II.

LABELLED INSECTIDE STUDIES: TECHNIQUES

SOME APPLICATIONS OF RADIOISOTOPES TO THE STUDY OF THE CONTAMINATION OF INSECTS BY INSECTICIDE SOLUTIONS*

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Abstract — Résumé — Аннотация — Resumen

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In subsequent investigations, dieldrin-C^{14} has been used in solution in oils labelled with tritiated hexadecane. In comparative experiments with Tribolium castaneum exposed to three solutions of different viscosity, appreciable differences in both the rates of diffusion over the insects and in absorption through the cuticle have been found. After an initial period, dieldrin is absorbed relatively faster than solvent, the magnitude of the differential absorption varying with viscosity.

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Au cours d'études ultérieures, la dieldrine marquée par ^{14}C a été utilisée en solution huileuse marquée à l'aide d'hexadécane tritié. Des expériences comparatives sur le Tribolium castaneum, exposé à trois solutions de viscosités différentes, ont révélé des différences sensibles en ce qui concerne les vitesses de dispersion sur l'insecte et l'absorption à travers la cuticule. Après une période initiale, la dieldrine est absorbée à une vitesse relativement plus grande que le solvant, la différence étant fonction du degré de viscosité.

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В последующих исследованиях использовался меченный C^{14} дильдрин в растворе в маслах, меченных насыщенным тритием гексадеканом. В сравнительных опытах с использованием Tribolium castaneum в виде трех растворов различной вязкости была обнаружена заметная разница как в отношении скоростей диффузии у насекомых, так и в отношении абсорбции через кутикулу. После начального периода дильдрин абсорбировался быстрее, чем раствор, а величина дифференциального поглощения менялась в зависимости от вязкости раствора.

EMPLEO DE LOS RADIOISÓTOPOS PARA ESTUDIAR LOS EFECTOS CONTAMINADORES DE LAS SOLUCIONES INSECTICIDAS. Las primeras investigaciones se hicieron utilizando como marcador diyodo-octadecano-I^{131} en solución oleosa. Esta sustancia ha resultado útil para estudiar la digresión de las películas de

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aceite sobre la epicutícula de los insectos, pero es inadecuada para investigar la absorción de aceites por la cutícula, pues una vez en los tejidos se transforma en uno o más derivados solubles en agua.

En investigaciones consecutivas se empleó Dieldrin-^{14}C disuelto en aceites marcados con hexadecano tritiado. Comparando los experimentos realizados con Tribolium castaneum tratado con tres soluciones de viscosidad diferente se han podido observar diferencias apreciables en los índices de difusión en los insectos y de absorción cuticular. Después de un período inicial, la velocidad de absorción del Dieldrín es relativamente más rápida que la del disolvente; la diferencia depende de la viscosidad.

INTRODUCTION

When an oily solution of an insecticide is picked up by an insect, the oil facilitates the action of the insecticide in at least two ways: firstly, by spreading the insecticide over the epicuticle of the insect and thus presenting it very intimately to the insect; secondly, by facilitating the diffusion of the insecticide through the cuticle. These processes have not hitherto been studied quantitatively, and indeed it is difficult to see how they could be without the employment of radioactive tracer techniques, as no chemical method of analysis for small quantities of hydrocarbon oils is known.

Two series of experiments on this subject have been carried out. The first series was principally concerned with the spread of oil over the cuticle of active insects, for which purpose iodine-131 in the form of an iodized hydrocarbon was employed as a tracer. The second series was particularly concerned with the penetration of both oily solvent and an insecticide, dieldrin, through the cuticle. In this series the oils and insecticide were labelled with tritium and carbon-14 respectively.

1. EXPERIMENTS WITH DI-IODO-OCTADECANE AS AN OIL TRACER

Materials and methods

Traces of di-iodo-octadecane-I^{131}, prepared by the addition of iodine-131 to the unsaturated hydrocarbon octadecene, were added to a highly refined mineral oil, Shell Risella 17, which consists of hydrocarbons of chain length varying from 17 to 20 carbon atoms. The specific activity of the oil sample was adjusted to 2 mc/ml. This tracer was selected because of its suitability for liquid and solid counting techniques using Geiger-Müller tubes, the only methods of radio-assay available in the department at that time.

The oil deposits used in the experiments were always of 3 μl/cm^2 evenly deposited on Whatman No. 1 filter papers. Blowflies of the species Phormia terraenovae R-D were exposed to the oiled substrate at 25°C for varying periods of time up to 60 min, by a method described previously [1].

Immediately after removal from the active substrate, some of the flies were rapidly dissected to enable assays of oil associated with different parts of the body to be carried out. The films of oil adhering to the epicuticle of whole or part insects were washed off by three successive rinses of petroleum hydrocarbons, boiling range 40-60°C. This treatment was shown by control experiments with untreated flies to remove the epicuticular waxes. It is therefore clear that the adherent oil was totally removed, together with the fraction taken up in the epicuticular wax.

The rinsed insects were then ground up in anhydrous sodium sulphite and extracted, first in ether, then in 10% NaOH to obtain the absorbed tracer. Washings and extracts were assayed with a Geiger-Müller liquid counter tube. The ground residue was finally dried and checked for any residual activity with a thin-end-window Geiger-Müller counter.

Other individuals were dissected and mounted between 2 layers of thin adhesive tape weighing 8.06 mg/cm^2, and the preparations were exposed on X-ray film for one week. From the resulting autoradiographs, the location of oil on the body surface could be determined and its rate of spread gauged qualitatively.

Results

A brief summary of the more significant results only will be given, to illustrate the scope and limitations of this tracer substance in the study of oily films on insects. Some aspects have been partially described elsewhere [2].

Two processes were distinguished in the contamination of an insect on an oily deposit. Firstly, there is the primary uptake of oil by direct transfer from the substrate to the tarsi of the insect; secondly, there follows a diffusion of oil away from the tarsi up the legs to the general body surface.

Initially, oil was taken up by the tarsi at a mean rate of 3×10^{-7} ml/min per fly, the rate of uptake remaining substantially constant so long as the fly was actively walking. The oil diffused over the surface of the insect away from the tarsi at the rate of approximately 1.5×10^{-7} ml/min to all parts of the body.

The wings accumulated oil at 15×10^{-8} ml/min. Since the molecular surface area of the wings of this strain of the species is known, having been calculated by gas-adsorption methods [3], it is possible to calculate the mean thickness of oil film on the wing for any given treatment. It appears that if the oil molecules are randomly oriented on the surface, a monomolecular layer is established in 5 - 15 min from the initial contact of insect with deposit.

Autoradiographs confirm that the wing membranes are covered with tracer molecules after this period. They also reveal higher concentrations of radioactivity, indicating oil, along veins and at the confluence of wing veins. Similarly, they show uneven distribution in other parts of the body, especially the legs, the tarsi being, of course, more heavily contaminated than the upper segments.

If the oil were spreading by normal surface-tension forces, these differences would certainly be lost and an even film would be established quite soon after the removal of fly from deposit. But the differences persist without change, as successive autoradiographs show. This evidence, coupled with the fact that uptake varies with the activity of the fly, suggests that the oil proceeds over the cuticle aided by the very small elastic deformations of the cuticle which occur during active movements of an insect [4]. When the insect is killed, the movement of the very thin oil film ceases.

The oil, advancing at first as a mono-molecular layer, is simultaneously being absorbed. When we consider the absorption of oil, the limitations

of this tracer compound as a guide to oil movement become apparent, for the halogenated hydrocarbon tracer may well be differentially absorbed from the oil. But a second disadvantage also became apparent when measurements of the rate of absorption of tracer were made. Not all the I^{131} could be recovered in ether extractions; for example, after 60 min only 12.8% of the I^{131} was extracted in ether, 55.6% of the residual I^{131} was recovered with boiling water and 98% of the remainder was extracted with 10% NaOH solution. Thus it is clear that the I^{131} moiety of the tracer molecule is converted after absorption to one or more water-soluble compounds.

To sum up, di-iodo-octadecane-I^{131} has proved useful as a tracer for oil movements on the surface of an insect, but is not suitable as a tracer for oil movements across the cuticle or within the tissues.

2. EXPERIMENTS WITH DIELDRIN-C^{14} AND HEXADECANE-1:2-H^3

Materials and Techniques

Dieldrin-C^{14} of specific activity 6.54 mc/mM, obtained from the Radiochemical Centre, Amersham, was dissolved in three solvents of differing viscosity to form 1% solutions. The three solvents were n-hexadecane and the Shell oils Risella 17 and Risella 33, having viscosities of 2.9, 22.1 and 187 cP, and hydrocarbon chain lengths of 16, 17-20 and 20-35, respectively. The labelled hydrocarbon n-hexadecane-1:2-H^3 of specific activity 207 mc/mM was also added to each solution to allow the tracing of the hydrocarbon solvent molecules. The specific activity of the n-hexadecane-1:2-H^3 in each oil sample was adjusted to 27.5 mc/ml.

Filter-paper slips were treated as before to provide a deposit of 3 ml/cm^2 of solution, and batches of insects of the species Tribolium castaneum Herbst. were allowed to crawl over the deposits of 25°C for periods ranging from 30 min to 24 h.

After removal from the deposit, batches of ten insects were washed with three rinses of petroleum hydrocarbons (boiling range 40-60°C) to remove the adherent solution. The washed insects were then ground up with anhydrous sodium sulphite and extracted first with hexane for 2 h, then with water to recover absorbed oil, insecticide and any water-soluble metabolites of the insecticide which might be formed.

The quantities of carbon-14 insecticide and tritiated hydrocarbon in each sample were assayed and discriminated by means of internal liquid scintillator techniques. The scintillation liquids, obtained already formulated from Nuclear Enterprises (G.B.) Ltd., were NE213 based on xylene for the hexane extracts and external washes, and NE220 based on dioxane for the aqueous extracts. In the xylene-based scintillator, the counting efficiency was 6.5% for H^3 and 73% for C^{14}, under the conditions of test. In the dioxane-based scintillator, the maximum efficiency for C^{14} was rather lower, though it should be mentioned that the presence of relatively high concentrations of sodium sulphite caused the formation of a flocculant precipitate when the sample was added to the scintillator solution. This introduced much noise of a very low energy level, possibly due to chemiluminescence, which would have interfered with tritium assay, but could be discriminated from the C^{14} response.

Results

Adherent film of solution

The insects became contaminated more rapidly with the hexadecane solution than with the more viscous oil solutions, differences becoming apparent from the first measurement, taken after 30 min, when less than 1×10^{-8} g of dieldrin had been accumulated.

In contrast, at first no significant differences between the rates of contamination with Risella 17 and Risella 33 solutions were found. Even after many hours exposure, differences between the adherent quantities of these two solutions were relatively small.

The faster rate of uptake of hexadecane solution is maintained for about 16 h. At this time the insects are moribund or very feeble, and the rate of uptake of all three solutions falls to a much lower level (Table I). As

TABLE I

ADHERENT FILMS: QUANTITIES OF SOLVENT AND DIELDRIN RECOVERED FROM THE EXTERIOR OF TRIBOLIUM CASTANEUM AFTER EXPOSURE TO 3-μl/cm^2 DEPOSITS OF 1% SOLUTIONS ON FILTER PAPER

Duration of exposure (h)	Hexadecane solution	Risella 17 solution	Risella 33 solution
	SOLVENT ($\times 10^{-6}$ ml)		
0.5	0.92	0.28	0.34
1.0	1.37	0.61	0.47
2.0	2.28	0.83	1.02
4.0	2.37	0.85	1.08
8.0	3.61	2.15	1.75
16.0	9.0	2.52	2.33
24.0	9.31	3.19	2.56
	DIELDRIN ($\times 10^{-8}$ g)		
0.5	0.75	0.31	0.22
1.0	1.15	0.62	0.37
2.0	1.67	0.76	0.91
4.0	2.10	0.92	1.19
8.0	4.34	2.01	2.29
16.0	8.70	3.18	2.80
24.0	9.24	3.3	2.95

would be expected, the ratio of insecticide to solvent in the adherent films does not differ from that in the original solutions applied to the substrate.

Absorption of solvent and insecticide

The three solvents were absorbed through the cuticle at a rate of approximately 2×10^{-7} ml/h at first, becoming progressively slower. There were no appreciable differences between the rates of absorption of the three solvents (Fig. 1).

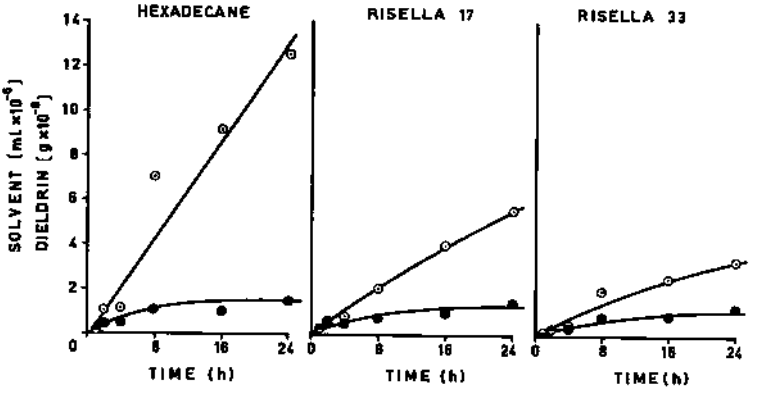

Fig. 1

Quantities of solvent and dieldrin extracted from the tissues of Tribolium castaneum Herbst. after exposure to 3-µl/cm² deposits of 1% solutions on filter paper

○ Dieldrin absorbed
● Solvent absorbed

However, the rates of absorption of dieldrin from the three solutions varied considerably. In the first hour, the average rate of uptake of dieldrin was relatively slower than that of the solvent. Subsequently, the insecticide was absorbed relatively faster than the solvent, the rate of uptake of dieldrin being approximately linear for each formulation (Fig. 1).

The magnitude of the differential absorption of dieldrin from each solution is illustrated by Table II, where the differential is expressed as the ratio (absorbed dieldrin over absorbed solvent) × 100. This ratio would be 1.0 for uniform absorption of both components of a 1% solution, but it will be seen that it rises steadily within 16 h to values of 8.36, 4.14 and 2.96 for solutions in hexadecane, Risella 17 and Risella 33 respectively.

Water-soluble metabolites

No evidence of water-soluble derivatives of dieldrin was found in the extracts.

TABLE II

DIFFERENTIAL ABSORPTION OF DIELDRIN FROM THREE 1% SOLUTIONS BY TRIBOLIUM CASTANEUM.

The differential is expressed as the ratio (absorbed dieldrin/absorbed oil) ×100

Treatment (h)	Hexadecane solution	Risella 17 solution	Risella 33 solution
0.5	1.09	0.25	0.63
1.0	0.88	0.79	0.82
2.0	2.87	1.16	1.01
4.0	2.74	1.65	1.40
8.0	6.45	2.81	2.43
16.0	8.36	4.14	2.96
24.0	8.46	3.96	2.74

Discussion

Although octadecane-di-iodide-I^{131} has certain limitations as a tracer for oil movements, significant results were obtained from its use; in particular, the importance of active movements of the cuticle in facilitating the uptake and spread of very thin oil films over an insect was demonstrated. However, the physical properties of the solvent also affect the rate of uptake, at least for films several molecules thick, for when crawling insects (Tribolium castaneum) were exposed to three solutions of different viscosity labelled with n-hexadecane-1:2-H^3, the least viscous was picked up appreciably faster than the more viscous (Table I).

The effects of solvent viscosity on the relative toxicities of different insecticide solutions has been noted previously from biological assays with Aëdes aegypti L. and Musca domestica L. [5]. The results of the present work permit the factors concerned to be analysed in greater detail.

The rates of absorption of the three solvents through the living cuticle were not significantly different, despite the differences in molecular size (Fig. 1). This result contrasts with earlier observations that light oils penetrate isolated pieces of cuticle faster than heavier oils [6]. The fact that no such differences were revealed in the present work suggests that the absorptive capacity of the hypodermal cells exercises a limiting effect on the rate of solvent absorption through living cuticle. The progressive decline in solvent absorption rate with time provides evidence of a saturation phenomenon.

The concentration of dieldrin in the film adhering to each insect and the rate of solvent absorption are the same for each formulation. Therefore, if the solvent facilitated the entry of dieldrin principally by carrying

the insecticide molecules in association with solvent molecules through the cuticular barriers, the quantities of dieldrin absorbed would be the same for each formulation.

But the quantities of dieldrin absorbed vary with solvent viscosity, and are much greater than would be expected on the basis of a direct solvent carrier effect (Fig. 1, Table II). Indeed, the capacity of the tissues to absorb dieldrin is considerable, and is not diminished by death, for the uptake of insecticide varied linearly with time and, unlike the solvent absorption, showed no appreciable indication of a saturation effect within a period of 24 h. Differential absorption of dieldrin from the adherent film was made good by diffusion from the substrate.

If neither concentration gradients nor direct carrier effects can explain the differences between the rates of dieldrin absorption from the different solvents, the facilitated absorption must depend almost entirely upon a modification of the cuticular barrier by the intercalation of solvent molecules, permitting faster transfusion of insecticide molecules than would otherwise occur. WIGGLESWORTH [6] has shown that the wax layer delays the absorption of pyrethrin solutions by Rhodnius nymphs. One likely effect of the oily solvents in the present experiment is to disturb the organization of the epicuticular wax. The smaller are the molecules of the solvent hydrocarbons dispersed in the wax layer, the faster the diffusion of dieldrin would be expected to take place.

In this context, it would be of interest to compare the effects of different solvents on insecticide absorption by a species having a structurally different type of epicuticle, e.g. blowfly larvae. Indeed, much remains to be done before the mechanisms of insecticide absorption are fully understood; it is certain that radioactive tracer techniques will be of great value in such studies.

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DISCUSSION

J. HALBERSTADT: The author reports no significant difference in the cuticle absorption rates as between hexadecane, Risella 17 and Risella 33, using tritiated hexadecane as tracer for all three solutions. But is he not in fact measuring the absorption of hexadecane in all three cases, so that there may be a difference of absorption between hexadecane and the two Risella oils? If that were the case, then there might be a much smaller difference in spreading rates between hexadecane and the two Risella oils,

which could be explained by assuming that the tracer disappears out of the Risella oil by absorption in the cuticle. That would convey the impression that the Risella oil spreads more slowly, while in fact the oil, now less active or inactive, might go on spreading at much the same rate as hexadecane.

C. T. LEWIS: The tracer must be giving a faithful measure of the absorption of the hexadecane solvent as it is chemically identical, and we are led to the conclusion that the facilitated absorption of dieldrin depends on a modification of the cuticular barriers and on this result alone; for much more dieldrin is absorbed than could be transported directly in the volume of solvent absorbed. The smaller absolute quantities of dieldrin-C^{14} absorbed from the Risella oils show that the cuticular barrier is less affected by the longer oil molecules, independently of the effect on the volumes of oil absorbed. Thus my principal conclusions still obtain.

If, as you suggest, the tracer is absorbed differentially from the Risella oils, the longer but otherwise similar Risella molecules must be penetrating more slowly than the tracer. In that event, the differential absorption of dieldrin relative to Risella oils would be greater than Table II suggests.

However, I still favour the view that the hexadecane-H^3 tracer moves proportionally with the oils in which it is dissolved, or very nearly so. That is certainly the case for movements of oil on the cuticle surface, to take your second point, which was that a differential absorption of H^3 tracer from the Risella oils would give an impression that the oils were spreading more slowly than the hexadecane solvent. That could be true only if the H^3 tracer was absorbed faster from the Risella oils than from the hexadecane; but the results of Fig. 1 show that this is not the case.

Moreover, if the totals of H^3 tracer, absorbed plus adherent, are added for the three solvents, it will be seen that the total for the hexadecane solvent taken up on the insect is much greater than the total for the Risella oils. Thus the H^3 tracer is not behaving independently of the oils in which it is dissolved.

D. A. CROSSLEY: I recall reading a paper published by Cumpel and Casida in 1957 on the metabolism of I^{131} by the cockroach Periplaneta, in which ingested I^{131} was found to be concentrated in the cuticle. If that is really a site of iodine concentration, it might explain some of your difficulties with I^{131}.

C. T. LEWIS: Yes, I think that the use of the iodized hydrocarbon for experiments on absorption is not acceptable.

F. T. PHILLIPS: I would like to ask the author whether any humidity effects could be observed. The evidence suggests that movement of the monolayer of oil is caused by small elastic deformations of the cuticle. Is this true for the wings, or could the spread of oil be facilitated or affected in any way by a presorbed layer of moisture? This could perhaps be shown in experiments with different humidities. Also, was it possible in your experiments that some of the oil was picked up on the wings by contact with the body?

C. T. LEWIS: Elastic stresses do occur in the wings, which possess several groups of campaniform sensilla which respond to such stresses. I have no information about the influence of adsorbed moisture on the creep of oil. It is possible that some of the oil passing to the folded wings may

have been transferred by incipient contact of the membranes proximally with the dorsal thorax, but the autoradiographs indicate the oil movement is principally by way of the articulations. If we assume oil was transferred by contact with the body, that still leaves unexplained the creep of oil over the whole of the wing. For that, one must still accept some suggestion of active cuticular movements which are on a sub-microscopic scale. Working with Dysdercus, which has much more rigid forewings than hindwings, I found that the spread occurs much more readily on the forewings than on the flimsy hindwings and this might very well be correlated with the elasticity of two types of wings.

K. van ASPEREN: Could the difference in absorption of dieldrin possibly depend on a difference of solubility in the oils used?

C. T. LEWIS: No; dieldrin has practically the same solubility in all three solvents used. Moreover Hadaway and Barlow (1958) found that the relative toxicities of solutions of DDT, and, I believe, of dieldrin also, did not vary with solubility in different solvents when topical applications were applied to Musca and Aëdes.

W. KLOFT: The ideal subject for this type of studies might be coccids (scales) of the type of Diaspididae, and this for two reasons. First, the females are more or less immobile, since they have no legs to allow screening movements and spreading of the insecticide over the cuticle in that way. Secondly, Diaspididae are well protected and covered by their scutum (scale) and must be controlled with oily solutions (or preparations in the vapour phase) since they suck in the plant's parenchyma and do not therefore pick up systemic insecticides. Your interesting method could be used to study how the oily film spreads from the scutum over the scale-body.

C. T. LEWIS: Your suggestion is very appropriate. A study of the uptake of insecticide from oily solutions and from the vapour phase by a scale insect is now in progress in my department. You mentioned that the oil could not be spread around by the legs. I would like to clear up any misunderstanding here. In the experiments on the creep of oil over the blow-fly, the insects were exposed only to oil and iodized tracer. There was nothing irritant in the solution and therefore no cleaning movements were performed. If a fly did perform random cleaning movements during the course of the experiment then it was noted and the results were not used for determining the rate of spread of oil, so that the spread that I refer to is not interfered with nor in any way assisted by the cleaning movements of an insect. Of course, with an insecticide solution in the field cleaning movements would be important.

J. R. OGLE: Like an earlier questioner, I would be inclined to doubt the validity of using hexadecane-H^3 to trace the rate of passage of a much heavier oil through the insect cuticle. Could you check this point by repeating the experiment with another tracer of rather higher or lower molecular weight? If the ratio of uptake of activity were then identical for the three oils, but different from those obtained with hexadecane, it would in fact suggest that you were measuring the rate of uptake of the tracer rather than the oil as a whole, and conversely, if the results were all in agreement, that would be confirmatory evidence that the nature of the solvent had little effect on its rate of uptake.

C. T. LEWIS: I would have liked to carry out such experiments. But a hydrocarbon of lower molecular weight than hexadecane would be too volatile for accurate work. No tritium-labelled paraffinic hydrocarbon of higher molecular weight is at present available, and the cost of synthesizing one would be considerable.

THE APPLICATION AND MEASUREMENT OF LABELLED RESIDUAL INSECTICIDES IN SOME PHYSICO-CHEMICAL STUDIES*

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Abstract — Résumé — Аннотация — Resumen

THE APPLICATION AND MEASUREMENT OF LABELLED RESIDUAL INSECTICIDES IN SOME PHYSICO-CHEMICAL STUDIES. The disappearance of residual films of insecticides from plant and other surfaces may be conveniently studied if the insecticide is labelled with a radioisotope of sufficient radiant energy to allow for a simple measuring technique.

Methods of application of insecticide solutions on different surfaces led to the design of a spray chamber suitable for the distribution of very small amounts (a few drops) of radioactive liquid formulations over a 35-cm² circular area.

Some measurements of the rates of volatilization of Cl^{36}-labelled dieldrin and aldrin crystals from glass surfaces are included.

APPLICATION ET MESURE DES INSECTICIDES RÉMANENTS MARQUÉS DANS CERTAINES ÉTUDES PHYSICO-CHIMIQUES. Pour étudier la disparition des pellicules que forment les insecticides rémanents sur les végétaux et d'autres surfaces, on a avantage à les marquer au moyen de radioisotopes ayant une énergie radiante suffisamment élevée pour pouvoir utiliser une méthode de mesure simple.

L'étude des méthodes d'applications des insecticides en solutions sur diverses surfaces a conduit à la mise au point d'un vaporisateur permettant de répartir de très faibles quantités (quelques gouttes) de liquide radioactif sur une surface circulaire de 35 cm².

Le mémoire donne les résultats de quelques mesures des vitesses de volatilisation de cristaux de dieldrine et d'aldrine marqués au ^{36}Cl déposés sur des surfaces de verre.

ПРИМЕНЕНИЕ И ИЗМЕРЕНИЕ МЕЧЕНЫХ ОСТАТОЧНЫХ ИНСЕКТИЦИДОВ ПРИ НЕКОТОРЫХ ФИЗИКО-ХИМИЧЕСКИХ ИССЛЕДОВАНИЯХ. Исчезновение остаточных пленок инсектицидов с растений и других поверхностей может быть легко изучено, если инсектициды мечены радиоизотопами с достаточной энергией излучения, позволяющей применять простые методы измерения.

В результате исследования методов нанесения растворов инсектицидов на различные поверхности была сконструирована камера, позволяющая распылять очень небольшие количества (несколько капель) радиоактивных жидкостей на круговую поверхность площадью 35 см².

Приводятся некоторые результаты измерений скоростей улетучивания со стеклянных поверхностей, меченных Cl^{36} кристаллов дильдрина и алдрина.

APLICACIÓN DE INSECTICIDAS MARCADOS Y MEDICIÓN DE SUS RESIDUOS EN ALGUNOS ESTUDIOS FISICOQUÍMICOS. La desaparición de las películas residuales de insecticidas que quedan en la superficie de las plantas y en otras superficies puede estudiarse fácilmente si los insecticidas se marcan con radioisótopos de una energía radiante que permita utilizar una técnica de medición sencilla.

Los métodos de aplicación de soluciones insecticidas sobre varias superficies han llevado al diseño de una cámara de pulverización capaz de distribuir cantidades sumamente pequeñas de solución (unas gotas) sobre una superficie circular de 35 cm².

La memoria reproduce los valores hallados para los índices de volatilización de cristales de Dieldrin y Aldrin marcados con ^{36}Cl, aplicados sobre superficies de vidrio.

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INTRODUCTION

In the study of the persistence of residual films of insecticides it is an obvious advantage to be able to follow the variation by a method which does not destroy or disturb the sample which is being measured. This may be done if a radiotracer is incorporated in the insecticide molecule, provided that this tracer truly follows the course of the molecule and does not exchange with similar atoms in different types of molecules and that further conditions are observed, namely, that the radioisotope has a half-life of sufficient duration to enable practical measurements to be made of the amount of radiation present, and that the type and strength of the radiant energy allows for a simple measuring technique. Thus, the chlorinated hydrocarbon residual insecticides may be conveniently studied if they are labelled with Cl^{36}, which has one of the longest half-lives known (3×10^5 yr) and whose β-radiation (0.714 MeV) can be measured simply and with high efficiency by means of a G-M end-window counter.

The laboratory application of thin and even films of insecticides on to a variety of surfaces may be accomplished in several ways with varying degrees of difficulty, but when the insecticides are radioactive (and in addition may have a high chemical toxicity to humans) either the difficulty of the method or the contamination hazards involved may preclude its use. For example, the sublimation of an insecticide from a warm to a cool surface [1], the controlled deposition of which would be especially difficult when using plant surfaces, or the spraying of the insecticide in an apparatus such as the Potter Tower [2], where special precautions would have to be taken, cannot be wholly satisfactory even under the best of conditions. The laboratory methods described in this paper involve a simple spreading method which has often been used but is suitable only for smooth surfaces, and a new spraying method which has been evolved for the distribution of a radioactive insecticide over a leaf surface or other plant surface.

THE VOLATILIZATION RATES OF PURE CRYSTALS OF DIELDRIN AND ALDRIN FROM GLASS SURFACE

Both the dieldrin and aldrin samples, containing Cl^{36} as radiotracer, were of specific activity 100 μc/g. The insecticide was dissolved in a suitable solvent (as described later) at a concentration of 4 mg/ml, and usually a 10 μl portion of this solution (containing 40 μg of insecticide) was spotted with a micro-pipette on to a 20-cm^2 circular area at the centre of a glass plate and spread evenly over this area with the edge of a thin glass coverslip. With experience, reproducible results giving an even coverage of 2 $\mu g/cm^2$ of insecticide crystals after evaporation of the solvent could be obtained by this method. Attempts to obtain an even coverage by wetting the glass surface with the pure solvent and then adding the radioactive solution dropwise, allowing it to spread by diffusion, usually resulted in an uneven coverage.

The crystalline insecticide film was measured radiometrically by placing the glass plate in a specially constructed Perspex castle containing an end-window G-M counter with a window area of 4.5 cm^2. The whole was

enclosed within lead shielding to reduce background counts to approximately 10 counts/min. The upper surface of the glass plate was 3 mm from the end-window, so that the central portion of the 20-cm^2 treated area was presented close to the end-window and a counting efficiency of over 20% was achieved. End-windows made of thin mica are often covered with a fine layer of graphite to eliminate photosensitivity. It is important to remove this layer (by wiping with cotton-wool moistened with alcohol), otherwise contamination of the counter-window by sorption of vapour from the radioactive insecticide will ensue.

Some results obtained for the rates of volatilization of these insecticides from glass surfaces are summarized below. The two ranges of crystal size were obtained by choosing a suitable solvent and allowing crystallization to proceed at room temperature (circa 20°C). "Cellosolve" (ethylene glycol monoethyl ether) gave large crystals and dioxan solvent gave small crystals.

(i) Dieldrin crystals, forming needles 1 - 10 mm long (average 5 mm long) at an initial deposit density of 2 $\mu g/cm^2$, gave a rate of volatilization of 0.06 - 0.08 $\mu g/cm^2 d$ at 20°C.
(ii) Dieldrin crystals, forming a milky-white deposit of needles <0.01-0.05 mm long (average, approx. 0.02 mm long) at initial deposit densities of 2 $\mu g/cm^2$ or 5 $\mu g/cm^2$, gave rates of volatilization of 0.12 - 0.14 $\mu g/cm^2/d$ at 20°C.
(iii) Aldrin, forming a milky-white deposit of rod-shaped crystals of average length approx. 0.01 mm at an initial deposit density of 2 $\mu g/cm^2$ lost most of this deposit at 20°C. after 1 d.

Thus, a reduction in the crystal size of dieldrin of the order of a hundredfold gave a twofold increase in the volatilization rate, although the volatilization rate was independent of the initial deposit density. Furthermore, the volatilization rates were linear, except for very low deposit densities. This shows that the rate of volatilization appears to be partly dependent on the surface area of the crystals forming the deposit, and also, in agreement with a similar observation of HOSKINS [3] to be independent of the initial weight per unit area of the deposit, except for very low deposit levels. The results also show the crystalline deposit of aldrin to be at least 15 times more volatile from glass surfaces than a similar deposit of dieldrin.

Volatilization rates of these crystalline deposits were sensitive to air movement and temperature change. To give extreme examples, whereas a 2 $\mu g/cm^2$ deposit of dieldrin crystals (1 - 10 mm long) at 20°C in still air took 3 - 4 weeks to fall to a deposit density of 0.2 $\mu g/cm^2$, the same tenfold fall was accomplished in 4 - 5 h at a temperature of 40°C and a wind speed of 2.0-2.5 mile/h.

It was noticed that although the volatilization rates of dieldrin and aldrin were linear, at very low deposit levels (circa 0.2 $\mu g/cm^2$) these rates decreased progressively, the curves of deposit density/time tending to approach the time axis asymptotically. This again is apparently in agreement with the observations of Hoskins.

THE APPLICATION OF SMALL AMOUNTS OF RESIDUAL RADIOACTIVE INSECTICIDES TO SURFACES BY A LABORATORY SPRAYING TECHNIQUE

The apparatus, shown in Fig. 1, consists of a glass tube of length 23 cm, internal diameter 6.6 cm and wall thickness 3 mm, with flat ground edges. The tube is gripped by nylon screws which allow for adjustment of its position, and the whole is supported by a stand. A brass plate, which bears the spray nozzle and 6 brass sorption tubes, is positioned on the top of the tube by a locating groove which is packed with a suitable grease (e.g.silicone high-vacuum grease) to form a seal. A brass platform at the bottom is fitted to a rack and pinion which moves the platform into the spraying position. A ball-and-socket joint which can be clamped in any position and joins the platform to the rack allows for adjustment of any tilt in the platform.

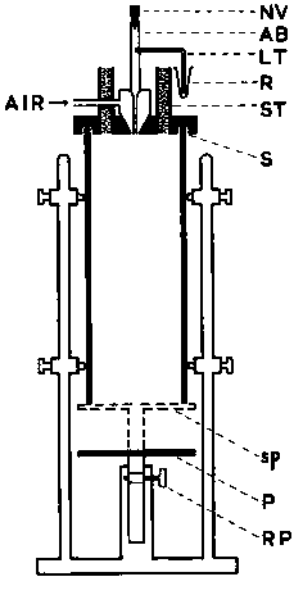

Fig.1

Apparatus for laboratory spraying technique

NV needle valve	AB air bleed	LT liquid tube
R reservoir	ST sorption tube	S seal
P platform	RP rack and pinion	sp spraying position

The atomizing nozzle consists of a brass body with an inlet for air under pressure and a 1.5-mm-diameter aperture into which a monel-metal liquid jet of external diameter 1.25 mm and internal diameter 0.75 mm fits concentrically. The tip of the liquid jet, which has a flat edge, is flush with the surrounding air orifice. A stainless-steel liquid tube of internal diameter 0.7 mm leads from the bottom of a small glass reservoir to the centre axis of a stainless-steel tube of internal diameter 1.75 mm. This latter tube allows an air bleed controlled by a needle valve to pass across the mouth of the liquid tube, and thus some atomization of the liquid occurs before it

reaches the main atomizing nozzle. This was found to be essential in order to prevent the formation of coarse liquid droplets in the spray chamber. The atomizing nozzle is inserted through a conical hole in the top brass plate and is clamped to the plate by screws which are also used for slight tilt adjustment in the nozzle. Silicone high-vacuum grease is again used to form an air-tight seal between the nozzle and brass plate. The six brass sorption tubes, each of 8-cm length and 8-mm internal diameter, slide into holes spaced equidistantly around the central atomizing nozzle and are soldered to the brass plate. They serve as exit tubes for the air during spraying and contain cotton-wool plugs which are used to trap any spray solution.

To obtain the optimum spraying conditions, the following procedure is adopted : a glass plate is placed on the platform so as to cover it completely and a filter-paper of somewhat larger diameter than the glass tube is placed on top of the plate. The platform is racked up firmly against the lower flat-ground edge of the tube so that the filter-paper forms a seal between the glass plate and the edge of the glass-tube. Tests have shown that no spray solution escapes past this seal. The air pressure to the atomizing nozzle is adjusted to 52 cm Hg (10 lb/in^2) by means of a mercury manometer in the air-line and cotton-wool plugs are inserted in the sorption tubes. The needle valve is adjusted so as to give an air-bleed flow-rate of 400 - 500 cm^3 air/min, adjustment being made at the same time in the resistance of the plugs to the air issuing from the spray chamber. This is achieved by teasing out or compressing the cotton-wool so that a water manometer records a positive pressure of 7 cm water inside the spray chamber or a suction pressure of 12 cm water in the liquid tube.

These spraying conditions are established quite easily, and spraying is quickly carried out by first racking down the platform, placing the leaf or other plant surface on the filter-paper (fixing with adhesive if necessary)and racking up again, and then presenting the reservoir containing the spray liquid to the liquid tube. As a precaution the apparatus, when in use, is placed in a well-vented fume chamber.

As is common with all spraying procedures, there were spray losses caused by air turbulence on the walls of the chamber and also entrainment of spray to the top of the chamber. Using both aqueous dye solutions and radioactive solutions of dieldrin in "Cellosolve", it was shown that the cotton-wool plugs in the sorption tubes were completely successful in holding back entrained spray solution. When water or "Cellosolve" was sprayed in the apparatus, approximately 15% of the spray solution showed a very fine droplet size and good coverage was obtained over the 35-cm^2 circular area at the bottom of the chamber. When small glass discs were placed on the platform and sprayed with radioactive solutions of dieldrin in "Cellosolve" and the crystalline deposits were assayed radiometrically, it was found that the central circular area of 5 cm^2 received an even coverage of the insecticide. The mean deposit density of the surrounding area was found to be slightly more than half that of the central area. The crystal sizes of the dieldrin deposits were in the range 0.1 - 1.5 mm (average 0.3 mm long), somewhat smaller than those obtained when similar solutions were sprayed in the Potter Tower (range 0.1 - 1.5 mm, average 1 mm long).

This apparatus has been developed for spraying small amounts of liquid; reproducible results have been obtained with as little as 0.05 ml liquid in the reservoir.

ACKNOWLEDGEMENTS

I wish to thank Dr. C. Potter, Head of the Department of Insecticides & Fungicides, for helpful advice and Mr. A.J. Arnold of the same Department for valuable technical help and the manufacture of the nozzle.

The samples of radioactive dieldrin and aldrin were a gift from Shell Research Ltd.

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DISCUSSION

G. F. BURNETT: Your deposit on the spray chamber is described as about 15% of that expended. How reproducible was this?

F. T. PHILLIPS: For five consecutive sprayings the amount of spray solution deposited ranged from $13\frac{1}{2}$ to $16\frac{1}{2}$% approximately (the approximate mean being 15%).

G. F. BURNETT: Did the deposit increase with repeated sprayings in the chamber?

F. T. PHILLIPS: Except for the initial spraying, which usually appeared to be less, there was no visible increase in the amount of spray deposited when 0.05 ml aqueous dye solutions were sprayed consecutively on to consecutive filter-papers until eventually run-off of spray solution occurred down the inside of the chamber.

D. F HEATH: Did you analyse the evaporation rates from glass slides in terms of the formula: rate = evaporation area × wind-speed, the effect of the temperature being exponential as usual? Your results seem on the whole to indicate that a formula of this sort might apply.

F. T. PHILLIPS: No, I have not used this or any similar formula showing correlations with wind-speed, because at the moment insufficient data have been obtained using different wind-speeds. Experiments using a range of wind-speeds are, however, to be conducted in the near future.

D. F. HEATH: What I had in mind was that you might have analysed your curve by determining, for example, the surface areas of crystals under the microscope.

F. T. PHILLIPS: No, I have not really done a proper analysis, though I can say that under the microscope at the very beginning, at a high deposit density, the crystals tend to be all joined together like fern-trees in arrangement. They touch at certain points. As time goes on, in a few weeks perhaps, these slowly disappear, until you get a very dispersed crystal system. The crystals themselves diminish in size. I did not measure the

diameters of the crystals. They are long needle crystals; all I measured was the length, which diminished with time. Finally they were not visible at all. After several weeks I still got a very low activity from the plates, although no crystals were observable in the microscope. The background was 10 counts/min in my operation, and I was getting counts of between 10 and 15 for long periods afterwards. So whether that was actually a radioactive count-out, or a slight contamination, I don't know. Certainly after five or six weeks, at any rate, one could say that the curves had reached the maximum and that practically no further radioactivity was observable.

J. W. MILES: Were evaporation rates measured on any surfaces other than glass?

F. T. PHILLIPS: No. Experiments using leaf or other plant surfaces were held up because of lack of a suitable method of applying insecticide solutions to these surfaces. With the design of the spray chamber described in this paper I hope now to go ahead with this work.

G. F. BURNETT: It is relevant, I think, to Mr. Miles' remarks that regular work is being done by HADAWAY and BARLOW on the disappearance of spread on mud surfaces*. They have obtained much bigger discrepancies in the rate of disappearance of crystals of different sizes than you have at Rothamsted. They graded the crystals into 1 μm, 10 μm and, I think, 100 μm. There the vapour phase disappears into the mud, which suggests that the removal of the vapour is extremely important in determining the rate of volatilization. You may find that if you increase your wind-speed above two miles an hour and completely remove your extracted air from contact with your surfaces, you considerably increase your rates of volatilization.

F. T. PHILLIPS: That is certainly true. At the moment I have only used two rates of air movement but, as you say, the evaporation rate increases quite remarkably with the rate of change of air movement.

Again, when we place the radioactive plates in still air, we have to be careful that although we shield the plates from draughts, we do not cover them: otherwise the concentration of vapour builds up in a covered container. Although there is some egress into the atmosphere, there is a considerable concentration above the plates and this diminishes the rate of volatilization. We have to find a happy medium between the two.

The suggestion that increased wind-speed might increase not only the evaporation rate but also the difference in evaporation rates due to different crystal sizes is a most interesting one. It seems also to agree with the formula cited by Dr. Heath and I shall certainly put it to the test when I come to do the work on variations in wind-speed.

K. von ASPEREN: In our own and other laboratories evidence has been obtained that chemical changes may occur under normal atmospheric conditions. Did you consider or exclude this possibility?

F T. PHILLIPS: These possibilities were considered, Any degradation by ultraviolet light could be excluded because of the conditions of the experiments; the glass plates with the insecticide deposits were held in a dark constant-temperature room at 20°C. Any oxidation of aldrin to dieldrin would probably be shown by a change in the rate of volatilization

as the experiment proceeded. This did not occur, except for the progressive decrease in rates at very low deposit levels which was noted for both aldrin and dieldrin. Also, most of the aldrin in this experiment volatilized during a short time (one day). Thus it was considered that no measurable amounts of aldrin oxidized to dieldrin.

K. von ASPEREN: I believe that other conversions could occur, but even then the occurrence of straight lines must be an indication that there is no chemical change in your experiment; is that right?

F. T. PHILLIPS: Not necessarily, but I do not think that there could be any other chemical changes except, as I said, a very slight change of aldrin into dieldrin. These are very stable compounds compared with most insecticides - that us why they are residual insecticides. The only strong instability is the fact that aldrine oxidizes to dieldrin, but that occurs generally when we spray into soil or any organic material; on glass plates there is very little opportunity for oxidation to take place.

RADIOAUTOGRAPHY IN THE STUDY OF RADIOISOTOPICALLY-TAGGED SUBSTANCES IN INSECT CONTROL

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Abstract — Résumé — Аннотация — Resumen

RADIOAUTOGRAPHY IN THE STUDY OF RADIOISOTOPICALLY TAGGED SUBSTANCES IN INSECT CONTROL. In return for a relatively small expenditure of effort, the newer, simplified techniques of radioautography offer great sensitivity and precision at the microscopic level in many phases of the investigations of singly or doubly radioisotopically-tagged insecticides: e.g., uptake, translocation, metabolism and determination of residues in insects, plants and animals. The physiology and biochemistry of irradiated insects are other phases which can be studied by radioautography. Radiation effects on the generative organs, especially, are being studied with tritiated thymidine. Whereas counting techniques yield gross information on the presence and quantity of radioisotopes, radioautography can provide information on actual sites of deposition in tissues or even parts of cells. This resolution is available on ordinary permanent histological preparations, in contrast to counting techniques in which accuracy of counting demands the destruction of the sample. Standard radioautography is especially useful for conveniently following fractional microcurie amounts of very weak emitters such as radiocarbon (C^{14}) and tritium (H^3), as opposed to the very expensive, highly sophisticated apparatus which is required for counting techniques when very weak emitters are employed.

The technique places no restriction on the isotope used for labelling, since even the radiation from tritium and C^{14} will register on the nuclear emulsion. In fact, tritium and C^{14} (which are desirable since nearly every insecticide will contain carbon and hydrogen) have the advantage of yielding high-resolution radioautograms and have long half-lives, so that if very long exposure times are necessary, there would be no difficulty. Long exposure times may be required since only tiny amounts of radioactivity, even of high-specific-activity insecticides, can be expected to be taken up. It is necessary to be certain that the insecticides are tagged at such a point in the molecule that the radioactive tag remains bound to the moiety which it is desired to study. Once the insecticide has been taken up or administered, the specimen preparation follows standard histological techniques of fixation, embedding, cutting and attaching to glass slides. The tissues can then be deparaffinized and hydrated and are then ready for application of the nuclear emulsion. If the radioactive compounds in the tissues are in a soluble state, freeze-drying techniques can be used to prevent loss or mobilization of the radioisotope. Dry-emulsion can be used for these preparations.

Staining can be done either before or after application of the nuclear emulsion, depending on the nature of the stain desired. In general, it is preferable to stain after the emulsion has been applied and photographically processed. With sufficiently energetic isotopes, whole mounts of plants or plant parts can be radioautographed, in which case histological preparation can be eliminated; the part to be radioautographed is simply compressed with the film in a plant press or other suitable device.

The most convenient method of applying nuclear emulsions to histological preparations is to dip them into melted bulk emulsion in the dark and then clean the bottom of the slide. The slides are then placed in a dark chamber, in a dry, inert atmosphere and allowed to expose for a time which must be empirically determined. At the expiration of this period, the slides are photographically processed and thoroughly washed and coverslips are mounted. The slides are then ready for study just as regular histological specimens at all magnifications. A slight upward adjustment of the focus brings into view the radioautographic pattern which can be thus easily studied in relation to the tissue. The recommended techniques and the simple, generally available laboratory equipment required are described.

L'AUTORADIOGRAPHIE DANS L'ÉTUDE DE SUBSTANCES MARQUÉES AU MOYEN DE RADIOISOTOPES UTILISÉES DANS LA LUTTE CONTRE LES INSECTES. Les nouvelles techniques simplifiées d'autoradiographie — d'une application relativement facile — sont très sensibles et précises au niveau microscopique dans de nombreuses phases des recherches sur les insecticides marqués au moyen de un ou deux radioindicateurs: recherches

sur l'absorption, la translocation, le métabolisme et les résidus chez les insectes, les végétaux et les animaux. L'autoradiographie peut aussi servir à étudier la physiologie et la biochimie d'insectes irradiés. On étudie notamment les effets des rayonnements sur les organes reproducteurs au moyen de thymidine tritiée. Alors que les méthodes de comptage fournissent des renseignements approximatifs sur la présence et la quantité de radioisotopes, l'autoradiographie permet d'obtenir des données sur l'emplacement exact du dépôt dans les tissus, voire dans des parties de cellules. Cette détermination peut se faire sur des préparations histologiques normales permanentes, alors que les méthodes de comptage impliquent la destruction de l'échantillon pour obtenir des résultats exacts. L'autoradiographie courante est particulièrement utile pour suivre des quantités de l'ordre d'une fraction de microcurie d'émetteurs très faibles tels que le radiocarbone (^{14}C) et le tritium (^{3}H) ; le comptage des mêmes émetteurs exige des appareils très coûteux et hautement perfectionnés.

