_2}{O_2}\right)$ of the extract. Note the ratio 0.4 above, which is much below that found for healthy beetles in pure air—the latter ratio being about 0.75. The last three results given in Table II are precautionary checks on the manner in which hydroquinone is oxidized by extract of the tissues of *P. cornutus*. To each of the stock

TABLE II.—INFLUENCE OF GASOLINE AND CS_2 VAPORS UPON THE ABSORPTION ACTIVITY FOR OXYGEN OF INSECT TISSUE-EXTRACT PLUS M-10 HYDROQUINONE SOLUTION.*

*(1.1 grams of hydroquinone in 100 c. c. of dist. water.)

Exp No.	Stock extract from:—	Insecticide.	Period.	Insecticide chamber.		Check gas-chamber.	
				O_2 used.	CO_2 given off.	O_2 used.	CO_2 given off.
1.	3 large white grubs and 20 c. c. water	CS_2, rather low concentration	29 hrs	5.5 c. c.	2.2 c. c.	6.4 c. c.	1.6 c. c.
2.	3 white grubs, 15 c. c. water. (1 hr. old).	CS_2, high concentration	21 hrs	0.3 c. c.	1.9 c. c.	4.9 c. c.	2.4 c. c.
3.	4 white grubs, 20 c. c. water (Ext. 5 hrs. old)	CS_2, nearly saturated	16 hrs	0.3 c. c.	2.8 c. c.	3.9 c. c.	1.4 c. c.
4.	4 white grubs, 20 c. c. water (ext. 6 hrs. old)	CS_2, nearly saturated	24½ hrs	0.6 c. c.	0.5 c. c.	3.9 c. c.	0.9 c. c.
	Totals			6.7 c. c.		19.1 c. c.	
5.	1 large and 6 small white grubs, 18 c. c. water	Gasoline vapor, low concentration	6 hrs	1.9 c. c.	1.3 c. c.	1.8 c. c.	1.3 c. c.
6.	8 *Allorhina* grubs, 20 c. c. water	Gasoline vapor, high concentration	16 hrs	2.5 c. c.	2.0 c. c.	4.5 c. c.	4.0 c. c.
7.	6 *Allorhina* grubs, 20 c. c. water	Gasoline vapor about ⅔ concentration	17 hrs	2.1 c. c.	2.8 c. c.	3.3 c. c.	2.3 c. c.
8.	6 large *Allorhina* grubs, 20 c. c. water	Gasoline vapor, low concentration	4 hrs	1.2 c. c.	0.8 c. c.	1.1 c. c.	0.7 c. c.
9.	8 *Passalus cornutus*, 30 c. c. water	Gasoline treated	8½ hrs	0.6 c. c.	1.0 c. c.	1.6 c. c.	0.4 c. c.
10.	8 *Passalus cornutus*, 30 c. c. water	Gasoline treated, but no insecticide present during the experiment	16 hrs	1.9 c. c.	0.5 c. c.	1.6 c. c.	0.6 c. c.
	Totals			10.2 c. c.		13.9 c. c.	
				Check extract — no hydroquinone.		Extract plus hydroquinone.	
				O_2 used.	CO_2 given off.	O_2 used.	CO_2 given off.
11.	5 *Passalus cornutus*, 20 c. c. water	No insecticide used	2½ hrs	0.7 c. c.	0.4 c. c.	0.9 c. c.	0.3 c. c.
12.	4 *Passalus cornutus*, 15 c. c. water	No insecticide used	15 hrs	0.7 c. c.	0.4 c. c.	3.4 c. c.	1.0 c. c.
13.	4 *Passalus cornutus*, 10 c. c. water	No insecticide used	6 hrs	2.0 c. c.	0.4 c. c.	4.4 c. c.	0.9 c. c.
	Totals			3.4 c. c.		8.7 c. c.	

extracts (before samples for the tests were taken) four drops of weak ammonia (0.9 sp. gr. diluted five times) were added. This ammonia had the effect to reduce the carbon dioxide excreted to a very small amount.* The influence of oxidase in causing the hydroquinone to take up oxygen in the manner described might therefore stand out prominently. The total oxygen taken up and the total carbon dioxide given off in each case is given in the table, and although the ammonia used kept the ratio $\left(\frac{CO_2}{O_2} = \frac{1.2}{3.4}\right)$ down to 0.35 in the extract containing no hydroquinone, the value was brought down lower still $\left(\frac{CO_2}{O_2} = \frac{2.2}{8.7}\right)$ to 0.25 by the increased amount of oxygen used in the extract plus hydroquinone mixture. The larger value of the ratio $\frac{CO_2}{O_2} = 0.8$, as found above for the "extract plus hydroquinone" in the insecticide chamber, must therefore point to the fact that the gasoline and carbon disulphide vapors used were able in some unknown manner to inhibit or lessen the accelerating (catalytic) action of the extract-oxydase in bringing about the oxidation of hydroquinone. A further precaution which was observed should be explained here. Tissue-extract was brought to a boiling temperature (about 15 to 20 minutes) to destroy the oxidasic enzymes. It was then allowed to cool and the regular amount of hydroquinone solution was added. Tests were then made using the apparatus as in other experiments, and no measurable amount of oxygen was taken up during longer periods than any used in the regular experiments. It has already been explained that the hydroquinone stock solution would remain clear for days. However, if the heated "extract plus hydroquinone mixture" had been scarcely brought to the boiling temperature or had been heated only a short time before it was allowed to cool, it often showed a very slight reddish or cherry tinge after twenty-four to thirty hours—indicating that the heat had not been sufficient to destroy quite all of the oxidasic activity. Thus, the appearance of this peculiar reddish brown color was an even more delicate test of the oxidation of hydroquinone than the volumetric measurement of the oxygen used, and mention has already been made of how the reddish brown tinge deepened progressively as hydroquinone became oxidized to quinone. It was interesting and confirmatory, therefore, to note that in all ten of the experiments listed in table II the deeper color was found to be in the stender dish of that gas container where most oxygen had been taken up. One must see that in the extracts used, a slight respiratory exchange exists, and the influence of the insecticide agents upon this exchange has not been eliminated; but the respiratory exchange in such a treated extract (a few hours old) is very low indeed as shown by the CO_2 given off—and the evidence just enumerated leaves no doubt that in studying the comparable oxygen measurements, we see mainly the influence of the agents in question upon the oxidase activity toward hydroquinone. Two experiments (not recorded in table II), carried at once for short periods with gasoline vapor in high concentration, gave results contradictory to all the rest as far as gas measurements were concerned—that is, the measurements seemed to show that most oxygen had been taken up in the

*See effect of ammonia on $\left(\frac{CO_2}{O_2}\right)$. Tech. Bulletin 11.

gasoline-treated chamber. Nevertheless, when run for longer periods afterward, the "extract plus hydroquinone" mixture in the gasoline chamber showed a reddish brown color not nearly so deep as that in the check gas-chamber containing air only, and the volumetric results then agreed with the results of the other experiments. The reason for the apparently contradictory results of the first and second parts of these two experiments was not understood until later. About a year and a half after these first experiments had been made (i. e. after the effect of gasoline upon the tissue reductase had been studied) it was decided to run a few more tests with gasoline-vapor, using a different method, in order to find out more surely if possible, the influence of this insecticide upon the absorption activity for oxygen of "insect tissue-extract plus hydroquinone" solution. The method decided upon was the old one (described in Part 1, Tech. Bull. II) in which the percentages of the gases present were determined at the beginning and at the end of the test period; but a different arrangement of gas-containers and gas- burettes was made to adapt the method to this problem.

The arrangement of containers and burettes in the apparatus used is represented in Fig. III. As may be seen, the apparatus is an adaptation of that shown in Fig. 1, with the circulating pumps and absorption flasks left out. There were two duplicate systems—gasoline-vapor-air was used in the gas-chamber of one system and pure air in the other. Hydroquinone burettes enabled one to introduce the hydroquinone as described in the case of apparatus Fig. I. The two gas-containers had each two two-way cocks, and these enabled one to adjust the mercury manometers and to take gas samples for estimation from each container at the beginning and at the end of test-periods. The gas-burettes permitted one to keep close check upon changes in gas-volume in each system, the readings being made at the known temperature and barometric pressure. From the percentage determinations (since the cubic contents of each system was known) one could figure the amount of oxygen used and of carbon dioxide given off at 760 m. m. pressure and O°C., as well as the exact percentage of gasoline to which the "insect-extract plus hydroquinone" solution had been subjected in the test. A set of results obtained with this arrangement of apparatus is given in table III. The total oxygen absorbed in the pure air chamber exceeded the total oxygen absorbed in the gasoline-air chamber, and the ratio $\frac{CO_2}{O_2}$ was a little higher in the gasoline-air chamber. By this method, as with the former method, however, it was found that if fresh extract were used for a short period, the result might be contradictory to that given for a much longer or for a later period with the same extract.

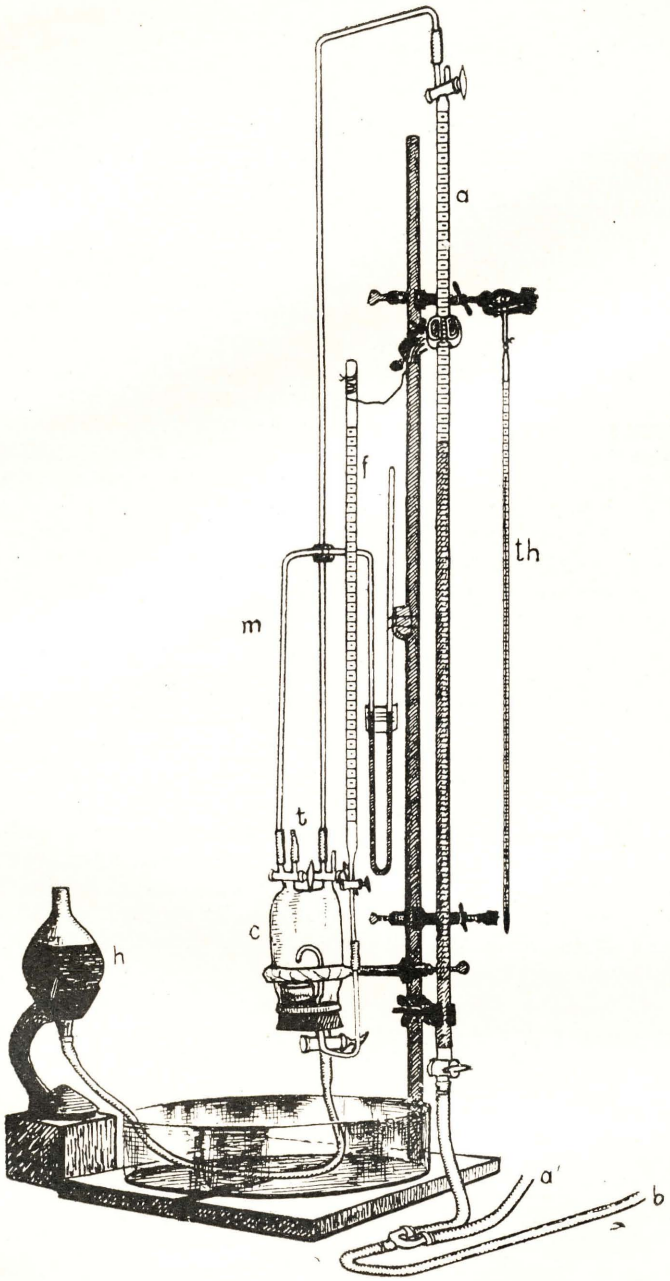

Fig. III. Shows half of a duplicate gas apparatus used for studying the influence of various agencies on certain activities of insects and insect tissue extracts: a = gas burette; b = rubber tube connected with mercury mug; a' = tube leading to duplicate gas burette; f = burette for hydroquinone solution or hydrogen peroxide or etc.; m = tube connecting gas container "c" with a mercury manometer; th.= thermometer; t = outlet of gas-cock.

TABLE III.—EFFECT OF GASOLINE VAPOR ON THE AMOUNT OF OXYGEN ABSORBED BY INSECT TISSUE-EXTRACT PLUS HYDROQUINONE-SOLUTION.
(Method of percentage estimations).

Exp. No.	Stock extract from:—	Gasoline vapor.	Period.	Insecticide chamber.		Check gas chamber.		Remarks.
				O_2.	CO_2.	O_2.	CO_2.	
1.	8 *P. cornutus*, 20 c. c. water.....	4.6%.....	16½ hrs...	2.2 c. c...	0.6 c. c...	2.8 c. c...	0.1 c. c...	0.6 c. c. more O_2 absorbed in the air chamber.
2.	a. 9 *P. cornutus*, 15 c. c. water....	4.37%.....	5 hrs.....	2.69 c. c..	0.2 c. c...	1.7 c. c...	0.4 c. c...	0.9 +c. c. more O_2 used in the gasoline chamber.
	b. 9 *P. cornutus*, 15 c. c. water....	.....	19 hrs....	2.3 c. c...	0.96 c. c..	2.72 c. c..	0.5 c. c...	0.4 +c. c. more O_2 used in the air chamber.
3.	9 *P. cornutus*, 15 c. c. water.....	6.7%.....	17 hrs....	2.7 c. c...	0.4 c. c...	2.9 c. c...	0.5 c. c...	0.2 c. c. more O_2 used in the air chamber.
4.	a. 15 *P. cornutus*, 22 c. c. water...	6.96%.....	6 hrs.....	1.31 c. c..	0.9 c. c...	1.73 c. c..	0.39 c. c..	0.42 c. c. more O_2 used in pure air.
	b. 15 *P. cornutus*, 22 c. c. water...	.....	14½ hrs...	0.72 c. c..	0.0 c. c...	1.6 c. c...	0.9 c. c...	0.88 c. c. more O_2 used in the pure air.
5.	15 *P. cornutus*, 22 c. c. water.....	5.9%.....	17 hrs....	1.1 c. c...	0.72 c. c..	1.6 c. c...	0.52 c. c..	0.5 c. c. more O_2 used in pure air.
	Totals.....			13.02 c.c..	3.78 c. c..	15.05 c.c..	3.31 c. c..	
	Totals, leaving out No. 2, a.....			10.33 c.c..	3.58 c. c..	13.35 c.c..	2.91 c. c..	

This is illustrated in table III by No. 2, where in the first five hours, the fresh extract used 0.9 c. c. more O_2 in the gasoline chamber than was used by its duplicate sample in pure air. When the same samples had then run undisturbed for nineteen hours, it was found on the other hand, that the pure air sample had taken up the most oxygen by 0.4 c. c. In No. 4 the stock extract was about five hours old when the samples were taken for the tests. Note that in this case, while the first test period of six hours showed more oxygen used in pure air, the longer second period gave an increased excess of oxygen used by the pure air sample. These results, taken with those obtained by the former method, seem to justify the conclusion that carbon disulphide and gasoline inhibit or lessen the rate of oxidation of hydroquinone in solution in the tissue extract of *Passalus cornutus*. Furthermore, in the case of gasoline, the effects on the rate of oxidation are not marked, unless the extract is a few hours old at the beginning of the test; and if perfectly fresh extract is used for a short period the results may be contradictory—apparently. As has already been mentioned, however, the series of experiments, results of which are recorded in Table III, were run after a study had been made of insect tissue reductase and of the effect of gasoline upon the reductase. This latter study, it is believed, affords an explanation of the apparantely contradictory results obtained when gasoline-vapor acts for short intervals upon freshly made "insect-tissue extract plus hydroquinone" solution. (See page 28.)

(b) INFLUENCE UPON THE REDUCING ACTIVITY.

Some evidence has already been given of the existence of a strong reducing power in the fresh extract of *Passalus cornutus;* namely, the reduction (when air is excluded) of the dark melanic pigment which develops in the extract through the influence of the oxidasic enzymes when tissue-extract has been exposed to the air for a while, and the reduction of methylene blue to leuco-methylene blue when air is excluded from extract to which a little methylene blue has been added. Still other evidence may be given. If comparatively fresh "extract plus hydroquinone" which has already oxidized enough of the hydroquinone to quinone to give the solution a reddish-brown hue, is then confined in the absence of air or free oxygen for a few hours, the reddish-brown quinone will all be reduced; and if a little perfectly fresh extract is added before excluding the air, the reduction takes place more rapidly. Fresh extract stained blue with a little indigo carmine will reduce the stain to leuco-indigo carmine in the absence of free oxygen; and then, the reduced color body will oxidize and turn the extract solution blue again, if air is admitted—just as happens also when confined extract, holding leuco-methylene blue, is once more exposed to the air. After fresh extract has caused alcoholic guaiac to be oxidized to guaiac blue, if the entire mixture is then confined from free oxygen, the reducing bodies show their presence by reducing all guaiac-blue back to its former condition. Likewise, after tyrosin has been oxidized to the black melanic pigment, the latter can then be reduced by the extract, if air is excluded. In every case after reduction, oxidation may again be brought about if the confined extract containing the reduced body is once more exposed to air.

It should be explained here, perhaps, that the strength and persistence of the reduction-activity varied a little with different lots of beetles, and that it always seemed to be best in the case of beetles showing the greatest strength and vitality.

