ROTHAMSTED RESEARCH

Report for 1956

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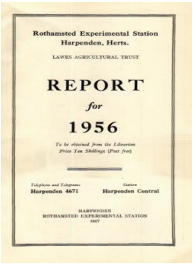

Insecticides and Fungicides Department

C. Potter

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INSECTICIDES AND FUNGICIDES DEPARTMENT

C. POTTER

During the current year Dr. Helen Salkeld and Miss Jill Kerridge left to go to Canada and R. van Baer left to return to Australia. F. T. Last has been seconded to the Department of Agriculture, Sudan, for two years to work on blackarm disease of cotton. The following appointments have been made: R. Sawicki to work on pyrethrum, T. Doherty to assist M. J. Way and supervise work on the rearing of the test insects, Frances Molloy to work on the histological and histochemical aspects of the mode of action of insecticides and Elaine Fairey to work on the problem of strains of insects resistant to insecticides.

K. A. Lord attended the 3rd International Congress of Biochemistry in Belgium. A grant offered by the committee of the 10th International Congress of Entomology to M. J. Way enabled him to attend this Congress in Montreal, where he read two papers. After the Congress the committee financed a five weeks' tour of Science Service and University Laboratories.

Mr. M. A. Nour from the Sudan returned to work in the department, and Mr. R. A. Harrison of the Department of Scientific and Industrial Research, Auckland, New Zealand, arrived for a stay of six months. Shorter stays for training purposes were made by P. S. Cheema of the Punjab, India, and H. Stevenson of the Kenya Pyrethrum Board.

INSECTICIDES

Time, temperature and toxicity of insecticides

M. Das has continued his work on the effects of after-treatment temperature and time on the toxicity of contact insecticides.

The test subjects were adults of *Oryzaephilus surinamensis*, *Tribolium castaneum*, *Tenebrio molitor* and *Musca domestica*. Insects were treated by dipping them in aqueous suspensions of poison (*O. surinamensis*) or by applying measured drops of solutions of poison (all other species). In each test insects were kept after treatment at each of two temperatures, usually 28° and 10° C. In all cases except one the percentage kills increased as time passed. Counts of kill were made repeatedly on the same insects until the percentage kill from the poison stopped increasing (at the " end-point " of the test), or until the control insects themselves died.

In this way, the effect of the passage of time on the temperature coefficient of kill by each poison could be followed. Each poison gave a characteristic pattern, which was roughly the same with all the species it was tested on. Some details of the results are given in the following paragraphs. All figures are approximate.

Temperature coefficients of kill changing in size and sign. With α-chlordane on *T. castaneum* and *T. molitor*, the temperature

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coefficient changed from positive to negative on the fifth day after treatment; with toxaphene on *T. molitor*, it changed from positive to negative on the fourth day; with "Valone" on *T. castaneum* it changed from positive to negative at the twentieth hour.

With rotenone on *O. surinamensis*, the temperature coefficient changed from negative to positive on the third day after treatment. Rotenone was unique in that, although there was a steady increase in kill at 28° C., there was merely a paralysis on the first day after treatment at 10° C., followed by recovery of the insects.

Temperature coefficient changing in size only. With 2-bromomercurithiophen on *O. surinamensis*, the temperature coefficient between 23° and 10° C. was +10 on the first day after treatment; it decreased to zero on the fifth day, but did not become negative. The results with *T. molitor* were similar, but the coefficient never became less than +2.

Nearly constant temperature coefficients. With "Dimetan" on *O. surinamensis*, the temperature coefficient was +1·5 on the first day after treatment. After this it remained unchanged, even on the sixth day.

With DDT the temperature coefficients on all four species were clearly negative (about —10), and remained almost unchanged throughout the tests. Other properties of DDT are noted below.

Special tests with DDT. Although DDT is more toxic at 10° C. than at 28° C., the toxicity does not continue to increase indefinitely as the after-treatment temperature is lowered still further. Tests on *T. molitor* showed that there was a maximum toxicity at about 6° C.; below 6° C. the temperature coefficient of kill became very strongly positive.

This positive coefficient, like the negative coefficient above 6° C., was reversible. Poisoned adult *T. molitor*, which had marked DDT symptoms after being kept at 10° C. for 2 days, showed some recovery when transferred to either —1° or 28° C. for a further 3 days; control insects were unaffected by the low temperature. Poisoned insects, kept at —1° C. for 3 days, were prostated by the low temperature, but showed no symptoms of DDT poisoning. However, they developed the usual symptoms when changed to 10° C.

Other tests have shown that the positive temperature coefficient was not due to failure of DDT to penetrate the cuticle at —1° C.; nor was it due to failure of DDT to reach its site of action at —1° C. after it had penetrated the cuticle.

It is not yet known whether the positive temperature coefficient changes in size as time passes.

Isolation and properties of insect esterases

K. A. Lord and S. R. B. Solly have continued the examination of esterases from *Blatella germanica*. Most time has been spent on fractions hydrolysing acetyl choline. The preparative methods have been improved in detail to give better yields of the acetyl choline hydrolysing preparations and to remove considerable amounts of what appears to be nucleic acid. Preparations have been examined by zone electrophoresis. It has been established that paper is not a suitable support, since the enzymes are absorbed strongly at useful pH and ionic strengths. Alternative supporting

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media have been examined, and starch paste and starch gels have been utilized. It has been shown that the best cholinesterase preparations to date appear to contain other types of esterase, but the results are not inconsistent with the presence of only one enzyme hydrolysing acetyl choline. Similar results have been obtained by chromatography using ion-exchange paper (a sample of experimental material received from W. & R. Balston Ltd.) prepared from modified cellulose. The results are of sufficient promise to warrant the investigation of the method using a column, before proceeding to the examination of the properties of cholinesterase from *B. germanica*.

Preliminary investigation of the properties of the cholinesterase preparation from *B. germanica* has shown it to have a high affinity for acetyl choline. The rate of hydrolysis of acetyl choline is not reduced at concentrations of acetyl choline too low for satisfactory measurements to be made in the Warburg. A pH comparator as described by J. Burch (*Bioch. J.* **58**, 415, (1954)) has therefore been constructed and modified to improve its stability, in order to make valid enzyme estimations at lower substrate concentrations under controlled conditions and without the addition of indicators.

