Translocation and Metabolism of Dodecylguanidine Acetate (Dodine) Fungicide in Apple Trees, Using C^{14} Radiotagged Dodine

ARTHUR N. CURRY
Agricultural Division,
American Cyanamid Co.,
Princeton, N. J.

The leaves along two branches of a bearing apple tree were painted repeatedly throughout the growing season with a solution of dodine tagged with C^{14} on the guanidine carbon. Radiocounting and chemical analysis of apples harvested from these branches indicated that there is no translocation of dodine from foliage into the fruits of the tree. The degradation products of dodine are translocated only very slightly and appear to be simple amino acid– and guanidine-type moieties bound in proteins and peptides.

THE fungicide *n*-dodecylguanidine acetate (coined name dodine) has exhibited utility in the control of various fungus diseases of apple, cherry, pear, pecan, and strawberry. Throughout the developmental field work on this compound there have been several inconclusive reports that, judged from biological activity, dodine was readily translocated from treated to untreated portions of a tree (*3*). In view of the known surface-active properties of long-chain guanidine compounds, some doubt existed as to whether surface creep and rain splash had been adequately excluded in these rough experiments as possible causes of the observed dodine migration. Since translocation, together with its implied penetration into the plant by dodine, might have important implications in the toxicological picture, and little background information existed on the detailed behavior of these alkylguanidine fungicides, it was decided to perform a carefully designed experiment which might throw some light on this alleged translocation phenomenon. Radioactive dodine, C^{14}-tagged in the guanidine carbon, was chosen as the instrument capable of yielding the most information.

Materials and Methods

Radioactive Dodine. This material was synthesized from $BaC^{14}O_3$, and the final product possessed an activity of 8 μc. per mg. It was chromatographed in acetone–acetic acid–water (10:10:80) and in pyridine–isoamyl alcohol–water (80:40:70) and found to be about 95% pure (Figure 1). (The severe tailing in the acetone–acetic acid–water system is dodine itself.) The three minor components, each amounting to no more than 1 or 2% of the total radioactivity of the sample, were deemed insignificant for the purpose of this experiment.

A repeat of the above chromatograms on the same water solution of dodine 14 months later gave identical results, but chromatography at this time in a third solvent system (butanol–acetic acid–water = 73:10:17) has introduced an element of uncertainty (Figure 1). Although this butanol system does not resolve as many impurities as the other two, one of the lesser peaks it produces contains 17% of the radioactivity. It could not be established whether this peak was due to partial decomposition of the sample during the 14 months in water solution or was simply unresolved by the other two developing systems during the initial investigation. Its chemical identity is unknown. If it were indeed present as one sixth of the total radioactivity at the time the apple trees were painted with the tagged material, its possible contribution to the degradation and translocation picture obtained cannot be entirely ruled out.

Radiocounting Equipment. Gas flow counter tube (Nuclear-Chicago Corp. Model D-47), 2-inch lead shield, 98.7% helium–1.3% butane gas mixture. Used with a Micromil window for planchet counting, or windowless in the Actigraph II.

Decade scaler (Nuclear-Chicago Model 151). Ratemeter (Nuclear-Chicago Model 1620A). Portable radiation counter (Heathkit Model RC-1) equipped with thin mica window tube (Nuclear-Chicago D-35) in P16 probe.

Chromatogram scanner (Nuclear-Chicago Actigraph II) with recorder (Texas Instruments Recti-Riter). At the maximum sensitivity of this setup, about 0.002 μg. of dodine could be detected over background on a paper chromatogram.

Chromatographic Systems. The chromatographic developing systems used were either developed for this study or were drawn from the work of Roche, Thoai, and Hatt (*7*) or Berry *et al.* (*1*). All chromatograms described were performed in ascending fashion at about 26° C., on 1.5-inch wide strips of Whatman No. 1 paper in glass jars 6 inches in diameter and 20 inches high, with equilibration times of 1 to $1^1/_2$ hours. Developing solvent was generally allowed to run 12 to 18 cm.

