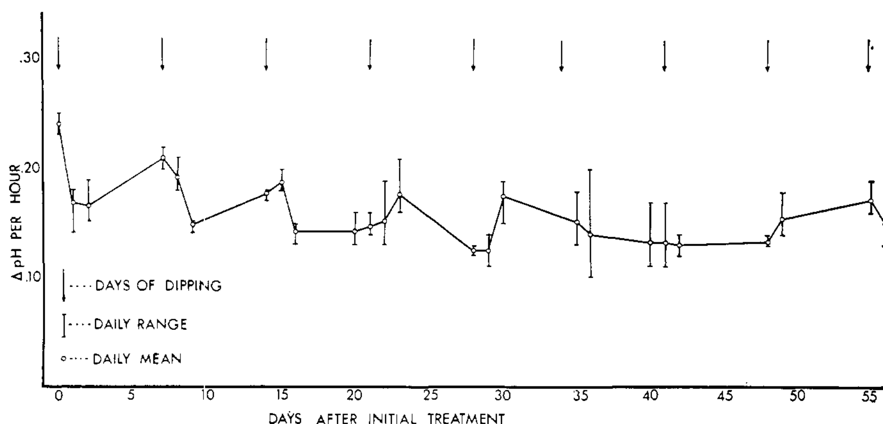

Figure 2. Effect of 9 weekly dippings in 0.5% bromophos emulsion on whole blood cholinesterase (means and range) of four lambs

Cholinesterase expressed in Δ pH per hour per ml. of blood and time in days after initial treatment

proportional to the nanograms of bromophos injected and were found to be more reliable than area under the peak as determined by the recorder disk integrator.

Results

No clinical signs of toxicosis were observed in any of the test animals. The only apparent effect was temporary reddening of the skin immediately after dipping. The effect of dipping on the daily cholinesterase activities is shown in Figure 2.

Bromophos residues found in the omental fat samples are given in Table I. The figures are the residues actually found, not corrected for extraction loss. The recoveries of bromophos which was added to omental fat from nontreated animals are shown in Table II. The oxygen analog of bromophos was not analyzed for specifically; however, under the chromatographic conditions used, there was only one peak corresponding to a halogenated compound.

Chlorinated hydrocarbon and some of the organophosphorus insecticides, when applied dermally, may be absorbed through the skin and stored in the fatty tissues of the animals. The residues found in other tissues can usually be attributed to the fat content of the tissues (*1,5*).

In view of the low toxicity, as demonstrated by the lack of clinical symptoms and low cholinesterase inhibition, and the fairly rapid depletion of residues, bromophos appears to offer advantages that many other halogenated and organophosphorus insecticides do not.

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RESIDUE ESTIMATION

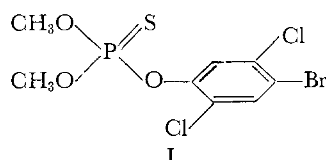
Determination of Bromophos Residues

PERETZ BRACHA[1] and JEAN P. BONARD

Insecticide Testing Unit, World Health Organization, Lagos, Nigeria

Procedures for the estimation of bromophos residues on mud, the commonest surface in the West African village, are described. 4-Aminoantipyrine coupling was used in the colorimetric procedure. The possible determination of its metabolite, 2,5-dichloro-4-bromophenol, in urine as a means of determining the degree of exposure to the insecticide is discussed. It was possible to separate bromophos from its hydrolysis products and to determine both quantitatively by using a spot area-to-weight relationship in thin-layer chromatography.

In a search for insecticides to replace DDT and dieldrin (to which resistance is developing) in antimalarial campaigns, a collaborative scheme for insecticide testing was launched by the World Health Organization. The Insecticide Testing Unit in Lagos has evaluated a few compounds suitable for mosquito control (*1, 6, 7*). Bromophos [*O,O*-dimethyl *O*-(2,5-dichloro-4-bromophenyl) phosphorothioate (I), Cela G.m.b.H., Ingelheim, West Germany] has been selected as a candidate com-



pound for evaluation of its mosquito control properties. This paper deals with the chemical analysis of bromophos and the estimation of its residues on mud, the commonest surface encountered in the West African village. The authors' main concern was the accurate determination of the initial deposit of bromophos, and the possible estimation of its residues at intervals thereafter. Entomological aspects of this work will be covered separately. A method is also proposed for the estimation of residues absorbed by people exposed to this insecticide.

