Phytochemistry, Vol. 25, No. 3, pp. 683–689, 1986.
Printed in Great Britain.

0031–9422/86 $3.00 + 0.00

OCCURRENCE IN HIGHER PLANTS OF 1-(3-AMINOPROPYL)-PYRROLINIUM AND PYRROLINE: PRODUCTS OF POLYAMINE OXIDATION

TERENCE A. SMITH, STEPHEN J. CROKER and R. S. THOMAS LOEFFLER

(*Received* 23 *May* 1985)

Long Ashton Research Station, University of Bristol, Long Ashton, Bristol, BS18 9AF, U.K.

Key Word Index—Gramineae; Leguminosae; 1-(3-aminopropyl)-2-pyrrolinium; 1-pyrroline; 1-piperideine; 2-aminobenzaldehyde adduct; 2,3-trimethylene-4-quinazolone; spermine; diaminopropane; amine oxidases.

Abstract—The presence of 1-(3-aminopropyl)pyrrolinium (App) has been established in the leaves of oats, maize, barley and wheat seedlings. In oat leaves, concentrations of 1,3-diaminopropane (Dap), putrescine (Put) and App were greatest in the youngest plants. Changes in Dap and App could not be correlated with changes in polyamine oxidase activity. Concentrations of the amines were smaller in maize than in oats, and smallest in barley and wheat. Pyrroline, an oxidation product of Put in pea seedlings and of spermidine in oat and maize seedlings, has been demonstrated in extracts of these plants, and also in spinach leaves and in radish shoots, following distillation, derivatization with 2-aminobenzaldehyde, oxidation of the adduct and GC-MS. Piperideine was also identified in pea seedlings.

INTRODUCTION

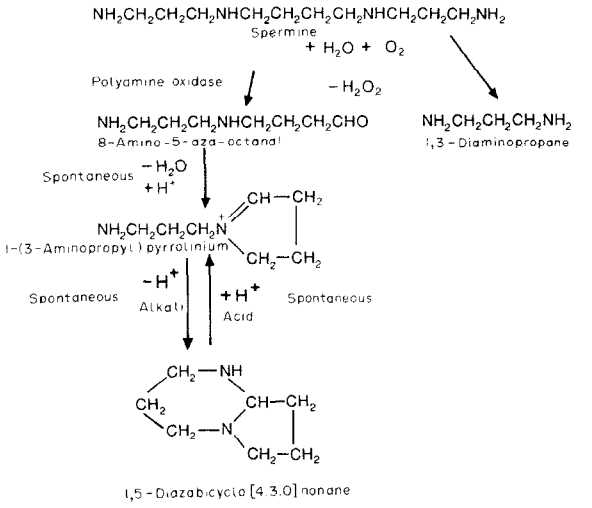
The enzyme polyamine oxidase (PAO) which is widespread in the Gramineae is especially active in the vascular system of maize [1] and in the stomata and cell walls of oats [2]. In a range of cereals the enzyme showed greatest activity in oat leaves, although it was also detected in leaves of maize, barley, wheat and rye [3]. In 14-day-old dark-grown oat shoots, PAO activity was 43 nkat/g fr. wt [4]. By comparison, activity of arginine decarboxylase (ADC), a key enzyme in polyamine biosynthesis, was very weak. The greatest ADC activity (in potassium-deficient light-grown seedlings) was only 2.7 nkat/g fr. wt [5]. Since the concentration of the polyamines may limit growth processes [6], PAO is a potential growth regulating enzyme.

The *in vitro* oxidation of spermine by PAO produces 1-(3-aminopropyl)pyrrolinium (App) and 1,3-diaminopropane (Dap). Preliminary studies had indicated the presence of both of these products in barley seedling leaves in which they had been detected on the basis of GC retention times and R_f on TLC [7]. In early work, App had also been characterized as a product of the *in vitro* oxidation of spermidine by pea seedling diamine oxidase [8, 9]. Dap has now been found in several higher plants including cucumber [10], pea [11], *Amaranthus* [12] and in various cereals [1, 7, 12–15]. Polyamines appear to be the only source of Dap and the occurrence of this amine in these plants and in algae [16] and slime moulds [17] indicates the presence of PAO in these species. One of the objectives of the present work was to characterize App, to demonstrate its presence in cereal seedlings and to study the changes in concentration of this amine and of Put and Dap with age.