The pH comparator has proved of value in other experiments of this nature, and in some preliminary experiments with an esterase preparation obtained from *B. germanica* which did not hydrolyse acetyl choline; it was shown that the rate of liberation of nitrophenol from nitrophenyl acetate was increased in the presence of ethanol and butanol. At the same time the rate of liberation of acid was shown to be decreased. Glycol and glycerol decreased the rate of liberation of acid in the reaction mixture but did not appear to change the rate of liberation of nitrophenol under the experimental conditions used. It is therefore concluded that in the presence of some alcohols trans-esterification may occur.

Examination by starch block electrophoresis of preparations of *B. germanica*, largely freed from cholinesterase, indicated that there were a number of other esterases. In one experiment it seemed likely that up to six esterases were present.

Esterases in relation to the development of eggs of Pieris brassicae *and* Gryllus domesticus *and the toxicity of organophosphorus compounds*

P. brassicae *eggs.* Work on the toxicity of TEPP to *Pieris brassicae* L. has been continued by C. Potter, K. A. Lord, D. V. Holbrook and H. Salkeld. It has been shown that low doses of TEPP permit full development of the embryo to the stage where it moves, although the larvae may not emerge. Higher doses arrest development at progressively earlier stages. The dose of TEPP which permits full development of the embryo but does not allow hatching decreases only slowly as the interval between the egg being laid and treated is increased. The decrease in dosage is small compared with the expected aqueous decomposition of TEPP, even allowing for protection by solution in non-aqueous parts of the egg. It has therefore been tentatively concluded that the toxic reaction of TEPP must occur soon after its application. The overall toxicological effects, however, are not observed until the time when the

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substances or their derivatives with which the TEPP has reacted are required for the metabolism of the embryo.

Preliminary experiments indicate that the enzymes hydrolysing acetyl choline and phenyl acetate in *P. brassicae* eggs are inhibited *in vitro* to about the same extent by TEPP under our experimental conditions. No definite conclusions can safely be drawn about which enzyme systems are affected in our present state of knowledge. However, since if big doses of poison are used, eggs may be killed in the earlier stages of development, before cholinesterase can be detected, there are indications that death may be produced by the TEPP by mechanisms other than the inhibition of cholinesterase.

G. domesticus *eggs.* Helen Salkeld has examined the eggs of *G. domesticus*, and S. R. B. Solly has examined the hydrolysis of three esters through the development of the eggs. The hydrolysis of acetyl choline is negligible in the early stages of development of the eggs, and only becomes apparent at the time the nervous system becomes organized, after which it continues to increase with the development of the eggs. Phenyl acetate is hydrolysed at all stages of development, but it increases as development occurs. Triacetin is hydrolysed at a fairly high and uniform rate throughout all stages of development of the egg.

A number of buffer solutions have been examined for use in connection with TEPP when poisoning *G. domesticus* eggs. These are necessary to control the pH of the aqueous solutions to which *G. domesticus* eggs must be exposed in order to ensure development under normal test conditions. Sodium succinate buffers have proved to be effective. Unfortunately aqueous solutions of 1 per cent TEPP show little toxicity to *G. domesticus* eggs when the pH of the surrounding medium is controlled.

Toxicity of organophosphorous compounds to various strains of Musca domestica

It was thought that some information on the process of poisoning by organophosphorous compounds might be gained by studying their effects on normal and resistant strains of insects.

The resistance of various strains of *M. domestica* to a number of organophosphorous compounds has been compared. Four strains are under examination, a normal strain as usually reared in the laboratory and three so-called phosphorus-resistant strains. One resistant strain was kindly supplied by Dr. Busvine and the two others (strains 79 and 150) by Dr. Keiding of Denmark. Only small differences in the resistance of the strains has been detected by the techniques of bioassay that were used, although strains 79 and 150 were reported to show considerable resistance. Paraoxon, Bayer 21/199, " Diazinon ", " Chlorothion ", malathion and " Dipterex " have been selected as a range of insecticides for including in the tests. In view of the apparent lack of resistance of the insects when tested by us, an exchange of insects with Dr. Keiding has been arranged so that check assays may be made. An attempt is also being made to breed resistant strains in the laboratory.

Although the so-called phosphorus-resistant strain of houseflies supplied by Dr. Busvine was only twice as resistant to poisoning

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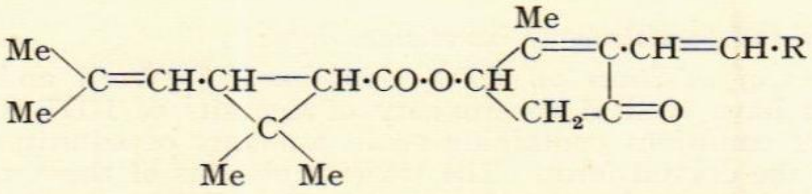
by paraoxon, the two strains were examined for differences in the *in vitro* inhibition of enzymes which hydrolysed acetyl choline, triacetin and phenyl acetate respectively. No differences could be detected. *In vitro* tests failed to demonstrate the hydrolysis of paraoxon by extracts of housefly tissues. Tests on whole flies treated with paraoxon showed a rapid disappearance of the poison at apparently the same rate in both strains of flies. The rate of disappearance of paraoxon was followed both by the inhibition of added cholinesterase and by chromatographic methods.

The relationship between insecticidal activity and chemical structure in pyrethrin-like compounds

Although many compounds related to the pyrethrins have been synthesized and tested in recent years, so far none have contained one or more double bonds in the alcoholic side chain conjugated with the *cyclo*pentenolone ring. Work has therefore been carried out by M. Elliott to explore synthetic routes to compounds such as ((I), R = alkyl or alkenyl) below.



(I)

Apart from interest in their insecticidal potency, such compounds would contain a cross-conjugated ketonic system, which it was desired to investigate spectrophotometrically. This system was discussed by Gillam and West,* and an unsuccessful attempt to synthesize 2-but-1′-enyl-3-methyl*cyclo*pent-2-enone was made by Harper.† The following routes have been investigated in the present work :

(*a*) It is known ‡ that 2-alkyl-4-bromo-3-methyl*cyclo*pentenones can be made from the corresponding ketones by reaction with *N*-bromo-succinimide (NBS) and that 2 more bromine atoms may be introduced into the molecule by further bromination with the same reagent. Therefore, 4-acetoxy-2-butyl-3-methyl*cyclo*pent-2-enone was brominated with one mole of NBS to see whether bromine had been introduced into the α-methylene group (1′-position) in the side chain and whether it could be removed as hydrogen bromide with trimethylamine. However, all fractions so far isolated have contained up to 10 per cent of bromine, and it has not proved possible, therefore, to obtain the bromine-free keto-ester ((I), R = Et) required.

