OC1 462/A

CONTINUED STUDIES ON THE PHARMACOKINETICS/METABOLISM OF VINYL CHLORIDE IN MAMMALS

Toxicology Research Laboratory
Health and Environmental Research
Dow Chemical, U.S.A.
Midland, Michigan 48640

Subject: Progress Report

Period Covered: September 1, 1974 to May 1, 1975

Prepared for the companies sponsoring
The Technical Task Group on Vinyl Chloride Research
under the auspices of The Manufacturing
Chemists Association

TABLE OF CONTENTS

SECTION		PAGE
I.	Work Completed or in Progress	v
II.	Problems Encountered	107
III.	Current Status	107-108
IV.	Work to be Completed	109-110

P E R S O N N E L

PHARMACOKINETICS/METABOLISM

P. J. Gehring
P. G. Watanabe
R. E. Hefner, Jr.

ANALYTICAL

G. R. McGowan
W. H. Braun
G. R. Wagner
W. M. Muelder

INHALATION

B.K.J. Leong

COMPUTATION LABORATORY

G. Blau

a $t_{\frac{1}{2}}$ of 63.0 minutes. The percent of the dose remaining in the carcass after 72 hours was 11 and 2% for the 1 and 100 mg/kg doses, respectively. The urinary radioactivity was separated by thin layer chromatography into three major regions corresponding to S-(2-hydroxyethyl)-N-acetyl cysteine, thiodiglycolic acid, and a third unidentified metabolite. The proportions of the urinary metabolites were not influenced by the dose.

The fate of VC following 1 and 100 mg/kg doses is clearly dose dependent. Consistent with our previous studies on the fate of VC following inhalation exposure in rats, the metabolism of VC appears to be a saturable process. The identification of sulfur containing cysteine conjugates in the urine supports the hypothesis that the carcinogenicity of VC is related to metabolic formation of alkylating metabolites. The conjugation of these metabolites with glutathione appears to be the primary mechanism of detoxification. The relation of the present work to other recently reported studies on VC are discussed.

Initial studies on the radiochemical separation of urinary metabolites by liquid chromatographic techniques have been completed. Three major metabolites and one minor metabolite of ^{14}C-VC were separated from the urine of rats exposed to an iniital concentration of 7855 ppm ^{14}C-VC in air for 62 minutes. One of the major urinary metabolites appeared to be a mercapturic acid. A single minor urinary metabolite was confirmed to be ^{14}C-urea by thin layer chromatography techniques. Formation of ^{14}C-urea is probably secondary to the formation of $^{14}CO_2$. Continued analytical studies on urinary metabolites from rats orally administered 20 mg/kg ^{14}C-VC showed the presence of 3 major metabolites which is consistent with results of the initial separation of urinary metabolites of ^{14}C-VC after exposure by inhalation. The primary metabolite, containing about 45% of the total ^{14}C activity in the urine has been identified by GC-MS as S-(2-hydroxyethyl)-N-acetyl-cysteine. A second metabolite containing about 25% of the total ^{14}C activity was identified as thiodiglycolic acid by high resolution GC-MS and comparison with an authentic sample. Preliminary evidence for the third metabolite indicates that it is S-(2-hydroxyethyl)-cysteine.

ABSTRACT

Since the last reporting period the majority of studies conducted have been centered around the utilization of ^{14}C-labeled vinyl chloride (VC). The previous published synthesis of ^{14}C-VC from 1,2-dichloroethane has been adapted to an analytical gas chromatograph (Hewlett-Packard, Model 5750). The conversion of 1,2-dichloroethane to vinyl chloride by this modified procedure was calculated to be 48% (molar basis). The specific activity of the ^{14}C-VC was 2.67 mCi/mmole. The radioactive VC-helium mixture (gaseous) proved to be stable for several hours. This technique provides a convenient method for preparation of VC prior to use.

The radiochemical and chemical purity of a representative sample of 1,2-^{14}C-vinyl chloride (^{14}C-VC) was determined by gas chromatography-mass spectrometry. Sequential fractions were trapped from gas chromatographic columns in a toluene based liquid scintillator. The retention time for the radioactivity correlated with the retention time of authentic VC, and a radiochemical purity of 96% was established. A mass spectrum and a high resolution infrared spectrum obtained on the same sample of ^{14}C-VC were identical to spectra of authentic VC. The major detected inpurity consisting of 2% of the radioactivity appeared to be ^{14}C-labeled acetylene.

The fate of ^{14}C-VC after oral administration of 1 and 100 mg/kg has been studied. Male rats were given single oral doses of 1 and 100 mg/kg ^{14}C-VC and the routes and rates of elimination of ^{14}C-activity followed for 72 hours. Following 1 mg/kg VC, excretion in the urine as non-volatile metabolites and as $^{14}CO_2$ in expired air accounted for 59 and 13%, respectively, of the administered dose. Only 2% of the dose was expired by the lungs as VC. Conversely, after 100 mg/kg, 67% of the dose was eliminated by the lungs as VC, while non-volatile urinary metabolites and $^{14}CO_2$ comprised 11 and 3% respectively.

Pulmonary elimination after 100 mg/kg VC showed an apparent biphasic clearance with half-lives ($t_{\frac{1}{2}}$) of 14.4 and 49.5 minutes for the respective fast and slow phases. Following 1 mg/kg the pulmonary clearance of VC was monophasic with

SUPPLEMENT TO SECTION I

		Page
A.	Procedure for preparing ^{14}C-labeled vinyl chloride	1-13
B.	Radiochemical purity of ^{14}C-labeled vinyl chloride	14-23
C.	Fate of ^{14}C-vinyl chloride after single oral administration in rats	24-53
D.	Radiochemical separation of urinary metabolites of ^{14}C-vinyl chloride by liquid chromatography	54-72
E.	Isolation and identification of urinary metabolites of vinyl chloride	73-106

SECTION I

WORK COMPLETED OR IN PROGRESS

		In Progress	Completed
A.	Procedure for preparing ^{14}C-labeled vinyl chloride		X
B.	Radiochemical purity of ^{14}C-labeled vinyl chloride		X
C.	Fate of ^{14}C-vinyl chloride after single oral administration in rats	X	
D.	Radiochemical separation of urinary metabolites of ^{14}C-vinyl chloride by liquid chromatographic techniques		X
E.	The isolation and identification of urinary metabolites of vinyl chloride	X	

modifying a gas chromatographic column it can be used to generate ^{14}C-VC from 1,2-dichloroethane (1,2-^{14}C). The precursor, 1,2-^{14}C-1,2-dichloroethane (DCE), is stable, relatively inexpensive, and commercially available.(2)

PROCEDURE

Several different sizes of columns have been prepared and all have been effective. As a general description, (see experimental portion for a more detailed description of columns) the initial portion of a glass column was packed with a mixture of Chromosorb W and Darco G-60 charcaol (2:1) and this section, 30 to 90 cm long, was wound with heating wire to allow it to be heated to 400-500°C, separately from the remainder of the column. The chromatographic portion of the column was packed with 80-90 mesh 25% Dow Corning 410 Gum on Chromosorb W (acid washed), but any similar agent could be used. This section was maintained at near room temperature or at whatever temperature was necessary to achieve resolution of the reaction components.

PROCEDURE FOR PREPARING ^{14}C-LABELED VINYL CHLORIDE

INTRODUCTION

Exposure to high levels of vinyl chloride has been implicated as the cause of liver cancer in some industrial workers and for this reason there has been a great interest in understanding the metabolism of this important product. To facilitate these studies ^{14}C-labeled vinyl chloride was required, but a commercially prepared ^{14}C-labeled sample had polymerized during shipment. A convenient, direct method for synthesis of radioactive vinyl chloride was required so that ^{14}C-VC could be synthesized quickly just prior to each experiment. Since the initial studies were conducted using inhalation exposure, and since ^{14}C-VC was likely to be quite stable as a gas, a method was sought which provided for the preparation of gaseous ^{14}C-VC.

The literature describes a procedure for the dehydrohalogenation of 1,2-dichlorethane over a carbon catalyst at elevated temperatures.[1] We have found that by

No attempt was made to determine the maximum load of dichloroethane that could be used, but the yield of vinyl chloride obtained in the conversion was determined by several factors, the first being the amount of dichloroethane injected. No doubt a maximum percent conversion point could be reached, but this was not determined.

A second factor in the converted yield was the temperature of the dehydrohalogenator portion. As the temperature was increased above 350°C, the percent yield of vinyl chloride increased with a slight concomitant increase in the second by-product peak and of course the HCl. This was measured for column 1 (see Table I).

TABLE I

Peak Heights After Injection
of 10 µl DCE

Pyrolysis Temp.	Impurity Peak 1	V.C. Peak 2
320°	0	2.2
350°	0.2	11.6
375°	1.0	30
400°	2.5	55.5

The temperature for maximum conversion on column 2 was about 480°C.

Both flame ionization and thermal conductivity detectors have been used, but the latter is preferred because it allows detection of the HCl by-product as well as not requiring a stream splitter with the resultant loss of product.

Injection of 10 µl samples of dichloroethane on column 2 with the dehydrohalogenation reactor portion at 470°C, the separation column at ambient temperature, and a helium carrier gas flow rate of about 30 ml per minute produced 5 peaks in the thermal conductivity detector. See Figure 1. The first was merely a trace with a rentention time of about 1.8 minutes. The second though still minor component appeared at approximately 2 minutes. These by-products remain unidentified. The third peak was HCl with a rentention time of 2.3 minutes and this was followed by a nearly equivalent vinyl chloride peak at 3.3 minutes. The unreacted dichloroethane was detected only after 16 minutes. This large separation allowed as many as three successive injections and collections before the first dichloroethane appeared so larger quantities of vinyl chloride could be prepared in a short time.

Alternatley, one could collect the gas by bubbling through a solvent (toluene) or by trapping in a cold trap.

Attempts to use a solvent carrier for the dichloroethane precursor failed. Heptane or toluene caused formation of a multitude of decomposition products under these conditions and were found unsuitable.

Determination of Identity and Amount of Vinyl Chloride Produced. Samples of the collected gas were analyzed by gas chromatography using a siliconized 5% OV-17 on 80-100 mesh gas Chrom Q. The column was used at ambient temperature and peaks were compared with a standard curve prepared with authentic vinyl chloride. The gas was also analyzed by infrared spectroscopy and shown to be identical with a standard vinyl chloride spectrum.

Radioactivity Measurements. The most convenient and reproducible measurement of the radioactivity of the vinyl chloride was obtained by bubbling an aliquot of the gas mixture through toluene based scintillation cocktail (Liquiflor 1X). Direct counting of the solutions at various time intervals revealed the level of radioactivity

A third factor controlling the yield was the helium flow rate and like the others, this showed a direct relationship to the time the gas remained in the reaction chamber. It was found to be convenient to operate so that an incomplete conversion would verify the completion of the run by the appearance of unreacted dichloroethane.

Collection. The exit port of the chromatograph was modified to connect to a 100 ml syringe which was fitted with a stainless steel stopcock. The vinyl chloride peak was collected and the stopcock closed. This system showed no loss of unlabeled vinyl chloride over several days. It could be fitted conveniently with a serum cap over the end to allow repeated samplings through the stopcock or with a needle to allow direct injection into another system. The movability of the plunger automatically maintained the contents at atmospheric pressure.

If it were necessary to remove the small amount of HCl present, one could partially fill the syringe with dilute aqueous caustic, displace with the collected gas and then sample the HCl-free vinyl chloride mixture from the gaseous portion. This gas could presumably then be dried by passing through some drying agent.

(acid washed) except for the top 36 cm. This upper portion was packed with a charcoal mixture prepared by mixing 20 g Chromosorb W and 10 g Darco G-60 charcoal powder. This catalyst section was then fitted with an external thermocouple and wrapped with asbestos tape. Around this tape was wound asbestos covered Chromel A wire (22 gauge) and then a final covering of asbestos tape for insulation. The heating wires were connected to a Variac to allow independent heating of the pyrolyzer portion of the column. The column was installed in a Barber Coleman preparative gas chromatograph and heated at 200° overnight before use. The exit gases were passed through a splitter valve 85:15 and analyzed by flame ionization. The separation portion of the column was maintained at 150°C with a helium flow rate of about 20 ml per minute. The pyrolyzer portion was heated to 400-480°C.

Up to 30 µl samples of dichloroethane could be converted to vinyl chloride in about 65-85% yield based on the peak areas of by-product, product, and unchanged DCE.

The exit gases were either passed through dry ice traps or better collected in entirety in a 100 ml glass syringe fitted with a stainless steel stopcock (Becton Dickinson).

in the gas phase to be relatively constant over a 2-3 hour period. After 9 hours it had decreased by 50% (see Figure 3). The only explanation for this loss could be polymerization of the vinyl chloride on the walls of the syringe and removal from the gas phase since mechanical losses have been shown to be negligible. Attempts to wash the syringe to recover the lost activity were only marginally successful.

Purity. Gas chromatographic analysis indicated less than 5% organic impurities in the gas mixture which of course contained helium and hydrogen chloride. These analyses will be reported in detail by the pharmacokinetics group. It can be assumed that these organic impurities are also radioactive.

Safety. This study was carried out using the established practices of the Dow Radiation Protection Manual.

EXPERIMENTAL

Column 1. A glass U-shaped chromatographic column, 8' long and 1/4" in diameter (see Figure 2) was packed with 80-90 mesh 25% Dow Corning 410 Gum on Chromosorb W

packed with siliconized 5% OV-17 on 80-100 mesh gas Chrom Q which had been conditioned 4 days at 220° with 50 µl silyl 8 added before use. The column was run at ambient temperatures.

Column 2. To allow adaptation to a Hewlett Packard model 5750 gas chromatograph, a glass column was prepared as shown in Figure 2. The large separation coil was 8 feet long and the smaller pyrolysis coil was 90 cm in length. The inside diameter of the tubing was 3 mm. This was packed with the same materials as for column 1 above. The pyrolysis coil was wound with heating wire as before. The column could then be conveniently connected to the metal liner tube in the injection port and the terminal end to the thermal conductivity detector. No heat was applied to the column and the lid to the column oven was maintained open to keep the column at 30-50°C during operation. The pyrolysis coil was heated to 470-480°C. This column was also heated at 200°C overnight before use.

Samples of up to 10 µl of DCE were injected conveniently and conversions were essentially complete if the pyrolysis temperature were sufficiently higher. Detector and injection port temperatures were varied from 100 to 300° and lower temperatures showed no particular advantage.

Analytical Gas Chromatograph. The gaseous vinyl chloride samples were analyzed on a 6 feet by 3 mm glass column

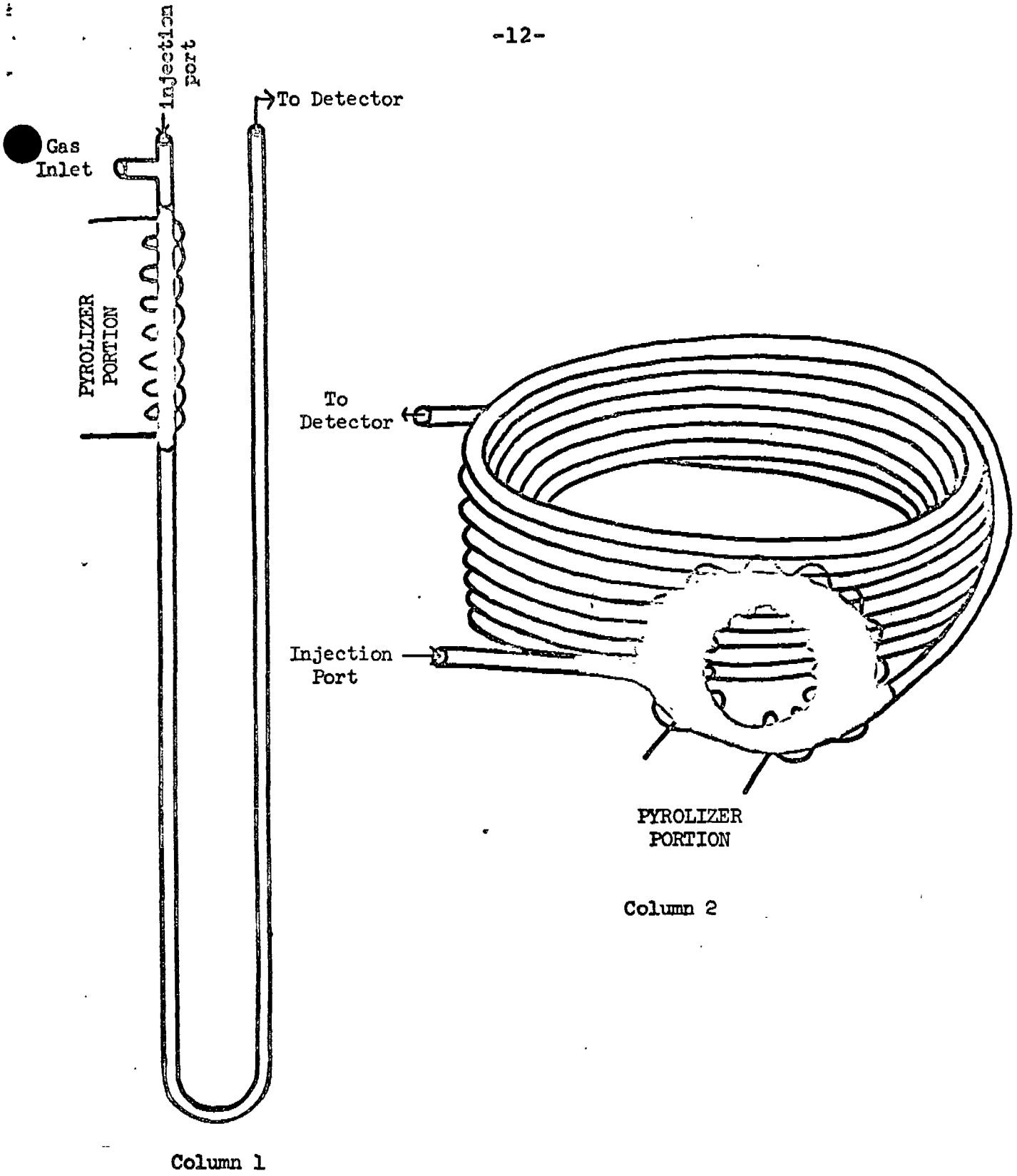
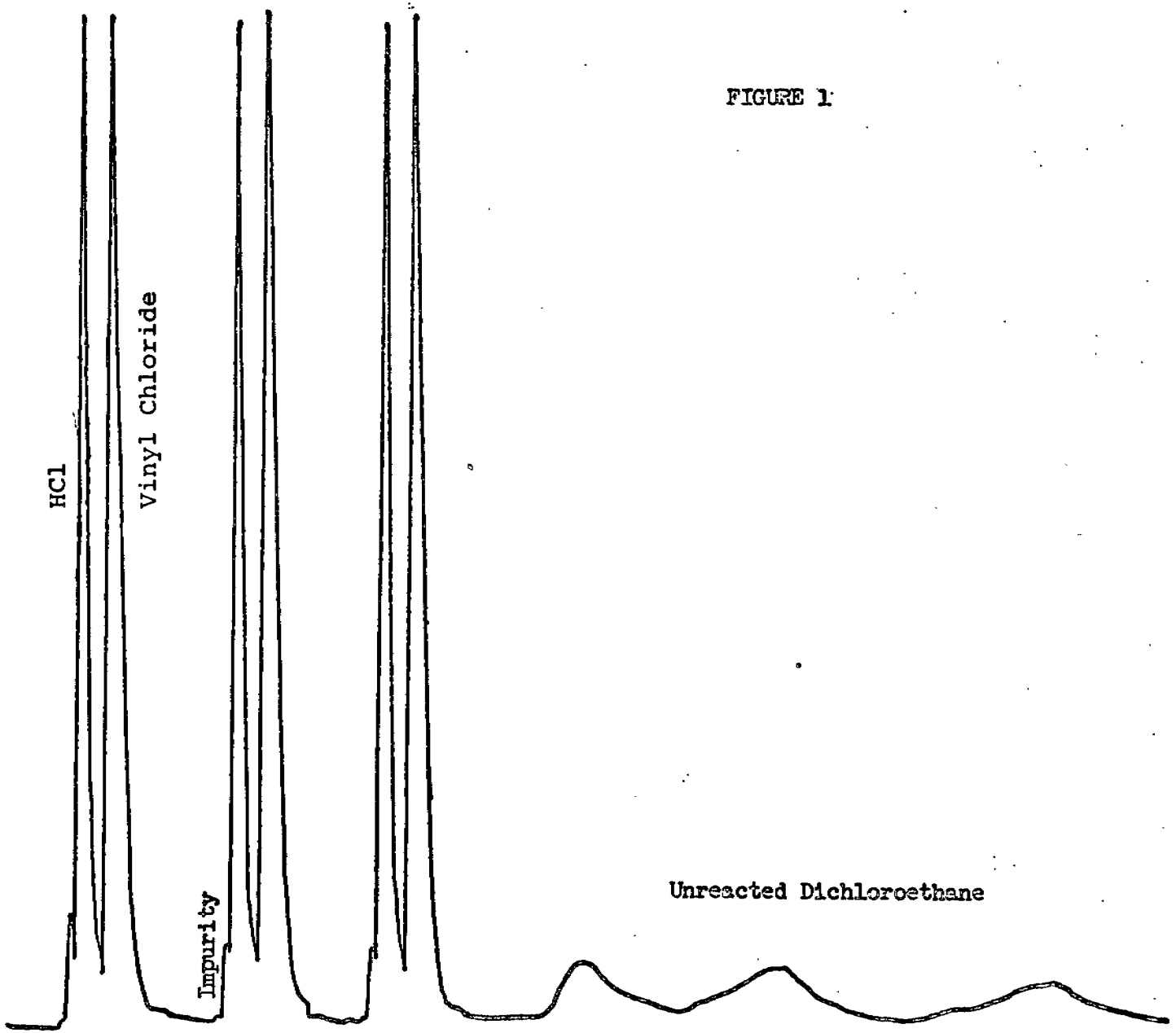

Injection port
To Detector
Gas Inlet
PYROLIZER PORTION
Column 1
To Detector
Injection Port
PYROLIZER PORTION
Column 2

FIGURE 2

FIGURE 1



RADIOCHEMICAL PURITY OF ^{14}C-LABELED VINYL CHLORIDE

INTRODUCTION

1,2-^{14}C-vinyl chloride (^{14}C-VC) is currently being used to study the kinetic and metabolic characteristics of VC in rats. The present work was conducted to establish the radiochemical and chemical purity of the synthesized ^{14}C-VC.

MATERIALS AND METHODS

Non-labeled VC (Matheson Gas Products, Joliet, Ill.) of 99.9% minimum purity was used as an authentic gas standard for comparison to the synthesized ^{14}C material.

1,2-^{14}C-dichloroethane (^{14}C-DCE) was purchased from the New England Nuclear Co. (Boston, Mass). The specific activity of the ^{14}C-DCE (lot #819-021) was 4.1 mCi/mmole. The purity reported by New England Nuclear was >97% as determined by ratio of gas chromatographic peak area measurements. Six µl (liquid) of ^{14}C-DCE was injected on the synthesis column (Wagner and Muelder, 1974) and the ^{14}C-VC produced was collected in a total volume of 40 ml with an airtight glass syringe.

