HILGARDIA

A Journal of Agricultural Science Published by the California Agricultural Experiment Station

VOLUME 30 JULY, 1960 NUMBER 5

THE ADAPTATION OF QUALITATIVE AND QUANTITATIVE TECHNIQUES FOR DETERMINATION OF RADIOACTIVE DALAPON IN PLANT TISSUES

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In studying the absorption, distribution, and metabolism of dalapon (2,2-dichloropropionic acid) in relation to phytotoxicity, a number of simplified techniques were developed, and other established procedures were usefully modified. The techniques of autoradiography, extraction and fractionation, counting, and paper partition cochromatography were effectively combined, thus permitting qualitative and quantitative determination of distribution patterns and, to some extent, of the metabolic fate of translocated herbicides. Although this report is concerned specifically with dalapon, the approach employed with that compound should be applicable, with minor changes, to the study of others.

Physiological findings resulting from application of these techniques will be published separately.

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THE ADAPTATION OF QUALITATIVE AND QUANTITATIVE TECHNIQUES FOR DETERMINATION OF RADIOACTIVE DALAPON IN PLANT TISSUES[1]

CHESTER L. FOY[2]

INTRODUCTION

RADIOISOTOPES have greatly facilitated research on the absorption, translocation, and mechanisms of toxic action of herbicides. Because of its relatively low-energy beta emissions and long half-life, C^{14} has been employed most extensively in phytotoxicity studies. Other beta emitters, such as Cl^{36} and S^{35}, are now being used in an increasing number of situations.

General procedures on the use of radioisotopes in biological research have been published (Aronoff, 1956; Boyd, 1955, Calvin *et al.*, 1949; Comar, 1955).[3] In many instances, however, the maximum benefit from these valuable research tools is not realized. Too often, for example, the sole approach used by plant scientists is that of gross autoradiography. That technique, although put to excellent use in the past (Arnon *et al.*, 1940; Boyd, 1955; Colwell, 1942; Crafts, 1953, 1956) and variously improved in more recent herbicidal studies (Pallas and Crafts, 1957; Yamaguchi and Crafts, 1958), permits only semiquantitative interpretation of results, and does not characterize the translocated radioactive substance(s) either as the original herbicide or some degradation products thereof. Moreover, when counting is done, the data are usually expressed in relative rather than absolute terms. Results are often more meaningful if several approaches are used concurrently.

In recent studies on the absorption, distribution, and metabolism of 2,2-dichloropropionic acid (dalapon) in relation to phytotoxicity, the techniques of (a) autoradiography, (b) extraction and fractionation, (c) counting, and (d) paper partition cochromatography were effectively combined. This combination of techniques permits the determination of distributional patterns and, to some extent, the metabolic fate of translocated herbicides, both qualitatively and quantitatively.

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[3] See "Literature Cited" for citations referred to in text by author and date.

One of the most important limitations in such studies is the time involved in obtaining quantitative results. This report describes a fast, simple method for quantifying data obtained by direct counting of ground plant tissue containing the radioactive herbicide. Also, certain other useful modifications of established techniques, e.g., extraction and fractionation, are briefly discussed. No claim is made for originality in the use of any standard individual procedure. However, the desirability of adapting and using all of these techniques concurrently, to solve a given problem, is emphasized. Although the information reported here relates to dalapon specifically, certain of the techniques and principles should be readily adaptable to the study of other compounds.

EXPERIMENTAL PROCEDURES AND RESULTS

Preliminary

Volatility. The loss of volatile radioactive substances during experimentation may introduce serious errors. Since dalapon (acid) was expected to be somewhat volatile, two preliminary tests were conducted to determine the relative importance of this property. As a precaution, dalapon-Cl^{36} (in acetone), before being converted to the sodium salt, was stored in a test tube stoppered with a cork coated lightly with stopcock grease. The whole top of the tube was then sealed with collodion. After several months in the refrigerator (8° C), the cork was cut into sections 1.5 to 2.0 mm thick, and counted in planchets, with the edge of cork that had been nearest the solution oriented upward. After exposure to the open air for 17 days, the same sections were counted again. Results of the initial and the final counts are shown in table 1. The precautions taken in storage were obviously justified. Dalapon was moving slowly through the cork by vaporous diffusion, but once absorbed, it was held rather tenaciously.

TABLE 1

VAPOROUS DIFFUSION OF 2,2-DICHLOROPROPIONIC ACID-Cl^{36} INTO THE CORK STOPPER FROM REFRIGERATED STOCK SOLUTION

Section of stopper	Net activity, thin-window G.M. tube	
	Initial	Final 17 days
	cpm	*cpm*
1 (nearest solution)	177	137
2	83	63
3	31	27
4	18	16
5	1	1
6	1	0
Rim of the test tube		
7	0	0
All other sections	0	0

The second preliminary run was made to determine how fast, and to what extent, loss from a single drop of dalapon might be when air currents were involved, as in a greenhouse environment. One drop of dalapon (acid) was deposited on a flat aluminum disk, then positioned once under a thin-window G.M. tube, and counted at intervals for 64 hours without change in geometry. Counts were taken under a laboratory hood at 27° C ± 3°, under continuous operation of a gentle fan. The procedure was repeated with a similar drop of the equivalent sodium salt. The trends of the two sets of data are compared in figure 1. The data represent 10-minute counts, taken continuously at first, and then at infrequent intervals. This procedure is a simple yet lucid means of determining the relative importance of volatility of pesticides.

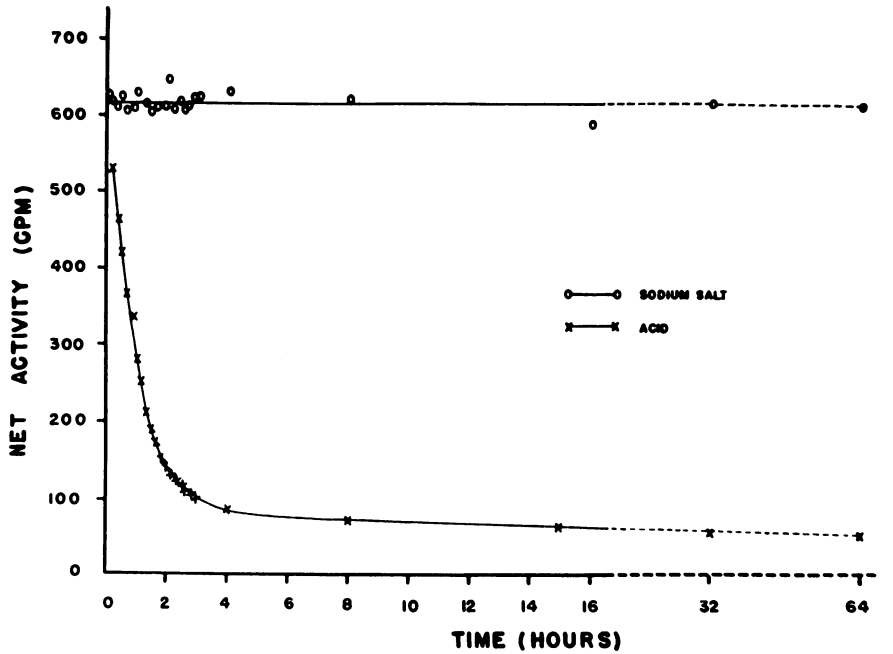

Fig. 1. Comparison of volatilization losses of the acid and the sodium salt of dalapon-Cl^{36} from an inert surface. Counts were taken continuously or at intervals, in a laboratory hood, under a gentle fan, without change in geometry.

