Aq2; a highly water-soluble plant growth regulator from the pollen of *Pinus radiata*.

A thesis presented for the degree of
Master of Science in Chemistry
at Massey University

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March 1974

ABSTRACT

Aq2; a highly water-soluble plant growth regulator from the pollen of *Pinus radiata*

Aq2 was detected in crude aqueous extracts from the pollen of *Pinus radiata* by Sweet and Lewis (1971). These workers noted Aq2 possessed some properties of both gibberellins and cytokinin-like compounds and was probably involved in the regulation of pollen tube growth. A further study was undertaken by Gallagher and Aldersley (1972) and these workers concluded after a preliminary investigation that Aq2 was most probably a cytokinin.

The purpose of this thesis was to further investigate the nature of Aq2. The physiological role and chemical composition of pollen, with special reference to *P. radiata* was studied, and a review of plant growth regulators carried out with a view to classifying Aq2 into one of the four groups. A survey of possible isolation techniques was also made.

Anion and cation exchange columns were run at various pH's and a portion of the activity attributable to Aq2 was found to bind to an anion column at pH 8.5. The remainder passed straight through the column. An aqueous alcohol treatment has been employed to remove some of the excess carbohydrate material, and freeze drying was shown not to affect the activity of Aq2.

Chemical evidence tends to mitigate against Aq2 being a gibberellin; however, the possibility that Aq2 is a cytokinin has not yet been ruled out. No additional physiological studies have been carried out since those of Sweet and Lewis (1971); however, good responses are still obtained in the radish cotyledon assay for cytokinins.

If Aq2 is indeed a cytokinin it does not appear to resemble any of those known to date.

errata

Page 15. Figure 1, gluocbrassicin,
Should read glucobrassicin.

ACKNOWLEDGEMENTS

Dr. R.T. Gallagher for his guidance and assistance; Mrs. J. Davis for her help with the large scale extractions and bioassays; Dr. E. Wong and Dr. L.N. Nixion for running the combined GLC-MS examination of the gibberellin derivatives; Professor R. Hodges for his help in interpreting the above spectra; Miss E.O. Campbell for kindly assisting with the account of the life cycle of Pinus radiata; Mrs. V. Tam for the drawings appearing in the same section; to various people who have given advice, encouragement and assistance during the course of this thesis. Mrs. S.E. Brennen and Mr. N.G. Williams for proof reading; Mrs. G. Percy for her typing; and last but not least my wife who has made this possible by her help and support.

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INTRODUCTION

A study of plant growth regulators occurring in the pollen of Pinus radiata D. Don was recently carried out by Sweet and Lewis (1971). In this investigation, both a qualitative and quantitative comparison between the levels occurring in germinated and ungerminated pollen was carried out, and thirteen growth regulating compounds were detected. These included five auxins, three inhibitors, four gibberellins and one compound with some properties characteristic of both gibberellins and cytokinins.

This latter substance was designated as Aq2 by Sweet and Lewis (1971), as it remained in the aqueous phase after extraction at pH 8.5 and pH 2.7 with diethyl ether and was the second growth regulating substance to be found in this fraction.

Aq2 was first detected in the radish cotyledon expansion assay used primarily for the detection of cytokinins; responses were also obtained in the barley endosperm assay and in the Rumex assay for gibberellins. No response was obtained in the carrot bioassay or the Spirodela bioassays for cytokinins. On the other hand, Aq2 appeared to parallel kinetin in its effect on pollen tube growth. Thus circumstantial evidence seemed to indicate Aq2 was a cytokinin. Sweet and Lewis found evidence to suggest Aq2 was involved in the modulation of pollen tube growth. Further work was undertaken by Gallagher and Aldersley (1972).

Initially Gallagher and Aldersley's work encountered a troublesome toxicity problem. Having solved this, these workers developed a large scale extraction procedure based upon mechanical disintegration of pollen grains in water.

Plant growth regulators are typically present in plants in extremely low concentrations. Thus the isolation and characterisation of such compounds usually involves large quantities of plant extract. For example, the best reported yield obtained for a cytokinin was by Letham (1966a), where 0.7 mg of zeatin was obtained from 60 Kg of maize kernels.

Preliminary results of Gallagher and Aldersley (1972) indicated that Aq2 was a low molecular weight, highly polar, water-soluble compound. Bearing in mind the biological activity

attributed to Aq2, Gallagher and Aldersley decided that it was possible that Aq2 was a cytokinin nucleotide or a "bound" form (e.g. glycoside) of a cytokinin. However, further investigation by these workers mitigated against the possible nucleotide nature of Aq2. Thus, it is known that nucleotides can be absorbed onto charcoal and eluted with aqueous base; attempts to absorb Aq2 onto activated charcoal were unsuccessful, whereas under the same conditions, the nucleotide, AMP absorbed qu ntitatively onto the charcoal. Again, ion exchange chromatography has been widely used in the purification of cytokinins; nucleotides binding strongly to anion exchange columns under neutral conditions. Attempts to purify Aq2 by anion exchange chromatography (Dowex-1) proved unsuccessful; no activity could be detected in the column eluates, however, a toxic effect in the radish cotyledon assay was obtained from the initial column effluent. From these latter results the possibility could not be excluded that a strong synergism existed in the partly purified extract.

It was the purpose of this thesis to investigate further, aspects of purification, isolation and characterisation of Aq2.

POLLEN

As the hormone Aq2 investigated in this thesis was extracted from pollen, it is clearly pertinent to consider the nature of pollen grains and their general chemical composition. However, before doing this, it was thought worthwhile to briefly consider the biological role of pollen, especially since it has been proposed (Sweet and Lewis, 1971) that the physiological role of the hormone appears to be the modulation of pollen tube growth.

A great deal of research has been carried out on pollens; their morphology and physiology being well studied. In addition to research of a purely botanical nature, pollen has been of interest in the studies of nutrition, and allergies (Nielsen et al., 1955; Kapp, 1969; Barbier, 1970; Heslop-Harrison, 1971; Wodehouse, 1965).

Pollen Function

Pollen is derived from the seed bearing plants which can be divided into two well defined groups: gymnosperms and angiosperms. The gymnosperms are woody perennial plants and while there are relatively few species, these are of great abundance and of economic importance as sources of timber.

The reproductive cycle of Pinus radiata D. Don (a gymnosperm) is illustrated in Figure 1.

Pinus produces both male and female cones. The male cones are produced in autumn, in groups, and fall from the tree at the end of the following spring, having remained fairly small in size.. The cone contains many microsporophylls arranged spirally about the long axis. Each microsporophyll contains two pollen sacs on the under side, see Figure 2. Pollen, which is partly germinated microspore, is released by these sacs splitting longitudinally during spring. The pollen from Pinus is wind borne and released in very large quantities.

The female cones grow considerably larger than the male cones and occur singly. Scales are arranged spirally about the long axis of the cone. Each scale has two ovules on the upper side. The ovule has archegonia embedded in the endosperm. The latter is completely surrounded by the nucellus which in turn is

