Plant Growth Regulant

Biochemical Behavior of 2-Chloroethyl Trimethylammonium Chloride in Wheat and in Rats

Roger C. Blinn

Studies of the biochemical behavior of C^{14}-labeled chlorocholine chloride in wheat plants and in rats have indicated that this plant growth regulant is metabolically inert in these species. The unchanged compound was the only radiolabeled material found as residues in wheat foliage, roots, and grain, and in rat urine. In wheat plants, it was absorbed slowly from foliar surface deposits, and small amounts were translocated to the roots. In rats, absorption was very rapid, followed by elimination of radioactivity in the urine. Small amounts were eliminated in the respiratory gases and in the feces.

Cycocel plant growth regulant, 2-chloroethyl trimethylammonium chloride (also referred to as CCC and chlorocholine chloride), is helpful in preventing lodging of wheat, and its use for this purpose is promising, especially in Europe. Before a meaningful residue analytical program can be developed, including the development of the analytical procedure, information as to the metabolic fate of chlorocholine chloride in wheat must be available, so that the residual behavior of all resulting compounds of toxicological interest can be evaluated. Mitchell (1962) found no evidence of metabolism when cucurbits were grown in a solution of a similar compound, 2-bromoethyl-1,2-C^{14} trimethylammonium bromide. Rothenberger (1965) found no evidence of metabolism when he applied C^{14}-labeled *N*-dimethylaminosuccinamic acid, a growth retardant which has a similar biological response, to chrysanthemums. However, Mitchell (1962) and Linser *et al.* (1965) showed, respectively, that bromocholine bromide and chlorocholine chloride are decomposed by soil microorganisms.

In the studies reported here, chlorocholine-1,2-C^{14} chloride was applied to actively growing wheat plants, and the radioactive materials resulting from this treatment were isolated and identified from several portions of the wheat plants at various intervals after treatment. The fate of chlorocholine-1,2-C^{14} chloride was studied in a representative mammal, the rat.

EXPERIMENTAL

Materials and Standard Methods. RADIOLABELED CHLOROCHOLINE CHLORIDE. Chlorocholine-1,2-C^{14} chloride was obtained from New England Nuclear Corp., Boston, Mass. This material had a specific activity of 4.81 mc. per millimole (30.5 μc. per mg.). Radiopurity was established with eight chromatographic systems described later. The infrared spectrum was in good agreement with that from authentic nonradiolabeled material.

COUNTING. Liquid Scintillation Counting. All extract solutions and urine were counted in a Packard TriCarb scintillation spectrometer. Aliquots of 0.1 to 1.0 ml. of aqueous or methanolic solutions were dissolved or suspended in DAM-611 scintillator solution [600 ml. of dioxane, 100 ml. of anisole, 100 ml. of dimethoxyethane, 6.0 grams of 2,5-diphenyloxazole (POP), and 200 mg. of 1,4-bis-2-(5-phenyloxazolyl)benzene (POPOP)] containing 5% Cab-O-Sil thixotropic gelling agent. Efficiency of counting was determined by internal standardization, using a commercial standard of C^{14}-labeled toluene.

Van Slyke Combustion. Fresh tissue from plant or animal parts was combusted by means of Van Slyke reagent (Van Slyke *et al.*, 1951), using the procedure of Smith *et al.* (1964). Liberated $C^{14}O_2$ was trapped in ethanolamine for evaluation of radioactivity by liquid scintillation counting.

Schöniger Flask Combustion. Dried marc from plant parts was burned in an oxygen atmosphere in a Schöniger flask (Kelly *et al.*, 1961). Ethanolamine was introduced into the cooled flask to trap the $C^{14}O_2$. This solution then was evaluated for radioactivity by liquid scintillation counting.

CHROMATOGRAPHY. The chromatographic systems used for the identification and purification of chlorocholine chloride are presented in Table I. The R_f values of choline chloride, a compound of similar structure which occurs naturally, are included to illustrate the separabilities achieved. The systems most useful for isolation and purification purposes were systems 1, 2, 4, and 5. Chromatograms were run as streaks on 8 × 10 inch sheets of paper or 8 × 8 inch thin-layer plates. All stationary phases were prewashed with methanol and air-dried before use. Solvents were of reagent grade quality and used without further purification.

