Discussion

Shell Compound 4072 is quantitatively hydrolyzed by boiling with 12*N* sulfuric acid for 30 minutes However, the heating time is not critical and a heating period of 1 hour neither increased or decreased the amount of 2,2′,4′-trichloroacetophenone produced. The concentration of sulfuric acid is not too critical, either, since 9*N* sulfuric gave complete hydrolysis in 1 hour.

In the description of the chromatographic columns used for cleanup, specific volumes of solvents are given. However, because of the variation in adsorbents these volumes may vary. All chromatographic adsorbents used should be calibrated.

The column temperature of 200° C. is higher than necessary for determination of 2,2′,4′-trichloroacetophenone; however, this temperature is required to remove DDT and other less volatile unknown compounds in a reasonable length of time. Under some circumstances a lower column temperature might be better for separation of interfering materials.

Trichloroacetophenone is very volatile and care must be taken to prevent its loss. Solutions should be concentrated by distillation through a Snyder column, or a *n*-hexane solution may be concentrated by evaporation at room temperature with a jet of air. Care should be taken to remove dichloromethane completely before the gas chromatographic analysis is performed.

When Shell Compound 4072 was added to the body tissues at the 0.05-p.p.m. level and to milk at the 0.1-p.p.m. level, and carried through the complete procedure described above, 71% was obtained from fat, 95% from milk, and 82 to 92% from the other tissues.

Recoveries of 2,2′,4′-trichloroacetophenone added to milk and the various body tissues amounted to 68%. By using the method as described above, maximum sensitivity would not be achieved, since the analysis is run on aliquots of a 5-ml. solution. With a more efficient cleanup procedure, this volume could be decreased to 1 ml. with a corresponding increase of sensitivity. However, this amount of solution is not recommended for analysis with the cleanup methods described. With the range setting at 10^{-9} ampere, 0.1 nanogram of Shell Compound 4072 or 0.062 nanogram of 2,2′,4′-trichloroacetophenone will give a response of 4% on the recorder. Control samples give no peaks at the retention time of 2,2′,4′-trichloroacetophenone, so that these amounts are readily detected. The amounts detectable represent residues of 0.005 p.p.m. of Shell Compound 4072 and 0.003 p.p.m. of trichloroacetophenone in the tissues, and 0.001 and 0.0006 p.p.m., respectively, in milk.

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METABOLISM OF INSECTICIDE RESIDUES

Fate of Inhaled C^{14}-TDE in Rabbits

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New Zealand Red rabbits, selected for their tolerance to mainstream tobacco smoke, were exposed to smoke from cigarettes containing 12 and 48 μg. of C^{14}-TDE per cigarette in Holland smoking boxes. The animals received smoke from 20 cigarettes per day for periods ranging from 2 weeks to 6 months, after which times they were sacrificed and 20 tissues examined for total and organosoluble radioactivity. The deposition via inhalation appears to follow that of oral ingestion with accumulation of TDE in the fat, followed by slow metabolism and elimination. There was no evidence of accumulation in the inhalation system or other vital organs. Human noninhaling smokers appear to exhale all TDE components of main-stream smoke, whereas inhaling smokers appear to retain (for subsequent storage and metabolism) about 3% of the TDE contained in a cigarette.

The fate of TDE [1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane] on tobacco from application through the various phases of production and utilization has been reported (*2*). Commercial cigarettes (70 mm. long) contained approximately 12 p.p.m. of TDE, and the mainstream smoke from these cigarettes contained 1.6 μg. of TDE and 1.4 μg. of the major heat-induced degradation product TDEE (1-chloro-2,2-bis - *p* - chlorophenylethylene) per "smoked" cigarette (one 35-ml. puff per minute, 2-second duration for seven puffs). The presence of TDE in commercial cigarette smoke was confirmed subsequently (*11*). Although the toxicity and fate of ingested and dermal doses of TDE in mammalian systems have been investigated (*3, 8, 9*), similar investigations have not been made with inhaled TDE. A report has been made of the vapor toxicity of chlordan (*6*) and hexachlorocyclopentadiene, an intermediate in the manufacture of aldrin (*12*). Studies on the toxicity of lindane, aldrin, and DDT dispensed from vaporizers have been reported (*1*).

