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GIBBERELLIN–PHENOL INTERACTIONS IN PLANT EXTRACTS

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Abstract—Radioactive materials, with low mobilities on PC, occurred in extracts of barley that had been dosed with gibberellic acid ([^{14}C]GA_3) before germination. The radioactive areas coincided with a coloured, phenol-containing zone on PC. The [^{14}C]GA_3 and phenolic components were not separated by a variety of chromatographic procedures unless an 'acid treatment' was used. At least part of the material recovered following 'acid treatment' was free GA_3. It is proposed that the materials with low mobilities were GA_3–phenol complexes of unknown nature. Similar products were formed *in vitro* between [^{14}C]GA_3 and various phenols. Complex formation was greater when [^{14}C]GA_3 reacted with a mixture of phenolics. The formation of GA_3–phenol complexes may explain a number of observations in the literature in connection with the extraction, separation and bioassay of gibberellins in plant extracts, the study of gibberellin binding sites, and the growth regulating properties of phenols.

INTRODUCTION

Gibberellins regulate the mobilization of nutrient reserves in germinating barley. There is disagreement as to whether GA_1 or GA_3 (gibberellic acid) is the more important in the initial stages of germination [1–6], when gibberellin is produced from stored precursor(s) such as *ent*-kaurene [2, 3, 5, 7]. In the later stages of germination gibberellins are produced from mevalonic acid. Wheat grains contain a 'bound' form of a gibberellin-like hormone [8] which is hydrolysed during germination to release 'free' gibberellin. The 'bound' gibberellins found in dicotyledonous plants have been identified as glucosyl esters and glycosyl ethers [9]. No such 'bound' gibberellins have been detected in ungerminated barley [1, 10], but isolated aleurone layers supplied with [^{3}H]GA_1 reportedly produce radioactive GA_1-glucoside [11], and other biologically inactive metabolites, i.e. GA_8 and its glucoside. However the major product, which had amphoteric properties, was not fully characterized [12].

In germinating grain the levels of biologically active 'gibberellin-like substances' rise and subsequently fall [1] indicating that inactivation mechanisms operate in whole grains. Extracts from barley, germinated in the presence of exogenous GA_3, contained enhanced levels of biologically active 'gibberellin-like substances' following incubation with a mixture of hydrolytic enzymes [13], suggesting that excess gibberellins may have been converted into biologically inactive enzyme-hydrolysable conjugates. The hypothesis that the reduction in the supply of active gibberellin from barley embryos, caused by increased availability of carbohydrates [1, 3, 14], might be due to the conversion of gibberellins into biologically inactive glycosides or other conjugates, was examined.

The amounts of biologically active hormone which were recovered from the surfaces of malting barley grains, treated with exogenous GA_3, declined to zero over a period of 2 days [15]. Microbes might have caused this loss of activity, but organisms isolated from the grains did not metabolize GA_3 [Brookes, P. A., personal communication]. In addition the chromatographic properties of the biologically active gibberellin, recovered from the surface of decorticated, sterilized grain, initially dosed with GA_3 changed with increasing germination time [13]. Thus alterations to applied GA_3 do not only occur internally in grains. During the present study it became apparent that [^{14}C]GA_3 became associated with polyphenols, an important finding because it has been shown previously that some phenols inhibit GA-induced responses, but were without effect on IAA-induced responses [16]. Thus they were not unspecifically toxic. In addition some phenols are apparently able to prevent the inhibitory effects of ABA [17], a result that might be due to the formation of phenol–ABA adducts. It has long been suspected that polyphenols may serve as growth regulators in plants. Due to their widespread occurrence they are probably generally important in confusing the results of experiments involving the extraction and bioassay of gibberellins or perhaps ABA.

