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Abstract. In this work the three theses below are discussed:

1) Identification and quantitative determination of the very strong plant hormone, the auxin 4-chloroindole-3-acetic acid methyl ester, in immature seeds of *Pisum*, *Vicia*, *Lathyrus*, and *Lens* spp. by incorporation of radioactive ^{36}Cl, thin layer chromatography, autoradiography, colour reactions, and gas chromatography/mass spectrometry.
2) The strong biological activity of 4-chloroindole-3-acetic acid and its analogues and its ability to induce strong, almost irreversible, ethylene evolution.
3) The possible role of chloroindole auxin in plants, particularly if it might be the hypothetical death hormone, secreted from developing seeds, which induces senescence and kills the mother plant at maturity; if plants generally have several auxin types, growth promoters and endogenous herbicides; and if other chlorine-containing plant hormones occur in developing seeds of other crop species.

»Denne afhandling er den 30. juni 1993 antaget til forsvar for den naturvidenskabelige doktorgrad ved Roskilde Universitetscenter.

Henrik Toft Jensen
Rektor«

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Contents

Preface

The present work reviews and extends the work on verifying the presence of natural chlorine-containing plant hormones and their quantitative determination in plants related to pea, *Pisum sativum* L. The experimental part of the work has been published previously, as have reviews on chloroindole auxins, on chlorine containing natural compounds in higher plants and on the death hormone hypothesis. The review is extended with speculation on the biosynthesis of chloroindole auxins, on their function in the plant, on the presence of natural chlorinated auxins in other plant tribes than the *Vicieae*, on the possible presence of endogenous herbicides in plants, and on the possible identity of chloroindole auxins and the monocarpic senescence factors or death hormones of pea. The following papers are included in the thesis as appendices I-XI:

I. Engvild, K.C. (1974): Cnloroindolyl-3-acetic acid and its methyl ester. Incorporation of ^{36}Cl in immature seeds of pea and barley. - *Physiol. Plant.* 32, 84-88.

II. Engvild, K.C. (1975): Natural chlorinated auxins labelled with radioactive chloride in immature seeds. - *Physiol. Plant.* 34, 286-287.

III. Engvild, K.C. (1977): Preparation of chlorinated 3-indolylacetic acids. - *Acta Chem. Scand.* B 31, 338-339.

IV. Engvild, K.C. (1978): Substituted indoleacetic acids tested in tissue cultures. - *Physiol. Plant.* 44, 345-346.

V. Engvild, K.C. (1980): Simple identification of the neutral chlorinated auxin in pea by thin layer chromatography. - *Physiol. Plant.* 48, 435-437.

VI. Engvild, K.C. (1985): The chloroindole auxins of pea and related species. In: Hormonal regulation of Plant Growth and Development, Volume II (S.S. Purohit, ed.) pp. 221-234. Martinus Nijhoff, Dordrecht.

VII. Engvild, K.C. (1986): Chlorine-containing natural compounds in higher plants. - *Phytochemistry* 25, 781-791.

VIII. Engvild, K.C. (1989): The death hormone hypothesis. - *Physiol. Plant.* 77, 282-285.

IX. Engvild, K.C., Egsgaard, H. and Larsen, E. (1978): Gas chromatographic-mass spectrometric identification of 4-chloroindolyl-3-acetic acid methyl ester in immature green peas. - *Physiol. Plant.* 42, 365-368.

X. Engvild, K.C., Egsgaard, H. and Larsen, E. (1980): Determination of 4-chloroindole-3-acetic acid methyl ester in *Lathyrus*, *Vicia* and *Pisum* by gas chromatography-mass spectrometry. - *Physiol. Plant.* 48, 499-503.

XI. Engvild, K.C., Egsgaard, H. and Larsen, E. (1981): Determination of 4-chloroindole-acetic acid methyl ester in *Vicieae* species by gas chromatography-mass spectrometry. - *Physiol. Plant.* 53, 79-81.

In contrast to the works above the work in the following papers has been done in other laboratories, but I have supplied the auxins and have been involved in the discussions and also in writing up of the material. The work is not part of the thesis proper, but I am drawing heavily on some of the results, particularly the work by Ahmad, Andersen and Engvild (1987). Thus it would be helpful for my arguments if the papers were included as appendices. This has become possible by the generous permission of the authors.

XII. Ahmad, A., Andersen, A.S. and Engvild, K.C. (1987): Rooting, growth and ethylene evolution of pea cuttings in response to chloroindole auxins. - *Physiol. Plant.* **69**, 137-140.

XIII. Stenlid, G. and Engvild, K.C. (1987): Effects of chlorosubstituted indoleacetic acids on root growth, ethylene and ATP formation. - *Physiol. Plant.* **70**, 109-113.

These references are referred to by their roman numbers, but they are also included in the reference list, which is intended to be an almost complete list of the work on chloroindole auxins to date.

At vide
hvad man ikke ved
er dog en slags
alvidenhed

Piet Hein

Knowing what
thou knowest not
is in a sense
omniscience

1 Introduction

Auxin was the first plant hormone discovered. Charles Darwin (1880) assisted by his son, Francis Darwin, made the initial observations on dark grown grass seedlings bending towards light. They showed that the light perception occurred at the tip of the seedling, but the bending further down the stem. Boysen Jensen and several other plant physiologists showed that a stimulus was transported down from the tip, and that the stimulus was probably a chemical substance as it could pass through gelatin (Boysen Jensen 1943). Went showed that the substance could be captured in small agar blocks, which could induce bending in decapitated oat seedlings, when the blocks were placed asymmetrically on the cut end. Finally, after the false starts with auxin a and b, auxin was identified as indoleacetic acid (IAA). It was first isolated from urine and fungi, and only later from plants.

The word »auxin« derives from greek »auxein«, to increase, and an auxin is a substance which 1) promotes extension growth, 2) promotes bending in coleoptiles (although perhaps not *in vivo*), 3) is polarly transported downwards, 4) promotes root formation, and 5) promotes growth of cells in tissue culture. To qualify as an auxin, a compound should possess most, preferably all of these characteristics.

For many years indoleacetic acid was the only plant hormone known. That is, many compounds had auxin activity, but they were either synthetic products, not naturally present in plants, or they were precursors or metabolites of IAA, *e.g.* indoleacetonitrile.

Plant hormones provide good illustration of the famous statement by Pasteur »Chance favours the prepared mind«. Plants do not have endocrine glands in the animal sense; the classical methods of demonstrating hormone activity by supplying gland extracts to an organism where the gland has been removed are only rarely applicable. So, plant physiologists have often found plant hormones only after keen observation of peculiar biological activity seen under strange circumstances.

Today, five families of plant hormones are recognized: The auxins (Davies 1988, Marumo 1986); the gibberellins (which were first isolated as fungal toxins), the cytokinins (which were discovered as autoclaving artefacts of DNA), the maturing hormone ethylene (first identified as the plant toxic component of coal gas), and the abscisic acid family. In this last case the plant biochemists were not helped by the lucky break of having a readily available source of material with high biological activity.

Several other families of substances await »citizenship« as plant hormones: the brassinosteroids isolated from pollen (Mandava 1988), the jasmonic acid inhibitors, which were isolated originally as perfume constituents and are involved in potato tuberization and plant senescence (Koda 1992), the turgorins, involved in the movements of mimosas (Kallas *et al.* 1990), and several others such as oligosaccharins (Ryan and Farmer 1991), polyamines (Evans and Malmberg 1989), salicylic acid (Raskin 1992), and the sadly neglected vitamin B_1 or thiamin (Mozafar and Oertli 1992).

During the second world war, some of the synthetic auxins turned out to be quite toxic to dicotyledonous plants and fairly harmless to cereals. This was immediately useful and formed the foundation of the very large herbicide industry. All research in plant physiology has already been paid for by the additional income generated by the yield increases on farms and the turnover within the agrochemical industry due to herbicides.

The invention of herbicides created a boom in auxin research. The auxins have now been investigated intensively for many years. Some people feel that the field has been overresearched. This is probably true in some areas, where the needs for hormone type herbicides initiated very large efforts in screening and much speculation about the mechanism of action. Many compounds were examined, and a large number of the active compounds contained chlorine or other halogens in the molecule.

After a while, however, organically bound chlorine came to be associated with unnaturalness and pollution catastrophes. All of the halogenated compounds were synthesized in ugly factories and nobody, including the people who isolated natural chlorine-containing auxins, quite believed that the old plant physiology workhorse, pea or *Pisum sativum* L., contains large amounts of 4-chloroindole-3-acetic acid methyl ester in the young seeds.

1.1 Organic Chlorinated Compounds in Nature

Organic chlorinated compounds are actually quite common in nature; they are not only nasty pollutants left in pristine areas by greedy chemical industry. Organic chlorine compounds are most common in the sea where many organisms contain up to several per cent of their body dry weight as halogen, typically bromine and chlorine but sometimes iodine (Scheuer and Darias 1978-83). Soils and sweet waters contain about 0.1% organically bound chlorine in the organic matter (Asplund and Grimvall 1991). The air contains large amounts of chloromethane, which is primarily liberated by organisms in the sea (Wuosmaa and Hager 1990). Wood rotting fungi also liberate copious amounts of chloromethane, which they seem to use as a methyl source in the extracellular detoxification of phenols (Harper *et al.* 1989).

Generally, many bacteria and fungi contain substantial amounts of chlorinated metabolites (Turner and Aldridge 1983). Chlorinated compounds are rarely found in higher plants (Engvild 1986, VII). In higher animals one finds thyroxine and other members of the iodinated hormone family, but other halogenated compounds are extremely rare. Perhaps animals contain a natural brominated sleep inducing hormone (Yanagisawa and Yoshikawa 1973) and there are natural analogues of the chlorine-containing valium (Klotz 1991). Some poisonous frogs contain an extremely potent chlorinated painkiller, which may have a future in medicine (Spande *et al.* 1992).

1.2 The Discovery of Chloroindole Auxins in France

Developing seeds are standard sources for plant hormones, especially gibberellins, cytokinins and auxins. In Nitsch's laboratory Jean-Claude Gandar investigated six different auxins in immature green peas (Gandar 1960, Gandar and Nitsch 1964). The compounds were not identical with IAA or any other known auxins and they were tentatively named auxins A to F. Compound F was chosen for further examination; it was either the strongest auxin or it was present in very large amounts. It was not acidic and very lipophilic. Substance F showed the highest mobility in Nitsch's one dimensional double paper chromatography system (Nitsch 1960). 350 kg of immature pea seeds, harvested about 14-20 days after fertilization, were extracted with methanol at -15°C. After extraction of the neutral fraction into ethyl acetate, it was purified by column chromatography on »magnesol« and Sephadex LH 20 and by paper chromatography. Auxin activity was followed by Nitsch' avena mesocotyl elongation assay (Gandar and Nitsch 1964). The UV spectrum showed

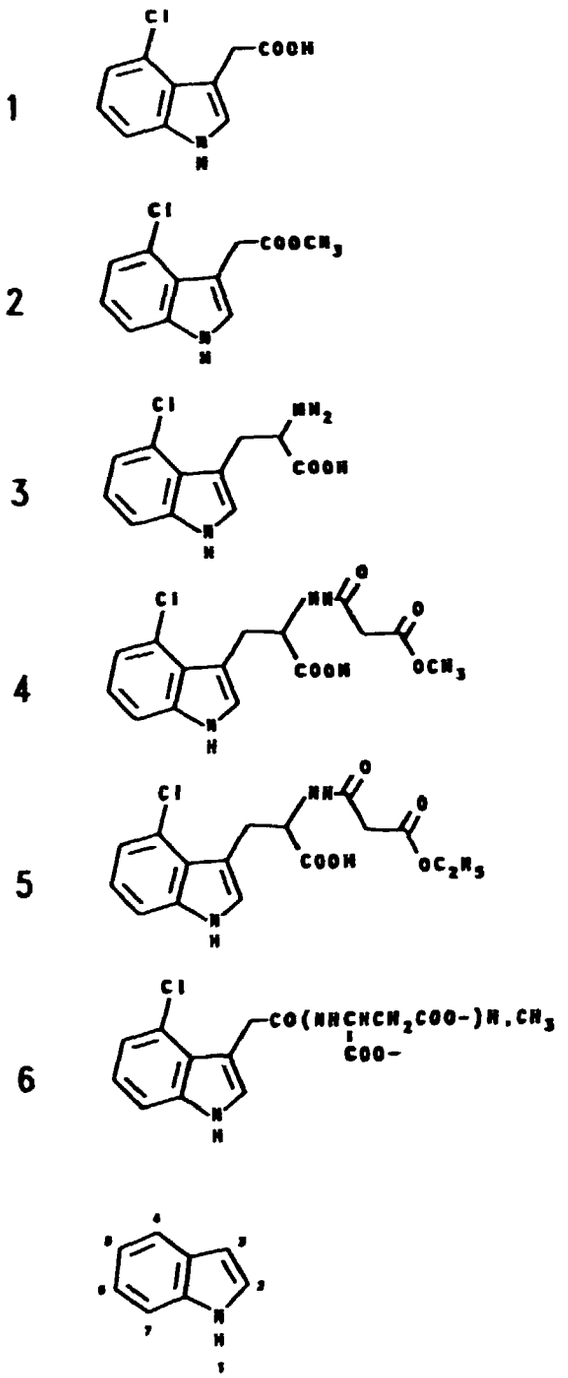


Figure 1.1. The family of chloroindole auxins found in pea. 1) 4-chloroindole-3-acetic acid. 2) 4-chloroindole-3-acetic acid methyl ester. 3) 4-chlorotryptophan. 4) N-carbomethoxyacetyl-4-chlorotryptophan, the malonyl amide methyl ester. 5) N-carboethoxyacetyl-4-chlorotryptophan, the malonyl amide ethyl ester. 6) 4-chloroindole-3-acetyl-aspartic acid mono methyl ester. The numbering of the indole ring is given below.

that substance F was probably indolic, but it was not IAA or an IAA ester. The infrared spectrum showed the presence of C=O, probably in the ester configuration. In the following years Nitsch's group continued their efforts to identify substance F. After several purification steps they obtained 6 mg of crystals which were examined by mass spectrometry, as well as infrared, ultraviolet and NMR spectroscopy. They concluded that substance F was a chlorine-containing indoleacetic acid methylester, – and they did not believe it! (Gandar and Nitsch 1967). They thought they had obtained an artefact.

At the time very few chlorine-containing compounds had been isolated from higher plants. To nonchemists chlorine compounds were known as antibiotics, such as chloramphenicol and chlortetracycline, herbicides such as 2,4-D, and pesticides such as DDT, all toxic compounds that should not be present in a proper plant, and in the edible parts at that. Therefore Gandar and Nitsch (1967) did a control experiment where they were careful to exclude chlorinated pesticides and herbicides when growing the peas, and they did not use chlorinated solvents such as $CHCl_3$ during the isolation. The inescapable conclusion was that green peas do contain a chlorinated indoleacetic acid methylester, but they did not know the position of the chlorine atom.

1.3 The Identification of Chloroindole Auxins in Japan

Meanwhile, Marumo's group of natural compound chemists in Japan had become interested in the pea auxins. For screening purposes it is often better to use a rapid, if only semiquantitative assay; they used an extremely simple mung bean hypocotyl swelling assay, where test solutions were added to intact mung bean seedlings 1 day after germination (Marumo *et al.* 1968a, Hatano *et al.* 1987a). For many purposes, visual evaluation of the shortened, thickened seedlings was enough. With their extensive background in the chemistry of natural substances they were able to deduce the structure of substance F and four other chlorine-containing auxins within a few years (Marumo *et al.* 1968a, b, Marumo and Hattori 1970, Hattori and Marumo 1972).

Substance F (25 mg) was first isolated from 276 kg of immature peas by extraction in methanol, transfer of the neutral fraction into acetonitrile, aluminium oxide column chromatography with ethyl acetate:hexane and recrystallization. The compound contained chlorine as demonstrated by the Beilstein test, a green colour when a sample is burned on a copper wire. A mass spectrum showed the typical 3:1 ratio of ^{35}Cl and ^{37}Cl of the molecular ions and the base peaks (Marumo *et al.* 1968a, Abe and Marumo 1974). The indole nucleus was suggested by the UV spectrum, and an acetic acid methyl ester sidechain by the infrared and proton NMR spectra. The proof that the compound was 4-chloroindole-3-acetic acid methyl ester (Fig. 1.1, 2) was obtained by synthesis (Fox and Bullock 1951). The natural and synthetic compounds were compared and found identical in all properties.

By similar procedures Marumo *et al.* (1968b) isolated 3.8 mg of an auxin from the acid fraction which was identified as 4-chloroindole-3-acetic acid (Fig. 1.1, 1). Later Marumo and Hattori (1970) isolated three acidic auxins, found in lower quantities, namely two N-malonylderivatives of D-4-chlorotryptophan esterified with methanol or ethanol (Fig. 1.1, 4, 5), and 4-chloroindoleacetylaspartic acid monomethyl ester (Fig. 1.1, 6) (Hattori and Maru-

mo 1972). This immediately suggested that D-4-chlorotryptophan (3) was a precursor and that the aspartic acid derivative was a metabolite or storage form of 4-chloroindoleacetic acid. Marumo's group did not check if the chlorine incorporation was an artefact. They did, however, do a control experiment to eliminate the possibility that the methyl ester was an artefact of methanol extraction.

The chloroindole auxins have very strong biological activity. This was already known from the early 1950s where a number of chloroindoleacetic acids were examined in the large analogue investigations of IAA (Hoffmann *et al.* 1952, Sell *et al.* 1952, Porter and Thimann 1965). These results agreed with the results of Gandar and Nitsch (1964, 1967) and of Marumo's group (1968a, b). The biological activity of 4-chloroindoleacetic acid was greater than that of IAA, whereas the methyl ester was about as strong as IAA.

1.4 The Chloroindole Auxin Scepticism

In spite of the dramatic biological activity of the natural, endogenous chloroindole auxins little happened afterwards. Many auxinologists were not aware of the developments, and many others did not believe in natural chlorinated organic compounds in plants at all, and much less in chlorinated auxins in pea. Nitsch's group dropped out of the investigations, partly because they became heavily involved in the exciting development of haploid plants from microspores by anther culture. Marumo's group went on to the brassinosteroids. The chloroindole auxins were also not commercially available, hence it was quite difficult for plant physiologists to do research in the area.

This was the situation when I became interested in the chloroindoles. Few people knew the chloroindoles existed, and fewer believed in them. In the beginning I was interested in 4-chloroindoleacetic acid as a potential very strong auxin in my work on anther cultures and shoot regeneration, and decided to synthesize it myself. I made a mixture of 6- and 4-chloroindole-3-acetic acid by a simple Fischer indolization (Engvild 1974, I). It then occurred to me that one good way to prove the natural occurrence of chloroindoles would be the incorporation of radioactive $^{36}Cl^-$ by living plants.

1.5 Abbreviations

ClIAA, 4-chloroindole-3-acetic acid; ClIAM, 4-chloroindole-3-acetic acid methylester; IAA, indoleacetic acid; 2,4-D; 2,4-dichlorophenoxyacetic acid; GC/MS gas chromatography-mass spectrometry; TLC, thin layer chromatography; DIF, differentiation inducing factor; HPLC, high performance liquid chromatography.

2 Studies on Identification of 4-Chloroindole-3-Acetic Acid Methyl Ester

2.1 Incorporation of Radioactive $^{36}Cl^-$ in Pea

Radioactive chloride was fed to pea *(Pisum sativum)* and barley in water culture. Butanol extracts of the developing seeds were separated by thin layer chromatography in several systems, followed by autoradiography. This proved that some of the radioactivity moved in organic solvents and co-chromatographed with the mixtures of 6- and 4-chloroindole-3-acetic acids and their methyl esters. This convinced me that chloroindole auxins were real. Furthermore, the incorporation of radioactivity into organic compounds (which might or might not be chloroindoles), from developing barley grains, indicated that natural chlorine-containing plant hormones might be widespread in nature (Engvild 1974, I; 1975, II).

2.2 Identification Strategy and Purpose

It was reasonable to assume that the chlorinated compounds played a central role in the growth and development of the seeds. It was decided to do a complete verification of the presence of 4-chloroindole-3-acetic acid methyl ester in pea, to study the distribution of the chloroindole auxins among cultivated plant species, to see if the chlorinated compounds of barley were chloroindoles or perhaps other types of auxin and to try to establish if such auxins were important in grain development and grain filling of barley. The biological activity of the chloroindole auxins would be studied in collaboration with other groups. To do that it was necessary to synthesize not only 4-chloroindole-3-acetic acid and its methyl ester, but preferably also a number of analogues (Engvild 1977, III) to be used as standards, in case other plants had different chloroindole auxins.

The work of Gandar and Nitsch (1967) and of Marumo *et al.* (1968a, b) had required several hundred kg of young green pea seeds, about 20 days after fertilization. My own verification by incorporation of radioactive chloride was done on a few grams of material, but the plants had to be grown in water culture with radioactivity for several months. After extraction and thin layer chromatography, the exposure needed for autoradiography lasted weeks. This was due to the very low specific radioactivity of the long lived ^{36}Cl isotope (half life 300,000 years). These methods were not suitable for routine work. I wanted therefore to develop methods of 4-chloroindole-3-acetic acid identification and quantitative analysis which were simpler and easier to use. One set of methods should involve rigorous identification, another set should be cheap and very easy to use in any plant physiology laboratory.

2.3 Rigorous Identification by Gas Chromatography/Mass Spectrometry in Pea

Rigorous identification was based on 4-chloroindole-3-acetic acid methyl ester labelled by growing plants with $^{36}Cl^-$ in water culture. The radioactivity made it straight forward to follow the substance through the various cleanup

procedures (Engvild *et al.* 1978, IX). The peas were grown in half or full strength Johnson's chloride free nutrient medium plus 50 μCi $^{36}Cl^-$ in an 18 litre container in a growth chamber. 100 μCi $^{36}Cl^-$ was added when flowering started. The radioactive peas were harvested 3-4 weeks after flowering and lyophilized. These peas were used together with commercial frozen green peas which were likewise lyophilized. 25 g lyophilized peas (5-10 g radioactive peas) were extracted with a mixture of 1-butanol and phosphate buffer (0.1 M, pH 2.5). This extraction procedure was chosen because the alternative, extraction with methanol, would have increased the risk of methyl ester artefacts. The butanol and water phases were separated by centrifugation. After a water wash the butanol was evaporated *in vacuo* and the residue was taken up in acetonitrile, leaving a voluminous precipitate. Most of the chlorophyll was removed from the acetonitrile by a heptane wash and the acetonitrile solution was concentrated *in vacuo*. The concentrate was chromatographed on a Sephadex LH 20 column with dichloromethane:methanol and fractions collected in glass scintillation counting vials. The beta radiation from ^{36}Cl is so hard that the Čerenkov radiation can be counted in the scintillation counter without scintillator liquid. Radioactive fractions were pooled and chromatographed on a silicagel column in tetrachloromethane:methanol 4:1. The radioactive fractions were evaporated and taken up in a few μl of chloroform and analysed by gas chromatography/mass spectrometry on a glass column. At this purification stage the extract was pure enough for GC/MS but not pure enough for direct inlet mass spectrometry.

The mass spectrum proved to be remarkably simple, especially when taken at 20 eV. There were molecular ion peaks at 223/225, and base peaks at m/z 164/166. This indicated:

1) that the compound contained one and only one chlorine atom, because the mass spectrum showed the characteristic 3:1 ratio of $^{35}Cl/^{37}Cl$ at m/z values 2 units apart.
2) The compound contained one N (or an uneven number of N and P) because the mass of the molecular ion is odd.
3) The compound is probably an indole. The base peaks corresponds to the very stable quinolinium ion characteristic of 3-substituted indoles. Metastable peaks at m/z 120.6 and 122.5 confirmed that the base peaks are formed directly from the molecular ions. Comparison between the radioactive pea extract and synthetic 4-chloroindole-3-acetic acid methyl ester showed that the mass spectra of the two compounds were identical.

The GC/MS system could not, however, separate the conceivable isomers. The radioactive compound was identified unambiguously as the 4-chloroindole isomer by co-chromatography of radiactivity and standards on TLC in 12 different solvent systems by autoradiography.

In the same chromatograms it was possible to identify 4-chloroindole-3-acetic acid by co-chromatography of radioactivity with standards.

In a later work (Engvild *et al.* 1980, X) 4-chloroindole-3-acetic acid methyl ester was also identified by a gas chromatographic procedure which separated the 4-, 5-, 6-, and 7-chloroindole-3-acetic acid methylesters. In this work the unknown co-chromatographed with a deuterium labelled internal standard, pentadeutero-4-chloroindole-3-acetic acid methyl ester. The mass spectrometer worked as a detector, showing simultaneously the 223 m/z peak of the natural compound and the 228 m/z peak of the pentadeuterated internal standard. All these methods have established, when taken together,

that 4-chloroindole-3-acetic acid methyl ester is a natural component of developing pea seeds.

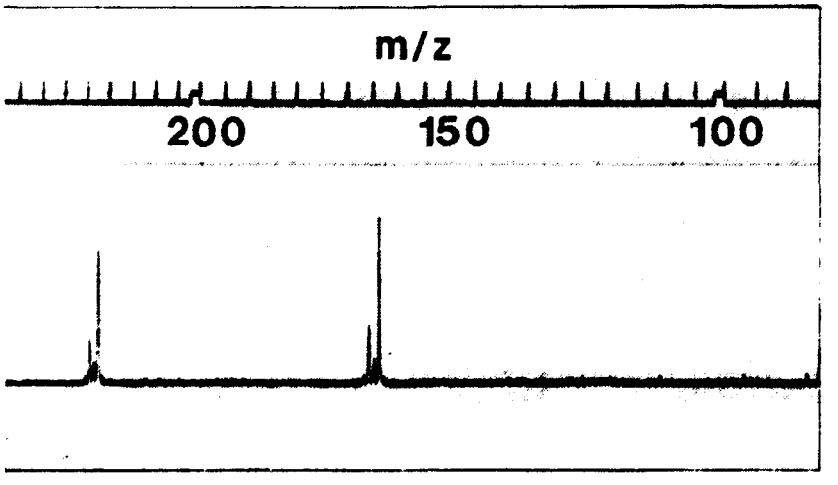
2.4 Simple Identification by Thin Layer Chromatography

The rigorous methods were time consuming and access to sophisticated expensive machinery was required. Simpler, rapid methods, suitable *e.g.* for teaching laboratories have also been developed, using frozen green peas from the local super market (Engvild 1980, V). 50 g of frozen green peas were extracted with acetone in a Wareing blender. After filtering, the extract was evaporated on a steam bath or rotary evaporator, until an unclear waterphase remained. An ethyl acetate extract of this water phase was evaporated, taken up in acetonitrile, filtered and concentrated to 100 µl acetonitrile solution. 20 µl extract was chromatographed on commercial Silicagel plates in chloroform:ethyl acetate (4:1) together with appropriate standards. The best colour reaction was obtained with Ehmann's reagent (Ehmann 1977). Several other solvents and colour reactions were available. Taken together they were almost sufficient by themselves as identification of 4-chloroindole-3-acetic acid methyl ester in a given plant material.

2.5 Identification of Chloroindole-3-Acetic Acid Methyl Ester in Pea by Other Groups

Besides the large scale isolation by Gandar and Nitsch and by Marumo's group and our own GC/MS work, 4-chloroindole-3-acetic acid methyl ester (ClIAM) has been identified in pea by several other methods by other groups. Eeuwens and Schwabe (1975) extracted a neutral auxin which was not indoleacetonitrile or the ethyl or methyl ester of IAA; they identified it as 4-chloroindole-3-acetic acid methylester by its UV spectrum. Taking in account the

Figure 2.1. The very simple mass spectrum of 4-chloroindole-3-acetic acid methyl ester isolated from frozen pea seeds, taken at 20 eV. The chlorine content is proven by the 3:1 ratio of the ions at 223/225 and at 164/166 reflecting the 3:1 natural abundance of $^{35}Cl/^{37}Cl$. The great stability of the base peak at mass 164 with very few ions at lower mass points to a very stable aromatic ion which still contains Cl.



work of other groups they have most likely isolated ClIAM. Heikes (1980) found a large unidentified peak when he examined canned peas for chlorinated pesticide residues by gas chromatography using microcoulometry as detection. He identified ClIAM by GC/MS and comparison with a synthesized sample. This paper shows that ClIAM is actually quite stable in the peas, surviving long autoclaving. Schneider *et al.* (1985) identified ClIAM in pea seeds and the free acid in the roots, cotyledons and shoots of young etiolated seedlings. They used high pressure liquid chromatography (HPLC) on a C18 column and water, methanol, formic acid gradients and UV detection at 254 nm. Katayama *et al.* (1988) identified IAA and ClIAA in pea plants at various growth stages by GC/MS.

2.6 Incorporation of ^{36}Cl in Chlorinated Compounds in Cultivated Plants

The original autoradiograms of thin layer chromatograms from immature seeds of pea and barley, labelled *in vivo* with ^{36}Cl (Engvild 1974, I) showed that both pea and barley contained organic radioactive compounds (defined as compounds moving in lipophilic solvents on TLC). In pea the compounds co-chromatographed with ClIAA and ClIAM, and the presence of these compounds in peas have been amply proven both before and since by many different methods. Barley contained ^{36}Cl labelled compounds, of which one moved very close to ClIAM. I therefore made a survey of 15 cultivated species to see if they also contained chlorinated organic compounds which might be similar to the chloroindoles. The plants were grown in vermiculite in 20 litre pots and 35 μCi ^{36}Cl was added just before flowering (Engvild 1975, II). Immature seeds were harvested and lyophilized, and extracts prepared from 0.5 g of material. Autoradiograms were prepared after one and 2 dimensional thin layer chromatography together with standards. The survey of the 15 cultivated plants showed that all species except maize, soybean, flax and sunflower incorporated detectable amounts of ^{36}Cl into organic compounds. However, pea incorporated more than the other species and only pea incorporated ^{36}Cl into ClIAA and ClIAM. The other species incorporated Cl into other compounds, which chromatographed in similar ways as the chloroindoles, but were not identical with them. These compounds might be other chlorine-containing plant hormones, and again they might not. They might just be artefacts such as *e.g.* chlorinated chlorophyll (Senge *et al.* 1988), or lipid soluble chloride complexes, although this is unlikely. At least the result showed that it would be worthwhile to investigate other species for chloroindoles as well as for other chlorine containing hormones. This I proceeded to do after we had developed additional methods of chloroindole identification (Engvild *et al.* 1978, IX).

Table 2.1. Radioactive $^{36}Cl^-$ incorporation in »organic« compounds moving on thin layer plates in $CHCl_3$/acetic acid 19:1.

Detectable			Not detectable
Barley	Tobacco	Pea	Maize
Oat	Tomato	Bean	Soybean
Wheat	Cress	Chives	Flax
Rye	Rape seed		Sunflower

Table 2.2. Species in which chloroindole auxins have been searched for with rigorous methods, and either identified or not found (Engvild et al. 1980, X; 1981, XI). The asterisk means work by other groups, see text.

Identified		Not found
Pisum sativum	*Lathyrus sativus*	*Phaseolus vulgaris**
Lens culinaris	*Lathyrus odoratus*	*Glycine soja**
Vicia faba	*Lathyrus maritimus*	*Vigna catiang**
Vicia sativa	*Lathyrus latifolius*	*Lablab purpureus**
*Vicia amurensis**	*Pinus sylvestris**	*Cicer arietinum*

2.7 Chloroindole Auxins in *Vicieae* Species

4-Chloroindole-3-acetic acid methyl ester was identified in eight species of the tribe *Vicieae (Fabaceae), i.e.* in species very closely related to pea, *Pisum sativum*. The identification itself was done by GC/MS, using gas chromatography which separated the 4, 5, 6, and 7 substituted chloroindoleacetic acid isomers. The localization of fractions containing the ClIAM was done by thin layer chromatography (Engvild *et al.* 1980, X; 1981, XI). In this way chloroindole auxin was identified in the eight *Vicieae* species given in the table 2.2.

However, I did not find chloroindoles in *Cicer arietinum*, chickpea, which is also placed in the tribus *Vicieae*. Chickpea differs from the other *Vicieae* in this respect as well as by not forming nodules with rhizobia of the leguminosarum bv. *Vicieae* group, but with different rhizobia of the »chick pea miscellany«.

Other groups have also identified chloroindoles in *Vicieae*. Hofinger and Böttger (1979) and Pless *et al.* (1984) found them in *Vicia faba*, and Katayama *et al.* (1987) found them in *Vicia amurensis*. Büchi *et al.* (1986) have investigated 4-chloro-6-methoxyindole as a carcinogenic compound in *Vicia faba*.

2.8 Chloroindole Auxins in *Pinus*

In one case ClIAA has been identified in a species outside the *Fabaceae*. Ernstsen and Sandberg (1986) found ClIAA in immature and mature seeds of scots pine, *Pinus sylvestris*. After cleaning up the acidic extracts from 2 kg of pine seed by reverse phase HPLC they identified the derivatized substance by GC/MS.

2.9 Chloroindole Auxins in Other Higher Plants

Intensive search by Hofinger and Böttger (1979) and Katayama *et al.* (1987) failed to find chloroindole auxins in four species of *Phaseolae*, the bean tribe: *Phaseolus vulgaris, Vigna catiang, Glycine Soja*, and *Lablab purpureus*.

Besides the ^{36}Cl incorporation experiment, I have also looked for chloroindoles in immature seeds of a number of other species by thin layer chromatography. This method is not very sensitive, so the presence of less than *e.g.* 0.5 mg/kg of chloroindole auxins may well have remained undetected. However, the results from the ^{36}Cl incorporation in the 15 species do suggest that 4-chloroindole auxins cannot be found in all species. And there are certainly large gaps in our knowledge of chloroindole auxin distribution in plants.

Table 2.3. Determination of chloroindole auxins

	Amount of auxin	Plant material	References
Isolation and chemical characterization	1-25 mg	30-300 kg	Gandar and Nitsch 1967 Marumo *et al.* 1968a, b, Hattori and Marumo 1972 Marumo and Hattori 1970 Park *et al.* 1987
Bioassay and paper chromatography			Gandar 1960, Gandar and Nitsch 1964 Eeuwens and Schwabe 1975 Hatano *et al.* 1987a
Paper chromatography and colour reaction	1-100 µg		Marumo *et al.* 1971
Thin layer chromatography and colour reaction	0.2-100 µg	5-50 g	Engvild 1980, V, Engvild *et al.* 1980, X, 1981, XI, Iino *et al.* 1980
Thin layer chromatography ^{36}Cl incorporation and autoradiography	0.1 µg	1 g	Engvild 1974, I, 1975, II, Engvild *et al.* 1978, IX
Gas chromatography/ mass spectrometry	0.1-100 µg	5-1000 g	Engvild *et al.* 1978, IX, 1980, X, 1981, XI Hofinger and Böttger 1979, Heikes 1980, Pless *et al.* 1984, Schneider *et al.* 1985, Ernstsen and Sandberg 1986, Katayama *et al.* 1988, Sandberg *et al.* 1987
High pressure liquid chromatography	0.001-10 µg	25-1000 g	Sjut 1981, Sandberg *et al.* 1981, Blakesley *et al.* 1983, Schneider *et al.* 1985, Ernstsen and Sandberg 1986
Chloroindolopyrone fluorescence	0.001 µg up		Iino *et al.* 1980, Böttger *et al.* 1978a Blakesley *et al.* 1983, 1984
Immunological assay			Marcussen *et al.* 1989, Ulvskov *et al.* 1992

2.10 Chloroindoles in Bacteria and Marine Organisms

In the sea halogenated indolecompounds have been known for a very long time; tyrian purple is a 6-bromoindolecompound (Scheuer and Darias 1978-1983). 7-Chloroindoleacetic acid and its amide have been isolated from a bacterium, *Pseudomonas* species (Salcher and Lingens 1978); it seems to be involved in the formation of the antibiotic pyrrolnitrin. It is not known whether it plays any role for the bacterium as a plant pathogen. 7-Chloroindole-acetic acid has some antiauxin characteristics (Engvild 1985, VI, Stenlid and Engvild 1987, XIII).

2.11 Quantitative Determination of Chloroindole Auxins

In the preceding sections I have described many of the methods that have been used in the qualitative identification of the chloroindoles. In several cases these methods can be used directly or after small modifications, *e.g.* the use of internal standards, for quantitative determination of the amounts of chloroindole auxins present. The Table 2.1. which is an up-dated version of Table 2 in Engvild (1985, VI) gives an overview of the methods available today (see Sandberg *et al.* 1987, for general methods of auxin determination).

Some of the methods have been used for determinations on plant material, while others have been tested on pure compounds only. The HPLC was used originally for extract clean-up, and the determination proper was done by GC/MS.

2.12 Quantitative Gas Chromatography-Mass Spectrometry

The method of choice is GC/MS using isotopically labelled internal standards. Here are several different possibilities. We have used deuterium labelled ClIAM (Engvild *et al.* 1980, X, 1981, XI). Pless *et al.* (1984) used ^{14}C labelled ClIAA. However, Ernstsen and Sandberg (1986) used non-labelled 7-chloroindoleacetic acid as a chemical internal standard.

The idea with internal standards is that a known quantity of a isotopically labelled substance is added to the sample at the beginning of the analysis. The standard behaves exactly like the unknown during extraction and clean-up. Losses, at least moderate losses, become unimportant. The determination is reduced to a relative determination of the unknown and the standard. The use of chloroindoleacetic acid methyl labelled with five deuterium atoms for the quantitative determination of ClIAM in *Pisum sativum, Vicia faba* and *Lathyrus latifolius* is described in Engvild *et al.* (1980, X) and the determinations on other *Vicieae* are given in Engvild *et al.* (1981, XI).

However, very much depends on the stability and reproducibility of the internal standard; if the internal standard fails, everything fails. The best internal standard is the one most like the unknown, which can still be resolved in the analysis.

Pentadeuterated ClIAM was prepared from ClIAM dissolved in D_4 methanol by deuterated sodium methoxide catalyzed proton exchange and transesterification. In the mass spectrometer the pentadeuterated ClIAM has a

Table 2.4. Typical contents of 4-chloroindole-3-acetic acid methyl ester in the immature seeds of eight Vicieae species.

	mg/kg		mg/kg
Pisum sativum	*5*	*Vicia faba*	*0.5*
Lens culinaris	*0.02*	*Vicia sativa*	*0.05*
Lathyrus sativus	*1*	*Lathyrus latifolius*	*0.5*
Lathyrus maritimus	*0.5*	*Lathyrus odoratus*	*0.2*

molecular ion of m/z 228, the unlabelled compound an ion of m/z 223. Known quantities were added to the samples of immature seeds.

The determination of the internal standard and the unknown was done simultaneously on the mass spectrometer by selected ion monitoring using the m/z 223 of ClIAM and m/z 228 of the deuterated compound. In Engvild *et al.* (1980, X) control experiments show that the deuterated 4-chloroindole-3-acetic acid methyl ester standard behaves as the nondeuterated compound and is gas chromatograpically well separated from the 5-, 6-, and 7-substituted isomers. At lower column temperatures there was a very slight shift in retention of standard and unknown, showing a slight isotope effect. Other controls showed that the internal standard was stable during extraction and purification with no isotope exchange, and that there was no interference from background peaks in the m/z range 220-230.