These two tests clearly demonstrated the advisability of making all applications to plants as the sodium or other involatile salt rather than as the acid. Consequently, except when formulation was the variable under consideration, only the sodium salt of dalapon was applied to plants or plant material.

Treatment of Plants. Depending on the objectives of a particular experiment, treatment was made by (a) spraying the foliage, (b) drop application to one or more leaves, (c) feeding through a cut vein or petiole from a sleeve of Tygon tubing, or (d) introducing labeled herbicide into the soil or nutrient solution. The advantages and disadvantages of each approach are apparent, and are not discussed further. The importance of proper selection of a test species has been pointed out (Yamaguchi and Crafts, 1958).

Autoradiography

The autoradiographic technique has been amply described as a valuable research tool (Boyd, 1955; Pallas and Crafts, 1957; Yamaguchi and Crafts, 1958). Its principal usefulness is the providing of a vivid graphic record of gross distribution of the herbicide under a prescribed set of conditions; its principal weakness is a tendency of researchers toward overinterpretation of results based on pictures of gross distribution alone. Autoradiography is clearly used to best advantage in combination with other, more refined methods of analysis. Figure 2, for example, shows regions of high and low

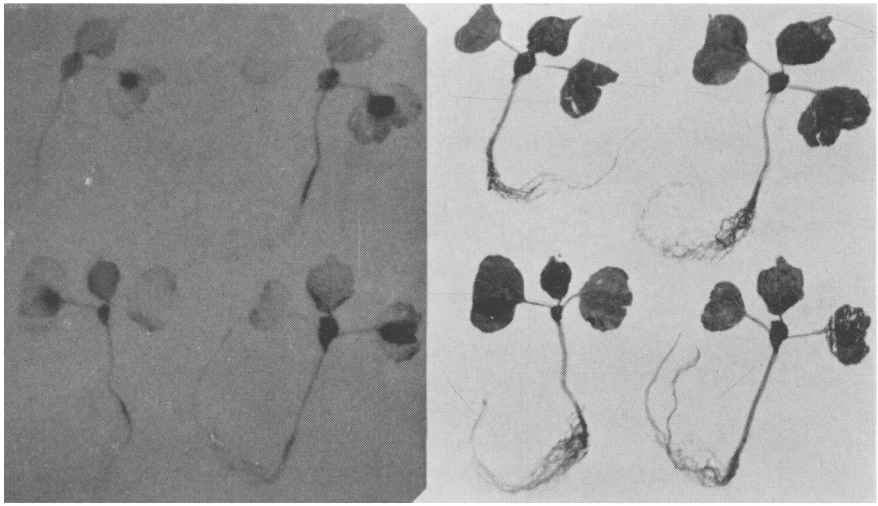

Fig. 2. Autoradiographs showing foliar absorption and translocation of dalapon-Cl^{36} in cotton three days after treatment. Note the degree of uniformity among replications.

concentrations of dalapon. Such autographs permit intelligent sampling of plant tissues and organs for later extraction, counting, and chromatography. Figure 2 also illustrates the degree of reproducibility obtainable among replications.

Figure 3 and table 2 also demonstrate that such autographs (especially in the case of thin plant masses such as leaves) are susceptible to at least semiquantitative interpretation, within the limits of resolution of the X-ray film and the human eye. Notice the very close correlation between extent and density of the images on the autoradiogram and the quantitative count data obtained directly from the same leaves.

In some cases, it may be desirable to autograph only one or two of several replications, and use the remainder for extraction and counting. However, processing and autoradiography do not preclude the use of the same plants for quantitative count data and/or chromatography of extracts later. In most experiments with dalapon, using sorghum, cotton, *Tradescantia,* and corn, the entire plants or plant parts were autographed, then removed from the paper mounting for grinding and counting directly, or for extraction,

counting, and/or chromatography. Usually the treated spot was first removed to reduce contamination and to ensure against drawing erroneous conclusions based on surface-absorbed herbicides. Dry, ground tissue of plants so treated may be conveniently stored in snap-cap glass vials for further study.

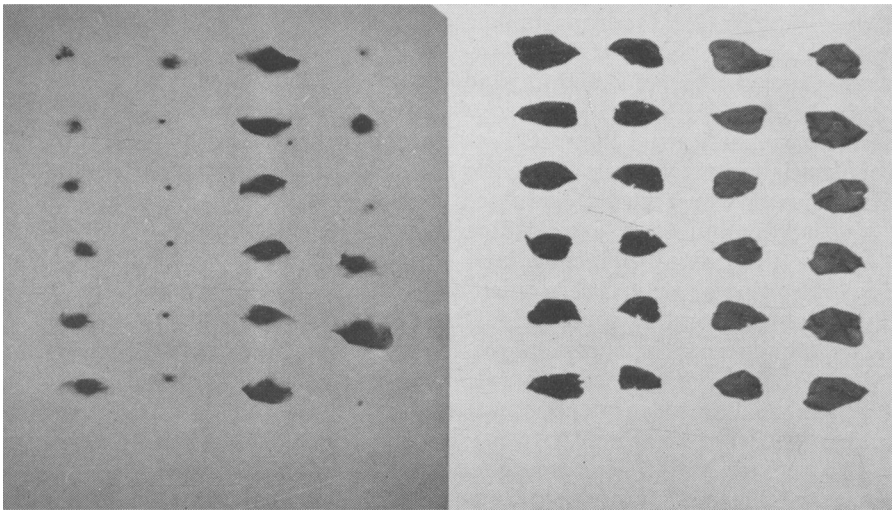

Fig. 3. Cuticular and stomatal penetration of 78 per cent dalapon-2-C^{14} into the upper or lower surface of *Tradescantia* leaves, two hours after drop application of 5 μl of herbicide, either alone or containing 0.1 per cent Vatsol OT. Six replications are shown. First row (at left), upper surface, surfactant; second row, upper surface, no surfactant; third row, lower surface, surfactant; fourth row, lower surface, no surfactant. Autographs are at left, plants at right.

TABLE 2

PENETRATION OF DALAPON-2-C^{14} INTO *TRADESCANTIA* LEAVES (FIGURE 3) WITHIN TWO HOURS*

Upper surface		Lower surface	
Surfactant	No surfactant	Surfactant	No surfactant
cpm	*cpm*	*cpm*	*cpm*
13	26	445	9
13	12	510	102
24	12	470	6
54	9	630	141
83	8	393	801
90	13	635	2
Average 46	13	514	177

* Droplet applied represents approximately 21 mg dalapon (0.05 μc) and is equivalent to 10,185 cpm (thin-window G.M. tube).