Before discussing the effect of temperature, gasoline and other agencies upon the reductase, it will be best to describe briefly the method used in confining insect tissue-extract in the absence of air so that a study of its reductase activity under the influence of the various agents and conditions could be carried on in a manner which would permit of comparisons being made. As a rule, it was necessary to use small amounts of the extract and a method was needed by which the air could be excluded as completely as possible—and excluded quickly. After various means had been tried, the following plan was found to serve well. Small glass tubes of the same diameter and of practically the same length were heated and drawn out at each end to a fine capillary bore. The extract to be studied could be quickly drawn up to fill a tube, and then the capillary ends could be sealed at once by touching them in a hot gas flame. Fig. 2, Plate I, is a photograph of such a sealed tube. Methylene blue was the "substrate and indicator" most often used in making a study of the reduction activity. A standard solution of 0.1 gram of methylene blue in 100 c. c. of distilled water was employed. Unless otherwise stated in giving the results, six drops of this standard solution from the methylene blue pipette were used to 4 c. c. of the extract. (In most cases this ratio was found to be best.) The stain was thoroughly mixed with the extract and then the extract was divided into two equal parts—the one part to be treated for a certain time with the insecticide and the other, held as a check. Or, if two equal portions of the extract were taken at first, an equal number of drops of the standard methylene blue was added to each from a graduated pipette at practically the same time. At the end of the treatment period both parts were again shaken until they became a uniform blue. Then, from the treated and untreated stained extracts, tubes were quickly drawn full and sealed. Note was made of the period of time required for each tube to become reduced entirely free of the blue color. The time intervals given by this method for tubes of treated and untreated extract thus furnished the means for an instructive study of the influence of insecticides (or temperature, etc.) upon the reductase activity of any extract. Methylene blue showed a very slight deteriorating influence upon the reductase, it was found, but in the method this is not to be regarded since the stain was added to the treated (with insecticide) and the untreated portions of the extract at the same time in equal amounts as explained—all other conditions being the same for both portions of the extract, except the one condition under consideration.

By this method the influence of temperature, chloroform, ether, alcohol, gasoline, formaldehyde, carbon disulphide, hydrocyanic acid gas, toluol, ammonium formate, sodium fluoride, borax, mercuric chloride, pyrethrum, to-bak-ine and white hellebore was studied. Representative results are given in Table IV where the ratio between the time required to reduce methylene blue in the check and the time requird in the case of the treated extract may easily be obtained.

TABLE IV.—INFLUENCE OF CERTAIN INSECTICIDE AGENTS ON THE REDUCING ACTIVITY (ON METHYLENE BLUE) OF EXTRACT FROM THE TISSUES OF *PASSALUS CORNUTUS*.

(Solution of Meth. Blue =0.1 gram in 100 c.c. of dist. water.)

Exp. No.	Agent.	Time extract was treated before being drawn into tube and sealed.	Time required to reduce Methylene Blue in the treated extract.	Time required to reduce Methylene Blue in the check extract.
1.	Gasoline, 0.5 c.c. to 4 c.c. of the extract	10 min.	35 min.	7 min.
2.	Gasoline, 1.0 c.c. in 3 c.c. of the extract	3 min.	17 hrs.	14 min.
3.	Carbon disulphide, 0.5 c.c. in 3 c.c. of extract	3 min.	1 hr., 16 min.	14 min.
4.	Carbon disulphide, 0.5 c.c. in 3 c.c. of extract	23 min.	3 hrs., 21 min.	19 min.
5.	Alcohol, 0.5 c.c. to 2 c.c. of extract	5 min.	6 min.	1½ min.
6.	Alcohol, 0.5 c.c. to 2 c.c. of extract	15 min.	10 min.	2 min.
7.	Chloroform, 0.5 c.c. to 2 c.c. of extract	5 min.	48 min.	8 min.
8.	Ether vapor, saturated	35 min.	5 min.	7 min.
9.	Ether, 0.5 c.c. in contact with 2 c.c. of the extract	5 min.	17 min.	9 min.
10.	Formaldehyde (40%) 3 drops in 2 c.c. of extract	5 min.	Still quite blue after 24 hrs.	10 min.
11.	Tobakine, 1 drop to 2 c.c. of extract	5 min.	10 min.	10 min.
12.	HCN., (from KCN and H_2SO_4) strong charge 10 min. [aired 20 min.]	10 min.	30 min.	4 min.
13.	Treated in a very strong Cyanide bottle, 2 hrs. (aired 5 min.)	2 hrs.	1 hr., 50 min.	55 min.
14.	Beetles treated for 15 hrs. in Cyanide bottle, and then aired ½ hr. before extraction	.....	18 min.	4 min.
15.	Toluol, 3 drops to 2 c.c. of extract	4 min.	5½ min.	2½ min.
16.	Toluol (same as above)	15 min.	30 min.	15 min.
17.	Pyrethrum, 0.5 c.c. dry powder to 2 c.c. of extract	5 min.	4 min.	1½ min.
18.	Pyrethrum (same as above)	27 min.	38 min.	6 min.
19.	Pyrethrum (same as above)	2 hrs.	18 hrs.	1 hr., 25 min.
20.	White Hellebore, 0.5 c.c. dry powder to 2 c.c. extract	5 min.	4½ min.	3 min.
21.	White Hellebore (same as above)	10 min.	16½ min.	5 min.
22.	White Hellebore (same as above)	97 min.	Between 16 and 18 hrs.	1 hr., 40 min.
23.	$HgCl_2$, 1 drop of saturated solution in 2 c.c. of extract	5 min.	No apparent reduction at all	20 min.
24.	NaF (sat. solution), 1 c.c. to 3 c.c. of extract; 5 drops of Meth. Blue	8 min.	6 min.	3 min.
25.	NaF (sat. solution), 1 c.c. to 3 c.c. of extract; 5 drops of Meth. Blue	25 min.	8 min.	10 min.
26.	NaF (sat. solution), 1 c.c. to 3 c.c. of extract; 5 drops of Meth. Blue	65 min.	21 min.	1 hr., 50 min.
27.	Borax (3 grms. in 100 c.c. water), 0.6 c.c. to 2 c.c. of extract	10 min.	27 min.	10 min.
28.	Borax (3 grms. in 100 c.c. water), 0.6 c.c. to 2 c.c. of extract	30 min.	1 hr., 50 min.	46 min.
29.	Am. Formate, 1 c.c. of $\frac{M}{1000}$ solution to 2 c.c. of extract	5 min.	15 min.	5 min.
30.	Temperature 58° C.	5 min.	About 10 hrs.	2 hrs.
31.	Temperature 0° F. for 3 hrs.	.....	After first warming, 3½ min.	Average of fresh extract, 1½ min.
32.	Temperature 70° F. stood for 3 hrs.	.....	6 hrs.	1 min.
33.	Carbon tetrachloride, 4 drops in 4 c.c. of extract	15 min.	70 min.	18 min.
34.	Air saturated with vapor of carbon tetrachloride at 70° F.	40 min.	3¼ hrs.	1¼ hrs.

The results as given here are designed only to show whether the influence of the agent upon the reducing power of the extract is marked or not. The reducing activity of the tissue extract of *P. Cornutus* may be entirely destroyed by a continuous temperature of 100° C. for ten to fifteen minutes. While this high continuous temperature is necessary for *complete* destruction, the greater part of the activity is quickly lost

at 58° to 60° C. A low temperature tends to conserve the reducing activity—indeed, it serves, at once, to conserve and to inhibit the reducing power of the extract. No. 31 of Table IV gives a trial with extract kept for three hours at 0° F. When this extract was quickly but gently warmed to room temperature, it reduced methylene blue in 3½ minutes, whereas extract that had stood exposed to the air at 70° for three hours required six hours to reduce the color. The checks of fresh extract at the beginning reduced the same amount of methylene blue in one minute and in 1½ minutes. The reducing activity of fresh extract seems to be best at a room temperature of about 70° to 80° F., but it rapidly deteriorates during the first five to six hours after extraction (at the room temperature) and gradually, more slowly afterward. This is true even when the fresh extract is confined from the air. For example, a certain check of perfectly fresh extract reduced its methylene blue more than six times faster than another portion of the extract (A) which was tested after its confinement from air for 1½ hours from the moment of its extraction. Nevertheless "A" was 2½ times faster in reducing its methylene blue than a third portion (B) of the same extract which had been exposed to the air during the 1½ hour period. One might think from this that confinement from air tended to conserve the reducing activity. It is necessary to remember, however, that a dark melanic pigment developed in any extract (as "B") exposed to the air, due to the influence of the oxydase present, and that a portion (as A) confined from air could develop no pigment. When "A" and "B" were finally given the reduction-test with methylene blue, then (at the end of 1½ hours in this example), the reductase in "A" had only methylene blue to reduce while that in "B" attacked the dark pigment as well as the methylene blue. It seems likely, therefore, that the longer time for the reduction of the methylene blue was required by "B" because of the greater work in reducing the melanic pigment in addition to the methylene blue, rather than because its reducing activity had become less than that in "A".

Mention has already been made of the fact that when fresh extract is exposed to the air, it darkens first, only in a thin film at the surface. Also, if the fresh extract is allowed to stand after it has been stained with methylene blue and shaken so that the color is uniform throughout, it will be but a few minutes until the surface only will be blue—a reduction of all color in the deeper portions of the container having taken place. After a time, however, the blue coloration will begin—slowly at first and then more and more rapidly—to extend deeper into the container; in the case of the unstained extract, the dark melanic pigment will gradually form more rapidly as time goes on. It was soon found that this increased rapidity in the reoxidation of the methylene blue and in the formation of the melanic pigment coincided or tallied with the deterioration in the activity of the reductase of the extract on standing. That is to say, when the extract was new and its reductase still remained strong, it visibly held in check the work of the oxydase in the same extract. This proved as well to be the case when alcoholic guaiac or hydroquinone solution was used in the extract as the agent to be oxidized. Now, as may be seen by Nos. 1 and 2 of Table IV, gasoline was found to be quickly and strongly deleterious to the reducing activity of

fresh extract of *Passalus cornutus*. The influence of strong gasoline-vapor upon the reductase was decided and much more marked than any influence upon the oxidase-activity—furthermore, the resultant-effect of gasoline-vapor upon the oxidasic action in very fresh extract was plainly due, in part, to the deleterious effect upon the reductase, since the latter was an inhibitor of the oxidasic processes. It was evident that in nearly elminating the reductase activity in the treated portion of a *fresh* extract, gasoline-vapor removed at once a greater inhibiting influence upon oxidasic action than it could itself exert—while in the check portion of the extract this inhibiting influence of the reductase continued for a few hours until it gradually disappeared through standing. Hence, a short period test of oxidase activity was sure to show that most oxygen had been taken up by the gasoline treated portion of a *fresh* extract. On the other hand, when the test with gasoline-vapor was made with extract that was a few hours old before the test began (and had thus already lost its strong reduction-activity) the inhibiting influence of the gasoline-vapor upon oxygen absorption in the "extract plus hydroquinone" solution became readily apparent in either long or short period tests. Thus, an explanation was afforded of those apparently contradictory results obtained when studying the action of gasoline-vapor upon hydroquinone-oxidase activity.

Table IV hardly shows the true extent of the deleterious effect of carbon disulphide upon the reduction-activity of fresh extract—since, in making check studies, it was found that methylene blue was slowly reduced by carbon disulphide alone, in the absence of air. However, that insecticide had little or no effect on indigo carmine, and when the latter was used as the test agent (½ grm. in 100 c. c. dist. water), it appeared that carbon disulphide was much more decidedly harmful to the reduction-activity of the extract than the study with methylene blue would lead one to believe.

It will be noticed from the table also that the amount of ether-vapor taken up by a water extract seemed to slightly increase or stimulate the reduction activity—whereas a small amount of liquid ether shaken up in the extract brought about noticeable deterioration in the reduction power. On the other hand, immediately upon being added in small amounts, a slight drop in the reduction-activity was caused by sodium fluoride, but after that it tended very markedly to preserve the reducing power of an extract (Nos. 24, 25 and 26 of Table IV).

The table (IV) itself, perhaps, gives sufficient explanation of the results for the remainder of the agents tested in this connection.

(c) EFFECT UPON THE CATALASES.

Brief mention has already been made of the presence of soluble and insoluble catalases in the tissue-extract of *Passalus cornutus*. Loew found that the alpha (i. e. insoluble) catalase of tobacco was slightly soluble in water after a time, and this appears to be true of the corresponding catalase in the insect tissue extract. The soluble and insoluble catalases seem to be affected alike by heat and other agents, except that the insoluble form is a little more resistant in most cases. The tissue-pulp or crude water extract of *P. cornutus* may be dried without destroying the ability to liberate oxygen from hydrogen peroxide, but

long continued drying injures that ability. A temperature of 59° C. for 10 minutes is sufficient, usually, to destroy almost all the catalase activity in the pulp or crude water-extract from this beetle, leaving the oxydasic power of the same pulp or extract still very active toward guaiac or tyrosin. Sometimes an extract was found to show a little greater resistance to heat; and in any case, if one wished to be sure of destroying absolutely all catalase activity toward hydrogen peroxide, so that small bubbles of oxygen would not appear even after several hours, a temperature very near the boiling point was required. It was possible to separate the soluble catalase from its solution but not to free it from proteins. The catalase was carried down with the protein precipitate by either half or full saturation with ammonium sulphate or with the precipitate by alcohol; and the precipitate from *full saturation* with ammonium sulphate was found to contain practically all of the catalase.

In making a quantitative study of the influence of insecticide agents on the catalases one could obtain a little more uniformly constant results by using a fairly clear solution in which any insoluble particles present would be extremely fine, so that they would remain in suspension for hours. The results given in Table V are therefore taken from a study of the influence of certain agents upon the catalases in such an extract solution from the tissues of *P. cornutus*. The beetles were extracted in the usual way (i. e. digestive tract removed, etc.), using distilled water at the rate of about 12 c. c. to 5 beetles. The crude extract was filtered through linen of very fine mesh. Two cubic centimeters of this filtrate were then measured out and diluted to 10 c. c. with distilled water. In this way an almost clear dilution was obtained in which the fine particles remained in suspension for hours; and by agitating the dilution just before dividing it, one could obtain entirely uniform samples. In every test, 2 c. c. of this diluted extract were measured out into a small stender dish and submitted to the treatment under consideration for the required time. A check of 2 c. c. was also measured out. In some cases where gases or vapors were used, the stender dish with treated extract was allowed to air for a certain period before the final test with hydrogen peroxide was made. For measuring the amount of oxygen liberated from hydrogen peroxide by the treated portion of the extract and by the check, the apparatus represented in Fig. III was used. As has already been explained, this apparatus was in duplicate so that the test with the treated extract could be run in one and the check in the other duplicate. After the stender dish of extract had been introduced into the gas-container. mercury seal was made at the mouth of the container. The mercury manometer was adjusted level, and the reading of the gas-burette taken. Then 5 c. c. of hydrogen peroxide were drawn in form burette "f". As oxygen was liberated and the volume of the gas in the container increased, the mercury in the gas-burette was lowered until the manometer was once more level just at the end of 10 minutes. Then the reading of the gas-burette was again taken. The increase in volume less 5 c. c. (i. e. 5 c. c. of H_2O_2), represented the oxygen liberated. Any variation in the volume of oxygen liberated by the treated extract from the volume liberated by the check, therefore, was due to the influence of the treating agent, since all the other conditions were the same for both extracts. After an extract was

TABLE V.—INFLUENCE OF INSECTICIDE AGENTS ON THE CATALASES FROM TISSUES OF *P. CORNUTUS*.