The quantitative determinations were done at recoveries around 25%. The reproducibility on individual extracts was very good, being less than the variability between samples. ClIAM has been determined quantitatively in eight species. Typical contents are given in table 2.4.

Using ^{14}C ClIAA labelled in the methylene group as standard Pless *et al.* (1984) found 16 mg/kg ClIAA in leaves of *Vicia faba*. This is a surprisingly large figure, which one would normally only expect in developing seeds.

The use of a radioactive internal standard is a simple way to follow the unknown hormones during the purification steps. I have also used this fact to great advantage when I used the *in vivo* ^{36}Cl labelled chloroindoles as markers. Ernstsen and Sandberg (1986) used 7-chloroindoleacetic acid as an internal standard. This compound has slightly different characteristics from 4-chloroindoleacetic acid, and moves with a different retention time in the gas chromatograph. If one checks that the differences are not important for losses and recovery, such an internal standard is very good, because standard and unknown give separate peaks in an ordinary gas chromatograph.

2.13 Chloroindole-α-Pyrone Fluorescence

Generally speaking the GC/MS and gas chromatography systems work well. However, they require considerable sample purification, so they are too laborious for routine determinations of chloroindoles. One reason for the paucity of data on chloroindole auxins is the lack of rapid routine procedures. Such procedures are feasible; 4-chloroindole-3-acetic acid reacts with acetic acid anhydride resulting in the formation of a strongly fluorescent compound (Böttger *et al.* 1978a, Iino *et al.* 1980, Blakesley *et al.* 1983). If IAA is present at the same time, both compounds react (Böttger *et al.* 1978a) and it is necessary to separate the two, *e.g.* by thin layer chromatography before the reaction (Iino et al. 1980) or by high pressure liquid chromatography with fluorescence detection after the reaction (Blakesley et al. 1984).

2.14 Quantification by Immunoassays

To my knowledge nobody has tried to estimate chloroindole auxins by radioimmunoassay or ELISA. The work of Marcussen *et al.* (1989) and Ulvskov *et al.* (1992) shows considerable cross reactivity between 4-chloroindoleacetic acid and IAA with several antibodies, so one would expect difficulties in using immunoassays in plants where both auxins occur.

2.15 Quantification by Bioassays

The quality of the individual measurements by the sophisticated new methods is most often very good, but there have been few attempts to put the measurements into a physiological context. Nobody knows if the chloroindole auxins at the time of measurement were rising or falling or staying at a continuously high level. Physiological measurements have been made by Gandar (1960) and Nitsch (1964) with the *Avena* mesocotyl assay. They measured the rise and fall of ClIAM in developing pea seeds from fertilization to maturity. Although they did not know the identity of ClIAM, and therefore had to express their results as IAA equivalents, their results have not since been bettered by any of the newer techniques.

The *Avena* curvature assay is generally recognized as the »defining« assay for auxins. Such assays require a good darkroom with temperature control and appropriate safelights, meticulous attention to growing proper plants, cutting good sections and so on. Not many people run good *Avena* assays any more. For some purposes they are essential, but often it is not necessary to use such elaborate procedures.

This is illustrated best by the success enjoyed by the very simple mung bean hypocotyl swelling assay used by Marumo's group in their isolations of the five known chloroindole auxins. Their method is so simple that it has actually never been published as more than a few lines (Marumo *et al.* 1974, Hatano *et al.* 1987a, b). Mung beans (*Vigna radiata* (L) Wilczek) were germinated for 24 hours in darkness and placed in solutions of the compounds to be tested. The hypocotyls swelled up and the roots shortened severely. The responses were recorded on photographs/shadowgraphs, but formal quantification was not always used.

2.16 Does Pea Contain Both IAA and ClIAA?

Pea contains auxins of both the IAA and the ClIAA family. IAA is found primarily in the vegetative plant parts, ClIAA in the developing seeds. There is some disagreement about the finer details. This disagreement turns on whether there are traces of chloroindole auxins in the vegetative parts (Hattori and Marumo 1972, Katayama *et al.* 1988).

Schneider *et al.* (1985) investigated 3-days old seedlings and found ClIAA in both roots, cotyledons and stems, together with IAA and phenylacetic acid as well as other auxins. They were not able to quantify the chloroindole auxins, because of lack of a suitable internal standard, but the levels seem to be around one µg/kg fresh weight. The ClIAM was found in the roots on one occasion, but not in other experiments. Ulvskov *et al.* (1992) did not see ClIAA in elongation zones of epicotyls of Alaska pea.

Katayama *et al.* (1988) measured ClIAA and IAA by gas chromatography in pea at various stages of development: after 20 days, vegetative development, 40 days, flowering, 47 days, seed development, 1 week after anthesis, 54 days,

mid stage seed development, and 70 days, maturity. ClIAA was detected only in the developing seeds, from 0.7 mg/kg at 47 days decreasing to very low levels at maturity. It was not seen in any other plant organ. IAA was detected in all plant organs examined, at all developmental stages, at levels from 0.6 mg/kg in flowers and very young pods down to 0.01 mg/kg.

2.17 Chloroindole Auxin Content in Different Plant Organs

Besides the experiments of Schneider *et al.* (1985) and Katayama *et al.* (1988) chloroindole auxin content has been examined in pea, *Pisum sativum*, and *Vicia faba*, and a little in scots pine, *Pinus silvestris*. Some measurements were made by bioassays by Gandar and Nitsch (1964). At the time it was still not known that peas contain chloroindole auxins, so the data are given in IAA equivalents, but it is substance F (alias 4-chloroindole-3-acetic acid methyl ester) that has been quantified. They found very little ClIAM in pea embryos 10 days after fertilization, an increase to a 1,000 to 10,000 fold higher level on days 18-26 after fertilization, and a decrease to non-detectable levels at maturity. The endosperm contained much smaller amounts, as did the seed coats. The pod walls also contained very little substance F.

Pless *et al.* (1984) have investigated *Vicia faba*, and found about 15 mg/kg fresh weight in the developing seeds at maximum and similar amounts in young, but fully developed leaves. Thus there are considerable differences between pea and *Vicia faba* in the way ClIAA is distributed between organs. However, the measurements by Pless *et al.* (1984) need independent confirmation.

Ernstsen and Sandberg (1986) found ClIAA in both developing and mature seeds, but not in germinating seedlings of scots pine, *Pinus sylvestris*. The levels ranged between 0.3-0.7 μg/kg fresh weight.

3 Biosynthesis of Chloroindoles?

Generally speaking very little is known about incorporation of Cl into organic compounds in higher plants and nothing is known about the biosynthesis of the chloroindole auxins. In general biology one operates with four chlorination processes:

1) Attack by free halogen, Cl_2 or by ClOH generated by haem-containing chloroperoxidases acting on halogen ions and H_2O_2 in processes similar to the iodination of tyrosine in the biosynthesis of thyroxine (Neidleman and Geigert 1986, 1987);
2) Cl incorporation in indole by a non-haem-chloroperoxidase + H_2O_2 by an unknown mechanism (van Pée 1990, Wiesner *et al.* 1988);
3) Attack by Cl^- (or HCl) on an epoxide formed by epoxidation of a double bond under the formation of a chlorohydrin, processes very common in marine natural substance biochemistry (Neidleman and Geigert 1986, 1987).
4) The methylation of Cl^- by adenosylmethionine, where the methylchloride forming enzyme has been found in fungi, marine algae, and a higher plant quite recently (Wuosmaa and Hager 1990).

Peroxidase associated halogenation is seen in the halogenation of aromatic rings and of double bonds. When double bonds are halogenated, dihalogen compounds usually result, but sometimes also Br, Cl compounds (or halogenohydrins) are formed, especially in marine systems, where bromoperoxidase generated Br^+ attacks first followed by the ubiquitous Cl^- (Geigert *et al.* 1984).

3.1 Types of Chlorine Compounds in Higher Plants

After thoroughly educating myself about the types of chlorine compounds actually known in higher plants, I wrote a review (Engvild 1986, VII). By 1985 the number of chlorine compounds known had increased to about 150 from the approximately 30 given in the previous review by Siuda and DeBernardis (1973). Most of the newly discovered compounds belong to groups already mentioned by Siuda and DeBernardis, and most are hydrophobic secondary plant substances. The uncertainty in the number of known compounds is caused by the large numbers of potential artefacts. Only in very few cases has any verification been given that a new chlorine-containing compound is a natural substance, and not an artefact.

The chlorine containing compounds in plants are most often polyacetylenes, thiophenes, and sesquiterpenoids with 7-carbon rings, often isolated from the composites, *Asteraceae*. Many of them were isolated by the group of Ferdinand Bohlmann in Berlin. In most cases the chlorine containing part was a chlorohydrin:

$$R_1\text{-CHCl-CHOH-}R_2$$

Many of these compounds will be formed spontaneously from epoxides after an attack by Cl^- or HCl.

Sometimes the OH group in the chlorohydrins is esterified *e.g.* with acetic acid or internally into ring structures:

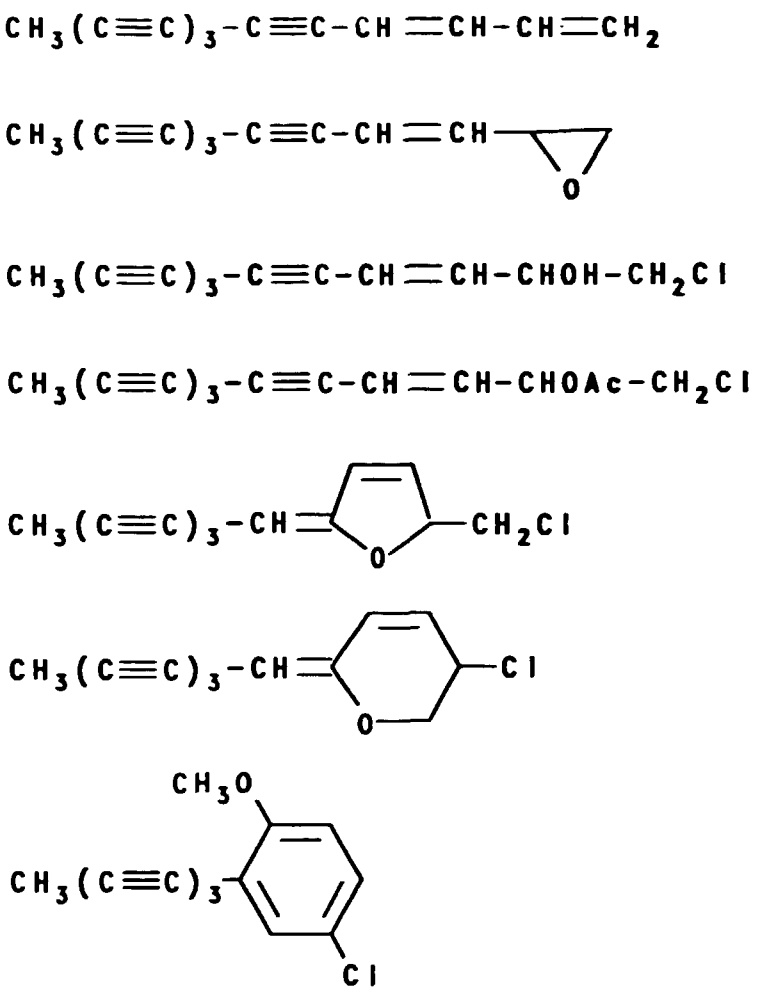


Figure 3.1. Chlorine containing polyacetylenes from cornflowers, knapweeds and everlasting flowers (Centaurea and Helichrysum spp.). Epoxides have probably formed chlorohydrins with chloride ions, and the chlorohydrins have been folded into ring structures.

The first chlorinated compounds in Figure 3.1. have been isolated from cornflowers and knapweeds, (Bohlmann *et al.* 1961, Andersen *et al.* 1977), the last three from *Anaphalis, Gnaphalium* and *Heliochrysum* spp., the everlasting composite flowers (Bohlmann *et al.* 1966, Bohlmann *et al.* 1984). It is probable in all cases that the chlorine has become incorporated as Cl^- into an epoxide, and that the other compounds have been formed by modification of the chlorohydrin (Bohlmann *et al.* 1984).

I know of no cases among higher plants where it is obvious that Cl_2 generated via H_2O_2 and chloroperoxidase is involved in the formation of a product. However, the di- and tetrabrominated fats of *Eremostachys molucelloides* (Gusakova and Umarov 1976) are most easily explained by bromination by Br_2.

Until recently tryptophan, especially D-tryptophan, has been considered the precursor of IAA. The presence of 4-chlorotryptophan in pea (Thiruvikraman *et al.* 1988) and *Vicia faba* (Fock *et al.* 1992) suggests this to be the precursor of ClIAA.

It has, however, been shown that tryptophan is probably not the crucial IAA precursor in higher plants. In both maize (Wright *et al.* 1991) and *Arabidopsis* (Fink 1991) mutants have been found which cannot make tryptophan, but produce large quantities of IAA. The maize mutant accumulates so much indole that it is visible as a bronze colour on the seeds.

Hence, the question of IAA and thus ClIAA biosynthesis is now completely open again. It is also completely open whether Cl is incorporated early or late in the biosynthetic pathway. If the biosynthesis is analogous to that of the chlorine containing polyacetylenes (via epoxide and chlorohydrin), one would expect early incorporation. If biosynthesis is analogous to the specific formation of 7-chloroindole from indole in *Pseudomonas sp.* (from Cl^- and H_2O_2 by means of a vanadium containing non-haem peroxidase, Wiesner *et al.* 1988), one would expect late incorporation, perhaps directly into IAA and tryptophan. Pea also seems to incorporate Cl into other compounds such as a chloro-aminobutyric acid which has been isolated from xylem sap (Fortier and MacKenzie 1986).

3.2 Artifactual Chlorine Compounds in Plants

Artefacts are a difficult aspect of natural substance chemistry, and this is very much the case for chlorinated compounds. It has become clear that many of the substances mentioned in the phytochemistry review (Engvild 1986, VII) are probably artefacts. These include the chlorohydrins, the pterosinoids (chlorinated compounds from ferns), and chlorochlorophyll (Senge *et al.* 1988).

The most common type of artefact is the epoxide/chlorohydrin pair, as already mentioned. There are numerous examples where similar substances have been isolated together; one has a double bond, the other is the corresponding epoxide and one contains the corresponding -Cl and -OH-groups. Usually, epoxides are formed by enzymatic oxygenation of double bonds, but epoxidation will also happen spontaneously (rancid butter). Some epoxides will convert to chlorohydrins at room temperature in a NaCl solution. *Vice versa* chlorohydrins may be converted to epoxides or double bonds by chromatography, e.g. on aluminium oxide or silicagel.

The chlorinated pterosinoids of Bracken fern were isolated during searches for the carcinogenic and toxic principles which made Bracken fern dangerous for cattle. After long searches, ptaquiloside, an unstable sesquiterpenoid glucoside containing a cyclopropane ring was isolated (Saito *et al.* 1989). This compound is mutagenic in the Ames test, and it is easily converted to other pterosinoids upon treatment with acid, base, light etc. It is very likely that the chlorinated pterosinoids will be formed during some of the isolation steps. To my knowledge this has not yet been examined.

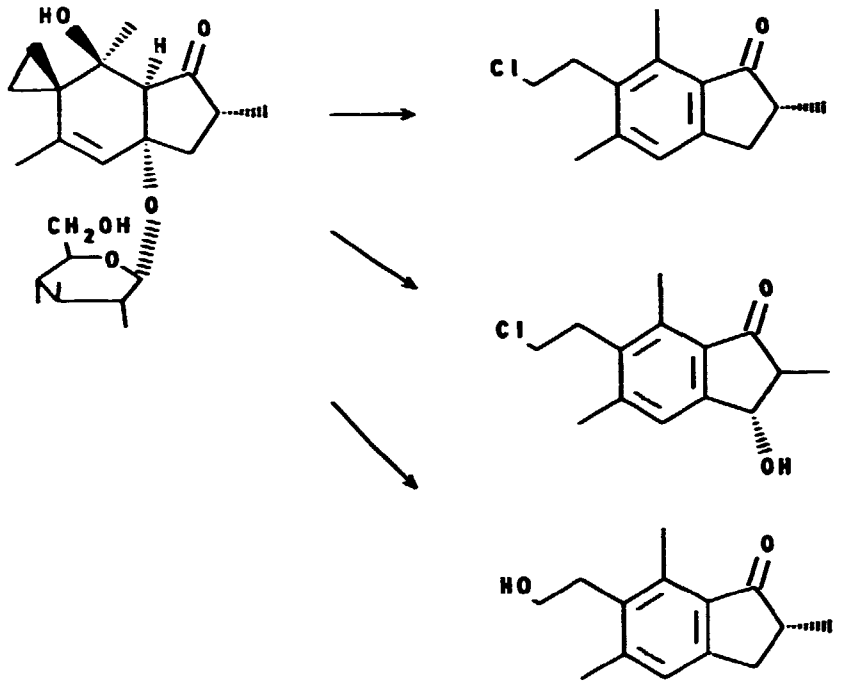


Figure 3.2. The chlorine containing pterosinoids of Bracken fern are probably cell crushing artefacts, formed from the toxic principle of Bracken fern, the highly unstable, mutagenic ptaquiloside.

4 The Biological Activity of 4-Chloroindole-3-Acetic Acid and Related Compounds

A summary of the biological activity is given in Table 4.1. The details up to 1984 are given in the review article (Engvild 1985, VI). More recent work is summarized here.

As described in the review article (Engvild 1985, VI), the chloroindole auxins are extremely potent, more in some assay systems, less in others. 4-Chloroindoleacetic acid is a little stronger than IAA in a pea hypocotyl section elongation assay, and 50 times stronger than IAA in the mung bean hypocotyl elongation assay. Most of the assay systems described in the review paper are elongation assays, where many unphysiological auxins are also active.

Curvature of the *Avena* coleoptile toward light has been the classical auxin manifestation, ever since the experiments by Charles Darwin. Most known natural auxins are active in curvature assays, and those that are not, tend not to be considered as active functional auxins under the assay conditions. Activity in the curvature assay indicates polar transport of the compound, a key feature in the concept of auxin activity. Gerhold and Muir (1989) showed that ClIAA is very active in the *Avena* curvature assay, more so than IAA. Even if few details are given in the published abstract, the results show that ClIAA is probably transported and hence a member of the »real« auxins. The work confirms a short note in Gandar and Nitsch (1964) that substance F is active in coleoptile curvature (shown by Poul Larsen).

Thomson *et al.* (1988) have examined the effect of ClIAA on stomata of *Commelina communis,* in order to see if the chloroindole auxins promoted stomata opening (like IAA), or stomata closing (like the synthetic auxins 2,4-D and NAA). The interaction of abscisic acid and IAA was not simulated by ClIAA. The conclusion is that for *Commelina* the chloroindole auxins behave as synthetic auxins. Unfortunately, the study did not include similar experiments on *e.g. Vicia faba,* which does contain chloroindole auxins.

Zažímalová and Kutáček (1985a, b) showed that there were parallel effects of the chloroindole auxins on wheat coleoptile elongation and the displacement of radioactive IAA from IAA binding sites in a wheat coleoptile homogenate. ClIAA was 9 times stronger than IAA in coleoptile elongation and 16 times stronger in radioactive IAA displacement.

Ahmad, Andersen and Engvild (1987, XII) obtained very strange responses of pea cuttings to ClIAA. When treated basally with auxin the cuttings rooted profusely in response to ClIAA, but the apical bud was completely inhibited at 10^{-3} M auxin and strongly inhibited at 10^{-6} M. After some time, growth of the cuttings resumed, primarily by outgrowth of laterals from the third and fourth node. The treatment of the cuttings with ClIAA caused very strong ethylene formation, which lasted for 6 days, while ethylene evolution after treatment with IAA lasted only about one day. This almost herbicidal effect of ClIAA on the plant from which it was isolated was completely unexpected. It prompted the notion that perhaps the principal effects of ClIAA was not growth promotion, but rather growth inhibition.

Stenlid and Engvild (1987, XIII) investigated root growth, ethylene evolution, and ATP formation in wheat and cucumber. ClIAA inhibited root growth less than IAA in wheat, but more than IAA in cucumber. ClIAA at

Table 4.1. Biological activity of 4-chloroindoleacetic acid.

	ClIAA	IAA	References
Avena coleoptile curvature	+++	++	Gerhold and Muir 1989
Avena coleoptile elongation	1.4	1	Muir and Hansch 1953
Avena coleoptile elongation	7	1	Marumo *et al.* 1974
Avena coleoptile elongation	10	1	Böttger *et al.* 1978b
Avena coleoptile elongation	3	1	Hatano *et al.* 1987a
Wheat coleoptile elongation	13	1	Marumo *et al.* 1974
Wheat coleoptile elongation	4	1	Katekar and Geissler 1983
Wheat coleoptile elongation	9	1	Zažímalová and Kutáček 1985b
Pea stem elongation	1.3	1	Katekar and Geissler 1982
Split pea curvature	7	1	Porter and Thimann 1965
Split pea curvature	+++	++	Hoffmann *et al.* 1952
Mung bean elongation	50	1	Marumo *et al.* 1974
Mung bean hypocotyl swelling	++++	?	Marumo *et al.* 1968a, b, 1974
Mung bean hypocotyl swelling	++++	++	Hatano *et al.* 1987a
Brassica stem growth inhib.	50	1	Hatano *et al.* 1987a
Inhibition of shoots in callus	+	++	Engvild 1978, IV
Tomato epinasty	+++	++	Hoffmann *et al.* 1952
Tomato callusing	++++	+	Hoffmann *et al.* 1952
Tomato parthenocarpy	+++	+	Sell *et al.* 1952
Rooting in pea cuttings	+++	++	Ahmad *et al.* 1987, XII
Side root primordia in pea	+++	+	Wightman *et al.* 1980
Ethylene from pea	++++	++	Ahmad *et al.* 1987, XII
Ethylene from cucumber	++++	++	Stenlid and Engvild 1987, XIII
Ethylene from mung bean	++++	++	Hatano *et al.* 1987b
Wheat root inhibition	(++++)	(+++)	Hansen 1954
Wheat root inhibition	3	1	Stenlid and Engvild 1987, XIII
Cucumber root inhibition	10	1	Stenlid and Engvild 1987, XIII
Hydrogen ion extrusion	++++	+++	Böttger *et al.* 1978b
Hydrogen ion extrusion, roots	+++	+++	Lüthen and Böttger 1988
ATP inhibition in mitochondria	++	+	Stenlid and Engvild 1987, XIII
Binding site displacement	16	1	Zažímalova and Kutáček 1985a, b
Amylase induction in pea	10	1	Hirasawa 1989
Cytokinin metabolism inhibition	+++	+	Parker *et al.* 1986
Stomata opening	close	open	Thomson *et al.* 1989
Rice lamina inclination	+		Park *et al.* 1987
Alkaloids in root culture	++	++	Kamata and Marumo 1988
Alkaloids in root culture	++	++	Yoshimatsu *et al.* 1990
Flower bud formation	2	1	Hatori *et al.* 1991
Pea pod growth	++		Ozga and Brenner 1992

10^{-7} M induced strong ethylene formation in cucumber seedlings after 18 hours; wheat seedlings evolved little ethylene.

Wightman *et al.* (1980) showed that ClIAA induces very large numbers of side root primordia on pea roots; but only few of these extra primordia develop into side roots proper. Lüthen and Böttger (1988) confirmed that ClIAA induces hydrogen ion extrusion, this time in maize roots.

Enzyme induction by ClIAA has been observed in mature cotyledons of pea seeds (Hirasawa 1989). Previously, it was believed that the hydrolytic enzymes involved in seed germination were induced primarily by gibberellic acid, especially in the cereal systems. Failure to find analog responses in pea may have been caused by poor oxygen supply and non-standardized conditions. In Hirasawas's hands naked cotyledons with good air supply increased amylase 5 fold with 10^{-5} M ClIAA, 10 fold with 10^{-5} M 2,4-D, and 10 fold with 10^{-3} M IAA after 3 days of incubation. The pea system is not so easy to

work with as the cereal systems, because the enzyme is not excreted and must be extracted.

ClIAA inhibits an enzyme which metabolizes cytokinins: 9-cytokinin-alanine synthase, at 10^{-6} M (Parker *et al.* 1986).

ClIAM is active in the defining test for brassinosteroid activity, the rice lamina inclination test (Park *et al.* 1987). When they tried to isolate brassinosteroids from *Vicia faba*, they found three fractions active in this assay. Two of them were castasterone and brassinolide, but the third was ClIAM. In this assay the brassinosteroids are active at ppb levels, ClIAM is active at the ppm level and ClIAA is almost inactive.

The influence of IAA, ClIAA and 5,6-Cl_2IAA on the formation of scopolamine and hyoscyamine was studied in root cultures of a *Duboisia* hybrid (Yoshimatsu *et al.* 1990). IAA induced the highest amounts of alkaloids, but ClIAA was active at the lowest concentration.

Flower induction in tissue cultures of cortical layers of tobacco has been studied by Hatori et al. (1991). The largest number of flower buds was formed with IAA (1 μM), but the optimum concentration obtained using ClIAA was a little lower. The best »flowering« was obtained with superoptimal IAA and the antiauxin 5,6-dichloroindoleisobutyric acid + extracts from short day treated plants.

4.1 Biological Activity of 4-Chloroindole-3-Acetic Acid Analogues

The activity of a number of halogenated analogues of ClIAA is given in Table 4.2., where more extensive investigations from recent years are summarized. The activity is given relative to IAA and is calculated as:

$$\frac{\text{conc. of IAA giving half maximal response}}{\text{conc. of analogue giving half maximal response}}$$

some of the older measurements, primarily on the monochlorinated IAAs are given in Table 4 in the review (Engvild 1986, VI). There are quite large differences in the responses to the different compounds in the various systems. It is also difficult to give good rationalizations of the responses. Some of the rules of thumb given in the review should be modified in the light of new information.

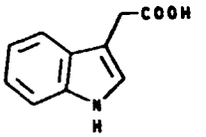
1) Indoleacetic acids substituted with halogen in the 4, 5, or 6 positions of the indole ring are strong auxins, stronger than, or comparable to IAA.
2) 7-halogenoindoleacetic acids are weak auxins, and in some assays they are antiauxins, *e.g.* promoting root growth.
3) Most dichlorosubstituted indoleacetic acids are weak auxins, except for 5,6-dichloroindoleacetic acid, which is a very strong auxin (Hatano *et al.* 1987a, b).
4) Disubstituted auxins with a substituent in the 7 position tend to be antiauxins and promote root growth.

Trihalogenated indoleacetic acids are now being investigated by Marumos's group and some of the results are published in the Japanese patents literature (Marumo *et al.* 1987). The methyl ester of 4,5,7-trichloroindole-3-acetic acid has been prepared.

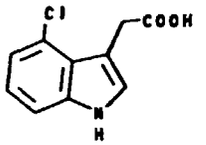
Table 4.2. Biological activity of analogues of 4-chloroindole-3-acetic acid relative to IAA in different systems

	Avena[1] elong.	Avena[2] elong.	Wheat[3] elong.	Wheat[4] elong.	Pea[3,5] elong.	Split[6] pea assay	Inhib.[7] shoot tissue	Inhib.[8] wheat roots	Wheat[4] binding assay
IAA	1	1	1	1	1	1	1	1	1
4-ClIAA	10	3	4	9	1.3	7	0.1	0.3	16
4-ClIAM	1.2								
5-ClIAA	3		0.7	4	0.7	3	10	0.1	4
6-ClIAA	19		1.5	5	1.4	5	1	1	7
7-ClIAA	0.1		0.1	0.1	0.1	2	0.1	promote	0.2
2-ClIAA			0.8		0.8	4			
5-FIAA	0.2		0.4	0.4	1.1	1.2	10		0.3
2-BrIAA						1.6			
5-BrIAA	0.3		0.4	0.6	0.9		1		0.3
7-BrIAA	0.1		0.04	0.02	0.02		0.01		0.1
4,5-Cl_2IAA		0.4							
4,6-Cl_2IAA	0.1	0.2	0.05	0.1	0.8		0.001	0.1	0.1
4,7-Cl_2IAA	0.01	0.02	0	0	0.01		0	promote	0.04
5,6-Cl_2IAA		5					promote[9]		
5,7-Cl_2IAA	0.01	0.02	0.03	0	0.01	0.15	0.001	promote	0.01
6,7-Cl_2IAA	0.1	1	0.01	0.1	0.1		0.01	promote	0.1
5-Cl, 7-MeIAA	0.1		0.03		0.02		0.001		
5,7-Cl_2IIBA		inhibit							
5,7-F_2IIBA		inhibit							

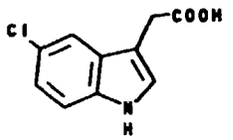
[1] Böttger, Engvild and Soll 1978b, [2] Hatano *et al.* 1987a, b, 1989, Marumo and Katayama 1988, 1989, [3] Katekar and Geissler 1983, [4] Zažímalová and Kutáček 1985b, [5] Katekar and Geissler 1982, [6] Porter and Thimann 1965, [7] Engvild 1978, IV, [8] Stenlid and Engvild 1987, XIII, [9] Mii *et al.* 1992.



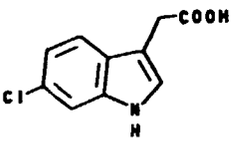
Standard.



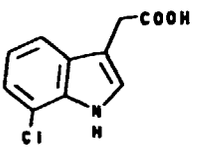
Natural in *Pisum sativum*.
Very strong auxin.
Perhaps death hormone?



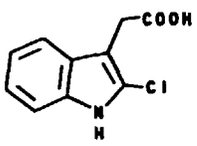
Very strong auxin.



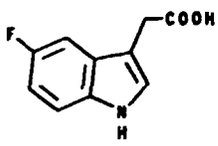
Very strong auxin.
6-bromoindoles common in marine organisms.



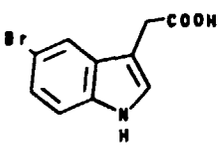
Activity less than IAA; antiauxin in promoting root growth.
Natural in *Pseudomonas* sp. as precursor for the antibiotic pyrrolnitrin.



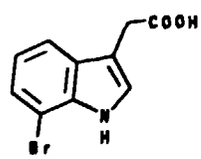
As IAA.



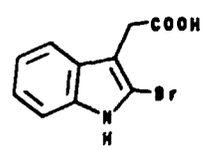
As IAA.



As IAA.

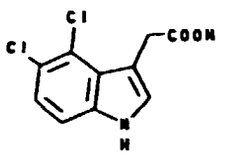


Weaker than IAA.

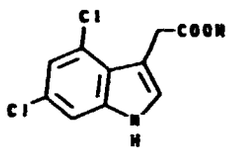


As IAA.

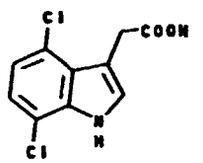
Figure 4.1a.



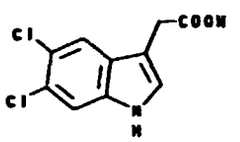
Weak auxin.



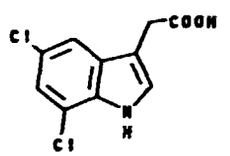
Weak auxin.



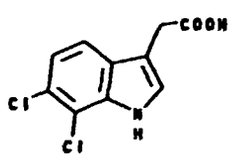
Very weak auxin.
Antiauxin in promoting root growth.



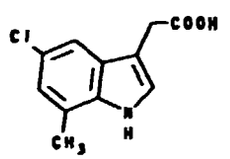
Very strong auxin.
Useful for shoot induction in tissue culture.



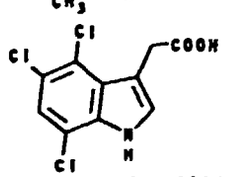
Very weak auxin.
Antiauxin in promoting root growth.



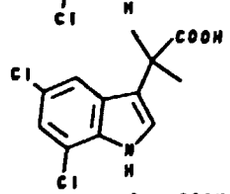
Very weak auxin.
Antiauxin in promoting root growth.



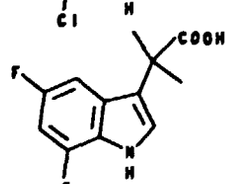
Very weak auxin.



Very strong auxin.



Very strong antiauxin.
Combining antiauxin character of 7-Cl and indoleisobutyric acid.



Strong antiauxin.

Figure 4.1b.

One very interesting development has been the parallel growth response to halogenated indoleacetic acids in the wheat coleoptile elongation assay and the displacement of radioactive IAA from an IAA binding preparation from wheat. (Zažímalová and Kutáček 1985a, b) as given in Table 4.2.

Halogenated indoleisobutyric acids were investigated by Hatano *et al.* 1987b, 1989, Hatori et al. 1991, Marumo and Katayama 1989 and by Yoshimatsu *et al.* 1990. They are very strong antiauxins.

Antiauxins like 4-chlorophenoxyisobutyric acid have become important as anticholesterol medicine in the treatment of heart attacks, and antiauxins in human medicine may become very important in auxin receptor research. Many of the compounds induce peroxisome proliferation and liver cell division or liver cancer in mice and rats. Receptor genes are being cloned, and they seem to be found in all the biological kingdoms, from bacteria to plants and animals. The medical people now talk of »growth signal pathways«, and the antiauxin receptor seems to be a member of the steroid hormone receptor superfamily (Nebert 1990, Issemann and Green 1990).

5 The Possible Role of 4-Chloroindole-3-Acetic Acid in Plants

When the chloroindole auxins were first isolated, they were considered to have more or less the same function as the well known IAA, with slight differences in degree and intensity of the various typical auxin manifestations in plants: Promotion of elongation growth, rooting, tissue culture growth, phototropism, gravitropism, ethylene evolution above a threshold, H^+ extrusion and so on.

Slowly it became apparent that ClIAA was often too strong to use for *e.g.* tissue cultures or the rooting of cuttings. When ClIAA was used in tissue culture of tobacco and soybean, there was little advantage over IAA or NAA (Engvild 1978, IV). In cuttings of pea ClIAA (Ahmad *et al.* 1987, XII) induced very strong, quite permanent epinasty. Many roots were formed, but the apical meristem did not grow for several weeks. Eventually, there was growth, not from the apical bud, but from the lowest adventitious bud, which formed an almost new plant. ClIAA treated cuttings evolved very large amounts of ethylene, and this ethylene evolution continued for 6 days, versus 1 day after IAA treatment.

This almost herbicidal quality of ClIAA on the plant from which it was isolated, has made me wonder if there might be two classes of endogenous auxins: growth promoters, typified by IAA, and autoherbicides, typified by ClIAA.

5.1 The Death Hormone Hypothesis

A hypothetical death hormone has a long history in plant physiology without really being accepted, such as the hypothetical flowering hormone has become accepted. The death hormone is a substance sent out from the developing seeds of monocarpic plants, *i.e.* plants that only flower once, as signals to

induce senescence in vegetative organs and remobilize nutrients to the seeds (Engvild 1989, VIII, Noodén and Leopold 1978, 1988, Noodén 1980, Kelly and Davies 1988).

In monocarpic plants seed set is usually accompanied by complete senescence of the vegetative parts. The typical exampie is the crop plants, where monocarpic senescence is demonstrated vividly every autumn. Monocarpic senescence is a very orderly phenomenon, where the degradation and remobilization of nutrients from vegetative parts to developing seeds is often tuned to provide maximum transfer of nutrients to the seeds.

Many mechanisms have been proposed to explain the phenomenon of monocarpic senescence *e.g.* Molisch (1929), Noodén and Leopold (1978, 1988), Noodén (1980), and Kelly and Davies (1988). They fall in four categories:

1) Nutrient drain or a remobilization of organic compounds from the vegetative parts to the developing seeds, caused by a very strong sink strength in the young seeds.
2) Nutrient competition or diversion. The reproductive structures monopolize all photosynthate and inorganic nutrients, making long term maintenance of the vegetative part impossible.
3) Limited lifespan or genetically programmed death of *e.g.* the apical meristem.
4) Production and export of a death hormone by the developing seeds.

Today most plant physiologists tend to favour the nutrient drain hypothesis, even those groups that earlier favoured a death hormone hypothesis (Kelly and Davies 1988, Gianfagna and Davies 1983, Hamilton and Davies 1988). Many plant physiologists also believe that neither hypothesis alone will explain monocarpic senescence (Woolhouse 1983, Kelly and Davies 1988). I tend to agree with this belief.

In many cases it is not possible to distinguish nutrient drain from nutrient competition or diversion. The two hypotheses will be treated as one in this chapter. Furthermore, hypothesis 3, limited lifespan or programmed cell death will always become true eventually. The laws of genetics stipulate that there will be no selection against deleterious mutations that act so late in development that they do not interfere with successful reproduction. So all organisms, whether they use nutrient drain, death hormones, something else, or nothing, will over the generations end up with a set of deleterious 'late' mutations. Those mutations will all look as programmed cell death to the experimentator.

The various types of experiments done on monocarpic senescence have not effectively been able to point out which of the models above is the most likely. It is quite certain that prevention of seed maturation by deflowering, defruiting and deseeding stops or postpones senescence. This can be explained both by nutrient drain and by death hormone action.

Grafting experiments, where a developing fruit is grafted onto a vegetative plant, have been quite difficult to do, and the results have sometimes been difficult to interpret. But the outcome: fruit-induced senescence can still be explained both by nutrient drain and death hormones.

Searches for death hormone candidates among radioactive substances transported from the fruit to the vegetative tissue have not given clearcut results. Sucrose and malate are major transported compounds, but only few other substances have been identified (Hamilton and Davies 1988). Death hormone bioassays are under development (Noodén *et al.* 1990), but definitive results are lacking.

5.2 The Need for Both Nutrient Drain and Death Hormone

I wish to propose that:

1) Death hormones or monocarpic senescence factors do exist. They are excreted from maturing seeds and promote senescence of vegetative plant parts.
2) Death hormones alone will not explain monocarpic senescence.

A major reason for advocating a monocarpic senescence promoter lies in the need of any regulated system for a speeder and a brake. The regulatory portion of a gene very often contains promoting and inhibitory sequences; an allosteric, regulating enzyme is often inhibited by some effectors and released from inhibition by others. There are often two pathways to important metabolites, one for synthesis involving ATP and energy expenditure, and another for breakdown.

In plant hormones similar antagonistic effects are known in many cases, for example between gibberellic acid and abscisic acid in the barley endosperm system; or the influence of the auxin/cytokinin ratio on shoot formation in tissue cultures, or the opposing effects of abscisic acid and cytokinins in senescence assays.

A very interesting parallel is the experience of Anton Lang, well known for his work on the probable existence of a flowering hormone. Many years later he showed by similar grafting experiments between long and short day plants, that there are probably also moving flowering inhibitors. When Lang returned to the records of his early work he saw that he had overlooked evidence for flowering inhibitors in his own controls (Lang 1980).

In a similar manner I think it is reasonable to assume that monocarpic senescence is governed both by senescence promoters and senescence inhibitors. In other words I think that neither the death hormone hypothesis alone nor the strong sink effect of developing seeds alone can explain monocarpic senescence. Rather both mechanisms operate simultaneously; under some circumstances one mechanism is evident, under other circumstances the other predominates.