Quantitative Aspects

General. The following radioactive chemicals were employed in the tracer studies: purified 2,2-dichloropropionic acid-Cl^{36} in acetone (12.78 μc/mM), converted to the sodium salt in aqueous solution before application to plants; 96 per cent Na 2,2-dichloropropionate-2-C^{14} (0.98 mc/mM); 78 per cent Na 2,2-dichloropropionate-2-C^{14} (0.98 mc/mM). Aqueous stock solutions of each were prepared to convenient volumes, both with and without surfactant (0.1 per cent Vatsol OT). Vatsol OT was chosen because of its superior wetting properties and the fact that it is a known chemical compound. (Most commercial wetting agents are poorly characterized.) Unless otherwise stated, all radioactive treatments contained the surfactant.

Initially, aliquots of each radioactive stock solution were deposited on 24-mm stainless-steel planchets, and counted with both a Tracerlab "1000" Scaler, equipped with a thin mica end-window Geiger-Muller tube, and a Tracerlab Autoscaler, equipped with an SC-16 windowless gas-flow apparatus. This precaution permits one to know the number of net counts per minute (cpm) represented in an aliquot (or application dose) of any stock solution used in subsequent experiments; it further permits quantitative interpretation of the count data obtained by either instrument. Both instruments were used on various occasions, depending on suitability for the experiment and availability for use. For example, the gas-flow counter, which is several times as sensitive as the G.M. tube, was especially useful at low levels of activity. For any given experiment, however, counts were obtained with one instrument. The total time of counting a sample and total counts recorded were variable among experiments, depending on the precision desired. The number of counts (totalized) required to obtain the desired degree of reproducibility can be predetermined from charts with each instrument. These tests were usually gauged to obtain an over-all reproducibility (including counting and sampling errors, but not biological variations) of 6 to 8 per cent. At the levels of activity used, coincidence loss was negligible.

Results are presented as counts per minute, appropriately corrected for background, self-absorption, and aliquot factors. These may be conveniently compared with the total counts represented in the applied dose, which is listed with each experiment.

Self-absorption. If the activity of a series of samples of varying weight but constant area is measured, a deviation from linear dependence upon the mass of sample material will be noted. This effect is caused by self-absorption of radiations not sufficiently penetrating to leave the surface of the sample. Self-absorption losses introduce large errors in activity measurements (especially with weak beta radiations) for which corrections must be made. Calvin *et al.* (1949) list four procedures by which this correction can be made or a need for it can be eliminated. First, it may be possible to mount the sample in such thin layers that the self-absorption error is negligible in comparison with other errors in the experiment. The sample is then at infinite thinness. Application of this procedure in measuring C^{14} and Cl^{36} is limited because of the frequency with which samples of low specific activity must be measured. Second, an attempt can be made to reproduce a standard

sample thickness accurately. This is difficult in practice, but with care can be done within allowable margins of error. Third, samples can be prepared at infinite thickness, i.e., so thick that saturation activity is observed. This procedure is of quite general applicability when low-energy particles are to be detected. Fourth, the relation between the observable activity and the thickness of the sample can be obtained, and by its use the activity observed at any thickness can be related to that which would have been observed at some standard thickness. This is clearly the most general approach since no limits are placed on the amount of activity or of material that can or must be used in preparing the radioactive substance for analysis.

All four of the described approaches were employed at one time or another in these investigations. The fourth and the first, in that order, were used most extensively. The terms "infinite thinness," "negligible self-absorption," "standard sample thickness," "infinite thickness," and "self-absorption correction curves" are therefore used where appropriate to the discussion.

Extraction of Intact Plant Tissue for Plancheting and Counting. It is frequently desirable to count samples of tissue extracts directly. The liquid is commonly applied to glass, aluminum, nickel, or stainless-steel planchets as a drop, or, by use of a sample spinner, as a spiral line (Calvin *et al.*, 1949). Smith (1958) recently described some simple improvements on this method. In most instances, the extracts are obtained by macerating the tissues. Direct extraction of intact tissues, e.g., leaf discs or stem sections, is also possible. In using this technique, considerable difficulty has been encountered in achieving reproducible results.

In short-term experiments (15 seconds to 3 hours), wherein the treated leaf of corn or *Tradescantia* was the only concern, one or more replicates were autographed and four others were liquid-extracted, plancheted, and counted. Several preliminary experiments were required before a satisfactorily sensitive method was finally decided upon. The results that aided in developing the final procedures are presented briefly.

In an early test, a uniform 5-μl drop of 78 per cent C^{14}-labeled dalapon was applied to fresh leaf discs (13 mm) of corn. The discs were allowed to absorb the droplet in a humid atmosphere. They were then stored overnight in a freezer at –7° C, and either 1 ml of distilled water, 95 per cent ethanol, or 1 N sodium hydroxide was added after thawing. Extraction was accomplished by allowing the tubes to sit overnight in a 50 to 60° C oven. One-fifth of the volumes added, or 200-μl aliquots, were plated smoothly in 24-mm stainless-steel planchets with a Nuclear-Chicago PMI Sample Spinner, and the samples were counted in a sensitive gas-flow counter. Another set of tubes (five replications) was treated identically except that no leaf discs were involved, the chemical being added directly to the tubes. The recovery results are summarized in table 3. It was learned from this test that water as the extracting medium showed less variability among replications, gave the highest per cent recovery, and required little or no correction for self-absorption loss. In later tests a lower percentage of ethanol (e.g., 50 per cent) was shown to be more effective than 95 per cent ethanol. The respective solubilities of dalapon (Na salt) in water and in ethanol at 25° C are 90.0 gm and 18.5 gm per 100 gm of solvent. Sodium hydroxide produced variable

results because of its hygroscopicity, and total counts were reduced by self-absorption. Even with plancheted samples in a constant-temperature oven, an error is unavoidably introduced when different counting periods are used. Also, self-absorption loss is a serious limitation in dealing with low activities. Impregnation of filter paper, similarly tested, was abandoned because, although reproducible, it decreased total counts in comparison with direct plancheting.

TABLE 3

RECOVERY OF DALAPON-2-C^{14} FROM CORN LEAF DISCS BY EXTRACTION WITH VARIOUS SOLVENTS

(Counts are averages of five replications.)

Extraction solvent	Net activity		Amount extracted	Weight of precipitate of extract
	No leaf	Leaf disc		
	cpm	*cpm*	*per cent*	*mg*
Water	38,800	38,157	98.3	0.1
Ethanol, 95%	35,814	23,052	64.3	0.1
Sodium hydroxide, 1 N	14,631	10,682	73.0	37.4
				21.2*

* Hygroscopic moisture removed by drying the precipitate thoroughly under an infrared heat lamp before counting.

TABLE 4

EFFECT OF VARIOUS PREPARATORY TECHNIQUES UPON THE PER CENT RECOVERABILITY OF DALAPON-2-C^{14} EXTRACTED FROM CORN LEAF DISCS WITH WATER

(Counts are averages of five replications.)

Preparatory practices*	Net activity	Recovery†
	cpm	*per cent*
Freezing, oven, and surfactant	39,963	88.7
Freezing and oven	43,294	96.1
Freezing and surfactant	39,907	88.6
Oven and surfactant	44,392	98.6
Oven only	29,880	66.3

* Freezing at −7° C, 24 hours; oven at 50 to 60° C, 24 hours; surfactant, Vatsol OT, 0.1 per cent.
† Standard counted directly equivalent to 45,045 cpm.