AUTORADIOGRAPHY. Developed chromatograms of plant or animal extracts containing radiolabeled materials were pressed against the emulsion surface of Kodak Royal Pan sheet film in Kodak x-ray exposure holders (Eastman Kodak Co., Rochester, N. Y.). After a suitable period of exposure, the film was developed using Baumann's Diafine two-bath film developer (Baumann Photo-Chemical Corp., Chicago, Ill.) according to the manufacturer's directions. All handling of the undeveloped film was in total darkness.

Studies with Wheat. APPLICATION PROCEDURE. In order to account for all of the radioactivity from the applied C^{14}-labeled chlorocholine chloride, the method of application must be precise so that the exact amount of material deposited on the plants can be calculated. It is also desirable for the deposit to be reasonably uniform throughout the treated area and in a concentration appropriate for biological response. The waxy nature and small

Metabolism Laboratory, Agricultural Division, American Cyanamid Co., Princeton, N. J.

Table I. Typical R_f Values for Choline Chloride and Chlorocholine Chloride in Selected Chromatographic Systems

Paper Chromatography	Stationary Phase	Mobile Phase	R_f Values: Choline chloride	R_f Values: Chlorocholine chloride
System 1	Whatman 3MM	*n*-BuOH–HOAc–H_2O (100:30:85)	0.54	0.65
System 2	Whatman 3MM	*n*-BuOH–EtOH–HOAc–H_2O (8:2:1:3)	0.32	0.46
System 3	Whatman 3MM	*n*-BuOH–propionic acid–H_2O (100:30:85)	0.24	0.36
System 4	Whatman 3MM	EtOH–NH_4OH (95:5)	0.44	0.49
Thin-Layer Chromatography				
System 5	Alumina	MeOH–acetone–H_2O (30:70:2)	0.26	0.46
System 6	Alumina	MeOH–acetone–HOAc (30:70:2)	0.38	0.46
System 7	Alumina	MeOH–$MeCl_2$–HOAc (45:37.5:3)	0.64	0.79
System 8	Cellulose	Et_2O–MeOH–0.1*N* HCl (3:2:1)	0.45	0.55

surface area of the young wheat plants made precise application by brush or micropipet impractical. The method of application finally adopted involved dipping the foliage into a solution of the radiolabeled chlorocholine chloride, and determining the amount deposited by weight difference and the difference of radioactivity in the solution.

A solution of chlorocholine-1,2-C^{14} chloride in 25 ml. of water (60 μg. or 23.1 μc. per ml.) with 1% Surfonic LF-7 surfactant added was placed in a 12 × 20 cm. double-thickness polyethylene bag. An aliquot was taken for counting and the weight of the solution determined. All but the foliage of 2- to 3-week-old wheat plants, planted in 2 × 2 × 2 inch peat pots, was wrapped in a sheet of thin plastic film to prevent soil particles from dropping into the solution bag while the plants were being treated. For the foliage, soil, and root experiments, 22 winter wheat plants per pot were used; for the grain experiments, two spring wheat plants per pot were used. Foliage of plants was dipped into the solution in the polyethylene bag and wet thoroughly with the radiolabeled solution by manipulating the bag until all portions of the plant surface had been covered. The foliage was allowed to remain in the bag, out of solution, until dripping ceased, then removed for air drying. The weight and activity of the chlorocholine-1,2-C^{14} chloride solution was determined again before subsequent treatment of other plants. The amount of C^{14}-labeled material remaining on the plant was determined from these weight and activity differences. The plants then were held until processing in a greenhouse with acrylic sheet ceiling windows which transmit radiation from 370 to 1620 mμ. The plants were watered by subirrigation to prevent surface leaching of the residue.

Sample Preparation. At 1, 3, or 12 weeks after treatment, the plants were removed from the greenhouse and the foliage was cut with scissors at soil level. With the 12-week sample, which had grain heads at the "milk" stage of development, the grain heads were removed separately. The soil in the pot was allowed to air-dry and then was worked loose from the roots. Soil-free roots and soil plus peat pots were processed separately. The soil, peat pot, and roots of the 12-week sample were discarded.