5.3 Auxin as a Death Hormone in Xylem Differentiation

In a very special case IAA is known as a death hormone: the induction of vascular bundles, where both xylem and phloem cells are dead. The differentiation of conductive tissue is governed by a complicated interaction between hormones and nutrients, involving among others auxin, ethylene, gibberellic acid, cytokinin, and sucrose, whereby auxin is considered the critical hormone. Often phloem differentiation is induced by low levels of auxin in the presence of sucrose, while the diffentiation of xylem requires higher levels of auxin (Aloni 1987, Roberts *et al.* 1988). In the synchronized cell cultures of *Zinnia* the auxin/cytokinin ratio determines the differentiation of the cells to tracheid elements (Church and Galston 1988, Fukuda and Komamine 1981). The cytokinin must be present for 24 hours and auxin for 56 hours for tracheid formation at 72 hours (Church and Galston 1988).

Exactly what happens when a cell differentiates into a dead empty xylem

element with a strongly thickened and reinforced wall is not understood. However the fact remains that a high auxin level induces cell death, albeit under very special circumstances and through a long series of steps.

5.4 The Auxin-Export Governed Sink Strength Hypothesis

Bangerth (1989) has proposed an interesting hypothesis on what governs the relative sink strength between different fruits and between fruits and vegetative sinks. Bangerth states that the earlier developed sink inhibits later developed organs, and calls it »Primigenic dominance«, the dominance of the first born.

Some of the experiments on relative sink strength reviewed in the paper cannot be explained by competition for limited assimilate supply. Often sink dominance effects are expressed very early, long before serious assimilate accumulation has started.

According to Bangerth primigenic dominance is governed by auxin export in a subtle manner: The firstborn sink establishes a strong auxin export which inhibits the auxin export of sinks developing later. This lack of auxin export inhibits growth/sink strength. Bangerth supplies nice evidence that dominant fruits export much auxin, and younger fruits export little.

Bangerth also supplies evidence that the inhibition of IAA export probably takes place at the junctions of the vascular bundles. In this matter the hypothesis is close to that of auxin regulation of apical dominance and leaf abscission. When Bangerth added labelled IAA to one arm of a tomato pedicel and unlabelled IAA to the other arm, the transport of radioactivity was much reduced when the unlabelled material was applied first.

Bangerth's hypothesis can be rationalized in terms of auxin regulated formation of vascular bundles. Auxin production starts in the first formed fruits, induces full vascularization to those fruits and prevents complete vascularization of fruits formed later.

5.5 4-Chloroindole-3-Acetic Acid as a Death Hormone

Good evidence that 4-chloroindole-3-acetic acid or its derivatives might be the hypothetical death hormone in pea and members of its tribe does not exist, but there are some indications as discussed in Engvild (1989, VIII).

1. The chloroindole auxins are found primarily in the developing seeds. They reach maximum 2-3 weeks after fertilization and disappear again towards maturity (Gandar 1960, Gandar and Nitsch 1964, Katayama *et al.* 1988).
2. ClIAA induces very strong, almost irreversible ethylene production (Ahmad *et al.* 1987, XII).
3. ClIAA induces inactivation or death of the apical meristem (Ahmad *et al.* 1987, XII).
4. Auxin in lanolin placed in deseeded bean pods induces leaf senescence (Tamas and Engels 1981).
5. Auxins are transported back into the plant from developing fruits and seeds (Bangerth 1989).

The experiments of Ahmad *et al.* (1987, XII) showed that a concentration of 10^{-6} M was sufficient for inducing prolonged ethylene evolution in young cuttings of pea. Assuming that a pea plant weighs 25 g dryweight and produces 10 g of seeds, they will contain about 50 μg 4-chloroindoleacetic acid methyl ester 2-3 weeks after fertilization (Engvild *et al.* 1980). At maturity almost all has disappeared (Gandar 1960, Gandar and Nitsch 1964, Katayama *et al.* 1988); if one assumes that it has been exported to the 50 g (15 g dryweight) vegetative part over a period of 2 weeks, this would mean about 4 μg exported per day. If one assumes also that the breakdown rate is less than 75% per day there would be 5 μg in 50 g of plant corresponding to a continuous concentration of 10^{-7} M. Although this is less than the concentration used by Ahmad *et al.* (1987), they used only a 5 minutes dip. So the content of chloroindole auxins in maturing pea seeds might very well be sufficient to induce lethal ethylene evolution in the vegetative plant parts.

6 Do Plants Contain Two Auxin Types, Growth Promoters and Endogenous Herbicides?

In the early days of plant hormone research auxin was considered a universal growth regulator; several chapters in early plant hormone books were devoted to the inhibitory effects of auxin (Audus 1959). This emphasis on the inhibitory effects has decreased after the isolation of specific inhibitory plant growth regulators such as abscisic acid and jasmonic acid and the elucidation of the role of ethylene. Today not many people would believe that some natural endogeneous auxins actually might function as herbicides secreted by the maturing seeds.

A major argument against an endogeneous auxin herbicide is that most people think of auxins as one substance, indoleacetic acid, and its precursors and metabolites. For some purposes this compound is found in inhibitory concentrations, inhibiting axillary bud growth and root elongation, and inducing ethylene synthesis; but all this is considered part of the normal auxin action repertoire. The presence of the chloroindole auxins in pea and closely related species is today recognized by many plant physiologists, but the fact is perceived as a curiosity which does not have important implications for auxinology in general.

I wish to propose that: There may be two auxin types, growth promoters and endogenous herbicides.

Evidence for this is the presence of indoleacetic acid in the vegetative stages of pea development, and of chloroindoleacetic acid in the generative stages of the pea development (Gandar and Nitsch 1964, Schneider *et al.* 1985, Katayama *et al.* 1988) and the inhibitory, strongly ethylene inducing qualities of ClIAA (Ahmad *et al.* 1987, XII).

A serious weakness of the proposal is of course that two auxin types are not commonly observed in plants, if one excludes phenylacetic acid, because of its low biological activity and non-transportability. However, many plants seem to contain other chlorine-containing compounds which might be candidates for endogeneous herbicide activity (Engvild 1975, II, Engvild 1986, VII).

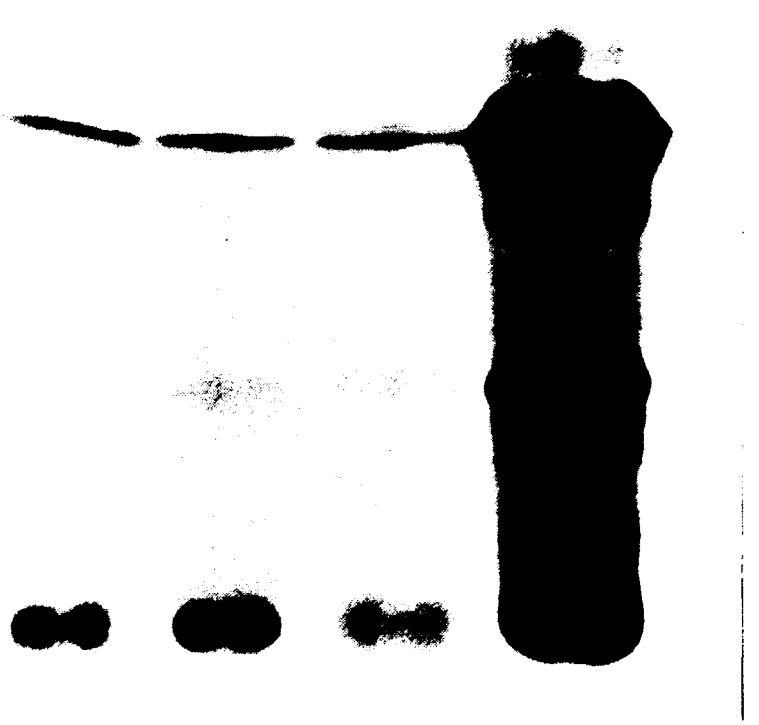
6.1 Are There Other Chlorine-Containing Natural Plant Hormones?

At present the answer is no. No other chlorine-containing natural plant hormones are known. However, there are chlorine-containing compounds in many plants and not just in the exotic, secondary plant compounds reviewed in Engvild (1986, VII). Many crop species incorporate radioactive chloride into compounds that move in organic solvents on thin layer chromatograms. The compounds are detectable on thin layer chromatograms by autoradiography (Engvild 1975, II). Their mobility indicates that they might be related to the chloroindoles, but they are not identical to the pea compounds.

So, chlorine-containing compounds are there. Are they for real, or are they artefacts? And if they are there for real, are they hormones?

Efforts have been done to isolate some of these compounds in barley, but without much success. Radioactive ^{36}Cl was incorporated into the maturing seeds of barley, and the radioactivity was used as a marker during attempts of extraction and purification of chlorine-containing substances, primarily by column chromatography on Sephadex LH-20. Fractions were collected directly in glass scintillations vials and screened for radioactivity by scintillation counting of the Cerenkov radiation. Fractionation was possible, but the peaks were broad, and the radioactivity disappeared subsequently, either when the fractions were concentrated, or during other purification steps, such as thin layer chromatography.

Figure 6.1. Autoradiogram of thin layer chromatogram of extracts of immature barley seeds from plants grown in radioactive chloride. The barley was extracted in four different solvents: hexane, ether, ethyl acetate and n-butanol. Butanol extracts much more radioactivity than the other solvents. This may be due to better solubility in butanol, or it may be artefacts formed by peroxidase still active in the presence of butanol.



During such studies I observed that the amount of radioactive ^{36}Cl incorporated in »organic linkage« was dependent on the extraction solvent. Much larger amounts of »organic« chlorine (defined as radioactivity moving in the very apolar thin layer chromatographic system) was extracted in butanol than in ether, ethyl acetate or hexane (Figure 6.1.). Such observations should eventually make it possible to isolate and identify natural chlorinated compounds in some of the important crop species.

The overextraction of chlorinated compounds into butanol may be a question of solubility, but it may also indicate that artefacts are formed, *e.g.* chlorinating peroxidases could be active in butanol. This may or may not be important, but should be checked. The formation of some halogenated animal hormones such as thyroxine is also »artifactual« and the contents can increase under artificial conditions.

For future work I should try to identify major chlorine-containing compounds in some important crop plant seeds: the grasses, the solaneaceaes and the crucifers, for example. When such identifications were made, and appropiate syntheses completed, I would test the compounds for auxin and, if possible, for death hormone activity.

6.2 The Natural Chlorine Containing Auxin/Death Hormone of *Dictyostelium*

The slime mould *Dictyostelium discoideum* has for many years been a work horse for biologists (Berks 1989). It is most famous because cyclic AMP proved to be identical with acrasin, the aggregation signal that caused the individual amoebae to seek together to form fruiting bodies.

Taxonomically, *Dictyostelium* is not easy to put into a proper slot. The individual amoebae look like protists and animals. The fruiting body looks like a fungus forming spores. The presence of cellulose in the sheaths of the stalk cells suggests a higher plant.

In the following I am going to describe another set of data which suggests similarity to higher plants. For many years biologists have tried to isolate the morphogens of *Dictyostelium, i.e.* the substances that induce the formation of spores and of stalk cells. The isolation of the differentiation inducing factor (DIF), depended on the development of a bioassay where the amoebae were prevented from aggregating by being kept in a single cell layer on the agar below a sheet of cellophane. Under such conditions the cells will form individual stalk cells, surrounded by a cellulose wall and with a very prominent vacuole with cytoplasmic strands (Kay *et al.* 1983).

The DIF is present in very low amounts, and only 50 μg were isolated in the largest preparation. DIF was allowed to diffuse through cellophane from large batches of starved amoebae, and was collected on ion exchange beads. The DIF was extracted from the ion exchanger with ethanol and purified by solvent extraction steps and HPLC. The substance was very active in the nanomole range.

After beautiful chemical detective work on the mass spectra, and a considerable amount of luck in choosing a methylated phloroglucinol as a starting point for synthesis, the dichlorohexanophenone (Figure 6.2.) was identified (Morris *et al.* 1987, 1988).

This compound type was unknown in biological contexts. The closest relatives with activity in plant related systems are the acetosyringones involved in the *Agrobacterium* induced tumour formations. However, the sub-

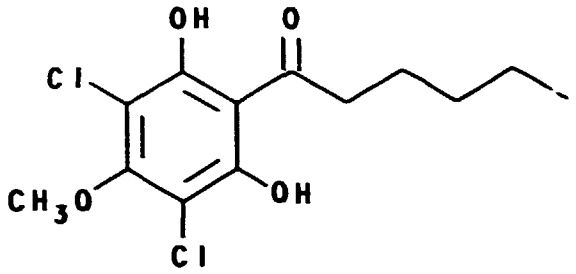


Figure 6.2. The death hormone or differentiation inducing factor of Dictyostelium discoides. The compound causes the differentiation of ameboid cells into elongated, vacuolated cells with a cellulose cell wall. These cells constitute the Dictyostelium stalk, and end up dying, while other ameboid cells differentiate into spores.

stance is not too far away from a 2,4-D type of compound in the sense that it is acidic and contains two chlorine atoms on a benzene ring.

The DIF has some auxin like characteristics: it induces cellulose formation, it induces cell elongation, and it induces vacuolization. DIF also has death hormone characteristics: the stalk cell dies while the spores are viable. However, nothing is known about the possible transfer of nutrients from the stalk cells to the spores.

7 Acknowledgements

Many people have helped me extensively through the various phases of the work on the chloroindole auxins. Most of the practical work was done from 1973 until 1982, but I have not stopped thinking about the chloroindole auxins and their possible significance since then.

I wish to express my sincere thanks to Poul Gade, Vagn Aage Nielsen, and Jørgen D. Thomsen who offered great assistance when I first grew plants in water cultures with radioactive chloride, as well as later.

I am also very grateful to Ole Jørgensen, Jan Rud Andersen and Bror Skytte Jensen at the former Chemistry Department for letting me synthesize all the different halogenated indoleacetic acids in their laboratory and their help with time and chemicals (unthinkable these days). Lone Dyrgaard Jensen and Claus Christensen helped also with synthesis and isolation of presumably chlorine-containing compounds from many species related to pea as well as many other crop plants. Beth Lemée and Liselotte Meltofte have helped with later stages.

This work would have been impossible without the expertise of the mass spectrometry people at the Chemistry Department: Helge Egsgaard, Jytte Funch and Elfinn Larsen. Helge Egsgaard introduced me to the use of internal deuterium-labelled standards, and did all the practical work on the gas chromatograph-mass spectrometer. It was a pleasure to share their great enthusiasm.

I am also grateful to the many plant hormone people, who have helped me at various stages; many of them by testing my halogenated indoleacetic acids in their particular systems. Especially, I am grateful to Michael Böttger who took an early interest in both biological activity and chemical determination, and extended kind hospitality under my visit in Germany. Also, Göran Stenlid of SLU, Uppsala, helped clear up many aspects of the biological activity of the analogues of chloroindoleacetic acid, particularly the antiauxin like activity of 7-substituted indoles. Finally, Aqil Ahmad and Arne Skytt Andersen sprang their great surprise on me that chloroindoleacetic acid is toxic to the plant which produces it, and caused me to flip-flop in my thinking on the compound. Without their observations, the content of the present work would have been very different.

I have had many fruitful discussions with Peter Ulvskov and the other auxinologists, both at the University of Aarhus and at the Royal Agricultural and Veterinary University and further afield in Scandinavia.

I wish to thank the former heads of my department Jens Sandfær and Arna Andersen for their help and support. They were not always convinced that chloroindole auxins were important in grain filling, which was the rationale for my work; and some of my negative experiments proved them right. I still believe that chloroindole auxins are important in grain filling, but now as endogenous herbicides in a way almost opposite to the hypotheses I used earlier.

I also wish to thank my colleagues at Risø for fruitful discussions and a general inspiring environment, and patience when having to read yet another manuscript. The staff at the Risø Library gave me important, cheerful assistance when I conducted some large literature searches. And finally, I wish to thank the octopuses who take care of typing and everything else, Lis Petersen, Anni Bækmark and Anette Frandsen.

8 Summary

The chloroindole auxins of pea, strong plant growth hormones or endogenous herbicides?

When the present work was started most people believed that the auxin group of plant hormones comprised indoleacetic acid, its precursors and metabolites. The hormone type herbicides were also auxins, but they were not naturally occurring compounds. Phenylacetic acid had also been identified, but it was uncertain whether it should be considered an auxin, as it did not appear to be transported.

The methyl ester of 4-chloroindole-3-acetic acid was identified in immature peas in 1967-68 by Nitsch's group in France and by Marumo's group in Japan. However, few people really accepted that there were naturally occurring auxins that contained chlorine. Even Gandar and Nitsch themselves were not quite convinced and performed several control experiments without undermining their conclusions.

A potent naturally occurring auxin should be an ideal substance for experiments in *e.g.* plant tissue culture. As the substance was difficult to obtain I decided to synthesize it myself. During this work it occurred to me that there was a very simple way to confirm the natural occurrence of 4-chloroindoleacetic acid and its methyl ester. One could let the plants take up radioactive ^{36}Cl and show by thin layer chromatography of extracts followed by autoradiography that the chlorine had been incorporated in compounds with exactly the same mobility as the chloroindole compounds.

This experiment was done on pea and barley with a mixture of 4- and 6-chloroindoleacetic acids and their methyl esters as standards. The results was a confirmation that immature pea seeds contained chloroindole auxins. Also barley contained chlorinated compounds, chromatographing close to the chloroindoles.

This experiment was extended with 13 other important crop plants to a total of 15 species: barley, oat, wheat, rye and maize, chives and flax, bean, soybean and pea, cress, rapeseed, tobacco, tomato and sunflower. Many of these incorporated radioactive ^{36}Cl into compounds that chromatographed close to the chloroindoles on thin layer chromatograms, but it was also clear that they were not identical to the standards in many of the plants. There was no visible ^{36}Cl incorporation in maize, flax, soybean and sunflower.

After synthesis of a number of chloroindole-3-acetic acids and their methyl esters, an identification study of 4-chloroindole-3-acetic acid methyl ester in lyophilized green peas was performed. The auxin was extracted with butanol, taken up into acetonitrile, chromatographed on Sephadex-LH20 and silicagel columns, and monitored by the Čerenkov radiation from ^{36}Cl. Final identification was done by gas chromatography/mass spectrometry, where the $^{35}Cl/^{37}Cl$ ratio of 3:1 and the characteristic fragmentation pattern of indoles showed the presence of a chloroindole compound, and by co-chromatography on thin layer chromatograms of ^{36}Cl radioactivity and 4-chloroindole-3-acetic acid methyl ester in 12 solvent systems.

4-Chloroindole-3-acetic acid methyl ester was subsequently identified in *Vicia faba, Lens culinaris,* and other species of *Vicia* and *Lathyrus.* The compound was monitored by its characteristic Ehmann colour reactions on thin layer plates, and verified by gas chromatography/mass spectrometry, using pentadeuterated 4-chloroindole-3-acetic acid methyl ester as internal standard.

The use of an internal standard made quantitative determinations of 4-chloroindoleacetic acid methyl ester in immature seeds possible. In pea there were mg/kg amounts, in the other species, a little less.

There were also attempts to identify chloroindole auxins in immature seeds of other species. However, in no other species was the Ehmann colour reaction for chloroindoles detected on thin layer plates, and no further attempts were made.

Several investigators have worked on the biological activity of the set of chloroindoleacetic acid analogues synthesized. All the monochloroindole acetic acids are strong auxins, although 7-chloroindole acetic acid is about 10 times weaker than the others and sometimes has antiauxin character, *e.g.* in promoting root growth. Most dichloroindoleacetic acids are weak auxins, and 5,7-dichloroindole-3-acetic acid is an antiauxin.

4-Chloroindole-3-acetic acid is a very active auxin in all assays; its activity varies from 50 times the activity of IAA to a little stronger than IAA. The compound promotes curvature in decapitated coleoptiles, which indicates that it is transported in the plant.

Ahmad, Andersen and Engvild (1987, XII) showed that pea cuttings formed many roots after treatment with 4-chloroindole-3-acetic acid. However, the cuttings nearly died. They evolved large amounts of ethylene over long time spans. The apical meristem stopped growing, but after several weeks the cuttings formed new shoots from the lowest bud.

The fact that 4-chloroindoleacetic acid is sometimes toxic in the very plant, where it occurs in large quantities under other circumstances has led me to propose the hypothesis that chloroindole auxin metabolites could be identical to the hypothetical death hormone or senescence factor, which is excreted from maturing seeds and signals to the mother plant that all nutrients be mobilized to the seeds and that »time is up« for the mother plant.

At present this is only hypothesis. On the other hand it is quite difficult experimentally to distinguish between the various models of monocarpic senescence: the mother plant is sucked dry by the sink strength of the seeds, or the mother plant is poisoned by the seeds, so to speak.

If the death hormone hypothesis is true (or partly true, as I believe both sucking dry and poisoning operate) it is at the same time postulated that there are endogenous herbicides with important roles in the lives of many plants, and that herbicide activity is a central function of many auxins. At the same time it opens up the possibilities of a different model of action for the hormone herbicides: Hormone herbicides simulate endogenous death hormones.

9 Dansk resumé

Klorindolauxinerne i ært, stærke plantevæksthormoner eller endogene herbicider?

Da dette arbejde blev startet, opfattede de fleste hormongruppen auxiner som indoleddikesyre (IAA) samt de stoffer, der kunne omdannes til indoleddikesyre, eller som indoleddikesyre kunne omdannes til. Man kendte ganske vist også de syntetiske auxiner, for eksempel hormonmidlerne til ukrudtsbekæmpelse; men de var ikke naturligt forekommende stoffer. Man havde også identificeret phenyleddikesyre, som man tøver med at kalde et ægte auxin, fordi det ikke ser ud til at blive transporteret i planten.

Metylesteren af 4-klorindol-3-eddikesyre var blevet identificeret i umodne ærter i 1967-68 af Nitsch's gruppe i Frankrig og af Marumo's gruppe i Japan. Selvom de to arbejder støttede hinanden, var der ikke generel accept af, at der fandtes naturligt klorholdige auxiner i planter. Gandar og Nitsch havde svært ved at tro på deres egne resultater og gennemførte adskillige kontrolforsøg for at udelukke, at de klorholdige stoffer kom fra sprøjtemidler eller var artefacter.

Et meget kraftigt naturligt forekommende plantehormon måtte være ideelt at bruge, fx i plantevævskultur. Da stoffet var vanskeligt at skaffe, besluttede jeg at syntetisere det selv. Under dette arbejde gik det op for mig, at der fandtes en enkel måde at bekræfte den naturlige forekomst af 4-klorindoleddikesyre og dens metylester på. Man kunne lade planterne optage radioaktiv $^{36}Cl^-$ og ved tyndtlagskromatografi og autoradiografi vise, at kloret var blevet inkorporeret i stoffer, som havde nøjagtig samme mobilitet som de to klorerede indoler ved tyndtlagskromatografi.

Dette forsøg blev i første omgang lavet på ært og byg med en blanding af 4- og 6-klorindoleddikesyre og deres metylestre som standarder. I ært opnåedes en smuk bekræftelse på, at der var klorindolauxiner til stede. Også byg indeholdt klorerede stoffer, som kromatograferede tæt ved klorindolerne.

Disse forsøg blev udvidet med 13 andre vigtige afgrøder til i alt 15: byg, havre, hvede, rug, majs; purløg, hør, bønne, soyabønne, ært, karse, raps, tobak, tomat og solsikke. Mange inkorporerede radioaktiv ^{36}Cl i stoffer som tyndtlagskromatografisk lignede klorindolauxinerne. Der var dog ingen synlig inkorporering af ^{36}Cl i majs, hør, soyabønne og solsikke.

Der blev lavet en fuldstændig identifikation af 4-klorindoleddikesyre metylester i frysetørrede grønærter. Auxinet blev ekstraheret fra ærterne med butanol, overført til acetonitril, kromatograferet på Sephadex-LH20 og silicagel søjler, altsammen fulgt med Čerenkov strålingen fra ^{36}Cl. Endelig identifikation skete ved gaskromatografi/massespektrometri, hvor $^{35}Cl/^{37}Cl$ forholdet 3:1 og det karakteristiske fragmenteringsmønster for indoler viste tilstedeværelsen af en klorindol forbindelse, og ved co-kromatografi på tyndtlag af ^{36}Cl radioaktivitet og 4-klorindol-3-eddikesyremetylester i 12 solvent systemer.

Derefter blev 4-klorindol-3-eddikesyre metylester identificeret i hestebønne, linse og andre arter af vikke og fladbælg. Stoffet blev lokaliseret ved den karakteristiske Ehmann farvereaktion på tyndtlagsplader, og verificeret ved gaskromatografi/massespektrometri, hvor der som intern standard blev brugt pentadeutereret 4-klorindoleddikesyre metylester efter forslag af Egsgaard og Larsen. Dette muliggjorde også en kvantitativ bestemmelse af indholdet af 4-klorindoleddikesyre metylester i de umodne frø. I ært var der adskillige mg/kg og i de øvrige undersøgte arter lidt mindre.

Der blev også gjort forsøg på at identificere klorindolauxiner i umodne frø af en række andre arter end bælgplanter. Men dette arbejde blev ikke forfulgt yderligere, hvis ikke der var synlig Ehmann farvereaktion ved tyndtlagskromatografi. Klorindolauxiner kan muligvis findes i planter, hvor jeg ikke har påvist dem; beviskraften i negative resultater af tyndtlagskromatografi er trods alt begrænset.

En række forskere har arbejdet med de forskellige klorindoleddikesyrer, der er blevet fremstillet. Alle monoklorforbindelserne er stærke auxiner, idet 7-klorindoleddikesyre dog er ca. 10 gange svagere end de andre, og i visse situationer har antiauxinkarakter. Diklorindoleddikesyrerne er næsten alle svage auxiner, og 5,7-diklorforbindelsen er et decideret antiauxin.

4-Klorindoleddikesyre er et meget aktivt auxin i alle assays; dets aktivitet varierer fra 50 gange IAA til lidt stærkere end IAA. 4-Klorindoleddikesyre fremmer koleoptilebøjning, dvs det transporteres sandsynligvis.

Ahmad, Andersen og Engvild (1987, XII) påviste, at ærtestiklinger dannede mange rødder under påvirkning af 4-klorindol-3-eddikesyre. Derefter så det næsten ud til, at stiklingerne døde. De udviklede store mængder ætylen i meget lang tid. Det apikale meristem standsede væksten totalt, men efter flere uger skød stiklingerne fra den nederste knop.

Det faktum, at 4-klorindol-3-eddikesyre er toksisk i den plante, hvori det under andre omstændigheder findes i meget store mængder, har fået mig til at fremsætte den hypotese, at klorindolauxin eller dets metabolitter kunne være identiske med det hypotetiske dødshormon eller senescensfaktor, som udskilles fra modnende frø, og som signalerer til moderplanten, at alle næringsstoffer skal mobiliseres til frøene, og at moderplantens dage er talte.

Dette er på nuværende tidspunkt kun hypotese. Det er meget vanskeligt eksperimentelt at skelne mellem de forskellige modeller for senescens induceret af frømodning: om moderplanten suges ud eller om moderplanten forgives.

Hvis dødshormonhypotesen er rigtig (eller delvis rigtig, fordi jeg tror, at både forgiftning og udsugning finder sted), er det samtidig postuleret, at der findes endogene herbicider, som spiller en fysiologisk rolle i mange planters liv, og at herbicideffekt er en meget central funktion for nogle auxiner. Samtidig giver det en helt ny mulighed for en model for de hormonale herbicider: Hormonmidlerne simulerer planternes naturlige dødshormoner.

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Appendix A

Chemistry and synthesis of chloroindole auxins

A.1 Chemistry of 4-chloroindole-3-acetic acid

The compound is rather unstable and should be kept in the refrigerator in the dark. Solutions should be freshly prepared. Aqueous solutions tend to redden, *e.g.* during recrystallization. Solutions of the salts are more stable. Yang *et al.* (1989) have shown that 4-chloroindoleacetic acid methyl ester is photolyzed by UV light with a high quantum yield to form indoleacetic acid methyl ester, two dimers and some polymeric material. Hence, one would expect some dehalogenation in sunlight or fluorescence lamp light. The light sensitivity is especially critical when labelled samples are used as internal standards. It may explain the difficulties in preparing radioactive ClIAA.

A few halogenated chloroindole-3-acetic acids were available commercially, 5-fluoroindole-3-acetic acid and 5-bromoindole-3-acetic acid. 5-Chloroindoleacetic acid, 7-chloroindole-3-acetic acid and four different dichloroindole-3-acetic acids were synthesized by a Fischer indole synthesis from chloro substituted phenylhydrazine condensed with succinic acid semialdehyde prepared from glutamic acid by NaOCl (Engvild 1977, III). Also 7-bromoindole-3-acetic acid and a few others were prepared this way (Engvild 1977, III). 4-Chloroindole-3-acetic acid was synthesized from 4-chloroindole (Engvild 1977, III) and 6-chloroindole-3-acetic acid in an analogous manner.

A.2 Synthesis of 4-chloroindole (German Patent 2057840, Hengartner *et al.* 1979)

6-Chloro-2-nitrotoluene (34.3 g 0.2 mole) was dissolved in 200 ml N,N-dimethylformamide and 50 ml 95% N,N-dimethylformamidediethylacetal (0.28 mole) and heated under argon for 6 hours at 140°C under magnetic stirring. The produced ethanol distilled off continuously. The solvent was evaporated at 35-40°C, 6 mm Hg and the intensely deep red trans-6-chloro-beta-dimethyl amino-2-nitrostyrene was distilled at 0.2-0.3 mm Hg at 110-116°C. Yield 40 g (88%). This compound was dissolved in 350 ml benzene with 1 g of Raney Nickel and hydrogenated in a Parr apparatus at 3-3.5 atm until no more hydrogen was taken up. After filtering the light red solution was washed 3 × 200 ml 1 M H_2SO_4, twice with 200 ml water and once with 100 ml 10% $NaHCO_3$ and finally with water and dried over $MgSO_4$. The solvent was distilled off and 15 g (50%) 4-chloroindole obtained after distillation *in vacuo* at 86-92°C, 0.2-0.3 mm Hg.

A.3 Synthesis of 4-chlorogramine

To a solution prepared under ice cooling of 3.0 g (0.1 mole) CH_2O and 7.3 g (0.1 mole) $(C_2H_5)_2NH$ in 20 ml acetic acid was added a cooled solution of 15 g (0.1 mole) 4-chloroindole in 30 ml acetic acid. The mixture was left at room temperature overnight. Under stirring and cooling the mixture was slowly added to 1 litre 1.5 M NaOH. The crude white precipitate, 3-diethylaminomethyl-4-chlcroindole was filtered off, washed with water and dried *in vacuum* overnight. Yield 22.2 g (93%).

A.4 Preparation of 4-oxo-butanoic acid solution

Glutamic acid (14.7 g, 0.1 mole) was dissolved in 250 ml water with 4 g NaOH and heated to 60°C; 0.1 mole (82 ml) of a NaOCl solution (concentration determined by iodine titration with thiosulphate) was added under mag-

netic stirring; the temperature rose to 75-80°C. The solution was acidified with 25 ml concentrated HCl to pH below 2; large quantities of CO_2 were evolved. The solution was used directly for Fischer indolization (Engvild 1977, III).

A.5 Synthesis of radioactive 4-chloroindole-3-acetic acid

Radioactive 4-chloroindoleacetic acid labelled with ^{14}C in the methylene carbon of the acetic acid was prepared from 4-chloroindole and radioactive formaldehyde. In a 25 ml flask with a B19 joint was placed consecutively 20 µl (0.19 mmole) $HN(C_2H_5)_2$, 15 µl (0.26 mmole) acetic acid, 10 µl 35% formaldehyde (0.13 mmole), 500 µCi ^{14}C labelled CH_2O = 61 µl = 0.03 mmole (17.3 mCi/mmole), and 20 µl = 25 mg (0.165 mmole) 4-chloroindole. The set-up was turned occasionally for 2 hours, while the chloroindole dissolved, and left overnight in the hood. One ml of water and 2 drops of concentrated NH_3 was added. The water and solvents were removed from the precipitated radioactive 4-chlorogramine *in vacuo*, the gramine was dissolved in 200 µl ethanol and 50 µl water containing 40 mg NaCN (0.8 mmole) and the mixture refluxed for 5 h. 75 µl 10 M NaOH was added and reflux continued overnight. 200 µl ethanol was added and reflux continued for 5 more hours. The contents were evaporated to dryness *in vacuo*. ½ ml of water + 2 drops of 2% NaOH was added and the contents were extracted with a pasteur pipet with 3 × 2 ml of ether which were discarded. The salt was acidified with eight drops of conc. HCl and the solvents evaporated in vacuum. The radioactive 4-chloroindoleacetic acid was purified by sublimation *in vacuo* onto a cold finger at 130-150°C and 1 mm Hg. Yield 6.6 mg, 60 µCi, 1.9 mCi/mmol, 19% for indole, 12% for radioactivity. The product was stored in two glass ampoules in 2:3 ethanol:benzene. The radioactive purity was approximately 96% estimated by thin layer chromatography and autoradiography. The structural identity was confirmed by thin layer chromatography, colour reactions and UV spectroscopy. Radioactive chloroindoleacetic acids have also been prepared by Baldi *et al.* (1985) from chlorophenylhydrazines and ^{14}C labelled 2-oxoglutaric acid.

A.6 Synthesis of 4-chloroindole-3-acetic acid methyl ester

To 90 ml ice-cold solution of Diazomethane (explosive, highly toxic) in ether prepared in an efficient hood from N-methyl-N-nitroso-p-toluenesulfoneamide (30 mmole, 6.4 g) according to Organic syntheses, **36**, 16-19, 1956, was added, slowly under cooling, a solution of 2 g (9.5 mmole) 4-chloroindoleacetic acid in ether. The ether was rotaevaporated. The residue was recrystallized from CCl_4. Yield 80%, mp 117-118°C.

Preparation has also been done with diazomethane from N-methyl-nitro-N-nitrosoguanidin (Organic syntheses, Coll. Vol **2**, 165, 1943). Acid catalyzed esterification with methanol has not been successful.

A.7 Preparation of pentadeuterated 4-chloroindole-3-acetic acid methyl ester

by Helge Egsgaard, Mass spectrometry laboratory.

50 mg (0.23 mmole) 4-chloroindole-3-acetic acid methyl ester was dissolved in 1 ml of a 5% solution of sodium deuteromethoxide in CD_3OD in an ampoule which was evacuated and sealed. The vial was heated at 50°C overnight. The contents were neutralized with HCl and the methanol was evacuated. After dilution with 3 ml of water, the ester was extracted with ether, the ether solution washed. The ester was characterized with mass spectrometry and stored dissolved in ethanol. It is important that the starting material is pure and dry.

A.8 Synthesis of 4-chloroindole-3-acetic acid nitrophenyl ester
by Susanne Petersen and Ole Jørgensen, Synthesis laboratory.
To a 0-5°C solution of 4-chloroindoleacetic acid (4.0 g, 19 mmole) and p-nitrophenol (2.65 g, 19 mmole) in 75 ml ethylacetate was added 3.94 g (19 mmole) dicyclohexylcarbodiimide in 15 ml ethylacetate and the mixture stirred under cooling for an hour and at room temperature for another hour (Mollan *et al.* 1972) The precipitated dicyclohexylurea was filtered off; the filtrate was rotaevaporated to 15 ml and refiltered. The filtrate was evaporated to dryness and 6.7 g crude nitrophenyl ester was isolated. Yield after recrystallization from ethylacetate/hexane 4.4 g, 70%, mp 166-168°C.

A.9 Synthesis of 4-chloroindole-3-acetyl-D,L-aspartic acid
by Susanne Petersen and Ole Jørgensen, Synthesis laboratory.
1.71 g (13 mmole) D,L-aspartic acid was dissolved in 9.4 g 25% tetramethylammoniumhydroxide (26 mmole). The solution was lyophilized to an oil. The tetramethylammonium salt was dissolved in 65 ml DMSO, and 4.25 g (13 mmole) 4-chloroindoleacetic acid nitrophenyl ester was added and stirred overnight (19 hours). The DMSO was rotaevaporated, and the product was dissolved in 130 ml 5% $NaHCO_3$. The solution was extracted with two 130 ml portions of ether, pH was adjusted to 5 and the solution was extracted with additional 2 × 130 ml portions of ether. After concentration to half, the solution was adjusted to pH 2 with conc. HCl and kept overnight in the refrigerator. The white precipitate was washed with cold water and twice with ether. Yield 2.85 g, 68%, mp 194-196°C.

A.10 Synthesis of α-N-carbomethoxyacetyl-D,L-4-chlorotryptophan methyl ester
by Paw Block and Ole Jørgensen, Synthesis laboratory.
An impure preparation of malonated D,L-4-chlorotryptophan dimethylester (Marumo and Hattori 1970) was obtained from D,L-4-chlorotryptophan methyl ester hydrochloride prepared according to Hengartner *et al.* (1979) (4-Chlorotryptophan was also prepared according to Rydon and Tweddle (1955) from 4-chloroindole). 4-Chlorotryptophan methyl ester (1.4 mmole) in 6 ml of dioxane was mixed with triethylamine (4.2 mmol) and methyl malonylchloride (1.4 mmol) with cooling and moisture exclusion. Purification beyond rotaevaporation of the dioxane and washing with water was not successful. Redbrown semicrystalline mass, 56%.

Table A.1. Synthesis of 4-chloroindole-3-acetic acid and related compounds.

Carbon-14 labelled chloroindole-3-acetic acid by Fischer indolization from phenyl-hydrazines and labelled α-oxoglutarate. Separation by chromatography	Baldi *et al.* 1985
Fischer indolization from glutamic acid and phenylhydrazines	Cocordano *et al.* 1970
From 4-chloroindole via the gramine NaCN reaction; analogues by Fischer indolization	Engvild 1977
From 4-chloroindole via the gramine and chloroacetonitrile	Fox and Bullock 1951 analogues by Fischer
From 4-chloroindole via the gramine KCN reaction	Hansch and Godfrey 1951
Fischer indolization with dimethoxybutyric acid in xylene, $ZnCl_2$	Hatano *et al.* 1987a, b
From the chloroindole, HCHO and KCN by autoclaving	Szewczyk and Lesiak 1972
Sandmeyer reaction of 4-aminoindole compounds	Nissan 1983 Somei *et al.* 1985
Trichloroindole-3-acetic acids by Fischer indolization ($ZnCl_2$) and 4.4-dimethoxybutyric acid	Marumo *et al.* 1987
2,X-dichloroindole-3-acetic acid from x-ClIAA by chlorination	Marumo and Katayama 1989
Halogenoindole-3-isobutyric acid from halogenoindole-3-acetic acids	Hatano *et al.* 1989 Marumo and Katayama 1989
4-Chlorotryptophan from 2-chloro-6-nitrotoluene	Thiruvikraman *et al.* 1988 Hengartner *et al.* 1979

Appendix I

Chloroindolyl-3-acetic Acid and Its Methyl Ester Incorporation of ^{36}Cl in Immature Seeds of Pea and Barley

By

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(Received February 25, 1974)

Abstract

Immature seeds of pea and barley were harvested on plants grown in solutions containing $^{36}Cl^-$, but no other chlorides. Autoradiography of two-dimensional thin layer chromatograms (silicagel) of butanol extracts of freeze-dried seeds showed the presence in both species of several radioactive compounds besides Cl^-. One compound, present in pea and probably in barley, cochromatographed with a mixture of 4- and 6-chloroindolyl-3-acetic acid methyl esters. Another, detected in pea, but probably not in barley, cochromatographed with a mixture of 4- and 6-chloroindolyl-3-acetic acids.

