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# DISTRIBUTION OF THE HORDATINES IN BARLEY

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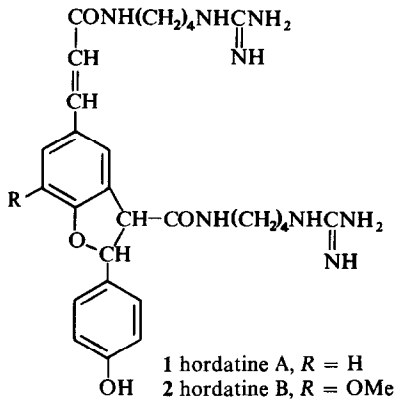
**Key Word Index**—*Hordeum vulgare*; *H. bulbosum*; *H. distichon*; *H. jubatum*; *H. murinum*; *H. spontaneum*; Gramineae; barley seedlings; coumarylagmatine; hordatines; fungal spore germination inhibitors; arginine decarboxylase.

**Abstract**—Concentrations of agmatine, coumarylagmatine and the antifungal hordatines in the shoots of barley seedlings have been determined at various stages of growth. Coumarylagmatine declined with age on a fresh weight basis, both in diurnal illumination and in continuous darkness. Hordatines A and B (estimated together) declined in the light to the 30th day after germination but their concentrations were stable in the dark to the 12th day. Hordatine M declined in the light to the 30th day and in the dark to the 12th day from germination. Agmatine declined in both light and dark to the 12th day. On the 30th day from germination potassium deficiency caused an increase in hordatines A + B (×6), hordatine M (×2) and agmatine (×13). Infection of the 11-day-old seedlings with mildew (*Erysiphe graminis*) caused an increase in the content of hordatine A + B (×6), hordatine M (×2) and agmatine (×2) 13 days later. Hordatines occurred in seedlings of *H. bulbosum*, *H. distichon*, *H. murinum* and *H. spontaneum*, though not in seedlings of *H. jubatum*, maize, millet, oats, rice, rye or wheat. Arginine decarboxylase activity declined with age in barley seedlings grown in the light or dark from the 3rd to the 12th day.

## INTRODUCTION

The basic antifungal compounds occurring in barley seedlings were first noted by Ludwig *et al.* [1] and were subsequently characterized as coumarylagmatine derivatives by Stoessl [2, 3]. Free coumarylagmatine is found in barley leaves together with its dimers, the hordatines A and B (**1**) and (**2**), which are probably derived from coumarylagmatine by peroxidative oxidation, a process which was demonstrated by Stoessl *in vitro* [2, 4]. Hordatine B (**2**) differs from hordatine A (**1**) only in the possession of a OMe group on the coumaran skeleton and hordatine B may therefore be formed by condensation of coumarylagmatine with ferulylagmatine. Hordatine M is a mixture of the glycosides of hordatines A and B. The *trans* forms of hordatines A, B and M inhibit spore germination in a wide range of fungi (*Monilinia* 100% at 5 ppm, $7 \times 10^{-6}$ M). However, they show relatively little activity against mycelium growing on agar and this has been attributed to adsorption of the hordatines on acidic groups on the agar gel [5]. Coumarylagmatine itself is a relatively weak inhibitor of spore germination (*Monilinia* 100% at $8 \times 10^{-4}$ M) [6].




1 hordatine A, $R$ = H
2 hordatine B, $R$ = OMe

In the present work the occurrence of the hordatines in *Hordeum* species and in other members of the Gramineae has been studied, and an investigation made of the changes in concentration of these compounds in the barley plant with tissue age, mineral nutrition and mildew infection. Activity of the arginine decarboxylase (arginine carboxy-lyase, EC 4.1.1.19), the enzyme responsible for the formation of agmatine in barley [7] has also been estimated in barley seedlings to determine if there is a correlation with hordatine content.