Experimental Procedure

Preparation and Treatment of Apple Tree. A Northern Spy apple tree, about 20 years old and with an excellent set of fruit, was chosen for the experiment. It was maintained throughout the duration of the experiment on a weekly spray schedule with a mixture of Captan, methoxychlor, and malathion. Two branches of the tree were selected for treatment. Small bags about 5 inches square were fashioned by heat-sealing 2-mil polyethylene sheeting. On June 17, 1958, when the apples were about $^3/_4$ inch in diameter, the 16 apples on these two branches were bagged. Plastic electrician's tape was used to seal the mouths of the bags tightly closed and to the supporting twigs. All seals held excellently throughout the experiment. Apple growth was normal, and, other than an unusually cool month of June, weather during the growing season was normal.

The radioactive dodine was dissolved in water, 1 mg. per ml., as needed. The resulting solution was carefully painted, with a small camel's-hair brush, onto the lower surfaces only of all the leaves along the two branches chosen for the experiment, except that leaf areas at the ends of the two branches were left unpainted for subsequent monitoring purposes. A small amount of solution was also painted onto the bark. Seven radiododine treatments were applied, at 10- to 14-day intervals from June 18 to September 3. Harvest was October 3. Approximately 5 ml. of the 1 mg. per ml. radiododine solution was applied at each treatment. Total dodine applied during the season was 35,000 μg. (280 μc.).