Introduction

Compounds with covalently linked Cl are not as rare in nature as once thought; more than 25 have already been isolated from higher plants (18). Isolation of 4-chloroindolyl-3-acetic acid methyl ester from young green peas was reported by Gandar and Nitsch in 1967 (5) and by Marumo *et al.* in 1968 (11). Marumo *et al.* (11) established the exact position of the chlorine atom. This discovery has had surprisingly little impact. After all it represents one of the first identifications of a potent auxin in plant material without prior knowledge of the chemical nature of the compound. Indoleacetic acid itself was first isolated from urine and fungi and only after that detected in higher plants. Most subsequent auxin identifications were done after educated guesses of likely indoleacetic acid derivatives, tests of derivative activity and subsequent chromatographic identification.

Few papers have been published on naturally occurring chloroindoles. 4-Chloroindoleacetic acid, its methyl ester, its aspartic acid amide and two derivatives of D-4-chlorotryptophan have been isolated from green peas by the group around Marumo (8, 11, 12, 13, 14; see Figure 4). This gives ample room for speculations on biosynthesis and metabolism. The auxin properties of natural Cl-IAM were investigated by Gandar and Nitsch (6), before its chemical nature was known. That Cl-IAA is a stronger auxin than IAA was shown in large scale experiments on IAA analogs (10, 15, 16, 17). Some attempts have been made to correlate auxin activity with theoretical chemical properties of IAA, Cl-IAA and other analogs (1, 16).

The purpose of this work was to confirm the presence of Cl-IAM and Cl-IAA by a method, which does not require sophisticated chemistry. The work was warranted by the lack of physiological information and was inspired by the success of autoradiography in photosynthesis research and by Fowden's (3) autoradiographic observation that mung beans and barley incorporate radioactive iodine into organic compounds.

Abbreviations: Cl-IAM, 4-chloroindolyl-3-acetic acid methyl ester; Cl-IAA, 4-chloroindolyl-3-acetic acid.

Material and Methods

Incorporation of ^{36}Cl. Peas (*Pisum sativum* L. cv. Lysima) and barley (*Hordeum vulgare* L. cv. Carlsberg II) were grown in solution culture in 18 litre tanks on a chloride free nutrient solution of 2.5 m*M* $Ca(NO_3)_2$, 1 m*M* KNO_3, 1 m*M* KH_2PO_4 and 1 m*M* $MgSO_4$, 23.6 mg/l FeNaEDTA and Arnon and Hoagland's micronutrients (9). The peas were grown during the spring and early summer in the greenhouse at about 20°C, while the barley was grown in a growth chamber with an 18 h artificial light day at 18°C and 6 h night at 12°C. The nutrient solutions were changed once a week.

When the first flower buds (or the first awns in barley) were visible, 25 μCi of ^{36}Cl as KCl (20 μCi in case of barley) were added corresponding to 4–5 mg Cl per tank (^{36}Cl: specific activity 4.6 mCi/g Cl). After ^{36}Cl addition the solutions were not changed, only replenished with distilled water and supplied with appropriate amounts of the essential nutrients.

Young pea pods were harvested 15–20 days after the flower buds had opened, while immature barley spikes were harvested about 8-12 days after flowering. Pods or spikes were frozen in liquid nitrogen and lyophilized.

Extraction. 1 g of lyophilized peas or barley kernels were powdered in a mortar and extracted with a mixture of 7 ml *n*-butanol and 7 ml 0.05 *M* citrate-phosphate buffer, pH 3, by stirring vigorously for 2 min on a test-tube stirrer. The contents were centrifuged for 5 min at 3000 r/min. The green butanol phase was separated and evaporated almost to dryness on a rotavapor under reduced pressure. The residue was dissolved (as much as possible) in 100 μl of ethanol.

Thin layer chromatography. Commercial ready-made (non-activated) Merck preparative (PSC Fertigplatten Kieselgel 60 F_{254} 20 × 20 cm, 2 mm layer) and analytical (DC Fertigplatten Kieselgel 60 F_{254} 20 × 20 cm, 0.25 mm layer) silicagel plates were used. 50 μl concentrated pea or barley extract were applied to the 2mm plates together with 15 μl 1 % solutions in ethanol of 4- and 6-Cl-IAA and 4- and 6-Cl-IAM, all in the same spot. 10 μl concentrate + 2 μl each of Cl-IAA and Cl-IAM were spotted on the 0.25 mm plates. Chromatograms were developed in two dimensions in vapour saturated tanks with the fresh solvent pairs: (A) Chloroform : acetic acid 19 : 1 v/v and (B) *n*-butanol : ammonia : water 4 : 1 : 1 v/v; (C) heptane : benzene : *n*-butanol 2 : 1 : 1 v/v and (D) ethylacetate : benzene 1 : 1 v/v; (E) heptane : benzene : ethanol 50 : 10 : 3 v/v and (A) chloroform : acetic acid 19 : 1 v/v. (Chromatography on analytical Merck cellulose plates with 1) *n*-butanol : ammonia : water 4 : 1 : 1 v/v and 2) water alone gave serious tailing problems and was given up.)

After thorough drying overnight the plates were put next to Kodak X-ray film in the original envelope. The films were exposed autoradiographically for 2 months. The Cl-IAA and Cl-IAM spots on the thin layer plates were then coloured by spraying the plates (13) until soaking wet with a mixture of 2 ml 0.05 *M* $FeCl_3$ and 50 ml 5% $HClO_4$ in water and heating at 85°C until the plates were dry. The Cl-IAA's were purple; one Cl-IAM was purple, the other blue (separated in *e.g.* (D) ethylacetate : benzene 1 : 1).

It was necessary to run controls and extracts in the same spot, because R_f values varied some especially due to tailing, and 0.25 mm chromatograms could not be compared directly to 2 mm chromatograms.

Synthesis of 4- and 6-Cl-IAA and 4- and 6-Cl-IAM. Attempts to synthesize 4-chloroindolyl-3-acetic acid by unambiguous methods (4, 7) from 4-chloroindole failed several times. Therefore a simple Fischer indole synthesis modified from Fox and Bullock (4), yielding a mixture of 4-Cl-IAA and 6-Cl-IAA, was adopted. 29.4 g (0.2 mol) of glutamic acid and 6 g of NaOH were dissolved in 400 ml of distilled water at 50°C by magnetic stirring. 0.2 mol of NaOCl (recently prepared, strength determined by iodometry) was added over 10 minutes, during which the temperature rose to about 65°C and CO_2 escaped. Under continued stirring the solution was acidified to pH 1–2 with about 150 ml 3 *N* HCl and kept until CO_2 development had practically ceased. 21.4 g (0.12 mol) of 3-chlorophenylhydrazine hydrochloride, dissolved in 100 ml of 25% acetic acid in water were added to this solution of formylpropionic acid. A yellow oil separated. (Separation of crystals means that the α-ketoglutaric acid derivative has been formed.) pH was raised to 3–4 with NaOH and stirring continued for 2 h while the mixture cooled to room temperature. The oil was separated from the reaction mixture by careful decantation and dissolved in 150 ml pyridine in a 500 ml flask. 150 ml concentrated HCl and 50 ml 85% H_3PO_4 was added and the mixture refluxed under nitrogen for 5 h. The reaction mixture was diluted to 1.5 l with water and the sticky precipitate filtered off and discarded. The mother liquor was extracted with one 400 and three 200 ml portions of ether. The combined ether extracts were washed twice with water and the Cl-IAA extracted by one 200 and two 100 ml portions of 2% NaOH. Crude 4- and 6-Cl-IAA precipitated after acidification as an oil which was dissolved by boiling and crystallized on cooling. Crude yields of 3 to 18% based on the 3-chlorophenylhydrazine were obtained. The Cl-IAA mixture was recrystallized from toluene and subsequently from water. M.p. 150–153°C. The identity was confirmed by a typical Cl-IAA UV spectrum (11) and by determination of equivalent weight by acid-base titration.

4- and 6-Cl-IAM was prepared from 4- and 6-Cl-IAA by methylation with diazomethane in ether (2). The sticky crystals obtained after evaporation of the ether were not further purified.

Results

Figures 1, 2 and 3 show photographs of some of the best autoradiograms and of the corresponding two-dimensional non-tailing thin layer chromatograms of pea and barley extracts. The intensity of the ^{36}Cl containing spots has been enhanced by photography on high contrast film to make them more visible after reproduction. The Cl-IAM and Cl-IAA positions on the chromatograms have been marked in pencil. Other coloured sports are mainly chlorophyllous and phenolic compounds. Cl-IAM travels very close to the chlorophyll(ides) which causes its identification to be a little difficult.

There is no doubt about the presence of at least two radioactive compounds besides $^{36}Cl^-$ in both pea and barley. The position of the radioactivity coincides well with the

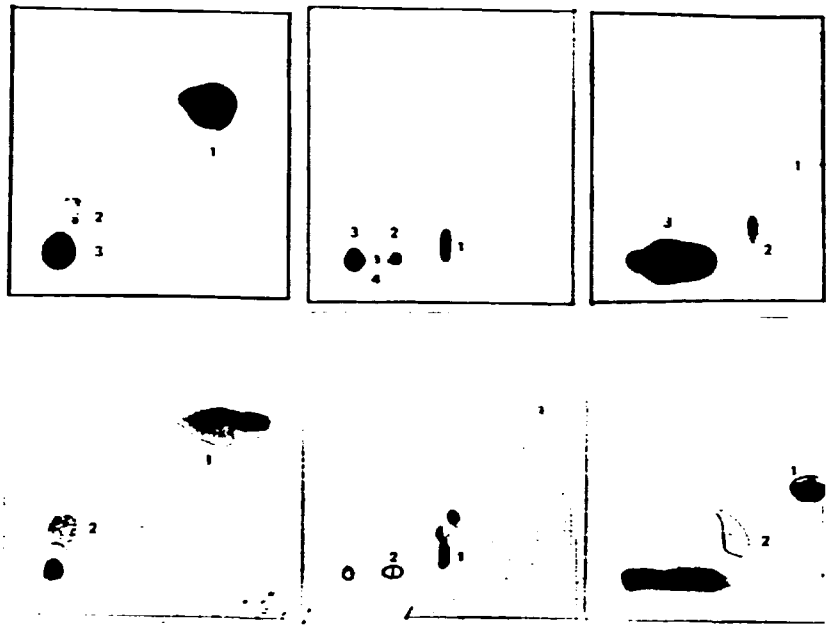

Figure 1. *Pea. Autoradiogram* (top) *and chromatogram* (bottom) *of* 50 *µl concentrated extract + Cl-IAA and Cl-IAM on a* 2 *mm silicagel plate.* Solvents: (C) heptane : benzene : *n*-butanol 2 : 1 : 1 (up) and (D) ethyl-acetate : benzene 1 : 1 (to the right). Exposure 2 months. (1) Cl-IAM, (2) Cl-IAA, (3) Cl^-.

Figure 2. *Pea. Autoradiogram* (top) *and chromatogram* (bottom) *of* 10 *µl concentrated extract + Cl-IAM and Cl-IAA on a* 0.25 *mm silicagel plate.* Solvents: (E) heptane : benzene : ethanol 50 : 10 : 3 (up) and (A) chloroform : acetic acid 19 : 1 (to the right). Exposure 2 months. (1) Cl-IAM, (2) Cl-IAA, (3) Cl^-, (4) unknown.

Figure 3. *Barley. Autoradiogram* (top) *and chromatogram* (bottom) of 50 *µl concentrated radioactive extract + Cl-IAA and Cl-IAM on a* 2 *mm silicagel plate.* Solvents: (A) chloroform : acetic acid 19 : 1 (up) and (B) *n*-butanol ammonia : water 4 : 1 : 1 (to the right). Exposure 2 months. (1) Cl-IAM, (2) unknown, chromatographing very close to Cl-IAA, 2 on the chromatogram, (3) Cl^-.

position of 4- and 6-Cl-IAA (pea) and of 4- and 6-Cl-IAM (pea and barley) on the chromatograms. Tables 1 and 2 show R_f values of the best autoradiogram, well darkened, but not tailing, of each solvent combination. About 20 autoradiograms have been made with varying extraction procedures, spot sizes and exposure times. Cl-IAM was always seen in pea, but the tables show that Cl-IAM was not detectable in all solvent systems with barley. Table 3 shows R_f values of synthetic 4- and 6-Cl-IAA and 4- and 6-Cl-IAM chromatographed alone.

Table 1. *R_f values from autoradiograms of ^{36}Cl compounds chromatographed on* 2 *mm silicagel plates.* Solvents: (A) chloroform : acetic acid 19 : 1, (B) *n*-butanol : ammonia : water 4 : 1 : 1, (C) heptane : benzene : *n*-butanol 2 : 1 : 1 and (D) ethylacetate : benzene 1 : 1 (n.d. = not detected). The barley unknown may or may not be identical to Cl-IAA.

Plant	Solvent	Compounds			
		Cl-IAM	Cl-IAA	Cl^-	Unknown
Pea	A	0.40	0.16	0.00	—
	B	0.93	0.48	0.0–0.5	—
	C	0.62	0.16	0.02	—
	D	0.65	0.04	0.00	—
Barley	A	0.38	n.d.	0.00	0.14
	B	0.98	n.d.	0.0–0.4	0.62
	C	n.d.	n.d.	0.02	—
	D	n.d.	n.d.	0.00	—

Table 2. *R_f values from autoradiograms of ^{36}Cl compounds chromatographed on* 0.25 *mm silicagel plates.* Solvents: (E) heptane : benzene : ethanol 50 : 10 : 3 and (A) chloroform : acetic acid 19 : 1. The barley unknown may or may not be identical to Cl-IAA.

Plant	Solvents	Compounds			
		Cl-IAM	Cl-IAA	Cl^-	Unknown
Pea	E	0.06	0.02	0.00	0.00
	A	0.41	0.20	0.00	0.12
Barley	E	0.08	n.d.	0.00	0.02
	A	0.40	n.d.	0.00	0.16

Table 3. *R_f values of the synthetic mixtures* (2 µl 1 % solutions in ethanol) *of 4- and 6- chloroindolyl acetic acids and 4- and 6-chloroindolyl acetic acid methyl esters chromatographed on* 0.25 *mm silicagel plates.* Solvents as in Tables 1 and 2. The isomers have been separated where two R_f values are given.

Solvents	4- and 6-Cl-IAM		4- and 6-Cl-IAA	
A	0.53		0.25	0.29
B	0.80		0.24	0.27
C	0.62	0.66	0.31	
D	0.68	0.71	0.09	
E	0.11		0.00	

Two unknown compounds have been seen (Figures 2 and 3). They may be some of the other compounds identified by Marumo *et al.* (see Figure 4). Another unknown may have been detected in peas on cellulose plates. It seems to be fairly water-soluble (4-chlorotryptophan?).

Discussion

This work describes substances in pea and barley that a) incorporate radioactive chlorine and b) cochromatograph in several solvent systems with 4- and 6-Cl-IAM or with 4- and 6-Cl-IAA. Taken together with the work of Gandar and Nitsch (5) and Marumo *et al.* (11, 14), this is evidence for the natural occurrence of 4-chloroindolyl-3-acetic acid and its methyl ester. It is unlikely that the two unusual chlorine spots contain chloride ions because of the lipid solubility, especially of Cl-IAM. However, in this work it is probably not possible to distinguish Cl-IAA or Cl-IAM from closely related analogs like, say an ethyl ester or an indole compound with chlorine in another position. It should be nice with more work on the isolation and chemical identification of Cl-IAM in plants other than pea.

Marumo's group has pursued the chemical side of chlorine containing compounds in peas (see Figure 4). With very few publications they have succeeded in suggesting both possible precursors (*e.g.* α-*N*-(β-methoxymalonyl)-D-4-chlorotryptophan) (12) and metabolites (monomethyl-4-chloroindolyl-3-acetyl-L-aspartate) (8).

Little quantitative information is available on the physiological properties of Cl-IAM. Cl-IAM was detected by means of a mung bean hypocotyl swelling assay (11, 14) and by an Avena mesocotyl assay (5, 6). Cl-IAM, at the time known as substance F (6), was more active than IAA at optimal concentrations. It promoted the curvature of Avena coleoptiles and callus growth of the Jerusalem artichoke, but had very little or no cytokinin or gibberellin effect. The content of Cl-IAM varied quite dramatically with the age of the immature peas, rising many orders of magnitude over 3 weeks after pollination and declining to zero at maturity (6). Therefore the differences in the contents of Cl-IAM in pea and barley observed in the autoradiograms need not mean anything; they may simply reflect that the barley was harvested at a wrong time.

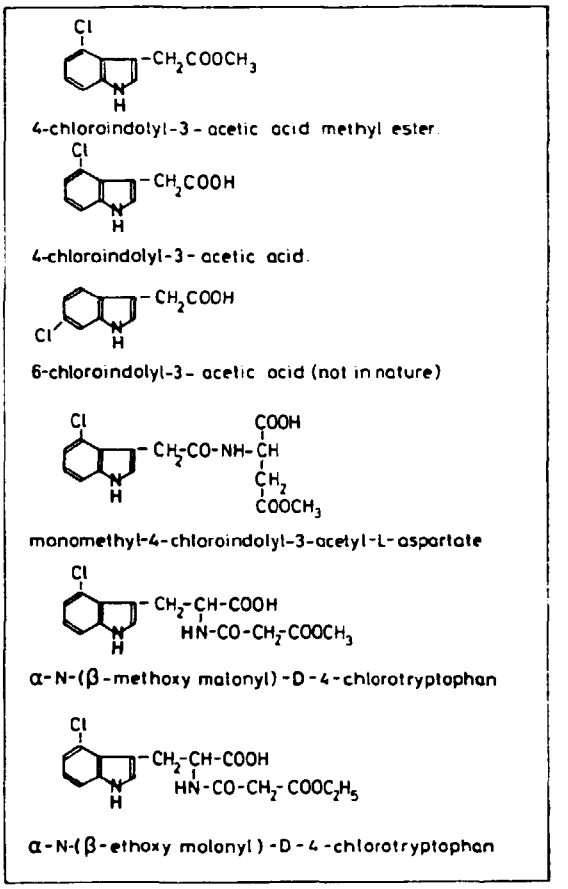

Figure 4. *Chemical formulas of the compounds* (except 6-Cl-IAA) *isolated by Marumo et al.* (8, 11, 12, 14).

Table 4. *The activity of IAA, Cl-IAA and IAA methyl ester in different assay systems.*

Source	Assay	Approximate relative activity		
		IAA	Cl-IAA	IAA methyl ester
Hoffmann *et al.* (10)	Split pea curvature	1	3	—
Hoffmann *et al.* (10)	Tomato callusing	1	30	—
Muir & Hansch (15)	Avena straight growth	1	1.4	—
Porter & Thimann (16)	Split pea curvature	1	7	1.3
Sell *et al.* (17)	Tomato parthenocarpy	1	5	30

Cl-IAA and indolylacetic acid methyl ester are both stronger auxins than IAA itself as is illustrated in Table 4.

A few observations were made on the approximate amounts of Cl-IAM in peas by scintillation counting on the scraped off radioactive spots under the not very valid assumption, that all the chlorine in the plant was the ^{36}Cl added. This gave about 25 dpm in the Cl-IAM spot corresponding to about 0.1 mg Cl-IAM per kg fresh weight. With the present technique, limits of detection will probably be about 1/10 of that value, corresponding to about 0.01 mg per kg.

^{36}Cl is a useful, but not ideal isotope for this kind of work. It has a reasonably strong β-radiation, but its very long half-life of 310,000 years means that even pure ^{36}Cl has a very low specific activity.

I am grateful to J. D. Thomsen for help with the water cultures.

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Appendix II

Natural Chlorinated Auxins Labelled with Radioactive Chloride in Immature Seeds

By

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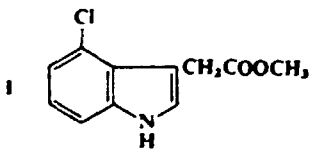
Abstract

Immature seeds were harvested from 15 species grown in perlite vermiculite containing $^{36}Cl^-$, but with very low levels of cold Cl^-. Autoradiograms of one- and two-dimensional thin layer chromatograms of butanol extracts of lyophilized seeds indicated several radioactive compounds besides the $^{36}Cl^-$ in many species. In pea the radioactivity cochromatographed with 4-(or 6-)chloroindolyl-3-acetic acid and its methyl ester; in other species radioactivity was found near these chlorinated indolyl-acetic acid markers.

Introduction

A new group of auxins was discovered when Marumo *et al.* (6) and Gandar and Nitsch (2, 3) isolated the methyl ester of 4-chloroindolyl-3-acetic acid (I) from immature seeds of pea. Since then Marumo's group (4, 7, 8) has isolated a number of related compounds from the same source with activity in the mung bean hypocotyl swelling assay: the 4-chloroindolyl-3-acetic acid itself, its aspartic acid amide and two malonyl derivatives of D-4-chlorotryptophan.



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These developments have not yet been generally recognized. They were confirmed by autoradiography of chromatograms of extracts of young peas harvested from plants grown in radioactive chloride (1). This is analogous to the detection of the animal hormone thyroxine after feeding with radioactive iodide. The purpose of the present work was to find out if chlorinated compounds were widespread among higher plants.

Materials and Methods

Pea (*Pisum sativum* L. cv. Lysima) was grown in ^{36}Cl water cultures (1). Barley (*Hordeum vulgare* L. cv. Bomi), oat (*Avena sativa* L. cv. Stål), wheat (*Triticum aestivum* L. cv. Heine Kleiber), rye (*Secale cereale* L. cv. Petkus Spring), maize (*Zea mays* L. cv. Københavns Torve), chives (*Allium schoenoprasum* L.), bean (*Phaseolus vulgaris* L. cv. Carlos Favorit), soybean (*Glycine max* (L.) Merrill cv. Fiskeby V), cress (*Lepidium sativum* L.), rape (*Brassica napus* L. cv. Svalöf Gyllen), flax (*Linum usitatissimum* L. cv. Trifolium), tobacco (*Nicotiana tabacum* L. cv. Samsun), tomato (*Lycopersicum esculentum* Mill. cv. Portia F_1), and sunflower (*Helianthus annuus* L. cv. Herbstschönheit) were grown in the greenhouse at about 20°C from the end of February until maturity, in perlite/vermiculite 2:1 in 20 l vessels equipped with an automatic circulating watering system (5). To each vessel was added 20 g $CaCO_3$, 15 g KNO_3, 10 g $MgSO_4 \cdot 7H_2O$, 7.5 g $NH_4H_2PO_4$, 1 g $MnSO_4 \cdot 4H_2O$, 1 g $CuSO_4 \cdot 5H_2O$ and 1 g ferric citrate. The nutrients were not changed. Problems with lime-induced chlorosis were overcome with FeEDTA, and Arnon and Hoagland micronutrients (5) were added as required. 35 μCi $^{36}Cl^-$ (7 μCi/mg Cl^-) was added as NaCl to each vessel just before flowering.

Two- to four-week-old seeds were harvested, frozen in liquid N_2 and lyophilized. 0.5 g ground seed was extracted with 6 ml *n*-butanol + 6 ml 0.1 *M* Na-citrate buffer, pH 3, by vigorous stirring for 90 s. The green butanol phase was separated with a pasteur pipette after centrifugation at 3000 r/min for 5 min, washed with 6 ml of water, evaporated *in vacuo*, and the residue dissolved as much as possible in 75 μl of ethanol or methanol. Avoiding the insoluble oil droplets, 5 μl alcoholic extract was spotted on 0.25 mm precoated silica gel 60 F_{254} thinlayer plates (Merck) together with 20 μg of standards (a 1:1:1:1 mixture of

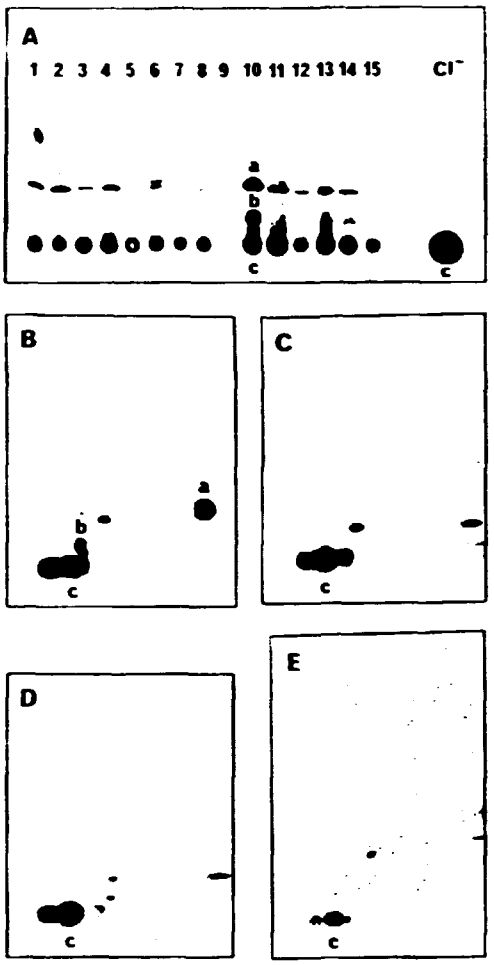

Figure 1. *Autoradiograms exposed 4 months from TLC chromatograms of 5 µl radioactive extracts of immature seeds from plants grown in $^{36}Cl^-$.* Intensified by photography on high contrast film. Extracts applied in (A): 1, barley; 2, oats; 3, wheat; 4, rye; 5, maize; 6, chives; 7, flax; 8, bean; 9, soybean; 10, pea; 11, cress; 12, rape; 13, tobacco; 14, tomato; 15, sunflower. Known substances: a = 4-chloroindolyl-3-acetic acid methyl ester, b = 4-chloroindolyl-3-acetic acid, c = $^{36}Cl^-$. Solvent $CHCl_3$: CH_3COOH 50:1. Second solvent—to the right—in the two-dimensional chromatograms: *n*-butanol:NH_3:H_2O 4:1:1. (B) pea; (C) cress; (D) tobacco; (E) barley.

4-chloroindolyl-3-acetic acid, 6-chloroindolyl-3-acetic acid and their methyl esters (1)). Chromatography with saturation in $CHCl_3$:CH_3COOH 50:1 and *n*-butanol:NH_3:H_2O 4:1:1. Autoradiograms (Kodak x-ray film) were exposed for 4 months. Standards were coloured after heavy spraying with 0.002 *M* $FeCl_3$ in 5% $HClO_4$ in water and heating at 85°C. Three separate extractions were made of each species. Barley, oat, wheat and rye were grown twice in separate experiments. About 80 autoradiograms were examined.

Results and Discussion

Figure 1A shows that at least 11 of the 15 species incorporated radioactive chloride into compounds moving in non-polar solvents. In pea (Figure 1A and B) two of these compounds were 4-chloroindolyl-3-acetic acid and its methyl ester (or the 6-chloro derivatives). In other species radioactive compounds moved close to these indoles, but in many cases (exemplified by cress, tobacco and barley, Figure 1C, D, and E) they did not seem identical with the pea auxins. It remains to be seen whether they are auxins or not, and whether they are different chloroindole derivatives. To improve sensitivity, experiments with ^{36}Cl should be run in water cultures (1) with higher activities than used in the present work.

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Appendix III

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Preparation of Chlorinated 3-Indolylacetic Acids

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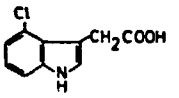
The interest in chlorinated 3-indolylacetic acids as plant hormones has increased since 4-chloro-3-indolylacetic acid [1] (*I*), its methyl ester,[2,3] its aspartic acid amide,[4] and two derivatives of 4-chlorotryptophan [5] were isolated from immature green pea seeds. Many other plants may contain similar hormones. When fed $^{36}Cl^-$ at least



I

ten cultivated species incorporate radioactivity into compounds migrating close to 4-chloro-3-indolylacetic acid methyl ester on thin layer chromatograms.[6]

Published procedures of preparation are tedious or require rare chemicals or equipment.[7–10] Therefore, simplified procedures giving similar or better yields were adopted. Five new compounds were synthesized. 4-Chloro-3-indolylacetic acid and 6-chloro-3-indolylacetic acid were made from the corresponding indoles by refluxing 3-diethylaminomethyl-chloroindoles with sodium cyanide overnight instead of four days.[9,11] The other compounds were prepared by a two-step Fischer indole synthesis from 3-formylpropionic acid (from glutamic acid) and the appropriate phenylhydrazine in pyridine/HCl [12] avoiding distillation and saponification of the ethyl esters.[7,8] A few compounds could also be prepared in one step from a phenylhydrazine and α-oxoglutaric acid in pyridine/HCl,[13] but this method was not generally applicable. The results are shown in Table 1.

All new compounds showed satisfactory elemental analyses (Novo microanalytical laboratory). Infra-red spectra (Perkin Elmer Spectrophotometer 221, KBr) were very similar to the spectra of 5-bromo- and 5-fluoro-3-indolylacetic acids.[14] The electron impact mass spectra (Varian MAT CH5) gave the following values of *m/e* for chloro-3-indolylacetic acids: 209 (M+), 164 (100 %), 128, 129 and 101; for 7-bromo-3-indolylacetic acid: 253 (M+), 208 (100 %), 128, 129 and 101; for dichloro-3-indolylacetic acids: 243 (M+), 198 (100 %), 162, 163, 127, 128 and 99; for 5-chloro-7-methyl-3-indolylacetic acid: 223 (M+), 178 (100 %), 142 and 115; all in good agreement with the mechanism of fragmentation proposed for 4-chloro-3-indolylacetic acid methyl ester.[15]

4-Chloro-3-indolylacetic acid. To a solution in ethanol (225 ml) of crude 3-diethylaminomethyl-4-chloroindole [16] (22.2 g) prepared from 4-chloroindole (15 g, 0.1 mol; Zion Chemical Products, POB 51 Yavne, Israel) was added a solution of NaCN (0.5 mol) in water (50 ml). The mixture was refluxed for 3 h and 100 ml distilled to remove diethylamine. Water (50 ml) was added and reflux continued for 16 h. The mixture was concentrated to 75 ml and water (80 ml) was added. The precipitate of nitrile + amide was filtered off. From the filtrate crude 4-chloro-3-indolylacetic acid (4.4 g) was isolated after acidification (hood!) with conc. HCl.

A mixture of nitrile + amide in ethanol (50 ml) and NaOH (20 g) in water (70 ml) was refluxed for 6 h. After concentration to about 60 ml, addition of water (80 ml) and filtration, more crude acid (10.6 g) was isolated after acidification with conc. HCl.

Crude 4-chloro-3-indolylacetic acid (15 g) was reprecipitated with conc. HCl from 40 ml of 10 % NaOH, recrystallized from water (1500 ml, a red insoluble fraction discarded) and from ethyl acetate:hexane 1:3. Yield 8.4 g (40 %), m.p. 184–187 °C.

Fischer indolization. To a fresh solution (60 °C) of 3-formylpropionic acid prepared from glutamic acid [17] (0.1 mol) was added the appropriate phenylhydrazine.HCl (0.05 mol, Aldrich) dissolved in 30 % acetic acid. The pH was adjusted to 4 with NaOH (5 M). After cooling the precipitate was collected and dissolved in pyridine (75 ml). Conc. HCl (100 ml) and 85 % H_3PO_4 (25 ml) were added and the mixture refluxed for 16 h protected from light under N_2.

The reaction mixture was diluted with water (600 ml), the tars filtered off, and the filtrate extracted repeatedly with ether. The combined ether fractions (600 ml) were washed with water and the indolylacetic acid extracted back into 0.5 M NaOH (200 ml).

Table 1. Yields, corrected melting points and ^1H NMR spectral data.[13] δ measured in CD_3COCD_3 on a Jeol JNM-PMX 60 instrument at 60 MHz. All compounds had broad peaks between δ 8 and 11 (—COOH and >NH).

3-Indolylacetic acid derivative	Starting material phenylhydrazine.HCl	Yield %	M.p. °C (dec.)	δ s (—CH_2—)	m (arom.)
4-Chloro-	(4-Chloroindole)	40	184–187 [a]	4.00	7.0–7.4 (4 H)
6-Chloro-	(6-Chloroindole)	38	182–184 [b]	3.76	6.9–7.7 (4 H)
5-Chloro-	4-Chloro-	28	156–160 [c]	3.70	7.0–7.7 (4 H)
7-Chloro-	2-Chloro-	23	163–166 [d]	3.77	6.9–7.7 (4 H)
4,6-Dichloro-	3,5-Dichloro-	7	210–214	3.97	7.0 (1 H) 7.4 (2 H)
4,7-Dichloro-	2,5-Dichloro-	0	214–217	4.00	7.1 (2 H) 7.4 (1 H)
5,7-Dichloro-	2,4-Dichloro-	22	202–204 [e]	3.76	7.2 (1 H) 7.4 (1 H) 7.6 (1 H)
6,7-Dichloro-	2,3-Dichloro-	11	170–176	3.76	7.1–7.6 (3 H)
5-Chloro-7-methyl-	4-Chloro-2-methyl-	4	178–180	3.73 [f]	6.9 (1 H) 7.3 (1 H) 7.5 (1 H)
7-Bromo-	2-Bromo-	7	180–182	3.77	6.8–7.7 (4 H)

Literature [a] m.p.'s: [a] 185–187 °C, [b] 187–188 °C, [c] 158–160 °C, [d] 164–166 °C, [e] 194–197 °C. [f] 5-Chloro-7-methyl-3-indolylacetic acid also had a peak at δ 2.40 (s, —CH_3).

After boiling the indolylacetic acid was precipitated with conc. HCl (pH 1). If possible, the soapy tars separating first were filtered or decanted off, before crystals appeared. Recrystallization twice from water, water/ethanol, toluene, or ethyl acetate/hexane.

Acknowledgements. I wish to thank Jan Rud Andersen and Ole Jorgensen for help, time and space, Helge Egsgaard for recording the mass spectra and Dr. E. C. Kornfeld for valuable information on 6-chloro-3-indolylacetic acid.

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Appendix IV

372

Substituted Indoleacetic Acids Tested in Tissue Cultures

By

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Abstract

Monochloro substituted indole-3-acetic acids inhibited shoot induction in tobacco tissue cultures about as much as IAA. Dichloro substituted indole-3-acetic acids inhibited shoot formation less. Other substituted indoleacetic acids except 5-fluoro- and 5-bromoindole-3-acetic acid were less active than IAA. Callus growth was quite variable and not correlated with auxin strength measured in the *Avena* coleoptile test.

Introduction

A naturally occurring auxin, 4-chloroindole-3-acetic acid methyl ester, was isolated from immature pea seeds by Gandar and Nitsch (1967) and by Marumo *et al.* (1968a). Also the free acid has been identified (Marumo *et al.* 1968b, Engvild *et al.* 1978). Chlorinated auxins may occur widely in nature; at least 10 cultivated species incorporated radioactive chloride into compounds migrating very close to 4-chloroindole-3-acetic acid methyl ester on thin layer chromatograms (Engvild 1975).

In this work 4-chloroindole-3-acetic acid was compared with standard auxins and a number of other substituted indole-3-acetic acids to see if the structure/activity relations found by Böttger *et al.* (1978) in *Avena* coleoptile tests apply to tissue cultures and to see if some of the substances might be useful in tissue culture work.

Material and Methods

Four monochloro-, four dichloro-, 7-bromo-, and 5-chloro-7-methylindole-3-acetic acids were synthesized (Engvild 1977). 5-bromo- and 5-fluoroindole-3-acetic acids were obtained from Ega Chemie (Aldrich); 5-methoxy- and 5-hydroxyindole-3-acetic acids were obtained from Sigma.

Tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38) tissue cultures were initiated from stem pith.

The tissue cultures were grown in plastic dishes, 5 x 7 x 2 cm, at 25°C (8 h of low intensity fluorescent light per day) on 30 ml of a slightly modified Murashige and Skoog medium (1962) containing 0.5 ml/l thiamin, HCl and 10^{-6} *M* benzyladenine. The 17 different auxins were added after sterilization by filtration through millipore filters. The auxins were tested in three concentrations, 10^{-7} *M*, 10^{-6} *M*, and 10^{-5} *M*.

Inocula (*c.* 30 mg) were taken from the 10^{-6} *M* treatment with the same hormone. A treatment included four dishes with three calluses in each. After incubation for 4 weeks the fresh weights and the number of shoots per callus were determined. The experiments were run through three passages and the results are the means of passages 2 and 3.

Table 1. *Shoot formation in tobacco callus cultures on 10^{-6} M benzyladenine and 17 different auxins tested on Murashige and Skoog's medium for 4 weeks.* 0 = no shoots, + = 0–0.5 shoots/callus, ++ = 0.5–2 shoots/callus, +++ = > 2 shoots/callus.

Indole-3-acetic acid derivative	10^{-7} *M*	10^{-6} *M*	10^{-5} *M*
IAA	+	0	0
4-Chloro-	++	+	0
5-Chloro-	0	0	0
6-Chloro-	+	0	0
7-Chloro-	++	+	0
4,6-Dichloro-	+++	+++	++
4,7-Dichloro-	+++	+++	+++
5,7-Dichloro-	+	+++	++
6,7-Dichloro-	+++	+++	+
5-Bromo-	+	0	0
7-Bromo-	+++	+++	+
5-Fluoro-	0	0	0
5-Chloro-7-methyl-	+++	+– +	++
5-Methoxy-	+++	+++	++
5-Hydroxy-	+++	+++	+++
NAA	+++	+++	0
2,4-D	0	0	0
No auxin	+++	+++	+++

Results and Discussion

Table 1 shows the shoot formation in the presence of the different auxins. The 4-chloro-, 5-chloro-, and 6-chloroindoleacetic acids were stronger auxins than IAA in the *Avena* coleoptile straight growth test (Böttger *et al.* 1978) but only about as strong as IAA in inhibiting shoot formation. The pea hormone 4-chloroindoleacetic acid was less inhibitory than IAA. Dichlorinated indoleacetic acids were weak auxins or even antiauxins (Böttger *et al.* 1978) and they inhibited shoot formation only at high concentrations. 2,4-D and NAA were about as strong as IAA in the coleoptile test; 2,4-D inhibited shoot formation completely, but NAA inhibited much less than IAA.

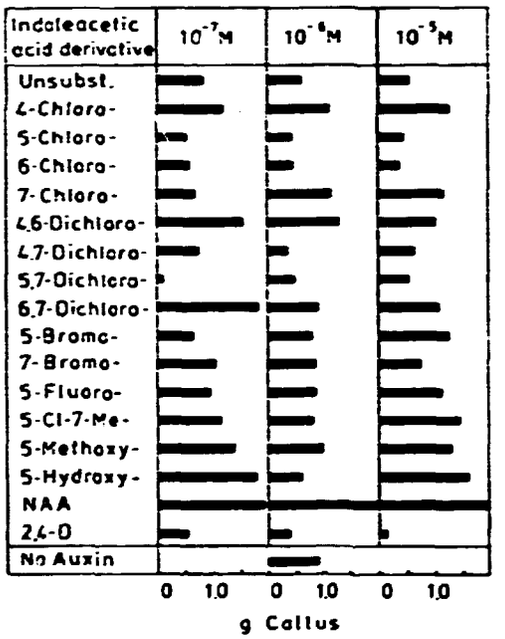

Figure 1. *Variable growth of tobacco callus cultures on 10^{-6} M benzyladenine and 17 different auxins on Murashige and Skoog's medium for 4 weeks.* Standard deviation on each value about 30%. Growth on NAA 10^{-6} *M*: 2.6 g, NAA 10^{-5} *M*: 2.7 g.