L'autoradiographie n'exclut l'usage d'aucun radioisotope pour le marquage puisque même les rayonnements de ^{3}H et de ^{14}C impressionnent l'émulsion nucléaire. En fait, ^{3}H et ^{14}C (qui sont particulièrement intéressants du fait que tous les insecticides contiennent du carbone et de l'hydrogène) ont l'avantage de donner des autoradiogrammes à haute résolution et d'avoir de longues périodes; il s'ensuit qu'il n'y a pas de difficultés lorsqu'un très long temps d'exposition est nécessaire. C'est souvent le cas, parce que même pour des insecticides ayant une forte activité spécifique, les quantités de radioactivité que l'on peut espérer absorber sont généralement très faibles. Il faut veiller à ce que l'insecticide soit marqué en un point de la molécule tel que le radio-indicateur demeure lié à la portion que l'on désire étudier. Une fois que l'insecticide a été absorbé ou administré, on prépare les échantillons en appliquant les méthodes histologiques courantes: fixation, enrobage, découpage et pose sur lamelle de verre. Les tissus sont ensuite déparaffinés et hydratés; ils sont alors prêts pour l'application de l'émulsion nucléaire. Si les composés radioactifs se trouvent à l'état soluble dans les tissus, on peut faire appel aux méthodes de dessication par congélation pour éviter toute perte ou tout mouvement du radioisotope. Des films à émulsion sèche peuvent être utilisés pour ces préparations.

La coloration peut être faite avant ou après application de l'émulsion nucléaire, selon la nature de la couleur désirée. Il est préférable, en général, que la coloration intervienne après l'application et le traitement photographique de l'émulsion. Si l'on utilise des radioisotopes ayant une énergie suffisante, on peut procéder à l'autoradiographie de plantes entières ou de parties de plantes, et supprimer ainsi la préparation histologique; la partie à autoradiographier est simplement comprimée avec le film dans une presse ou dans tout autre appareil approprié.

La méthode la plus pratique pour appliquer les émulsions nucléaires aux préparations histologiques consiste à plonger l'échantillon dans une émulsion fondue et à nettoyer ensuite la partie inférieure de la lamelle. Les lamelles sont ensuite placées en chambre noire, dans une atmosphère sèche et inerte, et exposées pendant une durée qui doit être déterminée empiriquement. Après quoi, elles font l'objet d'un traitement photographique, sont soigneusement lavées et revêtues d'une protection. On peut alors les étudier comme des échantillons histologiques normaux, avec le grossissement voulu. Un léger déplacement du foyer vers le haut permet de voir l'autoradiographie, que l'on peut ainsi facilement étudier en regard du tissu. Le mémoire décrit en détail les méthodes recommandées ainsi que le matériel nécessaire, qui est simple et dont disposent généralement les laboratoires.

ПРИМЕНЕНИЕ РАДИОАВТОГРАФИИ ПРИ ИЗУЧЕНИИ СУБСТАНЦИЙ, МЕЧЕННЫХ РАДИОИЗОТОПАМИ, В БОРЬБЕ С НАСЕКОМЫМИ. При относительно небольших затратах труда более новые, упрощенные методы радиоавтографии обеспечивают большую чувствительность и точность на микроскопическом уровне во многих фазах исследований однократно и двукратно меченных радиоизотопами инсектицидов, например при исследовании поглощения, перемещения, метаболизма и определении остатков инсектицидов в растениях, у насекомых и животных. Физиология и биохимия облученных насекомых являются другими областями применения радиоавтографии. Воздействие облучения, особенно на генеративные органы, изучается с помощью меченного тритием тимидина. В то время как методы счета дают много информации о наличии и количестве радиоизотопов, с помощью радиоавтографии можно получить данные о фактических местах их отложения в тканях или даже в частях клеток. Получение такой разрешающей способности возможно на обычных постоянных гистологических препаратах, в то время как при применении методов счета для получения точного результата требуется разрушение образца. Стандартная радиоавтография особенно удобна для прослеживания малых количеств изотопов, составляющих доли микрокюри, очень слабых излучателей, таких, как радиоактивный углерод (C^{14}) и тритий (H^{3}), тогда как для работ с очень слабыми излучателями с применением методов счета требуются очень дорогие и сложные приборы.

Метод не имеет ограничений в отношении изотопов, применяемых для мечения, так как даже излучения трития и C^{14} будут фиксироваться на ядерной фотоэмульсии. Действительно, тритий и C^{14} (которые желательны, поскольку почти каждый инсектицид будет содержать углерод и водород) имеют то преимущество, что дают радиоавтограммы с высокой разрешающей способностью и имеют большой период полураспада и поэтому не возникает трудностей в тех случаях, когда необходим очень длительный срок экспозиции. Продолжительная экспозиция может потребоваться, когда предполагается наличие очень небольших количеств радиоактивных веществ даже при высокой удельной активности инсектицида. Необходимо быть уверенным в том, что инсектицид попадает в ту часть молекулы, где радиоактивный индикатор остается связанным с той половиной, которую желательно изучить. После того как инсектицид поглощен или введен, подготовка препарата осуществляется обычными гистологическими методами фиксации, заливки, приготовления срезов и наложения на предметное стекло. Затем ткани могут быть депарафинированы и гидратированы, после чего они готовы для покрытия ядерной фотоэмульсией. Если радиоактивные составляющие в тканях находятся в растворимом состоянии, то для избежания потери или мобилизации радиоизотопа могут быть применены методы сушки замораживанием. Для таких препаратов можно применять сухие эмульсионные пленки.

Окраска может производиться как до, так и после применения ядерной фотоэмульсии, в зависимости от характера требующейся окраски. В общем, предпочтительнее окрашивать после того, как фотоэмульсия нанесена и проявлена. При применении изотопов с достаточной энергией излучения радиоавтографированию могут подвергнуться растения или их части целиком, и в этом случае гистологическая обработка может быть исключена; та часть, которая должна подвергнуться радиоавтографии, прижимается к пленке в прессе для растительных образцов или в другом, пригодном для этого устройстве.

Наиболее удобным методом применения ядерных фотоэмульсий для гистологических препаратов является погружение их в расплавленную массу фотоэмульсии в темноте и затем очистка нижней поверхности предметного стекла. После этого предметные стекла помещаются в темную камеру с сухой инертной атмосферой и выдерживаются там в течение времени, определяемого эмпирически. Затем предметные стекла проявляются, тщательно промываются, и на них надеваются защитные стекла. После этого предметные стекла готовы для исследования, точно так же, как обычные гистологические препараты при всех увеличениях. Незначительное смещение фокуса вверх вводит в поле зрения радиоавтографический образец, который может быть таким образом легко изучен при сравнении с тканью. Дается подробное описание методов и необходимого лабораторного оборудования.

LA AUTORRADIOGRAFÍA EN EL ESTUDIO DE LAS SUSTANCIAS MARCADAS CON RADIOISÓTOPOS UTILIZADAS EN LA LUCHA CONTRA LOS INSECTOS. Las nuevas técnicas autorradiográficas simplificadas requieren cierto esfuerzo pero permiten obtener una gran sensibilidad y exactitud al nivel microscópico en el estudio de los insecticidas marcados con uno o dos radioisótopos, y, en particular, en el de la absorción, traslocación, metabolismo y determinación de residuos en plantas, insectos y otros animales. Con ellas se pueden estudiar también la fisiología y la bioquímica de los insectos irradiados. Se están investigando con timidina tritiada los efectos de las radiaciones sobre los órganos reproductores. Las técnicas de recuento dan información general sobre la presencia y la cantidad de los radioisótopos, mientras que la autorradiografía proporciona datos sobre el lugar exacto de fijación en los tejidos e incluso en partes de la célula. Este poder de resolución se alcanza con preparados histológicos ordinarios que pueden conservarse, contrariamente a lo que sucede con las técnicas de recuento que exigen, si se quiere lograr mayor exactitud, la destrucción de la muestra. La autorradiografía corriente es particularmente útil para analizar por métodos muy sencillos fracciones de microcurie de emisores muy débiles como el radiocarbono (^{14}C) y el tritio (^{3}H), mientras que las técnicas de recuento requieren en estos casos un instrumental muy complicado y costoso.

Esta técnica no impone limitación alguna en cuanto a los isótopos que pueden emplearse como indicadores, porque la emulsión nuclear registra incluso las radiaciones del tritio y del ^{14}C. En realidad, el tritio y el ^{14}C (que son convenientes puesto que casi todos los insecticidas contienen carbono o hidrógeno) tienen la ventaja de dar autorradiografías de elevado poder de resolución y, como son de período largo, permiten tiempos de exposición muy prolongados. Estos tiempos de exposición pueden resultar necesarios debido a las pequeñas cantidades de radiactividad que se absorben incluso empleando insecticidas de elevada actividad específica. Es preciso cerciorarse de que el insecticida esté marcado en un punto tal de la molécula que el átomo trazador permanezca ligado a la parte que se desea estudiar. Una vez absorbido o administrado el insecticida, la preparación de las muestras se efectúa siguiendo las técnicas histológicas corrientes de fijación, inclusión, microtomía, y colocación sobre portaobjetos. Seguidamente se elimina la parafina y se hidratan los

tejidos, que quedan así preparados para la aplicación de la emulsión nuclear. Si los compuestos radiactivos del tejido se encuentran en estado soluble, se pueden emplear técnicas de liofilización para evitar las pérdidas y el desplazamiento de los radioisótopos. Para estos preparados pueden utilizarse películas de emulsión seca.

El tinte puede efectuarse antes o después de aplicar la emulsión nuclear, según el tipo de coloración que se desee obtener. En general es preferible efectuar la operación después de haber aplicado y revelado la emulsión. Si se emplean isótopos de energía suficiente, es posible autorradiografiar plantas enteras o partes de plantas, en cuyo caso se evita la preparación histológica; la parte que se desee autorradiografiar se comprime con la película en una prensa o en otro dispositivo adecuado.

El método más conveniente para aplicar las emulsiones nucleares a los preparados histológicos consiste en introducirlas en la emulsión fundida y limpiar después el reverso del portaobjetos; evidentemente esta operación tiene que efectuarse en la oscuridad. Los preparados se colocan en una cámara oscura, de atmósfera seca e inerte, y se exponen durante un tiempo que se determina empíricamente. Transcurrido este tiempo, la emulsión se revela, se lava cuidadosamente y se colocan los cubreobjetos. Los portaobjetos pueden entonces estudiarse en cualquier ampliación, como los preparados histológicos corrientes. Un ligero desplazamiento ascendente del foco pone de relieve los detalles de la autorradiografía que, de este modo, se puede estudiar en relación con el tejido. En la memoria se describen en detalle las técnicas recomendadas y el sencillo equipo necesario, que suele encontrarse en todos los laboratorios.

INTRODUCTION

Participants in previous symposia [1, 2] which were closely related to this one in subject matter have repeatedly mentioned the usefulness of radioautography in entomological research. In spite of this, examination of publications reveals that only a few investigators are taking advantage of this tool. Perhaps this is due to the fact that in the past radioautography had been difficult to perform and often yielded equivocal results. Recent development of more sensitive and dependable emulsions, which are commercially available, and simpler techniques and apparatus now place radioautography in the category of a routine tool which should be considered in any research problem where the fate of tracers in an organism is of interest. This is especially true if the research is to be done in the field or in areas remote from the amenities of highly-refined counting equipment and the repair personnel and other support items which such equipment requires. Furthermore, the amounts of radioactivity required are smaller than for other procedures, and so handling the tagged compounds can be more convenient.

Even in laboratories where all the refinements are available, radioautography offers a type of information which cannot be obtained in any other way. While it is now possible to detect extraordinarily small quantities of even tritium and radiocarbon by liquid scintillation counting, only radioautography makes it possible to visualize the sites of deposition within a tissue (or with tritium-tagging, within cells) of a radioisotopically-tagged compound. The purpose of this communication is to provide general background information necessary for using radioautography.

Within the area of the special interests of those attending this Symposium, radioautography is applicable in many ways. It is ideally suited to studies of the uptake, translocation and metabolism of tagged insecticides in insects, animals and plants; in studies of the physiology and biochemistry of insects under normal conditions and following radiation; the response of the reproductive processes in insects in response to radiation; the transport of

radioactive parasites from host to host through the insect vector; location of radioactive parasites in the tissues of the host, including insect parasites such as Empusa muscae; sites of deposition in studies of the influence on resistance of radioactive trace elements in plants; movements of ions across membranes; and many others.

SELECTION OF THE RADIOISOTOPE TO BE USED

Radioautography itself does not place any restrictions on the radioisotope to be used, since the nuclear emulsions now available respond to even the very low energies emanating from tritium, the weakest of the radioisotopes. The decision as to which isotope will be used then is made on the basis of the physiological, biochemical and metabolic factors under study and the chemical constituents of the compound it is desired to investigate. The effective half-life and the mean path length in tissue and emulsion must also be considered, as well as type of radiation – alpha, beta or gamma – and degree of avidity of the organism for the labelled compound. The radiation dose delivered to the organism should be kept as low as possible. The possibility of an isotope effect where the mass of the radioisotope is very different from that of the non-radioactive sister, as in the case of tritium, which has three times the mass of hydrogen, must also be taken into account [3].

LABELLING

Insects have been labelled in many different ways. They have been exposed to radioactive gas, to artificial nutrients contaminated with radioisotopes, dipped into, injected, perfused, sprayed or painted with radioactive solutions, or allowed to feed on their natural food which has been made radioactive. Radioactive insecticides can be produced by substituting a radioactive isotope in the original synthesis [4], or by chemical manipulation of the insecticide, or by exchange, as with tritium [3].

In using a labelled insecticide, there are several factors to consider: half-life, decay products produced, isotopic effects, position in the molecule, specific activity attainable, radiochemical purity, radiochemical stability and the radiation dose to the animal. The half-life is important because when the radioisotope decays to a daughter product the chemical composition changes, and the insecticide may change its properties or the insecticide will be metabolized differently, or may become undetectable if the daughter product is not radioactive. The position on the molecule should be known, since the metabolism of the insecticide may break up the molecule and it may be necessary to know which moiety of the original molecule is being followed. In such a case, the insecticide per se may disappear, but the toxic metabolite may persist for weeks, and be demonstrated by the radioautogram. Reversible or irreversible exchange may also occur, particularly with tritium or radiocarbon. If the insecticide is not radiostable, the analysis of results will be confused because the original material will have changed. Very high specific activities are ordinarily desirable

unless they significantly reduce radiostability and/or deliver radiation doses to the insect sufficient to cause undesired radiation effects. Radiostability can sometimes be preserved by storing the insecticide in a solvent which helps absorb some of the radiant energy.

HISTOLOGICAL PROCESSING

The method of administration of the radioisotope or of the tagged insecticide will be determined by the experimental work which is being done and the kind of information being sought, so little can be said about this. However, the time allowed between administration of the isotope and fixation of tissue for radioautography and the time spent in histological processing must be kept as short as possible relative to the half-life of the radioisotope. Except for reagents which dissolve the radioisotope-bearing compounds out of the tissues, ordinary fixatives and embedding techniques can be used. Alternative techniques can be found if necessary; for instance, if the compounds of interest are soluble in organic solvents but not in water, one can use water-soluble carbowax instead of paraffin or celloidin for embedding [5]. If the compounds are water-soluble, freeze-dry techniques may be employed.

The thickness at which the sections are cut is determined by the cytological resolution required. Of course, thin sections will contain less isotope than thicker ones, but then there are problems with absorption within the specimen, so, in general, one simply cuts at the same thickness as for non-radioautographic work.

Since the stains used may also dissolve the radioisotope-bearing compounds and since some stains are decolorized by the photographic processing which the radioautogram must undergo, it is generally preferable to stain after the photographic processing. However, there is considerable leeway in selecting stains. In our laboratory, we have found [6] that some stains, such as Feulgen-fast green, chromium hematoxylin phloxine and aldehyde fuchsin-PAS, work best when applied before application of the nuclear emulsion. Others, such as celestin blue-Mayer's haemalum, metanil yellow-iron hematoxylin, lithium carmine-picric acid, Weigert acid-iron haematoxylin, alum cochineal, methyl green pyronin, indigo carmine-picric acid, methylene blue-azure A, toluidine blue, Nissl and Cason, are best when applied after the photographic processing. It was also found that some combination stains, such as trichrome-PAS, luxol-fast blue-PAS, haematoxylin-eosin and aniline blue-carbol fuchsin, worked best when part of the staining was performed before application of the nuclear emulsion and the remainder applied after the photographic processing.

NUCLEAR EMULSIONS

Photographic emulsions intended for use in radioautography contain up to 95% silver halide, mostly bromide, by weight, with an average grain size of 0.2 to 0.3 μm and a maximum of 0.5 μm. X-ray film has much larger grain size, up to 6 μm, which yields high sensitivity but poor resolution.

The response of the emulsion to radiation depends upon the characteristics of the radiation traversing it. Alpha particles, which have a large mass and move relatively slowly, give up their energy to atoms in or near their path but do not tend to be deflected from an approximately straight course. Therefore, passage of an alpha particle through the emulsion results in a fairly straight line defined by a series of silver granules further apart at the origin than at the end of the path. The track may actually consist of a cylinder composed of granules if the local ionization is sufficient in quantity. The track of a beta particle is much more complicated, since it has little mass and is easily deflected by atoms in its path. The beta track is tortuous and the individual granules composing it are relatively far apart. It is impossible to unravel a group of beta tracks if they pass through a small volume. X or gamma rays, which are very fast and of negligible mass, register in nuclear emulsion by eliciting secondary electrons, which yield tracks similar to beta particles.

Nuclear emulsions are available from Eastman Kodak in the United States of America and Kodak, Ltd. and Ilford, Ltd. of England in a variety of forms. American Kodak provides nuclear emulsions in a variety of sensitivities and thicknesses coated on glass plates the size of microscope slides in several thicknesses, with and without a gelatin protective coating. In order of ascending sensitivity these emulsions are designated: NTA, NTB, NTB2 and NTM3. NTB2 and NTB3 are the most commonly used in biological work. These emulsions may be obtained in bulk, also. Kodak also supplies stripping film in several forms. British Kodak supplies several types of emulsion, of which their AR10 stripping film is quite popular in the United States and is now being distributed by the American branch, but it is not available in bulk. Ilford also provides a variety of emulsions as stripping film or in bulk. When not in use, nuclear emulsion should be stored at 5°C. It will keep up to six months or longer if not exposed to too much radiation. The ways in which the various forms of nuclear emulsion are utilized will be discussed below. HERZ [7] and YAGODA [8] have discussed the fundamental interactions of radiation and nuclear emulsions.

RADIOAUTOGRAPHIC PROCEDURES

Dozens of modifications of the few basic methods of radioautography have been evolved, and many of them have been described by BOYD [9]. There are also extensive bibliographies gathered by PASSALAQUA [10] and JOHNSTON [11, 12]. A few of the most generally applicable methods will be described to indicate the possibilities which in practice are limited only by the ingenuity of the investigator.

The two most widely used methods have come to be known as the dipping technique [13, 14] and the stripping technique [15]. Both yield excellent results with characteristic high resolution and dependability. Of the two, the dipping technique, which was developed in this laboratory, is the simpler and more convenient. All techniques have many manipulations in common, and the dipping technique will be described in some detail as a model for the others.

In our laboratory the procedure is as follows. If it is feasible, the mounted tissues should be rehydrated after paraffin or other embedding medium has been removed. This step will aid in obtaining even layers of emulsion which adhere to the tissue and glass much better than if the slides are not rehydrated, and it should be performed unless there is danger of loss of the radioisotope. While the tissue slides are going through the last stages of processing, the light-tight [14] container of bulk nuclear emulsion is taken from the refrigerator and placed in a water bath which is set to bring the emulsion to a temperature of approximately 40°C, in the darkroom. If thinner emulsion layers are desired, the temperature may be raised but not above 50°C (to avoid heat sensitization artifacts). When the emulsion is at temperature and the slides are ready, the slides are taken from the water in which they were rehydrated and arranged in a convenient array where they may be easily grasped at the label end in the dark. If too many slides are removed at once, the last ones to be done will be dried out and may lose some cytological detail. Groups of ten are convenient. The lights are shut off and the series 1 or 2 safelight turned on. Nuclear emulsions are quite insensitive to red light. The cover is then removed from the emulsion container and a clean glass rod is used to stir the emulsion thoroughly but gently without introducing bubbles. Stirring is necessary because during standing of the melted emulsion silver halide settles out of the gelatin. Once the stirring is completed, the first slide is picked up and immersed long axis vertical so that the tissue goes about half an inch below the surface. It is immediately withdrawn and allowed to drain for a few seconds to a minute on to a gauze pad held in the other hand. Within limits, the longer the drainage, the thinner the emulsion layer and the better the resolution. With 10-sec drainage, the dry layer thickness will be about 5 μm. Emulsion must not be allowed to drain back into the container. After draining, the gauze pad is used to remove all emulsion from the bottom of the slide. The slide is then placed horizontally in a tray to gel and be exposed. The slides need be kept horizontal no longer than the ten to twenty minutes it takes the emulsion to gel, after which any position of the slide will be all right. When the tray is full, it is placed in an exposure chamber specially made for the purpose* and other trays used if the number of slides require it. The proper compartments of the chamber are then charged with Drierite and solid CO_2, and the cover is sealed on. The chamber seals itself by means of an oil valve after all the CO_2 has sublimed. As the gas evolves, it is led through the chamber in such a manner that the air is completely swept out. If precautions to eliminate oxygen and reduce moisture below 15% relative humidity are not taken, serious losses of latent image may occur during the exposure period.

There has been much discussion about the proper temperature in the exposure chamber [13], but the best temperature to use is between 18 and 24°C. The argument that less background or fogging occurs at low temperatures has been advanced. In reality, the reduction of fog is due to the reduced sensitivity of the emulsion in the cold and the ratio of useful image

* The complete equipment for the dipping technique, consisting of an exposure chamber and the necessary ancillary equipment, is available from Controls for Radiation, Inc., 130 Alewife Brook Parkway, Cambridge 40, Mass.

to fog probably remains the same. With the dipping technique, fog is very seldom a problem and, therefore, it is preferable to expose at room temperature to obtain a more useful image. The fog level averages three to five grains per 100 μm^2 in this laboratory. However, the dipping technique is perfectly amenable to low-temperature exposure, should an investigator prefer to do so, or if there is an experimental reason for it, the exposure box may be placed in a cold room after it has been sealed.

Exposure time varies according to sensitivity of the emulsion, the total dose of isotope administered, its effective half-life, the length of time between administration and sacrifice of the animal, the degree of concentration, the pattern of distribution in the tissues under study, the thickness of the tissue slices and the energy of the radiation. These factors are difficult to reduce to a formula which could indicate actual exposure times. It has been found simpler to use replicate slides and to develop a few at intervals of several days until optimum exposure times become apparent. One soon gains experience enough to estimate the initial exposure period.

When the initial exposure period has elapsed, photographic development is performed with ordinary glass or plastic staining dishes containing the developing solutions. Ten to twenty slides are placed in the usual staining rack for immersion in the solutions. The solutions used are Kodak D 19 developer, Kodak SB5a stop bath and Kodak Fixer. D 19 is a rapid, fine-grain, fairly stable developer in wide use in radioautography, although others are in use also. The SB5a stop bath is non-swelling, thereby reducing the probability of dimensional changes and silver grain shifting. The acid fixer hardens the emulsion layer as it fixes, which helps prevent damage to, or loss of, the emulsion layer during washing or subsequent handling.

The development time can be as short as one minute or less or prolonged to ten minutes or more. The longer the time, the more granules appear, but increasing the development time too much will frequently increase the background without increasing the useful image proportionately. The photographic processing schedule is as follows:

Kodak D19 developer	1 to 10 min
Kodak SB5a Stop Bath	15 s
Kodak Fixer	2 × clearing time
Wash, running tap water	1 h.

All solutions should be 18 ± 1°C. If the individual solutions vary appreciably in temperature, the likelihood of reticulation increases. Higher temperatures increase the likelihood of the emulsion layer washing off. If it is impossible to achieve 18°C or lower, tropical processing methods can be used. After the slides have been in the fixer for a minute, sufficient white light may be used to observe the time it takes to clear the emulsion, and then the slides should be left in the fixer for an additional period equal to twice the clearing time. Thorough washing in running tap water at 18°C removes all the fixer and insures that no image loss or crystallization of hypo will occur if the preparations are stored for long periods. After thorough washing (or staining) has been completed, cover-glasses can be mounted in the usual way and the preparations can then be studied as soon as the mounting medium is hardened.

The radioautograms prepared this way can be studied under the microscope as though they were ordinary histological preparations. Under low and high dry magnification, only slight adjustment of the fine focus enables one to correlate the histologic with the radioautographic image. Oil immersion requires somewhat greater adjustment of focus, but it is still quite convenient. Similarly, photomicrography is quite simple under the lower magnifications by using a compromised focus, but it is necessary to make two negatives if oil-immersion magnification is required because the focal plane is too thin to focus clearly on both the tissue and the emulsion layers at once. A very simple registration frame suggested by Hoecker and described in BOYD [9] permits very accurate registration of the negatives for printing. Thus, study and recording of the data obtained by radioautography are simple.

BASERGA [16] has very cleverly modified the dipping technique to permit radioautography with doubly-labelled material using tritium and radiocarbon. The modification consists of proceeding essentially as described above but adding a layer of celloidin thick enough to absorb the weak tritium emanation over the developed radioautogram and redipping and developing to obtain a second radioautogram over the first, consisting of the granules exposed only by the radiocarbon.

HAMPTON and QUASTLER [17] use fluid emulsion in conjunction with electron microscopy.

Stripping-film technique differs (as do the other techniques to be described) from dipping primarily in the method of application of the nuclear emulsion to the specimen and also because a film of emulsion is used rather than a fluid emulsion. Specimen preparation and photographic development are similar to the dipping technique. The emulsion is applied by detaching or "stripping" the nuclear emulsion from the temporary glass or acetate support upon which it is placed by the manufacturer and floating it on a water bath. The specimen on the glass slide is then dipped under the floating film and brought up out of the bath with the film adhering to it. When the emulsion has dried, it usually adheres quite tightly to the slide and specimen because it shrinks as it loses water. In this method resolution is excellent, and the radiographic image retains its original relationship to the specimen. The only drawbacks to this technique are the difficulty most people encounter in handling the thin unsupported emulsion: it is quite time-consuming and there is a possibility of radioisotope loss in the water bath.

Stripping film in its dry state has been used by FITZGERALD [18] to apply to dry specimens where it was desirable to avoid loss of water-soluble isotopes. While this technique may create some artifacts due to the rather cavalier handling of the emulsion film which is necessary, no other method will serve when very soluble compounds are involved.

The methods described so far are used for high-resolution work with sections of specimens. For radioautography of whole mounts, for instance of a whole plant or a leaf or of a whole small insect, the simple apposition technique is very useful. In this technique the specimen is positioned on a cut film or a plate film protected from light and exposed, usually under pressure (as in a plant press or other device) to obtain close contact. After exposure, the specimen is separated from the film and the film is developed. The main drawbacks to this method are that it is very difficult to correlate

the radioautographic image with the specimen at the microscopic level once the two are separated, and resolution is not good, but it serves well for gross survey work. Several apposition methods are described in BOYD's book [9].

CONTROLS AND ARTIFACTS

Each slide should be used as its own control by observing the number of developed silver granules in natural or artifactual tissue spaces as well as in areas relatively remote from the tissue. In evaluating the slides this background is subtracted. It is also necessary to use non-radioactive control tissue slides in order to account for silver granules which may be due to the presence of reducing substances such as sulphydryl compounds or even reducing reagents which have not been completely washed out of the tissues. Other sources of background are: accidental exposure to white light, cosmic rays, naturally occurring radioisotopes in the containers, slides and tissues, irradiation of emulsion during immersion of the radioisotope-bearing tissue, radiation from adjacent slides in the exposure chamber, mechanical factors and heating. From time to time blank slides should be included to check the fog level of the emulsion; when it gets too high the emulsion should be discarded.

CONCLUSION

It is hoped that this short review will serve as a general guide to any investigator who wishes to take advantage of radioautography to obtain unique information. The techniques are not difficult if the fundamentals are understood, and the variety of techniques available make it possible to utilize radioautography in many types of research problems. Perhaps it should be emphasized that radioautography, as are radioisotopes in general, is a tool and should not be considered as an end in itself. However, for detecting the cytological positions of very small quantities of radioisotopes, radioautography is a very powerful tool, and it can serve the purposes for which this Symposium was convened admirably.

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DISCUSSION

K.K. NAIR: Is it not true that a mere blackening of the autoradiographs need not be an indication of radioactivity in the sections, but could be an artefact resulting from the precipitation of silver halide in certain niches in the section?

D.L. JOFTES: Yes, certainly. The emulsion is heir to many ills. There are usually radioisotopes in the structures which we use to manipulate the emulsion. Even the glass slides frequently contain some K^{40}, which then interferes, and mechanical manipulations heat it up too.

There are many many sources of error in this sort of thing and it is necessary for that reason to use adequate controls, to be very ruthless in analysis and to eliminate radioautograms which seem to be questionable. We have been doing radioautography now extensively for about eight years, and generally there is no question about really good positive radioautographs. The questions always arise when they are powerfully negative. Non-radioactive control tissues should always be included in the early runs to any new experiment involving radioautography. I have previously published a report on the type of artefact to which you refer. I believe it to be the result of differential drying of the emulsion, which is thicker over these tissue interstices; this differential drying causes mechanical strain in the emulsion and so "exposes" it.

M. HASCOËT: What limits of definition can one hope to obtain by using dry stripping films?

D.L. JOFTES: We have not used this technique in my laboratory but I believe the resolution achievable should be only slightly less than with standard stripping film techniques.

M. HASCOËT: Is it possible to work at the cellular level?

D.L. JOFTES: With low-energy isotopes such as H^3 or C^{14}, probably. It seems to me that one cannot achieve an intimate contact between tissue and a dry film as one can by dipping or standard stripping techniques, and this will adversely affect the resolution.

M. HASCOËT: I have had great difficulties in using a similar method with S^{35}. It is quite difficult to localize sulphur finally at the cellular scale and I would have been happy to know what methods you used to facilitate this.

D. L. JOFTES: That is partly a function of the mean path length of the radiation in the tissue and in the emulsion. I would expect you to get results with S^{35} comparable to those of C^{14}. Unfortunately I myself have no experience with the dry-film technique.

M. FRIED: I wondered if you had any experience of using film slides with fresh frozen sections, and the kind of artefact you get under these conditions.

D. L. JOFTES: We have not done this ourselves but we collaborate with another laboratory which has done work on these lines, and my guess is that the main problem would be associated with the melting, if you will, of the tissue fluid unless care was taken to dry the tissue into position. In the dipping system we put these things immediately into a chamber where humidity is very low and, the sections of tissue and the emulsion being very thin, there was very rapid drying, whereas in the fresh-frozen section there is a fair amount of water; and if you have a soluble isotope there, in compound, when this is warmed you may get some motion or some passage into the emulsion at some distance from the tissue.

The reason that I suggest drying and then rewarming is that you lower the sensitivity of a film considerably by chilling it. With the dipping technique and frozen dry sections one sometimes gets a build-up of emulsion in the interstices which occur there and then in the differential drying of the emulsion film, we often see streaks of silver granules running around the edges of the tissues and in the interstices, but this is such a fine kind of artefact that I am sure you would have no trouble.

III.

LABELLED INSECTIDE STUDIES: TOXICOLOGY AND RESIDUES

RADIOISOTOPES IN THE STUDY OF THE FATE OF INSECTICIDES APPLIED TO ANIMALS AND PLANTS

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Abstract — Résumé — Аннотация — Resumen

RADIOISOTOPES IN THE STUDY OF THE FATE OF INSECTICIDES APPLIED TO ANIMALS AND PLANTS. The use of radioisotopes has been of great value in the development of systemic insecticides for the control of insect pests of both plants and animals. General patterns of absorption, circulation and localization, metabolism, and the site and nature of residues have been determined more rapidly through the use of labelled materials than would have been possible by other available methods of analysis.

Information gained in studies with labelled insecticides has enabled entomologists to learn why some chemicals are relatively more toxic to insects than to mammals and why some are safer for use with certain types of mammal than with others. Results of these studies have led to the development of new methods for controlling insect pests. Hitherto unsuspected metabolic pathways have been elucidated and new methods of analysis have been developed.

Labelled insecticides have been essential in developing basic information on plant systemics. Radioisotopes have made it possible to follow the absorption, translocation and metabolism of systemic insecticides in plants. Also, the chemicals can be followed into insects that feed on treated plants and their metabolism and excretion can then be studied in the insects.

By utilizing labelled materials it has been shown that cotton plants grown from seed treated with systemic insecticides absorb less than 5% of the applied dose. Other studies have demonstrated that systemic insecticides are not readily translocated from treated leaves to new growth.

The use of radioisotopes in studying systemic insecticides will increase in the future. More emphasis is being placed on basic physiological studies on the fate of insecticides in plants, animals, and insects. Another area where isotopes should be used rather extensively is in studies designed to improve methods of application of systemic insecticides.

EMPLOI DES RADIOISOTOPES DANS L'ÉTUDE DE LA TRANSFORMATION DES INSECTICIDES ADMINISTRÉS AUX ANIMAUX ET AUX VÉGÉTAUX. L'emploi de radioisotopes s'est révélé très utile pour la mise au point d'insecticides endothérapiques pour la lutte contre les insectes nuisibles aux végétaux et aux animaux. On a pu déterminer plus rapidement divers processus généraux - absorption, circulation, localisation, métabolisme, emplacement et nature des résidus - grâce à des produits marqués que par les méthodes d'analyse utilisées auparavant.

Les renseignements obtenus à la suite des études faites au moyen d'insecticides marqués ont permis aux entomologistes de découvrir pourquoi certains produits chimiques sont plus toxiques pour les insectes que pour les mammifères et pour quelles raisons l'emploi de quelques-uns d'entre eux présente moins de danger pour certains mammifères que pour d'autres. Les résultats de ces études ont permis de mettre au point de nouvelles méthodes de lutte contre les insectes nuisibles. On a pu aussi expliquer certaines transformations métaboliques insoupçonnées jusqu'à présent et élaborer de nouvelles méthodes d'analyse.

L'emploi d'insecticides marqués a joué un rôle essentiel dans le rassemblement de données fondamentales sur les propriétés endothérapiques des plantes. Les radioisotopes ont permis, en effet, de suivre l'absorption, la translocation et le métabolisme d'insecticides endothérapiques dans les plantes. On peut aussi suivre les produits chimiques dans les insectes qui se nourrissent sur les plantes traitées et étudier ainsi leur métabolisme et leur excrétion.

En utilisant des produits marqués, on a pu montrer que les cotonniers issus de semences traitées au moyen d'insecticides endothérapiques absorbent moins de 5% de la dose appliquée. D'autres études ont prouvé que les insecticides endothérapiques ne sont pas facilement transportés des feuilles traitées aux nouvelles pousses.

L'emploi de radioisotopes dans l'étude des insecticides endothérapiques est appelé à s'étendre, en particulier pour les études physiologiques fondamentales sur la transformation des insecticides dans les plantes, les animaux et les insectes. Les radioisotopes devraient aussi être largement utilisés dans les études visant à améliorer les modes d'application des insecticides endothérapiques.

РОЛЬ РАДИОИЗОТОПОВ В ИЗУЧЕНИИ СУДЬБЫ ИНСЕКТИЦИДОВ ПРИ ОБРАБОТКЕ ИМИ ЖИВОТНЫХ И РАСТЕНИЙ. Использование радиоизотопов оказалось весьма ценным при выработке системных инсектицидов для истребления насекомых-вредителей растений и животных. Общая картина поглощения, циркуляции и локализации, метаболизма, а также местонахождения и характера остаточных количеств инсектицидов были более быстро определены с помощью меченых материалов, чем это было возможно при других методах анализа.

Сведения, приобретенные энтомологами с помощью меченых инсектицидов, дали им возможность выяснить, почему некоторые из химикалий относительно более токсичны для насекомых, чем для млекопитающих, и почему некоторые из них представляются более безопасными для одних видов млекопитающих, чем для других. Результаты этих исследований привели к выработке новых методов истребления вредных насекомых. Были выявлены новые неизвестные до сих пор пути метаболизма и выработаны новые методы анализа.

Меченые инсектициды оказались чрезвычайно важными для получения основной информации о растительных системах. Применение радиоизотопов дало возможность проследить за поглощением, перемещением и метаболизмом системных инсектицидов в растениях. Помимо этого, удалось проследить пути попадания химикалий в организм насекомых, питающихся обработанными химикалиями растениями, а на насекомых могут быть изучены метаболизм этих химикалий и их выделение.

Использование меченых материалов дало возможность показать, что хлопковое растение, выращенное из обработанных системными инсектицидами семян, поглощает менее 5% примененной дозы. Другие исследования показали, что системные инсектициды не легко переносятся из листьев, обработанных инсектицидами, в новые ростки.

Использование радиоизотопов для изучения системных инсектицидов получит в будущем дальнейшее распространение. В настоящее время уделяется больше внимания основным физиологическим исследованиям относительно судьбы инсектицидов в растениях, у животных и насекомых. Другой областью, в которой радиоизотопы должны широко использоваться, являются исследования, направленные на улучшение методов применения системных инсектицидов.

EMPLEO DE LOS RADIOISÓTOPOS PARA ESTUDIAR LOS INSECTICIDAS APLICADOS A PLANTAS Y ANIMALES. Los radioisótopos han sido de gran utilidad en la preparación de insecticidas de acción indirecta para combatir los insectos nocivos para las plantas y los animales. Gracias al empleo de sustancias marcadas se han podido determinar los esquemas generales de absorción, circulación y localización, el metabolismo, el lugar donde se depositan los residuos y el carácter de éstos, con más rapidez que con otros métodos de análisis.

Gracias a los estudios hechos con insecticidas marcados, los entomólogos han podido averiguar por qué ciertos productos son relativamente más tóxicos para los insectos que para los mamíferos, y por qué algunos son menos peligrosos para ciertos tipos de mamíferos que para otros. Los resultados han permitido desarrollar nuevos métodos de lucha contra los insectos. Se han descubierto procesos metabólicos hasta ahora ignorados y se han desarrollado nuevos métodos de análisis.

El empleo de insecticidas marcados ha sido esencial para averiguar datos fundamentales de fitofisiología. Con los radioisótopos se han podido seguir en las plantas los procesos de absorción, translocación y metabolismo de los insecticidas de acción indirecta. También es posible seguirlos en los insectos que se alimenten con las plantas tratadas, y estudiar su metabolismo y excreción.

Por ejemplo, se ha demostrado que las plantas de algodón nacidas de semillas tratadas con insecticidas de acción indirecta absorben menos del 5% de la dosis aplicada. Otros estudios han demostrado que estos insecticidas no pasan fácilmente de las hojas tratadas a los brotes nuevos.

En el futuro se emplearán cada vez más los radioisótopos para estudiar los insecticidas de acción indirecta. Los estudios fisiológicos básicos de los procesos que sufren los insecticidas en las plantas, animales e insectos adquieren cada vez más importancia. También convendría emplear más intensamente los radioisótopos en los estudios destinados a mejorar los métodos de aplicación de los insecticidas de acción indirecta.

INTRODUCTION

Entomologists have used radioisotopes in many areas of research. One of the major fields in which this research has been productive has been that of the fate of insecticides in various biological systems. Through the use of radioactive samples, our understanding of the metabolic fate of insecticides under in vivo conditions has been greatly broadened.

The use of radioisotopes has given entomologists a tool of unparalleled sensitivity for measuring factors such as the rate of penetration or uptake of insecticides by plants, animals and insects. The various and complex reactions, whether intoxications or detoxications, undergone by pesticides in biological tissues have been studied most effectively in efforts in which labelled samples of insecticides have been used. The determination of general patterns of the rates at which insecticides are lost by excretion, or stored in the form of residues, has been greatly aided through the use of labelled materials. Differences in toxicity to different forms, i.e. selective toxicity, have become understood in part through the use of isotopes.

In the present paper, we shall attempt a review of the general principles and patterns of the fate of certain insecticides, as determined by means of these studies.

1. FATE OF INSECTICIDES IN ANIMALS

A. Introduction

Within the necessarily brief confines of the present review, the discussion will be restricted largely to comments on the fate of selected organophosphorus insecticides in warm-blooded animals. The entire area of this subject matter, particularly as it has to do with the development of systemic insecticides, has been reviewed extensively in recent years [8, 39, 40, 41, 55, 57]. Perhaps the most thorough recent review is that of O'BRIEN in his book "Toxic Phosphorus Esters" [38]. For the most part, the present paper will be restricted to general comments on work published before 1960, with somewhat more detailed comments on certain papers published in the past two years.

B. General patterns of the fate of systemic insecticides in animals

Early in their development, organophosphate insecticides were found to be more satisfactory for use on domestic animals than the chlorinated insecticides. The chief advantages of the organophosphates were the lack of persistent insecticide residues stored in the tissues of treated animals and the low levels or absence of insecticide in milk. Early analyses were generally made by means of chemical methods, each method being specific for the compound studied.

Extensive work with radiolabelled samples of organophosphates, for the most part prepared with P^{32}, verified the earlier findings. A long series of papers on the fate of labelled organophosphates in large animals has been

published in the United States in the last seven years. These studies have indicated that, in most instances, the organophosphates are rapidly absorbed by animals after dermal, oral, or intramuscular treatment. Following absorption, small amounts of the insecticides may be found in the blood of treated animals for a few hours. Degradation to non-toxic water-soluble compounds, which are then excreted via the urine, is rapid. The entire process is usually 90% or more complete within 48 h. Small amounts of radioactivity may partition into fat or be excreted in the milk. Generally, only a portion of this radioactivity is in the form of non-degraded insecticide, and even this portion is rapidly dissipated.

Within this overall pattern, considerable variation has occurred in the rates at which the insecticides undergo destructive metabolism. The variations observed have, for the most part, been a reflection of the relative solubility of the compounds studied. The more soluble the compounds are found to be in polar solvents such as water, and the lower their solubility in the more inert and non-polar solvents such as chloroform and hexane, the more rapidly the compounds and residues associated with them are dissipated.

C. Selected examples

Dipterex (dimethyl 2, 2, 2-trichloro-1-hydroxyethyl phosphonate) is an example of one of the organophosphates which undergo extremely rapid degradation in biological systems. The insecticide is soluble to the extent of about 20% in water. Partitioning data have shown that when a sample of the radioactive insecticide is partitioned with equal volumes of chloroform and water, about 45% remains in the aqueous layer [2]. This result is in contrast to what occurs with most of the organophosphates, which can be recovered from aqueous solvents in a near-quantitative manner by a single extraction with chloroform.

Dipterex was the first of the insecticidal organophosphates to be studied in detail following administration to cattle. In 1956, ROBBINS et al. [46] reported on the fate of P^{32}-labelled Dipterex in a lactating cow following administration of a single oral dosage of 25 mg/kg. Radioactivity equivalent to 15 ppm appeared in the blood at 2 h after treatment and then declined to less than 1 ppm at 18 h. However, of the peak level of 15 ppm in the blood, only 7.5% behaved as Dipterex; the other 92.5% represented material degraded within 2 h of administration. There was no evidence that any unmetabolized insecticide was excreted in the milk, although 0.2% of the administered radioactivity was recovered in it. The major means of elimination of the insecticide was via the urine, the peak of excretion occurring 2.5-5.5 h after treatment. Only 3% of the administered radioactivity was recovered in the faeces, an indication that the insecticide was readily absorbed from the gut.

Similar studies on the fate of Dipterex in a dog and in rats have been reported [2, 3]. In these studies, evidence was obtained that degradation in smaller mammals, as in the cow, proceeds rapidly and is virtually complete within two days of treatment.

Co-ral (O, O-diethyl O-3-chloro-4-methyl-2-oxo-2H-1-benzopyran-7-yl phosphorothioate) is an insecticidal organophosphate with solubility properties much different from those of Dipterex. Unlike Dipterex, Co-ral is almost

completely insoluble in polar solvents; further, it is not even very soluble in many of the more common non-polar solvents. This difference in solubility properties of Co-ral results in a metabolic picture in animals significantly different from that of Dipterex.

When radioactive samples of Co-ral were sprayed on Hereford bulls, the major portions of the radioactivity were still present on the hide of treated animals 14 d after treatment [46].

Poor absorption of Co-ral was also demonstrated by analysing urine and faeces for radioactivity; less than 10% of the applied dosage was excreted via these routes. With Dipterex, absorbed radioactivity was almost quantitatively excreted; with Co-ral, considerable amounts of residual radioactivity were present in normal phosphorus-containing tissues and organs, evidence that the compound was degraded completely to phosphoric acid. Residues behaving as unmetabolized insecticide were less than 1 ppm in all tissues within two weeks of treatment.

In another study, the fate of radioactive Co-ral in cattle and goats following dermal treatment was investigated [29]. Again large radioactive residues behaving as inorganic phosphate were found in several tissues. Species differences were observed. For example, bone contained high levels of radioactivity in the cow, but low residues in the goat. Poor absorption of Co-ral following oral administration was found in the rat [29, 30]. In both studies, considerable unmetabolized insecticide was excreted in the faeces, evidently never having been absorbed from the intestinal tract.

D. Recent studies

Recently published work has tended to verify the results of earlier studies as to the nature of the metabolism of organophosphates in warm-blooded animals. The occurrence of interspecific metabolic differences has been investigated and the nature of residues present in other domestic animals studied.

In a recent study, the fate of P^{32}-dimethoate (O, O-dimethyl S-(N-methylcarbamoylmethyl) phosphorodithioate) in sheep was determined [11]. As in earlier studies involving cattle [12, 28], the insecticide was rapidly degraded to non-toxic products. In this study, paper chromatographic evidence was obtained which indicated that cleavage of the carbonyl-nitrogen bond was the main site of metabolic detoxication and confirmed previous findings of studies in which ion-exchange chromatographic procedures had been utilized. Residues behaving as unmetabolized insecticide were practically nil in tissues of animals sacrificed 2 and 4 weeks after treatment.

There are no published papers on the fate of P^{32}-labelled Ruelene (O-4-tert-butyl-2-chlorophenyl O-methyl methyl-phosphoramidate), an insecticide with systemic properties, in large animals. However, in poultry, it was determined that the insecticide was rapidly degraded after oral administration [10]. No evidence was obtained for the presence of insecticide in the eggs of treated hens. Similar work in poultry with P^{32}-labelled Co-ral [16] has indicated that small insecticide residues may be present in eggs after dust treatment. As in work with cattle, these differences are probably a reflection of differences in solubility properties and ease of metabolic detoxication of the two insecticides.

Another material of solubility properties similar to Co-ral is Bayer 22408 (O, O-diethyl O-naphthalimido phosphorothioate). In cattle, following oral treatment with radioactive samples of the insecticide, GATTERDAM et al. [22] found that more P^{32} was eliminated via the faeces than via the urine. Significant portions of the radioactivity present in the faeces occurred as undegraded insecticide and its oxygen analogue. The authors suggested that the insecticide might be effective against coprophagous insects, although it had proved to be ineffective as a systemic insecticide against several species of livestock pests.

Following dermal administration of the same insecticide to Holstein cows, BUTTRAM et al. [9] determined that residues of less than 0.1 ppm were present in the milk. Curiously, from 32 to 40% of the applied dosage was excreted in the faeces, with as much as 22% occurring as non-degraded insecticide. The residues of insecticide in the faeces were toxic to stable-fly larvae introduced into samples collected 2-7 d after treatment. The toxicity data obtained agreed well with those of radioactive analyses.

E. Future uses of radioisotopes in animal systemic research

The use of labelled samples of insecticides has proved an excellent tool in systemic insecticide research. The methods of analysis developed in these studies and the general pattern of results elucidated have been applicable to other studies involving the fate of labelled insecticides in insects and mammals. Future utilization of labelled insecticides will probably be made in studies involving isotopes other than P^{32}. Thus, the World Health Organization is currently making samples of C^{14}-labelled malathion available to interested entomologists, and a study on the fate of a tritiated sample of Famophos (O-p-(dimethylsulfamoyl) phenyl O, O-dimethyl phosphorothionate) in mammals and insects has been reported [49].