LIFE CYCLE OF PINUS

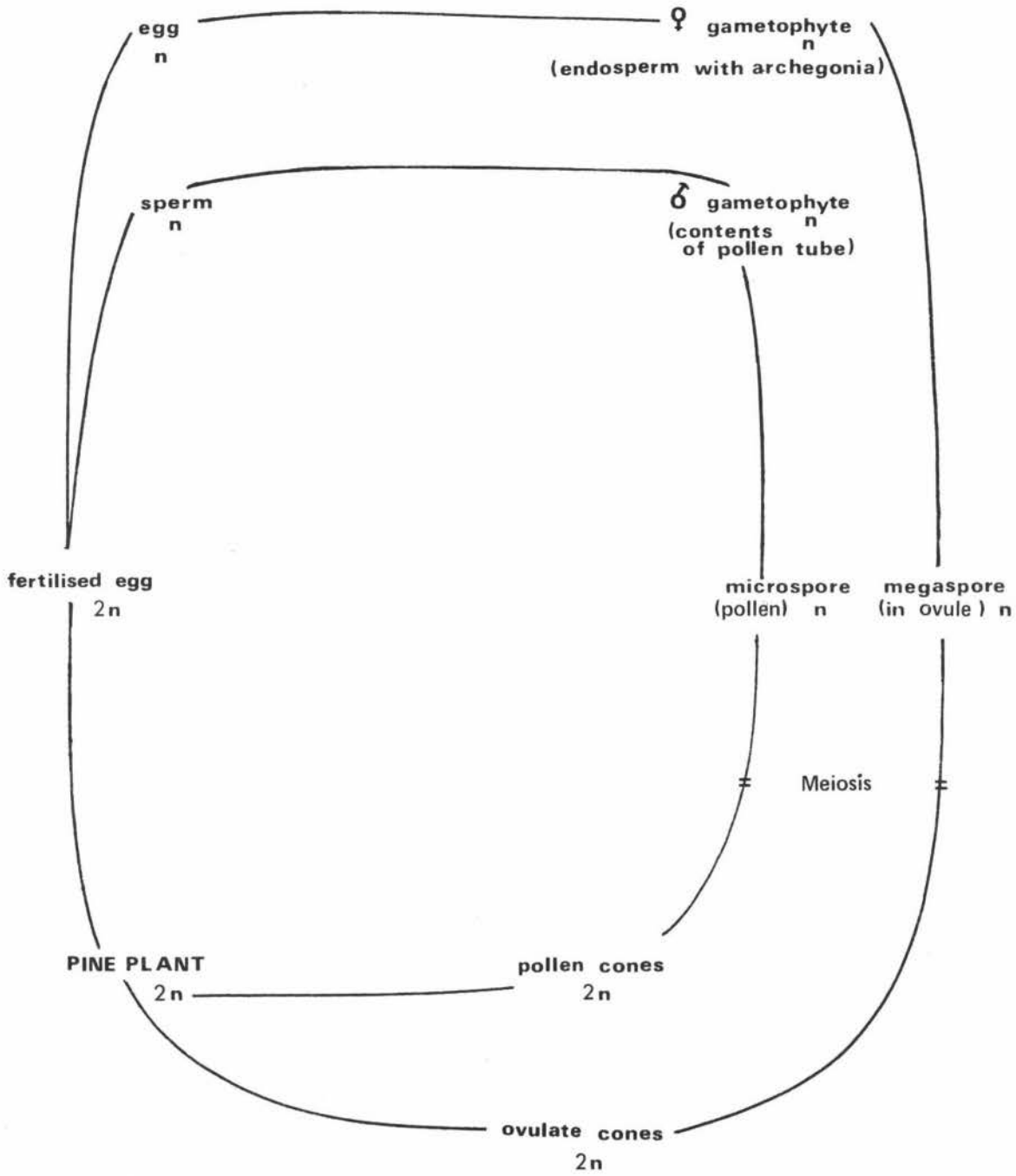
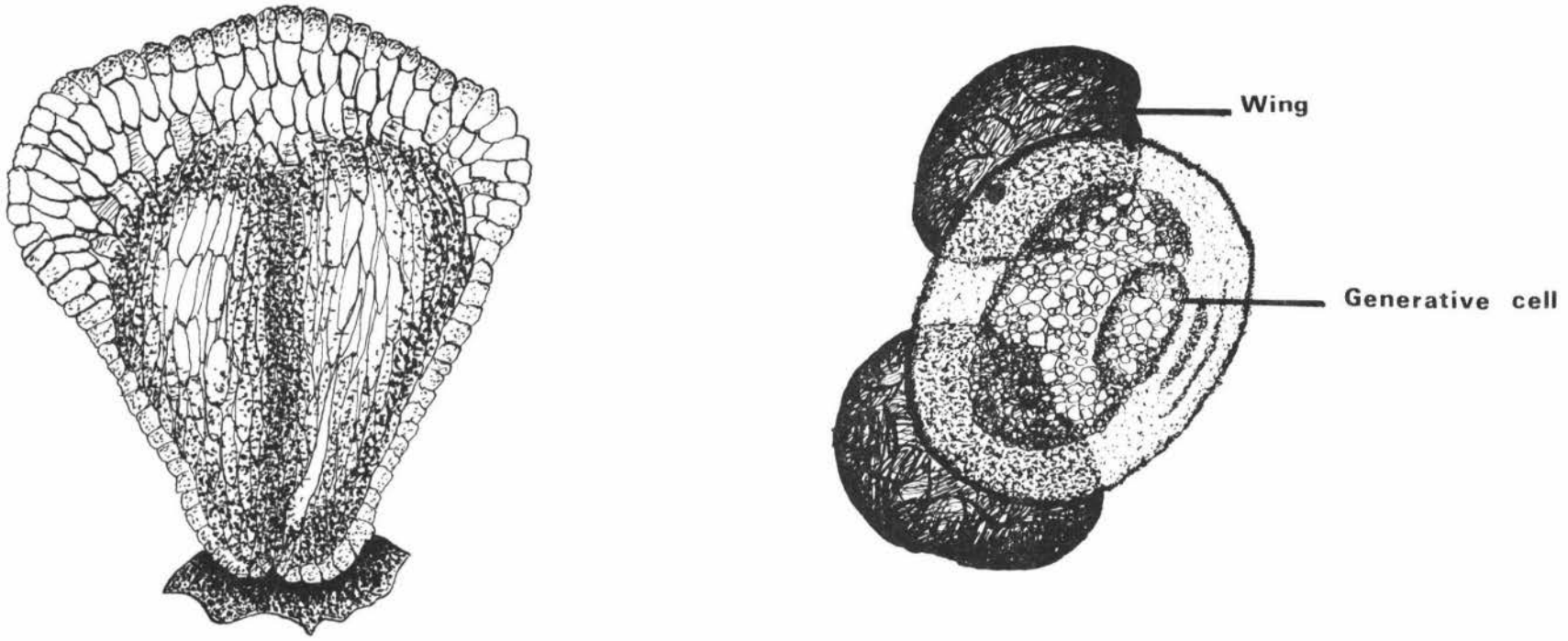


Figure 1



ENLARGED DRAWINGS OF A MICROSPOROPHYLL SEEN FROM THE LOWER SIDE, SHOWING THE POLLEN SACS, AND A MATURE POLLEN GRAIN

Figure 2

surrounded by the integument except at the micropyle (the point of access for the pollen grain), see Figure 3. Each archegonium has one ovum (egg).

At pollination, the scales of the female ovulate cone are slightly separated. Pollen settles between these and comes to rest close to the micropyle of the ovule. It is subsequently drawn in through this opening and comes into contact with the nucellus. The pollen grains now form a short pollen tube which starts to grow down through the nucellus. It remains dormant during the winter and recommences growth in the following spring, at which time the male gametophyte completes its development. Simultaneously, in the ovule the female gametophyte (endosperm and archegonia) completes its development. When the pollen tube reaches and grows into the egg fertilisation occurs by the fusion of a sperm, from the pollen tube, with the egg. Subsequently the fertilised egg develops into an embryo and the whole ovule forms a seed. The scales separate when the seed is mature and permit it to fall from the cone.(1)

Pollen Structure

Pollen grains vary in size from about 5 μ to 250 μ (Shaw, 1970). Many wind pollinated forms have walls modified to enhance bouyancy, while insect pollinated forms, the presence of surface rods, spines, and other sculptured features, serve to improve dissemination (Kapp, 1969).

The pollen grains of the genus Pinus are characterised by the possession of two large, conspicuous, air-filled bladders.(2) The grains are rather large, ranging in the different species from about 45 μ to 65 μ in diameter, the bladders measuring about two-thirds the diameter of the body of the grain (Wodehouse, 1965).

The wall of the pollen grain is composed of two basic layers: the intine and the exine. The intine is the cell wall, which immediately surrounds the living protoplasm. It is partly cellulose, but appears to have a different chemical composition from most cell walls, with higher proportions of pectic substances, callose, and other polysaccharides (Kapp, 1969). In some pollens,

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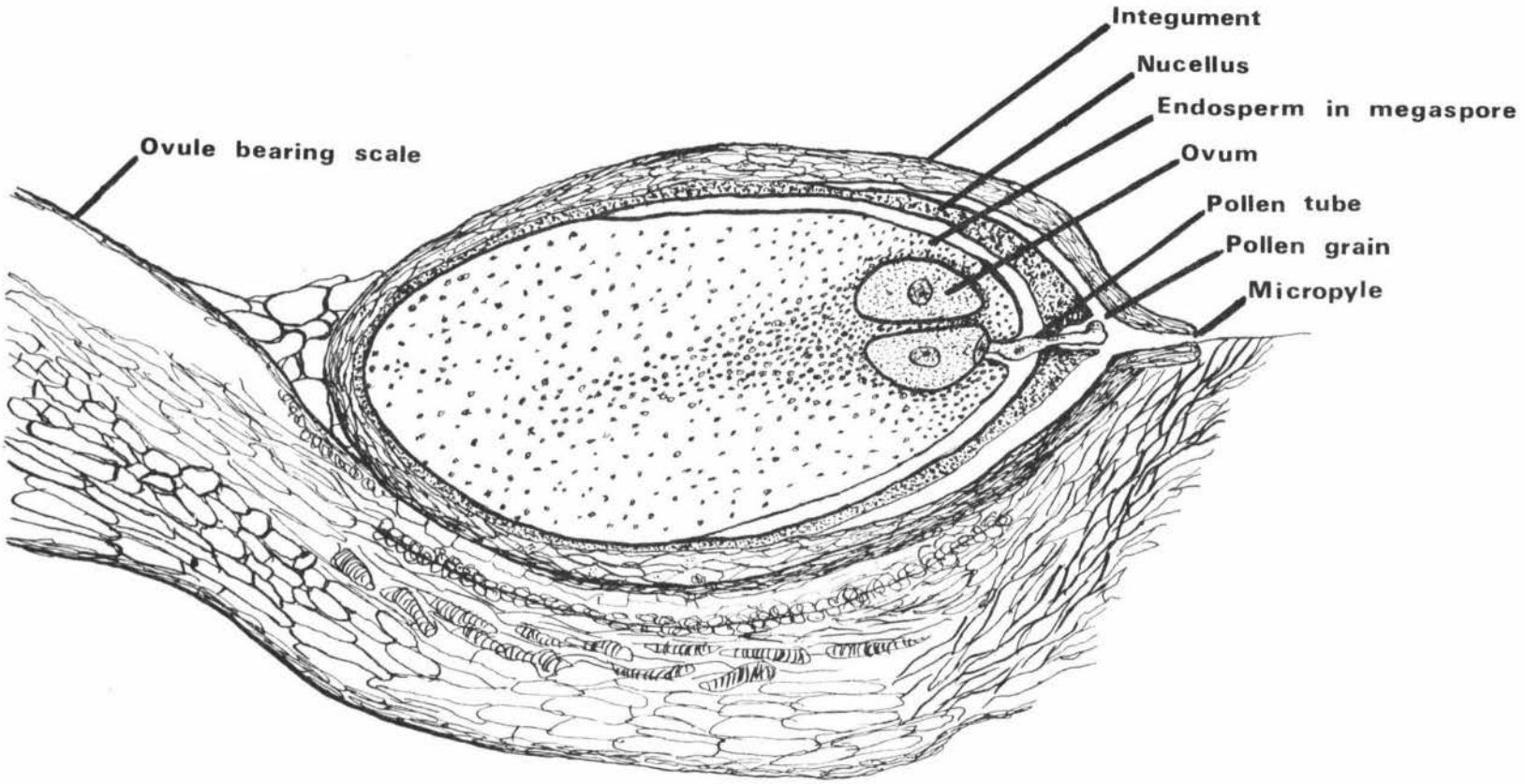
(1) Priestley et.al. (1964)

(2) To enhance buoyancy.