(*b*) Attempts have been made to isomerize the double bond in allethrin [(±)-2-allyl-3-methyl*cyclo*pent-2-en-4-ol-1-one (+)-*cis-trans*-chrysanthemate] into conjugation with the *cyclo*-

* Gillam, E. M. & West (1942). *J. chem. Soc.* 674.
† Harper (1946). *Ibid.* 892.
‡ Crombie, Elliott & Harper (1950). *Ibid.* 971.

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pentenone ring. Both alkaline and acidic reagents tried so far have failed.

(*c*) 2-Alkyl-4-ethoxycarbonyl-3-methyl*cyclo*pent-2-enones, and thence the parent ketones, are obtained by cyclization of the products of Stobbe condensations of succinic esters with alkyl methyl ketones.* Some progress has been made towards the extension of this route to use alkenyl methyl ketones. Whereas allyl acetone condensed with diethyl succinate in the presence of sodium hydride in benzene, or with potassium *t*-butoxide in *t*-butanol without double-bond migration (half-ester, $\lambda_{max.}$ 2200 Å., ϵ, 4260), the product from reaction with potassium *t*-butoxide suspended in ether appeared to have undergone a double-bond shift ($\lambda_{max.}$ 2400 Å., ϵ, 13,600), producing what may be 3-ethoxycarbonyl-4-methylocta-4 : 6-dienoic acid. Cyclization of this compound should produce a *cyclo*pentenone with a double bond in the 1′-position of the side chain, but experiments have not yet proceeded far enough to confirm whether this can be carried out.

Toxicity and persistence of insecticidal deposits

Effect of additives on DDT emulsions. J. Ward and E. M. Gillham have studied the toxicity of deposits of DDT made by spraying emulsions containing small amounts of additives which modify the crystal form. The toxicity of films of these materials produced by spraying them on to wax-coated glass plates has been measured, using the technique of 6-day exposure periods with *Tribolium castaneum* Hbst., described below in the bioassay section. Deposits from standard emulsions on wax-coated and on plain glass plates have been used for comparison. So far, despite the considerable effect of the additives on crystal form, no great differences in toxicity between the standard and the modified emulsions have been found. This may be because of the inadequacy of the biological assessment technique. Although the technique of long exposure periods gives satisfactorily precise results, it is not an adequate substitute for a test using a shorter period, as previous work has shown that differences in toxicity of different types of deposit tend to become less as the exposure period is increased. For this reason an attempt has been made to discover the causes of the irregular results obtained with the same test method when shorter exposure periods are used. This work is described in the section on bioassay. Work has also been started on an alternative method using *Musca domestica*, which is also described in the section on bioassay.

Rate of evaporation of insecticide films. Previous attempts to measure the rates of evaporation of DDT films under standard conditions have shown that reliable results can only be obtained if the rate of ventilation is controlled. This has been achieved by constructing a ventilated cabinet in which the air-flow is made laminar by a number of " honeycomb " screens. The plates are carried in slots in a metal rack which has been carefully made so that the plates are parallel with one another and at right-angles to the air-stream. The apparatus is kept in a well-ventilated constant-temperature

* Elliott (1956). *J. chem. Soc.* 2231.

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room at 24–25° C. With a wind speed of 8 m.p.h. (which at present cannot be varied) the variation in rates of evaporation between plates in different positions in the rack has been found to be satisfactorily small.

Using the wind tunnel, it has been shown that evaporation from the emulsion-sprayed plates used is linear with time until about two-thirds of the deposit has evaporated. At this stage most of the long outgrowths from the centres of crystallization have been lost, and only compact clumps at the centres remain. Thereafter, evaporation continues at a lower rate. Previous work had suggested that the rate of evaporation was independent of the initial density of the deposit. Recent results have confirmed that this is so during the initial linear period of evaporation for the deposits of emulsions of DDT on glass so far examined. For the conditions in the ventilated cabinet, i.e., 24–25° C. and 8 m.p.h. windspeed, the rate of loss is about 0·4 μg./sq. cm. per day.

Persistence of benzene hexachloride in soil

It was mentioned in last year's report that an experiment on the tainting of crops by applications of insecticide to soil was being conducted by the National Vegetable Research Station, that Rothamsted had agreed to analyse soil samples for benzene hexachloride and that a suitably sensitive method of analysis was being sought. J. Ward and J. Samuel have continued this work, and have devised an analytical method. The method devised is capable of estimating down to 2 parts of BHC in 10^8 parts of soil, but the precision of the results still leaves something to be desired, and it is hoped to improve the method in this respect. An outline of the method is given in the section on analytical techniques.

Sixty-six soil samples have been analysed using this method. The results show that BHC applied in March and worked into the top 4 inches of soil does not penetrate farther into the ground, though during the hot summer of 1955 about 70 per cent of the BHC was lost. After the vegetables had been harvested, the land was ploughed to a depth of 8 inches. As might be expected, this operation redistributed the insecticide to the ploughing depth. The soil was again sampled in April 1956, when it was found that there had been no significant change in the total BHC content of the soil during the winter.

Analytical and bioassay techniques

Estimation of BHC in soil. Steam is passed through a sample of the soil and carries with it the BHC, which is appreciably volatile. The BHC is extracted from the condensate with carbon tetrachloride which has been specially treated to remove all traces of aromatic substances. Impurities from the soil are removed by passing the carbon tetrachloride solution through a column, the top part of which is packed with nitrating mixture supported on kieselguhr, the lower with activated alumina. Impurities are nitrated in the upper part of the column, and the nitro-compounds formed are adsorbed on the alumina. BHC passes through without loss.

The carbon tetrachloride solution is concentrated under reduced pressure in a flask immersed in a bath at 10° C. When the volume

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has been reduced to 1 ml., a small quantity of piperidine is added and the solution is heated under reflux in semi-micro equipment with standard ground-glass joints. The piperidine dehydrochlorinates BHC to 1 : 2 : 4-trichlorobenzene. Nitrating acid is added, which destroys the excess piperidine and nitrates the trichlorobenzene. After boiling off the carbon tetrachloride and diluting the acid mixture, the dinitrotrichlorobenzene is extracted with benzene. An aliquot portion of the benzene is evaporated down to 0·2 ml., and a colour is formed by adding benzyl cyanide and acetone and shaking with concentrated aqueous potassium hydroxide. The depth of colour is measured using micro-cells which give a 1-cm. light-path with 0·3 ml. liquid.

