of impurities which might interfere with the quantitative analyses, particularly of the minor component.

Acknowledgment

The authors thank E. F. Shelberg and associates for microanalyses.

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GIBBERELLINS ANALYSIS

Determination of Gibberellins by Derivative Labeling with Diazomethane-C^{14} and by Isotopic Dilution Analysis with Tritium-Labeled Gibberellins

WALTER E. BAUMGARTNER, LaVERA S. LAZER, ANN M. DALZIEL, EARL V. CARDINAL, and ESTIE L. VARNER

Research and Development Divisions of Abbott Laboratories, North Chicago, Ill.

In fermentation broths, the concentration of gibberellic acid and of gibberellin A_1 is determined by derivative labeling with diazomethane-C^{14}. In pilot plant studies, step by step recovery data are obtained by the addition of tritium-labeled gibberellic acid. Complex mixtures are analyzed by a combination of these two methods.

VARIOUS PROCEDURES have been worked out for determining the concentration of gibberellic acid and of gibberellin A_1 in various media (*4, 5*). During the same period of time, an isotopic dilution method using deuterium-labeled gibberellic acid was published (*1*). In the current paper, three radiochemical procedures are outlined which were used routinely for the screening of broth samples and for residue studies in this laboratory's gibberellin program.

Experimental

Materials and Equipment The gibberellic acid and gibberellin A_1, which were used for the purpose of standardization, were Abbott products with an isomeric purity of at least 95% and a chemical purity of at least 98% as determined by radiochemical assay and by infrared analysis (*5*).

Diazomethane-C^{14} was prepared following the method of Stoll (*3*) from *N*-methyl-C^{14}, *N*-nitroso-*p*-toluenesulfonylamide. This nitroso intermediate could be stored at room temperature for extended periods of time.

Tritium-labeled gibberellins were prepared by exposure of the isomerically pure gibberellins to tritium gas (*6*). This preparation will be discussed in detail.

For the assay procedure, a Forro radiochromatogram scanner with an NRD precision ratemeter and a Packard Tricarb liquid scintillation spectrometer were used.

Methods

Determination of Gibberellic Acid and of Gibberellin A_1 in Broths

Sample Preparation. Small volumes, 1 to 5 ml., of the broth samples were extracted three times at a pH of 2.5 with 10-ml. portions of ethyl acetate. The washed extracts were combined, and the volumes were adjusted to contain an estimated 0.01 to 0.1 mg. of gibberellin per ml. of solution. Samples, 1-ml., were then transferred to the 5-ml. round reaction flasks. The solvent was removed under reduced pressure and the residues were redissolved in 1 ml. of ethyl alcohol.

Reaction with Diazomethane-C^{14}. An ether solution of diazomethane-C^{14} was prepared from an amount of the intermediate nitrosomethyl-C^{14}-sulfonamide (specific activity, 1 μc. per mg.) equivalent to 0.5 mg. per sample. Volume equivalents of the chilled solution were then pipetted into the various sample flasks. After standing in ice water for a few minutes, the excess reagent and the solvent were removed under reduced pressure. Excess activity was trapped by reaction with an organic acid.

Parallel with each series of test samples, one to three solutions of known composition were treated identically for calibration purposes.

Chromatographic Separation. The labeled sample residues were applied to Whatman No. I paper strips. The strips were developed overnight by descending flow chromatography with the upper phase of a mixture of water, methanol, petroleum, petroleum ether, and toluene (7:3:10:20 volume ratios). The lower phase of this mixture was used to saturate the atmosphere in the cabinet.

Assay. The dried strips were scanned at a speed of 0.75 inch per minute—time constant selection at 5% error—and the counting rates were recorded. The areas under the various peaks were measured with a planimeter and compared with the areas of the standard samples. These planimetric data were usually precise enough for the routine evaluation of broth samples. If higher accuracy was desired, the spots were extracted in the presence of unlabeled ester. Final specimens could then be isolated and assayed by liquid scintillation counting. As the specific activity of the reagent was known, the results were not influenced by the purity of the standard samples.

Results. Figure 1 shows the position of the various peaks in a typical radiochromatogram. The distance traveled by the methyl-C^{14} ester of gibberellic acid was assigned the R_{GA} value of 1.00, the locations of the other spots were expressed correspondingly.