AN ENLARGED SECTION OF AN OVULE FROM A SECOND-YEAR *Pinus* CONE

Figure 3

the cellulose layer is embedded with layers of protein (Heslop-Harrison, 1971).

The resistant outer wall layer, the exine,[3] of pollen grains, is a chemically resistant layer which has the function of the protection of the organism from injury by external agencies, such as excessive dessication, destruction by light, and mechanical injury. This wall invariably possesses pores and these appear to allow a certain degree of diffusion of water (Kapp, 1969; Wodehouse, 1965) and a diffusion of enzymes and other soluble material (Stanley and Search, 1971).

The Chemical Composition of Pollen

A number of reviews have been published on the chemical composition of pollen (e.g. Stanley, 1971; Barbier, 1970; Neilsen et al., 1955).

At dehiscence time grass pollen, including Zea mays, has a water content above 50%; other pollens, e.g. Pinus, usually contain about 20% or less water at time of shedding.

Some components, such as carbohydrates, vary more between and within species than other chemical constituents. The carbohydrate content of grass pollen (corn) may be more than twice that of other angiosperm pollens; carbohydrates in pine and most other gymnosperms are considerably lower. Typical results of analyses are shown in Table 1 (reproduced from Heslop-Harrison loc. cit.), Table 2 (reproduced from Nielsen et al., loc. cit.), and Table 3 (reproduced from Barbier loc. cit.)

It can be seen that proteins vary widely with species, usually accounting for 10-30% of the pollen dry weight. Lipid contents average 1.5-4% and may be higher in those with oils on the grain surface. Ash content is usually about 2-4%, but may be as high as 7% in some species.

Variation in chemical constituents of pollen is accounted for by:

(1) species differences, and

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(3) The exine layer of the pollen wall is almost completely composed of sporopollenins - a diverse group of biopolymers with similar empirical formulae (e.g. $C_{90}H_{144}O_{20}$ Pinus sylvestris). A source of suitable monomers has been suggested by Brooks and Shaw (1971).

Table 1

Gross Chemical Analysis of Pollen

Species	% dry weight Ash	Carbohydrates	Protein	Lipid	Reference
Zea mays	2.55	36.59	20.32	3.67	(2)
Zea mays	3.46	34.26	28.30	1.48	(3)
Typha latifolia	3.70	17.78	18.90	1,16	(4)
Pinus sabiniana	2.59	13.15	11.36	2.73	(2)
Pinus radiata	2.35	13.92	13,45	1.80	(2)

Table 2

	Zea mays 1953	Zea mays 1954	Alnus glutinosa	Alnus incana	Pinus montana
N %	4.1	4.2	4.1	4.2	2.2
Protein % (Nx6.25)	25.6	26.3	25.6	26.2	13.8
Sulphate ash %	4.9	4.9	2.4	2.8	3.0
P %	0.58	0.75	0.42	0.28	0.30
S %	0.43	0.30	0.24	0.32	0.18
Reducing sugars (as glucose) %	10.3	7.3	8.4	5.7	2.7
Total carbohydrates (as glucose) %	35.1	34.6	27.4	22.5	29.5
Water-soluble substances %	35.9	49.7	41.2	33.3	31.9
Ether-soluble substances %	5.0	1.8	9.4	13.2	7.1

Table 3

The Percentage Composition of 6 Hand-collected Pollens and 18 Bee-gathered Pollens (Todd and Bretherick)

	Proteins	Ether extract	Sugars	Water	Ashes	Indeterminate
Hand-collected pollens						
Pinus sabiniana	11.36	2.73	13.15	14.8	2.59	56.09
Pinus radiata	13.45	1.80	13.92	11.25	2.35	57.23
Typha latifolia	18.83	1.28	31.93	6.43	3.82	37.71
Mais	20.32	3.67	36.59	5.53	2.55	31.31
Juglans nigra	23.15	17.55	13.72	3.91	3.07	39.60
Phoenix dactylifera	35.50	3.08	1.20	17.14	6.36	36.73
Bee-gathered pollens						
Pinus contorta	7.02	2.04	48.35	7.01	1.32	34.26
Taraxacum vulgare	11.12	14.44	34.93	10.96	0.91	27.64
Salix sp. I	15.38	5.25	41.92	13.61	2.19	21.65
Salix nigra	22.33	4.15	33.18	12.30	2.61	26.43

(2) environmental differences during maturation and after dehiscence.

During maturation excessively high temperatures tend to reduce the pollen carbohydrate; low light also results in less carbohydrate accumulating in mature pollen, probably as a direct effect of reduced photosynthesis. If the plant nutrient supply, particularly microelements, is below optimum, pollen mineral content may be reduced, modifying the protein-enzyme levels.

Carbohydrates

Simple sugars are the principal metabolic substrates used by germinating pollen. The total sugar composition for P. radiata is given in Table 3 as 13.92%. While other soluble sugars occur in most pollens a high percentage of the free sugar in pine pollen is sucrose. Thus soluble carbohydrates can be seen to significantly contribute to the solids content of aqueous extracts of pollens. The separation of the hormone Aq2 from this carbohydrate material has been a major difficulty in this study.

Proteins and Enzymes

Again from Table 3 it is seen that a significant amount of proteinaceous material would occur in the aqueous extract. While no detailed information on the enzyme content of P. radiata is available, Brewbaker (1971) has published a list of enzymes known to occur in the pollens of higher plants. In all, thirty-nine are listed consisting of dehydrogenases, oxidases, transferases, hydrolases, lyases, and ligases. Knox (1971) reports the localised presence of certain enzymes such as acid phosphatase, ribonuclease, esterase, amylase, and protease in the pollen wall of many species. Stanley and Search (1971) report on protein and enzyme constituents which rapidly diffuse from germinating pollen. Pinus species were noted to undergo a smaller loss of weight when eluted with water than many other species. The rapidity and ease of loss of these proteins and enzymes suggest that some enzymes are surface localised or very near the surface of exine (Stanley and Search, 1971).

While the pollen used in this investigation for this thesis study was ungerminated it is likely that readily diffusable substances such as those above may be leached out by water washing. Indeed, Sweet and Gallagher (1972) proposed that such material was the cause of troublesome toxic effects experienced

in early extraction work.

Organic Acids

All the Krebs cycle acids have been found in pollen, but quantities vary markedly with the stage of development and handling. Phenolic acids such as p-hydroxybenzoic, p-coumaric and vanillic can be extracted and their levels can be measured. Fatty acids are also common in pollen. Large quantities of certain fatty acids, particularly palmitic, linoleic and linolenic acids exist in many pollens. Pine and other gymnosperm pollens are very high in linolenic acid. The other major fatty acids in Pinus are oleic, palmitic and stearic acid. The major portion of fatty acids exists in pollen as esters combined with sugars, phosphates or other constituents which have not yet been studied in any great detail (Stanley, 1971). Phospholipids from P. ponderosa were examined by McIlwain and Ballou (1966). Phosphatidyl-choline, phosphatidyl-ethanolamine, phosphatidyl-glycerol, phosphatidyl-myoinositol, phosphatidylserine and biphosphatidylglycerol were identified. The major fatty acids of each were palmitic, oleic and linoleic.

Callose and Sporopollenin

Callose is a β -1,3-glucose polymer present in high levels in pollen at maturity. This is a fairly common carbohydrate in plants, however its exact role is not known. The low water solubility of such polymers (compare cellulose) indicates that they are not likely to be present to any significant extent in aqueous pollen extracts.

The chemical composition of sporopollenins and possible precursors for synthesis are now known (Brooks and Shaw, 1971). In addition, the degradation products have been well characterised, giving mainly dicarboxylic acids and aromatic hydroxy acids. As significant degradation is not likely to occur under the mild extraction procedure used in the present investigation, the sporopollenins will not be further considered in this discussion.

Pigments

The chemistry of pollen pigments has been relatively well investigated, but their physiological role is not well understood. Pollens are predominantly yellow in colour and this is due to the presence of carotenoids or flavonoids. However, free carotenes

or their derivatives have never been isolated from pine pollen and thus whilst it is possible that carotenes exist in these pollens in some highly modified form, the visible pigments in the exine are primarily flavonoids. Flavenoids frequently occur as glucosides. Some pigments, in particular flavones, are water soluble and readily diffuse from pollen into water.

Hormones and Plant Growth Regulators

Many pollens have been assayed for their nutritive value, especially pollens which are bee-gathered. Stanley (1971), provides data on the vitamin content of Pinus montana pollen. Four B group vitamins, ascorbic acid, and biotin have been found. In addition, steroids similar in structure to some animal hormones have been found. The function of these is as yet unknown.

