THE DETERMINATION OF TRACE AMOUNT OF CARBON TETRACHLORIDE BY COLORIMETRIC AND RADIOMETRIC METHODS

by

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INTRODUCTION

In order to study the anthelmintic action of carbon tetrachloride on ascardia a sensitive method of analysis for carbon tetrachloride and its metabolites in biological samples was necessary. Because of known sensitivity of colorimetric and radiometric techniques, it appeared that these two methods used in conjunction would facilitate the proposed studies.

There were a number of other methods described in the literature on the estimation of organohalides. Nicloux (43) had determined the quantity of chloroform by its hydrolysis with alcoholic potash and the resultant chloride ion was titrated volumetrically by the Mohr method. Wells (67) estimated carbon tetrachloride in the same way and reported that the determination of quantities of carbon tetrachloride less than 1 mg was inaccurate. Schtschigol (56) found that the above method also gave low results for chloroform. A combustion method for the estimation of carbon tetrachloride had been used by Robbins (50), but the quantity determined was also relatively large, with the same order of 1 mg.

Colorimetric methods for carbon tetrachloride reported in the literature were modifications of a reaction first reported by Fujiwara (22) in 1914. A trace amount of chloroform gave a sharp color reaction when it was added to a hot solution of pyridine and sodium hydroxide. The minimal detectable concentration of $CHCl_3$ in water was reported as 1 ppm. Subsequent investigations by Webb, et al. (65) reported that a large number of organohalides were sensitive in the Fujiwara reaction and the minimal detectable concentration of these organohalides were given. By this color reaction carbon tetrachloride could be determined down to 2 ppm.

In the radiometric method of determination of carbon tetrachloride, C^{14} labeled carbon tetrachloride was the compound to be determined by counting the radioactivity of C^{14}. The sensitivity of the radiometric method was extremely high for the determination of labeled elements and compounds. If a Geiger-Muller counter with an efficiency of 5 per cent is used as a radioactivity measuring instrument, a thousand disintegrations per minute could easily be measured. The corresponding amount of radioactive carbon was only about 6×10^{-8} mg or 6.5×10^{-7} mg of radioactive carbon tetrachloride. This was about 10^5 or 10^4 times better than that of the colorimetric method. Biological samples which were taken from animals after the administration of CCl_4, could be estimated even if the starting material had been changed, because the total amount of carbon tetrachloride in the sample could be easily calculated from the specific activity of the C^{14}. However, in order to determine whether the labeled carbon present in the sample was still in the form of carbon tetrachloride, a colorimetric determination was necessary.

Techniques and procedures for the determination of trace amounts of carbon tetrachloride by the two methods were investigated. The colorimetric determination was based on a modification of the sensitive Fujiwara reaction and methods of isolating CCl_4 from biological samples were also studied. In the radiometric method, various methods of carbon-14 countings were studied in order to choose a counting method suitable for certain working conditions. Gaseous counting was the choice in practice. In this case, it was necessary to establish oxidation methods for CCl_4 and tissues containing CCl_4 and to perfect the techniques for handling and counting of the resulting gaseous $C^{14}O_2$.

LITERATURE SURVEY

Colorimetric Method

General. Early in 1914, Fujiwara (22) reported a sensitive color reaction when a trace amount of chloroform was added to a reagent consisting of 3 cc of a 10 per cent solution of sodium hydroxide with about 2 cc of pyridine and heating to boiling. In addition to chloroform, he indicated that chloral hydrate, bromoform and iodoform were also sensitive to this test. Based upon this reaction many qualitative analyses of chloroform and other chlorinated organic compounds were developed in the last 40 years (53, 66). Reports of quantitative determinations of some of the organohalides, especially chloroform (11, 69), carbon tetrachloride, and trichloroethylene, frequently appeared in the literature. For example, Fujiwara (22), Cole (14), McCollum (39), and Gettler & Blume (23) used this reaction to determine a small amount of chloroform in blood and in organs; Daroga and Pollard (16) investigated the reaction to determine chloroform and carbon tetrachloride in air and soil; Bruning and Schnetka (10), Barrett, et al. (5) applied this reaction to the determination of trichloroethylene in tissue samples. A large number of other halogenated organic compounds were investigated by Webb, et al. (65) for their sensitivity to the Fujiwara reaction. The compounds included: 1,1,2-trichloroethane, methylene bromide, dichloroacetic acid, ethylidene bromide, methylene chloride, ethylidene chloride, s-tetrachloroethylene, 1,1,1-trichloroethane, 1,1,2-tribromoethane, bromoform, tribromoethylene, benzotrichloride, s-tetrabromoethane, trichloro-tert-butyl acetate, s-tetrachloroethane, trichloroacetic acid, trichloroethylene, carbon

tetrachloride, trichloro-tert-butyl alcohol, and chloroform; in the order of increasing sensitivity to the Fujiwara reaction. It was indicated that the compounds containing only two halogen atoms per molecule or a maximum of two on any one carbon atom when more than two halogen atoms per molecule were present, in general, showed much less sensitivity than the compounds having three halogen atoms attached to the same carbon atom. Exceptions were trichloroethylene and s-tetrachloroethane which gave a much more sensitive test than 1,1,1-trichloroethane although Fujiwara and Ross (55) originally described the reaction as specific for trichlorinated hydrocarbons with three chlorine atoms on one carbon atom. It was demonstrated, however, that the compounds containing more than two halogens per molecule gave, generally, more sensitive tests than those containing only two halogens per molecule. Compounds containing only one halogen atom per molecule do not generally undergo the Fujiwara reaction (Ross, 55). An exception was 2,4-dinitrochloro benzene (10, 70). The bromine containing compounds were generally less sensitive toward the test than the corresponding chlorine compounds.

Factors That Influence Color Development. No information was available in the literature concerning the structure of the colored complex formed by the Fujiwara reaction although it has been used as a tool to analyze for some of the organohalides for many years. The sensitive color production due to a trace amount of an organohalide encouraged the investigators to find a working condition for a stable and reproducible color production. The color developed varied with different organohalides. Furthermore, it might change with reaction temperature, amount of compound present, the length of reaction time and also the reagents. For example

(4, 5), chloroform normally produced a red color but turned pale yellow or colorless with a long heating period. Trichloroethylene was a different shade of red than chloroform and on heating a longer time it turned to orange. With carbon tetrachloride or 1,1,2-chloroethane, a longer period of heating might show a negative Fujiwara test even though it was positive for a shorter time of reaction. The reaction temperature, also, was another factor for the color production. The reactants which Rogers and Kay (52) described began to boil between 76° and 80°C and that color production occurred shortly before the boiling point was attained. Heating to 100°C caused a wide variation in color production between tubes of the same carbon tetrachloride content. The working temperature was 70°C and 15 minutes of reaction was said to give a maximal color. Morris, et al. (41) developed the color at 82°±5°C with ether extract in the pyridine-sodium hydroxide solution. Eight minutes was needed to reach the maximal color production. Most of the other investigators developed the color at 100°C or in a boiling water bath. One to three minutes was necessary to reach the maximal color production (5, 19, 21, 60); a longer time of heating caused a change of color (4, 5). Thus the reaction time was temperature dependent.

For a steady, reproducible color production in a Fujiwara reaction the use of pure reagents was essential (19). As technical grade pyridine does develop a color with alkali, hence it was necessary to pretreat the pyridine until it was color stable. This was done by refluxing with a 25 per cent solution of NaOH followed by fractionation of the pyridine. The concentration of sodium hydroxide solution also influenced the color

intensity. According to the report of Webb, et al. (65), the optimum concentration of sodium hydroxide solution for carbon tetrachloride determination was 20 per cent by weight; good red colors were also obtained for the concentrations from 5 to 20 per cent in steps of 5 per cent. Salmon pink and pale yellow colors were obtained with 25 and 30 per cent concentrations of sodium hydroxide respectively. The concentration of sodium hydroxide solution necessary for maximal color development varied for other organohalides. When acetone was used as a solvent, over 75 per cent of the tested organohalides gave the maximal color when sodium hydroxide solution was in the range 10 to 20 per cent. Furthermore, other solvents of the organohalides in the reaction required some other specific concentration of sodium hydroxide solution for maximal color production. Therefore it was apparent that in the Fujiwara reaction, solvents played an important role. For a good color production of carbon tetrachloride, acetone appeared to be the best solvent to give maximal sensitivity (16, 19, 26, 65). In general, Beer's law was followed in a low concentration range of the organohalides. A higher concentration would develop a very unstable and non-reproducible color and might even change the hue (5).

