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## INSECTICIDE LABELING

# A Simple Technique for Tritiation of Aromatic Insecticides

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The tritiating technique of Yavorsky and Gorin has been used to label aromatic insecticides, and the degree of labeling, which varies greatly with compounds, has been tentatively accounted for. The method is simple and requires little space and no expensive reagents or apparatus. The cost per labeled compound is low, on the average \$7.00 for 100 to 200 mg. of labeled compound. Activities up to 15.5 mc. per mmole were obtained.

YAVORSKY and Gorin (11, 12) have shown that simple aromatic hydrocarbons can be tritiated by direct contact with the tritiating reagent  $H_2TPO_4 \cdot BF_3$ . Exchange is effected primarily at aromatic sites, although there is some evidence that exchange also takes place very slowly at a tertiary carbon. The method is simple, does not involve the handling of tritium gas, does not produce large quantities of unwanted side-products as in the Wilzbach technique, and requires no elaborate precautions. It uses a relatively inexpensive source of tritium—tritiated water.

The authors felt that this technique might be applicable to the labeling of biologically active compounds providing the method, which was originally applied to 70-gram quantities, could be scaled down sufficiently, and provided that reactive compounds could tolerate the conditions. Accordingly, the tritiation of two chlorinated hydrocarbons and several carbamates and phosphates was attempted in the hope that perhaps some general rule might be formulated by which the degree of labeling for different compounds could be predicted.

### Methods

Approximately 180 mg. of phosphoric anhydride ( $P_2O_5$ ), calculated as a small excess over the theoretical 0.0012 mole—i.e., 171 mg.—were weighed quickly into a 4-dram vial (Kimble Opticlear 60975-L), and the polyethylene top was snapped into place to prevent reaction of moisture from the air with the  $P_2O_5$ . The vial was cooled in an ice-water or dry ice-acetone mixture, and 65  $\mu$ l. (0.0036 mole) of tritiated water (specific activity 1 mc. per  $\mu$ l.) were added with a disposable micropipet. The top was quickly

replaced and the vial rotated slowly until the water had taken up all the  $P_2O_5$ . When the vial reached room temperature, a  $3/8$ -inch, Teflon-coated magnetic stirrer bar was used to stir the sirupy mixture rapidly for 5 minutes to ensure complete reaction of the water with the  $P_2O_5$ . An  $1/8$ -inch air space was left between the vial and the stirrer, otherwise heat from the stirrer influenced the reaction.

While stirring was continued, boron trifluoride was introduced at a rate of one bubble per second for about 7 minutes until the tritiated  $H_3PO_4$  became thoroughly saturated (Figure 1). Although stoichiometrically about 300 bubbles of  $BF_3$  were required, some was lost because of the poor physical conditions of the mixing. The reagent  $H_3^*PO_4 \cdot BF_3$  is a heavy, colorless liquid. The cap was replaced and stirring continued for about 5 minutes more. Two-hundred milligrams of the compound to be labeled was dissolved in 3 to 5 ml. of cyclohexane (if the compound was apolar) and added to the tritiating reagent. Cyclohexane (Matheson, Coleman and Bell No. 2825) was satisfactory without sodium treatment. The cap was again replaced and stirring continued for 20 hours. Polar compounds were rinsed into the tritiating mixture directly with a few milliliters of cyclohexane, and appeared to dissolve in the reagent mixture; the apolar compounds did not so dissolve.

After the reaction was complete, the contents of the vial were transferred to a 100-ml. separatory funnel. The vial was rinsed two or three times, first with cyclohexane, then with water, and finally with chloroform if the compound was not very soluble in cyclohexane. The rinses were transferred to the separatory funnel; the solvent layer was washed four times with

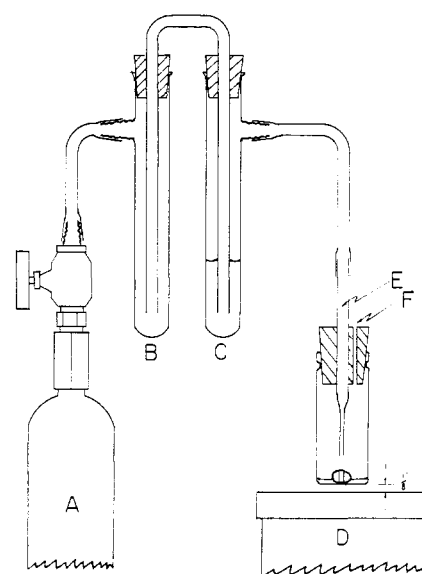


Figure 1. Apparatus for semimicro tritiation

(A) Lecture bottle of  $BF_3$ , (B) safety trap, (C) trap with concd.  $H_2SO_4$  for washing  $BF_3$ , (D) magnetic stirrer, (E) disposable pipet, (F) cork slotted to vent excess  $BF_3$

