Determination of Endogenous Gibberellins in Green Malt by Isotopic, Derivative Dilution Procedures

LAVERA LAZER and WALTER E. BAUMGARTNER[1]
Physical Chemistry Department, Research and Development Division, Abbott Laboratories, North Chicago, Ill.
ROBERT V. DAHLSTROM
Research Laboratory, Rahr Malting Co., Manitowoc, Wis.

The concentration of endogenous gibberellic acid in sprouting barley was determined by isotopic, derivative dilution procedures. Larger than expected variations in the results suggested that at this particular time of plant development endogenous gibberellic acid occurs predominantly in a bound form. The unidentified complex, derivative, or precursor apparently was broken up by a mild acid treatment to yield gibberellic acid at a concentration which was dependent upon extraction conditions. This finding raises questions as to the biochemically active structure of the gibberellins.

EXTRANEOUS GIBBERELLIC ACID accelerates the germination of barley seeds, and the resulting green malt has an increased enzyme content (*2*). However, very little is known about the biochemical mechanism of this growth-stimulating action. The extremely low, effective concentrations, coupled with the relatively poor chemical stability of the gibberellins, have made detailed studies difficult. Of the various analytical methods, only fluorometric assay (*6*) and bioassay (*4*) are sensitive enough to operate at natural levels. However, neither method lends itself readily to distinguish between the various gibberellins or gibberellin-like substances, or to quantitative work within the part per billion range.

An attempt was, therefore, made to gain a better understanding on the action of these plant hormones by means of isotopic derivative labeling techniques. Using labeled reagents of high specific activity, in combination with chromatographic methods, these techniques can bring phenomena into a measurable range which would otherwise be difficult to observe.

Isotopic Procedures and Reagents

A general outline of the isotopic procedures has been given (*1*). In order to minimize the effect of statistical errors in counting upon the result, the amount of labeled gibberellic acid added should be smaller than the expected amount of endogenous material. The unavoidable error, resulting from the added statistical errors at a minimum of 10,000 counts per sample and per channel, is summarized in Table I.

The tritium-labeled gibberellic acid used throughout this study had a specific activity of 144 μc. per mmole, the C^{14}-labeled diazomethane a specific activity of 6.68 μc. per mmole. Both materials were prepared as outlined (*1*). As the counting efficiency for C^{14} is considerably higher than the counting efficiency for tritium, this ratio of specific activities resulted in a satisfactory ratio in the observed counting rates for a two-channel analyzer. Approximate values for the counting efficiencies of the two isotopes are given in Table II.

[1] Present address, Grand Central Rocket Co., Redlands, Calif.

Handling of Green Malt

Extraction of Samples. The barley variety used throughout this study was L. Kindred. It was allowed to germinate for 5 days, and the resulting green malt was sampled for analysis prior to the usual kilning operation. For each analysis, amounts varying from 2.5 to 10 kg. were extracted in a Waring Blendor with aqueous acetone (33% water v./v.) at a pH of 2.5. The labeled gibberellic acid was added with the solvent. The resulting slurries were filtered, and the filtrates concentrated under reduced pressure at 35° C. (pH 6.0). The concentrates were extracted exhaustively with ethyl acetate (pH 2.5). Assay of volume equivalents at this point indicated an average material recovery of approximately 65%, varying somewhat with sample weight.

Purification of Extracts. Using the tritium activity of the samples as a guide, and the tritium activity of the standard samples as a reference, the extracts were purified first by column chromatography on aluminum oxide (*1*), then by adsorption on activated carbon (*7*), followed by a second chromatographic separation on aluminum oxide. Peak fractions of this latter chromatogram were chromatographed individually on paper in a butyl alcohol-ammonia system (*5*). The strips were scanned with a windowless gas flow counter, and sections carrying the gibberellin-bound tritium peaks were extracted.

Table I. Effect of Weight of Added, Labeled-Gibberellic Acid upon Accuracy

(Acid added, 5.0 μg./1000 grams)

Endogenous Gibberellic Acid, Expected Concn., P.P.B.	Expected Unavoidable Error,[a] ±%
0.25	100
1.0	30
10	7.5
100	5.2

[a] At a statistical accuracy of ±1% for each activity determination.