RESULTS

Initial experiments

Barley, sprayed with a solution of gibberellic acid, ([^{14}C]GA_3, 35 pmol/grain), was allowed to germinate for 4 days. The grain was washed with sodium bicarbonate solution to remove unassimilated GA_3 (23–31% of the applied dose) [13], homogenized and extracted with aqueous methanol. The extract was reduced to an aqueous concentrate containing 53–

67% of the applied radioactivity. Both washings and extractives had dry matter contents that were too high to allow direct fractionation by PC so in the initial experiments preparations were acidified and extracted with ethyl acetate. Of the radioactivity in the washings 55% entered the ethyl acetate, whereas 82% of that in the extractives entered this phase. These extracts should have contained any unchanged GA_3 and many of the known products of gibberellin metabolism [18]. However, glycosides and glycoside esters would have remained in the respective aqueous phases. A radioscan of a paper chromatogram (System 1) of the ethyl acetate-soluble fraction of the extractives (Fig. 1a) showed a faster moving peak ($R_{GA_3} = 1$) which may have contained unchanged GA_3; a second peak occurred at the origin. Re-extraction of the acidified aqueous residue with *n*-butanol removed a further 4% of the total radioactivity. On PC (System 1) this fraction gave a single peak at the origin which was not a known gibberellin glycoside since these have higher mobilities R_{GA_3} (Fig. 1b) [19]. A brown colour, present in the original solutions, which was probably due to phenolic oxidation products [6, 20] was also retained at the origins of these paper chromatograms. Various chromatographic systems were employed in attempts (mostly unsuccessful) to separate the brown coloured material(s) from the radioactivity. Materials eluted from the origins of the above paper chromatograms were subjected to further PC (System 2). The brown material then migrated to produce an irregularly shaped pattern ($R_{GA_3} = 0.1$–0.8), which turned blue with the ferric ferricyanide reagent [21] and purple–brown (fringed with green) with the DMAC (dimethylaminocinnamaldehyde) reagent [22]. Thus the brown coloured material co-chromatographed

