AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D C 20523

BIBLIOGRAPHIC INPUT SHEET

FOR AID USE ONLY
Batch 69

1. SUBJECT CLASSIFICATION
A. PRIMARY: Health
NS00-0000-0000
B. SECONDARY: Tropical diseases

2. TITLE AND SUBTITLE
Quantitative method for determination of DDT and DDT metabolites in blood serum

3. AUTHOR(S)
Dale,W.E.; Miles,J.E.; Gaines,T.B.

4. DOCUMENT DATE: 1970
5. NUMBER OF PAGES: 7p.
6. ARC NUMBER: ARC

7. REFERENCE ORGANIZATION NAME AND ADDRESS
HEW/PHS

8. SUPPLEMENTARY NOTES (Sponsoring Organization, Publishers, Availability)
(In J.of Assn.of Official Analytical Chemists,v.53,p.1287-1292)

9. ABSTRACT

10. CONTROL NUMBER
PN-AAE-570

11. PRICE OF DOCUMENT

12. DESCRIPTORS
Blood
Insecticides
Serodiagnosis
Tests

13. PROJECT NUMBER

14. CONTRACT NUMBER
PASA RA(HA)-7-00 Res.

15. TYPE OF DOCUMENT

AID 590-1 (4-74)

REPRINT NUMBER

486

Quantitative Method for Determination of DDT and DDT Metabolites in Blood Serum

Technical Development Laboratories
Laboratory Division
Center for Disease Control
P. O. Box 2167 · Savannah, Ga. 31402

By WILLIAM E. DALE and JAMES W. MILES (Technical Development Laboratories, Center for Disease Control, Health Services and Mental Health Administration, Public Health Service, Savannah, Ga. 31402) and THOMAS B. GAINES (Atlanta Toxicology Branch, Division of Pesticide Chemistry and Toxicology, Food and Drug Administration, 4770 Buford Highway, Chamblee, Ga. 30341)

A new method for the extraction and determination of DDT and related materials in blood has been developed. Rats were given a single oral dose of ^{14}C-labeled DDT, and it was demonstrated by parallel gas chromatographic and radiometric measurements that *in vivo*-introduced DDT and related materials could not be removed quantitatively from serum by simple hexane extraction. Pre-treatment of the serum with equal volumes of formic acid released the binding of DDT and DDT metabolites and permitted the extraction of these materials with *n*-hexane, yielding 95% recovery. When applied to sera of men from the general population and of men who had been exposed to DDT for more than 10 years in a manufacturing plant, this method gave consistently higher results than did the simple hexane extraction method.

DDT is so widely distributed in nature that traces can be found in almost all living organisms. Because of its solubility in fat, higher concentrations of DDT are found in adipose tissues than in other tissues. When an animal receives a toxic dose of DDT, the site of action is the central nervous system (1, 2). In rats, the toxic manifestations have been shown to be related to brain concentrations (1). It has also been shown that the concentration of DDT in the brain increased with increases of DDT in blood plasma (1). Brown *et al.* (3) reported a correlation between the levels of dieldrin in the blood and clinical signs of poisoning in humans. Significant correlations between the concentrations of DDT-related materials in the fat and in the blood have also been reported (4, 5). In view of the difficulties experienced in obtaining tissues other than blood from human subjects, blood serum has served as the tissue of choice in surveys designed to monitor the levels of concentration of chlorinated pesticides in human populations. Invariably, persons with high exposures to chlorinated hydrocarbon pesticides have relatively high concentrations of those pesticides in their blood (5–7).

With the advent of highly sensitive detectors, a number of gas chromatographic (GLC) methods have been developed for the determination of DDT and related materials in blood (4, 6–12); however, none has been completely satisfactory. Lengthy cleanup procedures were used in the methods described by Kadis and Jonasson (8), Saschenbrecker and Ecobichon (9), and Morgan and Roan (10). Procedures destructive to DDT were recommended in the methods described by Schafer and Campbell (4), Crosby and Archer (11), and Robinson and Hunter (12). To obtain reproducible results, the steam distillation method of Dale *et al.* (6) requires extreme care in preparation of the distillation apparatus as well as a high degree of technical skill. For these reasons, it is not suitable for routine analyses which are required in a monitoring program. The simple hexane extraction method described by Dale *et al.* (7) is probably the most widely used method for the determination of chlorinated hydrocarbon pesticides in blood, even though the authors show that quantitative recovery of added DDT and other related compounds is not obtained. They attributed this to *in vitro* binding of the chlorinated pesticides to serum proteins and suggested the possibility of *in vivo* binding. More recently Dale *et al.* (6) recognized that *in vivo* binding takes place with all chlorinated insecticides in blood and that the 2 kinds of binding are not identical. Gunther *et al.* (13) observed the binding of DDT and similar compounds to protein molecules and attributed this to van der Waals' forces. Bonderman *et al.* (14) made a statistical examination of the Dale *et al.* (7) method with respect to *p,p'*-DDE and concluded that aging the sample seriously reduced recovery of added DDE. This was attributed to degradation of lipoprotein, which affected the chromatograms.