Table II. Per Cent Counting Efficiencies[a]

Element	Lower Channel, 10 to 30 Volts	Upper Channel, 50 to ∞ Volts
C^{14}	12.7	47.1
Tritium	8.83	0.42

[a] Packard liquid scintillation spectrometer, operated at 1060 volts with split channels; data taken from third analysis.

Radioisotope Assay

Reaction with Diazomethane-C^{14}. Solutions, 1 ml. each, of the samples in ethyl alcohol were reacted with an excess of diazomethane-C^{14} in ether solution, and the products were chromatographed on paper (*1*). The dried strips were scanned with and without window to locate the various C^{14} and tritium activity peaks. The papers were then cut accordingly, and the segments were extracted in the presence of 300 mg. of the corresponding unlabeled esters. The carrier-diluted samples were recrystallized in ethyl acetate–petroleum ether

until the C^{14} to tritium ratio leveled out at a constant value. Between each crystallization, the solutions were treated with Norite A to remove trace amounts of labeled impurities.

Preliminary studies had shown that this latter treatment removes trace amounts of labeled gibberellic acid methyl-C^{14} ester from a sample of gibberellin-A_1 methyl ester and vice versa, making it a sensitive method of identification.

Sample Assay

The final samples were assayed by liquid scintillation counting [1 ml. of ethyl alcohol, 10 ml. of toluene containing 0.3% of 2,5-diphenyloxazole (DPO), and 0.01% of 1,4-bis-2 (5-phenyloxazole) benzene (POPOP)] at a discriminator setting as outlined above. Since an automatic sample changer (Packard Tri-Carb) was used, each series of samples and standards could be counted several times, minimizing minor instrument fluctuations.

The concentration of the endogenous gibberellic acid was calculated as follows: μg. gibberellic acid = $\left(\frac{R}{r} - 1\right)$ $\times$ μg. gibberellic acid-H^3 added, where R represents the C^{14} to tritium ratio of the analytical sample, and r represents the C^{14} to tritium ratio of the standard sample. Since only ratios are involved, neither the weight of the assay specimens nor the exact value of the specific activity of the reagents had to be known.

Experimental Results

The results for the concentration of endogenous gibberellic acid as obtained in a first and a second analysis are summarized in Table III. They showed a larger than expected spread, even considering possible sample variations. There was no indication of the presence of any measurable amount of gibberellin A_1 in either sample. On the other hand, radiochromatograms of the two samples showed a radiopeak at R_{ga} 1.25 (Figure 1), which is the R_{ga} value usually observed for gibberellin A_1. (R_{ga}, the distance traveled by the methyl-C^{14} ester of gibberellic acid, was assigned the R_{ga} value of 1.00; the location of the other spots was expressed correspondingly.) The C^{14} activity contained within this area, however, was lost upon dilution with carrier gibberellin A_1 methyl ester. There was a marked difference between the radiochromatograms at R_{ga} 0.65 and R_{ga} 2.33.

Both samples had been treated identically, except for the time element. In the first analysis, a larger sample was used and difficulties were encountered during filtration of the initial extracts. As a result, the materials had been exposed to an acidic pH for a longer time, and the idea occurred that gradual hydrolysis of a natural derivative, or precursor, might have influenced the results.

Consequently, a third analysis was carried out to test this possibility. In this third analysis, one half of the material was extracted in blendors, and the slurry was allowed to stand at room temperature (pH 2.5) for 24 hours. The other half was extracted, and the materials were processed in 8 hours.

The results for this third pair of analyses are given in Table III (III-a and -b). They indicated that the time factor indeed markedly influences results. Corresponding radiochromatograms are shown in Figure 1 (*C* and *D*). The sample with a shorter exposure to an acidic pH (Sample III-b) showed a marked radiopeak at R_{ga} 0.65, but it contained, according to the analysis, little or no gibberellic acid. The radiopeaks at R_{ga} 1.25 and R_{ga} 2.33 were absent. On the other hand, the sample which had been allowed to stand at an acidic pH (Sample III-a) showed a high concentration of gibberellic acid, but little or no radioactivity within the R_{ga} 0.65 range.

Neither sample contained measurable amounts of gibberellin-A_1.