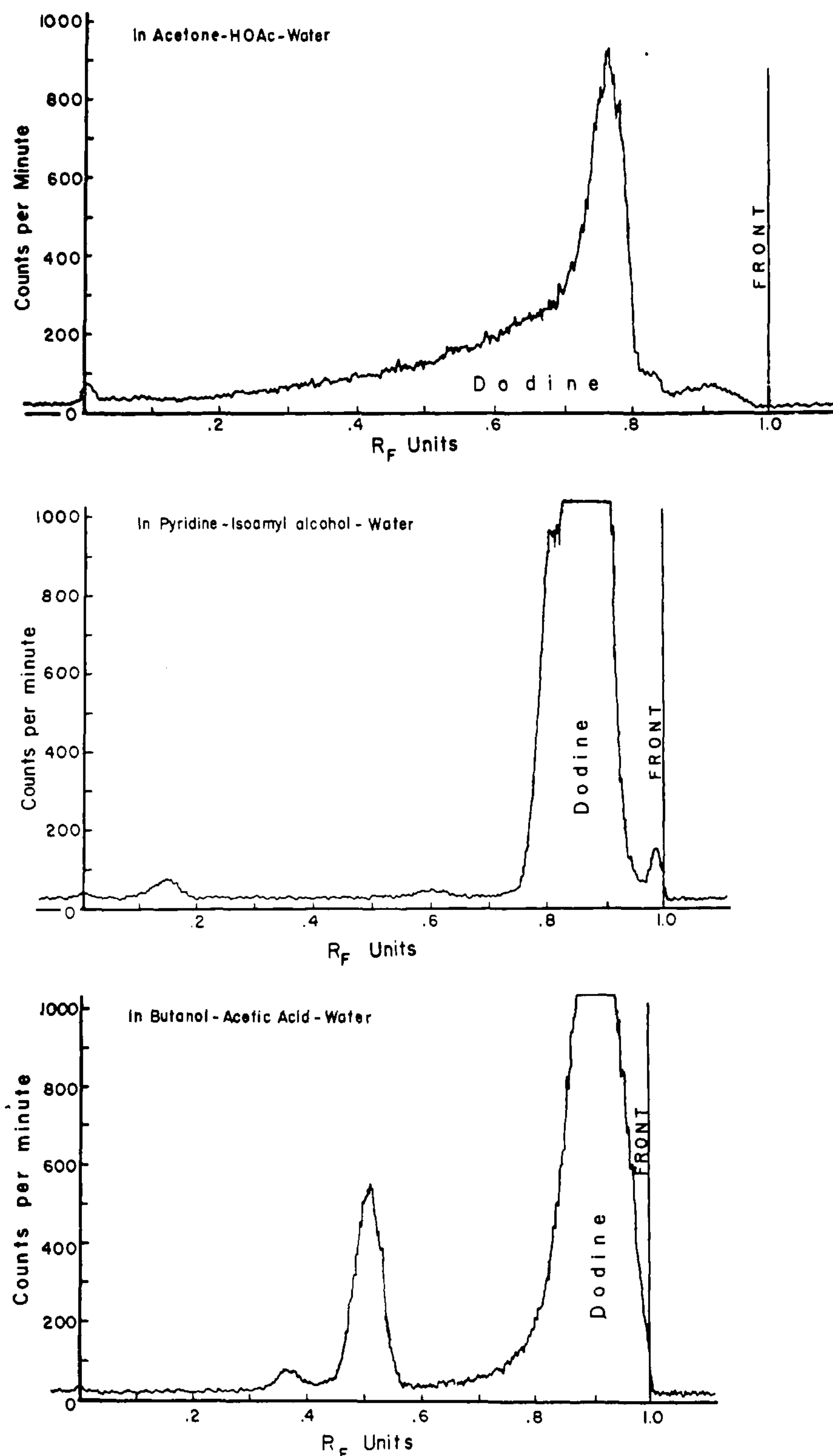

Figure 1. Chromatography in three systems of tagged dodine preparation

1/8-inch collimating slit
Full scale, 1000 c.p.m.
Sample weight (solids), approximately 5 μg.

Monitoring of Apple Tree. The area was monitored from time to time with a portable Geiger counter with thin mica window tube. (This tube, with window thickness of 1.4 mg. per sq. cm., has about one tenth the carbon-14 sensitivity of the gas flow tube used in the laboratory.) Described here is the monitoring of October 2—1 month after final treatment, and 1 day before harvest. The bottom surfaces of treated leaves showed 15,000 to 75,000 c.p.m. and the top surface (untreated) counts were about 5% of the corresponding bottom surface counts. The outsides of the apple bags usually showed about 120 c.p.m. above background, with a range of 30 to 400 c.p.m. This was presumably caused by the bags' rubbing against treated leaves on windy days. The insides of the bags, when measured after the harvest of the following day, were in all cases completely clean of radioactivity—indicating freedom from contamination in the experiment. The untreated foliage of the surrounding area was found contaminated (mainly below) with around 30 to 150 c.p.m. (net) of activity, by rain drip and wind whip, but there was no evidence—at least from surface counts with the portable survey meter—of any preferential migration of activity along the treated branches as opposed to nearby untreated branches.

Drying and Counting Apples. Eight of the 16 treated apples were taken for examination, and samples of skin, meat, and seeds were dried to constant weight at 70° C., pulverized with a glass bead in a Wig-L-Bug vibrator such as is used by dentists for preparing amalgams, and counted at infinite thickness *vs.* controls consisting of similar apples taken from the other side of the tree (see Table I). The figures for parts per million on dried material were derived with the aid of a self-absorption curve run on a known mixture of the tagged dodine and control pulverized apple meat and calculated to wet weight basis. Whole apple figures were calculated using measured weight ratios of meat to skin to seeds.

Separating Radioactive Materials from Apples. Undried portions of various of the above apples were processed by the bromocresol purple total macerate method of Steller *et al.* (*8*) for determining dodine residues in apples. The following fractions resulted from application of this method to the apples:

1. A Büchner filter cake, left after extraction of the dodine in methanol-chloroform
2. CCl_4 washes, used to extract waxes from an acidified aqueous methanol solution of the dodecylguanidine
3. Aqueous discard, left after extraction of the dodecylguanidine–bromocresol purple complex from aqueous methanol into
4. $CHCl_3$ washes
5. Aqueous buffer washes, used to back-extract the chloroform washes
6. Aqueous caustic, used to extract the bromocresol purple from the chloroform washes

The results presented in Table II for apples 7 and 8 are typical of the two runs made by this method. The microgram figures in the table are calculated from counts made on dried portions of the various fractions listed. The uncertainty indicated for the aqueous discard is due to the difficulty of drying and counting this 5% salt solution. Empirical correction, with the indicated uncertainty, had to be made for the fractional crystallization of inorganic salts and radioactive compounds in the planchet.