The method of recovering BHC from soil by steaming gives quantitative results with some types of soil, but with soil from the Fens it was found to give a low recovery. An alternative method of extraction is being developed. The method of analysis may also prove useful for potatoes and other vegetables; using the steam extraction process, we have found that 0·06 p.p.m. of BHC can be recovered from potatoes grown in soil treated with 1½ lb./acre of gamma BHC, but this must be regarded as a minimum figure, as we have, has yet, no information on the efficiency of recovery of BHC from potatoes.

Bioassay of contact toxicity of insecticide deposits

Assessment using adult Tribolium castaneum. A deposit is produced by spraying the surface with the insecticide in aqueous medium using a Potter Tower. The method used up to the present to assay the toxicity of deposits has been as follows: groups of 10 *Tribolium castaneum* (Hbst.) are confined on the surface to be tested in glass rings, for a period of either 4 or 24 hours, at a temperature of 20° C., R.H. 60 per cent. During exposure to the deposits the insects are kept in the dark. They are then removed from the deposits by means of a drawn-out glass tube attached to the vacuum-line, with which they can be picked up individually and transferred to specimen tubes. After a further period of 5 days at the same temperature and humidity, the dead are counted. Four groups, each of 10 insects, are used as replicates for each level of deposit density. As was mentioned in the last report, this method of assessment suffers from the disadvantage that the kills in the replicate groups of 10 are more variable than can be accounted for by random sampling-error, and consequently the confidence limits of the LD50s obtained in this way are wide, unless the number of replicates is greatly increased.

An attempt was made to list all the likely causes of variation and examine them. The following were considered :

1. The insects are not selected in a random manner when the groups of ten are counted out from the culture.
2. The deposits are unpredictably variable in character, so that the replicate groups of ten insects do not receive identical treatments.
3. The environments of the groups during exposure are not identical.

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4. It has been found that if insects are active the mean amount of insecticide picked up is greater than if they were inactive. If different groups differ in the presence or absence of active individuals and active individuals stimulate the group, this may prove a source of variation.

5. An occasional exceptional insect might pick up a dose very much greater than the average, then transfer it to the other members of its group during the waiting period before mortality assessment.

Experiments in which groups of insects were washed with acetone after removal from the deposit, and then the acetone analysed for DDT, showed that the variations in mortality are accompanied by variations in the amount picked up by the insects. Further evidence of the relevance of the amount picked up was obtained by exposing the insects to the deposits for 6 days instead of 24 hours and counting the dead at once. The longer exposure period resulted in the variations in mortality between groups being much reduced.

Non-random selection of insects from the culture is avoided by putting the insects into the glass circles one at a time, putting one into each ring before putting the second into the first ring and so on, so this source of variation can be ruled out.

Variability of the deposits has been investigated by using two successive batches of insects on the same deposits. If the deposits were irregular, it would be expected that an area of deposit which caused a low kill in the first batch of 10 insects would also cause a low kill in the second. The experiment showed that in many instances an area of film that gave a low kill with the first batch of insects gave a high kill with the second. This showed either that the toxicity of the deposit was altering between exposures or it was not the main cause of the variability in kill between replicates. There is some evidence that the physical state of the deposit could have altered considerably between exposures, and this still remains as a possible source of variation.

Variations in environment during exposure were as far as possible avoided by covering the trays of plates with opaque paper to exclude light, and storing at constant temperature and humidity.

The explanation that the insects of a group have some influence on one another during the period of exposure is being studied by exposing insects singly, instead of in groups, and comparing both the mean kill and the variability with those obtained by the standard method.

The possibility of transfer of insecticide from one insect to another during the storage-period before counting the kill was also eliminated as an explanation of the variation by the following experiment : Two sets, each of 10 groups of 10 insects, were exposed to a DDT deposit which would give about 50 per cent kill. One set was removed and kept in the usual manner, the other was randomized at the time of removal from the deposit, so that the insects which were exposed to the deposit as a group were distributed among the 10 groups during storage. The variability of kill was much greater for the groups of insects which had not been randomized than for those

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which had, and among the latter it was no greater than was to be expected on statistical grounds.

Assessment using adult Musca domestica. Insects may be killed by the penetration into them of insecticide, the particles of which continue to adhere to the substrate; or they may be killed by particles, originally adhering loosely to the substrate, which they pick up and carry away when they are removed from the deposit. The two methods may be distinguished as killing by direct action and by pick-up. Differences in ease of pick-up between different deposits may be expected to show up more clearly if the test-method uses a short exposure period rather than a relatively long one, as required by *T. castaneum*. A technique is therefore being developed by R. A. Harrison for comparing the biological activity of deposits prepared from different formulations and on different substrates, using *Musca domestica* as the test insect. Tests are being carried out to determine the performance of an apparatus which gives flies no choice but to walk on the insecticidal deposit and to determine the factors influencing the results. The apparatus confines flies between two surfaces, the lower surface being interchangeable with a test surface at will. The distance between the surfaces has a considerable effect on fly mortality. When the apparatus is inverted and flies are forced to walk upside down on the deposit the mortality is lower than when the apparatus is normally orientated and the flies are in a normal upright position.

Deposits of DDT (approx. 5·0 μg./sq. cm.) prepared on glass plates from a DDT emulsion show an initial rapid decline in toxicity when successive test samples of flies are exposed to them.

Bioassay using a measured-drop technique and Musca domestica *adults*

A convenient procedure for handling *M. domestica* for bioassay purposes, particularly where a measured-drop technique is used, has been elaborated during the course of work on the action of synergists of pyrethrins. It has already been applied to other insecticides. The adult flies are normally sexed the day before a test is made. The adult flies are reared in 9-inch-cube cages which are placed on trays of ice in a cold cabinet at 5° C. (approx.). The live flies migrate to the top of the cage in a torpid condition. The cage is then lifted from the tray which forms the base of the cage and quickly placed on a clean tray. In this way the live flies are separated from the dead and the normal debris of a rearing cage. The cage is then thoroughly cooled in a deep freeze to completely immobilize the flies, which are then shaken down into a clean tray. The top of the cage is removed and the tray placed in a cold cabinet fitted with a window and hand hole. The flies are then sexed and placed in petri-dishes whilst immobilized. The insects are then allowed to recover and are kept 24 hours at the temperature of treatment, usually 25° C. The following day, immediately prior to treatment, the petri-dishes containing the flies are cooled in the ice-box of a refrigerator at —8° C. for 5 minutes before applying the insecticide as a measured drop to the ventral surface of the thorax. When the solvent is volatile, e.g., acetone, it evaporates whilst the flies lie on their backs before they recover sufficiently to turn over and walk.