1-Pyrroline, which is formed by the spontaneous cyclization of 4-aminobutyraldehyde, may be produced in plants by di- or polyamine oxidases. Diamine oxidase, found notably in the legumes, oxidizes putrescine to 4-aminobutyraldehyde, while PAO, so far found exclusively in the Gramineae, oxidizes spermidine to 4-aminobutyraldehyde and Dap [18]. Further metabolism of pyrroline may take place via a recently characterized pyrroline dehydrogenase which has been demonstrated in bean, pea and oats [19, 20]. Previous reports of the occurrence of 1-pyrroline in biological samples have been few. The presence of pyrroline was established in wine [21] by GC-MS of the trifluoroacetyl (TFA) derivative. 1-Pyrroline was tentatively identified in Latakia tobacco leaves by the GC R_t of the TFA derivative [22], and a similar technique suggested the presence of 1-pyrroline at 20 ppm (290 nmol/g) in red radish [23]. In the present paper the unequivocal presence of pyrroline has been demonstrated in spinach leaves and in the shoots of oats, maize, pea and radish seedlings.

RESULTS AND DISCUSSION

Oxidation of spermine by PAO gives Dap, hydrogen peroxide and, initially, 8-amino-5-aza-octanal (Scheme 1). The latter spontaneously cyclizes to 1-(3-aminopropyl)pyrrolinium (App). A similar cyclic intermediate is probably formed from the oxidation product of homospermidine in the biosynthesis of trachelanthamidine and retronecine [24]. Although the cyclic product of the oxidation of spermine has usually been referred to as 1-(3-aminopropyl)-2-pyrroline, this structure is unlikely to exist in aqueous solution because it corresponds to a highly reactive species, an enamine; there is no authenticated report in the chemical literature for monomeric 2-pyrrolines unsubstituted at the 2-position. It has been shown recently [25] that the correct structure for the isolated amine is that of a bicyclic compound, formed by addition of the amino group to the activated double bond of App (Scheme 1); in aqueous solution above *ca* pH 6 the bicyclic form predominates [26]. Below pH 4, however,



Scheme 1.

the compound exists as the iminium cation and interconversion between these forms is rapid [26]. In this paper we shall refer to App, meaning the equilibrium mixture of these two forms.

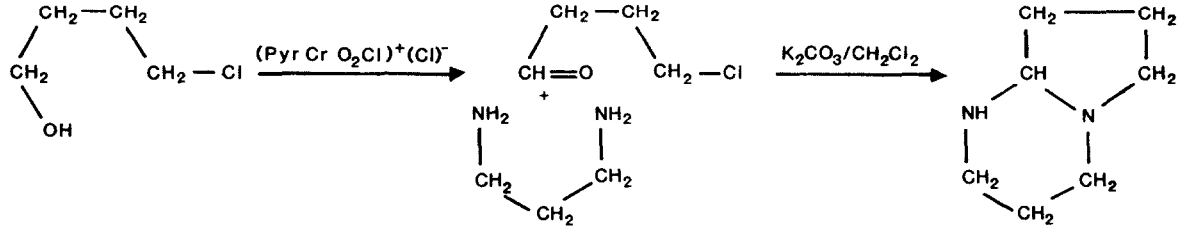
In earlier work, App was synthesized by oxidation of spermine in alkaline solution in the presence of metallic copper [27]. We have improved this method by shaking the mixture in air instead of bubbling air through the solution, and by substituting extraction into diethyl ether for the earlier steam distillation procedure. Sampling during the oxidation indicated that the yield of App was 93% after 2 hr; ether extraction recovered about 89%, while only 66% was recovered after removal of the ether, owing to the volatility of App. The yield of App by this process, after purification by distillation, was 30%. Although one mole of Dap should be formed per mole of spermine oxidized, as in the enzymatic oxidation, the amount of Dap obtained in the above oxidation was disproportionately small, and it was found that under the same conditions Dap itself was oxidised rapidly to a ninhydrin-positive compound (R_t 2.5 min on GC using Carbowax 20 M/KOH at 145°). This product was not 3-aminopropionaldehyde, which has been demonstrated as an enzymatic oxidation product of Dap [28, 29].

An alternative method of preparing App was devised (Scheme 2), which also provided support for the bicyclic structure. This synthesis starts from 4-chlorobutanol and Dap, both cheap, commercially available materials. Unlike the preparation from spermine, this lends itself to the synthesis of App analogues and of labelled App. The yield, although low (13% overall), was not optimized, and particularly the first step, the oxidation of 4-chlorobutanol to the aldehyde, should be amenable to improvements in purity of the product by the replacement of pyridinium chlorochromate with an alternative oxidizing agent. The products of both methods of App preparation were found to be identical with the product of the enzymatic oxidation using a range of physical methods [25].