an equal volume of water and dried over anhydrous sodium sulfate for 24 hours. After filtration, the solvent was removed by rotary evaporation. The residue was taken up in a few milliliters of the appropriate solvent for chromatographic separation. If very polar compounds were to be labeled, alternative procedures would be required.

For labeling at elevated temperatures, the stoppered vial was placed in a 75-mm. porcelain evaporating dish containing paraffin oil and a thermometer, and the reaction was carried out over a magnetic stirrer-hot plate combination. A trial was made to calibrate the temperature

of the oil bath with that of the reaction mixture. The cap was supported loosely until the desired temperature was reached and then pushed in to make a tight seal.

All compounds (even pure *p,p'*-DDT) degraded to some extent and were therefore cleaned up by the appropriate chromatographic system. The purity of each compound was checked by its infrared spectrum and its  $R_f$  on a paper chromatographic system both before and after tritiation. In addition, radio purity was checked by scanning the paper chromatograms with a Vanguard 880 Autoscaner. All purified compounds had only one radioactive spot, the  $R_f$  of which corresponded to that of the unlabeled compound. Possible side-products—usually phenols—were also compared by paper chromatography to ensure no overlapping of spots from the parent and its side-products.

Carbamates were chromatographed on a 10-gram activated Florisil column using ether-hexane mixtures as the eluant (2). In all cases, phenolic by-products came out in the first 30 to 40 ml.; the carbamates followed in the next 100 ml. Thirty grams of activity grade II (3% H<sub>2</sub>O) Woelm alumina was used to chromatograph phosphates and chlorinated hydrocarbons. The column was eluted initially with 100 ml. of benzene (10-ml. fractions), then with 50 ml. of chloroform, and finally with 100 ml. of acetone. A drop from each fraction was spotted on a long strip of filter paper and the whole strip, without further treatment, scanned by the Autoscaner to determine which fractions were radioactive. The appropriate fractions were then combined and checked by infrared for the parent compound.

For paper chromatography of the carbamates, 5% silicone (Dow-Corning 550 Silicone Fluid in acetone) treated paper was used with 35 : 65 (v./v.) pyridine-water as the solvent. The  $R_f$ 's of the carbamates were quite sensitive to changes in pyridine concentration and sometimes varied from bottle to bottle of pyridine depending on its age. The carbamates and phenolic by-products were detected by spraying the papers first with 15% KOH, heating for about 1 minute in steam, respraying with 1*N* acetic acid, and finally spraying with 0.01% methanolic *p*-nitrobenzene diazonium fluoroborate (Eastman No. P7078) (5). Spots due to phenolic constituents alone were detected by omitting the alkaline spray and steaming.

The usual paper chromatographic systems were used for the detection of phosphates (3, 7). For example, with parathion 20 : 80 (v./v.) dimethyl formamide-water was the immobile phase with trimethyl pentane as the mobile phase. Spots were developed either with a Hanes-Isherwood spray (7) using 0.01% SnCl<sub>2</sub> in 3*N* HCl as a reducing agent, or