A new usage of organophosphate insecticides, derived as a direct result of the radioactive studies, was developed for the control of faeces-breeding flies; such control is obtained by feeding the insecticides to the animals so that their faecal deposits will be toxic to insects breeding therein. Thus, both Co-ral and Bayer 22408, materials of such poor solubility that they are never absorbed from the digestive tract, have proved effective in controlling faeces-breeding flies [1, 19]. Similar studies on the control of flies breeding in poultry droppings have been reported [17, 50].

That the use of labelled compounds is not the sine qua non of metabolic studies has been shown recently in work by HOPKINS et al. [26]. These workers have demonstrated through the use of enzymatic rather than radioassay techniques that active antiesterase materials other than the oxygen analogues of phosphorothioates may be produced and be responsible for toxic effects. Working with ronnel (O, O-dimethyl O-(2, 4, 5-trichlorophenyl) phosphorothioate), they found the parent insecticide and its oxygen analogue present in the blood of treated cattle; in addition cattle grubs from the same animal contained ronnel and an unidentified anticholinesterase other than the oxygen analogue. The authors did not speculate on the nature of the unknown material.

As in plant systemic work, no problems of technique have arisen in the use of labelled systemic insecticides. Not all radioactivity present in tissues

is insecticide; indeed, not even all radioactivity soluble in organic solvents is insecticide. These problems of analysis have been solved in part through the use of a hexane-acetonitrile clean-up partitioning procedure. However, even this tool has proved to be less than perfect, since it has been shown that feeding labelled phosphoric acid to animals results in the occurrence of acetonitrile-soluble radioactive residues [18]. Thus, in order to prove the presence of toxic residues the employment of paper and column chromatographic techniques of analysis as well as enzymatic assays has been necessary. The extreme sensitivity of these alternative methods can, in certain instances, negate the advantages of sensitivity obtained through the use of labelled samples of insecticide.

2. FATE OF INSECTICIDES APPLIED TO PLANTS

A. Introduction

The portion of this report dealing with the fate of insecticides in plants will be limited to a general review of organophosphorus systemic insecticides. Systemic insecticides, as used to combat phytophagous insect pests, are compounds which are absorbed by a plant and translocated to other parts of the plant in large enough amounts to be toxic to insects feeding thereon. The first practical demonstration of this method of insect control was reported by HURD-KARRER et al. [27], who found that selenium was absorbed by the roots of wheat plants and translocated to the aerial plant parts in sufficient quantities to control aphids. However, little interest was shown in this area of research until SCHRADER [48] discovered the systemic activity of certain organic insecticides. Since this time, considerable work has been done on plant systemic insecticides.

Nearly all of the organic insecticides used at the present time possess some degree of systemic action. Most of the insecticides which are commonly called non-systemic will penetrate into the leaf, stem or roots of plants. This property has been called local systemic or Tiefenwirkung action.

METCALF [34] has stated that systemic action in plants is controlled by the following characteristics of an insecticide: (1) ability of the compound to penetrate into the plant; (2) sufficient water-solubility to move with the transpiration stream; and (3) sufficient stability in the plant so that the insecticide or its toxic metabolic products can exert the desired insecticidal action. These criteria are related to the physical and chemical properties of the insecticide as interrelated with the physiological action of the plant.

Several excellent reviews have been published recently on systemic insecticides [4, 37, 44]. Recent books by O'BRIEN [38] and HEATH [25] contain much information on systemic insecticides. In the following discussion some of the major findings in the areas of absorption, translocation and metabolism of systemic insecticides by plants will be briefly reviewed. A large portion of the data pertaining to these studies was obtained with the use of radiolabelled compounds. Without radioisotopes, much of these data would not be available because precise quantitative and qualitative chemical analytical methods are also not available. More information is available on

metabolism of systemic insecticides than on absorption and translocation, principally because of the emphasis recently placed on insecticide residues on and in food and fibre. Nearly all countries require residue data of some kind before an insecticide can be marketed.

B. Absorption

As previously mentioned, the absorption of organic insecticides by plant tissue is not uncommon. This absorption is undoubtedly due to the lipoid solubility of these compounds. In practice, systemic insecticides are usually applied so that they are absorbed by either the roots or leaves. Some experimental work has been done on stem treatment.

Using P^{32}-labelled compounds, DAVID [13, 14] demonstrated that Schradan (octamethyl pyrophosphoramide) was selectively rejected by bean roots but Dimefox (tetramethylphosphorodiamidic fluoride) was selectively absorbed. METCALF et al. [35] reported that P^{32}-labelled Schradan was absorbed by lemon roots at the same rate as $H_3P^{32}O_4$ from water cultures. TIETZ in a comprehensive report of studies with P^{32}-labelled demeton (Systox) (O, O-diethyl S(and O)-2-(ethylthio)ethyl phosphorothioate) [54], concluded that the toxicant was absorbed readily by the intact root. The most rapid absorption was observed in plants growing in water culture; slower absorption took place in sand and soil cultures. Using beans grown in water culture, this worker found that the roots selectively absorbed the insecticide during the first day but thereafter absorbed water and the insecticide at about the same rate.

Recent reports by HACSKAYLO et al. [23, 24] with P^{32}-labelled phorate (O, O-diethyl S-(ethylthio) methyl phosphorodithioate) and P^{32}-labelled dimethoate (O, O-dimethyl S-(N-methylcarbamoylmethyl) phosphorodithioate) discussed the absorption of these insecticides by the roots of cotton plants. The absorption of P^{32}-labelled phorate from nutrient solutions by cotton roots was very rapid initially but decreased with time. A leakage of the absorbed phorate from the roots back into the nutrient solution was observed. This leakage indicated that the insecticide caused some damage to the root system of the treated plants. In studies with P^{32}-labelled dimethoate, it was found that this compound was selectively rejected by the roots of cotton plants. The roots of cotton plants grown in complete and phosphorus-deficient water cultures absorbed essentially the same quantities of dimethoate.

LINDQUIST et al. [32] exposed small sections of the roots of intact cotton seedlings to P^{32}-labelled phorate and $H_3P^{32}O_4$. They found that the amount of phorate absorbed was in direct proportion to the volume of the root exposed to the insecticide. No evidence was found to indicate that any portion of the root absorbed phorate preferentially. However, $H_3P^{32}O_4$ was actively absorbed by the cotton root from 20 to 40 mm above the root tip. The volume of the root exposed to $H_3P^{32}O_4$ did not influence the amount absorbed. These workers concluded that the mechanism of absorption of phorate and that of $H_3P^{32}O_4$ by cotton roots were not similar.

Seed treatment with systemic insecticide for the control of insects attacking young plants has received considerable attention during the past few years. This method of applying systemic insecticides has been recently reviewed [42]. DAVID et al. [15] studied the absorption of P^{32}-labelled

demeton thiol isomer by seeds. These workers concluded that the demeton thiol isomer was absorbed directly into the cotyledons as the broad-bean seeds germinated and that some of the toxicant was absorbed by the roots of the seedling after emergence. REYNOLDS et al. [43] reported on a series of experiments with cotton, alfalfa and sugar-beet seed treated with P^{32}-labelled phorate, demeton thiol isomer and Di-syston (O, O-diethyl S-[2-(ethylthio) ethyl] phosphorodithioate). These workers demonstrated that the amount of toxicant absorbed by the plants following seed treatment was about the same as that following application of granules in the seed row. From 3 to 9% of the dosage applied was recovered in the resulting plants 16 d after planting. Similar studies with P^{32}-labelled phorate [33] demonstrated that absorption of this insecticide following seed treatment takes place primarily during the first few days after planting. In standard laboratory germination tests, phorate-treated intact cotton seeds absorbed 0.7% of the applied dose in 5 d. However, with the seed coats removed before treatment, the seeds absorbed 11.9% of the applied dose. Furthermore, more dehulled seeds germinated in the test, and germinated faster, than intact seeds. For comparison, intact and dehulled seeds were treated with $H_3P^{32}O_4$ and the radioactivity absorbed by the germinating seeds was measured. The presence of the cotton seed hull did not reduce the absorption of the $H_3P^{32}O_4$ as it did the absorption of phorate. Dimethoate is absorbed by cotton plants grown from dimethoate-treated seeds to about the same extent as phorate [31]. From 4 to 6% of the applied dose of phorate or dimethoate was found in the cotton plant. Bidrin (Shell 3562; 2-dimethylcarbamoyl-1-methylvinyl dimethyl phosphate) is absorbed by cotton seeds to approximately the same extent (LINDQUIST et al., unpublished data). In conclusion it can be stated that with the aid of radio-isotopes it has been found that the seed-treatment method is not a very efficient way to get a systemic insecticide into a plant.

MITCHELL et al. [37] discussed the absorption of systemic insecticides following stem or bark application. In general, this method of applying the toxicants is as effective or more effective than root treatment. Preliminary results with P^{32}-labelled Bidrin indicate that the toxicant is very rapidly absorbed by cotton stems (LINDQUIST et al., unpublished data). Data presented by BOWMAN et al. [6] indicated that phorate and Chipman R-6200 (O, O-diethyl S-(2-diethylaminoethyl) phosphorothioate p-toluenesulfonate) were readily absorbed by cacao trees when the materials were implanted into the tree trunks. SANTI [47] demonstrated that dimethoate (Rogor) with tri-n-butylphosphate added was rapidly absorbed by lemon-tree trunks.

Foliar sprays of systemic insecticides probably are more widely used than any other method of application. However, most of the insecticidal activity of these insecticides is local systemic action, i.e., penetration into the leaf. It is apparent, therefore, that absorption of systemic insecticides by leaves is quite important. MITCHELL et al. [37] compiled data from several sources relative to the rate of penetration of systemic insecticides into lemon leaves. Dimefox was absorbed more rapidly than Schradan, demeton, or amiton (O, O-diethyl S-2(diethylaminoethyl) phosphorothioate hydrogen oxalate). Several studies have been conducted on the absorption of P^{32}-labelled systemic insecticides by leaves [5, 13, 14, 52, 53, 54]. In general, these authors found that the compounds were not absorbed through the stomata and that light and temperature influenced the rate of absorption.

Although methyl parathion (O, O-dimethyl O-p-nitrophenyl phosphorothioate) is not regarded as a systemic insecticide, recent studies [51] have shown that this compound is readily absorbed by cotton leaves. By using P^{32}-labelled material, maximum absorption was found to take place within 2 h, after which the concentration within the leaf gradually decreased. Studies on the penetration of P^{32}-labelled Bidrin into excised cotton leaves indicated that maximum absorption was effected after 4 h when the leaves were held in the light, but took 24 h when the leaves were held in the dark (LINDQUIST et al., unpublished data). No difference in the rate or amount of insecticide absorbed was found when leaves were treated on the upper or lower surfaces.

C. Translocation

The translocation of systemic insecticides from the roots to the aerial plant parts appears to be via the transpiration stream in the xylem. Recent reports by HACSKAYLO et al. [23, 24] supported this observation. These authors also demonstrated that, following the initial rapid absorption of phorate by cotton roots, the insecticide was rapidly translocated upward into the leaves, thus reducing the phorate concentration in the roots. Factors which governed transpiration, i.e., temperature and humidity, also governed the amount of phorate accumulated in the leaves. LINDQUIST et al. (unpublished data) have shown that the translocation of dimethoate to cotton leaves, after application of the toxicant to the soil, follows the transpiration stream. Experiments were conducted in which half of the plant was held under conditions which slowed transpiration and the other half under conditions which were favourable to transpiration. The results of these tests demonstrated that dimethoate accumulated in much larger quantities in leaves held under favourable transpiration conditions.

Translocation of systemic insecticides following seed treatment appears to be similar to translocation following root treatment [37]. However, as previously stated, considerable amounts of the insecticide are absorbed directly by the germinating embryo. In fact, LINDQUIST et al. [33] have shown that phorate-treated cotton seeds absorb most of the toxicant that will eventually be absorbed before the young seedlings emerge. This result indicated that there is little actual translocation of the toxicant via the root system following seed treatment.

The translocation of systemic insecticides following stem treatment has not been investigated very thoroughly. WEEDING [56] demonstrated that S^{35}-labelled demeton was translocated upward and downward in lemon seedlings after stem application. Most of the movement was upward. Translocation took place initially in the xylem but gradually diffused into the phloem. LINDQUIST et al. (unpublished data) found that P^{32}-labelled Bidrin was translocated almost entirely upward when applied to the stems of cotton plants. METCALF et al. [36] found that P^{32}-labelled demeton thiono isomer was translocated to the leaves of lemon seedlings after stem treatment in a pattern similar to that noted after root treatment.

More research has been reported on the translocation of systemic insecticides after foliar application than on translocation after other methods

of treatment. In general, these insecticides are not translocated in large enough quantities to be insecticidally active. However, if the entire plant is treated, sufficient translocation takes place to the new growth to give insect protection. Upward translocation takes place much more readily than downward translocation. THOMAS et al. [52] reported that both temperature and light are important in translocation. Light appeared to be the most important factor. The translocation of these toxicants following foliar application probably is related to photosynthate movement in the plant [37]

D. Metabolism

Since the metabolism of systemic insecticides has been thoroughly reviewed [20, 21, 25, 38], the discussion of this subject here will be rather brief. Almost all of the metabolism studies have been carried out with radiolabelled insecticides. This research has revealed the relatively complex metabolic degradation pathways of many of the systemic insecticides. In a recent report on dimethoate [7], 11 metabolites were detected in various insect and plant extracts. Without the use of radiolabelled compounds research of this nature would not be possible.

One of the major difficulties in measuring the absorption and translocation of systemic insecticides is the rapid metabolism of many of these compounds by the plant [37]. Care must be taken before assuming that all radioactivity found in a plant after treatment with a labelled systemic insecticide is associated with the parent molecule. BULL et al. [7] found that cotton seedlings degraded more than 60% of the absorbed dimethoate in 5 d following root treatment in solution culture. Insects feeding on the treated plants further degraded the toxicants. Additional work along this line is needed to establish the relationship between plant and insect metabolism.

E Future uses of radioisotopes in plant systemic research

Radioisotopes have been used quite extensively in the relatively few investigations concerned with systemic insecticides. In the areas of absorption and translocation, very few critical studies have been done in which the metabolism of a compound has been taken into consideration. This effect of the plant on the insecticide must be known before any real knowledge of the systemic behaviour can be studied. Metabolism, however, is a major study in itself.

It is the writers' belief that the use of radiolabelled systemic insecticides would greatly aid the development of methods of applying these chemicals. Very little work has been done in this area. Nothing is known about the effect of various surfactants on the systemic action of these compounds. Very little has been done to elucidate the movement of systemic insecticides in the soil after granular application. Stem treatment is a very promising method of application. All of these topics can be investigated with much greater precision with radiolabelled compounds than without. In all studies discussed above, however, metabolism of the insecticide must be taken into account.

Other studies which can best be done with the aid of radiolabelled compounds include a determination of the mode of action of the absorption of

systemic insecticides by plants and the physical and chemical processes involved in translocation. Considerably more information must be available in this area before we can understand much about systemic insecticide behaviour in plants.

3. SUMMARY

It should be obvious from the preceding report that radioisotopes have been used quite extensively in studies associated with the fate of insecticides applied to animals and plants. Although residues as such have not been discussed at length, all of the topics covered relate directly to residues. In fact, much of the information cited was obtained during residue studies.

The use of radioisotopes has made it possible to study precisely the absorption, translocation, metabolism and excretion of insecticides by animals and plants. As more data are accumulated on the topics discussed in this paper, it will be possible for chemists to synthesize insecticides for use against specific insect pests. Also, insecticides which are extremely toxic to insects but virtually non-toxic to mammals should be the result of this type of research.

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DISCUSSION

J.W. MILES: I would like to thank the authors for their acknowledgement of the support given by the World Health Organization (WHO) to the work done with C^{14}-labelled malathion. I would also like to mention that WHO has supplies of C^{14}-labelled DDT, dieldrin and malathion for distribution to laboratories for projects involving insects of public health importance. In addition a new carbamate insecticide, O-isopropoxyphenyl-N-methyl carbamate, labelled in the isopropyl group, will soon be available for distribution. Further information can be obtained by writing to the Vector Control Section, Division of Environmental Health, WHO.

W. KLOFT: It is stated in the paper that any systemic insecticide labelled with P^{32} is transported first upwards into the xylem of a plant, and then transported into the phloem sap. Could you please indicate the methods that could be used to prove that?

W. E. ROBBINS (on behalf of F. W. Plapp and D. A. Lindquist): This is dealt with very fully in the work by Mitchell et al cited as reference [37] to the paper.

G. F. BURNETT (Chairman): What is the chance of charging a cow with sufficient radioactivity to mark tsetse flies (Glossina spp.) feeding on it when driven through the bush? These flies feed on nothing but blood and are very difficult to raise in the laboratory There is no convenient way of marking them without capture.

W. E. ROBBINS: It would probably be possible. There are quite a number of studies on the fate of radioisotopes in large animals, and certain of these studies might aid you in selecting the appropriate isotope for high blood levels in the animal and a long biological half-life. About 1952, for instance, Dahm did similar work on a goat. He was comparing parathion and inorganic phosphorus. His work might be of assistance as regards the maintenance of blood levels.

F. T. PHILLIPS: I would like to comment on the use of excised cotton leaves in penetration studies of P^{32}-labelled Bidrin. I have been told that the metabolic processes of excised leaves are different from those of leaves attached to the plant, so perhaps the figures obtained for the excised leaves would not be the same as for leaves which were attached to the plant? Photosynthesis and production of proteins in the plant are not going on, for instance, and that may affect your figures.

W. E. ROBBINS: I think Dr. Casida would like to comment.

J. E. CASIDA: Yes, Dr. Phillips is quite correct, but the intact plant can be treated by injection through the stem to minimize this difficulty. We take a very fine pulled-out glass needle, which is easily inserted into the stem with no apparent damage to the plant. Between 5 and 50 μl of solution put in are taken up by the plant within half an hour to two hours. Then we continue with the study.

SOME PROBLEMS IN THE DETERMINATION OF RESIDUES IN PLANTS AND MAMMALS

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Abstract — Résumé — Аннотация — Resumen

SOME PROBLEMS IN THE DETERMINATION OF RESIDUES IN PLANTS AND MAMMALS. In most instances residues must be determined by chemical or biochemical methods without the use of radioisotopes. These methods assume that the nature of the toxic compounds present is known, and that they can be extracted in known yields. Neither assumption is easily validated except by using radioisotopes. The use of radioisotopes to investigate these problems is described, with examples taken from work on demeton, dieldrin and the fungicide triphenyltin acetate.

PROBLÈMES RELATIFS A LA DÉTERMINATION DES RÉSIDUS DANS LES VÉGÉTAUX ET CHEX LES MAMMIFÈRES. Dans la plupart des cas, les résidus doivent être déterminés par des méthodes chimiques ou biochimiques, sans recourir aux radioisotopes. Ces méthodes sont fondées sur l'hypothèse que l'on connaît la nature des composés toxiques présents et que l'on peut les extraire en quantités connues. Aucune de ces deux conditions n'est facile à réaliser si ce n'est à l'aide de radioisotopes. L'auteur décrit l'utilisation des radioisotopes dans l'étude de ces problèmes et donne des exemples tirés de travaux sur le déméton, la dieldrine et un fongicide (l'acétate de triphényltine).

НЕКОТОРЫЕ ПРОБЛЕМЫ ОПРЕДЕЛЕНИЯ ОСТАТОЧНЫХ ИНСЕКТИЦИДОВ В РАСТЕНИЯХ И У МЛЕКОПИТАЮЩИХ. В большинстве случаев остаточные инсектициды должны определяться химическими и биохимическими способами без применения радиоизотопов. При применении этих методов предполагается, что известен характер присутствующих токсических смесей и что они могут быть экстрагированы в известных количествах. Ни одно предположение не может быть свободно принято, если оно не подтверждено радиоизотопной методикой. Описывается применение радиоизотопов для исследования этих проблем и приводятся примеры, взятые из работ, связанных с деметоном, дильдрином и фунгицидным трифенилацетатом олова.

DETERMINACIÓN DE RESIDUOS DE INSECTICIDAS EN PLANTAS Y MAMÍFEROS. En la mayor parte de los casos la determinación de los residuos de insecticidas debe efectuarse por métodos químicos o bioquímicos, sin emplear radioisótopos. Para estos métodos se parte de dos supuestos: que se conoce la naturaleza de los compuestos tóxicos presentes y que esos pueden extraerse con rendimientos conocidos. Ninguno de estos supuestos puede justificarse fácilmente si no es mediante el empleo de radioisótopos. El autor de la memoria examina este empleo de los radioisótopos y cita ejemplos tomados de trabajos efectuados con los insecticidas Demetón y Dieldrín, y con el fungicida acetato de trifenilestaño.

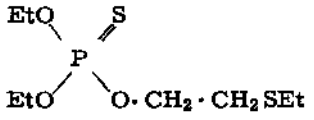
Much of the work in assessing the safety of a pesticide depends upon analysis of residues in crops and animal tissues. A high degree of reliability in methods is obviously essential – residue analysis is not a field in which speculation can be allowed much part – and this sets problems. Some of these problems, and the use of labelled compounds in solving them, are considered in this paper, with examples from work with which the author has been concerned. Not all of this work is new, but little of it has been described in this context.

A problem of very general interest is that of the reliability of recovery tests as usually carried out. In the instance to be described, an apparently reliable method gave substantially nil residues when used on a treated crop, a result which is very acceptable, but also, naturally, suspect. The example is that of Dimefox in cocoa beans.

Dimefox is the organophosphorus systemic insecticide N, N'-tetramethylphosphorodiamidic fluoride, $(Me_2N)_2PO \cdot F$. It was proposed at one time for the control of mealy-bugs on cocoa trees (Cacao theobroma) [1]. In view of its high toxicity, only levels below 0.1 mg/kg would be acceptable in the beans at harvest. An adequate analytical method, sensitive to 0.01 mg/kg, was developed. The beans were macerated with an oil of high boiling point. The oil was distilled under reduced pressure, and the relatively volatile Dimefox was carried over quantitatively with the first few per cent of the oil, from which it could be extracted, and estimated as phosphate [2]. This method was applied to beans from treated trees and gave residues of 0.05 mg/kg or less, even when the trees as a whole contained enough Dimefox to kill the mealy-bugs feeding on their foliage. It seemed possible that the recovery tests were at fault: Dimefox might be recovered quantitatively when added to beans just before maceration, but might not be recovered when it reached the beans by a systemic route, when it might either become irreversibly adsorbed or enclosed in plant matrices not destroyed by the distillation process. Pods were therefore left for a few days with their stems dipped in a dilute aqueous solution of Dimefox labelled with P^{32}. The pods were then opened, and the beans were divided into seven samples and analysed for Dimefox. Four samples were analysed in the usual way, except that the phosphate was estimated as P^{32} in a liquid counting tube, and were found to contain 0.044 ± 0.002 mg/kg (means ± SD). The remaining samples were macerated with water or refluxed with chloroform, and the macerates were counted. The extracts were then bulked, and the Dimefox was separated by partitioning between water and chloroform and estimated by counting. The Dimefox content of the beans as found by this completely different method was 0.046 mg/kg, in excellent agreement with the results of the distillation method. The distillation method was thus fully vindicated. These experiments also provided an explanation for the very low quantities found. The macerates in water contained P^{32} equivalent to 4 mg/kg of Dimefox, but 99% of this was as ionic compounds of very low toxicity, i.e. fruit tissue metabolized Dimefox very much more rapidly than leaf tissue did. This conclusion was later confirmed by analysing pods from a tree treated with P^{32} Dimefox. In these experiments the residual Dimefox concentration was too low to measure, but ionic decomposition products were found.

In this type of study the advantage conferred by using the labelled compound is that it can be estimated reliably after a method of extraction which does not separate it from naturally occurring compounds. The necessity for this separation is, of course, the controlling factor in most procedures. When it does not exist, as with labelled compounds, very much more drastic procedures can often be used, and a reliable check to a normal, non-isotopic method can be provided. It would be desirable always to have such checks; and where organophosphorus compounds are concerned this could easily be done almost always.

Another type of problem may arise when there is an obvious discrepancy between the behaviour of an insecticide and the methods suggested. This was the case when Demeton was first marketed under the names Systox and Bayer 8169. Analysis showed that Demeton consisted of two isomers: diethyl 2-ethylthioethyl phosphorothionate, I and O, O-diethyl S-2-ethylthioethyl phosphorothiolate, II [3].



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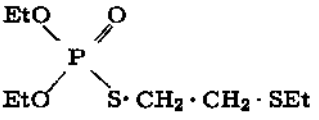
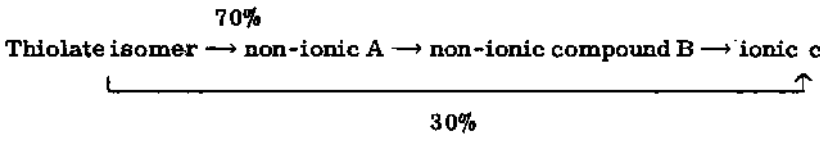


II

Tests on toxicity to insects and mammals indicated that the thiolate, II, was about ten times more active than the thionate, I. Both compounds could be extracted from vegetable material by hydrocarbon solvents, and the methods of analysis proposed depended on this type of extraction, followed by biological estimation using insects. Such methods gave very low residues within a few days of spraying, although insects feeding on treated plants continued to die for several weeks. In this case the results could not be explained in the same way as the low residues of Dimefox in cocoa beans, as the insects killed by Demeton were feeding on the actual foliage analysed. Some sugar beet (Beta sativa) and other plants were therefore treated with the thiolate isomer, II, labelled with P^{32}. Foliage was taken at various times after treatment and macerated with water. The macerate was then extracted successively with solvents of increasing polarity, and these extracts were in turn subjected to a simplified form of counter-current extraction. Four discoveries were made. Firstly, the thiolate itself rapidly disappeared, as already indicated by other methods. Secondly, two non-ionic compounds were produced. Thirdly, these apparently fitted a metabolic scheme which can be represented as follows:-



The non-ionic metabolites persisted for a few weeks, so that, if they were insecticidal, their formation could account for the prolonged systemic action of Demeton. Fourthly, neither of the two non-ionic metabolites could be extracted by most hydrocarbon solvents, and would not, therefore, be detected by the methods of analysis suggested. The metabolites were extracted in sufficient quantity for their toxicity to be tested, and as they were nearly as toxic as the parent compound both to insects and mammals it was clear that any worth-while method of residue analysis must record them. This work, which was reported briefly by HARTLEY [4], did not show what the metabolites were, nor did it supply a method of residue analysis; these problems were solved later [5, 6, 7, 8]. Nonetheless, the basis of much further work on those phosphorus insecticides which metabolize to active

compounds in plants was laid down in this preliminary study, which occupied only about 10 weeks. It is now common practice to use tracers in order to establish the presence or absence of toxic metabolites in plants, and their use for this purpose constitutes an essential step in the justification of analytical methods for most compounds with systemic properties.

Methods of analysis of animal tissues and products, meat and milk, do not differ in principle from the analysis of crops, and the value of using labelled compounds is the same. New problems appear, however, in the toxicological studies which are an essential part of the work involved in clearing a pesticide for use.

The initial stage in such investigations consists in determining the acute and chronic toxic effects of the compound in question, and of observations on signs and symptoms of poisoning. After this, some knowledge of the behaviour of the compound in mammals is desirable, with the eventual aim of explaining its mechanism of action. This degree of success is very rare. It has been substantially achieved with organophosphorus pesticides, and is of practical value: recommendations on permissible levels, handling and so on can be made with much more confidence about these compounds than about others whose mechanism of action is largely unknown, such as the chlorinated hydrocarbons. It is clear, however, when the studies on phosphorus anticholinesterases are considered as a whole [9, 10, 11] that tracer studies formed only a small part of the work of establishing the mechanism of action: the contribution of biochemists, pharmacologists and physiologists who did not need to use tracers was far greater. It is in the first stages of an investigation that tracers overcome difficult problems: before one asks "How does this act?", and when the questions are: "Where does this lodge in the body, and for how long; how rapidly is it excreted; and is it metabolized at all?" These questions can be answered only by analyses, and they raise acute problems, because the toxicologist wants to start work with small animals in order to correlate easily measurable effects such as the LD50 values with concentrations in vivo, and the sample sizes are then usually so small that most chemical methods are not sensitive enough. Some work still in progress on the fungicide triphenyltin illustrates these points.

Triphenyltin salts have the structure $(C_6H_5)_3SnX$, where X is an acid group. The acetate, under the name Brestan, has been used to control blight (Phytophthora infestans) on potatoes, and other fungus diseases on celery and sugar beet. The compound is very toxic by intraperitoneal injection, with LD50 values of 10 mg/kg in the rat and 4 mg/kg in the guinea-pig. Oral LD50 values are higher – 450 mg/kg for the rat and 30 mg/kg for the guinea-pig when the compound is given in arachis oil [12] – but the LD50 value in the rat is probably only about 70 mg/kg when the compound is given in dimethylformamide. The animals die 2-7 d after a lethal dose, with no specific symptoms and no obvious pathological lesions. Two questions arose. Firstly, does the compound persist in the body? Delayed death is not good evidence for persistence. Secondly, is the rat less sensitive than the guinea-pig to oral dosing because it absorbs the compound less readily, or because it metabolizes it rapidly before absorption can take place?

A first attempt was made to answer these questions by means of chemical methods of analysis, which appeared sensitive enough [13, 14]. When, however, triphenyltin acetate was left in tissues for 30 min before extraction

was attempted, only one method of extraction was found useful, namely maceration with a mixture of chloroform, methanol and conc. hydrochloric acid (200/100/2 by volume) similar to that used for the extraction of proteolipids [15, 16]. This had to be followed by tedious processing, and then only worked reliably on rat brain.

A specimen of triphenyltin chloride labelled with Sn^{113} (500μc/g) was then obtained from the Radiochemical Centre, Amersham, England. Experiments were carried out on rats and guinea-pigs, but only those on the rat are reported here. Essentially, albino male rats (Porton Wistar strain) were dosed with triphenyltin chloride intraperitoneally in arachis oil or orally in arachis oil or dimethylformamide. Animals were killed at intervals after injection, and specimens were taken for analysis. Urine and faeces were also collected from animals given the compounds orally. The total Sn^{113} content was estimated by scintillation counting in a 5-cm well-type crystal. The efficiency was 27% for a 1-ml specimen and 18% for a 5-ml specimen. Specimens had to be left about 12 h between extraction and counting, to allow equilibration between Sn^{113} and its indium daughter-isotope, In^{113m}, with a half-life of 104 min. Most of the recorded radiation is due to In^{113m}, and the equilibrium is disturbed by many extraction procedures.

The results of distribution studies on rats given triphenyltin chloride intraperitoneally are shown in Table I. The Sn^{113} was distributed throughout several tissues, including the brain, the concentration fell only slowly, and the distribution did not change much with time. In brain tissue measurable concentrations could be found 38 d after treatment. The high concentrations found 2 h after injection in specimens taken from the abdominal cavity probably represent material on the surface from the injection and should be discounted.

TABLE I

CONCENTRATIONS OF TIN IN THE TISSUES OF RATS TREATED WITH TRIPHENYLTIN CHLORIDE INTRAPERITONEALLY

Concentrations are expressed as μg triphenyltin chloride per g tissue. Columns headed 'A' refer to rats given 8.8 mg/kg, columns headed 'B' to rats given 3.5 mg/kg, and column C to rats given 4.4 mg/kg

Tissue	Times after injection						
	2 h	1 d		2 d	4 d		10 d
	A	A	B	A	A	B*	C*
Brain	3.2	5.8	2.6	3.6	4.4	1.2	0.7
Liver	41	28	8.7	10	13	2.2	1.1
Kidney	15	20	8.0	19	14	6.8	4.4
Fat	16	10	3.0	21	-	1.2	0.7
Muscle	2.2	2.7	1.2	2.1	-	0.6	0.4
Heart	8.7	2.2	2.2	3.3	-	-	-
Blood	2.3	1.1	0.5	0.8	-	0.14	0.05

* Average of 3 rats

The results did not show that the compound recorded was triphenyltin. Two methods have been used to investigate this, isotope dilution analysis and the separation procedure described by BOCK et al. [17]. Both agreed in showing that, of the Sn^{113} in rats 3 d after peritoneal injection of 6 mg/kg, 85% in the brain and 25% in the liver and kidney were present as triphenyltin. Bock's method also indicated that 25% in all those tissues was present as triphenyltin 18 d after injection. The method may not achieve complete separation in the presence of animal tissues, and this result is being checked by the isotope dilution method. If it is correct, triphenyltin persists considerably longer in the rat than it was found by HEROK and GÖTTE [18] to persist in the sheep; their evidence for the rapid excretion of triphenyltin was not, however, entirely convincing.

Excretion studies showed that Sn^{113} was retained after oral injection (Table II). The rate of excretion seemed to depend on the dose and medium, but more work is required to establish this. Most of the Sn^{113} excreted was apparently triphenyltin until half of the tin had been excreted. Thereafter some tin was probably excreted as metabolites.

Rats treated orally were also analysed for triphenyltin, with the results shown in Table III. The tin concentrations in most tissues were very similar to those found after equitoxic doses given intraperitoneally, except that high concentrations were found in the stomach.

A feature not brought out in the Tables is that the concentrations in internal organs (except the gastrointestinal tract) of rats that died were always about the same, however the rats had received the dose. In the brain the critical level was about 5 μg/g. The only exceptions were the two rats that received 185 mg/kg orally and were killed two days after injection; these

TABLE II

EXCRETION OF TIN FROM RATS GIVEN TRIPHENYLTIN CHLORIDE ORALLY

The total amount excreted is expressed as the percentage of the dose given for the following treatments:

A 185 mg/ kg in arachis oil to 2 rats
B 50 mg/ kg in arachis oil to 3 rats
C 67 mg/ kg in dimethylformamide to 3 rats. (One died 2 days after injection, so the experiment was continued with the two remaining, and amounts excreted after 2 d are expressed as percentages of the dose given to those two.)

The probable error was about 10% in every experiment.

Treatment	Time after treatment (d)								
	1	2	3	4	5	6	7	8	9
A	10	25	41	55	73	86	88	88	88
B	17	36	62	78	79	80	80	-	-
C	2	12	20	21	48	105*	110	111	111

* The high values are probably due to a sampling error on the sixth day.

rats might have died of triphenyltin poisoning had they been left longer. Their brains contained 11 μg/kg. (Table III, A). This observation is consistent with the assumption that most of the tin was present as triphenyltin and is difficult to explain in any other likely way.

TABLE III

TIN CONCENTRATIONS IN RATS TREATED ORALLY WITH TRIPHENYLTIN CHLORIDE

The column headings refer to rats treated as follows:
A Given 185 mg/kg in arachis oil and killed 2 d after injection; average of two rats.
B Same, but killed 9 d after treatment.
C Given 67 mg/kg in dimethylformamide, average of 3 rats, which died 2 d after treatment.

Tissue	Concentrations (μg triphenyltin chloride per g tissue) A	B	C
Brain	11	3.3	7.1
Liver	25	3.1	21
Kidney	22	7.5	26
Fat	9.2	0.7	7.6
Muscle	3.9	1.1	-
Stomach	331	0.6	1230
Small intestine	331	1.5	35
Large intestine	542	2.3	61

The results show that probably triphenyltin persists in the rat at appreciable concentrations for several days, and that the rat is not less susceptible than the guinea-pig to oral dosing because it decomposes triphenyltin rapidly in the gut. Under these circumstances, triphenyltin salts taken by mouth may well be very toxic to man.

A general view of the part played by tracers in the study of residues can now emerge. In the development of methods of analysis of residues in crops, tracer studies should in almost all cases be obligatory, although, of course, they are not a substitute for non-isotopic methods, which are essential for routine analysis. Tracer studies remove any doubts as to the validity of normal methods and, even in complicated cases, often give a nearly complete picture of the action of the plant on the pesticide. The work of METCALF et al. on Disyston is a particularly good example [19]. In toxicological studies tracers can often be helpful, but in most instances they are likely to yield the most useful results at an early stage in an investigation, to give way subsequently to biochemical, pharmacological and physio-

logical techniques. The main reason for this difference in utility is the difference in the relationship between a pesticide and a plant on the one hand and between a pesticide and a mammal on the other. The plant-pesticide interaction is nearly all in one direction: the plant acts on the compound, and not vice versa. Consequently when the nature, concentration and persistence of the products formed in the plant are known, the problems of interest are solved. By definition, however, in a poisoned mammal the pesticide acts on the mammal as well as the mammal on the pesticide. The problems raised by the first, toxic, action are very much more complex than those raised by the second, metabolic, action, and are not usually to be solved by labelling the pesticide.

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DISCUSSION

G.F. BURNETT (Chairman): I think we will see a good deal of the triphenyltin compounds, both as fungicides and molluscicides. Do you think we should go with care until the results of your work are fully clarified?

D.F. HEATH: Yes, unless it can be shown that the residues are in fact very low indeed. This possibility could easily be checked by using Sn^{113} -labelled triphenyltin.

C. H. SCHMIDT: Is there any evidence that triphenyltin may break down when it is applied on foliage and plants under field conditions?

D. F. HEATH: The evidence is not very good. Triphenyltin seems to break down on foliage, but break-down may be mainly due to the action of ultra-violet light. How fast triphenyltin which had run down the stalk into shady places would decompose I do not know – perhaps very slowly.

J. R. OGLE: With reference to your remarks on Demeton, there is, I think, one aspect in which the use of radioisotopes could lead to misleading results. It is a well-known fact that many dialkyl sulphides will undergo facile radiolytic oxidation to sulphoxides and sulphones. If this occurred in Demeton and related compounds in which the sulphoxides and sulphones are as toxic as the parent compound or more so, the results obtained might not be representative of the behaviour of the inactive material used under similar conditions. The rate of decomposition varies enormously from compound to compound and even between steroisomers, and is influenced by external factors such as physical state, temperature, presence or absence of moisture, oxygen, etc. It would seem that in using materials under these conditions it would be necessary to check, as in fact you did in your own work, that there was not an extra effect due to the radioisotope. For example, diisopropyl fluorophosphate-P^{32} at 40 mc/mM undergoes 10% decomposition per week but is stable in arachis oil or propylene glycol. Methionine-S^{35} at 60-100 mc/mM may undergo 20% decomposition within four weeks, depending upon the method of storage.

D. F. HEATH: In these experiments we used Demeton less than one hundredth as active as the specimens you refer to. In view of this it is perhaps not surprising that solvent distributions showed that our specimens did not break down measurably on storage. Are you normally producing pest control compounds of specific activities at which decomposition is likely to be important?

J. R. OGLE: We have recently been synthesizing insecticides at 60 to 100 mc/mM for various of our clients.

ÉVOLUTION DES DÉPÔTS SUPERFICIELS, DIFFUSION ET DÉGRADATION DE DEUX INSECTICIDES ENDOTHÉRAPIQUES: LE DÉMÉTON-S ET L'ENDOTHION DANS QUELQUES PLANTES MARAÎCHÈRES

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Abstract — Résumé — Аннотация — Resumen

CHARACTERISTICS OF SURFACE DEPOSITS, DIFFUSION AND DEGRADATION IN A NUMBER OF MARKET-GARDEN PLANTS OF TWO ENDOTHERAPIC INSECTICIDES: DEMETON-S AND ENDOTHION. In the absence of atmospheric precipitation, detoxication of plants treated with endotherapic insecticides depends on both inactivation of the surface deposits and degradation of the pesticide within the plant. Both these phenomena have been followed in a number of market-garden plants treated with demeton-S and endothion labelled with P^{32} and S^{35} respectively.

The rate of inactivation of the surface deposits is comparable as between endothion and demeton-S, where external conditions are themselves similar.

In the foliage, the persistence of demeton-S and its active metabolites varies with the plant. Normally, it is higher than that of endothion, whose disintegration pattern includes only non-toxic hydrolysis products.

In fruits treated with demeton-S, apparent degradation seems to be very rapid, whereas for endothion it remains comparable with that observed in the foliage and is apparently not affected by the maturity of the fruits. A hypothesis is put forward to explain this difference in behaviour.

Toxic residues found in the fruits may be due either to diffusion of the insecticide from the foliage or to direct contamination. A comparative study of treated and untreated fruits borne by a treated plant has made it possible to define the relative importance of these two phenomena.

Studies were made on penetration and diffusion of Demeton-S and Endothion in asparagus shoots, in an effort to explain the behaviour of these two insecticides in the control of the fly Platyparea poecilоptera. The importance of the part that may be played by the wetting agent is demonstrated.

ÉVOLUTION DES DÉPÔTS SUPERFICIELS, DIFFUSION ET DÉGRADATION DE DEUX INSECTICIDES ENDOTHÉRAPIQUES: LE DÉMÉTON-S ET L'ENDOTHION, DANS QUELQUES PLANTES MARAÎCHÈRES. En l'absence de précipitations atmosphériques, la détoxication des plantes traitées à l'aide d'insecticides endothérapiques dépend à la fois de l'inactivation des dépôts superficiels et de la dégradation du pesticide dans le végétal. Ces deux phénomènes ont été suivis sur plusieurs plantes maraîchères traitées au déméton-S et à l'endothion, marqués respectivement avec ^{32}P et ^{35}S.

La vitesse d'inactivation des dépôts superficiels est comparable pour l'endothion et le déméton-S, lorsque les conditions extérieures sont elles-mêmes voisines.

Au niveau du feuillage, la persistance du déméton-S et de ses métabolites actifs varie selon la plante traitée. Elle est en général supérieure à celle de l'endothion dont le schéma de désintégration ne comprend que des produits d'hydrolyse atoxiques.

Dans les fruits traités au déméton-S, la dégradation apparente semble très rapide; pour l'endothion, au contraire, elle reste comparable à celle que l'on observe dans le feuillage et ne semble pas affectée par la maturité des fruits. Une hypothèse est émise pour expliquer cette différence de comportement.

Les résidus toxiques retrouvés dans les fruits peuvent être dus, soit à une diffusion de l'insecticide provenant du feuillage, soit à une contamination directe. L'importance relative de ces deux phénomènes a pu être précisée grâce à une étude comparative des fruits, traités ou non, portés sur une plante elle-même traitée.

La pénétration et la diffusion du déméton-S et de l'endothion dans les pousses d'asperges sont étudiées afin de tenter d'expliquer le comportement de ces deux insecticides dans la lutte contre la mouche (Platyparea poecilоptera). Le mémoire met en évidence le rôle important que peut jouer le mouillant.

ЭВОЛЮЦИЯ ПОВЕРХНОСТНЫХ ОТЛОЖЕНИЙ, ДИФФУЗИЯ И ДЕГРАДАЦИЯ ДВУХ ЭНДОТЕРАПЕВТИЧЕСКИХ ИНСЕКТИЦИДОВ: ДЕМЕТОНА-S И ЭНДОТИОНА, В НЕКОТОРЫХ ОГОРОДНЫХ РАСТЕНИЯХ. При отсутствии атмосферных осадков детоксикация растений, обработанных с помощью эндотерапевтических инсектицидов, зависит одновременно от инактивации поверхностных отложений и от деградации пестицида в растении. Эти два феномена изучались на многих огородных растениях, обработанных деметоном-S и эндотионом, которые соответственно метились фосфором-32 и серой-35.

Скорость инактивации поверхностных отложений сходна для эндотиона и деметона-S при одинаковых внешних условиях.

На уровне листвы стойкость деметона-S и его активных метаболитов зависит от обрабатываемого растения. В основном, она выше стойкости эндотиона, схема распада которого включает лишь неядовитые продукты гидролиза.

Во фруктах, обработанных деметоном-S, видимая деградация, насколько это представляется, протекает очень быстро; и, напротив, по отношению к эндотиону она остается сравнимой с наблюдаемой в листве деградацией и не связана со зрелостью фруктов. Для объяснения этого различия подготовлена гипотеза.

Обнаруженные во фруктах ядовитые остатки могут появиться либо в результате диффузии инсектицида из листьев, либо прямого загрязнения. Сравнительное значение этих двух явлений может быть уточнено в результате сравнительного изучения обработанных или необработанных фруктов, находящихся на растении, которое также обработано.

Изучаются проникновение и диффузия деметона-S и эндотиона в побеге спаржи для объяснения способа действия этих двух инсектицидов в борьбе с мухой (Platyparea poeciloptera). Приводятся данные, показывающие важную роль влажности.

DIFUSIÓN Y DEGRADACIÓN EN ALGUNAS HORTALIZAS DE LOS DEPÓSITOS SUPERFICIALES DE DOS INSECTICIDAS ENDOTERÁPICOS: EL DEMETÓN-S Y EL ENDOTHIÓN. En ausencia de precipitaciones atmosféricas, la eliminación de los residuos tóxicos de las plantas tratadas con insecticidas endotherápicos depende a la vez de la inactivación de los depósitos superficiales y de la degradación del pesticida en el vegetal. Estos dos fenómenos han sido estudiados en varias hortalizas tratadas con Demetón-S y Endothión, marcados respectivamente con ^{32}P y ^{35}S.

Cuando las condiciones exteriores son semejantes, los depósitos superficiales del Endothión y del Demetón-S tienen una velocidad de inactivación comparable.

En el follaje, la persistencia del Demetón-S y de sus metabolitos activos varía según la planta tratada. En general, es superior a la del Endothión, cuyo esquema de desintegración sólo comprende productos de hidrólisis atóxicos.

En los frutos tratados con Demetón-S la degradación aparente parece muy rápida; en cambio, para el Endothión sigue siendo comparable a la que se observa en el follaje y no parece afectada por la madurez de los frutos. El autor de la memoria formula una hipótesis para explicar esta diferencia de comportamiento.

Los residuos tóxicos hallados en los frutos pueden deberse a una difusión del insecticida proveniente del follaje o a una contaminación directa. La importancia relativa de estos dos fenómenos se ha podido precisar gracias a un estudio comparativo de los frutos, tratados o sin tratar, dados por una planta sometida a tratamiento.

Se estudia la penetración y la difusión del Demetón-S y del Endothión en brotes de espárrago para tratar de comprender el comportamiento de estos dos insecticidas en la lucha contra la mosca (Platyparea poeciloptera). El autor de la memoria subraya la importante función que puede desempeñar el agente impregnante.

Le caractère endothérapique d'un pesticide contribue tout à la fois à l'augmentation de sa persistance et de la sélectivité de son action. Malheureusement cet avantage peut être en partie compensé par le danger dû à la présence de résidus toxiques à l'intérieur de la plante.

En effet pour les insecticides de surface, l'essentiel des résidus est constitué de dépôts extérieurs dont l'élimination peut être assurée par les facteurs climatiques: insolation, précipitations atmosphériques.

Lorsque le produit pénètre dans la plante, il se trouve de ce fait soustrait à cette action mais par contre peut servir de substrat à l'activité enzymatique du végétal.

On observe alors l'apparition de nouveaux composés dont la toxicité est très variable selon la nature du pesticide d'origine et l'évolution plus ou moins rapide selon la plante traitée.

Ainsi pour un insecticide endothérapique la contamination superficielle se trouve doublée d'une pollution interne dont l'évolution peut être complètement différente.

Enfin lorsque cet insecticide est télétoxique, les parties non atteintes directement peuvent être polluées par voie vasculaire.