Two Phase Reaction and One Phase Reaction. The Fujiwara reaction was carried out by most of the previous investigators in a two-phase system, the lower layer was colorless sodium hydroxide solution. After a separation of phases (1, 16, 26), the transmittance was measured by a colorimeter. The two phases were formed due to the partial missible nature of pyridine and sodium hydroxide solution. Oettler and Blume (23) and Adams (1) resolved this problem by adding water or alcohol after the color developed

to produce a homogeneous solution. Rogers and Kay in 1947 reported a modified Fujiwara reaction (52) which produced color in a single phase system when a special mixture of pyridine and sodium hydroxide solution was employed, hence no phase separation procedure was involved.

Colorimeter, Filter and Working Techniques. The transmittance of the color solution was measured previously by several types of colorimetric instruments such as Pulfrich visual photometer (19, 26), Evelyn photoelectric colorimeter (1, 41), and Coleman spectrophotometer (52). In general, visual photometers were not suitable for routine work and involved personal error. The Coleman Universal Spectrophotometer was a moderately priced instrument employing a plane transmission type grating and served either as photovoltaic type photocell or potentiometer null point method of measurement. The null point method was capable of giving a precision of about ±0.5 per cent, while values of 1 to 2 per cent were obtained with the galvanometer method. The spectra slit width was about 35 millimicrons (7).

In order to reduce the stray radiation a filter was employed in most types of colorimeters. For the color produced by carbon tetrachloride in the Fujiwara reaction, a 540 millimicrons filter was used by most of the investigators. Adams (1) indicated that the 540 millimicrons filter was more satisfactory than filters of 420, 440, 520, 580, 620, and 660 millimicrons. In other words, the maximal absorption range of radiation by the colored solution was very close to the wave length of 540 millimicrons. Frequently distilled water was chosen as a reference solution.

Sensitivity and Reproducibility. Chloroform was the most sensitive compound to the Fujiwara reaction among the organohalides (65). Its detectable concentrations were 1 ppm and 1.3 ppm according to the reports

of Fujiwara and Webb, et al. respectively. When acetone was used as a solvent the minimal detectable concentration of CCl_4 was 2 ppm (65). Habgood and Powell (26) reported a 25 ml blood sample containing 12.5 μg of CCl_4 could be detected. The reproducibility that was given by Rogers and Kay in their single-phase reaction was ±3% of the transmittance reading. Beer's law could be applied in a certain range of concentration. According to the calibration curve obtained by Habgood and Powell (26) for chloroform, trichloethylene and carbon tetrachloride, the linear relationship was maintained from zero up to 0.4 mg.

Techniques Related to the Determination of CCl_4 in Blood and Tissues. The essential point of the determination of carbon tetrachloride in blood and tissues depended on the method of extracting the carbon tetrachloride from tissue. Habgood and Powell carried out the step by steam distillation, and extracted carbon tetrachloride from the bulky aqueous solution by toluene and then developed the color with pyridine and sodium hydroxide solution. Fabre, et al. (19) used silica gel to absorb the carbon tetrachloride which was evaporated from the biological tissues at 100°C. Specially designed apparatus was needed for all of these methods. Fujiwara had indicated that a satisfactory method of extracting chloroform from fresh blood was merely by shaking it with ether. Morris, et al. (41) reported that carbon tetrachloride could be recovered from 94 to 105 per cent by extraction with ether. After the CCl_4 was isolated from the sample, its characteristic color was produced by the Fujiwara reaction with necessary modifications.

Radiometric Method

General. C^{14} is a soft β^- - ray emitter with a maximal beta energy of about 155 kev. and a half life of 5568 years (31). Because of the long half life, decay corrections are unnecessary but on the other hand the low energy beta-rays need special considerations to measure them quantitatively. C^{14} labeled compounds behave very similarly to the non-radioactive analogs and hence can be used in many chemical and biological studies.

Low Energy Beta-ray Counting Methods. In general, there are three methods of measuring the low energy beta-ray of C^{14}, namely, solid sample measurement, liquid scintillation method of measurement, and gaseous sample measurement. The choice between the three methods is based upon the required sensitivity, the self-absorption characteristics, the C^{14} to mass ratio in the sample available and the equipment available.

For solid sample counting (15, 57), the compounds containing C^{14} were combusted and all the carbon isotopes were converted into carbon dioxide which was in turn precipitated as $BaCO_3$, plated and counted. Two types of instruments are used for counting the $BaCO_3$ or solid sample; the thin-mica-end-window counter with a window thickness of 1 to 3 mg/cm^2 and the windowless gas-flow counters operating in either the Geiger or proportional region with the solid sample inset directly in the tube and with a gas flow to prevent air contamination. In general, if a high specific activity sample was present, the ordinary thin-mica-window tube was chosen because of simplicity. The windowless gas-flow counter increased operating difficulties but had greater sensitivity than the former due to lack of loss by air and window absorption and to the high geometric factor. The efficiency of

internal counters was about four times greater than that of the mica-window counter.

In measuring beta-emitting solids of any appreciable thickness, the phenomenon of self-absorption was more important than the other factors related to the efficiency of end-window counting. Beta-particles have a definite range and are stopped by relatively small amounts of material. In the radioactivity measurement of a sample, many of the beta particles originating from within the sample would be absorbed by the sample itself and therefore would not be counted. This behavior is known as self-absorption. In the case of counting C^{14}, 8 mg/cm^2 of sample caused a 50 per cent reduction of true counting rate. The commonly used thicknesses for reference were zero thickness and infinite thickness. The efficiency of thin-mica window counters with $BaCO_3$ sample was usually of the order of 5% and approximately 0.3-0.4 millimole of carbon may be used in the sample (infinite thickness).

Based on the fact that certain solutions emitted light when exposed to an externally placed radioactive source (49), the liquid scintillation counting was developed by dissolving or suspending radioactive material in a solvent containing the scintillator, a substance capable of fluorescence. Energetic particles emitted by the radioactive compound colliding with the solvent caused dismutation into a molecular cation and a high-energy electron. The collision of the latter with other solvent molecules produces many ions and electrons. The solvent cations eventually transferred their energy to a scintillator, and the scintillator cations combined with electrons, releasing their combining energy as light on return to the ground state (35). The fluorescent light is thus quantitatively related

to the amount of radioactivity in the sample. The collimated light flashes were made to impinge upon a photo-multiplier tube and there converted to electronic pulses which were preamplified and sent to a scaler. The scintillation solution employed must be transparent at the temperature employed. The liquid scintillation counting method has been developed recently. Since it possesses good sensitivity for counting and sample preparation is simple (3), it should become a very important counting method in the near future. Efficiencies for the liquid scintillation counting of 33 - 67 per cent were reported by some investigators (3, 46) and values as high as 75 - 90 per cent were given by Farmer and Berstein (20).

Most biological and organic material which contain C^{14} are assayed by combustion and counting of the $C^{14}O_2$ produced. If high sensitivity is required, the vibrating-reed-electrometer in combination with an ionization chamber for gas counting is probably the method of choice. The electrometer measurements involved the amount of current which flowed between the center electrode and the outer wall of an ion chamber due to a potential gradient across the chamber as this amount of current was proportional to the number of ions and therefore to the amount of radioactivity within the chamber. Vibrating reed electrometer was one type of dynamic-condenser electrometer and was commercially available. The precision of the vibrating-reed-electrometer was reported by Raaen and Ropp (47) as $\pm$ 3 per cent (95 per cent confidence). Ionization chambers could handle an active CO_2 sample, as large as 11 millimoles of CO_2 (9). On the other hand, very low-activity sample counting was also quite possible. Therefore,

flexibility was another advantage of vibrating-reed-electrometer other than the high sensitivity and precision.

A pulse counter instrument is also used for gas counting (6). With any type of pulse-measurements, one counts events and these events may be recorded with the aid of a scaler. The pulse counting was possible in either the Geiger-Muller or proportional voltage region. In either counting region, a suitable amount of other gas must be added with the carbon dioxide sample in order to make the tube function properly. The efficiency of the Geiger-Muller region counting was about 85 per cent and a sample of about 0.3 - 0.8 millimole of carbon might be used (9).

Sample Preparation Considerations. The preparation of either solid or gaseous samples for counting necessarily meant that most biological samples would have to be oxidized by some method prior to the sample preparation step. Consequently, a literature survey on combustion methods was done.

