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A BIO-ASSAY METHOD FOR THE ESTIMATION OF INSECTICIDE RESIDUES,
USING FIRST INSTAR LARVAE OF AEDES AEGYPTI

by

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1. INTRODUCTION

The problem of estimating the rate of loss of active principle of the toxicants used in residual spraying programmes is of great importance. There are two main lines of approach; chemical estimation and estimation of the biological activity of the residues.

A number of useful simple chemical tests have been devised. In the case of DDT for instance, colorimetric estimation (Alessandrini 1950) has been found satisfactory where great accuracy is not required, while dehydrochlorination and estimation of the hydrolysable chlorine has been used for more exact work. The DDT deposits normally used in malaria control are such that a reasonable amount of material is present on sprayed surfaces and available for estimation. The same applies to BHC deposits where the formulation used is one with a gamma isomer content of 6.5% to 10%. Here a residue of 10 mg (0.1 gramme/sq.m) per square foot of gamma isomer provides 154 mg or 100 mg of total benzene hexachloride available for dehydrochlorination, and assuming that the other isomers present are lost from the deposits at the same rate as the gamma isomer, estimation of total benzene hexachloride will provide a measure of the amount of the toxic isomer present. If this assumption does not hold good, so that direct analysis of gamma isomer is required, more advanced techniques must be employed. These include infra-red spectroscopy, polarography, or even the use of radioactive isotopes.

Where lindane grade BHC formulations have been used, or dieldrin formulations at the usual dosages, the quantity of material available for analysis is very much less than with DDT and low grade BHC formulations. For dieldrin as for lindane, the usual method for the analysis of small quantities has been an infra-red spectroscopic technique. More recently a method has been devised whereby small amounts of dieldrin can be dealt with by means of dehydrochlorination and estimation of total chlorine turbidimetrically as silver chloride (Fuell, 1955, private communication). This method also can only be carried out in a well equipped laboratory, as an absorptiometer is required.

It has therefore been thought desirable to develop some simpler procedures by means of which an indication of the fate of insecticidal residues may be determined in the absence of a chemist, or where a fully equipped laboratory is not available. The procedures to be described were developed during the course of the Ilaro Experimental Malaria Control Scheme (Bruce-Chwatt et al. 1955) and during the early stages of the Western Sokoto Malaria Control Pilot Project, and it is hoped that they may be of use to workers operating under similar field conditions.

Certain parts of the technique originated in the laboratories of the Jeallot's Hill, United Kingdom, experimental station of Imperial Chemical Industries, under Mr J. F. Newman. The work reported in section 7 was carried out in close collaboration with Dr Langbridge, Colonial Research Service Chemist to the Western Sokoto Project and the sampling, extraction and chemical analysis of the material were carried out by him, aliquot portions being reserved for bio-assay.

2. SAMPLING TECHNIQUE

For the removal of residues left on sprayed mud wall surfaces the use of a scraping tool working in an area defined by a metal stencil has been found satisfactory. The best of several tools tried was a steel paint scraper with a triangular head; an effective stencil consisted of a flat sheet of tin about 10 inches wide rolled at the lower edge to form a trough 3 inches wide. In the flat face a 3 x 3 inch hole defined the scraped area. The back, i.e. the side touching the wall, was padded with

adhesive tape round the edges of the hole to prevent scraped material dropping between the stencil and the wall and being lost. Four scrapings from a sampled room gave, from an area 6 inches square, a sample weighing from 20 to 50 g, which fitted conveniently into the thimble of a 100 ml Soxhlet's extraction apparatus.

The method of sampling described by Barlow (1953) whereby cellophane adhesive tape is applied to the mud surface can also be used and has the advantage of removing only the toxicant immediately available for pick-up by insects, but its use is confined to wall surfaces of a smoother texture than those commonly found in African huts. In the case of BHC, where toxicity depends on the fumigant action of deeply absorbed material, and not solely on pick-up of solid particles, this method of sampling might be misleading.

3. EXTRACTION OF SAMPLES

Cold extraction of samples with acetone or other organic solvent has been used with some success although there is usually an appreciable loss of insecticide. The sample of mud, placed in a filter paper in a funnel, is washed with successive quantities of acetone, and the resultant extract concentrated or evaporated to dryness and redissolved. More reliable and economical of solvent is extraction by use of the Soxhlet's apparatus. The more manageable sample obtained by Barlow's method may also be extracted cold by covering with solvent in a shallow dish.