Discussion

In this particular method of analysis, the various unavoidable errors are more likely to influence the results in the direction of high rather than low values. Incomplete removal of foreign acids will increase the C^{14} to tritium ratio in favor of high results. However, both the chromatographic procedure in the separation of esters and the subsequent carrier dilution and macroscopic purification are believed to be specific, making interference by foreign acids an unlikely source of error. High values could also be obtained by a partial elimination of the tritium label from the added, labeled gibberellic acid under the influence of an acidic pH. Such a possibility was ruled out by numerous stability tests. Especially, at an acidic pH, the molecule itself was found to be less stable than the label. On the other hand, partial degradation of the added, labeled gibberellic acid could cause an error in the direction of high values, if endogenous material is present in a bound form. This error, however, would be relatively small since at a pH of 2.5, gibberellic acid is degraded at an approximate rate of only 10% per day.

The only likely source of error resulting in low values is an incomplete equilibration between the added, labeled gibberellin and the endogenous tissue-bound material. As all the extractions were carried out under identical conditions yielding finely divided suspensions, this factor can be ruled out as a possible source of major error.

The authors are inclined to attribute the relatively large differences in results to a cause other than analytical error. Thus, the appearance of a pronounced radiopeak at R_{ga} 0.65 in the radiochromatograms of extracts yielding relatively low values for gibberellic acid, seems to indicate a chemical cause, such as the existence of a precursor or natural derivative of gibberellic acid yielding gradually, upon acid treatment, gibberellin in a free form. It remains to be determined whether or not one is

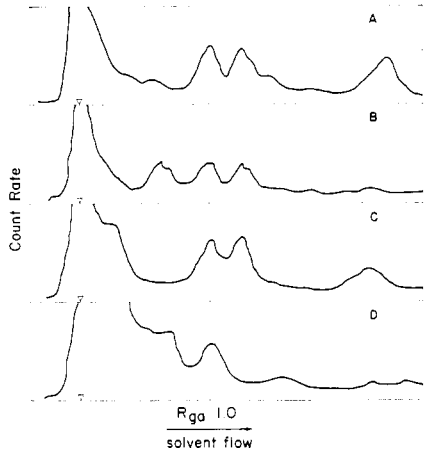

Figure 1. Radiochromatograms of the gibberellin carbon-14 esters

A. First determination
B. Second determination
C. Third determination, *a*
D. Third determination, *b*

Table III. Results of Determination of Endogenous Gibberellic Acid in Green Malt

Analysis	Sample Wt., Grams	Moisture, %	Labeled Material Added, μg.	C^{14}/H^3 Standard, r	C^{14}/H^3 Sample, R	$\frac{R}{r} - 1$	Endogenous Gibberellic Acid	
							μg.	P.p.b.[a]
I	10,000	44.8	51.0	0.235[b]	0.640	1.74	88.7	16
II	5,000	49.0	25.5	0.279[b]	0.440	0.58	14.8	6
III-a	2,500	36.6	17.0	0.259[c]	1.346	4.196	71.3	45
III-b	2,500	36.6	17.0	0.259[c]	0.263	0.015	0.3	less than 1

[a] Values based on dry weight.
[b] Samples assayed at different times and slightly different discriminator voltages.
[c] Calculated value, based upon specific activity of reagents and actual counting efficiencies of 0.249.

dealing with a pH-sensitive enzymatic reaction, or simply an acid effect.

This observation also raises some question as to the biochemically active form of the gibberellins. The fact that barley responds to treatment with extraneous gibberellic acid would indicate that this compound, or a derivative, constitutes part of the growth regulatory mechanism during this stage of plant development. On the other hand, the absence of free endogenous gibberellic acid, as observed under mild extraction conditions, favors a derivative, or precursor, as the biochemically active component. This view is supported by the appearance of the marked radiopeak at R_{ga} 0.65 in Sample III-b which disappears upon exposure to an acidic pH (Sample III-a) for 24 hours. Additional data will be needed to resolve this question.

In another study (*3*) various plants were treated with tritium-labeled gibberellic acid, and were analyzed for residual gibberellic acid after the end of the growth period. The results obtained there indicated a higher than expected stability of the tissue-bound gibberellic acid, suggesting the possibility of stabilization by complex formation. On the other hand, in most samples relatively large amounts of residual tritium activity were associated with materials other than gibberellic acid, which at that time were considered to be decomposition products. This would have to be re-examined in the light of the existence of possible conjugated forms of gibberellic acid.