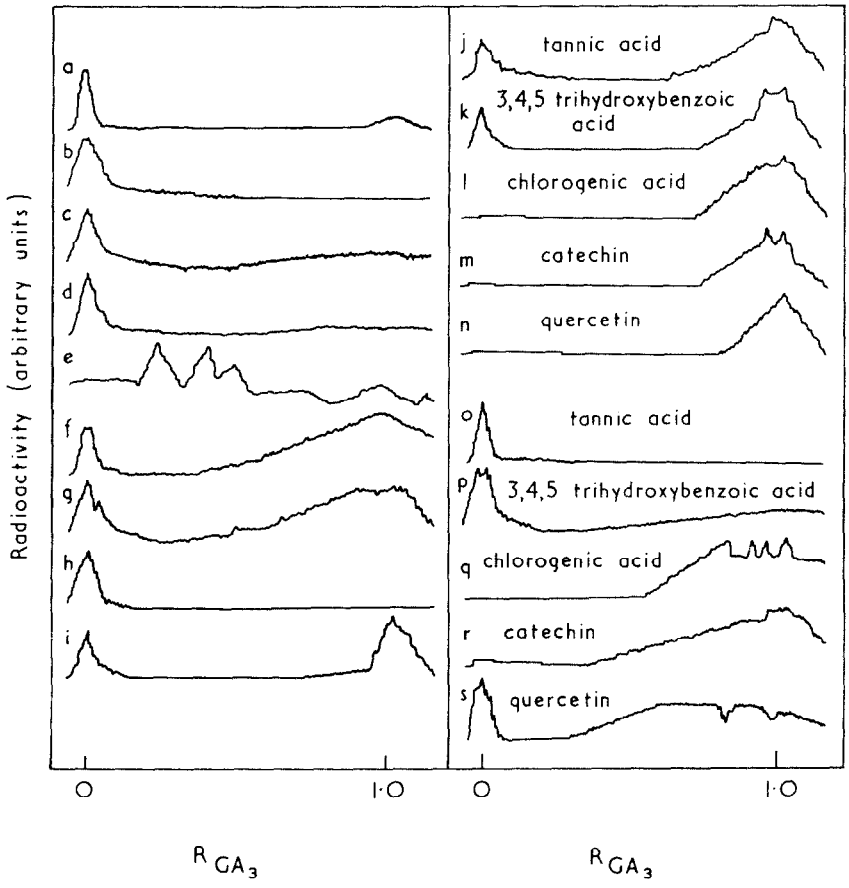

Fig. 1. Radioscans of PCs of various samples. (a) Extractives of germinated barley dosed with [^{14}C]GA_3, (EtOAc-soluble fraction; PC System 1). (b) *n*-BuOH-soluble fraction of barley extractives (PC System 1). (c) Me_2CO eluate of an Amberlite XAD-4 column loaded with $NaHCO_3$ washings (PC System 1). (d) Acidic Me_2CO eluate of Amberlite XAD-4 column loaded with $NaHCO_3$ washings (PC System 1); i.e. the fraction following that used in (c), above. (e) MeOH-soluble extractives of barley (EtOAc-soluble fraction; PC System 3). (f) Me_2CO eluate of XAD-4 column [as in (c) above] loaded on PC with unlabelled GA_3 (5 mg; PC System 1). (g) Me_2CO eluate of XAD-4 column [as in (c) above] loaded on PC with citric acid (5 mg; PC System 1). (h) Me_2CO eluate of XAD-4 column [as in (c) above; PC System 4]. (i) Standard [^{14}C]GA_3 eluted from a chromatogram run with a phenol-containing solvent (PC System 4), re-run with PC System 1. (j–n) Standard [^{14}C]GA_3 loaded with 1000 fold excess (w/w) of phenol indicated on diagram, then chromatographed (PC System 1). (o–s) [^{14}C]GA_3 loaded with the same phenols, (as in j–n), but chromatographed using PC System 4.

with phenols. Autoradiography showed that radioactivity also exactly coincided with the irregularly shaped brown areas.

The use of adsorbents

The affinities of a range of adsorbents for [^{14}C]GA_3 were tested. All the polystyrene-based resins retained some GA_3, but most (99.7%) was held by Amberlite XAD-4. The most effective eluents were methanol–cyclohexanol–water (1:1:2) and acetone (50%) which was preferred because it did not damage a chromatographic assembly.

A column of XAD-4 was used to adsorb the radioactive compounds from solutions (concentrates of washings or extractives) and so separate them from the large quantities of sugars and other unadsorbed compounds that were also present. Three fractions were collected from bicarbonate washings applied to the column: (1) an aqueous eluate (containing 37% of the radioactivity), (2) acetone (10%) eluate (61% radioactivity) and (3) an acidic acetone (10%) eluate (2% radioactivity). The aqueous eluate could not be analysed by PC due to its high dry matter content. When this was refractionated by the XAD-4 column three similar fractions, each containing a similar proportion of the applied radioactivity, were obtained. Evidently radioactivity in the aqueous eluate represented unadsorbed material. When materials from the acetone and acidic acetone eluates were analysed by PC (System 1) most of the radioactivity remained at the origin (Figs. 1c and 1d). This was unexpected as it was thought that the sodium bicarbonate washings would contain mostly unaltered GA_3. When PCs of the acidic acetone eluate were tested with DMAC reagent a purple–brown colour appeared, concentrated at the origin, while PCs of the acetone eluate were bright purple in the region $R_{GA_3} = 0$–0.4 suggesting the presence of indoles [23]. Upon electrophoresis at pH 6.5 the radioactive component(s) of the acetone eluate migrated towards the anode, relative to sucrose, (70–120 mm), while that of the acidic acetone eluate migrated towards the cathode (5–40 mm).