Exp. No.	Agent.	Time agent was used.	After treatment.	O_2 liberated from H_2O_2 by treated ext. in 10 min. period.	O_2 liberated from H_2O_2 by check in 10 min. period.	Ratio of treated to untreated.
1.	Strong Cyanide bottle	17 hrs.	Aired 5 min.	5.5 c. c.	55.0 c. c.	0.1
2.	Strong Cyanide bottle	Same 17 hrs.	Aired 2 hrs.	17 c. c.		0.3
3.	Strong Cyanide bottle	Same 17 hrs.	Aired 8 hrs.	26.2 c. c.	55.0 c. c.	0.47
4.	CS_2 (Air—Sat. vapor)	2 hrs.	15 min.	2.0 c. c.	55.0 c. c.	0.03
5.	CS_2 (Air—Sat. vapor)	Same 2 hrs.	16 hrs.	1.0 c. c.	55.0 c. c.	0.01
6.	Air sat. with gasoline vapor, 4½%	1 hr.		40.0 c. c.	38.0 c. c.	1.05
7.	Air sat. with gasoline vapor, 4½%	16 hrs.		36.0 c. c.	36.0 c. c.	1.0
8.	Carbon tetra-chloride vapor. Sat. air	16 hrs.	Aired 10 min.	56.5 c. c.	62.0 c. c.	0.92
9.	Carbon tetra-chloride vapor. Sat. air	Same 16 hrs.	Aired 1½ hrs.	57.5 c. c.	62.0 c. c.	0.94
10.	Chloroform vapor (air sat. at 68° F.)	16 hrs.	Aired 5 min.	40.5 c. c.	50.0 c. c.	0.81
11.	Chloroform vapor (air sat. at 68° F.)	Same 16 hrs.	Aired 5 hrs.	41.5 c. c.	50.0 c. c.	0.83
12.	Ether vap. air sat. at 68° F.	¾ hr.	20 min. airing.	60.5 c. c.	60.0 c. c.	1.0
13.	Ether, 0.5 c. c. shaken up with 2 c. c. of ext	15 min.	Aired 2 hrs. until ether was evaporated.	58.0 c. c.	59.0 c. c.	0.98
14.	Ammonia (0.9 sp. gr. —2 c. c. diluted to 100 c. c.) 0.5 c. c. of dilution to 2 c. c. of ext	5 min.		47.2 c. c.	56 c. c.	0.84
15.	Am. vap. (100 c. c. air sat'd. from 5 c. c. am.—water of 0.9 sp. gr.)	1 hr.	Aired 30 min.	0.5 c. c.	56 c. c.	0.008
16.	Tobakine, 1 drop to 2 c. c. of ext	10 min.		65.0 c. c.	63.5 c. c.	1.02
17.	Tobakine, 1 drop to 2 c. c. of ext	¾ hr.		54.0 c. c.	61.0 c. c.	0.88
18.	Alcohol abs., .05 c. c. to 2 c. c. of ext	10 min.		50.0 c. c.	49.0 c. c.	1.02
19.	Alcohol abs., .05 c. c. to 2 c. c. of ext	60 min.		48.0 c. c.	53.5 c. c.	0.89
20.	Alcohol abs., 1 c. c. to 2 c. c. of ext	45 min.		4.8 c. c.	53.5 c. c.	0.089
21.	$HgCl_2$, 1 drop of sat. sol. to 2 c. c. of ext	5 min.		No measurable amount.	54.4 c. c.	
22.	1 c. c. of N/100 HCl to 2 c. c. of ext	10 min.		2.5 c. c.	54.0 c. c.	0.04
23.	Same treatment as No. 22	Kept cold 19 hrs.		0.8 c. c.	54.0 c. c.	0.01
24.	NaOH 0.2 c. c. N/100 to 2 c. c. of ext	3½ hrs.		35.5 c. c.	32.5 c. c.	1.09
25.	Am. Formate (M/1000) 1 c. c. with 2 c. c. of ext	20 min.		32.0 c. c.	61.0 c. c.	0.52
26.	Am. Formate (M/1000) 1 c. c. with 2 c. c. of ext	55 min.		31.0 c. c.	61.0 c. c.	0.50
27.	Formaldehyde 0.3 c. c. of 40% to 2 c. c. of ext	15 min.		0.5 c. c.	41.0 c. c.	0.01
28.	Heat 52° C.	5 min.		47.0 c. c.	54.0 c. c.	0.87
29.	Heat 56° C.	5 min.		13.0 c. c.	54.0 c. c.	0.24
30.	Heat 59° C.	2 min.		11.0 c. c.	61.4 c. c.	0.17
31.	Heat 59° C.	10 min.		Amt. scarcely measurable.	61.4 c. c.	
32.	Pyrethrum powder, 0.5 c. c. by measure to 2 c. c. of ext	40 min.		23 c. c.	51.0 c. c.	0.45
33.	White Hellebore, 0.5 c. c. powder to 2 c. c. of ext	5 min.		57 c. c.	55.5 c. c.	1.02
34.	White Hellebore, 0.5 c. c. powder to 2 c. c. of ext	30 min.		53.5 c. c.	55.5 c. c.	0.96

TABLE V.—Concluded.—INFLUENCE OF INSECTICIDE AGENTS ON THE CATALASES FROM TISSUES OF *P. CORNUTUS*.

Exp. No.	Agent.	Time agent was used.	After treatment.	O_2 liberated from H_2O_2 by treated ext. in 10 min. period.	O_2 liberated from H_2O_2 by check in 10 min. period.	Ratio of treated to untreated.
35.	Borax (3 grms. in 100 c. c. water) 0.5 c. c. to 2 c. c. ext	Used 20 min. before testing		49.0 c. c.	55.0 c. c.	0.89
36.	Borax (3 grms. in 100 c. c. water) 0.5 c. c. to 2 c. c. ext	½ hr.		60.5 c. c.	61.4 c. c.	0.96
37.	NaF, 0.5 c. c. sat. sol. to 2 c. c. of ext	15 min.		11.7 c. c.	60.4 c. c.	0.19
38.	NaF, 0.5 c. c. sat. sol. to 2 c. c. of ext	45 min.		12.0 c. c.	56.0 c. c.	0.21

a few hours old (i. e. after the reductase had become weak) there was little change in the catalase activity during the next 24 hours (or even 48 hours if the extract were kept covered at near freezing). On this account, it was not always necessary to run a check for each test in a series when treated extract-samples from the same dilution were used. Instead, a check could be made at the beginning of the series and another check held until the end. If a perfectly fresh extract were used, however, the checks had to be run more often, because the strong reductase present inhibited the catalase a little; and as the former weakened on standing, the catalase was able to liberate more oxygen in a ten-minute period. For example, in one extreme case, 2 c. c. of a fresh extract dilution gave (with 5 c. c. of hydrogen peroxide) in 10 minutes, 54.5 c. c. of oxygen, while 2 c. c. of the same dilution kept at 40° F. over night liberated 61 c. c. of oxygen in 10 minutes. In another case using the same method, 2 c. c. of a fresh extract-dilution liberated 32 c. c. of oxygen while its check 3 hours later (at room temperature) liberated 34 c. c. of oxygen. It should be added that *after* this rise due to the passing of the reductase activity, the ability to liberate oxygen from hydrogen peroxide gradually diminished during longer periods of time.

About 75 quantitative tests were carried out by the methods described above to determine the effects of certain insecticide agents upon catalase activity. Representative results of these tests are recorded in Table V.

In discussing the influence of hydrocyanic acid gas on the oxidasic activity of the insect tissue-extract toward alcoholic guaiac, it was stated that, when the test was made immediately after treatment, the guaiac turned blue very much more slowly than in the check. On the other hand when the treated extract was allowed to stand in the air for a while, the influence of the cyanide gas gradually passed off until the treated extract would oxidize the guaiac almost as rapidly as the check. In other words, there was a partial progressive recovery. Table V shows that this insecticide agent behaved in the same manner toward the hydrogen peroxide catalases. In the experiment, results of which are given in the table, a little more than 6 c. c. of extract was kept in a strong cyanide bottle for 17 hours. At the end of that time, it was removed to the open air. After airing only 5 minutes, a test was made with 5 c. c. of hydrogen peroxide and in the following ten-minute period 2 c. c. of this extract liberated only 5.5 c. c. of oxygen—the check, in the same time, liberated 55 c. c. of oxygen. After airing for 2 hours, 2 c. c. of the same treated extract liberated 17 c. c. of oxygen; and after standing in the air for 8 hours until almost all odor of the cyanide gas had disappeared, 2 c. c. of the treated extract caused 26.2 c. c. of oxygen to separate from the 5 c. c. of hydrogen peroxide in a ten-minute period —the check giving 55 c. c. of oxygen. Other tests gave similar results, so that there is no doubt of a partial, progressive recovery of the catalase activity toward hydrogen peroxide in an extract treated with cyanide gas upon standing in the open air. Furthermore, the results of these tests seemed to show that both the soluble and insoluble catalases in the dilute extract had been affected and that both had recovered the greater part of their activity after the passing of the poison. In order to make more certain that this was the case, a few experiments were tried in which hydrocyanic acid gas (from KNC + dilute H_2SO_4) was

made to act upon the two catalases separately. The crude extract was filtered. Then the clear filtrate, containing the soluble catalase, was diluted and treated by the method already described. The residue on the filter was washed as nearly free from the soluble catalase as possible, after which it was shaken up in a small amount of distilled water. This water containing the insoluble catalase in suspension was then divided into equal samples to be treated as in the other experiments. Charges of hydrocyanic acid gas sufficient to render specimens of *P. cornutus* helpless and quiet in 3 to 4 minutes were used. Such a charge of cyanide gas is much stronger than that used in ordinary fumigation. The charges were applied 40 to 45 minutes. (This was a longer time than was really necessary to kill specimens of the beetle.) The experiments showed that over 95% of the activity of the soluble catalase and only about 52% of the activity of the insoluble catalase was suspended at the end of a 45-minute period. There was 88% recovery, or more, (after the treated extract stood 15 hours at about 34° F.) in case of the soluble catalase, and about 21% recovery of the activity of the insoluble catalase toward hydrogen peroxide. The insoluble catalase seemed to show the greater resistance, but once its activity had been hindered by the poison, greater permanent injury resulted than in the similar event with soluble catalase.

A number of tests were made to determine whether any such recovery of the catalases in an extract would obtain, after treatment with hydrochloric acid instead of with hydrocyanic acid gas. In preparing a treated extract and its check for this test, when 1 c. c. of $\frac{N}{100}$ acid was added to 2 c. c. of extract, then 1 c. c. of distilled water of the same temperature as the acid was added to the 2 c. c. check extract in order to keep the same dilution in the two portions. The tests were run in the apparatus already described. Numbers 22 and 23 in Table V show that $\frac{N}{100}$ hydrochloric acid was quite destructive to the catalase; and there was no apparent recovery, even upon long standing, if the treated extract and its check were kept at a temperature not far above freezing during the long period of treatment. Upon standing after the first decrease in catalase activity, three cases of increased activity for liberating oxygen from hydrogen peroxide *did* occur in some of the early tests with hydrochloric acid. However, the increase in these three cases was really brought about by decay which (in a warm room) the weak acid used did not entirely prevent, for long, in the extract. With the precaution of keeping the extracts in the cold (near freezing) during the treatment period, no example of increase in catalase activity at any time after treatment with hydrochloric acid, up to 19 hours, was observed. It may be added that the deleterious action of this acid upon the reducing power of the extract was not so great as it was upon the catalase activity.

The series of tests, results of which are recorded in Table V to show the effect of heat upon the catalases, are for short-interval periods only. Other experiments showed that when the treatment-period was lengthened, temperatures yet lower than 52° C. showed appreciable injury to the catalase activity. For example, at a temperature ranging from 45° to 46° C. for two hours, the ratio of the volume of oxygen liberated in

a test by the heat-treated extract to that liberated by its check was $\frac{75}{86} = 0.87$.

Some of the agents readily showed a slight acceleration of the catalase action, when used in small amounts. Attention may be called to gasoline and alcohol in the table (V) as illustrating this fact. The manner in which some of the insecticides (especially gasoline) appear to hasten oxidasic activity because of their deleterious action on the reducing power, when they were first applied to fresh extract, has already been described. It seems very likely that the slight acceleration of the catalase-action, at first, when an extract was treated with small amounts of the above insecticides, may have (in part at least) much the same explanation. That is, small amounts of gasoline, alcohol, and perhaps a few others, in attacking the reductases deleteriously before they attacked the catalases, removed a stronger inhibiting influence over the catalase than they themselves exerted, so that a resulting initial acceleration of the catalase action immediately followed. *Strong* treatment with the insecticides, however, so greatly hindered the catalase (as well as the reductase) activity, that there was a lessening of the volume of oxygen liberated from hydrogen peroxide. In the case of alcohol the deleterious effect of strong or more prolonged treatment was quite decided. Nos. 18 and 20 of Table V show that while 0.5 c. c. of absolute alcohol added to 2 c. c. of extract caused more oxygen to be liberated from hydrogen peroxide after a period of 10 minutes treatment, 1 c. c. of alcohol in 2 c. c. of extract for 45 minutes before the test brought the ratio of oxygen liberated by the treated to that liberated by the untreated extract down to 0.089. That is, the stronger treatment not only overcame the lead of the first increase, but reduced the catalase activity much below that of the check. The effect of strong, prolonged treatment with gasoline vapor was not so marked; it was sometimes barely able to overcome the first lead or increase in catalase activity—(the lead or increase which was due, as has already been suggested, to the removal of the inhibiting reductases). This is illustrated by the two experiments recorded in Table V, Nos. 6 and 7. In no case of strong treatment with gasoline vapor was the amount of oxygen liberated brought much below that of the check.

Ammonium formate and ammonium oxalate were used in the series of tests because they are known to be products formed when hydrocyanic acid gas breaks down in air. Ammonium formate, in any amount tried, permanently injured the catalytic action of the extract toward hydrogen peroxide, as illustrated in the table (V). Ammonium oxalate, on the other hand, served to slightly accelerate the liberation of oxygen from hydrogen peroxide by the catalases.

The influence of the other agents, so far as tested, are either made clear in the table or may be referred to later in discussing powdered solid contact insecticides.

DO THE CONTACT INSECTICIDES UNDER CONSIDERATION ACT UPON OXIDASES, CATALASES OR REDUCTASES IN THE LIVING TISSUES OF INSECTS TO CAUSE DEATH?

The experiments just described showed that the contact insecticides under consideration did interfere with the activity of the oxidases, catalases and reductases in the tissue extract of *P. cornutus*. In the concentrated form, such insecticides affected all three of the enzymes (if they may be so called) but usually not in the same degree. It was clear that carbon disulphide, for example, affected the oxidases and catalases more strongly than it affected the reductases—while gasoline, on the other hand, had its most deleterious influence upon the reductases.

Trial showed that oxidases, catalases, and reductases existed in the tissue-extract of many caterpillars, pupae and adult moths, as well as in the adults and grubs of various beetles. Also, reference has already been made to the work of Batelli and Stern* in relation to oxydones in insects. These authors, in the same year (1913), again described tyrosine-oxidase, polyphenol-oxidase, and oxydones in several forms of insects. Besides, a few other scattered references in literature briefly (often covertly) relate the occurrence of oxidases in the blood or in extracts from various lepidoptera, beetles and diptera. Loew has recorded the presence of catalase in three different beetles. In fact, the occurrence of oxidases, catalases and reductases in extracts appears to be as general among insects as among other forms of life.

Further experiments showed that extracts from tomato-worms did not give the test for oxidase with alcoholic guaiac but hydroquinone-oxidase and tyrosin-oxidase were clearly present as well as reductases and catalases. Moreover, the influence of gasoline and carbon disulphide upon the activity of extracts from white grubs (both *Lachnosterna* and *Allorhina*) and tomato worms was similar to that already recorded in connection with extracts from specimens of adult *P. cornutus*.

With these facts established, the question remained as to whether oxidases, catalases and reductases in the *living* tissues of *P. cornutus* became deleteriously affected by contact insecticides used in such amounts and for such periods only as were necessary to kill. That catalases and reductases, as found in the tissue extract of the insect, really existed in the living tissues (and were not after-products of the tissues in the extract) seemed reasonably certain. If hydrogen peroxide were introduced directly into the tissues in any part of the body of a living beetle, oxygen became liberated *at once* with great energy; and the reducing power of living animal tissues, generally, for methylene blue is well known. In technical bulletin 11 (this station), page 52 is reference to the fact that living beetles, in the absence of oxygen gas, reduced large amounts of indigo carmine and methylene blue which had been injected into the body tissues by means of a fine hypodermic needle. Even in the open air, a small amount of methylene blue, injected into the tissues of a beetle's body, became entirely reduced. When the reduced (leuco-) methylene blue was collected for excretion by the cells of the Malpighian tubules, however, it became oxidized to the blue color again in those cells. Still, this reoxidation of the color-body did not neces-

*Batelli and Stern (Biochem. Zeits., 1913, 56, 59-77). Rev., J. of the Chem. Soc., Vol. 103-104, part i, p. 1272.

sarily imply or prove the presence of oxidase activity, and no entirely satisfactory method of *visibly demonstrating* oxidase activity in the tissues of living uninjured insects was found. Upon studying the case, however, there was nevertheless found to be visible evidence of almost positive oxidase activity naturally present in certain living cells of most insects. The facts which set forth this evidence follow:—

The wing covers of newly emerged adults of *P. cornutus* were almost without color—pure white. They gradually began to brown, becoming darker and darker until they finally appeared jet black—and by that time, they were entirely dry and hard. When the dry, black wing covers were crushed and tested they were found to contain catalase and an (almost) insoluble tyrosin-oxidase (see Fig. 3, Plate I); reductase activity was practically absent. The dark coloring matter of the crushed wing-covers appeared to be identical with the dark pigment which formed when the insect-extract or blood was exposed to air. Moreover, this melanic pigment in both cases was like the melanic pigment obtained when tyrosin, in solution, was oxidized by insect tissue-extract. (Tyrosin is a protein derivative). The wing-cover pigment, as well as both the other pigments just mentioned, could be reduced by confining it in the absence of oxygen with fresh tissue-extract which was rich in reductase activity. The coloration of newly emerged insects could be entirely stopped at any stage by dropping the insects into boiling water and keeping them at that temperature for about 20 minutes. Again, if white, newly moulted insects (*Periplaneta Americana* was used) were submerged in CS_2 and kept there, almost no dark coloration developed. Also (as has been generally found by experience) in order to preserve white grubs and pupae in alcohol without darkening or discoloring, they first had to be treated with boiling water. That is to say, some means (such as heat) appeared to be necessary to destroy the oxidase activity before placing the white adults, grubs or pupae in alcohol; otherwise, the inhibiting action of the strong reductase, present in the living body, rapidly passed after death, and the oxidase (which is injured but not entirely destroyed by alcohol) then brought about oxidation and blackening of the tissues. Likewise, since an oxidase which could oxidize tyrosin was present in the wing-covers and outer integument of *P. cornutus* and the cockroach (*Periplaneta Americana*) it appeared that the cuticular excretion poured out from the hypodermal cells beneath the old integument before it became moulted—the excretion which *went to form* the integument of the newly moulted insect—contained not only the tyrosin-oxidase but also a tyrosin-like derivative (i. e. some similar organic derivative) which oxidized, under the influence of the oxidase, to form the brown or melanic coloration of the integument. These added instances of the activity of an oxidase did not, so far, prove its presence in living cells. The evidence showed, however, that in blood exposed to the air, in extract of the tissues exposed to air, in the cuticular integument of the newly moulted adult beetle and in the tissues of the dead grub and pupæ (killed by some means that does not destroy an oxidase) a very similar, if not an absolutely identical, melanic pigment developed under the influence of an oxidase in every case—and more rapidly as the reductase activity passed. Moreover, these instances of melanic pigment formation served to call to mind the fact that in certain cells of the

compound eyes of insects, and of the ocelli of insects and spiders, and in the hypodermal cells forming the circular boundary of ocelli, there was to be quite commonly found a melanic pigment which had its origin *in the cells* during ontogenic development. In view of the proven cases of melanic pigment formation through oxidase action cited above, the inference that the development of melanic pigment in certain eye and hypodermal cells was an example of a similar oxidase acting upon some constituent of the cells, themselves, seemed to have justification. Other similar evidence might be given all tending to show that melanic pigment, in animal tissues, develops through the oxidation of some organic constituent of the tissues—under the influence of an oxidase* acting after the tissue-reductase has been either weakened or destroyed by some means.