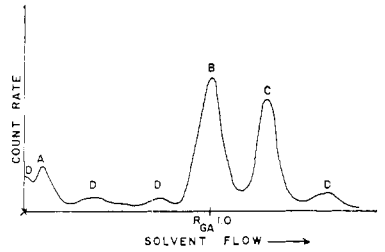

Figure 1. Radiochromatogram of a typical broth sample

X. Application point
A. Gibberellin A_1 methyl-C^{14} ester
B. Gibberellic acid methyl-C^{14}
C. Gibberellin A_1 methyl-C^{14} ester
D. Unknowns

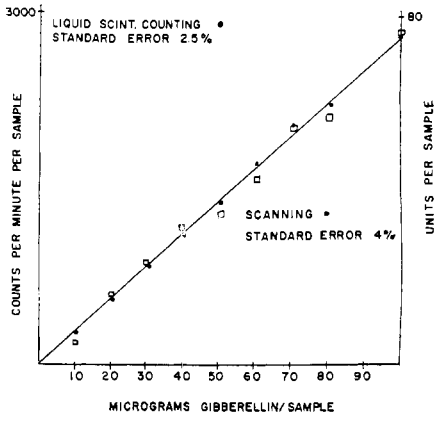

Figure 2. Standard curves for known amounts of gibberellins labeled, chromatographed, and assayed

Table II. Analysis of Synthetic Mixtures of Gibberellic Acid and Gibberellin A_1 by Conversion to the Methyl-C^{14} Esters

Ratio of Gibberellic Acid/ Gibberellin A_1	*Ratio Found by Analysis*
90/10	90/10
70/30	72/28
50/50	52/48
20/80	25/75
10/90	11/89

Table I summarizes the results with samples of known composition using the same batch of nitrosomethyl-C^{14}-sulfonamide. These data were accumulated over a period of several weeks and are indicative of the accuracy of the method, the daily variations, and the day to day variations. The data on reagent yield were determined by adding a standard volume of the reagent solution to a solution containing an excess of gibberellic acid.

Tables II to IV and Figure 2 present further data on this procedure and comparative data with other assay procedures.

Determination of Gibberellins in Process Liquors

Preparation of Tritium - Labeled Gibberellic Acid. Isomerically pure gibberellic acid, 1.6 grams, was exposed to 5 curies of tritium gas at a pressure of 420 mm. of mercury for 20 days. The spent gas was pumped off, and the acid was dissolved repeatedly in a mixture of ethyl alcohol and acetic acid to remove labile tritium. The material was then chromatographed over 150 grams of acid-washed aluminum oxide. The column was washed first with ethyl acetate and then with ethyl acetate containing 5% acetic acid. The major fractions were combined and subjected to a pH high of 8.0 and a pH low of 2.0 to complete the back exchange. The product was recovered by extraction with ethyl acetate and chromatographed again as outlined above. Final crystallization of the major fractions yielded 300 mg. of pure tritium-labeled gibberellic acid (specific activity 108 ± 5 μc. per mg.). What appears to be an isotope effect in the chromatography of the methyl-C^{14} esters of the tritium-labeled gibberellins will be outlined in the discussion part.

Isolation Yields Determination in Pilot Plant Studies. Tritium-labeled gibberellic acid (5.50 mg., specific activity 108 μc. per mg.) was added to 10 gallons of fermentation broth to be processed in pilot plant equipment. During the entire operation, all the solvent volumes and the adsorbent weights were recorded accurately, and representative samples were collected after each step during the procedures. Specimens, 1 ml., of all the collected test samples were assayed by liquid scintillation counting applying the method of internal standardization. The scintillation solution consisted of 25 ml. of a mixture of toluene (1200 ml.), ethyl alcohol (700 ml.), and diphenyloxazole (8 grams).

Results. As only one actual isolation was required for determining the specific activity of the final product, and as the counting was done in an automatic sample changer, very little manual work was necessary in obtaining complete data on each individual step over the entire process.