[1] Present address, Section of Neurobiology and Behavior, Cornell University, Ithaca, N. Y.

PART I. DETERMINATION OF BROMOPHOS RESIDUES ON MUD

Materials and Methods

Apparatus. Visible region spectrophotometer.

Reagents. 4-AMINOANTIPYRINE. 1.0% in water w./v. Prepare a fresh solution weekly. Store refrigerated in dark bottle.

POTASSIUM FERRICYANIDE, 1.4% in water w./v. Prepare fresh weekly, store refrigerated in dark bottle.

PHOSPHATE BUFFER. Mix 70 ml. of 0.7*M* dibasic potassium phosphate solution (K_2HPO_4) with 30 ml. of 0.7*M* potassium dihydrogen phosphate (KH_2PO_4) in a 1-liter volumetric flask. Add 500 ml. of water, 200 ml. of ethyl alcohol, and dilute to 1000 ml. with water.

SODIUM HYDROXIDE. Solutions, 0.5 and 2.0*N*.

BROMOPHOS. Stock solutions: 150 mg. dissolved in 100 ml. of acetone (solution A), and 200 mg. dissolved in 10 ml. of acetone (solution B).

2,5-DICHLORO-4-BROMOPHENOL. 100 mg. dissolved in 100 ml. of acetone.

Procedure

Colorimetric Method. STANDARD CURVE. Ten milliliters of the bromophos stock solution (A) were diluted to 100 ml. with acetone. Twenty-five milliliters of this solution were diluted to 50 ml. with acetone and 1 ml. of this solution was in turn diluted to 25 ml. Aliquots of 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 ml. (corresponding to 0.0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 μg. of 2,5-dichloro-4-bromophenol) were placed in 25-ml. volumetric flasks. Solvent was evaporated on a water bath and 1 ml. of 0.1*N* sodium hydroxide was added and the mixture heated on the water bath for 15 minutes, after which 1 ml. of 50% ethyl alcohol was added, and heating continued for an additional period of 15 minutes. After cooling, the mixture was neutralized with 1 ml. of 0.1*N* hydrochloric acid, and 15 ml. of phosphate buffer solution were added. Aliquots of 0.25 ml. of 1.0% 4-aminoantipyrine solution and the 1.4% potassium ferricyanide solution were added at clocked intervals to the flasks. Care was taken to shake the flasks after the addition of each reagent. The flasks were then made up to the mark with buffer solution. Absorbance at 500 mμ was recorded (at exactly 20 minutes after the addition of the potassium ferricyanide) and corrected for the reagent blank. Results were plotted against concentrations and showed a linear relationship over the range tested. Inasmuch as the colored complex is sensitive to oxidation and ultraviolet light, it should be kept in a closed, filled flask, away from direct sunlight, and its absorbance recorded immediately after it is placed in the cell.

Recovery Experiments. In the above procedure, the method outlined for the estimation of ronnel [*O,O*-dimethyl *O*-(2,4,5-trichlorophenyl) phosphorothioate)] in dips (*11*) usually was followed. However, 0.5*N* sodium hydroxide was used for the hydrolysis of bromophos. After the hydrolysis was completed, the excess alkali was neutralized. Fifteen minutes were found sufficient for complete hydrolysis.