Received June 15, 1970.

None of the above methods offers a truly accurate means of determination of *in vivo*-bound DDT and related metabolites.

The purpose of this study was to determine the efficiency of extraction of DDT and related materials by the Dale *et al.* (7) method and to improve this method, if possible. This was accomplished by dosing animals with ^{14}C-labeled DDT and conducting parallel chemical and radiometric measurements of the concentrations of DDT and related materials in the extracts obtained from serum samples by various extraction procedures. The bound pesticidal materials remaining in the residue after extraction by the various techniques were measured radiometrically.

METHOD

Apparatus and Reagents

(a) *Gas chromatograph.* Varian Aerograph Series 200 (Varian Aerograph, Walnut Creek, Calif.), with electron capture detector, 8 mCi ^{63}Ni source, and 1 mv full scale strip chart recorder (Sargent SRG, Chicago, Ill.). *GLC conditions:* detector 265°C, inlet and outlet blocks 240°C, column oven 205°C, column 4'8" × $^1/_4$" od aluminum tube packed with mixture of equal parts of column packing A and column packing B (*see* (b) below), nitrogen carrier gas 35 ml/min at 70 psi, and standing current 1×10^{-9} amp.

(b) *Column packings.* *A:* 3% OV-17 on 100–120 mesh acid-washed, DMCS-treated Chromosorb W (Applied Science Laboratories, Inc., State College, Pa.). *B:* 5% OV-210 on 100–120 mesh acid-washed, DMCS-treated Chromosorb W.

(c) *Vortex mixer.* Catalog No. 12-812B1 (Fisher Scientific Co.).

(d) *Pesticide standard solutions.*—Prepare standards (obtained as 99–100% pure from Pesticide Repository, Pesticides Research Laboratory, Perrine, Fla.) by accurately weighing ca 20 mg each of *o,p'*-DDE, *p,p'*-DDE, *o,p'*-DDD, *p,p'*-DDD, *o,p'*-DDT, and *p,p'*-DDT into separate 25 ml volumetric flasks and making to volume with isooctane. By serial dilution, prepare standard mixture to contain ca 10 pg/μl each of *o,p'*-DDE and *p,p'*-DDE; 15 pg/μl each of *o,p'*-DDD, *p,p'*-DDD, and *o,p'*-DDT; and 30 pg/μl of *p,p'*-DDT.

(e) *n-Hexane.* Redistill over 200 ml methyl alcohol plus 2 g metallic sodium 4 L hexane. Discard first 1 L distillate.

(f) *Formic acid.* Practical 97% (Eastman Chemical Co., Rochester, N.Y.).

The use of trade names is for identification purposes only and does not constitute endorsement by the Public Health Service or the U.S. Department of Health, Education, and Welfare.

Preparation of Standard Curve

Inject 1–5 μl mixed standard into gas chromatograph, using conditions described above. Plot peak heights vs. quantities injected to obtain standard curve for each compound, which is linear over range of concentrations used.

Procedure

Collect 5–10 ml blood from each subject and allow to clot. Centrifuge 10 min at 3000 rpm. With Pasteur pipet, carefully remove serum and place in 15 ml Teflon-lined, screw-cap centrifuge tube. (If serum is to be shipped to another point for analysis at this time, it should be packed in Dry Ice in Styrofoam container.) Pipet 1 ml serum plus 1 ml 97% formic acid into 15 ml centrifuge tube and shake tube 1 min on Vortex mixer. Pipet 5 ml *n*-hexane, shake 1 min, let phases separate, and then shake again 1 min. Centrifuge 3–5 min to insure separation of phases. Carefully remove *n*-hexane phase and place in second centrifuge tube. Again pipet 5 ml *n*-hexane into tube containing serum and repeat extraction as described. Combine extracts, wash combined extract once with 1 ml 5% potassium carbonate for 1 min by shaking on Vortex mixer, and discard aqueous phase. If subject has known history of high exposure to DDT, make preliminary analysis at this time by injecting 3–5 μl combined extract directly into gas chromatograph. For samples from general population, pipet 9 ml aliquot into 15 ml centrifuge tube. Place melting point tube sealed ca 1 cm from end into centrifuge tube to prevent bumping during evaporation. Place tube in 80°C water bath and carefully evaporate *just* to dryness. Add 1 ml *n*-hexane to tube and shake tube 1 min. Inject appropriate aliquots into gas chromatograph operated as described.