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Using this technique, a series of very satisfactory probit lines were obtained.

Detection and identification of toxic substances in poisoned bees

From time to time during the summer months, samples of dead bees are received by the Bee Advisory Service of the National Agricultural Advisory Service for determination of the cause of death. The beekeeper generally wants to know whether his bees have been poisoned, and if so, the identity of the poison. This year, samples of bees which did not show signs of disease have been passed to this department by the National Agricultural Advisory Service, and an attempt has been made by J. Ward and P. H. Needham to develop methods for identifying the toxic agent, using a combination of chemical and biological methods. A method capable of doing this should be quite generally useful for identifying traces of insecticides in other materials.

Published work on this subject originates mainly in Germany and Switzerland, and the methods described are all tentative. Several of these depend on bioassay with larvae of *Aëdes aegypti* as test species, and these methods were taken as a starting point. None of them is capable of separating the insecticide from all traces of the waxy material which is obtained when bees are extracted with fat-solvents, and it was found that the wax interfered with the bioassay, either by causing mortality among larvae treated with extracts from control (unpoisoned) bees or by suppressing the toxicity of added insecticide, presumably by preventing it from dissolving in the water in which the larvae were swimming. Attempts were made to remove the wax by partitioning the extracts between petroleum ether and wet alcohol, but this procedure was only partially successful. Two methods of separation employing chromatographic columns were tried; that described by Erwin *et al.* (*J. Agric. Fd Chem.* **3** (1955), 676) as a means of separating insecticides from other matter in vegetable extracts appeared to be promising.

When a chromatographic column is used to dewax the bee extract, some indication of the nature of the insecticide present is obtained by observing its rate of progress through the column. A further indication can be obtained by treating the dewaxed extract with reagents which destroy some insecticides but not others. Treatment with oleum, for instance, will destroy all commonly used insecticides except lindane, DDT and chlordane. If an extract remains toxic to mosquito larvae after treatment with oleum one of these three insecticides is present. The method of separation and identification is being further developed.

The biological material used for indicating the presence of poison in the extracts of the bees was kindly supplied by the Cooper Technical Bureau, Berkhamsted, Herts. Eggs of *Aëdes aegypti* were obtained as required.

Larvae were obtained from the eggs and cultured in distilled water on " rat cube " (M.R.C. diet 41) at a temperature of 27·5° C. Three-day-old larvae were used for detecting the poisons.

The method of conducting the tests on the extract was rather rough, as it was devised at very short notice. A more elaborate and precise method has been developed for next season.

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Of the 10 samples received from the Bee Advisory Service 8 were found to contain insecticidal material. The insecticide was tentatively identified in 5 samples, 3 as lindane, 1 as dieldrin or endrin and 1 as a nitrophenol.

Factors influencing the resistance of Drosophila melanogaster *to insecticides*

This work has been continued by F. Tattersfield, J. Kerridge and E. Fairey. It has previously been noted that the addition of certain protein-containing materials, such as yeast and casein, to a sugar- or honey-containing medium increased significantly the resistance to DDT of adult *D. melanogaster*. During this year's work the result was confirmed that the addition to a glucose–agar–nipagin medium of live yeast or casein (vitamin-free) or casein (soluble) increased the resistance to DDT of the *D. melanogaster* adults which were fed on it to about the same degree; dead yeast was not so effective. It was also confirmed that, whereas the adults fed on this medium plus yeast, dead or alive, produced eggs and larvae which passed through the whole life cycle, those fed on it plus casein did not.

Preliminary work was also started on the effect of population density of larvae upon the resistance of the resulting adults. It has been shown in the previous years' work that very high densities of adult *D. melanogaster* either males or females (separately or mixed) reduced their resistance, but that the pattern depended on the sex. The problem of the effect of the population of the larvae proved more complicated. The size of the resulting adults in general declined with the population density, but this was not correlated in any simple way with the resistance to DDT. The work is to be repeated and an attempt made to ascertain whether a selection for higher resistance is possible by increasing the population per unit of food available. The slopes of the respective log concentration–probit lines, in addition to their relative positions with respect to each other, emerged as a matter of importance in these investigations, as they indicate the relative degree of variation in resistance within the respective populations, and the extent to which the slopes may have been affected by the death-rate caused by the relation between population densities and available food.

During the year Miss Jill Kerridge left the department to go to Canada. This caused some delay in pursuing the work. It has been possible, however, with the help of other assistants in the department, to carry out a survey in some detail of the effects of the B-vitamins upon resistance. It was found in preliminary tests that neutralized nucleic acid and sodium nucleate derived from yeast gave a definite increase in the resistance to DDT of adult *D. melanogaster* fed upon a glucose–agar medium containing it. The sulphates of iron, manganese and magnesium and the potassium phosphates K_2HPO_4 and KH_2PO_4, important constituents of Tatum's salt mixture, have either little or no effect that could be regarded as significant. If anything, particularly in the case of the males, the phosphates at the concentrations tested slightly reduced resistance. A few amino acids tested gave uncertain results.

With *D. melanogaster* the B-vitamins are much more important

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than any others, as they determine whether the life cycle—larvae, pupae and adults—will be carried through successfully. Many of these vitamins act as co-enzymes. Tests were made with vitamin B_1 (aneurin, thiamine), B_2 riboflavin, B_6 pyridoxin, nicotinamide, folic acid, calcium D-pantothenate and choline chloride. None of them when used in a glucose–agar medium at relatively high concentrations gave significant increases in the resistance to DDT of adults fed for a week upon them. The problem arose, however, whether the effect produced might not depend upon the concentration of DDT used in the spray tests.

The work is being continued using media containing nucleic acid, with both a number of salts and certain vitamin-B mixtures.

It is proposed that Miss Elaine Fairey undertakes the selection of DDT-resistant strains by feeding the larvae of *D. melanogaster* upon DDT-containing media.

Pyrethrum and other plant products

Pyrethrum synergists. Experimental work by C. Potter, K. A. Lord and D. V. Holbrook to compare the effects of piperonyl butoxide and sulfoxide on the toxicity of pyrethrins to *Musca domestica* has now been completed. The results are being examined in conjunction with those obtained using *Tenebrio molitor* to determine if specificity occurs and, if so, the nature of it.

A major difficulty in comparing the action of either a number of synergists on one species or a single synergist on a number of species arises from the difficulty of summarizing the experimental data. We believe that we have elaborated a convenient method of obtaining and expressing data for the comparison of the effects of different synergists.