## RESULTS AND DISCUSSION

In earlier work [5], hordatines were extracted from barley tissue by boiling in water and separated on IRC-50 resin prior to TLC. In the present work, elution of the plant material with cold 17 M acetic acid doubled the hordatine yield. The hordatines extracted by the acetic acid were probably displaced from insoluble polyanions, possibly in the cell walls or from precipitated nucleic acid. Since satisfactory separations were obtained in extracts prepared without the use of an IRC-50 resin step before TLC, a procedure involving direct TLC of concentrated extracts of the plant material with 17 M acetic acid was adopted. The hordatines in the plant material were normally extracted by soaking intact tissue for 24 hr in 17 M acetic acid, since substances were extracted on blending which reduced the resolution of TLC separations. In order to test for completion of extraction, intact samples of the shoots of 6-day-old barley seedlings grown in the light were extracted by soaking for 18 hr in 17 M acetic acid at 2°. After filtration the residue was blended on addition of 17 M acetic

Table 1. Effects of age, illumination and potassium deficiency on hordatine levels (in nmol/g fr. wt) in barley seedlings. Seedlings were grown in intermittent light (light) or continuous dark (dark). Values for the 6-day-old plants were duplicates from seedlings grown on different occasions

| Age (days) | 3 | | | | 6 | | 12 | | 30 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Potassium nutrition | +K | | +K | | +K | +K | +K | +K | +K | −K |
| Illumination | Light | | Dark | | Light | Dark | Light | Dark | Light | Light |
| Tissue | shoots | roots* | shoots | roots* | shoots | shoots | shoots | shoots | shoots | shoots |
| Coumarylagmatine | 350 | <30 | 250 | <30 | 80, 85 | 120, 100 | 75 | 75 | <50 | <50 |
| Hordatines A and B | 680 | 15 | 400 | 15 | 430, 400 | 470, 500 | 350 | 450 | <50 | 315 |
| Hordatine M | 350 | 15 | 480 | 50 | 120, 130 | 300, 300 | 180 | 150 | 175 | 340 |
| Agmatine | 100 | <30 | 150 | <30 | <30, <30 | <30, <30 | <30 | <30 | 44 | 580 |

* Values assessed by eye. For older roots—coumarylagmatine <30 nmol/g fr. wt; all hordatines <15 nmol/g fr. wt each.

acid and the macerate soaked for a further 18 hr at 2°. On filtration, concentration at 40° under vacuum and TLC, all Sakaguchi positive material was found in the first eluate. No hydrolysis was detected in 17 M acetic acid, even on heating for 30 min at 100° in a sealed tube. On concentrating acetic acid extracts to dryness, precipitates were formed which adsorbed the hordatines. The hordatines could be eluted from these with acetic acid and concentrates were therefore taken up in 17 M acetic acid for TLC.

In barley shoots grown in both light and dark, concentrations of coumarylagmatine and hordatines A + B were high in the early stages of germination and declined progressively to the 12th day (Table 1). Hordatine M showed a similar overall decline though a slight recovery occurred on the 12th day in the light. Barley shoots grown in the light for 30 days showed a continuing decline in the content of coumarylagmatine and hordatines A + B, though hordatine M remained relatively unchanged during this period. In roots, these conjugates could be found only in 3-day-old seedlings and then at only low concentrations. Illumination appeared to have little influence on the hordatine content, as already indicated by Stoessl and Unwin [5]. No hordatines could be detected in the ungerminated grain.

In 12-day-old shoots grown in the light, the coumarylagmatine and hordatine content appeared to be much higher in the stems than in the leaves. This difference was particularly marked with hordatine M; this was barely detectable in the leaves but was relatively abundant (×20) in the stems. In the dark-grown shoots the hordatine M content was again much higher in the stems than in the leaves, though the coumarylagmatine and hordatine A + B contents were similar in both tissues.