In the classical Liebig method of combustion (40) analysis for estimation of hydrogen and carbon of an organic substance, the sample was put in a porcelain boat, and was heated in a slow oxygen or air stream until there was no residue, and the oxidation of the carbonaceous matter was completed by passing the hot gases over a column of red-hot copper oxide. Water was trapped and weighed in sulphuric acid or calcium chloride and carbon dioxide was collected in strong caustic potash solution. If elements other than C, H, O are present, then the procedure must be modified. Nitrogen, halogens and sulfur were the elements which appeared frequently in the organic or biological samples and must be retained in the combustion tube to avoid errors on absorption. Compounds used to

absorb these interferring elements had been suggested. Oxygen gas pressure control and the applied temperature were also important factors for quantitative recovery of hydrogen and carbon. The Pregl's dry combustion method had been known as a very successful combustion process. His "universal filling" of a combustion tube could be used for all types of organic compounds. Usually, the combustion was completed at red heat (600° - 700°) by a column of mixed copper oxide wire and lead chromate granules, retained between short asbestos plugs. The lead chromate served to retain sulfur, arsenic, etc. Halogens were retained by plugs of silver-wool. Finally, oxides of nitrogen were retained by passing the gases through the granular lead peroxide maintained at 170° - 200°C. In addition, Pregl standardized his combustion procedure by maintaining a constant gas flow rate through the tube, thus minimizing the chance of errors due to irregular burning, inflammation, or explosion of the organic substance.

The microanalysis of organic substances by wet combustion had been carried out first by Van Slyke and Folch's manometric determination (61) which was based on the oxidation of the organic compound to carbon dioxide and the measuring of the pressure exerted by this gas at a constant volume and at room temperature. This method was accurate and was applicable even if nitrogen, sulfur, halogen and alkali metals were present.

Claycomb, et al. (13) had developed a wet combustion procedure which was very simple and rapid. The apparatus (Plate III) was composed of two compartments; one of them used for the combustion reaction and the other one for absorption of the produced CO_2. Van Slyke-Folch's combustion

solution was used as the oxidizing agent. As the combustion was carried out under reduced pressure (20 - 30 mm) only non-volatile substances could be combusted by this method with an accuracy of 94 - 95 per cent or better. The procedure takes 15 minutes.

The requirements of the solid sample for low energy beta-ray counting were that it must be geometrically and physically uniform. Therefore a method that would prepare a smooth, crackfree, uniformly thick sample was essential. Labeled carbon was converted to carbon dioxide by combustion and precipitated as $BaCO_3$ by adding Ba^{++} solution. The fine precipitate of $BaCO_3$ was generally plated by one of three ways; evaporation (15, 17), filtration (2, 30, 68), or centrifugation (12, 13). The evaporation method involved the suspension of the $BaCO_3$ in a volatile inert liquid and through the evaporation of the liquid, the $BaCO_3$ solid plate was left behind. In the centrifugation, variously designed containers were described to give a flat bottom. With thorough agitation of the suspended sample, it was transferred into the vessel and centrifuged at high speeds. Then it was dried and counted. Plating of $BaCO_3$ by filtration was the most common method and had been studied and reported frequently (32, 51). Techniques involving the use of filter paper suffered from the general disadvantage of variable texture, difficulty in reproducing constant weights, tendency of the paper and precipitate to buckle, and the necessity for careful handling of the final sample to avoid damaging the precipitate. When a sintered-glass Gooch crucible was used in this case, however, a thorough decontamination was necessary before reuse, and it was not practical to hold a large number of samples until the results had been studied. To dry

the $BaCO_3$ plate completely and keep it dry was important because in moist air, C^{14} in $BaCO_3$ would exchange with the gaseous CO_2 in air and thus cause a large error (55, 68).

The preparation of samples for liquid scintillation counting was simple. However, samples could be counted only if they dissolved in a certain liquid scintillator. Various liquid scintillators were studied by the previous investigators in order that a high light efficiency (49) and solubility in all kinds of substances could be attained. A liquid scintillator of terphenyl in xylene was reported suitable for organic materials (48) and a saturated solution of terphenyl in dioxane was suitable for both organic and water soluble materials (20). 2,5-diphenyloxazole in toluene (27, 28), was used to determine the naturally occurring C^{14} content of compounds such as p-cymene and also used for the determination of $C^{14}O_2$, H_2S^{35}, $S^{35}O_2$ by dissolving them in a solution of toluene and a quaternary ammonium hydroxide (46). The factors contributing to the count were, first, the light from the sample, second, light from the background radiation, and third, tube noise. Several liquid scintillation counters had been reported (3, 49, 54) so modified as to reduce the magnitude of the second and the third factors.

In the counting of $C^{14}O_2$ in an ionization chamber with a vibrating reed electrometer, quantitative transferring of the labeled carbon dioxide into the ion chamber was first necessary. For a reproducible counting, the pressure inside the ion chamber must be maintained constant and kept the same for all the measurements. Thus the labeled carbon dioxide was usually swept into the chamber by non-active CO_2 until the required pressure was

reached. With the sweeping method, the measurement of activity at different pressures have been reported, e.g. 50 cm of Hg and atmospheric pressure (9, 42). Radioactive CO_2 could also be diffused into the chamber from a small liquid-nitrogen trap (33). In general, the system consisted of a pumping line to evacuate the ion chamber; a carbon dioxide generator to release the labeled CO_2 by acidifying either sodium or barium carbonate; a non-active carbon dioxide sweeping line with the necessary drying tubes and bubble-rate counting devices and a pressure regulator to control the pressure as desired. An assembly of apparatus to fill the ion chamber to atmospheric pressure is shown in Plate IV (42).

EXPERIMENTAL WORK AND RESULTS

Colorimetric Method

Examination of Sensitivity and Color Behavior of CCl_4. In the preliminary studies on the color behavior and on the sensitivity of the color reaction of CCl_4, the single-phase reaction procedure described by Rogers and Kay (52) was followed rather closely. The procedure is given in the following paragraph.

Procedure. Five ml of pyridine-NaOH solution was mixed with 2.5 ml of an acetone solution of CCl_4 in a 18 x 145 mm test tube stoppered by a tissue paper-wrapped cork. After being heated for 15 minutes in a water bath held at $70^{\circ} \pm 0.5^{\circ}C$, the tube was immersed in cold water for one minute, and then cooled in air for 11 minutes. Transmission of the solution was determined with a Coleman Universal Spectrophometer at a wave length of 540 millimicrons.

Reagents. Purified reagents were necessary in the experiment. The method of purification of carbon tetrachloride as described by Klein (36) involved the refluxing of 40 ml of carbon tetrachloride and 30 ml of 5 per cent sodium hydroxide solution for two hours. The carbon tetrachloride layer was then washed with water thoroughly, dried over calcium chloride, filtered and distilled over calcium oxide.

A method for preparing pyridine with a good color stability was described by Waldron (64). Pyridine was refluxed for 0.5 hour with 20 per cent of its volume of 28 per cent sodium hydroxide solution and then fractionated.

A pyridine-NaOH reagent was made by mixing all of the constituents in volume ratio, i.e. 100 parts of purified pyridine, 40 parts water and 0.48 part of 15 per cent sodium solution.

Since carbon tetrachloride was only slightly soluble in water (about 0.8 gms per liter), it was necessary to employ some other solvent to make a suitable dilution for reference test. Thus acetone-CCl_4 solution was prepared; first 1.0 ml of CCl_4 was diluted to 50.0 ml with acetone, then 5.0 ml of this solution were diluted to 50.0 ml and finally 6.28 ml of the second dilution were made up to a volume of 100 ml. This stock solution (I) was then diluted as required. Its concentration should be:

$$\frac{5 \times 6.28}{50 \times 50 \times 100} = 1.26 \times 10^{-4} \text{ ml of } CCl_4/\text{ml of (I)}$$

(density of CCl_4 at 20°C = 1.595 gm/ml)

$$1.595 \times 1.26 \times 10 = 2.01 \times 10^{-4} \text{ gm of } CCl_4/\text{ml of (I)}$$

$$= 201 \text{ mg of } CCl_4/\text{ml of (I)}$$

also = 201 ppm

The relationship of concentration and transmittance percentage (T%) was of considerable importance. In order to determine this relationship various amounts of the above stock solution were added to a number of solutions in which all the other components were kept constant. The color was developed under the same conditions for each concentration and the T% recorded. If the colored solution obeyed Beer's law, then a straight line should result from plotting the logarithm of per cent transmittance against concentration (ppm). The resulting curve appeared to be very close to a straight line for the concentration range from zero to 45 ppm. High concentrations showed considerable deviation from this relationship. The limit of color detection was about 1 ppm. The experimental data are given in Table 1 and the corresponding plot of transmittance per cent versus concentrations are presented in Plate I.