Callus growth (Figure 1) was apparently determined mainly by the cytokinin. The growth was very variable and not a good measure of auxin activity. Variation was caused (1) by adaptation phenomena with very different growth in different passages as described by Gautheret (1959), (2) by release from growth inhibition at variable times after subculture, and (3) in a few cases latent yeast contamination (*e.g.* 5,7-dichloroindoleacetic acid at 10^{-7} *M*). 4,7-dichloro- and 5,7-dichloroindoleacetic acid gave much necrotic callus, which started growth only after shoots had formed. This confirmed the antiauxin character of the two compounds (Böttger *et al.* 1978).

A similar series of experiments were run on soybeans. Callus growth was determined by the cytokinin. None of the substituted indoleacetic acids induced substantial callusing without cytokinin as did 2,4-D.

I am grateful to Mr. Klaus Christensen for skilful technical assistance.

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Appendix V

Physiol. Plant. 48: 435–437. 1980

Simple identification of the neutral chlorinated auxin in pea by thin layer chromatography

By

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Abstract

One of the neutral chlorinated auxins of immature pea seeds was readily identified by thin layer procedures simple enough to serve in student's laboratory courses.

4-Chloroindole-3-acetic acid methyl ester was extracted from 50 g of commercial, frozen peas by either water or acetone, concentrated to small volumes and chromatographed in $CHCl_3$ or CCl_4 solvent systems separating the chlorinated auxin from indoleacetonitrile and the methyl or ethyl esters of indoleacetic acid.

Colour reaction was carried out with some of the Salkowski $FeCl_3$ sprays of which Ehmann's $FeCl_3$/dimethylaminobenzaldehyde modification gave the most stable blue colour.

Key words: Identification, thin layer chromatography, TLC, auxin, pea, *Pisum sativum*, 4-chloroindole-3-acetic acid methyl ester.

Introduction

The auxin 4-chloroindoleacetic acid methyl ester has been identified in immature pea seeds by several methods, all of which have certain drawbacks. The isolation of crystals by Gandar and Nitsch (1967) and Marumo *et al.* (1968a, b) involved several hundred kg of material. The identification by thin layer chromatography/autoradiography after incorporation of radioactive chloride was difficult because long exposure times were necessary due to the low specific activity of the isotope (Engvild 1975), and the identification by gas chromatography/mass spectrometry (Engvild *et al.* 1978, Hofinger and Böttger 1979) involved very expensive equipment. It is the purpose of this paper to show that identification can be done with simple procedures and inexpensive equipment during *e.g.* a 2×3 h laboratory course.

Material and methods

Plant material: Frozen green peas, preferably a coarse, large seeded quality (dry matter about 25%) obtained in local supermarkets.

Chemicals: Solvents were Merck pro analysi quality. The diethylether was stabilized from the factory. Indole-3-acetic acid ethyl ester and indoleacetonitrile were obtained from Sigma and indoleacetic acid methylester from ICN Pharmaceuticals. Indoleacetic acids substituted with Cl in the 4, 5, 6, and 7 position in the ring were synthesized (Engvild 1977) and the corresponding methyl esters prepared by methylation with diazomethane. Precoated Merck silicagel 60 F_{254} plates, 10×20 cm or 5×10 cm, thickness 0.25 mm were used.

Extraction with water. 50 g of frozen green peas were homogenized in a Waring blender for 30 s with 100 ml 0.1 mol l^{-1} Na_2CO_3 in distilled water containing 5 mmol l^{-1} ascorbic acid. The homogenate was centrifuged at 3600 *g* (5000 r/min 13 cm radius) for 10 min.

The supernatant was extracted with 100 ml of ether in a separatory funnel. The ether phase was washed with 50 ml of water and evaporated to dryness on a steambath or on a rotary evaporator at a bath temperature of 45°C. The residue was dissolved in 0.5 ml of acetonitrile, transferred to a small tube, evaporated to dryness under an air stream, and taken up in 25 µl acetonitrile for chromatography. (NB: Don't use stopcock grease).

Extraction with acetone. 50 g of frozen green peas were homogenized with 100 ml of acetone for 30 s in a Waring blender. The homogenate was filtered and the acetone evaporated on a steambath or on a rotary evaporator at a bath temperature of 75°C until 25–35 ml unclear water phase remained. The water phase was extracted with 25

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ml ethyl acetate, which in its turn was evaporated until about 0.5 ml water remained (bath temperature 90°C). 0.5 ml acetonitrile was added, the resulting precipitate was filtered off and the filtrate collected in a small tube. The filtrate was evaporated under an air stream and the residue dissolved in 100 µl acetonitrile for chromatography. (NB: Don't use stopcock grease).

Thin layer chromatography. Five µl of water extract and/or 20 µl acetone extract were spotted on four 10×20 cm precoated plates together with 5 µl of standards (0.1% in ethanol) of indoleacetonitrile, indoleacetic acid methyl ester, indoleacetic acid ethylester, indoleacetic acid, and the four monosubstituted chloroindoleacetic acid methyl esters. The spots should not be spaced less than 15 mm apart. After drying, the plates were chromatographed in

1. $CHCl_3$: ethyl acetate 4:1 (2 plates)
2. CCl_4: acetone 4:1
3. $CHCl_3$: ethyl acetate: formic acid 5:4:1

Colour formation. The plates were sprayed with Ehmann's (1977) mixture of a) the Salkowski reagent: 2.03 g $FeCl_3 \cdot 6H_2O$ dissolved in 500 ml water and 300 ml conc. H_2SO_4 and b) the van Urk reagent: 1 g dimethylaminobenzaldehyde in 50 ml ethanol and 50 ml conc. HCl. The reagents were mixed 3 parts a) to 1 part b). The plates were sprayed in a well ventilated hood until transparent and dried at 80°C for about 10 min until blue colours had developed. The plates were then washed in a tank filled with water for a couple of minutes, air dried overnight and, if desired, wrapped in plastic foil. The colours are stable for years, if not exposed to sunlight. One plate was sprayed with the 20 mmol l^{-1} $FeCl_3$ in 35% perchloric acid and heated to 80°C for 10 min. The yellow, purplish and blue-brown colours were good for identification, but changed quickly to nondiagnostic browns.

Colour development by wetting. If spraying with the corrosive acid solutions is not desirable, it is possible to develop colours by firmly pressing the plate against a pile of thick filter paper soaked with Ehmann's mixture in a flat tray, heating for 10 min at 80°C and washing. It is necessary to check filter paper and chemicals before use, because some samples interfered with the colour reaction.

Results and discussion

Figure 1 shows a picture of a typical chromatogram with a water extract, an acetone extract and standards. It is quite clear that the compound present in pea chromatographs is 4-chloroindole-3-acetic acid methyl ester. Most of the solvent systems described in the literature do not separate the chlorinated auxin from indoleacetonitrile and the methyl or ethyl ester of indoleacetic acid. Solvents containing $CHCl_3$ and CCl_4 usually give quite good separations.

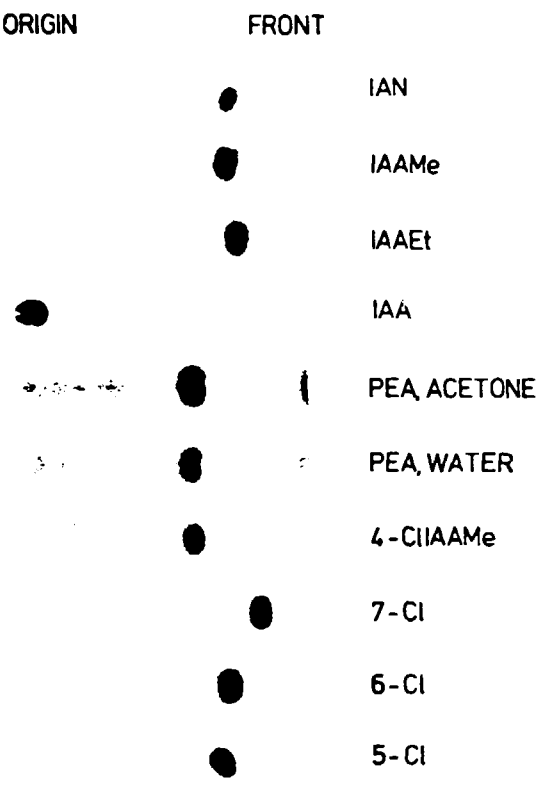

Figure 1. *Thin layer chromatogram of extracts and standards chromatographed in $CHCl_3$:ethyl acetate 4:1 and sprayed with Ehmann's mixture.*

Table 1 gives Rf-values of three solvent systems for the compounds which are most easily confused with 4-chloroindole-3-acetic acid methyl ester on chromatograms. Other indoles like tryptophol, tryptamine, indoleacetamide and tryptophan are easy to distinguish from the 4-chloroindole-3-acetic acid methyl ester (Marumo *et al.* 1971).

Table 1 also gives the colour reactions of the Ehmann's mixture (1977) and of the $FeCl_3$/35% $HClO_4$. Also 10 mmol l^{-1} $FeCl_3$/37% H_2SO_4, 1 mmol l^{-1} $FeCl_3$/5% $HClO_4$ and 10 mmol l^{-1} $Fe(NO_3)_3/HNO_3$ give good diagnostic colour differences (data not shown) but most of the colours change quickly (Stahl 1967).

The sodium carbonate/water extraction and transfer of the neutrals into ether recover only small amounts, about 5%, of the total chlorinated auxin present. However, the water extract is remarkably low in other substances and is often pure enough for direct analysis by gas chromatography/mass spectrometry (Hofinger and Böttger 1979).

Table 1. *Rf values and colours on silicagel thin layer chromatograms of auxins easily confused with 4-chloroindole-3-acetic acid methyl ester in standard systems* (Stahl 1967). Chromatographed in tank without filter paper.

Compound	Solvent			Spray	
	$CHCl_3$: ethyl acetate 4:1	CCl_4: acetone 4:1	$CHCl_3$: ethyl acetate: HCOOH 5:4:1	Ehmann's after wash	$FeCl_3/HClO_4$ just dried
Water extract	0.54	0.37	0.63	blue	yellow brown
Acetone extract	0.57	0.40	0.65	blue	yellow brown
4-Chloroindoleacetic acid methyl ester	0.56	0.38	0.65	blue	yellow brown
5-Chloroindoleacetic acid methyl ester	0.65	0.46	0.67	blue	red purple
6-Chloroindoleacetic acid methyl ester	0.68	0.49	0.69	blue	brownish
7-Chloroindoleacetic acid methyl ester	0.81	0.60	0.73	blue	red purple
Indoleacetic acid	0.02	0.02	0.55	dark blue	yellow brown
Indoleacetic acid ethyl ester	0.73	0.53	0.72	dark blue	yellow brown
Indoleacetic acid methyl ester	0.69	0.49	0.68	dark blue	yellow brown
Indoleacetonitrile	0.70	0.41	0.68	blue brown	blue brown

The acetone extraction gives much higher yields, about 20%, but the concentrate contains many other substances which often cause tailing or interfere with colour development.

With the simple extraction procedures described one does not see the many other chlorinated indole auxins present in immature green peas (Gandar 1960, Marumo *et al.* 1974), namely the 4-chloroindole-3-acetic acid itself, its aspartic acid amide, and two chlorotryptophan derivatives. Their concentration in the acetone extract is probably too low for detection.

To search for chlorinated compounds in other species it is important to select seed of the right age. The auxin content varied more than 10,000-fold (Gandar 1960, Gandar and Nitsch 1964) during the seed development. There was no auxin activity in the mature seeds.

I am grateful to Ms. Lone Dyrgaard Jensen for skilful technical assistance.

Edited by L.E.

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Appendix VI

The Chloroindole Auxins of Pea and Related Species

Kjeld C. Engvild

Introduction

The chloroindole auxins of *Pisum*, *Vicia*, *Lathyrus* and *Lens* species are the strongest natural auxins known. 4-Chloroindole -3-acetic acid is an order of magnitude stronger than IAA. The chloroindole auxins occur in immature seeds to above 10 mg per kg. They are also found in vegetative parts. Five compounds: 4-chloroindole-3-acetic acid (ClIAA), its methyl ester (ClIAM), an aspartate derivative, and two chlorotryptophan derivatives have been found in pea, *Pisum sativum* L.

Chloroindoleacetic acids were investigated already around 1950 during the thorough investigations on auxin analogues, the hormone type herbicides, and antiauxins. Nobody anticipated, however, that 4-chloroindoleacetic acid should be a naturally occurring compound in one of the classical species of plant physiology - pea.

Special auxins in immature seeds of pea, *Pisum sativum* L., was reported by Gandar (1960). Paper chromatography in two solvents consecutively (Nitsch, 1960) and *Avena* mesocotyl elongation assay revealed 6 (or 7) different auxins, A, B, C, D, E, and F. Most of these auxins were also present in the legume, but in much lower concentrations.

Chloroindole Isolation

Substance F, the least polar compound, was isolated from several hundred kg of young pea seeds (Gandar and Nitsch, 1964; Gandar and Nitsch, 1967) by methanol extraction, purification by partitioning and chromatography on Sephadex LH 20 with two different solvent mixtures. The structure was determined by IR, NMR, and mass spectroscopy. The presence of a chlorine atom was proven by the $^{35}Cl/^{37}Cl$ ratio 3:1 at m/z 223 and 225 (Abe and Marumo, 1974). Gandar and Nitsch concluded that substance F was a chlorinated indoleacetic acid methyl ester.

Almost simultaneously, Marumo *et al.* (1968a) published a complete

structure of a neutral auxin of pea (25 mg) isolated from 276 kg of pea using the mung bean hypocotyl swelling assay. After hexane partitioning

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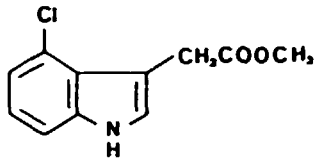

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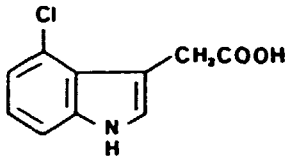

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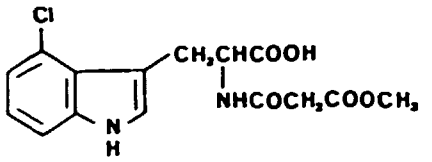

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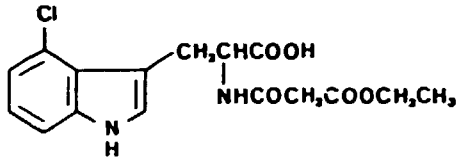

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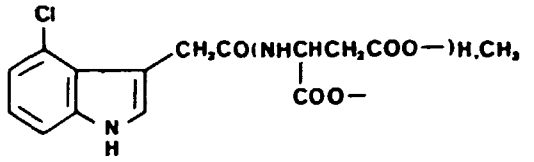

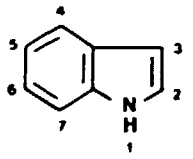

Fig. 12.1 Chloroindole Auxins

222

the methanol extract was chromatographed on alumina and the compound eluted with ethyl acetate:hexane 15:85. This compound was ClIAM, 4-chloroindole-acetic acid methyl ester (1), proven by IR, UV, NMR and mass spectroscopy, and by synthesis.

Having identified the methyl ester they proceeded to isolate 3.8 mg ClIAA, chloroindoleacetic acid (2) from the acidic fraction of the pea extract (Marumo *et al.* 1968b). They used counter current distribution followed by chromatography on Sephadex LH 20 and silica.

Two polar acidic compounds probably corresponding to substance A and B of Gandar (1960) was isolated using similar procedures (Marumo and Hattori, 1970). They were identified as derivatives of D-4-chlorotryptophan, where the amino group is coupled to a malonic acid monomethyl or monoethyl ester (3, 4). The structures were established UV, IR, NMR, and mass spectroscopy, and by synthesis. It is interesting that the 4-chlorotryptophan is from the "unnatural" D series, where protein amino acids are of the L series.

A third polar acidic auxin was also isolated. This was proven to be 4-chloroindole-3-acetyl-L-aspartic acid monomethyl ester (5). The structure was proven by spectroscopy, paper chromatography and by synthesis of the dimethyl ester (Hattori and Marumo, 1972). It is not known which aspartic acid carboxyl carries the methyl group.

Chemistry

4-Chloroindoleacetic acid has been synthesized from 4-chloroindole (Fox and Bullock, 1951; Hansch and Godfrey, 1951; Engvild 1975). The compound is fairly unstable in acid solutions and forms red degradation products on recrystallization from water. The stability is much better in salt form in basic solution. The compound should be stored in the refrigerator in darkness, and solutions should be prepared fresh.

Occurrence

The occurrence of 4-chloroindoleacetic acid methyl ester in *Pisum sativum* has been confirmed by several workers using different methods. Besides the chemical isolations of Gandar and Nitsch and Marumos group the presence of ClIAM in young pea seeds has been proven by incorporation of radioactive $^{36}Cl^-$ using thin layer chromatography and autoradiography (Engvild 1974, 1975; Engvild *et al*, 1978). ClIAM was found by thin

layer chromatography after spraying with Ehmanns reagent, a mixture of the Salkowski and the van Urk reagent (Engvild 1980). Eeuwens and Schwabe (1975) identified ClIAM by its characteristic UV spectrum and bioassay. Engvild *et al.* (1978) and Heikes (1980) found ClIAM in canned and frozen peas by gas chromatography-mass spectrometry with or without a deuterated internal standard.

ClIAM has also been identified in other species of the tribus *Vicieae*, all closely related to *Pisum* (Table 1). The identifications were performed by gas chromatography-mass spectrometry after some purification steps using internal standards. In this way ClIAM was found in *Pisum sativum*, two *Vicia* species: *Vicia faba*, *V. sativa*, four *Lathyrus* species: *Lathyrus latifolius*, *L. odoratus*, *L. sativus*, and *L. maritimus*, and in *Lens culinaris* (Engvild *et al.*, 1980; Hofinger and Böttger, 1979; Engvild *et al.*, 1981; Pless *et al.*, 1984).

Table 1. Species where chloroindole auxins have been identified and measured

	ClIAM mg/kg	ClIAA mg/kg	Organ	References
Pisum sativum	7	+	immature seeds	Gandar and Nitsch 1964, Gandar and Nitsch 1967, Marumo *et al.* 1968a, b, Engvild 1974, 1975, Engvild *et al.* 1978, 1980
Vicia faba	0.5		immature seeds	Hofinger and Bottger 1980, Engvild *et al.* 1980
		16	young leaves	Pless *et al.* 1984
Vicia sativa	0.06		immature seeds	Engvild *et al.* 1981
Lathyrus latifolius	0.6		,, ,,	Engvild *et al.* 1980
Lathyrus maritimus	0.4		,, ,,	Engvild *et al.* 1981
Lathyrus odoratus	0.2		,, ,,	Engvild *et al.* 1981
Lathyrus sativus	1		,, ,,	Engvild *et al.* 1981
Lens culinaris	0.02		,, ,,	Engvild *et al.* 1981

At first, chloroinodole auxins were believed to occur in many species, because most cultivated plants which were tested incorporated radioactive $^{36}Cl^-$ into compounds which moved close to ClIAM on thin layer chromatograms (Engvild, 1975). Radioactive compounds were found in barley, oat, wheat, rye, chives, bean, pea, cress, rapeseed, tobacco, and

tomato. However, these compounds were perhaps not chloroindole auxins after all. A search for ClIAM in barley, oat, wheat, rye, poppy, and rapeseed (Engvild *et al.*, 1980) or ClIAA in bean and maize (Hofinger and Böttger 1980) did not give any positive results. The power of proof in negative results is not very strong, but the distribution of chloroindole auxins in plants is perhaps quite narrow. Not even all *Vicieae* contain chloroindoles, as I have not found any in *Cicer arietinum* (Engvild, unpubl.).

In pea and *Vicia faba* chloroindole auxins were the only auxins detectable; that is, IAA could not be detected by gas chromatography (Hattori and Marumo, 1972; Pless *et al.*, 1984). In *Lathyrus maritimus*, on the other hand, indoleacetic acid methyl ester and chloroindoleacetic acid methyl ester occurred together in equal amounts (Engvild *et al.*, 1981).

Chloroindole auxins occur in large quantities in immature seeds of *Pisum*, *Vicia*, *Lathyrus*, and *Lens* (Table 1). The figures are only indicative, because the ClIAM content is very dependent of the developmental stage of the seed. In pea the ClIAM may increase more than 10,000 times during the first 20 days after fertilization (Gandar and Nitsch 1963); then it decreases almost to zero at maturity. In *Vicia faba* the ClIAA concentration in the young seeds begins at more than 15 mg/kg 13 days after fertilization, then it falls. However, the amount of ClIAAreaches a maximum of 1.3 µg per seed at 25 days after fertilization (Pless *et al.* 1984). Young, but fully grown leaves of *Vicia faba* contain 16 mg/kg ClIAA, a surprisingly high figure (Pless *et al.*, 1984).

Determination

Table 2 gives an overview of the methods which have been used for identification and quantitative determination. Some of the methods have been used on plant material, while others have been investigated with pure chloroindoles in order to show that the method might be useful.

The simplest methods are paper and thin layer chromatography combined with biological assays or colour formation (Nitsch, 1960; Gandar 1960, Gandar and Nitsch, 1963; Marumo *et al.*, 1971; Eeuwens and Schwabe 1975, Engvild, 1980; Engvild *et al.* 1980, 1981, Iino *et al.*, 1980). Usually it is simple to find ClIAM on thin layer chromatograms by colour

formation with Ehmann's mixture (1977) of the Salkowski and van Urk reagents (Engvild, 1980), but ClIAA is not often seen by this method.

Table 2. Methods of identification and determination of chloroindole auxins

	Amount of auxin	Plant material	References
Isolation and chemical characterization	2-25 mg	100-300 kg	Gandar and Nitsch, 1967 Marumo et *al.* 1968a, b. 1970; Hattori *et al.* 1972
Paper chromatography and bioassay			Gandar 1960, Gandar and Nitsch 1963, Euwens and Schwabe 1975.
Paper chromatography and colour	1-100 μg		Marumo *et al.* 1971
Thin layer chromatography	1-100 μg	50 g	Engvild, 1980 Engvild *et al.* 1080, 1981 Iino *et al.* 1980
Thin layer chromatography $^{36}Cl^-$ + autoradiography	0.1 μg	1 g	Engvild 1974, 1975 Engvild *et al.* 1975
Gas chromatography mass spectrometry	1-1000 μg	50 g	Engvild *et al.* 1978, 1980, 1981, Hofinger and Böttger 1980, Heikes 1980, Piess *et al.* 1984
High pressure liquid chromatography	0.001-10 μg		Blakesley *et al.* 1982 Sjut 1981, Sandberg et *al.* 1981
Chloroindolepyrone fluorescence	0.001 μg up		Böttger *et al.* 1981 Blakesley *et al.* 1982 Iino *et al.* 1980

In spite of the acknowledged limitations in bio-assay determinations, the measurements of Gandar (1960), Gandar and Nitsch (1963), and Euwens and Schwabe (1975) using paper chromatography with bio-assay give a very informative picture of the ClIAM content during the development of the pea seed. Although these measurements are expressed in IAA equivalents, they have rarely been surpassed by the more sophisticated modern methods.

Thin layer chromatography combined with incorporation of radioactive $^{36}Cl^-$ is a good method for proving the existence of chlorine containing natural compounds (Engvild 1974, 1975, Engvild *et al.* 1978), but the method is too cumbersome to use for routine purpose. ^{36}Cl has a very

long half-life and therefor, a low specific activity. It is sometimes difficult to obtain sufficiently high counts in a sample.

Determinations by gas chromatography most often combined with mass spectroscopy have been used extensively in recent investigations on chloroindole auxins (Engvild *et al.* 1978, 1980, 1981, Heikes 1980, Hofinger and Böttger 1980, Pless *et al.* 1984). The ClIAM is sufficiently volatile to be analysed directly (Engvild *et al* 1978, 1980, Heikes 1980), but derivatization is necessary for the determination of ClIAA (Hofinger and Böttger 1980; Pless *et al.*, 1984).

Gas Chromatography-mass spectrometry are very good for identification and quantitative analysis, especially when combined with internal standards. There may, however, be some pitfalls with the stability and behavior of internal standards which have not yet been recognized. The methods require expensive equipment and careful clean-up procedures. The methods have some limitations in routine applications when many samples need to be analysed.

ClIAA can be separated from IAA and 5-OH-IAA by high performance liquid chromatography (Sandberg *et al.* 1981, Sjut 1981, Blakesley *et al.* 1981). The method seems not yet to have been used for determinations of chloroindole auxins in plant material.

ClIAA produces a fluorescing indole-2-pyrone after reaction with acetic anhydride and trifluoroacetic acid. The fluorescence is about 40% of IAA indolopyrone. If chloroindole auxins and IAA occur together it will be necessary to separate them before using auxin determinations by indolepyrone fluorescence (Böttger *et al.* 1978, Blakesley *et al.* 1981, Iino *et al.* 1980).

Most of the known methods for determination of plant hormones have already been tried on the chloroindole auxins, but rapid routine methods are still needed. It would be useful with immunoassays for ClIAA and ClIAM (Weiler, 1982).

Biological Activity

The 4-chloroindoleacetic acid is the strongest natural auxin known. It has been investigated in many different assay systems, and the activity varies much with the particular assay used. ClIAA and ClIAM often have very steep dose response curves (Gandar and Nitsch 1964, Böttger

et al. 1978, Katekar and Geissler 1982, 1983); this makes it difficult to compare the biological activity of chloroindole auxins with that of IAA (Böttger *et al.* 1978). The relative activity, defined as:

$$\frac{\text{conc. IAA giving half maximal elongation}}{\text{conc. substituted IAA giving the same elongation}}$$

was used in the comparisons (Böttger *et al.*, 1978).

Table 3. Activity of 4-Chloroindolacetic acid relative to IAA in various assay systems.

Assay system	4-Cl IAA	References
Avena coleoptile elong.	1.4	Muir and Hansch 1953
Avena coleoptile elong.	7	Marumo *et al.*, 1973
Avena coleoptile elong.	10	Böttger *et al.*, 1978
Wheat coleoptile elong.	13	Marumo *et al.*, 1973
Wheat coleoptile elong.	4	Katekar and Geissler 1983
Pea hypocotyl elong.	1.3	Katekar and Geissler 1982
Split pea curvature	7	Porter and Thimann 1967
Mung bean elong.	50	Marumo *et al.*, 1973

Table 3 shows the relative activity of ClIAA in several assay systems. The *Avena* coleoptile elongation has been studied by three groups. The low figure of Muir and Hansch (1953) is probably due to their particular definition of relative activity using the begining of the dose response curve. There is good agreement between the measurements of Marumo *et al.* (1973) and Böttger *et al.* (1978). Also the figures on wheat elongation by Marumo *et al.* (1973) and Katekar and Geissler (1983) are probably not significantly different. In most systems ClIAA is an order of magnitude stronger than IAA. In the mung bean elongation assay (Marumo *et al.* 1973) ClIAA is about 50 times stronger than IAA. Therefore, a mung bean assay was a fortunate choice by Marumo's group during their work on chloroindole auxin isolation.

ClIAA has also been tested in a number of other systems (Table 4). ClIAA is stronger than IAA in inducing epinasty, parthenocarpy and callusing in tomato (Hoffmann *et al.* 1952, Sell *et al.* 1952). ClIAA did not, however, inhibit shoot formation in tobacco callus cultures as much as IAA (Engvild, 1978). It had no significant advantages over IAA in callus cultures of tobacco and growth of pea callus cultures (Engvild, unpubl.). In experiments on rooting in pea cuttings, dipping in 10^{-3} M ClIAA induced more roots than IAA. ClIAA also induced strong and

Table 4. Activity of chlorinated indoleacetic acids relative to IAA.

Assay	IAA	4-ClIAA	5-ClIAA	6-ClIAA	7-ClIAA	References
Tomato epinasty	++	+++	++	+++	+	Hoffmann *et al.* 1952
Tomato callusing	+	++++	+++	+++	+	Hoffmann *et al.* 1952
Tomato parthenocarpy	+	+++	+++	+++	+++	Sell *et al.* 1952
Split pea curvature	++	+++	+++	+++	+	Hoffmann *et al.* 1952
Split pea curvature	1	7	3	5	2	Porter and Thimann 1967
Avena coleoptile elongation	++++		+++	++++	++	Cocordano *et al.* 1970
Inhibition of shoots in callus	++	+	+++	++	+	Engvild 1978
Rooting in pea cutting	++	+++				Ahmad *et al.*, 1985
Ethylene induction in pea	++	++++				Ahmad *et al.*, 1985
Wheat root growth inhibition	+++			++++		Hansen 1954
Wheat root growth inhibition	++++	+++	++	++++	-	Stenlid and Engvild, unpubl.
Flax root growth inhibition	+++	+-	++	++++	+	Stenlid and Engvild, unpubl.
Hydrogen ion extrusion	+++	++++				Böttger *et al.*, 1978

protracted ethylene evolution in the cuttings, which became epinastic and stopped growing at the tip, but formed new shoots from low lateral buds instead (Ahmad *et al.* 1985). ClIAA inhibited growth of wheat and flax roots a little less than IAA (Stenlid and Engvild, unpubl.).

Table 5. Relative activity of halogenated indoleacetic acid in pea, oat and triticum elongation assays. Data on oats from Bottger *et al.* (1978), data on peas and wheat recalculated from Katekar and Geissler (1982, 1983).

Auxin	*Avena*	*Pisum*	*Triticum*
IAA	1	1	1
4-ClIAA	10	1.3	4
4-ClIAAM	1.2		
5-ClIAA	3.3	0.7	0.7
6-ClIAA	19	1.4	1.5
7-ClIAA	0.13	0.11	0.12
4,6-Cl_2IAA	0.11	0.8	0.05
4,7-Cl_2IAA	<0.01	0.01	0
5,7-Cl_2IAA	<0.01	0.01	0.03
6,7-Cl_2IAA	0.11	0.14	0.01
5-Cl,7-MeIAA	*0.07*	*0.02*	*0.03*
5-FIAA	0.2	1.1	0.4
5-BrIAA	0.3	0.9	0.4
7-BrIAA	0.08	0.02	0.84
2-ClIAA		0.8	0.8

Analogue Activity

Chlorine containing analogues of IAA were synthesized (Fox and Bullock, 1951) and tested by several groups (Table 4), before anybody suspected that chloroindole auxins be natural compounds. The tests were part of the general investigations of auxins, their mode of action and possible use as herbicides.

A wider range of chloroindoles were synthesized (Engvild, 1977) and tested (Table 5) in Avena coleoptile assay (Böttger *et al.* 1978), the *Triticum* coleoptile assay, and the *Pisum* epicotyl assay (Katekar and Geissler 1982, 1983). The relative activity as defined previously is used as measure. There is some variation in the activity of the same compound in different assays. In spite of this it is possible to discern a few rules of thumb:

(1) Indoleacetic acids substituted with halogen in positions 4, 5, and 6 are strong auxins, stronger than or comparable to IAA.

(2) 7-halogenoindoleacetic acids are weak auxins.
(3) dichlorosubstituted indoleacetic acids are weak auxins.
(4) 5,7- and 4,7-dichloroindoleacetic acids are probably strong antiauxins, inhibiting the effect of IAA (Böttger *et al.*, 1978).

The data on auxin analogs have been used in deliberations on the mode of action of the auxin and the properties of the active site of auxin receptors. Porter and Thimann (1965) found a correlation between auxin activity and a fractional positive charge on the -NH 5.5 A away from the carboxylic group. This was denied by Block and Clements (1975) who found correlation between auxin activity and decreasing energy in the lowest unoccupied molecular orbitals. Cocordano *et al.* (1970) related auxin activity to the electronic structure calculated according to Huckel's method. Katekar and Geissler (1982, 1983) tested a hypothesis of the structure of the auxin receptor site. On the basis of dose response curves they concluded that there were perhaps differences in the receptor sites in pea and wheat. The question will probably only be resolved, when receptors or their parent DNA/RNA have been isolated and the detailed structures of the active site determined.

Biosynthesis

Nothing is known about the biosynthesis of the chloroindole auxins. It is, however, possible to make certain guesses based on analogy with IAA biosynthesis, on the immediate versus delayed response in bioassays, and on the time course of the appearance of the auxins. D-4-chlorotryptophan derivatives appear early in development (Gandar 1960) and the maximum response in mungbean assays comes after 2.5 days compared to 1 day for ClIAA (Marumo ann Hattori 1970). It would therefore be reasonable to assume that D-4-chlorotryptophan is a precursor for ClIAA, and that the Cl atom is incorporated at an earlier stage. A biosynthetic pathway is proposed by Marumo *et al.* (1973).

Biological Role

Nothing is known about the biological role of chloroindole auxins. One would assume that they play the role of IAA in the *Vicieae* species where they belong, but there is no experimental evidence for this. Nothing is known of ClIAA transport. The ClIAA induced ethylene formation and growth inhibition in pea cuttings led to the hypothesis that ClIAA be an autoherbicide or death hormone, by means of which the maturing seeds slowly kill the mother plant and mobilize the nutrients for the seeds.

However, attempts to prove this have met with little success (Engvild, unpubl.).

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Appendix VII

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REVIEW ARTICLE NUMBER 15

CHLORINE-CONTAINING NATURAL COMPOUNDS IN HIGHER PLANTS

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Key Word Index—Asteraceae; chlorine-containing compounds; halogenated compounds; acetylenes; terpenoids; sesquiterpenoids; alkaloids; maytansinoids; phenolics.

Abstract—More than 130 chlorine-containing compounds have been isolated from higher plants and ferns; about half are polyacetylenes, thiophenes and sesquiterpene lactones from the Asteraceae. A chlorinated chlorophyll may be an important part of photosystem 1. High biological activity is found in 4-chloroindoleacetic acid from pea and in the cancerostatic maytansinoids. Many compounds are chlorohydrins isolated along with the related epoxides. Some compounds, like gibberellin A_6 hydrochloride from bean, are perhaps artefacts.

INTRODUCTION

Natural chlorine-containing compounds of higher plants were last reviewed in 1973 [1–3]. They are not very common, although halogen compounds are frequently found in certain marine algae and in fungi [4, 5]. However, they may be more important than the low frequency of their isolation suggests. Chlorine-containing compounds often show strong biological activity. Many antibiotics, fungicides, herbicides and pesticides contain chlorine or other halogens. Man and higher animals contain the iodinated hormone thyroxine and the bromine-containing sleep-related substance 1 [6, 7].

Some of the natural chlorine-containing compounds of plants also have strong biological activity, either within the plant itself as plant hormones or against other organisms as antifeedants or toxins. Chlorine-containing compounds are probably more common in plants than realized today since at least 11 out of 15 crop species incorporated $^{36}Cl^-$ into lipid-soluble compounds migrating on TLC [8].

The presence of covalently bound chlorine in a plant compound was probably first established in 1951 in sceleratinic acid lactone from the highly toxic *Senecio sceleratus* [9]. However a detailed chemical structure was first published in 1958 on an acetylenic chlorohydrin by Bohlmann's group [10].

POLYACETYLENES AND THIOPHENES

The chlorine-containing acetylenes and thiophenes are found in secretory canals in the Asteraceae. Some of the straight chain acetylenic chlorohydrins (2–8) are found in gram quantities in *Centaurea ruthenica* [10–13], and *C. scabiosa* [14] and 6 in *Carthamus tinctorius* [15]. The structures 4 and 5 have been confirmed by synthesis [16]. Polyacetylenes with one thiophene ring (9–16) are found in several genera: 10, 16 and 19 in *Eclipta erecta* [18], 11 and 12 in *Echinops sphaerocephalus* [17], 12 and 13 in *Pluchea dioscoridis* [19], and 14, 15 and 17 in *Pterocaulon virgatum* [20]. Dithiophenes (17–20) have been found in *Tagetes minuta* [21], *Berkheya adlamii* [22] and in *Epaltes brasiliensis* [23].

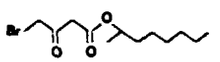
A number of cyclic compounds (21–28) probably derived from chlorohydrins have been isolated from species of *Anaphalis* [24, 25], *Gnaphalium* [25] and *Dicoma* [26]. The structures of some of these compounds have been confirmed by synthesis [27, 28]. Very interesting chlorophenols (29–32) were isolated from *Helichrysum* species [29, 30]. Further information about the distribution of chlorine-containing polyacetylenes can be found in ref. [31].

IRIDOIDS

The known chlorine-containing monoterpenoids are iridoids: linarioside (33) from *Linaria japonica* [32] and *Cymbalaria muralis* [33], both Scrophulariaceae; 7-chlorodeutziol (34) of *Mentzelia decapetala* (Loasaceae) [34], valechlorine (35) and valeridine (36) of *Valeriana officinalis* [35]; eustoside (37) of *Eustoma russellianum* (Gentianaceae) [36]; and cistachlorin (38) of *Cistanche salsa*, Orobanchaceae [37].

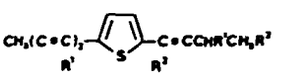
SESQUITERPENE LACTONES

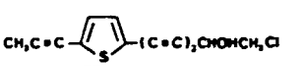
Chlorine-containing sesquiterpenoids are found in the Asteraceae. The first, eupachlorin (39), eupachlorin acetate (40) and eupachloroxin (42), were isolated from *Eupatorium rotundifolium* by Kupchan's group [38, 39] with extensive controls showing that the compounds were not artefacts. Eupachloroxin and eupachifolin-D (41) were isolated from other species of *Eupatorium* [40, 41].