On the basis of the fresh weight, arginine decarboxylase activity of 12-day-old shoots grown in light or dark was only 20 to 30% of that at 3 days old (Table 2). In dark-grown tissue, part of this decrease was associated with the increase in water content with age (see Table 7). However, in light-grown tissue there was an increase in dry wt with age, and activity declined both in terms of dry wt and protein over the 12-day period. Activity on the basis of fr. wt was about the same in light and dark-grown seedlings, though in terms of protein, activity was *ca* 2× greater in the shoots of seedlings grown in the dark than in the light. The arginine decarboxylase activity of the roots on a fr. wt basis was 30–60% of that of the shoots, though it was comparable with the activity of the shoots on a protein basis (Table 2). Arginine decarboxylase activity was highest in the base of the stem (Table 3) and this may be associated with the higher concentrations of hordatines found here (see above). In previous work [7] arginine decarboxylase activity in 10-day-old seedlings grown in the light (mean of 3 varieties) was 210 pkat/g fr. wt. In the present work activity in the shoots of 12-day-old seedlings grown in the light was 330 pkat/g fr. wt.

Stoessl and Unwin [5] extracted essentially pure hordatine M present at a mean concentration of 277 mg/kg (380 nmol/g fr. wt) from the shoots of barley seedlings after 5–6 days' grown in the dark. They found that barley seedlings grown in the light contained *ca* 45 mg/kg (80 nmol/g fr. wt) of hordatines A + B and 75 mg/kg (100 nmol/g fr. wt) of hordatine M. The coumarylagmatine content of 6-day-old dark-grown coleoptiles was 4.9 nmol/g ft. wt [6]. The concentration of hordatine M found in barley seedlings by Stoessl and Unwin [5] using cv. Herta was similar to that found in the present work using cv. Zephyr. However the concentrations of hordatine A + B and of coumarylagmatine were considerably greater (×5 and ×22 respectively) in the present study. These differences may be related to the different method of extraction used.

In earlier work [7, 8] estimations of agmatine may

Table 2. Arginine decarboxylase activity in barley seedlings. Seedlings were grown in intermittent light (light grown) or continuous dark (dark grown) and arginine decarboxylase was determined by the method described in Experimental. The values are means of two estimations

| Tissue | Age (days) | Light-grown | | Dark-grown | |
|---|---|---|---|---|---|
| | | pkat/g fr. wt | pkat/mg protein | pkat/g fr. wt | pkat/mg protein |
| Shoots | 3 | 1030 | 110 | 1450 | 220 |
| Shoots | 6 | 620 | 80 | 730 | 170 |
| Roots | 6 | 220 | 90 | 520 | 200 |
| Shoots | 12 | 330 | 40 | 290 | 90 |

Table 3. The distribution of arginine decarboxylase activity in the tissues of 21-day-old barley grown in the light with the normal nutrient solution

| | pkat/g fr. wt | wt of one unit |
|---|---|---|
| 1st leaf (youngest) | 120 | 32 mg |
| 2nd leaf | 188 | 48 mg |
| Upper half of stem | 109 | 88 mg |
| Lower half of stem | 338 | 90 mg |

Table 4. Effect of mineral nutrition on arginine decarboxylase of 9-day-old barley seedlings. Plants were grown with a normal nutrient supply, in water, and in a nutrient lacking potassium (see Experimental). Duplicate values are given for 9-day-old plants

| Nutrition | pkat/g fr. wt | pkat/mg protein | wt of one unit (mg) |
|---|---|---|---|
| Normal | 260, 260 | 31, 30 | 104 |
| K deficient | 350, 450 | 55, 70 | 134 |
| Water only | 440, 510 | 57, 49 | 64 |

have partially included agmatine derived from the hordatines; for 16-day-old barley seedlings grown in the light the agmatine content was found to be 260 nmol/g fr. wt in the normal nutrient plants and 630 nmol/g when nutrient deficient in potassium was given [8]. In the present work 12-day-old seedlings grown in the light in normal (plus potassium) nutrients contained *ca* 1100 nmol/g fr. wt of free and combined (amide) agmatine. By the 30th day this had decreased to *ca* 400 nmol/g fr. wt.