Attempts were made to crystallize salts of App, but without success. Acidification of an App solution with hydrochloric acid and evaporation at 40° under reduced pressure gave a complex mixture of products together with unchanged App. It is likely that a considerable amount of polymerization had occurred; self-condensation of 1- or 2-pyrrolines is a general phenomenon, e.g. an attempted preparation of 1-methyl-2-pyrroline has been reported to give the dimer and the trimer, but no 1-methyl-2-pyrroline [30], and 1-pyrroline readily forms a trimer [31]. Subsequently, it has been reported [26] that App forms a stable hydrodiperchlorate.

Derivatization of App proved difficult. It did not form a dinitrophenylhydrazone, indicating that no carbonyl groups were present, and that the formation of App from 8-amino-5-azaoctanal in aqueous solution was irreversible, as shown in Scheme 1. Although App did react with ninhydrin, as would be expected for a compound with a free $-CHR-NH_2$ group, dansylation by the method of ref. [32] was unsuccessful; several products appeared to be formed: a weak spot was found (R_f 0.18, TLC on silica gel G using cyclohexane–ethyl acetate, 5:4 v/v) at about the position of dansylated spermine, together with a large amount of free dansylamide. Other reagents for primary amines were no better; derivatization with trifluoroacetic anhydride by the method of ref. [33] gave no product detectable by GC on an OV-17 column, while the dark brown colour obtained with the fluorodinitrobenzene reagent of ref. [34] is characteristic of certain heterocyclic compounds. These results show that App does not behave as a typical primary amine, in accordance with the equilibrium postulated (see Scheme 1). Cyclic iminium salts, such as 1-pyrroline, react with 2-aminobenzaldehyde to give condensation products which can be oxidized to stable substituted quinazolones, and this has been used as a method for detecting and estimating 1-pyrroline and related compounds [35]. However, App did not react with 2-aminobenzaldehyde, indicating that App does not occur in aqueous solution in the monocyclic pyrrolinium ion form (Scheme 1), but undergoes spontaneous cyclization to 1,5-diazabicyclo[4.3.0]nonane, which is also the only form of the compound existing in solution in organic solvents [25].

In earlier work Dap has been detected in seedlings of barley (2–25 nmol per g fr. wt) [1, 7], wheat (unquantified)



Scheme 2.

[13] and oats (1.3–11.5 nmol per g fr. wt [12], 82–202 nmol per g fr. wt [14] and 50–220 nmol per g fr. wt [15]). App has previously been detected only in barley (4–37 nmol per g fr. wt) [7]. Concentrations of Dap, Put and App in the present work were greatest in the leaves of the youngest (6-day old) light-grown oat seedlings (Table 1). In these, Dap was *ca* 2.5 times more abundant than Put, though in older leaves and especially in the flag leaf and developing ears, Put was much greater than Dap. Out of the three amines in both light- and dark-grown oat seedlings, App was consistently in the smallest concentrations. In the dark-grown seedlings each of the three amines appeared to reach a peak after day 6 and then declined. In the light-grown shoots the ratio of the concentrations of each amine on the 6th and the 21st day was roughly constant (3.5:3.6:3.4 for Dap:Put:App). A similar relationship was found in the dark-grown material. These changes with age were independent of the percentage dry wt, which remained almost constant with age (Table 2).

By contrast with the light-grown seedlings of comparable age, Put was the predominant amine. Cadaverine was not detected, in agreement with the work of Flores and Galston [12]. Amounts of Dap and Put were similar to those found by Kaur-Sawhney *et al.* [15]. In their work, Dap declined from 220 nmol per g fr. wt on the 4th day to 59 nmol per g fr. wt on the 21st day and Put fell from 52 nmol per g fr. wt to 32 nmol per g fr. wt over the same period.

Only relatively small changes in PAO with age were found in the light-grown oat seedlings (Table 2). Estimates of PAO made in the leaves from dark-grown oat seedlings 14–21 days old [4] were comparable with those found in the present studies. In other work PAO activities were reduced by half in one to three weeks' growth in light-grown leaves of oat seedlings [2], confirming the downward trend with age suggested by the present work. A significant peak of activity occurred on day 14 and the activity declined to a minimum value on day 20.