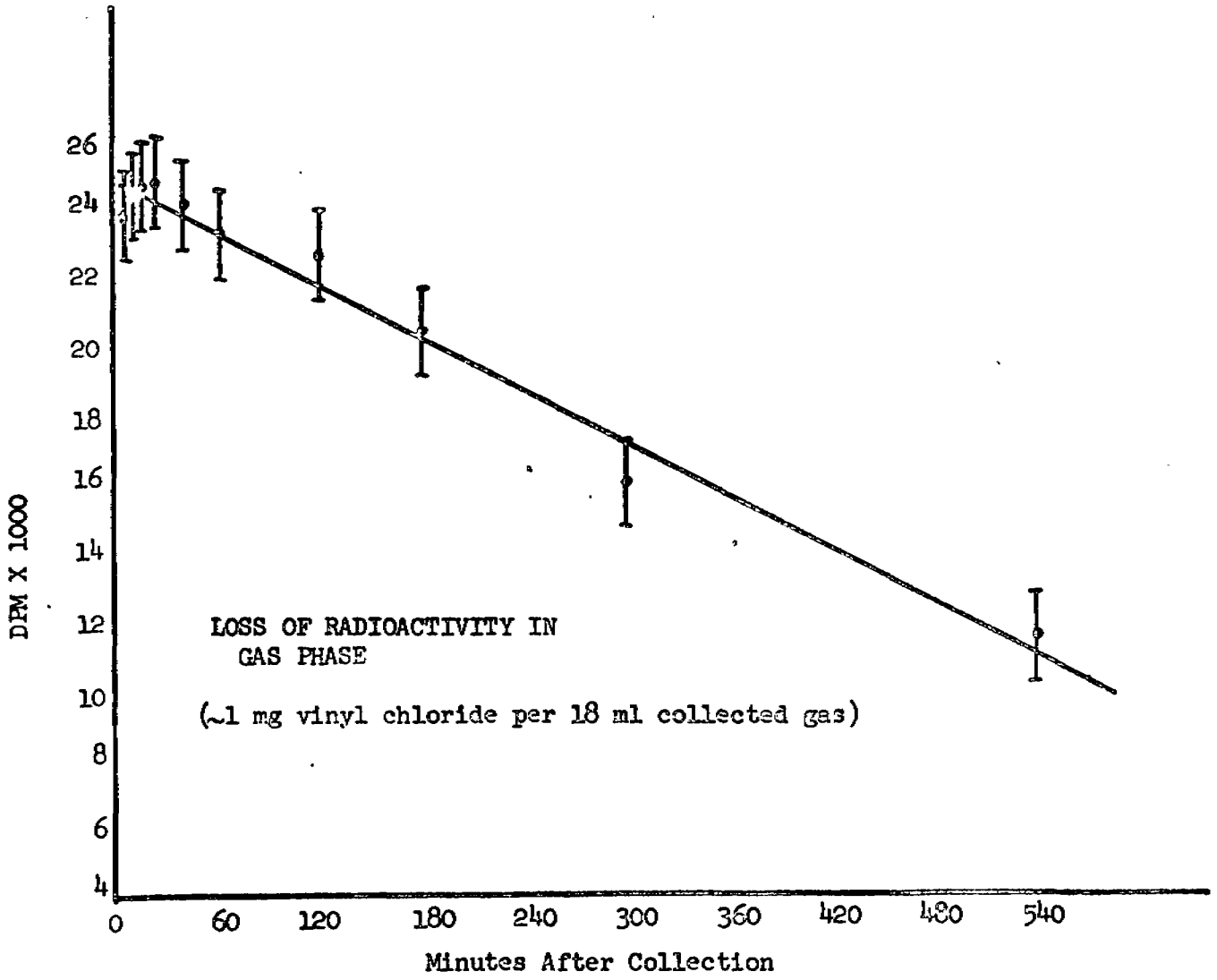

DPM X 1000
26
24
22
20
18
16
14
12
10
8
6
4
0
60
120
180
240
300
360
420
480
540
Minutes After Collection
LOSS OF RADIOACTIVITY IN
GAS PHASE
(~1 mg vinyl chloride per 18 ml collected gas)

FIGURE 3

GC-MS analysis. Infrared analysis was conducted by introducing the ^{14}C-VC into an evacuated 10 cm gas cell. The infrared spectrum was recorded at a pressure of 150 mm Hg in a normal and expanded mode to detect small quantities of impurities.

One ml aliquots of the ^{14}C-VC (4.332×10^6 DPM) were analyzed by gas chromatography (Hewlett-Packard, Model 5750) on two different columns (Porapak Q, 20% Carbowax 20M). Table 1 gives the specific conditions of the analysis. The effluent gases, after passing through the thermal conductivity detector, were trapped by bubbling direct into scintillation vials containing 20 ml of scintillator. The scintillator consisted of Concifluor (New England Nuclear, Boston, Mass.), 2-methoxyethanol, and toluene (1:1.8:11). The vials were changed every 15 seconds for 9 minutes. The efficiency of trapping VC by bubbling through toluene had been checked in this laboratory previously and shown to be 90% efficient. The radioactivity in the various fractions were determined by counting with a Nuclear Chicago Mark II liquid scintillation spectrometer. External standard channels ratio was used to determine the counting efficiency.

Additional aliquots of the ^{14}C-VC were subjected to GC-MS analysis (Finnigan Quadrupole GC-MS, Sunnyvale, California) and infrared spectrometry (FTS-14, Digilab, Cambridge, Mass.). Table 1 gives conditions of the

RESULTS

The profile of radioactivity trapped from both gas chromatographic systems indicated that the ^{14}C-activity corresponded with the retention time of the standard VC gas. The ^{14}C-VC constituted 97% (Porapak Q, Figure 1) and 96% (Carbowax) of the total radioactivity trapped from the respective columns. A ^{14}C contaminant eluting prior to VC in both systems comprised from 1-2% of the radioactivity. The mean total radioactivity recovered was 98% of that applied. This was well within the limits of accuracy in measuring the 1 ml aliquot introduced onto the columns.

The total ion chromatogram (TIC) obtained from the GC-MS analysis is shown in Figure 2. Individual mass spectra of the peaks indicated the presence of N_2, CO_2, acetylene, and VC. The ratio of peak areas of VC to acetylene was 70:1. The mass spectrum of the synthesized ^{14}C-VC (Figure 3) was identical when compared to the spectrum of the authentic VC standard.

Fourier-transform infrared spectrophotometric analysis indicated that the synthesized ^{14}C-VC was identical to a VC standard gas sample. Under the given conditions, there was no evidence for the presence of any contaminants.

TABLE 1

Conditions of GC and GC-MS Analysis

Instrument	Finnigan Quadrupole GC-MS	Hewlett-Packard GC	Hewlett-Packard GC
Col. Type	Porapak Q (80/100 mesh)	Porapak Q (80/100 mesh)	20% Carbowax 20M (60/80 mesh on Chromosorb W,AW)
Col. Dimensions	6' x 1/4" stainless steel	5' x 1/4" stainless steel	10' x 1/4" stainless steel
Detector	Mass Spectrometer	Thermal Conductivity	Thermal Conductivity
Col. Oven Temp (°C)	100-200, 30/min	150	60
Injection Temp (°C)	250	270	270
Thermal Conductivity Detector Temp (°C)		100	100
Bridge Current		150 mA	150 mA
Flow Rate	14 (Rotometer	20 ml/min	20 ml/min
Separator Vacuum	5×10^{-1} torr		
Source Vacuum	5×10^{-5} torr		
Separator Temp (°C)	220		
Ion Source Temp (°C)	40		
Ion Energy	70 eV		
Retention Time Standard VC (sec)	150	313	259

contaminant; however, it seems a very likely possibility based on the GC-MS data.

The infrared analysis did not confirm further the presence of acetylene. However, after theoretically calculating the concentration of acetylene present, this concentration was below the detection limits under the specified conditions.

The present study serves as an example for determining chemical and radiochemical purity of a gaseous compound utilizing a combination of GC, GC-MS, and infrared spectrophotometric techniques. The general approach may be useful for other compounds with similar physical characteristics.

DISCUSSION

The data indicated that the procedure used to synthesize ^{14}C-VC yields a product of 96-97% minimum radiochemical purity. Close inspection of the radioactivity-time profile (Figure 1), reveals that the retention time for the peak radioactivity was slightly longer than that of the VC standard. This may be due to a delay in transit through the thermal conductivity detector prior to trapping. This presumed artifact caused a slow "bleeding effect" of ^{14}C-activity from the detector as evidenced by the tailing in the resultant VC peak. Since the radioactivity associated with ^{14}C-VC was calculated over the time elution period of the standard gas, the radiochemical purity may actually be slightly higher than reported here due to this trapping artifact.

The ^{14}C-labeled contaminant which eluted prior to VC in both GC systems may be ^{14}C-acetylene, because the only other organic compound evidenced by GC-MS analysis in the preparation was acetylene. The VC/acetylene ratio of 70:1 would suggest a 1.4% acetylene content which coincides very well with the 1-2% radiochemical contamination. Due to the trapping artifact, it is not possible to state absolutely that acetylene is the major

Figure 2

TOTAL ION CHROMATOGRAM (TIC) FROM GC-MS ANALYSIS, SPECTRA WERE SCANNED EVERY 2 SECONDS

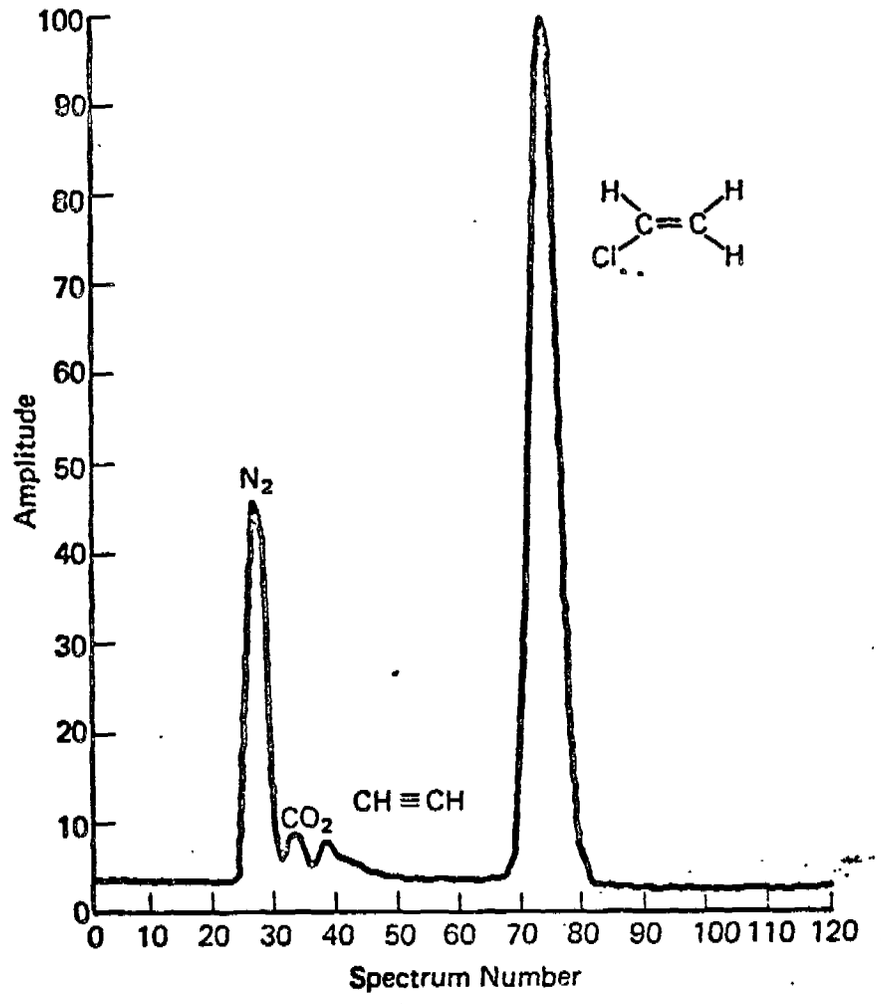
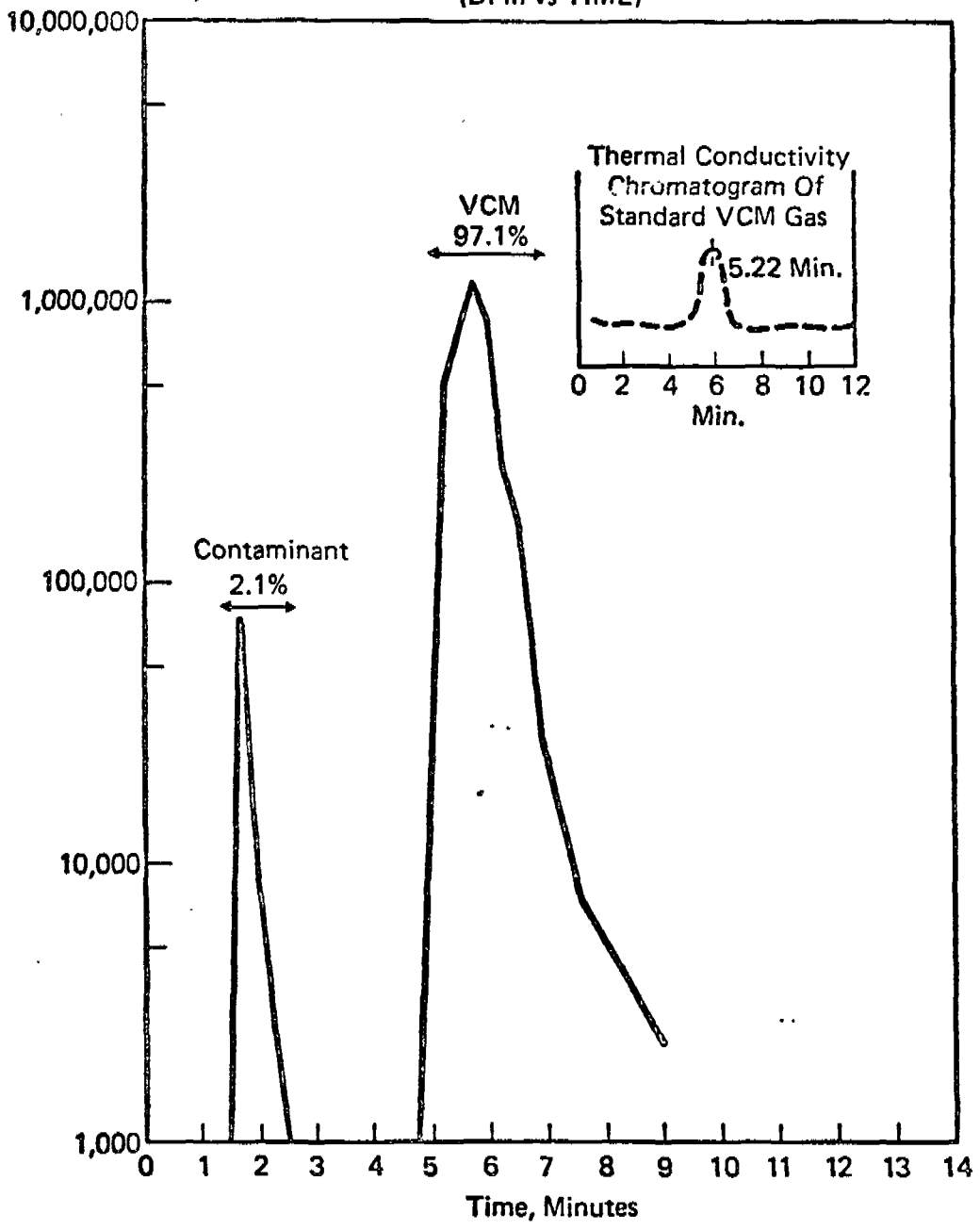


Figure 1

TRAPPING RADIOACTIVITY OF SYNTHESIZED ^{14}C-VCM FROM A PORAPAK Q COLUMN (DPM vs TIME)



FATE OF ^{14}C-VINYL CHLORIDE AFTER SINGLE ORAL ADMINISTRATION IN RATS

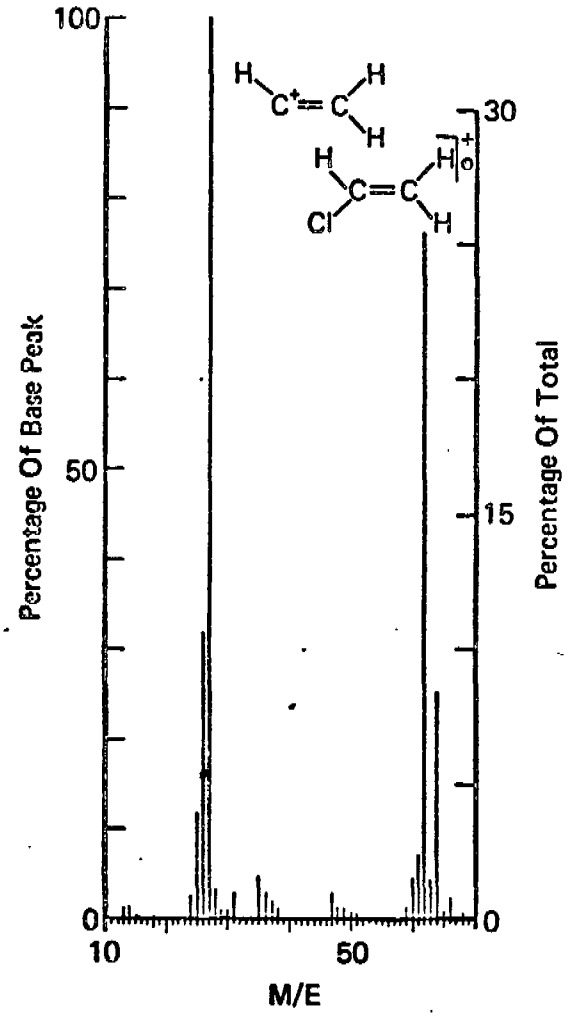
INTRODUCTION

The hazard of industrial exposure to vinyl chloride (VC) during the production of polyvinyl chloride and other plastics has received considerable attention. Maltoni and Lefemine (1974) demonstrated the carcinogenic potential of VC in rats exposed daily by inhalation to concentrations ranging from 50-10,000 ppm. Subsequent epidemiologic data obtained from industrial workers with long term exposure to high concentrations of VC demonstrated an association between exposure and various hepatic abnormalities including induction of angiosarcoma (Creech and Johnson, 1974).

The fate of inhaled VC has been previously studied in rats (Hefner, et al., 1975). Although some aspects of the fate of inhaled VC remain to be elucidated, the data indicated that in rats exposed to 50 ppm for 1 hour the inhaled VC was metabolized to polar products which were excreted predominantly in the urine. Using kinetic parameters and inhibitors of drug metabolism, additional evidence indicated that the fate of inhaled VC was concentration dependent and that at least two metabolic pathways were involved in its metabolism. Consideration of these data, led to the speculation that the carcinogenic

Figure 3

MASS SPECTRUM OF SYNTHESIZED
^{14}C–VCM



METHODS

Compound. 1,2-^{14}C-vinyl chloride (^{14}C-VC) was synthesized directly from 1,2-dichloroethane (1,2-^{14}C, lot # 819-021, 4.1 mCi/mmole, New England Nuclear, Corp.) by the method of Wagner and Muelder (1975). The radiochemical purity of a representative sample of ^{14}C-VC from this synthesis has been reported to be 95-96% pure (Wagner et al., 1975). The primary ^{14}C-containing contaminant (4-5%) in the ^{14}C-VC preparation was ^{14}C-acetylene. Non-labeled VC (Matheson Gas Products) of 99.9% minimum purity was mixed with the ^{14}C material to obtain the desired specific activity.

Preparation of Dose. The ^{14}C-VC was synthesized immediately prior to use. Typically, 20 ml of the ^{14}C-VC, helium mixture (approximately 2.6 mCi/mmole) was bubbled directly into 15 g of USP corn oil in a sealed septum vial. An appropriate quantity of non-labeled VC was then bubbled into the corn oil to obtain the desired concentration. One µl of the corn oil dosing solution was subjected to gas chromatography and the final concentration of VC in the corn oil was determined by comparison to standard gas samples. The specific conditions of the analysis were as follows:

activity of VC may be mediated through formation of alkylating metabolites such as chloroacetaldehyde and chloroethylene oxide. Even more important is the fact that the production of carcinogenic metabolites may increase disproportionately with the extent of exposure; or conversely, the capacity to detoxify alkylating metabolites may decrease disproportionately with the extent of exposure. Resolution of these possibilities is critical in assessing the hazard of exposure to low levels of VC.

In the production of polyvinylchloride (PVC) small amounts of unreacted monomer may be retained in the polymer. Because residual monomer may be leached from PVC used in food wrapping and packaging material, it is important to evaluate the toxicological potential of ingested VC. The objective of this study was to assess the fate of orally administered VC in rats.

The rats were housed in glass Roth-type metabolism chambers designed for the separate collection of urine, feces, and expired air. Room air was drawn by vacuum through the chambers at 400-500 ml/minute. The exiting air was passed through a series of traps to collect the expired ^{14}C-VC and $^{14}CO_2$. Initially the routinely used urine and feces traps were replaced by a straight glass tube. This modification reduced the dead space in the chamber and allowed air to be drawn directely through the metabolism chamber. Pilot studies indicated that the majority of the expired VC was eliminated during the first 2 hours after dosing; therefore, the conventional urine and feces traps were used after 4 hours.

The air exiting the chamber was first passed through a glass tube containing about 40 g of Drierite® (W. A. Hammond Drierite Co.) to remove moisture. Subsequent transit through a series of 2 cold finger traps containing 50 ml of toluene, 2-methoxyethanol (80:20) and a single trap containing 120 ml of 5M ethanolamine in 2-methoxyethanol enabled the collection of ^{14}C-VC and $^{14}CO_2$ respectively. The cold finger traps were immersed in 2-methoxyethanol, dry-ice baths throughout the collection periods. The trap for CO_2 was maintained at room temperature.

Gas Chromatograph	Hewlett Packard (Model 5750)
Column	Porapak Q (80/100 mesh, 6 ' x 1/4" stainless steel)
Column Temperature	180°C
Helium Flow	15 ml/min
Detector	Flame Ionization (250°C)
Injection Temperature	250°C

The radioactivity of the dosing solution was determined by placing aliquots (0.050 - 0.100 g) into pre-filled scintillation vials containing 20 ml scintillant, Concifluor® (Mallinckrodt Chemical Works), 2-methoxyethanol:toluene, 6:11:83. The radioactivity was determined in a Nuclear Chicago Mark II liquid scintillation spectrometer (Searle Inc.). The specific activities for the 1 and 100 mg/kg dose solutions were 20.50 and 0.18 μCi/mg VC respectively. The VC-corn oil solution was administered by oral gavage with a glass syringe and stainless steel dosing needle in a volume not exceeding 5 ml/kg.

Animals and Apparatus. Male albino Sprague-Dawley (Spartan substrain) rats weighing from 180-225 g purchased from Spartan Research were used throughout the studies. All animals were fasted overnight and the VC administered between 8 and 10 AM the following morning.

Five ml samples of the solution from the CO_2 trap were added to 5 ml of 5M ethanolamine in 2-methoxyethanol and 10 ml of the scintillant described above. The urine samples, 250 mg, were prepared by adding 1 ml of distilled water and 12 ml of Aquasol® (New England Nuclear).

Aqueous homogenates (33 or 50%, w/v) of feces and tissues were oxidized to CO_2 and H_2O in a Biological Material Oxidizer (Beckman Instruments). The $^{14}CO_2$ from the combustion was trapped in 8 ml of 5M ethanolamine in 2-methoxyethanol and added to the Concifluor® scintillant as described previously. Samples of skin and fat were combusted without homogenization.

Carbon-14 activity in all samples was determined by counting in a Mark II liquid scintillation spectrometer. External standard channel ratios were used to determine the counting efficiency. The counts per minute were converted to disintegrations per minute using a standard quench curve.

Radiochemical Separation of Urinary Metabolites.

Urine samples from each animal were pooled individually through the 24 hour collection period. Aliquots (10 ml) of

Experimental Procedure. The animals were placed in the modified metabolism cages immediately after dosing. The VC traps were changed at 30 minute intervals for the first 4 hours. Two subsequent changes at 4 hour intervals (8 and 12 hours) completed the collection of expired VC. The CO_2 trap, urine, and feces, receptacles were changed at 12 hour intervals for 72 hours. The urine trap was immersed in a 2-methoxyethanol, dry-ice bath throughout the collection periods.

At termination of the study the animals were decapitated, exsanguanated, and samples of tissue (liver, lung, perirenal fat, muscle, plasma) were collected for analysis of ^{14}C-activity. The remaining carcass was skinned and homogenized (50%, w/v) in distilled water.

Sample Preparation and Assay. Aliquots of the VC traps (5 ml) were prepared for counting by addition of an equal volume of scintillant containing Concifluor® (Mallinckrodt Chemical Works) 2-methoxyethanol, and toluene (6:11:33).

overall recovery of ^{14}C-activity was 88.8% and 82.3% at the 1 and 100 mg/kg dose levels, respectively. The primary radiochemical contaminant in the ^{14}C-VC preparation was ^{14}C-labeled acetylene (4-5%). Due to the physicochemical properties of acetylene, the solvent cold traps used for the collection of expired VC would not trap the highly volatile ^{14}C-acetylene. Based on this assumption the total recovery of ^{14}C-activity due solely to VC would be slightly higher than expressed in Table 1.

The characteristic pattern of pulmonary elimination of VC differed greatly between rats given 1 and 100 mg/kg. (Figure 1, Table 2). During the first 4 hours after administration of 100 mg/kg, the pulmonary elimination of VC was biphasic. The two linear portions of the curves were determined by regression analysis of the logarithmically transformed data. The data were feathered by inspection to obtain an approximation of the rate constant for the rapid phase of elimination. The apparent first order rate constants for the rapid and slow phases were 0.043 and 0.014 min^{-1} (95% confidence intervals, ±0.012 and ±0.010, respectively).

these pooled samples were lyophilized and reconstituted in 15 ml of methanol. The methanol extracts were evaporated under dry N_2 to approximately 1 ml. Five µl of the methanol extract was spotted on a thin layer chromatography plate composed of DOWEX® 50 ion exchange resin (Dow Chemical Co.). The plates were developed in n-propanol, H_2O, NH_4OH (70:21:9). The developed plates were scraped in 0.5 cm segments and the scrapings suspended in an Aquasol® gel (New England Nuclear) for scintillation counting.