Potassium deficiency is known to cause accumulation of agmatine and putrescine in barley plants [7], and in the present study 30-day-old plants contained a considerably enhanced concentration of agmatine ($\times 13$), hordatines A + B ($> \times 6$) and hordatine M ($\times 2$) (Table 1). The increased activity of the arginine decarboxylase ($\times 2$) with potassium deficiency was detected even in 9-day-old seedlings (Table 4). However the content of the agmatine conjugates was not changed significantly by potassium deficiency on the 6th day of germination. The enhanced weight of the seedling units may have been due to succulence induced by the sodium ions used to substitute for potassium. In the absence of nutrients (water only) seedlings were only half the size of the controls. The arginine decarboxylase activity was comparable to that in the potassium deficient seedlings. Increases in the activity of this enzyme with potassium deficiency in 6- to 14-week-old barley have been demonstrated in previous work, probably accounting for the increased putrescine content of these plants [7, 9].

Sakaguchi-positive spots corresponding to the hordatimes were present in *Hordeum bulbosum*, *H. distichon*, *H. murinum* and *H. spontaneum* though none were detectable in *H. jubatum* (Table 5). The content of coumarylagmatine was very high in *H. bulbosum*. In *H. bulbosum* and *H. murinum* a Sakaguchi-positive compound was found at very high concentration having a slightly lower $R_f$ than that of the mixture of hordatines A + B. The seedlings used to study the hordatines in these *Hordeum* species were older than 14 days in order to obtain sufficient material, as the plants were small. On the basis of the results with *H. vulgare*, younger plants may have contained even higher concentrations. Even so the concentrations of hordatines A + B were in general greater in the wild species than in *H. vulgare*. The low concentration of the hordatines in *H. jubatum* may be related to its different geographical distribution *H. jubatum* is from North America; the remaining species are Eurasian [10].

Extracts of the leaves of other cereals indicated that the hordatines are confined to the genus *Hordeum*. In earlier work Stoessl [3] could find no hordatines in wheat, oat or maize seedlings. Their absence was confirmed in the present work with shoots of wheat (grown 6 days in light or dark), oat (11 days in light or 10 days in dark), and maize (23 days in light). Neither could the hordatines be found in shoots of millet (30 days in light) or rice (30 days in light). A Sakaguchi-positive compound present in trace amounts in rye shoots corresponded in $R_f$ to hordatine M. This compound, which is probably not related to the hordatines, was also found in the roots.

Arginine decarboxylase occurs in wheat, rye and maize with less activity than in barley, but in oats activity is up to $10\times$ greater than in barley (Smith, unpublished). Hordatine formation is not therefore solely determined by the presence of arginine decarboxylase. However, the increase in hordatine content in potassium deficient barley indicates that arginine decarboxylase, and not coumarylagmatine synthetase, may be limiting the formation of the hordatines.

To test the effects of fungal infection on the hordatine content, 11-day-old barley seedlings were infected with *Erysiphe graminis* (powdery mildew) in a greenhouse. Samples taken from infected and control leaves 3 days after inoculation showed no significant change in the hordatine content (Table 6). However, 13 days after inoculation increases were shown in hordatines A + B ($\times 6$), hordatine M ($\times 2.5$) and agmatine ($\times 2$) on infection.

Table 5. Coumarylagmatine, hordatine and agmatine content of barley species (nmol/g fr. wt)

| | *H. bulbosum* L. | *H. distichon* L. | *H. jubatum* L. | *H. murinum* L. | *H. spontaneum* Koch |
|---|---|---|---|---|---|
| Age (days) | 18 | 15 | 27 | 18 | 14 |
| Units/g | 21 | 5 | >50 | >50 | 5.2 |
| Coumarylagmatine | 960 | 100 | <100 | <100 | 167 |
| Hordatines A and B | 1320* | 1100 | <10 | 1150* | 1260 |
| Hordatine M | 11 | 448 | <10 | 61 | 386 |
| Agmatine | 480 | 150 | 100 | <100 | <100 |

* $R_f$ 0.46, main hordatine A + B spot in remainder of species have $R_f$ 0.56.