Table I. Diazomethane-C^{14} Yields and Standard Values

Nitroso-Intermediate Used, Mg.	*Adjusted Volume of CH_2N_2-C^{14} Ether Solution, Ml.*	*Volume Equiv. Used per Sample, Ml.*	*Gibberellin Labeled in Excess Sample, γ*	*CH_2N_2-C^{14} Yield, %*	*Surface Area per Standard Arbitrary Units[a]*
23.2	25	1	177	12	0.69 ± 0.05 (3)
12.5	25	2	500	31	0.65 ± 0.01 (3)
6.2	20	1	Not determined	..	0.63 ± 0.03 (3)
6.9	25	2	335	38	0.69 ± 0.03 (3)
5.0	25	2	260	40	0.70 ± 0.04 (3)
6.0	10	1	280	29	0.74 ± 0.05 (2)
6.1	25	2	360	51	0.74 ± 0.05 (6)
11.6	25	1	296	39	0.72 ± 0.03 (2)
12.0	25	1	254	33	0.74 ± 0.01 (2)
12.0	25	1	215	29	0.69 ± 0.05 (2)
11.5	25	1	226	33	0.68 ± 0.05 (6)
12.9	25	1	232	31	0.68 ± 0.07 (11)
12.5	25	1	210	29	0.68 ± 0.04 (2)
				Average	0.69 ± 0.04 (13 series)

[a] Number in parenthesis indicates the number of standard samples varying in weight between 10 and 100 γ. All values determined by scanning at 0.75 inch per minute, time constant at 5% error.

Table III. Comparison of Data Obtained by Fluorometric Analysis of Broth Samples with the Data Obtained by Conversion to the Methyl-C^{14} Esters (Scanning)

Sample No.	*Analysis by the Carbon-14 Method: Ratio gibberellic acid/ gibberellin A_1*	*Analysis by the Carbon-14 Method: γ Gibberellic acid/ ml. broth*	*Fluorometric Analysis, γ Gibberellic Acid/ Ml. Brath*
1	88/12	185	218
2	90/10	139	159
3	86/14	164	168
4	85/15	220	244
5	88/12	80	80
6	89/11	171	198
7	88/12	259	210
8	86/14	188	161
9	94/6	96	99
10	93/7	169	175
		Average deviation ±12%	

Table IV. Comparison of Data Obtained by the Carbon-14 Method on Broth Samples with the Data Obtained by Infrared Analysis of the Isolated Products

Sample No.	*Ratio of Gibberellic Acid/Gibberellin A_1 Found: Carbon-14 analysis*	*Ratio of Gibberellic Acid/Gibberellin A_1 Found: Infrared analysis[a]*
1	24/76	25/75
2	38/56	41/59
3	94/6	95/trace
4	80/20;79/21	77/23
5	82/18;81/19	78/22
6	87/13;80/20	87/13
7	50/50	44/56

[a] IR data corrected to $A_1 + A_3 = 100\%$.

Gibberellic Acid Determination by Double Isotopic Dilution Analysis

Test Determinations. The purification and separation steps used in these test determinations are the same operations used later for determining the concentration of gibberellic acid in plant tissues. Three solutions containing 0.10 mg. of gibberellic acid were prepared. To each solution, a small but accurately known amount of tritium-labeled gibberellic acid was added, and the mixtures were chromatographed over 15 grams of aluminum oxide (Merck, acid-washed). Each column was washed first with ethyl acetate and then with a mixture of ethyl acetate and 5 volume % acetic acid. The effluent was collected in 10-ml. fractions of which 0.1-ml. portions were assayed by liquid scintillation counting. The fractions around the radioactivity peak value were combined, and the solvents were evaporated under reduced pressure. The thus purified samples were then reacted with an excess of diazomethane-C^{14}. For further purification, the double-labeled esters were chromatographed on paper strips as outlined earlier. After location of the spots by scanning, the strips were cut, and the segments containing the tritium activity were immersed in an alcoholic solution of 200 mg. of unlabeled methyl gibberellate. Solid samples could then be isolated and purified by repeated crystallization until the carbon-14–to-tritium ratio remained constant.

Assay Procedure. Portions, 5- to 10-mg., of the various specimens were dissolved in 2 ml. of absolute methanol, and 10-ml. portions of toluene containing 0.3% of diphenyloxazole were added. The sample vials were then counted in a liquid scintillation counter using pulse height analysis as described by Okita (*2*). The carbon-14–to-tritium activity ratios of the test samples were then compared with the ratio in an undiluted standard sample of gibberellic acid-H^3 methyl-C^{14} ester.

Calculations. The following equation was used to compute the concentration of gibberellic acid in the original sample:

$$\text{Mg. gibberellic acid} = (r/P - 1) \times A \text{ mg.}$$

where:

r represents the ratio $\frac{C^{14}\text{ c.p.m.}}{H^3\text{ c.p.m.}}$ of the test sample

and P represents the ratio $\frac{C^{14}\text{ c.p.m.}}{H^3\text{ c.p.m.}}$ of the standard sample

and A represents the amount of tritium-labeled gibberellic acid (mg.) added to each sample.