So far the processes are similar to those which would be used were chemical analysis to follow. As has been said, in the case of DDT and low gamma content BHC, chemical analysis is comparatively simple and probably the most suitable method. In the case of colorimetric tests such as the Alessandrini method, a complication may now appear. Walls bearing a deposit of soot and wood-tar are common, and the resulting extracts are often so dark as to interfere with the judgement of the colours developed. In the case of dieldrin analysis using the sodium reduction technique followed by Volhard titration to estimate chloride ion, the end-point colour of the final titration is often identical with that of the interfering matter (Langbridge, private communication). The process of removal of the interference by

absorption may, of course, also remove some toxicant. For purposes of bio-assay these extraneous colours can be ignored; the substances causing them are comparatively non-toxic and have been found completely so in final dilution.

If bio-assay is to follow directly on extraction, the original solvent, usually acetone, may be used for presentation to the biological material. If it is intended to keep samples for future reference they may be evaporated to dryness and redissolved in a less volatile solvent such as ethyl alcohol. Where samples are to be sent by post it has been found convenient to allow acetone solutions to evaporate in 3 x 1 inch tubes and to redissolve the toxicant on arrival.

4. BIOLOGICAL MATERIAL

Bio-assay using adult mosquitos or other adult insects is of course highly informative, being the closest approach to nature obtainable under controlled conditions. Unfortunately the cumbersome techniques required, and the great difficulty in transporting adult insects, especially anopheles mosquitos, limits the amount of use that can be made of them for the estimation of residual deposits in the field.

The laboratory use of Aedes aegypti larvae for estimation of insecticides employed in antimalarial work has been described (Pal and Sharma 1952). In this case fourth instar larvae were used and a standard curve constructed for the regression of percentage mortality on dosage; from this the DDT content of alcoholic extracts of mud scrapings was determined. It has been found, however, that there is much variation in sensitivity between one batch of old larvae and another, especially when rearing takes place under field conditions. The use of first instar larvae is therefore preferable as these, under reasonably stable conditions of temperature etc. are remarkably uniform in their reactions, especially if drawn in successive sub-grow from the same egg batch. The original batch of course consists of the progeny of a large number of females in the parent colony laid over a period of a day or so, and has sufficient variation within the batch to provide the range of sensitivity on which the mathematical treatment of bio-assay results depends. The other great advantage of this type of material is that it can be sent by post, and stored for long periods.

Stored Aedes eggs, therefore, soaked in warm water which provokes a complete hatch over a period of about 30 minutes, provide a very satisfactory source of biological material.

5. PRESENTATION OF TOXICANT AND EXPOSURE TECHNIQUE

For presentation to newly hatched larvae the toxicant must be in the form of an aqueous solution or of an aqueous dilution of a solution in another solvent. In the case of DDT this will give a colloidal suspension; BHC and dieldrin are sufficiently soluble for the final dilution to be a true solution in water. Either ethanol or acetone may be used as the solvent. Ginsburg (1949) used one per cent. ethanol, while Wharton (1955) found acetone at one part in 250 satisfactory; in the present work these proportions of solvent to water have not been exceeded.

Aqueous dilutions of the unknown extracts are therefore made: at the same time a set of standards prepared from known amounts of toxicant dissolved in the same solvent is set up. It is important that all glassware used at this stage and thereafter shall be chemically clean, as minute quantities of grease can preferentially absorb sufficient insecticide from an aqueous solution to introduce a considerable error.

Small aliquot portions - usually one millilitre - of the aqueous dilutions of both the standards and the unknown extracts, are placed with a pipette into small glass tubes or vials of about 4 ml capacity. The larvae are added in batches of about 20, each batch being pipetted into the tube along with a similar measured volume of water. The period of exposure should ideally be 24 hours or very close to it. This gives sufficient time for a clear distinction to be made between moribund or dead larvae and actively swimming survivors. With stronger solutions the shorter period does not give time for this distinction to develop, as all the larvae become more or less moribund. After 24 hours natural mortality due to starvation begins to appear and to affect the result.

The strength of the final dilution should therefore be so adjusted as to produce a suitable range of mortalities. The insecticide to be estimated and the natural

susceptibility of the strain of Aedes in use determine the required strength. The median concentration of the standard series usually lies between 3 and 15 parts of toxicant per 100 million of water with DDT, dielêrin and lindane.

6. DETERMINATION OF MORTALITY

When the experiment has proceeded for 20-28 hours counts are made of the numbers in each batch either alive (i.e. capable of normal swimming movements) or dead and moribund (i.e. capable of disordered twitching only). This is best done by removing water and larvae from the exposure tubes with a pipette and placing them on a sheet of perspex or of glass treated with silicone polish. This keeps the larvae in a rounded drop in which they can readily be counted. For the most informative results, mortalities should run from about 80% at the highest concentration to about 20% at the lowest, distributed more or less equally about the 50% mark. This is desirable because correlation of dosage with mortality is closest near to the median lethal concentration (LD_{50}) of the toxicant.