The bulk of any dodecylguanidine present would be expected to show up as free base in the final chloroform phase. The fact that only 0.1% of the total radioactivity present appeared here indicates absence of this compound. Since the filter cake was washed with only one small portion of methanol, the 11% found in it is assumed to be primarily material adsorbed on it and/or not washed out of it. It is possible that some of the radioactivity has been incorporated into the pulp.

The major portion of the radioactivity is seen to be preferentially soluble, on the acid side, in 1:1 water-methanol, even in the presence of 5% salt. It is not extracted therefrom by carbon tetrachloride, and the 6% that is extracted by chloroform is readily back-extracted by aqueous pH 5.5 buffer. Apparently, then, the radioactivity is associated with compounds that possess a considerable proportion of polar, hydrophilic groups. The ready removal of the radioactivity from the chloroform washes by back-extraction rules out dodecylurea as a possible constituent, since it should remain predominantly in the chloroform phase.

Characterization of Radioactive Materials. The radioactivity available for characterization amounted to 8 μg. (calculated as parent compound), or 64 mμc.; and this was dispersed through 250 ml. of an aqueous 5% salt solution (the "aqueous discard"). Neither the osazones formed with phenylhydrazine nor the ether-extractables at pH <1 contained any radioactivity, indicating the absence of conversion of the guanidine carbon to monosaccharides or organic acids.

Three aliquots of this aqueous discard, ranging from 10 to 70 ml., were diluted with twice their volume of water, acidified with hydrochloric acid to a pH of 2.3, and passed through suitable columns of Amberlite IR-120 cation exchange resin (H^+ form). About 1.8 to 2.0 ml. of resin (column volume) was used for each 1 ml. of the aqueous discard. The columns were then washed with 15 column volumes of water (negative for radioactivity, discarded) and the radioactivity was then eluted from them with 15 to 20 column volumes of 2*M* ammonia. The eluate was concentrated in a rotating film evaporator at 45° C. and the residue was transferred to weighed planchets, dried at 65° C., weighed, and counted. Essentially all the radioactivity that could be recovered was found to come off in about 12 column volumes of eluate. Recoveries (counts) on three separate runs were 128 (a small orienting run), 76, and 104%.

The first two of the above IR-120 runs gave 39 mg. of a waxy material containing 10.1 mμc. of activity (≡1.26 μg. of parent compound). A portion of this, equivalent to 0.24 μg. of parent compound, was dissolved in a small portion of aqueous 3*M* ammonia, and half was spotted onto each of two strips of Whatman No. 1 paper for chromatography in butanol–acetic acid–water (60:15:25). The two identical strips were air-dried after the chromatographic development. Strip 120-A was sprayed with ninhydrin (*4*), 0.4% in water-saturated butanol, and heated, to detect amino acids. After drying overnight, it was then given the benzidine blue peptide test of Reindel and Hoppe (*6*). The purpose of these and the following color tests was not to pick up the dodine degradation products themselves, since they were present at levels (<0.1 μg.) much too low to be detected directly by color-developing sprays, but rather to detect the naturally occurring apple components with which they were associated in the various chromatographic systems, in hopes of thereby gaining some clues toward their identity.

Strip 120-B was scanned with a Geiger tube, and the radioactivity was found to be concentrated in two of the six discrete ninhydrin-responsive bands (Figure 2). There was also a very faint trace of radioactivity extending from band 5 back to the origin—possibly due to smearing of polypeptides. The benzidine blue peptide test showed a smear from R_f 0.04 to 0.14.

Another portion of the residue from the IR-120 eluate was heated for 20 hours on the steam bath in 6*N* hydrochloric acid, the acid was removed by evaporation, and the residue was dissolved in water and passed again through an IR-120 column, to remove salts. The ammonia eluate was concentrated and chromatographed successively in buffered phenol, then 2,6-lutidine–water (65:35), then butanol-ethanol-water (80:20:20), and then in lutidine–water again. The material in the radioactive areas was transferred from one strip to the next with water by Dent's method (*2*), a small IR-120 column being used for salt removal following the buffered phenol system. The remaining nonradioactive areas of the strips were sprayed with ninhydrin and gave discrete bands, as shown. The Geiger scans of the first two of these strips, together with the ninhydrin-sprayed portions of the strips,