In another test, an attempt was made to refine the procedure by evaluating the effects of freezing, incubation at 50 to 60° C for 24 hours, and the addition of a surfactant (Vatsol OT, 0.1 per cent). Other procedures were the same as described above. The results are summarized in table 4. Conclusions from these experiments were: Freezing and thawing, and the use of Vatsol OT individually increased per cent of recovery, presumably through increases in the permeability of the leaf discs. Incubation in the oven did not alter the results strikingly. When all three practices were used, or when only the period in the oven was omitted, total counts tended to decrease, probably because of their cumulative effects on permeability. Leakage was apparently sufficient to introduce a slight amount of self-absorption loss resulting from the precipitate left after plancheting. Despite the necessity for this small correction, all three practices were used in subsequent tests, for the following

reasons: Freezing was a convenient means of rapidly suspending any possible enzymatic activity, and storage in the frozen condition prevented contamination by microörganisms in case extraction should be delayed. The period in the oven provided a uniform thawing while approaching equilibrium, but otherwise was of no apparent advantage. Finally, the surfactant was considered essential to ensure thorough wetting of the tissue (otherwise, grass leaf sections tend to float). The self-absorption loss, although slight, may be reduced by using only freezing or surfactant, instead of both.

Fig. 4. Sequence of steps represented in short-term absorption and translocation studies with radioactive dalapon on corn leaves. The procedure consisted of drop application to the second leaf, excising the leaf after a predetermined time, washing in three water baths, blotting free of excess moisture, and sectioning into five approximately equal parts. One replication of all sections was autoradiographed, and four others were liquid-extracted, plancheted, and counted.

In subsequent experiments, corn was grown, and treated by drop application on a single leaf in the conventional manner. At varying intervals of time, the treated leaf was excised and washed by being plunged five times into each of three separate wash-water baths, blotted with Kleenex tissue, and sectioned into five approximately equal parts. The treated spot was made to fall in the middle of the center section. It is almost certain from this and other work that the washing removed all of the unabsorbed dalapon. Extraction was carried out as described, in 75-mm bacteriological culture tubes. This sequence is illustrated in figure 4. In addition to counting the extracts in quantitative studies of dalapon movement, aliquots were also chromatographed, and autoradiograms were prepared from the developed chromatograms. The latter was accomplished simply by placing the chromatogram in close contact with regular medical X-ray film and exposing for a suitable period, usually one to four weeks. In the case of radio-pure compound, radioactivity was detected as dalapon only; in the case of impure chemical, as dalapon plus impurities already present in the stock solution (in about the original ratios). Thus the results indicated no rapid metabolic changes in the herbicide under these conditions.

Tradescantia leaves may be treated similarly as single sections.

Standardization for Direct Counting of Ground Plant Material. Both wet and dry combustion methods are available for analysis of materials containing C^{14} (Calvin *et al.*, 1949). Radioactive carbon dioxide is precipitated and counted as barium carbonate. Also, an adaptation of a plating technique described by the same authors has proved satisfactory for analysis of dalapon containing Cl^{36} (Redemann and Meikle, 1958). Dalapon, in sodium carbonate solution, is hydrolyzed to pyruvate and radioactive inorganic chloride, and an aliquot is plated and counted. Quantitative data can be obtained by

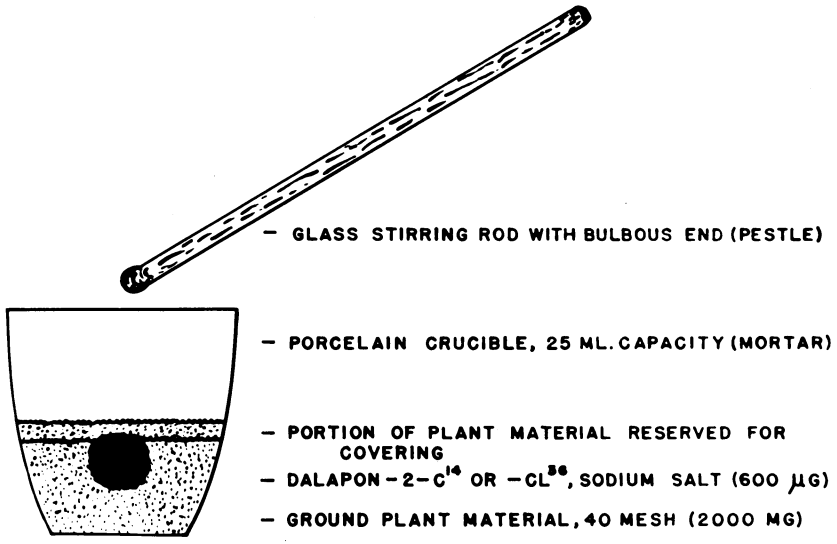

Fig. 5. Equipment used to prepare standard plant samples, from which self-absorption correction curves were constructed. Aliquot samples taken after mixing were varied in thickness but uniform in concentration.

either method, but both are rather laborious and time-consuming, and have other limitations not encountered in the procedure described below. Moreover, in those studies in which C^{14}- and Cl^{36}-labeled dalapon were used, two analytical techniques would have been required rather than one.

Instead, standardization curves were constructed for direct counting of dry, ground plant material containing either Na-dalapon-Cl^{36} or Na-dalapon-2-C^{14}. Whole cotton plants comparable with those used in the experiments were dried and ground to 40 mesh in a Wiley mill. Then 600 μg (approximately 115 μl) of either Cl^{36}- or C^{14}-labeled dalapon were added to 2 gm of plant material and mixed thoroughly, as shown in figure 5. With dalapon-Cl^{36}, the amount added was equivalent to 0.054 μc (6,635 cpm, G.M. tube); with dalapon-2-C^{14}, 1.130 μc (46,869 cpm, G.M. tube). Care was taken to prevent movement to the external surfaces of the plant mass until drying was complete. Vaporization loss was negligible during the drying process (40° C for one hour) (see fig. 1). Aliquot samples ranging from 20 to 1,000 mg, but uniform in concentration, were spread smoothly in stainless-steel planchets, tamped firmly with a special tamping device that just fitted

inside the planchet, and counted (G.M. tube). The curves in figure 6 were constructed from these data. After calculation, the self-absorption correction curves shown in figure 7 were constructed. The per cent of self-absorption in any size of sample of the plant material could then be read directly from the latter pair of curves. Variations in the consistency of different plant