RESULTS

Excretion of ^{14}C-activity within 72 hours following a single oral dose of 1 and 100 mg/kg ^{14}C-VC is shown in Table 1. The percentage of the dose expired as VC per se was 2 and 67% respectively. Due to the disproportionate pulmonary elimination of VC, a greater percentage of the dose was metabolized and eliminated in the urine, feces, and as expired $^{14}CO_2$ by rats given 1 mg/kg than by rats given 100 mg/kg. No special precautions were taken to insure detection of volatile compounds when collecting and processing the urine, feces, and carcass. Thus, the ^{14}C-activity in the excreta and carcass represent non-volatile metabolites of ^{14}C-VC. The

respectively, estimates of the apparent first order rate constants for the initial phase of elimination were 0.134 and 0.153 hours^{-1}. These correspond to half-lives of 5.2 and 4.5 hours. During this initial phase of elimination which lasted through 36 hours, 57 and 11% of the administered ^{14}C-activity had been excreted by rats receiving the low and high dose, respectively.

At both dose levels of ^{14}C-VC the liver contained the highest concentration of ^{14}C-activity after 72 hours (Table 4). The concentration in the liver expressed on a percent dose per gram tissue basis was 3-5 fold greater than muscle lung or fat. Consistent with the proportionally greater metabolism at the 1 mg/kg level, the proportion of the dose remaining in the tissues after 72 hours was considerably higher in rats given 1 mg/kg than those given 100 mg/kg.

Carbon-14 activity in the urine was separated into three regions of radioactivity by thin layer chromatography (Table 5). These three regions represent three ^{14}C-containing polar metabolites of VC. Regions II and III correspond to thiodiglycolic acid and S-(2-hydroxyethyl)-N-acetyl cysteine as identified by McGowan et al. (1975). The proportions

These rate constants correspond to half-lives of 14.4 and 49.5 minutes. Following a dose of 1 mg/kg, pulmonary elimination of VC was monophasic with an apparent first order rate constant of 0.011 min^{-1} (95% confidence interval, ±0.001) corresponding to a half-life of 63.0 minutes. To assure that the ^{14}C-activity collected in the cold traps was VC, the trapping solutions were analyzed by gas chromatography. Other than the components of the trapping solution the only compound detected had an identical retention time as a standard gas sample of VC.

The elimination of ^{14}C-activity in the urine as a function of time after 1 and 100 mg/kg ^{14}C-VC is shown in Table 3 and Figure 2. Table 3 also shows the expiration of $^{14}CO_2$ as a function of time. At both doses, the excretion of urinary and expired $^{14}CO_2$ was rapid. Greater than 80% of the total excreted by these routes was eliminated within 24 hours.

In Figure 2 shows the urinary excretion of ^{14}C-containing metabolites of VC (the curves were drawn by inspection). A similar biphasic elimination was evident at both dose levels. For rats given 1 and 100 mg/kg VC

DISCUSSION

The results of the current study establish that the fate of VC following ingestion by rats is dose dependent. Following a dose of 1 mg/kg ^{14}C-VC, most of the ^{14}C activity was excreted in the urine as non-volatile metabolites and as $^{14}CO_2$ in expired air. After 100 mg/kg, the predominant mode of excretion was by expiration of VC. Therefore, it appears that the metabolism of VC is a dose-dependent, saturable process. Similar results on the excretion of ^{14}C-VC following oral ingestion in rats have been reported recently by Green and Hathway (1975).

The fate of VC following oral administration is consistent with its fate after inhalation (Hefner et al., 1975). In those studies, the rate of VC metabolism was more rapid in rats exposed to 100 ppm or less than in rats exposed to 220 ppm or greater. Because of the experimental procedure used by Hefner et al. (1975), evidence was obtained indicating that at least two pathways may be involved in the metabolism of VC and that the degree of their involvement was concentration dependent.

of radioactivity determined in the three regions were not influenced by dose. The 3 regions do not represent one-hundred percent of the ^{14}C-activity since streaking of radioactivity occurred and it was not possible to separate these areas into discreet peaks.

In any case, the results of this study and those of Withey (1975) appear compatable for interpretive purposes.

Of paramount importance to assessing the hazard of exposure to VC is the fate of that portion metabolized. In the previous study (Hefner et al., 1975) it was speculated that potential alkylating metabolites such as chloroacetaldehyde and chloroethylene oxide may be formed in vivo from VC. A concurrent analytical chemistry study in this laboratory has identified two of the three polar urinary metabolites of VC as thiodiglycolic acid and S-2-(hydroxyethyl)-N-acetyl cysteine (McGowan et al., 1975). The identification of these metabolites is consistent with the proposed pathways for metabolism of VC. Chloroacetaldehyde and chloroethylene oxide will conjugate with glutathione and cysteine leading ultimately to the metabolites identified in the urine. Göthe (1975) reported recently the trapping of acetaldehyde formed from VC by an in vitro microsomal preparation. This provides additional support for the formation of chloroacetaldehyde.

Finally, work in progress and that reported previously by Hefner et al. (1975) shows that exposure of rats to

Interestingly, the predominant pathway for the metabolism of VC by rats exposed to 100 ppm or less was inhibited almost completely in rats pre-treated with ethanol. This suggested that the pathway for the metabolism of ethanol, which is also saturable, may be involved in the metabolism of VC. At low blood levels of ethanol, its elimination is solely a function of metabolism. As the blood concentration increases, a greater proportion of ethanol is excreted via the lungs (Lundquist and Wolthers, 1958).

Recently, Withey (1975) reported a biphasic clearance of vinyl chloride from the plasma of rats after cessation of inhalation exposure to concentrations of 500 to 7000 ppm or after intravenous injection of 50 to 75 mg/kg VC. The half-lives of the biphasic process were 4 to 9 min and approximately 40 minutes. These results deviate to some degree from those reported here. In this study the biphasic pulmonary excretion of VC following a dose of 100 mg/kg had half-lives of 14.4 and 49.5 minutes for the two phases. All considered, the half-lives for the slow component, 40 min vs 49.5 min appears equivacol. With regard to the differences for the half-lives of the first phase, 4 to 9 min vs 14.4 min, our value may be somewhat slower because of delayed absorption from the gastrointestinal tract.

Contrary to our identification of S-(2-hydroxyethyl)-N-acetyl cysteine as a major ^{14}C-metabolite of VC, Green and Hathway (1975) reported S-2-chloroethylcysteine and S-(2-chloroethyl)N-acetyl cysteine as major urinary metabolites. S-2-chloroethylcysteine is a monofunctional sulfur mustard and has been shown to be mutagenic (Fahmy and Fahmy, 1958). Due to its chemical properties this compound would be extremely unstable and has been reported to have a half-life of only 7 minutes in an aqueous solution at 37°C and pH 7 (Ross, 1962). Therefore, it seems unlikely that the S-2-chloroethyl compounds would be stable in the urine. Nachtomi (1970) studying the metabolism of 1,2-dibromoethane (EDB) *in vitro* demonstrated the enzymatic conjugation of EDB with glutathione (GSH) to form β-bromoethyl-SG and its subsequent spontaneous hydrolysis to produce the isolated hydroxyethyl-SG. This is additional evidence for the instability of halo-ethyl sulfur derivatives.

Since it is possible that S-hydroxyethyl cysteine as identified in the current work is derived from a totally different pathway other than via chloroethyl cysteine, additional studies may be necessary to clarify the

VC reduces the non-protein sulfhydryl content of the liver. Thus the metabolites of VC appear to react with and deplete the hepatic non-protein free sulfhydryls in vivo.

Green and Hathway (1975) recently reported the identification of 3 major metabolites in rats given 3 doses of 50 mg/kg VC at 3 hr intervals. The major urinary metabolite comprising 47% of all ^{14}C metabolites excreted in the urine was thiodiglycolic acid. In our study, the percentage of the total urinary metabolites identified as thiodiglycolic acid was 15.1±2.0 and 21.5±1.5% for rats given 1 and 100 mg/kg, respectively. Although these results appear inconsistent with those of Green and Hathway, it is likely that more thiodiglycolic acid is produced when repeated metabolically saturating doses of 50 mg/kg are given.

The metabolites of VC identified in the urine indicate that the primary deactivating mechanism is by conjugation with the non-protein free sulfhydryl compounds, glutathione and cysteine. Studies in progress in this laboratory show that the non-protein free sulfhydryls of the liver are depleted in rats exposed to VC both as a function of concentration and exposure duration. As the levels of non-protein free sulfhydryls are depleted, the alkylating metabolites are more likely to react with protein, DNA, and RNA, eliciting proportionally greater toxicity including carcinogenicity. This phenomena has been demonstrated to markedly influence the toxicity of compounds such as N-acetylaminofluorene, bromobenzene, and furosemide (Gillette 1974a and 1975b). The threshold for the toxicity of these materials coincides with their reaction with tissue protein, DNA, and RNA. Reactions with these cellular components and discernible toxicity occurs only after the glutathione levels are sufficiently depleted to preclude deactivation of the reactive metabolites of these agents.

discrepancy between the types of cysteine conjugates excreted in the urine. The metabolic scheme proposed by Green and Hathway (1975) does not include enzymatic processes. Chemical processes such as the free radical mediated mechanisms proposed by Green and Hathway for the metabolism of VC are governed solely by laws of mass action. Therefore, their proposed mechanism is inconsistent with the saturation phenomena demonstrated in our work and effects of various enzyme inhibitors (pyrazole, ethanol, SKF525A) on VC metabolism as reported previously (Hefner, et al., 1975). The ultimate isolation of the reactive intermediates of VC may require further in vitro studies.

As a final point for discussion, it is important to consider, how the data gathered by this laboratory and others may relate to assessing the hazard of exposure to VC. Such a discussion has been made previously (Hefner et al. 1975). Data gathered since that report continue to support the hypothesis that the carcinogenicity of VC is related to the metabolic formation of alkylating metabolites. Rannug et al. (1974) have reported a positive mutagenic response in Salmonella typhimurium exposed to VC if microsomal enzymes are present but not in their absence.

REFERENCES

Creech, J. L. and Johnson, M. N. (1974). Angiosarcoma of liver in the manufacture of polyvinyl chloride. J. Occup. Med. 16, 150-151.

Fahmy, O. G. and Fahmy, M. J. (1970). Gene elimination in carcinogenesis:reinterpretation of the somatic mutation theory. Cancer Res., 30, 195-205.

Gillette, J. R. (1974a). A perspective on the role of chemically reactive metabolites of foreign compounds in toxicity - I. Biochem. Pharmacol. 23, 2785-2794.

Gillette, J. R.(1974b). A perspective on the role of chemically reactive metabolites of foreign compounds in toxicity - II. Biochem. Pharmacol. 23, 2927-2938.

Göthe, R., Calleman, C. J., Ehrenberg, L. and Wachtmeister, C. A. (1974). Trapping with 3,4-dichlorobenzenethiol of reactive metabolites formed in vitro from the carcinogen vinyl chloride Ambio, 3, 224-226.

LEGENDS

Figure 1 Expired Vinyl Chloride expressed as % of the dose administered (1 and 100 mg/kg) versus hours. Each point represents the mean ± standard error of the mean of 5 rats. The linear phases of the curves were fit by linear regression analysis.

Figure 2 ^{14}C-activity excreted in the urine expressed as % of the dose administered (1 and 100 mg/kg) versus hours. Each point represents the mean ± standard error of the mean for 5 rats. The curves were drawn by inspection.

McGowan, G. R., Watanabe, P. G. and Gehring, P. J. (1975). The isolation and identification of urinary metabolites of vinyl chloride. (in manuscript).

Nachtomi, E. (1970). The metabolism of ethylene dibromide in the rat. Biochem. Pharmacol., 19, 2853-2860.

Rannug, U., Johansson, A., Ramel, C. and Wachtmeister, C. A. (1974). The mutagenicity of vinyl chloride after metabolic activation.Ambio, 3, 194-197.

Ross, W.C.J. (1962). Biological Alkylating Agents, pg. 173, Butterworth, Inc., Washington, D.C.

Wagner, E. R. and Muelder, W. M. (1975). A procedure for preparing ^{14}C-labeled vinyl chloride Ann. N.Y. Acad. Sci., 246, 152-153.

Wagner, E. R., Muelder, W. M.Watanabe, P. G., Hefner, R. E. Jr., Braun, W. H. and Gehring, P. J. (1975). Gas chromatographic method for the preparation of ^{14}C-labeled vinyl chloride. (in manuscript).

Withey, R. J. (1975). Uptake and pharmacodynamics of vinyl chloride administered to rats by different routes. Toxicol. Appl. Pharmacol., to be published.

Green, T. and Hathway, D. E. (1975). The biological fate in rats of vinyl chloride in relation to its oncogenicity. Imperial Chemical Industries, Central Toxicology Laboratories, Alderley Park, Chesire, England. (personal communication)

Hefner, R. E. Jr., Watanabe, P. G., and Gehring, P. J. (1975). Studies of the fate of inhaled vinyl chloride monomer (VCM) in rats Ann. N.Y. Acad. Sci., 246, 135-148.

Jones, A. R. (1973). The metabolism of biological alkylating agents. Drug Metabolism Rev., 2, 71-100.

Lundquist, F. and Wolthers, H. (1958). The kinetics of alcohol elimination in man. Acta Pharmacol. et. Toxicol., 14, 256-289.

Maltoni, C. and Lefemine, G. (1974). La potenzialita die saggi spermentali nella predizione dei rischi oncogeni ambientali. Un esempio: Il chloruro di vinile. Accad. National Dei Lincei (Roma), Series VIII 56, 1-11.

FIGURE 1

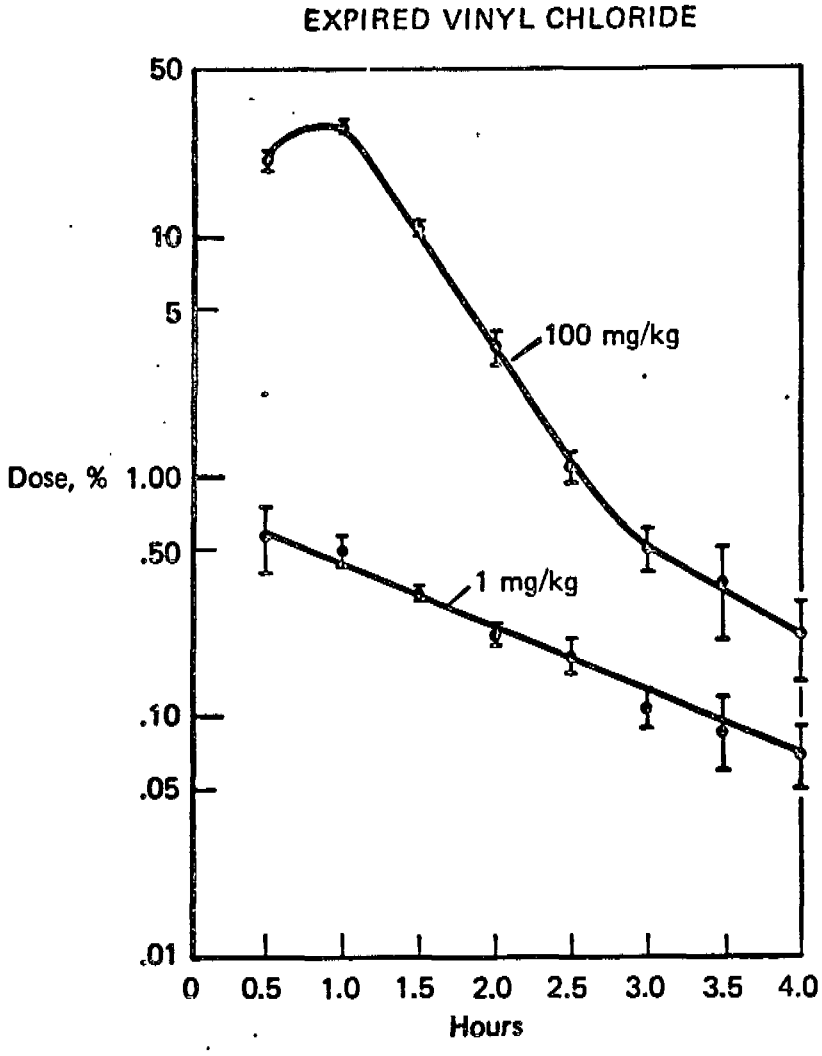

EXPIRED VINYL CHLORIDE
Dose, %
50
10
5
1.00
.50
.10
.05
.01
100 mg/kg
1 mg/kg
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
Hours

TABLE 1

PERCENT OF ADMINISTERED ^{14}C-ACTIVITY RECOVERED FOLLOWING A SINGLE ORAL DOSE OF VINYL CHLORIDE (VC)[a]

	Dose Level	
	1 mg/kg	100 mg/kg
Expired:		
As VC	2.13±0.22[b]	66.64±0.67
As CO_2	13.26±0.47	2.52±0.13
Urine	59.30±2.75	10.84±0.95
Feces	2.20±0.39	0.47±0.06
Carcass & Tissues	11.10±0.47	1.83±0.14
Cage Wash[c]	0.84±0.45	0
Total Recovery	88.83±1.98	82.32±0.43

[a]Percentage of dose excreted over 72 hours. Only the ^{14}C-activity associated with the expired VC can be attributed to VC per se.

[b]Mean ± standard error of the mean, 5 rats/dose

[c]Distilled water wash of metabolism cage at termination of the study.

FIGURE 2

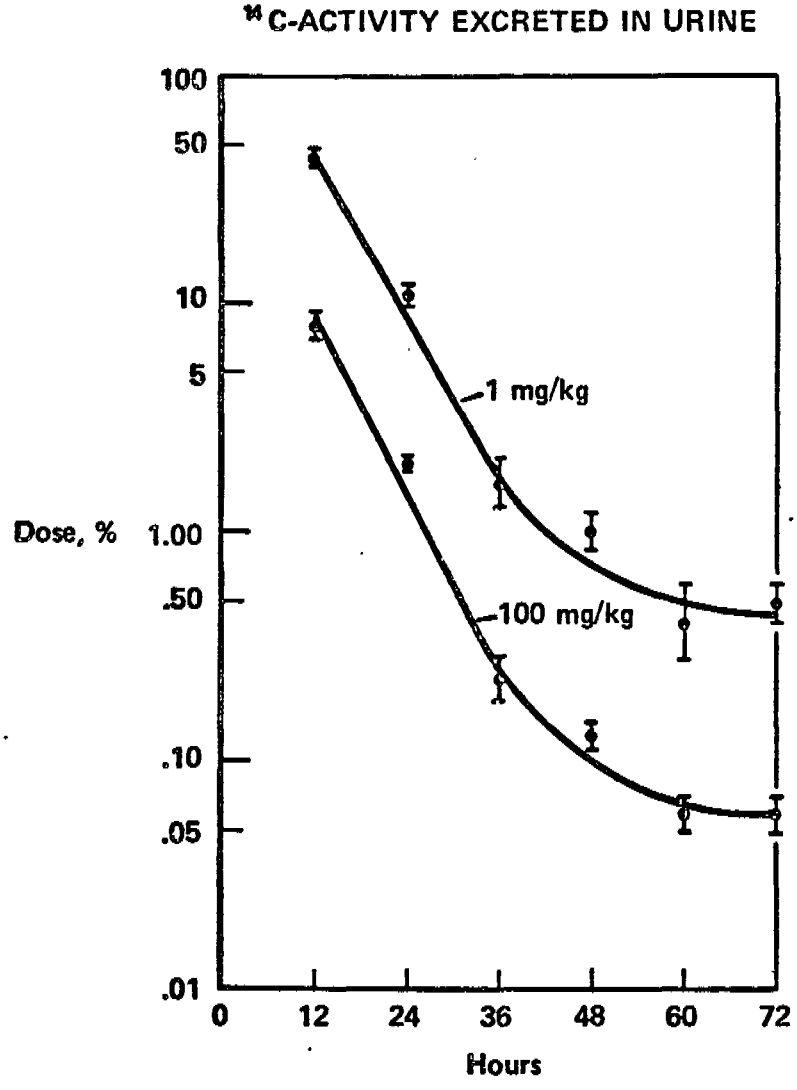

^{14}C-ACTIVITY EXCRETED IN URINE
Dose, %
100
50
10
5
1.00
.50
.10
.05
.01
1 mg/kg
100 mg/kg
0
12
24
36
48
60
72
Hours

TABLE 2

PERCENT OF ADMINISTERED ^{14}C-VINYL CHLORIDE EXCRETED BY THE LUNGS AS A FUNCTION OF TIME FOLLOWING SINGLE ORAL ADMINISTRATION[a]

Time (hr)	Dose Level 1 mg/kg	100 mg/kg
0-0.5	0.58±0.18 [b]	20.90±1.56
0.5-1.0	0.50±0.07	28.93±1.19
1.0-1.5	0.33±0.02	10.96±0.69
1.5-2.0	0.22±0.02	3.46±0.53
2.0-2.5	0.18±0.03	1.10±0.16
2.5-3.0	0.11±0.02	0.51±0.10
3.0-3.5	0.09±0.03	0.36±0.15
3.5-4.0	0.07±0.02	0.22±0.08
4.0-8.0	0.04±0.01	0.18±0.04
8.0-12.0	0.01±0.00	0.02±0.01
Total	2.13±0.22	66.64±0.68

[a] VC in expired air was trapped exclusively of $^{14}CO_2$, see Methods. The ^{14}C VC in the trapping solution was confirmed by gas chromatographic analysis.

[b] Mean ± standard error of the mean, 5 rats/dose

TABLE 4

PERCENT OF THE ADMINISTERED ^{14}C-ACTIVITY PER GRAM TISSUE AFTER ADMINISTRATION OF ^{14}C-VINYL CHLORIDE[a]

	Dose Level	
Tissue	1 mg/kg	100 mg/kg
Liver	0.182±0.005[b]	0.029±0.002
Skin	0.076±0.010	0.010±0.002
Carcass	0.046±0.002	0.007±0.001
Plasma	0.053±0.007	ND[c]
Muscle	0.031±0.003	0.006±0.001
Lung	0.061±0.003	0.011±0.001
Fat	0.045±0.008	0.006±0.001

[a]Remaining in the body after 72 hours.

[b]Mean ± standard error of the mean, 5 rats/dose

[c]Not detectable above background

TABLE 3

PERCENT OF ADMINISTERED ^{14}C-ACTIVITY EXCRETED IN THE URINE AND AS $^{14}CO_2$ FOLLOWING A SINGLE ORAL DOSE OF ^{14}C-VINYL CHLORIDE

	Dose Level			
	Urine		CO_2	
Time (hr)	1 mg/kg	100 mg/kg	1 mg/kg	100 mg/kg
0-12	44.47±3.19[a]	8.30±0.95	9.21±0.23	1.79±0.11
12-24	11.14±0.92	2.05±0.09	1.63±0.16	0.34±0.02
24-36	1.72±0.40	0.24±0.05	0.94±0.07	0.16±0.02
36-48	1.02±0.17	0.13±0.02	0.59±0.09	0.09±0.01
48-60	0.44±0.16	0.06±0.01	0.54±0.02	0.07±0.01
60-72	0.51±0.09	0.06±0.01	0.35±0.01	0.07±0.01
Total	59.30±2.75	10.84±0.95	13.26±0.47	2.52±0.13

[a]Mean ± standard error of the mean, 5 rats/dose

RADIOCHEMICAL SEPARATION OF URINARY METABOLITES OF ^{14}C-VINYL CHLORIDE BY LIQUID CHROMATOGRAPHY

INTRODUCTION

The kinetics and fate of inhaled vinyl chloride (VC) in rats have been extensively studied in this laboratory. Using the methods and inhalation apparatus described in our previous studies, 4 rats were exposed to 7855 ppm of ^{14}C-VC for 62 minutes. The urine collected for up to 12 hours post exposure was pooled and subjected to the various chromatographic techniques described.

METHODS

Column Chromatography. Various types of liquid chromatography packings (silica, DEAE cellulose, Dowex® 1 and 50) were screened in an attempt to separate ^{14}C-activity from the urine. Dowex 50 columns provided adequate resolution of the various ^{14}C-labeled urinary metabolites. The column characteristics used in the present investigation are defined as follows:

TABLE 5

THIN LAYER CHROMATOGRAPHIC SEPARATION OF ^{14}C-CONTAINING METABOLITES IN THE URINE OF RATS GIVEN ^{14}C-VINYL CHLORIDE[a]

	R_f	Dose Level	
		1 mg/kg	100 mg/kg
Region I	0.18	19.1±4.6[b]	17.5±4.4
Region II[c]	0.45	15.1±2.0	21.5±1.5
Region III[d]	0.60	53.4±4.8	47.8±6.8

[a]Percent of total ^{14}C-activity recovered from the chromatography plate. The plates were composed of DOWEX® 50 ion exchange resin and developed in n-propanol, H_2O, NH_4OH (70:21:9).