In order, then, to learn something finally concerning the effect of a few contact insecticides (when used only in such amounts as were necessary to kill) upon oxidases, catalases and reductases in the living tissues of *P. cornutus,* the method of study was varied as follows:

Insects were killed by the insecticide agent to be studied, using various amounts of the agent for the length of time necessary to kill. The bodies were then extracted by the method already described, as soon as the insects were considered dead or beyond recovery, and the strength of the oxidase, catalase and reductase activities in this extract was compared with that in a fresh extract similarly prepared from the same number of untreated insects. Tests were made in this manner using heat, hydrocyanic acid gas, chloroform and carbon disulphide. It will be appreciated that it was impossible to check the factors of time, original strengths of catalase, oxidase and reductase in the extracts, etc. by this method as accurately as they could be checked in the former method where samples were taken at the same time, from a single extract, for study. Nevertheless, the results showed certain things very satisfactorily.

The extract from gasoline-vapor-killed beetles was always much weaker in reductase activity than the check, while the catalase and oxidase activity remained much the same. For example, when two beetles were treated with about 3% gasoline vapor for 20 hours, it required 4 hours for the treated extract to reduce a certain amount of methylene blue —the check accomplishing the same reduction in 10 minutes. Practically no difference could be seen in the rapidity with which alcoholic guaiac was oxidized; and a check-extract-sample liberated only 2 c. c. more of oxygen from the standard hydrogen peroxide than a similar sample-extract of the insects killed by gasoline.

The extract from beetles killed with carbon disulphide was always quite white or very light straw color at first and it remained so for two or three hours, usually, before it began to darken. That is, oxidase action always appeared to be almost entirely absent, at first. After a few hours, however, this activity became partly recovered. Catalase-action was usually injured one-half or more, and the reductase much less, so that it was relatively strong, at first, as compared with the oxidase activity.

Reductase in the extract of beetles treated 2½ hours with strong

*Hammarsten (Mandel) in his "Physiological Chemistry" quotes Fürth with Schneider and Prisbram, Gessard, Neuberg and Dewitz as having shown the presence of tyrosinases in insect-tissue and sepia, in melanotic tumors and in pigmented skin.

chloroform-air was found to be, at the end of that time, 16½ times weaker than the check. The oxidase was apparently uninjured; sometimes it seemed a little more active than in the check. The catalase showed but little injury.

Hydrocyanic acid gas caused great interference with the activity of the oxidase. For example, three beetles were treated in a cyanide bottle 15 hours and then aired one-half hour before being extracted. This extract remained light or whitish in appearance for 2 hours before it began to darken at the surface in contact with the air, and before it would begin to show any indication of bluing guaiac. The check required only about 2 minutes to bring about a decided bluing of guaiac. The catalase-activity was quite strongly interfered with, at first; but the reductase required only about 9 times longer to reduce its methylene blue than the check.

Extract from beetles kept at 44° to 46° C. for 2½ hours, until dead, showed little loss in catalase activity—but more in its oxidasic and reductase activity. The oxidase was about 7 times slower, and the reductase about 21 times slower than the check.

Clearly, the results given by the last method corresponded very well with results obtained by the former method where the effect of the insecticide upon oxidase, catalase and reductase in the ready-prepared extract was studied. It is true that in every case the beetles were killed by the insecticide agent without the entire destruction of any one of the three enzymes—but never without greatly injuring one or more of them. Also, as will be observed, if the reductase were strongly injured, the oxidasic enzymes were usually little injured or apparently not at all; and conversely, if the oxidase and catalase were greatly injured by the insecticide, the reductase was little injured. Thus, the natural or normal balance of catalase, oxidase and reductase activity was disturbed.

The question may now be repeated: Is this deleterious effect of the insecticides studied, upon the oxidases, catalases or reductases, the cause of death? It is hardly possible that the three enzyme-like bodies named in the question could be so universally present (with activities so apparently coordinated) in tissue extracts and in the living tissues, if they were not of vital importance. They surely accomplish oxidations and reductions in the living tissues and much evidence indicates that they are a part, at least, of the machinery of internal respiration. It has been shown that *P. cornutus* cannot be killed or rendered beyond recovery, by certain contact insecticides studied, without decided injury to one or more of the three activities—oxidase, catalase or reductase. This fact, taken in connection with what has been shown to be true of the influence of those same insecticides upon the respiratory rate and the respiratory-quotient, supports the view that oxidase, catalases and reductases have to do with the starting and carrying forward of cell respiration. If that view is true, then the deleterious effect of the insecticides (named in this connection) upon oxidases, catalases or reductases in insects must be an important factor—perhaps, in some cases, the *determining* factor—in causing death.

No special study has been made of the influence of various contact insecticides upon the protoplasmic activities which carry forward life-processes in the nervous tissue cells of insects. Such a study in this connection would appear to be of next importance, since all the volatile

insect fumigants are more or less narcotic in their action. Shiro Tashiro has found, by means of his new apparatus for estimating exceedingly minute quantities of carbon dioxide produced in nerve fibres, that ether and urathane "diminish CO_2 production from isolated nerve fibre"*—although he was not working with insects.

CONCERNING THE INFLUENCE OF GASOLINE-VAPOR, ETC., AND LIME-SULPHUR SOLUTION UPON THE PASSAGE OF OXYGEN, RESPECTIVELY, THROUGH FAT OR LIPOID, AND CERTAIN WAX MEMBRANES.

It has been made clear that gasoline deleteriously affected the reductase activity far more quickly and severely than it did that of the oxidases; and further that, since the presence of a strong reductase tended to partly hold in check oxidase-activity, the quick lowering of reductase-activity in a gasoline-treated extract caused the latter to show greater oxidase power for a while than an untreated check of the same extract. This was true when the test was made with fresh extract in the presence of gasoline vapor. But in such tests as the last, made with extract that had already lost the greater part of its reductase-activity, the fact will be remembered that a less amount of oxygen was used by the "extract plus hydroquinone solution" (and less quinone was formed) in the presence of air containing gasoline vapor than by the check in pure air.

When an extract a few hours old was treated very strongly with gasoline and then test was afterward carried on in fresh air, however, oxidase activity did not appear to have been greatly lessened. The suspicion therefore presented itself that the lessened oxidation of hydroquinone occasioned by the extract in the presence of gasoline-vapor, as well as also the lessened use of oxygen by insects deeply under the influence of gasoline-vapor, was partly due to some other cause than injury of the oxidase or reductase by gasoline.

As has been mentioned several times, the first and most noticeable fact in connection with the tissues of an insect strongly treated with gasoline vapor, as well as with tissue-extract so treated, was the solvent action of the gasoline upon the fats and fat-like bodies (lipoids); or, looked at from the other point of view—the insistent fact was the solubility of the tissue-fats and lipoids for gasoline vapor. Lipoids were found to be present in or around the cells of insect tissues. For example, a semi-transparent lipoid residue was obtained by extracting the *washed* muscles of *P. cornutus* with ether containing a little alcohol. This was perhaps to be expected, since the almost universal occurrence of lipoids, especially such as lecithin, in animal cells and animal fluids has been recognized for several years. This lipoid residue obtained from the washed muscles of *P. cornutus* absorbed gasoline readily. The condition therefore seemed to obtain that wherever the lipoids might be found, in the body fluids (blood)—where they certainly were present—in the protoplasm boundary layer of the cells (Overton) or in the complex of the cell-protoplasm itself, gasoline would surely also be present in an insect under the influence of that insecticide. In any such case the insect tissue cells would be enveloped by blood holding gasoline in solution; the ends of the trachaeoles would be permeated by gasoline. Could

*Am. Jour. of Physiol., Vol. 32, 1913, No. 11, p. 117.
See also same Journal pp. 137-145.
See also same Journal Vol. 34, 1914, pp. 405-413.

it be that this ready solubility for gasoline of the fats and lipoids of insect-tissues furnished a means for hindering the *absorption* of oxygen by the tissues, or lessening the oxidation of hydroquinone by the oxidase of insect-tissue extract in the presence of gasoline? It seemed that such might be the case, if in fact it should be found that gasoline, held in solution by fats and lipoids, lessened their solubility for oxygen—and by so doing, lowered the rate at which atmospheric oxygen might pass into an extract, or from the trachaeoles (or by any path) through the fluids bathing the cells, on into the cells to those agencies which (in life) utilize the incoming oxygen. Accordingly plans were made to determine what change the absorption of gasoline-vapor by a lipoid membrane, such as a lecithin membrane (or by a fat membrane) would have upon the rate at which oxygen passed through it. For the series of experiments to be made in this connection, it did not seem necessary or even desirable to use the fat or lipoids derived from insects. Clearly, the advantage would lie in eliminating all possible variable quantities. On that account, a comparatively pure fat and lipoid, each of which would be uniform and easily obtained, was desired. Several preliminary quantitative absorptions of gasoline-vapor, using definite amounts (3 grams) of lard, lanolin, lecithin, cholesterin, and (for comparison) egg-yolk and egg-albumen were first carried out. Some representative results of a few of these absorption tests are given in Table VI. The apparatus used was that represented by Fig. III, except that the hooked U-tube "u" and the burette "b" were not needed. The 3-gram quantity of lecithin (or eggwhite, etc.) was placed in a standard stender dish—so that the same absorbing-surface was exposed always—and the dish was then floated upon mercury in the gas-container "g. c." Most of the tests were made over rather short periods, during which the barometer and thermometer remained practically constant. Checks showed that in pure air the volume of gas enclosed with the absorbent (fat, etc.) which was being tested remained unchanged over similar periods of time. In the tests, a loss in volume as shown by the gas-burette was therefore the measure of the "gasoline-vapor" that had been absorbed. At the end of each test the percentage of gasoline-vapor still present was determined from a sample taken from the gas-container—using the "Nordhausen sulphuric acid" method of estimation already described in the former bulletin.

TABLE VI.—COMPARATIVE SOLUBILITY OF GASOLINE-VAPOR IN 3 GRAMS, RESPECTIVELY, OF LECITHIN, EGG-WHITE, ETC.

Exp. No.	Material.	Temperature.	Per cent gasoline vapor at end of test.	Volume measure of gasoline-vapor absorbed.	Time used.
1.	a. Lecithin	22.2° C.	3.0%	2.7 c. c.	30 min.
	b. Egg-albumen	22.2° C.	3.0%	0.6 c. c.	
2.	a. Egg-albumen	24.1° C.	3.0%	0.7 c. c.	40 min.
	b. Egg-yolk (fresh)	23.7° C.	3.0%	0.1 c. c.	
3.	Egg-yolk (fresh)	25.0° C.	3.5%	0.1 c. c.	60 min.
4.	Cholesterin	26.8° C.	3.0%	0.6 c. c.	20 min.
5.	Egg-yolk (fertile egg) after 4 days incubation	24.0° C.	4.4%	1.0 c. c.	60 min.
6.	Egg-yolk (fertile egg) after 5 days incubation	22.4° C.	4.0%	1.8 c. c.	30 min.

The lecithin (H. P. from egg, Merck) was used, as received from Merck, in the form of a paste; and in the case of cholesterin the soft dry powder

was used. The ready capacity of lecithin to absorb gasoline-vapor, from air containing it, is illustrated in the table. In connection with the solubility of egg-white for gasoline, it must be remembered that even egg-white is not pure albumen, but that it contains a mesh of extremely fine membranes of keratin and a very small amount of fats, lecithin, and cholesterin. The largest absorption of (3%) gasoline-vapor by 3 grms. of egg-albumen was that given in Exp. No. 2 (0.7 c. c.). A surprise came in finding the comparative solubility of fresh egg-yolk for gasoline-vapor as shown in the table. The results obtained in connection with fresh and incubated egg-yolk will be referred to again under the next heading.

After these preliminary tests and other trials, lard was selected as the fat and lecithin as the lipoid which seemed best adapted for the purpose (according to the plan mentioned above) of being spread into membranes to be used in determining the effect of gasoline-vapor upon their permeability to oxygen of the air. However, a few tests were made with membranes composed of a mixture of lard and lecithin, and also with lanoline membranes. All these membranes were prepared, and manipulated to obtain the results required by the plan, as follows:

A very light India muslin was used for the sustaining fabric, and over this the lecithin (or lard, etc.) was spread in a thin sheet entirely covering the fabric and filling its meshes to form essentially a lecithin (or lard, etc.) membrane. In making use of such a lecithin membrane, for example, it was placed as a cover over the mouth of a small preparation dish (4 cm. in diameter), which had been almost but not quite filled with concentrated lime-sulphur solution. The cover was then fastened down "lecithin tight" with a thin metal band, thus enclosing a small air space above the lime-sulphur in the preparation dish. Great care was used in order that the lime-sulphur might not wet the lecithin cover, and the dish was floated up on mercury in the gas container of the apparatus, Fig. III. In this position, the covered preparation dish was allowed to stand a few hours. Then the mercury manometer "m" was adjusted level. The thermometer and barometer readings, the time and the reading of the gas-burette "a" were all recorded. After a certain definite period all these readings were recorded again, the mercury-manometer having been first leveled once more through adjusting the height of the mercury column in the gas-burette "a". In this manner, the rate of volume-decrease of the air in the gas-container "c" was determined. The decrease was due to a decrease in the amount of oxygen in the air of the container holding the membrane-covered dish of lime-sulphur solution. This was definitely proven by determining the percentage of oxygen in the air from samples taken from the container immediately before the tests began and immediately after the last set of readings were recorded—the volume of air used in the container during the test being known. Since the conditions of the experiment have been outlined, it will be clear that this rate of decrease in oxygen was the rate at which oxygen was passing through the lecithin membrane—or whatever membrane was used to cover the preparation dish. From the beginning of the test, the lime-sulphur solution absorbed oxygen gradually and steadily from the small air-space above it. Just as constantly, oxygen was given off from the under side of the membrane (to the small air-space above the lime-sulphur) and *taken up* or *absorbed* by the upper surface of the membrane from the air of the container—the volume of which was

comparatively large. The prime condition was (it will be remembered) that the lecithin cover (or lard, etc.) closed the preparation dish "lecithin tight." No oxygen could pass into the air space of the dish except it passed through the lecithin itself. When the rate at which oxygen passed through the membrane from air had thus been determined, the mercury mug "b" was raised, cocks "a" and "c" were opened and as much of the air as possible was carefully expelled.* Then, from a stock preparation, gasoline-air was drawn into the container as the mercury mug "b" was lowered. At first, gasoline-vapor was absorbed rapidly by the lecithin membrane. By making tests (as already described) at intervals, it was found that the rate of decrease in volume would finally settle down to a fairly uniform rate. That is, the membrane would finally become saturated with the gasoline-vapor, and the decrease in volume after that was due to the loss in oxygen absorbed by the lime-sulphur solution. This last rate was then determined as accurately as possible (just as has been explained when the cup was in air.) Here also, the direct method of measuring the loss in volume of oxygen could be checked by taking samples of gasoline-air at the beginning and at the end—making determinations of the percentage of gasoline and of oxygen present at each time and thus figuring the rate at which oxygen had been absorbed. Now this last rate in the loss of oxygen, it will be observed, was the rate at which oxygen passed through the same lecithin membrane after the latter had become saturated with gasoline-vapor in the presence of gasoline-vapor-air. The rate at which oxygen passed through the lecithin membrane in air might therefore be compared with the corresponding rate when the membrane was under the influence of gasoline-vapor. In many of the experiments, the covered preparation dish was taken from the container and aired until as much as possible of the gasoline-vapor had evaporated from the lecithin cover. After that the dish was carefully replaced in fresh air in the container and the rate at which oxygen passed through the membrane again determined. This was done, as a check, to learn whether the lime-sulphur solution was still in condition to absorb oxygen at a practically uniform rate when the membrane was exposed to pure air.. Further precautionary checks were made by finding the rate at which the uncovered dish of lime-sulphur absorbed oxygen in pure air and in gasoline air. These checks showed conclusively that at the end of the series of tests the lime-sulphur solution was still active enough to absorb oxygen much more rapidly than the lecithin or other membranes could take up that gas and pass it through to the absorbing solution, either in pure air or gasoline-vapor-air. In all cases, the temperature during any series of tests was kept as uniform as possible. When the thermometer or barometer changed appreciably, or whenever the tests extended over more than three hours, all gas-volume measurements were changed to 0° C, and 760 m. m. mercury pressure before comparisons were made.

Over 35 trial experiments were made with the apparatus in learning the best thickness of membrane to use, and the time-limits (in order that the lime-sulphur solution should not be weakened too much), as well as many other details. Thereafter, twelve series of tests were run to determine the influence of gasoline-vapor upon the rate of oxygen transfer

*The edge of the metal band, which was used around the top of the preparation dish to fasten the cover-membrane tightly, projected above the surface of the latter enough to entirely protect it from damage by the top of the container when the air was being expelled.

through lecithin, lecithin-lard and lanolin membranes. The lecithin-lard mixture was made of about equal parts of lecithin and lard thoroughly mixed together. In half of these both methods mentioned were run together—i. e. the method of total gas-volume measurements, and the method of percentage determinations of the oxygen, gasoline-vapor, etc., present at the start and the end of each separate test in the series. The cautionary checks were also observed. Four series of tests were run to determine the influence of chloroform-vapor upon the rate of oxygen transfer through lecithin membranes. These four series of tests were carried out by the one method only—namely, that of the total gas-volume measurements at the beginning and end of each test.