Dap, App and Put were also found in barley, wheat and maize (Table 3). The concentrations of Dap and App in 6-day old seedlings of maize were smaller than in comparable oat seedlings, though the Put concentrations were similar. The concentration of the three amines was significantly smaller in barley and wheat than in maize and oats. Neither Dap nor App could be detected in pea seedlings.

Oxidation of spermidine by the polyamine oxidase yields Dap, hydrogen peroxide and 1-pyrroline. In the present work, utilizing the technique of Sakamoto and Samejima [35], the presence of 1-pyrroline has been investigated in distillates of alkaline leaf extracts containing *ca* 2.5 M sodium hydroxide. Since in maize, labelled spermine is metabolized to 4-aminobutyric acid, it appears likely that 1-pyrroline is an intermediate in the degradation of spermine via App [36]. Dap is degraded to 3-aminopropionic acid (β-alanine) in maize [37].

Experiments using Dowex 50 resin for pyrroline estimation, unlike App, gave low recoveries, and a distillation technique was therefore developed for purifying this amine. Using this method (see Experimental) recoveries of 250 nmol pyrroline/g fr. wt were 89, 81 and 84% in triplicate determinations in the presence of oat leaf extracts. For characterization of pyrroline [35], the 2-aminobenzaldehyde adduct was oxidized by chromium trioxide to 2,3-trimethylene-4-quinazolone, a reaction which is stoichiometric. The product is stable and was found to give a mass spectrum with a large $[M]^+$ yield (see Experimental).

The Strecker degradation of proline [38], and oxidation of Put in alkaline conditions were considered to be potential artefactual sources of pyrroline. However on adding proline or Put to the plant extracts in amounts equivalent to 20 μmol/g fr. wt prior to distillation, these potential precursors were without effect on pyrroline concentrations.

Pyrroline was detected by GC on the Carbowax 20 M/KOH column in the leaves of spinach and in the shoots of oat, maize, pea and radish seedlings (Table 4). Concentrations in oat and pea seedlings grown in potassium-deficient (−K) media were not significantly increased. Pyrroline was further characterized by GC-MS

Table 1. Concentrations of diaminopropane, putrescine and 1-(3-aminopropyl)-pyrrolinium in the leaves of oat seedlings grown at 23° in diurnal light or darkness

			nmol/g fr. wt		
Growth conditions	Age (days)	Tissue	Diamino-propane	Putrescine	Aminopropyl-pyrrolinium
Diurnal light	6	Shoot	153.8	66.6	21.3
	10	Shoot	63.3	34.3	15.1
	14	Shoot	46.5	18.7	6.2
	21	Shoot	43.9	18.5	6.2
	46	4th leaf	17.4	12.5	3.2
	83	Flag leaf	<0.5	11.2	<0.5
	83	Developing ears	<0.5	23.3	<0.5
Darkness	6	Shoot	15.8	48.7	5.0
	10	Shoot	29.4	70.9	12.5
	14	Shoot	22.8	51.4	6.0
S.E.			6.5	6.5	3.5

Values given are the means of three independent estimates (S.E. = standard error).

Table 2. Dry weights and polyamine oxidase activity in oat seedlings grown in diurnal light at 23°

Age (days)	Dry wt (%)	Polyamine oxidase (nkat/g fr. wt)
6	8.0	48.3
10	8.2	45.6
14	8.4	55.5
20	8.7	34.4
S.E.	0.86	2.64
L.S.D. (5%)		7.3

Values shown are means of duplicates from independent extracts for the enzyme estimations.

Table 3. Concentrations of diaminopropane, putrescine and 1-(3-aminopropyl)pyrrolinium in the leaves of barley, wheat and maize seedlings grown for 6 days in a greenhouse

	nmol/g fr. wt		
	Diaminopropane	Putrescine	Aminopropyl-pyrrolinium
Maize	38.9	79.4	8.7
Barley	2.5	34.7	1.4
Wheat	6.0	28.5	<0.5

Values given are means of three independent estimates. Standard error 1.15 nmol/g.

Table 4. The pyrroline concentration of plants

Plant		Age (days)	Pyrroline (nmol/g fr. wt)
Oats	Shoots	7	10
		14	14*
		23	22
		42	7
Maize	Shoots	70	15†
Pea	Shoots	8–26	<2‡
Radish	Shoots	12	20
Spinach	Leaf	Mature	21
Carrot	Root	Mature	<2
Lettuce	Leaf	Mature	<2

The pyrroline was estimated by GC as the free base after distillation (see Experimental).