[b]Mean percent ± S.E., 5 rats/group

[c]Corresponds to thiodiglycolic acid

[d]Corresponds to S-(2-hydroxyethyl)-N-acetyl cysteine

or 30% acetylated cellulose plate. Two µg of S-(2-carboxymethyl)cysteine and S-(2-hydroxyethyl)cysteine were spotted on the plates as standards. The chromatograms were developed in a sealed glass tank containing 30% NH_4OH; 70% N-propanol solution. After air drying, the plates were analyzed for ^{14}C activity using a Panax TLC radioscanner and/or by scrapping the plates at 0.5 cm intervals and suspending the powdered scrappings in Aquasol gels. These were counted in a Mark II Nuclear Chicago liquid scintillation counter. The S-substituted standards were visualized using Ninspray ninhydrin reagent. R_f values were calculated for all standards and all regions of ^{14}C activity.

Mercapturic Acid Assay. After separation of the ^{14}C-labeled metabolites by column chromatography, they were analyzed for characteristics of mercapturic acids by two methods, 1) caustic hydrolysis, mercuric chloride precipitation of Stekol (1935) and 2) dicyclohexylamine (DCHA) reaction similar to Barnsley and Young (1965). The ^{14}C-labeled metabolites were subjected to thin layer chromatography before and after the respective reaction to determine if the procedure had altered the characteristics of the metabolite tested.

System

	A	B
Resin	Dowex 50WX4 200/400 mesh, H form	Dowex 50WX4 200/400 mesh, H form
Column Dimensions	22 x 1.5 cm	22 x 1.5 cm
Column Wash	Tris (0.01M) pH 7.0 Batch Process until pH = 7.0	Tris (0.01M) pH 7.0 Wash once pack column, wash column (100 ml) with same buffer until effluent pH = 2-3.5
Column Elution	Tris (0.01M) pH 7.0	Tris (0.01M) pH 7
Sample Size (urine)	4 ml lyophlized to 0.2 ml	4 ml lyophlized to 0.2 ml
Void Volume	10 ml	10 ml
Fraction Size	1 ml	1 ml
Flow Rate	0.3-0.8 ml/min	0.4 ml/min

After terminating elution of each column, 0.5 or 0.05 ml of each fraction was added to 12 ml Aquasol (New England Nuclear) and counted in a liquid scintillation spectrometer (Mark II, Nuclear Chicago).

Thin-layer Chromatography (TLC). ^{14}C containing fractions eluted from the various columns were examined using thin-layer chromatography (TLC). Ten to 12 µl of the chosen fraction was spotted on either a 5 by 20 cm silica plate

RESULTS

Column and Thin-Layer Chromatography. The Dowex 50 column (system A) resolved the ^{14}C activity in the urine into 3 peaks containing 97.9, 1.2, and 0.9% of the recovered ^{14}C activity (Figure 1). Over 90% of the ^{14}C activity applied to the Dowex 50 column was recovered. The large fraction of ^{14}C activity, (97.9%) was resolved further into 3 distinct components by TLC on 30% acetylated cellulose plates. None of these components had R_f values matching those of the S-(2-hydroxyethyl)-cysteine or S-(2-carboxymethyl)-cysteine standards. Subsequently a Dowex 50 column (system B) also resolved this major peak of ^{14}C activity into three metabolites (Figure 2). The ratio of these metabolites were approximatley 1 to 1 to 1 (labeled A, B, C).

Mercapturic Acid Assay ($HgCl_2$ precipitation). Metabolite C formed a precipitate with $HgCl_2$. After the reaction metabolite C was no longer evident as a region of ^{14}C activity using TLC (Figure 3). Likewise, metabolite A reacted in a similar manner (Figure 4). The apparent decrease in peak height of the ^{14}C labeled metabolite A before and after the reaction was not due entirely to reaction with $HgCl_2$. This decrease occurred because about 4 fold less

Isolation of ^{14}C-Urea. Fractions containing minor ^{14}C activity (1.2%) were obtained from a Dowex 50 column and lyophilized to dryness. Lyophilization produced a mass of white crystalline material which was dissolved in a minimum of distilled water. Silica plates were spotted with 5-10 µg of an authentic urea standard, and 24 to 42 µl (6 µl per spot) of the sample. The chromatograms were developed in sealed glass tanks using the following solvent systems:

30% NH_4OH, 70% n-propanol; 30% NH_4OH, 70% ethanol (95%); 50% ethyl acetate, 50% acetone, 10% acetic acid, 90% n-propanol; and 60% N-butanol, 20% acetic acid, 20% distilled water. After air drying, the chromatograms were sprayed with 4-dimethylaminobenzaldehyde reagent and exposed to HCl fumes. This produced a characteristic bright yellow color in both the urea standard, as well as the spot containing the ^{14}C activity. Dimensions of the colored urea spots were measured and R_f values calculated. Distribution of ^{14}C activity was determined by scraping the chromatograms at 0.5 cm intervals and suspending the powdered scrapings in Aquasol gels which were counted as previously described.

Based on the procedure described in the methods, reaction of a mercapturic acid with DCHA should give rise to a cyclic complex which generally crystallizes from the acetone solution following DCHA addition. Thus, one major urinary metabolite (A) of ^{14}C-VC gave the expected reaction of a mercapturic acid.

Isolation of ^{14}C-Urea as a Minor Urinary Metabolite. ^{14}C-urea appeared to be present as a minor urinary metabolite of VC comprising 1.2% of the recovered ^{14}C activity, (Figure 1). Several of the TLC solvent systems used, especially those without ammonium hydroxide as a component, smeared both the sample and the urea standard on the chromatograms. In spite of this, R_f values for the colored urea standard and sample spots and the ^{14}C activity always correlated in all of the chromatograms (Figure 9). No other ^{14}C-labeled components other than urea were detected in this minor fraction of the total recovered ^{14}C activity.

sample from the reaction supernatant was spotted on the TLC plate as compared to the unreacted sample. It was necessary to apply less sample due to the limited capacity of the TLC plate for the sample. In spite of this, an 8 fold decrease in ^{14}C activity after the addition of $HgCl_2$ occurred. Therefore, some derivatization with $HgCl_2$ very likely occurred. The remaining metabolite B, (Figure 5), did not react with $HgCl_2$ and was present as an unchanged region of ^{14}C activity detected by TLC before and after the reaction. Thus, two major urinary metabolites of ^{14}C-VC (C, A, Refer to Figure 2) gave the expected reaction of a mercapturic acid.

Dicyclohexylamine (DCHA) Assay for Mercapturic Acids. Of the 3 major urinary metabolites of ^{14}C-VC (A, B, C, Refer to Figure 2), metabolite C reacted with DCHA to yield a new ^{14}C-labeled compound of slightly higher R_f, (Figure 6). Interestingly, this new compound did not form a crystalline precipitate, but rather stayed in solution. Metabolite A, (Figure 7), reacted with DCHA to form a crystalline precipitate. After precipitation with DCHA, this metabolite was no longer evident as a region of ^{14}C activity using TLC. Metabolite B (Figure 8) did not react with DCHA and was present as an unchanged region of ^{14}C activity using TLC.

products from this route may be hydroxyethyl or 1,2 hydroxyethyl cysteine or their respective mercapturic acids.

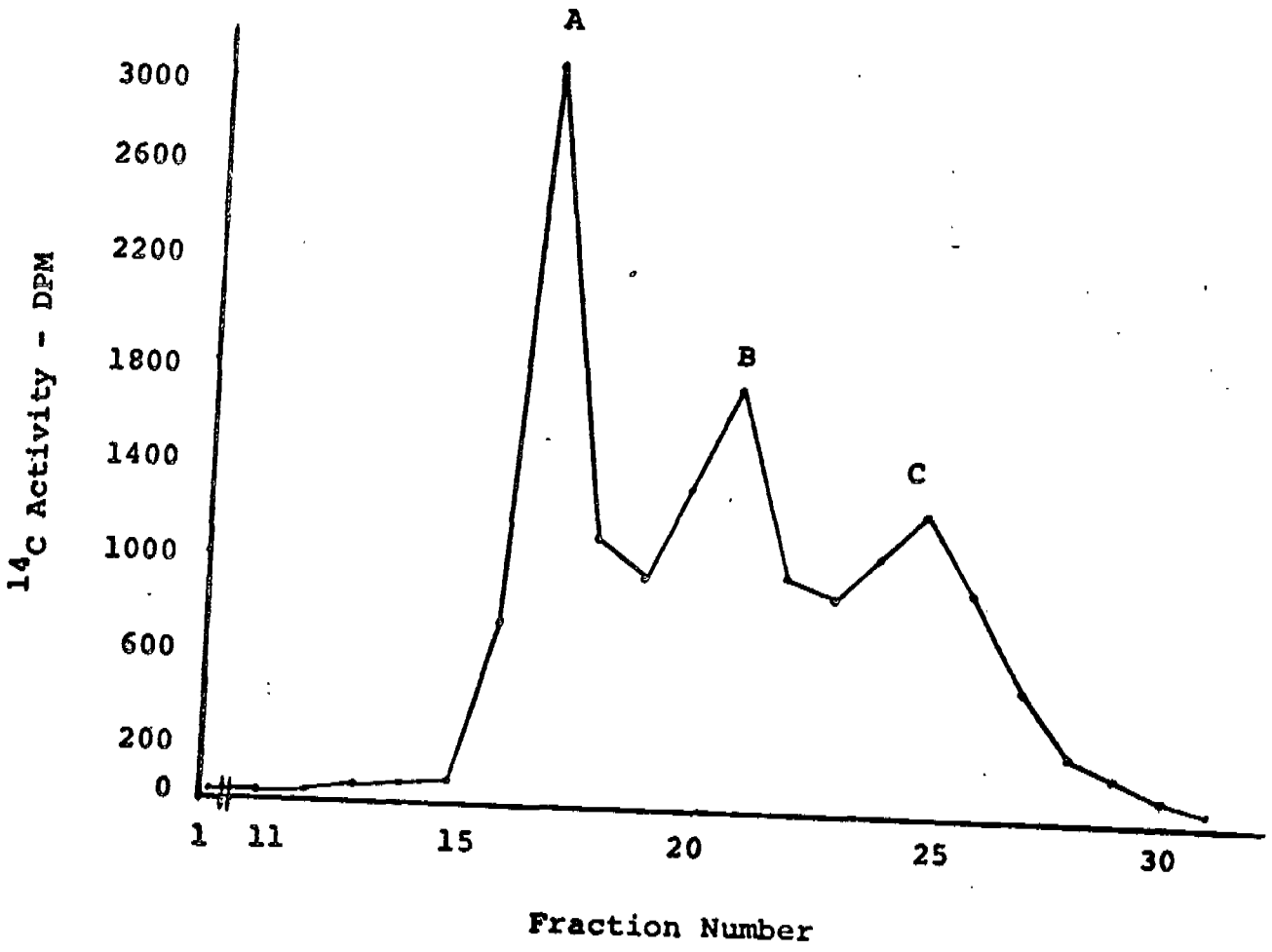
Formation of ^{14}C-urea as a minor urinary metabolite is not totally unexpected, as our previous studies demonstrated appreciable $^{14}CO_2$ formation following inhalation of ^{14}C-VC.

Additional experiments are in progress to aid in the elucidation of all metabolites of VC. As firm mass spectral and instrumental verification of the structure of the various urinary metabolites are obtained, additional reports will be issued.

DISCUSSION

Using the column and thin-layer chromatographic techniques described in the methods, 3 major labeled urinary metabolites of VC have been separated. One of the urinary metabolites showed characteristic reactions of a mercapturic acid in two systems tested. Formation of mercapturic acids is in accordance with our previous experimental observations which demonstrated marked depletion of liver non-protein sulfhydryl levels of rats exposed to VC. Although the specific structure of the possible mercapturic acid remains undetermined at this time, several possibilities exist. Johnson (1967) has shown that *in vivo* oxidation of 2-chloroethanol to chloroacetaldehyde and subsequent conjugation of chloroacetaldehyde with glutathione (GSH) results in the formation of *S*-(2-carboxymethyl)-cysteine, in rats. The expected urinary metabolite from this set of reactions would be *N*-acetyl-*S*-(2-carboxymethyl)-cysteine. However, at the relatively high (7855 ppm) VCM exposure concentrations used in these studies, our previous experiments indicated that the alcohol dehydrogenase pathway was saturated via which 2-chloroethanol is converted to chloroacetaldehyde. Therefore, an alternant pathway involving a direct microsomal oxidation of VC followed by conjugation with GSH may occur. Possible metabolic

Figure 2: Three Major ^{14}C-Labeled Urinary Metabolites of VC Resolved with a Dowex 50 Ion-Exchange Column.



EC- 0546

Figure 1: ^{14}C-Labeled Urinary Metabolites of VC Resolved Using a Dowex 50 Ion-Exchange Column.

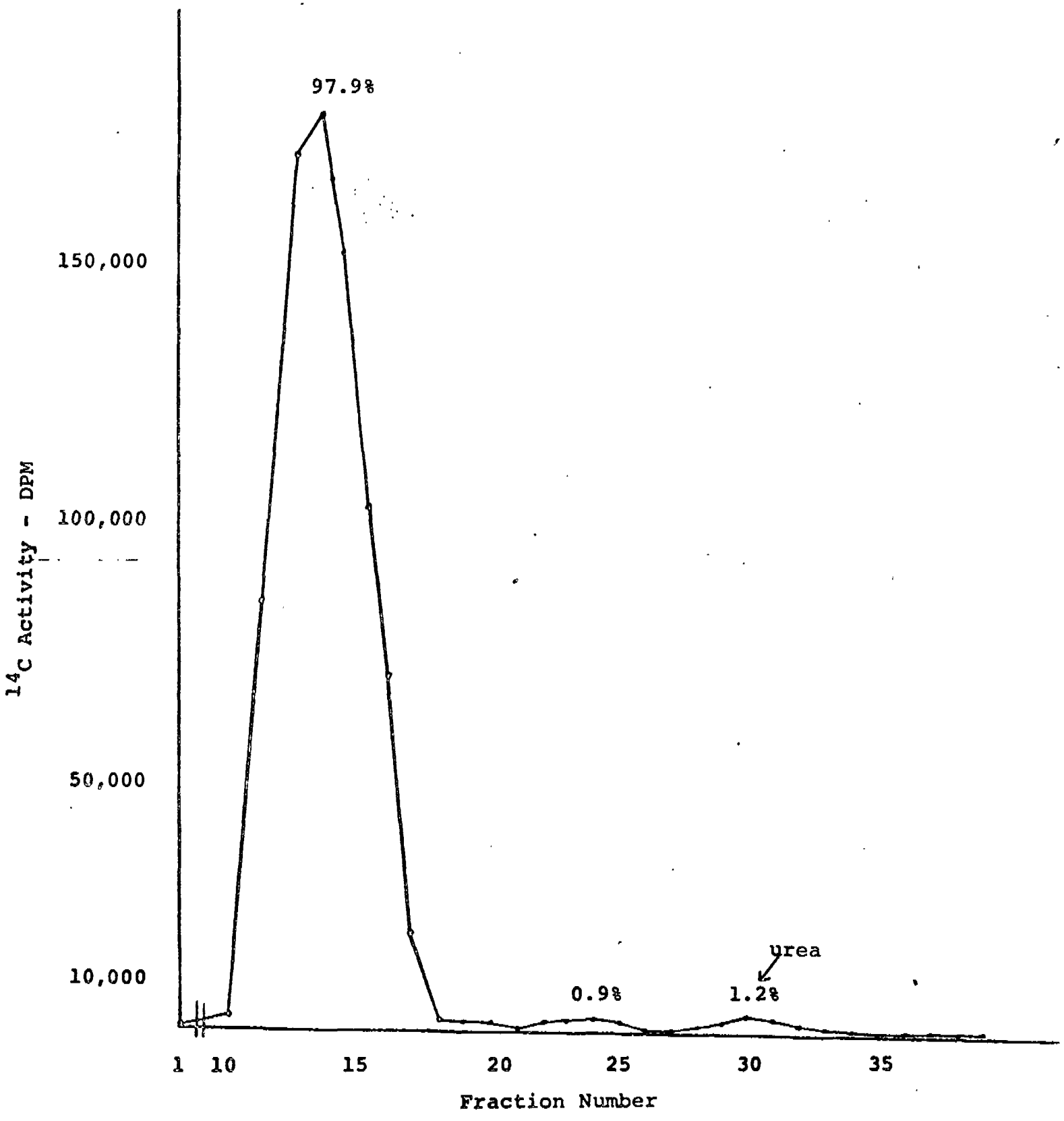


Figure 4: Thin-layer Chromatography of Metabolite A Before and After Mercuric Chloride Mercapturic Acid Assay.

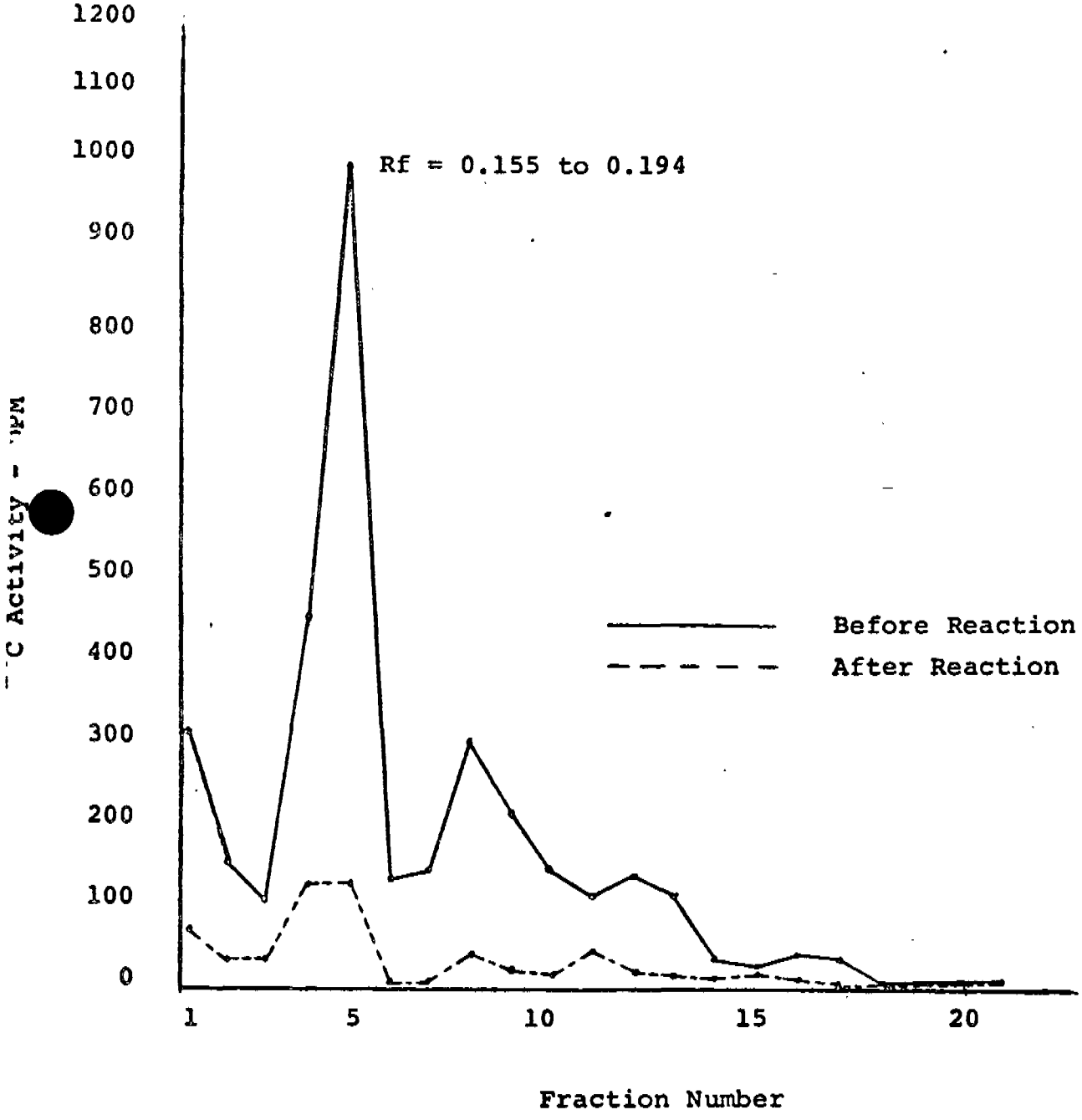


Figure 3: Thin-layer Chromatography of Metabolite C Before and After a Mercuric Chloride Mercapturic Acid Assay.

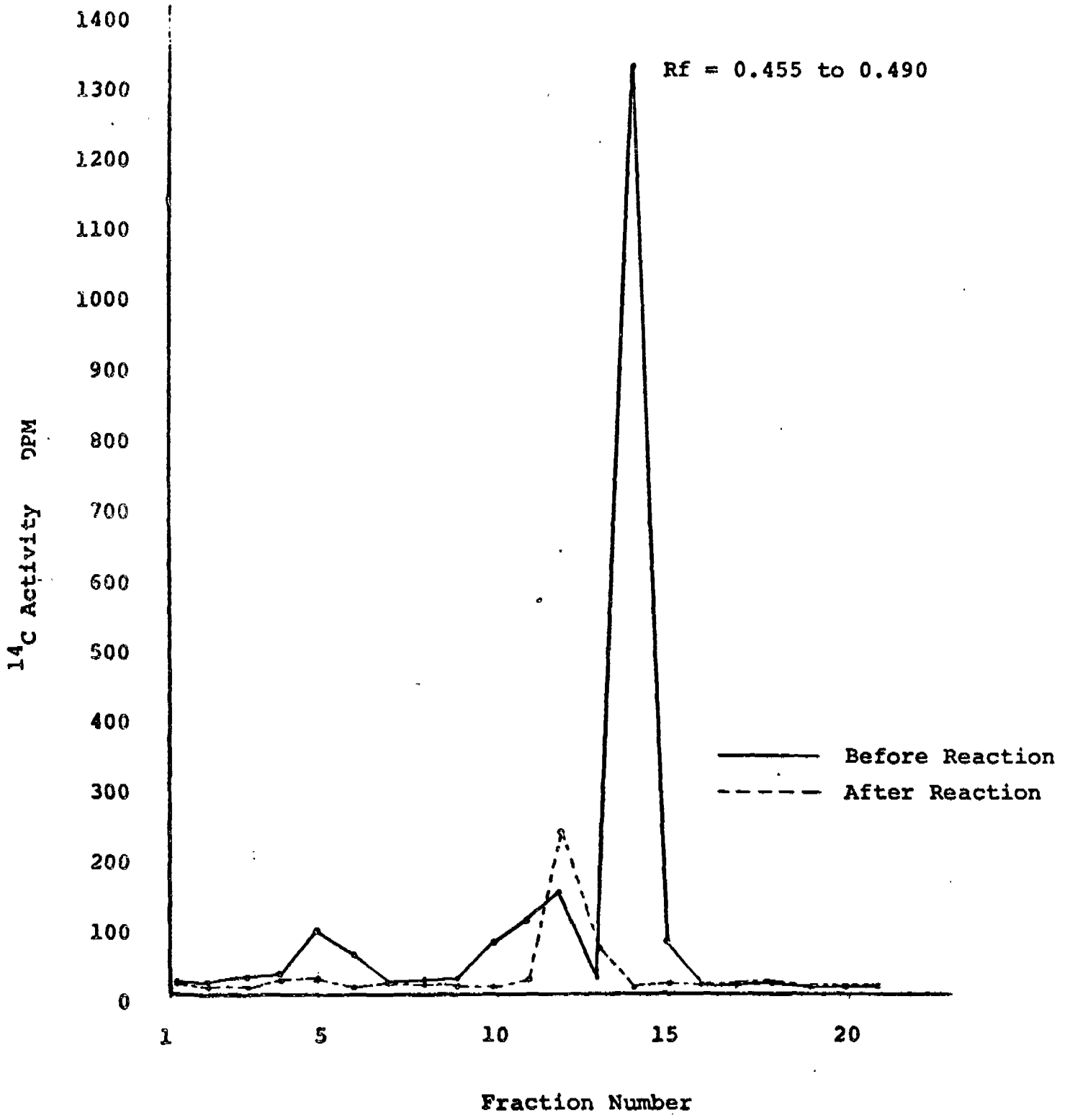


Figure 6: Thin-layer Chromatography of Metabolite C Before and After DCHA Mercapturic Acid Assay.

