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Gibberellin Metabolism: Objectives and Methodology

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Abstract. The objectives and methodology of metabolic studies of the gibberellins are discussed with particular reference to the rôle of gibberellins in the promotion of stem elongation in gibberellin-responding dwarf mutants of pea.

The gibberellins (GAs) occur in most, if not all, higher plants. When applied to plants the GAs promote normal processes of plant growth and development. Thus the GAs have been classified as plant hormones. However, the use of the description "plant hormone" for the GAs (and for abscisic acid, auxin, cytokinin and ethylene) has been questioned from time to time and most recently by Trewavas (1981, 1982) and Weyers (1984). To apply the term "hormone" in plant biochemistry as it was strictly defined (Starling 1905) in mammalian biochemistry is arguable. However its continued use in the simple etymological sense as a natural "impeller" can be justified. The most important point, however, is that a debate on semantics does not obscure serious scientific enquiry.

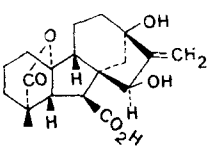
Trewavas (1981, 1982) has theoretised that the responses of plants to natural plant hormones is not regulated by quantitative changes in hormone concentrations but by changes in tissue sensitivity (amounts of a receptor molecule). Recent work on GA-responding dwarf mutants of maize (Spray *et al.* 1984) and pea (Ingram *et al.* 1984) have an important bearing on this point. These studies have been built upon the secure foundations of previous metabolic studies. For example, the *in vivo* investigations of the metabolism of GAs in developing seeds of pea have identified the native GAs and established the steps $GA_{20} \rightarrow GA_{29} \rightarrow GA_{29}$-catabolite (for leading references see Sponsel 1983a,b). In addition the work of Kamiya and Graebe (1983), using cell-free preparations from pea cotyledons, established the "early 13-hydroxylation pathway" from GA_{12} aldehyde to GA_{29}. This whole body of work provides an excellent example of the value of metabolic studies. The opportunity is therefore taken to discuss our general approach to GA-metabolism, exemplifying it by reference to GA-metabolism in peas (Ingram *et al.* 1984, Gaskin *et al.* 1984) and, at the same time, providing a background to the following paper on maize mutants.

OBJECTIVES AND EXPERIMENTAL SYSTEMS

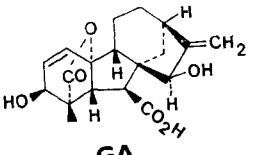
The objectives of metabolic studies are to determine the pathways, to characterise the enzymes of these pathways, to locate the sites of metabolism, to investigate the control mechanisms of the metabolic steps and finally to relate these findings to GA-function. Specific questions must be posed and the appropriate plant system selected. One important question is "Do GAs play a qualitative and/or quantitative rôle in promoting stem elongation?" This question can be posed in relation to photoperiodic (GIANFAGNA *et al.* 1983) or genetic (INGRAM *et al.* 1984, SPRAY *et al.* 1984) factors. To answer this question, single gene mutants which respond to applied GAs by tall phenotypic growth (BRIAN and HEMMING 1955, PHINNEY 1956) provide an excellent plant system.

IDENTIFICATION OF NATIVE GIBBERELLINS

The first step is to identify the native GAs in the selected plant system. The criteria required for the identification of a plant constituent have been vigorously debated recently. REEVE and CROZIER (1980, 1983) have advocated the application of information theory and have concluded that chromatographic methods such as HPLC can provide sufficient bits of information for the identification of plant hormones. SCOTT (1982, 1983) and MACMILLAN (1984) have put the view that, in practice, no method provides sufficient bits of independent (non-correlated) information for identification and that identification is still best made by correlation of analytical data in terms of a chemical structure. The best proven empirical method is still capillary gas chromatography coupled to a mass spectrometer linked to a dedicated data processor (C-GC-MS-C). This method provides both retention time and mass spectra by which data all 68 known GAs can be distinguished. The technique can also detect new GAs for which tentative chemical structures can be deduced from the empirical correlation between mass spectral fragmentation and known chemical structures. The structures of these new GAs can then be confirmed by synthesis. For example GA_{67} and GA_{68}, the latest GAs to be characterised, were detected by C-GC-MS-C in extracts from sunflower and apple seed respectively. The structures, shown below, were deduced from the mass spectra of their Me ester bis-TMSi ethers and were confirmed (DOLAN and MACMILLAN, unpublished results) by synthesis from GA_3 and GA_7 respectively.