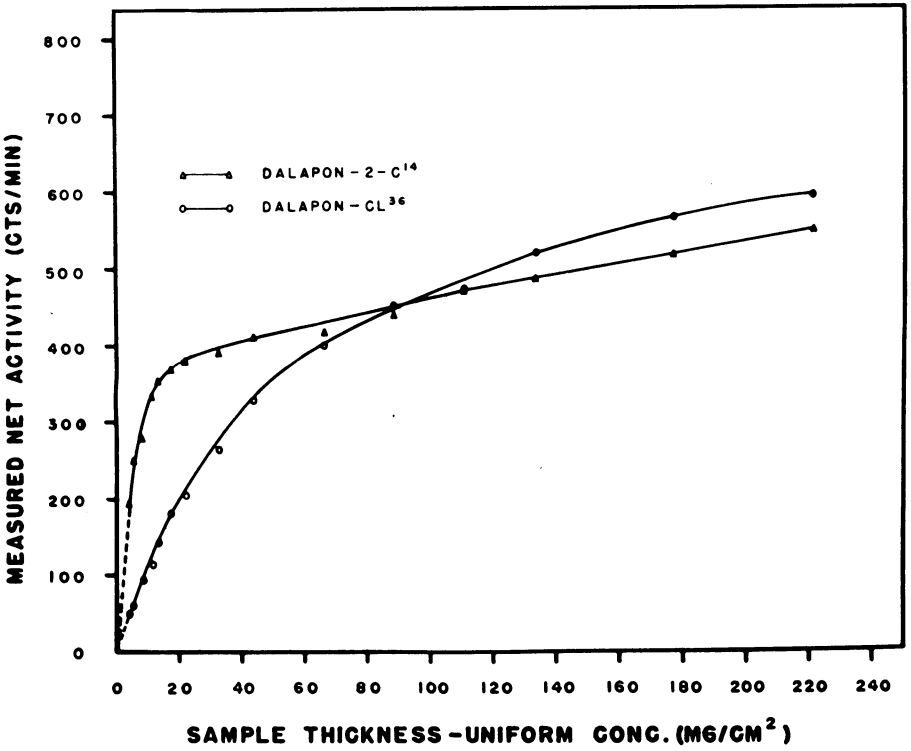

Fig. 6. Activity saturation curve of dry plant tissue ground to 40 mesh and containing either dalapon-2-C^{14} or -Cl^{36} in uniform concentration. (TGC thin-window GM tube data, efficiency for infinite thickness C^{14} source approximately 3 per cent; samples mounted in stainless-steel planchets.)

tissues could conceivably alter the absorption patterns shown, but, on the basis of counting, cotton and sorghum were not considered to differ enough to warrant another set of curves. On the other hand, changing from one isotope to another, e.g., from C^{14} to Cl^{36}, would absolutely necessitate the construction of a new standardization curve.

The lower specific activity, but higher energy of radiation of Cl^{36} in comparison with C^{14} is readily apparent. The reliability of the technique is shown by the fact that relatively smooth curves were obtained, and individual points on the curves were reproducible within 6 per cent variability. Also, both curves extrapolate well back to zero at infinite thinness. The observation that the curves are not absolutely flat at infinite thickness may be the result of the slightly decreasing distance between surface of the

sample and the G.M. tube as mass of the sample increases. It is clear that infinite thickness is achieved much more rapidly with C^{14} than with Cl^{36}. Curves similar to those shown are invaluable in quantifying count data from tissue samples of different sizes. Such curves are also useful even if a standard sample thickness is maintained for a given experiment. The over-all error in this simple yet convenient procedure is not considered excessive for most purposes, and is probably no greater than the combined errors encountered in other methods.

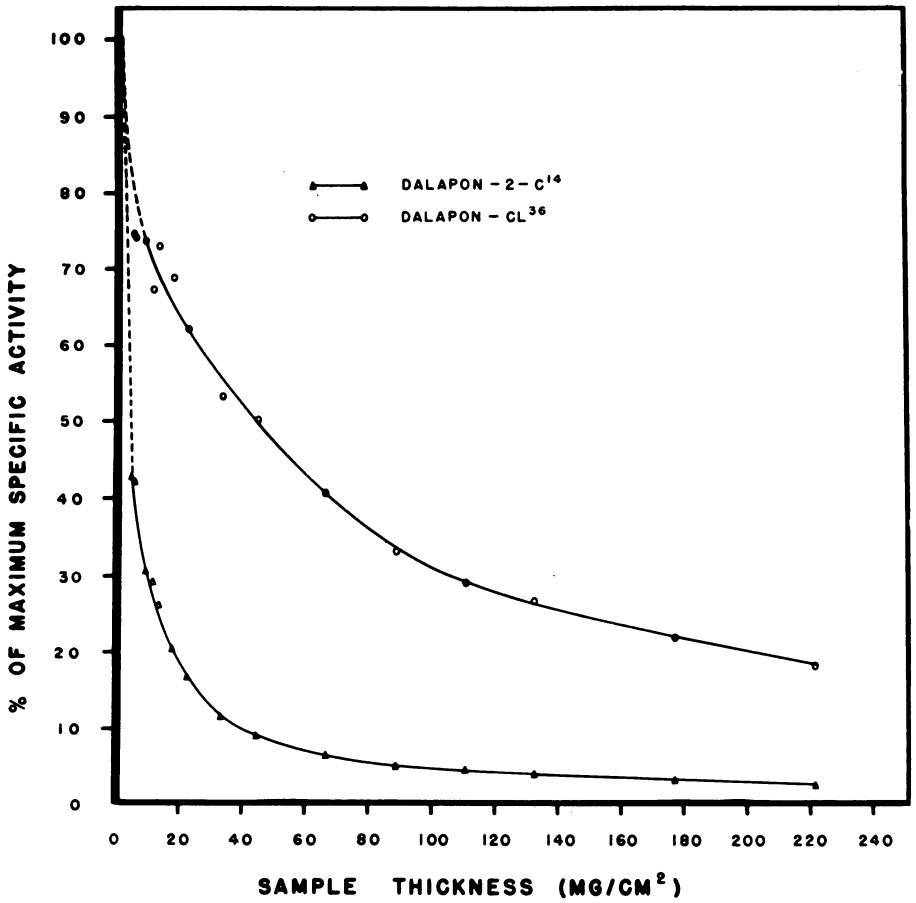

Fig. 7. Self-absorption correction curves for C^{14} or Cl^{36} radiation in dry, ground plant tissue. Computed from data shown in figure 6.

Technique for Studying the Metabolic Fate of Dalapon

Two basic approaches may be adopted in studying metabolic changes in plants treated with herbicides. One is to determine the effects of the herbicide upon the normal chemical constituents of the plant. This method has been used widely (e.g., with 2,4-D), but generally with little success in elucidating the mechanisms of action of herbicides. A second approach, the one described here, is to investigate the metabolic fate of the applied herbicide in the hope that this may offer clues to its herbicidal action.

Studies consisted of two types: (a) paper chromatography and autoradiography of undiluted plant sap and extracts of whole plants, fresh and dry plant parts, or nutrient solutions; (b) extraction and separation of plant material by known analytical procedures to establish a broad categorization of the compounds found to contain radioactivity originally present in the dalapon.

Paper Chromatography and Autoradiography. At the time the work was undertaken, no procedures were available for chromatographic identification of dalapon from plant tissues. Since general techniques, e.g., as employed by Benson *et al.* (1950), were found applicable to such a wide array of chemical substances, it was thought probable that similar procedures could be profitably employed in this study. Chromatographic methods for the determination of volatile fatty acids (Roberts and Bucek, 1957; Manganelli and Brofazi, 1957) and chlorinated fatty acids (Renard, 1949) have been described. However, the following two solvent systems were considered the most logical choices for the separation of dalapon: (a) 1-pentanol and 5 M formic acid (V/V), which was used by Blanchard (1954) in detecting trichloroacetic acid (TCA); (b) n-butanol and 1.5 N ammonium hydroxide (V/V), suggested by Meikle (1956).