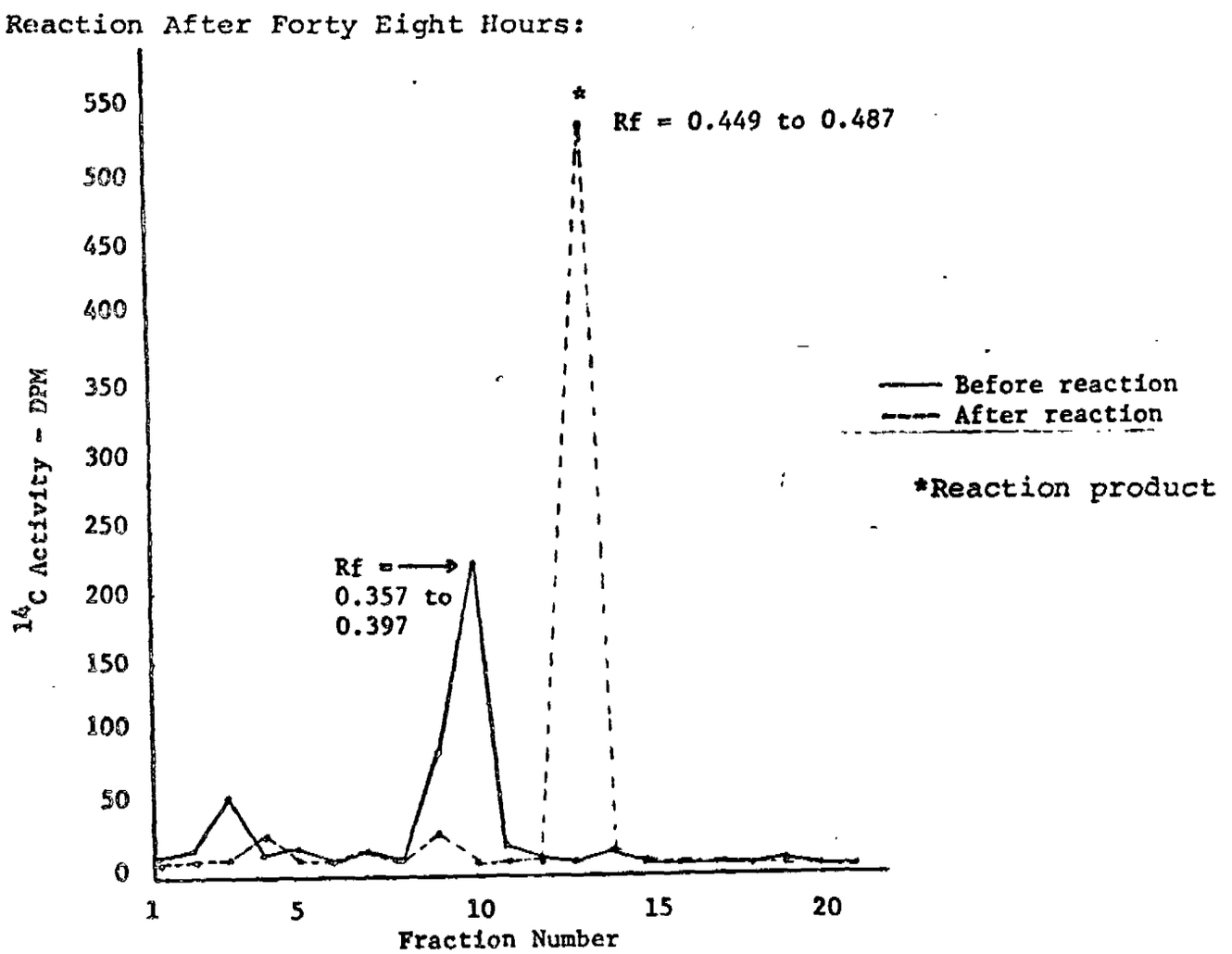
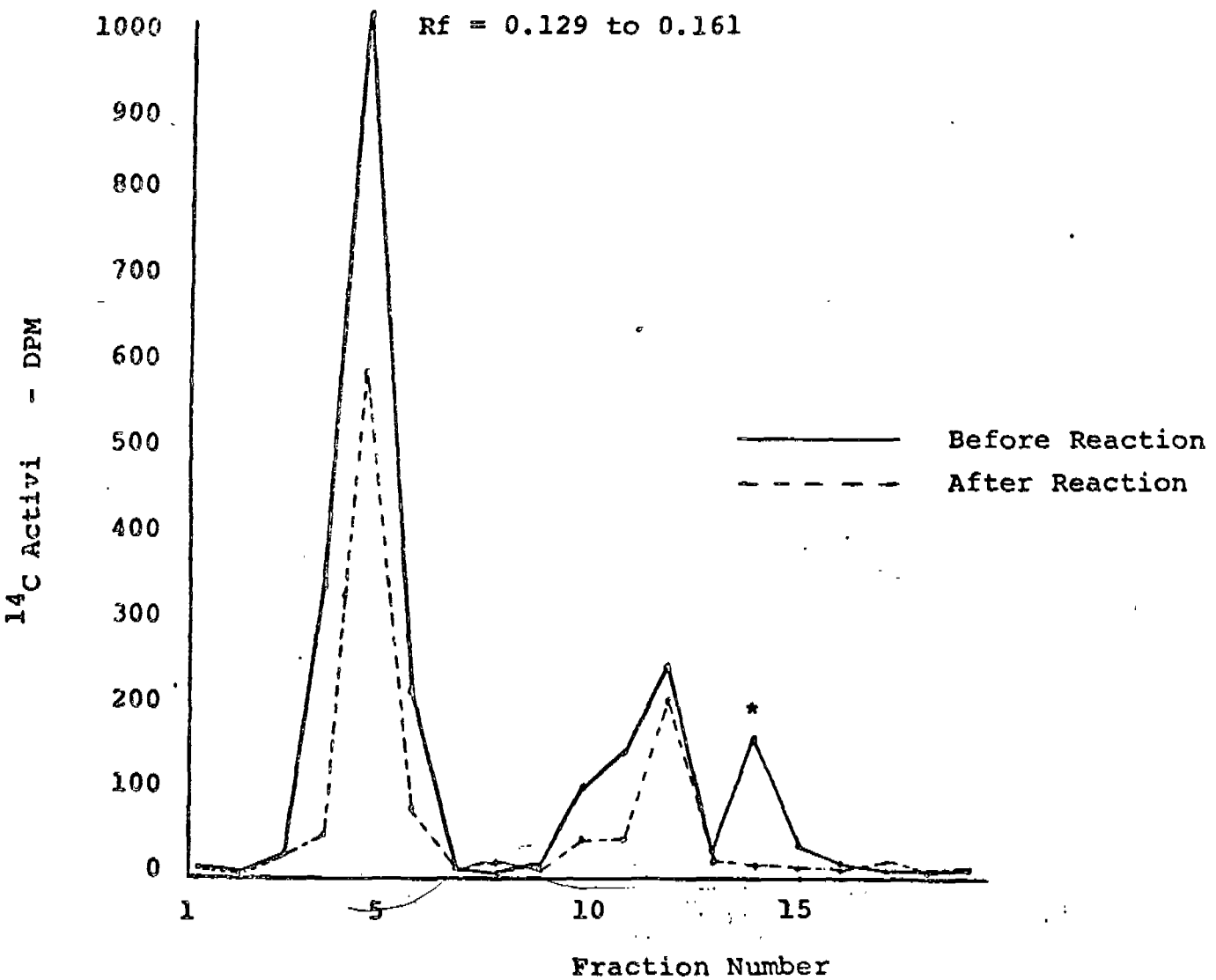
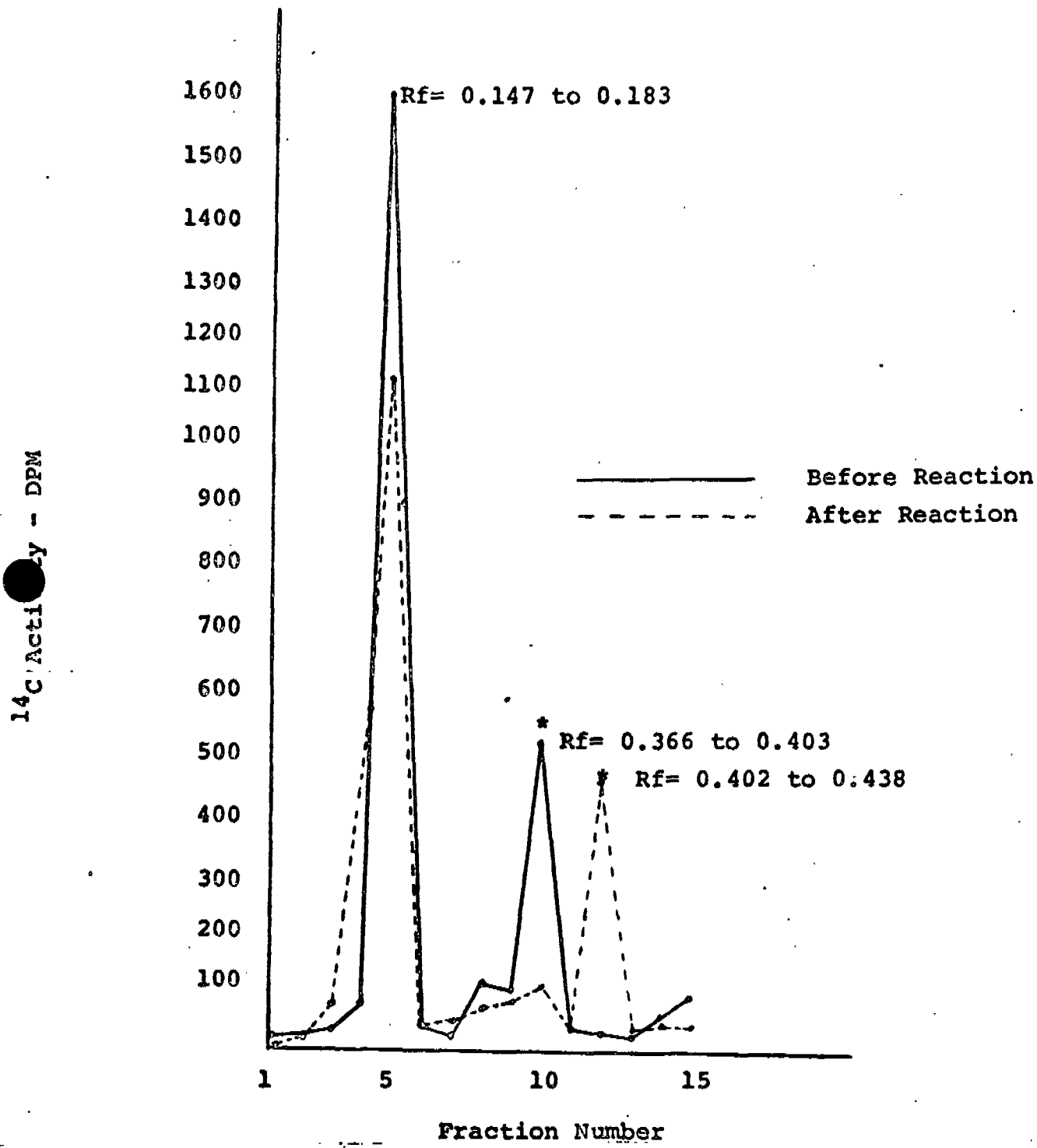


Figure 5: Thin-layer Chromatography of Metabolite B Before and After Mercuric Chloride Mercapturic Acid Assay.



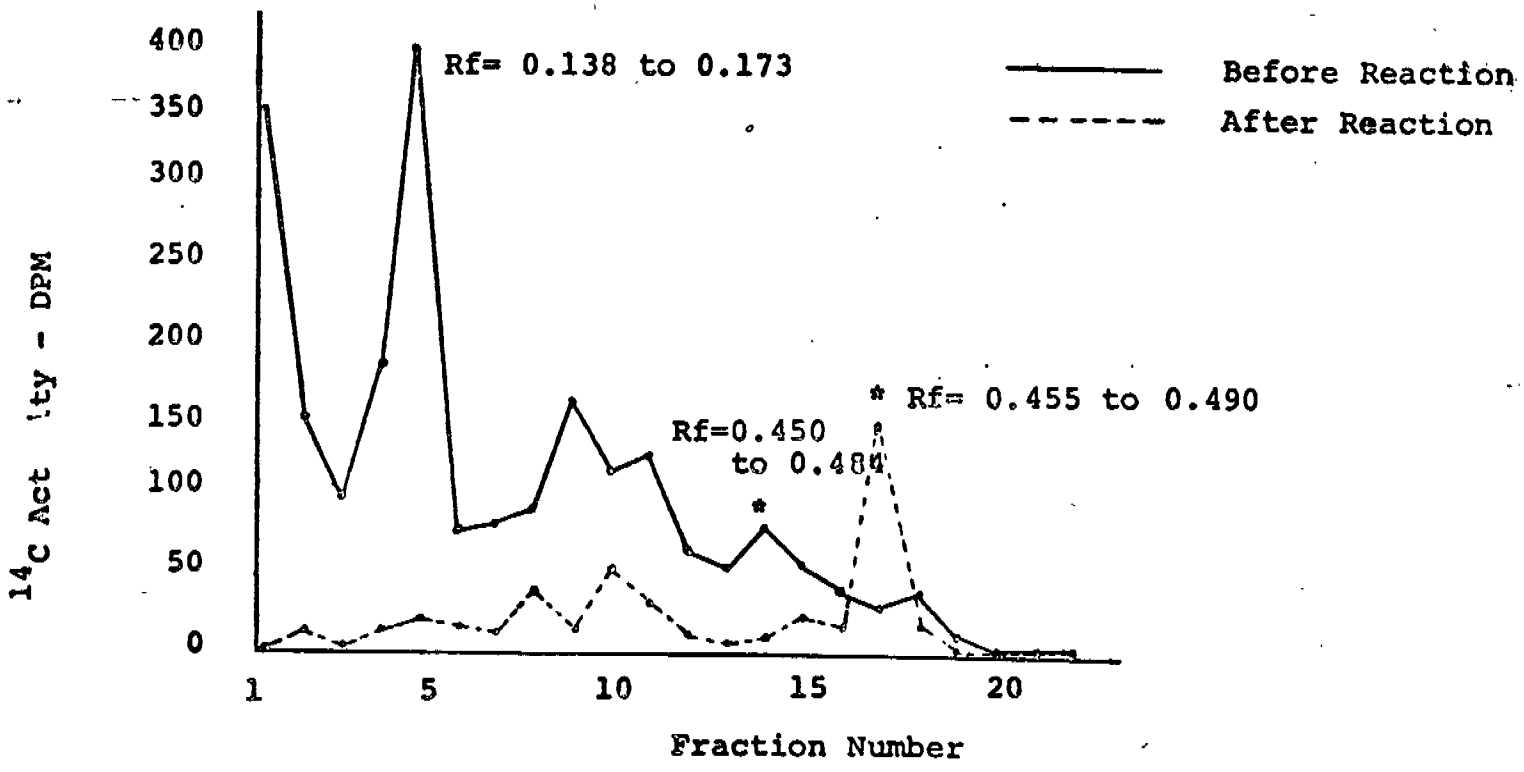
*(The peak at fraction 14 which disappears after reaction corresponds to the metabolite present at a 1.00 ratio, see Figure 3.)

Figure 8: Thin-layer Chromatography of Metabolite B Before and After DCHA Mercapturic Acid Assay



*(These peaks correspond to metabolite C and are shifted by DCHA treatment in a fashion similar to that occurring in Figure 6).

Figure 7: Thin-layer Chromatography of Metabolite A Before and After DCHA Mercapturic Acid Assay



*(Contaminant peak present before and after reaction)

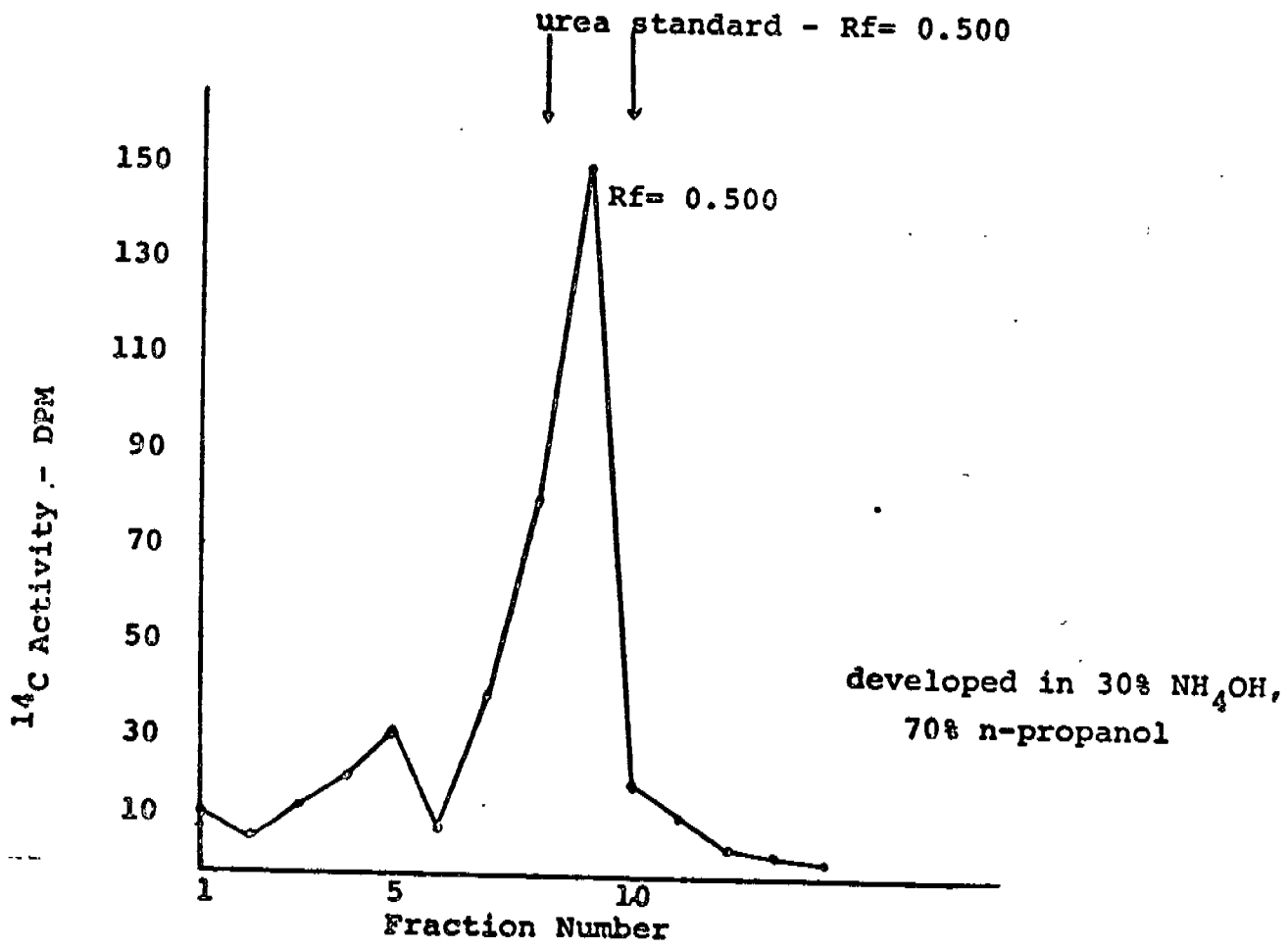
REFERENCES

1) Johnson, M. K., Biochem. Pharmacol., 16 185,199, (1967).

2) Stekol, J. A., J. Biol. Chem., 113 279-288, (1936).

3) Barnsley, E. A. and Young, L., Biochem. J., 95 77-81 (1965).

Figure 9: Thin-layer Chromatograms of a Minor ^{14}C-Labeled Urinary Metabolite of VC: ^{14}C-Urea



METHODS

Porasil Liquid Chromatography. Six ml of urine containing about $3 \cdot 10^6$ dpm of ^{14}C activity was lyophylized to a volume of about 2 ml, transferred to a 2 dram vial and evaporated to dryness under a stream of nitrogen at room temperature. One ml of methanol was added and the sample shaken to dissolve the organic material. The sample was centrifuged and the methanol solution transferred to a clean vial. About 90% of the ^{14}C activity in the original sample was found in the recovered methanol solution.

The liquid chromatography column was prepared by packing a 1/4" OD x 1 meter stainless steel column with Porasil B®(250), 37-75 μ (Waters Associates, Framington, Ma.). The pumping system used was a Waters Model 660 Solvent Programmer with two Waters Model 6000 Pumps. The flow rate was maintained at 5.0 ml/min while the solvent was programmed to form a linear gradient from hexane:chloroform (1:1) to methanol over 30 minutes. The column temperature was ambient, about 23°C. The sample was injected in 50 μl aliquots and washed onto the column with hexane:chloroform (1:1) for about 30 seconds between injections. Normally from 100-300 μl of methanol solution containing from 250,000-750,000 dpm of ^{14}C activity were injected for each run.

THE ISOLATION AND IDENTIFICATION OF URINARY METABOLITES OF VINYL CHLORIDE

INTRODUCTION

Earlier work in this laboratory with rats exposed to 7900 ppm of ^{14}C-vinyl chloride (VC) for one hour had indicated the presence of 3 major and several minor urinary metabolites which could be separated by TLC and column chromatography. One of the major metabolites reacted as a mercapturic acid in several chemical derivitizations but it could not be identified as such by instrumental techniques.

In this study urine collected from 6 rats for 24 hours after administration of 20 mg/kg of ^{14}C-1,2-VC was pooled and used for the isolation and identification of the ^{14}C labeled metabolites.

Gas Chromatography. The collected eluant fractions from the Porasil liquid chromatography step were combined to give 3 fractions containing the 3 peaks of ^{14}C activity. These were evaporated to dryness under N_2, dissolved in methanol: diethylether (1:1) and methylated using diazomethane. They were then evaporated to dryness and dissolved in methanol to give a concentration of 500-1200 dpm of ^{14}C activity/µl.

The methylated fractions were chromatographed on one of two columns, (A); 6' x 2 mm ID glass packed with 10% UCW-98 on 80/100 Gas Chrom Q or (B); 6' x 2 mm ID glass packed with 3% OV-210 on 80/100 Chromsorb 750. Column A was programmed from 100° to 250°C at 10°/minute and Column B was run at 125°C isothermally.

The outlet of the column was routed into a Hewlett Packard 19034A splitter using a 5:1 split ratio. One part was fed into the Flame Ionization Detector (FID) while five parts exited through a 1/8" OD stainless steel heated exit line to a fraction trapping apparatus (Figure 1). A Hewlett-Packard 5750B gas chromatograph was used with the injection port and FID maintained at 250 and 275°C, respectively. The helium carrier gas flow rate was 35 ml/min.

The eluant was monitored at 254 or 280 nm using a Chromatronix 220 UV monitor and collected in 5 ml fractions. An aliquot of each fraction (0.1 to 0.5 ml) was counted for ^{14}C activity by adding 10 ml of Aquasol® (New England Nuclear, Boston, Mass.) and counting in a Searle MK III liquid scintillation spectrometer using external standardization. The collected eluant fractions making up individual peaks of ^{14}C activity were combined, evaporated to dryness under N_2 and picked up in a small volume of methanol.

Alumina Liquid Chromatography. The column was prepared by packing a 2 mm ID x 1 meter glass column with acidic alumina AG-4 (40 μ) (Bio-Rad Laboratories, Richmond, Ca.). The column was repacked for each run. The packed column was washed with methanol until a stable baseline was indicated by the UV monitor. About 100,000 dpm of ^{14}C activity of a single peak from the initial Porasil separation was injected on the column and eluted with 6% concentrated aqueous NH_4OH in methanol at a flow of 1 ml/min. The eluant was monitored at 254 nm at a sensitivity of 0.16 absorbance full scale and collected in fractions of from 1-2.5 ml. The fractions making up peaks of ^{14}C activity were combined and evaporated to dryness under N_2.

Synthetic Metabolite Samples. Samples of S-(2-hydroxyethyl)-N-acetyl-cysteine and thiodiglycolic acid were synthesized by Dr. Norton Peet of Dow Lepetit Pharmaceutical R&D.

RESULTS

Separation of Three Major Metabolites by Liquid Chromatography. Using liquid chromatography on a Porasil (porous silica gel) column, urine from rats given 20 mg/kg ^{14}C-VC orally was separated into three major peaks containing over 90% of the applied ^{14}C activity and several minor peaks. The major peaks are shown in Figure 2. The separation between the peaks and consequently the relative amounts of each varied, depending apparently on the amount of water in the sample and on the column. Under average condition about 45% of the ^{14}C activity was contained in the first peak (Metabolite A).