The results of all the experiments by both methods pointed directly to the conclusion that, in the presence of gasoline-vapor air or chloroform-vapor air, the membranes named permitted less oxygen to pass per unit of time than they permitted in air only.

Note that the experiments with the covered preparation dish of lime-sulphur were carried out under the conditions encountered regularly by the tissue cells of an insect subjected to fumigation by gasoline-vapor or chloroform-vapor. Just as oxygen reached the lime-sulphur for absorption at a lower rate, when passing through the lecithin membrane in the presence of gasoline-vapor or chloroform-vapor, so, it may be judged, less oxygen would be able to reach the oxygen-absorbing protoplasm of the cells through the surrounding body fluids (blood) etc., the lipoids of which held gasoline-vapor. So also, in the presence of air containing that vapor, less oxygen would be able to pass through the surface of an insect-tissue extract (containing hydroquinone, for example) into the extract where it could be utilized by the oxidase in oxidizing hydroquinone.

Now, the actual percentage of oxygen present in the gasoline-vapor air would naturally be lower than in normal, pure air through dilution by the gasoline-vapor, but it appeared that this alone could not fully account for the lower rate of oxygen transfer through the membranes. Attention may be called to the fact that when the percentage of oxygen in the air of the container, for the "air only" tests, was made as nearly equal as possible to the percentage of oxygen in the gasoline air used (see Nos. 3 and 4, Table VII), the rate of oxygen transfer through the membrane remained lower, nevertheless, in the presence of gasoline-vapor. In Exp. 3 (Table VII) the oxygen percentage was 20.2 at the beginning, and 18.26 at the end of test No. 1. In test No. 2, carried out in "gasoline-vapor air" mixture, 18.26 per cent oxygen was present at the start and 17.5 per cent, at the end. Test No. 3 of Exp. 3 was started after the covered preparation dish had been aired several hours. All the gasoline had not been given up by the membrane, however—and by the end of the test, the small percentage of gasoline-vapor shown in the table had been given off to the air of the container. A very thick lecithin-lard membrane had been used. In Exp. 4 (test No. 1) of the same table, the percentage of oxygen at the beginning was 15.13, and at the end, 13.2. In test No. 2 of this experiment, the gasoline-vapor air contained 14.6 per cent oxygen at the start, and 13.11 per cent at the end. The conclusion in regard to this point must be, therefore, that the gasoline-vapor which was taken up by the membranes rendered them less permeable to oxygen. Representative results are given in Table VII.

TABLE VII.—SHOWING THE INFLUENCE OF GASOLINE-VAPOR, CHLOROFORM-VAPOR AND LIME-SULPHUR SOLUTION UPON THE PASSAGE OF O_2 THROUGH CERTAIN SPECIFIED MEMBRANES.

Exp. No.	Test No.	Membrane cover used.	Insecticide-vapor used.	Per cent of Insecticide-vapor at end of test.	Temp. Cent.	Total time exp. ran.	Total O_2 absorbed.	O_2 absorbed, or O_2 passing through membrane per period of 10 hrs.	Excess of O_2 passing through membrance per 10 hr. period in pure air over that in insecticide-vapor-air mixture.
1.	1.	Lecithin	Air only		23.2° C. to 22.6° C.	2¾ hrs.	3.8 c. c.	13.8 c. c. Average	
	2.	Lecithin	Air only		23.3° C.	1 hr.	1.5 c. c.	15.0 c. c. 14.4 c. c.	10.4 c. c.
	3.	Lecithin	Gasoline vapor	2.8%	24.2° to 21.4°	15 hrs.	6.1 c. c.	4.0 c. c.	
	4.	Lecithin	Gasoline vapor	3.5%	21.4° to 20.4°	3½ hrs.	2.42 c. c.	7.6 c. c.	6.8 c. c.
	5.	No cover	Gasoline vapor	4.4%	26.0° to 25.6°	3½ hrs.	3.8 c. c.	10.8 c. c.	
	6.	No cover	Gasoline vapor	4.4%	25.6°	¾ hrs.	0.8 c. c.	10.6 c. c.	
2.	1.	Lecithin	Air only		25.6° to 27.4°	1⅔ hrs.	1.6 c. c.	9.6 c. c.	
	2.	Lecithin	Gasoline vapor	2.7%	24.8° to 25.4°	3 hrs.	1.47 c. c.	4.9 c. c.	4.7 c. c.
	3.	No cover	Gasoline vapor	3.7%	28.2° to 27.4°	2 hrs.	1.6 c. c.	8.0 c. c.	
3.	1.	A thick Lecithin—lard cover	Air only		25.2° to 24.4°	24 hrs.	2.4 c. c.	1.0 c. c.	0.809 c. c.
	2.	A thick Lecithin—lard cover	Gasoline vapor	5.37%	25.0° to 27.2°	24 hrs.	0.46 c. c.	0.191 c. c.	
	3.	A thick Lecithin—lard cover	Air (gasoline vapor)	0.55%	27.2° to 24.0°	24 hrs.	1.17 c. c.	0.48 c. c.	0.52 c. c.
	4.	No cover	Gasoline vapor	5.7%	22.4° to 21.4°	4 hrs.	2.07 c. c.	5.17 c. c.	
4.	1.	Lecithin—lard	Air only		25.0°	6 hrs.	3.5 c. c.	5.83 c. c.	3.45 c. c.
	2.	Lecithin—lard	Gasoline vapor	4.54%	26.6°	6 hrs.	1.43 c. c.	2.38 c. c.	
	3.	Lecithin—lard	Air again		27.2° to 26.6°	6 hrs.	2.05 c. c.	3.4 c. c.	
	4.	No cover	Gasoline vapor	5.1%	22.8° to 22.0°	4 hrs.	1.98 c. c.	4.95 c. c.	
5.	1.	No cover	Air only		24.2°	4-5 hr.	1.8 c. c.	22.5 c. c.	
	2.	No cover	Chloroform vapor		25.4°	1½ hr.	1.5 c. c.	10.0 c. c.	
	3.	Lecithin	Chloroform vapor		25.0°	1 hr.	0.5 c. c.	5.0 c. c.	11.0 c. c.
	4.	Lecithin	Air only		25.2°	1 hr.	1.6 c. c.	16.0 c. c.	
	5.	No cover	Air only		25.0°	2 hrs.	3.7 c. c.	18.5 c. c.	
6.	1.	No cover	Air only		20.0°	1 11-12 hrs.	2.3 c. c.	12.0 c. c.	
	2.	Lecithin	Air only		20.0°	2 hrs.	2.0 c. c.	10.0 c. c.	4.0 c. c.
	3.	Lecithin	Chloroform vapor		21.0° to 21.4°	1 hr.	0.6 c. c.	6.0 c. c.	
	4.	No cover	Chloroform vapor		19.8° to 20.0°	1½ hrs.	1.5 c. c.	11.2 c. c.	
7.	1.	Bees wax membrane	Air only		27.0°	⅔ hr.	1.2 c. c.	18.0 c. c.	
	2.	Bees wax membrance wet with lime-sulphur solution	Air only		25.2°	48 hrs.	18.46 c. c.	3.8 c. c.	14.2 c. c.
	3.	No cover	Air		25.2°	1 hr.	1.2 c. c.	12.0 c. c.	
8.	1.	Shellac	Air		26.0°	1 hr.	1.4 c. c.	14.0 c. c.	
	2.	Shellac wet with lime-sulphur	Air		25.6° to 25.4°	2⅔ hrs.	1.6 c. c.	6.0 c. c.	14.0—6=8.0 c. c.
	3.	Shellac wet with lime-sulphur	Air		25°	7-12 hrs.	0.1 c. c.	1.7 c. c.	14.0—1.7=12.3 c. c.
	4.	Shellac wet with lime-sulphur	Air		25.0° to 24.8°	50 hrs.	1.1 c. c.	0.22 c. c.	14—0.22=13.78 c. c.
	5.	No cover at end	Air		24.8° to 25.0°	1 hr.	1.7 c. c.	17.0 c. c.	

Before figuring the rates given in Table VII, all gas-volumes were reduced to 0° C, and 760 m. m. mercury pressure. Since all tests were not run for equal periods of time, it was necessary to figure results to some certain period—a ten-hour period was used. Results of experiments with gasoline-vapor are those obtained through estimating the actual amount of oxygen present at the beginning and end of each test. Where two or three determinations were made, the value given under any test number in the table is the value which came nearest the average. As has already been mentioned, however, all determinations (by both methods) led to the same general conclusion already stated.

In this connection, it occurred to the author that a good opportunity had presented itself for testing the effect of lime-sulphur, itself, upon the permeability of a wax membrane to oxygen. Accordingly two series of tests, results of which are given under Nos. 7 and 8 of Table VII, were carried out, using bees wax and shellac membranes. The membranes were formed by dipping thin India linen in melted beeswax, and in melted shellac, until membranes of the desired thickness were obtained. After the tests with a dry membrane had been carried out, the preparation dish (over which the membrane was fastened as a cover) was inverted and shaken until the lime-sulphur in the dish had thoroughly wet the surface of the membrane inside the dish. Before test No. 2 of Exp. 8 was tried, the preparation dish was only shaken. It was again thoroughly shaken, before test No. 3; and before the fourth test the dish was inverted—the lime-sulphur solution being left in contact with the shellac membrane, thus, for 25 minutes. In that time, the solution had "taken hold" and thoroughly wet the membrane. Note the decline in the rate of oxygen transfer. Both sets of tests showed that much less oxygen was absorbed (i. e. passed through the membrane) after the membrane-surface, inside the preparation dish, had been wet with the solution. That is, the lime-sulphur solution rendered the wax membranes decidedly less permeable to oxygen—less able to transmit oxygen. There seems to be no reason why lime-sulphur* would not have the same effect upon the wax covering of a scale insect, such as that of the *San Jose* scale.

A few experiments had been made a long while ago with living grasshoppers (*Melanoplus*) in air containing very strong fumes of osmic acid. These insects were darkened, and were rendered clumsy in their movements, but they lived for many hours in the fumes of the acid. It seemed a wonder that they could thus withstand the fumes—and that the fumes did not penetrate. In addition to the membrane-experiments just recited, therefore, one set of tests was carried out to determine the rate at which oxygen from air could pass through a lecithin-lard membrane before and after the membrane had been exposed to strong osmic acid fumes. Oxygen passed through the membrane at the rate of 1.3 c. c. per hour before treatment with the fumes. The membrane was treated (from one side) with the fumes for over three hours. It was then aired over night, and on the next day, in the three tests made, the darkened membrane did not transmit oxygen at a greater rate than 0.2 c. c. per hour—i. e. the rate was about six times slower than before the treatment. The lecithin-lard membrane was penetrated by the fumes but slowly.

*Some other scalecide properties of lime-sulphur are given in Tech. Bull. 11.

(The very poor penetrating power of osmic acid fumes, when used as a histological killing agent, are well known.) It may only be suggested here that perhaps the first effect of osmic acid fumes in blackening lipoids and fats in the experiment-membrane (or the surface of a living grasshopper, etc.) is to establish a black surface-membrane at once which is not only a little less permeable to oxygen, but also much less permeable to the osmic acid fumes, themselves.

THE EFFECT OF TEMPERATURE ON THE CAPACITY OF LIVING INSECT TISSUES FOR THE ABSORPTION OF CERTAIN GASES OR VAPORS.

In Table VI results are given of gasoline-vapor absorptions by 3-gram samples of egg-albumen, and also by the same amounts, respectively, of fresh unincubated egg-yolk and of fertile egg-yolk after four and five days of incubation. It was a surprise, in these tests, to find that gasoline vapor was about six times more soluble in egg-albumen than in the fresh egg-yolk, although the latter contained fats and fat-like material in the greater amounts by far. All other tests, so far carried out, had shown fats and lecithin to have greater capacity for the absorption of gasoline-vapor than egg-albumen. The exception, therefore, seemed so interesting and so possibly significant that it was decided to test the absorption capacity of the yolk for gasoline-vapor after certain periods of incubation. The yolk of infertile eggs, even after several days of incubation, was found to give the same results as yolk taken from fresh unincubated eggs. In the case of incubated fertile eggs the young, developing embryo was stirred up with the yolk before the 3-gram sample for the absorption test was taken. A comparison of such results of yolk-absorptions as are given in Nos. 2 and 3 with those given in Nos. 5 and 6 of Table VI seemed to show that the solubility, for gasoline-vapor, of fats and lipoids in the yolk changed greatly in the four and five days of incubation. The first few hours of incubation did not bring about a measurable change in the capacity of the yolk to dissolve gasoline-vapor. All the tests with yolk samples as given in the table were made at a room temperature of 25° to 26° C. Four tests with yolk samples of only a few hours incubation were made in a small incubation room at 103° F. These gave practically the same results, as to absorption capacity, as similar tests at the ordinary room temperature; if there was any increase in that capacity, it was not great enough to measure. As is well known, on about the fourth and fifth days of incubation the embryo of the hen's egg begins to show quite decided respiratory activity, and a period of rapid development begins. It was at this period of incubation that the decided increase in the capacity for absorbing gasoline vapor was shown by the embryo-yolk-mixture.* Here was an interesting change, (an increase) in the absorption capacity of the yolk-mixture for gasoline vapor, which seemed to be related with the changes that made for active development in the embryo. As long as the embryo remained comparatively dormant (or, was unable to develop through non-fertilization,

*Attention should be called to the fact that absorption results in 5 and 6 are much greater than the absorptions given by egg-albumen (i. e. egg-white). It would seem that the change in the embryo-yolk-mixture, which occasioned an increase in the gasoline-vapor absorptions, was probably a change in the fats and lipoids. The amount of alcohol-soluble material in the embryo is said to increase rapidly with development.

when incubated) the capacity for absorbing gasoline vapor remained comparatively low. These facts surely seemed significant in connection with the behavior of gasoline vapor, and the like, toward dormant insects.

It is a well known rule that dormant insects are harder to kill with fumigants (such as gasoline vapor, carbon disulphide, etc.), and that they are more resistant to sprays of kerosene emulsion and the miscible oils in general than are active insects of the same species. The question naturally arose in this connection, therefore, as to whether insects in the dormant state, from cold say, might not absorb less gasoline vapor under a given fumigation charge than they would absorb when in the active condition. In view of the close relation which the question bore to the main problem of how contact insecticides kill, experiments were planned to determine whether dormant insects would take up less of such a fumigant as gasoline vapor than would be taken up by the same insects after they had been brought into an active condition.

Hibernating Luna moth pupae were selected as the first insects to be used in these experiments, and gasoline vapor was the fumigant chosen. This fumigant was used again in this case for the reason, already mentioned several times, that it permitted of accurate percentage determinations of itself and of the oxygen and carbon dioxide present in the air in which it was used. The pupae had been kept in the cold room of the insectary all fall and winter until the experiments were first undertaken December 17, 1913. The apparatus represented in Figure III (except for the hooked U-tube, and the burette "f") was moved into the cold room six to eight hours before an experiment was to be begun, in order that everything might reach the temperature of that room. Among the accessories was a separate gas container with a stock supply of gasoline-vapor-air, and a little water to maintain saturation with water vapor. The dormant pupae were floated up on mercury in the gas container "c", Fig. III. The mercury in the container was raised until almost all air was expelled and the pupae were near the top. The outlet cock "t" of the container "c" was then connected with the stock-container of gasoline-vapor-air and the required amount of gasoline-vapor-air was quickly drawn in with the pupae. Within the next half minute, a sample of the gasoline-vapor-air with the pupae was drawn off above mercury into a separate gas-container, to be kept for percentage estimations. The mercury manometer, "m" Fig. III, was quickly leveled and the apparatus was then watched until the first rapid loss in volume had passed, and the manometer showed that no more gasoline vapor was being absorbed. Then, a second sample of the gasoline-vapor-air confined with the insects was drawn off for estimation in the same manner as the first. Both the samples (one taken at the beginning and one at the end of the test) were removed to the laboratory and allowed to stand until they reached the temperature of the room and of the apparatus (i. e. gas-pipettes and compensation burette;* (see Fig. 4, p. 27, Tech. Bull. 11, this station) used in making the percentage estimations. After that, the percentages of gasoline vapor, of carbon dioxide, and of oxygen present at the beginning and at the end of the absorption test with the pupae were determined. From these results, knowing the volume of gas present with

*By means of this burette, direct volume measurements at 0°C. and 760 m. m. mercury pressure, with proper water vapor-tension corrections, could be made.

the insects at the beginning, the amount of gasoline vapor present at the start, and the amount absorbed, could be figured.

At the end of the absorption test, the pupæ were carefully removed from the container and aired. They were then brought into the laboratory, where they might warm up and start active development. After their actions, taken in connection with respiration tests, showed that they were in an "active state", an absorption test with gasoline-vapor-air (using as nearly as possible the same percentage and the same amount of gasoline vapor) was carried out in the warm room by the same method as in the former case.

Two separate experiments with Luna moth pupae, entailing the complete sets of estimations just outlined, were carried out by this method. Results of these two experiments are given in Table VIII, Nos. 1 and 2.

TABLE VIII.—INSECTICIDE-VAPOR ABSORPTIONS OF COLD (DORMANT) AND WARM (ACTIVE) INSECTS.