Extraction Procedure. Foliage Surface Residue. Surface residues on the excised wheat leaves were removed by immersing successively into two 50-ml. aliquots of water in graduated cylinders. The combined water washes were evaporated to dryness in a rotating film evaporator under vacuum at 40° C. The residue was dissolved in 20 ml. of 50% aqueous methanol and 0.1-ml. aliquots were taken for radioactivity determination.

Foliage Internal Residue, Root Residue, and Grain Head Residue. The water-washed foliage, soil-free roots, or grain heads were minced with four 100-ml. portions of methanol using an Omni-mixer. The alcohol was filtered through a medium-porosity fritted-glass funnel after each mincing operation. The resulting light tan residue was air-dried and radioactivity determined by Van Slyke or Schöniger flask combustion. The filtered alcoholic extract was evaporated to dryness in a rotating film evaporator under vacuum at 40° C. The residue was dissolved in 20 ml. of 50% aqueous methanol and 0.1-ml. aliquots were taken for radioactivity determination.

Soil Residue. The soil was extracted in a Soxhlet extractor with methanol for about 3½ hours. The extract was evaporated to dryness, as described above, and aliquots were taken for radioactivity determination. The radioactivity of the extracted soil was determined by Van Slyke combustion.

Isolation Procedures. The 50% aqueous methanol solutions of the various tissue extracts were diluted with 75 ml. of water, acidified with a few drops of dilute hydrochloric acid, and extracted four times with 100-ml. portions of carbon tetrachloride. Centrifugation facilitated separation of the two phases. These aqueous solutions were filtered through pads of Nuchar vegetable charcoal overlaid with diatomaceous earth in medium-porosity fritted-glass funnels. All of the radioactivity remained in the aqueous solutions.

The volumes of the aqueous solutions were reduced to 25 ml. in a rotating film evaporator under vacuum at 40° C. The solutions were saturated with potassium carbonate and extracted four times with 25-ml. portions of 95% ethanol. Centrifugation facilitated separation of the layers. At this point, the radioactivity had been transferred to the alcoholic phases.

The alcoholic solutions were evaporated to dryness in a rotating film evaporator under vacuum at 40° C. The residues were dissolved in 10 ml. of methanol. Then 90 ml. of methylene chloride was added and the white precipitates were removed by filtering through medium-porosity, fritted-glass funnels, washing with several portions of 10% methanol in methylene chloride. The radioactivity remained in the solutions.

The solutions were evaporated to dryness and the residues were dissolved in 1 to 2 ml. of methanol acidified with a few drops of dilute hydrochloric acid. They were chromatographed as streaks on 8 × 8 inch sheets of Whatman No. 17 paper with ethanol–ammonium hydroxide (95:5). The areas of radioactivity were located by autoradiography, cut from the sheets, and eluted with methanol. This chromatographic purification was repeated once. Further chromatographic purification was by means of Whatman 3MM paper with butanol–acetic acid–water (100:30:85) and aluminum oxide thin-layer chromatography with methanol-acetone-water (30:70:2). All chromatograms resulted in only one radioactive zone, which was identified by cochromatography as parent chlorocholine chloride.

CHROMATOGRAPHIC IDENTIFICATION PROCEDURES. Following the carbon tetrachloride washes of the acidified aqueous extract from the foliage, roots, grain heads, and soil, the chromatographic properties of the radioactive material present in the extracts were determined using systems 1, 2, 4, and 5 (Table I). Authentic chlorocholine-1,2-C^{14} chloride was mixed with each of the extracts and the mixtures were chromatographed with the same systems to determine whether tissue extractives influenced the migration of chlorocholine chloride, and to show whether the tissue-extracted radioactivity and chlorocholine chloride migrated similarly.

Studies with Rats. Two 200-gram male rats (CFN No. 1 grade, Carworth Co., New York, N.Y.) were given an oral dosage of chlorocholine-1,2-C^{14} chloride dissolved in 0.8 ml. of water. One was placed immediately in a Delmar metabolism cage (Delmar Co., Maywood, Ill.), and the other in a stainless steel metabolism cage (Acme Metal Products, Inc., Chicago, Ill.). Liberal amounts of rat chow and drinking water were available. Temperature was maintained between 25–8° C. A commercial grade of breathing air was passed through the Delmar cage, dried by passage through anhydrous calcium sulfate, and C^{14}-labeled respiration gases were detected by passage through a 1000-ml. flow-type ionization chamber coupled with a Dynacon Model 6000 electrometer (Nuclear-Chicago Corp., Des Plaines, Ill.). The $C^{14}O_2$ contained in the respiration gases was then removed by scrubbing through 3*N* sodium hydroxide solution, and quantitatively determined by liquid scintillation counting. Urine and feces from each rat experiment were separated and collected at intervals for radioactivity determination. The feces were homogenized in water with a Potter-Elvehjem tissue grinder before aliquots were taken for determination of C^{14} content by Van Slyke combustion, whereas the radioactivity in the urine was determined directly by liquid scintillation counting.