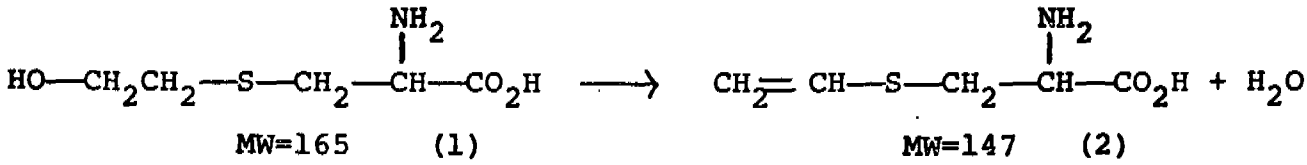
A typical series of successive preparative runs using the same sample with a column loading of about 750,000 dpm (equivalent to about 0.7 ml of urine) is shown in Figure 3. The recovery of ^{14}C activity from the Porasil column was essentially 100% and the column could be reused several times before repacking. When a Corasil II column was substituted for the Porasil column the results were similar but the column capacity was decreased.

When fractions were trapped from the GC for counting of ^{14}C labeled metabolites using the trapping apparatus shown in Figure 1, the glass capillary was washed into a scintillation vial using 2 ml of Aquasol from a syringe. Eight additional ml of Aquasol were added to the vial. When fractions were trapped for IR analysis, multiple GC runs were made using the same capillary and the metabolite removed with 5 µl of solvent and placed in a cavity cell.

Mass Spectroscopy. Low resolution mass spectra were run on a Finnigan Model 3000D GC-MS with the Model 6000 MS data system using both probe and GC inlets. When using the GC inlet the columns and GC conditions were identical to those previously given. Probe samples were run by placing sample containing about 1000 dpm of ^{14}C activity in a quartz cup in the probe and slowly raising the temperature from ambient to 250°C.

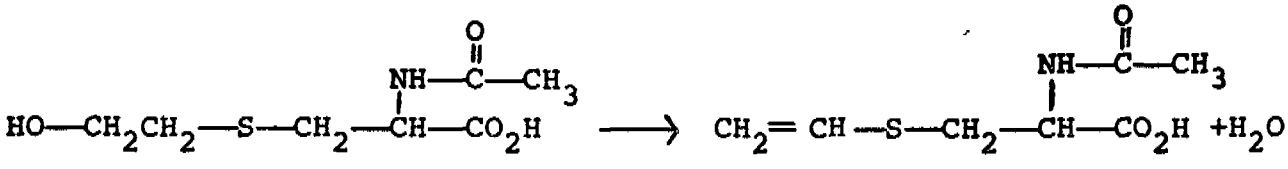
High resolution GC-MS were run on an AEI MS-30/DS-50 double beam mass spectrometer at Dow Corning Analytical Services. The resolution was 4300 and the mass measuring accuracy was generally within ±0.005 mass units over the range of interest. The GC conditions were as previously described.

Direct Probe MS Identification of Metabolite A . A probe mass spectrum was run on the combined fractions from the alumina column containing the bulk of the ^{14}C activity. The sample was shown to be a mixture of several components including one which had an apparent weak molecular ion of m/e = 189 and an important peak at m/e = 130 (Figure 6). Earlier work with S-(2-hydroxyethyl)-cysteine(1) had shown that the highest mass peak found corresponded to the dehydrated molecular ion (M-18) and that the spectrum fit that expected for S-ethylene-cysteine.(2)



MW=165 (1) MW=147 (2)

If a similar dehydration had occurred in the metabolite the mass spectrum obtained corresponded to that expected for S-(2-hydroxyethyl)-N-acetyl-cysteine (3)



MW=207 (3) MW=189

The mass spectrum of a synthesized sample of S-(2-hydroxyethyl)-N-acetyl-cysteine was found to be virtually identical to that of the material found in the metabolite A fraction (Figure 7).

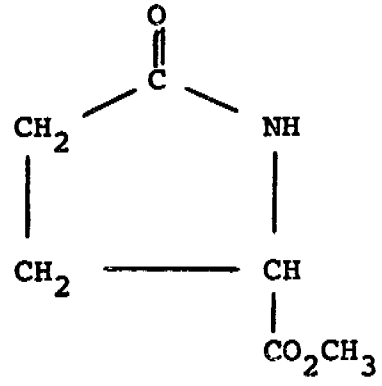
Attempts were made to purify metabolite A by additional liquid chromatography. Ion exchange was tried using a strong anion exchange packing and eluting with methanol: water containing acetic acid, tri-fluoroacetic acid or ammonium hydroxide without success. A Varian micropak SI-10 silica gel column (Varian Instrument Division, Palo Alto, Ca.) offered improved resolution compared to Porasil with very light column loadings but lost resolution when the loadings were increased to normal preparative amounts.

Alumina in basic, neutral, and acidic forms was tried using methanol:water containing various acids and bases to elute the metabolites. The most successful separation was obtained using an acidic alumina packing and a methanol: aqueous NH_4OH eluant. When the metabolite A fraction from the Porasil column was run using a gradient, the ^{14}C activity eluted as a single band containing over 90% of the ^{14}C activity put on the column (Figure 4). Using 6% aqueous NH_4OH in methanol as the eluant, the ^{14}C activity eluted as single peak partially resolved from a UV absorbing material eluting about 7 minutes later (Figure 5).

Structure (4) was theorized to arise from the elimination of mercaptoethanol from S-(2-hydroxyethyl)-N-acetyl-cysteine. A peak in the metabolite A fraction with a similar retention time to mercaptoethanol was observed by gas chromatography using a Chromosorb 101 column but at a barely detectable level. Its structure has not been confirmed.

Trapping of ^{14}C labeled metabolites from the Gas Chromatograph. Attempts at trapping metabolite A were relatively unsuccessful due to its unstable nature and the low trapping efficiency of the original trapping system. In the most successful attempt it was found that 30% of the activity trapped was in the solvent peak, 20% was in the fraction associated with metabolite B and 45% was contained in the fractions associated with metabolite A as identified earlier. In those samples which had decomposed so that the peak associated with metabolite A had disappeared, the ^{14}C activity was found only in the solvent peak and with the metabolite B fraction. In any case only about 15% of the ^{14}C activity in the metabolite A fraction could actually be trapped at the outlet of the column.

GC-MS Identification of Metabolite A. The methylated metabolite A fraction was prepared as described in the Methods section. Using a 10% UCW-98 column programmed from 120° to 250°C, metabolite A was shown to contain a peak which had the identical retention time and mass spectrum as that of the methyl ester of the previously synthesized S-(2-hydroxyethyl)-N-acetyl-cysteine (Figures 8 and 9). It was found that only fresh samples of the metabolite A fraction contained this peak. When the methylated sample was allowed to sit for several days at room temperature the peak would disappear, probably due to decomposition of the metabolite. It was found that the chromatogram of both the metabolite A fraction and the synthesized S-(2-hydroxyethyl)-N-acetyl-cysteine contained a significant peak with a molecular ion at m/e=143 (Figure 10). The mass spectrum corresponded to that expected for:

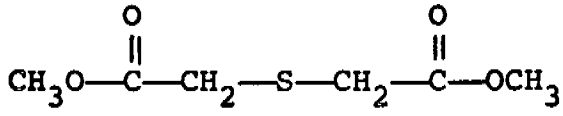


(4)

The methylated metabolite B fraction was run on an AEI-MS-30/DS-50 high-resolution GC-MS to aid in the assignment of a molecular formula. The GC conditions were similar to those described earlier.

Metabolite B was shown to be a carboxylic acid (methyl ester) with a nominal molecular weight 178 and molecular formula $C_6H_{10}O_4S$. There was a prominent loss of CH_3OH to a peak at 146. The base peak was 45 (C_2H_5O). A listing of the important peaks is shown in Figure 13.

A sample of thiodiglycolic acid (dimethyl ester) (5) was run on the Finnigan GC-MS. The mass spectrum was found to be identical to that of Metabolite B (Figure 14).



(5)

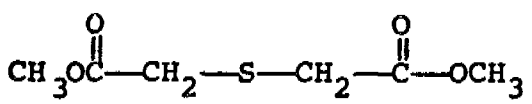
Infrared Spectroscopy of Metabolite B. Sufficient material was trapped from the GC column to obtain an IR spectrum of Metabolite B. About 12 μg of the metabolite dissolved in 5 μl of CCl_4 and run in a Beckman IR-9 infrared spectrophotometer gave the spectrum shown in Figure 15.

The ^{14}C activity in the metabolite B Porasil fraction could be associated with a single peak in the GC which eluted at relatively low temperature from either the UCW-98 or OV-210 column. It was possible to trap about 55% of the ^{14}C activity put on the column. Of the trapped material 20% eluted with the solvent peak and 50% with the single peak associated with metabolite B (Figure 11). Because of the higher efficiency of trapping metabolite B compared to metabolite A, the apparent high levels of metabolite B in the metabolite A fraction as reported above are misleading.

GC-MS Identification of Metabolite B. The methylated metabolite B fraction used in the GC trapping was also run under identical conditions in the Finnigan GC-MS to obtain the mass spectrum of the peak associated with the ^{14}C activity trapped previously (Figure 12). This same peak was also observed in the metabolite A and metabolite C fraction from the Porasil separation but at lower levels than in the metabolite B fraction.

Several ambiguous areas remain in the positive identification of metabolite A which should be pointed out. Quantitative interpretation was difficult because of its instability at room temperature. In GC considerable ^{14}C activity eluted as a low MW component with the solvent peak. In addition, only a small portion of the ^{14}C activity put on column was trapped coming off the column. These inconsistencies could be explained by sample degradation on column leading to the observed m/e 143 peak (Figure 10). Additional work is now in progress using synthetic ^{14}C-labeled S-2-(hydroxyethyl)-N-acetyl cysteine on LC and TLC. Preliminary results strongly support our original identification of metabolite A and will be reported when complete.

Metabolite B, containing about 25% of the total ^{14}C activity in the urine has been identified as thiodiglycolic acid (5)



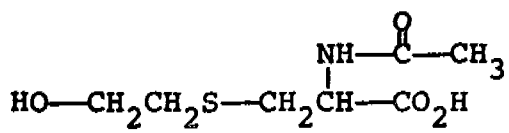
(5) (methyl ester)

Its structure has been confirmed by high resolution GC-MS, by IR spectroscopy and by comparison with an authentic synthesized sample.

The band at 1435 cm^{-1} would indicate a $-S-CH_2-\overset{O}{\overset{\|}{C}}-$ structure and that at 1745 c^{-1} an ester. An authenic sample of thiodiglycolic acid (dimethyl ester) was run for comparison and was found to contain major bands identical to those obtained in Metabolite B (Figure 15).

DISCUSSION

Three major urinary metabolites of VC have been separated by liquid chromatography. The major metabolite, containing about 45% of the total ^{14}C activity in the urine and designated metabolite A, appears to be S-(2-hydroxyethyl)-N-acetyl cysteine:



It has been identified by probe MS in the fraction of ^{14}C activity which was eluted as a single peak on Porasil and Alumina LC columns successively. Its methyl ester as found in the metabolite A fraction had the identical retention time and mass spectrum as an authentic synthesized sample. In addition, it has been associated with at least a portion of the ^{14}C activity within metabolite A by trapping and counting a peak corresponding to its methyl ester from a gas chromatograph.

hydrolysis would be expected for the chloroethyl conjugate. More direct evidence for the instability of S-(2-chloroethyl)-cysteine is its reported half-life of 7 minutes in aqueous solution at 37°C and pH 7 (Ross, 1962).

Green and Hathway (1975) identified the vinyl chloride metabolites by preparing the N-trifluoroacetyl n-butyl esters by the method of Gehrke and Stalling (1967). This involves the formation of the methyl ester using 1.25 M HCl (gas) in methanol at room temperature followed by the transesterification to the n-butyl ester using 1.25 M HCl (gas) in n-butanol at 100°C. It has been reported by Connors and Ross (1958) and Carson and Wong (1964) that S-(2-chloroethyl)-cysteine can be prepared by heating S-(2-hydroxyethyl)-cysteine with concentrated HCl. It seems likely, therefore, that if the urinary vinyl chloride metabolites were present as S-(2-hydroxyethyl)-cysteine and its mercapturic acid they would be converted into the corresponding chloroethyl compounds by the derivatization procedure used by Green and Hathway.

While the identification of Metabolite C has not been completed at this time, we have a tenative GC-MS

In a study reported recently, Green and Hathway (1975) indicated that thiodiglycolic was the major metabolite of vinyl chloride (about 50% of the total urinary ^{14}C activity). Our work confirms that thiodiglycolic acid is one of the major metabolites of vinyl chloride but in our study it is not the major metabolite and accounts for only about 25% of ^{14}C activity in the urine.

Two other major metabolites identified by Green and Hathway (1975) were S-(2-chloroethyl)cysteine and its acetylated analog, S-(2-chloroethyl)-N-acetyl-cysteine. Since we have identified the major metabolite as S-(2-hydroxyethyl)-N-acetyl-cysteine the primary question now is whether the vinyl chloride metabolite exists as a hydroxyethyl or chloroethyl conjugate.

It seems doubtful that S-(2-chloroethyl)-cysteine could be detected intact in the urine even if formed because of its susceptability to hydrolysis to S-(2-hydroxyethyl)-cysteine. Jones (1973) reviewing the metabolism of 1,2-dibromoethane reported that the initially formed S-(2-bromoethyl)-glutathione is unstable and is hydrolyzed non-enzymatically to S-(2-hydroxyethyl)-glutathione producing S-(2-hydroxyethyl)-cysteine as the primary metabolite. A similar

REFERENCES

Carson, J. F. and F. F. Wong, The Synthesis of L-1,4-Thiazane-3-Carboxylic Acid 1-Oxide, Journal of Organic Chemistry, 29, 2203 (1964).

Connors, T. A. and W.C.J. Ross, α-Methylcysteine and S-2-chloroethyl-cysteine, Chemistry and Industry, 366, (1958).

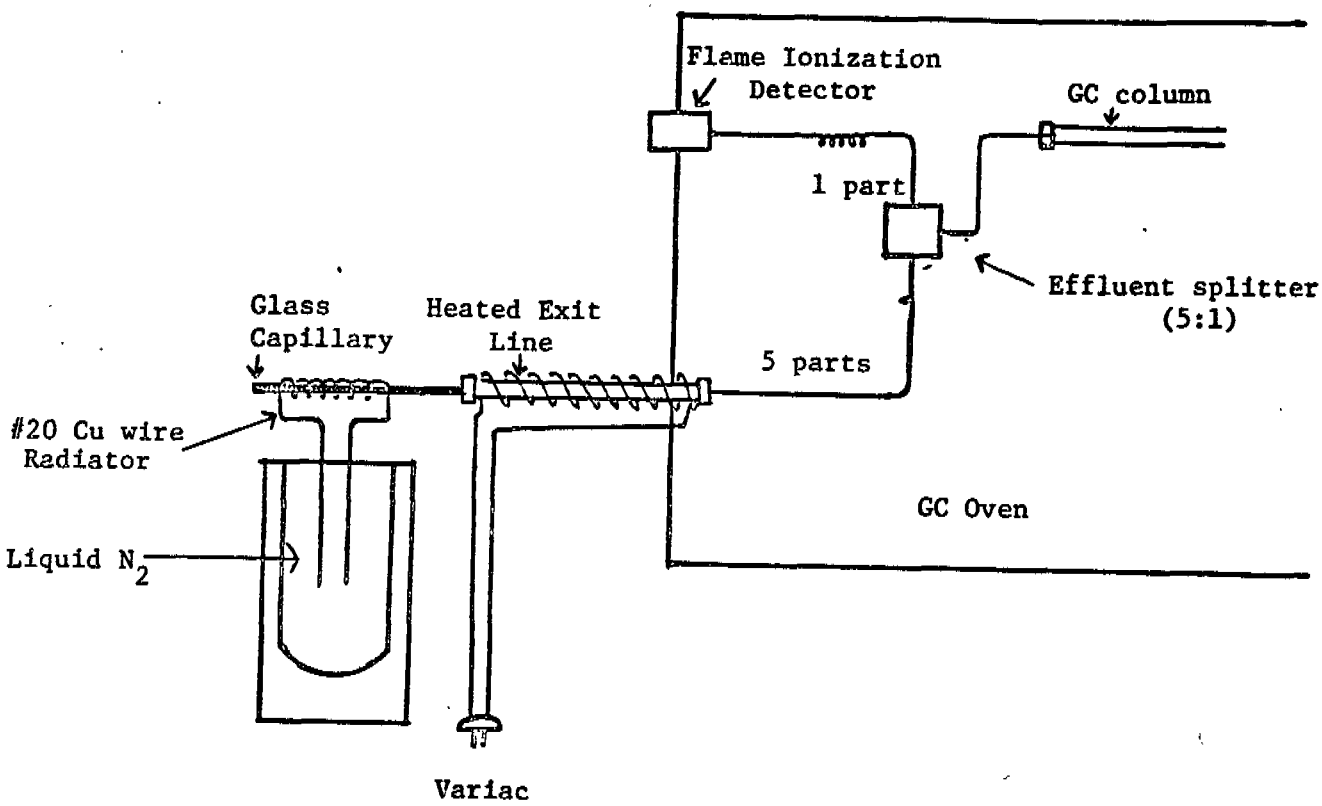
Green, T. and D. E. Hathway, The Biological Fate in Rats of Vinyl Chloride in Relation to its Oncogenicity, Imperial Chemical Industries, Central Toxicology Laboratories, Alderley Park, Cheshire, England (1975), Personal Communication.

Gehrke, C. W. and D. L. Stalling, Quantitative Analysis of Twenty Natural Protein Amino Acid by Gas-Liquid Chromatography, Separation Science, 2(1), 101-130, (1967).

Jones, A. R., The Metabolism of Biological Alkylating Agents, Drug Metabolism Reviews, 2(1), 71-100 (1973).

identification of the N,O-trifluoroacetyl, methyl ester of S-(2-hydroxyethyl)-cysteine in a metabolite C fraction separated by an improved LC procedure. This would be consistent with our interpretation of the results reported by Green and Hathway (1975).

FIGURE 1: Gas Chromatography Fraction Trapping Apparatus



Glass capillaries were 50 µl micro-pipettes pre-cooled in Liquid Nitrogen

Ross, W.C.J., Biological Alkylating Agents, p. 173, Butterworth, Inc., Washington, D.C. (1962).

Watanabe, P. G. and P. J. Gehring, Excretion of ^{14}C-Vinyl Chloride After Single Oral Administration Dow Chemical Report NBK-104 (1975).

FIGURE 3: Preparative Liquid Chromatography of ^{14}C-VC Metabolites on Porasil B(250)