Dans ce dernier cas, qui est celui du déméton-S et de l'endothion,
- la contamination superficielle,
- la contamination interne directe, et
- la contamination interne par voie vasculaire constituent trois sources de pollution dont l'importance respective et l'évolution dans le temps ne sauraient être identiques. C'est ce que nous allons étudier dans le cas particulier de quelques plantes maraîchères. Cette question revêt dans ce cas une acuité particulière, soit parce que le feuillage est consommé (salades), soit parce que la fructification étant échelonnée, il risque d'y avoir des interférences entre les traitements et les récoltes (c'est le cas en particulier des haricots cueillis en vert).

Nous envisageons ensuite quelques aspects de la pénétration et de la diffusion de ces deux insecticides dans les pousses d'asperges afin de tenter d'expliquer leur comportement dans la lutte contre Platyparea poeciloptera.

MATÉRIEL ET MÉTHODES

Plantes utilisées

L'expérimentation a porté sur les espèces suivantes: tomates, haricots nains, pois nains, et laitues. Toutes ces plantes ont été maintenues en serre tempérée pendant la totalité de leur cycle végétatif.

Produits et doses

Les insecticides choisis étaient le déméton-S et l'endothion.

L'o, o-diméthyl-thiolophosphate de S (éthyl thio 2 éthyle) ou déméton-S marqué au ^{32}P nous avait été obligeamment fourni par les établissements Bayer.

La synthèse radioactive du phosphorothiolate de ortho ortho diméthyl et de S-méthyl 2 méthoxy 5 pyrone 4 ou endothion fut réalisé au CEN de Saclay. L'élément traceur était dans ce dernier cas le ^{35}S.

Les deux insecticides ont été utilisés à une dose quatre fois supérieure à la dose efficace, soit 100 g/hl pour le demeton-S et 200 g/hl pour l'endothion.

Modalité de traitement

Etant donné la haute radioactivité spécifique de ces composés et le manque d'installation spécialisée, la pulvérisation insecticide a été remplacée par un badigeonnage de feuillage.

Lorsque cela était possible, les fruits d'un même plant ont été partagés en deux lots, l'un traité, l'autre protégé par une feuille de cellophane; ceci afin d'évaluer séparément la contamination directe (à partir de la surface traitée) et celle d'origine télétoxique.

Afin de suivre l'évolution des résidus, nous avons divisé les plantes traitées en trois lots dont la récolte était échelonnée selon le programme suivant:
déméton-S: récolte après 8, 15 et 22 j,
endothion: récolte après 5, 10 et 15 j.

La récolte des végétaux traités au déméton a été légèrement déoalée pour tenir compte de la rémanence plus grande de ce produit.

Dosage des résidus

Dans le cas du déméton-S, le dosage des résidus a été effectué selon une technique voisine de celle décrite par MÜHLMAN et TIETZ [1]:
- broyage des plantes en milieu acétonique à 80%,
- évaporation de l'acétone sous pression réduite,
- extraction chloroformique de la phase aqueuse.

La phase chloroformique ainsi obtenue contenait l'insecticide et ses dérivés toxiques (sulfoxyde et sulfone).

Dans le cas présent ce traitement était précédé d'un lavage des feuilles et des fruits traités afin d'estimer séparément l'importance des dépôts superficiels. Ce lavage a été réalisé en trois opérations successives, par agitation dans une solution de mouillant à 0,5‰. Les eaux de lavage réunies ont été soumises à une extraction chloroformique dans les mêmes conditions que précédemment.

Le degré de détoxication a été établi dans tous les cas par comparaison de la radioactivité de la phase chloroformique seule à celle du total: phase chloroformique + phase aqueuse.

L'étude de la dégradation de l'endothion a été réalisée suivant une méthode analogue; toutefois le broyage des plantes a été effectué en milieu alcoolique à 80%.

Nous signalons également que pour ce produit, seul l'insecticide non décomposé passe dans la phase chloroformique.

ÉVOLUTION DES DÉPÔTS SUPERFICIELS (tableau I)

Comme nous l'avons signalé, la dégradation du film insecticide déposé à la surface de la plante dépend essentiellement des conditions climatiques. Cependant la feuille ne saurait être considérée comme un support inerte puisqu'elle est le centre d'échanges actifs entre la plante et l'atmosphère. D'autre part la texture de la surface foliaire, tomenteuse ou lisse, varie d'une plante à l'autre; chez une même plante des différences analogues peuvent également être observées entre feuilles et fruits (tomates par exemple). On pouvait donc espérer quelques variations entre les vitesses de dégradations mesurées à la surface des différentes plantes expérimentées.

Or, pour un traitement réalisé dans des conditions extérieures identiques (série A), les différences constatées sont extrêmement réduites; qu'il

TABLEAU I

ÉVOLUTION DES DÉPÔTS SUPERFICIELS* EN FONCTION DU TEMPS

	Plantes traitées		Temps écoulé entre le traitement et la récolte				
			5 j	8 j	10 j	15 j	22 j
ENDOTHION	Tomates	Feuilles	71		47,6	35,3	
		Fruits	74,9		60	46,8	
Série A	Haricots	Feuilles	69,9		58	35,9	
		Fruits	67,5		63	47,8	
	Petits Pois	Feuilles	73,2		55	30	
		Fruits	56		49,5	21,3	
Série B	Tomates	Feuilles	78		66,3	54,2	
		Fruits	76,1		61	43	
	Haricots	Feuilles	88,2		70,6	42,2	
		Fruits	86,4		74,4	54	
DÉMÉTON-S	Tomates	Feuilles et fruits		57		49,3	31,2
	Salades	Feuilles		68,2		55	42,3

* Pourcentage de la radioactivité présente dans la phase chloroformique

s'agisse de surfaces foliaires d'espèces différentes ou bien que la comparaison porte sur les feuilles et les fruits d'une même espèce.

Dans nos conditions expérimentales, la nature de la surface traitée (feuille ou fruit) est donc sans influence pratique sur la vitesse de dégradation sauf peut-être chez le pois où l'on note une décomposition accrue à la surface du fruit.

Il y a une seule exception, semble-t-il: la disparition des résidus de déméton-S paraît retardée sur les feuilles de salade; ceci s'explique dans une certaine mesure par la disposition particulière du feuillage de cette plante qui protège l'insecticide de l'action des agents extérieurs.

Si nous comparons maintenant les vitesses de dégradation respectives des deux insecticides expérimentés, on constate que, pour une même plante, la tomate, les dépôts superficiels d'endothion sont un peu moins tenaces que ne le sont les métabolites actifs du déméton-S.

En l'absence de certaines conditions extérieures, pluie abondante ou insolation intense, la détoxication des dépôts superficiels d'endothion ou

de déméton-S semble assez lente. Après 15 jours il reste encore 20 à 50% de l'endothion et 50 à 55% du déméton-S; après trois semaines 30-40% du déméton-S sont encore présent sur le feuillage.

RÉMANENCE DES RÉSIDUS TOXIQUES DANS LE FEUILLAGE DES PLANTES TRAITÉES (tableau II)

La persistance de la toxicité chez les plantes traitées dépend avant tout de la nature chimique du pesticide qui conditionne l'existence et la nature de métabolites toxiques éventuels.

Or, nous l'avons déjà signalé, le schéma de dégradation de l'endothion est totalement différent de celui du déméton-S et ne comprend que des produits d'hydrolyse atoxiques; ceci se traduit pratiquement par une rémanence moins accentuée. C'est ce que l'on peut constater en comparant les résultats obtenus sur tomates pour ces deux insecticides. 15 jours après le traitement le déméton-S et ses métabolites toxiques constituent encore 66% des résidus phosphorés présents dans la plante tandis que 90% environ de l'endothion a déjà disparu.

TABLEAU II

RÉMANENCE* DE L'ENDOTHION ET DU DÉMÉTON-S DANS LES PLANTES TRAITÉES

	Plantes traitées	Temps écoulé entre le traitement et la récolte				
		5 j	8 j	10 j	15 j	22j
ENDOTHION						
Feuilles (série A)	Tomates	41,9		17,6	11,8	
	Haricots	25		21,8	13,3	
	Pois	45		38	14,2	
Feuilles (moyennes)	Tomates	52,6		13,8	12	
	Haricots	39,7		20,5	12	
Fruits (moyennes)	Tomates	58,1		32,6	12,9	
	Haricots	34,6		24,1	7,9	
DÉMÉTON-S						
Feuilles	Laitue		91,2		86,5	83,7
	Tomate		89,7		66,3	42,7
Fruits	Tomate		34		14	4 4

* Pourcentage de la radioactivité présente dans la phase chloroformique

La nature de la plante traitée peut elle aussi, affecter la vitesse de dégradation d'un insecticide; BENNETT et THOMAS ont démontré que le schradan se décompose plus rapidement chez le haricot que dans le chrysanthème ou le coleus [2]. METCALF et autres ont constaté pour le disyston une métabolisation accélérée chez la tomate [3]. Une observation analogue peut être faite pour le déméton-S et l'endothion.

Appliqué sur les feuilles de salades, le déméton-S se dégrade très lentement. 21 jours après le traitement le pourcentage de destruction ne dépasse pas 16%; durant le même temps il atteint 53% chez la tomate.

Pour l'endothion, la vitesse de métabolisation est à peu près la même chez la tomate et le petit pois, mais plus élévée chez le haricot.

PERSISTANCE DES RÉSIDUS TOXIQUES DANS LES FRUITS

En premier lieu, il convenait d'établir si la disparition des résidus toxiques était aussi rapide dans les fruits que dans les feuilles. Ce problème était important car pour trois des plantes étudiées le fruit constituait la partie consommable de la plante.

Il semble y avoir sur ce point précis une différence notable entre l'endothion et le déméton-S.

Chez les plantes traitées à l'endothion, la rapidité de la dégradation semble voisine dans le feuillage et les fruits traités.

Dans les fruits non traités l'évolution des résidus toxiques est comparable pendant les premiers jours (tableau III, série C). On assiste ensuite à une détoxication extrêmement rapide.

Pour le déméton-S, la vitesse apparente de détoxication s'accentue immédiatement quand on passe des feuilles aux fruits traités; elle atteint son maximum dans les fruits n'ayant reçu aucun traitement. Après 14 jours la proportion de résidus toxiques est la suivante:

Feuilles: 66%
Fruits traités: 14%
Fruits non traités: 7%.

TABLEAU III

VITESSE DE DÉGRADATION* DES DEUX PESTICIDES DANS LES DIFFÉRENTES PARTIES DU VÉGÉTAL

Temps écoulé entre le traitement et la récolte	Endothion (série C)			Déméton-S	
	5 j	10 j	15 j	8 j	15 j
Feuilles traitées	66	10	7,3	89,7	66,3
Fruits traités	59	32,6	19,6	34	14
Fruits non traités	73	3,4	0,66	21	7

* Pourcentage de la radioactivité présente dans la phase chloroformique

La feuille et le fruit étant des organes dont le métabolisme est différent, on conçoit fort bien que les vitesses de destruction d'un insecticide y soient elles-mêmes différentes.

Ce qui semble moins évident est la disparité que l'on constate entre fruits traités et non traités. Que l'insecticide parvienne par voie vasculaire ou à partir de l'épiderme ne change rien à sa vitesse de dégradation.

On est donc conduit à admettre que ce qui parvient au fruit par la voie vasculaire est surtout composé de produits d'hydrolyse voire même de restes phosphorés ou soufrés réutilisés par la plante et drainés vers les centres d'activité métabolique que constituent les fruits en croissance.

Pour le déméton-S ce transport de métabolites non extractibles par le chloroforme doit commencer très tôt, ce qui expliquerait la faible proportion de résidus toxiques retrouvée dès le septième jour et l'accentuation du phénomène dans les fruits non traités (voir tableau III).

Au contraire, c'est l'endothion qui constitue l'essentiel des substances soufrées qui parviennent aux fruits durant les cinq premiers jours qui suivent le traitement; puis l'insecticide est rapidement remplacé par des dérivés atoxiques et dénués de pouvoir insecticide.

Comparons maintenant, pour chacun des insecticides, leurs vitesses de dégradation à la surface et à l'intérieur de la plante traitée.

Pour le déméton-S, au niveau du feuillage, c'est le film superficiel qui se dégrade le plus rapidement; au niveau des fruits, c'est la pollution interne qui disparaît la première.

En ce qui concerne l'endothion dans nos conditions expérimentales, la dégradation est toujours plus rapide à l'intérieur de la plante qu'à l'extérieur.

Dans l'une des séries expérimentales nous avons étudié séparément la persistance de l'endothion dans les fruits mûrs ou encore verts.

Au 5^{e} jour la détoxication s'est révélée beaucoup plus grande dans les fruits verts. Ce fait pouvant présenter une certaine importance au point de vue toxicologie, nous avons repris l'étude sur des broyats de fruits réalisés dans divers tampons allant de pH 6 à pH 8. Dans toutes les expériences réalisées le taux de dégradation du pesticide était très voisin pour les deux types de fruits.

IMPORTANCE RELATIVE DES DÉPÔTS SUPERFICIELS, DE LA CONTAMINATION DIRECTE ET DU TRANSPORT INTERNE DANS LA POLLUTION DES PLANTES TRAITÉES

Les modalités de la dénaturation du pesticide ne constitue évidemment qu'une donnée dans le problème des résidus. Nous allons maintenant en aborder l'aspect quantitatif. La technique de traitement que nous avons dû adopter pour des raisons de sécurité s'est révélée assez imparfaite et il existe une irrégularité évidente entre les dépôts. Les comparaisons qui vont suivre ont donc été établies sur les moyennes de trois traitements.

Feuillage

Les dépôts foliaires d'endothion sont très importants, 150 à 270 ppm, soit environ 15 à 27 γ/cm^2 (à peu près quatre fois la dose normale). Cette

saturation a été recherchée afin d'accentuer au maximum la pénétration du produit. Dans ces conditions on retrouve dans le feuillage de 60 à 120 ppm de produits marqués.

Ceci est sans intérêt pour les tomates et les haricots dont le feuillage n'est pas consommé, mais on doit en tenir compte dans le cas des pois car le feuillage de cette plante est parfois utilisé (après cueillette en vert et battage mécanique) pour la nourriture du bétail. Etant donné qu'une pulvérisation normale réduirait au quart les résidus laissés à la surface et dans la plante, la contamination quinze jours après le traitement serait la suivante:

Dépôts superficiels: $\frac{153,8}{4} = 40$ ppm $\times$ 33,3 = 13 ppm

Résidus internes : $\frac{121,2}{4} = 30$ ppm $\times$ 14,2 = 4 ppm

Même si l'on envisage une réduction importante de la pollution superficielle par les intempéries, la quantité de pesticide présente dans le tissu foliaire rend celui-ci dangereux pour l'alimentation du bétail deux semaines après le traitement.

Les quantités de déméton-S déposées à la surface des deux plantes expérimentées sont assez réduites et correspondent sensiblement à l'apport d'une application normale. Pour la tomate le coefficient de pénétration est toujours sensiblement plus élevé que celui de l'endothion, mais ceci peut être une conséquence du caractère limité de l'application. Il atteint une valeur très grande lorsque le traitement est réalisé sur les salades. Un coefficient de pénétration aussi important joint à une détoxication très lente chez ce végétal rend impropre l'emploi de cet insecticide dans ce cas particulier.

Fruits

La teneur en résidus des fruits traités dépend à la fois de la contamination directe à partir de la surface et de l'apport interne, conséquence du caractère télétoxique des pesticides.

Il est évidemment difficile d'avoir une idée exacte de l'importance relative de ces deux apports.

Afin d'obtenir un ordre de grandeur, nous avons utilisé un artifice expérimental en abritant un fruit sur deux lorsque ceux-ci étaient voisins et de préférence disposés sur la même « grappe ». Dans ces conditions nous avons admis que l'apport interne était comparable pour l'un et l'autre fruit, compte tenu évidemment de leur masse respective. Ceci n'est d'ailleurs qu'une approximation, car la répartition d'un pesticide dans une plante est largement hétérogène.

Ces conventions étant admises, la comparaison des résultats portés dans les colonne 6 et 7 du tableau IV nous permet de juger l'importance de l'apport vasculaire dans la contamination interne des fruits traités.

On constate que la totalité des substances marquées, drainées vers les fruits, pourrait, si elle était constituée uniquement de métabolites toxiques, assurer 50 à 70% de leur contamination interne. En fait, on s'aperçoit que la pollution réelle due à l'effet télétoxique va en s'amenuisant ra-

pidement (tableau IV). Après 15 jours, elle peut être tenue pour négligeable pour toutes les plantes étudiées, qu'il s'agisse de l'endothion ou du déméton.

Il n'en est pas de même pour la contamination externe. Dans les conditions de nos essais, les dépôts superficiels constituent parfois la source de pollution essentielle des fruits traités. Nous avons pu l'établir pour l'endothion. Quelle que soit la plante que nous ayons traitée, cet insecticide persistait toujours plus longtemps à la surface qu'à l'intérieur du fruit; l'importance relative de la contamination externe croît donc avec le temps.

Ainsi chez la tomate elle représente 83% de la pollution totale au 5^{e} jour et 91% après 15 jours.

En ce qui concerne le déméton, l'évolution est encore plus nette; toujours chez la tomate, la contamination externe assure 54% de la contamination totale au quinzième jour et 76% au vingt-et-unième jour.

Quel que soit d'ailleurs le pesticide employé, la quantité de résidus retrouvée dans les fruits de tomate paraît réduite, surtout si nous la comparons à celle présente dans les gousses de haricots et de pois (voir tableau IV, colonnes 8 et 9).

Ceci est lié, d'une part, à la faible contamination superficielle, due elle-même à la surface lisse de ce fruit et aussi à la dilution que subit l'apport interne; chez la tomate, les fruits constituaient, dans nos essais, une masse beaucoup plus importante que les feuilles (500 à 600%).

Inversement, la pollution élevée des gousses de pois et de haricots s'explique autant par une lourde contamination superficielle que par un apport interne notable dû à la masse assez réduite que les fruits représentent par rapport aux feuilles (voir tableau IV, colonne 14).

PÉNÉTRATION ET DIFFUSION DU DÉMÉTON-S ET DE L'ENDOTHION DANS LES POUSSES D'ASPERGES

A la suite d'essais réalisés dans le cadre de la lutte contre la mouche de l'asperge (Platyparea poeciloptera), une différence d'efficacité avait été remarquée en faveur de l'endothion.

En dehors de toute spécificité vis-à-vis de l'insecte lui-même, nous avons tenté de vérifier si l'efficacité de l'endothion ne pouvait être expliquée par une meilleure pénétration à travers l'écorce ou une diffusion plus importante dans la pousse d'asperge.

Dans ce but deux types d'expérimentations ont été réalisés:

1° Essais de «pénétration»: toute la surface de la pousse étant traitée, nous avons évalué:
   a) la pénétration dans la plante,
   b) la diffusion de la zone corticale vers le centre.

2° Essais de «diffusion»: dans ce type d'expérimentation seule la partie médiane de la pousse recevait l'insecticide. Nous avons ensuite suivi le passage de ce composé:
   a) vers le centre,
   b) vers les extrémités apicales et basales.

TABLEAU IV

IMPORTANCE RELATIVE DES DÉPÔTS SUPERFICIELS, DE LA CONTAMINATION DIRECTE ET DU TRANSPORT INTERNE DANS LA POLLUTION DES PLANTES TRAITÉES

		FEUILLAGE TRAITÉ				FRUITS TRAITÉS				FRUITS NON TRAITÉS			
		Contamination externe		Contamination interne	I/T(1)	Contamination externe		Contamination interne	I/T	(Effet télétoxique)		ET/I(3)	Poids des fruits / Poids des feuilles
		RT(2)	E.C.(5)	RT E.C.	(%)	RT	E.C.	RT E.C.	(%)	RT	E.C.	(%)	(%)
ENDOTHION (série A)													
	5 j		149(4)	34	18		2,4	0,5	17		0,22	30	
Tomates		210(4)		81		3,2		1,25		0,9			608
	15 j		74	13	15		1,5	0,15	9		0,02	11	
	5 j		188	14	7		8,4	3,3	28		0,62	28	
Haricots		268		58		12,5		11,7		6,2			180
	15 j		95	11	10		6	1,1	15		0,02	18	
	5 j		112	54	32		5,1	3,5	40		1,4	28	
Pois		154		121		9,2		12,3		9			92
	15 j		51	46	47		1,9				0,01		
DÉMÉTON-S													
	15 j		4,75	8,4	64		0,45	0,39	46		0,012	3,1	
Tomates		9,6		12,7		0,92		2,48		1,8			
	21 j		3,97	5,4	57		0,38	0,12	24		0,017	14	
	15 j		1,65	9,7	85								
Salades		3		11,2									
	21 j		1,27	9,4	88								

(1) - I/T: contamination interne/contamination totale.
(2) - RT: radioactivité totale exprimée en équivalent insecticide.
(3) - ET/I: effet télétoxique/contamination interne des fruits traités.
(4) - concentrations exprimées en ppm.
(5) - E.C.: extrait chloroformique.

De plus, pour le déméton seulement, nous avons complété ce travail en étudiant:
a) l'influence de l'addition d'un émulsifiant,
b) l'efficacité comparée de 2 émulsifiants (formule A et B).
L'endothion a toujours été utilisé avec l'adjuvant A.

Les modes opératoires étaient les suivants:
- l'expérimentation a été réalisée en serre,
- les traitements et l'évaluation des résidus ont été effectués selon les méthodes décrites dans l'essai précédent,
- les deux insecticides ont été utilisés à la dose de 1 mg/cc soit 100 g/hl,
- la durée de l'essai était de 48 h.

Résultats obtenus

1. Pénétration dans la plante (voir tableau V)

a) Influence de l'émulsifiant (déméton seulement)

L'hétérogénéité des résultats de la série «sans émulsifiant» ne permet pas de conclure avec certitude. Il semble toutefois que l'adjuvant utilisé accroît la concentration de l'insecticide dans les plantes. La teneur importante (2,4 ppm) constatée dans un lot d'asperges traitées en l'absence d'adjuvant pourrait s'expliquer ainsi: ce lot étant constitué de pousses de faible section, le rapport surface/volume se trouvait donc augmenté, ce qui a entraîné un accroissement du taux de pénétration apparente.

TABLEAU V

PÉNÉTRATION DE L'ENDOTHION ET DU DÉMÉTON-S A TRAVERS L'ÉCORCE DES POUSSES D'ASPERGES.

	Poids(1) (g)	Conc.(2) (ppm)	Poids (g)	Conc. (ppm)
DÉMÉTON-S				
Emulsifiant B	20,3	3,9	29,7	2,6
Emulsifiant A	28,2	1,93	19,5	2,2
Sans émulsifiant	25,3	0,77	12,2	2,4
ENDOTHION				
Emulsifiant A	28,2	5,9	12,2	38,6

(1) Poids moyen de la partie traitée
(2) Concentration en insecticide (radioactivité totale mesurée dans la pousse, exprimée en équivalent d'insecticide)

b) Influence de la nature de l'insecticide.

L'endothion semble pénétrer en quantité beaucoup plus importante que ne le fait le déméton. Qu'il s'agisse des essais de «pénétration» ou de «diffusion», les teneurs élevées en insecticide sont toujours en faveur de l'endothion.

TABLEAU VI

DIFFUSION DE L'ENDOTHION ET DU DÉMÉTON-S VERS LA ZONE CENTRALE DE L'ASPERGE

	Teneur en insecticide*		Taux de diffusion (%)
	Ecorce	Centre	
DÉMÉTON-S			
Emulsifiant B	45,72	6,44	12,3
Emulsifiant A	30,39	3,75	11
Sans émulsifiant	34,35	8,59	20
ENDOTHION			
Emulsifiant A	116,00	36,36	23,8

* Radioactivité totale exprimé en équivalent insecticide.

2. Diffusion vers le centre de la pousse d'asperge (voir tableau VI)

a) Influence du mouillant (déméton seulement).

L'addition de l'un ou de l'autre des adjuvants expérimentés influence défavorablement la diffusion vers la zone centrale, comme on peut le constater dans le tableau VI.

b) Influence de la nature de l'insecticide.

Le même tableau met en évidence une diffusion plus marquée de l'endothion vers le centre de la pousse d'asperge.

3. Diffusion vers les extrémités non traitées (voir tableau VII)

a) Influence du mouillant (déméton seulement).

Les résultats sont comparables à ceux que nous avons observé pour la diffusion vers la partie centrale. C'est le déméton exempt d'adjuvant qui migre en quantité la plus importante.

La diffusion est d'ailleurs essentiellement dirigée vers le haut. La partie basale de l'asperge ne reçoit pratiquement rien.

TABLEAU VII

DIFFUSION DE L'ENDOTHION ET DU DÉMÉTON-S VERS LES EXTRÉMITÉS NON TRAITÉES.

	Teneur en insecticide			Taux de diffusion * (%)	
	Base non traitée (ppm)	Partie traitée (ppm)	Haut non traitée (ppm)	haut	bas
DÉMÉTON-S					
Emulsifiant B	0,02	0,78	0,72	36,7	1,6
Emulsifiant A	0,06	0,72	0,77	40,2	5,4
Sans émulsifiant	0,09	0,47	0,69	38,2	5,4
ENDOTHION					
Emulsifiant A		6,13	2,32	17,4	

* Etabli sur les teneurs mesurées en valeur absolue.

b) Influence de la nature de l'insecticide

Dans la partie haute de la pousse, la teneur en endothion se montre toujours supérieure à la teneur en déméton. Ceci est simplement dû à la concentration plus forte de l'endothion dans la totalité de la plante, car dans le cas présent c'est le déméton qui possède le taux de diffusion le plus élevé.

L'endothion, comme le déméton, ne migre pratiquement pas vers la base des pousses.

4. Dégradation des insecticides dans les différentes parties de la pousse (voir tableau VIII)

Il convenait de s'assurer que les molécules marquées sillonnant la plante correspondaient effectivement à des produits actifs et non à des métabolites sans intérêt sur le plan insecticide. Ceci nous a amenés à suivre au cours de l'essai précédent les taux de dégradation dans les différentes parties de la pousse d'asperge.

Pour le déméton le taux de dégradation reste assez faible mais croît malgré tout de l'écorce traitée vers l'intérieur et les extrémités. Dans le cas de l'endothion le taux de destruction de l'insecticide paraît légèrement plus élevé, mais il ne semble pas que la migration du produit s'accompagne d'une dégradation plus accentuée.

En résumé la nature de l'émulsifiant stabilisateur ne semble pas modifier de façon notable la pénétration ou la diffusion du déméton. L'action la plus nette des deux formules d'adjuvants serait de réduire légèrement la diffusion de l'insecticide.

TABLEAU VIII

DÉGRADATION* DES DEUX INSECTICIDES DANS LES DIFFÉRENTES PARTIES DE L'ASPERGE

	Partie traitée		Haut non traité
	Ecorce	Centre	Total
Essai «diffusion»			
DÉMÉTON-S			
Emulsifiant B	78,5	49,7	58,5
Emulsifiant A	72,5	68	59,5
Sans émulsifiant	92,7	67,5	76,7
ENDOTHION			
Emulsifiant A	64	58,5	60
Essai «pénétration»			
DÉMÉTON-S			
Emulsifiant B	86,3	76,9	
Emulsifiant A	80,6	63,5	
Sans émulsifiant	83,9	86,9	

* Dégradation exprimé en pourcentage de la radioactivité présente dans la phase chloroformique

En dehors de toute spécificité vis-à-vis de l'insecte l'efficacité accrue de l'endothion pourrait donc s'expliquer par une concentration plus élevée dans les pousses d'asperges.

CONCLUSION

Nous avons étudié le problème des résidus toxiques laissés dans quelques plantes maraîchères par deux insecticides endothérapiques, le déméton-S et l'endothion.

En dehors de toute précipitation atmosphérique, l'importance relative des dépôts superficiels et de la contamination interne évolue de façon différente selon l'insecticide, la plante, et pour une même plante selon qu'il s'agit des feuilles ou des fruits.

La voie vasculaire peut assurer une part importante de la contamination des fruits lorsqu'on utilise l'endothion.

Par contre ce type de pollution reste tout à fait secondaire dans le cas du déméton.

Quinze jours après le traitement l'effet télétoxique de ces deux insecticides semble sans influence pratique sur le taux de contamination interne des fruits traités.

Dans les conditions de nos essais, la contamination interne décroît très lentement chez les salades traitées au déméton.

Par contre quel que soit l'insecticide utilisé, la pollution externe et interne des fruits de tomate reste faible.

La persistance de l'endothion dans les plantes traitées est inférieure à celle du déméton-S.

L'efficacité plus grande de l'endothion dans la lutte contre la mouche de l'asperge (Platyparea poeciloptera) pourrait s'expliquer par une meilleure pénétration dans la pousse d'asperge.

L'absence de diffusion vers l'extrémité basale de la pousse, qui est l'endroit où les larves du 3^{e} âge creusent leurs galeries, serait également en accord avec l'inefficacité des deux insecticides contre les larves ayant atteint ce stade.

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DISCUSSION

J.E. CASIDA: Did you subject any of your extracts to chromatographic studies?

M. HASCOËT: In view of the large number of extractions which this work required it was not possible for us to carry out a chromatographic test in every case. We did, however, carry out this study for endothion, where only the non-degraded insecticide passes into the chloroform phase. Endothion has three degradation products. One of them, a monomethylated product, is now known. The two others we have not yet been able to identify exactly. One of them does not contain any phosphorus; they are probably hydrolysis products but they do not correspond to the products obtained by normal chemical hydrolysis.

Although these were the only degradation products we subjected to a chromatographic test, each insecticide was given a chromatographic test for chemical purity before being used.

L'ÉTABLISSEMENT DES PROCESSUS D'ABSORPTION ET DIFFUSION DES INSECTICIDES SYSTÉMIQUES AU POPULUS x EURAMERICANA DODE GUINIER «ROBUSTA»

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Abstract — Résumé — Аннотация — Resumen

ESTABLISHMENT OF THE PROCESSES OF ABSORPTION AND DIFFUSION OF SYSTEMIC INSECTICIDES IN POPULUS EURAMERICANA DODE GUINIER "ROBUSTA". The organophosphoric insecticides having systemic properties due to their ability to penetrate into the sap flow of plants act on insects which spend part of their lives either in the cambial region between wood and bark, or in the wood itself. Until the introduction of these new insecticides chemical control of xylophagous insects in tree-trunks was almost impossible.

The mechanism of absorption, diffusion and concentration of systemic insecticides in species of poplar and willow, which are frequently attacked by xylophagous insects, was studied using labelled Dipterex on the poplar Robusta R20.

The insecticide was labelled in the reactor, using powdered Dipterex (1.5 g) as target, at a flux $\Phi = 10^{11}$ n/cm² s and at a temperature of 30-40°C. Irradiation was carried on until an absolute target activity of ≅1 mc was obtained.

The insecticide was administered, in the form of a solution, by pouring it on to the soil, except in one case in which the insecticide was sprayed on to the leaves.

One series of experiments was conducted in the laboratory using seedlings grown in pots, and another series was perfonned in a nursery, using one- and two-year-old saplings.

One to two months after administration of the labelled insecticide solutions the trees were subjected to radiometric analysis.

In the laboratory experiments the insecticide accumulated in considerable quantities in the leaves, and in smaller amounts in the wood.

Both in the laboratory and in the nursery a greater amount of insecticide accumulated in the leaves. In the nursery experiment, however, a considerable increase was observed in the accumulation in the branches and in the wood of the stem, especially at the extremities.

The mean accumulation of insecticide amounted to 1.65 mg/g green matter in the nursery as against 0.24 mg/g in pots.

It was shown that under field conditions, in dry weather, two-year-old Robusta saplings take up Dipterex administered in an aqueous solution at the rate of 9.3% of the amount applied to the oil, as compared with 1.35% in laboratory experiments.

Application of solution to the leaves by spraying yields a low insecticide absorption rate owing both to the difficult process of uptake through the leaves and to the removal of the insecticide by rain, dew and mist.

Much insecticide was found on the ground and the authors therefore concluded that the method of administering systemic insecticide solutions through the soil should be adopted as a priority measure in the campaign against tree-species pests.

L'ÉTABLISSEMENT DES PROCESSUS D'ABSORPTION ET DIFFUSION DES INSECTICIDES SYSTÉMIQUES AU POPULUS X EURAMERICANA DODE GUINIER «ROBUSTA» Les insecticides organophosphoriques à propriétés systémiques, par leur faculté de pénétrer dans le courant de sève des plantes, ont une action sur les insectes qui mènent une partie de leur vie soit dans la zone cambiale entre le bois et l'écorce, soit dans le bois. Jusqu'à l'apparition de ces nouveaux insecticides, la lutte chimique contre les insectes xylophages de la tige des arbres était presque impossible.

Pour étudier le mécanisme d'absorption, de diffusion et de localisation des insecticides systémiques en ce qui concerne les peupliers et les saules, espèces fréquemment attaquées par les insectes xylophages, on a fait des recherches en employant le «Dipterex» marqué sur le peuplier Robusta R20.

Le marquage de l'insecticide a été fait dans un réacteur, en utilisant comme cible le Dipterex en poudre (1,5 g), avec un flux $\Phi = 10^{11}$ n/cm^2 · s et à une température de 30 à 40°C. L'irradiation a été effectuée jusqu'à l'obtention d'une activité absolue de la cible ≅ 1 mc.

L'administration de l'insecticide a été faite par verse des solutions dans le sol, sauf dans un cas où l'on a pulvérisé l'insecticide sur l'appareil foliacé.

Une série d'expériences a été faite en laboratoire, en utilisant des plants cultivés en pots; une autre série a été faite, en pépinière, avec des plants de un et deux ans.

En un laps de temps de un à deux mois après l'administration des solutions d'insecticide marqué, les plants ont été soumis à l'analyse radiométrique.

Dans les expériences en laboratoire, l'insecticide a été accumulé en grandes quantités dans les feuilles et en plus petites quantités dans le bois.

Tant en laboratoire qu'en pépinière, l'insecticide a été accumulé en plus grande proportion dans les feuilles. Pourtant, on constate une augmentation importante des accumulations d'insecticide dans les rameaux et dans le bois de la tige, surtout au bout des plants, dans l'expérience en pépinière.

En général, l'accumulation d'insecticide a été de 1,65 mg/g substance verte en pépinière, contre 0,24 mg/g en pots.

Il en résulte que, dans les conditions de terrain, les plants de peuplier Robusta de 2 ans, pendant le temps sec, peuvent mobiliser le Dipterex administré en solution aqueuse à raison de 9,3% de la quantité versée dans le sol, contre 1,35% dans les expériences en laboratoire.

L'administration des solutions sur les feuilles par pulvérisation a un rendement faible d'absorption de l'insecticide, grâce aussi bien au mécanisme difficile d'absorption des feuilles qu'au lavage de l'insecticide par l'eau de pluie, la rosée et le brouillard.

On a décelé une grande quantité d'insecticide sur le sol; c'est pourquoi les auteurs considèrent que la méthode d'administration des solutions d'insecticides systémiques dans le sol doit être adoptée en priorité dans la lutte contre les ennemis des essences ligneuses.

ОПРЕДЕЛЕНИЕ ПРОЦЕССОВ ПОГЛОЩЕНИЯ И ДИФФУЗИИ СОМАТИЧЕСКИХ ИНСЕКТИЦИДОВ У POPULUS X EURAMERICANA DODE GUINIER "ROBUSTA". Фосфорооргапические инсектициды с соматическими свойствами, связанными с их способностью проникать в сок растений, воздействуют на насекомых, которые проводят часть своей жизни либо в камбиальной зоне между древесиной и корой, либо в древесине. До появления этих инсектицидов химическая борьба против ксилофаговых насекомых, находящихся в стволе растений, была почти невозможной.

Для изучения механизма поглощения, диффузии и локализации соматических инсектицидов у тополя и ивы, которые часто подвергаются нападению ксилофаговых насекомых, были проведены исследования с применением меченого "Dipterex" на тополе Robusta R-20.

Мечение инсектицида было произведено в реакторе с использованием в качестве мишени "Dipterex" в порошке (1,5 г) при потоке в $\Phi = 10^{11}$ н/см^{2}сек и при температуре в 30 - 40°С. Это продолжалось до получения абсолютной активности мишени ≈ 1 мкк.

Введение инсектицида производилось путем полива земли, за исключением одного эксперимента, когда инсектицид разбрызгивался на листья.

Часть опытов проводилась в лабораториях, где использовались растения, выращенные в вегетационных сосудах. Другая часть опытов проводилась на почве, в питомниках, с использованием однолетних и двухлетних растений.

В течение 1 - 2 месяцев после введения растворов меченого инсектицида растения подвергались радиометрическому анализу.

При проведении опытов в лабораторных условиях инсектицид накапливался в значительных количествах в листве и в незначительных количествах - в древесине.

При проведении опытов как в лабораторных условиях, так и на почве инсектицид накапливался в большем процентном отношении в листве. Вместе с тем отмечается значительный рост накоплений инсектицида в ветвях и в древесине ствола, особенно в концевых частях растений.

В общем, при данном опыте накопление инсектицида составило 1,65 мг/г зеленого вещества по сравнению с 0,24 мг/г этого же вещества у растений, выращенных в лабораторных условиях.

Из этого следует, что на почве двухлетние тополи Robusta могут в сухое время года накапливать "Dipterex", который был введен в водном растворе в отношении 9,3% к количеству, внесенному в землю. При проведении опытов в лабораторных условиях это отношение составляло 1,35%.

Нанесение растворов на листву путем разбрызгивания дает слабый коэффициент поглощения ин-

сектицида, что объясняется также трудным процессом поглощения у листьев и смыванием инсектицида дождевой водой, росой и туманом.

На земле обнаружено большое количество инсектицида. В связи с этим мы считаем, что методу внесения растворов соматических инсектицидов в почву должно быть отдано предпочтение в борьбе с вредителями древесных пород.

DETERMINACIÓN DE LOS PROCESOS DE ABSORCIÓN Y DIFUSIÓN DE LOS INSECTICIDAS SISTÉMICOS EN EL POPULUS X EURAMERICANA DODE GUINIER «ROBUSTA». Los insecticidas organofosfóricos de acción indirecta penetran en la corriente de savia de las plantas y actúan así sobre los insectos que viven en el leño o en la zona cambial situada entre el leño y la corteza. Hasta la aparición de estos nuevos insecticidas era casi imposible luchar por medios químicos contra los insectos xilófagos del tronco de los árboles.

Para estudiar el mecanismo de absorción, difusión y localización de los insecticidas de acción indirecta en las distintas especies de álamos y sauces que suelen verse atacados por insectos xilófagos, se han hecho investigaciones con el álamo Robusta R 20 empleando «Dipterex» marcado.

La marcación del insecticida se hizo en un reactor, sometiendo el Dipterex en polvo (1,5 g) a un flujo $\Phi = 10^{11}$ n/cm^2·s, a una temperatura de 30-40°C. Se prosiguió la irradiación hasta obtener una actividad absoluta de ≅1 mc.

Salvo en un experimento, en el que se pulverizó el insecticida en el follaje, en todos los demás se regó el suelo con soluciones insecticidas.

Parte de los experimentos se efectuaron en laboratorio, con plántulas cultivadas en macetas, y los demás en semillero y en las condiciones normales del terreno, con plántulas de uno y dos años.

Un mes o dos después de haber administrado las soluciones de insecticida marcado se realizó el análisis radiométrico de las plántulas.

En los experimentos de laboratorio, el insecticida se había acumulado en grandes cantidades en las hojas y en cantidades más pequeñas en el leño.

En los experimentos hechos en semillero y en las condiciones normales de terreno, el insecticida se acumuló en mayor proporción en las hojas, pero se pudo observar un aumento importante de la cantidad de insecticida acumulado en las ramas y en el leño del tronco, en especial en las extremidades de las plántulas.

Por regla general, en estos experimentos la acumulación de insecticida ha sido de 1,65 mg/g de sustancia verde, mientras que en las plántulas cultivadas en laboratorio fue de 0,24 mg/g.

De ello se desprende que en las condiciones normales de terreno, las plántulas de álamo Robusta de dos años pueden movilizar durante la estación seca el 9,3% del Dipterex vertido en el suelo en solución acuosa, mientras que en los experimentos de laboratorio sólo movilizan el 1,35%.

Si la solución se pulveriza en las hojas, la cantidad de insecticida absorbido es relativamente baja, debido a que la absorción se produce difícilmente en las hojas y a que la lluvia, el rocío y la niebla arrastran al insecticida.

Como se ha observado que en el suelo queda gran cantidad de insecticida, los autores consideran que en la lucha contra los insectos que atacan a las especies leñosas es preferible verter en el suelo las soluciones de insecticidas de acción indirecta.

1. INTRODUCTION

Par leur propriété de s'intégrer dans le courant de sève des plantes, les insecticides organophosphoriques à propriétés systémiques immunisent, d'une part, les plantes aux attaques des insectes nuisibles et, d'autre part, détruisent les insectes nuisibles dans les organes divers de la plante. La fonction systémique assure la pénétration de l'insecticide dans les tissus vivants, évitant le danger de lavage de la pellicule d'insecticide sur la surface des organes de la plante. Aussi, on attend que la pénétration, ainsi que le degré d'utilisation des insecticides, soient plus grands que dans le cas des insecticides qui sont administrés par pulvérisation ou arrosage.

Les insecticides à fonctions systémiques présentent une importance particulière dans la protection des forêts, parce que les espèces ligneuses sont attaquées par des insectes qui se localisent soit dans le bois, soit entre

l'écorce et le bois. La lutte contre ces insectes nuisibles est donc impossible avec des insecticides sans fonction systémique.

Parmi les espèces ligneuses cultivées dans la République Populaire Roumaine, ce sont les peupliers euraméricains ainsi que les peupliers indigènes qui souffrent le plus des attaques d'insectes se localisant dans les bois. La Saperda populnea L., Paranthrenae tabaniformis Rott., Crypthorrinchus lapathi L., etc. appartiennent aux insectes nuisibles les plus répandus. Les larves de ces insectes se développent dans le bois de peuplier. En détruisant les exemplaires attaqués elles compromettent les jeunes peuplements.

L'application des insecticides systémiques dans ce domaine est devenue plus qu'une nécessité. Mais, vu la difficulté de suivre la pénétration de l'insecticide dans la plante, les méthodes d'administration n'ont pas été explorées scientifiquement, ce qui a empêché leur application sur une grande échelle.

La possibilité de marquer les insecticides systémiques avec des isotopes radioactifs a ouvert une nouvelle perspective aux recherches dans ce domaine. Vu l'importance de la lutte contre les insectes nuisibles du peuplier et afin d'assurer un état phytosanitaire optimum des cultures de peupliers dans notre pays, on a entrepris une série de recherches pour étudier en détails l'efficacité des insecticides systémiques par rapport à la méthode d'administration aux insectes nuisibles, aux espèces et aux clônes de peupliers et aux facteurs écologiques.

Les premières recherches qui font l'objet du présent mémoire ont comme but l'éclaircissement des processus d'absorption et diffusion du dipterex marqué dans les plants de Populus x euramericana Dode Guinier, cv. «robusta».

2. MÉTHODES DE RECHERCHES

2.1. La conception de base des recherches entreprises par le Laboratoire d'isotopes radioactifs de notre Institut, a été déterminée par le but précis des recherches en espèce, et par l'analyse des méthodes de travail utilisées jusqu'à présent.

Le degré de pénétration des insecticides systémiques dans le flux de la sève des espèces ligneuses a été établi d'après la mortalité des insectes qui attaquent les espèces respectives. Comme méthode d'administration de ces insecticides, on a eu recours soit à la pulvérisation fine sur les feuilles et les branches, soit à l'introduction de l'insecticide dans le sol. Parfois on a essayé le bandage de la tige avec de la ouate imbibée d'une solution d'insecticide.

Dans les premières expériences faites dans notre pays en vue de déterminer l'efficacité des insecticides systémiques, on a choisi comme méthode d'administration la pulvérisation de l'insecticide sur les feuilles, considérant qu'une proportion importante s'intègre dans le courant descendant de la sève.

JEPPSON a recours à une méthode moins pratique mais plus efficace. Il a appliqué des bandages de ouate imbibée avec metasystox d'une concen-

tration de 2,5% et 5%, autour de la tige des exemplaires de mélèze attaqués par Taeniothrips laricivorus Krat [1].

Ces méthodes ont permis la détermination de l'efficacité de l'insecticide basée sur les observations faites sur la mortalité des insectes et les différences de croissance enregistrées par les exemplaires des variantes traitées avec des insecticides systémiques et celles non traitées (contrôle).

Les progrès réalisés dans le domaine du marquage des molécules de divers composés chimiques, ainsi que la présence du phosphore, du soufre et du carbone dans la molécule des insecticides systémiques, ont permis aisément le marquage des insecticides organophosphoriques.

ANDREEV [2], CASIDA [3] et HOPKINS [4] ont utilisé dans les recherches effectuées, des insecticides systémiques marqués par une synthèse chimique. Andreev a poursuivi l'absorption du mercaptophos marqué par les plantes et a établi les concentrations létales et la localisation de l'insecticide dans les plantes.

En général, les recherches dans ce domaine ont pour objet les cycles métaboliques des insecticides systémiques dans les plantes et dans l'organisme des insectes, poursuivis à l'aide des composés marqués aux isotopes radioactifs et mis en évidence par chromatographie radiochimique [2, 3, 4, 5].

2.2. Les recherches entreprises par notre Institut ont le but d'éclaircir tout d'abord la quantité d'insecticides systémiques qui s'accumule dans les plants de Populus x euramericana, cv. «robusta». On a utilisé du dipterex marqué au réacteur nucléaire de l'Institut de physique atomique de l'Académie de la République Populaire Roumaine.

On a obtenu le dipterex radioactif par marquage du phosphore, avec une activité spécifique initiale de 1 mc/g. On a effectué les expériences, soit seulement avec l'insecticide marqué, soit avec l'insecticide marqué auquel on a ajouté l'insecticide stable, par rapport aux concentrations établies. On a travaillé d'habitude avec des concentrations de 1-2% dans une solution aqueuse.

On a administré les solutions de dipterex dans la première dilution (10 μc/ml) dans le sol à la racine des plants de peuplier, on a poussé une partie dans de vases de végétation, et une autre partie dans la pépinière. Pour la mobilisation de l'insecticide par les plantes, on a assuré une percolation intense du sol avec de l'eau.

On a observé le degré d'absorption de l'insecticide à l'aide de l'intensimètre «Luci» portable.

Au moment où les mesures radiométriques indiquaient une activité maximale à la cime des plants, on a enlevé celle-ci pour préparer des échantillons afin de procéder à des mesures exactes à l'aide du dispositif de comptage BSP, avec compteur G-M, type MST-17, à la fenêtre mica de 5 mg/cm^2.

L'activité des échantillons prélevés en diverses parties de la plante a été déterminée à l'aide d'échantillons calcinés. Les résultats des mesures permettent de déterminer avec une précision suffisante la diffusion du dipterex dans diverses parties de la plante, la localisation et la quantité de l'insecticide absorbé par rapport à la quantité administrée.

3. RÉSULTATS DES RECHERCHES

Les recherches ont été effectuées en deux étapes, à savoir, une étape au laboratoire et une étape sur le terrain. Les résultats obtenus diffèrent évidemment surtout en ce qui concerne la quantité de dipterex absorbé du sol par les plants de peuplier.

3.1. Les expériences de laboratoire ont été effectuées avec des plants âgés d'une année, plantés dans de vases de végétation Mitscherlich avec une capacité de 20 l. Dans les vases on a utilisé un sol alluvionaire à texture sablonneuse, riche en humus.

Les solutions de dipterex marqué ont été administrées à la racine des plants, en concentration de 1,5%. L'activité spécifique des solutions a été $R_0 = 5340$ cpm/ml. L'activité totale sur la plante a été de $5,34 \cdot 10^6$ cpm.