Table 1. Sensitivity and color intensity behavior of CCl_4 in Fujiwara reaction.

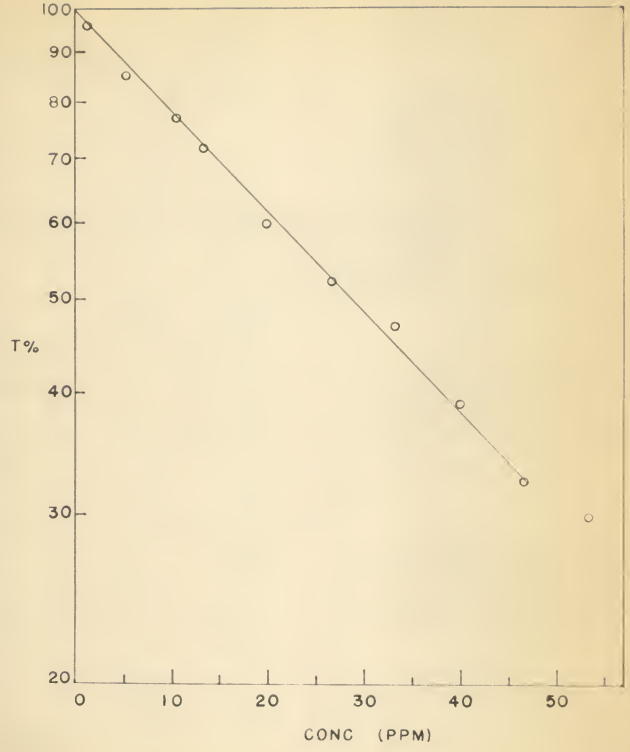
Experiment number	ppm	Volume of stock solution (CCl_4 acetone) (ml)	Acetone (ml)	Pyridine NaOH rgt (ml)	Transmittance % observed readings	Transmittance % readings corrected by blank reading
1	0	0	2.5	5	94.8	100
2	1.3	0.05	2.45	5	91.0	96.2
3	5.4	0.20	2.30	5	80.2	86.4
4	10.7	0.40	2.10	5	71.9	77.1
5	13.4	0.50	2.00	5	66.6	71.8
6	20.1	0.75	1.75	5	54.5	59.7
7	26.8	1.00	1.50	5	47.0	52.2
8	33.5	1.25	1.25	5	41.9	47.1
9	40.1	1.50	1.00	5	33.8	39.0
10	46.9	1.75	0.75	5	27.2	32.4
11	53.6	2.00	0.50	5	24.4	29.6

EXPLANATION OF PLATE I

The Relationship of Concentration of
CCl_4 and Logarithm Per cent Transmittance.

This is a plot of concentration of CCl_4 versus logarithm percent transmittance. A linear relationship was maintained when the concentration was varied from zero to 45 ppm. Points of higher concentration deviated from this relationship and were not reproducible.

PLATE I



Method Development. Observations of Effects of Different Extractive Solvents of CCl_4 to the Fujiwara Reaction. It was necessary to find a solvent which would extract CCl_4 from aqueous solution without hindrance of the color development in the Fujiwara reaction. According to the report of Habgood and Powell (26), toluene was used as the carbon tetrachloride extracting reagent from a bulky aqueous steam distillate of blood. The color developed with pyridine-20 per cent NaOH by heating at 100°C for 5 minutes. However, on attempting to repeat this work a good color was not obtained when toluene was used as a solvent for carbon tetrachloride until 1 ml of acetone was added. Thus acetone could enhance the color development which agreed with the previous observations (19, 26, 65). There were two other observations also. First, the pink colored complex developed by carbon tetrachloride, pyridine, sodium hydroxide and acetone faded on addition of toluene after 5 minutes at 70°C. Second, as the amount of toluene increased in a pyridine and sodium hydroxide solution, the intensity of the color developed was decreased even though acetone was present.

The color did not develop well when hexane was used as a solvent for carbon tetrachloride. If a curve of CCl_4 concentration versus logarithm per cent transmittance was plotted, an approximately straight line resulted with a very small slope. When 0.01 ml of CCl_4, 5 ml of pyridine-NaOH reagent and 5 ml hexane were heated together at 98 - 100°C for 5 minutes, a faint color formed. When the top hexane layer was drawn off, 5 ml of acetone added, and the solution heated again for 2 minutes at 80°C, the solution developed a very deep color. This proved also that acetone enhances the production of color.

When ether was used as an extracting reagent, its presence in the pyridine and NaOH solution retarded the color reaction. However, the boiling point of ether was low enough to distill from the immiscible solution when heated with an air condenser at a temperature higher than the boiling point of ether but lower than the boiling point of any other component in the solution. After most of the ether escaped, the color developed nicely.

Determination of CCl_4 in Aqueous Solution or Small Amount of Blood. The procedure to determine CCl_4 in aqueous solution with some modifications was similar to that of Morris, et al. (41). The samples containing CCl_4 were added to 18 ml of diethyl ether and shaken vigorously. Aliquots of 5 ml or more were taken from the supernatant layer and the CCl_4 concentration was determined as follows:

Procedure. Five ml of pyridine-NaOH reagent was mixed with 5 ml of ether-CCl_4 extract in a 22 x 145 mm test tube which was mounted on a 15 inch air condenser by ground-glass joint. A red wax pencil line was marked on the test tube for the 5 ml level. Then the solution was well shaken for 5 minutes and immediately placed in a water bath maintained at a temperature of $68 \pm 2^{\circ}C$. After heating for 10 - 15 minutes the volume of solution was reduced almost to the 5 ml mark. The test tube was taken from the water bath and 2.5 ml of acetone was added through the air condenser which was thus rinsed at the same time. The test tube was heated again with the air condenser for 20 minutes at $79 \pm 1^{\circ}C$. The tube was then immersed in cold water for one minute, and cooled in the air for 11 minutes. A few drops of distilled water were added to make the total solution to 8 ml. Then the transmittance was determined by the Coleman Universal Spectrophotometer employing a 540 millimicron filter.

Reagents and Other Preparations. All reagents were prepared as described (page 17) with two exceptions. First, ether was fractionated at 34.6°C and second, pyridine-NaOH reagent was prepared by shaking two volumes of pyridine with one volume of 15 per cent NaOH by a mechanical shaker for 15 minutes. The speed of the machine was constant for all the pyridine-NaOH reagent preparations. After standing overnight, 19 ml of distilled water was added for each 89 ml of the upper layer of the reagent.

Working Curve and Reproducibility. Three separate experiments with standard solutions of known concentrations were run at different times but under the same operating conditions to study the reproducibility of the results as well as for making a working curve. The results are shown in Tables 2, 3, and 4.

Table 2. Relation of color intensity and concentration of CCl_4 - Run 1.

Experiment number	ppm	Stock solution CCl_4-ether (ml)	ether (ml)	acetone (ml)	Transmittance % observed reading	Transmittance % reading corrected by blank reading
1	0	0	5	2.5	100.5	100
2	4	0.1	4.9	2.5	91.5	91
3	8	0.2	4.8	2.5	84.9	84.4
4	12	0.3	4.7	2.5	76.6	76.1
5	16	0.4	4.6	2.5	74.1	73.6
6	20	0.5	4.5	2.5	66	65.5
7	24	0.6	4.4	2.5	63.2	62.7
8	32	0.7	4.3	2.5	49.5	49.0
9	40	1.0	4.0	2.5	48	47.5

Table 3. Relation of color intensity and concentration of CCl_4 - Run 2.

Experiment number	ppm	Stock solution CCl_4-ether (ml)	ether (ml)	acetone (ml)	Transmittance % observed reading	Transmittance % reading corrected by blank reading
1	0	0	5	2.5	100.5	100
2	4	0.1	4.9	2.5	90	89.5
3	8	0.2	4.8	2.5	84	83.5
4	12	0.3	4.7	2.5	73	72.5
5	16	0.4	4.6	2.5	68	67.5
6	20	0.5	4.5	2.5	62.8	62.3
7	24	0.6	4.4	2.5	57	56.5
8	32	0.8	4.2	2.5	47.4	46.9

Table 4. Relation of color intensity and concentration of CCl_4 - Run 3.