Indole acetic acid, auxins, inhibitors, and gibberellins have been detected in pollens. Sweet and Lewis (1971) carried out a comprehensive hormone assay analysis of P. radiata pollen. In a series of papers Mitchell et al. (1970, 1971a, 1971b, 1972) claim the isolation of a new class of hormones, "Brassins", from pollen of rape (Brassica napus Goertn). These hormones were isolated from ether extracts and were of a lipid nature. However, in a recent paper Milborrow and Pryce (1973) have made major criticisms of the work of Mitchell et al. Milborrow and Pryce maintain that as the brassins were not assayed using any of the "reasonably specific bioassays for auxins (oat coleoptile elongation), gibberellins (growth stimulation of dwarf maize or amylase synthesis in barley aleurone), or cytokinins (tobacco pith callus growth), therefore a contribution of these hormones to the activity of the brassin fraction has not been excluded". These authors also contest much of the other data presented by Mitchell et al.

Mineral content

The predominant elements found in pollens are potassium, phosphorus, calcium and magnesium. Stanley (1971) presents data for the mineral content of Pinus sabiniana and P. radiata. These have an ash content of 2.59 and 2.35 per cent of their dry weight. Aluminium, copper, iron, manganese, nickle, titanium and zinc occur in trace amounts. Estimations of chloride and boron are difficult as these may volatilise during ashing.

PLANT GROWTH REGULATORS

These may be divided into naturally occurring and synthetic regulators. The term hormone has been reserved for the naturally occurring growth promoting substances. These have traditionally been divided into three groups: auxins, gibberellins, and cytokinins; depending upon chemical structure and physiological effects. Inhibitors comprise a separate class of growth regulators.

Auxins

This group of hormones is characterised by their ability to induce elongation in shoot cells. Auxins have traditionally been assayed for by the 'Avena' curvature test developed by Went (1926). The isolation of the first chemically pure growth promoting substances, auxin a, auxin b, and indole-3-acetic acid (IAA), see Figure 1, was carried out by Kagl et al., 1934. These substances were not at first thought to be naturally occurring in higher plants, however IAA has since been shown to be very widespread. Shortly after its isolation, IAA was synthesised and was thus readily available for study.

In low concentrations IAA was found to be growth promoting, while at higher concentrations it was found to inhibit growth. Following the synthesis of IAA a search for similar, more potent compounds was undertaken. As a consequence the herbicide 2,4-D (2,4-dichlorophenoxyacetic acid) and the selective herbicide[1] MCPA (4-chloro-2-methylphenoxyacetic acid, methoxane) were developed in the mid-forties. A wide range of naturally occurring and synthetic auxins are now known. Many of the synthetic auxins are of great importance in horticulture and agriculture as herbicides and weedicides.

Since Went developed the 'Avena' curvature test for auxins several other bioassays for auxins have been developed, some more sensitive. Possibly the most sensitive is the Avena first internode test which is capable of detecting auxin at between 0.34 μg/L. and 1 μg/L, (Weaver, 1972). In addition, this test is sensitive to inhibitors and gibberellins.

There are several chemical tests which may be used to detect

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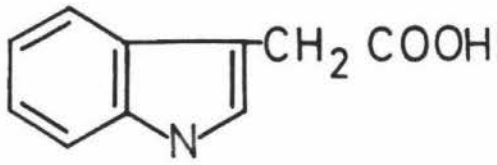
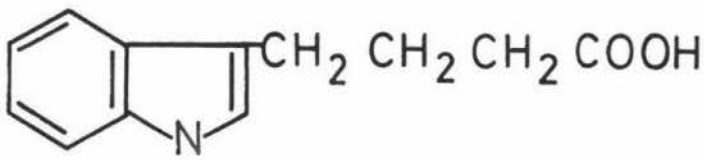
(1) Selective for broad leaf weeds - dicotyledons

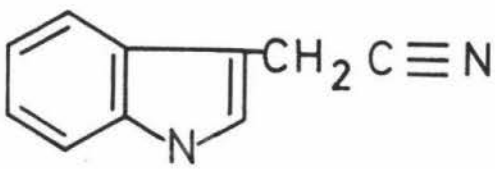
Figure 1 gives the structures of some of the known auxins.



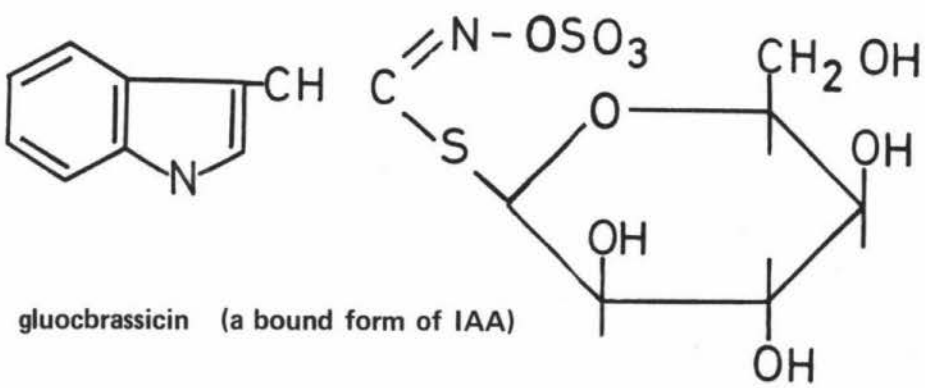
indole–3–acetic acid (IAA)



indole–3–butyric acid (IBA)



indole–3–acetonitrile (IAN)



gluocbrassicin (a bound form of IAA)

Figure 1

auxins (Weaver, 1972); however these are generally less sensitive than the biological assays. In addition, quantitative estimations can be made on purified extracts only, as there is the possibility of other plant material causing interference.

Gibberellins

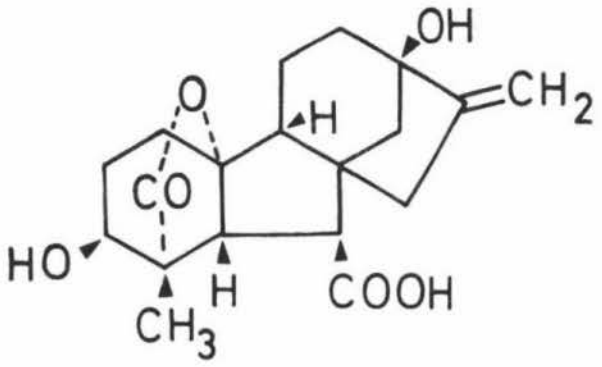
This group of hormones can be defined as having a gibbane skeleton and the ability of modifying the growth of dwarf mutants of maize and peas to that of normal plants. Physiologically, gibberellins may stimulate cell elongation, or cell division, or both. The first crystalline gibberellin material was obtained in 1939 by Yabata from the fungus Gibberella fujikuroi. This subsequently was shown to be a mixture of three Gibberellins (GA_1, GA_2 and GA_3), Figure 2. Following this, several gibberellins were isolated but it was not until 1954 that the first structural determination was carried out on Gibberellic acid (GA_3). It was not until 1957 that gibberellins were found to occur in higher plants. At the time of writing this thesis, there are some thirty-seven known naturally occurring gibberellins. Figure 3 gives the structures of iso-Gibberellic acid, Gibberellin A_{12} and Gibberellin A_{23}.

Several very specific bioassays are known for gibberellins (Weaver, 1972); amongst the most sensitive are the Barley Endosperm Test and the Retardation of Leaf Senescence in Rumex. These will be briefly described here as Aq2 has given positive responses in these tests.

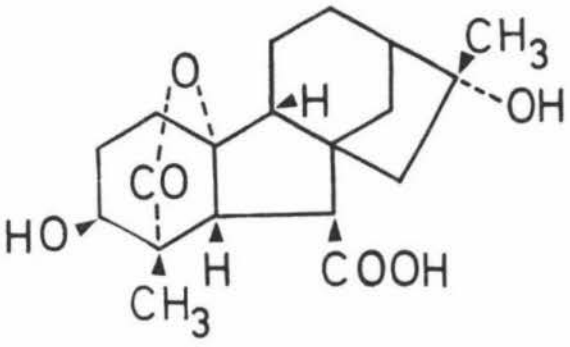
In the barley endosperm test gibberellins cause the release of amylase in embryoless endosperms and starch is hydrolysed to reducing sugars. The amount of gibberellin is proportional to the amount of amylase released and the amount of reducing sugar produced. Thus the amount of gibberellin present may be estimated by estimating the amount of amylase or the amount of reducing sugar. This bioassay can be used over a very wide concentration range and is extremely sensitive; $4 \times 10^{-3}\mu g/L$ to 4mg/L of Gibberellic Acid may be detected. An added advantage of this bioassay is that it is a relatively rapid test requiring only 5 days. This assay has been applied successfully to crude as well as pure extracts.