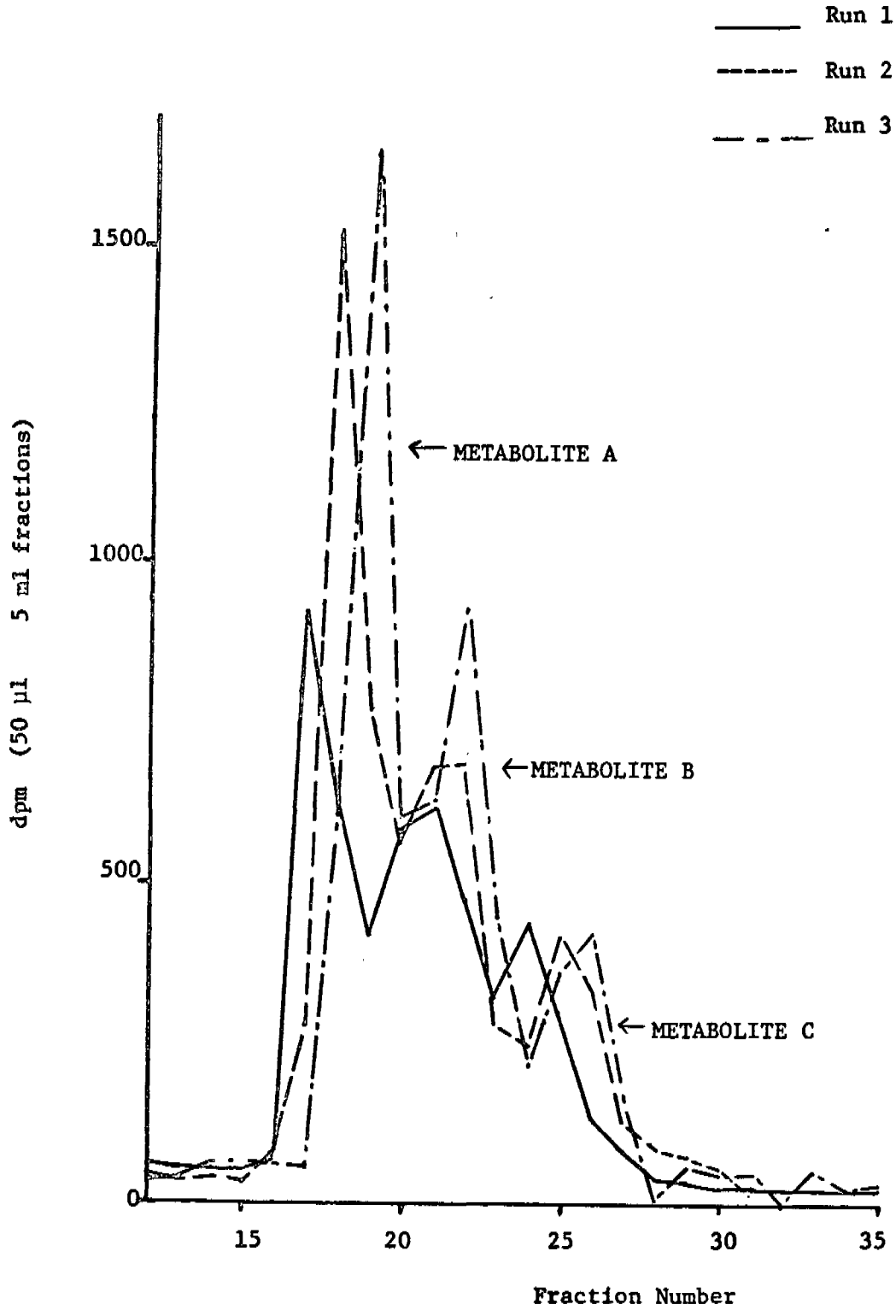


FIGURE 2: Porasil Liquid Chromatography of Urinary ^{14}C-VC Metabolites

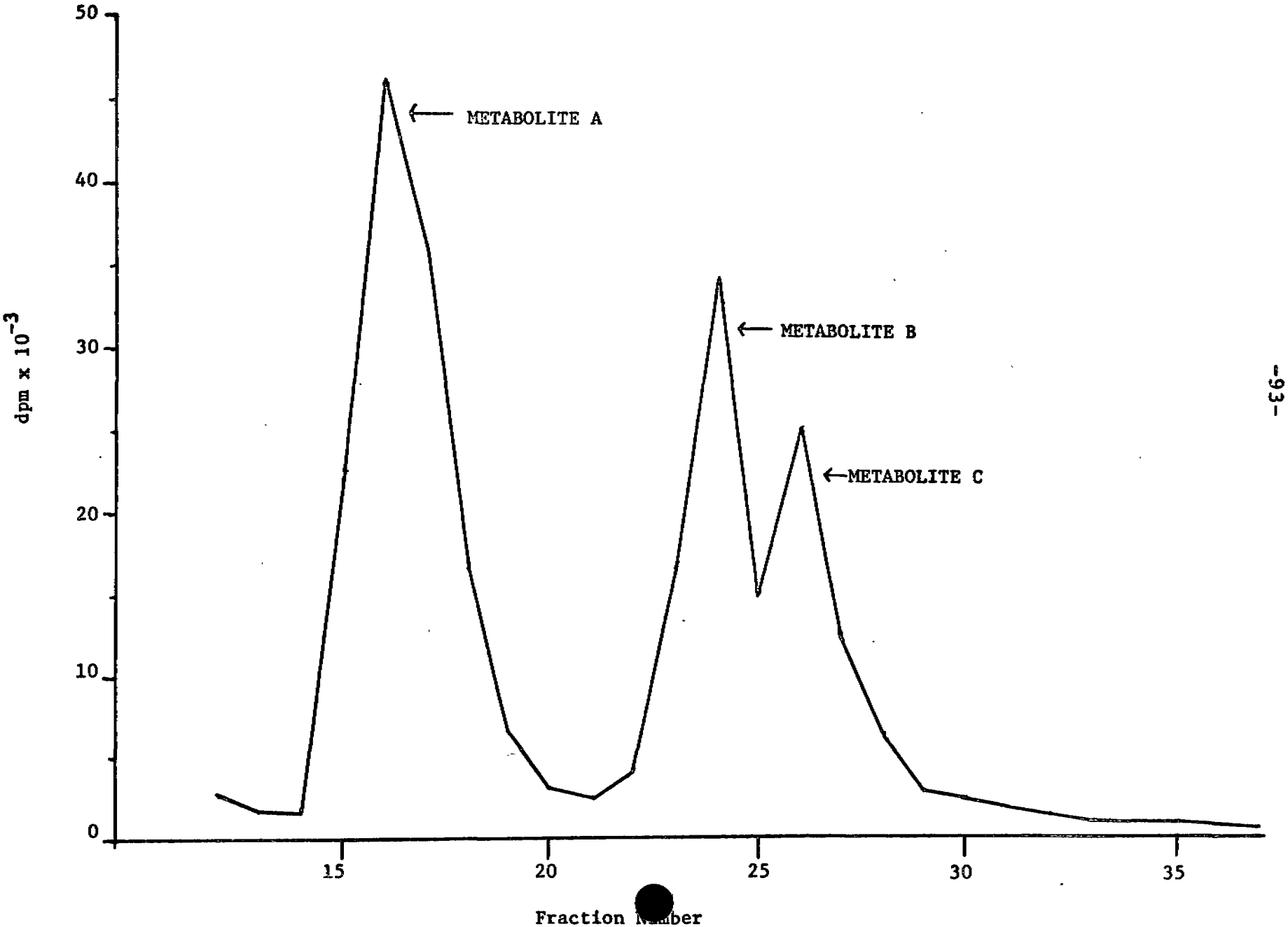


FIGURE 5: Alumina Liquid Chromatography of Metabolite A

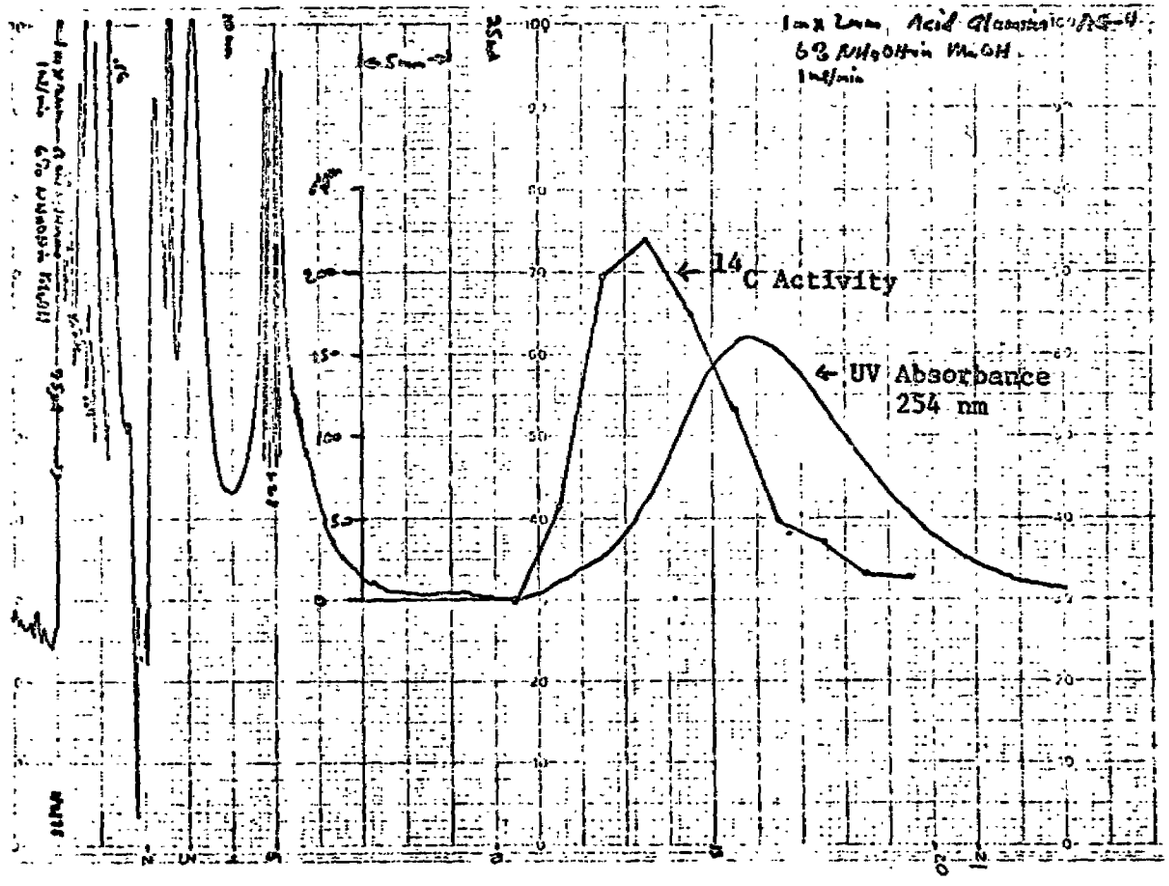


FIGURE 4: Alumina Liquid Chromatography of Metabolite A

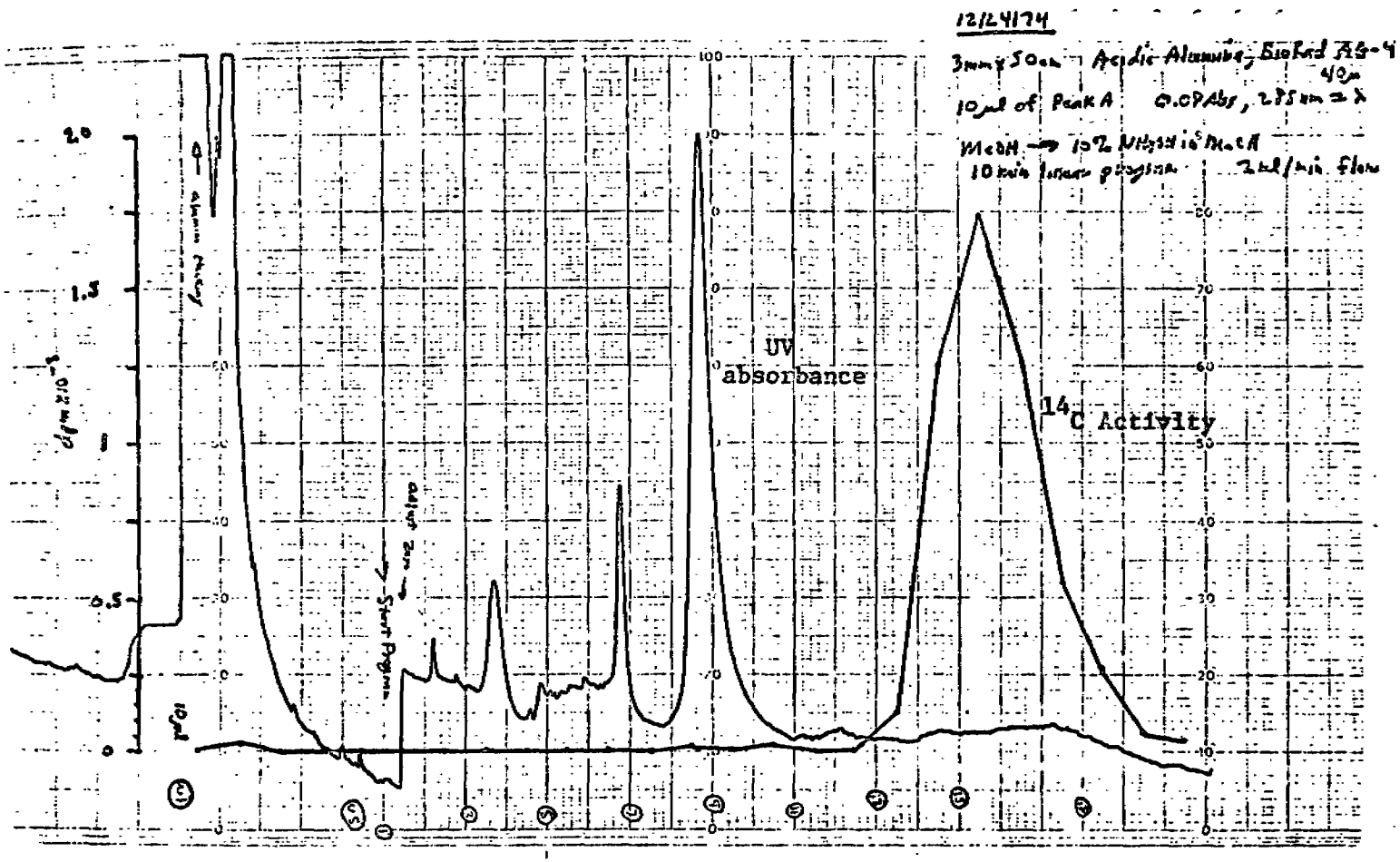
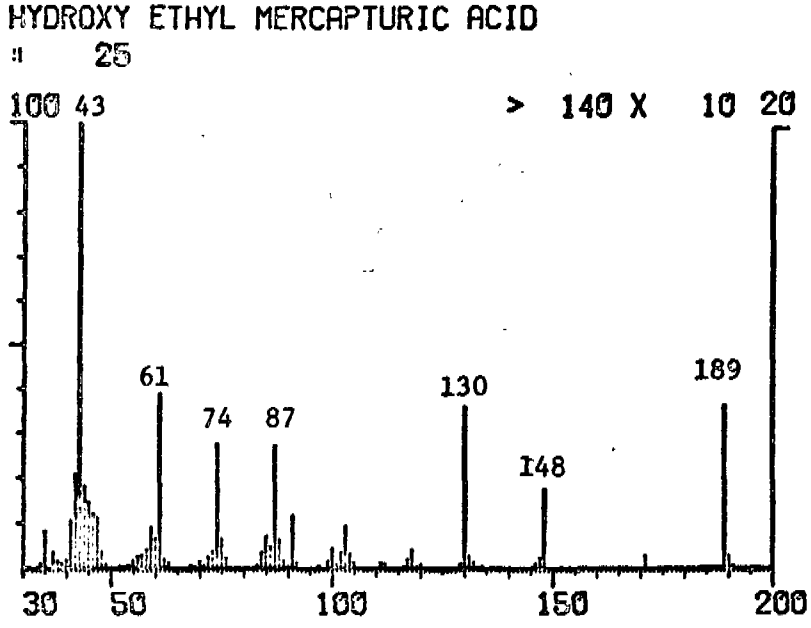


FIGURE 7: Probe Mass Spectra

S-(2-hydroxyethyl)-N-acetyl-cysteine



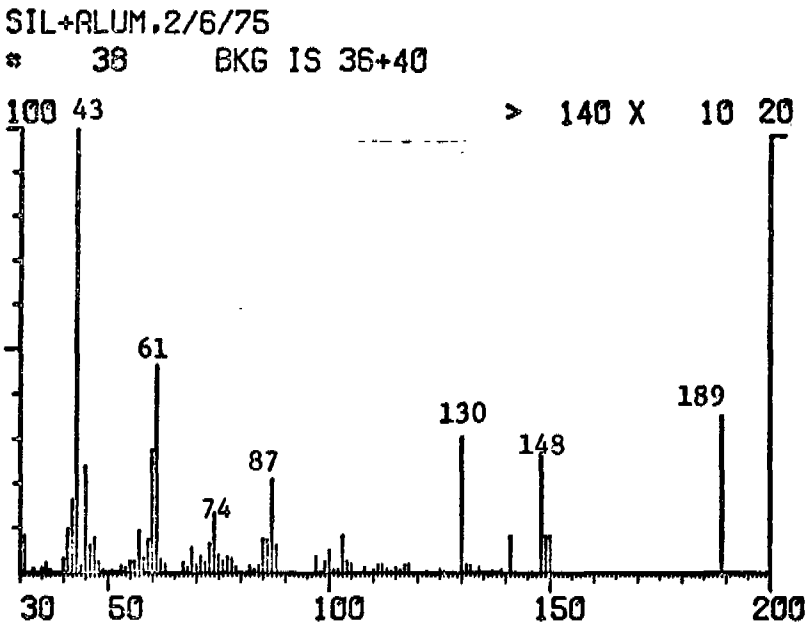


FIGURE 6: Probe Mass Spectrum of Metabolite A From Alumina Column

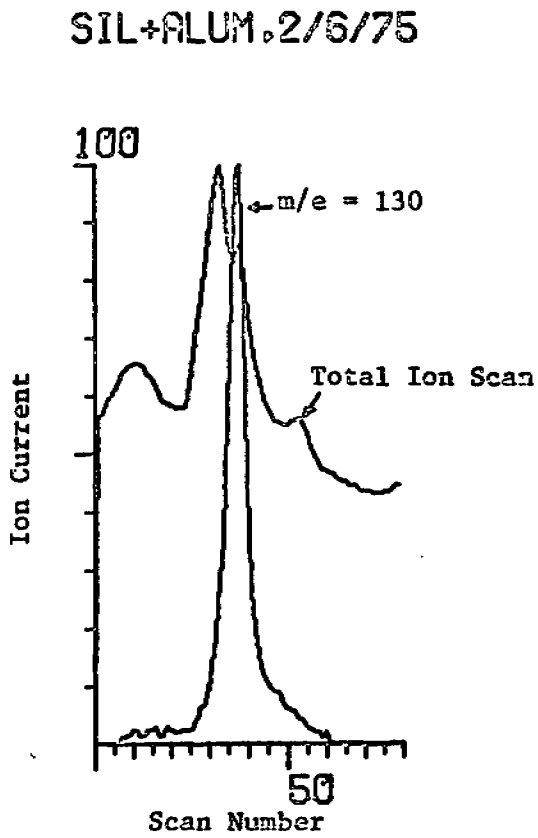
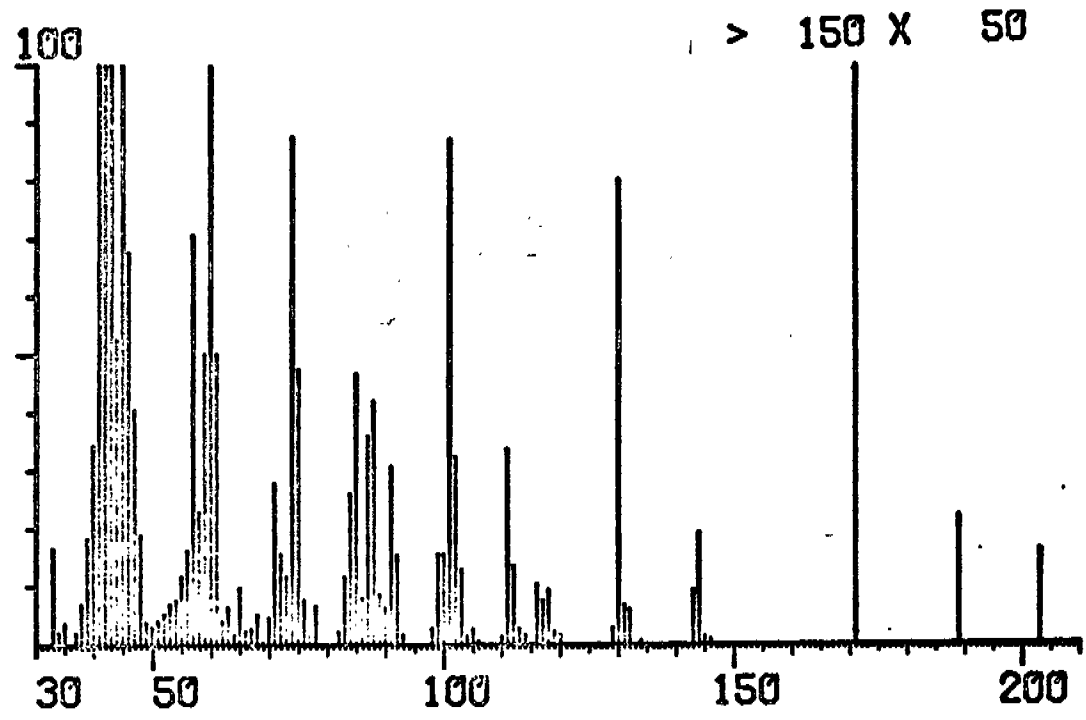
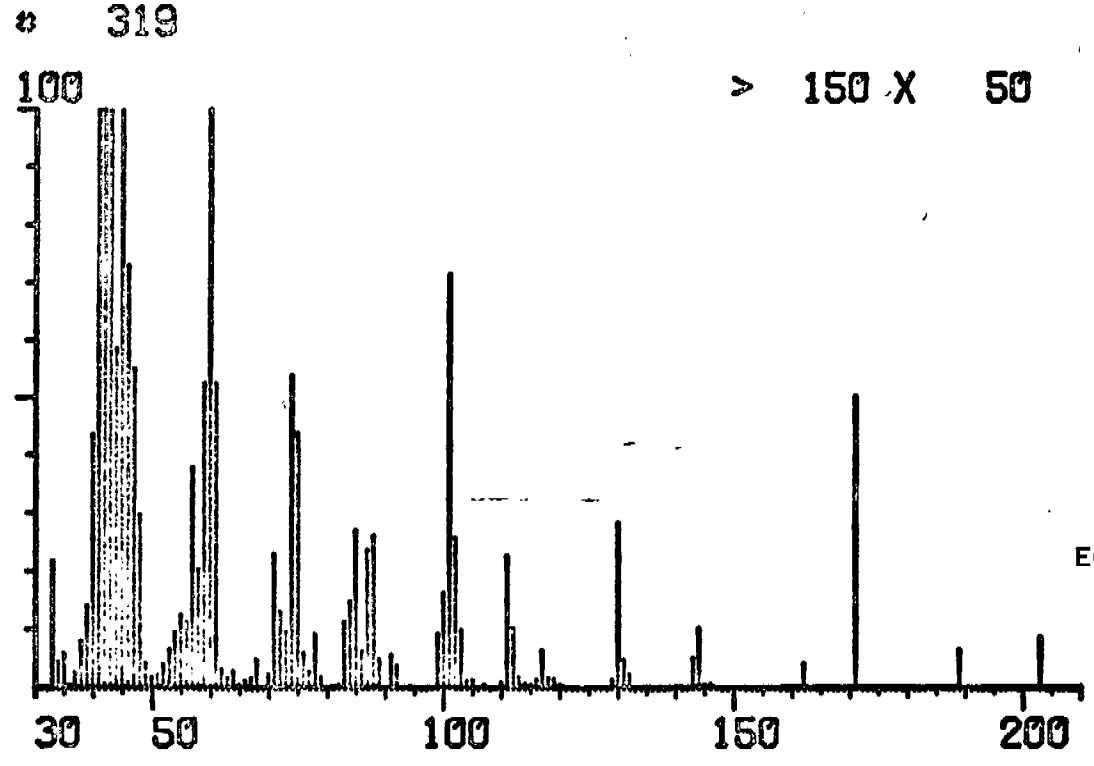


FIGURE 9: GC-MS of Metabolite A and
S-(2-hydroxyethyl)-N-acetyl-cysteine Methyl Esters





EC- 0564

FIGURE 8: GC-MS of Methylated Metabolite A Fraction

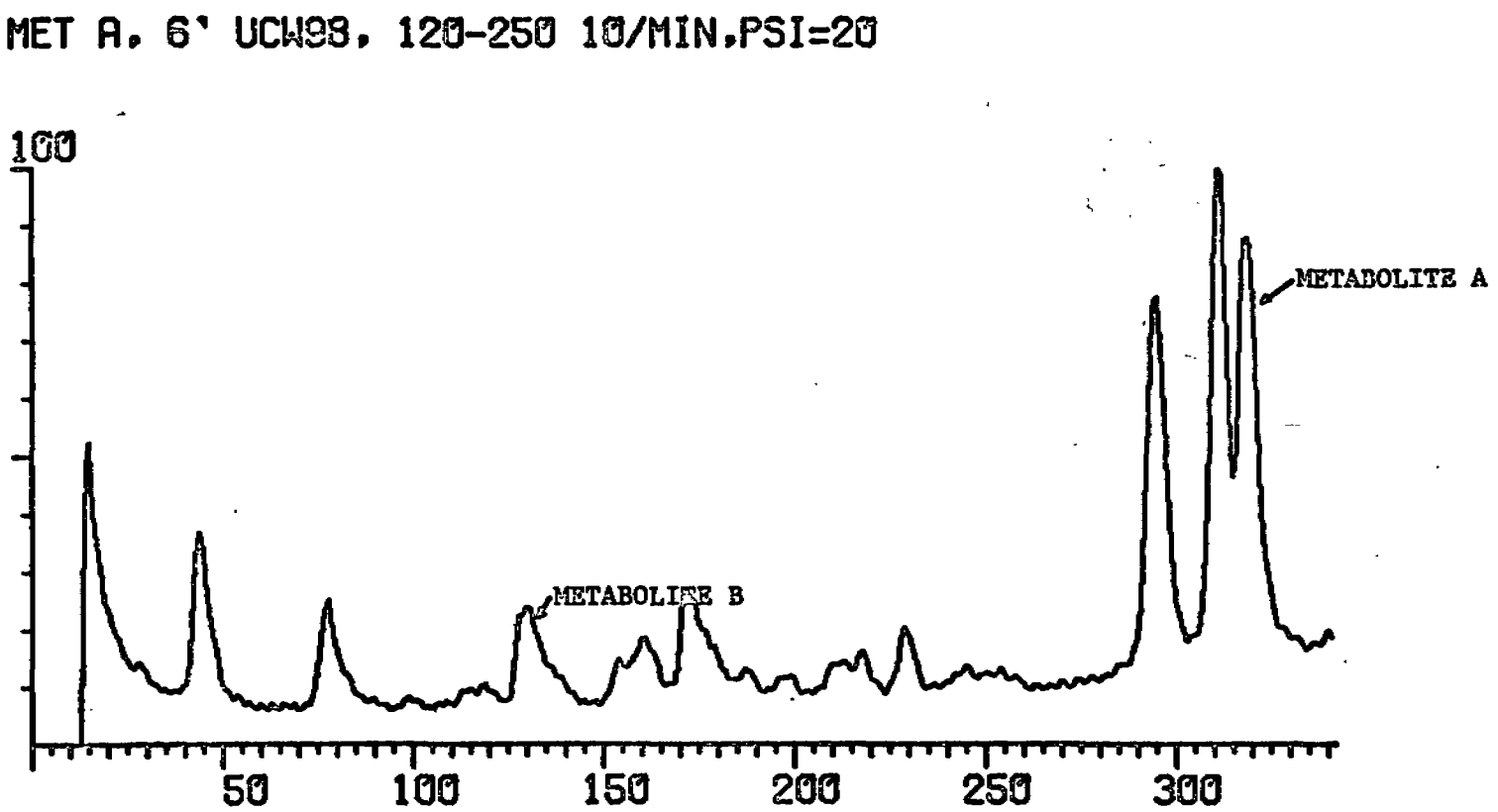


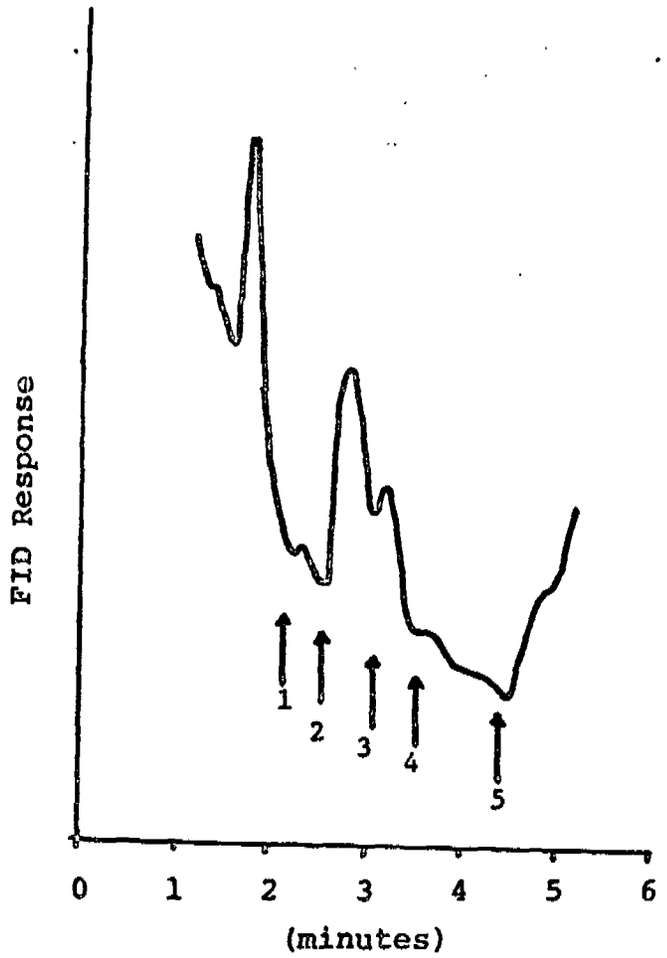
FIGURE 11: Trapping of ^{14}C Labeled Metabolite B From a Gas Chromatograph