Experimental

To study the efficiency of various extraction methods for recovery of DDT-related materials in serum and to compare these methods with the simple hexane extraction method, it was necessary to have a source of serum containing DDT and related materials at measurable levels. This source was obtained by dosing rats with *p,p'*-DDT.

Six adult male rats of the Sherman strain, weighing 302–337 g, were used. The rats were individually caged and were non-fasting. Three rats were dosed by stomach tube with *p,p'*-DDT (99+%) dissolved in peanut oil at a concentration of 3% (w/v). The solutions were prepared just prior to administration and were given at the rate of 5 ml/kg body weight to achieve a dosage level of 150 mg *p,p'*-DDT/kg body weight. Three control rats were given peanut oil only as described.

The rats were bled 6 hr after dosing, at which time all of the *p,p'*-DDT-dosed rats were exhibiting mod-

erate tremors. As much blood as possible was taken from each rat by cardiac puncture with a 20 ml syringe equipped with a 20 gauge needle. The blood was transferred to a 15 ml centrifuge tube and allowed to clot. After centrifugation the serum was removed as described. The sera from the dosed rats were combined and stored in a 25 ml screw-cap test tube, and the sera from the control rats were combined and stored in a similar tube for not more than 3 to 4 days before analysis. This gave about 20 ml each of control and dosed serum for preliminary studies.

A modification of the Nawa *et al.* (15) performic acid method for the release of protein-bound lipoic acid has been described by Perry *et al.* (16) for the extraction and isolation of conjugated metabolites of DDT from body lice. Attempts were made to use the performic acid treatment of serum, but foaming of the samples made the method unworkable. However, when the hydrogen peroxide was omitted, no foaming occurred, and consistently higher results were obtained when the serum was mixed with an equal volume of 97% formic acid at room temperature prior to extraction with *n*-hexane. Even though higher results were obtained with the formic acid addition, there was no way to determine whether the recovery was quantitative.

Although the analysis of serum samples spiked with known concentrations of DDT has been useful in studying extraction techniques, it was felt that an absolute method for determination of extraction efficiency would require *in vivo* studies with radiolabeled material. Therefore the experiment described above was repeated, except that ^{14}C-labeled *p,p'*-DDT was substituted for *p,p'*-DDT. The ^{14}C-DDT used was that prepared by Pearce and Jensen (17) and was labeled in the tertiary position. Some of this product was recrystallized from ethanol and dried to yield crystals which melted at 108–108.5°C. The specific activity of the resulting *p,p'*-DDT was 0.5 mCi/g. Three Sherman strain rats were dosed with the labeled DDT at 150 mg/kg in the same manner as described above and were bled and sacrificed after 6 hr. The total ^{14}C-DDT concentration in the serum of the treated rats was determined by direct liquid scintillation counting on 0.2 ml samples of serum according to the method described by Gjone *et al.* (18), using the scintillation solution recommended by Hall and Cocking (19). The concentrations of ^{14}C-DDT remaining in the serum samples after extraction with hexane were determined in the same manner with the exception of those which had been treated with formic acid. Samples to which formic acid had been added were neutralized with 5*N* alcoholic potassium hydroxide before solubilization and counting by the Gjone *et al.* method. Aliquots of hexane extracts of samples with and without formic acid treatment were transferred to 20 ml counting vials and evaporated to dryness. After the addition of counting solution, the samples were counted in a routine manner. Aliquots of the same extracts were analyzed for DDT and DDT metabolites by GLC.

Known quantities of ^{14}C-DDT were added to serum from control rats to produce standard solutions for comparison with unknown radioactive serum samples. Known solutions of ^{14}C-DDT in hexane were prepared for use as standards for determination of concentrations of ^{14}C-DDT in hexane extracts of radioactive serum samples. Preparation of 2 series of standard solutions was necessary because of the lower counting efficiency obtained by the Gjone *et al.* method for ^{14}C in serum samples. Control rat serum samples and *n*-hexane samples without added ^{14}C-DDT were carried through appropriate procedures to obtain background counting rates. All radioactive samples were counted on a Nuclear-Chicago Mark I liquid scintillation counter, Model 6860.