On a vérifié périodiquement l'accroissement de l'activité dans les feuilles, et 30 jours après l'administration de la solution de dipterex marqué on a prélevé des échantillons de bois et de feuilles soumis ultérieurement aux mesures radiométriques, à l'état calciné.

Les déterminations faites montrent que le dipterex marqué a été absorbé par les plants et qu'il est distribué différemment dans le bois et dans les feuilles, sur les secteurs de 20 cm mesurés sur la tige.

L'activité totale (cpm) est plus grande dans les secteurs dans lesquels la masse ligneuse ou la masse de feuilles est plus grande et en général décroît vers la tête des plants (tableau I).

En échange, l'activité spécifique exprimée en cpm/g (cendre), montre une accumulation plus intense de l'insecticide dans les racines et dans la moitié supérieure de la tige, surtout aux 3/5 de la hauteur. Dans les feuilles l'activité spécifique en cpm/g (cendre) est distinctement plus grande que dans le bois et accumule une quantité plus grande d'insecticide aussi aux 3/5 de la hauteur de la plante et vers le bout de la plante. On remarque

TABLEAU I

LA VARIATION DE L'ACTIVITÉ TOTALE EN cpm DANS LES DIVERSES PARTIES DES PLANTS DE PEUPLIER (POPULUS ROBUSTA) TRAITÉS AU DIPTEREX MARQUÉ (EXPÉRIENCE DE LABORATOIRE)

Partie de la plante	La hauteur à partir du sol (cm)				
	0-20	20-40	40-60	60-80	80-100
Feuilles	-	723	1518	854	652
Bois	254	184	274	137	57
Bouttures	1990				
Racines	580				

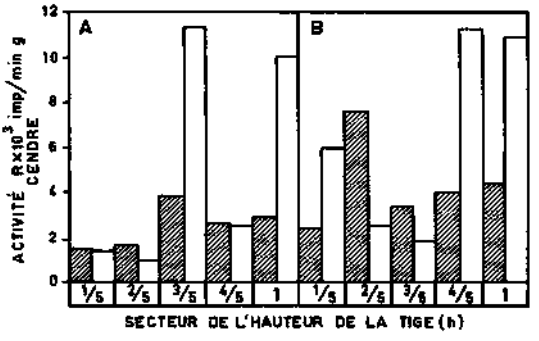

Figure 1

La variation de l'activité rapportée à l'unité de masse calcinée, en cpm/g (cendre), dans les diverses parties des plants de peuplier traités avec dipterex marqué, sur divers secteurs de la hauteur de la tige.

▨ - Bois tige; □ - Feuilles.

A. Expérience de laboratoire; B. Expérience de terrain.

une tendance de polarité du dipterex marqué vers les extrémités de la plante, avec la présence d'un troisième maximum aux 3/5 de la hauteur des plants (fig. 1).

Dans le bois de la tige, l'activité varie entre 1500-3800 cpm/g (cendre) et dans les feuilles entre 2400-7600 cpm/g (cendre). Par conséquent, l'accumulation d'insecticide dans les feuilles s'avère être deux fois plus grande que dans le bois. Les différences sont encore plus grandes si l'on prend en considération les valeurs de l'activité rapportée à la substance sèche, parce que le contenu en cendre du bois varie entre 3-4%, et celui des feuilles entre 8-9%.

Il résulte des expériences de laboratoire que l'absorption du dipterex par les plants de P. robusta représente 1,34% de la quantité administrée; la mobilisation de l'insecticide en proportion réduite dans ces expériences est due, d'une part, à la percolation intense du sol et à la présence de l'insecticide dans l'eau d'arrosage et, d'autre part, aux conditions écologiques du laboratoire, qui ne permettent pas une activité physiologique intense des plantes.

De la dernière analyse il résulte que l'accumulation d'insecticide dans le bois est de 0,172 mg/g, et dans les feuilles de 0,373 mg/g, substance verte.

3.2. Les expériences sur le terrain montrent qu'en général, l'absorption et la diffusion du dipterex dans les plants de P. robusta se produisent de la même manière que dans l'expérience de laboratoire, à la différence que ces processus sont beaucoup plus intenses.

L'expérience s'est déroulée avec la même hypothèse et la méthode de travail est semblable à celle utilisée dans les expériences de laboratoire.

La solution active de dipterex dans la première dilution (d_1) a eu une activité $R_{01} = 22\,200$ cpm/ml. A chaque exemplaire de peuplier on a administré $R_{02} = 666$ cpm/ml. L'activité totale de la solution administrée à un exemplaire de peuplier a été de $6{,}66 \cdot 10^5$ cpm, donc du même ordre de grandeur que dans le cas de l'expérience de laboratoire.

Au dipterex marqué on a ajouté aussi le dipterex stable de telle manière que la concentration de la solution d'insecticide fut de 1%. L'activité du dipterex a été de $6,6 \cdot 10^4$ cpm/g (dipterex).

On a observé le progrès de l'absorption de l'insecticide et lors d'une accumulation d'activité facilement mesurable on a prélevé les échantillons pour les mesures radiométriques.

L'activité de la substance, à l'état calciné, montre le même phénomène de polarité dans l'accumulation du dipterex marqué que dans le cas de l'expérience de laboratoire. Dans la tige l'activité maximum apparaît aux 3/5 de la hauteur et est de 11 340 cpm/g (cendre), dans les rameaux aux 2/5 et au sommet avec des valeurs de 28 400 et de 23 100 cpm/g (cendre), respectivement, dans le pétiole des feuilles aux 4/5 et 1/5 avec des valeurs de 13 200 et de 7400 cpm/g (cendre), respectivement, et dans les feuilles aux 2/5 et au bout avec des valeurs de 11 250 et 5870 cpm/g (cendre), respectivement (tableau II).

TABLEAU II

LA VARIATION DE L'ACTIVITÉ EN cpm/g (CENDRE) DANS LES DIVERSES PARTIES DES PLANTS DE PEUPLIER TRAITÉS AU DIPTEREX MARQUÉ (EXPÉRIENCE DE TERRAIN)

La hauteur à partir du sol (cm)	Parties de la plante			
	Bois	Branches	Pétiole feuilles	Feuilles
0-50	10 000	-	7400	10 850
50-100	2500	28400	4150	11250
100-150	11 340	2500	4160	1830
150-200	960	1430	13200	2490
200-250	1400	23100	1700	5870

En comparaison avec les mêmes valeurs trouvées dans l'expérience de laboratoire, les plants de peupliers élevés dans des conditions de terrain en pleine saison de végétation mobilisent l'insecticide dans une proportion 3-4 fois plus grande sur l'unité de masse.

Contrairement à l'expérience précédente, on constate une augmentation de la radioactivité dans le bois de la tige et les rameaux qui dépasse les valeurs enregistrées dans les feuilles, phénomène inverse à celui enregistré auprès des plants en pleine croissance dans des conditions de laboratoire. Il en résulte que les insecticides systémiques diffusent aussi dans de divers organes des plantes, selon la même loi que la sève élaborée et en concordance parfaite avec les composés de biosynthèse. Dans la première phase d'accroissement, au début de la saison de végétation, le dipterex s'accumule plus fortement dans les feuilles, et dans la seconde partie de la saison

de végétation dans le bois. Ce phénomène est d'un grand intérêt d'ordre pratique.

L'accumulation de l'insecticide dans les plants vigoureux de P. robusta de deux ans se produit à raison de 10,8% dans le bois et de 8,6% dans les feuilles par rapport à la quantité totale de dipterex administrée à un exemplaire.

En unité de masse on peut dire que dans le bois s'est accumulée une quantité de dipterex de 1,1 g, et dans les feuilles de 0,9 g, donc, au total, approximativement 2 g des 10 g administrées à une plante.

L'absorption d'environ 20% du dipterex par plants de P. robusta au mois d'août de l'année 1962 montre que l'utilisation des insecticides systémiques administrés dans le sol peut donner des résultats remarquables dans la lutte contre les insectes. Nous considérons cependant que, dans ce cas, il est utile d'augmenter la concentration des solutions jusqu'à 5%, fait relevé d'ailleurs aussi par JEPPSON [1] dans un autre genre d'expériences.

On a observé qu'à la suite de l'infestation artificielle intense des plants de peuplier avec Saperda populnea, les plants ont été faiblement attaqués et les larves dans le bois sont mortes. La quantité d'insecticide accumulée dans le bois a été de 1,81 mg/g et dans les feuilles de 2,18 mg/g de substance verte, presque plus grande donc que dans le cas de l'expérience de laboratoire. On a observé également une faible absorption du dipterex chez les plants auxquels on a administré l'insecticide par voie extraradiculaire, grâce au lent processus d'absorption par les feuilles ainsi qu'au lavage de celles-ci par les eaux de pluies.

Il résulte donc que les processus d'absorption et diffusion des insecticides systémiques sont déterminés dans une mesure appréciable par les conditions météorologiques, par l'intensité des processus physiologiques et par la vigueur des cultures de peupliers.

4. CONCLUSIONS

4.1. L'utilisation des traceurs radioactifs permet l'étude de l'absorption et de la circulation des insecticides organo-phosphoriques aux fonctions systémiques dans le système sol-plante.

4.2. Les plants de P. robusta absorbent, dans les limites convenables, le dipterex administré dans le sol, et la localisation de l'insecticide dans les organes divers peut assurer une immunité de la plante envers les divers insectes nuisibles.

4.3. Dans les conditions de laboratoire et dans la première phase de croissance, l'absorption de l'insecticide est plus faible, tandis que dans les conditions de terrain en cultures intensives, environ 20% de la quantité administrée sont absorbés.

4.4. Dans la première partie de la saison de végétation, l'accumulation de l'insecticide est plus intense dans les feuilles que dans le bois, tandis que dans la seconde partie de la saison, l'accumulation de celui-ci dans le bois augmente d'une manière considérable (dans une plus grande proportion).

4.5. On remarque un phénomène distinct de polarité dans la diffusion de l'insecticide dans les plants. Toutefois, il apparaît une zone d'accumulation intense de l'insecticide dans la partie inférieure de la couronne des plants, tant dans les feuilles que dans le bois, plus prononcée dans le tiers moyen de la tige des plants.

4.6. Les aspects concernant la stabilité du dipterex et des autres insecticides systémiques constituent l'étape nouvelle des recherches entreprises, et l'établissement des concentrations optima à administrer dans le sol est un problème qu'on est en train d'étudier. Bien que les dates obtenues jusqu'à présent tendent de montrer que certains insecticides systémiques se décomposent après 19-24 jours [2], nous considérons que ce problème doit encore être examiné, surtout dans l'hypothèse de l'administration des solutions de l'insecticide dans le sol. Le phénomène doit être relié surtout au pH de la solution du sol et de la phase liquide contenue dans les plantes.

4.7. Les préoccupations concernant l'utilisation sur une large échelle des insecticides systémiques dans la sylviculture pratique sont en plein développement, et nous considérons que la protection des forêts offre le plus important domaine d'application de ces substances.

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DISCUSSION

J.E. CASIDA: Dipterex is known to be a fairly good systemic in certain trees. It is also somewhat unstable biologically under these circumstances - the period of stability can be estimated at 2 to 10 days, on the basis of bioassays not restricted by localization. I am wondering if this might have some effect on the interpretation of your results. In other words, do you know for sure that you are analysing the active insecticide? Was any attempt made to ascertain that the radioactivity measured was due to Dipterex per se and not to non-insecticide metabolites?

C.H. SCHMIDT: Yes, I also wanted to ask this question.

I. CATRINA: As regards the period of stability of Dipterex, the research carried out in my country has indicated a rather longer period, i.e. about 30 days. Research work on the problem is still going on. You are certainly quite right, Dr. Casida, in saying that Dipterex decomposes, especially when it is applied topically to plants. The main cause of this phenomenon is the ultra-violet ray effect. In the case of injection of Dipterex into the soil, however, this particular phenomenon is eliminated.

There is no doubt that the radioactivity we measured was due to the Dipterex itself. The biological test used showed it to be Dipterex and not non-insecticide metabolites. The absorption rate was too rapid, the activity appeared too soon for it to have been otherwise. The dead larvae in the wood and the mortality among larvae fed on the leaves suffice to show the presence of Dipterex itself.

G. F. BURNETT (Chairman): It seems to me you are using very large amounts of Dipterex, 10 g to a very small tree. Your 20% utilization is high, and I assume in practice you will use very much smaller quantities. Have you considered that in such a case the relative distribution of Dipterex in wood and leaves may be different and fall below the effective concentration in the wood? Are you pursuing any studies along this line to see whether with, say 1 g of Dipterex you will get the same type of distribution throughout the tree?

I. CATRINA: The 10-g dose which we used for a two-year-old sapling of Populus robusta was not too large because the insecticide was administered into the soil. We have not studied dosage from the practical point of view. That is for the future.

T. SAITO: Do you do purification of chemical assays of used radioactive Dipterex?

I. CATRINA: After labelling we do no purification or chemical assays of Dipterex because we receive the radioactive insecticide from a radiochemical laboratory as pure labelled Dipterex, and its purity is guaranteed.

I might add that paper-chromatographic studies made at the Institute of Atomic Physics of the Romanian Academy of Sciences aim at establishing the quality of labelled insecticides as we use them in our experiments.

RADIOTRACER APPROACHES TO CARBAMATE INSECTICIDE TOXICOLOGY

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Abstract — Résumé — Аннотация — Resumen

RADIOTRACER APPROACHES TO CARBAMATE INSECTICIDE TOXICOLOGY. Methylcarbamates constitute one of the major groups of insecticides. Many unresolved problems in their toxicology may be readily approached with radiotracer studies. Dimethylcarbamates have been prepared with carbonyl-C^{14}-labelling and methylcarbamates with methyl-, carbonyl- and ring-labelling utilizing carbon-14. The pharmacological action of these compounds presumably results from acetylcholinesterase inhibition and may involve carbamylation. Reaction of carbonyl- or methyl-labelled carbamates with purified cholinesterase or other esterases would allow a critical examination of this carbamylation reaction and the ease of spontaneous and induced reactivation or decarbamylation. The physiological significance of cholinesterase inhibition might be examined by administering acetate-C^{14} and analysis for radiolabelled acetylcholine accumulation in nervous tissue, or by utilizing acetyl-C^{14}-choline as the substrate for in vitro determination of the degree of cholinesterase inhibition in tissues of poisoned animals with minimal dilution of the inhibitors and enzymes during analysis. Some progress has been made with radiolabelled materials in investigating the metabolism of carbamate insecticides. Sevin (1-naphthyl methylcarbamate) has been most extensively studied along with its potential hydrolysis products. The assumption that the metabolism of Sevin involves an initial hydrolysis and then further decomposition of the fragments was not supported by carbon-14 studies. The major detoxification mechanism in mammals, and probably also in insects, results from initial oxidative attack on the carbamate by the microsomes in the presence of reduced nicotinamide-adenine dinucleotide phosphate. Sevin is rapidly metabolized in mammals, but the fate of certain of the fragments has not been resolved. Some of the metabolites appear in the milk of lactating animals. One step in the metabolism appears to be formation of the N-methylol derivative. Preliminary studies on the metabolism of radiolabelled Dimetilan (2-dimethylcarbamyl-3-methylpyrazolyl-(5)-dimethylcarbamate) and a related compound in cockroaches also indicate that oxidative attack forms N-methyl N-methylol derivatives. Much remains to be done on the relationship of these detoxification reactions to the resistance mechanism, the action of synergists, the selective toxicity in this group of insecticides, and the nature and significance of residues. Metabolism of Sevin following injection into plants is probably also oxidative rather than hydrolytic, but the nature of the products and the enzymatic mechanism have not yet been established.

EMPLOI DES RADIOINDICATEURS POUR L'ÉTUDE DE LA TOXICOLOGIE DES INSECTICIDES A BASE DE CARBAMATES. Les méthylcarbamates constituent l'un des principaux groupes d'insecticides. Les radioindicateurs permettent d'étudier facilement de nombreux problèmes que pose leur toxicologie. On a préparé des dimethylcarbamates en marquant le groupe carbonyle par ^{14}C, et des méthylcarbamates en marquant par ^{14}C les groupes méthyle et carbonyle et le noyau. L'action pharmacologique de ces composés résulte vraisemblablement de l'inhibition de l'acétylcholinestérase et peut impliquer une carbamylation. La réaction, avec de la cholinestérase ou d'autres estérases purifiées, des carbamates dont on a marqué les groupe carbonyle et méthyle permettrait de faire une étude critique de cette carbamylation et de la facilité avec laquelle se produit la réactivation ou décarbamylation, spontanée ou induite. L'importance physiologique de l'inhibition de la cholinestérase peut être étudiée en administrant de l'acétate marqué par ^{14}C et en analysant l'accumulation de l'acétylcholine marquée dans les tissus nerveux, ou bien en utilisant de l'acétyl-^{14}C-choline comme substrat pour déterminer in vitro le degré d'inhibition de la cholinestérase dans les tissus d'animaux traités, avec une dilution minimum des inhibiteurs et des enzymes pendant l'analyse. Les produits marqués ont permis certains progrès dans l'étude du métabolisme des insecticides à base de carbamates. On a étudié de manière très approfondie le Sevin (1-naphtyl méthylcarbamate), en même temps que ses produits d'hydrolyse éventuels. L'hypothèse selon laquelle le métabolisme du Sevin implique une hydrolyse initiale, puis une nouvelle décomposition des produits d'hydrolyse, n'a pas été confirmée par les études faites avec le carbone-14. Chez les mammifères et probablement aussi chez les insectes, la détoxification résulte principalement d'une oxydation initiale du carbamate par les microsomes en présence de phosphate de nicotinamide adénide dinucléotide réduit. La

métabolisation du Sevin est rapide chez les mammifères, mais on n'a pas encore pu déterminer le sort de certains des fragments. Quelques métabolites apparaissent dans le lait des animaux en lactation. L'une des étapes du métabolisme semble être la formation d'un dérivé de N-méthylol. Des études préliminaires sur le métabolisme du Dimétilan radiomarqué (diméthylcarbamate de 5-[3-méthyl-2 diméthylcarbamyl]-pyrazoline) et d'un composé voisin chez les blattes a montré également que l'oxydation forme des dérivés de N-méthyl-N-méthylol. Il reste encore beaucoup à faire pour déterminer le rapport qui existe entre ces réactions de détoxification et la résistance, l'action des agents synergétiques et la toxicité sélective dans ce groupe d'insecticides, ainsi que pour déterminer la nature et l'importance des résidus. Il est également probable que le métabolisme du Sevin, à la suite d'injection dans des plantes, suit une voie oxydante plutôt qu'une voie hydrolytique, mais la nature des produits et le mécanisme enzymatique n'ont pas encore été déterminés.

ПРИМЕНЕНИЕ РАДИОАКТИВНЫХ ИНДИКАТОРОВ ДЛЯ ИЗУЧЕНИЯ ТОКСИКОЛОГИИ КАРБАМАТНЫХ ИНСЕКТИЦИДОВ. Метилкарбаматы являются одной из главных групп инсектицидов. Многие неразрешенные проблемы токсикологии этих веществ могут быть легко изучены при помощи радиоактивных индикаторов. Были изготовлены N, N-диметилкарбаматы, карбонильная группа которых метилась C^{14}, а также N-метилкарбаматы, метильная, карбонильная группа и кольца которых были мечены C^{14}. Фармакологическое действие этих сложных соединений, вероятно, связано с торможением ацетилхолинэстеразы и может включать карбамилацию. Мечение карбонильной или метильной группы карбаматов дает возможность в их реакциях с очищенной холинэстеразой или с другими эстеразами критически изучить реакцию карбамилации, а также легкость самопроизвольной или вызванной реактивации или декарбамилации. Физиологическое значение торможения холинэстеразы может быть изучено при помощи введения меченного C^{14} ацетата и анализа меченого ацетилхолина, накопившегося в нервных тканях, или же путем использования ацетил-C^{14}-холина в качестве субстрата для определения in vitro степени торможения холинэстеразы в тканях отравленных животных при минимальном растворении во время анализа ингибиторов и ферментов. Некоторые успехи были достигнуты при помощи меченных радиоизотопами веществ в исследовании метаболизма карбаматных инсектицидов. Севин (1-naphtyl N-methylcarbamate) был наиболее тщательно изучен параллельно с возможными продуктами его гидролиза. Исследования с помощью углерода-14 не подтвердили гипотезы о том, что при метаболизме севина происходит первоначальный гидролиз, а затем дальнейшее разложение продуктов гидролиза. Основной механизм обезвреживания инсектицида в организме млекопитающих и, вероятно, насекомых заключается в первоначальном окислящем воздействии микросом на карбаматы в присутствии восстановленного никотинамид-аденинди-нуклеотид фосфата. Севин быстро подвергается распаду у млекопитающих, но судьба некоторых продуктов распада еще не выяснена. Некоторые из продуктов метаболизма могут быть обнаружены в молоке животных. Одной из ступеней метаболизма, по-видимому, является образование производного N-метилола. Предварительные исследования метаболизма меченного радиоактивными веществами диметилана (2-диметилкарбамид-3-метилпиразолил-(5)-диметилкарбамат) и метаболизма подобного соединения у тараканов указывает также на то, что при окислении образуются производные N-метила и N-метилола. Многое остается сделать для выяснения связи этих реакций обезвреживания с механизмом сопротивляемости, действием синергистов, селективной токсичностью этой группы инсектицидов, а также с природой и значением остатков инсектицида. Метаболизм севина, введенного в растения, носит, вероятно, также скорее окислительный, а не гидролитический характер, но характер продуктов и ферментативные механизмы еще не установлены.

ESTUDIO CON RADIOINDICADORES DE LA TOXICOLOGÍA DE LOS INSECTICIDAS A BASE DE CARBAMATOS. Los metilcarbamatos constituyen uno de los principales grupos de insecticidas. Muchos de los problemas que su toxicología plantea se pueden estudiar fácilmente mediante radioindicadores. Se han preparado dimetilcarbamatos marcados con ^{14}C en el grupo carbonilo, y metilcarbamatos marcados con ^{14}C en los grupos metilo, carbonilo y, en el caso de derivados cíclicos, en el anillo. La acción farmacológica de estos compuestos se debe probablemente a la inhibición de la acetilcolinesterasa y es posible que implique una carbamilación. La reacción con colinesterasa purificada u otras esterasas de los carbamatos marcados en los grupos carbonilo o metilo permitiría un examen crítico de esta reacción de carbamilación y de la tendencia a la reactivación o descarbamilación espontánea e inducida. La importancia fisiológica de la inhibición de la colinesterasa se podría estudiar administrando acetato-^{14}C y analizando la acumulación de acetilcolina marcada en el tejido nervioso, o bien utilizando acetil-^{14}C-colina como substrato para la determinación in vitro del grado de inhibición de la colinesterasa en tejidos de animales envenenados, con una dilución mínima de los inhibidores y las enzimas durante el análisis. El empleo de sustancias marcadas ha permitido realizar

algunos progresos en la investigación del metabolismo de los insecticidas a base de carbamato. Se ha estudiado principalmente el Sevin (metilcarbamato de 1-naftilo) y sus posibles productos de hidrólisis. Los estudios con ^{14}C no corroboraron la hipótesis de que el metabolismo del Sevin implica una hidrólisis inicial seguida de una mayor descomposición de los fragmentos. La principal desintoxicación en los mamíferos, y probablemente también en los insectos, es el resultado de un ataque oxidante inicial del carbamato por los microsomas en presencia de fosfato de nicotinamida-adenina-dinucleótido reducido. Los mamíferos metabolizan rápidamente el Sevin, pero no se ha averiguado todavía la evolución ulterior de algunos de sus fragmentos. Algunos metabolitos aparecen en la leche de animales lactantes. Al parecer, una de las etapas metabólicas es la formación de un derivado N-metilol. Estudios preliminares, efectuados en la cucaracha, del metabolismo del Dimetilan radiomarcado (dimetilcarbamato de 5-[3-metil-2-dimetilcarbamil]- pirazolina) y un compuesto comparable indican también que el ataque oxidante forma derivados N-metilo y N-metilol. Faltan todavía muchos datos sobre la relación entre esas reacciones de desintoxicación y el mecanismo de resistencia, la acción de las sustancias sinérgicas, la toxicidad selectiva de este grupo de insecticidas, y la naturaleza y el significado de los residuos. Es probable también que el metabolismo del Sevin inyectado en las plantas sea oxidativo más que hidrolítico, pero no se ha establecido todavía la naturaleza de los productos y el mecanismo enzimático.

INTRODUCTION

Insecticidal carbamic acid esters have been extensively investigated during the past decade. Two dimethylcarbamates, Isolan (1-isopropyl-3-3-methylpyrazolyl-(5)-dimethylcarbamate, compound VIII of Fig. 1) and Dimetilan (2-dimethylcarbamyl-3-methylpyrazolyl-(5)-dimethylcarbamate, compound VII of Fig. 1), are in limited use, and one monomethylcarbamate, Sevin (1-naphthyl methylcarbamate, compound I of Fig. 1), is extensively utilized for pest control. Several other methylcarbamates have been thoroughly field-tested for insecticidal activity, and their residual persistence and toxicology are now being evaluated. Recent reviews have considered the development, mode of action and present status of these carbamates [1-8].

Investigations of certain aspects of the mode of action of the carbamate insecticides, particularly those dealing with their detoxification mechanisms, are greatly facilitated by radiotracer approaches. Although few such studies have been reported, many are now in progress. An attempt will be made to review the information obtained to date from such studies, and to indicate where tagged molecules might further assist in clarifying unresolved or incompletely elucidated facets of the mode of action of the carbamate insecticides.

1. RADIOSYNTHESIS

The insecticidal carbamates of current interest are all methyl- or dimethylcarbamates of substituted phenols or heterocyclic enols. Two routes of radiosynthesis are obvious for the dimethylcarbamates and three for the methylcarbamates:

1. Via the carbamyl chloride

$$COCl_2 + 2\,(CH_3)(R)NH \rightarrow (CH_3)(R)NC(O)Cl + (CH_3)(R)NH \cdot HCl$$

$$\downarrow \text{ ROH or RONa}$$

$$(CH_3)(R)NC(O)OR + HCl \text{ or } NaCl$$

2. Via the chloroformate

$$COCl_2 + ROH \xrightarrow{-HCl} ClC(O)OR \xrightarrow{2(CH_3)(R)NH} (CH_3)(R)NC(O)OR + (CH_3)(R)NH \cdot HCl$$

2. Via the isocyanate

$$CH_3NCO + ROH \rightarrow (CH\ )(H)NC(O)OR$$

The first route has been used by Dr. D. P. Ryskiewich (Geigy Chemical Corporation, Ardsley, New York) to prepare carbonyl-C^{14}-dimethylcarbamyl chloride in almost quantitative yields. A total of 3 mM of phosgene-C^{14} in 3.6 ml dry benzene was transferred to a 25-ml reaction flask equipped with a condenser, drying tube and magnetic pellet in an ice bath. A total of 6 mM of dimethylamine in 6.6 ml dry benzene was added with stirring in 1-ml portions at 10-min intervals. The resulting slurry was then stirred at room temperature for one hour. The benzene solution of carbonyl-C^{14}-dimethylcarbamyl chloride was separated from the insoluble dimethylamine hydrochloride by filtration and used directly by Dr. Ryskiewich in the preparation of the dimethylcarbamates, Dimetilan (VII) and Isolan (VIII). By substituting anhydrous methylamine for the dimethylamine, it should be possible to prepare carbonyl-C^{14}-methylcarbamyl chloride under similar reaction conditions. This reaction could also be used to prepare the methyl-C^{14}- or methyl-H^3-labelled methylcarbamyl chlorides by providing a means of regenerating the amines from the hydrochloride for completion of the reaction of methyl-C^{14}-amine or methyl-H^3-amine with phosgene. Reaction conditions suitable for the final coupling to form the carbamate ester have been reported [9–15].

The second route has been used for radiolabelling carbamates of pharmacological interest but not for the methylcarbamate insecticides. For example, in the preparation of 1-ethynylcyclohexyl carbamate-carbonyl-C^{14}, the reactions were carried out in collidine at -10°C without isolation of the intermediate chloroformate [16].

Sevin (I) has been prepared by the reaction of 1-naphthol-1-C^{14} with methyl isocyanate [17, 18]. Detailed conditions for this reaction conducted in a solution of 2% pyridine in dry benzene at 80°C for 5 h have been reported (17). The product after recrystallization from hot p-xylene was recovered in 82% yield and characterized by melting point and isotopic dilution analysis. Methyl isocyanate-C^{14} has also been prepared and reacted to yield labelled methylcarbamate insecticides [19] using the following reaction sequence and conditions adapted from reported procedures for non-labelled materials [20, 21]:

$$CH_3C(O)Cl + NaN_3 \xrightarrow[140°C,\ 2\ h]{\text{benzene}} CH_3NCO + NaCl + N_2$$

$$CH_3NCO \xrightarrow{100°C,\ 2\ h,\ ROH,\ (C_2H_5)_3N} CH_3NHC(O)OR$$

Acetyl chloride, labelled with carbon-14 in either the methyl or carbonyl position, and sodium azide were reacted to yield methyl isocyanate-C^{14}, which was then reacted with the appropriate phenol. A two-compartment reaction tube with a break-seal was utilized. Yields on a 0.5-mM scale were routinely 40-70%. The methylcarbamates were isolated by chromatography on Florisil and crystallization. Their purity and structure were confirmed by infra-red spectra, co-chromatography on Florisil columns and silica-gel thin-layer chromatograms, and by their melting points and mixed melting points. Acetyl-1-C^{14} chloride or bromide yielded carbonyl-C^{14}-methylcarbamates, and acetyl-2-C^{14} chloride or bromide yielded the methyl-C^{14}-carbamates. This method has been modified (Dr. Roy Everett, Chemagro Corporation, Kansas City, Missouri) by introducing the acetyl chloride into the reaction tube with a vacuum manifold rather than as a benzene solution to minimize water pick-up, and by completing the first reaction at the higher temperature before introducing the substituted phenol in the case of thermally unstable phenols. Further useful modifications of this procedure include preparation of the methyl isocyanate-C^{14} followed by introduction of the substituted phenol into the other compartment of the reaction tube without triethylamine, and reacting the phenol and isocyanate for either 2 h at 100°C or several days at room temperature [22]. Sevin-carbonyl-C^{14}, as prepared via methyl isocyanate-C^{14}, is available from Volk Radiochemical Co. (Skokie, Illinois). This break-seal tube and reaction sequence should also be appropriate for preparing methyl-H^3-carbamates from acetyl-H^3-chloride or acetic-H^3-anhydride.

The carbon-14-labelled carbamate insecticides currently under investigation are indicated in Fig. 1. The available evidence on the metabolism of these insecticides has emphasized the importance of working with multiple sites of labelling. The toxicological studies discussed later have already reached the point where it would be advantageous to utilize carbonyl-C^{14} and methyl-H^3 labels on the same methylcarbamate, or carbonyl-C^{14} and tritium labelling of the enolic moiety within the same molecule. The synthesis of certain of these insecticides with carbon-14 at selected sites in the rings or in a ring substituent should also be readily possible with available routes.

2. METABOLISM OF METHYLCARBAMATES

The first reported study utilizing radiolabelled carbamate insecticides dealt with a comparison of the metabolism of naphtyl-1-C^{14}-Sevin and 1-naphthol-1-C^{14} in three insect species [18]. The metabolites were separated by a paper chromatographic system, with glutaronitrile as the stationary phase and isopropyl ether saturated with glutaronitrile as the ascending mobile phase. With a normal susceptible (S) strain of house-fly, Musca domestica L., Sevin penetrated rapidly either alone or with the synergist, sesamex [2-(3,4-methylenedioxyphenoxy)-3,6,9-trioxaundecane], but sesamex interfered with the process or processes by which the absorbed Sevin was metabolized and excreted. Sevin was rapidly changed to a very polar substance that appeared both in the tissues and the excreta. With sesamex there was a much greater amount of unchanged Sevin in the body and much

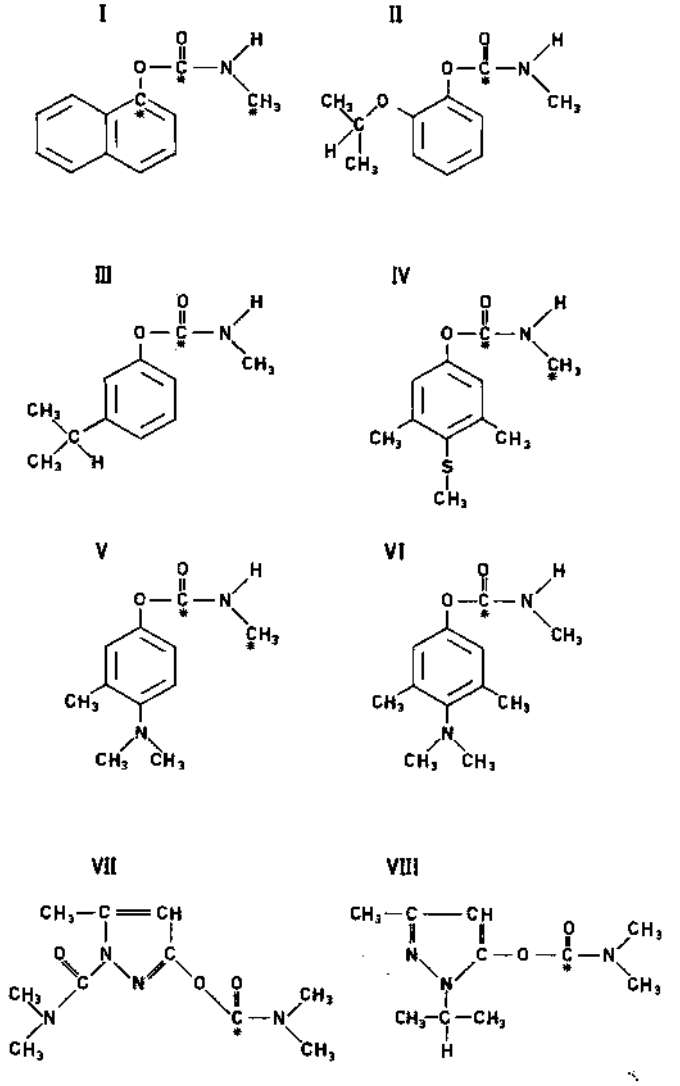

Fig. 1

Sites of C^{14}-labelling (marked *) in carbamate insecticides currently being used in radiotracer investigations

I. Sevin: 1-naphthyl methylcarbamate
II. Bayer 39007: o-isopropoxyphenyl methylcarbamate
III. Hercules 5727 or Union Carbide 10854: m-isopropylphenyl methylcarbamate
IV. Bayer 37344: 4-(methylthio)-3,5-xylyl methylcarbamate
V. Bayer 44646: 4-dimethylamino-m-tolyl methylcarbamate
VI. Zectran: 4-dimethylamino-3,5-xylyl methylcarbamate
VII. Dimetilan or GS-13332: 2-dimethylcarbamyl-3-methylpyrazolyl-(5)-dimethylcarbamate
VIII. Isolan or G 23611: 1-isopropyl-3-methylpyrazolyl-(5)-dimethyl-carbamate

less polar metabolite in the excreta. A resistant (R) house-fly strain was also investigated with and without synergist. Although the R strain was 25 times more resistant to Sevin than the S strain, the strain differences in susceptibility and the effect of the sesamex could not be accounted for on the basis of changes in absorption. However, a quantitative difference in rates of metabolism favoured survival of the R strain, since more of the polar metabolite was excreted and less Sevin remained in the body. Sesamex decreased the excretion of metabolites and increased the retention of unchanged Sevin in the bodies of the R flies in exactly the same manner as with the S flies. Sevin metabolism yielded at least three metabolites in the house-fly, only a very polar metabolite in the milkweed bug (Oncopeltus fasciatus Dall.), and six metabolites in the German cockroach (Blattella germanica L.). Sevin and 1-naphthol formed the same series of more polar products in these insects. The authors concluded that the metabolic pattern differed greatly in the three insects, that the rate of metabolism was related to the toxicity to a particular insect species and to the action of the synergist, and that the rate of formation of one key product probably controlled the overall metabolism. They proposed that the rate of hydrolysis of the ester bond by a carbamate esterase enzyme was the first and rate-limiting step in the metabolism of Sevin.

A recent investigation [23] has established that the metabolism of Sevin is not as simple as a carbamate esterase mediating hydrolysis and other systems then degrading the hydrolysed fragments. It appears instead that oxidative attack by enzymes in liver microsomes, and possibly also insect microsomes, in the presence of the co-factor, reduced nicotinamide-adenine dinucleotide phosphate ($NADPH_2$), may be the initial and critical detoxification mechanism and that hydrolysis of the carbamate ester may or may not follow oxidation.

Sevin was metabolized by rat-liver microsomes to yield at least five different carbamate metabolites with the C-O-C (O)-N-C skeleton intact, and two further metabolites that lacked the carbonyl and methyl group [23]. These conclusions resulted from studies where three samples of Sevin labelled with carbon-14 in the methyl, carbonyl and 1-naphthyl positions were used in separate but identical experiments. Metabolites were separated with column chromatography on Florisil using mixtures of hexane and ether, and on thin-layer chromatographic plates with silica gel, using hexane-ether mixtures. The carbonyl-C^{14}-labelled derivatives were utilized to ascertain suitable conditions for hydrolysis of the carbamate metabolites. Possible modification of the N-methyl group was then studied with metabolites from the methyl-C^{14}-labelled sample by hydrolysis and determination of formaldehyde-C^{14} as 3,3-methylene-C^{14}-bis (4-hydroxycoumarin) after reaction with 4 hydroxycoumarin [24], and of methyl-C^{14}-amine as methyl-C^{14}-phenylthiourea after reaction with phenylisothiocyanate. The structure of these labelled derivatives was verified by both isotopic dilution analysis and co-chromatography on silica-gel thin-layer chromatograms. Ring-labelled metabolites were subjected to hydrolysis and separated on thin-layer chromatograms as the phenolic derivatives or as the methylcarbamates after reaction of the hydrolysis products with methyl isocyanate. These techniques revealed that modifications of both the ring and methyl group

were involved within this series of metabolites. Characterization studies on these Sevin metabolites are in progress.

House-flies and American cockroaches (Periplaneta americana L.) yielded the same series of metabolites as those formed by the rat-liver microsomes, based on their chromatographic characteristics and experiments with the different sites of radiolabelling. Thus insects also formed at least five carbamate metabolites from Sevin [23]. The use of multiple-labelling of the insecticide allowed a more complete evaluation of the metabolism leading to a different interpretation of the metabolic pathway in insects as compared with a study [18] where only Sevin-naphthyl-C^{14} was investigated.

When Sevin has been fed to or sprayed on lactating animals, no significant residues of Sevin or metabolites responding to the p-nitrobenzenediazonium fluoborate chromogenic reagent after hydrolysis have been detected in the milk [25-28]. The complexity of the metabolism of Sevin by liver microsomes prompted the treatment of a lactating goat with carbonyl-C^{14}-Sevin, using a single oral dose of 1.3 mg/kg [23]. A method for the analysis of milk for Sevin and metabolites was developed by fortification of goat milk with the radiolabelled metabolites formed by rat-liver microsomes. The method involved extraction with acetonitrile and chloroform and chromatography of the organosolubles on a Florisil column. Sevin reached a peak level of 0.004 ppm in the milk after 2 h; a metabolite which appears to be a methylcarbamate with the ring modified, probably by hydroxylation, reached a peak level of 0.075 ppm at 2 h; and a metabolite not formed by rat-liver microsomes reached a peak level of 0.027 ppm at 16 h. The level of total Sevin-C^{14}-equivalents appearing as metabolites in milk reached a peak of 0.93 ppm at 8 h and diminished progressively thereafter to below 0.003 ppm by 60 h. The majority of these radiolabelled metabolites were not extractable into organic solvents and may represent conjugates of certain hydroxylation products of Sevin. An analysis of the urine of this goat, which contained about half of the administered radioactivity, revealed the presence of the same metabolites. These results indicate that analysis for free and conjugated 1-naphthol, as for detection of possible occupational or other exposure by urine analysis [28-30] or of possible milk contamination following ingestion of feed containing Sevin residues or direct application of Sevin to animals, may detect only a portion of the metabolites eliminated by these routes.

Sevin does not yield persistent tissue residues in animals exposed to this insecticide, according to analyses of tissue extracts using p-nitrobenzenediazonium fluoborate before and after hydrolysis [31-33]. In order to examine the mechanism of this rapid dissipation of Sevin residues further, the following six compounds (specific activity 1 mc/mM) were administered intraperitoneally to male rats at 7.5 μM/kg: Sevin labelled with carbon-14 in the carbonyl, methyl and 1-naphthyl positions; 1-naphthol-1-C^{14}; methyl-C^{14}-amine hydrochloride; and sodium carbonate-C^{14} [22]. Sodium carbonate yielded rapid (89% within one hour) and almost complete elimination of the radioactivity in the expired air, whereas with Sevin-carbonyl-C^{14} only about one-quarter of the radioactivity was eliminated by this route and the time for half-elimination of that which was expired was about 4 h. With methyl-C^{14}-amine, about half the radioactivity was eliminated as expired carbon-14

dioxide, whereas with Sevin-methyl-C^{14} this value was only about one-eighth of the administered radioactivity, the times for half-elimination of that which was expired being about 1.5 h for methyl-C^{14}-amine and 8 h for Sevin-methyl-C^{14}. No radioactivity appeared as carbon-14 dioxide with Sevin-naphthyl-C^{14} or with 1-naphthol-1-C^{14}. With all six labelled compounds the elimination of radioactivity in the faeces accounted for less than 1% of the administered dose. The radioactivity from Sevin-naphthyl-C^{14} and 1-naphthol-C^{14} was largely eliminated in the urine, leaving very little residual carbon-14 in the tissues after 48 h. The radioactivity in the tissues at 48 h, by which time the rate of elimination of carbon-14 from the rats with all six compounds was negligible, was higher with Sevin-carbonyl-C^{14} than with sodium carbonate-C^{14}, but was lower with Sevin-methyl-C^{14} than with methyl-C^{14}-amine. The total Sevin-C^{14}-equivalents for ten rat tissues ranged from about 0.1 to 0.5 μM/kg 48 h after administration of Sevin-carbonyl-C^{14}, but ranged to 1.7 μM/kg after administration of Sevin-methyl-C^{14}. The radioactivity was most persistent with both carbonyl- and methyl-labelled Sevin in the corpuscles, brain, heart, lung, and particularly the liver and kidney. The localization of the carbon-14 and specific activity of the tissues were quite different with labelled sodium carbonate and methylamine compared to carbonyl- and methyl-labelled Sevin. Since the radioactivity persisting in the tissues was very low for that from the Sevin-naphthyl-C^{14} compared with the other labels, the residual radioactivity was largely due to metabolites lacking the bicyclic ring. Tissue residue differences between administration of carbonyl- and methyl-labelled Sevin and their hydrolysis products may have resulted from variation in distribution of the fat-soluble Sevin compared with its water-soluble hydrolysis products and subsequent localization of metabolites at different sites. Much of the radioactivity (about 60%) appeared in the urine as carbamate metabolites of Sevin, but the identity of these has not been established.

The fate of Sevin after application to plants is incompletely understood. Many studies have been reported on the persistence of this insecticide on plants, based on colorimetric analysis for 1-naphthol after hydrolysis of the insecticide during the analytical procedure (see, e.g. [34-38]). Bean and cotton plants injected through the stem with Sevin-C^{14} slowly converted it to metabolites which could not be extracted into organic solvents from aqueous solution [23]. Five of the carbamate metabolites as detected with animals were not evident in the plants. It is not known whether the major water-soluble metabolite from the plants is the same as that from animals, although this seems unlikely. Little loss of carbon-14 occurred from the plants, even with the carbonyl-C^{14} material and after 28 days, when over 90% of the carbon-14 from Sevin was present as metabolites.

Experiments similar to those conducted with Sevin were also made with o-isopropoxyphenyl methylcarbamate (compound II of Fig. 1) C^{14}-labelled in the carbonyl position [23]. The microsomes of rat liver and the cockroaches yielded two organosoluble metabolites and one or more metabolites which were not readily extracted from water into organic solvents. The principal organosoluble metabolite appeared to be o-isopropoxyphenyl N-methylolcarbamate based on isolation, degradation and synthesis. The behaviour of o-isopropoxyphenyl methylcarbamate in plants was similar to that of Sevin.

Only limited information is available on the biological activity of these metabolites of the methylcarbamates. The organosoluble metabolites from the liver microsomes of 1-naphthyl and o-isopropoxyphenyl methylcarbamates were less than one-eight as potent as their methylcarbamate precursors when assayed in vitro for anticholinesterase activity with fly-head homogenates [23].

3. METABOLISM OF N,N-DIMETHYLCARBAMATES

The metabolism of Dimetilan-5-carbonyl-C^{14} (VII) was investigated in adult American and German cockroaches [39]. The half-life of the injected compound in the German cockroach was less than one hour, with the loss occurring from elimination as carbon-14 dioxide and formation of at least three organosoluble C^{14}-metabolites, none of which was 3-methylpyrazolyl-(5)-dimethylcarbamate. The metabolites in the American cockroach were examined in more detail using both thin-layer chromatography on silica gel with an ethyl acetate-ethanol (98:2) developing solvent, and column partition chromatography with silica gel developed with hexane-methylene chloride mixtures. Chromatography on the silica-gel column of an extract prepared from roaches 4 h after injection of 1,0 mg Dimetilan-C^{14} per roach resulted in recovery of about half of the original carbon-14 injected. About one-fourth of the injected dose was recovered as Dimetilan, and one-fourth as four metabolites which were partially resolved on the column and more completely so on the thin-layer chromatograms. Two of these metabolites yielded formaldehyde on acid degradation. The biological activity of these metabolites was less than that of the Dimetilan precursor on the basis both of toxicity to house-flies and of anticholinesterase activity as assayed in vitro with house-fly head acetylcholinesterase and human plasma butyrylcholinesterase. Infra-red spectra on the two major metabolites, those which yielded formaldehyde on acid degradation, were consistent with the presence of both carbamyl groups on the molecule, but with an alcoholic function which might result from the oxidation of a methyl to a methylol group. On the analogy of the metabolism of similar non-labelled compounds by rat-liver microsomes and insects [40, 41] it would appear likely that the formaldehyde-yielding metabolites from Dimetilan were N-methylol derivatives.

Mammals treated with Pyramat [2-propyl-4-methylpyrimidinyl-(6)-dimethylcarbamate] excreted three metabolites in the urine. One was identified as 2-propyl-4-methyl-6-hydroxypyrimidine and the others were presumed from limited evidence to be 2-propyl-4-methyl-5,6-dihydroxypyrimidine and a carbamate intermediate which might decompose to yield this latter enol [42]. Metabolism of this dimethylcarbamate insecticide thus appeared to involve ring hydroxylation, but methyl hydroxylation and some conjugation may also have occurred as acid hydrolysis increased the recovery of certain of the enolic products.

4. OTHER RADIOTRACER APPROACHES

The availability of methods of preparing C^{14}-labelled carbamates with specific activities of 1 to 5 mc/mM should facilitate, and perhaps even

stimulate, further critical investigations on the mode of action of this relatively new group of insecticides.

The insecticidal activity of the methylcarbamates is known to be related at least in part to their rate of metabolism by insects based on many types of experimental evidence, including a single study with radiolabelled compounds [18]. Species specificity in insecticidal activity may be a function of differences in rate of metabolism of the insecticides. Resistance acquired to the carbamates in strains pressured with agents of this type, or as a side-resistance where the insects were selected with non-carbamate compounds, appears to be related to the greater ability of the resistant strain to detoxify the carbamate. The action of methylenedioxyphenyl compounds and others that serve to synergize the insecticidal activity of the carbamates is probably associated with decreasing the efficiency of detoxification of the toxicants. Certain of these synergists may act by inhibiting the microsome-$NADPH_2$ system involved in hydroxylation of the N-methyl groups of the enolic portion of the molecule. The experimental evidence supporting these relationships has been recently reviewed [1, 2, 5, 18, 43, 44]. The ultimate testing of these hypotheses requires detailed knowledge of the rates of the various detoxification reactions in the appropriate insects. Radiotracer and chromatographic techniques adequate for such studies are now available.