The Rumex assay relies on the fact that gibberellins delay

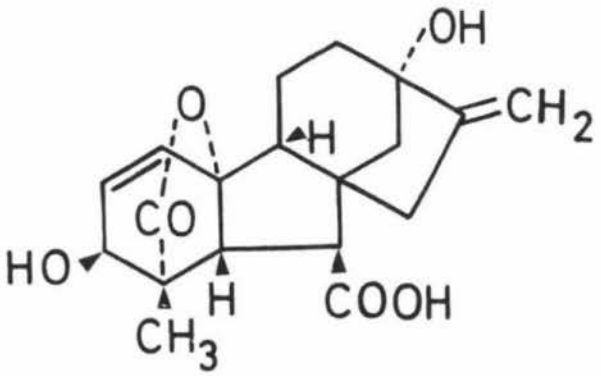
Figure 2 gives the structure of GA_1, GA_2, & GA_3, the components of Gibberellin A isolated by Yabata (1939)



Gibberellin A_1



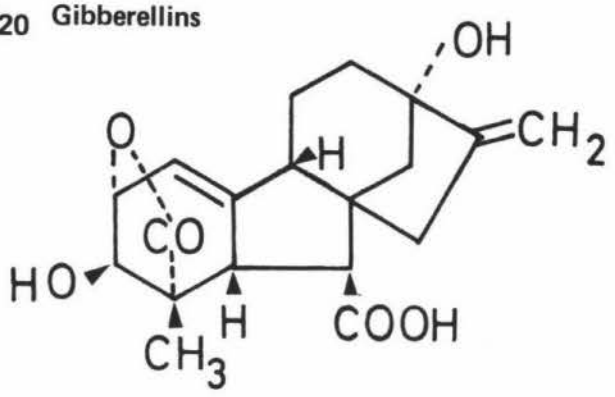
Gibberellin A_2



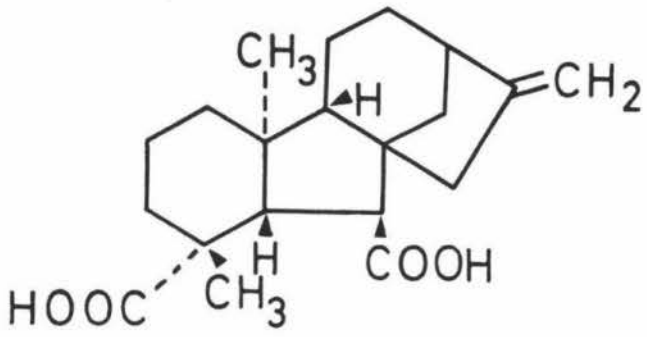
Gibberellin A_3

Figure 2

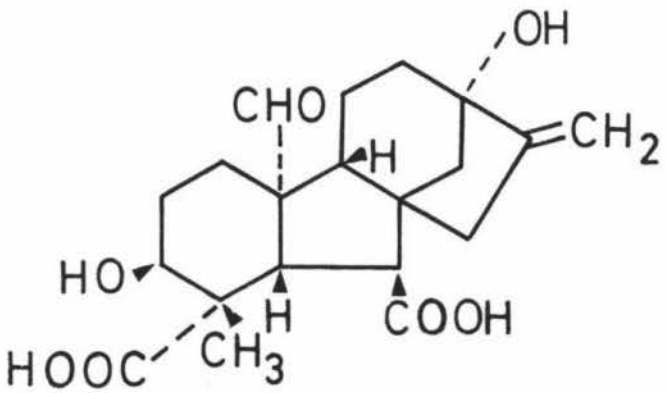
Figure 3 Gibberellin A_{12} and Gibberellin A_{23} are examples of C_{20} Gibberellins



Gibberellin - iso A_3



Gibberellin A_{12}



Gibberellin A_{23}

Figure 3

senescence in leaves of Rumex obtusifolius. This again is a highly specific test for gibberellins, being able to detect 0.01µg/L to 0.1mg/L .GA_3, while kinetin and IAA at concentrations of 6mg/L produce no response.

Cytokinins

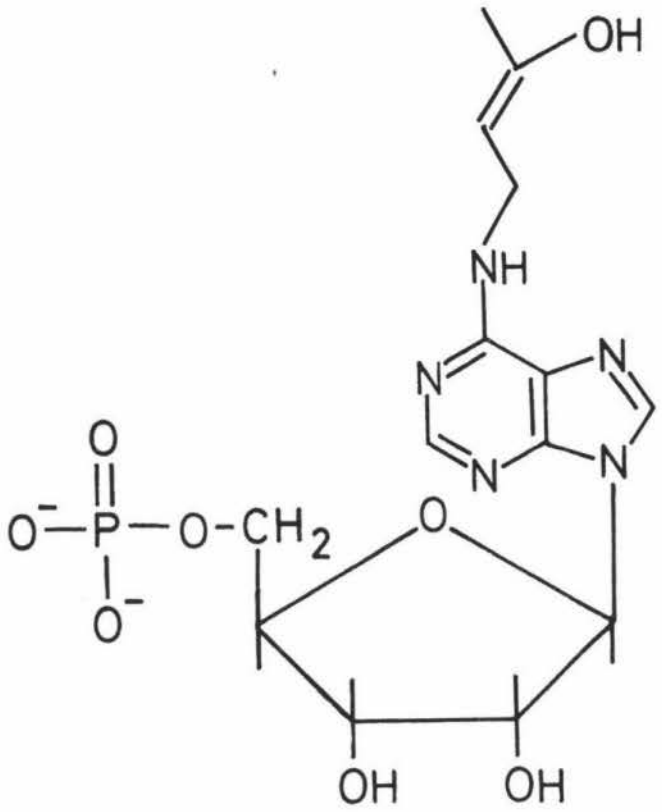
These plant growth regulators induce cell division. All known naturally occurring cytokinins are adenines substituted on the amino group.

Kinetin, Figure 4, the first isolated active cell division factor, was isolated from aged DNA, and subsequently synthesised by Miller et al. (1955a, 1955b).

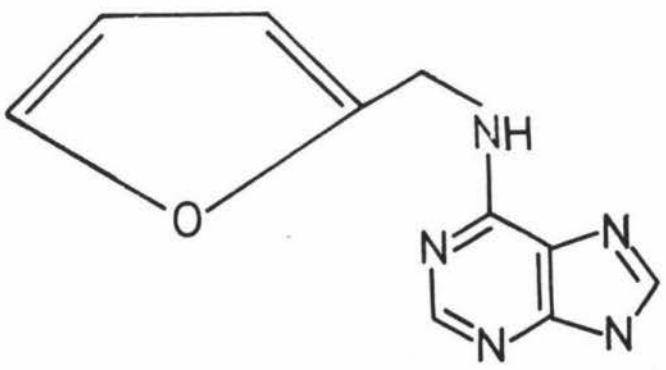
It was not until 1964 that a naturally occuring cytokinin was isolated and fully characterised. This was zeatin, extracted by Letham in 1964 from Zea mays. (However Miller in 1961 had extracted and partly characterised a similar compound from the same source). There are, to date, only a few known naturally occurring cytokinins: of these, zeatin, zeatin riboside and zeatin ribotide, Figure 4, have been found to be distributed widely among higher plants (of the three, zeatin is by far the most active). Dihydrozeatin and isopentenyladenine, Figure 5, have also been found to occur naturally. Very recently, 6-(o -hydroxybenzylamino)-9-β -D-ribofuranosylpurine, Figure 5, the first known naturally occurring cytokinin with an aromatic side chain, has been characterised by Horgan et al. (1973). Isopentenyladenine has also been derived from yeast serine and tyrosine transfer RNA, and from the plant pathogen Corynebacterium fascians. Kinetin has been isolated from aged DNA preparations, but is not considered to be a naturally occurring hormone. Numerous synthetic analogues such as benzyladenine have been synthesised and these have varying growth promoting activities. Skoog and Armstrong (1970) undertook a study of cytokinin analogues and determined which structural features are required for activity.

Other plant growth regulators active in traditional cytokinin bioassays

Plant hormones have been assayed for, and classified by, the response they cause in specific test systems. In addition to the synthetic analogues, there are other compounds which are capable

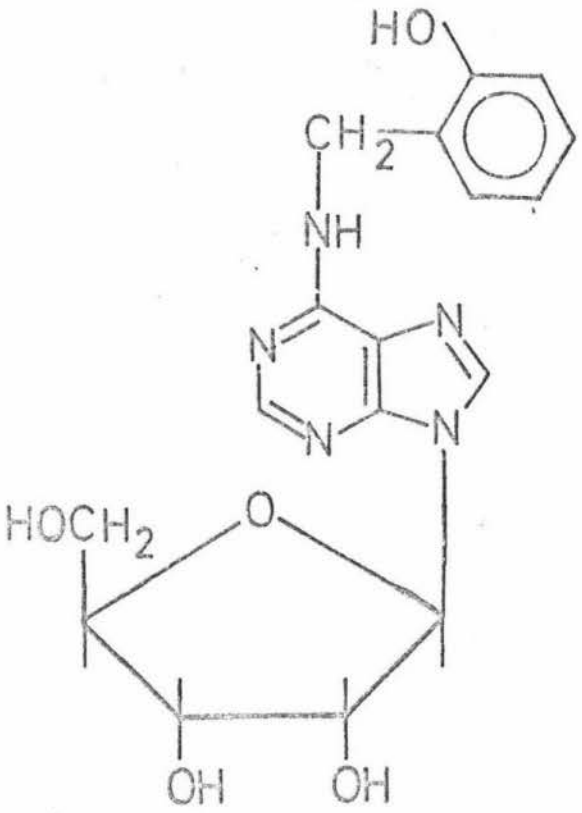


Zeatin ribotide

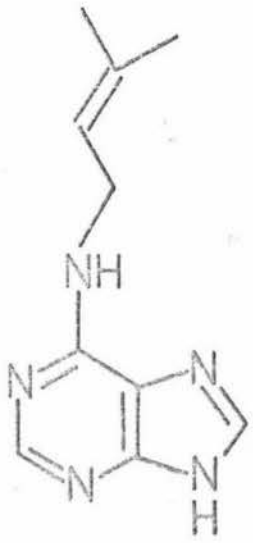


Kinetin

Figure 4



6–(o – hydroxybenzylamino) – 9 –β– D-ribofuranosylpurina
Horgan et al. (1973)