For each synergist a number of probit lines covering a wide range of pyrethrins–synergist ratios are constructed, either by varying the amount of pyrethrins and keeping a constant ratio of synergist to pyrethrins, or by keeping the concentration of synergist constant and varying the amount of pyrethrins, or by keeping the pyrethrins constant and varying the amount of synergist.

Any desired mortality level may then be selected, and for each of the probit lines the amount of synergist and pyrethrins giving this kill is obtained. From these data a curve is constructed which shows the amounts of pyrethrins and synergists, covering all possible ratios, which will produce the specified kill. When these curves have been constructed for each of the synergists to be compared the comparison of their effects over a whole range of pyrethrins–synergist mixtures may be made at a glance.

These curves may conveniently be used when comparison at fixed ratios of pyrethrins to synergist are required, since straight lines drawn through the origin of the graph give fixed ratios. These data are of practical importance, since, in the field, only one ratio is used and the amount of poisons received by individual insects is variable.

Further examination of the results has shown that in our experiments the results may be expressed by the equation

$$Y = Px + Sz + C$$

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where Y = probit mortality; x = log pyrethrins concentration; z = log synergist concentration; P, S and C are constants. In addition to the information obtained on the relative performance of piperonyl butoxide and sulfoxide on the two species of insects, the results show that there is a specificity of effect with both synergists, since it was possible to increase the toxicity of pyrethrins to a much greater extent with one of the species tested than with the other.

Toxicity of Millettia dura *extracts.* At the request of the Colonial Products Laboratory a preliminary assessment of the toxicity of various extracts of *Millettia dura* was made by P. H. Needham. The plant material was obtained from Kenya.

The extracts tested were: (*a*) chloroform extract of the seeds; (*b*) chloroform extract of the roots; (*c*) petroleum ether extract of the seeds.

The chloroform extract, which was the most toxic one, was found to be one-quarter to one-fifth as toxic as rotenone when applied by a measured-drop technique to adult *Phaedon cochleariae* (see Table 1). This extract was then separated into the alkali-soluble fraction and the neutral fraction. These two were tested against adult *P. cochleariae*, with the result that the neutral fraction showed the same toxicity as the original chloroform extract, and the alkali-soluble fraction was non-toxic at 0·5 per cent w/v.

TABLE 1

Toxicity of extracts of Millettia dura *to adult* Phaedon cochleariae *in comparison with rotenone*

	Relative toxicity
Rotenone	100
Chloroform extract of the seeds	23
Chloroform extract of the roots	4
Petroleum ether extract of the seeds	1
Neutral fraction of chloroform extract of seeds ...	24

Insect rearing

Species reared. The following insect species were reared in the department during the year. They include 10 plant-feeding species and 17 feeding on stored products.

Plant feeding

HEMIPTERA	*Megoura viciae* Buck.
	Aphis fabae Scop.
	Myzus persicae Sulz.
	Acyrthosiphon pisum Harris
LEPIDOPTERA	*Pieris brassicae* L.
	Diataraxia oleracea L.
	Actias selene Hubner.
COLEOPTERA	*Phaedon cochleariae* F.
DIPTERA	*Leptohylemyia coarctata* Fall.
	Hylemyia antiqua Meig.

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Stored product, domestic and medical

HEMIPTERA	*Dysdercus fasciatus* Sign.
ORTHOPTERA	*Blatella germanica* L.
	Blatta orientalis L.
	Periplaneta americana L.
	Gryllus domesticus L.
LEPIDOPTERA	*Achroia grisella* Fabr.
	Ephestia kühniella Zell.
COLEOPTERA	*Oryzaephilus surinamensis* L.
	Oryzaephilus mercator Fouv.
	Tribolium castaneum Hbst.
	Tribolium confusum Duval.
	Tenebrio molitor L.
	Calandra granaria L.
	Trogoderma granarium Everts.
DIPTERA	*Aëdes aegypti* L.
	Drosophila melanogaster Meig.
	Musca domestica L.

Four constant-temperature rooms are now working in the West Building, one with controlled lighting for the rearing of plant-feeding species. A number of different strains of *M. domestica* are being reared for work on organophosphorous resistance.

Diapause in Leptohylemyia coarctata *Fall.* This work, by M. J. Way, is nearing completion. Temperature requirements for termination of diapause in the egg have been determined, the range being from about +12° to about —24° C. Diapause is terminated slowly at +12° C., and there is also a high mortality. Within the range +12° to —6° C. it is terminated most rapidly and efficiently at +1° to +3° C. It is terminated slowly at —6° C., but at temperatures below this the rate increases rapidly, at any rate for the later stages of diapause. The eggs freeze at —26° to —28° C.; this kills them.

The treatment of *L. coarctata* eggs at temperatures around —20° C. makes it possible to shorten the length of this stage from about six to three months. This is useful for laboratory rearing, and it also greatly extends the period during which newly-hatched larvae can be made available for experimental work.

Wheat bulb fly Leptohylemyia coarctata *Fall.*

Laboratory rearing. A paper on this subject by R. Bardner and J. Kenten is now in the press. Bad weather consistently hindered the routine collection of adult females for breeding purposes, but finally about 16,000 eggs were obtained for experimental purposes. Research into a suitable method of rearing larvae on an artificial diet is continuing.

Insecticidal control (R. Bardner)

Box experiments. Thirteen different insecticides were formulated as seed dressings (γ-BHC, heptachlor, parathion, dieldrin, endrin, isodrin, aldrin, DDT, " Chlorothion ", " Metasystox ", " Systox ",

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" Dipterex ", " Diazinon ") and a comparison made of the protection they gave against wheat bulb fly attack. The technique used was similar to that described in previous annual reports. None was outstandingly better than γ-BHC, dieldrin or aldrin, though heptachlor and parathion were comparable, and our formulations of these and other materials are being compared in small field-plot trials by the National Agricultural Advisory Service. This work is being continued.

A comparison of different methods of applying dieldrin seed-dressing to wheat showed that the phytotoxicity experienced in last year's field experiment was largely due to treating the wheat with acetone, which was used as a solvent for the dieldrin.

Field experiment. In 1955–56 the experiment in Pennels Piece was in its second season. The general design was described in *Rep. Rothamst. exp. Sta. for* **1955**. There were 5 different treatments in 5 randomized blocks of 6 plots each, but it was observed that a drought in April was associated with a decline in larval numbers in all blocks except one, situated on very wet soil. This is the second year in succession that a drought in late spring appeared to kill larvae, and this may be an important factor in controlling the wheat bulb fly population on heavy clay soils.