Detoxification rates may also be related to certain aspects of the toxicity to mammals of the methylcarbamates, although adequate experimental evidence is not yet available on this points. Insecticidal carbamates vary in rat oral LD50 from less than 1 to more than 1000 mg/kg, although most of the compounds being considered for development as commercial insecticides fall in the range 10–500 mg/kg. The route of administration may have a large and differential effect on the toxicity, as illustrated by Isolan (VIII), which is more toxic to mammals following dermal than oral administration, and Dimetilan (VII), where the dermal is much less than the oral toxicity. Toxicity changes with different substituents on the molecule and with different routes of administration may be related in part to the stability of the compound in the body. With sufficient information on the relationship of structure to rates of metabolism of the carbamates in different organisms, it may be possible to design a molecule that would be readily detoxified by mammals and less readily decomposed by insects. This appears to be the case with Sevin, which has a considerable level of selectivity in its toxic action. This selectivity might be further increased by the use of suitable adjuvants, such as the methylenedioxyphenyl and other synergists, to reduce further the detoxification rate by insects without affecting the rate by mammals. The fundamental information leading to such approaches to selective toxicity will probably come in large part from radiotracer studies.

Radiotagged compounds have been extremely valuable in determining the distribution of pesticides among tissue and organ systems in plants and animals. Considerable fractionation will be necessary to define the localization of the original radiolabelled carbamate in insects and mammals, since metabolites of somewhat similar solubility properties are rapidly formed. The carbamates which have been investigated, by either radiotracer or colorimetric techniques, are not stored for prolonged periods

in any particular depots. Many ionized carbamates are almost inactive as insecticides, despite very high anticholinesterase activity, presumably because of their inability to penetrate into the insect nervous system at a sufficient rate to achieve the critical levels necessary for blocking impulse conduction. Data on the level of intact carbamate or its anticholinesterase metabolites in the insect nervous system might be advantageously compared with the degree of cholinesterase inhibition occurring within a portion of the nervous system, such as the thoracic ganglia or entire nerve cord. The correlation of the actual level of carbamate in the nervous system with the symptoms and degree of inhibition of acetylcholinesterase and other carbamate-sensitive esterases, such as aliesterases, would contribute to an evaluation of the mode of insecticidal action of these materials. Further studies are needed on the penetration rate into insects as affected by formulation and other adjuvants, and the rate of absorption and distribution following ingestion, particularly since considerable variation occurs in insecticidal activity depending on whether the compounds are applied topically, injected or ingested. Penetration and distribution studies in plants utilizing methyl-carbamates-C^{14} might be more readily approached than similar studies in animals, since the compounds studied so far are more stable in plants than animals and the plant metabolites vary greatly from the original compound in solubility characteristics.

Studies on the residual persistence of the carbamate insecticides have been made primarily by determination of the enolic or amine portion of the molecule as liberated by hydrolysis during analysis. The validity of any such method depends on the specificity in accounting for all toxic agents, with minimum interference from non-toxic metabolites. It is known that metabolism may modify both the amine and enolic portions of the molecule, and that certain of the metabolites are biologically active. Metabolism involves both oxidation and hydrolysis, with or without conjugation of certain of the hydroxylated derivatives. This same situation also occurs with other pharmacologically active but non-insecticidal carbamates [45]. Ethyl carbamate (urethane) as studied with a carbonyl-C^{14}-label and probably also methyl carbamate are rapidly hydrolysed in mammals [46-48]. Ethyl chloramate [ethyl 2,2,2-trichloro-1-hydroxyethylcarbamate] is partially hydrolysed and partially conjugated [49]. Mephenesin carbamate (3-o-tolyloxy-1,2-propanediol 1-carbamate) is quite stable in mammals, and its metabolism includes conjugation of a portion of the dose without hydrolysis of the carbamate ester group [50, 51]. In vivo hydrolysis of methocarbamol [3-(o-methoxyphenoxy)-1, 2-propanediol 1-carbamate] is negligible, according to a carbonyl-C^{14} study, and the excreted metabolites include the original compound and its glucuronide and four further metabolites, two of which are glucuronides [52]. Meprobamate (2-methyl-2-n-propyl-1, 3-propanediol dicarbamate) labelled with both tritium (Wilzbach technique) and carbon-14 has been investigated and its metabolism found to involve almost no hydrolysis but rather predominantly hydroxylation of the 2-methyl position with or without glucuronide formation before excretion [53, 54]. Metabolism of both 1-ethynylcyclohexyl carbamate-carbonyl-C^{14} and 1-ethylcyclohexyl carbamate-carbonyl-C^{14} involves ring hydroxylation. Hydrolytic cleavage was a minor catabolic route and excretion occurred as both ring-hydroxylated 1-ethynylcyclohexyl carbamate and its glucuronide [16]. The nature of the changes

in the enolic portion of the methylcarbamate insecticides during metabolism will determine the choice of the phenolic chromogenic reagent to differentiate only the toxic products. Radiotagged metabolites can be separated and the specificity of the cromogenic reagents can be used to assist in elucidating their structure and in selecting an appropriate method for residue analysis. The biological activity of the metabolites can be determined readily with labelled materials, as they need not be completely pure since their amount can be precisely determined on the basis of their initial specific activity.

Toxic manifestations with the carbamate insecticides, as with the organophosphate insecticides, presumably result from inhibition of nerve acetylcholinesterase activity. They differ, however, in the nature of the reaction with the cholinesterase, since the organophosphate inhibition by phosphorylation is only very slowly reversible, whereas the more ready reversibility of the carbamate inhibitors may lead to enzyme-inhibitor dissociation during tissue preparation and assay. The reaction of the carbamate with the esterase is complex, the rates of formation of the enzyme-inhibitor complex, carbamylation of the enzyme and hydrolysis of the carbamylated enzyme being affected by concentration of inhibitor, enzyme and substrate. The sequence of reactions at the esteratic site on the enzyme surface appears to be the same for the substrate and the carbamate and phosphate inhibitors, with the marked differences with these compounds resulting from variations in rate of different components of the reaction. Radiolabelled carbamates are now available with adequately high specific activity for use in studies on the nature of the reaction of the enzyme and inhibitor, an approach allowing possible verification and extension of the kinetic studies which have led to the information now available. Highly purified cholinesterase, from mammalian or insect sources, might be reacted with the radiolabelled carbamates to ascertain whether, for example, a radiolabel on the enolic moiety would be released and the radiolabelled methyl and carbonyl groups would be bound to the protein. The stability of the C^{14}-methylcarbamyl group at the esteratic site of the esterase and the nature of the peptide chain involved at the site of carbamylation might be investigated on similar lines to those adopted for P^{32}-phosphorylated esterases, using P^{32}-organophosphate inhibitors, with the exception that suitable conditions would have to be utilized to prevent spontaneous hydrolysis of the carbamyl group from the esterase or peptide suring the studies (for reviews and recent articles relating to this problem see [1, 55-66]). Certain esterases other than cholinesterase have also been observed to hydrolyse some insecticidal carbamates, such as esterases of rabbit plasma, liver and kidney, which catalyse the hydrolysis of dimethylcarbamyl fluoride, and the albumin fraction of mammalian plasmata which increases the hydrolysis rate of certain methylcarbamates, including Sevin [67-69]. These esterases presumably differ from those that are more sensitive to inhibition by carbamates in that the carbamyl esterases are more rapidly hydrolysed, an assumption susceptible to direct test with radiolabelled carbamate substrates. A recent proposal [10] to use acetyl-C^{14}-choline as the substrate in studying the cholinesterase inhibition from carbamates is particularly attractive as a means of minimizing enzyme and inhibitor dilution during assay and utilizing much lower substrate concentrations during analysis than has been possible with other procedures. This should result in

higher observed levels of inhibition. The degree of inhibition for tissue from carbamate-poisoned animals might then more closely approximate, although certainly not reproduce, the in vivo situation (for considerations related to this point see [1, 71-73]).

The labelled-pool technique, using phosphorus-32 and carbon-14 biochemicals, has proved very valuable in considering the mode of action of such insecticides as DDT, dieldrin and others (for review see [74]). No such studies have been reported as yet with animals poisoned with carbamate insecticides. The procedure for examining the rate of acetate-C^{14} incorporation into C^{14}-acetylcholine would be of particular interest, especially if applied to the insect nervous system.

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DISCUSSION

M. HASCOËT: What solvent system did you use for thin-layer chromatography?

J.E. CASIDA: We have a mixture of about half-a-dozen different solvent materials which works very well. Hexane-ether mixtures of varying proportion give good results, the proportion depending on the type of metabolites being used. We usually run these as two-dimensional thin-layer chromatograms in order to determine the stability of the metabolites on the thin layer and we run them with each radio-label.

M. HASCOËT: What is the capacity of the Florisil column that you use? How many milligrams per gram do you need to obtain a good resolution?

J.E. CASIDA: The capacity is about 50-150 mg. For further information I would refer you to my reference [19]. Actually you cannot obtain as good a resolution by this means as with thin-layer chromatography – this is the experience of many people who have tried both. You can probably run at about 5 mg/g but this is a top level. Optimum resolution would be obtained with about 1 mg/g.

M. HASCOËT: Is there no degradation of Sevin on the column?

J.E. CASIDA: This has been very carefully checked in a variety of experiments. There is no degradation on the column, but there is degradation for some of the metabolites if left for prolonged periods on the silica-gel thin-layer chromatogram.

M. HASCOËT: At what temperature is the silica gel activated?

J.E. CASIDA: Our silica gel is obtained in a large drum and we take it from the drum and add water. We do not activate it. With each new batch of silica gel we re-ascertain the optimal conditions for separating the material. These conditions, like the purity of the solvent, appear to play a large part in determining the quality of resolution obtained.

C.E. SEKERIS: With thin-layer chromatography I think you are able to see how many hydroxyl groups you have on your ring, if you choose the right elution system.

J.E. CASIDA: Yes. We have been using it in this manner to clarify the modifications occurring on the naphthol ring.

H. HUQUE: Insects are said to develop resistance very quickly in the case of Sevin. What are the factors responsible for this?

J.E. CASIDA: My references [1], [4] and [5] provide information on this point. The rate of development of resistance depends on the slope of the dosage-mortality curve, the type and level of field application made and the previous history of exposure of the insects in question to other insecticides. House-flies quickly develop resistance to Sevin, but the Mexican bean beetle does not. Synergists may reduce the rate of developing re-

sistance. The rate of metabolism of Sevin appears to be at least partially correlated with resistance in selected strains and this presumably results primarily from in vivo hydroxylation, as discussed. The techniques are now available for the critical studies on resistance which are needed.

H. I. PETERSEN: A problem of very great interest in this connection is the toxicity of the metabolites. Can you say anything about this?

J. E. CASIDA: The experiments that we ran were limited because we had a limited supply of metabolites. However, with Dimetilan it is very easy because we can put 1 mg into each cockroach and recover about 250 μg as pure metabolite by passing through one column – it will then crystallize so that we can get hundreds of milligrams of these metabolites. None of them are as potent as Sevin in inhibiting cholinesterase from its source in the house-fly brain. The anti-cholinesterase activity of the materials is, of course, no indication of their toxicity, and until we can make enough of these materials to study their toxicology independently the question remains unresolved. I think it is an important question because they appear in quite a number of foodstuffs produced by currently recommended procedures.

C. C. HASSETT (Chairman): Dr. Casida, would you care to comment on problems of cross-resistance with the carbamate insecticides?

J. E. CASIDA: I believe Dr. Meltzer investigated this rather closely. I think it would be more appropriate for him to answer.

J. MELTZER: We have had a lot of experience in the selection of strains of house-flies using phenyl-dimethyl-carbamate. Within a certain strain of house-flies, this compound induced a remarkable cross-resistance, in particular to chlorinated hydrocarbon insecticides, whereas the resistance to the carbamate was only slight. Work with other strains of house-flies showed that it was possible to obtain resistance to the phenyl-dimethyl-carbamate, but the maximum resistance did not exceed a factor of 10.

I should like to add, however, that our work with selections of resistant strains showed that we should be very careful in generalizing results. If we obtain a certain result by our selections, we can only say that the results apply to the particular strain used, and under the particular circumstances. Under other conditions, we might obtain contradictory results. I would, therefore, like to warn against generalizing the results obtained in this selection work.

J. E. CASIDA: I would like to make one final comment on this question of resistance, drawing attention in this connection to references [1], [4] and [5] to my paper. From the studies that have been made so far it appears that metabolism in insects is not by hydrolysis but by oxidation. There is considerable evidence in the literature that resistance is related to the rate of metabolism, so that one could deduce that the resistance is due to an increased ability to carry out this oxidation. There is no proof of this, however.

PROBLEMS OF APPLICATION AND ACTION OF THIODAN STUDIED WITH S^{35}-LABELLED INSECTICIDE

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Abstract — Résumé — Аннотация — Resumen

PROBLEMS OF APPLICATION AND ACTION OF THIODAN STUDIED WITH S^{35}- LABELLED INSECTICIDE. Thiodan (6,7,8,10,10-hexachloro - 1,5,5a,6,9,9a - hexahydro-6-9-methano -2,4,3 - benzodioxathiepin - 3 - oxide) is an insecticide developed by Farbwerke Hoechst AG. The technical product consists of two isomers with different melting points and different velocities of insecticidal effect. Using highly purified X- and S-isomers together with technical product, all labelled with S^{35} of the same specific activity, the authors studied problems of the application and mode of action of the isomers. Since the insecticide can be experimentally applied in the vapour phase, we tested the effect of different temperatures and air humidities on sublimation on the end penetration through the cuticula. As intoxication began, a reactive increase in respiration, followed by a remarkable elevation of the insects' body temperature, was found. It was shown with the labelled insecticides that this raising of body temperature after some time causes surface removal of the sublimated insecticidal substance by way of resublimation into the air. This mechanism influences the complex mechanism of penetration, intoxication and detoxication in connection with temperature and relative humidity of the air.

Further experiments are concerned with the penetration and distribution of the labelled insecticide in the insect organism.

MODES D'APPLICATION ET D'ACTION DU THIODAN ÉTUDIÉS PAR MARQUAGE DE L'INSECTICIDE PAR ^{35}S. Le Thiodan (6,7,8,10,10-hexachloro - 1,5,5a,6,9,9a - hexahydro-6-9-méthano -2,4,3 - benzodioxathiépine - 3 - oxyde) est un insecticide mis au point par la Farbwerke Hoechst Ag. Le produit fabriqué comprend deux isomères ayant des points de fusion différents et dont l'effet insecticide ne se manifeste pas avec la même rapidité. En utilisant simultanément les isomères α et β à l'état très pur et le produit fini, tous trois marqués par ^{35}S de même activité spécifique, on a étudié les problèmes relatifs au mode d'application du produit et au mode d'action des isomères. Comme l'insecticide peut être appliqué expérimentalement en phase vapeur, on a vérifié l'effet de diverses températures et de divers degrés d'humidité de l'air sur la sublimation à la surface de la cuticule et sur la pénétration à travers celle-ci. On a constaté que le début d'intoxication provoquait une accélération du rythme de la respiration, suivie d'une élévation sensible de la température du corps des insectes. Comme on a pu le constater en utilisant des insecticides marqués, cette augmentation de la température du corps provoque, après un certain délai, la disparition de l'insecticide sublimé à la surface, qui s'évapore de nouveau dans l'air. Ce phénomène influe sur le mécanisme complexe de la pénétration, de l'intoxication et de la désintoxication en fonction de la température et de l'humidité relative de l'air.

D'autres expériences ont porté sur la pénétration et la distribution de l'insecticide marqué dans l'organisme de l'insecte.

ПРОБЛЕМЫ ПРИМЕНЕНИЯ И ИЗУЧЕНИЕ СПОСОБА ДЕЙСТВИЯ ТИОДАНА ПРИ ПОМОЩИ МЕЧЕННЫХ S^{35} ИНСЕКТИЦИДОВ. Тиодан (6, 7, 8, 10, 10-гексахлоро-1, 5, 5а, 6, 9, 9а-гексагидро-6-9-метано-2, 4, 3-бензодиоксатиепин-3-оксид) - инсектицид, выработанный фирмой "Фарбверке Хехст АГ". Этот технический продукт состоит из двух изомеров с различными температурами плавления и с различной скоростью инсектицидного эффекта. Используя высокоочищенные альфа- и бета-изомеры и технический продукт, меченный S^{35} одинаковой удельной активности, удалось изучить проблемы применения и способы действия этих изомеров. Так как этот инсектицид может быть экспериментально применен в газообразном состоянии, авторы проследили за влиянием изменений температуры и влажности воздуха на сублимацию и проникновение инсектицида через кутикулу. Одновременно с начинающимся отравлением авторы обнаружили реакцию, выражающуюся в учащении дыхания, вслед за чем наблюдалось значитель-

ное повышение температуры тела насекомых. Как установлено при помощи меченых инсектицидов, это повышение температуры тела вызывает через некоторое время исчезновение сублимированного инсектицидного вещества с поверхности путем обратной сублимации в воздух. Этот процесс влияет на сложный механизм проникновения, отравления и устранения токсичности в зависимости от температуры и относительной влажности воздуха.

В дальнейших экспериментах рассматривается проникновение и распределение меченого инсектицида в организм насекомого.

ESTUDIO DE LA APLICACION Y DEL MODO DE ACTUAR DEL THIODAN MARCADO CON ^{35}S. El Thiodan (6,7,8,10,10-hexacloro - 1,5,5a,6,9,9a - hexahidro-6-9-metano -2,4,3 - benzodioxatiepina-3-óxido) es un insecticida preparado por la Farbwerke Hoechst AG. El producto comercial consiste en dos isómeros de diferente punto de fusión, que actúan con velocidades distintas. El autor de la memoria ha estudiado los problemas que plantean la aplicación y el modo de actuar de los isómeros empleando los isómeros α y β de elevado grado de pureza junto con el producto comercial, todos ellos marcados con ^{35}S de la misma actividad específica. Como el insecticida puede aplicarse experimentalmente en la fase vapor, se comprobó el efecto de la temperatura y de la humedad del aire sobre la sublimación y la penetración a través de la cutícula. Al comenzar la intoxicación se observó un aumento del ritmo respiratorio seguido de una notable elevación de la temperatura del cuerpo de los insectos. Los insecticidas marcados permitieron demostrar que este incremento de la temperatura se produce algo después de que el insecticida condensado en la cutícula ha podido comenzar a evaporarse. Este proceso ejerce influencia sobre el complejo mecanismo de la penetración, intoxicación y desintoxicación, y depende a su vez de la temperatura y de la humedad relativa del aire.

Se efectuaron nuevos experimentos para estudiar la penetración y la distribución del insecticida marcado en el organismo del insecto.

INTRODUCTION

Thiodan® is an insecticide developed by Farbwerke Hoechst AG, Federal Republic of Germany [1-2]. The commercial product (6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a, 6, 9, 9a-hexahydro-6, 9-methano-2,4,3-benzodioxathiepin-3-oxide) consists of two isomers with distinctly different melting points and different rates of insecticidal action [3]. The configuration of the stereoisomers was studied by RIEMSCHNEIDER et al. [5]; in this paper they are designated as the α-isomer (F = 108-110°C) and the β-isomer (F = 208-210°C). As shown in Fig. 1, the Thiodan molecule was labelled with radiosulphur (S^{35}); the

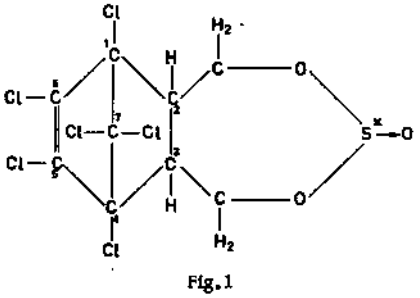

Fig. 1

Molecular structure of Thiodan-S^{35}

highly purified α- and β-isomers and the commercial product (F = 103°C) had the same specific activity (20.6 mc/g). It is generally postulated that a modern synthetic insecticide must possess two essential properties: first, it must be toxic to the insect and secondly it must be able to gain access to

its specific site of action either by penetration through the cuticle or by other paths. In this case, tracer methods are an excellent way of studying the problems of pick-up and penetration. This paper gives a brief account of a large field of experiments using tracer techniques as a part of complex investigations into the mode of action of Thiodan [3, 4, 6].

PICK-UP OF THIODAN BY THE SURFACE OF THE INTEGUMENT AND THE INFLUENCE OF RELATIVE AIR HUMIDITY

The first part of the work to be described attempts to answer the question: Do the varying relative humidities of the air influence the pick-up of Thiodan and its two isomers? Among the different application methods for testing insecticides, the use of the vapour phase is advantageous in our case, because the test insects are exposed to the pure insecticide in molecular form. The main experiments have been done with the granary weevil Calandra granaria L. on account of its relatively large body surface and small amount of body fat. The insects were kept in closed hygrostatic dishes with an inset made of copper gauze on which they were able to run about (Fig. 2). The bottoms of the dishes were filled with super-saturated inorganic salt

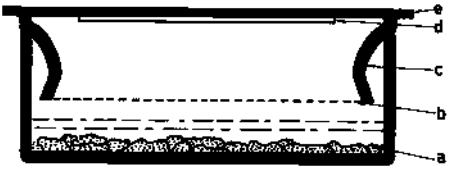

Fig. 2

Special hygrostatic dish for application of radio-Thiodan under controlled air humidity (9 cm diameter)
a = supersaturated salt solution
b = copper gauze
c = inset
d = filter paper impregnated with insecticide
e = glass lid

solutions providing the different relative humidities (K_2SO_4 = 98-100% r. h., Na Cl = 74% r. h., $Ca(NO_3)_2$ = 54% r. h., $Zn Cl_2$ = 17% r. h.).

Filter papers of 7-cm diameter had been previously impregnated with 1 ml of a Thiodan-acetone solution (500μg Thiodan) and were fixed under the glass lid after evaporation of the solvent. This method permitted the molecular insecticide particles to come into contact with the entire surface of the weevils from all directions. The exposure was done in a constant test temperature of 30°C. After 72 h 50 granary weevils in each case were collected from the dishes and washed five times with 2 ml cold methanol by means of a sprayer. By this means all the insecticide picked up was removed from the superficial layers. We preferred methanol to ether and chloroform for removing Thiodan from outside the insect, since, according to BEAMENT [7], it is a poor solvent for the wax of the insect cuticle. The five fractions were collected in small aluminium planchets. After evaporation of the solvent, they were measured for 10 min by means of a Frieseke

and Hoepfner electronic scaler in conjunction with an end-window counting-tube having a window thickness of 1.2 - 1.3 mg/cm².

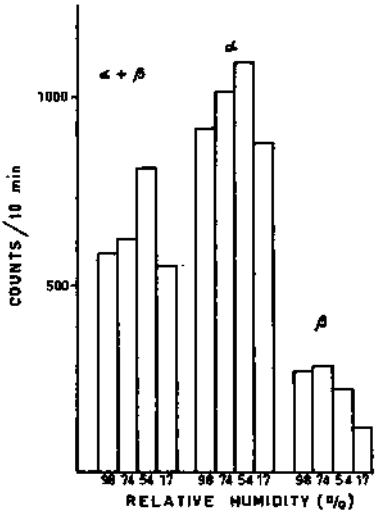

Fig. 3

Amount of Thiodan-S^{35} isomers picked up by 50 granary weevils exposed (72 h, 30°C) to the vapour phase of the insecticide under different air humidities

The first point of interest in Fig. 3 is that different amounts of Thiodan and its α- and β-isomers were picked up by the superficial layers of the integument. This had been anticipated, because the difference in the quantity picked up depends on physical properties, i.e. the different melting points of the α- and β-isomer, as well as on the composition of the commercial product ($\alpha:\beta = 3:1$) [8].

Further, it is apparent – and this is the main point of interest – that the pick-up of Thiodan on the epicuticle is considerably influenced by the relative humidity. These effects are likewise due to physico-chemical properties of the superficial layers. As the wax layers of the insect epicuticle consist of mixtures of paraffins, alcohols and fatty acids, i.e. a mixture of lipoids of varying reactivity [9], it has a certain solubility for Thiodan, which is a lipophilic insecticide. If a Thiodan molecule comes into contact with the epicuticle the surface and the particle will be subjected to attractive forces varying in intensity from primary valence bonds through hydrogen bonds to long-range intermolecular or van der Waals forces. The highest rate of solubility is shown in the middle range of relative humidities, at which the wax layer is not covered with a thin film of water and the transpiration rate is relatively low. In this case, we assume that the dissolving forces of the epicuticle are fully effective. At high humidities, a thin film of water covers the epicuticle, which reduces the solubility for the insecticide, while at very low humidities the rate of transpiration and loss of water through the cuticle act against the deposition and pick-up of the Thiodan molecules [6].

THE PICK-UP AND PENETRATION OF THIODAN AND ITS ISOMERS

In the second series of experiments we tried to clarify the problem of the relationship between pick-up and penetration through the integument and the symptomatology of the insecticidal action.

These studies were done with the same experimental arrangement as described above, but now the granary weevils (50 specimens) were exposed directly to deposits of the pure isomers or commercial product on filter paper impregnated with insecticide to 0.63$\mu g/cm^2$ for varying periods at 25°C and 54% r.h. After different periods the symptoms induced by the insecticide were evaluated. For the purposes of the following presentation the symptoms will be designated by numerals: 1 = latency phase; 2 = incipient excitation; 3 = strong excitation; 4 = convulsions; 5 = paralysis and death.

The weevils were collected and first of all washed five times with 2 ml cold methanol. This removed the Thiodan collected on the surface for determination. This fraction is termed the "outside" fraction. After this, the test insects were homogenized in a Potter-Evelhjem homogenizer with 5 times 2 ml acetone. This fraction is termed the "inside" fraction. Since the Thiodan was labelled with S^{35}, combustion and ash measurement was not a convenient procedure; it was therefore necessary to establish a standard curve for the absorption of β-radiation by the body substances which are also extracted. The amount of fat extracted from 50 granary weevils absorbs at least 31% of the β-radiation.

As Fig. 4 shows, different amounts of Thiodan and its isomers had been picked up by the insects from deposits of equal density during equal periods. If the pick-up had been no more than superficial contamination, it might

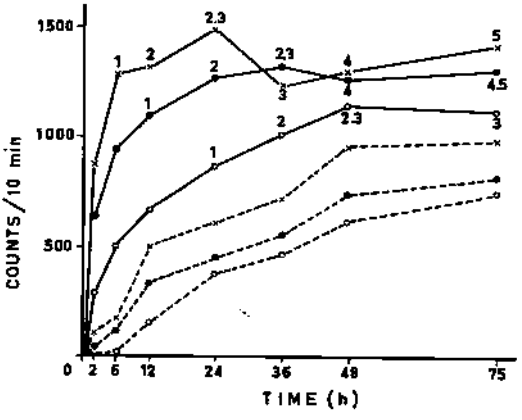

Fig. 4

The pick-up and penetration of Thiodan-S^{35} and its isomers in relation to the increasing insecticidal effect (designated by numerals 1–5 as described in the text)

Outside	Inside
X —— X α	X - - - X α
O —— O β	O - - - O β
● —— ● α + β	● - - - ● α + β

have been expected that the amounts of the three substances recovered in the "outside" fraction would have been equal. Since this is clearly not the case, however, it demonstrates that there must be differential solubility for the three substances in the epicuticle, depending on their different physico-chemical properties, in particular their stereoconfiguration [6]. These facts are confirmed if one also compares the times at which the visible symptoms evoked by the poison appear: For α-Thiodan, the stage of irreversible injury (symptoms 2 and 3) is reached after 24 h and for commercial Thiodan after 36 h, whereas for the β-isomer this stage is not reached for 48 h. It must be emphasized that all three substances are absolutely toxic for insects [3, 6]; the better initial toxicity of the α-isomer is merely a function of its physico-chemical properties. By comparing the symptoms induced by Thiodan intoxication and the amount of "outside" pick-up, it may be seen that up to the end of the incipient excitation phase (symptom 2) and beginning of the strong excitation (symptom 3) insecticide is picked up steadily and the curve has a distinct peak. During the strong excitation phase, however, no more insecticide is picked up; in fact the detectable activity of the outside falls. At a later stage (symptom 4) pick-up again rises. ARMSTRONG et al. [10], working with the isomers of benzene hexachloride, obtained curves of nearly the same type, but were unable to explain this effect. By means of an apparatus which enables us to measure the body temperature in the body cavities of insects [3, 6], we found that the temperature rises from incipient excitation until the end of the strong excitation (symptoms 2 and 3). Parallel to the rise in body temperature, the oxygen consumption curve also has its peak at the same intoxication stages [6].

Another experiment to confirm the fall of the outside curves after intoxication stages 2 and 3 was made by subjecting life weevils and freshly killed weevils under identical experimental conditions to radioactive Thiodan vapour. It was found that the pick-up of labelled Thiodan showed a linear increase in the dead weevils, whereas the live weevils demonstrated the typical fall of activity during excitation stage 3 [6]. These experiments represent an absolute proof that the fall of the "outside" curve is due to a physiological reaction on the part of the insects.

If one examines the shape of the "inside" curves it appears that the α-isomer penetrates the insect more rapidly than the commercial product and the β-isomer. After two hours' exposure, the α-isomer and commercial product already appear in substantial amounts "inside" the insects. Thus it is confirmed that the structural difference between the isomers and the quantitative composition of commercial Thiodan have a considerable effect on the ability to penetrate the insect cuticle [6]. Allowing for the different amounts of "outside" pick-up, the "inside" curves show nearly the same shape. There is a visible increase from the latency phase 1 through the incipient excitation stage 2, whereas during the strong excitation stage 3 the rise of the curves is very small. As we have stated before, all three substances possess absolute toxicity; a total amount of 0.4 μg of Thiodan or its isomers must be present "inside" the insects to cause irreversible injury in 50 granary weevils.

It has been shown, by working with the labelled insecticide, that Thiodan possesses the postulated properties of an active insecticide; it has an absolute and sufficient toxicity for the insect (0.008 μg per insect will kill granary weevils in 100% of the cases) and the ability to gain access to its specific site of action.

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DISCUSSION

B. DARIS: Did you count the number of dead weevils in different relative humidities after the period of 72 h?

W. KLOFT: Yes, naturally we did that, but the results will be published elsewhere.

B. DARIS: I am especially interested in the high relative humidity of 98-100%. Did you find live weevils at these high relative humidities?

W. KLOFT: Yes, even at 98-100% r. h. some weevils survive, depending on the insecticide concentration and on the exposure periods. The purpose of the experiments was in fact to find the best concentrations and exposure periods at different relative humidities, in order to establish control methods adapted to particular climatic conditions. We think that so far insufficient attention has been paid to this.

D.A. CROSSLEY: What method do you use to measure respiration for these insects?

W. KLOFT: We measured respiration using Warburg manometric techniques at different temperatures. The insects were put directly into the vessels and were intoxicated – this is important, I feel – within the vessel. First, the rate of oxygen consumption was measured for two hours, and then the respiratory quotient was measured throughout the whole course of intoxication until death.

C.T. LEWIS: I was very interested in the figures for the uptake of insecticide from the vapour phase. I wonder whether you have any information, or any views, on the possibility that sublimated insecticide might have been picked up by the weevils from the gauze on which they were walking. Do you agree that a significant fraction of the uptake might have come from insecticide that had condensed on the gauze surface?

W. KLOFT: Yes, we cannot totally exclude this factor. Only at the beginning of the exposure was the uptake exclusively from the vapour phase, and I am sure that after about two hours we had a supplementary intoxication of the type you mention.

F. T. PHILLIPS: Your results on the pick-up of Thiodan vapour by insect epicuticle under different relative humidities are interesting. Did you use other surfaces for comparison, for example a glass surface (as a standard surface) and a glass surface covered with a layer of insect wax? I realize that it probably would not be possible to obtain the same wax structure on a glass surface as on insect bodies, but this might be overcome by using excised cuticle from the insects.

W. KLOFT: We have not done any experiments exposing glass surfaces under different relative humidities, since we are of the opinion that the differences observed are mainly due to physiological processes, for the reasons explained in greater detail in the paper. In particular, the loss of "outside" activity after a rise in internal body temperature shows that physiological processes are involved, and this was proved by the simultaneous exposure of dead weevils. We were also able to show this resublimation effect using a glass model heated inside by an electric resistance.

E. HORBER: Is there no interaction to be expected between the insecticide vapour and the ions of the solutions in the bottom of the Zwölfer hygrostatic dishes?

W. KLOFT: No, because Thiodan is practically water-insoluble. In addition, the filter with the insecticide is fixed under the lid and the vapour phase comes from above, while the aqueous salt solution is underneath.

ПРИМЕНЕНИЕ РАДИОАКТИВНЫХ ИЗОТОПОВ В ИЗУЧЕНИИ ПРОЦЕССОВ ВСАСЫВАНИЯ, РАСПРЕДЕЛЕНИЯ И ВЫДЕЛЕНИЯ ИЗ ЖИВОТНОГО ОРГАНИЗМА НЕКОТОРЫХ ИНСЕКТИЦИДОВ

Г.В. ФИЛАТОВ, П.А. КАРТАШОВ, М.И. МУТИН, И.А. ЗАКАМЫРДИН, У.Я. УЗАКОВ
ВСЕСОЮЗНЫЙ НАУЧНО-ИССЛЕДОВАТЕЛЬСКИЙ ИНСТИТУТ ВЕТЕРИНАРНОЙ САНИТАЦИИ, МОСКВА, СССР

Abstract — Résumé — Аннотация — Resumen

THE USE OF RADIOISOTOPES TO STUDY THE ABSORPTION, DISTRIBUTION AND ELIMINATION OF VARIOUS INSECTICIDES IN ANIMALS. When insecticides are used against farm-animal parasites it is important to ensure that no harm is done to the health of the animal or the consumer. Radioisotopes provide a means of studying the behaviour of labelled insecticides in animal organisms and of obtaining extremely accurate data on residues of insecticides and insecticide decomposition products in meat and milk.

The paper gives details on the rate at which DDT-C^{14}, polychloropinene-Cl^{36} and chlorophos-P^{32} are absorbed through the skin, accumulated in the organs and tissues and eliminated from the organisms of farm and laboratory animals.

EMPLOI DES RADIOISOTOPES POUR L'ÉTUDE DES PROCESSUS D'ABSORPTION, DE DISTRIBUTION ET D'EXCRÉTION DE CERTAINS INSECTICIDES DANS L'ORGANISME VIVANT. L'emploi des insecticides dans la lutte contre les parasites des animaux de ferme ne doit pas compromettre la santé des animaux ni celle des personnes utilisant les produits animaux. Les radioisotopes permettent de suivre le comportement d'un insecticide marqué qui a été introduit dans l'organisme animal, et de déterminer par ailleurs, avec une grande précision, la teneur du lait et de la viande en insecticides et produits de désintégration.

Le mémoire fournit des données sur la vitesse d'absorption à travers l'épiderme, l'accumulation dans les organes et tissus et l'excrétion, par l'organisme d'animaux de laboratoire et d'animaux de ferme, de DDT-^{14}C, de polychlorure de pinène-^{36}C et de chlorure de phosphore-^{32}P.

ПРИМЕНЕНИЕ РАДИОАКТИВНЫХ ИЗОТОПОВ В ИЗУЧЕНИИ ПРОЦЕССОВ ВСАСЫВАНИЯ, РАСПРЕДЕЛЕНИЯ И ВЫДЕЛЕНИЯ ИЗ ЖИВОТНОГО ОРГАНИЗМА НЕКОТОРЫХ ИНСЕКТИЦИДОВ. Применение инсектицидов для борьбы с паразитами сельскохозяйственных животных не должно наносить ущерб здоровью как самих животных, так и человека, потребляющего животные продукты. Использование радиоактивных изотопов позволяет проследить за поведением меченого инсектицида в животном организме и с большой точностью определить остаточное содержание инсектицидов и продуктов их распада в молоке и мясе.

В работе приводятся данные о скорости всасывания через кожный покров, накоплении в органах и тканях и выделении из организма лабораторных и сельскохозяйственных животных препарата ДДТ-C^{14}, полихлорпинена-Cl^{3} и хлорофоса-P^{32}.

EMPLEO DE LOS RADIOISÓTOPOS EN EL ESTUDIO DE LOS PROCESOS DE ABSORCIÓN, DISTRIBUCIÓN Y ELIMINACIÓN DE CIERTOS INSECTICIDAS EN EL ORGANISMO ANIMAL. El empleo de los insecticidas en la lucha contra los parásitos del ganado no debe perjudicar a la salud de éste ni de las personas que utilizan los productos animales. Los radioisótopos permiten seguir en el organismo animal el comportamiento de un insecticida marcado y determinar con gran precisión el contenido de residuos de insecticidas y de productos de su desintegración en la leche y en la carne.

En la memoria se presentan datos sobre la velocidad de absorción a través de la epidermis, la acumulación en los órganos y tejidos y la eliminación del DDT-^{14}C, del policlorpineno-^{36}Cl y del Clorosfos-^{32}P por el organismo de animales de laboratorio y por el del ganado.

Всесоюзный научно-исследовательский институт ветеринарной санитарии применяет радиоактивные изотопы для изучения процессов

всасывания, распределения и выделения из организма инсектицидов, используемых для борьбы с эктопаразитами сельскохозяйственных животных, а также при кожноводовой инвазии.

ИЗУЧЕНИЕ ВСАСЫВАНИЯ, ДЕПОНИРОВАНИЯ И ВЫДЕЛЕНИЯ ИЗ ОРГАНИЗМА ЖИВОТНЫХ ПРЕПАРАТА ДДТ, МЕЧЕННОГО C^{14}.

Широкое использование препаратов ДДТ (дихлордифенилтрихлорэтан) в ветеринарии как средства борьбы с эктопаразитами сельскохозяйственных животных и разноречивые данные о поведении его в организме сельскохозяйственных животных заставили провести специальное исследование по изучению вопросов всасывания, депонирования и выделения из организма животных этого препарата при нанесении его на кожу в виде масляного раствора в обычно применяемых дозах.

Меченный C^{14} препарат ДДТ в количестве 8,6 г растворялся в 200 мл вазелинового масла при подогревании. На одну обработку лактирующей коровы брали 100 мл раствора, в котором содержалось 4,3 г ДДТ с общей активностью 19,35 мккюри, что составляло 62 мккюри на 1 кг веса животного или 14 мг ДДТ на 1 кг живого веса.

Раствор втирался щеткой в область спины коровы от переднего края лопатки до корня хвоста. Активности препарата ДДТ, меченного C^{14}, в образцах крови, кала, мочи и молока определялись радиометрически на установке Б-2 с торцовым счетчиком Т-25-БФЛ. Полученные результаты переводились в весовые количества ДДТ.

В соответствии с ветеринарным законодательством корова обрабатывалась 4,3%-ным масляным раствором ДДТ дважды, с промежутком в 20 дней.

После первой обработки коровы уже через 2 часа в крови обнаруживается наличие ДДТ. Через сутки оно достигает 0,37 мг/л. Максимальное содержание ДДТ в крови - 0,43 мг/л обнаруживается к 6-му или 7-му дню, после чего резко уменьшается и с 10-го дня не обнаруживается.

После второй обработки (через 20 дней) ДДТ появляется в крови вновь и достигает максимальной величины на 13-й день (0,1 мг/л). Через 19 дней ДДТ в крови не обнаруживается.

Через 2 часа после первого нанесения на кожу 4,3%-ного масляного раствора ДДТ в пробах молока можно обнаружить C^{14}.

На следующий день в утреннем удое содержалось 0,48, а в вечернем - 2,78 мг/л ДДТ.

Максимальное количество C^{14} - ДДТ выделяется в молоке через 36 часов и соответствует 8,44 мг/л. Через двое суток содержание ДДТ в молоке уменьшается, однако до 10 - 12 суток содержание его превышает 1 мг/л. На 20-й день после первой обработки в молоке еще содержалось до 0,48 мг/л ДДТ.

После второй обработки (на 21 день) количество C^{14} - ДДТ в молоке возросло до 0,67 мг/л. Максимальное содержание ДДТ в молоке, после второй обработки, обнаружено на 4 сутки - 7,69 мг/л,

после чего идет постепенное уменьшение. Однако до 130 дней после обработки в молоке можно обнаружить C^{14} - ДДТ.

Всего за время проведения опыта с молоком выделилось около 590 мг ДДТ, что составляет 7% от количества, нанесенного на кожу.

За опытный период (130 дней) с мочой выделилось около 1149 мг, или 13,3%, а с калом - 1353 мг, или 15,7% от нанесенного количества ДДТ.

В органах и тканях подопытной коровы, забитой через 5 месяцев с момента первой обработки, обнаружено содержание ДДТ преимущественно в жировой ткани.

Так, в жире брыжейки, жире подкожной клетчатки и жире перикарда содержание ДДТ составляло 1,2 - 1,47 мг/кг.

Таким образом, за 5 месяцев после обработки коровы ДДТ выделяется около половины нанесенного препарата, причем наибольшее количество его выделяется в первые 4 суток.

Оставшийся в организме ДДТ депонируется во всех органах с преимуществом в жировой ткани.

ИЗУЧЕНИЕ ПРОНИЦАЕМОСТИ ЧЕРЕЗ КОЖУ, ДЕПОНИРОВАНИЯ И ВЫДЕЛЕНИЯ ИЗ ОРГАНИЗМА ЖИВОТНЫХ ПОЛИХЛОРПИНЕНА, МЕЧЕННОГО Cl^{36}

В опытах на кроликах и дойной корове изучалось всасывание, депонирование и выделение из организма препарата полихлорпинена, применяемого в стране для защиты животных от гнуса.

В опытах с нанесением 3%-ной эмульсии полихлорпинена, меченного Cl^{36} на кожу подопытных кроликов из расчета 0,75 мккюри (150 мг ПХП) на 1 кг живого веса, показали, что Cl^{36} появляется в крови через 1 час и обнаруживается в ней до 24 часов. В моче и кале Cl^{36} полихлорпинена появляется через 3 часа и выделяется с мочой в течение 96 часов, а с калом - 144 часов.

После забоя подопытных кроликов определяли содержание Cl^{36} в органах и тканях. Наибольшее количество его зарегистрировано в печени и почках.

В опытах на дойной корове с нанесением на кожу 3%-ной водной эмульсии меченого полихлорпинена в количестве 1,5 л с содержанием 225 мккюри Cl^{36} было установлено, что препарат ПХП быстро обнаруживается в крови и в небольших количествах регистрируется в течение 72 часов. В моче и кале Cl^{36} выделяется соответственно в течение 144 - 166 часов.

В молоке следы Cl^{36} обнаруживаются на 48 - 72 часе.

После четырехкратной обработки коровы эмульсией полихлорпинена Cl^{36} не обнаруживается в крови спустя 120 часов, а в моче и кале - после 168 часов.

Учитывая, что полихлорпинен не растворяется в воде при определении активности органов и тканей подопытных кроликов и коровы, измерение проводилось в водных и спиртовых вытяжках, при этом, как правило, Cl^{36} регистрировался только в водном экстракте. Отсюда было сделано предположение о быстром расщеплении молекулы ПХП в организме и выделения его в виде продуктов распада.

Для подтверждения этого был проведен хроматографический анализ ПХП и продуктов его распада, содержащихся в органах и тканях и выделяющихся с молоком, мочой и калом. Хроматография проводилась на бумаге Ленинградской фабрики марки "М" по видоизмененной методике Кишнера, Джона, Миллера и Келлера. В качестве подвижного растворителя применялся гексан и этилацетон.

Проявление хроматограмм производилось опрыскиванием бумажных полос 0,01%-ным водным раствором флюоресцеина и последующим выдерживанием хроматограмм в парах брома.

При этом флюоресцеин, реагируя с парами брома, дает красную окраску (образование эозина). В месте расположения полихлорпинена флюоресцеин сохраняет свою нормальную окраску, давая желтые пятна.

Данные хроматографического анализа подтвердили, что полихлорпинен в организме животного быстро расщепляется, так как ни в одном случае не было выделено пятен, Rf которых соответствовал бы цельной молекуле полихлорпинена.

ИЗУЧЕНИЕ МЕТАБОЛИЗМА ХЛОРОФОСА В ЖИВОТНОМ ОРГАНИЗМЕ

Хлорофос как фосфорорганический инсектицид в настоящее время широко применяется для обработки сельскохозяйственных животных против эктопаразитов и подкожного овода. В нашей лаборатории был синтезирован препарат метилового хлорофоса, меченный P^{32}, с удельной активностью 250 мккюри.

При наружной обработке лабораторных животных, а также крупного рогатого скота было установлено, что хлорофос в первые минуты после нанесения на кожу обнаруживается в крови, через 30 минут достигает максимальной величины, затем постепенно снижается и к 6 - 7-му дню не обнаруживается в крови.

Исследование проб молока дойной коровы показало, что через 30 минут в молоке появляются следовые количества P^{32} хлорофоса, через 3 - 6 часов активность образцов достигает максимальной величины, а затем уменьшается. На 6 - 7-й день радиоактивный фосфор хлорофоса в молоке обнаружить не удалось.

Выделение P^{32} хлорофоса с мочой достигает максимальной величины через 3 часа после нанесения на кожу и держится на высоком уровне до 6 часов. После 6 часов содержание P^{32} хлорофоса в моче снижается и на 3-й день становится в 6 раз меньше максимальной величины.

Однако и через 16 дней после обработки активность 1 мл мочи достигает 900 расп/мин. Выделение P^{32} хлорофоса с калом зарегистрировано через 2 часа после наружной обработки коровы меченым хлорофосом.

Максимальное количество P^{32} в кале обнаруживается через 48 часов, после чего уменьшается, и к 16 дню кал имеет небольшую активность.

Наряду с радиометрическим методом определения P^{32} пробы крови, мочи, кала, молока и внутренних органов кроликов и коровы подвергали хроматографическому анализу. Хроматография хлорофоса

и продуктов его распада проводилась на бумаге марки "Б" Ленинградской фабрики. В качестве подвижного растворителя использовалась смесь, состоящая из 2,5 частей 0,2%-го раствора соляной кислоты и 7,5 частей 95%-ного этилового спирта.

Проявление пятен осуществлялось опрыскиванием хроматограмм реактивом Бурроуса (25 мл 4%-ного раствора молибденовокислого аммония, 10 мл 1N раствора соляной кислоты, 5 мл 60%-ной хлорной кислоты и 60 мл дистиллированой воды).

После высушивания хроматограммы облучались кварцевой лампой в течение 2 - 5 минут. Соединения, содержащие фосфорные эфиры проявлялись в виде голубых пятен на белом фоне. Неорганические фосфаты обнаруживались в виде желтых пятен. В качестве свидетелей использовались препараты химически чистого хлорофоса, диметилфосфористой кислоты и ДДВФ, Rf которых соответственно равнялся: 0,75, 0,82 и 0,90.

Хроматография мочи и экстрактов печени подопытных животных, забитых в разные сроки после наружной обработки хлорофосом, показала отсутствие пятен, Rf которых соответствовал бы хлорофосу и ДДВФ. Обнаружены пятна, Rf которых соответствовал диметилфосфористой кислоте и неизвестным метаболитам. Значительная часть P^{32} содержалась в пятнах, соответствующих неорганическим фосфатам.

Не удалось также обнаружить хлорофос при хроматографическом исследовании молока коровы, обработанной хлорофосом.