Table I. Results of Radiocounting of Dried Samples of Bagged Apples

(All counts at infinite thickness)

Apple	Net C.P.M., 90% Confidence Limits	P.P.M. on Dried Material[a]	Calcd. P.P.M. On wet material[a]	Calcd. P.P.M. On whole apple[a]	Calcd. Weight Present in Whole Apple, μg.
1				0.006	0.4
Meat	2.6 ± 0.8	0.052	0.006		
Skin	1.3 ± 0.8	0.026	0.005		
Seeds	0.0 ± 0.8	0 ± 0.016	n.f. (<0.009)		
5				0.010	0.7
Meat	3.9 ± 1.2	0.077	0.010		
7				0.011	0.7
Meat	4.4 ± 1.1	0.087	0.011		
6				0.022	1.5
Meat	8.1 ± 1.3	0.16	0.021		
2				0.027	1.8
Meat	10.8 ± 0.9	0.21	0.026		
Skin	9.5 ± 1.3	0.19	0.035		
Seeds	4.5 ± 1.0	0.09	0.047		
4				0.039	2.7
Meat	15.7 ± 1.3	0.31	0.038		
Skin	12.6 ± 1.3	0.25	0.046		
Seeds	...	...	...		
3				0.10	6.8
Meat	39.4 ± 1.6	0.77	0.094		
Skin	37.6 ± 1.7	0.74	0.137		
Seeds	15.5 ± 1.3	0.30	0.164		
8				0.21	14.3
Meat	83.4 ± 2.2	1.64	0.20		

[a] All figures calculated as parent compound (dodine). At infinite thickness, 1.0 p.p.m. added radiododine gave 51 c.p.m. net.

Table II. Recovery of Radioactivity from 50 Grams of Apple 7 (0.011 P.P.M.) plus 50 Grams of Apple 8 (0.21 P.P.M.)

(All results calculated as parent compound)

Fraction	Amount, μg.	% of 11 μg.
In 100 grams of apple, calcd. from total counts of dried residues	11	100
Aqueous discard	8.1 ± 3	74 (46–101)
Filter cake	1.2	11
Buffer washes	0.7	6
CCl_4 washes	0.12	1.1
$CHCl_3$ washes	0.015	0.1
Aqueous caustic	0.00	0.0
Total recovery	10.1 ± 3	92 (64–118)

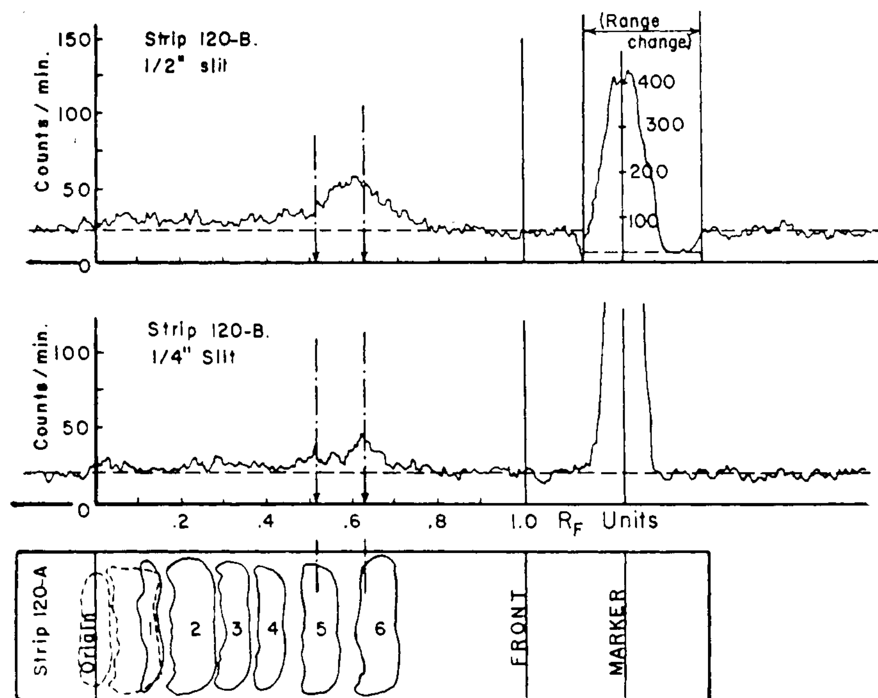

Figure 2. Chromatography in butanol–acetic acid–water (60:15:25) of ammonia eluate from Amberlite IR-120 resin column before hydrolysis