Bromophos was sprayed in the native huts in the vicinity of Lagos, and its residues on mud were determined in the following manner: initial insecticide concentration was found by determining the deposit on filter papers attached to the mud huts walls prior to the spraying operation (*7*), while residues on mud at intervals after the spraying date were found by attaching a large number of previously prepared mud bricks (2-inch diameter, ½-inch thickness) to the walls and collecting a given number of them, at random, on intervals after the spraying date. Extraction of the bricks and filter papers with acetone gave the best recoveries. The following procedures were used. Mud bricks: Aliquots of 0.1, 0.25, 0.5, 1.0, and 2.0 ml. of bromophos stock solution (B) (corresponding to 2.0, 5.0, 10.0, 20.0, and 40.0 mg. of the insecticide) were added to a series of mud bricks. After the solvent had evaporated the bricks were crushed and quantitatively transferred, with the aid of 100 ml. of acetone, into an extraction thimble placed in a Soxhlet apparatus. After boiling for 1 hour, the extract was cooled and transferred to a round-bottomed flask and concentrated in vacuo to about 20 ml. The concentrate was diluted in a volumetric flask to 25 ml., 1 ml. was taken for hydrolysis and subsequently treated as the standard curve aliquots. Filter papers: These were treated with aliquots of 0.1, 0.25, 0.5, 1.0, and 2.0 ml. of bromophos stock solution (B). After the solvent had evaporated, the papers were extracted twice with boiling acetone (10 ml.), washed (2 ml.), and the combined extracts and washings transferred to a 25-ml. volumetric flask. A suitable aliquot was taken for hydrolysis and subsequently treated as above.

Discussion

To analyze bromophos residues on mud, Smith's procedure (*11*) for the determination of ronnel was used. When subjected to alkaline hydrolysis followed by coupling with 4-aminoantipyrine, bromophos gave the same colored complex one would obtain upon similar treatment of ronnel. The halogen atom substituting the para position of the aromatic ring is eliminated in both cases (*4*). The colored complex obtained from bromophos had the same absorbance and stability characteristics which were reported earlier (*11*). There are some minor alterations in the authors' procedure, intended mainly to facilitate recovery of the insecticide from the surfaces dealt with in this work. Best recoveries were achieved when acetone was used as the recovery solvent and 0.5*N* sodium hydroxide for the hydrolysis process, with an optimal hydrolysis times of 15 minutes. Recovery results are summarized in Table I.

While the aminoantipyrine method was most suitable for micro quantities—i.e., down to 0.5 micron in the final dilution—it was also convenient to use the bromometric oxidative procedure (*7*) for semimicro and micro estimations. The bromophos residue was oxidized with an excess of acidified standard 0.1*N* bromide-bromate solution. The excess bromate was determined by titration, with standard sodium thiosulfate solution, of the iodine liberated on addition of excess potassium iodide.

By determination of the amount of 2,4-dichloro-4-bromophenol present prior to and after the hydrolysis step of bromophos residues from mud walls, it was found that 23% of the initial deposit had undergone hydrolysis in situ during the first 6 weeks after spraying at prevailing temperatures of 35–40° C. The stability of ronnel on clay has been discussed recently (*10*), and its hydrolysis to 2,4,5-trichlorophenol was explained in terms of interaction with carrier crystal-bound water. A similar process probably occurs on mud surfaces, especially if they are, as in this case, equilibrated with water vapors (prevailing relative humidity is 80–100%). Furthermore, an almost complete loss of bromophos surface activity was observed by the end of the mentioned period. Most of the recovered bromophos was found in the inner mud layers and, thus, was rendered biologically inactive to surface-resting mosquitoes.

Table I. Bromophos Recovery from Mud and Filter Papers

Amount Added, Mg.	Amount Found, Mg.	Error, %
	MUD BRICKS	
2.0	2.00	0.0
5.0	5.10	+2.0
10.0	10.4	−4.0
20.0	20.4	+2.0
40.0	39.6	−1.0
	FILTER PAPERS	
2.0	1.96	−2.0
5.0	4.92	−1.6
10.0	10.2	+2.0
20.0	20.3	+1.5
40.0	41.2	+3.0