Exp. No.	Insects.	Vapor used.	Temperature.	Volume of vapor absorbed.	Time.
1.	6 Pupae of Luna moth; 15 grms...	Gasoline vapor 2.6%.....	9.2° C.....	0.38 c. c.....	2 hrs.
	6 Pupae of Luna moth; 15 grms...	2.8%.....	24.6° C.....	1.13 c. c.....	2 hrs.
2.	9 Pupae of Luna moth; 20 grms...	Gasoline vapor 2.12%.....	7.2° C.....	0.35 c. c.....	1 hr.
	9 Pupae of Luna moth; 20 grms...	2.4%.....	24.8° C.....	0.89 c. c.....	1 hr.
3.	5 Passalus cornutus, 10 grms.....	Ether.....	0.6° C.....	1.2 c. c.....	10 min.
	5 Passalus cornutus, 10 grms.....	Ether.....	0.6° C.....	1.4 c. c.....	30 min.
	5 Passalus cornutus, 10 grms.....	Ether.....	22.2° C.....	6.0 c. c.....	10 min.
	5 Passalus cornutus, 10 grms.....	Ether.....	22.2° C.....	7.5 c. c.....	30 min.
4.	5 Passalus cornutus, 11.2 grms....	Ether.....	−1.0° C.....	2.40 c. c.....	10 min.
	5 Passalus cornutus, 11.2 grms....	Ether.....	−1.0° C.....	2.45 c. c.....	15 min.
	5 Passalus cornutus, 11.2 grms....	Ether.....	23.4° C.....	5.2 c. c.....	10 min.
	5 Passalus cornutus, 11.2 grms....	Ether.....	23.4° C.....	5.4 c. c.....	15 min.
5.	5 Passalus cornutus, 10.5 grms....	Ether.....	−2.0° C.....	0.7 c. c.....	10 min.
	5 Passalus cornutus, 10.5 grms....	Ether.....	−2.0° C.....	0.8 c. c.....	20 min.
	5 Passalus cornutus, 10.5 grms....	Ether.....	24.0° C.....	5.1 c. c.....	10 min.
	5 Passalus cornutus, 10.5 grms....	Ether.....	24.0° C.....	6.0 c. c.....	20 min.
6.	5 Passalus cornutus, 10 grms.....	Ether.....	−2.4° C.....	0.4 c. c.....	10 min.
	5 Passalus cornutus, 10 grms.....	Ether.....	−2.4° C.....	0.5 c. c.....	20 min.
	5 Passalus cornutus, 10 grms.....	Ether.....	About 20.0° C.	1.2 c. c.....	10 min.
	5 Passalus cornutus, 10 grms.....	Ether.....	About 20.0° C.	1.5 c. c.....	20 min.

At the beginning of Exp. No. 1, in the cold room, there were 2.08 c. c. of gasoline vapor present in the air with the pupæ. The table shows that 0.38 c. c. of the vapor was absorbed—that is, only 0.18 of all the gasoline vapor present at the start was absorbed by the six pupae. The latter were all alive when brought into the warm room. They were kept in moist air and their activity increased. By the third day, the respiration had increased to more than three times its rate in the cold room. On the sixth day, the rate was about the same or slightly less. The pupae were kept in the warm room 17 days, in all, before the last absorption test was made. Since, by the method used, not all the air was expelled from the container before gasoline-air was drawn in with the pupae above the mercury, it was impossible to make sure that the percentage of gasoline vapor used in the warm room was *just* the same as was used in the cold room test. As may be seen by the table, however, it was possible to make the percentages *almost* or *practically* the same. In the warm room test of Exp. 1, there were 2.24 c. c. of gasoline vapor present in the air used, and 0.5+ of all this was absorbed by the same

pupae which in the dormant stage had absorbed only 0.18 + of the 2.08 c. c. of gasoline vapor placed with the air used in the cold room test.

When the nine pupae, used in Exp. 2, were removed from the gasoline-vapor-air at the end of the test in the cold room, they had become quite sensitive to touch. They would wriggle actively when touched. The gasoline vapor seemed to have stimulated them. They were removed to the warm room and kept there about 15 days before the second absorption test was run. In this experiment 1.96 c. c. of gasoline vapor was present in the air at the beginning of the warm room test, and 0.45+ of this vapor was absorbed by the pupae as against only 0.2 + of the 1.7 c. c. of vapor present in the air during the cold room test. About 80 c. c. of gasoline-vapor-air was present at the end of each test. The ratio of the *absorbed* gasoline vapor to the whole amount present was, in both experiments, greatest in the warm room tests where the insects were undergoing active development. All the pupae recovered at the end of the tests, and the first moths began to emerge about five days after the last tests in each experiment. Adults emerged from all the pupae, although none were very perfect moths. After the above experiments, it was decided to use adult *Passalus cornutus* in absorption-tests made, first, when the beetles were quiet and dormant from cold, and again when they had become active in the warm room. These beetles were chosen because, as has been explained in Bulletin 11, all of the air could be safely forced out from around these insects by mercury. Thus, it was possible to introduce samples of the *same* stock of insecticide-vapor-air into the container, unmixed with other air, in the cold room and in the warm room. Air that was almost, but not quite, saturated with the insecticide vapor at the cold room temperature was kept in a gas container above mercury as the stock-supply. One could, in this way, be sure that the percentage of insecticide vapor present, in both tests, was exactly the same. With this assurance that the same percentage of the insecticide vapor could be placed with the insects in the two different tests, it was not necessary to use a vapor which would permit of accurate quantitative estimations of the percentage present at the beginning and end of each test. Thus, different apparatus and a different method might be employed. The method of measuring the total rapid decrease in volume could be depended upon, it appeared, to show the volume of insecticide vapor absorbed by the insects. Experience had shown that most of the vapor-absorption would take place in an interval too short for respiration to make an appreciable change in volume. Moreover, in the short interval necessary, barometer and therometer readings remained practically the same. The apparatus used for making the gas measurements was the compensation-burette just referred to (Shown in Fig. 4, Tech. Bull. 11, and a gas-container of the type shown at A., Fig. 7, Tech. Bull. 11 of this station.

A certain measure of the insecticide vapor to be used was transferred from the stock supply into the compensation burette. The beetles were floated, dorsal side up, on mercury in a gas-container, and the mercury was raised until all air was expelled from around them. The container was at once connected with the compensation gas-burette, and the accurately measured sample of insecticide vapor was quickly drawn over

with the beetles. After 10 minutes the whole remaining volume was returned to the compensation-burette and again accurately measured. The first reading minus the second reading of the compensation burette showed the amount of vapor that had been absorbed by the insects. The gas could thus be transferred back and forth at intervals to obtain absorption values at 10, 20 and 30 minute intervals, if desired. At the end of this test in the cold room, the beetles were removed to fresh air; and after an hour or two were brought into the warm room along with the apparatus and the stock supply of insecticide vapor. After 18 to 41 hours, when the beetles had become active and everything to be used had reached the room temperature, absorption-tests were again carried out with the same insects—using a sample of the same insecticide vapor under the new conditions. Now, gasoline did not produce a very high percentage of vapor at the cold room temperature; and a fluid which would give a higher percentage of vapor at the low temperature seemed desirable. On that account, ether was selected for the new trials instead of gasoline.

Four experiments were carried out in the manner just outlined, using *P. cornutus*, and the vapor of ether in air. The results of all four of the experiments are given in Table VIII. In no case, as may be seen, were the absorptions less than two times greater in the warm room, where the beetles were active, than when the latter were dormant in the cold room. In experiments 3, 4 and 5, in the cold room, the small amount of absorbed ether-vapor stimulated the dormant insects until they twitched the antennae and moved the legs. On the other hand, in the warm room tests of Exp. 3, the active beetles had become entirely motionless (anaesthetized) in 10 minutes; at the end of Exp. 4, only a bare movement of the antennae could be noticed, still; and in the warm room test of Exp. 5, the active beetles were completely anaesthetized by the end of 20 minutes. In the case of Exp. 6, the beetles were entirely stiff and helpless at minus 2.4° C., and they remained so even under the influence of the small amount of ether absorbed. When these insects were removed from the first test, they showed no movement whatever; and they recovered from the cold, only slowly, after being brought into the warm room. They had been kept in the cold (at minus 2.4° C.) only a few hours in all. A different stock supply of ether-vapor-air was used in Exp. 6, and the five beetles absorbed only 1.5 c. c. of the vapor even in the warm room in 20 minutes. This was barely enough of the ether to place the insects in the excitement state—they were more active at the end than at the beginning of the last test.

In order to show how nearly constant a certain volume of pure air remained during a period of 30 minutes with five beetles, a single check may be related. The five beetles used weighed 10 grams. The 50 c. c. of pure air was transferred from the compensation burette to the container with the beetles and back again three times during the check, with the following results:

50.0 c. c. at the beginning;
50.2 c. c. at end of 10 min. (after first transfer);
50.0 c. c. at end of 20 min. (after second transfer);
49.9 c. c. at end of 30 min. (after third transfer).

The six experiments (Table VIII) were carried out with such care,

and were checked in such a manner, it is believed, as to make the apparent conclusion seem undoubted. In the dormant condition, the insects absorbed less gasoline vapor and less of the vapor of ether than did the same insects when active. The difference in solubility of the insecticide vapors in the dormant and in the active insects, was so great that ordinary charges of the insecticides used did not bring the concentration of the insecticide, within the body tissue of the dormant insects, to the danger point. Thus, the lowered absorption capacity, which accompanied the dormant condition, may easily furnish the chief explanation of the fact that insects, dormant from cold, are harder to kill by ordinary fumigants and by ordinary miscible oil, contact sprays.

Evidence has been given that the tissue fats and lipoids of living insects should be considered as the principal solvents of such insecticides as gasoline and kerosene vapors, ether and the like. The suggestion made in connection with egg-yolk absorptions, therefore, seems proper here. Namely, the changes, which accompanied respectively an active or a dormant state, occasioning an increase or decrease in the capacity for absorption of insecticide vapors, was probably a change in the condition of the tissue fats and lipoids—or, in their relation to the other cell constituents..

IN REGARD TO THE MANNER IN WHICH BORAX AND SOME OTHER POWDERS (SOLID CONTACT INSECTICIDES) BECOME EFFECTIVE AGAINST CERTAIN INSECTS.

Mention has already been made (Technical Bulletin 11) of certain dry powdered solids that may be applied as contact insecticides. In the case of pyrethrum powder, attention was called to the fact that, as applied in most cases, the principal influence of this insecticide is due to the volatile bodies held in the powder. This is very clear when the insect is confined in a warm air-space with some of the powder. However, certain insects (for example diptera and grasshoppers) may sometimes be overcome—for a time, at least—by dusting their bodies with the powder, even in the open air. When such insects are examined, one may often observe with a binocular microscope that some of the powder becomes lodged in the dry vestibule of the tracheæ within the spiracles, and no doubt the volatile bodies from the powder charge the air in the tracheal system. The poison in such cases, therefore, really becomes absorbed as a gas or vapor. Nevertheless, it is possible in the case of some insects for certain dry non-volatile powders to become effective as contact insecticides. White hellebore (although it contains a slightly volatile body) does not kill insects confined in the air with it at ordinary temperatures, provided they do not come in contact with the poison—or do not eat it. It is well known that the fresh hellebore powder is a stomach poison to insects; it may be observed to be effective as a contact insecticide also, with moist rose slugs. I have watched many young rose slugs that did not *attempt* to eat after their bodies had been powdered with fresh hellebore. If they attempted to crawl, the body seemed to be clumsy as if the hypodermal sense organs had been numbed; and usually, the slugs would fall from the leaf. Slugs, in this condition, were kept for varying lengths of time (up to nearly two days) until they died.

It was clear that the body secretions had dissolved some of the hellebore, and had moistened more of it so that it stuck as a white crust on the body. The indications were that but very little of the hellebore, dissolved in the body secretions, had been absorbed through the cuticular covering—only enough to render the outer surface of the body numb and clumsy. In the final stages, the insect seemed to be dying, more from drying and from starvation than anything else—yet it was the hellebore which first rendered the animal in an unfit condition for normal life-processes.

Borax powder and finely powdered sodium fluoride have been used as effective agents against cockroaches. It was known to be not at all necessary to place these powders on a bait that should be eaten by the roaches; the dry powders became effective if they were dusted in locations where the insects were sure to run. There seemed to be nothing appetizing about the powders, in themselves, and it was a question *how* they became effective against roaches. The large American cockroach (*Periplaneta Americana*) and the smaller croton bug were available for study in this connection.

In the first tests, a little dry powder of borax, or of sodium fluoride was sprinkled on one-half the bottom of wide, flat, glass-covered culture dishes. Then single roaches were introduced and watched. It was soon noticed that when a roach ran through either the borax or the sodium fluoride, a little of the dry powder stuck to the lower part of the body and to the legs—especially at the bases of the legs, and between the sternal plates. Also, the insect was almost sure to get its antennae dusted with the powder as it waved them about. If left to itself, a roach that had become powdered in the manner just described would soon settle down and begin "cleaning up." In this latter process, the long antennae were drawn across the mouth and licked clean—the feet and legs and body were licked. After a time, a roach that had been cleaning itself of either of the powders named would begin to show uneasiness and irritability. It was apt to become sick, after a time, and exude a drop of saliva from the mouth. Finally, the insect would become dumpy, often remaining quiet for long intervals, only to start up suddenly and nervously, and then lapse again. Such actions were usually followed by partial paralysis of the hind and middle legs. The wings and feet twitched at intervals, and gradually the body became more helpless until death resulted. This might all take place in anywhere from four to forty-eight hours; and the length of time before death would result seemed to depend in great part upon the amount of powder the roach had cleaned from his body. As a rule, the sodium fluoride acted very much more rapidly than the borax—requiring only four to twelve hours. Sometimes a roach that had taken only a little borax, in cleaning the powder from its body, would pass through the first stages of uneasiness (and apparent stomach-sickness) and then recover so far as to eat, and act normal for four or five days, perhaps—after which it would become dumpy. It would then eat no more. The wings would quiver when the insect was disturbed; paralysis of the legs would finally set in, and gradually the body would become more helpless in every case until death resulted.

Observations were made, and record was kept (from treatment to

death) of the actions of 45 of the insects treated with borax, sodium fluoride, or borax and pyrethrum mixed. A few tests were made by compulsory feeding of the roaches with a very little borax or sodium fluoride from the point of a small knife-blade. In every case the symptoms were much like those described above and the insects finally died. These last tests made it clear, therefore, that both borax and sodium fluoride could act as stomach poisons in the case of the cockroach. Moreover, it seemed very likely that in cleaning either of the powdered poisons from antennae, feet and body, the insect would eat enough of the poison to kill. Finally, visible proof was obtained that powder, licked from various parts of the body, was swallowed. Sodium carmine was very thoroughly dried and pulverized with borax, and with sodium fluoride. Insects were placed with this stained powder, and several hours after they had cleaned it from their bodies, dissections were made; the red stain was found in the contents of the crop.

The question still remained, however, as to whether a little of the powdered insecticides might not be dissolved in the moist exudations at the bases of the legs and between the sternal plates, and then absorbed through the thinner body integument in the locations named. It seemed possible also that the very fine powder might enter the spiracles. That is, it was still a question as to whether the powders under consideration might not be contact insecticides as well as stomach poisons when used against roaches. In order to get at the solution of this question, it was found to be absolutely necessary to devise some way of preventing the insects from licking their bodies after they had become dusted with the powder—without, at the same time, inflicting any serious mechanical injury in the use of the preventative device. The desired result was accomplished by means of card board collars, which were prepared and used in the following manner:

The collars were made from single pieces of flat bristle board of about the weight of ordinary medium index cards. Each collar was four to four and one-half inches in diameter—i. e. large enough to form a cover over a shallow glass stender dish. A slit was cut in the circular card from the circumference to the center, where a small smooth hole was punched. This central opening was large enough to turn freely on the neck of a cockroach, but it was very much smaller than the animal's head. After the collar had been very carefully placed around the neck of a roach, a thin strip of paper was afterward pasted over the slit. The flat collar could then be placed over the stender dish, containing a little powder to be tested, so that the insect's body rested upon the bottom of the dish, and the head stuck up above the collar outside the dish. The fluttering wings and scrambling legs would thoroughly dust the animal's body without any of the powder's reaching the head or mouth. Fig. No. 4 of Plate I shows the position of the head of a cockroach wearing such a collar. In check tests, untreated roaches were made to wear collars for eight, eighteen, and even ninety-eight hours without any serious injury. After these specimens were released, they were kept under observation for ten to nineteen days, and during that time fed and behaved normally. In all these experiments the roaches were permitted to have moist food when no insecticide powder was present and they were not wearing a collar.

In order to be able to see just where the powder went, when it came in contact with the body of an insect wearing a collar, both borax and sodium fluoride were stained with either sodium carmine, or indigo carmine, after which they were thoroughly dried and pulverized to a powder. In a few cases, where dissections were to be made under water, Sudan III was used as the stain for the insecticide powder. Individual experiments were carried out with more than forty insects according to the outline just given, and all these were in agreement as to the question considered. It was found that powdered borax and powdered sodium fluoride could bring about the death of roaches through being dusted thoroughly on their bodies, without ever having come in contact with the mouth. The dusting of the body was done through the efforts of the insect itself, in scrambling and fluttering with the legs and wings in the manner already explained. Borax required two to ten days to cause death through contact of the powder alone. Sodium fluoride required from five to twenty-two hours. In each case the average time was much longer than was required to bring about death when the insect had eaten some of the powder in cleaning the same from its body. The symptoms following contact alone were usually very similar to those which followed when the poison had been eaten. As a rule, the period of partial paralysis and helplessness was much more prolonged when the powder had been used only as a contact insecticide. The exudation which caused the powder to stick to the roach's body was nearly always sufficient to dissolve enough of the borax or sodium fluoride to cause incrustation at the bases of the legs, between the sternal plates, etc.