On the basis of numerous preliminary investigations involving both radioactive and nonradioactive compounds, the following results and conclusions seem justified.

(1) Dalapon chromatographed cleanly in descending development on Whatman #1 filter paper with either solvent, moving as the acid in the former case and as the ammonium salt in the latter. Respective R_f values were approximately 0.9 and 0.4 to 0.6.

(2) Volatility loss was evident when the acidic solvent system was used or when the drops were applied to the paper as the acid, if several hours elapsed before the chromatogram was developed.

(3) Either bromphenol blue (0.04 per cent in 95 per cent ethanol, pH 6.75) or 2,4-dichlorophenol indophenol (1 per cent in ethanol, no pH adjustment) was satisfactory as a color reagent when the acidic solvent system was used. Dalapon sprayed with bromphenol blue appeared yellow against a pale-blue background; with indophenol, vivid pink against a purplish-blue background.

(4) When 1-pentanol and formic acid were used, the chromatographic properties of dalapon were similar whether applied in pure form (acid, Na salt or NH_4 salt) or as the technical or commercial (85 per cent) formula-

tions. The cations behaved differently in that ammonia eventually volatilized away, whereas sodium, lithium, et cetera, remained, and appeared as strongly basic spots of low R_f.

(5) The R_f did not change appreciably with variations in concentrations of dalapon. This is in contrast to the results of Renard (1949), in which the R_f was reported to be a function of the quantity of chloroacetic and chloropropionic acid (developed with dichloroethylene and water). He also reported that one acid exerted a displacing action on the other when they were chromatographed together.

(6) The R_f varied slightly between runs. The factors responsible for these variations were discussed in detail by Consden *et al.* (1944).

(7) When labeled dalapon was used, spraying with a color indicator did not affect subsequent autoradiography when such was desirable. Autoradiography and/or scanning proved much more sensitive than did detection by color indicators.

A communication was later received in which Smith *et al.* (1957) reported extensive work on the choice of a solvent system for chromatographic separation of dalapon from contaminants and possible metabolic products. These workers concluded that, of 10 solvent systems tested, only the n-butanol and 1.5 N ammonium hydroxide (V/V) system gave satisfactory separation of all compounds (monochloropropionate, dichloropropionate, trichloropropionate, pyruvate, and acetate). On the strength of this work and the preliminary findings reported above, it was decided to use this system exclusively thereafter.

In some experiments, sap was expressed and chromatographed directly. The sap was obtained either by rolling a glass cylinder over the tissue, which was backed with a microscope slide, and then collecting the juice with a micropipette, or (for larger samples) by quick freezing and thawing and squeezing in a hydraulic press. These procedures were replaced by more refined techniques in later experiments. Later, the plants or plant parts (whether fresh or dry) were finely pulverized in a ground-glass tissue homogenizer containing a small volume of water (or, rarely, 80 per cent ethanol) as the solvent. The homogenate was then centrifuged for 10 minutes at 1,000 to 1,500 × gravity. This gave a clear supernatant (essentially free of insoluble plant material), which was suitable for chromatographing or plancheting and counting. The plant residue was also washed exhaustively with several hundred volumes of water, plated, dried, and counted to test for incorporation of the radioactive atom into insoluble plant constituents. A Waring blender was used in a similar manner for one experiment involving several whole plants.

Dalapon was easily removable from the water extract, when this was desirable, by acidifying with HCl and extracting with diethyl ether, either repeatedly or continuously. The partition coefficient between ether and 1 N HCl is 6.5 in favor of the ether (Meikle, 1957). In most cases this extraction was not done because it was of interest to test for the presence of possible radioactive metabolites as well.

In some cases, the excessive volumes of solution were objectionable when chromatography and counting were to be undertaken. Both vacuum desic-

cation and lyophilization were used to concentrate the solutions, but these were slow processes when very large volumes were involved, e.g., several hundred milliliters of nutrient solution per treatment. Acidifying and extracting continuously with ether, as described by Smith *et al.* (1957), was a more satisfactory solution for recovering dalapon. The nutrient solution or plant extract was transferred to a continuous liquid-liquid extraction apparatus. Sufficient ether was added to permit continuous operation without boiling dry. Extraction was continued for eight hours at a constant rate to permit a steady flow of ether. It was possible to extract several solutions at once by using a six-unit extraction rack in which the temperature was rheostatically controlled. To avoid volatility loss, the extracted dalapon was converted to the sodium salt by extracting the ether solution with a small volume of sodium hydroxide or sodium carbonate (5 N).

The same general procedures were used for all solutions. The extracts were spotted in 5- or 10-μl aliquots 3 inches from one end of 7 × 17-inch Whatman #1 filter paper sheets, with time allowed for drying between applications. The final volumes applied varied according to the specific activity. The n-butanol and 1.5 N ammonium hydroxide system was prepared 24 hours before use by mutually saturating equal volumes of the two components in a separatory funnel. The sheets were equilibrated for three hours with the vapors from the solvent phases. After equilibration, the organic phase was added to the trays, and the chromatogram was developed one-dimensionally overnight. The solvent front normally moved 36 to 40 cm in 21 hours. After development, the solvent front was marked, and the sheets were allowed to air-dry in a ventilated laboratory at room temperature.

Radioautograms were made by placing the dry papers in contact with 7 × 17-inch Eastman Kodak No-Screen X-ray film for two to four weeks in light-proof X-ray exposure holders. Some of the chromatograms were scanned manually by cutting the chromatographic paper into strips and then individually counting 20-mm square sections in planchets. A more efficient procedure is to use automatic scanning equipment when it is available.

In addition to the labeled stock solutions and several nonradioactive substances, the following aqueous solutions also served as reference standards in the chromatography experiments: impure Na 2,2,3-trichloropropionate-2-^{14} and relatively pure Na acetate-1-C^{14} (5.18 mc/mM) and Na pyruvate-1-C^{14} (4.17 mc/mM). Preliminary identification of compounds was made by comparing R_f values with those of known pure compounds obtained in the same run or by superpositioning the R_f patterns of the known compounds over those of the unknowns. The identified compounds were confirmed by cochromatography with authentic samples of the suspected compound. Figure 8 is representative of the results obtainable by the above-mentioned procedure.