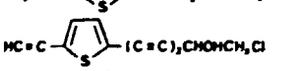
1 

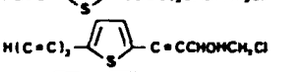
	$CH_3(C{\equiv}C)_4CH{-}CHCHR^1CH_2R^2$	
	R^1	R^2
2	OH	Cl
3	OAc	Cl
4	Cl	OH
5	Cl	OAc

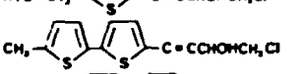
	$CH_3CH{-}CH(C{\equiv}C)_3CH{-}CHCHR^1CH_2R^2$	
	R^1	R^2
6	OH	Cl
7	Cl	OAc
8	Cl	OH

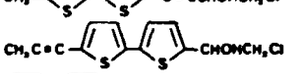
		
	R^1	R^2
9	OH	Cl
10	OAc	Cl
11	Cl	OH
12	Cl	OAc
13	Cl	OVal

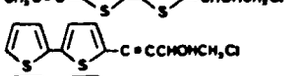
14 

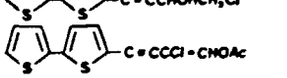
15 

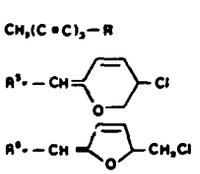
16 

17 

18 

19 

20 

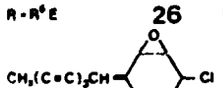


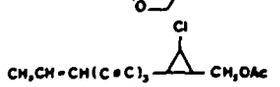
21 R = R^5 E 22 R = R^5 Z

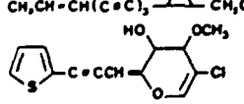
23 R = R^6 E 24 R = R^6 Z

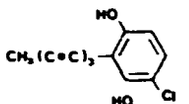
$CH_3CH{=}CH(C{\equiv}C)_2{-}R$

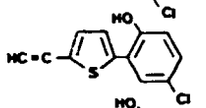
25 R = R^6 E 26 R = R^6 Z

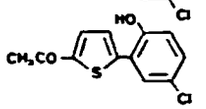
27 

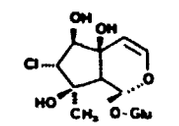
28 

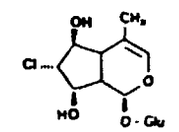
29 

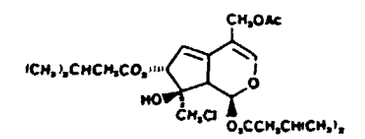
30 

31 

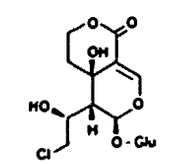
32 

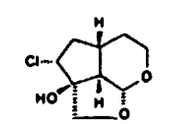
33 

34 

35 

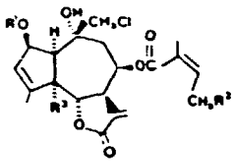
36 R = 35

37 

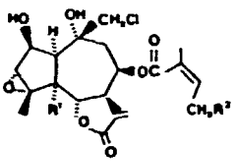
38 

Graminichlorin (43), spicatin hydrochloride (44) and desacetylspicatin hydrochloride (45) were isolated from *Liatris graminifolia* [42], *L. pychnostachya* [43] and *L. squarrosa* [44].

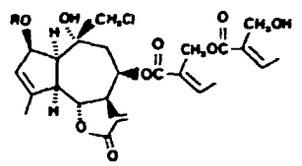
Centaurepensin (47; = chlorohyssopifolin A) was isolated from *Centaurea repens* [45–47], chlorohyssopifolin B (46) from *C. hyssopifolia* [45], as were chlorohyssopifolins D (48) and E (49) [47, 48]. Acroptilin (54; = chlorohyssopifolin C) was isolated from *Acroptilon repens* [49, 50]. Linichlorins A (51), B (55) and C (56) were isolated from *Centaurea linifolia* [51, 52], chlorojanerin (57) from *C. janeri* and *Jurinea derderioides* [53, 54], and elegin (52) and salegin (53) from *Saussurea elegans* [55–57]. Compound 53 was isolated from *Centaurea imperialis* [58]. There is confusion about the correct structure of some of these compounds [47, 48, 50] and it is not clear if linichlorin A (51) and elegin (52) are identical [51, 56].



	R¹	R²	R³
39	H	H	OH
40	Ac	H	OH
41	Ac	OH	H

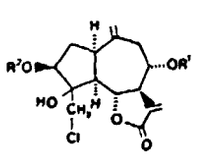


	R¹	R²
42	OH	H
43	H	OAc

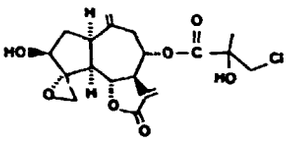


	R
44	Ac
45	H

Chlorodihydroatripliciolide derivatives (**58–61**) were isolated from *Calea pilosa*, *C. morii* [59] and *C. villosa* [60], and 3-chlorodihydroleucodins (**62–64**) from *Lasiolaena santosii* [61]. Compound **65** was isolated from *Lasiolaena morii* [62] and **66** from *Trichogonia gardneri* [63]; however, **65** may be deacetylated **66** [63]. The chlorodihydroleucodins are not chlorohydrins, but contain chlorine in vinylic linkage. Chlorfastin (**67**) occurs in *Ajania fastigiata* [64] and bibsanin (**68**) in *Achillea biebersteinii* and *A. santolina* [65]. Chlorochrymorin (**69**) was isolated from *Chrysanthemum morifolium* [66], while a chlorinated guaianolide lactone (**70**) was obtained from the artichoke, *Cynara scolymus* [67], and a chlorinated heliangolide (**71**) from *Liatris acidota* [68]. Some of the



	R¹	R²
46	H	H
47	—COC(CH₃)OHCH₂Cl	H
48	—COC(CH₃)OEtCH₂OH	H
49	—COCCH₂OHCH₂OH	H
50	—COC(CH₃)OHCH₂Cl	Ac
51	—COC(CH₃)=CH₂	H
52	—COC(CH₃)=CH₂	H
53	—COC(CH₃)=CHCH₂OH	H



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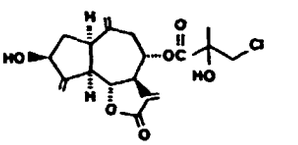

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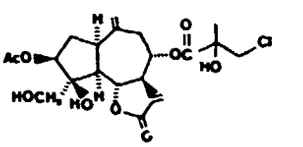

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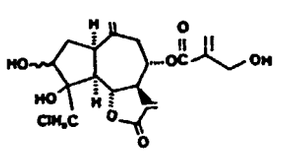

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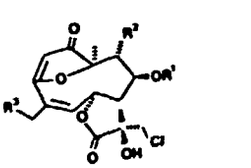
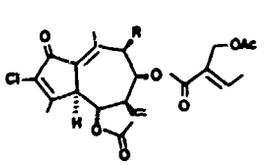
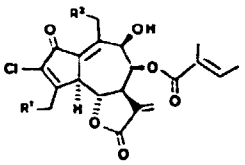


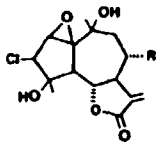
	R¹	R²	R³
58	Ang.	H	H
59	Ang.	OH	H
60	Tigl.	H	H
61	Ang.	H	OH



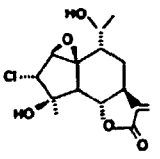
	R
62	OAc
63	H
64	OH



	R'	R²
65	OH	H
66	H	OAc



67 R=H
68 R=OH



69

chlorinated sesquiterpene lactones show anticancer activity [38, 39, 69], but are probably not as active as the maytansinoids.

PTEROSINOIDS

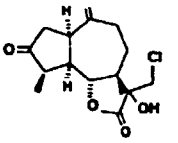
The pterosinoids are sesquiterpenoids containing one benzene ring which occur in ferns. The chlorine is bound to the aliphatic part of the molecule. Although most of the pterosinoids were isolated during a search for the toxic and carcinogenic compounds of Bracken fern [70, 71], they are not themselves toxic [72]. Pterosins F (72), J (73) and K (75) were isolated from *Pteridium aquilinum* [70, 71, 73, 74] and hypolepin A (74) from *Hypolepis punctata* [75].

DITERPENOIDS

Gutierolide (76) was isolated from *Gutierrezia dracunculoides* (Asteraceae) [76], ajugamarin chlorohydrin (77) from *Ajuga nipponensis* (Lamiaceae) [77] and tafricanins A (78) and B (79) from *Teucrium africanum* (Lamiaceae) in gram quantities [78].

STEROIDS AND GIBBERELLINS

Some Solanaceae contain gram quantities of steroidal lactone chlorohydrins. Jaborosalactones C (80) and E (81) were isolated from *Jaborosa integrifolia* [79], 6-chloro-5-hydroxywithaferin (82) from *Withania frutescens* [80], 6-chloro-5-hydroxywithanolide (83) from *Withania somnifera* [81], deoxyphysalolactone (84), physalolactone (85) and physalolactone C (86) from *Physalis peruviana* [82–84]. Bourjotinolone C (87) (a possible artefact) was isolated from *Flindersia bourjotiana* (Rutaceae) [85].



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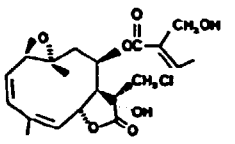

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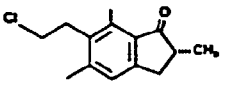

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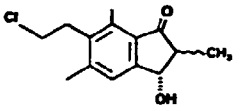

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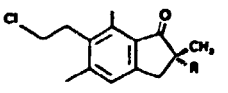
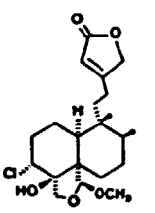


74 R = CH_3 75 R = CH_2OH



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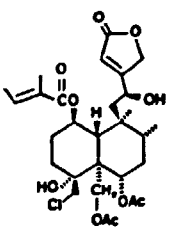

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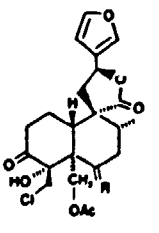
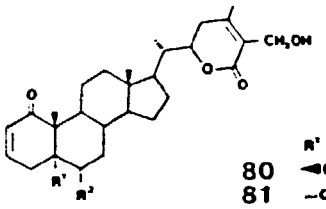
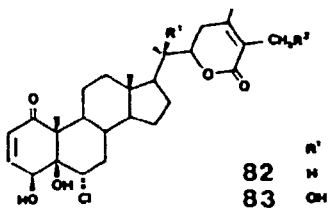


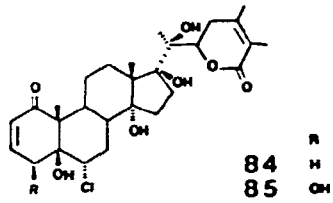
78 R=O
79 R=····OAc ◄H



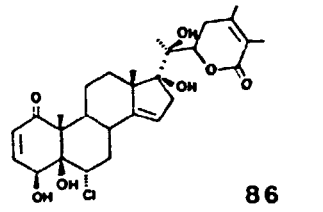
80 R¹ = OH, R² = Cl
81 R¹ = Cl, R² = OH



82 R¹ = H, R² = OH
83 R¹ = OH, R² = H



84 R = H
85 R = OH



86

A gibberellin from bean, compound b [86, 87], believed to be GA_6 chlorohydrin (**88**), is almost as active a hormone as GA_3 [86]. However, later work indicated that compound b is probably an artefact [88].

MAYTANSINOIDS

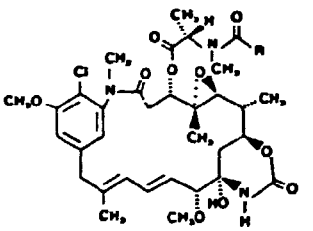
The maytansinoids are interesting macrolides with considerable anti-tumour activity. The chlorine is attached to a benzene ring *ortho* to a methoxy group and an amino group. The benzene ring and the nitrogen are part of the macrocyclic structure. Maytansinoids are found in several plant families, typically in very low concentrations. The scarcity and high cost have limited the clinical evaluation of these compounds [89], but the isolation of maytansinoids from the microorganism *Nocardia* may change this [90].

The first maytansinoids maytansine (**89**), maytanprine (**90**) and maytanbutine (**91**) were isolated by Kupchan's group [91, 92] from *Maytenus serrata* (syn. *M. ocatus*) and *Maytenus buchanii*, Celastraceae, in their search for anti-tumour agents. Maytanvaline (**92**), maytanbutacine (**103**) and maytanacine (**97**) were isolated from *Maytenus* sp. or *Putterlickia verrucosa*, Celastraceae [93, 94], as were the maytansides normaysine (**93**), maysine (**94**), maysenine (**95**) and maytansinol (**96**) which have much less biological activity.

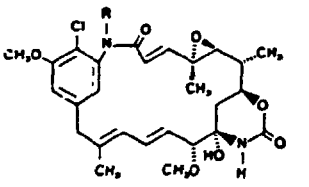
Colubrinol (**101**) and colubrinol acetate (**102**) were found in *Colubrina texensis*, Rhamnaceae [95] while normaytansine (**98**) and normantancyprine (**99**) occur in *P. verrucosa* [96, 97]. Many maytansinoids have recently been isolated from *Trewia nudiflora*, Euphorbiaceae [98–100], namely trewiasine (**104**), dehydrotrewiasine (**105**), demethyltrewiasine (**106**), 10-epitrewiasine (**107**), nortrewiasine (**108**), treflorine (**108**), trenudine (**110**) and *N*-methyltrenudone (**109**).

ALKALOIDS

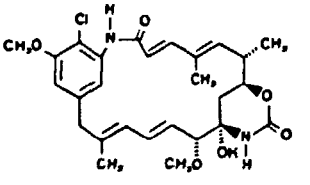
Four types of chlorinated alkaloids are known. The pyrrolizidine alkaloidal chlorohydrins of *Senecio* species



89 R = CH_3
90 R = C_2H_5
91 R = $-CH(CH_3)_2$
92 R = $-CH_2CH(CH_3)_2$



93 R = H
94 R = CH_3



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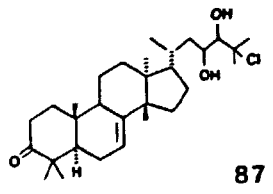

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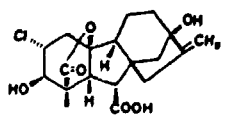

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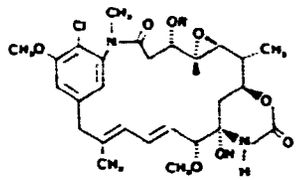
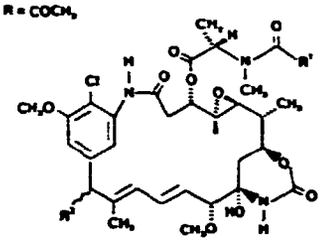
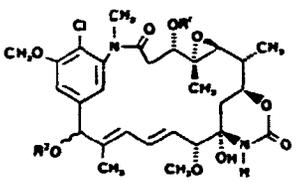
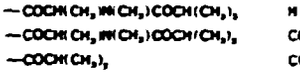


96 R = H
97 R = COCH$_3$



	R'	R²
98	CH$_3$	H
99		H
100	—CH(CH$_3$)$_2$	OCH$_3$



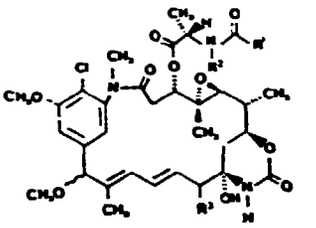
	R'	R³
101		H
102		COCH$_3$
103		COCH$_3$

(Asteraceae) are very toxic. Sceleratinic acid lactone, a degradation product of chlorodeoxysceleratine, was isolated in 1951 and found to contain chlorine [9], but its structure (**111**) was only clarified later [101, 105]. The structure of jaconine (**112**) from *Senecio jacobaea* was solved in 1959 [102]. A dehydro derivative of jaconine (**113**) also probably occurs naturally [103, 104]. Chlorodeoxysceleratine (**114**) was isolated from *Senecio sceleratus* [105] while doronine (**115**) was obtained from *Doronicum macrophyllum* (Asteraceae) [106].

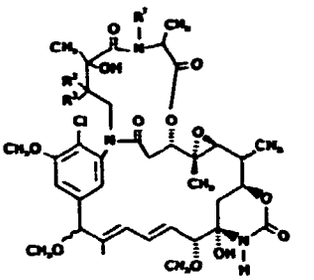
A pyrrolizidine alkaloid (lolidine, **116**) with chlorine attached to the ring has been isolated from *Lolium cuneatum* (Poaceae) [107]. Acutumidine (**117**) and acutumine (**118**) were found in *Sinomenium acutum*, *Menispermum dauricum* and *M. canadense* (all Menispermaceae). Acutuminine (**119**) was found in *M. dauricum* leaves [108–111]. From the roots of *Ruta graveolens* (Rutaceae) gravacridonchlorin (**120**), gravacridonolchlorin (**121**) and isogravacridonchlorin (**122**) were isolated [112, 113].

CHLORINATED CHLOROPHYLL

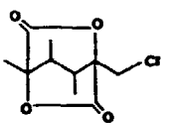
A chlorinated chlorophyll (**123**), also known as chlorophyll-RCI, has been isolated from algae in microgram quantities, but it is also present in spinach, *Spinacia oleracea* [114–116]. It seems to be closely related to P-700,

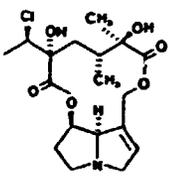


	R'	R²	R³
104	—CH(CH$_3$)$_2$	CH$_3$	—OCH$_3$
105	—C(CH$_3$)=CH$_2$	CH$_3$	—OCH$_3$
106	—CH(CH$_3$)$_2$	H	—OCH$_3$
107	—CH(CH$_3$)$_2$	CH$_3$	—OCH$_3$

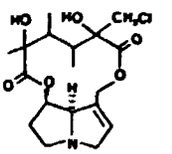


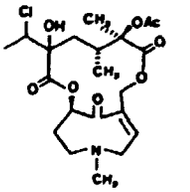
	R'	R²	R³
108	H	H	H
109	CH$_3$	=O	
110	H	OH	H

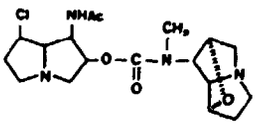
111 

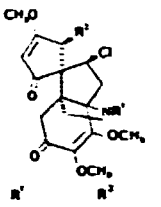
112 

113 112 - 2H

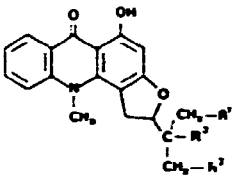
114 

115 

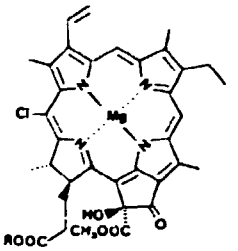
116 



	R'	R²
117	H	OH
118	CH_3	OH
119	CH_3	H

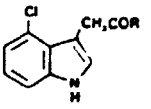


	R'	R²	R³
120	OH	Cl	H
121	OH	Cl	OH
122	Cl	OH	H



123

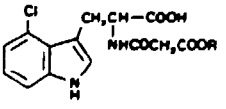
R = Phytyl ?



124 R = OH

125 R = OCH_3

126 R = —NH(CHCH$_2$COO—)H.CH$_3$ (COO—)



127 R = CH_3

128 R = C_2H_5

the reaction centre of photosystem I. However, chlorinated chlorophyll is a well known artefact [117], and much more work is needed before chlorochlorophyll can be finally accepted as a genuine natural compound with a well defined function in photosynthesis.

CHLOROINDOLES AND AMINO ACIDS

From immature seeds of pea, *Pisum sativum*, 4-chloroindoleacetic acid (124) and its methyl ester (125) [118–120], an aspartate conjugate (126) [121], and two derivatives of 4-chlorotryptophan (127, 128) [122] were isolated. 4-Chloroindoleacetic acid is the strongest natural auxin known, being about 10 times more active than IAA [123], but with interesting differences in sensitivity between species [124]. Compound 125 is present in tens of mg per kg in pea and *Vicia faba* [125, 126], and is present in *Lens culinaris* and *Lathyrus* and *Vicia* species [127], but it has not been found outside the tribe Vicieae [128; Engvild, unpublished].

PHENOLICS

One chlorinated flavonoid, 6-chloroapigeninin (129) has been isolated from *Equisetum arvense* (Equisetaceae) in small amounts [129]. The Apiaceae contain several chlorinated coumarins; peuchlorin (130), peuchloridin (131) and peuchloronin (132) in *Peucedanum arenarium* [130]; saxalin (133) in *Angelica saxatilis* [131–133]; (134) in *Prangos pabularia* and *Heracleum granatense* [134, 135]; and 135 in *Heracleum pyrenaicum* [136].

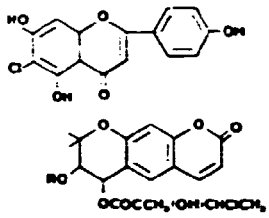
Two isocoumarins (136, 137) were isolated from *Swartzia laeticarpa* (Caesalpiniaceae) [137] and the antileukemic xanthone psorospermin chlorohydrin (138) was obtained from *Psorospermum febrifugum* (Clusiaceae) [138]. The naphthoquinone 3-chloroplumbagin (139) was isolated from species of *Drosera* (Droseraceae) [139] and *Plumbago zeylanica* (Plumbaginaceae) [140] and pipoxide chlorohydrin (140) was isolated from *Piper hookerii* (Piperaceae) [141]. The cyclic hydroxamic acid derivative (141) was isolated from young *Zea mays* (Poaceae) roots [142].

FATTY ACIDS

Seed oil of *Eremostachys moluccelloides* (Lamiaceae) [143] contains dibromo- and tetrabromostearic acids (142, 143). As some of the seed oil fractions also contain 0.1 % chlorine [144], one might suspect that chlorinated fatty acids also occur in the same plant.

BIOSYNTHESIS

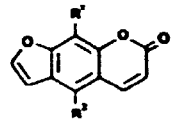
Feeding experiments with a ^{14}C-labelled polyacetylene show that a terminal double bond is converted into a chlorohydrin probably via epoxidation and introduction of chlorine as the chloride anion [145, 146]. Reasonable hypotheses have been proposed for the biosynthesis of most of the chlorine-containing heterocyclic polyacetylenes (21–29) by cyclizing chlorohydrins; but more experimental work is needed in this area. It is not known if all chlorine-containing compounds are formed from chloride ion, or if some are formed by chloroperoxidase [147, 148]-generated Cl_2.

129 

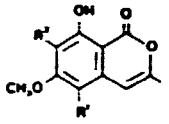
130 R = $CH_2CH{=}CCH_2CO-$

131 R = $CH_2CHOC(OH)CH_2CO-$

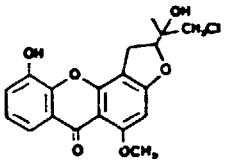
132 R = $CH_2CH(O)CH-CCH_2CO-$

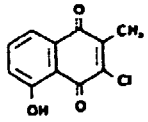


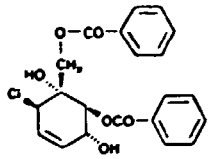
	R¹	R²
133	H	$(CH_3)_2CCOCH_2CH_2O-$
134	$(CH_3)_2COCHOHCH_2O-$	H
135	$(CH_3)_2COCHOHCH_2O-$	OCH_3

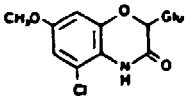


	R¹	R²
136	Cl	H
137	H	Cl

138 

139 

140 

141 

142 $CH_3(CH_2)_3CHBrCHBr(CH_2)_7COOH$

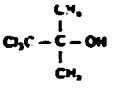
143 $CH_3(CH_2)_4CHBrCHBrCH_2CHBrCHBr(CH_2)_7COOH$

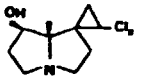
*The author would appreciate references or reprints on new chlorine-containing natural compounds in higher plants, as well as comments on omissions or errors.

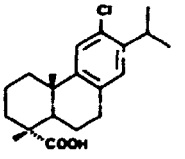
ARTEFACTS

Most of the compounds described in this paper are chlorohydrins and they are often isolated together with the corresponding epoxides. Some of them may be artefacts of isolation; some epoxides are very sensitive to acids and incorporate Cl^- from the plant, the silica gel [81] or the solvent, e.g. chloroform [138]. However, the chlorohydrins are sometimes also very labile and may be converted to the epoxides with traces of alkali or by chromatography [38, 39, 42] or silica gel [42]. In some cases it is not at all clear which is the artefact, the chlorohydrin or the epoxide. Controls for artefact formation have involved isolation without chlorine containing solvents [39] or presence of the chlorine-containing compound in crude extracts has been proven by specific tests [128, 134], or by the incorporation of radioactive $^{36}Cl^-$ [8, 116].

Some compounds are without doubt artefacts. Compound 144 found in *Ilex aquifolium* was only found in nursery plants [149]; its formation is not understood. The dichlorocyclopropyl compound (145) is probably an artefact formed from chloroform [150]. Chlorodehydroabietic acids (146) are formed in paper manufacture during the Kraft liquor cooking [151]; they are important fish poisons. Chlorine-containing compounds derived from fats (147, 148) are formed in the hydrolysis of proteins [152]. Senegenin (149) was isolated from *Polygala senega* [153] but later shown to be an artefact [154].*

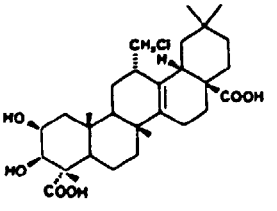
144 

145 

146 

147 $CH_2Cl-CHO-R-CH_2Cl$ R = fatty acids

148 $CH_2O-R-CHO-R-CH_2Cl$ R = H

149 

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Appendix VIII

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What's New in Plant Physiology

The death hormone hypothesis

K. C. Engvild

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The 'death hormones' or monocarpic senescence factors are hypothetical substances transported from the fruits to the vegetative parts of the mother plant, where they may stop growth, activate senescence, remobilize nutrients, and finally lead to the death of the plant. Death hormones could well be derivatives of jasmonic acid, 4-chloroindoleacetic acid, or other chlorine compounds.

Key words – Monocarpic senescence, senescence factor.

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Introduction

The term 'death hormone' has been used (Noodén and Leopold 1978) when discussing senescence in plants that flower only once (monocarpic plants), e.g., peas, soybeans, cereal grains, some bamboo species, and *Agave americana*, the century plant. Monocarpic senescence is a dramatic phenomenon, visible in most fields before harvest. Senescence is a highly regulated, ordered series of events involving loss of photosynthesis, disintegration of chloroplasts, breakdown of the CO_2 fixing enzyme and other proteins, loss of chlorophyll and removal of amino acids.

In most cases, but not all, senescence is inhibited or retarded if the plant is prevented from flowering or setting fruit. This can be done by keeping the plant at noninductive day lengths and temperatures, or by manual deflowering and defruiting (Noodén and Leopold 1988). Several theories have been proposed (Noodén and Leopold 1978), but at present mainly two hypotheses are competing as explanations of monocarpic senescence:

1) The death hormone hypothesis (Noodén and Leopold 1978, 1988, Kelly and Davies 1988): senescence factors produced by the flower, fruit, or seeds are transported to the vegetative parts. They induce cessation of growth, degradation of cell apparatus and remobilization of nutrients to the maturing seeds.
2) The nutrient drain hypothesis of Molisch modified by Kelly and Davies (1988): a pull from the strong sink of young reproductive tissue monopolizes all nutrients, helped by a reduced sink strength of the vegetative parts in the flowering plant.

Around 1980 there was much talk about death hormones or senescence factors (Noodén and Leopold 1978, Gianfagna and Davies 1983), but recently the pendulum seems to be swinging back to the nutrient drain theories (Hamilton and Davies 1988, Kelly and Davies 1988). The mechanism are probably not the same in various plants, with small differences between cultivars and large differences between plant families (Woolhouse 1983).

It is important to realize that the different hypotheses need not to be mutually exclusive. One might imagine situations, where a chemical signal from the young seeds stops cell division and cytokinin production in the root tips. This causes senescence of the leaves, redirection of nutrient flows, and leads the cells in the vegetative parts to self destruction. Other combinations of hormonal, nutritional, physical, and genetic mechanisms are just as possible. Most of the following discussion is based on the literature on pulses, particularly soybean, (*Glycine max*) and pea (*Pisum sativum*).

Abbreviation – ClIAA, 4-chloroindoleacetic acid.

Plant hormones and senescence

It is generally agreed that all plant hormone groups influence senescence (Kelly and Davies 1988, Noodén and Leopold 1988). In most plants cytokinins are anti-senescence compounds that can postpone plant death

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(Noodén et al. 1989); gibberellins also have similar effects, but in fewer plant species.

Auxins influence senescence and abscission in very complex manners, apparently depending on the auxin source, or depending on the age and receptivity of the tissue (Noodén and Leopold 1988). When applied to the leaf, auxin often retards senescence, but paradoxically when auxin enters the leaf from the stem, it sometimes promotes senescence (Tamas et al. 1981, Noodén and Leopold 1988).

Two plant hormones have been suggested as candidates for death hormones: ethylene and abscisic acid (Noodén and Leopold 1988). Ethylene is intimately involved in many senescence phenomena, especially flower wilting and fruit ripening. However, there is no clear correlation between ethylene and monocarpic plant death (Mattoo and Aharoni in Noodén and Leopold 1988). Also the mobility of ethylene as a gas does not fit that of a death hormone (Woolhouse 1983). However, one could imagine that the ethylene precursor, aminocyclopropane carboxylic acid, is transported, and that ethylene is a kind of secondary messenger within the individual plant tissue.

Abscisic acid was isolated on the basis of its promotion of abscission and dormancy. The compound is much less active on whole plants, and there is little correlation between ABA concentration and plant death (Tamas et al. 1981, Kelly and Davies 1988, Noodén and Leopold 1988). It is also difficult to reconcile the large fluctuations of ABA in response to drought and turgor changes with the protracted signalling at the stage of seed maturation.

Mutants and genetic variation

Few people including plant breeders realize that genetic variation in monocarpic senescence (Woolhouse 1983, Phillips et al. 1984, Thomas 1987) is a much exploited trait in plant breeding. Usually, the desired plant types are those with determinate flowering, high harvest index, and well defined, synchronized ripening, making the crop suitable for machine harvesting. The G_2 line in peas has been used extensively in death hormone research (Gianfagna and Davies 1983, Hamilton and Davies 1988). This line has a specific combination of genes, causing plant death induced by seed development in long days but not in short days. Genetic variation is also available in soybeans (Phillips et al. 1984), and it would be particularly useful to have a collection of senescence mutants in *Arabidopsis*, pea, barley, or tomato that could further studies on the genetics, physiology, hormone regulation, or molecular biology of monocarpic death.

Methodology

Death hormone research has followed several strategies:

1) Defloration, defruiting, or deseeding (Kelly and Davies 1988, Noodén and Leopold 1988).
2) Grafting experiments (Noodén and Leopold 1988).
3) Steam inactivation of phloem transport (Noodén and Murray 1982).
4) Guessing and testing compounds for death hormone characteristics (Noodén and Leopold 1978, 1988).
5) Identification of radioactive compounds transported back from the seeds or fruit to vegetative plant parts (Gianfagna and Davies 1983, Hamilton and Davies 1988).
6) Senescence bioassays such as induced chlorophyll loss (Noodén and Leopold 1988).
7) Death hormone senescence bioassays (Noodén et al. 1989).

Intuitively, the most logical method of search for death hormones would be the last alternative. Along this line, maturing soybean seeds were washed in water; soybean cuttings were allowed to suck up this wash water, and responded with yellowing of the leaves (Noodén et al. 1989). This recent bioassay was also used in tests for death hormone candidates, but as yet, little experience has been gained using it. More bioassays are needed, especially because each species may have its own combination of death hormone signals.

Assays for death hormones will always be difficult to design in principle, because inhibitory rather than promotive effects are measured. Plant extracts are full of toxic compounds, phytoalexins, or other allelochemicals so that specific death inducers are difficult to discern. The same is true for the general senescence bioassays which were instrumental in the discovery of the growth inhibitor and perfume, jasmonic acid (Sembdner and Klose 1985).

It is well established that young seeds produce or accumulate almost all plant hormones in very large quantities, and it is often assumed that some of these compounds move out into the plant as signals (references in Noodén and Leopold 1988). Little work has been done on this, but it is known that both hormones and other metabolites move out of the maturing fruit (Gianfagna and Davies 1983, Tamas et al. 1981, Hein et al. 1986, Hamilton and Davies 1988).

Although the chemical identity of death hormones is unknown, the deflowering, deseeding, and phloem killing experiments suggest certain features:

Death hormone characteristics

1) Death hormones are produced in developing seeds (Noodén and Leopold 1988).
2) They seem to be transported back to the plant in the xylem (Noodén and Murray 1982, Noodén and Leopold 1988).
3) They have limited mobility in the xylem, a little more in the phloem (Noodén and Leopold 1988).
4) In soybean they act primarily on the leaves, in peas

on the apical meristems (Gianfagna and Davies 1983, Noodén and Leopold 1988).

Maturing seeds seem to be the production sites; this has been proven by surgical experiments with excision of flowers, pods, or the developing seeds within the pods. About 40% of the maximum number of pods seem to induce full senescence (Noodén and Leopold 1988).

The senescence factors may move to the adjacent leaf in the xylem, because they move even when the petiole phloem has been killed with steam (Noodén and Murray 1982). It is important to note that seed filling is accompanied with a backflow in the xylem of surplus water from the seed (Noodén and Murray 1982, Pate in Noodén and Leopold 1988). The leaves are probably the most important senescence targets in soybeans (Noodén and Leopold 1988), but cessation of meristematic growth may be more important in peas (Gianfagna and Davies 1983). Assuming that neither ABA nor ethylene are the death hormones per se (Noodén and Leopold 1988) little is known about the possible identity of the putative death hormones. The case for a pea factor with a carboxyl group (Gianfagna and Davies 1983) was weakened when the compound was found to be malic acid (Hamilton and Davies 1988). Besides the ethylene precursors (Mattoo and Aharoni in Noodén and Leopold 1988), there are other naturally occurring compounds in pea and soybean with characteristics of death hormone candidates: the chloroindole auxins and jasmonic acid.

Chloroindole auxins in *Pisum*

4-chloroindoleacetic acid derivatives (references Engvild 1987, Katayama et al. 1988) may be death hormones in *Pisum*, *Vicia*, *Lens*, and *Lathyrus*:

1) ClIAA (Fig. 1) induces very strong, almost irreversible ethylene production (Ahmad et al. 1987).
2) ClIAA induces death of the apical meristem in pea cuttings (Ahmad et al. 1987).
3) Auxin in lanolin placed in deseeded bean pods induce leaf senescence (Tamas et al. 1981), and ClIAA is the strongest natural auxin known (Engvild 1987).
4) Five different chloroindole auxin derivatives are present in large amounts in maturing pea seeds, and reach concentrations of several mg kg^{-1} for methyl 4-chloroindoleacetate (references in Engvild 1987 and Katayama et al. 1988).

The five naturally occurring chloroindoles known are: 4-chloroindoleacetic acid (Fig. 1), its methyl ester, the methyl and ethyl esters of malonyl-4-chlorotryptophan, and a monomethyl ester of 4-chloroindoleacetyl aspartic acid (references Engvild 1987, Katayama et al. 1988) (Fig. 1).

There are of course problems with chloroindoles as death hormones or endogenous herbicides. One problem is the postulation of two distinct auxin groups with opposite physiological properties: growth inducers and endogenous herbicides. Another problem is that chloroindole auxins have so far only been found in pea and related species, but not in soybean or kidney bean (Engvild 1987). However, other chlorinated organic compounds are found in maturing seeds of kidney bean, tomato, rape seed, barley, wheat, and other crops (Engvild 1975). Despite some attempts, none of these have been identified yet (K. C. Engvild, unpublished results) and they may be death hormones. A chlorine-containing compound with both auxin and death hormone like characteristics has been found by Morris et al. (1987) in the slime mold *Dictyostelium discoideum*. The chlorinated phenolic compound (Fig. 1) was first named DIF-1 (differentiation inducing factor 1); together with cyclic AMP it induces stalk cell morphogenesis, including cellulose wall formation, cell elongation, vacuolization, and eventually the stalk cell dies. At present it is not clear if the compound induces cell death as such or just launches the cell on an irreversible path.

Jasmonic acid

Jasmonic acid derivatives may be death hormones:

1) Jasmonic acid methyl ester is a senescence inducer, in some assays it is stronger than ABA (Sembdner and Klose 1985).
2) Jasmonic acid (Fig. 1) is widely distributed in plants (Sembdner and Klose 1985).
3) Soybeans contain high concentrations in vascular bundles of the pericarp (Lopez et al. 1987).

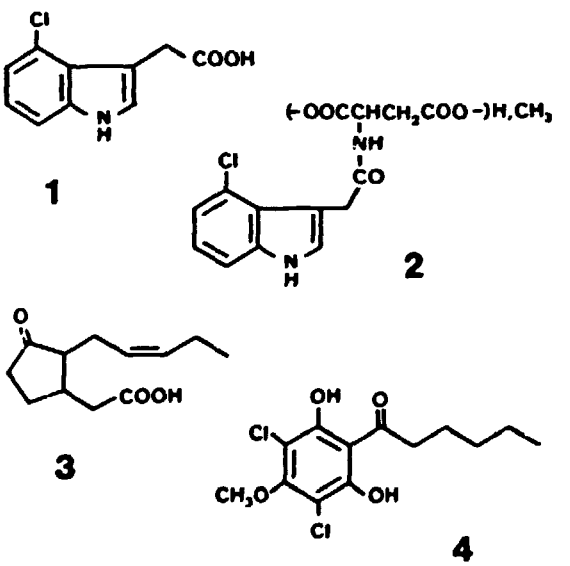

Fig. 1. Potential death hormones: 1. 4-Chloroindoleacetic acid. 2. 4-Chloroindoleacetyl aspartate monomethylester. 3. Jasmonic acid. 4. The chlorinated death hormone of *Dictyostelium*.

4) Jasmonic acid effects can often be alleviated or reversed by cytokinins. This is in agreement with effects of cytokinins on monocarpic senescence (Sembdner and Klose 1985).
5) Jasmonic acid methyl ester induces ethylene formation (Saniewski et al. 1987).

The recently isolated substances are not yet qualified for plant hormone status. It is not known if they regulate anything or if they are transported. However, jasmonic acid derivatives are active in very low concentrations typical of hormones and one would expect such compounds to have some function in the plant. Methyl jasmonate has already been tested by Guiamét (Noodén et al. 1989) for death hormone activity in the soybean cutting assay. It was the only compound tested which induced yellowing of the leaves, but Noodén et al. (1989) do not believe that jasmonic acid is the senescence factor in their system.

Conclusion

Most of the available evidence fits both a death hormone hypothesis and a nutrient drain hypothesis. However, nutrient drain does not easily explain two experiments: the senescence of male spinach plants after flowering (Noodén and Leopold 1978, 1988) and the fruit induced senescence of the soybean leaf when the phloem of the petiole has been destroyed by a jet of steam (Noodén and Murray 1982, Noodén and Leopold 1988). I believe therefore that there is very good reason to continue the search for death hormones, although their existence is certainly not yet proven beyond reasonable doubt. One reason for this is lack of adequate techniques. There is a need both for refinements of the classical physiological methods of plant surgery, hormone research, and radioactive labelling and for the introduction of molecular genetic methods. This is already well advanced in leaf senescence, but not in whole plant physiology.

One good starting point would be a systematic collection of monocarpic senescence mutants in one or more of the genetically well known species, such as *Arabidopsis*, maize, or pea.

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Appendix IX

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363

Gas Chromatographic–Mass Spectrometric Identification of 4-Chloroindolyl-3-acetic Acid Methyl Ester in Immature Green Peas

By

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Abstract

Immature green peas were harvested from plants grown in water culture on Johnson's nutrient solution with radioactive chloride. Twenty-five g of lyophilized peas were extracted with butanol, the extract chromatographed, first on Sephadex LH 20 and then on silica gel. The radioactive hormone was followed by scintillation counting without the use of any scintillant. The radioactive fractions were taken up in a few µl of chloroform and injected into a gas chromatograph interfaced with a mass spectrometer. Derivatization was not necessary. The electron impact mass spectrum showed the typical peaks of a chloroindolyl-3-acetic acid methyl ester: the molecular ion cluster at *m/e* 223, 225 and the characteristic indole fragment ions at *m/e* 164, 166 $(M\text{-}COOCH_3)^+$. The chlorine atom was assigned to the 4 position of the indole ring after thin layer chromatography of radioactive extract and standards of four different chlorinated indolyl-3-acetic acid methyl esters.