PART II. BROMOPHOS RESIDUES IN URINE

Dawson *et al.* (*2*) utilized the 4-aminoantipyrine color reaction for the estimation of volatile phenols such as *o*-isopropoxyphenol in urine. 2,5-Dichloro - 4 - bromophenol, obtainable from bromophos upon hydrolysis, is steam distillable and, as such, is capable of being separated from the nonvolatile phenols present in urine. Fur-

thermore, *p*-cresol, the major volatile phenol present in urine, does not react with 4-aminoantipyrine (*4*). However, other volatile phenols react with reagent, causing high blanks. Dawson *et. al.* (*2*) and Elliott *et al.* (*3*) point out that, although recovery of phenols from urine is never complete [because of either incomplete hydrolysis or incomplete excretion—i.e., 70% recovery of *p*-nitrophenol (*3, 5*), 30% recovery of *o*-isopropoxyphenol (*2*)], it was possible to use estimates of the phenol found as a general measure to the amount of exposure to the insecticides. This was of special importance during spraying operations in underdeveloped areas such as the one in which the present work was carried out. Inhabitants were more likely to be exposed to larger doses of the insecticide because of their primitive way of life. Sleeping and cooking on the floor enhance pick-up of the insecticide particles. Although floors are not sprayed directly, they acquire a heavy dose through fall-off of insecticide dust from sprayed walls and ceilings (*7*). For description of the treated dwellings, see (*6*). Such determinations are especially useful if carried out along with the micro Michel cholinesterase analysis (*8*). 2,5-Dichloro-4-bromophenol is less volatile in steam than *o*-isopropoxyphenol and, thus, would need a larger distillate for complete recovery. Urine samples usually were hydrolyzed in alkali, where a quicker hydrolysis was achieved. Acid hydrolysis gave somewhat erroneous results.

Purdy and Truter (*9*) found that, in thin-layer chromatography, the square root of the area of a given spot is proportional to the logarithm of the weight of material it contains. These authors outlined a simple analytical method by which a series of unknowns could be determined graphically by plotting the logarithm of the weight against the square root of standards which were run simultaneously with the unknowns. Also, assuming linear variation of $\sqrt{A}$ with log W, one could use Purdy's algebraic methods (*9*) and calculate unknowns in respect to standards by using Equation 1, where W and W_s are the weights of unknown and standard samples, respectively, A, A_d, and A_s are areas of unknown sample, diluted unknown sample, and standard sample, respectively, and d is the dilution factor of the diluted unknown solution.

$$\log W = \log W_s + \frac{\sqrt{A} - \sqrt{A_s}}{\sqrt{A_d} - \sqrt{A}} \log d \quad (1)$$

Materials and Methods

Apparatus. Visible region spectrophotometer. Thin-layer chromatography basic equipment. (Authors used the DESAGA TLC equipment; plates were coated with 250-micron layer of silica gel G).

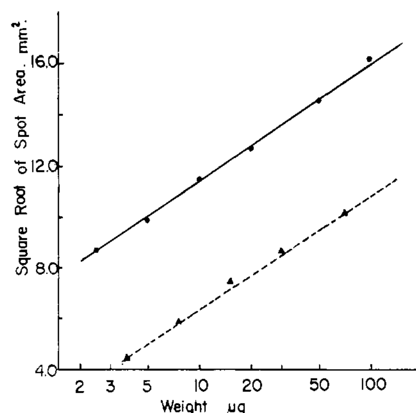


Figure 1. Sample weight and spot area relationship for bromophos (broken line) and 2,5-dichloro-4-bromophenol (solid line)

Reagents. Bromophos and 2,5-dichloro-4-bromophenol stock solutions (see Part I).

Chromatographic visualization reagents: Bromine, 5% in carbon tetrachloride. Fluorescein, 1 ml. of 0.25% w./v. in *N,N*-dimethyl formamide diluted to 50 ml. with ethyl alcohol.

Procedure

Recovery Experiments. In a typical run, 15 mg. of bromophos were hydrolyzed with excess of alkali and the resultant phenol was steam distilled after acidification of the mixture. Complete recovery of the phenol was secured by steam distilling a volume of 30 ml. Similarly, 10 mg. of 2,5-dichloro-4-bromophenol were completely recovered upon collection of a similar volume. The distillate was either extracted with ether-benzene (20 to 80%) or, if concentration permitted, determined directly: 1 ml. of either extract or distillate was diluted to a known volume, an aliquot was taken into a 25-ml. volumetric flask, organic solvents were evaporated, 15 ml. of buffer solution were added, and the procedure outlined in Part I was followed. Alternatively, the bromophenol could be separated from the interfering *p*-cresol very easily by thin-layer chromatography. (R_f = 0.47 and 0.32, respectively, when developed with chloroform on silica gel G plates.) The bromophenol spot was transferred quantitatively to a centrifuge tube and suspended in 3 ml. of 0.1*N* sodium hydroxide in 50% methanol, heated on a water bath for 15 minutes, and centrifuged. The residue was extracted twice more by this method, and the extracts were collected in a 10-ml. volumetric flask, neutralized with 0.3 ml. of 3*N* hydrochloric acid, and diluted to the mark with phosphate buffer. An aliquot of this solution was taken, phosphate buffer added, and analysis continued as outlined in Part I. How-