Isopentenyladenine

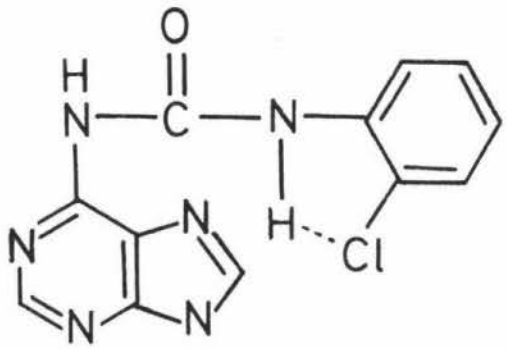
Figure 5

of eliciting a positive response in cytokinin cell division bioassays, such as the tobacco bioassay. The largest group in this class are the substituted ureas. Diphenyl urea, for example, was isolated from coconut milk and shown to have weak cytokinin activity by Shantz and Steward (1952, 1955). Some 500 urea derivatives have been examined by Bruce and Zwar (1966), and approximately half of these were active in the tobacco bioassay. Bruce and Zwar came to the general conclusion that an intact -NH-CO-NH- bridge with a phenyl group attached was required for activity. A second phenyl ring on the opposite side of the bridge increased activity. Substitution of a benzyl group produces a compound which is either inactive or has antagonistic properties to cytokinins. Ortho-chlorophenylureidopurine (I) and N-[9-(β-D-ribofuranosyl-9H) purin-6-ylcarbamoyl] threonine (II), Figure 6, have also been shown to be active in the tobacco bioassay. Kefford et al. (1968) suggest that the substituted ureas may act at the same site as the purine cytokinins, or that the effect arises from a modulation of the naturally occurring purine cytokinin levels. Hall (1973) indicates the possibility of hydrogen bonding in (I) and (II), and suggests that the similarity between (I) and (II), the substituted ureas, and the naturally occurring cytokinins, is that they are all planar molecules. Skoog and Armstrong (1970) points out that a possible similarity between the substituted ureas and purine cytokinins is in the N = C - N group in positions 7, 8 and 9 of the adenine derivatives, and the N - CO - N bridge of the substituted ureas. This similarity can be extended to compounds (I) and (II).

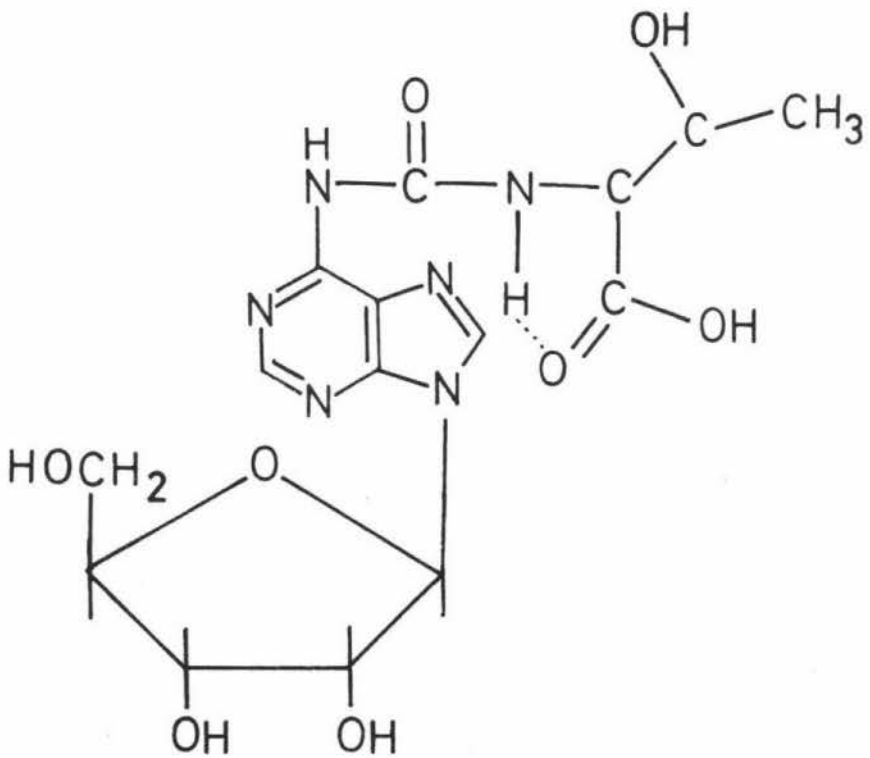
Wood et al. (1969) claim the existence of a new group of compounds having cytokinin-like activity. These were isolated from Vinca rosea but were not fully characterised and appeared to be nicotinamide derivatives containing glucose, sulphur and sulphate or sulphonate, and a straight chain fatty acid. These growth factors are claimed to be found in "dividing cells of certain dicotyledonous plant species that are far removed taxonomically from Vinca rosea, the plant species from which these substances were first isolated".

Site of synthesis and bound forms

The concept of a hormone is that of action at a distance, the active compound being synthesised in one part of the plant



ortho—chlorophenylureidopurine



N—[9—(β—D—Ribofuranosyl—9—H) Purin—6—ylcarbamoyl] Threonine

Figure 6

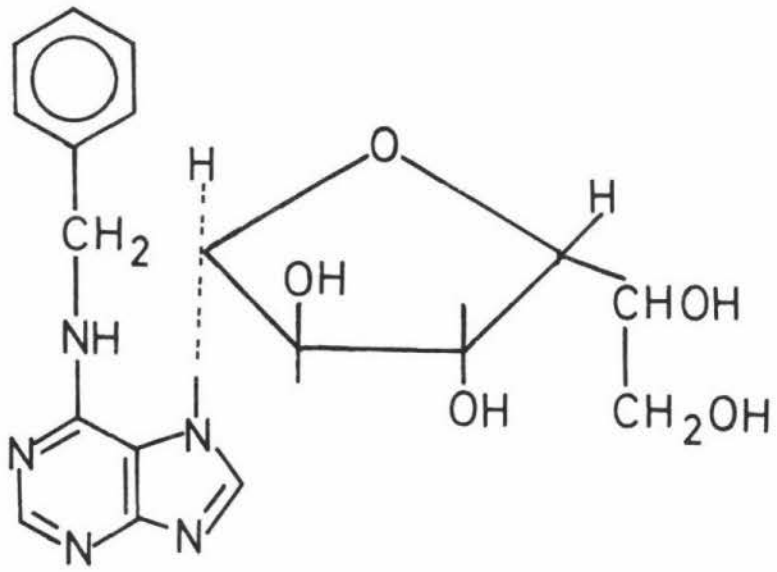
and then being translocated to its site of action. Hall (1973) states:

> The negative effects of removal of roots from a shoot can often be offset by application of a cytokinin, and such data lead to the suggestion that roots produce a cytokinin which is translocated to other parts of the plant. Bui-Dong-Ha and Nitsch were the first to positively identify such a compound, trans-ribosylzeatin, which they isolated from chicory roots. More recently Dyson et al. (unpublished data), working in our laboratory, obtained evidence that spring maple sap contains isopentenyladenine. The ribosylzeatin and isopentenyladenine isolated by the above workers were obtained under conditions which would yield only free nucleosides. There is always a possibility that elements of the cytokinin system are transported in a bound form. Kende (1965), working with a root exudate, demonstrated that a factor inactive in the cell division assay was converted to an active form by acid hydrolysis, which suggests the presence of a bound cytokinin form.

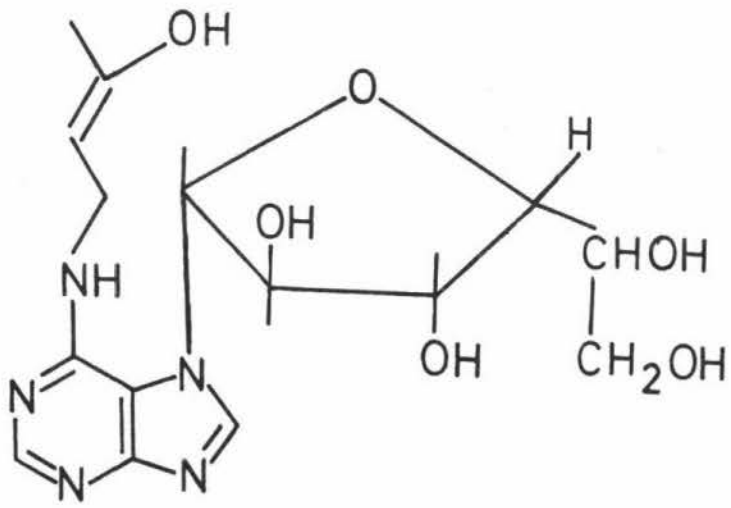
Yoshida and Oritani (1972) have found a highly water soluble cytokinin which remained in the aqueous fraction after exhaustive extractions with n-butanol or ethyl acetate. Evidence suggested that this was a zeatin glucoside. After hydrolysis with acid or β-glucosidase the activity in the cytokinin bioassay was greatly increased.

Glucosides of gibberellins have been isolated from immature seeds of Pharbitis nil by Yokota et al. (1969, 1969b, 1971). These have considerably reduced activity, but may be hydrolysed with acid to give an active gibberellin. Certain of these glucosides are partially resistant to enzymatic attack. It has been postulated that these may be a storage or transport form of the hormone. Recently Deleuze et al. (1972) and Parker et al. (1972) have found two cytokinin glycosides.