Table 6. Effects of mildew (*Erysiphe graminis*) on hordatines and related compounds in barley leaves. Plants inoculated after 11 days growth and extracted 3 days and 13 days after infection together with non-infected controls

| | nmol/g fr. wt | | | |
|---|---|---|---|---|
| | + 3 days | | + 13 days | |
| | Non-infected | Infected | Non-infected | Infected |
| Coumarylagmatine | < 100 | < 100 | < 100 | < 100 |
| Hordatines A and B | 305 | 288 | 89 | 502 |
| Hordatine M | 97 | 85 | 43 | 105 |
| Agmatine | < 30 | < 30 | 68 | 119 |

Ludwig *et al.* [1] found loss of antifungal activity in extracts of barley seedlings between the 5th and 8th day after germination which was not associated with the loss of the hordatines. This was ascribed to an increase in $Ca^{2+}$ and $Mg^{2+}$ ions in the plant, since addition of these ions at 40 ppm (1 mM $Ca^{2+}$ or *ca* 2 mM $Mg^{2+}$) to the active extracts caused loss of inhibition in the spore germination test [5]. Moreover on adding EDTA to the extracts of older leaves the ability to inhibit spore germination was restored [1].

In the present work the $Ca^{2+}$ and $Mg^{2+}$ levels in barley seedlings were estimated (Table 7). A considerable loss of antifungal activity in barley shoot extracts was found between the 5th and 6th days from germination by Stoessl *et al.* [5]. This was confirmed with water extracts of barley shoots assayed against *Helminthosporium sativum* in the present study. Indeed Table 7 shows that the $Ca^{2+}$ concentration increases significantly during this 24-hr period ($\times 2$ in the light and $\times 1.6$ in the dark, on a fr. wt basis). There was only a relatively small increase in $Mg^{2+}$ on ageing. Its concentration is less than 10% that of the $Ca^{2+}$ and it is much less effective in suppressing the inhibitory effect of the hordatines [5]. Therefore $Ca^{2+}$ is likely to be the more important cation in repressing hordatine activity. Manganese ($Mn^{2+}$) is $2\times$ more effective than $Ca^{2+}$ in preventing the antifungal effects of the hordatines [5] though its concentration within the plant is likely to be quite low.

The concentration of the hordatines A/B and M in the barley seedlings is greatly in excess of that required to inhibit spore germination. Stoessl and Unwin [5] found that the hordatines are effective against spores even at $7 \times 10^{-6}$ M, preparations with predominantly *trans*-hordatines causing 100% inhibition. This concentration (*ca* 5–10 nmol/g fr. wt) is *ca* 1% of that actually found in the leaves. However the adsorption of the hordatines to the polyanions of the cell walls, as well as the loss of activity caused by interaction with divalent cations, might considerably reduce their effectiveness.

Tests conducted by Stoessl and Unwin (Table 8) with concentrations of hordatine M up to 900 μM and $Ca^{2+}$ up to 16 mM showed that even in the youngest plants the concentration of $Ca^{2+}$ should be sufficient to reverse the spore germination inhibition due to the hordatines if both $Ca^{2+}$ and hordatines were distributed evenly throughout the tissue. It seems likely therefore that there is a higher concentration of the hordatines and/or a lower concentration of $Ca^{2+}$ at the surface of the young resistant seedlings than within the tissues. A redistribution of

Table 7. Percentage dry weight and calcium and magnesium concentrations in the shoots of barley seedlings. Duplicates of the 6-day samples are given

| | Light-grown | | | Dark-grown | | |
|---|---|---|---|---|---|---|
| | % | mM | | % | mM | |
| Age (days) | dry wt | Ca | Mg | dry wt | Ca | Mg |
| 3 | 8.7 | 2.2 | 0.58 | 11.3 | 2.0 | 0.71 |
| 4 | 6.2 | 2.8 | 0.46 | 9.3 | 2.3 | 0.50 |
| 5 | 6.4 | 5.5 | 0.71 | 7.1 | 3.2 | 0.50 |
| 6 | 8.4, 7.7 | 11.7, 10.7 | 1.21, 1.04 | 5.2, 5.2 | 5.5, 4.7 | 0.63, 0.58 |
| 7 | 7.1 | 11.9 | 1.04 | 6.4 | 6.7 | 0.79 |
| 9 | 6.5 | 12.7 | 1.08 | 6.3 | 9.5 | 0.96 |
| 12 | 10.0 | 14.8 | 1.42 | 5.3 | 9.0 | 0.63 |