**Table I. Reaction Conditions, Yields, and Specific Activities of Labeled Compounds**

Compound	Temp., ° C.	Time, Hours	$W_x/W_r^a$	Activity, mc./mmole	Theoretical Labeling, %	Yield, %
3,5-Diisopropylphenyl <i>N</i> -methylcarbamate	23	20	0.5	9.75	48.8	72.8
3-Isopropylphenyl <i>N</i> -methylcarbamate	23	20	0.5	0.90	3.9	76.2
1-Naphthyl <i>N</i> -methylcarbamate (Sevin)	23	20	0.5	3.26	10.2	70.0
2-Isopropoxyphenyl <i>N</i> -methylcarbamate	23	20	0.5	4.34	18.5	58.2
3,5-Dimethyl-4-methylthiophenyl <i>N</i> -methylcarbamate	23	20	0.5	5.86	40.4	69.7
2,2-Bis( <i>p</i> -chlorophenyl)-1,1,1-trichloroethane (DDT)	23	20	0.5	0.04	0.1	79.2
2,2-Bis( <i>p</i> -methoxyphenyl)-1,1,1-trichloroethane (methoxychlor)	23	20	0.5	15.5	35.4	60.1
Dimethyl 3-methyl-4-methylthiophenyl phosphorothionate (Baytex)	23	20	0.5	2.03	9.8	10.3
Diethyl 3-chloro-4-methyl-7-coumarinyl phosphorothionate (Co-ral)	23	20	0.5	0.42	1.7	40.0
Diethyl <i>p</i> -nitrophenyl phosphorothionate (parathion)	23	20	0.5	0.27	1.0	2.5
	23	20	0.25	1.10	3.6	2.0
Dimethyl <i>p</i> - <i>N</i> -dimethylsulfamoylphenyl phosphate	50	6	0.5	0.00045	0.0015	19.1
Naphthalene	23	20	0.5	11.28	42.7	65.3

<sup>a</sup>  $W_x/W_r$  is the weight ratio of insecticide to reagent; 65 mc. of HTO were used.

with 2,6-dibromo-*N*-chloro-*p*-quinoneimine (4).

Paper chromatography of the chlorinated hydrocarbons was carried out according to Mitchell (6).

The specific activity of each compound was determined with a Packard Tri-Carb Scintillation Counter. Because some of the compounds exhibited a self-quenching effect, counts were determined on several successive dilutions of the labeled compound. Values of those dilutions which gave counts that were a linear function of concentration were used to determine the true specific activity. Duplication of runs varied on the average  $\pm 15\%$ ; the more highly tagged compounds varied the least (1 to 5%), while compounds with the least tagging varied the most (20 to 40%).

Yields, as might be expected, were the lowest for phosphates (0 to 40%) and the best for chlorinated hydrocarbons (60 to 79%); carbamates lay in between (50 to 75%). The physical conditions of the reaction markedly influenced yields—parathion, for example, gave 0% at 50° C., but 2 to 5% at room temperature. Duplication of runs varied on the average  $\pm 5\%$ .

### Results and Discussion

In the tables, the theoretical labeling  $S_z$  is calculated by the formula of Yavor-sky and Gorin (7):

$$S_z = \frac{S_r}{\frac{3M}{166N} + \frac{W_x}{W_r}}$$

where  $S_r$  is the specific activity of the H<sub>3</sub>\*PO<sub>4</sub>·BF<sub>3</sub> complex;  $M$  is the molecular weight of insecticide;  $N$  is the number of sites per insecticide molecule available for tritiation—i.e., H-substituted aromatic carbons;  $W_x$  is the weight of insecticide; and  $W_r$  is the weight of complex.

Three aspects of molecular structure are of importance in controlling specific activity, which is presumably increased by substituents which promote electrophilic substitutions. Firstly, the activating effects of substituents are to be considered. Thus *m*-directing substituents (such as nitro or quaternary nitrogen) and halogens are ring-deactivating and therefore tend to decrease labeling, whereas *o,p*-directing substituents other than halogens—e.g., amino or alkoxy—are ring-activating and tend to increase labeling (8). Secondly, the directional influence has to be such that it directs towards a site available for substitution. For example, 3,5-dimethylaniline should label better than 2,4-dimethylaniline, in which two of the three favored positions are unavailable for tritiation. Thirdly, compounds with a large number of sites available for tritiation will be more labeled.

Although methoxy and chlorine are both *o,p*-directing, only the chlorine is ring-deactivating, and consequently methoxychlor gives 354 times higher specific activity than DDT (Table I). Comparing Sevin and naphthalene, the specific activity is reduced 3.5-fold by carbamoylation. This is attributable to the deactivation of the ring, caused by