*Detected by GC-MS after derivatization with 2-aminobenzaldehyde (see Experimental).

†In potassium-deficient maize.

‡Detected by GC-MS, together with piperideine (5 nmol/g fr. wt) in 14-day old potassium-deficient pea leaves.

of the oxidized 2-aminobenzaldehyde adduct in oat and pea extracts, and by electrophoresis of the 2-aminobenzaldehyde adduct using ninhydrin as chromogenic reagent (Table 5) in oat leaf extracts.

GC-MS of the amine fraction from +K pea seedlings also indicated the presence of isoamylamine ($Me_2CH(CH_2)_2NH_2$; R_t 3 min). Isoamylamine, which is probably formed as a decarboxylation product of leucine, has not previously been found in the pea plant [39]. Pyrrolidine (R_t 4.5 min) could not be found in any of the

Table 5. Electrophoretic mobilities on Whatman 3 MM paper of 1-(3-aminopropyl)pyrrolinium and related compounds

	Acetic acid, pH 2.4	0.1 M Pyridine–acetic acid, pH 4.6	0.1 M Pyridine–acetic acid, pH 6.1	Na citrate, pH 4.6	Ninhydrin colour
Diaminopropane	12.9	11.5	10.0	26.0	Brown-pink
Putrescine	11.8	10.8	9.6	26.4	Pink
Spermidine	10.9	10.1	8.8	18.0	Pink
Spermine	10.2	9.4	8.4	13.1	Red-pink
Aminopropylpyrrolinium	11.2	8.6	5.7	23.0	Pink
Aminopropylpyrrolidine	10.2*	9.2	8.1	22.0	Pink
Agmatine	10.7	9.0	8.6	23.0	Pink
Pyrroline	7.6	4.8	6.5	—*	Red-brown
Pyrroline adduct†	—	4.5	—	—	Pink
Pyrrolidine	8.0	7.4	7.3	—	Yellow
4-Aminobutanol	7.6*	5.9	4.5	—	Pink
4-Guanidinobutyraldehyde	—	—	5.6	—	—
Methyl green	4.6	3.6	3.6	7.5	—

Methyl green is a basic dye which serves as a visual mobility reference. All mobilities (in cm/hr) are normalized for 10 V/cm.

*Streaked.

†Condensation product of pyrroline with 2-aminobenzaldehyde.

plant extracts. Synthetic 4-guanidinobutyraldehyde could not be detected by GC on the Carbowax column, though it was detected by the dinitrophenylhydrazine reagent after electrophoresis. No evidence was found for the presence of this aldehyde in the plant extracts.

Dap, Put and App could be detected in the amine fractions of oat leaf extracts after electrophoresis, with ninhydrin as chromogenic reagent. Pyrroline could be identified by the ninhydrin reagent as the 2-aminobenzaldehyde adduct in the oat leaf extracts together with several unknowns.

The biological properties of App and pyrroline have not as yet been studied, but Dap is a strong *in vivo* inhibitor of ethylene biosynthesis [40] and Dap strongly promotes the growth of *Helianthus* tuber explants [41]. The function of the polyamine oxidase forming Dap, pyrroline and App may be to control the levels of the growth-promoting polyamines, though it is also possible that it could serve as a source of peroxide used in lignification, or as an antiseptic. The location of this enzyme in the cell walls would be compatible with both of these functions.

EXPERIMENTAL

Plant material. (a) For App estimations: oat seedlings (*Avena sativa* L., cv. Black Supreme) were grown in darkness or in the light (16 hr day, 300 $\mu E/m^2/sec$) at 23°, in vermiculite and watered daily with a nutrient soln consisting of KNO_3 (4 mM), $Ca(NO_3)_2$ (4 mM), $MgSO_4$ (1.5 mM) and NaH_2PO_4 (1.33 mM) with FeEDTA and micronutrients. Seedlings of barley (*Hordeum vulgare* L. cv. Proctor), wheat (*Triticum vulgare* L., cv. Musket), maize (*Zea mays* L. cv. Kelvedon Glory) and pea (*Pisum sativum* L. cv. Alaska) were grown in a greenhouse and watered with the above nutrient solns.