Deleuze et al. have found and have tentatively identified a metabolite of benzyladenine: 6-benzylamino-7-glucofuranosyl purine; Figure 7, this compound has some biological activity. The extent of this activity was not discussed. The stereochemistry of the glycosidic link has not been established. This compound was resistant to hydrolysis by both α- and β-glucosidases, but was hydrolysed by 2N HCl at 100° for 1 hr. It was found to persist in soya bean tissue for up to 60 days and this is thought to be due to resistance to enzymatic degradation. 6, 7-Disubstituted adenines are uncommon and this coupled with



6– benzylamino –7– glucofuranosylpurine
(the stereochemistry of the glycosidic bond is undetermined)
Deleuze et al. (1972)



Raphantin
Parker et al. (1972)

Figure 7

the furanose configuration of the glucose is possibly responsible for its resistance to enzymatic degradation.

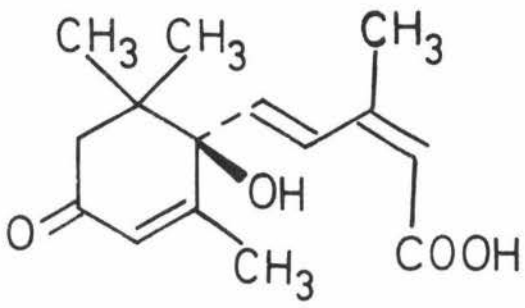
Parker et al. have reported a metabolite of zeatin, Figure 7, which appears to be a zeatin glucoside with the glucosidic linkage at position 7. This compound shows activity in the radish cotyledon bioassay.

The significance of the glucoside forms of the hormones is not yet clear; however, one might speculate that these forms may be more readily translocated than the free hormone.

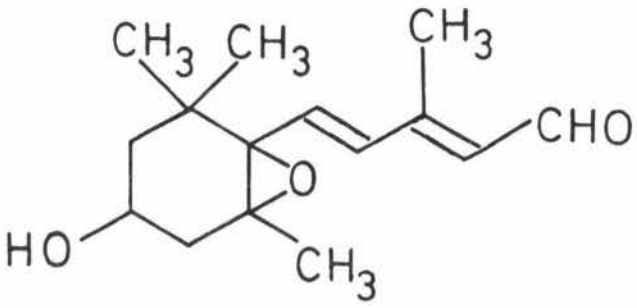
Other important plant growth regulators include classes of inhibitors such as the abscisions ; abscisic acid and xanthoxin, Figure 8; germination and growth inhibitors such as coumarin and scopaletin, Figure 8, and the phenolics, for example phloretin, and acids such as cinnamic and caffeic, Figure 9, are also known to be plant growth inhibitors.

Ethylene has also been shown to be a naturally occurring growth regulator ubiquitious among higher plants.

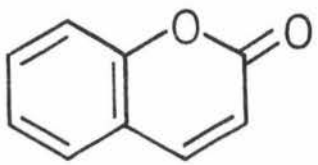
While it is obvious that Aq2 does not belong to this latter group of regulators, they have been included as they may be pertinent to the discussion of the toxic or inhibitory effect encountered when using the radish leaf expansion bioassay for cytokinins.



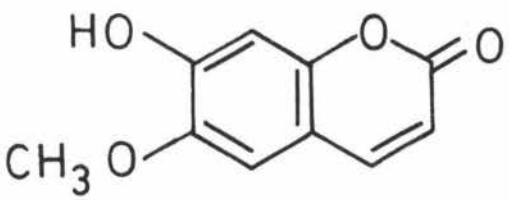
Abscisic acid



Xanthoxin

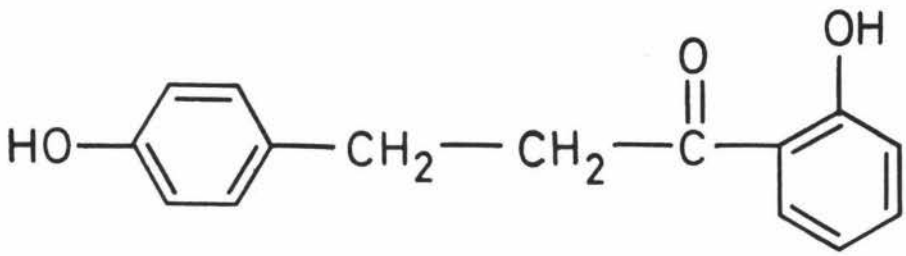


Coumarin

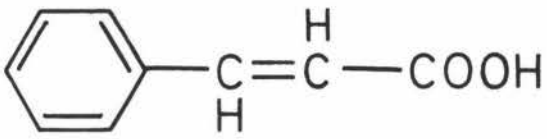


Scopaletin

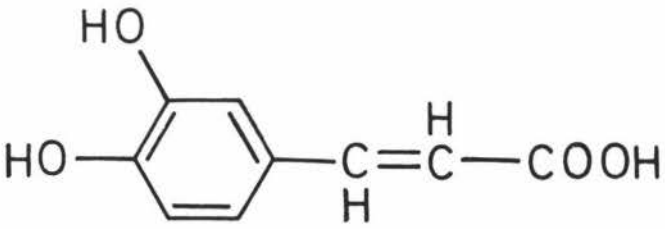
Figure 8



Phloretin



Cinnamic acid



Caffeic acid

Figure 9

METHODS OF SEPARATION AND PURIFICATION OF GIBBERELLINS AND CYTOKININS

As indicated earlier, it appeared that Aq2 was most likely a cytokinin or a gibberellin, and thus methods of separation and purification of these classes of growth regulators are briefly reviewed in this thesis.

The purification of plant growth regulators is most frequently achieved by utilisation of chromatography in one or more of its varied forms.

Gibberellins

All the known gibberellins and gibberellin glucosides are solvent extractable. The gibberellins partition into ethyl acetate under acidic conditions (Durley and Pharis, 1972) while all the glucosides so far identified extract into n-butanol under acidic conditions (Tamura et al., 1968; Yokota et al., 1969a, b). Thus it is possible to make a large class separation at an early stage of a purification procedure.

Gibberellin mixtures are frequently resolved by partition chromatography on celite-charcoal and celite-silica gel columns Harrisson and MacMillan (1971).

Separation of gibberellic acid, iso-gibberellic acid and gibberellin A_1, have been reported by Pitel et al. (1971) using Sephadex as a support for partition chromatography. GLC and GLC-MS combinations are now a powerful method of separating and identifying gibberellins (MacMillan and Pryce, 1968; Cavell et al., 1967).

Cytokinins

Ion Exchange chromatography has been a very useful technique for the separation and purification of the purine cytokinins. Nucleotides will bind to anion exchange columns at neutral pH's (Yoshida and Oritani, 1972), while nucleosides and the free bases should bind to cation exchange columns under moderately acidic conditions (Letham, 1966; Parker and Letham, 1973).

Nucleotides can also be purified by absorption onto charcoal (Nitsch, 1968; Letham, 1966; Smid and Vardjan, 1970).

In the earlier isolations, precipitation using silver and other heavy metals has been employed to purify purine derivatives unsubstituted in position 9 (Miller, 1961; Letham, 1963, 1965, 1966; Maheshwari and Prakash, 1967).

Substituted purine nucleotides can be precipitated as their barium salts (Miller, 1965; MacKenzie and Street, 1972) while nicotinamide-type cytokinins can be precipitated as their mercury salts (Wood, 1964; MacKenzie et al., 1972). Letham (1964) has formed a crystalline picrate of zeatin.

Paper and thin layer chromatography utilising various solvent systems have been used extensively as secondary stages in purification, often after initial column chromatography (Krasnick et al., 1971; Mizrahi et al., 1971; Maheshwari and Prakash, 1967; Letham, 1966).

Glen et al. (1972) have utilised Polyclar AT, an insoluble form of the polymer poly-N-vinylpyrrolidone (PVP) and have shown it to be reasonably specific in separating a phenolic fraction from plant tissue extracts by H-bonding. It has been employed for column chromatography of nucleotides, purines, pyrimidines, riboflavin and vitamin B_{12}. Glen et al. (1972) were able to demonstrate the separation of Gibberellins A_1, A_3, A_4, A_5, A_7, A_8, A_9, A_{10}, abscisic acid, indole-acetic acid and zeatin.

Sephadex LH 20 has been used for the separation of cytokinins by Armstrong et al. (1969); Hewett and Wareing (1973); Burrows et al. (1971). Recently Horgan et al. (1973) utilised Sephadex LH 20 as the support for partition chromatography of cytokinins.

CYTOKININ ASSAYS

Chemical and physiochemical methods of detection generally can only be applied meaningfully on purified or partly purified extracts; however, there are some exceptions. Thin layer and paper chromatography have been used mainly in purification and identification roles - bioassays being used to monitor activity. GLC can detect cytokinins in samples as low as 0.1μg, while individual components in mixtures of bases and ribosides can be separated. GLC-MS is a powerful analytical tool; however, bioassays are still one to two magnitudes of order more sensitive.