**Table II. Tritiation of Naphthalene under Various Reaction Conditions**

Run No.	Temp., ° C.	Time, Hours	Container	HTO Added, mc.	$W_z/W_r$	Specific Activity, mc./mmole	Theoretical Labeling, %
1	23	20	Glass	10	0.5	0.17	4.2
2	23	43	Glass	10	0.5	0.13	3.2
3	23	20	Polyethylene	10	0.5	0.06	1.5
4	65	5.5	Glass	10	0.5	1.43	35.2
5	65	20	Glass	10	0.5	1.56	38.4
6	23	20	Glass	65	0.5	11.3	42.7

the protonation of the nitrogen under the very acid conditions of the reaction. The 3-isopropylphenyl *N*-methylcarbamate is poorly labeled because the effect of the *m*-isopropyl group in making carbons 2, 4, and 6 partially negative is neutralized in part by the tendency of the carbamoyl group to make these same carbons positive. By contrast, the 3,5-diisopropylphenyl *N*-methylcarbamate labels very well because the two isopropyl groups reinforce the negativity of carbons 2, 4, and 6. Also 2-isopropoxyphenyl *N*-methylcarbamate labels quite well—the isopropoxy group is a better activator than the isopropyl. Furthermore, it makes carbons 3 and 5 partially negative, and this effect is not neutralized by the carbamoyl whose influence is on carbons 2, 4, and 6. The insecticidal organophosphates label relatively poorly, probably because a strongly electrophilic substituent is commonly a prerequisite for anticholinesterase activity (9).

The specific activity is also subject to variations in molecular weight. Since a fixed weight of reactant was used each time, the molar ratio of reagent to reactant varies.

Yavorsky and Gorin (11, 12) reported that the rate of labeling is a function of temperature. Accordingly, several runs with naphthalene were made under different conditions of temperature and time. When the reaction temperature was raised, a higher degree of labeling was indeed obtained, and a higher reaction temperature could therefore be used to increase the labeling of compounds which labeled poorly under normal conditions—providing, of course, that the compound was stable at the higher temperature (Table II). In fact, a poor compound, even though somewhat unstable at higher temperatures, could be labeled more strongly by allowing the reaction to go for only a short time at the higher temperature since most of the labeling takes part in the earlier part of the reac-

tion. The naphthalene runs also showed that no further labeling of naphthalene was obtained after 6 hours even though theoretical labeling had not been reached. There was a large discrepancy between the 10-mc. and 65-mc. runs. The 10-mc. run gave a per cent labeling of 4.2, but the 65-mc. run gave a per cent labeling of 42.7—10 times as great, although only a 6.5-fold increase was expected. This may be due to the difficulty of adding a small amount of tritiated water (10  $\mu$ l. for the low level runs) to  $P_2O_5$ . Even though the  $P_2O_5$  was cooled in a mixture of dry ice-acetone, a violent reaction took place when the tritiated water was added, and some tritiated water may have been lost in the form of steam. Yavorsky (10) loads  $P_2O_5$  into a rubber tube, and expels it slowly with a rod into the stirred water. This would be difficult on the micro scale.

Another discrepancy in labeling was noted when the weight of tritiating reagent was doubled. Parathion was labeled, first using the usual ratio  $W_z/W_r$  of  $1/2$ . The experiment was then repeated using the same specific activity for the reagent but with a weight ratio of  $1/4$ . This should have increased the specific activity of the parathion by 16%. In actual fact, however, the specific activity was increased 265%.

Let us consider how this semimicro method compares with the macro method of Yavorsky and Gorin (11, 12). The only direct comparison available is for naphthalene. In 6 hours at room temperature, Yavorsky and Gorin obtained 45.6% of theoretical labeling. The present authors obtained a comparable value (42.7%) with the 65-mc. run continued for 20 hours. With smaller quantities (10 mc.) poor labeling of naphthalene was found at room temperature—e.g., 4.2% at 20 hours—but was much better at 65° C.—e.g., 38.4% at 20 hours. Yavorsky and Gorin (working with benzene only) found that pro-

longing treatment to 80 hours gave 100% of theoretical, even though their standard 6-hour run gave only 17.2%. However, the present authors found that with naphthalene (in a 10-mc. run at 23° C.) increasing the time to 43 hours did not improve labeling (Table II); a similar observation was made with parathion.

Yavorsky (10) has pointed out that further restrictions on the method are that basic compounds form salts with the acid reagent and isotope exchange is prevented; and that readily dehydrated materials, such as carbohydrates, are dehydrated during the exchange. He also recommends use of a 5% excess of  $P_2O_5$  in making up the reagent, to scavenge moisture, and considers that this would bring naphthalene labeling to the calculated level, and would account for the great increase in effectiveness when more tritiating agent was used. An excess was in fact used in the present study (see Methods), but scavenging may be more difficult on the micro scale.

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