Extraction and Fractionation. In these studies, cotton was grown in large clay pots in the greenhouse, to the stage at which squares, flowers, and small bolls (fruits) were present. Various methods of application were tried (injection into petiole, feeding through severed vein, etc.), but the most satisfactory means of introducing a relatively large amount of chemical (Na 2,2-

dichloropropionate-2-C^{14}) in a short period was by loading a short section of Tygon tubing (3.5 mm inside diameter) with 100 or 200 μl of solution, and then slipping the tube as a sleeve over the cut end of the petiole. The lamina had been removed, but the petiole normally stayed attached to the plant for several days or weeks after treatment. To ensure a good fit, the inside diameter of the capillary tubing was chosen to correspond to the diameter of the petiole. If desired, the application may be apportioned in separate doses by leaving air space at intervals between the doses when loading the tubing. The sleeve is simply slipped farther onto the petiole for the second application. (This, of course, requires a fit loose enough to permit

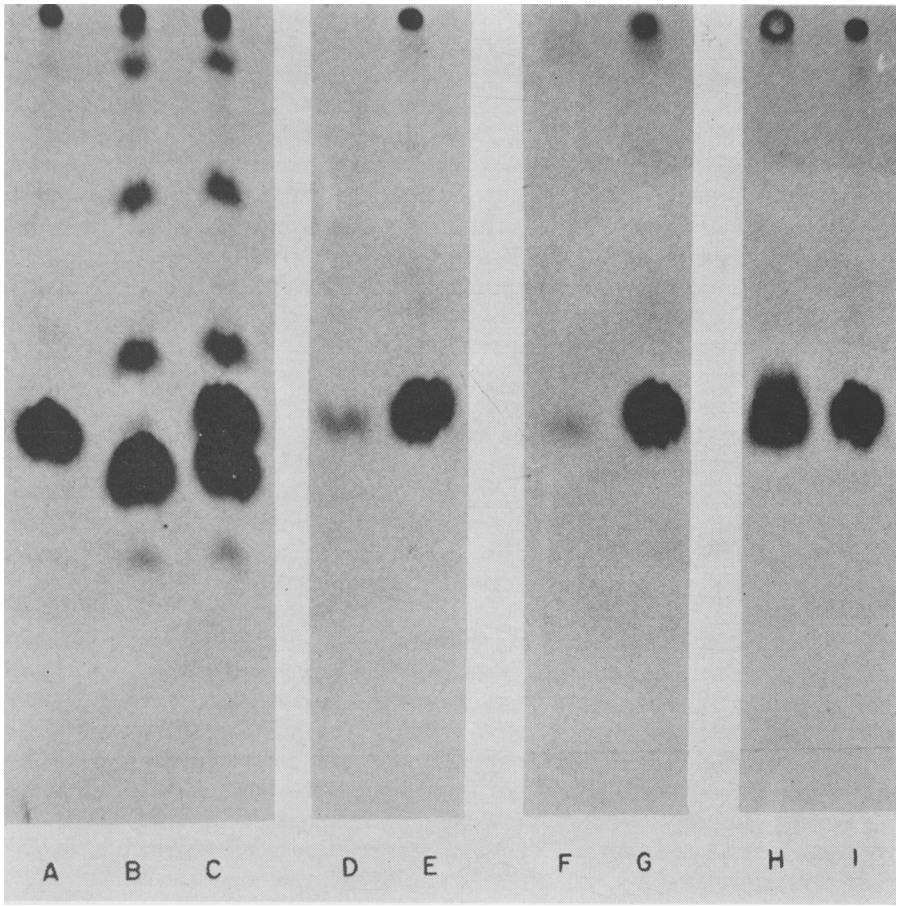

Fig. 8. Autoradiographs of representative cochromatograms: (A) 5 μl 96 per cent dalapon-2-C^{14} stock solution; (B) 5 μl impure 2,2,3-trichloropropionate-2-C^{14} (TCP); (C) dalapon plus TCP, 5 μl each; (D) aqueous extract from roots of dalapon-treated cotton plants; (E) extract in (D) plus dalapon; (F) aqueous drop (from ether extract) of nutrient solution in which dalapon-treated cotton was grown; (G) extract in (F) plus dalapon; (H) aqueous extract from dalapon-treated leaves of sorghum; (I) extract in (H) plus dalapon. See text for more detailed explanation.

some leakage of air between tube and petiole.) The rate of uptake was about 1 μl per minute. (This is the same figure reported by Nelson and Gorham [1957], who used a similar technique to introduce sugars into soybean seedlings.) This technique is particularly advantageous in such studies because it ensures rapid introduction of a relatively large amount of radioactivity into the xylem stream, simulating uptake through the soil, but without the complicating factors of soil dilution, adsorption, et cetera. Also, this procedure eliminated the possibility of metabolic degradation outside the plant and subsequent absorption of the degradation products, which are known to occur from nutrient solutions after several weeks. The plants were allowed to develop through the reproductive stages for eight to 10 weeks, until a few bolls were fully matured and open, yet all stages of reproductive development (squares, flowers, and bolls of different ages) were still represented. The plants were harvested, sectioned, and dried overnight in a 50° C oven. Representative samples of all plant parts were autoradiographed to determine the distribution and accumulation of the C^{14} in fruiting cotton plants. From the rest of the plants, composite samples of leaves and of whole flowers and fruits (i.e., seed, fiber, ovary wall, etc.) were collected and ground in a Wiley mill. Fruit tissue from the check plant was also included for comparison. Aliquots of fruits (30 gm) and leaves (15 gm) were then extracted and separated according to the scheme shown in figure 9, which represents an adaptation of the analytical procedure described by Aronoff *et al.* (1947). This procedure was used to ascertain whether the radioactivity was still represented in the form of dalapon or as other products of metabolic conversion.

Details of the procedures were as follows: Unless stated otherwise, all operations were performed at room temperature. The dry plant material was extracted three times with 25 ml of ether per gram of plant material, and the ether extracts were combined. Ether was driven off by passing a stream of air over the solution, and the dark, oily residue was plancheted and counted. This extract was resuspended in ether and washed with 2 ml of 10 per cent Na_2CO_3, and these two fractions were plancheted and counted. The ether-soluble fraction was again resuspended in a small volume of ether, and separated between petroleum ether and 95 per cent ethanol (approximately equal volumes). Both fractions were colored, but they were distinguishable in a separatory funnel. These were concentrated and counted also.

The ether-insoluble residue was next extracted twice for three hours on a 60° C steam bath with 80 per cent ethanol, and the ethanol extracts were combined. This solution was concentrated *in vacuo* (six hours at 55 to 65° C). Aliquots of this concentrate and the entire distillate were further concentrated in planchets and counted. Upon concentration *in vacuo,* the flask contained, in addition to the soluble portion, a dark, insoluble mass similar in appearance to the ether extract. (This residue was at least partially soluble in ether. The compounds may well have been some of the same compounds [e.g., lipids] obtained in ether extraction that were not removed by the cold ether but were slightly soluble in warm ethanol.) The concentrated extract (ethanol now removed) was successively separated via ion-exchange