GA_{67}



GA_{68}

Our methodology for free GAs is to isolate the total acids which are soluble in ethyl acetate by the usual extraction of the plant material with aqueous methanol, followed by partitioning at *ca.* pH 8.0 then at *ca.* pH 3.0. It should be noted that a preliminary extraction of the aqueous extract at pH 3.0 with chloroform before extraction with ethyl acetate (see KELLER and COUL-

TER 1982) results in the loss of 85 % GA_{20} and 35 % GA_1 (GASKIN *et al.* 1985). For C-GC-MS-C the concentration of each GA in the ethyl acetate-soluble acids must be increased in concentration to at least 1 part in 10^4. This achieved by the appropriate combination of methods such as partitioning between 80 % aqueous methanol and light petroleum (b.p. 60—80 °C), treatment

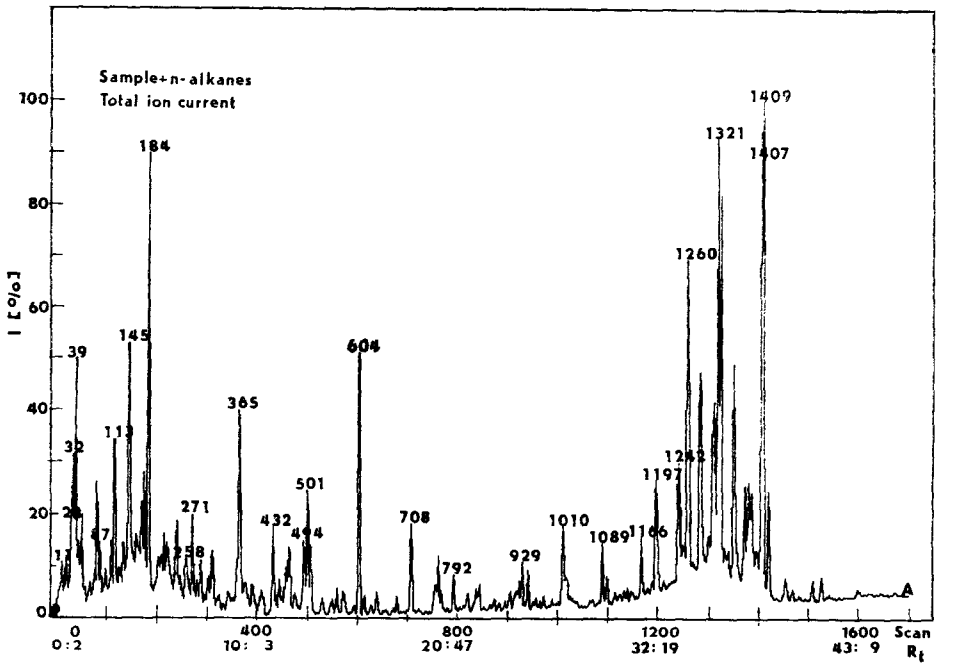


Fig. 1. Total ion current trace of a combined selection of RP—HPLC fractions from expanding apical tissue of an *Le*-genotype of pea.