Experiment number	ppm	Stock solution CCl_4-ether (ml)	ether (ml)	acetone (ml)	Transmittance % observed reading	Transmittance % reading corrected by blank reading
1	8	0.2	4.8	2.5	85.2	84.7
2	16	0.4	4.6	2.5	72.2	71.7
3	20	0.5	4.5	2.5	61.5	61
4	24	0.6	4.4	2.5	57	56.5
5	28	0.7	4.3	2.5	48.2	47.7

*Same blank correction as given in Table 2 and 3 since runs were identical.

The average values of transmittance against concentration from the three runs are plotted as Plate II. The plot was linear for concentrations from 0 to 33 ppm. This curve was then used as the calibration curve for the routine work of CCl_4 determination.

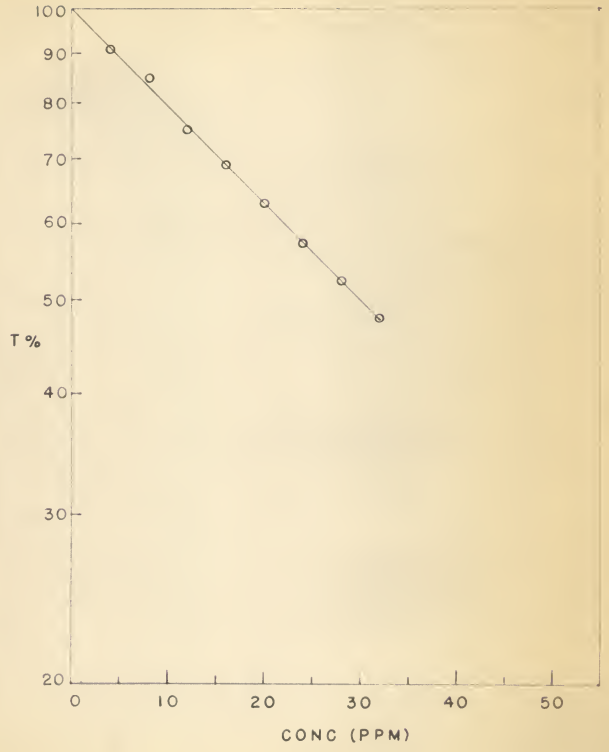
In order to show that the calibration curve could be used to determine the concentration of CCl_4 in whole blood or plasma, further studies were carried out to see if either blood or plasma interfered with the recovery of CCl_4 or the color intensity. Since CCl_4 dissolved only slightly in water, a calibration curve of concentration of CCl_4 in blood or plasma against

EXPLANATION OF PLATE II

Calibration Curve of CCl_4 Determination

This is the plot of concentration of CCl_4 versus logarithm percent transmittance. Values of transmittance were obtained from the average value of Table 2, Table 3 and Table 4. The concentration of unknown samples were determined by the interpolation of this curve.

PLATE II



transmittance per cent was difficult to determine. The recovery of CCl_4 from blood was compared to that from a 5 per cent aqueous solution of NaCl by adding the same quantity of CCl_4 to each. If there was no interaction between blood and CCl_4, the transmittance should be the same and the recovery of CCl_4 from blood was quantitative. This gave a correction factor for the above calibration curve. A 95 per cent recovery was obtained as indicated in Table 5. In this study plasma was used.

Table 5. Comparison of CCl_4 recovery from plasma with 5% NaOH.

Experiment number	CCl_4-H_2O (ml)	Plasma (ml)	5% NaOH (ml)	Vol. of CCl_4-H_2O & plasma (or 5% NaOH) mixture taken to be extracted by ether	T%	ppm cor. res. to T%
1	2	0	2	4	50.5	29.5
2	2	2	0	4	55.5	27
3	2	0	1	2	67.5	17
4	2	1	0	2	72	14
5	2	0	2	2	77	11.5
6	2	2	0	2	75	12.5

A Suggestion for the Determination of CCl_4 in a Large Amount of Blood. It was noted that a yellow color was present in the ether when more than 5 ml samples of blood were used for the subsequent color determination. The yellow color interfered with the development of the red color characteristic of the CCl_4-NaOH-pyridine system. The yellow color was probably due to the fats extracted from blood as the intensity changed with different blood samples. While some determinations of CCl_4 in plasma were made, these were discontinued because of the possibility of an appreciable loss of CCl_4 in the blood clot during the preparation of plasma. Because of these observations,

it was decided to eliminate the yellow color by distillation. The procedure is given in the following paragraph.

Ten ml of whole blood containing a known amount of CCl_4-H_2O stock solution was extracted with 20 ml of ether by shaking for 15 minutes. The layers were allowed to separate and 10 ml of the ether layer was pipetted into a distillation flask. Five ml of H_2O were added to the flask and the flask connected to a distillation column, condenser and receiving flask. At a pot temperature of 35°C most of the ether distilled out and the temperature was then raised to 100°C. After five minutes of distilling at 100°C (temperature at condenser head), the distillation was stopped. The distillate was collected in a receiver containing 5 ml of the pyridine-NaOH reagent. The color was then developed in the same manner as described before. Data are given in Table 6 for three runs each having the same amount of CCl_4 present but only run #1 having blood present. Runs #1 and #2 were distilled and the CCl_4 from run #3 was extracted.

The agreement between runs 1, 2 and 3 were fairly satisfactory. The advantages of this method were, first, that there is no color interference from blood or plasma, and second, it could be used for any amount of blood or presumably tissue samples, thus enabling one to detect small amounts of CCl_4 in large samples. Samples (worms, blood and tissues) may be placed directly into the ether and thus the loss of CCl_4 through volatilization would be avoided.

Table 6. Comparison of CCl_4 recovery - through extraction or distillation. (Blood was present in experiment number 1).

Experiment number	CCl_4-H_2O (ml)	Blood (ml)	5% NaCl solution	T%	ppm	method
1	1	10	0	80	9.5	distn
1	1	0	10	78.5	10.5	distn
1	1	0	10	76	12.5	extn

Radiometric Method

The development of a radiometric method for the analysis of trace amount of CCl_4 or its metabolites in organic or biological samples involved combustion and counting techniques. With C^{14} as a tracer, $C^{14}Cl_4$ containing specimens were combusted and the C^{14} and other carbon isotopes converted to carbon dioxide which was then collected in an ion chamber and the activity of C^{14} measured with a vibrating reed electrometer.

Combustion Procedures and Recovery of CO_2. Both dry and wet methods of combustion were studied.

Dry Combustion. This method was discussed in Steyermark (59) and was similar to that originally reported by Pregel (Grant 25). CO_2 from sample combustion was absorbed by standard NaOH solution and its amount was determined by back titration with hydrochloric acid to the phenolphthalein end point.

The combustion of volatile samples, such as CCl_4, was carried out by weighing the sample in a capillary tube (44), and combusted by the procedure as previously described. After weighing the amount of CCl_4 in the capillary and just prior to the combustion, the tip of the capillary was broken off and placed in a previously cleaned platinum foil. Both were

transfered to the combustion tube with the open end facing the heating unit. A small flame had to be used for the first combustion. The recovery percentages of carbon are given in Table 7.

Table 7. Results of dry combustion of liquid CCl_4.

Experiment number	wt. of CCl_4 (mg)	Theoretical mM of CCl_4 (mM)	Recovered mM of CCl_4 (mM)	% recovery
1	29.9	0.194	0.202	104.1
2	46.6	0.302	0.312	103.3
3	29.7	0.193	0.195	101.0
4	25.8	0.167	0.163	97.6
5	49.1	0.319	0.308	96.6
			Average	100.5

The errors were probably caused by the titration after the combustion.

Wet Combustion. The Van Slyke Folch oxidizing solution was prepared by placing 25 g chromium trioxide, 167 ml phosphoric acid (density 1.7) and 33 ml fuming sulfuric acid containing 20 per cent free sulfur trioxide in a one liter Erlenmeyer flask equipped with a ground glass stopper. The mixture was heated and occasionally swirled until the temperature reached 150°C (62). Standard CO_2-free NaOH solution was used as the absorbing reagent. The apparatus used is shown as Plate III.