Introduction

The methyl ester of 4-chloroindolyl-3-acetic acid (6 and 25 mg) was isolated from 100 and 276 kg of immature peas by Gandar and Nitsch (1967) and by Marumo *et al.* (1968a). Its structure was determined by ultra-violet, infra-red, and nuclear magnetic resonance spectroscopy, and mass spectrometry, but only Marumo *et al.* (1968a) established the position of the chlorine atom by synthesis of a series of isomers.

Until now chlorinated natural auxins have been identified with rigorous methods only in immature seeds of *Pisum*, where Marumo's group also identified 4-chloroindolyl-3-acetic acid (Marumo *et al.* 1968b), its aspartic acid amide (Hattori and Marumo 1972) and two derivatives of 4-chloro-tryptophan (Marumo and Hattori 1970). Many other species may, however, contain similar hormones, as the young seeds incorporate radioactive chloride into compounds migrating very close to 4-chloroindolyl-3-acetic acid methyl ester on thin layer chromatograms (Engvild 1975).

Structural determination and large-scale isolation are difficult to do in plant physiology laboratories. It is the purpose of this paper to show that structure identification on µg amounts of a chlorinated auxin is possible in collaboration with chemists with a gas chromatograph–mass spectrometer. This method has been used several times for identification or quantitative determination of IAA (Bandurski and Schulze 1974, Bridges *et al.* 1973, DeYoe and Zaerr 1976, Elliot and Greenwood 1974, Hopping and Bukovac 1975, Rivier and Pilet 1974).

Material and Methods

4-Chloroindolyl-3-acetic acid and three of its isomers were synthesized (Engvild 1977) and methylated by diazomethane in ether.

Plant material. Peas (*Pisum sativum* L. cv. Allround) were grown in water culture in an 18-litre container on half or full strength Johnson *et al.* (1957) chloride-free nutrient solution. The growth chamber was kept light (Sylvania fluorescent tubes, F96T12/CW/VHO + incandescent lamps, output 100 W m^{-2}), at 17°C, 60% rel. humidity for 18 h and dark at 10°C, 80% rel. humidity for 6 h. Three weeks after sowing 50 µCi $K^{36}Cl$ (specific activity 0.25 mCi/mmol Cl^-) was added. Water or diluted nutrient solution was added as required. The solution was changed only once, a few days after flowering started. At the same time a further 100 µCi $^{36}Cl^-$ was added. The radioactive peas were harvested 3–4 weeks after flowering and lyophilized.

Frozen green peas were obtained in local grocery stores and lyophilized.

Extraction. Twenty-five g of lyophilized peas (of which 5–10 g were $^{36}Cl^-$ labelled material) were crushed in a mortar and extracted with a mixture of 250 ml of butanol-1 and 250 ml of a 0.1 *M* sodium phosphate buffer, pH 2.5, by vigorous shaking for 2 min. After centrifugation (10 min at 1000–2000 *g*) the butanol fraction was separated, washed with

water and evaporated *in vacuo* almost to dryness. Ten ml of acetonitrile was added to the residue. After shaking, the acetonitrile was separated from a large precipitate and washed with 10 ml of *n*-heptane. The acetonitrile was concentrated *in vacuo* to about 1 ml.

Column chromatography. The acetonitrile solution was chromatographed on a Sephadex LH 20 column (35 × 1.5 cm) with dichloromethane : methanol 1 : 1. Fractions of 4 ml were collected directly in scintillation vials. The radioactive fractions from the scintillation counter were concentrated *in vacuo* and chromatographed a second time on a Merck pre-packed Lobar silica gel 60 column, size A, with tetrachloromethane : methanol 4 : 1 or chloroform.

Counting. The radioactive fractions were located in the scintillation counter at the standard tritium setting. No scintillant was needed as the β-radiation from ^{36}Cl is energetic enough to cause Čerenkov radiation. There were problems with colour quenching when the chlorinated auxin was not sufficiently separated from the chlorophylls.

Gas chromatography–mass spectrometry. After silica gel chromatography the radioactive fractions were evaporated to dryness and dissolved in a few μl of chloroform. 1 μl was on-column injected into a modified Perkin Elmer F11 gas chromatograph equipped with a 1 m × 3 mm i.d. glass column containing 3% OV17 on chromosorb W (80–100 mesh). The gas chromatograph was interfaced through a two stage all-glass jet separator to a Varian MAT CH5 mass spectrometer. Carrier gas: helium at 60 ml min^{-1}. Temperatures: column 260°C, injection port 260°C, separator 325°C, ion source 180°C. The mass spectrometer was used as a detector writing out the total ion current and the single ion current at *m/e* 223. When the *m/e* 223 peak appeared, two to four scans (*m/e* 10–400) were run. The electron impact mass spectra were obtained at 20 eV (80 μA) to avoid the ionization of the carrier gas, helium.

Thin layer chromatography and autoradiography. Radioactive extracts transferred to acetonitrile (20 μl) were chromatographed on thin layer silica gel 60 F_{254} in one or two dimensions together with standards of 4-chloroindolyl-3-acetic acid and its methyl ester and, sometimes, of their isomers. Radioactive auxins were visualized on autoradiograms (Kodirex X-ray film) exposed for 4 to 8 weeks. Standards were located after spraying with 0.002 *M* $FeCl_3$ in 5% $HClO_4$ and heating at 85°C (Marumo *et al.* 1971).

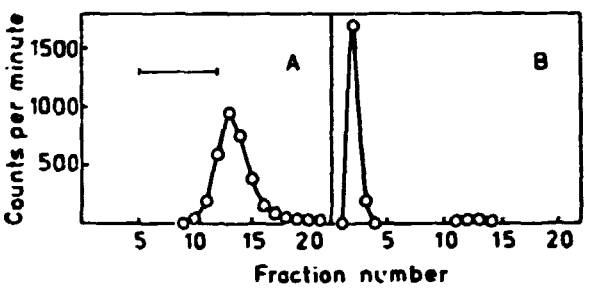

Figure 1. *Column chromatography of radioactive pea extract. A.* 4 ml fractions from Sephadex LH 20 (CH_2Cl_2 : CH_3OH, 1 : 1); ⊢——⊣ indicates the position of chlorophylls. Fraction 12–15 collected for silica gel chromatography. *B.* 6 ml fractions from silica gel column (CCl_4 : CH_3OH, 4 : 1). Fraction 2 and 3 collected for gas chromatography–mass spectrometry.

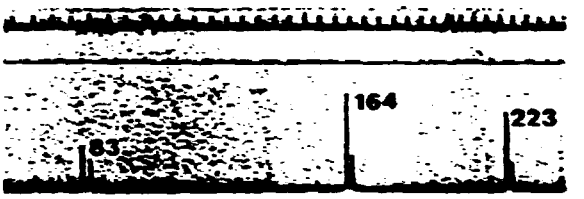

Figure 2. *Mass spectrum of pea extract obtained at 20 eV after gas chromatography.* There is a light background of the solvent, $CHCl_3$ (*m/e* 83, 85, 87). The spectrum corresponds to at least 1 ng 4-chloroindolyl-3-acetic acid methyl ester.

Results

Figure 1A shows the distribution of ^{36}Cl radioactivity after chromatography of a pea extract on Sephadex LH 20. The radioactive material was reasonably well separated from the chlorophylls in most cases. After the second chromatography on silica gel (Figure 1B) the samples could

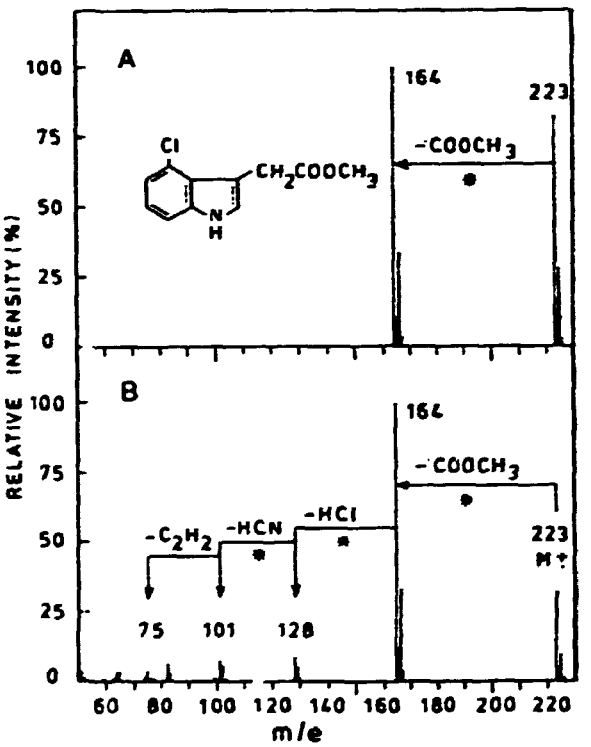

Figure 3. *Direct-inlet electron impact mass spectra of synthetic 4-chloroindolyl-3-acetic acid methyl ester. A.* 20 eV spectrum. *B.* 70 eV spectrum, agreeing well with the data of Abe and Marumo (1974).

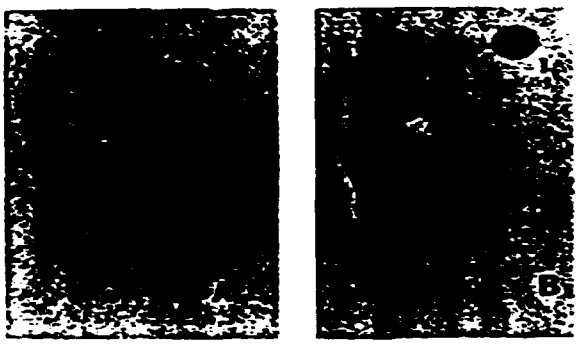

Figure 4. *Thin layer chromatogram* (A) *and the corresponding autoradiogram* (B) *of radioactive pea extract. 1.* 4-Chloroindolyl-3-acetic acid methyl ester and its radioactive counterpart. *2.* 4-Chloroindolyl-3-acetic acid and its radioactive counterpart. Solvents: $CHCl_3$:CH_3OH:H_2O, 3:5:2 (up) and CH_2Cl_2:$CH_3COOC_2H_5$, 2:1 (to the right). Spray: $FeCl_3$/$HClO_4$.

be used for gas chromatography–mass spectrometry, although they were not pure enough for direct-inlet mass spectrometry.

When a sample, dissolved in a few µl of $CHCl_3$, was applied to the gas chromatograph it showed a retention time of a few minutes. The mass spectrum (Figure 2), recorded at 20 eV, proved to be remarkably simple, showing only the peaks at *m/e* 223, 225 and *m/e* 164, 166. The peaks at *m/e* 83, 85, 87 are background from the solvent chloroform. The mass spectrum of the unknown was identical with the 20 eV mass spectrum of synthetic 4-chloroindolyl-3-acetic acid methyl ester taken either via direct-inlet (Figure 3A) or via the gas chromatograph.

Figure 3B shows the 70 eV direct-inlet mass spectrum of synthetic 4-chloroindolyl-3-acetic acid methyl ester. It confirms the mass spectral data obtained by Abe and Marumo (1974) on 4-, 5-, 6-, and 7-chloroindolyl-3-acetic acid methyl ester. The 70 eV mass spectra obtained via the gas chromatograph were not satisfactory because of background due to "bleeding" of the column material.

Thin layer chromatograms of radioactive extracts and the corresponding autoradiograms showed complete correspondence between the radioactivity and the standards both for 4-chloroindolyl-3-acetic acid and its methyl ester (Figure 4) in 12 different solvent systems (Table 1).

Table 1. *R_f values in 12 solvent systems for 4-chloroindolyl-3-acetic acid, its methyl ester and the ^{36}Cl radioactive compounds extracted from pea.*

Solvent	Methyl ester radioactive Spot 1	Free acid radioactive Spot 2
Ethyl acetate : pyridine : H_2O, 4 : 2 : 2	0.75	0.68
$CHCl_3$	0.27	0.00
CCl_4 : methanol, 4 : 1	0.34	0.15
Benzene : acetone : pyridine, 60 : 39 : 1	0.59	0.27
$CHCl_3$: methanol : H_2O, 3 : 5 : 2	0.87	0.66
CH_2Cl_2 : ethyl acetate, 2 : 1	0.62	0.15
Heptane : benzene : butanol, 2 : 1 : 1	0.52	0.30
Benzene : ethyl acetate, 1 : 1	0.34	0.24
$CHCl_3$: ethyl acetate : HCOOH, 5 : 4 : 1	0.59	0.45
Butanol : AcOH : ether : H_2O, 9 : 6 : 2 : 1	0.91	0.89
Heptane : benzene : ethanol, 25 : 15 : 1.5	0.03	0.00
$CHCl_3$: acetic acid, 19 : 1	0.35	0.22

Discussion

On the basis of the behaviour of the "unknown" compound the following conclusions can be drawn:

1. The compound contains one and only one chlorine atom. (a) because it is radioactive from ^{36}Cl and (b) because the mass spectrum shows the characteristic 3:1 ratio of $^{35}Cl/^{37}Cl$ at *m/e* values two units apart (Williams and Fleming 1973).
2. The compound contains one N (or an uneven number of N's and P's) because the mass of the molecular ions is uneven (Williams and Fleming 1973).
3. The compound is probably an indole. The base peak at *m/e* 164 corresponds to the very stable quinolinium ion characteristic of substituted indoles (Abe and Marumo 1974, Jamieson and Hutzinger 1970, Khmel'nitskiĭ *et al.* 1969, Williams and Fleming 1973). Metastable peaks at *m/e* 120.6 and 122.5 (not visible in Figure 2) show that the ions at *m/e* 164, 166 are formed directly from the molecular ions.

The identity of the "unknown" compound with the synthetic 4-chloroindolyl-3-acetic acid methyl ester is highly likely, but not certain on the basis of the mass spectra alone. The gas chromatographic column cannot separate the four different monochlorinated isomers of indolyl-3-acetic acid methyl esters (chlorinated in the 4, 5, 6, or 7 position) and the compounds cannot be distinguished by mass spectrometry (Abe and Marumo 1974).

The identity of the "unknown" radioactive compound as 4-chloroindolyl-3-acetic acid methyl ester was established by thin layer chromatography and autoradiography. The solvents chloroform and benzene : ethyl acetate 1 : 1 separated the 4-chloro- from the 5-, 6-, and 7-chloro-isomers. In most solvents the 7-chloro-isomer had the highest R_f value. It was very difficult to distinguish between the 5-chloro- and the 6-chloro-isomers.

It was shown that the methyl ester of 4-chloroindolyl-3-acetic acid was not an artifact of the methanol : dichloromethane chromatography because identical mass spectra were obtained after extraction with acetone and chromatography with CCl_4 : acetone on Sephadex LH 20 and with $CHCl_3$ on silica gel. The amount of 4-chloroindolyl-3-acetic acid methyl ester is at least 0.1 mg/kg dry weight. This is also the order of magnitude obtained in the large-scale isolations (Gandar and Nitsch 1967, Marumo *et al.* 1968a).

Thus, it has been possible to elucidate the chemical structure of a few µg of a chlorinated auxin, isolated from

25 g of lyophilized, ^{36}Cl-labelled material by following its radioactivity without having to use laborious bioassays. Similar identification of chlorinated auxins in other species should be possible eventually, but larger amounts of material will probably be necessary.

We are grateful to Mr. Klaus Christensen for competent technical assistance.

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Appendix X

406

Determination of 4-chloroindole-3-acetic acid methyl ester in Lathyrus, Vicia and Pisum by gas chromatography – mass spectrometry

By

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Abstract

4-Chloroindole-3-acetic acid methyl ester was identified unequivocally in *Lathyrus latifolius* L., *Vicia faba* L. and *Pisum sativum* L. by thin layer chromatography, gas chromatography and mass spectrometry. The gas chromatographic system was able to separate underivatized chloroindole-3-acetic acid methyl ester isomers.

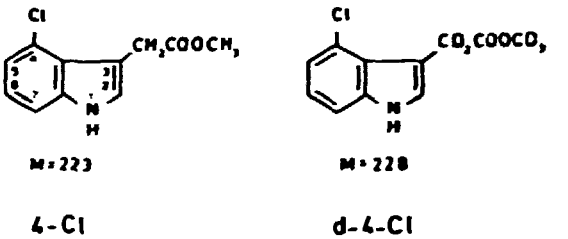
The quantitative determination of 4-chloroindole-3-acetic acid methyl ester in immature seeds of these three species was performed by gas chromatography – mass spectrometry using deuterium labelled 4-chloro-indole-3-acetic acid methyl ester as an internal standard. *P. sativum* contained approximately 25 mg kg^{-1}, *V. faba* 1–2 mg kg^{-1} and *L. latifolius* 2 mg kg^{-1} dry weight.

Key-words: Gas chromatography – mass spectrometry, isotopic dilution technique, auxin, 4-chloroindole-3-acetic acid methyl ester, *Pisum sativum*, *Lathyrus latifolius*, *Vicia faba*.

Introduction

A natural chlorinated auxin, 4-chloroindole-3-acetic acid methyl ester (see formula), was detected in immature pea seeds by Gandar and Nitsch (1967) and by Marumo's group (Marumo *et al.* 1968a). Later Marumo's group isolated smaller quantities of the free acid (Marumo *et al.* 1968b), of its aspartic acid amide (Hattori and Marumo 1972) and of two derivatives of 4-chlorotryptophan (Marumo and Hattori 1970). The occurrence of 4-chloroindole-3-acetic acid methyl ester in immature pea seeds has also been verified by radioactive isotopic experiments with ^{36}Cl (Engvild 1975) and by gas chromatography-mass spectrometry (Engvild *et al.* 1978).

Chlorinated indole compounds are very strong auxins (Bottger *et al.* 1978), and therefore their possible presence in other plant species would be of interest. Hofinger and Bottger (1979) have identified 4-chloroindole-3-acetic acid and an ester of this compound in *Vicia faba* by GC-MS. The present work reports the identification of 4-chloroindole-3-acetic acid methyl ester and its quantitative determination using stable isotopic dilution technique. Furthermore, an improved gas chromatographic separation of the chloroindole-3-acetic acid methyl esters is presented.



Abbreviations: ClIAM, 4-chloroindole-3-acetic acid methyl ester; GC-MS, gas chromatography-mass spectrometry; TLC, thin layer chromatography.

Materials and methods

Plant material

Immature seeds of *Lathyrus latifolius* L. were obtained from the garden at Risø and from a garden at Ryomgård, Jutland. Young seeds of *Vicia faba* L. cv. Dr. Francks Ackerperle were grown in the greenhouse during spring. Another sample of *Vicia faba* was obtained from a segregating F_2 population grown in the field at Højbakkegård by Dr. Helt Poulsen. The seeds were harvested about three weeks after flowering. Frozen green peas, *Pisum sativum* L., of a large grained quality of unknown cultivars, were obtained in local supermarkets. The immature seeds of the three species (dry matter about 25%) were frozen in liquid nitrogen and lyophilized.

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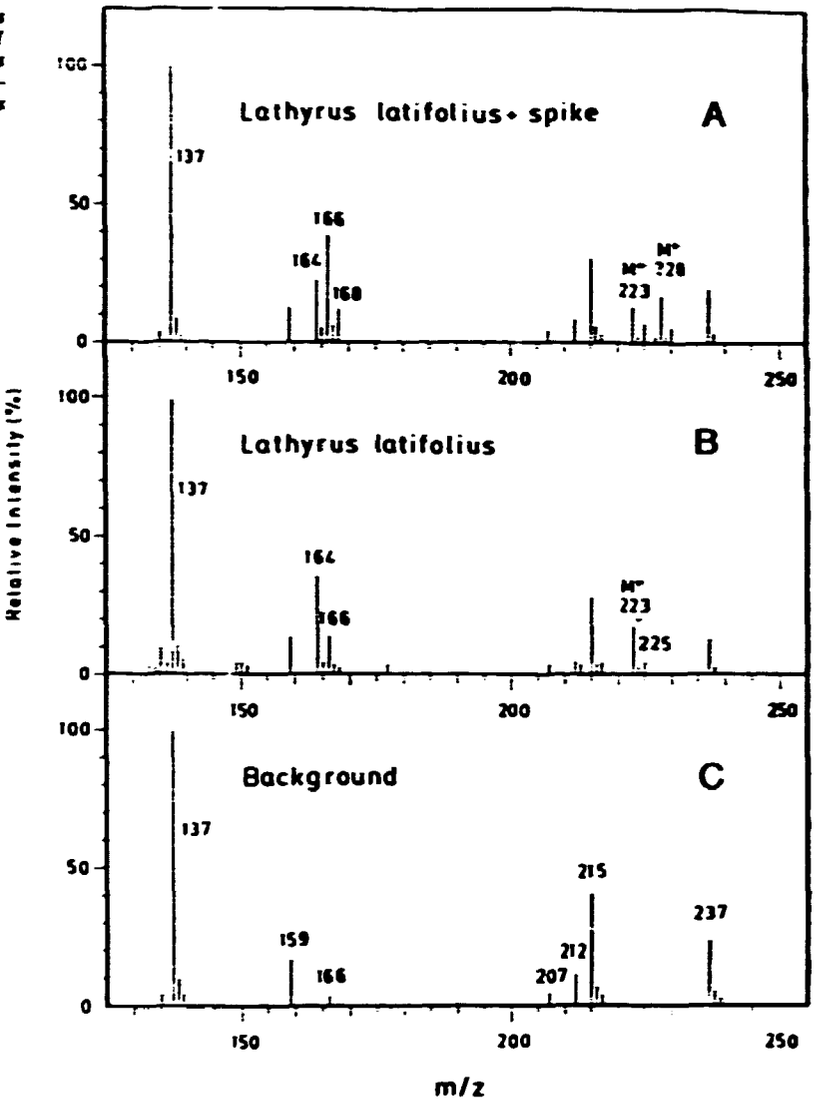


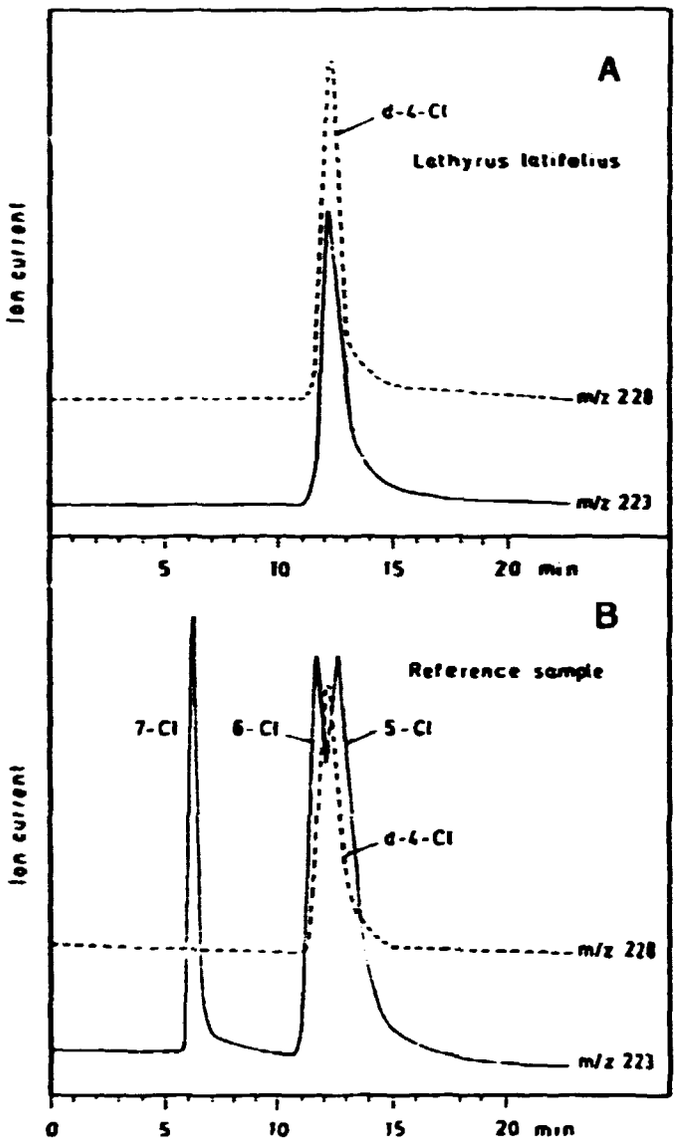
Figure 1. *20 eV mass spectra after the gas chromatographic separation on a QF 1 column at 220°C.* A. Sample of *Lathyrus latifolius* with deuterated internal standard (spike). B. Sample of *Lathyrus latifolius*. C. The background.

Extraction and purification

Ten to 20 g of powdered, lyophilized material were extracted with 100 ml *n*-butanol + 100 ml 0.1 *M* H_3PO_4 by shaking for 10 min. The suspension was centrifuged at 3500 *g* for 10 min; the lower phase including precipitate was extracted a second time with *n*-butanol only (10 min). The butanol phases were combined and washed with 100 ml of water. After centrifugation the butanol phase was isolated and evaporated to almost dryness *in vacuo* on a rotary evaporator. The viscous residue was dissolved in 10 ml acetonitrile. A large precipitate was filtered off and the filtrate was concentrated to dryness in an air stream. The residue was dissolved in 100 µl acetonitrile and applied to a chromatographic column, 15 cm × 1.2 cm, packed with 5 g Sephadex LH-20. The column was eluted with CH_2Cl_2:acetone 1:1 v/v and 2 ml fractions were collected. The solvent was evaporated in an air stream and the residues dissolved in 50 µl acetonitrile. The CIIAM was localized by TLC and usually appeared in fractions 12–18.

Thin layer chromatography

Ten µl from each fraction were applied to 10 cm × 20 cm precoated plates (Merck silicagel 60 F_{254}, thickness 0.25 mm) and chromatographed together with standards. The developing solvents were CH_2Cl_2:ethyl acetate 2:1 v/v or $CHCl_3$:ethyl acetate 4:1 v/v. CIIAM was visualized with Ehmann's reagent (1977): three parts by volume of 10 m*M* $FeCl_3$ in 37% H_2SO_4 and one part of a mixture of



Figur 2. (A) *Co-chromatography of "unknown" (m/z 223) from Lathyrus latifolius and the added deuterium labelled internal standard of 4-chloroindole-3-acetic acid methyl ester (m/z 228).* (B) *Partial separation of indole-3-acetic acid methyl esters with Cl in position 4, 5, 6, and 7 of the indolering.* The separation was performed on a QF 1 gas chromatographic column at 190°C and detected by selected ion monitoring.

1% dimethylaminobenzaldehyde in ethanol and conc. HCl 1:1 v/v. The fractions containing the auxin were pooled and the solvent evaporated in an air stream. The sample was dissolved in a few µl of $CHCl_3$.

Gas chromatography-mass spectrometry

One µl of the $CHCl_3$ samples were on-column injected into a modified Perkin-Elmer F-11 gas chromatograph connected to a Varian MAT CH 5D mass spectrometer through a two-stage all-glass jet separator. The glass column (2 m × 3 mm i.d.) was packed with 3% QF 1 on 80–100 mesh Chromosorb W. Carrier gas: helium with a flow rate of 65 ml min^{-1}. Temperatures: column 220°C, ion source 200°C, separator 280°C. To avoid ionization of the helium carrier gas the electron impact mass spectra were obtained at 20 eV (80 µA) using the mass spectrometer as a detector writing out the ion current at *m/z* 223. When the *m/z* 223 peak appeared, two to four mass scans (*m/z* 10-400) were run. Resolution was approximately 1000.

Quantitative determination

For the quantitative determination a known amount of a deuterium labelled ClIAM dissolved in ethanol was ad-

ded to the lyophilized plant material as an internal standard. The deuterated compound was prepared by the CD_3ONa catalyzed proton exchange/transesterification of ClIAM in CD_3OD (Engvild 1977, Engvild and Egsgaard, unpublished results). The deuterated ester was characterized by mass spectrometry and, dissolved in CD_3OD, by ^{1}H-NMR. It was found to be perdeuterated (> 99%) in the side chain (see formula). The concentration of the internal standard was calibrated against known amounts of unlabelled ClIAM. The mass spectrometer was focused on mass *m/z* 231 from perfluorotributylamine. Using selected ion monitoring the molecular ions of ClIAM (*m/z* 223) and of the labelled standard (*m/z* 228) were recorded on a two-channel recorder.

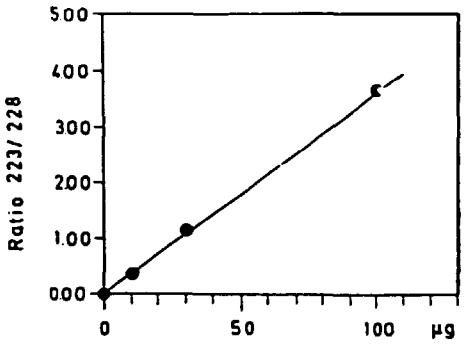


Figure 3. *Calibration curve showing the ratio of the ion currents of m/z 223 and m/z 228 versus the amount of 4-chloroindole-3-acetic acid methyl ester.*

Results

After TLC of purified extracts of young seeds, faint, but unmistakable blue spots at the Rf value of ClIAM were seen both in Vicia faba and Lathyrus latifolius. Analysis by GC-MS of two different sources of Lathyrus and of Vicia both with and without deuterated internal standard showed the presence of chloroindole-3-acetic acid methyl esters. Figure 1A shows the electron impact mass spectrum of an extract of Lathyrus latifolius treated with internal standard. The mass spectrum shows *m/z* 223/225 peaks of the molecular ions of the unknown and the *m/z* 228/230 peaks of the deuterated standard. The base quinolinium ion peaks at *m/z* 164/166 and at *m/z* 166/168 fuse together into the expected *m/z* 164/166/168 pattern. When mass spectra were run on extracts without internal standard, only peaks at *m/z* 223/225 and 164/166 were seen (Figure 1B). The background mass spectrum (Figure 1C) shows the bleeding of the QF 1 column (Spiteller and Spiteller 1973). There are virtually no peaks in the mass range *m/z* 220–230 which we used for the quantitative determination. When control experiments were run on extracts of powdered barley straw with added deuterated standard, GC-MS peaks appeared only at *m/z* 228/230 and at 166/168 corresponding to the standard. Therefore, there was no hydrogen isotope exchange during the extraction, purification or the GC-MS analysis. By decreasing the temperature of the gas chromatographic QF 1 column to 190°C it was possible to obtain a partial separation of the four chloroindole-3-acetic acid methyl ester isomers. Figure 2B shows the selected ion monitoring of a mixture of deuterated 4-chloroindole-3-acetic acid methyl ester (*m/z* 228) and 5-chloro-, 6-chloro- and 7-chloroindole-3-acetic acid methyl esters (*m/z* 223; Engvild 1977). Figure 2A shows the co-chromatography of the unknown extracted from Lathyrus latifolius (*m/z* 223) and of the internal standard (*m/z* 228). A chromatogram of an extract without addition of the deuterated internal standard shows only a response at *m/z* 223 in agreement with the mass spectrum (Figure 1B).

A calibration curve is shown in Figure 3. Known amounts of ClIAM were added to 500 µl standard solution (containing about 30 µg deuterated ClIAM). Table 1 shows the amounts of ClIAM found in the various samples. Each value represents the mean of two GC-MS measurements made on the same extract.

Table 1. *Content of 4-chloroindole-3-acetic acid methyl ester in three plant species.* Each result is the average of two GC-MS readings made on one extract.

Plant material	mg kg^{-1} dry weight
Vicia faba, Dr. Francks Ackerperle	2.6
Vicia faba, F_2 segregants	1.4
Lathyrus latifolius, Risø	2.3
Lathyrus latifolius, Ryomgård	2.3
Pisum sativum, Irma market	32
Pisum sativum, Favør market	25

Discussion

The presence of ClIAM in Lathyrus latifolius and Vicia faba has been proven by TLC and GC-MS. The compound co-chromatographed with ClIAM and showed the right colour after spraying with Ehmann's reagent. The compound contained one and only one chlorine atom (the 3:1 ratio of ^{35}Cl/^{37}Cl at *m/z* 223/225) and it had an indole type fragmentation.

The quantitative data show that the chlorinated auxin was present in about 10 times larger amounts in P. sativum than in V. faba and L. latifolius. The figures for pea are quite large for an auxin, about 25 mg kg^{-1} dry weight. It is actually surprising that the determinations in the different seed samples have given such similar values, since Gandar and Nitsch (1964) showed by bioassay that the amount of the substance "F" which was later identified as ClIAM varied many thousand fold during development, reaching a maximum 20 days after flowering and disappearing at maturity.

The isotopic dilution technique is independent of auxin recovery during extraction and purification, thus avoiding errors due to uncontrolled losses. A semiquantitative estimate based on the colour density on the TLC plates indicated that the recovery was below 25%.

Hofinger and Böttger (1979) have shown the presence of free 4-chloroindole-3-acetic acid in Vicia faba. Our procedures are not designed for the free acid but we did observe 4-chloroindole-3-acetic acid on thin layer chromatograms in Lathyrus latifolius.

As many plant species including the cereals incorporate $^{36}Cl^-$ into compounds chromatographing close to ClIAM on TLC (Engvild 1975) we expect to find chlorinated auxins in other species than those of the Vicia tribe. At the moment, however, it is not known whether the incorporation of radioactive chloride into organic compounds in seeds of other species was due to chlorinated indole auxins, to other chlorinated compounds or simply to artifacts (cf. Hofinger and Böttger 1979).

We have looked for ClIAM in immature seeds of a number of other cultivated plants by TLC. We did not see the ClIAM spot, so the content in the seed samples of the investigated species was at least below 0.5 mg kg^{-1} dry weight, the approximate sensitivity of the TLC. However, we did observe spots of indole-3-acetic acid (IAA) in young seeds of barley, oats, wheat, rye, poppy and rape, and we saw the spot of indole-3-acetonitrile (IAN) in rape. With proper controls for hydrogen isotope exchange of the added deuterated standard as a carrier it will be possible to use the method described in this paper to search for chlorinated indole auxins in these species by GC-MS with selected ion monitoring. The sensitivity of the method has been estimated to be approximately 10 μg kg^{-1}.

We are grateful to Ms. Lone Dyrgaard Jensen for skilful technical assistance with extractions and purifications and to Ms. Jytte Funck for drawings.

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Appendix XI

PHYSIOL. PLANT. 53: 79–81. Copenhagen 1981

451

Determination of 4-chloroindoleacetic acid methyl ester in Vicieae species by gas chromatography-mass spectrometry

Kjeld C. Engvild, Helge Egsgaard and Elfinn Larsen

Engvild, K. C., Egsgaard, H. and Larsen, E. 1981. Determination of 4-chloroindoleacetic acid methyl ester in *Vicieae* species by gas chromatography-mass spectrometry. – Physiol. Plant. 53: 79–81.

The natural chlorinated auxin, 4-chloroindoleacetic acid methyl ester, was identified in immature seeds of *Lathyrus sativus* L., *Lathyrus maritimus* (L.) Bigel and *Lathyrus odoratus* L. by thin layer chromatography and gas chromatography-mass spectrometry. In immature seeds of *Vicia sativa* L. and *Lens culinaris* Medik. the hormone was identified by selected ion monitoring. The hormone was determined quantitatively using pentadeuterated 4-chloroindoleacetic acid methyl ester as internal standard. Contents varied from 1 mg/kg fresh weight in *Lathyrus sativus* to 0.02 mg/kg in *Lens culinaris*. *Lathyrus maritimus* also contained indoleacetic acid methyl ester (0.3 mg/kg) besides the chlorinated analogue.

Additional key-words: 4-Chloroindoleacetic acid methyl ester, auxin, immature seeds, *Vicia*, *Lathyrus*, *Lens*, isotope dilution analysis, gas chromatography-mass spectrometry, selected ion monitoring.

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Introduction

Until now 4-chloroindoleacetic acid methyl ester has been identified in immature seeds of three species, all of the *Vicieae* tribe of the *Fabaceae*. *Pisum sativum* (Gandar and Nitsch 1967, Marumo *et al.* 1968, Engvild *et al.* 1978) contains large amounts, 5 mg/kg fresh weight (Engvild *et al.* 1980), *Vicia faba* (Hofinger and Böttger 1979, Engvild *et al.* 1980) and *Lathyrus latifolius* (Engvild *et al.* 1980) smaller amounts. Pea contains at least four compounds related to 4-chloroindoleacetic acid (Marumo *et al.* 1974), but it is not known if chlorinated auxins occur outside the *Vicieae* tribe. However, many species incorporate $^{36}Cl^-$ into organic compounds that chromatograph close to the chlorinated auxins on TLC (Engvild 1975). It is the purpose of the present work to see if ClIAM is common in *Vicieae* species.

Abbreviations: ClIAM, 4-chloroindoleacetic acid methyl ester; Rt, retention time; GC-MS, gas chromatography-mass spectrometry; SIM, selected ion monitoring; TLC, thin layer chromatography.

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Material and methods

Plant material: Immature seeds of all five species were collected 2–4 weeks after flowering; dry matter was about 25%. The seeds were boiled in water for 30 seconds and stored at –18°C. Three species were grown out of doors at Risø, namely *Lathyrus sativus* L. (grass-pea) and *Vicia sativa* L. (vetch) from seeds from the Botanical Garden in Copenhagen, *Lens culinaris* Medik. (lentil) from seeds from a health food store. *Lathyrus odoratus* L. (sweet pea) was grown from a seed mixture from Daehnfeldt's Frø, Odense, Denmark, in a growth chamber at 18°C/12°C for 18 h/6 h at 17,000 lux with no dark period. Light from 2000 installed W/m² Philips mercury HPLR lamps 1.5 m above the table supplemented with 200 W/m² incandescent light. *Lathyrus maritimus* (L.) Bigel (sea pea) was collected at Jersie Strand, Køge Bay, Zealand, Denmark.

Extraction, purification and GC-MS: 25–50 g of frozen material with or without a deuterium labelled ClIAM standard was extracted with butanol/H_3PO_4 0.4 *M* (4:1), transferred to acetonitrile, chromatographed on

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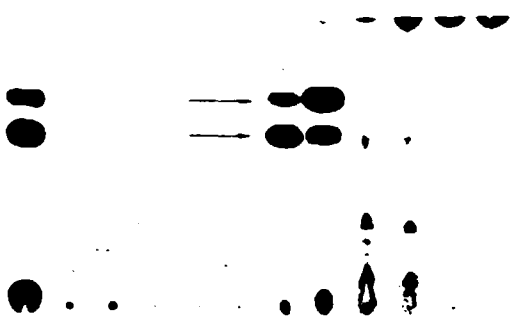

Fig. 1. Simultaneous presence of chloroindoleacetic acid methyl ester (Rf 0.58) and indoleacetic acid methyl ester (Rf 0.70) in a *Lathyrus maritimus* extract shown by thin layer chromatography of fractions from Sephadex LH-20 chromatography. Staining with Ehmann's (1977) reagent. Standards to the left.

Sephadex LH-20 with CH_2Cl_2:acetone (1:1), and ClIAM containing fractions located by TLC as described in detail by Engvild *et al.* (1980).

GC-MS was performed on an OV-1 column for identification by obtaining complete low background mass spectra, and on a QF-1 column for quantitative determinations involving stable isotope dilution analysis with pentadeuterated ClIAM as internal standard (Engvild *et al.* 1978, 1980). SIM of the molecular ions *m/z* 223 (ClIAM) and *m/z* 228 (pentadeuterated ClIAM) was used. The amount of ClIAM in *Vicia sativa* and *Lens culinaris* was too low to obtain complete mass spectra. Therefore, the identification was performed only by the much more sensitive SIM method. However, then both the characteristic ion pairs *m/z* 223, 225 and *m/z* 164, 166 were measured, and the ratio of the ion intensities at *m/z* 223 and *m/z* 164 was estimated using the ratios at *m/z* 223, 228 and *m/z* 164, 168 determined in the quantitative analysis.