with polyvinylpyrrolidone, partition chromatography and reversed phase HPLC (RP-HPLC). These purification methods need not separate GAs from each other (capillary GC does that). Indeed the less separation of GAs the smaller the number of fractions that have to be analysed by C-GC-MS-C. Purified fractions containing 1 part GA per 10^4 total dry mass are methylated with diazomethane in methanol, evaporated to dryness in a stream of nitrogen gas and heated with N-methyl-N-trimethylsilyltrifluoroacetamide. The solution of the Me esters TMSi ethers is adjusted, by adding dichloromethane, to a concentration of *ca.* 10 g^{-9} of each GA in 1.0 µl, a convenient volume for injection into the capillary GC. A mixture of *n*-alkanes (C_{16}—C_{34}) is co-injected with each sample. The GC and MS operating conditions are described in INGRAM *et al.* (1984). The MS data are acquired by computerised cyclic scanning over 1.2 s at 0.7 s per decade, usually m/z 660—60, and are processed as indicated in Figs. 1—3.

The basic data from which GA_1, GA_{29} and GA_8 were identified in expanding apical tissue of pea plants is shown in Fig. 1. The total ion current trace was obtained from a selected combination of fractions from the final purification

step of RP-HPLC. The amount injected corresponds to the acids, obtained from three-twentieths of one plant and was shown to contain 2×10^{-9} to 5×10^{-9} g of each GA by isotope dilution as described in the following section. Fig. 2 shows the selected ion current trace for the ion, m/z 85, characteristic of the *n*-alkanes. Thus the retention times of peaks in the sample can be

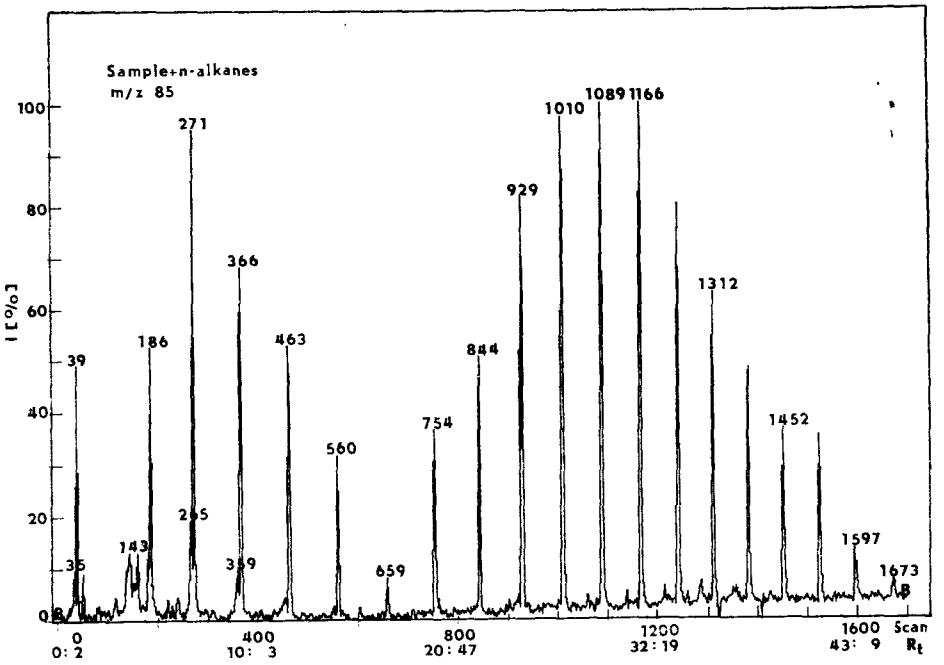


Fig. 2. Selected ion current trace for m/z 85 from total ion current trace, shown in Fig. 1.

measured and recorded as Kovats retention indices (KRI). Fig. 3 shows the selected ion current traces for m/z 506 (the molecular ion of GA_1 MeTMSi and GA_{29} MeTMSi) and for m/z 594 (the molecular ion of GA_8 MeTMSi). These molecular ion peaks have the correct KRI values for the MeTMSi derivatives of GA_1, GA_{29} and GA_8 and these identifications were further established from the full scan mass spectra, obtained from the appropriate scans and published in the paper by INGRAM *et al.* (1984).