Chromatography in acidic solvent systems

A PC system (3) which separates plant phenols [24], was applied to the material of low mobility (PC System 1) isolated from ethyl acetate-soluble material from grain extractives (Fig. 1e). Radioactivity was located from $R_{GA_3} = 0$–1. It was not clear whether radioactivity was associated with each partially-separated class of polyphenol or if continuous slow breakdown of a GA_3-polyphenol complex(es) had occurred during chromatography. The entire PC was eluted with methanol and the eluate was rechromatographed (PC System 2) giving a single peak of radioactivity at $R_{GA_3} = 1.0$. Evidently on PC in System 3, either a [^{14}C]GA_3–phenol complex with high mobility or free [^{14}C]GA_3 itself had appeared, possibly due to the acidity. Further results consistent with an acid dependent dissociation came from PCs (System 1) of the acetone eluate from a XAD-4 column, initially loaded with sodium bicarbonate washings. When samples were mixed with either unlabelled GA_3 (Fig. 1f) or with citric acid (Fig. 1g) and were chromatographed greater proportions of the radioactivity migrated with 'free' GA_3. On the other hand acetone eluates of the XAD-4 column (loaded with sodium bicarbonate washings) subjected to PC (System 3) showed no dissociation, yet after being made up in acetic acid (50%) and incubated at 30° a double peak of radioactivity was observed on PC (System 3) at $R_{GA_3} = ca$ 1.0. Pure [^{14}C]GA_3 gave only a single peak when treated similarly. Clearly after chromatography on a XAD-4 column the materials with low mobility from grain extractives have different properties from those obtained directly from sodium bicarbonate washings. The ethyl acetate-soluble materials from acidified sodium bicarbonate washings gave a single peak at $R_{GA_3} = 1.0$ (PC System 3) confirming that most of the interaction between [^{14}C]GA_3 and (phenolic) compounds from the grain's surfaces, occurred on the XAD-4 column. The compound obtained in this ethyl acetate-soluble fraction may have been either free GA_3, or a GA_3–phenol complex with high mobility on PC. The broad peaks of radioactivity at $R_{GA_3} = 1.0$ of citric acid and GA_3 treated samples (Figs. 1f and 1g) were eluted and mixed with carrier GA_3 (to give a sample of specific activity 122.0 dpm/mg) and subjected to repeated recrystallizations. For this sample and a control ([^{14}C]GA_3 and carrier GA_3) the specific activity became constant during four recrystallizations (i.e. 98.3, 101.2, 111.5 and 109.2 dpm/mg). Thus, at least the major part of the radioactivity with mobility of $R_{GA_3} = 1.0$ was composed of uncomplexed GA_3 or material which released GA_3 on recrystallization with carrier. The formation of the GA_3 complex is evidently reversible. The acidic properties of citric acid and GA_3 may have been sufficient to dissociate the [^{14}C]GA_3 complex. Such an effect could account for the reported reversal of inhibition of GA_3 stimulated responses by the addition of further GA_3 [16]. Alternatively the addition of more GA_3 may merely saturate the phenols' binding capacity.

GA_3 association with phenolics

The coincidence of the GA_3-complex with plant phenols during PC does not establish that the complexing compounds are themselves phenolic. Therefore in model experiments, various phenolics were loaded together with aliquots of [^{14}C]GA_3 onto the origins of PCs. After development (System 1) the chromatograms were scanned for radioactivity (Figs. 1j–1m). Significant amounts of radioactivity remained at the origins for samples mixed with tannic and gallic acids. All samples loaded with phenols showed broadening of the 'free' GA_3 peak.

This experiment was repeated using a solvent system (System 4) containing phenol (intended to perturb the GA_3–phenol association). With this system greater proportions of the radioactivity remained at the origins with the chromatograms involving tannic acid, gallic acid and quercetin (Figs. 1o, 1p and 1s), and also eluates of the XAD-4 column (loaded with sodium bicarbonate washings; Fig. 1h). In the experiments in which a significant amount of radioactivity moved with $R_{GA_3} = ca$ 1, this was distributed in broad peaks (Fig. 1p–1s). Samples eluted from chromatograms, developed in System 4, were red in colour due to the polymeric products which form when phenol is exposed to air and light [25].