Table 8. Effect of calcium ions on the inhibition of the germination of *Monilinia fructicola* spores by hordatine M diacetate. Results in this Table were provided by Dr. A. Stoessl and Mr. C. H. Unwin, Canada Department of Agriculture

| | ← Assay 1 → | | | | ← Assay 2 → | | | |
|---|---|---|---|---|---|---|---|---|
| $CaCl_2$ (mM) | 0 | 0.1 | 0.25 | 0.5 | 1 | 1 | 2 | 16 |
| Hordatine M (μM) | | | | | | | | |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 300 | 100 | 100 | 76 | 12 | 0 | 0 | 0 | 0 |
| 500 | 100 | 100 | 100 | 59 | 15 | 0 | 0 | 0 |
| 700 | 100 | 100 | 100 | 80 | 0 | 0 | 0 | 0 |
| 900 | 100 | 100 | 100 | 100 | 74 | 58 | 0 | 0 |

$Ca^{2+}$ and/or hordatines on ageing may explain the reduced resistance of the older seedlings.

Several other growth inhibitory guanidino compounds are known. Streptomycin has two guanidino groups, and both streptomycin and the hordatines inhibit protein synthesis in pea stem segments [11]. Streptomycin also inhibits germination of *Monilinia* spores (100% at $2 \times 10^{-4}$ M) [11]. A synthetic fungicide, Guazatine, NH $[(CH_2)_8NHC(NH)NH_2]_2$ which is used as a cereal seed dressing [12], has a marked structural similarity to the hordatines. The separation of the guanidino groups is by 16 carbon units and 2 nitrogen units in the hordatines, and by 16 carbon units and 1 nitrogen unit in Guazatine. However, the importance of steric relations in the antifungal activity of the hordatines is not easily reconciled with a direct analogy between these two classes of antifungal compounds. Even so, saturation of the ethylenic double bond reduces antifungal activity of the hordatines by only a factor of 10 [5], and the relative activity of the synthetic and natural fungicides in the same test is at present unknown. Other growth inhibitory compounds having structural similarities to the hordatines are also found. For example Dodine ($C_{12}H_{25}$-$NHC(NH)NH_2$) is a protective synthetic fungicide [12], probably functioning as a surface active agent, and the bleomycins and phleomycins are antitumour compounds which may bear one or more guanidino groups as agmatine moieties [13].

It is of interest that in wheat leaves the content of feruloyl- and coumaroyl-2-hydroxyputrescine, compounds related structurally to coumarylagmatine, increase in response to infection with *Puccinia graminis* or on physical or chemical damage [14, 15]. Putrescine amides are known to be formed on virus infection in *Nicotiana* [16]. These amides completely inhibit virus infection at 750 ppm ($3 \times 10^{-3}$ M). Several other aromatic amines and polyamines occur as conjugates of cinnamic acid and its derivatives in various unrelated families [17]. The function of these conjugates is still unknown. Other antifungal compounds have been recently found in barley plants on infection with *Selenophoma donacis*. These have been tentatively identified as benzoquinones [18].

## EXPERIMENTAL

*Barley* (*Hordeum vulgare* L. cv Zephyr) and seedlings of other species were grown in sand in polyethylene pots in the dark at 21° or in a 16-hr-day (10 klx) with the day temp 24° and the night temp 19°. The pots were watered daily with a nutrient medium containing (with concentrations in mM) $K_2SO_4$ (2), $MgSO_4$ (1.5), $CaCl_2$ (4), $Na_2HPO_4$ (0·33), $NaNO_3$ (4), $(NH_4)_2SO_4$ (4), with Fe EDTA and micronutrients. K deficient plants were grown in a medium in which $Na_2SO_4$ was substituted for $K_2SO_4$.

*Spore germination test.* Seedlings grown in the dark were extracted in boiling $H_2O$ for 10 min (2 ml/g) and the extracts evaporated to 0.5 ml/g. Spores of *Helminthosporium sativum* were applied to a microscope slide in *ca* 100 µl of the extract and a coverslip added. Germination was assessed after 4 days' incubation in the dark at 20° with 100% humidity.