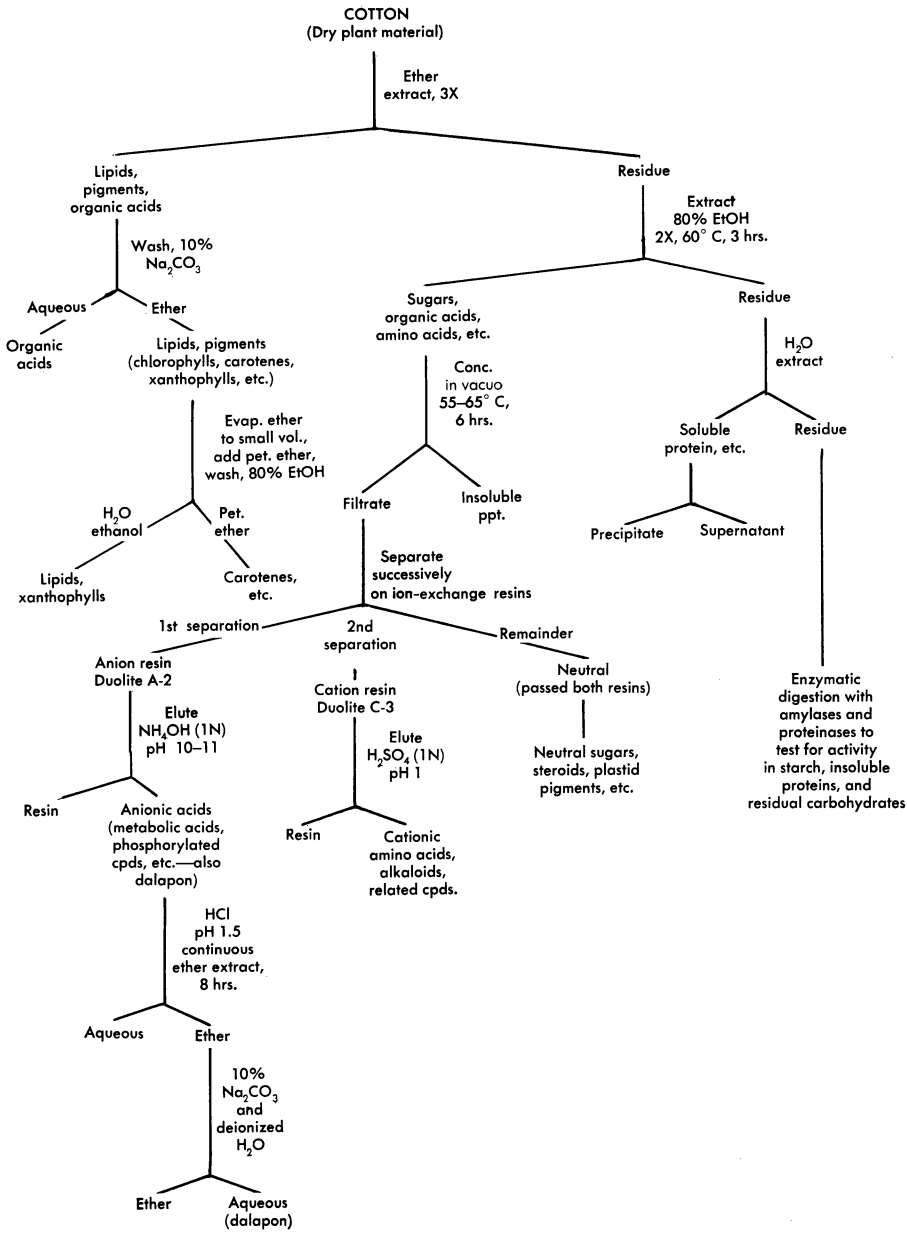

Fig. 9. Analytical scheme followed in categorizing radioactive chemical constituents from fruits and leaves of cotton eight to 10 weeks after treatment with 96 per cent Na 2,2-dichloropropionate-2-C^{14}.

resins into anionic, cationic, and neutral substances. In this way, substances were divided roughly into (1) anionic acids (metabolic acids, phosphorylated compounds, etc.), (2) cationic amino acids, alkaloids, and related compounds, and (3) the neutral sugars, steroids, plastid pigments, et cetera. Dalapon was adsorbed by the anion exchange resin, and could be eluted by means of dilute HCl (see Van Etten and McGrew, 1957). The resin beds used were approximately 30 ml in volume, and the rate of adding regenerant or unknown solution was 4 to 6 volumes per hour. The solution (approximately 30 ml) was first passed through a Duolite A-2 anion-exchange resin charged with NH_4OH. The liquid that passed through was then dripped through a Duolite C-3 cation-exchange resin charged with H_2SO_4. The neutral fraction that passed both resins was preserved, and the anionic and cationic fractions were respectively recovered from the resins by eluting with 1 N NH_4OH and H_2SO_4. The final volumes of the fractions, including eluate and wash water, were 75 to 125 ml each. These three fractions were then lyophilized to approximately 1 to 3 ml each, and aliquots of these containing sufficient radioactivity were chromatographed.

The ether- and ethanol-insoluble residue was washed with water in an attempt to remove any soluble proteins not already extracted in the 80 per cent ethanol. TCA (5 per cent) was used as a protein precipitant, but no precipitate was formed. Finally, aliquots of the residue were enzymatically digested with amylases and proteinases to test for possible activity in starch, insoluble proteins, and residual carbohydrates. Fresh enzyme preparations were used, and recommended conditions of pH, temperature, concentration, and reaction periods were observed.

Semiquantitative information was obtained at various stages in the fractionation schedule by counting resins, residues after each extraction, spots on paper, et cetera. All counts in this experiment, taken with the Autoscaler with gas-flow attachment, were made either at or near infinite thickness, or were approximately corrected for self-absorption, assuming similarity to barium carbonate in this respect. It was impractical to construct self-absorption correction curves for each conglomeration of compounds counted. Because of this, and the fact that some difficulty was encountered in obtaining quantitative recovery from the exchange resins, these data are relative and semiquantitative only, and are not to be considered as absolute values. The usefulness of such a fractionation scheme is apparent, however, in helping to determine the stability of the herbicide molecule, its net charge and adsorptive affinities, and finally, the fate of the radioisotope itself. Such results should be interpreted in conjunction with the findings obtained with techniques already described.

SUMMARY

During an investigation into the mechanisms of absorption, distribution, and metabolism of radioactive dalapon in relation to phytotoxicity, a number of simplified techniques were developed and other established procedures were usefully modified. Dalapon-2-C^{14} and -Cl^{36}, as well as nonradioactive materials, were employed in tracer and metabolic studies. By gross auto-

radiography and counting, both qualitative and quantitative data were obtained. A fast, simple method of quantifying data obtained by direct counting of dry, ground plant material containing radioactive herbicide is described. Extraction and fractionation procedures were developed, and the techniques of paper partition cochromatography and autoradiography were adapted for detecting dalapon and related metabolic degradation products.

This paper summarizes the various techniques and methods evolved and discusses the merits and shortcomings of each for use in physiological research. Although this report is specifically concerned with the herbicide dalapon, the techniques and approaches involved should be equally applicable, with only minor changes, to the study of other compounds. The desirability of employing, in combination, the techniques of (a) autoradiography, (b) extraction and fractionation, (c) counting, and (d) paper-partition cochromatography in the solution of a given problem is emphasized.

The principal physiological findings from application of these techniques in several recent studies will be published separately.

ACKNOWLEDGMENT

Encouragement and guidance were provided by various members of the Botany Department staff, University of California at Davis. Recognition is also due the Dow Chemical Company for gratuitously supplying labeled dalapon, and for offering continued interest and coöperation.

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