В моче и экстрактах печени кроликов, которым хлорофос вводился per os, были обнаружены соединения, Rf которых соответствовал хлорофосу.

Изучение холинэстеразной активности крови, молока и внутренних органов подопытных животных подтвердило динамику всасывания и выведения из организма препарата хлорофоса.

DISCUSSION

R. von BORSTEL: What other insecticides are you planning to use in your future research?

G.V. FILATOV: Phosphoric organic compounds, mainly of the Trolene and Rogor types, and also carbamates of the Sevin type.

J. HALBERSTADT: Were any specific assay techniques used for determining the original compound and its break-down products in tissues, organs and excreta?

G.V. FILATOV: For this purpose we used the chromatographic method, and also inhibition of the choline esterase of standard serum.

STUDIES ON THE SELECTIVE TOXICITY OF SCHRADAN

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Abstract — Résumé — Аннотация — Resumen

STUDIES ON THE SELECTIVE TOXICITY OF SCHRADAN. Schradan is of low toxicity for chewing insects, while it is highly toxic to sucking insects. The selective toxicity of Schradan may be associated with distribution of toxicants in insect bodies, susceptibilities of insect cholinesterases to toxicants and metabolism of toxicants.

The absorption, excretion and metabolic rates of P^{32}-Schradan in those insects varied considerably and no definite relationships were found between those factors and toxicities, nor found between susceptibility of cholinesterases to toxicants and toxicities; but quantitative differences in P^{32}-Schradan distribution patterns existed among those insects. Much more P^{32}-Schradan accumulated in the central nervous systems of sucking insects than in those of chewing insects. Isolated nervous tissues of sucking insects absorbed much more P^{32}-Schradan than those of chewing insects.

Electron microscope observations on the central nervous sheath disclosed that the thoracic ganglia of chewing insects were enclosed in a thick and robust sheath, while those of sucking insects were surrounded by simple, thin double membranes.

The author came to the conclusion that the selective toxicity of Schradan to insects was not due to the difference of their metabolizing activities of Schradan as regards activation and detoxication, nor to the sensitivity of insect cholinesterases to activated Schradan, but to the differences of Schradan distribution patterns in insect bodies. Thus the distribution of toxicants in insects and the character of the nerve sheath, which acts as a barrier against the penetration of toxicants, may be the most important factors responsible for the selective toxicity of Schradan.

ÉTUDE DE LA TOXICITÉ SÉLECTIVE DU SCHRADAN. Le Schradan est peu toxique pour les insectes broyeurs, alors qu'il l'est extrêmement pour les insectes suceurs. Sa toxicité sélective pourrait dépendre de la distribution des substances toxiques dans l'organisme de l'insecte, de leur action sur la cholinestérase et de leur métabolisme.

On a observé des différences considérables, selon les insectes, dans les taux d'absorption et d'excrétion et dans le métabolisme du Schradan marqué par ^{32}P, mais on n'a pas pu établir de rapport bien défini entre ces facteurs et la toxicité, pas plus qu'entre la sensibilité de la cholinestérase aux substances toxiques et leur toxicité. Par contre, on a relevé des différences quantitatives dans la distribution du Schradan-^{32}P. L'accumulation de Schradan-^{32}P a été beaucoup plus forte dans le système nerveux central des insectes suceurs que dans celui des insectes broyeurs. De même, les tissus nerveux isolés des premiers ont absorbé beaucoup plus de Schradan-^{32}P que ceux des insectes broyeurs.

L'observation au microscope électronique des gaines dans les centres nerveux a révélé que les ganglions thoraciques des insectes broyeurs sont revêtus d'une gaine épaisse et robuste, tandis que ceux des insectes suceurs sont entourés d'une membrane simple ou d'une membrane mince et double.

L'auteur est parvenu à la conclusion que la toxicité sélective du Schradan pour les insectes ne tient pas aux différences de métabolisme du Schradan au cours des phases d'activation et de détoxication, ni à la sensibilité de la cholinestérase au Schradan marqué, mais qu'elle est due aux différences de distribution du Schradan dans l'organisme des insectes. La distribution des substances toxiques dans l'organisme des insectes et la nature de la gaine des nerfs, qui s'oppose à la pénétration des substances toxiques, pourraient donc constituer les principaux facteurs de la toxicité sélective du Schradan.

ИЗУЧЕНИЕ СЕЛЕКТИВНОЙ ТОКСИЧНОСТИ ШРАДАНА. Шредан мало токсичен для жующих, но высоко токсичен для сосущих насекомых. Селективная токсичность шредана может быть связана с распределением токсических веществ в теле насекомых, с чувствительностью холинэстеразы насекомых к токсическим веществам и с метаболизмом последних.

Поглощение, выделение и скорости метаболизма прадана, меченного фосфором-32, значительно различались у этих насекомых, и определенных связей между этими факторами и токсичностью установлено не было, так же как не было установлено определенных связей между чувствительностью холинэстеразы к токсическим веществам и к токсичности. Однако у этих насекомых наблюдались количественные различия в распределении прадана, меченного фосфором-32. У сосущих насекомых в центральной нервной системе сосредоточивалось гораздо больше меченного фосфором-32 прадана, чем у жующих насекомых. Изолированные нервные ткани сосущих насекомых поглощали гораздо больше прадана, меченного фосфором-32, чем нервные ткани жующих насекомых.

Наблюдения под электронным микроскопом оболочки центральной нервной системы показали, что грудные ганглии жующих насекомых были покрыты толстой и прочной оболочкой, тогда как те же органы сосущих насекомых были окружены простой и тонкой двойной мембраной.

Авторы пришли к заключению, что селективная токсичность прадана для насекомых объясняется не разницей в метаболических процессах активации и детоксикации прадана и не чувствительностью холинэстеразы насекомых к активированному прадану, а разницей в распределении его в теле насекомых. Таким образом, распределение токсических веществ в теле насекомых и характер оболочки нервной системы, являющейся барьером для проникновения токсических веществ, являются, по-видимому, наиболее важными факторами, объединяющими селективную токсичность прадана.

ESTUDIO DE LA TOXICIDAD SELECTIVA DEL SCHRADAN. El Schradan es poco tóxico para los insectos masticadores mientras que lo es en alto grado para los chupadores. Su toxicidad selectiva quizá esté asociada a la distribución de los tóxicos en el organismo de los insectos, a la sensibilidad de las colinesterasas de los insectos a los tóxicos, y al metabolismo de éstos.

Las velocidades de absorción, excreción y metabolismo del Schradan-^{32}P varían mucho en estos dos tipos de insectos y no se pudo encontrar ninguna relación clara entre estos factores y la toxicidad, ni entre la sensibilidad de las colinesterasas a los tóxicos y la toxicidad. En cambio se registraron diferencias cuantitativas entre los esquemas de distribución del Schradan-^{32}P en estos dos tipos de insectos. Se acumula mucho más Schradan-^{32}P en el sistema nervioso central de los insectos chupadores que en el de los masticadores. Los tejidos nerviosos aislados de los insectos chupadores absorben mucho más Schradan-^{32}P que los de los insectos masticadores.

Observando con el microscopio electrónico la vaina del sistema nervioso central se pudo comprobar que los ganglios torácicos de los insectos masticadores poseen una vaina gruesa y robusta, mientras que los de los insectos chupadores están rodeados de una sola membrana o de membranas dobles muy delgadas.

El autor llegó a la conclusión de que la toxicidad selectiva del Schradan para estos insectos no se debe a la diferencia que hay entre la actividad metabólica del Schradan en la activación o la desintoxicación ni a la sensibilidad de las colinesterasas del insecto al Schradan activado, sino al diferente esquema de distribución del insecticida en el organismo del insecto. Así, pues, los factores más importantes que determinan la toxicidad selectiva del Schradan pueden ser la distribución del tóxico en los insectos y el carácter de la vaina nerviosa que hace de barrera contra su penetración.

INTRODUCTION

Generally, a given insecticide is not always equally effective against all kinds of insects. Systemic insecticides, which are absorbed by a plant and translocated to other parts of the plant, so that insecticidal activity is transferred to untreated parts, are effective against sucking insects, but not so effective against chewing insects.

Schradan, which is one of the systemic insecticides, was found to be of low toxicity for the larva of the rice stem-borer, Chilo suppressalis Walker, the adult of the American cockroach, Periplaneta americana L., and the adult of the house-fly, Musca domestica vicina Macq., while it was highly toxic for the adult of the rice bug, Leptocorisa varicornis Fabricius, the black rice bug, Scotinophara lurida Burmeister, and the green rice leafhopper, Nephotettix bipunctatus cincticeps Uhler (Table I).

TABLE I

TOXICITIES OF SCHRADAN APPLIED TOPICALLY TO VARIOUS INSECTS

Insect	LD50 (μg/g)	Regression coefficient
Rice stem-borer	>26 222	--
American cockroach	2 170	0.251
House-fly	1 932	0.242
Green rice leafhopper	160	0.649
Black rice bug	92	0.394
Rice bug	23>	-

Schradan is converted, by enzymatic oxidation in the insect body, into oxidized Schradan, a strong anticholinesterase. The inhibition of cholinesterase in the central nervous system may be the principal action of Schradan. Consequently the selective toxicity of Schradan may be associated with the following three main factors:

(1) Differences in the distribution of toxicants in insect bodies;
(2) Differences in the susceptibility of insect cholinesterase to toxicants; and
(3) Differences in the metabolism of toxicants as regards activation and detoxification.

TABLE II

ABSORPTION AND EXCRETION OF SCHRADAN BY VARIOUS INSECTS AFTER TOPICAL APPLICATION (36 μg Schradan per insect)

Insect	Absorption/excretion (%)					
	After 1 h			After 6 h		
	Outer	Internal	Excreta	Outer	Internal	Excreta
Rice stem-borer	26.5	3.7	69.8	11.4	12.1	76.5
American cockroach	31.1	27.7	41.2	8.8	42.8	48.4
House-fly	11.5	1.8	86.7	8.2	3.0	88.8
Green rice leafhopper	20.6	3.3	76.1	18.5	4.3	77.2
Black rice bug	17.8	18.7	63.5	13.4	10.4	76.2
Rice bug	19.6	3.1	77.3	11.8	11.3	76.9

RESULTS

(a) Distribution of Schradan in various insect species

No quantitative differences concerning the absorption and excretion capacities of Schradan were observed as between the susceptible and non-susceptible insects (Table II). There were, however, quantitative differences in the Schradan distribution patterns. Much more Schradan accumulated in the central nervous systems of susceptible insects than in those of non-susceptible insects (Tables III, IV).

(b) Cholinesterase inhibition of Schradan in various insect species

Schradan itself is a very weak anticholinesterase, but oxidized Schradan is a powerful anticholinesterase, oxidation enhancing the activity by as much as 1000-10 000 times. The susceptibility of the cholinesterases of the various insects to Schradan and to oxidized Schradan varied considerably, and no definite relationships were found between enzyme inhibition and toxicity (Table V).

(c) Metabolism of Schradan in various insect species

Schradan was activated and detoxicated by the species studied. The metabolic rates of Schradan in those species varied considerably and no definite relationships were found between metabolism and toxicity (Table VI).

(d) Penetration of Schradan and oxidized Schradan to isolated nervous tissues in various insect species

Isolated nervous tissues of susceptible insects absorbed much more Schradan than those of non-susceptible insects (Table VII), and desheathed nervous tissues of cockroach absorbed much more Schradan and oxidized Schradan than untreated tissues (Table VIII).

(e) Electron-microscope observation of the ganglionic sheaths of various insect species

Electron-microscope studies on the central nervous sheath showed that the thoracic ganglia of non-susceptible insects were enclosed in a thick and robust sheath, while those of susceptible insects were surrounded by single or thin double membranes.

DISCUSSION

Several hypotheses have been put forward to explain the physiological selectivity of Schradan. CASIDA et al. have shown that the susceptibility of insects to Schradan depends upon the sensitivity of the particular insect cholinesterase for the activated Schradan and that the cholinesterase from the non-susceptible insects required 6-24 times as much activated Schradan

TABLE III

DISTRIBUTION OF SCHRADAN IN THE TISSUES OF VARIOUS INSECTS TREATED WITH 500 $\mu g/g$ P^{32}-SCHRADAN BY TOPICAL APPLICATION
(Percentages)

Insect	Nerve cord	Thoracic ganglion	Gut	Fat	Coxa	Reproductive system	Remainder
Rice stem-borer	0.008	-	0.202	0.490	-	-	0.451
American cockroach	0.151	-	3.146	1.113	1.971	0.147	10.623
House-fly	-	0.053	0.403	0.090	0.105	0.158	2.153
Green rice leafhopper	-	0.583	0.583	0.292	-	-	16.917
Black rice bug	-	0.600	1.400	0.500	0.900	0.300	22.600
Rice bug	-	0.500	2.288	1.500	2.250	0.875	25.119

TABLE IV

DISTRIBUTION OF SCHRADAN IN THE TISSUES OF VARIOUS INSECTS TREATED WITH 500 μg/g P^{32}-SCHRADAN BY TOPICAL APPLICATION
(Amounts, μg/mg)

Insect	Nerve cord	Thoracic ganglion	Gut	Fat	Coxa	Reproductive system	Remainder
Rice stem-borer	0.019	-	0.011	0.008	-	-	0.003
American cockroach	0.068	-	0.135	0.058	0.055	0.044	0.073
House-fly	-	0.025	0.007	0.007	0.017	0.008	0.007
Green rice leafhopper	-	0.100	0.013	0.023	-	-	0.024
Black rice bug	-	1.500	0.046	0.085	0.188	0.142	0.121
Rice bug	-	0.500	0.087	0.100	0.300	0.350	0.095

TABLE V

MOLAR CONCENTRATIONS OF SCHRADAN AND OXIDIZED SCHRADAN FOR 50% AND 80% CHOLINESTERASE INHIBITIONS (mM)

Insect	Schradan		Oxidized Schradan	
	Inhibition		Inhibition	
	50%	80%	50%	80%
Rice stem-borer	800	2700	1.0	2.2
American cockroach	1200	2300	4.2	6.8
House-fly	40	380	0.0098	0.025
Green rice leafhopper	200	720	0.98	3.0
Black rice bug	410	1300	2.4	5.6
Rice bug	1100	2800	3.2	5.8

for inhibition as that of the susceptible insects [1,2]. O'BRIEN and SPENCER [3, 4, 5], on the other hand, considered that the physiological selectivity of Schradan was not due to the sensitivity of the particular insect cholinesterase to the Schradan metabolite, but that susceptible insects converted Schradan to the cholinergic oxidation product slowly within the nerve tissue, while in the non-susceptible species the conversion rate in the fat body of the insect was so great that little or no unconverted Schradan reached the nerve tissue and the oxidation product of Schradan was too unstable to penetrate the lipoid nerve sheath. They proposed the hypothesis that non-susceptible insects have a barrier which prevents the penetration of the converted Schradan to the site of action. TSUYUKI et al. suggested [6] that the active anti-cholinesterase agent was isomerized to a much more stable component, the methyl ether. They considered that the selective toxicity of Schradan might be associated with this isomerization.

CONCLUSION

The author came to the conclusion that the selective toxicity of Schradan to insects was not due to differences in their metabolism of Schradan as regards activation and detoxication, nor to the sensitivity of insect cholinesterases to activated Schradan, but to the differences of Schradan distribution patterns in insect bodies. Thus the distribution of toxicants in insects and the nature of the nerve sheath which acts as a barrier against the penetration of toxicants may be the most important factors responsible for the selective toxicity of Schradan.

TABLE VI

PER-CENT METABOLISM OF P^{32}-SCHRADAN INDICATED BY SLICE OF TISSUE IN VARIOUS INSECT SPECIES (30°C, 3 h)

Insect					Corrected	
	Schradan	Oxidized Schradan	Hydrozates	Protein incorporates	Hydrozates	Oxidized Schradan
Rice stem-borer	94.8	1.2	3.8	0.2	3.6	1.6
American cockroach	90.2	1.0	7.9	0.9	7.0	2.8
House-fly	93.4	1.7	4.5	0.4	4.2	2.4
Green rice leafhopper	92.6	1.2	5.6	0.6	5.0	2.4
Rice bug	97.0	0.8	1.8	0.2	1.7	1.3

TABLE VII

ABSORPTION OF P^{32}-SCHRADAN BY ISOLATED NERVE TISSUES OF VARIOUS INSECTS IMMERSED IN PHOSPHATE-BUFFER RINGER SOLUTION OF 0.1% SCHRADAN (pH 7.1, 30°C, after 60 min)

Nerve tissues	Tissue weight (mg)	Absorbed Schradan (μg/mg)
Rice stem-borer	0.16	0.313
American cockroach	11.20	0.528
House-fly	0.16	0.506
Green rice leafhopper	0.06	0.583
Black rice bug	0.10	0.770
Rice bug	0.14	0.621
Desheathed American cockroach	9.90	0.612

TABLE VIII

ABSORPTION OF SCHRADAN AND OXIDIZED SCHRADAN BY VENTRAL NERVE CORD OF THE AMERICAN COCKROACH

Insecticides	Schradan in Ringer solution					Oxidized Schradan
Concentrations (%)		0.1%		0.0114	0.0006	0.0114
Immersion times (min)	10	20	40	40	40	40
Absorption in normal nerve cord (μg/mg)	0.195	0.249	0.303	0.074	0.001	0.079
Absorption in desheathed nerve cord (μg/mg)	0.289	0.275	0.317	0.118	0.003	0.104

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DISCUSSION

S. FUZEAU-BRAESCH: Do you possess strains resistant to this insecticide, and if so can you compare insects of the same species, susceptible or non-susceptible, instead of comparing different species?

T. SAITO: The study reported compares species which were naturally susceptible or resistant to Schradan. No strains of any of the susceptible species which had acquired resistance through treatment with this compound were available for study.

J.E. CASIDA: How did you prepare oxidized Schradan?

T. SAITO: The oxidized P^{32}-Schradan was prepared by potassium permanganate oxidation from P^{32}-Schradan and separated by silica-gel chromatography, by the method described in ref. [6] to the paper. One fraction was then analysed by means of infra-red spectra, paper chromatography, anticholinesterase activity and chloroform-water partition coefficient.

K. van ASPEREN: I have an idea that O'Brien and Spencer suggested that resistant insects may oxidize the Schradan outside the nervous system so quickly that very little penetration of the now more polar substance through the nerve sheath could occur. If that is true, their explanation of differences in toxicity in different insects is more or less similar to yours, i.e. a difference in distribution. However, my memory may mislead me.

T. SAITO: I had some correspondence with O'Brien on this problem. I think that the different results may be due to the different methods. O'Brien used enzymatic analysis, but I am analysing radiometrically in situ.

C.C. HASSETT (Chairman): I might make a general comment on the problem of the insect nerve sheath in relation to Dr. Saito's work. This sheath is a relatively impermeable barrier to drugs and it was not until Roeder and colleagues devised the technique of removal that it was found that the insect nervous system responded to various agents in much the same way as the mammalian system.

J.E. CASIDA: Perhaps I could also add a comment. Dr. Saito is right in saying that O'Brien and Spencer postulated that the nerve sheath was a barrier in the resistant species more than in the susceptible species. However, it is easy for the author of a paper to throw out several hypotheses more or less in passing; as you know, the more one works with how a compound acts on an insect in relation to resistance, be it natural or acquired, the more one comes to the conclusion that the effects cannot be pinned down to a single factor. I think the evidence Dr. Saito has presented for the role of the nerve sheath shows better than anything else that this is a major contributing factor.

D. F. HEATH: Your results undoubtedly indicate that differences in distribution are important, but taking the results as a whole one would conclude that the house-fly should be the most susceptible species, since house-fly anticholinesterase was found to be inhibited at very much lower concentrations than the anticholinesterase of any other species, and this would quite outweigh the other effects you observed.

This does not necessarily mean that your conclusions are wrong. The concentration of inhibitor in the nervous system as a whole can only be a very crude indicator of the concentration at the synapses. De-sheathing a locust ganglion preparation increases the sensitivity of the preparation to eserine and acetylcholine, but the ganglion is still much less sensitive than mammalian ganglia. This and other evidence suggests that the synapses are protected by additional membranes. Consequently, the concentration of inhibitor in the synaptic region cannot be assumed to be similar to the concentration in a whole nerve. It would be very useful to determine inhibitor concentrations in the synaptic regions, but I have no idea of how this could be done.

IV.

INSECT METABOLISM: TRACER APPLICATIONS

STUDIES ON THE UTILIZATION, METABOLISM AND FUNCTION OF STEROLS IN THE HOUSE-FLY, MUSCA DOMESTICA

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Abstract — Résumé — Аннотация — Resumen

STUDIES ON THE UTILIZATION, METABOLISM AND FUNCTION OF STEROLS IN THE HOUSE-FLY, MUSCA DOMESTICA. Insects generally have been found to require a dietary source of sterol for normal larval growth and metamorphosis. Our work has pointed to two additional physiological roles for sterols in the house-fly, Musca domestica L.: (1) A dietary source of sterol is essential for sustained viable egg production in the female fly; on a sterol-deficient diet eggs are produced but hatch and viability are low. (2) Cholesterol is also involved in the mobilization and utilization of nutrient reserves associated with the initiation of ovarian maturation in the female fly.

The quantitative sterol requirements for the above physiological processes and the metabolic conversions that occur during growth, metamorphosis and reproduction have been studied in this insect, using C^{14}- and H^3-labelled sterols in conjunction with a variety of analytical tools, including reverse isotope dilution, gas-liquid chromatography and spectroscopy, and employing aseptic rearing techniques and semi-defined larval and adult diets.

Both C^{14}-cholesterol and H^3-β-sitosterol have been used as a source of sterol in either the larval or the adult diet of the house fly, and the pattern of utilization and metabolism was found to be almost identical for these two sterols. However, there was no detectable conversion of β-sitosterol to cholesterol.

Sub-minimal quantities of cholesterol have also been used in the larval diet in combination with "sparing sterols" such as cholestanol, which will fulfill in part but not entirely the sterol requirement of this insect. The utilization and fate of the "sparing sterol" has been investigated using C^{14}-cholestanol, and the metabolism of the minute quantity of essential cholesterol is currently under study using high-specific-activity C^{14}-cholesterol.

Other species of insects, including the German cockroach (Blattella germanica), have been examined in relation to the patterns of utilization and the metabolic pathways for sterols found in the house-fly.

UTILISATION, MÉTABOLISME ET FONCTIONS DES STÉROLS CHEZ LA MOUCHE DOMESTIQUE (MUSCA DOMESTICA). On a déterminé que, de façon générale, les insectes ont besoin de stérols dans leur régime alimentaire pour que leurs larves se développent et se métamorphosent normalement. Les travaux de l'auteur ont fait apparaître deux autres fonctions physiologiques des stérols chez la mouche domestique (Musca domestica L.): 1. il est indispensable que le régime alimentaire de la femelle contienne des stérols pour que celle-ci ponde régulièrement des œufs viables; s'il y a carence de stérols, la femelle continue à pondre mais seul un faible pourcentage des œufs parviennent à éclosion et sont viables; 2. le cholestérol joue également un rôle dans l'utilisation des réserves nutritives dont s'accompagne le début du développement des ovaires.

On a étudié chez cet insecte les quantités de stérols qui sont nécessaires pour que les processus physiologiques ci-dessus se déroulent, ainsi que les transformations métaboliques qui se produisent au cours du développement, de la métamorphose et de la reproduction. Cette étude a été faite à l'aide de stérols marqués par ^{14}C et ^3H, en appliquant diverses méthodes analytiques: dilution isotopique inversée, chromatographie à phase gazeuse et phase liquide, spectroscopie; on a eu recours à des méthodes d'élevage en milieu aseptique et on a établi des régimes alimentaires semi-contrôlés pour les larves et les adultes.

Le cholestérol marqué par ^{14}C et le sitostérol-β marqué par ^3H ont été utilisés comme sources de stérols dans le régime alimentaire de la mouche domestique aux stades larvaire et adulte; il s'est révélé que l'utilisation et le métabolisme de ces deux stérols étaient à peu près identiques. Toutefois, on n'a pas observé de transformation du sitostérol-β en cholestérol.

Dans le régime alimentaire des larves on a également fait entrer du cholestérol en quantité inférieure aux besoins minima et on y a ajouté des «stérols pauvres» comme le cholestanol, qui ne satisfont que partiellement les besoins en stérols de l'insecte. L'utilisation et le sort du «stérol pauvre» ont été étudiés à l'aide de cholestanol marqué par ^{14}C; à l'aide de cholestérol marqué par ^{14}C, d'activité spécifique élevée, on poursuit les recherches sur le métabolisme de la microquantité de cholestérol indispensable.

On a étudié chez d'autres espèces d'insectes, notamment la blatte allemande (Blattela germanica), l'utilisation et les voies métaboliques des stérols et on les a comparées à celles qui ont été observées chez la mouche domestique.

ИЗУЧЕНИЕ УСВОЕНИЯ, МЕТАБОЛИЗМА И ФУНКЦИИ СТЕРИНОВ В ОРГАНИЗМЕ ДОМАШНЕЙ МУХИ MUSCA DOMESTICA. Было обнаружено, что большинству насекомых нужен диетический источник стеринов для нормального роста и метаморфоза личинки. Работа посвящена двум дополнительным физиологическим функциям стеринов в организме домашней мухи Musca domestica L.: 1) Источник стеринового питания совершенно необходим для образования выносливых жизнеспособных яиц у женских особей мухи; при недостаточном стериновом питании яйца образуются, но их выносливость и жизнеспособность являются низкими. 2) Холестерин также играет роль в мобилизации и использовании резервов питания при созревании яичников у самок домашних мух.

Изучалась количественная потребность в стерине при вышеуказанных физиологических процессах и метаболических превращениях, происходящих во время роста, метаморфоза и размножения у этих насекомых. Исследования проводились с помощью меченных углеродом-14 и тритием стеринов в сочетании с различными аналитическими методами, включая обратное изотопное разбавление, газово-жидкостную хроматографию, спектроскопию, и с помощью методов асептического культивирования и пищевых режимов для полуоформировавшихся личинок и взрослых особей.

Холестерин, меченный C^{14}, и β-ситостерин, меченный тритием, использовались в качестве источника стерина в рационе или личинок, или взрослых особей домашней мухи. Было обнаружено, что характер усвоения и метаболизм этих двух стеринов почти одинаковы. Однако не было поддающегося обнаружению превращения меченного тритием β-ситостерина в холестерин.

В рационе личинки использовались также субминимальные количества холестерина в сочетании с "резервными стеринами", например с холестанолом, который удовлетворял частично, но не полностью потребности этого насекомого в стерине. Усвоение и судьба "резервного стерина" исследовались при помощи холестанола, меченного углеродом-14, а в настоящее время с помощью холестерина, меченного углеродом-14, с высокой удельной активностью, изучается метаболизм малых количеств исходного холестерина.

ESTUDIOS SOBRE LA ASIMILACIÓN, EL METABOLISMO Y LA FUNCIÓN DE LOS ESTEROLES EN LA MOSCA COMÚN (MUSCA DOMESTICA). Se ha descubierto que en general los insectos necesitan consumir habitualmente esterol para que el desarrollo de las larvas y las metamorfosis se efectúen en condiciones normales. El presente trabajo se refiere a otras dos funciones fisiológicas del esterol en la mosca común (Musca domestica L.), que pueden concretarse del modo siguiente: 1) Las hembras necesitan consumir habitualmente esterol para poder producir huevos fértiles; en caso de consumo insuficiente, los huevos son de escasa fertilidad y viabilidad; 2) El colesterol interviene también en el mecanismo de utilización de las reservas nutritivas en la mosca hembra cuando se inicia la maduración ovárica.

La cantidad de esterol necesaria para el proceso fisiológico indicado y las transformaciones metabólicas que se producen durante el desarrollo, la metamorfosis y la reproducción han sido estudiadas en este insecto empleando esteroles marcados con ^{14}C y ^{3}H; al mismo tiempo se ha hecho uso de diversos procedimientos de análisis, entre ellos la dilución isotópica recíproca, la cromatografía gas-líquido y la espectroscopía, y se han utilizado procedimientos de cría en medio aséptico y dietas semidefinidas de larvas y adultos.

Como fuente de esterol en la dieta de los individuos adultos o de las larvas de mosca común, se emplearon el colesterol-^{14}C y el sitosterol-β-^{3}H, habiendo sido casi idénticos en ambos casos el proceso de asimilación y metabolismo de estos esteroles. Sin embargo, no hubo transformación apreciable de β-sitosterol en colesterol.

En las dietas de las larvas se utilizaron también cantidades de colesterol inferiores al mínimo necesario, combinadas con «esteroles de ahorro», como el colestanol, que pueden suministrar sólo una parte del esterol que necesita el insecto. La asimilación y el destino final del «esterol de ahorro» se han estudiado empleando colestanol-^{14}C; también se está estudiando el metabolismo de la minúscula cantidad de colesterol esencial, empleando para ello colesterol-^{14}C de elevada actividad específica.

Se han utilizado también otras especies de insectos, entre ellos la cucaracha alemana (Blattella germanica), para completar el estudio de los modos de asimilación y los esquemas metabólicos de los esteroles en la mosca común.

INTRODUCTION

In 1935, HOBSON [1] reported that larvae of the blowfly, Phaenicia sericata, require a dietary source of sterol for normal growth and development. Since this discovery, the immature stages of a number of insects, representing several of the major orders, have been examined as to their sterol requirement and every insect studied has been found to require a dietary or exogenous source of sterol for normal larval growth and metamorphosis [2]. This nutritional requirement for sterols has generally been considered to indicate the absence of sterol biosynthesis in insects and this has been confirmed biochemically in several species, using C^{14}-labelled sterol precursors [3-10]. However, there is some indication that certain of the more primitive insects may be capable of some degree of cholesterol biosynthesis [11, 12].

Although cholesterol has generally been used as the dietary sterol and has been found to support growth in every species studied, many omnivorous and phytophagous insects can also use certain C-28 and C-29 phytosterols in lieu of cholesterol. For certain carnivorous insects, such as the hide beetle, Dermestes maculatus, however, only cholesterol and certain closely related C-27 sterols are satisfactory sterol sources [13, 4]. Using this insect, CLARK et al. [14], in a particularly significant study, recently provided us with a working hypothesis for investigating both the fate and function(s) of sterols in insects. These workers have shown that the hide beetle will grow and mature on a larval diet containing sub-minimal quantities of cholesterol if the diet is supplemented by certain "sparing-sterols" which alone will not fulfil the sterol requirement of this insect. Based on their work, they have proposed a dual role for sterols in insects. They postulate that the "sparing-sterols", which will replace up to 97% of the cholesterol requirement of Dermestes, serve in a structural capacity, as cellular or subcellular components. The remaining irreplaceable cholesterol is believed to serve a metabolic role, perhaps as a precursor for physiologically active sterol metabolites. However, in a subsequent study on the metabolism of C^{14}-cholesterol by the cockroach, Eurycotis floridana, under "sparing" conditions, CLAYTON et al. [15] found that the minimum essential cholesterol requirements of this insect were not completely metabolized and suggested that in insects cholesterol may also have a specific structural role which cannot be fulfilled by the "sparing-sterols".

In addition to larval growth and metamorphosis, sterols have been reported to play a role in several other physiological processes. Certain phytosterols appear to act as phagostimulants for silkworm larvae, Bombyx mori, [16, 17] and a dietary source of sterol has been reported to be essential for normal reproduction in the German cockroach, Blattella germanica [18]. In the house fly, Musca domestica, we have found sterols to be involved in the initiation of ovarian development [19] and to be necessary for sustained viable egg production [20, 21]. Finally, the recent reports by KOBAYASHI et al. [22, 23] that cholesterol has "brain hormone" activity

in debrained, diapausing silkworm pupae, suggest that in insects sterols may either serve as hormones per se or as precursors to hormones.

From this brief review it is apparent that sterols are either involved in or essential to a number of the important physiological and biochemical processes of insects.

Information on the biochemical and physiological function(s) of sterols and their metabolic fate in insects is not only important for a more complete knowledge of insect physiology and biochemistry, but should also add to our general understanding of the role(s) of neutral sterols in living systems generally.

Both the minute quantities of cholesterol required, especially when used in combination with other sterols, and the small size of many insects, make it difficult to follow the uptake, utilization, metabolism, and transfer of cholesterol from one development stage to another. Radioisotope-labelled sterols, which have been extensively used in this area of research, have not only provided an extremely sensitive and efficient technique for following the above processes, but have permitted certain experiments to be carried out with ease which otherwise would have been extremely difficult, if not impossible.

The purpose of this paper is to briefly report on certain facets of our research on the function(s) and metabolic fate of sterols in the house fly, Musca domestica, and in so doing to illustrate some applications of radioactive-tracer techniques to this area of insect physiology.

MATERIALS AND METHODS

The house flies used in these studies were from a 1948 NAIDM strain. The stock colonies were reared by the CSMA procedure [24] and fed an adult diet of dry defatted milk-sucrose (1:1). Unless otherwise noted, a semi-defined adult diet [21] and an aseptic semi-defined larval diet [25] were employed in these experiments. The "vitamin-free" casein used in preparing these diets was exhaustively extracted to remove sterols. The C^{14}- and H^3-labelled sterols were repurified just before use and their radiochemical purity checked by several analytical methods. All radioassays were made on replicated "weightless" samples using windowless gas-flow proportional counters. Gas-liquid chromatographic analyses of the sterols were made using the systems of VANDENHEUVEL et al. [26].

STEROLS AND REPRODUCTION

In contrast to the numerous studies on the requirement for sterols by immature insects, our knowledge of the role of sterols in the adult insect is extremely limited. MONROE [20, 21], in a study from our laboratory, has shown that flies fed on a sterol-deficient semi-defined diet lived as long and produced approximately the same number of eggs as flies fed on the same diet containing cholesterol. However, the cholesterol deficiency caused an overall reduction of about 80% in egg hatch or viable egg production. When flies were fed on the semi-defined diet containing cholesterol, egg hatch ranged from 82 to 98% throughout all the egg collections. When cholesterol

was omitted from the diet, egg hatch was initially lower and decreased rapidly until by the fourth egg collection it was only 5%. If at this time these flies were given a diet containing cholesterol, the egg hatch was found to increase progressively until by the ninth collection it was similar to that found for the eggs of flies fed the diet plus cholesterol. In addition, only about 50% of the larvae from the eggs from flies fed on the sterol-deficient diet produced adults as compared with 91% of those from the diet containing cholesterol.

The production of some viable eggs up to about the fourth egg collection suggested a physiological mechanism whereby the female fly may use sterols stored from the larval diet for the early groups of eggs produced. The presence of such a storage mechanism was confirmed in a subsequent study [27].

The above-mentioned finding prompted us to examine the relative contribution of the stored sterols and the sterols from the adult diet to the total sterol content of the eggs. This was accomplished by using either C^{14}-labelled or unlabelled cholesterol in the larval diet and C^{14}-cholesterol in the adult diets and then examining the first groups of eggs produced for both the sterol content and the specific activity of the sterols present. Under our rearing conditions, and using optimal concentrations of cholesterol in both the larval and adult diets, we have found that as much as 75% of the sterol present in the first group of eggs produced originates from the stored reserves of the female fly. However, when flies are reared on larval diets containing combinations of "sparing sterols" and sub-minimal quantities of cholesterol, the adult females, which contain very little, if any, excess cholesterol, then must depend almost wholly on the sterols from the adult diet for egg production [28].

STEROLS OF HOUSE-FLY EGGS

From the above studies, it is apparent that the sterols present in the house-fly egg are essential for both normal embryonic development and early larval growth. These findings have prompted us to undertake a thorough study on both the amount and nature of the sterols present in the eggs and to examine any qualitative or quantitative changes that may occur in the sterols during these early developmental stages.

An earlier study [29], in which we injected C^{14}-cholesterol into adult female flies reared by the CSMA procedure, provided us with some qualitative information on the egg sterols. The injected C^{14}-cholesterol was efficiently incorporated into the eggs where it was found to be present in both the free and esterified form. A portion of the C^{14}-cholesterol in the egg and adult fly had been converted to its 5,7-diene, 7-dehydro-cholesterol. However, because of the presence in these eggs of phytosterols from the adult flies and unlabelled cholesterol from the adult diet, the total sterol content could not be studied.

Eggs from flies reared aseptically on a semi-defined larval diet containing 0.2% C^{14}-cholesterol and fed on a semi-defined adult diet containing 0.1% C^{14}-cholesterol were used for the quantitative studies. These eggs were found to contain about 0.051 μg-equivalents of radioactive sterol per egg. When the crude total lipid from these eggs was fractionated into lipid classes by column chromatography on silicic acid, two major radioactive peaks, representing sterol esters and free sterols, were detected [30]. In

these eggs the sterol esters accounted for about 41% of the total C^{14}-sterols present, but in some of our other studies we have found ester fractions as high as 60%. Analysis by ultra-violet (UV) spectroscopy of either the total lipids before fractionation or of the free sterol and sterol ester fractions indicated the presence of an appreciable amount of a 5,7-diene. For confirmation of this finding, the total sterols, as their acetates, were fractionated by chromatography on an alumina column which separates Δ^5 and $\Delta^{5,7}$ sterols. Two major radioactive peaks were detected: The major peak (Δ^5 fraction), which accounted for about 86% of the radioactive material, was identified as cholesterol by reverse-isotope dilution and purification through the dibromide and by its relative retention time in several gas-liquid chromatographic systems. The minor, more polar, peak ($\Delta^{5,7}$ fraction) contained about 13.8% of the total radioactivity and, when analysed by UV spectroscopy, was found to have a spectrum and molecular extinction coefficient, computed from the micromoles of radioactive sterol present, identical to 7-dehydrocholesterol [28]. Further confirmation of the identity of the compound has been made using gas-liquid chromatography and reverse-isotope dilution. A summary of our findings on the sterol content of house-fly eggs is presented in Table I. Although we have not determined the minimum sterol requirement for egg viability, we have found that as little as 0.015 μg total sterol per egg, or 25 to 30% of the maximum sterol content we have observed, is sufficient to support normal embryonic development and early larval growth [28].

TABLE I

STEROL CONTENT OF HOUSE-FLY EGGS

	Equivalent per egg (μg)
Total C^{14}-sterols	0.051
Free sterols	0.029
Sterol esters	0.021
Polar compounds	0.001
Cholesterol	0.043
7-Dehydro-cholesterol	0.007

We have also examined the fatty acid moiety of the sterol esters of the eggs and found these to be composed of greater than 92% mono-unsaturated C-16 and C-18 fatty acids, with C-18:1 accounting for about 76% and C-16:1 another 16% [30]. Cholesteryl oleate has recently been reported to be the major sterol ester in the cockroach, Eurycotis floridana [31].

An examination of the sterol content of newly-hatched house-fly larvae has shown that there is little, if any, detectable quantitative change in the sterol content of the eggs and newly-hatched larvae. However, about 80%

of the sterol esters present in the egg are converted to free sterols during embryonic development. Both the cholesterol and 7-dehydro-cholesterol fractions appeared to remain constant [28]. These studies are being continued using high-specific-activity C^{14}-sterols which permit a more precise measurement of both the changes and overall balance of these sterols.

"SPARING" STUDIES

The house fly, like Dermestes [14], may be reared on a larval diet containing sub-minimal quantities of cholesterol plus a "sparing sterol" [32, 33]. We have found that a number of the sterols which "spare" cholesterol in Dermestes, including cholestanol, will perform this same function in house-fly larvae. When added to an aseptic semi-defined larval diet, these sterols may replace greater than 95% of the dietary cholesterol requirement and still provide for optimal growth and development. However, from a number of studies in which we have used different "sparing sterols" and tested various techniques for making the "essential" cholesterol available to the larvae, we have found that the actual "essential" cholesterol requirement may be more of the order of 0.5% of the total sterol requirement, or less than 0.1 μg per insect.

An interesting phenomenon observed in these sparing studies is the efficient selective uptake and/or accumulation of cholesterol from the larval diet by the house fly, even when the cholesterol is present at only a fraction of the concentration of the sparing sterol (Table II). An extreme example of this phenomenon was observed in a study in which we used cholesterol in a 1:10 000 ratio to the "sparing sterol" and found the pupae to contain 80% of the cholesterol available in the larval diet [28].

TABLE II

SELECTIVE UPTAKE AND/OR ACCUMULATION OF C^{14}-CHOLESTEROL FROM THE LARVAL DIET BY HOUSE-FLY LARVAE

Sterol concentration (Dry weight)	C^{14} - sterols per insect		
	Available in diet (μg)	Present in insect (μg)	Total in insects (%)
0.2% Cholestanol + 0.01% C^{14}-cholesterol	3.00	1.72	57.3
0.2% Cholestanol + 0.0005% C^{14}-cholesterol	0.150	0.106	70.7

We have studied the fate of a sparing sterol in house-fly larvae using C^{14}-cholestanol plus sub-minimal quantities of cholesterol in an aseptic semi-defined larval diet. Pupae reared in this manner were found to contain about 14.5 μg of C^{14}-sterol per insect, nearly all of which was present as free sterol. Analysis by reverse-isotope dilution and/or gas-liquid chromatography showed that there was no conversion of cholestanol to cholesterol

and that greater than 99% of the C^{14}-sterol present in the insects represented unchanged cholestanol [33]. The house-fly then differs from two species of cockroaches, Blattella germanica and Eurycotis floridana, which convert nearly half of the cholestanol to Δ^7 -cholestenol during its utilization as a "sparing sterol" [34, 35] and which contain a high percentage of both the cholestanol and Δ^7 -cholestenol in the tissues as sterol esters [15, 36]. It would appear that this conversion of a stanol to a Δ^7 -stenol is related to the dehydrogenation of Δ^5 -sterols such as cholesterol to form their corresponding 5, 7-dienes. We have found this metabolic pathway in both the adult house-fly [29, 37] and the nymphal German cockroach [38], but it occurs at an extremely low level in house-fly larvae [28].

We are currently using C^{14}-cholesterol to study the fate of the minute "essential" cholesterol requirement of house-fly larvae. Our preliminary findings confirm those of CLAYTON et al. [15] in that greater than 95% of the C^{14}-compounds present in pupae and adults reared under sparing conditions, using C^{14}-cholesterol, represent unchanged cholesterol. However, both balance studies on the different developmental stages and the presence of C^{14}-labelled polar metabolites in the larval medium and in the insects suggest that at least a portion of the "essential" cholesterol requirement of house-fly larvae is metabolized during its use for growth and metamorphosis [28], and it is in this area that we are currently concentrating our effort.

UTILIZATION AND METABOLISM OF PHYTOSTEROLS

The literature suggests that a number of the phytophagous and omnivorous insects which utilize the C-28 and C-29 phytosterols convert these sterols to cholestane derivatives. Much of the evidence for such conversions involves either the use of natural diets [39, 40] or identifications which rely on bioassay and/or paper chromatography [41, 42, 43]. However, the use of radiotracer-labelled plant sterols in semi-defined diets and the availability of gas-liquid chromatographic systems which separate with ease the various phytosterols and cholesterol now permit a more critical investigation of this problem. Recently CLARK et al. [44], using C^{14}-labelled ergosterol, have definitely established that the German cockroach (Blattella germanica) can convert this C-28 trienol to 22-dehydro-cholesterol by saturation of the Δ^7 double bond and demethylation of the side chain. In a subsequent study using H^3 -labelled β-sitosterol and gas-liquid chromatographic analysis, we have found the German cockroach to have also available the biochemical mechanism for dealkylating the β-sitosterol side chain to form cholesterol [45].

Since the house-fly is routinely reared on the standard CSMA larval medium, which consists solely of plant material, this insect then must be able to use certain plant sterols to fulfil at least a part of its dietary sterol requirement. Adult flies reared on this medium have been reported to contain a mixture of sterols, and the major sterol(s) present was found to differ from both cholesterol and β-sitosterol in its chemical and physical properties [46, 47]. In a recent study THOMPSON et al. [48] have shown the sterols present in CSMA-reared flies to consist primarily of a mixture of campesterol (74%) and β-sitosterol (21%) and that the campesterol origi-

nates from the CSMA medium through its selective uptake and/or retention by house-fly larvae.

The presence in these insects of a mixture of phytosterols is in contrast to the reports that larvae of Musca domestica vicini convert β-sitosterol to cholesterol [42, 43]. To resolve these differences, we have used H^3-β-sitosterol to study its metabolism in house-fly larvae and adults [37, 49]. When fed in a semi-defined adult diet, the H^3-β-sitosterol was efficiently utilized for egg production. Although the major portion of the radioactive compounds in both the adult flies and eggs behaved as free sterols, as much as 30% of the H^3-sterol in the eggs was esterified. Column chromatographic analysis of the total sterols indicated the presence of both Δ^5 and $\Delta^{5,7}$ sterols. The Δ^5 sterols, which accounted for 88 to 95% of the total H^3-labelled compounds, was found to consist of greater than 90% of β-sitosterol when analysed by gas-liquid chromatography and reverse-isotope dilution. The 5, 7-diene fraction was tentatively identified as 7-dehydro-β-sitosterol using the same techniques. Similar results were obtained with pupae reared aseptically on a semi-defined larval diet containing H^3-β-sitosterol in which greater than 99% of the sterols present behaved like β-sitosterol. There was no detectable conversion of β-sitosterol to cholesterol in either the larvae, the adult fly or the eggs. This was determined by reverse-isotope dilution and purification through the dibromide and by gas-liquid chromatographic analysis with concurrent trapping of the radioactive compounds. The house-fly then uses β-sitosterol directly and as a precursor for 7-dehydro-β-sitosterol, to fulfil at least a part of its sterol requirement, without detectable conversion of this phytosterol to cholesterol.

In the course of these investigations we have observed an interesting physiological difference between adult flies reared on larval diets containing phytosterols and those reared on larval diets containing cholesterol. The adult female house-fly has generally been found to require a complete diet for ovarian maturation and to show little or no ovarian growth on a diet consisting solely of carbohydrates. When flies from our NAIDM strain were reared on the CSMA larval diet, which contains a mixture of phytosterols [48], and were held on an adult diet of sucrose and water, only 1 to 2% of these flies developed mature ovaries. However, when the flies were reared on this larval medium supplemented with cholesterol and held on sucrose, then about half the flies developed mature ovaries [19]. This ovarian maturation occurred in the absence of any mortality, an indication that it was not brought about by the females feeding on dead flies, and eggs from these flies were viable and produced larvae which developed to adults. These results have been confirmed with four other laboratory strains of house flies.

In order to eliminate the possibility that the activity was brought about by micro-organisms, these tests were repeated using a semi-defined larval diet and aseptic rearing techniques. The phytosterol β-sitosterol, which is the major sterol present in the CSMA medium [48], was used for comparison. About 50% of the flies reared on the larval diet containing various concentrations of cholesterol and held on an adult diet of sucrose and water were found to contain mature ovaries, as compared with only about 5% of flies reared on the diet containing β-sitosterol. We are currently attempting to determine whether cholesterol, as such, is responsible for this effect or

whether it serves as a precursor for a sterol metabolite which regulates gonad development.

This involvement of cholesterol in ovarian maturation and yolk deposition points to an interesting role for this sterol in the house-fly, since these physiological processes are known to be under endocrine control in a number of insects [50, 51]. Both this effect, and the minute amount of "essential" cholesterol that is required for either embryonic development or complete metamorphosis, in which there is a 400-500 fold increase in weight, strongly support the current concept that at least a portion of the sterol required by insects serves in a metabolic capacity, perhaps as a hormone or a hormone precursor [14, 22].

(Since the preparation of this paper, the moulting hormone (ecdysone) of insects has been reported to be a sterol (KARLSON, P., HOFFMEISTER, H., HOPPE, W. and HUBER, R., Ann. Chem. Liebigs 662 (1963) 1).