Table II. Quantitative Determination by Thin-Layer Chromatography of 2,4-Dichloro-4-bromophenol and Bromophos

2,4-Dichloro-4-bromophenol, $R_f = 0.46 - 0.48$				Bromophos, $R_f = 0.82$				Mixtures			
Weight known per spot, μg.	Spot areas, sq. mm.	Weight found (graphic), μg.	Weight found (algeb.), μg.	Weight known per spot, μg.	Spot areas, sq. mm.	Weight found (graphic), μg.	Weight found (algeb.), μg.	Weight known per spot, μg.	Spot areas, sq. mm.	Weight found (graphic), μg.	Weight found (algeb.), μg.
W_1 = 100.0	262	100.1	96.1,[a] 98.7[b]	W_{s1} = 70	104	Standard	Standard	2.1 + 3.0	45, 15	2.3 + 2.7	1.7 + 2.6[e]
W_{1d1} = 25.0	162	23.8	24.0,[d] 24.7[d]	W_{s2} = 14	21	Standard	Standard	4.2 + 6.0	60, 27	4.2 + 5.2	3.4 + 5.2[e]
W_{s1} = 50.0	212	Standard	Standard	W_1 = 60.9	89	57.8	54.5,[f] 67.5[g]	10.6 + 15.0	85, 52	9.8 + 15.5	9.6 + 18.4[e]
W_{1d2} = 9.6	112	9.2	9.2,[d] 9.5[d]	W_2 = 24.9	47	25.6	26.6,[f] 27.1[g]	21.2 + 30.0	108, 71	20.0 + 28.5	20.0 + 30.1[e]
W_2 = 400.0	361	400.0	395[c]	W_{1d} = 12.2	20	12.2	10.9[d], 13.5[d]	— 60.0	— 96	— Stand.	—
W_{2d} = 100.0	254	100.0	98.7[d]	W_{2d} = 12.45	20	12.2	13.3[d], 13.5[d]	— 24.0	— 65	— Stand.	—
W_{s2} = 200.0	305	Standard	Standard					8.4 —	80 —	Stand. —	—
W_3 = 50.0	205	48.5	46.5[c]					21.2 —	110 —	Stand. —	—
W_{3d} = 25.0	168	26.0	23.2[d]								

W_{s1}, W_{s2}, ... weight of standard samples, W_1, W_2, ... Weight of unknown samples. W_{1d}, W_{2d}, ... Diluted unknown samples. [a] Calculated using W_{1d1} as diluted sample. [b] Calculated by using W_{1d2} as dilute sample. [c] Calculated by using W_{s2} as standard sample. [d] Calculated from undiluted found value. [e] Using previous value as diluted sample. [f] Using W_{s1} as standard. [g] Using W_{s2} as standard.

A solution containing 84.4 μg. per ml. of 2,4-dichloro-4-bromophenol and 120.0 μg. per ml. of bromophos was used. Aliquots containing different amounts of this mixture were applied to filter papers and recovery was carried out as described previously. Mixtures spotted along with spots of pure components.

ever, this procedure necessitated a large initial amount for the final dilution to be analyzable.