A full scan mass spectrum showing ions down to 5 % intensity of the base peak can be obtained from about 5×10^{-11} g of a GA MeTMSi. The ethyl-acetate-soluble acids from 1.0 g of plant material can usually be reduced by the normal purification methods to a mass which can be injected into the C-GC-MS. Assuming 50 % recovery of GAs during the extraction and purification processes, the limit of detection is approximately 1×10^{-10} g GA per g plant material. The usual levels of GAs are about 10^{-6} g per g seed and about 10^{-9} g per g non-reproductive tissue.

The quoted limits of sensitivity are average; they may be greater or less depending on the degree of GC-separation of individual GAs from other components in the fraction.

LABELLED GIBBERELLINS

Having identified the native GAs the next step is to obtain these native GAs, labelled with both a radio-isotope and a stable isotope. The reasons for using these doubly-labelled substrates have been given by SPONSEL and MACMILLAN (1977, 1978). Recoveries and percentage conversions can be

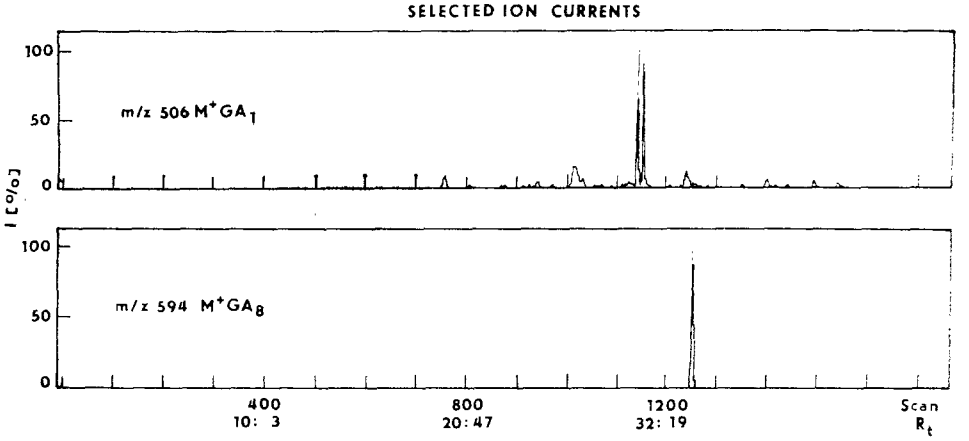


Fig. 3. Selected ion current traces for m/z 506 and m/z 594 from total ion current trace shown in Fig. 1.

determined from the radioactivity. The presence of the stable isotope, detected by C-GC-MS-C, in a metabolite establishes its formation from the substrate fed. From the dilution of the stable isotope in the metabolites and recovered substrate, the levels of endogenous unlabelled compounds can be measured. In time-course studies the rates of metabolism of the endogenous and applied GA can be compared by measuring the ratio of labelled and unlabelled substrate and metabolites. Gibberellins, labelled with stable isotopes, may also function as carriers for the unlabelled GA, present at too low an endogenous level for direct detection by full scan mass spectrum (GASKIN *et al.* 1985).

Methods of preparing labelled GAs have been reviewed by MACMILLAN (1980) and by TAKAHASHI and YAMAGUCHI (1983). Recently MACMILLAN and WILLIS (1984) have described the preparation of [1β, 2β-$^2H_2$]GA_4 (Fig. 4) by catalytic deuteriogenation, in which the 16,17-double bond is protected as the 16,17-epoxide. The same methodology has been applied to the preparation of [1β,2β-$^2H_2$]GA_1 (Fig. 4) and [1ξ,2ξ,3ξ-$^2H_3$]GA_{20} [Fig. 4] from GA_3 (MACMILLAN and WILLIS, unpublished work). Using these procedures the corresponding (1ξ,2ξ,3ξ-$^3H_3$]GA_{20} (Fig. 4) has been made with high specific activity and with 90 % radiochemical purity. The parallel preparation of [1β,2β-$^3H_2$]GA_4 and [1β,2β-$^3H_2$]GA_1 is being undertaken by Amersham International p.l.c.