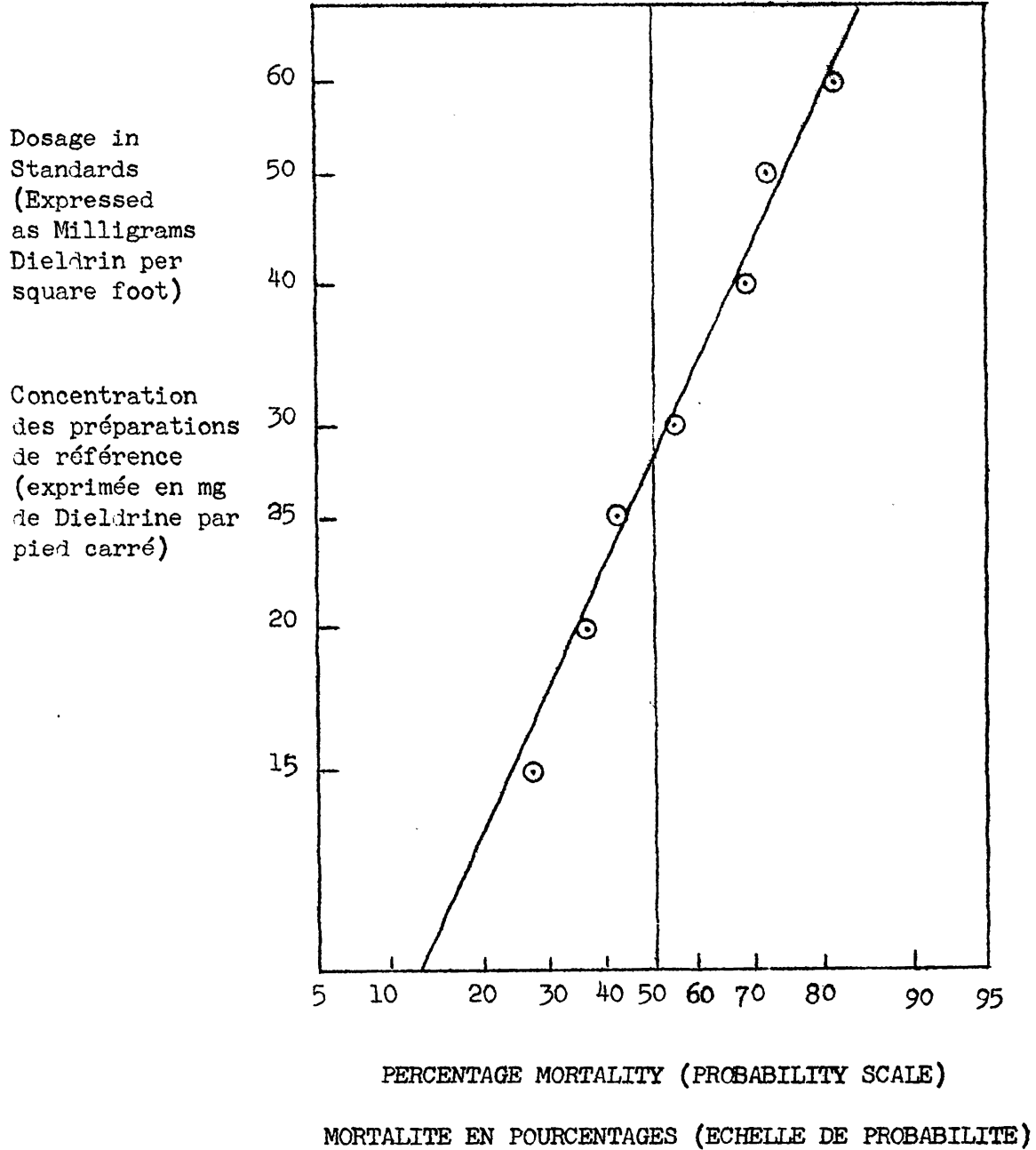
If necessary, samples covering a wide range of toxicant content must be dealt with in separate batches with different series of standards made up to suit each batch. This was done with the DDT samples described in section 7.

When a consistent set of results from the standards has been obtained a regression line of mortality against dosage can be constructed, either by plotting probit mortality against the logarithm of the dosage, or, more directly, by plotting percentage mortality against dosage on log-probability paper, as shown in Fig.1. The toxicant content of the standards may conveniently be expressed in terms of milligrammes toxicant per unit area of the original sprayed surface. The dosage present in the unknown samples can then be read off from the regression line, according to the percentage mortalities observed. One or more preliminary trials are needed to determine if the range of standards has been well chosen, and to decide the most suitable dilution for both standards and unknowns. After this three to six replicates should suffice to produce the required information.