(b) For pyrroline estimations: Oat (*Avena sativa* L. cv. Trafalgar), maize (*Zea mays* L. cv. Kelvedon Glory), pea (*Pisum sativum* L. cv. Meteor) and radish (*Raphanus sativus* L. cv. Crimson French Breakfast) were grown in the light (16 hr day 300 $\mu E/m^2/sec$) in a mixture of sand and perlite (1:1) and watered on alternate days with nutrient solns of ref. [42]. Spinach (*Spinacia oleracea* L. cv. Fabris) was grown in a glasshouse in a high nitrate nutrient soln. Other plants were obtained from a local supermarket.

Synthesis of 1,5-*diazabicyclo*[4.3.0]*nonane.* (1) *From spermine.* 0.5 M NaOH (30 ml) was added to 0.5 g spermine (4HCl) (Fluka), with 3–4 g of freshly prepared Cu powder [43] in a 250 ml round bottomed flask. The flask was stoppered and shaken vigorously for 4 hr, admitting air at intervals of 30 min. The contents were then centrifuged and the supernatant was extracted with 4 × 50 ml of Et_2O. The Et_2O was dried over Na_2SO_4 for 18 hr and after filtration, the Et_2O was removed under a stream of N_2. It is important not to prolong this step as the App can be lost by volatilization. The residue was fractionally distilled under red. pres. using a cold finger. Most of the App was recovered at 30–40° in high purity (yield 30%), though *ca* 50% of the distillate was H_2O. Since it was not easy to remove the H_2O by distillation without loss of App, the App concn was determined by GC. A standard soln of App was prepared by oxidation to completion of a known amount of spermidine, using pea seedling diamine oxidase [18]. The products in this oxidation are App, NH_3 and H_2O_2. The H_2O_2 is removed by catalase as it is formed. After standardization by GC, the soln of synthetic App was neutralized with HCl and diluted to prepare a 25 mM stock soln.

(2) *From 4-chlorobutanol and diaminopropane.* 4-Chlorobutanol (10 ml) in dry CH_2Cl_2 (30 ml) was added dropwise over 0.5 hr to a vigorously stirred mixture of pyridinium chlorochromate (32.4 g) and dry CH_2Cl_2 (100 ml). After stirring for another 1 hr, *n*-pentane (200 ml) was added, the soln decanted and the residual black sludge washed with *n*-pentane (2 × 100 ml). The combined *n*-pentane extracts were filtered through Celite and evaporated *in vacuo*. The residue was taken up in *n*-pentane (100 ml) and applied to a dry column of silica gel (13.4 g). Elution with *n*-pentane (200 ml) and evaporation gave the crude aldehyde as a colourless liquid. Crude yield: 5.36 g (quantitative). ^1H NMR ($CDCl_3$): δ9.70 (*s*), 3.56 (*t*, J = 6 Hz), 2.58 (*t*), 2.08 (*m*). The crude aldehyde was used without further purification.

Freshly distilled 1,3-diaminopropane (4.3 ml) was added to a stirred soln of 4-chlorobutanal (5.36 g) in CH_2Cl_2 (200 ml), followed by dry K_2CO_3 (25 g). The mixture was stirred for 18 hr at room temp., filtered to remove K salts and evaporated to dryness. The residue, a pale-coloured liquid, was distilled under red. pres. Fraction 1, bp 35–42°/5–6 mm Hg (0.33 g), and fraction 2, bp 46–48°/4.5–5 mm Hg (1.24 g), were both the desired product; combined yield 1.57 g (13% overall from 4-chlorobutanol). ^1H NMR ($CDCl_3$): δ2.22 (*s*) overlapping, 1.3–3.3 (complex *m*). ^{13}C NMR ($CDCl_3$): δ19.12, 25.95, 30.01, 45.51, 51.41, 51.68 and 78.66. MS *m/z* (rel. int.): 126 $[M]^+$ (63), 125 (98), 98 $[M-C_2H_4]^+$ (100), 97 (42), 84 $[M-C_3H_6]^+$ (35), 70 (77), 69 (51), 68 (38), 56 $[C_4H_8]^+$ (32), 44 $[C_2H_6N]^+$ (32), 43 (48), 41 $[C_3H_5]^+$ (73).

App and 1(3-aminopropyl)-2-pyrrolidinone (Aldrich) (20 μl of each) were reduced using Sn and 1 ml 11 M HCl to aminopropylpyrrolidine. This was identical (electrophoretic mobility, Table 5) with the product obtained by reduction of App using Na cyanoborohydride.