EXPERIMENTAL PROTOCOL FOR FEEDS

The doubly labelled GA is usually applied by injection or by surface application of a solution. It should, if possible, be applied in an amount which is experimentally determined to give an approximate 1 : 1 ratio of labelled

and unlabelled GA in the plant system. Thus perturbation of the normal metabolism of the endogenous GA is minimised and the precision of measurement of the ratio of labelled and unlabelled metabolites and recovered substrate is maximised. Immediate extraction gives the endogenous level of the GA at the beginning of the experiment and extraction at selected intervals of time gives a measure of the kinetics of metabolism.

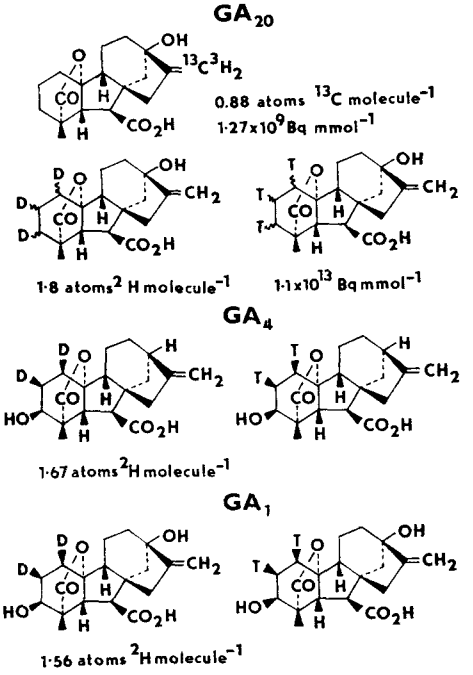


Fig. 4. Isotopically labelled GAs, referred to in the text.

GIBBERELLINS AND STEM ELONGATION IN PEA

Using the methodology outlined in the previous sections, INGRAM *et al.* (1984) have examined the rôle of the *Na* and *Le* genes on the levels of GAs and metabolism of [17-^{13}C, 17-$^{3}H_2$]GA_{20} (Fig. 4) in the expanding apical region of 20-day old light-grown seedlings of pea plants.

In the *Le* lines, the [^{13}C,^{3}H]GA_{20} was metabolised to [^{13}C,^{3}H]GA_1, [^{13}C,^{3}H]GA_8 and [^{13}C,^{3}H]GA_{29}. In contrast, no [^{13}C,^{3}H]GA_1 was detected as a metabolite of [^{13}C,^{3}H]GA_{20} in plants homozygous for the *le*-gene; the only products were [^{13}C,^{3}H]GA_{29} and, in one case, [^{13}C,^{3}H]GA_{29}-catabolite. These results confirm the earlier suggestion (POTTS and REID 1983, INGRAM *et al.* 1983) that the *Le*-gene controls the 3β-hydroxylation of GA_{20} to GA_1 in the expanding apical tissue of pea seedlings. Similar results, discussed in the following paper, have been obtained for the *dwarf*-1 mutation in maize (SPRAY *et al.* 1984). The simplest interpretation of these results is that GA_1 is a qualitative and quantitative requirement for stem elongation in these GA-responding dwarf mutants. It is difficult to explain these results in terms of a difference in the levels of a GA-receptor (TREWAVAS 1981, 1982).