Recovery from urine was checked by adding known amounts of bromophos to 'blank' urine samples. Urine samples were subjected to the following treatment: 5 ml. of 2*N* sodium hydroxide were added to 5 ml. of urine, followed by 40 ml. of water. The mixture was boiled for 15 minutes, cooled, and acidified with 5 ml. of concentrated hydrochloric acid, after which it was distilled until 30 ml. of distillate were collected. The distillate was extracted twice with 30-ml. portions of ether-benzene (20 to 80%). After being dried over sodium sulfate, 50 ml. of the organic phase were extracted with 5 ml. of 20% sodium hydroxide. The extract was centrifuged and the last traces of the solvent were removed by a stream of hot air. Finally, 4 ml. of the alkaline solution were extracted with two 3-ml. portions of acetonitrile. One milliliter of this extract was transferred to a 10-ml. volumetric flask; 5 ml. of buffer were added, followed by 0.25 ml. of 4-aminoantipyrine and 0.25 ml. of potassium ferricyanide, and the solution was diluted to the mark. The recovery, as determined colorimetrically, was over 90%.

Because of the limitations mentioned above, in extracting the spots of bromophos and its phenol from silica gel plates, Purdy's (*9*) work was referred to, as it enables one to estimate the amounts of the compound directly on the plate omitting its recovery from the adsorbent. Both bromophos and 2,5-dichloro-4-bromophenol obey the linear relationship between the logarithm of the weight spotted and the square root of the developed spot area (Figure 1).

Thin-Layer Chromatography Procedure. Silica gel G plates (250 microns) were prepared according to Stahl's (*12*) method. A series of solutions of varying concentrations, of both bromophos and 2,5-dichloro-4-bromophenol in benzene was prepared. Five-microliter aliquots were expressed from an Agla syringe—care was taken not to scratch the adsorbent. Stahl's color mixture was used to determine the activity of the layer. The spotted plates were developed with chloroform (ascending technique). The developing tank was lined with filter paper sheets to ensure a saturated atmosphere, and the plates were developed until the solvent front advanced for 10 cm. Spots were revealed after the plate had dried by hanging the plate for 30 seconds in a chamber containing a few milliliters of the bromine reagent, followed by spraying with the fluorescein solution (*13*) and exposing the plate to ultraviolet light. The area of the located spots (R_f values: 0.47 and 0.82 for the phenol and bromophos, respectively) was determined by laying a sheet of transparent millimetric graph paper on the plate, and tracing the outline of the spots. However, since spot size from different chromatograms might vary considerably because of variables such as the characteristics of the layer, development procedures, and temperature, it might become necessary to run a series of standards simultaneously with the unknown samples for each plate. The square roots of the standards were plotted against the logarithm of their weight and the unknowns determined graphically. Alternatively, unknown samples were diluted by a known factor, and determined with reference to a standard sample, using Equation 1.

Discussion

The accuracy of both of Purdy's methods depends very much on the accurate estimation of the spot area, the relative error becoming greater the smaller the spot is. Larger spots were obtained from 2,5-dichloro-4-bromophenol (Figure 1), and this compound was easier to determine. On a typical run, a 5-μl. spot of 100 μg. had an area of 262 sq. mm, and a similar spot containing 9.6 μg. had an area of 112 sq. mm. In comparison, a bromophos 5-μl. spot containing 60.9 μg. gave an area of 89 sq. mm., while a similar spot with 12.2 μg. had an area of 20 sq. mm. (Table II). The experimental error in determining bromophos low samples was considerable, and the lowest bromophos concentration determined directly on the plate, with reasonable accuracy (down to $\pm 10\%$), was 5 μg. With the phenol, one could go down to spot concentration of 1 μg. In general, an average $\pm 5\%$ error was noted; a better recovery was recorded for phenol spots than for bromophos spots, the error increasing with decreasing spot size. In Table II, the experimental results from representative chromatograms of single components or mixtures are summarized.

The on-the-plate procedure proved to be helpful also in rapidly determining the relative amounts of bromophos and its decomposition product, 2,5-dichloro-4-bromophenol, on the mud surfaces of the native huts. Recovery from both mud and filter papers by the above procedures was satisfactory when the final concentration of the test solution was not lower than 0.2 mg. per ml. for the phenol or 1.0 mg. per ml. for bromophos—i.e., 1.0 μg. and 5.0 μg. per 5-μl. spot, respectively. Although the accuracy of this method is limited in comparison with the 4-aminoantipyrine colorimetric method, it is still useful whenever a quick analysis of both bromophos and its phenol is required simultaneously.

Acknowledgment

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