After 48 hours, the rat in the Delmar cage was sacrificed, and liver, kidney, intestines, muscle tissue, and fat were removed for homogenization and determination of C^{14} content by the Van Slyke combustion technique. The remainder of the carcass was digested in a 1 to 1 mixture of glacial acetic acid and 70% perchloric acid (Stanley and LeVoure, 1965). Aliquots of this digest were neutralized with sodium hydroxide and C^{14} content determined by liquid scintillation counting.

Aliquots of the various urine samples were chromatographed directly using the four systems described for wheat, and chromatographic confirmation of identity by cochromatography with chlorocholine-1,2-C^{14} chloride was obtained.

RESULTS AND DISCUSSION

Studies with Wheat. In Table II are presented the proportions of the radioactivity found in the various plant parts at 1 and 3 weeks after treatment. Chlorocholine chloride is slowly absorbed from the foliar surface, and resides principally within the foliage. Some activity was translocated to the roots and possibly excreted into the soil. Faulty application techniques, evident in the 7-day sample, were corrected when application was made for the 0- and 21-day samples.

In Table III is presented the distribution of radioactivity found in grain heads and foliage for the 12-week study. The concentration of radioactivity in the grain heads and the foliage is essentially the same, and the radioactivity is largely extracted into methanol.

Typical examples of autoradiograms obtained from the chromatograms of the various plant extracts and mixtures of the extracts with chlorocholine-1,2-C^{14} chloride are shown in Figure 1. Similar results were obtained with the extracts from all plant parts; in each only one band was observed. This band had an R_f value in agreement with that of chlorocholine chloride. A large excess of chloride ion in the sample was essential to suppress anion effects caused by tissue extractives. In the absence of excess chloride ion, several bands were observed, as was reported

Table II. Distribution of Radioactivity in Plant Parts

Plant Part	Per Cent of Applied Activity		
	0 day[a, b]	7 days[b, c]	21 days[d]
Foliage			
Surface	...	28.6	6.6
Methanol extract	...	59.8	79.3
Marc	...	0.6	2.6
Roots			
Methanol extract	...	4.3	1.1
Marc	...	3.0	0.3
Soil			
Methanol extract	...	...[e]	1.6
Marc	...	...[e]	4.6
Total	98.3	96.3	96.1

[a] Processed within an hour after treatment. No attempt to segregate plant parts. [b] Average of two values. [c] Values normalized to 96.3%. Apparent low recovery of 64.4% due to errors in application procedure. [d] Average of six values. [e] Not evaluated in this experiment.

Table III. Distribution of Radioactivity in Wheat Grain Heads and Foliage

Plant Part	Weight, G.	μc. Found	%	Calculated as Chlorocholine Chloride	
				μg.	P.p.m.
Grain head	284				
Methanol extract		0.36	18.3	57	0.20
Marc		0.04	2.2	10	0.04
Foliage	1213				
Methanol extract		1.25	63.7	200	0.17
Marc		0.31	15.8	49	0.04