The samples to be oxidized containing 5 - 10 mg carbon, were placed in the bulb A. (Plate III). Solution may be pipetted into the flask or solids may be introduced in a porcelain boat of suitable dimension. Five ml of the Van Slyke-Folch reagent was carefully added to B with a bent tip pipette. The flask was attached to tube C. The absorption flask F, containing a measured amount of standard CO_2-free NaOH, was also connected to C, the

EXPLANATION OF PLATE III

Wet Combustion Assembly for C^{14} Assay

A---Reaction compartment.

B---Side arm for holding oxidizing solution before the reaction.

C---Delivery tube.

D---Stopcock.

E---Manometer.

F---Absorption flask.

PLATE III

system evacuated through D to about 20 mm pressure and stopcock D closed. Since maintaining adequate reduced pressure was essential, the apparatus was allowed to stand for a few minutes, and the manometer observed for pressure change. Flask A was rotated 180 degrees bringing the side arm B upright and spilling the oxidant onto the sample. The bulb of flask A was now immersed in the 160°C bath. An ice water bath was then placed so as to cool absorption flask F. After 10 minutes flask A was removed from the hot bath and the apparatus allowed to stand an additional 5 minutes. F remained in the ice bath. If no leakage had occurred and CO_2 absorption was adequate, the manometer reading returned to 20 - 30 mm. CO_2-free air or purified nitrogen was admitted through stopcock D to equalize the pressure. Absorption flask F was removed, and its contents titrated with 0.1 N HCl to a faint but definite phenolphthalein pink.

It was noted that for a constant amount of Van Slyke-Folch oxidizing reagent (5 ml), the per cent recovery decreased with increasing weight of benzoic acid. Perhaps if the amount of the oxidant had been increased, the per cent recovery would have been greater for the higher weights of benzoic acid. Because of the simplicity and rapidity of the procedure, the method is recommended for small organic samples. The combustion results are shown in Table 8.

Table 8. Results of wet combustion of benzoic acid.

Amount of benzoic acid (mg)	Theoretical mM of CO_2 containing in sample (mM)	mM of CO_2 absorbed by NaOH solution (mM)	% CO_2 recovery
5	0.292	0.290	99
7.4	0.421	0.409	97
8.9	0.510	0.479	94
10.9	0.625	0.574	92
11.9	0.682	0.626	91
12.8	0.734	0.676	92
13.8	0.791	0.735	93
16.3	0.934	0.811	87

C^{14} Labeled CO_2 Counting. Gaseous counting by vibrating-reed-electrometer was used in this experiment. Samples containing C^{14} were oxidized quantitatively to carbon dioxide either by the dry combustion or by the wet combustion method. The radioactive carbon dioxide, released by acidifying the absorbing solution, was swept directly into an ion chamber for assay. The apparatus is shown in Plate IV.

Procedure. The ion chamber, placed in position I was evacuated and flushed with non-radioactive carbon dioxide which was passed from a compressed CO_2 cylinder through a bubbler and drying tube F. When the pressure inside the chamber reached one atmosphere, the stopcock of the chamber was turned off. Five to ten minutes later the non-radioactive carbon dioxide in the chamber was counted as background.

The ion-chamber was evacuated again in position I and moved to position II. Ice water was circulated through the condenser. A measured sample was put into the flask C together with 1-1.5 millimoles of 0.5 N non-active Na_2CO_3 solution and enough distilled water so that the tip of the inlet tube was immersed in the solution. An 8 to 15 ml portion of concentrated perchloric acid was released into the flask from tube B and the stopcock

EXPLANATION OF PLATE IV

Assembly for Transferring of Labeled CO_2 into Ion Chamber

A---Mercury pressure regulator.

B---Acid introducing cup.

C---Reaction flask.

D---Ice water condenser.

E---Pressure regulator.

F---Drying tube for absorbing possible moisture from CO_2 tank.

G---Drying tube for absorbing possible moisture from reaction flask.

H---Ion chamber, position I.

I---Ion chamber, position II.

J---Non-active CO_2 inlet tube.

K---Connection to vacuum pump.

PLATE IV

was closed when about 0.5 ml of $HClO_4$ was left in B. The solution in flask C was boiled for one minute, then the stopcock connected to the non-radioactive CO_2 was opened and the gas was allowed to sweep through the solution for several minutes until the chamber was almost full. The chamber was moved to position I where CO_2 was admitted rapidly to bring the gas to one atmospheric pressure. If materials containing halogen or nitrogen were oxidized, a U tube containing 5 - 10 g of hydrated stannous chloride was inserted between the water condenser and the pressure regulator. The use of the pressure regulator was to permit the reaction in flask C to be carried out at atmospheric pressure.

Observations on Transferring of $C^{14}O_2$. It was found that the CO_2 released by acidifying the NaOH solution containing absorbed CO_2 was not complete at room temperature. The solution was brought to a boil and 1 - 1.5 millimoles of non-active Na_2CO_3 solution were added to give effective stirring by the evolution of a large amount of CO_2. A rather complete transfer of labeled CO_2 to the ion-chamber was then achieved. A dry ice and acetone cooled U tube replaced the drying tube G to condense any moisture which passed through the water-cooled condenser while boiling the solution in the flask. After filling the ion-chamber with $C^{14}O_2$, it should stand for about 10 minutes before reading the vibrating reed electrometer. Stresses and strains (9) within the chamber, especially on the center electrode, caused by the evacuation and refilling of the chamber, were apparently transformed into electrical currents producing a charge on the vibrating reed electrometer which would be read as pseudo-activity.

Properties Involving the Instrument and Working Technique. The Applied Physics Corporation, Model 30 vibrating reed electrometer together

with a 250 ml ion chamber were used in the laboratory to measure the radioactivity of gaseous $C^{14}O_2$. (Plate V).

The ion-chamber was a metal can containing an insulator, a center electrode, a guard ring and a gas inlet tube. The potential across the chamber was supplied by the appropriate number of batteries, arranged in series. The 250 ml chamber usually operated at 135 volts. Since the current drain was extremely low the battery life was quite long (42). Radiation impinging on the gas filling the ionization chamber resulted in the formation of positive and negative ions. The positive and negative ions were collected on the two electrodes when an electric field was applied between these electrodes. If the radiation level was constant, the collected ions produced a constant direct current at each electrode. The radioactivity of C^{14} may be calculated from the current. The relation between the observed current and the amount of C^{14} in the chamber was a function of four quantities: first, the average energy of the beta-ray emitted from C^{14}; second, the average energy necessary for the production of one ion pair in CO_2; third, the amount of radiation absorbed in the walls of the chamber; and fourth, the recombination of ions within the chamber gas. The first and second quantities could be determined (9, 58), but the third and fourth quantities were not readily measurable. This limited the ionization chamber technique as a primary method of determining the absolute disintegration rate of C^{14}. However, by measuring samples of known specific activity the resultant current observed versus true disintegration rate could be plotted. The activity of unknowns could be easily ascertained from their drift rate in the ion chamber.

EXPLANATION OF PLATE V

Ion Chamber, Vibrating-Reed-Electrometer and Recorder

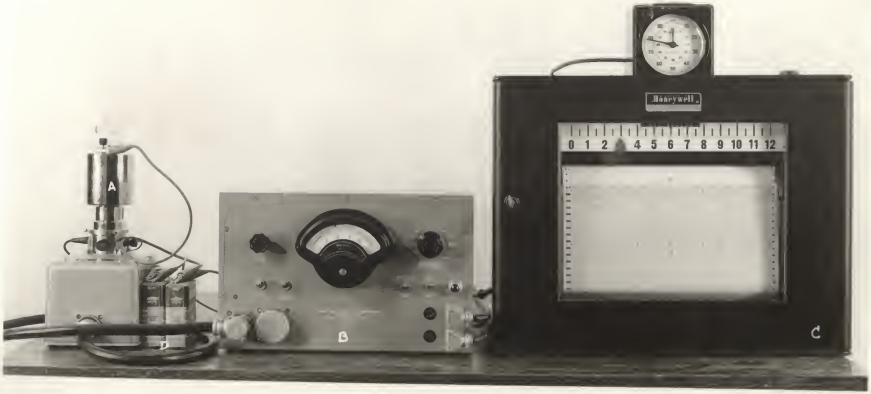
A---Ion chamber.

B---Vibrating-reed-electrometer.

C---Recorder.

D---Batteries.