The content of IAA-methyl ester in *Lathyrus maritimus* was also measured by isotope dilution analysis using as internal standard indoleacetic acid methyl ester, perdeuterated in the side chain. 250 µl of an ethanolic solution of this pentadeuterated ester (conc. 725 µg/ml) was added to 50 g of frozen material and the extraction procedure described for ClIAM (Engvild *et al.* 1980) was followed. SIM was performed measuring the molecular ions *m/z* 189 and *m/z* 194. The standard was prepared by CD_3ONa catalyzed proton exchange/transesterification of IAA methyl ester in CD_3OD. The isotopic purity measured by mass spectrometry was estimated to be 94% and the ion ratio of *m/z* 189: *m/z* 194 found to be lower than 0.005.

Results and discussion

In three of the species, *L. sativus*, *L. odoratus* and *L. maritimus*, it was possible to identify ClIAM after TLC of the fractions obtained after chromatography on Sephadex LH-20. In *L. maritimus* we identified both ClIAM and indoleacetic acid methyl ester on the same TLC plates as shown in Figure 1. We have not seen indoleacetic acid methyl ester in amounts detectable on TLC either in pea or in other *Vicieae* species.

The methyl ester of IAA in *Lathyrus maritimus* was also identified by GC-MS. Mass spectra showed prominent peaks at *m/z* 189, $M^{+\cdot}$, and *m/z* 130, $(M\text{-}COOCH_3)^+$ (Heller and Milne 1978). The content of IAA methyl ester measured by isotopic dilution was 0.3 mg/kg fresh weight (Tab. 2).

The identification of ClIAM in the three *Lathyrus* species proceeded smoothly. We obtained complete mass spectra where the 3/1 $^{35}Cl/^{37}Cl$ ratio was easily picked out at *m/z* 223, 225, $M^{+\cdot}$, and at the base peaks *m/z* 164, 166 $(M\text{-}COOCH_3)^+$. GC-MS of an extract with deuterated standard gave exactly the same retention time for *m/z* 223 and *m/z* 228 under gas chromatographic conditions where the four different monochloro isomers (positions 4, 5, 6, and 7) of indoleacetic acid methyl ester were partly separated, proving the presence of 4-chloroindoleacetic acid methyl ester.

Tab. 1. Identification of 4-chloroindoleacetic acid methyl ester in five *Vicieae* species and of indoleacetic acid methyl ester in *Lathyrus maritimus*. Positive signs indicate agreement with authentic standards. Where complete mass spectra were not obtainable, the presence of ClIAM was established by selected ion monitoring of the characteristic ions.

Species	TLC Rf, colour	GC-MS				
		Rt	Complete mass spectra	SIM		
				m/z 223:*m/z* 225	*m/z* 164:*m/z* 166	*m/z* 223:*m/z* 164
Lathyrus sativus	+	+	+			
Lathyrus odoratus	+	+	+			
Lathyrus maritimus	+	+	+			
Vicia sativa		+		+	+	+
Lens culinaris		+		+	+	+
Lathyrus maritimus IAA methyl ester	+	+	+			

Tab. 2. Quantitative determination of 4-chloroindoleacetic acid methyl ester in five *Vicieae* species. A stable isotope dilution technique was used. The final analysis was performed by selected ion monitoring by GC-MS.

Species	Amount mg/kg fr. wt.
Lathyrus sativus	1.11 0.94
Lathyrus odoratus	0.25 0.23
Lathyrus maritimus	0.39 0.37
Vicia sativa	0.068 0.042
Lens culinaris	0.017 0.024
Lathyrus maritimus IAA methyl ester	0.34 0.32

The identification of ClIAM in *Vicia sativa* and *Lens culinaris* rests on a slightly weaker basis. There was not enough hormone to show up on the thin layer plates after Sephadex chromatography. Therefore, pentadeuterated standard was added before extraction as carrier and marker. The Sephadex fractions containing the isotopic standard were analysed by GC-MS using SIM. There was exact chromatographic coincidence between *m/z* 223, *m/z* 228 (standard) and the base peaks, *m/z* 164 (unknown) and *m/z* 168 (standard, ^{37}Cl peak). Furthermore, the ratio between the intensities of the ions *m/z* 223 and *m/z* 164 was identical with that of the authentic ClIAM.

In other experiments IAA methyl ester was added as a marker for the relevant ClIAM containing Sephadex fractions. These were collected and analyzed by SIM for the ^{35}Cl and ^{37}Cl ratio at *m/z* 223, 225, $M^{+\cdot}$, and at *m/z* 164, 166 $(M\text{-}COOCH_3)^+$, which were found to be identical with ClIAM. The combined results are virtually certain evidence that ClIAM is a constituent of *Vicia sativa* and *Lens culinaris* (Tab. I).

With the present work ClIAM has been identified with rigorous chemical methods in immature seeds of eight species of the *Vicieae* tribe. The content of ClIAM varied widely from about 5 mg/kg fr.wt. in pea (Engvild *et al.* 1980) to about 0.02 mg/kg in lentil (Tab. 2).

As the method involves an isotopic internal standard, the determination is independent of known recovery and intrinsic errors are small. Variation (Tab. 2) is probably due to sample differences *(L. sativus)* and the uncertainties *(V. sativa, L. culinaris)* close to the detection limit of the method.

Acknowledgement – We are grateful to Ms. Lone Dyrgaard Jensen for skilful technical assistance.

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Appendix XII

PHYSIOL. PLANTARUM 69: 137–140. Copenhagen 1987

Rooting, growth and ethylene evolution of pea cuttings in response to chloroindole auxins

A. Ahmad, A. S. Andersen and K. Engvild

Ahmad, A., Andersen, A. S. and Engvild, K. C. 1987. Rooting, growth and ethylene evolution of pea cuttings in response to chloroindole auxins. – Physiol. Plantarum 69: 137–140.

In pea cuttings (*Pisum sativum* L. cv. Alaska) we measured shoot and root growth and ethylene production in response to 4-chloroindole-3-acetic acid (4-Cl-IAA) or 4,6-dichloroindole-3-acetic acid (4,6-Cl_2-IAA). Leafy cuttings treated basally with either of the chlorinated auxins in high concentrations showed permanent epinasty, loss of apical growth and dominance resulting in the outgrowth of laterals from the lower-most axillary bud. The naturally occurring 4-Cl-IAA was a better root promoter than the synthetic 4,6-Cl_2-IAA which inhibited rooting. Both chloroindole auxins induced very high ethylene evolution, which lasted much longer than the ethylene evolution after IAA treatment.

Additional key words – Apical dominance, 4-chloroindole-3-acetic acid, 4,6-dichloroindole-3-acetic acid, ethylene, rooting, shoot growth.

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Introduction

The methyl ester of a naturally occurring chlorinated auxin, 4-Cl-IAA was identified in immature pea seeds by Gandar and Nitsch (1967) and Marumo et al. (1968b). Later, the free acid was identified (Marumo et al. 1968a, Schneider et al. 1985). A number of chlorinated indoles have been tested for auxin activity in several assays (Böttger et al. 1978, Katekar and Geissler 1982, 1983; see Engvild 1985 for a review). All have reported that 4-Cl-IAA is one of the most active auxins, about ten times more active than IAA.

Auxin-induced ethylene production is well established (Abeles 1973). The resulting ethylene can inhibit elongation in young stems. The resulting ethylene can inhibit elongation in young stems. The reduction in the capacity of polar auxin transport was the main reason according to Apelbaum and Burg (1972).

Plants exposed to ethylene also lose apical dominance, e.g., in cotton (Hall et al. 1957), pea (Andersen 1976) and apple, crabapple and apricot (Zimmerman et al. 1977). The modification of auxin transport caused by ethylene has been suggested as the main reason for this (Morgan et al. 1968).

Ethylene has repeatedly been assigned a major role in the rooting of cuttings, but the experimental results have been contradictory. Roy et al. (1972) did not find any effect of ethylene on rooting, whereas Mullins (1972) reported inhibition, and Krishnamoorthy (1970) and Robbins et al. (1985) reported stimulation. As previously suggested there are many reasons for these discrepancies (Andersen 1977), among these light conditions of stock plants, timing of ethylene treatment (Robbins et al. 1985) and control solutions for rooting. The present experiment was carried out to investigate the relationship between rooting and ethylene evolution in leafy cuttings of pea when treated with the endogenous 4-Cl-IAA and the synthetic analogue 4,6-Cl_2-IAA.

Abbreviations – 4-Cl-IAA, 4-chloroindole-3-acetic acid; 4,6-Cl_2-IAA, 4,6-dichloroindole-3-acetic acid.

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Materials and methods

Pisum sativum L. cv. Alaska seeds obtained from FDB seed Production, Flakkebjerg, Denmark, were imbibed for 12 h with aeration in a 1% (w/v) solution of Captan fungicide. The seeds were sown in a standard soil mixture (K-jord, Plantin and Co., Sweden) and allowed to grow for 11 days in a controlled environment room under the following conditions: Irradiance 38 W m^{-2} from Philips HPI/T 375 W lamps supplemented with incandescent lamps, photoperiod 14 h, relative humidity about 60%, day and night air temperature 21±1°C. The plants were watered with tap water as required. On the eleventh day, the auxins were fed by dipping the lower 1 cm of the cutting 5 min and placed in standard nutrient solution under the same climatic conditions after a rinse in deionized water (Eriksen 1973).

Ethylene was trapped by enclosing the entire cuttings in sealed glass jars for 1 h. The jars remained in the controlled environment with light. Five ml gas samples were drawn with an airtight syringe and analysed on a gas chromatograph, and the results were expressed on fresh weight basis. A Hewlett-Packard 7620 A gas chromatograph was fitted with a stainless steel column packed with Porapack T, 80–100 mesh, 2 m × 2.4 mm i.d. and a flame-ionization detector. Nitrogen was used as the carrier gas at 40 ml h^{-1}. The column temperature was 65°C, injection port 100°C, and the detector 150°C. The retention time of ethylene under these conditions was 60–70 s.

The rooting experiment was terminated on the 13th day by counting the number of roots per cutting, the position and length of the laterals and the final length of the main shoot. The experiment was repeated once, taking three replicates of each treatment which included ten cuttings.

Results

Auxin feeding of pea cuttings resulted in epinastic growth, twisting and bending of the apical part of the stems. Cuttings treated with IAA (10^{-3} *M*) recovered within 24 h to the normal upright condition, whereas those treated with similar concentrations of either 4-Cl-IAA or 4,6-Cl_2-IAA did not show any sign of recovery during the first 13 days after the treatment. But they were not permanently damaged, since they were still alive and adventitious buds grew out later. Apical shoot growth was completely inhibited in cuttings treated with either of the chlorinated auxins (Tab. 1). The maximum inhibition of the rate of elongation was with 4,6-Cl_2-IAA followed by 4-Cl-IAA, while the rate of growth was identical for control cuttings and cuttings treated with IAA. Lack of aerial growth was also evident from the number of leaves per cutting (Tab. 1).

Apical dominance in the cuttings treated with either 4-Cl-IAA or 4,6-Cl_2-IAA was completely lost. These cuttings behaved similarly to decapitated cuttings, i.e., lateral buds started growth. However, the difference in these two types of cuttings was on the position of the growing laterals. In the cuttings where the apex was removed, lateral buds started growing randomly more than one at a time, but in the majority of the cuttings it was the second bud from the top. However, in the cuttings treated with 4-Cl-IAA or 4,6-Cl_2-IAA the lateral bud at the lowest node started growing (Tabs 1 and 2).

Treatment of cuttings with 4-Cl-IAA (10^{-3} *M*) was most effective for rooting and produced twice as many roots per cutting as the control. Removal of the apex or treatment of the cuttings with 4,6-Cl_2-IAA (10^{-3} *M*) both inhibited rooting by about 50% (Tab. 1).

A separate experiment (Tab. 2) with auxin treatments at 10^{-6} *M* showed similar patterns with interesting variations. The cuttings treated with 10^{-6} *M* 4-Cl-IAA recovered from initial epinasty after 48 h and resumed bud growth, but apical dominance was still weak. Cuttings treated with 10^{-6} *M* 4,6-Cl_2-IAA did resume slow growth, but showed reduced apical dominance and rooted poorly.

The production of ethylene was strongly stimulated by 4-Cl-IAA and 4,6-Cl_2-IAA both at 10^{-3} and 10^{-6} *M* (Fig. 1). A lag period of 1 h was found before the increased production of ethylene. A continuous increase in the ethylene production by 10^{-3} *M* 4-Cl-IAA was noted up to 2.9 nmol g^{-1} h^{-1} after 30 h and it started a slow decrease from the third day reaching 0.22 nmol g^{-1} h^{-1}, the level of the control, on the sixth day after treatment. The cuttings treated with 10^{-3} *M* 4,6-Cl_2-IAA followed a similar pattern reaching 3,79 nmol g^{-1} h^{-1}, 48 h after the treatment and decreasing slowly to 0.22 nmol

Tab. 1. Number of roots per cutting, rate of elongation of aerial part, number of mature leaves and distribution of laterals in pea cuttings treated by dipping, with IAA, 4-Cl-IAA or 4,6-Cl_2-IAA, all at a concentration of 10^{-3} *M*. Mean ± SE.

Conditions	Number of roots/cutting	Rate of shoot elongation cm day^{-1}	Number of mature leaves	% Distribution of laterals			
				1st node	2nd node	3rd node	4th node
Control	12.7±0.9	0.9±0.0	7.4±0.1	0	0	0	0
Decapitated	6.2±2.3	0.3±0.0	4.0±0.0	61±10	95±6	25±15	16±7
IAA	15.7±1.7	0.9±0.1	6.0±0.1	0	0	0	0
4-Cl-IAA	25.2±1.8	0.7±0.8	4.0±0.0	0	0	0	100±0
4,6-Cl_2-IAA	6.6±0.5	0.5±0.0	4.0±0.0	0	0	18±13	90±12

Tab. 2. Number of roots per cutting, shoot length, number of mature leaves, and length of laterals in pea cuttings treated at 10^{-3} *M* auxins by dipping. The readings were taken 13 days after treatment ± SE.

Conditions	Number of roots/cutting	Shoot length cm	Number of mature leaves	Length of the laterals, cm (average)
Control	12.0±1.0	29.6±2.5	7.3±0.6	0
IAA	12.2±1.4	26.8±3.0	6.3±0.7	0
4-Cl-IAA	13.2±2.2	24.7±1.0	5.6±0.3	4.0±1.2
4,6-Cl_2-IAA	0.7±0.2	21.3±1.2	3.9±0.2	3.4±0.4

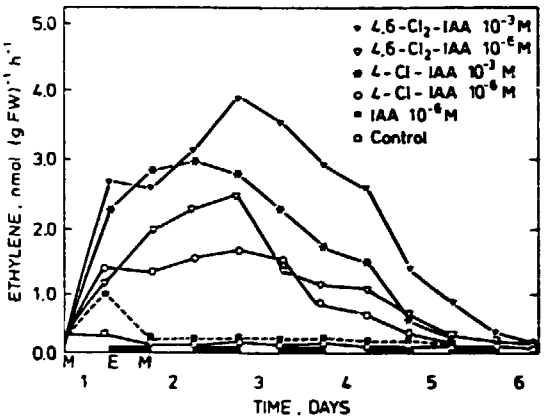

Fig. 1. Ethylene production induced in pea cuttings during the first six days after the treatment. The ethylene was trapped in the morning (M) and evening (E) each day.

g^{-1} h^{-1} on the sixth day. The ethylene formation at 10^{-6} *M* was only a little lower than production at 10^{-3} *M* and followed a similar 6-day time course, while the ethylene evolution after IAA treatment reached control levels within 48 h.

Discussion

Auxin activity (Böttger et al. 1978, Katekar and Geissler 1982, 1983; for a review, see Engvild 1985) of the naturally occurring 4-Cl-IAA is about 10 times stronger than IAA. The synthetic 4,6-Cl_2-IAA also has strong auxin effects. The strong auxin activity of 4-Cl-IAA and 4,6-Cl_2-IAA is further evident since they induced the production of very high quantities of ethylene (Fig. 1). The pattern of this induced ethylene production during the first six days after treatment is quite different from that noted in our earlier studies with IAA at the same concentration (Ahmad and Andersen 1986).

The high rate of ethylene production in the first few days of the rooting period induced by the chlorinated auxins compared with IAA at the same concentration reported (0.89 nmol g^{-1} h^{-1}; Ahmad and Andersen 1986) may be due to a higher degree of resistance to conjugation, as reported with IBA (Fernqvist 1966, Hess 1969) in addition to their high auxin activity.

High concentrations of auxins are known to bring about epinastic growth, twisting and bending of the apical stem which has been correlated with ethylene production; however, at a moderate level of ethylene induced by IAA, the plants were able to recover (Morgan and Hall 1964). In the present studies, treatment with 10^{-3} *M* of either of the two chlorinated auxins resulted in permanent epinasty, twisting and bending of the apical stem which may be caused by the very high and long-lasting ethylene evolution.

The loss of apical growth (Tab. 1) was also correlated with ethylene production (Fig. 1), where 4,6-Cl_2-IAA was more effective than 4-Cl-IAA. Ethylene has already been shown to cause complete inhibition of bud growth caused by auxins (Zimmermann and Wilcoxon 1935, Burg and Burg 1966).

Loss of apical dominance and the growth of the laterals from the lowermost bud (Tab. 1) was another effect induced by both chloroindoles and might be caused by the high ethylene production induced by these auxins. This is in agreement with Hall et al. (1957) who reported loss of apical dominance and outgrowth of laterals in cotton plants in the field exposed to ethylene as an air pollutant. In pea plants, Andersen (1976) found loss of apical dominance with the release of the laterals under irradiance or CO_2 after treatment with ethephon. The reason as proposed by Morgan et al. (1968) could again be reduced basipetal auxin transport in the young stems.

The naturally occurring 4-Cl-IAA was most effective in root promotion, producing twice as many roots per cutting as the control (Tab. 1). The other chlorinated (synthetic) auxin, 4,6-Cl_2-IAA was inhibitory to rooting. It is hard to explain why one of the chlorinated auxins stimulates rooting and the other inhibits. If we compare the induced ethylene production (Fig. 1) and rooting (Tab. 1), both the chlorinated auxins produce much more ethylene than IAA or IBA at similar concentrations. Therefore, it is likely that the ethylene induced by the auxins has no role to play in the rooting of the pea cuttings. This supports the findings of Kender et al. (1969) and Roy et al. (1972) but does not agree with the findings of Chadwick and Burg (1970), Krishnamoorthy

(1970), Mullins (1972) and Robbins et al. (1985) who found either promotion or inhibition.

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Appendix XIII

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Effects of chlorosubstituted indoleacetic acids on root growth, ethylene and ATP formation

Göran Stenlid and Kjeld C. Engvild

Stenlid, G. and Engvild, K. C. 1987. Effects of chlorosubstituted indoleacetic acids on root growth, ethylene and ATP formation. – Physiol. Plantarum 70: 109–113.

7-Chloroindoleacetic acid and dichloroindoleacetic acids with a Cl in the 7 position showed anti-auxinic activity and promoted root growth in wheat (*Triticum aestivum* L. cv. Diamant II). In contrast, 4-, 5- and 6-chloroindoleacetic acids acted as strong auxins inhibiting the growth of wheat roots. Flax (*Linum usitatissimum* L. cv. Concurrent) and cucumber (*Cucumis sativus* L. cv. Favör) roots showed similar, but less clear-cut responses. 7-Chloroindoleacetic acid and 4,7-dichloroindoleacetic acid alleviated root growth inhibition in wheat caused by IAA, monochloroindoleacetic acids and benzyladenine. 2,4-D. 4- and 6-chloroindoleacetic acids strongly induced ethylene formation in cucumber seedlings; 4,7- and 6,7-dichloroindoleacetic acids did not, except at high concentrations. The more lipid-soluble dichloroindoleacetic acids were stronger inhibitors of ATP formation in cucumber mitochondria than monochloroindoleacetic acids, while IAA itself had only a very slight effect.

Additional key words – antiauxin, auxin, 4-chloroindoleacetic acid, *Cucumis sativus*, *Linum usitatissimum*, *Triticum aestivum*.

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Introduction

Certain chlorosubstituted indole-3-acetic acids with auxinic activity occur in nature. 4-Chloroindoleacetic acid (4-Cl-IAA) and its methyl ester are very strong auxins found in pea and related species of the tribus Vicieae in Leguminosae (Gandar and Nitsch 1964, Marumo et al. 1968, Engvild et al. 1981). Recently 4-Cl-IAA was reported as a constituent also in seeds of *Pinus sylvestris* (Ernstsen and Sandberg 1986). 7-Chloroindoleacetic acid has been isolated from a medium on which *Pseudomonas aureofaciens* was cultivated, perhaps synthesized as an intermediate in the biosynthesis of the antibiotic pyrrolnitrin (Salcher and Lingens 1978).

Synthetic chloroindoleacetic acids were tested as auxins already in the 1950s when no one suspected that they were natural compounds (Hoffmann et al. 1952, Muir and Hansch 1953, Sell et al. 1953, Porter and Thimann 1965). Several of the monochlorosubstituted indoleacetic acids are highly active auxins, while most dichlorosubstituted indoleacetic acids are less active auxins or, perhaps, anti-auxins. 4-Cl-IAA and 6-Cl-IAA are e.g. more effective than IAA in stimulating coleoptile straight growth (Böttger et al. 1978) or pea stem elongation (Katekar and Geissler 1982, 1983). On the other hand 4,7-Cl_2-IAA and 5,7-Cl_2-IAA inhibit the IAA-induced growth of coleoptiles, indicating a possible antagonistic effect against auxins (Böttger et al. 1978).

The role of 4-Cl-IAA in the plant is not clear and little is known about its metabolism or the possibility that it can replace IAA. Due to the close structural similarity of the chlorosubstituted IAAs to IAA, some of them can be expected to act as true competitive antagonists to IAA. For a review of the chloroindoleacetic acids and their properties see Engvild 1985.

In the present paper the effects of chlorosubstituted IAAs on root growth are described. Some act as auxins, inhibiting root growth, while others stimulate root

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Tab. 1. Effect of chlorosubstituted indoleacetic acids on elongation of wheat roots. Values are presented as the percentage of the growth in the untreated controls.

Compound added	Concentration of compound, *M*				
	$3\cdot10^{-9}$	10^{-8}	$3\cdot10^{-8}$	10^{-7}	$3\cdot10^{-7}$
IAA	72	53	34	–	–
4-Cl-IAA	90	80	44	–	–
5-Cl-IAA	–	88	76	–	–
6-Cl-IAA	73	55	31	–	–
7-Cl-IAA	104±12	124±12	138±7	129±8	78
4,6-Cl_2-IAA	103	111	93	54	–
4,7-Cl_2-IAA	104	117±6	134±11	158±3	155±12
5,7-Cl_2-IAA	104	117±5	128±8	160±10	–
6,7-Cl_2-IAA	128±4	144±4	132±9	92±11	–

growth and counteract inhibitions caused by auxins. In addition, effects on ethylene liberation from seedlings and on ATP formation in mitochondria are reported.

Abbreviations – 2,4-D, 2,4-dichlorophenoxyacetic acid; -Cl-IAA, -chloroindoleacetic acid; BA, N^6-benzyladenine; iP, N^6-(2-isopentyl)adenine; 1-NMSP, α-(napht-1-ylmethylthio)-propionic acid.

Materials and methods

The root growth tests were made in the dark with seedlings of wheat (*Triticum aestivum* L. cv. Diamant II, spring wheat), flax (*Linum usitatissimum* L. cv. Concurrent) and cucumber (*Cucumis sativus* L. cv. Favör). At the start of the experiments, wheat and cucumber roots were 20–25 mm long, and flax roots 7 mm, and their increase in length during 20 h was determined. The growth of the control roots was 20–22 mm for wheat and cucumber and 17–20 mm for flax. In the experiments, three cork discs (floating on the test solutions) with 10 plants each were used for each treatment. The experiments were repeated at least once. Where data from more than two experiments are available the SE is given. For further details see Stenlid (1982).

Ethylene production was estimated as the amount of ethylene liberated from young cucumber seedlings during an 18-h-period as described earlier (Stenlid 1982). Ten control seedlings (root length at the start, 20–25 mm) liberated about 3 nmol ethylene during 18 h.

The conversion of ADP to ATP was determined with the firefly method as described by Stenlid (1970). The light emission was measured with a Wallac luminometer 1250. To each sample of 2 ml containing ca 0.5 mg (dry weight) mitochondria, 1 nmol ADP was added and the controls converted 70–80% of the ADP to ATP during 30 s.

All chloroindoleacetic acids were prepared by K. Engvild (see Engvild 1977). The cytokinins were obtained from Sigma Chem. Co.

Results

Root growth

The monosubstituted compounds 4-Cl-IAA, 5-Cl-IAA, and 6-Cl-IAA inhibited elongation of wheat and cucumber roots (Tabs 1 and 2). The activity was similar to (in wheat) or higher than (in cucumber) that of unsubstituted IAA, indicating auxine properties. Flax roots were also inhibited to a similar extent; 6-Cl-IAA was more active than IAA itself (data not given). These results agree with earlier data showing strong auxinic activity for these compounds (Porter and Thimann 1965, Böttger et al. 1978).

In contrast, 7-Cl-IAA stimulated root growth in wheat (Tab. 1), which might be due to antagonism

Tab. 2. Effect of chlorosubstituted indoleacetic acids on elongation of cucumber roots. Values presented as in Tab. 1.

Compound added	Concentration of compound, *M*					
	$3\cdot10^{-9}$	10^{-8}	$3\cdot10^{-8}$	10^{-7}	$3\cdot10^{-7}$	10^{-6}
IAA	–	–	78±10	51±7	33±6	–
4-Cl-IAA	79±6	46±5	28±8	11±2	–	–
5-Cl-IAA	–	–	55±9	–	–	–
6-Cl-IAA	92±8	67±4	33±7	14±3	–	–
7-Cl-IAA	–	103±5	101±6	94±6	61±8	12±3
4,7-Cl_2-IAA	–	103±6	113±6	119±4	88±8	44±11
6,7-Cl_2-IAA	–	108±6	116±6	95±13	57±15	36±7

Tab. 3. Effect of antagonistic chlorosubstituted indoleacetic acids on inhibition of root growth in wheat caused by auxins or cytokinins. Values are given as the percentage of the growth in the untreated controls.

Inhibitor added, *M*		Antagonistic Cl-IAA added	Conc. of antagonistic Cl-IAA, *M*			
			0	$3 \cdot 10^{-8}$	10^{-7}	$3 \cdot 10^{-7}$
IAA	$3 \cdot 10^{-8}$	7-Cl	34	74	92	–
4-Cl-IAA	$3 \cdot 10^{-8}$	"	43	–	100	–
6-Cl-IAA	$2 \cdot 10^{-8}$	"	42	–	79	–
6-Cl-IAA	$3 \cdot 10^{-8}$	"	33	–	55	–
2,4-D	$4 \cdot 10^{-7}$	"	54	65	67	–
BA	$3 \cdot 10^{-8}$	"	47	112	117	–
iP	10^{-7}	"	34	86	114	–
IAA	$3 \cdot 10^{-8}$	4,7-Cl_2	33	–	63	79
4-Cl-IAA	$3 \cdot 10^{-8}$	"	46	–	–	84
6-Cl-IAA	$2 \cdot 10^{-8}$	"	42	–	–	57
6-Cl-IAA	$3 \cdot 10^{-8}$	"	33	–	40	46
2,4-D	$4 \cdot 10^{-7}$	"	55	–	67	80
BA	$3 \cdot 10^{-8}$	"	43	47	84	129

against supra-optimal auxin. At higher concentrations, 7-Cl-IAA also was inhibitory.

If a Cl atom at the 4, 5 or 6 position gives an inhibitory auxin and at the 7 position a stimulatory compound, what happens with the dichloro-compounds? Table 1 shows that all three Cl_2-IAAs with Cl at the 7 position stimulated root growth in wheat, and especially 6,7-Cl_2-IAA was active at very low concentration. 4,6-Cl_2-IAA was inhibitory, but less so than the corresponding monochlorocompounds. As reported for antiauxins (see Åberg 1961), flax roots were not markedly stimulated, but the dichloro-IAAs were less inhibitory than the monochloro-compounds (data not given).

When the stimulatory compounds were tested together with auxins, a reversal of the auxin-induced inhibition of wheat root growth was observed (Tab. 3). In addition, also here the Cl_2-IAAs were more effective on wheat roots than on cucumber or flax roots (data not given).

Cytokinins applied from outside are potent inhibitors of root growth (Stenlid 1982), and some chlorosubstituted IAAs are even more antagonistic against cytokinins than against auxins (Tab. 3).

Ethylene formation

Auxins and cytokinins increase the rate of ethylene formation in seedlings. As ethylene inhibits root growth, the effect of the Cl-substituted IAAs was tested on ethylene liberation both alone and in the presence of the cytokinin benzyladenine (Tab. 4). Since wheat seedlings liberate only small amounts of ethylene (data not shown), cucumber seedlings were used. There was a clear difference in ethylene liberation between the seedlings treated with the auxinic acids 4-Cl-IAA and 6-Cl-IAA and those treated with the antagonistic acids 4,7-Cl_2-IAA and 6,7-Cl_2-IAA. The auxinic compounds were stimulatory at lower concentrations compared

Tab. 4. Effect of some chlorosubstituted indoleacetic acids on ethylene liberation from cucumber seedlings. Values are given as the percentage of the liberation in the controls (with or without benzyladenine). Ethylene liberation in controls with benzyladenine was ca 175% of that in controls without benzyladenine.

Compound added	BA, *M*	Concentration of compound, *M*			
		10^{-8}	$3 \cdot 10^{-8}$	10^{-7}	$3 \cdot 10^{-7}$
IAA	–	–	137±11	203±38	193±32
	$3 \cdot 10^{-7}$	–	141±18	182±32	162±14
4-Cl-IAA	–	126±12	179±24	211±21	180±28
	$3 \cdot 10^{-7}$	126±15	171±18	251±31	341±101
6-Cl-IAA	–	–	174±25	176±15	264±21
	$3 \cdot 10^{-7}$	–	222±49	–	244±48
4,7-Cl_2-IAA	–	90±7	116±11	98±11	118±19
	$3 \cdot 10^{-7}$	99±6	93±8	91±7	123±18
6,7-Cl_2-IAA	–	100±9	100±4	109±6	140±13
	$3 \cdot 10^{-7}$	101±5	98±8	112±12	143±10

Tab. 5. Effect of IAA, 2,4-D and chlorosubstituted indoleacetic acids on ATP formation in cucumber mitochondria. The results are given as percentage of control values.

Compound added	Concentration of compound, *M*	
	10^{-4}	$3 \cdot 10^{-4}$
IAA	96±4.4	95±3.3
4-Cl-IAA	92±3.5	66±2.6
5-Cl-IAA	89±2.6	79±2.0
6-Cl-IAA	78±4.5	64±1.9
7-Cl-IAA	83±0.9	60±2.6
4,6-Cl_2-IAA	75±3.9	48±4.7
4,7-Cl_2-IAA	78±3.0	61±4.3
5,7-Cl_2-IAA	77±3.6	56±2.8
6,7-Cl_2-IAA	57±5.0	35±2.6
2,4-D	-	76±3.9

with the antagonistic compounds. Antiauxins such as 1-NMSP inhibit ethylene liberation (Stenlid 1982), but no such effects were obtained with the dichloro-substituted IAAs, probably because the latter also have auxinic properties in contrast to 1-NMSP.

ATP formation

The formation of ATP by cucumber mitochondria was inhibited by the substituted IAAs (Tab. 5), but high concentrations (ca 10^{-4} *M*) were necessary. The dichloro-substituted compounds were more inhibitory than the monochloro substances and IAA itself produced no distinct effect. On the whole, the antagonistic compounds were more active than the auxinic ones. For other types of compounds some sort of connection between uncoupling and antiauxinic properties has also been observed (Stenlid 1963).

Discussion

The chlorosubstituted indoleacetic acids may interfere in various ways with IAA and other growth regulators. The monochloro-IAAs have auxinic properties similar to those of IAA; however, since their degradation, conjugation, and sequestration properties certainly differ from those of IAA, and since their side effects also differ, their behaviour in the plant is probably also different. In any case it seems plausible that the inhibitions caused by 4-Cl-IAA, 5-Cl-IAA and 6-Cl-IAA (Tab. 1 and 2) are of auxinic type. 7-Cl-IAA inhibited root growth only at much higher concentrations than the other monochloro-IAAs and in lower concentrations it stimulated root growth of wheat. Data presented by Porter and Thimann (1965), Böttger et al. (1978) and Katekar and Geissler (1982) also indicate that 7-Cl-IAA is a weaker auxin than the other monochloro acids. The dichloro acids 4,7-Cl_2-IAA, 5,7-Cl_2-IAA and 6,7-Cl_2-IAA stimulate root growth of wheat strongly (Tab. 1) and a Cl at position 7 is thus a structural characteristic for this activity.

The nature of the stimulatory effect is more uncertain than that of the inhibitions, but an antagonism against auxins seems possible. The reversal of inhibitions caused by auxins in the external solution (Tab. 3) gives further support to this view.

The terms antiauxin and auxin antagonist are ambiguous (see McGlasson et al. 1978 p. 452) and they have been used for a diversity of substances of various chemical structure that counteract auxins in one way or other. In this investigation, the classification as auxin antagonist or antiauxin is based on stimulations of root growth and reversal of inhibition of root growth caused by auxins. The effect of added auxins can be due to inhibition of the uptake of auxins, but stimulation of roots without addition of auxins shows that there is an effect in the root itself. The substances may of course also cause stimulations independent of the auxins. Further careful quantitative studies of the interaction with various concentrations of IAA and other auxins are necessary to decide if there is a real competition with auxins.

The chlorosubstituted IAAs are more effective reversing agents against IAA than against 2,4-D (Tab. 3), which is to be expected if the antagonism is due to structural resemblance. In contrast, most other substances are more efficient against 2,4-D (see e.g. Åberg 1961, Stenlid 1963). As observed for other antiauxins, the stimulatory effects on wheat roots are stronger than those on flax roots (see Åberg 1978 for data on other indole derivatives). Åberg reported antiauxinic activities for several indole derivatives, and especially strong effects were obtained with β-(indolyl-3)-ethylselenoacetic acid.

7-Cl-IAA and 4,7-Cl_2-IAA were more active against unsubstituted IAA and 4-Cl-IAA than against 6-Cl-IAA (Tab. 3). Until further data on combination experiments are available with other Cl-IAAs, it is difficult to explain these results.

Some experiments with oat coleoptile sections (data not given) confirm the results of Böttger et al. (1978). All chlorosubstituted IAAs tested stimulated elongation, but higher concentrations of 4,7-Cl_2-IAA, 5,7-Cl_2-IAA and 6,7-Cl_2-IAA rather than 4-Cl-IAA, 5-Cl-IAA and 6-Cl-IAA were necessary. Moreover, low concentrations of these dichloro acids slightly inhibited coleoptile elongation. The available data on coleoptile elongation thus agree with the results from the experiments with roots.

The ability of the chlorosubstituted IAAs to effectively reverse cytokinin-induced inhibition of root growth (Tab. 3) is especially interesting. The chlorosubstituted IAAs can be supposed to act mainly as structural antagonists against IAA; thus it is puzzling that they show pronounced activity against cytokinins, surpassing even that against auxins. One possible explanation would be that IAA is necessary for the inhibitory action of cytokinins; as a consequence, the antagonism against auxin would also reverse the inhibition caused by cytokinins. Similar effects have been obtained with

other substances acting against auxins (Stenlid 1982). A key reaction could be the production of ethylene, which is stimulated both by auxins and by cytokinins, often in a synergistic way. The experiments reported here (Tab. 4) do not support this view, even if the two types of chloro-IAAs differ in their effects on ethylene production. Ahmad et al. (1985) report that 4-Cl-IAA and 4,6-Cl$_2$-IAA strongly increased ethylene production in pea cuttings, but they found no correlation to root formation.

The effect on ATP formation in mitochondria (Tab. 5) seems to be correlated with lipophilicity, the dichloro-compounds being the most lipophilic and the unsubstituted IAA the least lipophilic (Katekar and Geissler 1982). This agrees with results obtained with flavonoids where the aglycones were more inhibitory than the less lipophilic glycosides (Stenlid 1970). The effects on ATP formation show that side effects may be important when discussing the physiological importance of both the natural 4-Cl-IAA and other indole derivatives. Another noteworthy reaction is oxidation by IAA oxidase, which can influence their stability.

The close structural similarity between IAA and the chloroindoleacetic acids gives the Cl-IAAs a special position among the auxin antagonists. The possibility to vary the substitution pattern provides interesting tools for the study of auxinic and antiauxinic properties.

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The Chloroindole Auxins of Pea, Strong Plant Growth Hormones or Endogenous Herbicides?

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121	7	8	144

Abstract (Max. 2000 characters)

In this work the three theses below are discussed:

1) Identification and quantitative determination of the very strong plant hormone, the auxin 4-chloroindole-3-acetic acid methyl ester, in immature seeds of *Pisum, Vicia, Lathyrus,* and *Lens* spp. by incorporation of radioactive ^{36}Cl, thin layer chromatography, autoradiography, colour reactions, and gas chromatography/ mass spectrometry.
2) The strong biological activity of 4-chloroindole-3-acetic acid and its analogues and its ability to induce strong, almost irreversible of ethylene evolution.
3) The possible role of chloroindole auxin in plants, particularly if it might be the hypothetical death hormone, secreted from developing seeds, which induces senescence and kills the mother plant at maturity; if plants generally have several auxin types, growth promoters and endogenous herbicides; and if other chlorine-containing plant hormones occur in developing seeds of other crop species.

Descriptors INIS/EDB

AUTORADIOGRAPHY; AUXINS; CHLORINE 36; ETHYLENE; HERBICIDES; INDOLES; INHIBITION; LABELLED COMPOUNDS; MASS SPECTROSCOPY; PISUM; QUANTITATIVE CHEMICAL ANALYSIS; SEEDS; THIN-LAYER CHROMOTOGRAPHY; VICIA

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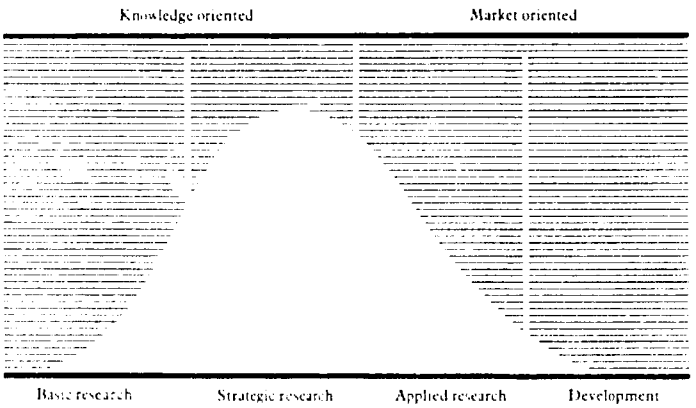

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