When samples with zero mobility were re-run in PC (System 1) they were retained at the origin. Thus the increased proportion of slow moving materials was due to a reaction taking place during chromatography in phenol. Rechromatography (System 1) of the eluate of the broad peak of [^{14}C]GA_3 previously run alone in System 4 gave two peaks of radioactivity (Fig. 1i) one of which moved with 'free' GA_3, while the other remained at the origin, with the red polymeric compounds.

A range of chromatographic solvents were used in attempts to achieve a complete separation of GA_3 from either tannic or gallic acids. To promote interaction between [^{14}C]GA_3 and the phenolic material placed with it on the origin of the PC, the chromatograph was hung in an atmosphere of ammonia for 24 hr and dried before development. Replacing phenol in system 4 with benzene (System 5), which does not readily produce oxidized polymeric products, gave two distinct peaks with [^{14}C]GA_3, loaded alone. The major peak contained free GA_3 and the minor peak, at $R_{GA_3} = 0.8$, contained 17% of the applied radioactivity. This result was not due to contamination of the GA_3 by GA_1 as a sample of [^{14}C]GA_3 ran as a single compound in a TLC system used for separating GA_1 and GA_3 [5]. The peaks were eluted from the PC and re-run (PC System 1). The minor peak was separated into three, a major peak at $R_{GA_3} = 0.84$ and others at $R_{GA} = 0.72$ and 0.49. The major peak ($R_{GA_3} = 1$, System 5), on rechromatography, gave two peaks with System 1, the larger at $R_{GA_3} = 0.6$. Non-volatile benzene residues were not responsible for the formation of material with reduced mobility, since they showed no effect upon the mobility of standard GA_3 (System 1). The use of recrystallized, thiophene-free benzene reduced, but did not abolish, the formation of peaks of radioactivity at low R_{GA_3} (System 5). Rechromatography (in thiophene-free System 5) of materials eluted from the previous chromatograms gave PCs with significant amounts of radioactivity retained near the origin, similar to samples eluted from PCs run in System 4. The benzene did not affect the association of [^{14}C]GA_3 with either tannic or gallic acids.

Both borate and formaldehyde interact with phenols [26, 27], but neither detectably altered the GA_3–phenol interactions which occurred during PC. Similarly, the occurrence of [^{14}C]GA_3–phenol complexes was not reduced by the use of acid solvent systems (Systems 7 and 8) or by using the eluent which was most effective in removing radioactivity from Amberlite XAD-4 (System 6). The above results suggest that complex formation might occur between GA_3 and substances other than phenols. However, when the aromatic hydrocarbons anthracene, indene and naphthalene; the aromatic amino acids phenylalanine, tryptophan and tyrosine; and the barley amines gramine and hordenine (which is also a phenol) [6] were tested, no complex formation was observed.

Effect of hydrolases on the complexes

When GA_3 was applied to germinating grain, a biologically inactive material was formed which released 'free' (i.e. biologically active) hormone when extracts were incubated with a mixture of hydrolytic enzymes [13]. To test whether such an incubation could dissociate a gibberellin–phenol adduct the aqueous phase remaining after concentrating a methanolic extract of germinating grain, previously dosed with [^{14}C]GA_3, was treated with a similar range of enzymes and some other agents. Separate aliquots of the aqueous concentrate were incubated with a mixture of hydrolytic enzymes, β-glucosidase, bovine serum albumin and 2-mercaptoethanol. At the end of the incubation period the mixtures were acidified (to pH 2.5) and extracted with ethyl acetate. These extracts were concentrated and analysed by PC (System 1). None of the treatments resulted in significantly more radioactivity entering the ethyl acetate or in reducing the proportion of radioactivity retained at the origins of the PC than occurred in the controls. Indeed mercaptoethanol and β-glucosidase appeared to increase the amount of radioactivity retained at the origins of PCs in this experiment.