Strip 120-A tested first with ninhydrin (——) and then with benzidine blue (– – –). Its twin, 120-B, was radiocounted. Note low-level smear of radioactivity extending from peaks back to origin

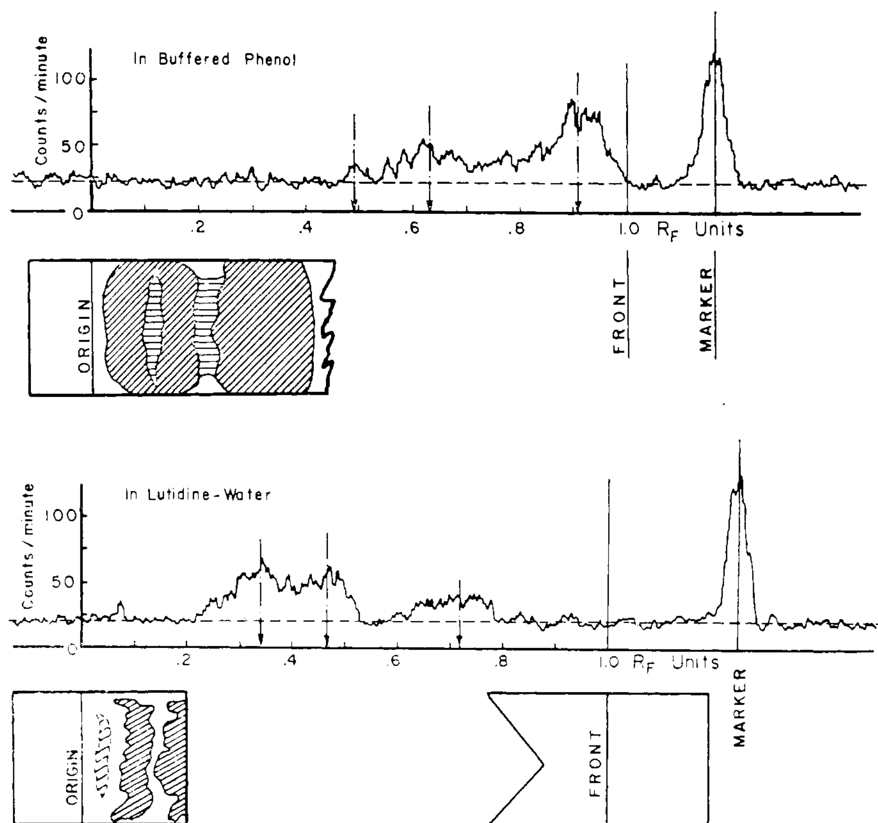

Figure 3. Chromatography in two systems of ammonia eluate from Amberlite IR-120 resin column after hydrolysis

Buffered phenol. 100 grams Baker's analytical reagent phenol saturated with aqueous solution containing 6.3% sodium citrate and 3.7% potassium dihydrogen phosphate
Lutidine-water. 65:35.
Radioactive material from buffered phenol run eluted and used for lutidine-water run

are depicted in Figure 3, and those of the other two are analogous. Three bands were detected on each strip.

Total amounts of radioactivity on each strip were of the order of 0.1 μg. (calculated as dodine), with individual peaks as small as 0.01 μg. and below. Cochromatography with likely knowns—in this case, probably amino acids and related compounds—seemed the only plausible technique likely to elucidate further information on the unknown compounds at these low levels.

The following knowns were chromatographed, in most cases, concurrently, with the above runs and in the same chambers:

Arginine	Glycocyamine
Lysine	Canavanine
Citrulline	Guanidine
Creatine	Urea
Creatinine	

Of these, only creatine and guanidine appeared to match any of the radio-tagged materials consistently in chromatographic behavior.