Results and Application. The results of the above three test determinations are summarized in Table V.

Table V. Gibberellic Acid Determination by Double Isotopic Dilution Analysis

Gibberellic Acid in Sample Solution, Mg.	Gibberellic Acid-H^3 Added, Mg.	Carbon-14, C.P.M./Sample	Tritium, C.P.M./Sample	Carbon-14–Tritium Ratio	Gibberellic Acid Found, Mg.	Expected Error, %
nil	0.1	10 890	80 506	0.135(P)	Standard	
0.100	0.104	16 820	61 555	0.273	0.106	7.9%
0.100	0.021	15 800	19 866	0.795	0.102	4.8%
0.100	0.010	22 700	15 800	1.437	0.096	4.4%

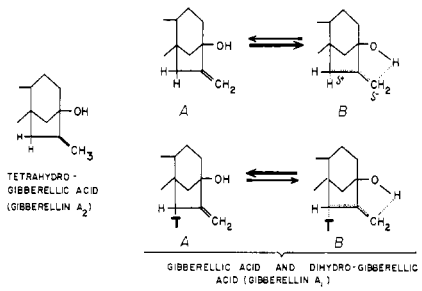

Figure 3. Postulated effect of tritium labeling upon hyperconjugation and R_{GA} values

This procedure was then applied to the determination of residues of gibberellic acid in various plants treated with this growth stimulant.

Discussion

Scope and Accuracy of the Various Procedures

Determination by Reaction with Diazomethane-C^{14}. The sensitivity of this method is determined by the specific activity of the labeling reagent and by the concentration of interfering acidic substances. For routine application, a gibberellin concentration of 0.01 to 0.1 mg. per sample is desirable to minimize the errors. Extraction and subsequent liquid scintillation counting in place of scanning will increase both sensitivity and accuracy. However, for the routine screening of a large number of broth samples, emphasis was on time economy, and a 10% error in the isomeric ratio was acceptable.

Isotopic Dilution Analysis with Tritium-Labeled Gibberellic Acid. The precision in this procedure is defined by the sum of the statistical errors in the counting operations and by the isotopic dilution ratio, R:

$$\text{Relative error } \% = (\text{sum of the statistical and the gravimetric errors}) \times \frac{R}{R-1}$$

Using an adequately high dilution ratio and a minimum of 10^4 counts per sample, the errors actually are a function of the accuracy of the pipets. For this reason, all counting vials were usually prepared in duplicate using different pipets for each vial. If the vial activity

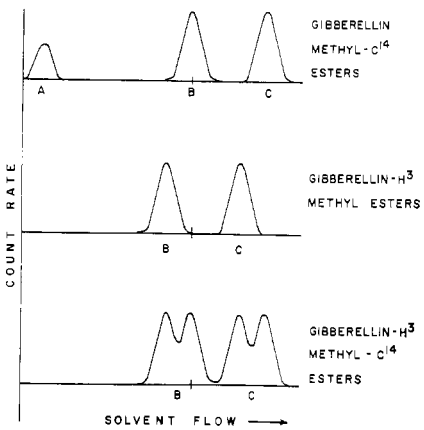

Figure 4. Effect of tritium substitution upon mobility of the esters

A. Tetrohydro gibberellic acid (gibberellin A_1) methyl ester
B. Gibberellic acid methyl ester
C. Dihydro gibberellic acid (gibberellin A_1) methyl ester

varied by more than 3%, an additional vial was prepared.

Determination of Gibberellic Acid by Double Isotopic Dilution. This procedure is capable of high sensitivity and high accuracy, especially as material losses are accounted for in the isotopic procedure. The main criteria are the specific activity of the labeling reagent and the degree to which the samples could be purified prior to the reaction with the labeling reagent. Insufficient purification will increase the strip background upon scanning and will make localization of the spots, and purification of the final specimens, difficult. The over-all error is defined by:

$$\text{Total error } \% = (\text{added errors in measurements}) \times \frac{r}{r-P}$$

where r represents the carbon-14–to-tritium activities ratio in the sample, and P represents the carbon-14–to-tritium activities ratio in the standard sample.