Synthesis of 1-(3-*aminopropyl*)-3-*pyrroline.* This was prepared by the method of ref. [44]. ^1H NMR ($CDCl_3$): δ1.55, (2H, *q*, J = 7 Hz), 2.72, (4H, *t*, J = 7 Hz), 3.42 (2H, *s*), 5.71 (2H, *s*). MS (probe), *m/z* (rel. int.): 126 $[M]^+$ (22), 82 $[C_5H_8N]^+$ (100), 81 (26), 80 (25), 68 $[C_4H_6N]^+$ (97), 55 $[C_3H_5N]^+$ (60), 44 $[C_2H_6N]^+$ (31), 41 $[C_3H_5]^+$ (30).

Polyamine oxidase activity was estimated in extracts prepared from 5 g samples in 15 ml 0.1 M citrate buffer, pH 6, containing 1 M NaCl at 4°. The solns were centrifuged and dialysed against the same buffer and activity was estimated by an O_2 electrode using 2 ml buffer, 0.1 ml extract and 0.1 ml catalase (1 mg/ml), and the reaction started with 0.1 ml of 25 mM spermine (4HCl). Activity was assessed by measurement of the slope of the line representing O_2 consumption within the first 2 min after adding spermine, since activity declines rapidly with time [18].

Estimation of App in plant extracts. The amine fraction was isolated in principle by the method of ref. [33]. Freshly harvested samples (10 g fr. wt) of plant material were extracted into 100 ml of 5% $Cl_3C \cdot CO_2H$ and allowed to stand for 18 hr at 4°. After centrifuging, the supernatant was applied to a column of Dowex cation exchange resin (50 W × 4, 200–400 mesh) (2 cm diam. × 3 cm). The column was then washed with 60 ml of H_2O, followed by 60 ml of NaPi buffer prepared by titrating together NaH_2PO_4 (0.1 M) and Na_2HPO_4 (0.1 M) to pH 7.6, and then adding NaCl to 0.25 M. After washing with 60 ml of 0.1 M HCl, the amine fraction was eluted with 60 ml of 6 M HCl. Recovery (mean of duplicate estimations) for App was 90% in the absence of plant material and 84% in its presence. After evaporation to dryness, the amines were dissolved in 2 ml 2.5 M NaOH. Samples (10 μl) were injected for GC, which was effected on a 2 m × 4 mm column of Carbowax 20 M (10%) and KOH (5%) on Chromosorb W (100–120 mesh) at 145° using FID with N_2 as carrier gas (20 ml/min) [1, 45]. The R_ts (min) were diaminopropane, 2.0; putrescine, 3.2; App, 3.9; cadaverine, 4.4; and 1-(3-aminopropyl)-3-pyrroline, 5.8. In early experiments, hexamethylene diamine (R_t 7.3 min) was used as an internal standard,

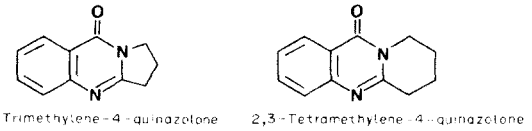
though this was omitted in later work since it tends to tail. The peak areas were electronically integrated. All estimations were made from triplicated extracts.

GC-MS of App. The shoots of light-grown 14-day old oat seedlings (300 g) were extracted into 0.5 M NaOH (1 l.) and the extracts stored at 4° for 5 days. After filtration and centrifuging, solid NaOH was added to provide a concn of 5 M and the App was extracted with 2 × 500 ml of Et_2O. On concn to 12 ml, 10 μl samples were injected for GC-MS with the Carbowax 20 M/KOH column.

Electrophoresis. The amine fraction of 290 g of 6-day-old light-grown oat leaves prepared by the ion exchange method, was dissolved in 10 ml of 5 M NaOH. The App was extracted into Et_2O (2 × 2.5 ml) and 50 μl samples applied to Whatman 3 MM paper. The pyridine buffer (pH 4.6) was prepared from 20 ml of C_5H_5N and 25 ml of HOAc made up to 1 l. with H_2O. The citrate buffer (pH 4.6) was prepared by titrating 0.1 M citric acid with 0.1 M NaOH. The amine spots were more diffuse after electrophoresis in the citrate buffer, although mobilities were much greater. The separation of the amines was effected at 2000 V (80 mA) with paper 56 cm long × 23 cm wide). After electrophoresis the paper was dried and the amines were detected with ninhydrin (0.25% in Me_2CO) used as a dip, followed by heating at 100° for 10 min. The mobilities of various amines are given in Table 5.