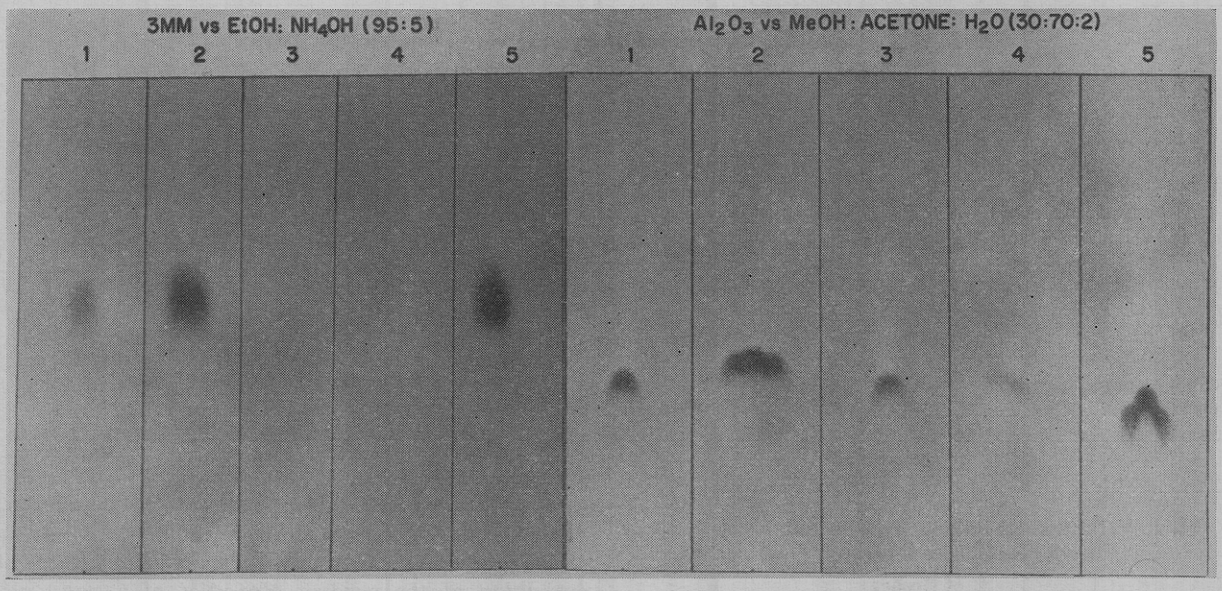

Figure 1. Radiochromatograms of wheat plant extracts

1. Foliage surface 2. Foliage extract 3. Soil extract 4. Root extract 5. Chlorocholine chloride

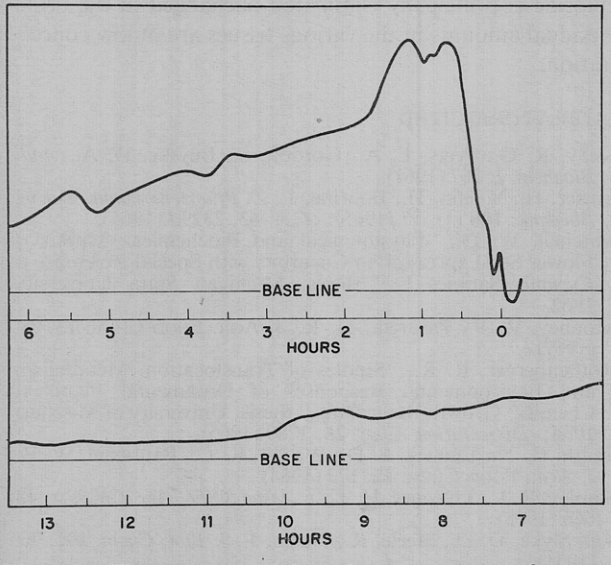

Figure 2. Electrometer recording of respiration gases from rat treated with chlorocholine-1,2-C^{14} chloride

by Mitchell (1962) working with bromocholine bromide. A weak anion column, Amberlite CG-45 (OH^-), also eliminated the anion effects in the chromatography.

Studies with Rats. Although only two animals were involved in this study, the elimination pattern of each animal was similar. The detailed data are presented as indicative of the metabolic fate of chlorocholine chloride in a mammalian system.

The Dynacon electrometer responded immediately to the respiration gases from the rat (Figure 2). C^{14}-labeled respiration gases were detected within a few minutes after treatment; the concentration of radioactivity then slowly decreased to slightly above background 12 hours after treatment. However, the amount of radioactivity in respiration gases was an insignificant proportion (0.44%) of the administered dose (Table IV). The table shows that the bulk of the administered radioactivity was excreted in the urine rapidly, 60.6% being eliminated in 4 hours and 95.6% in 46.5 hours. Excretion in the feces accounted for another 2.3%; thus, about 98% of the radioactive dose was