Effect of GA_3 concentration on complex formation

In this latter trial the proportions of total radioactivity associated with materials of low mobility were less than in previous experiments (average over treatments = 12.1% compared to 61%, Fig. 1a), but the amount of [^{14}C]GA_3 used had been increased from

Table 1. [^{14}C]GA_3 bound in compounds with low R_{GA_3} on PC (System 1) in washings and extracts of grains that had initially been dosed with different quantities of hormone

Amount of [^{14}C]GA_3 applied to grain		Relative amount of radioactivity in compounds with low R_{GA_3} (% of total recovered from PC).	
(pmol/grain)	(ng/grain)	Bicarbonate washings (EtOAc)	Methanolic extract (EtOAc)
100	34.6	2.4	6.1
50	17.3	5.7	17.5
10	3.5	9.9	20.8
5	1.7	16.1	45.5
1	0.3	88.4	27.8

35 to 200 pmol/grain. Thus an increase in the amount of GA_3 might have caused the decrease in the proportion recovered at the origins of PCs. The effect of altering the dose of [^{14}C]GA_3 on the relative amounts of radioactivity which were fast and slow running in the ethyl acetate extracts of sodium bicarbonate washings and grain extractives was tested. With one exception when samples were treated with smaller amounts of [^{14}C]GA_3, larger proportions of radioactivity were associated with substances having low mobilities (Table 1). Thus problems caused by the formation of gibberellin–phenol complexes are likely to be acute when dealing with the low levels of hormone which occur in plant tissues (e.g. 3–4 pmol GA_3 equivalent/barley grain; [1, 5].

DISCUSSION

In these experiments the coincidence of barley phenolic materials, brown phenolic oxidation products, and radioactivity from [^{14}C]GA_3 certainly indicates the formation of association product(s) and such products also form between GA_3 and model phenolics. It is likely that these products show reduced gibberellin biological activity as well as altered chromatographic properties [13], and their formation could be the mechanism which causes the disappearance of gibberellin activity from the surface of GA_3-dosed grains [15]. The formation of GA–phenol complexes may also account for the specific inhibitory effect of plant phenols on gibberellin-induced responses (IAA-induced responsed are not affected [16]). One of the proposed functions of endogenous phenolic compounds may be to maintain dormancy [28]. This might be achieved by an ability to 'sequester' endogenous gibberellins in seeds in which phenol contents decline to zero upon germination [28, 29]. However, this seems improbable in barley where the polyphenol content remains largely unchanged during germination [21, 30].

The nature of the association(s) between gibberellins and phenols is evidently complex as the formation of GA_3–phenol complexes was greatest when the reacting phenolic component consisted of a mixture. Tannic acid, barley phenols and the red products of phenol oxidation each bound GA_3 more effectively than catechin, chlorogenic acid and quercetin. Mixtures of ferulic, *p*-coumaric and vanillic acids are reportedly much more effective inhibitors of GA_3-stimulated germination in sorghum than the individual compounds [31] and tannic acid (a heterogeneous material) is one of the most effective inhibitors of GA_3-induced responses [32]. It is noteworthy that tannic acid and gallic acid effect ABA-induced inhibition only at concentrations greater than the effective dose of quercetin [17]. Thus there may be at least two classes of regulatory polyphenols, (a) those which promote, e.g. growth, by binding ABA, and (b) those which antagonize, e.g. growth, by binding gibberellins.

The nature of GA_3–phenol complexes differed depending upon the method of isolation used. Thus 'acid treatment' (PC System 3) readily dissociated complexes that were soluble in ethyl acetate, but was without effect on samples prepared by chromatography on Amerlite XAD-4. Such differences may account for the ineffectiveness of our enzyme treatments, to increase the amount of 'free' [^{14}C]GA_3, in grain extractives, in contrast to previous work in which the level of biologically active material was increased [13]. The differences were probably not due to different phenolic contents in the barley samples since these vary little in commercial samples of grain [30].