*Determination of the hordatines.* Intact plant material (10 g) was immersed in 40 ml of 17 M HOAc for 18 hr at 2°. After filtration, the HOAc was concentrated to *ca* 1 ml at 40° under vac, made up to 1 ml with HOAc and stored at −15°. Samples (1–10 µl, normally 2 µl) were applied to a TLC plate (cellulose CC41, 250 µm). The plate was then run in $Et_2O$ (30 min) to remove chlorophyll ($R_f$ 1.0), dried at room temp. and then run in BuOH/HOAc/$H_2O$ (4:1:5, upper) (3 hr) and dried at 100° for 1 hr. After cooling, the plate was sprayed with the Sakaguchi reagent. α-Naphthol (0.1%) in M NaOH (6 ml) was applied first, allowing to air dry for *ca* 30 min. Na hypobromite soln (0.3 ml $Br_2$ in 0.5 M NaOH, 100 ml) (3 ml) was then sprayed. Hordatines appeared as pink spots on a violet background. The background colour disappeared on drying 2–3 hr at room temp. The Sakaguchi spots were stable for at least 1 week. Qualitative standards (2 µl of *ca* 0.2% solns) were also applied to the plates. Coumarylagmatine, and hordatines A/B (unresolved) and M had $R_f$ values of 0.77, 0.59 and 0.47 respectively. Agmatine produces multiple spots in this solvent [19, 20] with a main spot at $R_f$ 0.40. Arginine had $R_f$ 0.19. The salts extended from the origin to $R_f$ 0.15. Arginine in plant extracts is normally dispersed in this salt spot. Extracts of barley seedling shoots grown in the light had flavonoids giving yellow colours with the alkaline reagents. These did not interfere in the subsequent scanning. On MN 300 cellulose coumarylagmatine and hordatine A/B have $R_f$ 0.8 and hordatine M has $R_f$ 0.4.

*Quantitative assessment of the TLC plates.* The Vitatron thin layer densitometer was used to measure the intensity of the Sakaguchi spots. Insufficient authentic hordatine material was available to act as quantitative standards and agmatine was not convenient for this purpose as it produces multiple spots on TLC. Arginine (10 nmol) was therefore run on each plate as a quantitative standard: the mean ratio of $A_{507}$ nm for agmatine: arginine (equivalent molar amounts) was 1.01 (15 determinations). Calibration curves of arginine and serial dilutions of coumarylagmatine and the hordatines gave similar results. The curve was linear to *ca* 10 nmol arginine/origin. 50 nmol/origin gave $A_{507}$ only 3.3 × as great, and at 200 nmol/origin $A_{507}$ value was 7 × as great as at 10 nmol. Settings on the scanner were; mode, -log; pre-amp, d; HT, 4; damp, 2; span, 9; filter 507 nm; traverse 3 cm/min. Densities were determined by measuring the areas on the recorder paper by tracing, cutting-out and weighing.

*Arginine decarboxylase assay.* This was determined by a modification of the method in ref. [7]. Tissue was extracted in 2 vol 0.1 M $Na_2HPO_4$ in a pestle and mortar with sand. After centrifuging and dialysis against 0.1 M pH 6.3 Pi buffer, samples (4 ml) were incubated at 30° with 1 ml 25 mM arginine. Samples (2 ml) were withdrawn at 0 and 2 hr and added to 10 ml of 2 M $NH_4OH$. The soln was centrifuged if necessary, no losses occurring on the ppt. The extract was applied to a column of IRC-50 CG type 1, 100–200 mesh (2 ml) in a column (1 × 30 cm). The flow rate for all solns was not greater than 3 ml/min. The columns were washed with $H_2O$ (2 × 20 ml) and then with 2 M $NH_4OH$ (3 × 10 ml). This removed the residual arginine. In earlier work [7] 4 M $NH_4OH$ was used for this purpose but in the present system this was found to elute a fraction of the agmatine. The agmatine was then eluted with saturated ammonium carbonate (2 × 5 ml) (25% saturated ammonium carbonate eluted only 60% of the agmatine). This ammonium carbonate soln was boiled to 1 ml. $H_2O$ (5 ml) was added and the soln reboiled to 1 ml. This eliminates residual $NH_4OH$ which otherwise interferes with the Sakaguchi estimation. The residue was made up to 2·5 ml and samples (0.1–0.5 ml) were used for the Sakaguchi assay.