Estimation of pyrroline. Authentic 1-pyrroline was obtained by hydrolysis of 4-aminobutyraldehydediethylacetal (Aldrich) in 0.1 M HCl [46]. GC of samples on the Carbowax 20 M/KOH column gave pyrroline (R_t 5 min) and EtOH (R_t 2.4 min) at 70°. Hydrolysis was complete in 30 min at room temp. 2-Aminobenzaldehyde was from Fluka and pyrrolidine from Aldrich. Authentic piperideine and 4-guanidinobutyraldehyde were formed on oxidation of cadaverine and agmatine respectively, using pea seedling diamine oxidase. Partially purified pea seedling diamine oxidase (0.2 ml, 100 nkat/ml) was incubated with 1 ml pH 7, 0.1 M NaPi buffer, 0.2 ml catalase (1 mg/ml) and 1 ml of 25 mM substrate for 3 hr at 30°. The reaction was then stopped by adding 250 μl of 1 M HCl. For GC, 50 μl of this soln was added to 50 μl of 1 M NaOH and 10 μl injected, using the Carbowax 20 M/KOH column described above. To determine the pyrroline in the plant material, samples (10 g) were extracted in 50 ml 0.1 M HCl and allowed to stand for 18 hr at 4°. After filtration, the extract was placed in a two-necked 100 ml round flask at 60°. One neck led to a trap and on to an absorption column containing 10 ml 0.1 M HCl. O_2-free N_2 gas was admitted to the other neck (150 ml/min). After addition of 50 ml of 5 M NaOH and two drops of silicone antifoam (Antifoam-A, Midland Silicones), N_2 gas was passed for 3 hr. To 50 μl of the HCl soln was added 50 μl 1 M NaOH, and 20 μl was injected immediately for GC (R_t pyrroline 5 min at 70°; isothermal; N_2, 20 ml/min) using the Carbowax 20 M/KOH column [45].

Characterization of pyrroline and piperideine. Since pyrroline was completely lost by adsorption to the separator on GC-MS using the Carbowax 20 M/KOH column, characterization was effected after derivatization according to a modification of the method of refs [35, 47]. Samples of the distillates (1 ml in 0.1 M HCl) and of the authentic amines were placed in screw top tubes. 1 ml of 50 mM 2-aminobenzaldehyde in 50 mM HCl was added and the tubes were then incubated for 15 min at 80° [48]. 1.5 ml of 0.64 M CrO_3 in 0.8 M H_2SO_4 was added to the tubes and these were then incubated for a further 15 min at 80°. The derivative was then extracted in Et_2O (1 ml) and 10 μl injected for GC using 2% OV 17 on Chromosorb W-HP (1 m × 4 mm) at 180° with N_2 at 20 ml per min. 2,3-Trimethylene-4-quinazolone (from pyrroline) had R_t 6.3 min and 2,3-tetramethylene-4-quinazolone (from piperideine) had R_t 8.5 min.



Scheme 3.

Mass spectra: 2,3-*Trimethylene-4-quinazolone* (product of pyrroline) *m/z*: 186 $[M]^+$ (86), 185 (100), 160 (5), 144 (3), 130 (10), 103 (81), 102 (8), 76 (10). 2,3-*Tetramethylene-4-quinazolone* (product of piperidcine) *m/z*: 200 $[M]^+$ (100), 199 (78), 185 (40), 184 (7), 173 (10), 160 (8), 144 (7), 119 (10). *Isoamylamine* (free base) *m/z*: 87 $[M]^+$ (10), 85 (9), 71 $[Me_2CH(CH_2)_2-]^+$ (15), 57 $[Me_2CH_2]^+$ or $[-CH(CH_2)_2NH_2]^+$ (100), 56 (20), 43 $[Me_2CH]^+$ (40).

Amine analyser. Using the system of ref. [49], App was eluted close to Dap and Put, while pyrroline was eluted with arginine. However, on elution with 0.2 M Na citrate buffer, pH 6.6, containing 1 M NaCl, App was eluted after 40 min while Dap and Put were eluted after 70 min and 90 min respectively.

Acknowledgements—The authors are grateful to David Puckey of the Meat Research Institute, Langford, for the GC-MS, and to Sally Todd for the statistical analysis of the results.

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