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DISCUSSION

K. HAGEN: Do you have any information on, or has your work shown any correlation or relationship between, choline and sterol requirements? It appears that we can get good egg production and fertility without cholesterol or sterol in the adult diet.

W.E. ROBBINS: No, we do not have such information. When it emerges, the female fly probably has from 10 to 15 μg of sterol. If we hold flies for a prolonged period and collect the eggs, we notice that the sterol content goes down. Probably at least half the sterol can be used for egg production. It might take some time to detect any cholesterol deficiency because the storage mechanism is very good, but the house-fly puts a lot of sterol into the first egg and if she runs out, you very soon get non-viable eggs. However, 13 000 μg for egg hatch and larval viability is not the minimum. You could probably go far below that. When Drosophila was studied, it was at first thought that it did not require sterol at all, but when the casein was very carefully extracted it was found that it did. In some of these Diptera, however, there may actually be enough in the protein source. It is a very active material, you know, and only a tenth of a microgram is required to carry an insect to a five- or six-hundredfold increase in mass. When we

extract it from the casein we do not refer to a sterol-free casein, but to a sterol-deficient casein, because though it contains less than one microgram per gram, it is even then not sterol-free. The sterol is firmly bound in it. The same is true of soya-bean protein. I do not know what you were using as a source of protein, but you may have been giving a little sterol along with it.

W. KLOFT: You mentioned that certain of the more primitive insects may be capable of some degree of cholesterol biosynthesis. Dr. G. H. Schmidt of Würzburg has worked out a qualitative and quantitative chemical analysis (proteins, carbohydrates and total lipoids) for the ant Formica polyctena Foerst. During the metamorphosis, which is divided into 10 stages, beginning with prenymphs and ending with young imagoes, he analysed females, workers and males. He found an increase in sterol content during metamorphosis only in workers, although they did not take up any external food. He had to conclude that in this differentiated social hymenopterum workers may have regained the possibility of biosynthesizing sterols, which appears to have been lost in the course of evolution to higher insects. We have no knowledge of the existence of symbiotic bacteria. They seem to have been lost, but I myself am not yet certain whether this is true. Your special tracer techniques may be able to throw light on this problem.

W.E. ROBBINS: That is a very interesting observation. However, as you are probably aware, the subject of cholesterol biosynthesis in primitive insects needs considerably more study. The use of radioactive precursors, I agree, should be very helpful in resolving your problem, but care should be taken to avoid contamination due to the biosynthesis of sterols by symbionts or intestinal micro-organisms. Axenic culture methods, antibiotics or the injection of labelled precursors should be employed.

In another series of experiments on the biosynthesis of cholesterol, we took another species of Thysanura and by-passed the intestinal tract by injection. In our preliminary studies we found no incorporation of injected C^{14}-acetate into the sterols. We did, however, obtain good incorporation into the total lipids.

J. HALBERSTADT: Was gas-liquid chromatography (GLC) used as a separation or isolation tool for the reverse isotope dilution analysis?

W.E. ROBBINS: Most of the gas-liquid chromatographic columns for sterols are low capacity, in the microgram to fraction-of-a-milligram range, because of the low percentages of liquid phase used. Because of this, adsorption chromatography is usually used for separation and isolation. However we do analyse our radioactive samples by GLC with concurrent trapping of the radioactive compounds.

J. HALBERSTADT: Is there any indication as to what the mechanisms might be that cause the efficient selection and/or accumulation of cholesterol as observed in sparing studies?

W.E. ROBBINS: We have not investigated this phenomenon as yet but plan to do so in the very near future.

TYROSINE METABOLISM IN THE BLOWFLY, CALLIPHORA ERYTHROCEPHALA

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Abstract — Résumé — Аннотация — Resumen

TYROSINE METABOLISM IN THE BLOWFLY, CALLIPHORA ERYTHROCEPHALA. Sclerotization, i. e. the transformation of the soft, white larval cuticle into the hardened, dark puparium, is due to interaction of o-quinones with the cuticle proteins. Using radioactively labelled amino-acids, it has been shown that N-acetyldopamine is the immediate precursor of the sclerotizing quinones in Calliphora erythrocephala. The intermediate steps in the biosynthesis of N-acetyldopamine are hydroxylation of tyrosine to dopa, decarboxylation of dopa to dopamine and N-acetylation of dopamine to N-acetyldopamine. This metabolic pathway of tyrosine is followed only in the final-instar larvae; early third-instar larvae catabolize tyrosine by transamination to p-hydroxyphenylpyruvic acid and reduction to p-hydroxyphenyllactic and -propionic acid. The metabolic shift from transamination to hydroxylation and decarboxylation is brought about by the hormone of the prothoracic gland, ecdysone, and can be inhibited by ligation or destruction of the ring gland.

Injection of ecdysone into the ligated animals leads within 10-14 h to activation of the dopadecarboxylase, presumably as the result of biosynthesis of enzyme protein. The action of the hormone is not a direct one on the biosynthetic mechanism, but indirect, the first action of the hormone being on the chromosomes (puffing phenomenon). The working hypothesis is that the hormone interacting with the genetic material leads to stimulation of the synthesis of specific (messenger-) RNA which is transferred to the cytoplasm and produces enzyme proteins.

MÉTABOLISME DE LA TYROSINE DANS LA CALLIPHORA ERYTHROCEPHALA. L'induration, c'est-à-dire la transformation de la cuticule larvaire, douce et blanche, en un puparium dur et sombre, est due à l'interaction d'ortho-quinones avec les protéines de la cuticule. Grâce à l'emploi d'acides aminés marqués au moyen de substances radioactives, on a montré que la N-acétyl-dopamine est le précurseur immédiat des quinones produisant l'induration dans la Calliphora erythrocephala. Les stades intermédiaires de la biosynthèse de la N-acétyl-dopamine sont l'hydroxylation de la tyrosine, qui se transforme en dioxyphénylalamine (dopa), la décarboxylation de la dopa en dopamine et la N-acétylation de la dopamine en N-acétyl-dopamine. Ce processus métabolique de la tyrosine ne se produit qu'au dernier stade de l'état larvaire; les trois premiers stades produisent le catabolisme de la tyrosine par transamination en acide p-hydroxyphényl-pyruvique et réduction en acide p-hydroxyphényl-lactique et propionique. Le processus métabolique de la transamination à l'hydroxylation est provoqué par l'hormone de la glande prothoracique, l'ecdyson, et peut être rendu impossible par la ligature ou l'ablation de la glande annulaire.

L'injection d'ecdyson aux animaux ligaturés entraîne, au bout de 10-14 h, l'activation de la décarboxylase de la dopa, due vraisemblablement à la bioxynthèse de la protéine de l'enzyme. L'action de l'hormone sur le mécanisme de biosynthèse est indirecte, le premier effet de l'hormone étant d'agir sur les chromosomes (phénomène de gonflage). L'hypothèse de travail est que l'hormone qui entre en interaction avec la matière génétique a pour effet de stimuler la synthèse de l'ARN spécifique (vecteur) qui est transmis au cytoplasme et produit les protéines d'enzymes.

МЕТАБОЛИЗМ ТИРОЗИНА У МЯСНОЙ МУХИ, CALLIPHORA ERYTHROCEPHALA. Склеротизация, т.е. превращение мягкой белой кутикулы личинки в затвердевший темный пупарий, происходит благодаря взаимодействию ортохинонов с белками кутикулы. С помощью меченных изотопами аминокислот было показано, что N-ацетилдопамин является ближайшим предшественником склеротизирующих хинонов у Calliphora erythrocephala. Промежуточными стадиями в биосинтезе N-ацетилдопамина являются гидроксилирование тирозина в допа, декарбоксилирование допа до допамина и N-ацетилирование допамина до N-ацетилдопамина. Этот метаболический процесс превращения тирозина происходит только у личинок на последней стадии развития. У личинок в ранней фазе третьей стадии происходит катаболизация ти-

розина путем переаминирования в пара-оксифенилпировиноградную кислоту и восстановление в пара-оксифенилмолочную пара-оксифенилпропионовую кислоту. Метаболическое смещение от переаминирования до гидроксилизации и декарбоксилизации вызывается гормоном переднегрудной железы, экдизоном, и может задерживаться наложением лигатуры и разрушением кольцевой железы.

Введение экдизона животным с наложенной лигатурой приводит в течение 10 - 14 часов к активации допадекарбоксилазы, вероятно в результате биосинтеза ферментативного белка. Гормон оказывает не прямое, а косвенное действие на механизм биосинтеза и, прежде всего, на хромосомы (явление отечности). Рабочая гипотеза заключается в том, что гормон, взаимодействуя с генетическим материалом, приводит к стимулированию синтеза особой (переносной) РНК, которая переносится в клеточную протоплазму и производит ферментативные белки.

METABOLISMO DE LA TIROSINA EN LA MOSCA CALLIPHORA ERYTHROCEPHALA. La esclerotización (transformación de la cutícula blanda y blanca de la larva en la cubierta dura y oscura de la ninfa) se debe a la acción recíproca de o-quinonas con las proteínas de la cutícula. Empleando aminoácidos marcados se ha comprobado que la N-acetildopamina es el precursor inmediato de las quinonas esclerotizantes en la Calliphora erythrocephala. Las etapas intermedias en la biosíntesis de la N-acetildopamina son la hidroxilación de la tirosina que da dioxifenil-alanina, la descarboxilación de ésta que da dopamina, y la N-acetilación de la dopamina que da la N-acetildopamina. Este proceso metabólico de la tirosina se produce solamente en las larvas del último estadio; al principio del tercer estadio las larvas catabolizan tirosina por transaminación, dando ácido p-hidroxifenilpirúvico, y por reducción, dando los ácidos p-hidroxifenillácticco y p-hidroxifenil propiónico. Esta transición metabólica desde la transaminación a la hidroxilación y descarboxilación se debe a la hormona de la glándula protorácica (ecdyson) y se puede inhibir ligando o destruyendo la glándula anular.

Si se inyecta ecdyson en los animales ligados se produce al cabo de 10 a 14 h una activación de la dopadecarboxilasa, debida probablemente a la biosíntesis de proteínas enzimáticas. La acción de la hormona sobre el mecanismo de biosíntesis no es directa sino indirecta, dejándose sentir sus primeros efectos sobre los cromosomas (fenómeno de turgencia). La hipótesis de que se partió es que la hormona que actúa sobre las sustancias genéticas y que sufre la acción de éstas estimula la síntesis de un ARN específico («vehículo») que se traslada al citoplasma y produce proteínas enzimáticas.

1. TYROSINE AND SCLEROTIZATION

Interest in tyrosine metabolism in insects arose mainly in connection with sclerotization, i.e. the hardening and darkening of the larval cuticle during pupation [1]. This process is brought about by the interaction of o-quinones with the cuticular proteins [2]. These quinones arise from the respective o-diphenols which are oxidized in the cuticle by the polyphenol-oxidase present there. Many such phenolic substances, particularly acids, were extracted from cuticles of different insects and their structural relationship to tyrosine pointed to this amino-acid as the precursor of the phenols. A more direct indication of the involvement of tyrosine in the sclerotization process was its high rate of incorporation in the cuticle of pupating larvae, whereas other amino-acids, for example leucine, were hardly incorporated at all [3].

2. TYROSINE METABOLISM IN LATE III-INSTAR LARVAE

We injected generally labelled C^{14}-tyrosine into final-instar Calliphora larvae and looked for radioactive metabolites that might be potential sclerotizing agents on paper chromatograms. The main metabolite was identified as N-acetyldopamine [4]. Its role in sclerotization was established

by the high incorporation of injected 2'C^{14}-labelled N-acetyldopamine in the cuticle of pupating larvae and also by following the in vivo concentration of this metabolite during larval development [5]. The concentration is low in early III-instar larvae, rises in the late III-instar larvae and disappears during pupation.

Using larval homogenates as the enzyme source, we studied the biosynthesis of N-acetyldopamine [6]. The first reaction is the hydroxylation of tyrosine to DOPA. This hydroxylation is brought about by a particle-bound enzyme system whose action is potentiated by the presence of H-donors, such as DPNH or ascorbic acid. The next step is the decarboxylation of DOPA to dopamine by a pyridoxal-phosphate-dependent decarboxylase in the epidermis cells. Dopamine is further acetylated by a transacetylase system and thus protected from ring closure, which is so common for this class of compounds [7]. Most of the N-acetyldopamine thus formed will be used for puparium formation, while a small part is being stored up as an o-glucoside which plays a role later on in the eclosion of the imago.

3. TYROSINE METABOLISM IN EARLY III-INSTAR LARVAE

The injection of tyrosine into early III-instar larvae showed a different "spectrum" of metabolites: phenol carbonic acids (such as p-hydroxyphenylpropionic acid) were isolated [6]. The incubation of tyrosine with Calliphora homogenates also gave p-hydroxyphenylpropionic acid as a main product. From the incubation mixtures p-hydroxyphenylpyruvic acid and p-hydroxyphenyllactic acid were isolated so that the pathway is tyrosine → p-hydroxyphenylpyruvic acid → p-hydroxyphenyllactic acid → p-hydroxyphenylpropionic acid. The first reaction was studied in greater detail and was shown to be a transamination reaction with α-ketoglutarate as amino-acceptor and pyridoxal phosphate as coenzyme. In Fig. 1 we have summarized the metabolism of tyrosine in early and late III-instar Calliphora larvae.

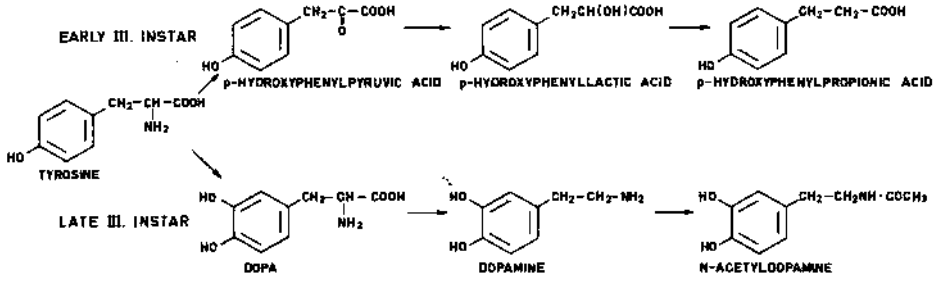


Fig. 1

Tyrosine metabolism in early and late III-instar Calliphora larvae (from KARLSON and SEKERIS [5]).

4. CONTROL OF TYROSINE METABOLISM BY ECDYSONE

The metabolic shift from transamination to hydroxylation and decarboxylation coincides with the time of secretion of the hormone ecdysone. Ecdy-

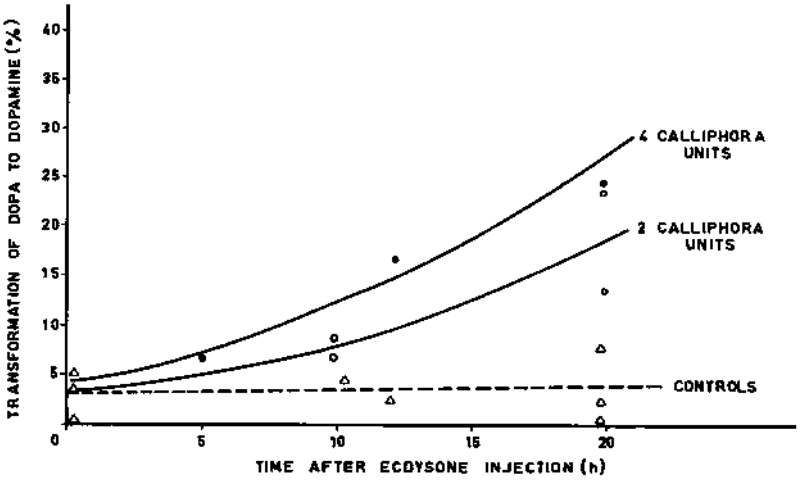


Fig. 2

DOPA decarboxylase activity in relation to time after injection of ecdysone
– – Δ – – Δ – – ligated control larvae
— o — o — injected with 2 Calliphora Units
— • — • — injected with 4 Calliphora Units
(from KARLSON and SEKERIS [9]).

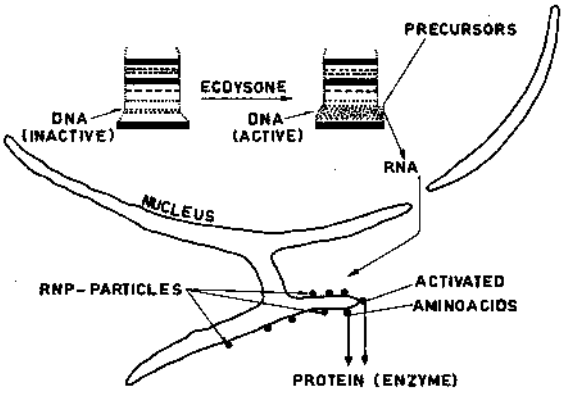
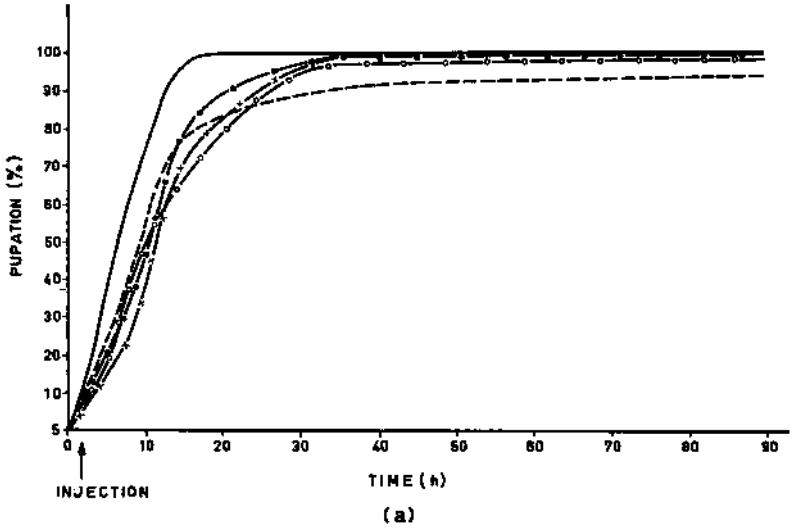


Fig. 3

Schematic representation of the mode of action of hormones
The primary action of the hormone is the activation of gene loci (DNA) which leads to formation of nuclear (messenger) RNA from nucleic acid precursors. The RNA formed enters the cytoplasm and attaches itself to the ribosomal surface, thus serving as a template for specific protein (enzyme) synthesis.
(from KARLSON [12]).



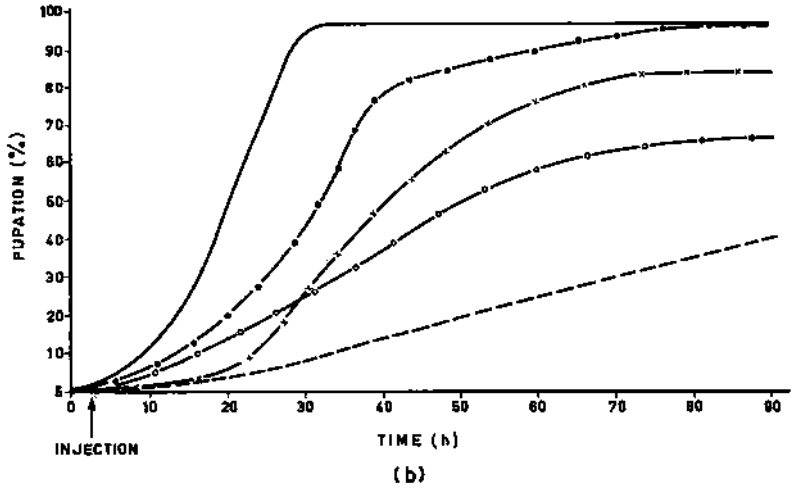


Fig. 4

Influence of antimetabolites on the pupation rate of Calliphora larvae
Groups of 40 larvae were injected with 4 different antimetabolites (concentration in brackets) and their pupation rate was followed in comparison with that of untreated larvae.

——— untreated larvae
—o—o— larvae injected with streptomycin (300 µg)
—x—x— larvae injected with puromycin (6 µg)
- - - - - - larvae injected with actinomycin (0.02 µg)
—•—•— larvae injected with mitomycin (0.1 µg)

In Fig. 4(a) the substances were injected during the last day of larval life, in Fig. 4(b) 1-2 days before pupation.

sone, the product of the prothoracic or ring gland, induces a series of changes which lead to pupation [8]. It is natural to conclude that the shift in the metabolism of tyrosine is related in some way with the secretion of the hormone. Ligation of late III-instar larvae prevents pupation; the cuticle remains white and soft, thus retaining its larval character. The concentration of the sclerotizing agent N-acetyldopamine in the ligated animals remains low in comparison with the normal larvae, which points to an impaired synthesis. We studied the activity of the enzymes involved in the biosynthesis of N-acetyldopamine in these animals and found that the activity of the hydroxylase and decarboxylase remained low [9]. The injection of ecdysone leads to activation of the enzymes, as seen in Fig. 2 with reference to decarboxylase. This activation is dose-dependent and cannot be clearly seen until 8-10 h after injection of the hormone, i.e. the effect is a late one.

5. NATURE OF ECDYSONE ACTION

This induction of enzyme activity by ecdysone brings up the question of the nature of the activation: does the hormone activate a pre-existing inactive proenzyme, is it a de novo synthesis of enzyme protein, an action on a coenzyme, etc.? A new approach to this problem has been opened up by the work of CLEVER and KARLSON [10]. Injection of ecdysone into final-instar Chironomus larvae leads within 30 min to modification of the structure of the salivary gland chromosomes. These morphologically modified gene loci, known as "puffs", are active centres of RNA formation. This was shown by autoradiography of salivary gland cells of Chironomus after injecting H^3-uridine or H^3-cytidine [11]. Recalling the role of nuclear RNA in protein synthesis, we can picture the action of the hormone indirectly on protein synthesis through activation of DNA → messenger RNA synthesis → protein (enzyme) synthesis (Fig. 3) [12].

We have indirect evidence that de novo synthesis of enzyme protein takes place. Injection into Calliphora of substances which inhibit protein formation (such as puromycin or streptomycin) leads to delayed pupation and also to inhibition of decarboxylase induction. No significant inhibition can be seen if the substances are given in the last 10-15 h of larval life, while maximum effectiveness is observed if they are injected 24-48 h before pupation, at the time of hormone secretion when the activation of the biosynthetic chain begins (see Fig. 4). Similar action is exhibited by some DNA and RNA inhibitors (such as actinomycin, mitomycin and 5-F-deoxyuridine), which fits in with the hormone action mechanism proposed.

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DISCUSSION

R. von BORSTEL: The conclusions from the work of Clever and Karlson must be approached with caution, since recent work by H. Kroeger in Zürich indicates that the same "puff" that can be activated by ecdysone can also be activated by several different narcotics as well as butanol. Even though the ecdysone activation of the puff occurs at extremely low concentrations of the compound, the effect may still be a secondary one.

C. E. SEKERIS: Ecdysone induces the puffing phenomenon in concentrations of about 0.01 μg, that is with minute amounts of substance, which points to the specific nature of the effect. There is a possibility that ecdysone does not act directly on the puff but indirectly by combining with a repressor (according to the theory of Jacob et al). This still remains to be proved.

S. FUZEAU-BRAESCH: Do you think that N-acetyldopamine is responsible for hardening and pigmentation or just for one of these? Can you give reasons?

C. E. SEKERIS: In Calliphora sclerotization and pigmentation cannot be separated. We believe that N-acetyldopamine is the sclerotizing agent in Calliphora and that, during the quinone tanning, it also gives the dark colour to the puparium. Of course this is not true of all insects and your paper has a bearing on this problem.

ÉTUDE DE LA PIGMENTATION TÉGUMENTAIRE DES INSECTES A L'AIDE DE RADIOÉLÉMENTS

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Abstract — Résumé — Аннотация — Resumen

RADIOISOTOPE STUDY OF TEGUMENTARY PIGMENTATION IN INSECTS. The nature of insect cuticle, which is made up in large part of scleroproteins, calls for the use of labelled isotopes to obtain answers to certain questions regarding pigmentation.

The following method, which has the advantage of being quick and easy to apply, has been developed.

The labelled substance chosen is injected into the animal at various phases of its skin-shedding cycle: before secretion of the cuticular proteins, i. e. when the cuticle is at rest; at the time these proteins are deposited; and, lastly, at the time their sclerification begins.

After a suitable interval the cuticle is removed, suitably treated, and subjected to full autoradiography. Photographic comparison of the results then indicates whether or not the substance chosen has been used for any formation of pigment, due account being taken of prior chemical processes involving the substance.

The findings presented in the paper relate to three labelled substances: two carbon-14 amino-acids - tyrosine and tryptophane - and inorganic sulphur-35 in the form of sodium sulphate. It has thus been possible to give direct proof of the origin of variously-coloured cuticular pigments and to discuss the role of tryptophane and sulphur in forming pigments in insect integument.

All cuticular pigments spring from the metabolism of tyrosine, thus confirming the term "melanic" hitherto applied to them without direct proof. Tryptophane, exceptionally integrated in the cuticle, is the substratum of the ommochromic red and black pigments in the hypodermis. Inorganic sulphur plays no regular specific role in the formation of cuticular pigments, contrary to what has been suggested by various hypotheses on the role of the sulphydryl group.

From the standpoint of comparative biochemistry, the melanins appear to be purely cuticular among insects, granular melanins being confined to the vertebrates. Dark hypodermic granules in insects are ommochromic, derived from tryptophane, and not melanic.

ÉTUDE DE LA PIGMENTATION TÉGUMENTAIRE DES INSECTES A L'AIDE DE RADIOÉLÉMENTS. En raison de la nature de la cuticule des insectes - constituée en grande partie de scléroprotéines - il est nécessaire, pour répondre à certaines questions relatives à la pigmentation, d'utiliser des éléments marqués.

La méthode suivante a été mise au point; elle présente l'avantage de la rapidité et de la facilité.

L'élément choisi est injecté à l'animal à différents moments de son cycle de mue: avant la sécrétion des protéines cuticulaires, la cuticule étant alors au repos; au moment du dépôt de ces protéines; à l'instant où commence le processus de sclérification de ces protéines.

Après un laps de temps adéquate la cuticule est prélevée, traitée de façon appropriée et autoradiographiée in toto. La comparaison photographique des résultats permet de conclure à l'utilisation ou non de l'élément choisi dans telle formation pigmentaire, compte tenu des processus chimiques antérieurs relatifs à l'élément.

Les résultats présentés concernent trois éléments marqués: deux acides aminés - tyrosine et tryptophane - au carbone-14, du soufre-35 inorganique sous forme de sulfate de sodium. On a pu ainsi démontrer directement l'origine des pigments cuticulaires de différentes couleurs et discuter le rôle du tryptophane et du soufre dans les formations pigmentaires du tégument des insectes.

Les pigments cuticulaires sont tous issus du métabolisme de la tyrosine, confirmant le terme de «mélaniques» qui leur était jusqu'à présent attribué sans preuve directe. Le tryptophane, exceptionnellement intégré dans la cuticule, constitue le substrat des pigments noirs et rouges ommochromiques de l'hypoderme. Le soufre inorganique ne joue pas de rôle spécifique régulier dans la formation des pigments cuticulaires, contrairement à ce que laissaient supposer différentes hypothèses sur le rôle des groupements sulfhydriles.

Du point de vue de la biochimie comparée, les mélanines semblent être chez les insectes exclusivement cuticulaires, les granulaires étant réservées aux vertébrés. Quant aux granules hypodermiques sombres, ils sont, chez les insectes, de nature ommochromique, dérivés du tryptophane et non mélaniques.

ИЗУЧЕНИЕ ПИГМЕНТАЦИИ ОБОЛОЧКИ НАСЕКОМЫХ С ПОМОЩЬЮ РАДИОЭЛЕМЕНТОВ. В связи с характером кутикулы насекомых, состоящей в значительной части из склеропротеина, возникает необходимость в использовании меченых элементов для изучения некоторых процессов пигментации.

Был использован следующий быстрый и легкий метод исследования. Определенный элемент вводится животному в различные периоды линьки: до выделения кутикулярных протеинов, когда кутикула находится в стадии покоя, в момент отложения этих протеинов и, наконец, в момент, когда начинается процесс склеротизации этих протеинов.

Через определенный промежуток времени кутикула снимается, обрабатывается соответствующим образом и в целом подвергается авторадиографии. Фотографическое сравнение полученных результатов позволяет прийти к выводу относительно использования или неиспользования данного элемента в образовании пигмента с учетом предыдущих химических процессов, касающихся элемента.

Представленные здесь результаты относятся к трем меченым элементам: двум аминокислотам - тирозину и триптофану, меченным углеродом-14, и неорганической сере-35 в виде сульфата натрия. Таким образом представилось возможным наглядно показать происхождение кожных пигментов различных цветов и обсудить роль триптофана и серы в образовании пигмента оболочки насекомых.

Кутикулярные пигменты образуются в результате метаболизма тирозина, подтверждая тем самым термин "меланиновые", который был им до сих пор приписан без прямого доказательства. Триптофан, который входит только в состав кутикулы, представляет собой оммохромный субстрат черных и красных пигментов гиподермы. Неорганическая сера не играет определенной специфической роли в образовании кутикулярных пигментов вопреки различным гипотезам о роли сероводородных соединений.

С точки зрения сравнительной биохимии меланины присутствуют, по-видимому, только у кутикулярных насекомых, а зернистость свойственна позвоночным. Что касается темных гиподермических гранул, то у насекомых они носят оммохромный характер, лишены триптофана и не относятся к меланинам.

ESTUDIO DE LA PIGMENTACIÓN TEGUMENTARIA DE LOS INSECTOS CON AYUDA DE RADIOELEMENTOS. La naturaleza de la cutícula de los insectos -constituida en gran parte por escleroproteínas- obliga a utilizar elementos marcados para resolver ciertas cuestiones relativas a la pigmentación.

Se ha preparado el método siguiente, que ofrece la ventaja de su rapidez y simplicidad.

El elemento elegido se inyecta en el animal en diferentes momentos de su ciclo de muda: antes de la secreción de las proteínas cuticulares, cuando la cutícula se encuentra en reposo; en el momento de depositarse estas proteínas, y, por último, en el instante en que comienza el proceso de esclerificación de dichas proteínas.

Después de un lapso de tiempo adecuado se retira la cutícula, se somete a un tratamiento apropiado y se autorradiografía in toto. La comparación fotográfica de los resultados permite determinar si el elemento elegido ha sido utilizado o no en la formación pigmentaria, teniendo en cuenta los procesos químicos anteriores que afectan a dicho elemento.

Los resultados presentados en la memoria se refieren a tres elementos marcados: dos aminoácidos -tirosina y triptófano- marcados con carbono-14, y azufre-35 inorgánico, en forma de sulfato de sodio. Con este procedimiento se ha podido demostrar directamente el origen de los pigmentos cuticulares de diferentes colores y analizar la función del triptófano y del azufre en las formaciones pigmentarias del tegumento de los insectos.

Los pigmentos cuticulares provienen todos del metabolismo de la tirosina, lo que confirma el término de «melánicos» que hasta ahora se les atribuía sin pruebas directas. El triptófano, excepcionalmente integrado en la cutícula, constituye el substrato de los pigmentos negros y rojos homocrómicos de la hipodermis. El azufre inorgánico no desempeña una función específica regular en la formación de los pigmentos cuticulares, contrariamente a lo que permitían suponer diferentes hipótesis sobre la función de los grupos sulfhidrilos.

Desde el punto de vista de la bioquímica comparada, las melaninas parecen ser exclusivamente cuticulares en los insectos, mientras que son granulares en los vertebrados. En cuanto a los gránulos hipodérmicos oscuros, en los insectos son de naturaleza homocrómica, derivados del triptófano, y no melánica.

Un certain nombre d'éléments radioactifs sont utilisés dans les marquages d'insectes. Il est intéressant de connaître le sort réservé à ces

éléments en réalisant la détection non globalement mais, pour la partie externe, par autoradiographie. Etant donné le cycle de mue fondamental de l'insecte et son métabolisme particulier en rapport avec la formation de la nouvelle cuticule, on doit distinguer des étapes et rechercher les variations dans l'incorporation des substances au cours de ce cycle. Une telle étude est réalisée ici pour le soufre à l'occasion d'une analyse du tégument dont les formations pigmentaires étudiées à l'aide de tyrosine et tryptophane radioactifs vont servir de base de comparaison.

MÉTHODE

La méthode utilisée d'autoradiographie in toto présente l'avantage de la rapidité et de la facilité. Si elle ne fournit pas de données quantitatives précises, elle permet d'atteindre par contre un niveau que l'analyse chimique ne peut approcher: celui des différences dans la structure fine, pigmentaire ou morphologique de la cuticule.

L'élément choisi est injecté à l'insecte dans la cavité générale, dissout dans du liquide de Ringer pour insecte, en quantité variant de 10 à 20 μl représentant une activité totale voisine du microcurie. L'animal est endormi au gaz carbonique et l'injection effectuée dans l'abdomen, à travers une membrane intersegmentaire, à l'aide d'une micropipette de verre.

Les injections sont réalisées à des moments variés du cycle de mue:

a) avant la sécrétion des protéines cuticulaires, lorsque la cuticule est au repos,
b) au moment du dépôt de ces protéines avant la mue,
c) à l'instant ou commence le processus de sclérification et de pigmentation de ces protéines.

Après un laps de temps adéquat, la cuticule est prélevée, nettoyée à la loupe binoculaire, traitée rapidement de façon appropriée par une solution de potasse bouillante qui élimine toute trace de tissus sous-jacents (hypoderme), puis lavée, séchée, et autoradiographiée sur film spécial «Radio dentaire KODAK». L'autoradiogramme donne l'image des régions radioactives, que l'on peut comparer avec la cuticule elle-même, ou avec une photographie de celle-ci lorsqu'il s'agit d'étudier de près les dessins pigmentaires. Un exemple des résultats obtenus est présenté en figure 1.

Les substances suivantes ont été utilisées:

1. 1 - Tyrosine, marquée uniformément au ^{14}C (Amersham, Radiochemical Centre).
2. Tryptophane, marqué au ^{14}C, premier atome de la chaîne (Amersham, Radiochemical Centre).
3. Sulfate de sodium marqué au ^{35}S (C.E.A.)
4. dl - méthionine marqué au ^{35}S (C.E.A.)
5. dl - cystine marqué au ^{35}S (C.E.A.)

Les animaux utilisés sont des insectes hétérométaboles: Gryllus bimaculatus de Geer (gryllidae, orthoptères), (substances 1- 5), et Locusta migratoria (acrididae) (substance 1).

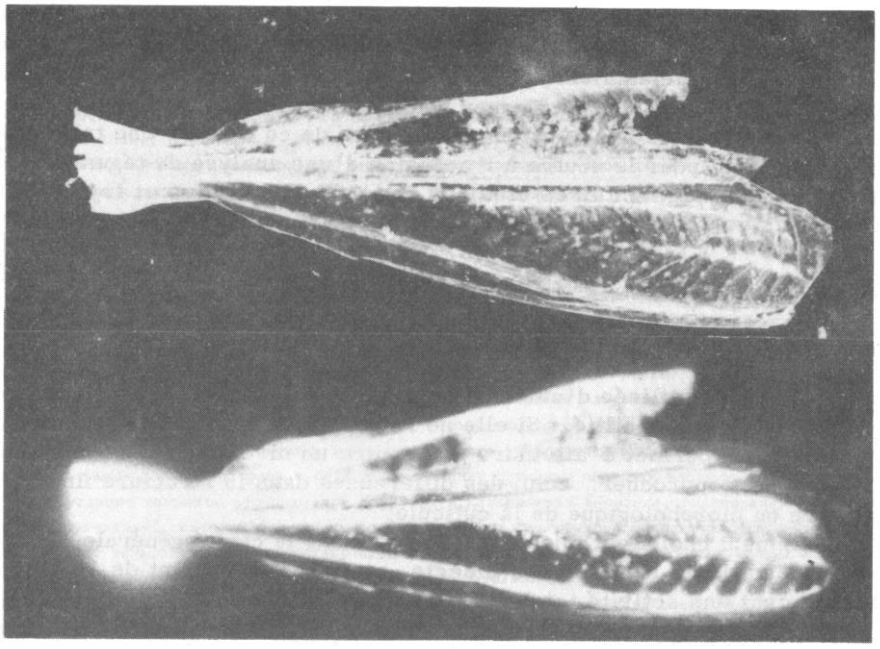

Figure 1

Exemple d'étude chromatographique de la cuticule: incorporation de tyrosine radioactive post-éxuviale dans le fémur postérieur de Locusta migratoria.
A gauche: image des deux faces du fémur; en blanc, les régions pigmentées.
A droite: autoradiogramme correspondant.

FORMATION DE LA CUTICULE ET PIGMENTATION

Il est bon de rappeler ici l'essentiel des processus de formation de la cuticule chez les insectes.

Avant la mue et sous l'ancienne cuticule se constitue une couche de protéines sécrétée par les cellules de l'hypoderme sous-jacent; ce sont elles qui deviendront la partie solide de la future cuticule. Molles et incolores avant la mue, aussitôt après le rejet de l'ancienne cuticule ces protéines sont durcies, sclérifiées selon un processus complexe de «tannage» par des substances quinoniques, en même temps qu'elles deviennent pigmentées.

Une étude de la cuticule générale de l'ensemble du corps a été effectuée; parallèlement à celle-ci une observation plus détaillée a été réalisée dans une région intéressante à cause du décalage chronologique de sa formation: il s'agit de deux processus latéraux du condyle de l'articulation fémur-postérieur/tibia, petites zones en forme de croissant renforçant l'articulation en rapport avec la fonction saltatrice de la patte, qui durcissent et se pigmentent avant la mue. Enfin, une attention spéciale a également été accordée aux grosses épines du tibia postérieur.

Il faut dès le départ distinguer l'incorporation des substances:

1° dans les protéines cuticulaires mises en place avant la mue (injection: un ou deux jours avant la mue, - prélèvement à l'exuviation ou après la mue, à condition de tenir compte éventuellement de l'activité propre à la période qui suit la mue);

2° après la mue au moment du durcissement et de la pigmentation (injection: à la mue, - prélèvement après durcissement: un jour).

3° dans la cuticule au repos (injection entre les deux mues plus de deux jours après la mue précédente et au moins trois jours avant la mue suivante, prélèvement un ou deux jours après).

Le tableau I schématise les résultats obtenus.

1. Tyrosine et tryptophane dans la cuticule

La tyrosine est incorporée dans les protéines cuticulaires d'une façon uniforme. Par contre, après la mue, elle l'est uniquement dans les zones pigmentées, quelle que soit la couleur du pigment. Les autoradiogrammes montrent une concordance parfaite entre les régions radioactives et les zones pigmentées de la cuticule, tandis que les zones durcies sans pigmentation sont dénuées de radioactivité quelle que soit l'espèce considérée, les animaux ayant vécus groupés ou isolés (fig. 1). La fraction pré-exuviale de la tyrosine est protéinogène, tandis que la fraction post-exuviale est pigmentogène.

Les zones non pigmentées de la cuticule durcissent apparemment autant que les zones pigmentées, bien qu'elles n'intègrent pas de tyrosine après la mue. Le processus chimique de ce durcissement n'est pas établi. On voit donc que la fraction post-exuviale pigmentogène de la tyrosine ne peut être mise en évidence que par la comparaison des autoradiogrammes pré- et post-exuviaux et doit passer inaperçue à l'analyse chimique globale. C'est peut-être la raison pour laquelle KARLSON et coll. [2] n'ait pas trouvé de différences très nettes entre les insectes mélanisés, Schistocerca gregaria et ceux d'une souche «albino» dépigmentés de la même espèce, tandis qu'ici la concordance est toujours parfaite entre tyrosine et mélanine lorsque l'étendue de cette dernière varie, par exemple sous l'influence du groupement.

Dans le processus du condyle, l'incorporation se fait uniquement et fortement avant la mue, attestant bien de l'achèvement précoce inhabituel de cette zone. Les épines du tibia ne présentent pas de caractères distinctifs.

Le tryptophane est lui aussi intégré dans les protéines cuticulaires; il joue, par contre, fort peu de rôle dans les processus qui se déroulent après la mue et n'est pas en rapport évident avec la pigmentation.

Dans la cuticule au repos, aucun des deux acides aminés n'est incorporé.

Par contre le tryptophane est fortement intégré aux granules d'ommochromes, pigments hypodermiques sous-jacents, dont il est la substance-mère, ce qui permet de distinguer parfaitement ces granules des granules mélaniques issus de la tyrosine tels qu'on les connaît chez les vertébrés, et qui jusqu'à présent n'ont pas été trouvés chez les insectes. Chez ces derniers les pigments polymérisés issus de la tyrosine semblent bien être

TABLEAU I

RADIOACTIVITÉ DE LA CUTICULE APRÈS INJECTIONS DE DIFFÉRENTES SUBSTANCES RADIOACTIVES EN FONCTION DU CYCLE DE MUE

Substance radioactive injectée	Radioactivité pré-exuviale des protéines cuticulaires (Injections avant la mue)			Radioactivité post-exuviale de la cuticule (Injections à la mue)			Radioactivité de la cuticule au repos (Injections entre deux mues)
	Cuticule générale	Processus du condyle	Epines tibiales	Cuticule générale	Processus du condyle	Epines tibiales	
Tyrosine (^{14}C)	+++	++++	++	++++ (Pigments)	0	+	0
Tryptophane (^{14}C)	++	+	+	+ 0	0	+ 0	0
Sulfate de sodium (^{35}S)	+	0	+	++	+	+++	+
Méthionine (^{35}S)	++++	++	+	++	++	+	+
Cystine (^{35}S)	+	++	+	++	++	+	+

Détection effectuée par autoradiographie: nombre de croix (+) = évaluation visuelle approximative des intensités.

l'unique apanage de la cuticule au sein de laquelle ils se trouvent à l'état diffus et non granulaire [1].

2. Analyse comparée du rôle du soufre dans la cuticule

1° Soufre inorganique

Il apparaît que le soufre inorganique sous forme de sulfate de sodium est uniformément incorporé à la cuticule, avant et après la mue. Seuls, les processus du condyle ne sont par radioactifs avant la mue, tandis que les épines tibiales le sont au contraire très fortement après la mue, et même en période de repos de la cuticule.

2° Soufre organique

La cuticule devient là aussi uniformément radioactive. Il est remarquable cependant que la méthionine provoque une radioactivité extrêmement forte lorsque l'injection est faite avant la mue, beaucoup plus intense que celle que l'on obtient dans les mêmes circonstances avec la cystine. Les deux substances sont - contrairement au soufre inorganique - présentes dans le processus du condyle avant comme après la mue. Les épines tibiales ne présentent pas ici d'activité particulièrement intense.

Il ressort de ces résultats que le soufre est souvent incorporé dans la cuticule, mais avec des modalités variées.

Le fait le plus frappant est la très forte radioactivité des protéines cuticulaires dans le cas d'injections de méthionine qui doit être l'acide aminé soufré essentiel pour cette formation.

Des différences entre soufre inorganique et soufre organique se font jour: au niveau des processus du condyle, seul le soufre organique est incorporé, de façon d'ailleurs importante, tandis que pour les épines tibiales, c'est le soufre inorganique qui paraît le plus utilisé, et cela même en période de repos.

CONCLUSIONS

Il faut tout d'abord souligner l'incorporation de soufre dans la cuticule au repos alors que la tyrosine et le tryptophane ne provoque pratiquement pas de rayonnement. Sauf dans le cas des épines du tibia, cette incorporation reste faible; on doit néanmoins en tenir compte dans toutes les expériences de ce type. L'interprétation n'en est pas aisée. La cuticule, durcie et pigmentée, est théoriquement inerte. En réalité, elle reste en relation avec l'hypoderme vivant sous-jacent qui conserve des prolongements cytoplasmiques dans les innombrables canalicules de la cuticule - relation qui reste à étudier.

Depuis longtemps la présence de soufre est connue par analyse chimique dans la cuticule et la possibilité de réactions de groupements sulfhydrilés des protéines cuticulaires avec des quinones est envisagée [3, 4]. Cette hypothèse n'est pas incompatible avec les résultats obtenus ici par autoradiographie. Comparés à ceux obtenus avec la tyrosine, ils attestent

que la présence de pigments issus de cet acide aminé n'est pas liée à une richesse différentielle en soufre ni que le soufre soit à l'origine des variations de coloration, l'incorporation étant ici la même dans une région pigmentée en noire ou une zone colorée en jaune.

Par contre, l'attention est attirée par le fait que seul le soufre organique est intégré notablement dans les processus du condyle dont on connaît l'accélération du durcissement et de la pigmentation et l'intégration également accélérée de la tyrosine. On peut peut-être évoquer ici les couplages obtenus in vitro entre dérivés quinoniques de la tyrosine et des thiols [5] pouvant donner naissance à des pigments sans que l'état hormonal de l'animal ne permette la libération des enzymes conditionnant l'évolution générale de la cuticule (voir le rôle de l'Ecdyson dans le déclenchement de l'activité décarboxylasique [6]).

Enfin, le fait que le soufre inorganique est incorporé à des structures organiques complexes telle que la cuticule amène à poser le problème de la conversion chez Gryllus du soufre inorganique en soufre organique, démontré chez quelques autres espèces [7]. Cet insecte possède en outre un segment intestinal à micro-organismes lequel, d'après MARTOJA [8], incorpore le soufre inorganique injecté dans la cavité générale, et peut jouer un rôle ici.

En conclusion, on peut dire que les pigments cuticulaires ont tous leur origine dans la tyrosine incorporée après la mue; le soufre est présent dans la cuticule de façon régulière, mais ne paraît pas jouer de rôle spécifique dans la pigmentation.

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DISCUSSION

C.E. SEKERIS: The autoradiographical method that you use is very interesting. In Munich, Frau Dr. Schlossberger is using similar techniques to study the incorporation of labelled tyrosine and tryptophane in the wild and albino strains of the grasshopper Schistocerca gregaria.

It would be interesting to use this technique for the incorporation in the cuticle not only of tyrosine but also of its metabolites, acids as well as dopamine in the different developmental stages. This could give some insight into the problems of sclerotization and pigmentation, especially in insects where these two processes are clearly separated.

Concerning your autoradiograms after injection of S-labelled compounds, is it possible that these substances are merely being inactivated and excreted in this way, as cuticulum?

S. FUZEAU-BRAESCH: Yes, that is certainly a possibility.

W. KLOFT: Mme Fuzeau-Braesch's method helps one to understand the mechanism of moulting in hemi-metabolic insects. The incorporation of the labelled substance in the femur condyle is most interesting from this point of view, and I am most grateful to her.

E. HORBER: I should like to mention that with certain Diptera, as Oscinis pumilionis or Meromyza americana, which develop in graminaceous plants, the phenomenon of sexual dimorphism has been observed in addition to very pronounced seasonal dimorphism. The males showed more melanine than the females, and the hibernating flies more than the generations produced during the summer. Perhaps these species could be considered in the studies as well as the Orthoptera which you have mentioned.

S. FUZEAU-BRAESCH: Thank you very much, that might be useful.

J. R. OGLE: I should like to say something with regard to your observations on the appearance of S^{35} from methionine an cystine into the cuticle. If, as Dr. Sekeris suggested, this could be caused by excretion into the cuticle, do you think that, in fact, it might be due to the deposition of the non-metabolized d-isomers, rather than a genuine incorporation of the sulphur from the l-isomer? Would it not be preferable to use the l-isomers of these labelled amino-acids?

S. FUZEAU-BRAESCH: Yes, this ought to be done. The substance we had at the time of the experiments was a mixture, not the pure l-isomer.