Studies with arsenic-74-supplemented mainstream tobacco smoke showed that rabbits retained 0.01% of the volatilized arsenic, and the distribution of the arsenic in the respiratory tract 2 hours after smoking was discussed (*5*). Following withdrawal from tobacco smoke containing As^{74}, the radioactivity decreased rapidly during the first 2 days and then tapered off slowly. The general distribution and elimination of

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arsenic have been reported by Lanz (7). The As^{74} work was later extended to smoking experiments with human volunteers, and it was found that when mainstream smoke from As^{74}-impregnated cigarettes was inhaled, levels of radioactivity found in the respiratory tract varied from 4.8 to 8.8% of that present in the smoke (4). The radioactivity was rapidly absorbed from the bronchial tree for the first several days, and by the 13th day about 10 to 15% of the total radioactivity had accumulated in this portion of the respiratory tract. Approximately 50% of the inhaled arsenic was eliminated within 10 days following treatment, primarily in the urine. The remainder was "assumed to have been deposited in the body, exhaled, and/or eliminated in body secretions and excreta over a long period of time."

To determine the site and level of deposition of inhaled TDE residue components in a mammalian system, New Zealand Red rabbits were exposed to mainstream smoke from experimental cigarettes which contained radioactive TDE. To ascertain the extent that TDE was absorbed or expelled during cigarette smoking, experiments utilizing cigarettes containing C^{14}-TDE were conducted with both inhaling and noninhaling human volunteers.

Materials and Methods

Radioactive Synthesis and Activity. Radiosynthesis of TDE was performed by the U. S. Nuclear Corp., Burbank, Calif. Radiocarbon as C^{14} was used to label C_1 of the ethane moiety of TDE. The specific activity of the pure labeled compound was 23 mcuries per mmole (34,300 c.p.m. per μg.), as determined by a Nuclear-Chicago D-47 detector with micromil window. Prior to incorporation into cigarettes, the radiolabeled compound was further purified by recrystallization in *n*-octane. The recrystallized product was found to be 95% *p,p'*-TDE as determined by paper chromatography.

Preparation of Test Cigarettes. Tobacco utilized for the experimental cigarettes was isolated from other crops to ensure no insecticidal contamination. The three varieties of insecticide-free tobacco (flue-cured, burley, and aromatic) were blended, manufactured, and packaged by a major tobacco company to produce a cigarette (70 mm. long) closely resembling a commercial brand. Appropriate amounts of labeled TDE were diluted in 100 ml. of acetone and 100 cigarettes simultaneously were dropped vertically into the insecticide solution. The treated cigarettes were air-dried at ambient room temperature for 24 hours, wrapped in aluminum foil, and smoked within the following 24 hours. Based on the absorption of 1 ml. of solution per cigarette, the final approximate concentrations of labeled TDE were 12 μg. (amount present in commercial cigarettes and hereafter referred to as the low level) and 48 μg. (high level) per cigarette. The actual radioactive dosages per cigarette were 0.916 and 3.66 μcuries for low and high level treatments, respectively.

Mainstream Smoke Exposure. Purebred New Zealand Red rabbits, specially selected for their acceptance of mainstream smoke (5), were purchased from two broods over a 1-year period from Mayfair Rabbitry, Taunton, Mass. The rabbits varied in age from 5 months to one year at the inception of the initial treatment. The animals were exposed to radioactive mainstream cigarette smoke by means of Holland smoking boxes (5) delivering 45-ml. puffs of a 2-second duration once every minute. One group of rabbits used in the 3-month smoking test received puffs of a 6-second duration. Preliminary smoking tests with insecticide-free cigarettes were made to eliminate those animals which reacted violently to the experimental conditions required for the tests.

The rabbits received smoke from 20 cigarettes per day, 5 days per week for three different exposure periods. One male was exposed for 2 weeks at the low level treatment; three males and one female were exposed at the high level for 3 months; and one male was exposed to the high level and one female to the low level for 6 months. Depending on the smoking schedules, the rabbits were exposed to smoke from 14 to 21 minutes out of each hour. The animals were either confined to the smoking box between exposure times or removed and returned to their respective cages. During exposure the rabbits were covered by plastic covers allowing only the nose and mouth to protrude to minimize contamination and subsequent oral ingestion of any contaminants during self-cleaning. The rabbits were returned to holding cages each night and the smoking boxes thoroughly cleaned subsequent to the start of each daily exposure. Control rabbits were subjected to the same conditions as exposed animals. Food, water, and other rearing facilities were in keeping with normal procedures for small animal rearing.

Analysis of Blood, Urine, and Feces. The animals were sacrificed, and 1-ml. samples of blood and urine plated directly into 1-inch aluminum planchets, dried under infrared heat, and counted. Feces samples were obtained from excised sections of the lower bowel, dried by infrared heat, ground in a Wiley mill, and pressed into 1-inch pellets (Carver laboratory press). Corrections for absorption and dilution were made with control biological extracts to which known amounts of radioactive TDE were added. Radioactive constituents were identified by extraction with chloroform and water and subsequent chromatography as described below.