FIGURE 1

STANDARD DOSAGE - MORTALITY CURVE FOR
DIELDRIN BIOASSAY REPORTED IN SECTION 7

DOSES DE LA PREPARATION DE REFERENCE : COURBE DE
MORTALITE CORRESPONDANT AUX ESSAIS BIOLOGIQUES EFFECTUES
AVEC DE LA DIELDRINE ET DECRITS A LA SECTION 7



7. DISCUSSION AND EXAMPLES OF THE METHOD

It is not claimed that the method described is quicker than chemical analysis, although a large number of extracts can be processed simultaneously, and a fair amount of information can be produced in a reasonable time. In accuracy it also falls short of the better established chemical methods, although it probably compares well with the cruder colorimetric techniques. Apart from the colouring matter referred to in section 3 above, the presence of non-toxic degradation products of the original insecticide may also interfere with the results of chemical analysis. The technique of bio-assay has as its main advantage the fact that the insecticidal efficiency of the extracted material is the sole criterion of the amount of toxicant present. It is therefore suggested that in situations where chemical methods cannot be operated, bio-assay will provide adequate information as to the fate of insecticide residues, and that it will always form a useful cross check on the results of chemical analysis.

Tables 1 and 2 show some results obtained by bio-assay compared with those for chemical analysis. In both cases the main sample was estimated chemically, while an aliquot was reserved and estimated by bio-assay. Table 1 shows a series of DDT samples estimated colorimetrically by nitration and treatment with sodium methoxide. This method uses a small portion only of the extract, being based on 40 to 200 microgrammes of DDT. In this case as many replicates as required can be estimated, and the chemical method is superior to the biological. In Table 2, for dieldrin samples, the chemical method used was estimation of total chloride turbidimetrically with silver nitrate after reduction with sodium. Here the reduction process was applied to 95% of the original extract, the remaining 5% being reserved for bio-assay, and a series of replicates could be obtained for the turbidimetric process but not for the original reduction.

The bio-assay method on the other hand, could afford a large number of replicates from only 5% of the extract. The two methods are probably capable of about equal accuracy, while the bio-assay method has the advantage that the use of expensive and delicate apparatus such as the absorptiometer is avoided. It is considered that,

while an exact correspondence cannot be shown, the two sets of results are reasonably compatible, and that either set would give an adequate picture of the rate of degradation of the residual deposit.

Table 1

Results of bio-assay of DDT samples compared with those
by chemical analysis

Sample No.	Bio-assay (mg per square foot)	Chemical analysis (mg per square foot)
12	280 - 360	378
9	280 - 360	344
8	280 - 360	320
10	200 - 280	266
1	200 - 280	258
11	160 - 200	240
7	160 - 200	198
3	120 - 160	216
6	80 - 120	126
5	80 - 120	104
4	40 - 80	54
2	40 - 80	38

Table 2

Results of bio-assay of dieldrin samples compared with those by chemical analysis

Age of deposit in days	50 mg per square foot theoretical deposit		25 mg per square foot theoretical deposit	
	Bio-assay	Chemical analysis	Bio-assay	Chemical analysis
0	42	50.1	33	33.9
4	26	24.2	27	15.8
11	14	16.2	17	15.6
26	9	11.0	11	10.7
76	9	9.6	3	2.8
120	7	5.2	8	4.0

Figure 1 shows the regression line of percentage mortality on dosage in the standards set up for the series of dieldrin samples reported in Table 2. The mortalities were plotted on logarithmic-probability paper and the best-fitting straight line drawn by eye through the points. From this line the dosages present in the unknown samples were read off directly.

8. SUMMARY

1. The estimation by chemical means of the rate of loss of toxicants used in residual spraying while satisfactory in the case of DDT formulations and BHC formulations of low gamma isomer content, may meet with difficulties through lack of available material in the case of dieldrin and lindane formulations. Moreover, the necessity for apparatus such as absorptiometers and spectrosopes means that a reasonably well equipped laboratory is necessary. It is therefore suggested that for field work methods of bio-assay may give adequate results with simple apparatus.

2. Methods of sampling wall surfaces, and of extraction and further treatment of samples are discussed.

3. Various types of biological material are considered and the advantages of using freshly hatched, first instar larvae of Aedes aegypti are described.

4. Methods of presentation of the toxicants to the larvae are discussed and a semi-micro technique is described.

5. The advantages and disadvantages of the method as against chemical analysis are compared and results are given from the assay of two sets of samples, one of DDT and one of dieldrin residues, by the two methods. It is considered that the results are reasonably compatible and that either method would give adequate information as to the behaviour of the insecticide deposits.

9. REFERENCES

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