In seventeen of the experiments in which the stained insecticide powder (sodium carmine was the stain mostly used) had been employed, the insects were dropped into a hot alcoholic fixing fluid at about the time of death. Afterward, parts of the bodies of these insects were embedded, and sectioned. A study was made of the miscroscopic sections prepared in this way, in order to learn by means of the stain if possible, whether the powder had entered the spiracles—or had passed, after solution, into the insect's body through the thinner portions of the body wall. The sections showed the incrustations of the stain at the bases of the legs, between the sternal plates, and (sometimes) just within the spiracles in the vestibule from which the tracheae arise; but no stain was found further within the tracheæ, and no sections were found which showed undoubted evidence that the stain had penetrated the integument to stain the hypodermal cells. If the cuticula was permeable to the stain and to the borax or sodium fluoride solutions in the same degree, then it is certain that only a very limited amount of the insecticide passed into the animal's body through the thinner portions of the body integument. The latter, however, seems to have been the possible and probable path. No indication was found that the stained insecticide powder had entered the bodies of cockroaches wearing the collars, in any other way. It must be remembered that the sodium fluoride rendered roaches helpless and practically dead in some cases within five hours—i. e., during the time the insects were wearing the collars, and sections showed no trace of stain in the crops of these insects. It took the borax so long to kill by contact that in the first tests, the roaches were brushed as clean as possible after six to eight hours treatment, and were then re-

leased from the collars. Proof was soon obtained that this would not do; the insects licked the joints of their legs and other rough parts of the body so thoroughly that the contents of the crop became decidedly stained. In other words, the insects did their cleaning so carefully that they might be eating enough of the borax powder to kill. The only safe method, therefore, was to leave the collars on the animals until they were no longer *able* to lick various parts of the body. That the death of an insect so treated was due primarily to the influence of borax powder brought in *contact* with the body, and not to injury from the collar (or otherwise) was shown by the checks carried out with untreated insects in collars, and by the absence of any stain in the digestive tract.

The evidence gained from the experiments carried out in this connection, therefore, may be summed up in the following statements:

When cockroaches run through powdered borax, sodium fluoride, or borax and pyrethrum mixed, the dry powder sticks to the legs, body, and often to the antennae. Some of the powder is moistened or dissolved in the exudation about the bases of the legs and on the thinner portions of the outer integument. This seems to cause some irritation and uneasiness; the insect soon begins to clean the moistened powder from the body by licking it. In doing this, enough of the poison may be brought into the mouth, and swallowed, to kill after a period varying from five hours to ten days. Borax acts much more slowly but kills with no less certainty than sodium fluoride. It is possible for both powdered borax and sodium fluoride to kill roaches by contact (without any of the poison's having been swallowed), but in actual practice it is hardly possible that any roach ever gets its body dusted with one of these powders without also licking and swallowing some of the powdered poison. Both powders are more rapid in their action as stomach poisons than they are as contact insecticides.

Tables IV and V contain representative results illustrating the influence of borax, sodium fluoride, pyrethrum and white hellebore on the reductases and catalases, respectively, of insect extract. Borax acted quite injuriously on the activity of the reductase, and very little on that of the catalase. The same was true of the hellebore and pyrethrum, except that the pyrethrum seemed to interfere a little more with the activity of the catalase than did either the borax or the hellebore. Sodium fluoride was most detrimental to the catalase. When added to perfectly fresh extract, the sodium fluoride caused a slight drop in reductase activity; after that however, such a treated extract retained its reductase activity very much better than did untreated extract. Both sodium fluoride and borax interfered with the oxidase activity slightly, if one may regard the alcoholic guaiac test as an indicator in this case.

An attempt was made to determine the influence of borax and sodium fluoride upon the respiratory ratio of cockroaches dying under the influence of these poisons, but the normal respiratory ratio, itself, varied so much for the roaches that no decided information could be gained. The intestinal tracts of the specimens used were apt to be gaseous, and the contents of the intestines were usually found to be well infested with infusoria, etc. This, it was believed, might account for the normally irregular respiratory ratio.

SUGGESTIONS FOR POSSIBLE PRACTICE.

(a) SOLID AND LIQUID APPLICATIONS.

It will be observed that when the insecticides gained entrance into the insect tissues as purely contact agents, they did so, ultimately, in the fluid condition. Powdered solid contact insecticides gave off volatile bodies to be absorbed by the insect, or else the powdered agent became moistened and partly dissolved in exudations on the insect integument. Experiments showed that gases or volatile insecticides gained entrance to the insect tissues most rapidly. The "miscible oils" usually combined volatile and purely liquid agents. The liquid or the finely powdered solid often gained entrance through the spiracles to the larger tracheae where any volatile bodies that might be carried by the insecticides could then charge the air of the tracheal system. In the case of the powdered solid contact insecticide, the advantage evidentally lay in having the powder so fine and dry that it would adhere well and could sift readily into all crevices—even into the stigmata of the insect. Similarly, a weak surface tension for a liquid insecticide enabled it to flow into all irregularities of the area treated, and to penetrate often into the larger tracheae of the insect. Even in the case of lime-sulphur wash—which kills scales, perhaps in most cases, without ever having actually come in contact with the body of the insect itself—it would seem to be advantageous if the wash could be given a lower surface tension. The solution could then flow into crevices of the bark and thoroughly wet the scale-covering of insects that might otherwise escape. As is well known, soap cannot be used for the purpose, since it causes in the lime-sulphur solution a greasy lime-soap precipitate. A number of tests were carried out with the idea of finding agents which might be added to lime-sulphur, without injury, to lower the surface tension and cause it to spread on the bark of plants more readily. In order to be able to compare the "spread" of different samples of lime-sulphur dilutions readily in the laboratory, resort was had to what may be termed the "drop method." For example, the number of drops in 5 c. c. of water were counted as they came from a pipette. The same pipette was then washed out with lime-sulphur prepared by diluting a concentrate with a water solution of the agent to be tested. The number of drops in 5 c. c. of the latter lime-sulphur dilution were then counted from the pipette, and the ratio of the number of drops of water to the number of drops of lime-sulphur dilution was obtained. As long as the temperature of the liquids was practically the same and the drops were counted from the same cleaned pipette, the ratio between the number of drops in equal volumes of water and *any* lime-sulphur dilutions of approximately equal densities afforded a direct means for comparison of spread in the various dilutions. Actual trial on a tree covered with scale insects, showed that when this ratio equalled about 0.85*, the "Agent plus L. S. solution" had sufficient spread; that is, the spray readily flowed into crevices of the bark and wet the waxen scale coverings. The method was adapted from

* (e. g. $\frac{\text{Number drops in 5 c. c. of water}}{\text{Number drops in 5 c. c. of "Saponin-water-dilution of Conc. L. S."}} = 0.85$.)

Mansier† who first suggested counting the drops in equal volumes of liquids under proper conditions of temperature, etc., for making a study of their surface tension in connection with insecticides. It was found that gelatine, glue, soluble albumen, and saponin could all be used in lime-surphur to increase the "spread" on the bark of a tree. The various agents were dissolved in the proper amount of warm water, after which the solution was added to concentrated lime-sulphur in making up the proper dilution. Glue and gelatine had to be used at the rate of about one pound to eleven gallons of lime-sulphur wash as diluted ready for use. The expense, therefore, would be too great to make their employment practical. This was true also in the case of albumen and saponin. Except for its expense, however, saponin seemed to be best of all the agents tried for the purpose. It dissolved readily, and a comparatively small amount added to the lime-sulphur increased the "spread" to the desired point without any apparent injury to the oxygen-absorbing power of the wash. Tests were made of this last point in the following manner:

A measured amount of the solution of saponin in water was added to a measured amount of concentrated lime-sulphur in a stender dish of standard volume and diameter; then, an equal sample of the same concentrated lime-sulphur, in a dish of the same diameter, was diluted with a volume of distilled water equal to the saponin solution used. The two dishes were introduced into exactly similar containers with equal volumes of air having the same known percentage of oxygen. At the end of a certain period of time (6 hours, for example) the oxygen percentage in each air-chamber was again determined, and from that, the volume of oxygen that had been absorbed. The volumes of oxygen absorbed in the saponin-lime-sulphur and in the check were practically the same—the greatest difference in three successive tests being no greater than the possible experimental error of the apparatus. When the dry powder was stirred directly into the lime-sulphur, however, the oxygen-absorbing ability of the solution was quite appreciably weakened. This was due no doubt to oxidation of the sulphides by air carried in with the dry powder. It was best, therefore, to dissolve the saponin in water, and then add that solution to the concentrated lime-sulphur.

Four tests were carried out to determine the influence of adding saponin, upon the rate at which water would evaporate from lime-sulphur (i. e. influence upon rate of drying). In every case, the evaporation was found to be a little more rapid from the lime-sulphur to which saponin had been added—as might be expected from the weakened surface tension. The difference, however, was not great—amounting to only 0.1 grm. of water from an area of 10.17 cm. in seven hours, on the average. This difference could be largely eliminated by adding salt (Na Cl) to the saponin-lime-sulphur solution. That is, salt had the effect of retarding the evaporation of water. It was used at about the rate first recommended in the original lime-sulphur salt-wash (i. e. about 18 lbs. to 100 gallons). Except in extremely dry weather or in a normally dry climate the salt would not serve a useful purpose.

Finally, all the tests tried seemed to confirm the first impression that saponin would be the ideal agent for lowering the surface tension of

†Mansier, M., 15 Jan. 1908, Bulletin de la Société des Agr. de France.

lime-sulphur to obtain "spread," if only it were not so expensive. At the price of crude saponin, when these tests were carried out, it would add about $1.50 to the expense of each fifty-five gallons of lime-sulphur wash diluted ready for use against San Jose scale. The crude saponin, however, was just as good for the purpose as the *pure* obtained from Merck at a cost of more than five times the price of the crude product. Now the dry plant, *Saponaria officinalis* or *"bouncing bet"* was known to contain from thirty to forty per cent of extractives and it occurred to the author that perhaps the plant, itself, could be used in this connection. *Bouncing bet* grows about many farm yards, orchards, or gardens, coming up year after year from underground stolons. It is quite hardy, escaping cultivation and growing by roadsides, often, quite thickly. If the *plant* could be used, therefore, the orchardist might have a plot planted to *bouncing bet,* and prepare his lime-sulphur-saponin wash with only the extra expense of cutting, storing and extracting—which need not be great, it was thought. Accordingly, some plants were cut and dried in the early fall and laboratory experiments were carried out to determine whether "bouncing bet hay" could be used to satisfactorily increase the spread of lime-sulphur wash. Stated briefly, results proved that an excellent lime-sulphur spray mixture, as regards spread, etc., could be obtained by the use of an extract of either the green or the dry stems and leaves of *bouncing bet.* A good method of extraction was as follows:—the plant material was soaked in lime-water over night (12 to 18 hours, say) after which it was boiled about 30 minutes and then pressed out and strained. Thus an extract was prepared by using the dry stems and leaves of *bouncing bet* at the rate of 22lbs. of the plant material and 15lbs.* of freshly slacked lime to 50 gallons of water. This extract could be used to dilute concentrated lime-sulphur (of 26° Baume' and up) the required amount for use against San Jose scale; giving the spray mixture a very satisfactory spread. The added lime in the extract should prove useful, also, in assisting the lime-sulphur to soften the wax about the margins of the scale-coverings—in addition to the fact that it serves as a first class marker to show whether all parts of a tree have been covered.

When extract water from *bouncing bet,* prepared as explained above, was used in the place of ordinary water in the old formula (lump lime 20 lbs., sulphur flour 15 lbs., water 50 gallons) a homemade wash having a very high degree of spread resulted. A little of such a homemade wash was filtered, and the number of drops in 5 c. c. of water divided by the number of drops in 5 c. c. of the filtrate equalled 0.72. One homemade lot of lime-sulphur was prepared (in a smaller amount than is called for by the formula) while the plant material was still in the water. The formula follows: lime 20 lbs., sulphur 15 lbs., water 50 gallons, and 22 lbs. of the dry leaves and stems of *bouncing bet* to which enough water to wet them thoroughly had first been added. The mixture was kept boiling for forty-five minutes, after which the liquid was pressed out, and strained through coarse cheese cloth. The lime-sulphur appeared to have cooked up in good shape and the "drop ratio" for the spray solution was 0.73.

Whether these suggestions would actually prove profitable in orchard

*Less lime may be used. It is not absolutely necessary but it assists in softening and digesting the plant and in other ways.

practice may be learned only by experience. If they should prove to be so, it is likely that commercial companies might produce a crude extract of *bouncing bet*—incorporating it in lime-sulphur concentrate, cheaply. A single test extending over several months indicated that saponin, in itself, did not cause lime-sulphur to deteriorate.

b. FUMIGANTS.

The advantage of the fumigant—i. e. vapor or gaseous—contact insecticides are generally recognized. Likewise, disadvantages are equally well known. Generally speaking, gases diffuse rapidly and often penetrate where non-volatile liquids could never penetrate without damage—as in stored grain, clothing, etc. Also many gases or vapors are more rapidly absorbed by active insects than are any non-volatile liquid insecticides. A disadvantage, such as difficulty of confining a gas for the necessary period of time, may be overcome; but danger from fire and explosion in the case of carbon disulphide, and difficulty of application because of its danger to the operator in the case of hydrocyanic acid gas are disadvantages never entirely eliminated. It was in the hope of finding fumigants which might be safely substituted, in some cases, for the two fumigant insecticides just named that test work was carried on with ammonia and carbon tetra-chloride.

Ammonia. Experience gained with ammonia as an insecticide, during the time its influence upon the respiration of insects was being studied, led to the belief that perhaps ammonia gas from compressed or liquified ammonia might be applied as an insect fumigant with greater ease and with less danger than hydrocyanic acid gas. Experiments carried out with *Calandra granaria, Silvanus surinamensis,* and larvæ of *Tenebroides mauritanicus* enclosed with a little dry wheat in tight glass receptacles showed that the insects could *all* be killed with three to four per cent ammonia in twenty to twenty-two hours, and with nine to ten per cent in six to seven hours. In fact, it was possible to kill in most cases at lower percentages, and in shorter periods when using the higher percentages of ammonia—but death was always certain when the gas was used at the rates given.

A few germination tests were made with dry wheat treated with different percentages of ammonia, 100 grains being used in each check and in each treated sample. It was found that where the percentage of ammonia was kept up to about 3.2 for 22 hours the germination was ruined. At 10 to 13.5 per cent for an interval of eight hours, however, the per cent of injury from ammonia to germination was less than one; for ten hours at 11.9 per cent the injury was over fifty per cent.

Following the various laboratory tests, two fumigations of the college mill-room were carried out with ammonia gas derived from cylinders of compressed and partly liquefied ammonia. The room was closed as tightly as any plastered room with closely fitting windows may be closed. The iron tube of ammonia was placed on a platform balance outside, and the ammonia gas was conducted through a tube (properly fitted under the door) almost to the center of the room. Two wire cages of insects were left in the open where they could be seen from the outside through the glass of the mill-room door. Two other cages were buried three and six inches, respectively, in dry wheat. There were

about fifteen bushels of sacked wheat, and a little corn in the ear, stored in the room. In the first test (Oct. 7, 1911) ten pounds of ammonia were passed through the tube. Since the air-space where the gas expanded was 7166 cubic feet, the percentage of ammonia was about 3.1 in the early part of the test. The experiment ran between nineteen and twenty hours. A caged specimen of the large American cockroach was still alive at the end of the test, but he was so under the influence of ammonia that he died from the effects later. *S. surinamensis* and *C. granaria* in one of the exposed cages were motionless. Some of the *Calandra* recovered in fresh air enough to move their legs, but none ever recovered entirely. All the beetles in the two cages buried in the wheat were active at the end of the test and were still alive and normal three days later. The next day living specimens of the saw-tooth grain beetle were found between the grains of the corn in the ear.

On November 21, 1911, the mill was again fumigated with ammonia in the same manner as in the first test; but this time 32.5 lbs. of the compressed ammonia were used—i. e., one pound of ammonia gas to 223.9 cubic feet of air space. This gave about ten per cent of ammonia in the room. One hour and fifteen minutes were used in introducing the charge, and the room was kept closed (over night) for seventeen hours. Wire-caged insects had been introduced again, as well as several large sacks of infested wheat. About twenty bushels of new corn that was not very dry was present also. The latter became very decidedly darkened during the test. Wheat was always more or less darkened by ammonia fumigation in the laboratory tests—as well as in the mill test—but the dryer the grain, the less noticeable was the darkening in every case. In this test, a caged cockroach (*P. americana*) became quiet by the time the charge had been two-thirds introduced. The roach was dead at the end of the test, as were also the caged beetles left in the open. Mice were killed. One mouse was seen to come out on the floor and die when fumigation had been going for forty-five minutes. Insects about one inch deep in the wheat were killed, but caged beetles (saw-tooth grain beetle and grain weevil) buried in the center of a sack of wheat were active at the end of the test. In both the mill tests brass and copper parts of the mill machinery were covered with vaseline, which protected them perfectly from action by the ammonia. The compressed ammonia was certainly convenient to apply; and at three to four per cent, fumigation could be carried on with the gas at a reasonable cost, since iron cylinders of liquefied ammonia could be obtained at that time for twenty-nine cents per pound. Considerable of the ammonia was absorbed by the grain; but after a few hours airing, the odor disappeared. The darkening of the grain seemed to be an undesirable feature, however. Mr. Spragg of the Farm Crops Department prepared flour from three kinds of wheat treated in the first mill experiment—and also, from the three corresponding check samples of wheat. There was no taste of ammonia on any of the flour, but that from two of the treated samples of wheat was quite noticeably darker than the corresponding checks. A Lansing miller, who was kind enough to slick the samples of flour and judge them, decided that the action of the ammonia on the wheat had undoubtedly injured its milling qualities commercially. To be sure, the grain was really darkened, noticeably, to a depth of only two or three inches

in a large container; but at that depth, the insects were not killed. For the reason just stated the mill experiments with ammonia were discouraging—although it appeared that the gas, in some instances, might prove to be a desirable fumigant for insects.