An amphoteric conjugate, reported to be the major product of GA_1 metabolism in isolated barley aleurone layers [11, 12], in some ways resembled the GA_3–phenol complexes reported here. The GA_1 amphoteric conjugate may have arisen from a GA_1–phenol complex binding to proteins or peptides as is known to occur with polyphenol complexes [33, 34]. The migration of radioactivity towards the cathode, of the acidic acetone eluate of a XAD-4 column that had been loaded with a preparation of a grain that had been dosed with [^{14}C]GA_3 may have been due to such a GA_3–phenol–protein complex.

Ways must be found to prevent the formation of complexes in solution, on columns and on paper chromatograms and to quantitatively release gibberellins from phenolic complexes in plant extracts for subsequent bioassay or chemical investigation. In this work, PC with acid solvent (System 3) was the most effective means of releasing GA_3 from at least some complexes. The recommended [18] procedure of adding a resin to plant extracts to remove phenolic compounds must be used with discretion, since it will also remove significant quantities of free gibberellins and gibberellin–phenol complexes. Indeed the concentration of phenol and hormone on the resin may facilitate complex formation. The existence of complexes, some of which are dissociable, also has implications in studies of 'gibberellin-binding sites'.

EXPERIMENTAL

Germination. Decorticated barley (*Hordeum distichon* cv Maris Otter) was micromalted [13]. Decorticated grain was dried before use in a rapid airflow (*ca* 18 hr, 40°). Samples (50 g) were placed in wide necked bottled (*ca* 500 ml) and surface sterilized with $Ca(OCl)_2$ (1%; 20 min at 4°). After repeated washings, samples were immersed in H_2O (steeped; 2 × 24 hr; 16°). After draining they were sprayed with a soln (1 ml) of streptomycin sulphate (0.8 mg), mycostatin (0.4 mg), benzyl penicillin (0.4 mg) and amphotericin B (0.4 mg), together with the appropriate amount of [^{14}C]GA_3 (Radiochemical Centre, Amersham). Grain was germinated in a moisture-satd atmosphere (16°; 4 days).

Preparation of extracts. Germinated grains were washed with $NaHCO_3$ (1%) then homogenized in MeOH (70%; 200 ml; −20°) in a Willem's Polytron (max. speed; 30 sec bursts). Solids were separated by centrifugation and re-extracted × 2. Pooled supernatants were concd by rotary evaporation to a viscous yellow–brown soln with some suspended solid. In some expts the extractives and the $NaHCO_3$ (1%) washings, treated separately, were brought to pH 2.5 and, where appropriate, were degassed under vacuum to remove CO_2, then extracted with an equal vol. of EtOAc (× 5). Pooled EtOAc fractions were concd under vacuum. Sometimes acidic aq. residues were re-extracted with *n*-BuOH.

Hydrolytic enzymes. Cellulase (Sigma Type II) and pectinase (Koch-Light) from *Aspergillus niger*, cellulase (BDH) from *Trichoderma viride*, Onozuka SS enzyme (all Japan Biochemicals) and β-D-glucoside glucanohydrolase (Sigma) from almonds, were separately purified [13].

Grain samples (50 g with 200 pmol [^{14}C]GA_3; 25.4 MBq/m mol) were washed and extracted. Aliquots of the concd extract (5 ml) were mixed with buffer (5 ml, pH 6 KH_2PO_4 10 mM) alone or containing either a mixture of enzymes [cellulases, pectinase and Onozuka SS (2 mg each)] or β-D-glucoside glucanohydrolase (8 mg), or bovine serum albumin (8 mg; Sigma Fraction V) or 2-mercaptoethanol (100 mM). After 2 hr at 30° they were acidified (pH 2.5 with H_3PO_4), and extracted with EtOAc (20 ml × 5). The pooled and concd EtOAc extracts were subjected to PC.