In order to determine the precision of the proposed GLC method, 6 replicate determinations for DDT and related materials were made on 1 serum sample from an exposed person. The mean, standard deviation, and relative standard deviation were calculated for each DDT-related component.

The method was then applied to serum samples from 5 men of the general population and from 4 men who had been exposed to DDT for more than 10 years in a DDT-manufacturing plant. A comparison was made between the formic acid-hexane extraction method and the simple hexane extraction method.

Results and Discussion

Data presented in Table 1 show that by radiometric analysis (RA) 1.3 ppm (8%) of the *in vitro* added DDT-derived material remained in the serum after 2 extractions with *n*-hexane and that none remained in the serum when it was treated with formic acid prior to extraction with *n*-hexane. When the simple hexane extraction method was used, material balances of 87 and 96% were obtained by GLC and RA, respectively. When the serum was treated with formic acid before extraction with *n*-hexane, material balances of 91 and 105% were obtained by GLC and RA, respectively. When rats were dosed with ^{14}C-labeled DDT and the serum was analyzed radiometrically, 4.30 ppm (37%) of the total DDT-derived material remained in the serum after extraction with *n*-hexane. However, when the serum was treated with formic acid before extraction with hexane, only 0.10 ppm (<1.0%) of the total DDT-derived material remained in

Table 1. Comparison of recoveries (in ppm) of *in vitro*- and *in vivo*-introduced ^{14}C-DDT from rat serum obtained by the hexane extraction method and the formic acid-hexane method

Determination	*In vitro* GLC	*In vitro* Radio-metric	*In vivo* GLC	*In vivo* Radio-metric
Source Studies				
A. ^{14}C-DDT[a]		15.6		11.71
B. Concn in serum after extn with *n*-hexane		1.3		4.30
C. Concn in serum after extn with HCOOH + *n*-hexane		0.0		0.10
Hexane Extraction				
1st extn	9.47	10.20	5.23	5.13
2nd extn	2.80	3.48	2.19	2.09
1st + 2nd extns	12.27	13.68	7.42	7.22
B	1.30	1.30	4.30	4.30
Total	13.57	14.98	11.72	11.52
Recovery, %	87	96	100	98
Formic Acid-Hexane Extraction				
1st extn	13.71	16.00	11.20	10.50
2nd extn	0.43	0.36	0.21	0.20
1st + 2nd extns	14.14	16.36	11.41	10.70
C	0.00	0.00	0.10	0.10
Total	14.14	16.36	11.51	10.80
Recovery, %	91	105	98	92

[a] For *in vitro* study, 15.6 ppm ^{14}C-DDT was added to serum; for *in vivo* study, ^{14}C-DDT refers to total concentration found by radioassay before extraction.

the serum. Material balances of 98 and 100% were obtained by RA and GLC, respectively, with the simple hexane extraction method. A material balance of 92 and 98% was obtained by RA and GLC, respectively, for *in vivo* added DDT and related materials, using the formic acid-hexane extraction method.

Both these data and those of Dale *et al.* (6) show that there is a difference in *in vitro* and *in vivo* binding and that DDT and related materials present *in vivo* are more difficult to extract from serum.

For monitoring purposes a rapid, sensitive, accurate, and precise method is needed. Although it is recommended that the serum be extracted twice, it can be readily seen that approximately 95% of the total DDT-related material was removed by one extraction with formic acid-hexane and that, in the interest of time, one may choose this option.

Table 2. Precision of formic acid-hexane extraction method for DDT and related materials

Compound	Range, ppm	$\bar{X}_6$	Std Dev.	Rel. Std Dev., %
p,p'-DDE	0.782 0.841	0.808	0.024	2.97
o,p'-DDT	0.240 0.300	0.270	0.020	7.41
p,p'-DDT	1.776 1.982	1.885	0.071	3.77

The range, mean, standard deviation, and relative standard deviation for the determination of *p,p'*-DDE, *o,p'*-DDT and *p,p'*-DDT in serum of an occupationally exposed man are presented in Table 2. These data show a relative standard deviation of 2.97 and 3.77% for *p,p'*-DDE and *p,p'*-DDT, respectively, which is well within the 5% reported by Schafer (20). The slightly higher relative standard deviation (7.41%) obtained for *o,p'*-DDT was undoubtedly due to the lower concentration of this isomer in serum, which made peak height measurements more difficult.