Carbon Tetrachloride. Early in the year 1910, Professor Pettit was urged in behalf of the Michigan Millers to find, if possible, some volatile non-inflamable liquid which might take the place of carbon disulphide as a fumigant for grain in bins. It was suggested to him by Professor Kedzie of the chemistry department that perhaps carbon tetrachloride might have the qualities desired. Following this suggestion, a series of comparative tests with carbon disulphide and carbon tetrachloride were carried out by Professor Pettit, Mr. M. A. Yothers (who was connected with the department at that time), and the writer, working together. The tests were made in large glass flasks with rubber stoppers. Among the insects in the infested grain used in these tests were the following beetles determined by Prof. F. H. Chittenden of the U. S. Bureau of Entomology:

Rhizopertha dominica,
Latheticus oryzæ.
Laemophlaeus pusillus,
Calandra oryzæ,
Tribolium ferrugineum.

A great many *Silvanus surinamensis* were also used. In these tests carried out in the tight glass flasks, practically all beetles were killed by carbon disulphide used at the rate of ¼ dram to a cubic foot of air space. It took 1½ drams of the tetrachloride of carbon to accomplish the same result (at about 70° F.). That is, compared with carbon disulphide, six times as much carbon tetrachloride was required to accomplish effective fumigation. The tests were run from seventeen to twenty-four hours. It should be stated that the liquid insecticide in every case was dropped upon a thin piece of absorbent cotton, which was suspended at the center of the flask. Thus, the broad surface of the cotton enabled the fluid to evaporate rapidly. The liquid carbon tetrachloride was found to be entirely non-inflammable—and its vapor, non-explosive.

Results of the experiments just described were not published at the time—mainly because the expense for effective fumigation with carbon tetrachloride seemed so great as to be almost prohibitive. Dr. Britton of the Connecticut Experiment Station had given, in 1908, his experiments with carbon tetrachloride used as a fumigant against scale insects on nursery trees.*

Later, Feb., 1910, Mr. Albert P. Morse published a note on "Carbon Tetrachloride vs. Carbon Bisulphid" as a fumigant used in the control of grain and natural history pests.† Mr. Morse stated that "for large cases a strength of one quart to fifty cubic feet is desirable—practically twice that of carbon bisulphid, of which a pint to fifty cubic feet is sufficent."

In October, 1911, Professor F. H. Chittenden and Mr. C. H. Popenoe of the U. S. Department of Agriculture published a paper on experiments carried out with the same chemical used as a substitute for car-

*Journal of Economic Entomology, Vol. 1, No. 2, p. 111.
†Journal of Economic Entomology Vol. 3, No. 1, p. 104.

bon bisulphid in fumigation against insects.* The authors found that "if the material were a cheap product, it might prove a substitute for carbon bisulphid if used in strength greater than ten pounds to one thousand cubic feet."

Later still (May, 1913), Professor H. Garman of the Kentucky station stated that, as the largest amount of carbon tetrachloride used in a series of tests, 20.8 ozs. to 100 cubic feet killed 100 per cent of the insects in nine cases out of twelve.†

In the Spring of 1914, a request came from the J. W. Knapp Department Store of Lansing, Michigan, asking for some safe means of insuring valuable furs, stored in a small room, against moths during the summer. The "fur room" was 4x6½x6½ feet—the side walls tightly built of matched lumber, the ceiling of glass. It was located in the large cloak and coat department room, where it was very accessible; but any fumigant used had to be as inoffensive as possible, without especial danger to human life, and without the entailment of extra fire risk. Carbon tetrachloride suggested itself as the possible, satisfactory fumigant in this case. Further laboratory tests had shown the writer that with ordinary wooden enclosures, which were not practically gas-tight (as was the case with the glass flasks), perfect fumigation could not be obtained at usual room temperatures (68° to 70° F), even when the carbon tetrachloride was exposed in a wide vessel. The vapor slowly escaped from the walls of such enclosures, and evaporation did not go on rapidly enough at even 70° F. to bring about the necessary concentration for perfect fumigation. It was necessary to use heat and vaporize the liquid quickly. Dr. Britton had experienced a somewhat similar difficulty in volatilizing this liquid in his experiments with nursery stock. He finally resorted to the use of heated cast iron pans for generating the vapor more quickly (citation given above). Recommendation was made in this instance, therefore, that six pounds of carbon tetrachloride be vaporized from wide, heated pans placed near the ceiling of the "fur room"—and that the charge be started immediately after closing hours in the evening. According to this recommendation, the liquid carbon tetrachloride was used at the rate of about 3.55 pounds to 100 cubic feet of room space. Heat for vaporization was supplied by hot, flat soapstones laid on iron brackets. The wide pans of carbon tetrachloride were set on the hot soapstones and the room was quickly closed. In the case of the first charge tried, the stones had not been made hot enough and not quite all the liquid insecticide had evaporated even after twenty-four hours. Several adult moths (*Tinea biselliella*) had been placed in a wire cage in the room. These were quiet when the cage was removed at the end of the twenty-four hours period, but after about four hours in fresh air two moths recovered enough to be able to move their legs. However, none ever recovered entirely. Another charge was made with six pounds of the carbon tetrachloride, and this time the soapstones were made very hot so that the liquid vaporized rapidly. A cage of moths could be observed through the glass roof of the room. They quickly became quiet. Before morning all the liquid insecticide had been vaporized. The cage was removed after thirty-six hours, and the moths were

*Bulletin No. 96, Part IV, 1911; Bureau of Entomology, U. S. Dept. of Agriculture.
†Kentucky Sta. Bulletin No. 172, p. 200.

kept under observation at intervals for one day; none of the insects showed any recovery. (See tables IV and V for the effect of carbon tetrachloride upon reductases and catalases). This last charge of insecticide vapor was considered satisfactory; and following it, similar fumigations were made at intervals of about five weeks, on the average, from May until October. The large room, in which the "fur room" was located, was well ventilated and nothing of a disagreeable nature was ever experienced from the fumigation. All the furs came through the Summer in fine condition. The company management was so well pleased that the same method is being used with the furs again in the Summer of 1915. Moreover, Mr. Armstrong, who was in charge, reported that this method of caring for the furs had proven to be cheaper than 'cold storage"; and in addition, it afforded the very great advantage of convenience in having the furs where they were easily accessible. Carbon tetrachloride cost the company twenty-seven cents per pound.

C. HEAT AS AN INSECTICIDE.

Finally, suggestions relating to possible practice should certainly call attention to heat as an insecticide. The effect of heat upon oxidases, catalases and reductases in the tissues of *P. cornutus* and various other insects has already been given earlier in this paper. No more efficient means than heat was found for destroying the activities of these three agencies of the tissue cells; it was shown that when specimens of *P. cornutus* were killed by the least degree of heat that could be effectively used, the activities of reductases, oxidases, and catalases were injured in a greater or less degree. The effect of different degrees of heat in coagulating the various soluble proteins in animal tissues is well known. It should be added here that fatal injury to the insects, and noticeable injury to one or more of the three kinds of enzyme-like bodies named, seemed to occur just before the first agglutination of protein took place. The reductase was the most susceptible. In case of clear, filtered water extracts of the tissues of *P. cornutus*, which were heated up very slowly and gradually, the soluble catalase showed its greatest loss in activity earlier than the guaiac oxidase. The latter lost its principal activity at the moment of separation of the chief or largest protein precipitate.

The fatal temperature, in the case of insects, varied considerably with the species and with the length of time the insects were exposed. No insects were tried, however (in small enclosures), which required a temperature of more than 122° F. maintained for a period of three hours to cause death; and at that temperature, death usually resulted quickly. Now, 122° F. is a comparatively low temperature and many plants can stand exposure to even higher temperatures for several minutes without appreciable injury. It is very well known that cabbage can endure hot water or hot soapsuds much better than can the common "cabbage worm." In fact, when cabbage is heading, hardly any safer or better method for killing cabbage-worms in the home garden is practiced than the use of hot soapsuds. During the last five or six years, Professor Dean of the Kansas State Agricultural College and Experiment Station has introduced heat as a comparatively cheap and efficient method of controlling all classes of mill-infesting in-

sects.* The method has been put to the test in several other states and is now recognized as the best method of controlling insects in well built, steam-heated mills.

In view of the above facts it has seemed to the author that perhaps heat might be applied to advantage as an insecticide for many insects in situations where it has never been the practice to use it. A few, more specific suggestions may be made. There have never been any extensive tests carried out (so far as we have been able to find) to determine whether heat might not be used profitably in certain instances to clean some kinds of nursery stock of insect pests. One opportunity was afforded the author of making a limited test of the possibilities along this line. Forty-four one year old apple-stocks were given by the Department of Horticulture for the test. It had been found by trial that San Jose scale on thickly infested twigs could be killed by submerging them for five minutes in water heated to 130° F. The green apple aphis was killed within one-half minute in water at that temperature. The test with the apple-stocks was carried out, therefore, to learn something of the effect, upon the stocks themselves, of dipping them in water at certain temperatures for different periods of time. The bundle of stocks, wrapped in sphagnum, had been kept all winter in the basement of the cold storage plant at the college. They were still almost entirely dormant on May 4th, 1914, when the test was started. Ten average stocks were picked out for a check—not to be treated, but planted just as they came from the storage. The remainder were given treatment as follows:—

(1) 5 stocks—tops, only, dipped at 130° F. for 5 mins.
(2) 5 stocks—tops, only, dipped at 130° F. for 10 mins.
(3) 5 stocks—tops and roots dipped at 130° F. for 6 mins.
(4) 5 stocks—tops, only, dipped at 140° F. for 5 mins.
(5) 5 stocks—tops, only, dipped at 140° F. for 15 mins.
(6) 5 stocks—tops and roots dipped at 140° F. for 20 mins.
(7) 4 stocks—tops and roots dipped at 150° F. for 10 minutes.

All the stocks were then planted in a row close together at the same depth in the same kind of soil.

The checks or untreated stocks leaved out several days ahead of the treated plants, but all of the latter finally came into leaf, and were still alive in the fall—except the four stocks which had been treated in water at 150° F. for 10 minutes. Those four plants had been killed, evidently by the treatment. Examined on May 21, 1915, most of the young apple trees were in almost full leaf. The dead and injured trees could be easily recognized. Nine of the trees in the check were alive and one dead. By numbers, the condition of the treated trees was as follows:—

(1) 5 trees alive.
(2) 4 trees alive; 1 tree dead.
(3) 3 trees alive; 2 trees dead.
(4) 3 trees alive; 2 trees dead.
(5) 5 trees alive; 3 of them with 3 or 4 inches of stock dead at the top.

*Bulletin No. 189, 1913, Kansas Agr. Expt. Station.

(6) 2 trees alive; 3 trees dead.
(7) These trees were all dead last Fall.

It will be observed that some of these plants stood exposure to a higher temperature for a longer period than would be necessary to kill San Jose scale. This test is not offered as in any way conclusive, however. It is described here in order to better call attention to the suggestion that possibly a careful and thorough investigation along this and similar lines might be worth while.

Most household pests succumb readily to heat. For example, adults of one of the common clothes-mothes (*Tinea biselliella*) may be quickly killed at 119° F. A hot flat iron has often been recommended as a means of killing the larvæ of moths in clothing—but might not some practical and safe means of applying heat be arranged to insure valuable clothing in chests or closets against moths? Thus, there are many possibilities for heat, as an insecticide, that have never been carefully and systematically tested as to their larger practicability.

GENERAL CONCLUSIONS.

Reductases, catalases and oxidases were found in water extracts and in the insoluble pulp of the tissues of *P. Cornutus* and other insects. Moreover, almost certain evidence indicated that the same three kinds of enzyme-like bodies exist in the intact tissues of living insects.

Heat of certain intensities, and the several contact insecticides studied (gasoline, carbon-disulphide, hydrocyanic acid gas, sodium fluoride, etc.), when used at a concentration sufficient to kill insects, deleteriously affected the activities of reductases, catalases and oxidases—usually in unequal degree, thus disturbing the natural or normal balance of such activities.

If the catalase, oxidase, and reductase activities are actually of as vital importance to the life processes of the tissue cells as certain evidence indicated, then the deleterious action of the contact insecticides studied in this connection must be an important factor—perhaps, in some cases, the *determining* factor—in causing the death of treated insects.

A study of the influence of the various contact insecticides upon the life processes in nervous tissue cells seems of next importance in this connection.

Fat or fat-like membrances (e. g., lard and lanoline) absorbed gasoline-vapor (and chloroform-vapor) from air charged with that vapor, and the absorbed vapor rendered the membranes less permeable to oxygen. This finding may, in part, account for the fact that less oxygen was used by an insect deeply under the influence of gasoline, since a similar condition existed—in that, under such circumstances, the lipoids of the living, oxygen-absorbing cells, and of the body fluids surrounding them, were impregnated with gasoline. So also, the same finding may help to explain the fact that, in the presence of air containing gasoline vapor, less hydroquinone was oxidized in an "insect tissue extract plus hydroquinone solution" (in which the reductase had mostly passed) than was the case when the same extract was in pure air.

Waxen membranes which had been thoroughly wet with lime-sulphur

solution were found to be less permeable to oxygen than before they were treated with the solution. Thus lime-sulphur, in addition to its effect as described in a former paper, would render the waxen covering of a scale insect less permeable to oxygen.

Pupæ of the Luna moth, and adults of *Passalus cornutus* in a dormant condition from cold, absorbed much less gasoline-vapor or vapor of ether in air than did the same insects when they were most active, at a warm room temperature, in air charged with practically the same percentage of vapor. This lowered absorption capacity, which was found to accompany the dormant condition, may furnish the chief explanation of the fact that insects, dormant from cold, are harder to kill by ordinary fumigants and by those contact sprays which depend partly upon volatile insecticide ingredients for their effectiveness.

It was found that certain non-volatile, powdered solids were able to act as effective contact insecticides when used on certain insects. Such dry, powdered insecticides stuck fast in exudations on portions of the insect body, where they became partly dissolved, after which they appeared to be slowly absorbed through the body-integument.

Both powdered borax and sodium fluoride may kill cockroaches in the manner of purely contact agents, but normally they become stomach poisons as well—since the roaches regularly lick and swallow some of the powder in cleaning it from their bodies.

In the case of powdered solid contact insecticides, the advantage seemed to lie in having the powder so fine and dry that it could sift readily into all crevices and could adhere well. Similarly, other things being equal, a weak surface tension gave a liquid contact insecticide an advantage, enabling it to thoroughly wet the bodies of insects and to flow into all irregularities of the area treated.

Among several substances which were found to increase the "spread" of lime-sulphur solution, saponin or extracts taken directly from the stems and leaves of *Saponaria officinalis* (*bouncing bet*) seemed to be best. It is suggested that perhaps the use of extracts from "bouncing-bet hay" with lime-sulphur solution might prove profitable in orchard spray-practice.

Experiments with ammonia (derived from dry liquefied ammonia) as a fumigant for mill insects were rather disappointing; but the liquified ammonia was easy to apply and might prove to be a desirable fumigant for insects in some instances.

When carbon tetrachloride was compared with carbon disulphide as to its action on grain insects in tight flasks, six times as much of the former was required for effective fumigation. The carbon tetrachloride was vaporized with heat and satisfactorily used, at the rate of 3.55 lbs. for 100 cubic feet of air space, to insure furs against moths—fumigation being repeated every five weeks during the summer months. The charge as used killed adults of *Tinea biselliella*.

Evidence indicates that heat might be applied to advantage as an insecticide in many situations where it has never been the practice to use it.

1.
3.
5 9 J. 1913.
2.
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PLATE I.

Fig. 1. Sample of thick creamy extract in the left stender dish was treated strongly with CS, and $\frac{M}{10}$ hydroquinone was added later in the presence of the CS_2.

Check extract to the right (not treated) had the same amount of $\frac{M}{10}$ hydroquinone added, at the same time as the treated extract.

The darker color of the check shows the greater oxidation of the hydroquinone to quinone.

Fig. 2. Shows the form of sealed tube in which the reducing action of insect-tissue extract toward methylene blue, indigo carmine, etc., was studied.

Fig. 3. First cup to the left shows the darkened solution of tyrosin, oxidized under the influence of a crushed elytra (thoroughly cleaned with alcohol) of an old adult *P. cornutus*.

Middle cup contains clear unoxidized solution of tyrosin with crushed elytra which had been heated to boiling for 10 minutes.

Cup to the right—check, containing only tyrosin solution which remained clear during the 7 days of the test.

Fig. 4. Cockroach (*P. americana*), showing the head and arrangement of the white bristol-board collar to prevent the insecticide powder, with which the body was treated, from reaching the insect's mouth.