The treatments were as follows: (1) untreated (2 plots per block); (2) 4 per cent dieldrin dust at 1 cwt./acre combine-drilled with seed on 1 November 1955; (3) 4 per cent dieldrin dust at 1 cwt./acre broadcast on surface on 16 February 1956; (4) sprayed with parathion on 8 March 1956 at 0·1 per cent v/v., 100 gal./acre; (5) sprayed with parathion on 12 April 1956 at 0·1 per cent v/v. at 100 gal./acre.

Though larval numbers were comparable with those recorded for 1955, the plants had tillered well by the time attack occurred and, as a consequence, the proportion of attacked tillers was lower. Table 2 gives the results of sampling on 27 April 1956.

TABLE 2

Effect of insecticides in reducing plant damage by wheat bulb fly *

	Un-treated	Dieldrin, combine-drilled	Dieldrin, broadcast	Parathion, early spray	Parathion, late spray
Damaged tillers	341	40	61	199	131
Decrease ...	—	301	280	142	210

Least significant difference at 5 per cent level between treatments, 88.

Least significant difference at 5 per cent level between untreated and treated, 76.

* All numbers in thousands per acre.

At the 5 per cent level all treatments showed a reduction of damaged tillers compared with the controls. There was no significant difference between the dieldrin treatments or between the parathion treatments. The broadcast treatment was significantly better than the early parathion spraying, but not the late spraying treatment and appeared to be rather slow in killing larvae compared with the combine-drill treatment. At harvest, however, the broadcast treatment was the only one to give a significant increase in yield, as shown in Table 3.

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TABLE 3

Effect of insecticides for wheat bulb fly control on grain yields

Grain yield, cwt./acre (85 per cent dry matter)	Untreated	Dieldrin, combine-drilled	Dieldrin, broadcast	Parathion, early spray	Parathion, late spray
Mean	46·2	45·9	52·3	45·7	48·5
Increase ...	—	−0·3	+6·1	−0·5	+2·4

Least significant difference at 5 per cent level between treatments, 4·5.
Least significant difference at 5 per cent level between untreated and treated, 3·9.

The possibility that the broadcast treatment was controlling a pest attacking the crop after the conclusion of the bulb-fly attack is being investigated. Further work on the optimum dosage rates and time of application of these treatments is in progress. The results of the first three seasons' work on insecticidal control are now being prepared for publication.

Mode of action of the insecticides. Further laboratory and pot experiments by M. J. Way and R. Bardner confirmed that γ-BHC-treated plants are distasteful to newly hatched larvae of *L. coarctata*. Aldrin-treated plants were attacked, but the larvae died after feeding for varying periods of time within the stems. Experiments to determine whether the γ-BHC-treated plant was toxic as well as repellent and whether the action of aldrin and dieldrin was due to their systemic properties gave inconclusive results. The work is being continued.

Bean aphid Aphis fabae *Scop.*

Effect of planting date on infestation and control. This experiment by M. J. Way and T. Doherty was essentially similar to one done in 1955. Field beans were planted at four different dates and, for each planting date, yields of unsprayed plots were compared with those of plots sprayed once and twice with "Metasystox" (0·05 per cent active ingredient and 100 gal./acre). Aphid counts were made at weekly intervals from the beginning of June to the end of September.

As in 1955, few aphids migrated to the bean crop in June, but the populations never built up even on the late-sown crops. The aphid numbers on unsprayed plots never rose above an average of 1·4 per plant, compared with a peak of 4,170 per plant in the 1955 experiment. The weather (hot and dry in 1955 and cold and wet in 1956) is considered to be the main cause of the different types of population which developed.

Table 4 shows that the insecticidal treatments gave some significant increases in yields of grain. It seems unlikely, however, that these result from control of the very few aphids present.

This series of experiments, which will probably be completed in 1957, shows that insecticidal control is always justified when there is a big migration of bean aphids from the winter host to beans in June. When there is a small migration, an insecticide should be applied if the crop is sown late. This ensures control of damaging

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aphid populations which might otherwise develop if the weather is favourable.

TABLE 4

Effect of insecticides to control Aphis fabae *on grain yield of field beans*

Time of spraying	Grain yield, cwt./acre, few different sowing dates			
	10 March	3 April	24 April	15 May
Untreated	26·7	26·9	20·9	13·7
22 June	28·5 *	29·5 *	21·6	13·9
11 July	27·6	28·9 *	22·1	12·9
22 June and 11 July ...	26·1	28·3 *	20·7	14·7

* Significantly different from untreated at 5 per cent level.

Relative importance of biological and chemical control. This work is being continued by M. J. Way and T. Doherty in collaboration with C. J. Banks of the Entomology Department.

Naturally occurring populations of *A. fabae* and its parasites and predators were estimated on various host plants, including *Euonymus europaeus*, field beans and *Chenopodium* sp. In addition, caged plots of these plants were artificially infested with aphids. One type of cage covered with dieldrin-treated terylene netting was used to exclude predators and parasites, while another type which gave comparable climatic conditions allowed free access to the natural enemies. The aphid populations developing in predator-free and predator-exposed plots were determined. With field beans the crop yields were compared with those of plots treated with an aphicide spray. These gave some measure of the relative efficiency of biological and chemical control.

Natural populations of *A. fabae* on *Euonymus* in spring were very small, and few alatae migrated to field beans in midsummer. Overwintering predators and their progeny which stopped build-up of aphids on *Euonymus* were unable to multiply on the few aphids which developed on beans and later on *Chenopodium*. However, between October and December, when most predators had begun hibernating, populations of the aphid developed on *Euonymus* and laid eggs.

The combination of fairly big overwintering aphid populations and small predator populations should lead to populations of aphids on beans during 1957 (at any rate in the Harpenden area), which should justify insecticidal control.

The control of the vectors of potato virus diseases

The experiments on the control of the spread of aphid-borne potato viruses carried out by P. Burt in collaboration with L. Broadbent of the Plant Pathology Department were continued this year.

Previous work (see *Rep. Rothamst. exp. Sta. for* **1955**, 129) had suggested that applying DDT emulsion at low volume might be as effective as application at high volume in controlling potato aphid populations. In the main experiment the ability of DDT emulsion applied at high or low volume 4 or 6 times during the season to prevent the spread of leaf-roll virus and virus Y was tested. A spraying machine with some unusual features, including an unorthodox arrangement of spray-jets, was lent us by courtesy of the Colorado

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Beetle Machinery Depot of the Ministry of Agriculture and used throughout the experiment; it performed very well. Aphid control was good, although the weather seriously interfered with the spraying programme (see report of the Plant Pathology Department).