Studies accomplished as part of a contractural agreement between the Center for Disease Control and the Agency for International Development.

Table 3. Comparison of formic acid-hexane method with hexane extraction method for determination of DDT and related materials in serum of general population (values expressed in ppm)

	Formic Acid-Hexane Method (FH)				Hexane Extraction Method (HE)				Per Cent Increase[a]
Sample	*p,p'*-DDE	*o,p'*-DDT	*p,p'*-DDT	Total as DDT	*p,p'*-DDE	*o,p'*-DDT	*p,p'*-DDT	Total as DDT	[(FH − HE)/HE] × 100
1	0.020	0.007	0.023	0.052	0.016	0.008	0.016	0.042	23.8
2	0.047	0.008	0.030	0.090	0.032	0.010	0.024	0.070	28.6
3	0.026	0.007	0.026	0.061	0.020	0.011	0.021	0.054	13.0
4	0.033	0.007	0.025	0.068	0.023	0.008	0.015	0.049	38.8
5	0.030	0.008	0.035	0.076	0.021	0.009	0.026	0.058	31.0

[a] Percentage increase in total DDT-derived materials as DDT obtained by hexane-formic acid method compared to the simple hexane extraction method.

Results obtained by the formic acid-hexane and by the simple hexane extraction methods for the determination of DDT-related materials in the serum of 5 men of the general population are reported in Table 3. In all cases, higher results were obtained for *p,p'*-DDE and *p,p'*-DDT by the formic acid-hexane method than by the simple hexane extraction method. Although very slightly higher results were obtained for *o,p'*-DDT by the hexane extraction method this may not be a true reflection of a difference in the 2 methods in that maximum sensitivity of the method for *o,p'*-DDT is 0.007 ppm. A comparison of results obtained by the 2 methods for total DDT-derived materials calculated as DDT showed that the formic acid-hexane extraction method was 13–38.8% more efficient than the hexane extraction method.

Results obtained for DDT and related materials from the serum of the 4 occupationally exposed men are presented in Table 4. Comparison of the results obtained by the formic acid-hexane method and the original hexane extraction method shows that higher results were obtained for DDT and all related materials by the formic acid-hexane method. Values for total DDT-derived materials obtained by the formic acid-hexane method increased from 15.6 to 66.1% over those found by the simple hexane extraction method.

A typical chromatogram of a serum extract from an occupationally exposed individual and a chromatogram of DDT and related compounds are shown in Fig. 1. Peaks corresponding to *p,p'*-DDE, *o,p'*-DDT, and *p,p'*-DDT were found in all samples analyzed. Weak peaks corresponding to *o,p'*-DDE and *p,p'*-DDD were observed occasionally in occupationally exposed individuals. No peak corresponding to *o,p'*-DDD was observed in any of the samples.

Conclusions

A modification of the Dale *et al.* (7) method for the determination of DDT and DDT metabolites in serum is described. The new method is rapid, simple, and more precise and accurate than the original method. A simple but important modification releases bound DDT and its metabolites without degradation. This permits the extraction of total DDT-related materials from serum. An additional advantage of the formic

Table 4. Comparison of formic acid-hexane method with hexane extraction method for determination of DDT and related materials in serum of occupationally exposed men (values expressed in ppm)

	Formic Acid-Hexane Method (FH)				Hexane Extraction Method (HE)				Per Cent Increase[a]
Sample	*p,p'*-DDE	*o,p'*-DDT	*p,p'*-DDT	Total as DDT	*p,p'*-DDE	*o,p'*-DDT	*p,p'*-DDT	Total as DDT	[(FH − HE)/HE] × 100
1	0.38	0.07	0.43	0.92	0.25	0.04	0.28	0.60	53.3
2	0.74	0.08	1.03	1.93	0.58	0.05	0.97	1.67	15.6
3	1.02	0.07	1.03	2.24	0.68	0.06	0.67	1.49	50.3
4	1.04	0.21	2.60	3.97	0.60	0.14	1.58	2.39	66.1

[a] Percentage increase in total DDT-derived materials as DDT obtained by hexane-formic acid method compared to the simple hexane extraction method.

acid-hexane method is the elimination of emulsions which frequently form when serum is extracted with hexane in the absence of formic acid.