Figure 4 depicts the R_f values observed in the various chromatograms described above. The first entry, on unhydrolyzed material, was run independently. Runs 2 through 5, on hydrolyzed material, are all on the identical sample, eluted from one strip to the next by Dent's method, as described. The Arabic numerals are the micrograms (calculated as dodine) of material present in the spot, as estimated by comparing the area (planimeter) of the Geiger scan of the spot with that of a standard spot. Conservation of total radioactivity throughout the series of four runs is excellent, considering the small amount present.

The letters C, U, and G in Figure 4 indicate where creatine, urea, and guanidine were observed to chromatograph in these systems. The five unknown compounds have been assigned Roman numerals, the correspondence from system to system being assigned by matching with the knowns, by matching relative amounts present, and by their association with apple materials which react in characteristic ways toward various spray reagents. Compound IV in run 1 may well be the same as compound III. It is not known if compound V matches any of the compounds found in the hydrolyzed material.

An attempt was made to establish the existence or absence of anionic groups (especially carboxyl) on these compounds, by seeing if they could be held on a strong base anion exchange resin (Amberlite IRA-400, OH form). Unfortunately, the acid-hydrolyzed IR-120 eluate used was apparently decomposed completely by the strongly alkaline column. The percolate (containing the bulk of the radioactivity) and the hydrochloric acid eluate are shown in Figure 5 chromatographed together. The peak

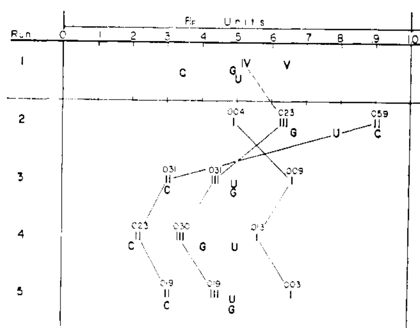

Figure 4. R_f values observed in chromatography of Amberlite IR-120 resin eluate, with values for appropriate knowns run concurrently

Solvents
- Unhydrolyzed material
  1. Butanol–acetic acid–water (60:15:25) run at 24° C.
- Acid-hydrolyzed material
  2. Buffered phenol at 24° C.
  3. Lutidine-water (65:35) at 26° C.
  4. Butanol-ethanol-water (80:20:20) at 29° C.
  5. Lutidine-water (65:35) at 26° C.

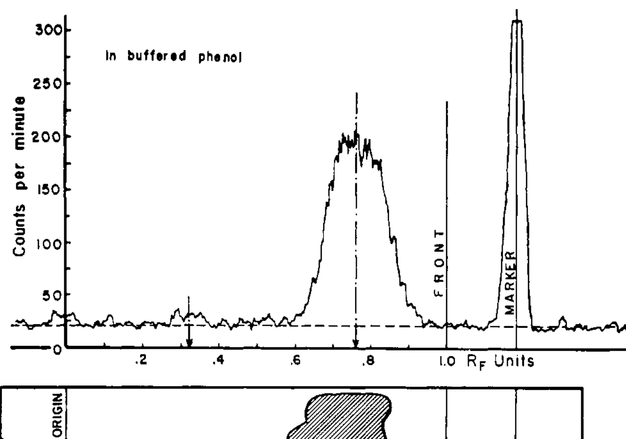

Figure 5. Chromatography in buffered phenol of IR-120 eluate after acid hydrolysis and passage through Amberlite IRA-400 resin

Radioactive strip (split in half) depicted below a scan after spraying with p-dimethylaminobenzaldehyde for detection of urea. Known urea, run concurrently on another strip gave an R_f of 0.75

at R_f 0.76 matches that of urea (R_f 0.75) and also gives a good yellow with the standard urea spray reagent, *p*-dimethylaminobenzaldehyde (*5*). This decomposition to urea under the above conditions is consistent with the presence not only of guanidine (or urea itself), but of creatine, as confirmed by a subsequent experiment on known creatine. The trace at R_f 0.32 (compound VI) (Figure 5) is not identified. It is associated with ninhydrin-positive apple materials. Total recovery from the IRA-400 column was 96±3%.