PLATE V



The vibrating reed electrometer was a d-c current measuring instrument capable of detecting a current as small as 1.0×10^{-17} amperes originating in a high impedance source. It was normally used in the measurement of currents smaller than 1.0×10^{-12} amperes by the rate-of-charge method by measuring the voltage increase across a small capacitor. Larger currents might be measured by employing a calibrated high value resistor. The main feature of this electrometer was a condenser whose capacitance varied cyclically by means of a grounded vibrating reed adjacent to an "anvil" connected to the a-c output. The d-c voltage across the vibrating reed generated a proportional a-c voltage which was amplified and recorded (45). It had the same frequency as the mechanical vibrating reed.

The relation of drift rate with voltage applied to the ion chamber was determined. The chamber was filled with a known amount of active material at atmospheric pressure. The voltages applied to the ion chamber were varied. All the readings were taken on the 500 mv scale and the values are given in Table 9. A plot of voltage versus drift rate is given in Plate VI.

Table 9. Relation of drift rate with voltage applied to the ion chamber.

Voltage applied	Time (min) for drift of 8 divisions	Drift rate (div/min)
0	5.52	1.45
1 1/2	4.35	1.84
3	3.77	2.12
6	3.14	2.35
9	3.36	2.38
22 1/2	3.22	2.48
67 1/2	3.18	2.52
135	3.16	2.53
180	3.13	2.56
270	3.07	2.61
337 1/2	3.10	2.58

The above results showed that the drift rate was nearly a constant only for applied voltages from 67 1/2 volts to 180 volts.

To determine the linearity of the vibrating reed electrometer scale reading, a constant amount of active material was placed in the ion chamber under one atmosphere pressure. Its drift rate was indicated by different millivolt scales on the electrometer. The applied voltage was 135 volts.

Scales (mv)	50	100	200	500	1000	2000
Time required for drifting 8 divisions in minutes.	0.315	0.645	1.263	3.163	6.28	12.443

In the usual calculations for drift rate, the time necessary to cover 8 divisions on the 500 mv scale was measured. Some corrections for conversion of drift rate to the 500 mv scale were needed when the measurement was actually read from other scales. The factors for converting from one scale to another are as follows:

EXPLANATION OF PLATE VI

Relationship of Drift Rate with Voltage
Applied to the Ion-Chamber

This is a plot of the drift rate versus the voltage applied between the two electrodes of an ionization chamber. The drift rate values obtained from a fixed activity source were nearly constant for applied voltages from 67.5 volts to 180 volts.

The data are plotted as two graphs, one is the higher voltage versus drift rate (divisions per minutes), and the other is the lower voltage versus drift rate (divisions per minutes).

PLATE VI

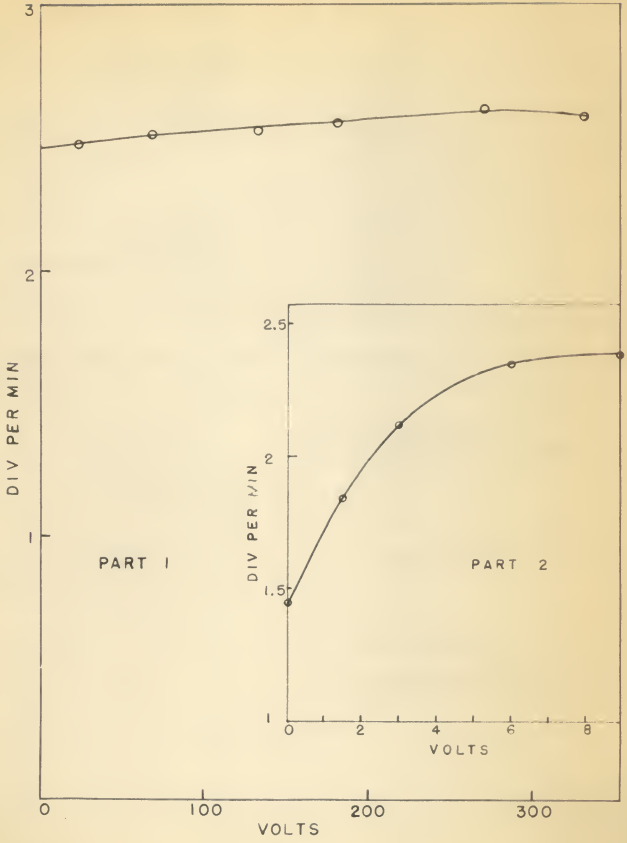

3
2
1
DIV PER MIN
PART 1
0
100
200
300
VOLTS
2.5
2
1.5
1
DIV PER MIN
PART 2
0
2
4
6
8
VOLTS

Table 10. Conversion factors of drift rate from different scales of vibrating reed electrometer.

Scale (mv)	Multiplying factors to convert the time for 8 divisions on 500 mv scale from other mv scale	
	without correction	additional correction factor
50	10	1.0041
100	5	0.9807
200	2 1/2	1.0025
500	1	1.
1000	1/2	1.0048
2000	1/4	1.017

If the reading varied eight divisions in 3 minutes on the 50 mv scale, then the time for travelling eight divisions on a 500 mv scale must be 3 x 10 x 1.0041 = 30.125 minutes.

To determine how easily an active sample could be removed quantitatively from an ion chamber by evacuation and flushing, some additional studies were carried out. An active sample of $C^{14}O_2$ corresponding to 1 ml of National Bureau Standard $Na_2C^{14}O_3$ solution (1280 dis/sec/ml) was collected in an ion chamber under atmospheric pressure. The applied voltage was 135 volts. The initial reading of the sample and each reading after evacuating and flushing are listed in Table 11.

Table 11. Efficiency of ion-chamber decontamination by vacuum pump.

Flushing times	Degree of vacuum evacuated (μ)	Scale (mv)	Time for 8 divisions (min)
Initial sample		1000	1.12
First flushing	250	50	6.64
Second flushing	250	50	8.70
Third flushing	250	50	7.22
Fourth flushing	250	50	7.53
Fifth flushing	250	50	7.98

From the preceding data, three cycles of evacuation and flushing with non-active CO_2 brought the chamber back to the background level. The time for each flushing was about 3 - 4 minutes and the total time needed to bring the chamber to background level and ready to read after each sampling was less than 30 minutes.

Reproducibility. The ion chamber could not be used in determining the absolute disintegration rate of C^{14} as stated before. It was necessary to know whether reproducible results could be obtained with the sampling techniques, apparatus and instruments used . As many factors were involved in the counting rate, such as air contamination, back diffusion during filling of chamber, cosmic rays, etc.

Therefore, three 100 μl samples of a National Bureau of Standards $Na_2C^{14}O_3$ solution were carried through the same acidification and transfer procedures to check the reproducibility of the system. The results are given in Table 12.

Table 12. Reproducibility of gaseous $C^{14}O_2$ counting by ion chamber.

Nat. Bur. Std. $Na_2C^{14}O_3$ Solution used (μl)	Activity given by Nat. Bur. Std. as dis/sec	div/min (sample-bkg)	div/min (bkg)	div/min (sample)
100	128	1.789	0.117	1.672
100	128	1.713	0.121	1.595
100	128	1.624	0.124	1.500
			Average	1.588

Calibration Curve Preparation. A standard $Na_2C^{14}O_3$ solution of a specific activity of 1280 disintegration per second per milliliter as

certified by National Standard Bureau was used to prepare a standard curve. The drift rate on the 500 mv scale of the vibrating reed electrometer for $C^{14}O_2$ from different volumes of the standard $Na_2C^{14}O_3$ and corresponding disintegration per second are given as follows in Table 13. The standard curve is shown in Plate VII.

Table 13. Relation of radioactivity and drift rate -- Data of calibration curve.

Experiment number	Std. sample (μl)	Radioactivity dis/sec	Drifting rate (corrected from bkg) (div/min)
1	50	64	0.789
2	100	128	1.588
3	200	384	4.236
4	500	640	7.536
5	1000	1280	14.176
6	3000	3840	42.629

Oxidation and Counting of $C^{14}Cl_4$. The activity of samples of $C^{14}Cl_4$ which was prepared by neutron irradiation of non-active CCl_4 dissolved in certain nitrogen containing compounds (29) were studied by the techniques previously described. After purification of CCl_4 following the irradiation, the $C^{14}Cl_4$ portions were oxidized by the dry combustion method in the manner previously described. The counting results were reproducible and from comparing the experimental drift rate with standard curve, the specific activity of the $C^{14}Cl_4$ was determined.