Analysis of Animal Tissues. Twenty tissues per animal were examined. Freshly prepared tissues were washed in cold water, dried on paper toweling, frozen, and pulverized in anhydrous sodium sulfate to a dry paste. The finely ground tissue was then blended for 5 minutes in a Waring Blendor with a 1 to 1 ether–chloroform mixture utilizing 25 ml. of solvent for each gram of tissue. The homogenized samples were transferred to 250-ml. Erlenmeyer flasks and mixed for 2 hours on a rotor-shaker. The samples were then filtered through anhydrous sodium sulfate and the filtrate was concentrated to dryness. The residue in the filter was re-extracted in a blender using an acetone–2-propanol 80 to 20 mixture and filtered. This filtrate was added to the dried residue from the ether–chloroform extraction and concentrated to dryness and the oily residue was taken up with *n*-hexane and extracted three times with acetonitrile. After the acetonitrile fraction had been concentrated to near-dryness, a suitable aliquot was taken for radioassay. The remainder of the acetonitrile fraction was concentrated to dryness, taken up in 5 ml. of petroleum ether, and transferred to a glass column (25 × 300 mm.) packed to a depth of 15 cm. with unactivated 60- to 100-mesh Florisil (Floridin Co., Tallahassee, Fla.). The column was wet with the eluting mixture (petroleum ether–ethyl ether, 96 to 4) and 200 ml. of eluting mixture passed through the column. The eluent was collected in a 250-cc. beaker, and the contents were dried over anhydrous sodium sulfate and concentrated to a final volume of 1 to 5 ml. for subsequent chromatography. The residue remaining after the second tissue extraction (acetone–2-propanol) was shredded in a Wiley mill and prepared for radioassay in a manner identical to that for feces. Total radioactivity for each tissue fraction as well as organosoluble radioactivity was measured on the basis of combined tissue extracts.

Nature of Radioactive Substances in Tissue Extracts. Extracts of all tissues containing sufficient radioactivity were chromatographed for further determination. The chromatographic system utilized most extensively was the iso-octane plus 2-phenoxyethanol system of Mitchell (10). One-inch paper strips (Whatman No. 1) were coated with the immobile phase, and aliquots of the tissue extracts spotted on the treated papers. The strips were placed in 1000-cc. glass-stoppered glass cylinders containing 50 ml. of mobile solvent and a 4-inch section of Whatman No. 1 filter paper extending the length of the cylinder to equilibrate the chamber contents. All the strips were developed for 2 to 3 hours at 23° C. The developed chromatograms were then air-dried and sectioned into 1-cm. strips for radioassay in a windowless Nuclear-Chicago D-47 detector. The radioactivity in the various tissue extracts was identified by cochromatography of the radioactive substance with known standard compounds. The counting system used throughout these experiments permitted a counting efficiency of ±10%. Autopsies were performed by the N. C. State College Veterinary Research Section. Gross examination was made of all major organs and tissues as well as histological examination of lungs, heart, liver, and kidney.

Following sacrifice, the urine of certain animals was taken from the bladder and extracted with equal amounts of chloroform. The mixture was centrifuged at 200 r.p.m. for 5 minutes and the organic layer removed, dried over anhydrous sodium sulfate, and prepared for radioassay and chromatography.

Human Smoking of C^{14}-TDE Cigarettes. One group of noninhalers and one group of inhalers smoked one radioactive cigarette each on two separate dates. Each smoker puffed the cigarette seven times. The exhaled smoke from each group was collected by having the smokers exhale into a glass filter connected to three traps, each containing 100 ml. of acetone. A slight vacuum on the system aided the absorption of the exhaled smoke. The solvent contained in the collecting traps was concentrated to 10 ml. and 1-ml. aliquots were taken for radioassay by liquid scintillation in a Packard Tri-Carb system or windowless proportional counting in aluminum planchets.

Results and Discussion

In a preliminary experiment, it was determined that a rabbit inhaling smoke from 20 cigarettes per day for 2 weeks at the low level (0.916 μcurie per cigarette) contained measurable levels of radioactivity in all tissues (Table I). The lungs, nasal parts, larynx, and trachea showed the highest total and organosoluble radioactivity levels. Since these tissues were in close contact with the inhaled smoke during the short-term experiment, this result was expected. No attempt was made to ascertain the exact nature of the tissue components in the preliminary test. Trace amounts of radioactivity were detected on the fur of all animals, but this was considered to be an inconsequential source of error.