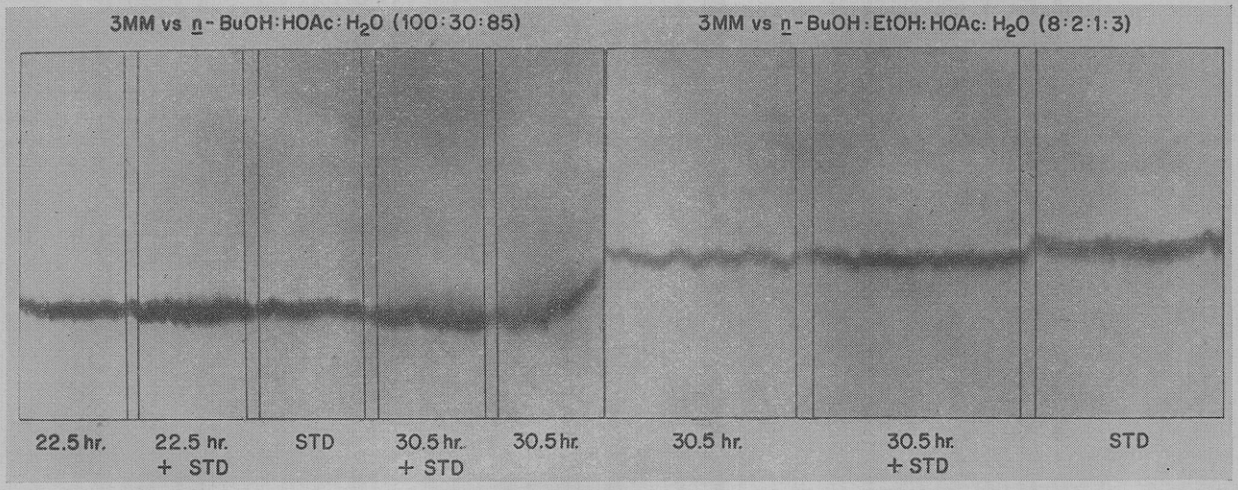

Figure 3. Radiochromatograms of cochromatograms of rat urine with chlorocholine-1,2-C^{14} chloride

Table IV. Percentage Distribution of Radioactivity Found in Rat Study

Sample	Hours after Dosage Administration								
Rat 1	**4**	**5**	**6.5**	**22.5**	**30.5**	**46.5**	**48**	**(Post-mortem)**	**Total**
Respiration gases	...	0.2	...	0.2	0.04	...	...		0.4
Urine	60.6	...	19.1	13.9	1.4	0.6	...		95.6
Feces	...	...	...	1.9	...[a]	0.4	0.1[b]		2.3
Tissues	...	...	...	...	...	...	0.3		0.3
Total	60.6	0.2	19.1	16.0	1.44	1.0	0.4		98.6
Rat 2				24			48	120	Total
Urine				70.9			1.7	0.8	73.4
Feces				2.8			0.3	0.2	3.3
Total				73.7			2.0	1.0	76.7

[a] Sample lost. [b] Intestines.

Table V. Residual Radioactivity in Rat Tissue 48 Hours after Dosage

Tissue	% of Dosage Radioactivity	P.P.M.[a]
Liver	0.08	0.53
Kidney	0.01	0.32
Intestines	0.11	0.36
Muscle	0.004	0.06
Fat	0.002	0.04
Carcass	0.25	0.09

[a] Calculated as chlorocholine chloride from specific activity of dosage solution.

excreted within 46.5 hours. The 0.5% of the administered radioactivity remaining in the various tissues of the rat at sacrifice was distributed as shown in Table V. The residual amounts remaining in the tissues were present in low concentrations.

Chromatography and cochromatography with chlorocholine-1,2-C^{14} chloride of the various urine samples using four systems showed no evidence of radioactive materials other than chlorocholine chloride. Illustrative autoradiograms are presented in Figure 3.

CONCLUSIONS

On the basis of the work reported here, it appears that chlorocholine chloride is absorbed by wheat foliage but undergoes no significant metabolism and very little translocation to the roots. Therefore, the residue analytical procedure developed by Mooney (1967) which responds to parent chlorocholine chloride should provide realistic evaluation of the residual properties of Cycocel plant growth regulant in wheat foliage and grain.

Mammalian studies suggest that ingested chlorocholine chloride is principally eliminated unchanged in the urine. Residual amounts in the various tissues are in low concentration.

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