EXPLANATION OF PLATE VII

Standard Curve of Radioactivity of $C^{14}O_2$
Versus Drift Rate

This graph is the result of plotting the drift rates against known activities from a standard sample. The activity of unknown samples were determined by the interpolation of this standard curve.

Part 1 is the plot of higher activity versus drift rate and part 2 is the plot of lower activity versus drift rate.

PLATE VII

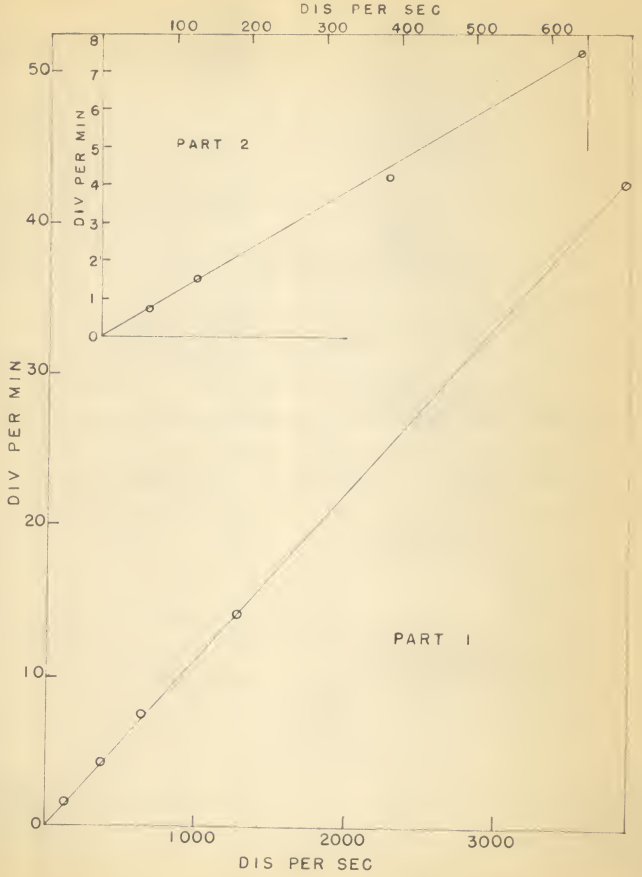

DIS PER SEC
100
200
300
400
500
600
DIV PER MIN
8
7
6
5
4
3
2
1
0
PART 2
50
40
30
20
10
0
DIV PER MIN
PART 1
1000
2000
3000
DIS PER SEC

Table 14. Counting results of $C^{14}Cl_4$.

	Fractions	No. of samples measured	Boiling point range	Volume	Disintegration per sec per 50 μl
$C^{14}Cl_4$ as irradiation product of pyridine-CCl_4 solution	1st	2	70-74°C	2.1 ml	9.6
	2nd	2	74-75°C	5.3 ml	10.5
	3rd	3	75°C	1.1 ml	10.4
	original	2	---	---	32.5
$C^{14}Cl_4$ as irradiation product of aniline CCl_4 solution	1st	3	45-72°C	2.0 ml	7.4
	2nd	2	72-75°C	4.4 ml	6.7
	3rd	2	75°C	1.9 ml	4.5
	original	4	---	---	20.0

SUMMARY AND DISCUSSION

The determination of trace amounts of carbon tetrachloride was studied using colorimetric and radiometric methods.

The colorimetric method was based upon the reaction of CCl_4 and a hot pyridine-sodium hydroxide solution which produced a red color. By using a standard calibration curve the intensity of the color was related to the concentration of CCl_4. In general, the color produced was not very stable. Factors involving the stability and reproducibility of color production were studied. The determination of CCl_4 in aqueous solution and in a small amount of blood was carried out in two steps; first CCl_4 was isolated from samples by ether extractions and second, the color was developed in a hot pyridine-sodium hydroxide solution. The minimum detectable concentration of CCl_4 was 1 ppm. Quantitative determinations of CCl_4 were possible from 1 ppm up to 33 ppm, as in this range, the concentration follows Beer's law. Color produced by higher

concentrations of CCl_4 deviated considerably from the linear relationship and also were not reproducible. The reproducibility of the measurement was found to be ± 3%.

In the radiometric method of determination, C^{14} labeled CCl_4 was used. The measurement of radioactivity of C^{14} was the basis for measuring the amount of CCl_4 or its metabolites. Samples were oxidized quantitatively to carbon dioxide either by the dry combustion method or by a wet combustion method. The resultant $C^{14}O_2$ was transferred into an ionization chamber and counted by a vibrating reed electrometer.

The recovery of carbon by the dry combustion method was about 99 per cent for both volatile liquid samples and solid samples. For the wet combustion, the recovery of carbon depended on the amount of sample present relative to the volume of oxidizing solution. As an example, for 5 ml of the oxidizing solution, 97 - 99 per cent of the carbon could be recovered from the benzoic acid sample providing 7 mg or less of benzoic acid was used. However, when a 9 - 14 mg sample was oxidized, only 91 - 94 per cent of carbon was recovered. Wet combustion requires much less time than the dry combustion which is advantageous. For the combustion of non-volatile compounds, wet combustion is recommended, while for the combustion of volatile samples, dry combustion is the better method.

With the vibrating reed electrometer and the $C^{14}O_2$ transfer techniques perfected, it is possible to detect the order of 10^{-6} mg of $C^{14}Cl_4$. The counting results from a standard sample of $Na_2C^{14}O_3$ were reproducible within ± 5 per cent. While the radiometric method is more sensitive than the colorimetric method, it suffers from the disadvantage of not discriminating between C^{14} in CCl_4 or in some metabolite. However, the radio-

metric method can identify the maximum amount of CCl_4 that would be present through a knowledge of the specific activity and the measured activity.

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THE DETERMINATION OF TRACE AMOUNT OF CARBON TETRACHLORIDE BY COLORIMETRIC AND RADIOMETRIC METHODS

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In order to study the anthelmintic action of carbon tetrachloride on ascardia, a sensitive method of analysis for carbon tetrachloride and its metabolites in biological samples was necessary. Because of the known sensitivity of colorimetric and radiometric techniques, it appeared that a combination of these two methods would facilitate the proposed studies.

The colorimetric method was based upon the reaction of CCl_4 and a hot pyridine-sodium hydroxide solution which produced a red color. The intensity of the color could be related to the concentration of CCl_4 by using a standard calibration curve. In general, the color produced was not very stable. Factors involving the stability and reproducibility of color production were studied. The determination of CCl_4 in aqueous solution and in small amounts of blood was carried out in two steps, first, CCl_4 was isolated from samples by ether extraction and second, the color was developed in a hot pyridine-NaOH solution. The minimum detectable concentration of CCl_4 was 1 ppm. Quantitative determinations of CCl_4 were possible from 1 ppm to 33 ppm, as in this range, the concentration follows Beer's law. Color produced by higher concentrations of CCl_4 deviated considerably from the linear relationship and were not reproducible. The reproducibility of the measurement was found to be $\pm 3\%$.

In the radiometric method of determination, C^{14} labeled CCl_4 was used. The measurement of the radioactivity of C^{14} was the basis for measuring the amount of CCl_4 or its metabolites. Samples were oxidized quantitatively to carbon dioxide either by the dry combustion method or by a wet combustion method. The resultant $C^{14}O_2$ was transferred to an ionization chamber and counted by a vibrating reed electrometer.

The recovery of carbon by the dry combustion method was about 99 per cent for both volatile liquid samples and solid samples. For the wet combustion, the recovery of carbon depended on the amount of sample present relative to the volume of oxidizing solution. As an example, for 5 ml of the oxidizing solution, 97 - 99 per cent of the carbon could be recovered from the benzoic acid sample providing 7 mg or less of benzoic acid was used. However, only 91 - 94 per cent of carbon was recovered when 9 - 14 mg of sample was oxidized.

With the vibrating reed electrometer and the $C^{14}O_2$ transfer techniques that were perfected, it was possible to detect the order of 10^{-6} mg of $C^{14}Cl_4$. The counting results from a standard sample of $Na_2C^{14}O_3$ were reproducible within ± 5 per cent. While the radiometric method is more sensitive than the colorimetric method, it suffers from the disadvantage of not discriminating between C^{14} in CCl_4 or in some metabolite. However, the radiometric method can identify the maximum amount of CCl_4 that would be present through a knowledge of the specific activity and the measured activity.