Analysis of Blood, Urine, and Feces. Animals on the high-level dosage and a 3-month exposure period were examined for radioactivity level in blood and excreta (Table II). Urine samples generally contained the highest levels of radioactivity, with only trace amounts found in blood or feces. There was noticeable variation in levels of activity in the same sex but no differences due to sex or brood can be established because of the lack of a sufficient number of animals in this treatment group. Chloroform extracts of pooled urine from all animals in the 3-month high-level group showed about 4.3% organosoluble radioactivity based on total radioactivity in the pooled urine samples. Chromatograms of the organosoluble fraction indicated that about 85% was present as *p,p'*-TDE with minor amounts of TDEE and a material having chromatographic and partition coefficient characteristics similar to bis(*p*-chlorophenyl) acetic acid (DDA).

Analysis of Animal Tissues. Residues of TDE or equivalents in various tissues of rabbits following exposure to cigarette smoke containing radioactive TDE at two treatment levels are indicated in Table III. A total of 20 tissues were analyzed for total and organosoluble radioactivity. In 3-month-exposure animals (high level), the average of the selected body fats contained two to three times as many TDE equivalents as any one other tissue, with the exception of the spleen and pancreas. In 6-month-exposure animals, however, this was less evident. Organosoluble radioactivity levels varied appreciably among tissues, with the greatest levels appearing in the vital organs, notably the spleen and pancreas. A general distribution of TDE equivalents is apparent in most organs of the exposed animal, but because of a lack of significant numbers of animals in these experiments and low radiotracer sensitivity, other trends regarding rates of

Table I. Concentration of Radioactivity in Rabbit Tissues Following 2-Week Exposure to Smoke from 20 Cigarettes per Day Containing 12 μg. of C^{14}-TDE per Cigarette

Tissue	Concn. of Radioactivity Found, P.P.M. Total	Apparent TDE	% Radioactivity Organosoluble
Lung	0.016	0.015	90
Larynx and trachea	0.004	0.003	61
Nasal parts	0.010	0.009	90
Kidney	0.004	0.002	55
Liver	0.003	0.002	57
Brain	0.003	0.000	10
Heart	0.002	0.000	5
Fat	...	0.000	...
Urine	...	0.001% of daily exposure	...

Table II. Radioactivity Levels in Blood, Urine, and Feces of Rabbits Following 3-Month Exposure to Smoke from 20 Cigarettes per Day Containing 48 μg. of C^{14}-TDE per Cigarette

Sex	Concn. of Radioactivity Calcd. as C^{14}-TDE: Blood, μg./ml.	Urine, μg./ml.	Feces, μg./g.
Male	0.017	0.014	0.001
	0.004	0.780	0.003
	0.013	0.709	0.011
Female	0.012	0.135	0.013
Av.	0.011	0.409	0.007

Table III. Residues of TDE in Rabbit Tissues Following Exposures of 3 and 6 Months to Mainstream Cigarette Smoke from Cigarettes Containing 12 and 48 μg. of C^{14}-TDE per Cigarette

(Mean values of concentration of radioactivity calculated as C^{14}-TDE)