Farmers carrying out commercial seed-retention trials have questioned the necessity of using underleaf as well as overhead jets when spraying potato crops to control aphids. A small, preliminary, non-replicated experiment was done to see what would be the effect on an aphid population of: (1) applying DDT emulsion to a potato crop from overhead jets only (plot A), and (2) from overhead and underleaf jets (plot B); in both treatments 2 lb. DDT was applied per acre. The sprayed plots were 8 rows wide and about 30 yards long, and were separated by 18 unsprayed rows which served as a control (plot C). The crop had not been sprayed previously and was only sprayed once during the experiment (23 July). Aphid counts on upper, middle and lower leaves were done immediately before spraying and at intervals afterwards until 25 days after the crop was sprayed, 5 counts being carried out in all.

After spraying, on plot B the number of aphids fell rapidly to a fairly low level, thereafter remaining nearly constant until the end of the experiment. The number on plot A fell much more slowly, so that even after a week the populations on plots B, A and C were 265, 696 and 1,290 per 100 leaves. The population on plot A continued to fall steadily and finally coincided with that on plot B on the seventeenth day after spraying (9 August); numbers on B, A and C were then 158, 158 and 1,162. The populations on all plots then declined rapidly from natural causes.

From the third day after spraying onwards, the difference in numbers of aphids found in the two sprayed plots was confined almost entirely to those on the lower leaves; as the lower leaves carried most of the aphids in the crop (during the 25 days the experiment lasted, aphids were between 1·1 and 1·9 times as numerous on the lower leaves as they were on the upper and middle leaves taken together) the difference was important.

The results suggest that to reduce aphid numbers in a potato crop quickly with contact insecticides, the plants must be thoroughly covered with insecticide, and that results obtained with underleaf spray jets as well as overhead jets are superior to those obtained with overhead jets only. Further information is needed about the distribution of aphids on the plants throughout the season and on the effect of repeated overhead sprays.

The percentages of virus spread which occurred in the 1955 experiment are given in the report of the Plant Pathology Department, together with information on other potato virus experiments.

Fungicides

Time, temperature and toxicity of fungicides (A. H. McIntosh)

The slide-germination method for testing fungicides *in vitro* has been adapted for use with Böttcher's slides. The cells are filled to the top and then closed with cover glasses. In this way, drops of spore suspensions can be kept for up to 3 days without any evaporation; if there is no cover glass, evaporation from a 0·2-ml. drop may

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be as much as 47 per cent in 2 days at 10° C., even in an atmosphere " saturated " with water vapour.

This slide method was used with spores of *Botrytis fabae*, which were exposed continuously to solutions of water-soluble fungicides for up to 3 days at each of three temperatures (10°, 20° and 25° C.). Counts of the numbers of spores germinating were made each day. The " optimum " temperature for germination of this species is about 25° C. Germination occurred more quickly at 25° C. than at any lower temperature; but the percentage of spores which finally germinated was hardly affected by temperature in the range 10–25° C. It was common to find that at least 90 per cent of the spores had germinated within 24 hours at 10° C. The upper lethal temperature is about 28° C.

The effects of temperature on toxicity seem to be much more important than those of time. The following compounds had positive coefficients of toxicity between 25° and 10° C. : *n*-butyl alcohol (3·1 in 24 hours), *tert*-butyl alcohol (3·0 in 24 hours), mercuric chloride (6·0 in 24 hours) and mercuric bromide (3·4 in 24 hours). These poisons were all at their most toxic at the " optimum " temperature for germination and not, as might have been expected, at 10° C. In contrast, the toxicities of the following compounds were almost independent of temperature in the same range : 8-hydroxyquinoline sulphate, sodium pentachlorophenate, phenyl mercuric acetate, acetic acid, copper sulphate, silver nitrate and mercuric acetate.

The difference between the temperature coefficients of the mercuric halides and that of mercuric acetate may be associated with the fact that the halides are unionized in solution, whereas mercuric acetate is partly ionized.

The alcohols are " physical " poisons. *n*-Butyl alcohol is only partly soluble in water, and is less soluble at 25° C. than at 10° C. The increased toxicity of *n*-butyl alcohol at 25° C. was not a " Ferguson " effect, because *tert*-butyl alcohol, which is soluble in water in all proportions, gave a temperature coefficient of the same size.

The effects of time on toxicity were very small. There was often a slight increase in median lethal concentration between the first and third days. This increase, if it occurred at all, was always greater at 10° C. than at the other temperatures. Mercuric bromide showed the greatest increase of this type (1·7 times); there was a corresponding increase in the size of the temperature coefficient of toxicity.

Fungal physiology in relation to bioassay of fungicides

The relation between spore germination and infectivity has been studied by F. T. Last. With increasing age of culture, the ability of *Botrytis fabae* conidia to infect broad bean leaves, *Vicia faba*, is lost more rapidly than their ability to germinate on glass slides. Although 80 per cent of conidia from 40 day-old cultures germinated, only 10 per cent from 20 day-old cultures could infect.

Similarly, a shorter exposure to ultra-violet is necessary to reduce the infectivity of *B. fabae* conidia than to reduce the ability to form colonies on agar. When the logarithm of the percentage survival is plotted on the ordinate against the duration of exposure

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to ultra-violet, and when the straight part of the curve is extrapolated to the ordinate, the point of intersection gives a measure of the number of viable centres which have to be inactivated. Four and 8 centres were inactivated to prevent infection and germination respectively.

As with *B. fabae*, ultra-violet reduces the ability of *Erysiphe graminis* conidia to infect barley more readily than the ability to germinate on glass slides.

Griseofulvin and club-root of cabbages caused by Plasmodiophora brassicae

In more precise experiments by F. T. Last in collaboration with I. Macfarlane of the Plant Pathology Department, the retardation of club development when griseofulvin was sprayed on to the foliage was not found to be caused by griseofulvin translocated after absorption through the leaves. It was, however, caused by the absorption through the roots of griseofulvin which had reached the soil.

In addition to retarding club development, soil applications reduced the number of plants clubbed. As before, there was a strong interaction between inoculum concentration and the percentage of plants clubbed—the lower the inoculum concentration, the fewer the plants clubbed.

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