GA_3 binding to adsorbents. Adsorbents (2 ml) tested were: (1) Amberlite XAD-4; (2) Amberlite IR-45 (analytical grade); (3) insoluble polyvinylpyrrolidone [Polyclar AT; given by Allied Breweries (U.K.) Ltd]; (4) polystyrene beads (2% divinyl benzene; Eastman Kodak Co.); (5) Dowex 50–X8; and (6) DEAE-cellulose (Whatman DE52). [^{14}C]GA_3 soln was added (2 ml; 0.1 μmol; 127 MBq/mmol), the mixtures were shaken and an aliquot of liquid was removed for scintillation counting. Eluents under test (2 ml) were added to give the final concns shown: (1) H_2O; (2) NH_4OH (5%); (3) HCl (0.5 M); (4) MeOH (50%); (5) MeOH (25%) and cyclohexanol (25%); (6) Me_2CO (50%); (7) C_5H_5N (50%); and (8) MeCOEt (50%). Mixtures were shaken, and then radioactivity was measured in the supernatants.

Column chromatography. A column (120 mm long, 10 mm diam.) was packed with pre-washed Amberlite XAD-4 [35]. H_2O was added to a conc. MeOH extract of grain to dissolve solids then the soln was applied to the column. $NaHCO_3$ washings were applied without any treatment. Three fractions (each of six column vols.) were collected; (1) eluted with H_2O; (2) eluted with Me_2CO (10%); and (3) eluted with Me_2CO (50%) containing HCl (0.5 M).

Paper chromatography. All PCs were on Whatman No. 1 paper. The solvents used were: System 1, *iso*-PrOH–NH_4OH–H_2O (10:1:1) (210 mm, 24 hr); System 2, MeOH (320 mm, 24 hr); System 3, HOAc (2%) (320 mm, 24 hr); System 4, PhOH–*iso*-PrOH–NH_4OH–H_2O (5:5:1:1) (200 mm, 24 hr); System 5, C_6H_6–*iso*-PrOH–NH_4OH–H_2O (5:5:1:1, upper phase) (240 mm, 30 hr); System 6, MeOH–cyclohexanol–H_2O (1:1:2) (230 mm, 30 hr); System 7, Me_2CO–HCl (0.5 M) (1:1) (310 mm, 9 hr); System 8, *iso*-PrOH–H_2O (5:1) satd with citric acid (270 mm, 24 hr); System 9, *iso*-PrOH–H_2O (5:1) satd with H_3BO_3 (210 mm, 30 hr); System 10, *iso*-PrOH–HCHO (40%; commercial formalin, B.D.H.) (5:1) (420 mm, 20 hr). Figures in brackets give mobilities of GA_3 and time of development. As in some cases this involved running solvent off the end of the papers, mobilities are given as R_{GA_3}, i.e. relative to standard [^{14}C]GA_3.

Air-dried PCs were cut longitudinally into strips (50 mm). Radioactivity was located with a Packard 7201 radiochromatogram scanner. To test for phenolic substances strips were either (a) dipped in $FeCl_3$ (0.2%) and $K_3Fe(CN)_6$ (0.2%) then, after H_2O washing, were heated (45°, 20 min), or (b) were sprayed with dimethylaminocinnamaldehyde (DMAC; 0.1%) in acidic MeOH (25% HCl) [21].

High voltage electrophoresis. On Whatman 3MM PC paper satd with buffer (C_5H_5N–HOAc–H_2O, 25:1:225; pH 6.5) at 3 kV for 45 min. Standard GA_3 migrated 100 mm towards the anode relative to the sucrose electro-endosmotic marker.

Radioactivity. For scintillation counting samples (to 1 ml with MeOH) were added to 10 ml scintillant (PPO 5 g with POPOP 0.1 g and Triton X-100, 0.5 l. made up to 1.5 l. with toluene). Counting was in a Philips PW 4532 liquid scintillation analyser. Autoradiographs were exposed for 30 days using Kodak Blue brand medical X-ray film.

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