Results of the above characterization procedures are (refer to Figure 4):

COMPOUND I, present in smallest amount of the three compounds appearing after acid hydrolysis, is present in all developing systems after, but not before, acid hydrolysis; it disappears on passage through IRA-400. It matches no knowns tried.

COMPOUND II, not present before acid hydrolysis, gives R_f's consistent with those of creatine in the three solvent systems used and, like creatine, is converted to urea by IRA-400 treatment. It is associated on the strips with compounds that are positive to ninhydrin and positive to a Sakaguchi test for the guanidyl group (*7*).

COMPOUND III does not match exactly any knowns tried, but lies very close to guanidine in the three developing systems used—and in a fourth, the one used on the unhydrolyzed material, if indeed compound IV is identical with compound III. It is not present after IRA-400 treatment. It is associated on the strip with compounds that are positive to ninhydrin, but not with any compounds positive to the Sakaguchi test. It very probably is guanidine (present at too low a level to be picked up by color test), or guanidine monosubstituted with a small group.

COMPOUND V, appearing before acid hydrolysis but not after, matches a ninhydrin band, but is not otherwise identified. Similarly, a trace of compound VI, appearing only after IRA-400 treatment, matches a ninhydrin band, but is not otherwise identified.

Conclusions

The absence of translocation of dodine from foliage into the fruit of apple trees has been demonstrated by using C^{14} guanidine-tagged dodine. There is some degradation of the dodine, however, and traces of the radiotagged carbon (<0.2% of the amount applied) appear in the apple. Except for a small amount remaining with the pulp, all of the radioactivity can be adsorbed on Amberlite IR-120 cation exchange resin, indicating that all tagged carbon is apparently still attached to nitrogen. It presumably has settled largely or entirely in the protein or peptide portion of the fruit. This latter supposition is strengthened, in part, by the unresolved smear of radioactivity obtained on chromatographing the unhydrolyzed material, and its replacement by discrete chromatographic bands when the chromatography is preceded by a standard peptide acid hydrolysis treatment.

The peptide hydrolysis treatment of the IR-120 eluate yields three compounds whose identity has been only partially characterized. One would appear to be creatine—or is at least remarkably consistent with creatine's behavior. Another, from its close but not exact association with guanidine through several chromatographic systems, would appear to be guanidine substituted with a small group (or groups). All decompose to urea on contact with Amberlite IRA-400 anion exchange resin (OH form). The mechanism of derivation of these three compounds which result from acid hydrolysis, or of their peptide precursors, is not known.

With the possible exception of compound I, which appears in lowest concentration in the peptide hydrolyzate, all the compounds, both before and after peptide hydrolysis, are associated on the chromatograms with ninydrin-positive materials naturally occurring in the apple itself. This latter fact would suggest that the guanidino moiety of the dodine molecule, in so far as it appears in the apples, is being incorporated by the plant into normal components of its pool of amino acids, peptides, and closely related compounds.

Acknowledgment

The radiotagged dodine used in this experiment was synthesized by Teresa Ziegler. The helpful advice of Robert Deems is gratefully acknowledged.

Literature Cited

(1) Berry, H. K., Sutton, H. E., Cain, L., Berry, J. S., *Univ. Texas Publ.* **5109,** 22–55 (1951).
(2) Dent, C. E., *Biochem. J.* **41,** 240 (1947).
(3) Hamilton, J. M., Szkolnik, M., *Phytopathology* **48,** 262 (1958).
(4) McFarren, E. F., Mills, J. A., *Anal. Chem.* **24,** 650 (1952).
(5) Marini-Bettolo, G. B., Trabacchi, G., *Biochim. et Biophys. Acta* **21,** 258 (1956).
(6) Reindel, F., Hoppe, W., *Naturwissenschaften* **40,** 221 (1953).
(7) Roche, J., Thoai, Nguyen-van, Hatt, J. L., *Biochim. et Biophys. Acta* **14,** 71 (1954).
(8) Steller, W. A. Klotsas, K., Kuchar, E. J., Norris, M. V., J. AGR. FOOD CHEM. **8,** 460 (1960).

Received for review April 17, 1961. Accepted May 12, 1961.