Isotope Effect. Upon chromatography of the methyl-C^{14} esters of tritium-labeled gibberellic acid and of tritium-labeled gibberellin A_1, a slight displacement between the tritium and the carbon-14 peaks was observed. As the hydrogen-tritium exchange operating in the labeling reaction produces ran-

domly labeled materials, and as it is usually accompanied by material degradation, the possibility existed that the tritium-labeled materials were degradation or decomposition products.

Small quantities of the tritium-labeled materials were then reacted with an excess of diazomethane-C^{14}, and the resulting double-labeled products were subjected to various methods of purification:

Addition of trace amounts of the double-labeled material to a large excess of unlabeled ester and repeated crystallization.

Paper chromatography of the double-labeled material, extraction of the spots, addition of unlabeled ester in large excess, followed by repeated crystallization.

After each operation, the carbon-14-to-tritium ratio was determined. This value remained constant within the limits of error. The entire procedure was then repeated by reacting mixtures of the tritium-labeled material and of unlabeled material in various ratios with diazomethane-C^{14}. Again, subsequent fractionation of the carrier diluted specimens did not alter the expected ratios of the samples.

Finally, the tritium-labeled materials were applied to plants and were biochemically nondistinguishable from the unlabeled materials as judged by concentration time and translocation data (*7*). Therefore, the slight difference in R_{GA} values is assumed to be caused by an isotope effect based upon a hyperconjugation phenomenon (Figure 3). Substitution of the alpha-hydrogens with tritium reduces the contribution of *B*, thereby enancing the activity of the hydroxyl hydrogen. As a result, the tritium-labeled materials should exhibit a somewhat more hydrophilic behavior than the unlabeled compounds. Consequently, the R_{GA} values of the tritium-labeled compounds should be in between the R_{GA} values of the unlabeled compounds and the R_{GA} value of gibberellin A_2 (tetrahydrogibberellic acid, structure *C*). This is observed and shown in Figure 4.

On the practical side, this isotope effect could be used for the preparation of small quantities of tritium-labeled esters of very high specific activities.

Acknowledgment

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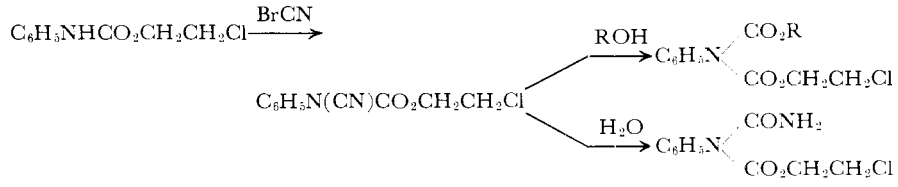
HERBICIDAL ACTIVITY AND STRUCTURE

Comparative Herbicidal Activities of Carbamates and *N*-Substituted Derivatives

DAVID STEFANYE and H. R. DeROSE
Biological Warfare Laboratories, Fort Detrick, Frederick, Md.

Comparative tests between isopropyl carbamates and their *N*-substituted derivatives on oat plants indicate that a hydrogen atom is not necessary on the nitrogen to produce strong phytotoxic effects. Substitution with other groups alters, but does not destroy the toxicity. Relatively greater carbamate activity occurs if the phenyl ring contains a halogen substituent in the meta position. Phytotoxicity data on a series of these compounds are presented.

The exploratory work of Templeman and Sexton (*8*) on the herbicidal activity of isopropyl carbamates (IPC) dwelt briefly with the replacement of the amide hydrogen by other functional groups. Thus, the growth-regulating properties of ethyl phenylimidodicarboxylate, which may be regarded as an *N*-substituted carbamate, were mentioned, but no conclusions were drawn as to its activity relative to other carbamates. As an extension of this work the synthesis of a homologous series of halogenated isopropyl phenylimidocarboxylates was recently described (*7*). The amide hydrogen of the carbamate had been replaced by cyano and carbamyl groups during an unsuccessful attempt to synthesize alkyl phenylimidodicarboxylates by the following reactions:



The investigations reported herein were carried out to obtain phytotoxicity data on this series of carbamates and their corresponding isopropyl phenylimidodicarboxylates, which they closely resemble structurally. It was further the purpose of this study to correlate chemical structure with the effectiveness of the two types of compounds.

Methods and Materials

Ennis (*2*) described the effect of isopropyl carbamate on oats, barley, and other grasses. He demonstrated greater activity when the compound