*Sakaguchi assay* was conducted by a modification of the method of ref. [7]. This method using sodium hypobromite as oxidant was 1.5x more sensitive than the method [21] in which *N*-bromosuccinimide is used. To a 2 ml sample containing up to 400 nmol of agmatine was added 0.5 ml 0.2% urea, 0.5 ml 10 M NaOH, 0.5 ml 0.05% 8-hydroxyquinoline $K_2SO_4$, mixing after each addition. Adding 1.5 ml of these reagents, premixed in equal vols, showed no loss of A after standing for 3 days at room temp. Na hypobromite soln (0.3 ml $Br_2$ in 100 ml 0.5 M NaOH) (0.5 ml) was then added forcibly. The $A_{490}$ nm was read after 10 min. Maximum $A$ was developed on mixing and was reduced to 70% and 65% of maximum after 10 min and 30 min resp. Allowing up to 4 min between the addition of each reagent did not affect the absorbance. The $A$ obtained with the reagents at 0° was 1.6 × greater than at 30°, and it was linear for up to

200 nmol agmatine (in 1 cm cells, $A_{490}$ nm for 200 nmol = 0.4; for 400 nmol $A_{490}$ nm = 0.7).

Applying arginine (12.5 μmol) to the resin, the residual Sakaguchi colour consistently corresponded to *ca* 30 nmol. In the presence of arginine (12.5 μmol) recovery of 0.4 μmol agmatine was 108, 89 and 91% in the absence of plant extract and 89 and 87% in the presence of an extract of 0.3 g barley leaves. *Ca and Mg* were determined by atomic absorption spectrophotometry after dry ashing and *protein* was estimated by the method of ref. [22].

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## REFERENCES

1. Ludwig, R. A., Spencer, E. Y. and Unwin, C. H. (1960) *Can. J. Botany* **38**, 21.
2. Stoessl, A. (1967) *Can. J. Chem.* **45**, 1745.
3. Stoessl, A. (1970) *Recent Adv. Phytochem.* **3**, 143.
4. Stoessl, A. (1966) *Tetrahedron Lett.* (25), 2849.
5. Stoessl, A. and Unwin, C. H. (1970) *Can. J. Botany* **48**, 465.
6. Stoessl, A. (1965) *Phytochemistry* **4**, 973.
7. Smith, T. A. (1963) *Phytochemistry* **2**, 241.
8. Basso, L. C. and Smith, T. A. (1974) *Phytochemistry* **13**, 875.
9. Smith, T. A. (1965) *Phytochemistry* **4**, 599.
10. Bowden, W. M. (1965) *Can. J. Genet. Cytol.* **7**, 394.
11. Venis, M. A. (1969) *Phytochemistry* **8**, 1193.
12. Martin, H. and Worthing, C. R. (eds.) (1977) *Pesticide Manual* (5th edn). British Crop Protection Council.
13. Umezawa, H. (1977) *Lloydia* **40**, 67.
14. Stoessl, A., Rohringer, R. and Samborski, D. J. (1969) *Tetrahedron Lett.* (33), 2807.
15. Samborski, D. J. and Rohringer, R. (1970) *Phytochemistry* **9**, 1939.
16. Martin-Tanguy, J., Martin, C., Gallet, M. and Vernoy, R. (1976) *Compte Rend.* **282**, 2231.
17. Smith, T. A. (1977) *Progr. Phytochem.* **4**, 27.
18. Evans, R. L. and Pluck, D. J. (1978) *Ann. Appl. Biol.* (in press).
19. Miranda, F. and Lissitzky, S. (1956) *Compte Rend.* **243**, 1458.
20. Lepri, L., Desideri, P. G. and Goas, V. (1973) *J. Chrom.* **79**, 129.
21. Satake, K. and Luck, J. M. (1958) *Bull. Soc. Chim. Biol.* **40**, 1743.
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.