Conditions: Column 6'-3% OV-210 on 80/100 Chromsorb WHP

Temp. 125°C

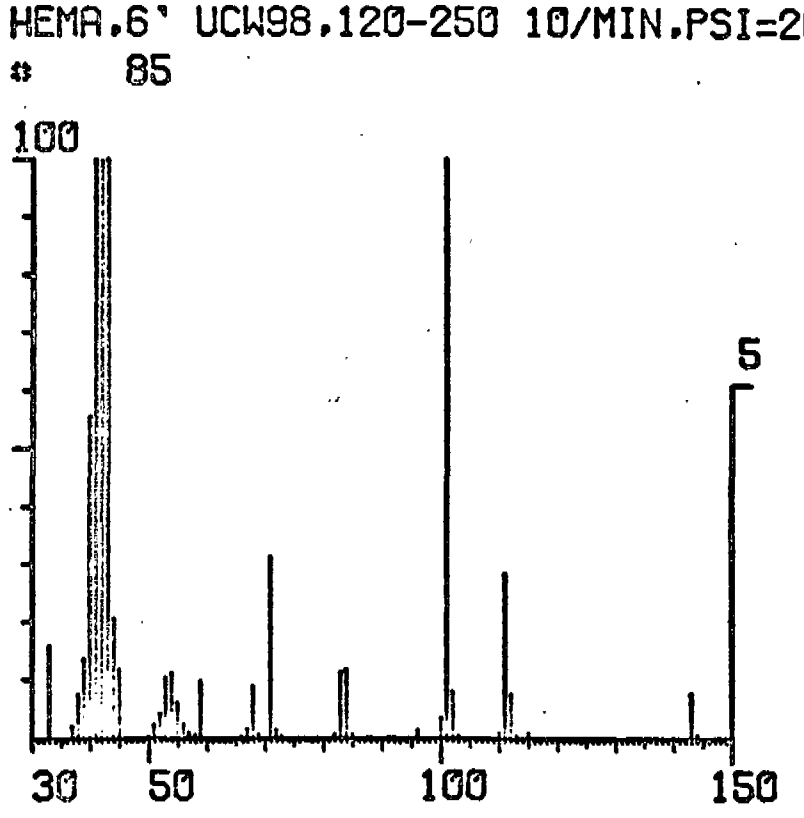
Injection 3.7 µl containing 3600 dpm

Splitter 5:1



Fraction	dpm	%
1	91	3.0
2	94	3.1
3	756	25.2
4	192	6.4

FIGURE 10: MS of Decomposition Peak (mass =143)
in Metabolite A and Synthesized S-(2-hydroxyethyl)-N-acetyl-cysteine



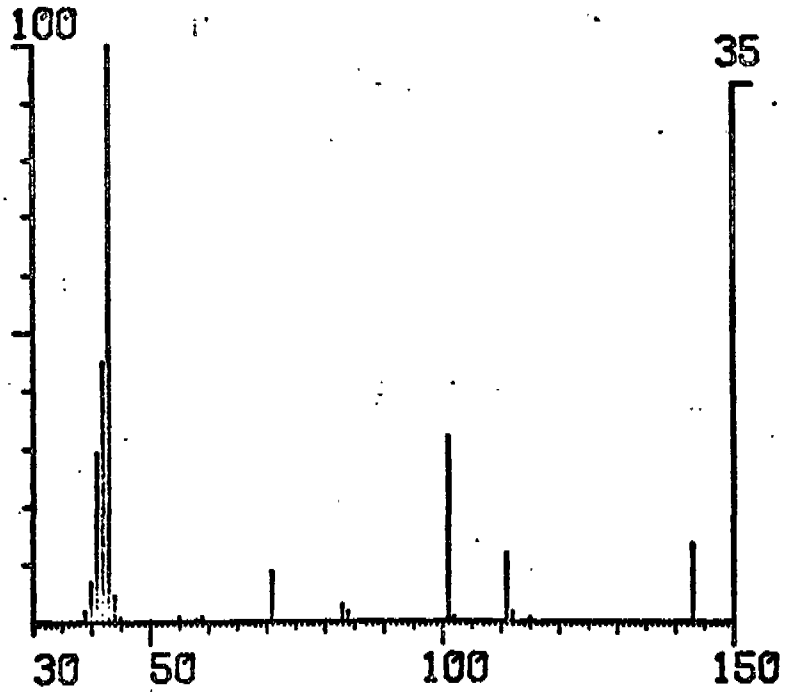
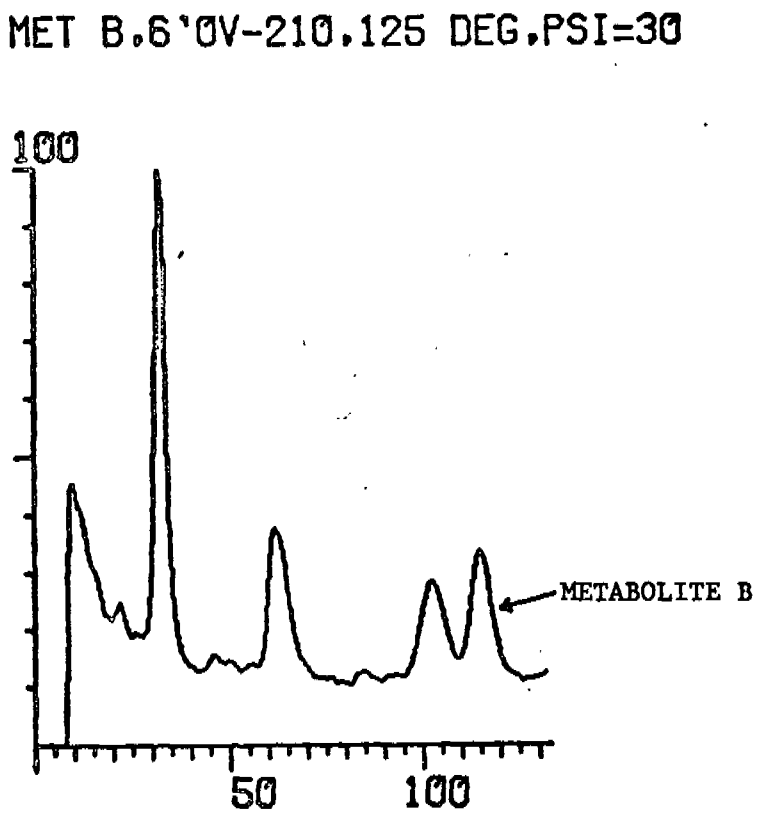


FIGURE 13: High Resolution GC-MS of Metabolite B

m/e	Molecular Formula	Structure	Comments
178.0302	$C_6H_{10}O_4S$		$CH_3O-\overset{O}{\overset{\|}{C}}-CH_2-S-CH_2\overset{O}{\overset{\|}{C}}-OCH_3$
146.0064	$C_5H_6O_3S$		178 minus CH_3OH
119.0165	$C_4H_7O_2S$		178 minus - $\overset{O}{\overset{\|}{C}}-OCH_3$
118.0127	$C_4H_6O_2S$		
101.0293	$C_4H_5O_3$	$-\overset{O}{\overset{\|}{C}}-\overset{O}{\overset{\|}{C}}-OCH_3$	
91.0247	C_3H_7OS	$-CH_2-S-CH_2-O-CH_3$	119 minus $>C=O$
86.9913	C_3H_3OS		
74.0393	$C_3H_6O_2$	$CH_3\overset{O}{\overset{\|}{C}}-OCH_3$	McLafferty rearrangement
73.9862	C_2H_2OS		
61.0142	C_2H_5S		
59.0118	$C_2H_3O_2$	$-\overset{O}{\overset{\|}{C}}-OCH_3$	
45.9905	CH_2S		
45.0379	C_2H_5O	$-CH_2OCH_3$	
43.9961	CO_2		
43.0231	C_2H_3O		

FIGURE 12: GC-MS of Metabolite B



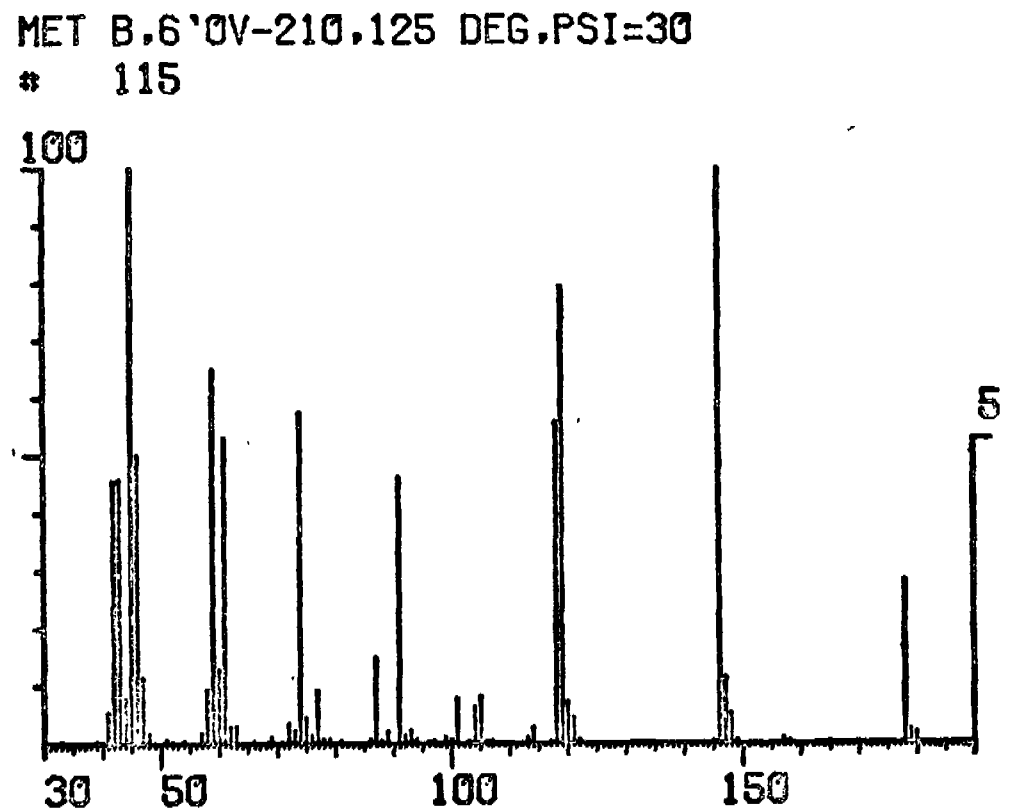
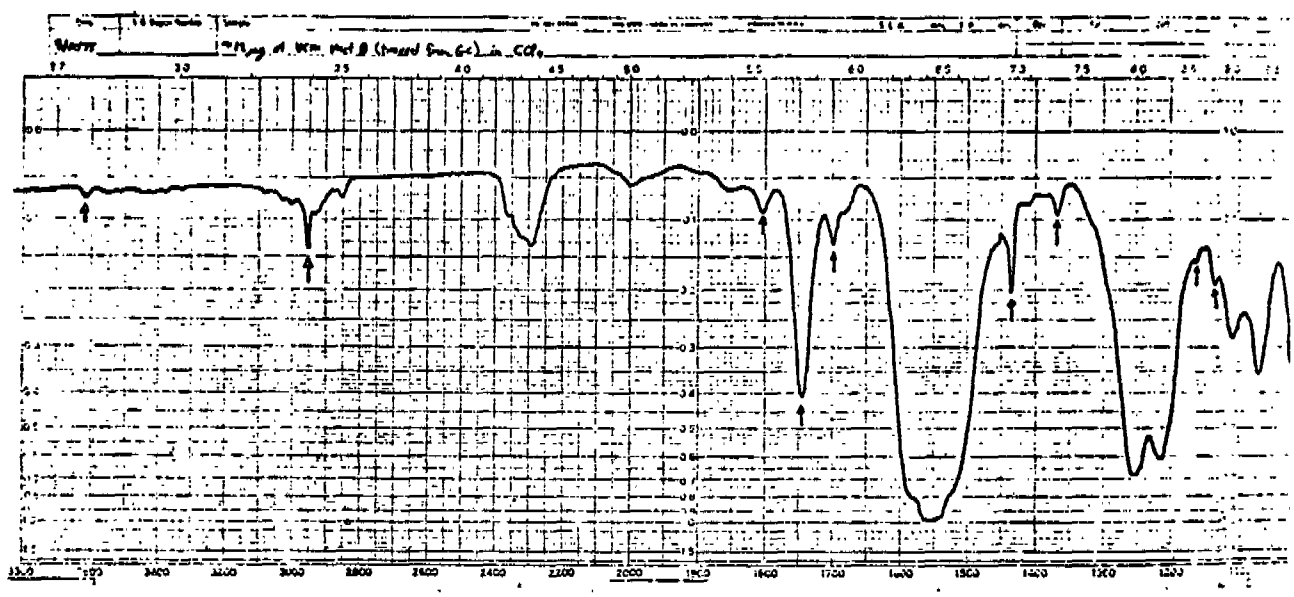


FIGURE 15: IR Spectrum of Metabolite B in CCl_4

METABOLITE B



STANDARD

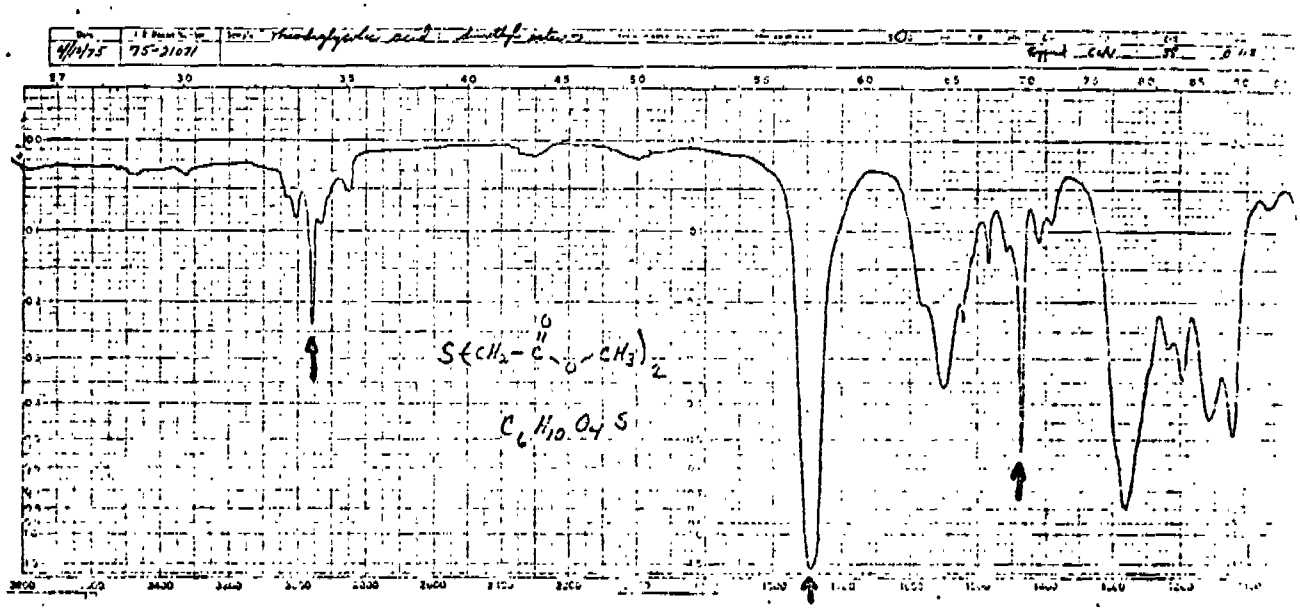
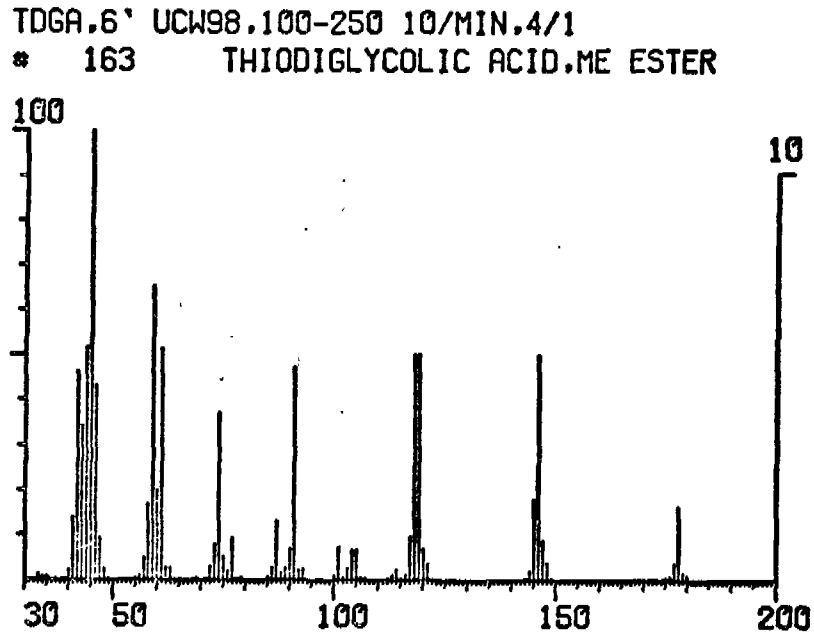
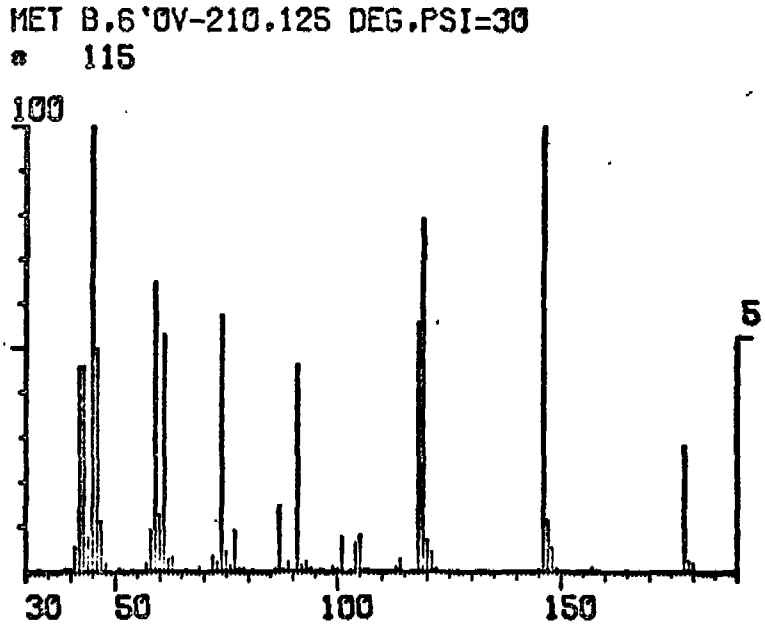


FIGURE 14: MS of Thiodiglycolic Acid (Methyl Ester)





liver homogenates and microsomal enzyme preparations (Rannug et al., 1974; Malaveille et al., 1975; Bartsch et al., 1975).

The identification of cysteine conjugates as the primary urinary metabolites of VC indicates that the principal deactivating mechanism is by conjugation with the non-protein sulfhydryl compounds glutathione and cysteine. Studies in progress in this laboratory have shown that the VC-induced depletion of hepatic non-protein sulfhydryl groups in rats is a function of concentration and duration of exposure. As the non-protein sulfhydryls are depleted, the alkylating metabolites are likely to react with protein, DNA, and RNA eliciting toxicity and carcinogenicity. This phenomena has been demonstrated to markedly influence the toxicity of compounds such as N-acetylaminofluorene, bromobenzene, and furosemide (Gillette, 1974a,b). The hepatic toxicity of these chemicals coincides with the covalent binding to tissue macromolecules.

Additional studies on the metabolic conversion of VC to alkylating metabolites will very likely provide valuable insights into the ultimate mechanism of VC-induced carcinogenesis. Characterizing the formation of these reactive products and their potential to covalently bind to tissue macromolecules would significantly contribute to explaining:

1) The dose-response relationship between the extent of exposure to VC and carcinogenicity.

2) Species differences in the susceptibility to VC induced carcinogenesis.

3) The influence of factors such as drug interactions in altering the sensitivity to carcinogenesis by VC.

SECTION II

PROBLEMS ENCOUNTERED

Considerable effort was expended in developing a system to trap expired vinyl chloride for the excretion studies. This problem has been surmounted and we plan to continue the excretion studies after inhalation exposure to ^{14}C-VC.

The separation and identification of the urinary metabolites by gas chromatography-mass spectrometry (GC-MS) has entailed substantial effort. Two of the three major urinary metabolites and one minor urinary metabolite have been identified while preliminary identification of the third major metabolite has been obtained.

SECTION III

CURRENT STATUS

The major accomplishments during the past 8 months were the development of procedures for identification and quantitation of the urinary metabolites of VC and of methodology to assess the fate of ^{14}C-VC after oral ingestion. Completion of the studies on the fate of ^{14}C-VC after inhalation exposure will conclude the initial phase of investigation as stated in the pharmacokinetics/metabolism protocol (Dec. 16, 1974).

Considering the results gathered to date by this laboratory and other recently published studies on VC, it is appropriate at this juncture to assess which additional studies as outlined in the previous protocol should be continued. Current information continues to support the hypothesis that the carcinogenicity of VC is mediated through the production of alkylating metabolites. Göthe (1975) reported the identification of acetaldehyde formed from VC by an *in vitro* microsomal enzyme preparation. In addition, numerous reports have been published which demonstrate that the mutagenicity of VC to various strains of *Salmonella typhimurium* is enhanced when incubated in the presence of fortified

Having satisfactorily accomplished identification of urinary metabolites and the kinetics of metabolism, we plan to continue our studies as outlined in the previous section. However, in order to accomplish this, it is anticipated that an additional 1 to 1.5 man years will be required. The estimated additional cost will be $50,000 to $75,000. The work will be done at cost and any funds not spent will be returned. A complete report will be issued at the completion of the study.

REFERENCES

Bartsch, H., Malavielle, C. and Montesano, R. (1975). Human, rat, and mouse liver mediated mutagenicity of vinyl chloride in Salmonella typhimurium strains. Int. J. Cancer 15, 429-437.

Gillette, J. R. (1974a). A perspective on the role of chemically reactive metabolites of foreign compounds in toxicity - I. Biochem. Pharmacol. 23, 2785-2794.

Gillette, J. R. (1974b). A perspective on the role of chemically reactive metabolites of foreign compounds in toxicity - II. Biochem. Pharmacol. 23, 2927-2938.

Göthe, R., Calleman, C. J., Ehrenberg, L. and Wactmeister, C. A. (1974). Trapping with 3,4-dichlorobenzenethiol of reactive metabolites formed in vitro from the carcinogen vinyl chloride. Ambio 3, 224-226.

Malaveille, C., Bartsch, H., Barbin, A., Camus, A. M. and Montesano, R. (1975). Mutagenicity of vinyl chloride, chloroethyleneoxide, chloroacetaldehyde, and chloroethanol. Biochem. Biophys. Res. Comm. 63, 363-370.

Rannug, U., Johansson, A., Ramel, C. and Wachtmeister, C. A. (1974). The mutagenicity of vinyl chloride after metabolic activation. Ambio 3, 194-197.

SECTION IV

WORK TO BE COMPLETED

A. Studies on the fate of VC after single oral administration to rats will be completed within the next month. The fate of ^{14}C-VC after an oral dose of 0.05 mg/kg has been completed and the data are being analyzed.

B. The fate of ^{14}C-VC after exposure via inhalation at several concentrations will be started within the next month.

C. Studies on the in vivo hepatic protein binding of alkylating metabolites of VC relative to dose and route of administration have been started.

D. Studies on the comparative metabolism of VC between species have not been started. Since the mouse has shown an increased sensitivity to the carcinogenic potential of VC, it is proposed that the composition of urinary metabolites and extent of hepatic protein binding be studied in the mouse and compared to the rat.

E. Studies of the effects of metabolic inhibitors and inducers of drug metabolism (phenobarbital, SKF-525A, ethanol, 3-methylcholanthrene, diethyl maleate) in vivo on the composition of urinary metabolites have been started.

F. Initial studies to determine the feasibility of trapping and identifying reactive VC metabolites in vitro have not been started.

A major proportion of the previous funding was utilized in developing methods for the studies on the excretion of ^{14}C-VC and identification of urinary metabolites. This has been slow and tedious but we feel absolutely esential to the ultimate resolution of the mechanism of VC carcinogenicity and even more important assessing the hazard of low level exposures in man.