Acknowledgment

The authors thank Ann M. Dalziel for preparing all the samples with C^{14}-labeled diazomethane. Thanks are also due to Edward G. Martin, Rahr Malting Co., for his able assistance in the initial extraction work.

Literature Cited

(1) Baumgartner, W. E., Lazer, L. S., Dalziel, A. M., Cardinal, E. V., Varner, E. L., J. AGR. FOOD CHEM. **7**, 422–5 (1959).
(2) Bowden, R. F., Dahlstrom, R. V., Sfat, M. R., *Am. Soc. Brewing Chemists Proc.* **1959**, 137–41.
(3) Lazer, L. S., Dalziel, A. M., Baumgartner, W. E., Dahlstrom, R. V., Morton, B. J., *Advances in Chem. Ser.*, in press.
(4) Phinney, B. O., *Proc. Natl. Acad. Sci.* **42**, 185–9 (1956).
(5) Phinney, B. O., West, C. B., Ritzel, M., Neely, P. N., *Ibid.*, **43**, 398–404 (1957).
(6) Theriault, R. J., Friedland, W. C., Peterson, M. H., Sylvester, J. C., J. AGR. FOOD CHEM. **9**, 21 (1961).
(7) West, C. A., Phinney, B. O., *J. Am. Chem. Soc.* **81**, 2424–7 (1959).

Received for review April 4, 1960. Accepted November 3, 1960.

FERTILIZER-PESTICIDE MIXTURES

Homogeneity of Fertilizer-Pesticide Mixtures

ROBERT M. CREAMER

Fertilizer Investigations Research Branch, Soil and Water Conservation Research Division, U. S. Department of Agriculture, Beltsville, Md.

The use of fertilizer-pesticide mixtures has grown rapidly. Because mixtures prepared in the laboratory were relatively nonuniform, factors influencing their uniformity were evaluated. The mixtures were more uniform if insecticide were added as a solution than if added on a solid carrier. Mixing during addition of solution and use of closely sized fertilizer increased uniformity. The insecticide solution was preferentially adsorbed by the fine particles and by the phosphatic portions of a mixed fertilizer. The variation in insecticide content of random samples was large enough to cause poor analytical precision. Concentration of the toxicant on the fine portions of the fertilizer contributes to nonuniformity and forms toxic dusts. Preferential adsorption of toxicant by certain portions of a mixed fertilizer may accelerate decomposition of the toxicant.

FERTILIZER-PESTICIDE MIXTURES, introduced about 1950, have rapidly attained importance in agricultural practice. Their use was made possible by the development of toxic, chlorinated hydrocarbon insecticides that are stable in the soil and reasonably stable in mixtures with fertilizers. The latest data available indicate that 109,956 tons, equivalent to 0.74% of all mixed fertilizers, were used in the United States in the year ending June 30, 1956 (*6*).

Two general methods are used to formulate fertilizer-pesticide mixtures. Either solid carriers containing the insecticide or solutions of insecticide are incorporated into well cured fertilizers. Carriers provide a simple method of producing fertilizer-pesticide mixtures, because the mixtures can be formulated in conventional fertilizer-mixing equipment. Solution addition of toxicant can cause less dilution of the plant nutrient content of the fertilizer, and is generally recommended for introduction of toxicant into granular fertilizers.

Most suggested methods of preparation of the mixtures (*3*, *7*) are based primarily on plant-scale experience.

This paper presents data on the uniformity of mixing of toxicant with fertilizers by the above two methods, and the relative distribution of pesticides among the ingredients of mixed fertilizers.

Materials and Methods of Analysis

The chemical and screen analyses of the fertilizer materials used in this study are listed in Table I. With the exception of the 5–20–20 fertilizer, all were commercial products. The 5–10–5 mixture was a well cured, nongranular material. The granular 5–20–20 was an experimental product formulated from ordinary and triple superphosphates, potassium chloride, and an ammonia–ammonium nitrate solution.

The insecticides were undiluted technical grade products. Laboratory reagent quality benzene and acetone were used as solvents.

Carrier-insecticide concentrates (25%