It is important to note, however, that the influence of the *Le* gene may be confined to the immature expanding tissue of the pea shoot (POTTS *et al.* 1982, REID *et al.* 1983). Furthermore, seeds of the tall cultivar, Alaska, which carries the dominant allele, *Le*, contain GA_{20} (EEWENS *et al.* 1973) but no detectable amounts ($<10^{-10}$ g per g fresh mass) of GA_1 (GASKIN *et al.* 1985). These results may, however, be clarified by further experimentation on the control of expression of the *Le* gene. More difficult to explain is the observation (GASKIN *et al.* 1985) that dark-grown seedlings of both Alaska and Progress No. 9 which is homozygous for the recessive allele, *le*, contain the same amount of GA_1 (0.3—1.8 ng per g fresh mass) as judged by C-GC-MS-C measurement of the dilution of label in the added internal standard 3α-^{2}H]GA_1 (GASKIN *et al.* 1985).

CONCLUDING REMARKS

Metabolic studies are charting metabolic pathways through the maze of 68 GAs. From these pathways rational enquiries into the function of the GAs can be based. In the case of stem elongation, studies with single-gene mutants of pea and maize have revealed the importance of GA_1. As in all scientific endeavour, the results pose further questions such as: (a) the changes in GA status during the ontogeny of the pea plant; (b) the comparison of GA-metabolism in light- and dark-grown plants; (c) the nature of the enzymes which catalyse the conversion of GA_{20} to GA_1 and GA_{29} and of GA_1 to GA_8; (d) the subcellular location of these conversions; and (e) the regulation of gene expression of the *Le* and D_1 genes for the formation of the enzyme required for the formation of GA_1 from GA_{20}. The answers to these questions are currently being sought.

REFERENCES

BRIAN, P. W., HEMMING, H. G.: The effects of gibberellic acid on shoot growth and pea seedlings — Physiol. Plant. **8**: 669—681, 1955.

EEWENS, C. J., GASKIN, P., MACMILLAN, J.: Gibberellin A_{20} in seeds of *Pisum sativum* cv. Alaska. — Planta **115**: 73—76, 1973.

GASKIN, P., GILMOUR, S. J., MACMILLAN, J., SPONSEL, V. M.: Gibberellins (GAs) in immature seeds and dark-grown shoots of *Pisum sativum*: GAs identified in the tall cultivar Alaska in comparison with those in the dwarf Progress No. 9. — Planta **63** : 283—289, 1985.

GIANFAGNA, T., ZEEWAART, J. A. D., LUSK, W. J.: Effect of photoperiod on the metabolism of deuterium-labelled gibberellin A_{53} in spinach. — Plant Physiol. **72**: 86—89, 1983.

INGRAM, T. J., REID, J. B., POTTS, W. C., MURFET, I. C.: Internode length in *Pisum* IV. The effect of the gene *Le* on gibberellin metabolism. — Physiol. Plant. **59**: 607—616, 1983.

INGRAM, T. J., REID, J. B., MURFET, I. C., GASKIN, P., WILLIS, C. L., MACMILLAN, J.: Internode length in *Pisum*. The *Le* gene controls the 3β-hydroxylation of gibberellin A_{20} to gibberellin A_1. — Planta **160**: 455—463, 1984.

KAMIYA, Y., GRAEBE, J. E.: The biosynthesis of all major pea gibberellins in a cell-free system from *Pisum sativum*. — Phytochemistry **22**: 681—689, 1983.

KELLER, P. L., COULTER, M. W.: The relationship of endogenous gibberellins in light-regulated stem elongation rates in dwarf and normal cultivars of *Pisum sativum* L. — Plant Cell Physiol. **23**: 409—416, 1982.

MACMILLAN, J.: Partial synthesis of isotopically labelled gibberellins. — In: SKOOG, F. (ed.): Plant Growth Substances 1979. Pp. 161—164. Springer-Verlag, Berlin—Heidelberg—New York 1980.

MACMILLAN, J.: Analysis of plant hormones and metabolism of gibberellins. — In: CROZIER, A., HILLMAN, J. R. (ed.): The Biosynthesis and Metabolism of Plant Hormones. Society of Experimental Biology, Seminar Series Vol. 23. Pp. 1—16. Cambridge University Press, Cambridge 1984.