Tissue	Low Level Exposure: 3 Months[a] Organosoluble, p.p.m.	3 Months[a] Total, p.p.m.	6 Months[a] Organosoluble, p.p.m.	6 Months[a] Total, p.p.m.	High Level Exposure: 3 Months[b] Organosoluble, p.p.m.	3 Months[b] Total, p.p.m.	6 Months[a] Organosoluble, p.p.m.	6 Months[a] Total, p.p.m.
Perirenal fat	...	...	0.016	0.017	0.025	0.026	0.011	0.022
Omental fat	...	...	0.020	0.021	0.027	0.028	0.033	0.073
Groin fat	...	...	0.034	0.036	0.026	0.026	0.023	0.031
Axillary fat	...	...	0.032	0.039	...	...	0.027	0.043
Av. fats	...	...	0.026	0.028	0.026	0.027	0.023	0.042
Kidneys	0.019	0.033	0.006	0.011	0.008	0.009	0.010	0.023
Liver	0.019	0.033	0.002	0.003	0.004	0.005	0.000	0.003
Stomach	...	...	0.010	0.016	0.004	0.005	0.006	0.008
Esophagus	...	...	0.005	0.005	0.005	0.006	0.024	0.093
Spleen	...	...	0.023	0.035	0.025	0.027	0.024	0.190
Heart	...	...	0.000	0.010	0.004	0.005	0.012	0.066
Pancreas	...	...	0.037	0.037	0.024	0.025	0.033	0.104
Brain	...	...	0.000	0.002	0.002	0.002	0.002	0.015
Av., vital organs			0.010	0.015	0.010	0.011	0.014	0.062
Lungs	0.002	0.006	0.001	0.003	0.006	0.011	0.006	0.024
Trachea and larynx	0.000	0.008	0.022	0.038	0.010	0.013	0.009	0.037
Nasal parts	0.010	0.011	0.003	0.007	0.003	0.004	0.003	0.034
Jaw muscle	...	...	0.026	0.054	0.004	0.006	0.014	0.054
Thigh muscle	...	...	0.001	0.001	0.002	0.002	0.004	0.018
Adrenal glands	...	...	0.050	0.065	0.010	0.012	0.044	0.385
Bladder	...	...	0.008	0.071	0.013	0.013	0.032	0.095
Reproductive tract	...	...	0.011	0.013	0.007	0.008	...	...
Testicles	...	...	...	...	0.003	0.007	0.016	0.028
Av., other tissues		...	0.019	0.027	0.006	0.008	0.022	0.116

[a] Results from 1 animal only. [b] Average of 4 animals

Table IV. Tentative Identification of Inhaled C^{14}-TDE or Equivalents in Tissues of Rabbits[a]

System	% Organosoluble Components Present as TDE	TDEE
Fat	43	56
Inhalation	59	41
Vital organs[b]	33	49
Other tissues	43	57
Mainstream smoke (*2*)	54	47

[a] Results from composite tissue of all treated animals containing sufficient radioactivity levels for organic solvent extraction. [b] About 18% of organosoluble radioactivity behaved as metabolic more polar in nature than TDE or TDEE.

Table V. Expulsion of Mainstream Tobacco Smoke by Machine and Noninhaling and Inhaling Smokers

Sample	Av. %[a] of Total Radioactivity Found in Exhaled Smoke[b]
Machine-smoked	4.7
Human, noninhalers	5.0
Human, inhalers	1.3

[a] Variability between replicate tests was less than 1%. [b] Whole cigarettes extracted in acetone contained approximately 1608 c.p.m. per cigarette and were used as reference total.

appearance, elimination, and deposition cannot be accurately defined.

There were sufficient radioactivity levels in certain tissues for co-chromatography of organosoluble extracts. These results based on composite organ systems from different animals are shown in Table IV. The mainstream smoke, as previously determined (*2*), contained TDE and TDEE in a ratio of approximately 60 to 40. Organosoluble radioactivity from the inhalation system showed a similar ratio of the two components, indicating that no significant change or metabolism took place on the surface or within the respiratory tract within the time limits of these experiments. In fat, vital organs, and other tissues, however, different ratios of TDE to TDEE were noted. The ratio of these two components is nearly the reverse in fat and the other tissues, with the greatest alteration occurring in the vital organs (principally in liver) where 33% of the organosoluble radioactivity was identified as TDE and 49% as TDEE.

Although there appears to be some storage of TDE and its less toxic analog TDEE in certain tissues following inhalation of cigarette smoke by rabbits, the mainstream smoke can be considered relatively unimportant as a source of possible contamination with TDE when a tolerance of 7 p.p.m. is permissible on food crops. The level of deposition of TDE or TDE equivalents from inhalation through cigarette smoke appears to parallel the level and route following oral ingestion of the insecticide (*3*).

Autopsy. No significant changes or abnormalities were found in any treated animals when compared to control animals receiving smoke without added insecticide. Although several animals died during the experimental period, reasons other than those imposed by the experimental conditions were responsible.

Human Smoking of C^{14}-TDE Cigarettes. Table V shows the approximate amounts of radioactivity exhaled by human inhalers and noninhalers following exposure to C^{14}TDE-treated cigarettes. Mainstream smoke contained about 4.7% of the total radioactivity as determined by a machine smoker. Noninhalers appeared to exhale the radioactive TDE present in the smoke, whereas the inhalers retained about 70% of the radioactive TDE (about 3% of the TDE found in the total cigarette). Thus, retention of TDE by human smokers in this limited experiment appeared to agree with the reports of Holland (*5*) on radioactive arsenic. Although there appears to be a three- to fourfold difference in retention of TDE by the inhaler, it was beyond the experimental intent to relate this retention to storage or deposition of TDE or its components in human fat or other tissues.

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