Acknowledgment

The authors gratefully acknowledge the technical assistance of Lee M. Alderman.

REFERENCES

(1) Dale, W. E., Gaines, T. B., Hayes, W. J., Jr., and Pearce, G. W., *Science* **142**, 1474–1476 (1963).
(2) O'Brien, R. D., and Matsumura, F., *Science* **146**, 657–658 (1964).
(3) Brown, V. K. H., Hunter, C. G., and Richardson, A., *Brit. J. Ind. Med.* **21**, 283–286 (1964).
(4) Schafer, M. L., and Campbell, J. E., *Organic Pesticides in the Environment*, R. F. Gould (Ed.), American Chemical Society, Washington, D.C., 1966, pp. 89–98 (Chapter 7).
(5) Laws, E. R., Curley, A., and Biros, F. J., *Arch. Environ. Health* **15**, 766–775 (1967).
(6) Dale, W. E., Curley, A., and Hayes, W. J., Jr., *Ind. Med. Surg.* **36**, 275–280 (1967).
(7) Dale, W. E., Curley, A., and Cueto, C., Jr., *Life Sci.* **5**, 47–54 (1966).
(8) Kadis, V. W., and Jonasson, O. J., *Can. J. Public Health* **56**, 433–436 (1965).
(9) Saschenbrecker, P. W., and Ecobichon, D. J., *J. Agr. Food Chem.* **15**, 168–170 (1967).
(10) Morgan, D. P., and Roan, C. C., *Arch. Environ. Health* **20**, 452–457 (1970).
(11) Crosby, D. G., and Archer, T. E., *Bull. Exp. Contam. Toxicol.* **1**, 16–20 (1966).
(12) Robinson, J., and Hunter, C. G., *Arch. Environ. Health* **13**, 558–563 (1966).
(13) Gunther, F. A., Blinn, R. C., Carmen, G. E., and Metcalf, R. C., *Arch. Biochem. Biophys.* **50**, 504–505 (1954).
(14) Bouderman, D. P., Choi, U. Y., Hetzler, H. L., and Long, K. R., *JAOAC* **52**, 1063–1066 (1969).
(15) Nawa, H., Brady, W. T., Koike, M., and Reed, L. J., *J. Amer. Chem. Soc.* **82**, 896–903 (1960).
(16) Perry, A. S., Miller, S., and Buckner, A. J., *J. Agr. Food Chem.* **11**, 457–462 (1963).

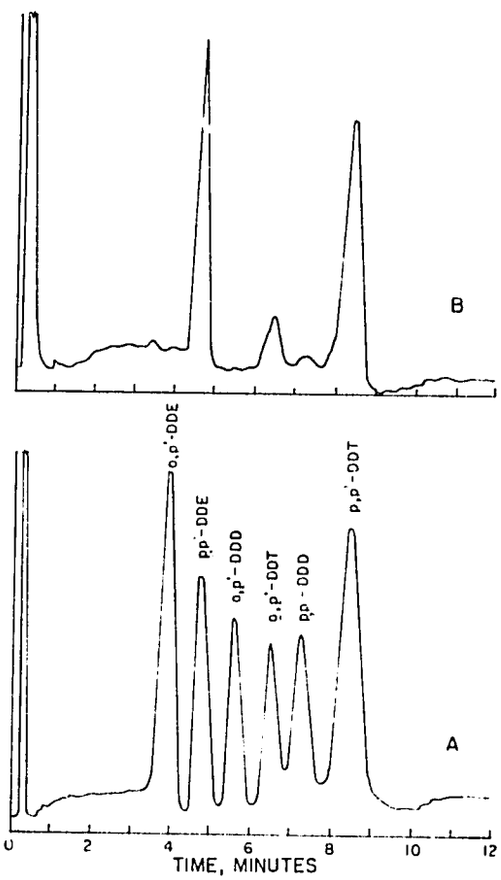

FIG. 1—Gas chromatograms of standard DDT-type compounds (A) and of a serum extract from a person occupationally exposed to DDT (B).

(17) Pearce, G. W., and Jensen, J. A., *J. Agr. Food Chem.* **1**, 776–778 (1953).
(18) Gjone, E., Vance, H. G., and Turner, D. A., *Int. J. Appl. Rad. Isotopes* **8**, 95–97 (1960).
(19) Hall, T. C., and Cocking, E. C., *Biochem. J.* **96**, 626–633 (1965).
(20) Schafer, M. L., *Residue Rev.* **24**, 19–39 (1968).