MacMillan, J., Willis, C. L.: N.m.r. assignments of ring A hydrogens in gibberellin A_4 methyl esters and some derivatives. — J. chem. Soc. Perkin I: 351—355, 1984.

Phinney, B. O.: Growth response of single gene mutants of maize to gibberellic acid. — Proc. nat. Acad. Sci. USA **42**: 185—189, 1956.

Potts, W. C., Reid, J. B.: Internode length in *Pisum* III. The effect and interaction of the *Na/na* and *Le/le* gene differences on endogenous gibberellin-like substances. — Physiol. Plant. **55**: 323—328, 1983.

Potts, W. C., Reid, J. B., Murfet, I. C.: Internode length in *Pisum*. I. The effect of the *Le/le* gene difference on endogenous gibberellin-like substances. — Physiol. Plant. **55**: 323—328, 1982.

Reeve, D. R., Crozier, A.: Qualitative analysis of plant hormones. — In: MacMillan, J. (ed.): Encyclopedia of Plant Physiology, New Series, Vol. 9. Pp. 203—280. Spinger-Verlag Berlin-Heidelberg-New York 1980.

Reeve, D. R., Crozier, A.: A reply to "Information theory and plant growth substance analysis" by I. M. Scott. — Plant Cell Environ. **6** : 365—367, 1983.

Reid, J. B., Murfet, I. C. Potts, W. C.: Internode length in *Pisum* II. Additional information on the relationships and action of loci, *Le*, *La*, *Cry*, *Na* and *Lm*. — J. exp. Bot. **34**: 349—364, 1983.

Scott, I. M.: Information theory and plant growth substance analysis. — Plant Cell Environ. **5**: 339—342, 1982.

Scott, I. M.: An answer to Reeve and Crozier's reply. — Plant Cell Environ. **6**: 367—368, 1983.

Sponsel, V. M.: *In vivo* gibberellin metabolism in higher plants. — In: Crozier, A. (ed.): The Biochemistry and Physiology of Gibberellins. Vol. 1. Pp. 151—250. Praeger Publ., New York 1983a.

Sponsel, V. M.: The localization, metabolism and biological activity of gibberellins in maturing and germinating seeds of *Pisum sativum* cv. Progress No. 9 — Planta **159**: 454—468, 1983b.

Sponsel, V. M., MacMillan, J.: Further studies on the metabolism of gibberellins (GAs) A_9, A_{20} and A_{29} in immature seeds of *Pisum sativum* cv. Progress No. 9. — Planta **135**: 129—136, 1977.

Sponsel, V. M., MacMillan, J.: Metabolism of gibberellin A_{29} in seeds of *Pisum sativum* cv. Progress No. 9; use of [^{2}H] and [^{3}H] GAs and the identification of a new GA catabolite. — Planta **144**: 69—78, 1978.

Spray, C., Phinney, B. O., Gaskin, P., Gilmour, S. J., MacMillan, J.: Internode length in *Zea mays* L. The dwarf-1 mutation controls the 3β-hydroxylation of gibberellin A_{20} to gibberellin A_1. — Planta **160**: 464—468, 1984.

Starling, E. H.:The chemical correlation of the functions of the body. — Lancet **2**: 339—341, 1905.

Takahashi, N., Yamaguchi, I.: Preparation and isotopic labelling of gibberellins. — In: Crozier, A. (ed.): The Biochemistry and Physiology of Gibberellins, Vol. 1. Pp. 457—484, Praeger Publ., New York 1983.

Trewavas, A.: How do plant growth substances work? — Plant Cell Environ. **4**: 203—228, 1981.

Trewavas, A.: Growth substance sensitivity. The limiting factor in plant development. — Physiol. Plant. **55**: 60—72, 1982.

Weyers, J.: Do plants really have hormones? — New Scientist **1410**: 9—13, 1984.