Cytokinin Bioassays

There are three well known bioassays essentially involving cell or tissue cultures and one utilising whole cotyledons, which rely on the ability of cytokinins to cause cell division. These are the tobacco callus test, the soya bean callus test, the carrot root phloem test and the radish cotyledon enlargement test. The tobacco and soya bean callus tests rely on a synergistic effect between cytokinins and auxins to produce the callus (a mass of undifferentiated cells). These are both highly specific and sensitive assays, the former being capable of detecting isopentenyladenine at concentrations down to 5×10^{-11}M and produces a linear response over the concentration range 10^{-10} to 10^{-8}M. The soya bean assay is slightly less sensitive, but gives a measurable response over a wider concentration range. The carrot phloem test appears to be slightly less specialised, allowing one also to test the effects of plant growth substances and their inhibitors on such parameters as protein nitrogen content and other biochemical criteria (Steward and Kirkorian, 1971). The radish cotyledon expansion test developed by Letham (1968) is claimed to be able to detect kinetin at 10μg/L ($\sim 5 \times 10^{-8}$M). This assay has been shown to be sensitive to gibberellic acid in high concentrations, however "a gibberellin-induced cotyledon enlargement can be distinguished from a cytokinin induced enlargement by placing radish hypocotyl sections on the test solution beside the cotyledons. Gibberellic

acid promotes marked hypocotyl section elongation while cytokinin slightly suppresses elongation" (Letham, 1968). The advantage of this assay is the short length of time required to complete it (2-3 days), and its sensitivity is claimed to be comparable to that of the other rapid assays.

Among the rapid bioassays which have been described to date, are those which have relied upon the ability of cytokinins to delay chlorophyll degradation in leaf material; using radish or xanthium leaves, between 0.063 to 16 mg/L of kinetin can be detected (Weaver, 1972). Other rapid bioassays have relied upon the ability of cytokinins to increase the length of the lateral buds in peas, mung beans and other plants, and to increase the size and weight of leaf discs from radish and bean leaves.

The Biological Activity of Aq2

Aq2 was found to give a response in the radish cotyledon enlargement bioassay while no response was obtained in the carrot phloem assay; however, responses were obtained in both the barley endosperm and Rumex assays for gibberellins (Sweet and Lewis, 1971) from their crude extract. The extracts of Gallagher and Aldersley, and that of the author, have been tested for their response in the radish cotyledon bioassay only.

DISCUSSION AND CONCLUSIONS

GLC of Gibberellins

At the time the author became involved with this project the probable chemical nature of Aq2 was still undetermined. Sweet and Lewis (1971) indicated that Aq2 appeared to have some properties of both cytokinin and gibberellin type compounds. While the physiological effect on pollen tube growth paralleled that of the cytokinin kinetin, the responses in the bioassays tend to indicate a gibberellin type compound.

Cavell et al. (1966) reported the direct identification of gibberellins from crude plant extracts by gas-liquid chromatography. Furthermore, MacMillan and Pryce (1968), Binks et al. (1969), and Crozier et al. (1971), have all reported the characterisation of gibberellins by combined gas-liquid chromatography-mass spectrometry.

It is for these reasons that a preliminary study of a GLC method for the identification of gibberellins was undertaken. The conditions of Cavell et al. (1966) were used as a guide and gibberellic acid used to gain experience with the techniques.

A sample of gibberellic acid was methylated, as described in the experimental section, and injected onto a 2% SE33 column. Two major overlapping peaks were obtained; these were suspected to be the methyl ester derivatives of gibberellic acid and either gibberellin A_1, A_2 or iso-A_3. The reason for the appearance of the second major peak here was uncertain. It seemed improbable that such a large amount of impurity would be present in the original Koch Light sample. It was therefore assumed that the second peak was probably due to the presence of iso-GA_3.

It is known that base will cause the lactone ring of gibberellic acid to isomerise (Cross et al., 1961), and as alcoholic 10% KOH was used to generate the diazomethane the possibility that some base may have been carried over with the diazomethane was considered, although thought unlikely. It appeared more likely that catalytic isomerisation was

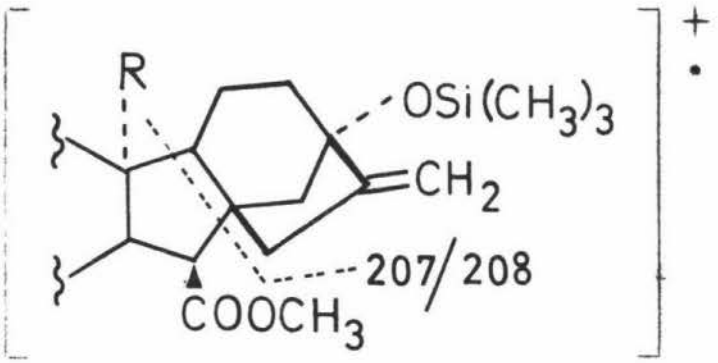
caused by the presence of metal, metal ions or traces of acid in the GLC column, especially in the region of the injector, (MacMillan, 1973). The presence of the latter agent was thought to be improbable. Subsequent use of a repacked column produced one peak only.(1)

The trimethyl silyl ether of this sample of methyl gibberellate was formed, as Cavell et al. (1966) indicated that gibberellins A_1, A_2, and A_3 were not resolved as their methyl ester but were as their trimethyl silyl ether-methyl ester derivatives. Chromatography of this sample on a 2% SE33 column yielded one major peak and two minor peaks. The retention times, when taken relative to the major peak, which was assumed to be gibberellic acid, did not appear to correspond to any of those published by Cavell et al. (1966) and are thought to be artifacts of the silylation procedure used.

A sample of the trimethylsilyl ether - methyl ester derivative was chromatographed on a SE30 column, yielding one minor and one major peak, the former having the shorter retention time. Mass spectra were run from samples taken at the leading and trailing portions of the minor peak and from the leading, middle and trailing portions of the major peak. The mass spectrum from the centre of the major peak was very similar to that published by Binks et al. (1968) of the methyl ester - trimethylsilyl ether of gibberellin A_3. However, additional peaks are evident in the authors sample. "The significant peaks at m/e 207/208, shown to be $C_{12}H_{19/20}OSi$ in the case of Me A_5 TMSi, are characteristic of the 7-TMSi ethers; they must contain rings C and D and probably arise by the cleavage shown in figure 1, with or without additional loss of hydrogen." (Binks et al., 1968). The remaining mass spectra were not readily identifiable and were not further analysed.

At this point solvent extraction data obtained by Callagher and Aldersley (1972) indicated that Aq2 was probably not a gibberellin or gibberellin glucoside, and hence this aspect of the project was not further pursued.

(1) The column producing two peaks was unpacked and traces of metal filings were found in the region of the injector. This column was repacked with fresh support prepared at the time the column was initially packed.



Significant peaks at m/e 207/208 are characteristic of the 7 TMSi ethers

Binks et al. (1968)

Figure 1

Aq2 as a Cytokinin

Following the work of Sweet and Lewis (1971), Gallagher and Aldersley (1972) carried out further investigations into the nature of Aq2. These workers had developed a large scale extraction procedure, and came to the following conclusions:

i. That Aq2 consisted of at least one major active factor which is a low molecular weight, polar, highly water-soluble, non-solvent extractable compound.
ii. That Aq2 was almost definitely not a gibberellin or gibberellin glycoside.
iii. That if Aq2 was a cytokinin, it was not zeatin, zeatin riboside, isopentenyladenine or isopentenyl-adenosine.
iv. The possibility existed that Aq2 was a ribotide, possibly zeatin ribotide. However failure to absorb onto charcoal did not support this.

An anion exchange column, BIO-RAD AG 1-x8, 200-400 mesh, formate form had been run by these workers and this led to a dramatic loss in activity which led the workers to suggest the possibility of a synergisim being present.

This was the situation which existed when this author became involved in the study of Aq2 as a cytokinin.

Column Chromatography

An anion column was prepared, as discussed in the experimental section. Seven 80 cm^3 fractions were collected and bioassayed. Fraction one caused the cotyledons to die while no activity could be found in any of the remaining fractions. This result paralleled the results of Gallagher and Aldersley. The toxicity observed for fraction one was thought to be due to material released from the column, the nature of which was unknown. An attempt to remove this toxicity by solvent extraction with ethyl acetate or n-butanol at acid and basic pH's was made. Extractions with n-butanol at pH 2.5 proved successful. This indicated that Aq2 did not bind to the anion column under neutral conditions mitigating against Aq2 being a ribotide as these would be expected to bind under these conditions.

Cohn (1955) has reported the separation of a mixture

of free bases, ribosides and nucleotides from nucleic acids. As all the naturally occurring cytokinins (with the exceptions of substituted ureas and thio-ureas) are adenine derivatives it was decided to try and bind Aq2 under the conditions described by Cohn (1955). Two columns were run at these moderately basic pH's. In both cases no activity could be found in the separate fractions or when the fractions were combined. Aq2 had been shown to be stable to base and therefore these results indicated that either Aq2 was binding irreversibly at these pH's or that Aq2 was being degraded catalytically by the resin.

After discussions with my supervisor and various delegates at the International Plant Physiology Symposium 1973, notably Dr. G. B. Sweet and Dr. D. S. Letham, it was decided that cation exchange chromatography under acidic conditions might yield more results. If Aq2 was amphoteric and binding strongly under mildly basic conditions then it may be possible under acidic conditions to form a cation. Furthermore, Sweet and Lewis (1971) had reported binding Aq2 onto a Dowex 50-W, H^+ column and subsequently eluting it off again with an ammonia solution. Gallagher and Alersley (1972), however, were not able to reproduce these results.

Parker et al. (1972) successfully used cellulose phosphate in the isolation of a cytokinin glucoside. Cellulose based ion exchanges are claimed to give superior performance with poly-electrolytes allowing faster absorption and desorption than the polystyrene base resins.(1)

It is for these reasons that Whatman cellulose phosphate P11 cation exchange cellulose was chosen for this study. The results from these column runs were difficult to interpret, neither really positive or negative results being obtained. No toxicity effects were encountered in any of these column effluents.

Following the lack of success with the cellulose based ion exchanger a further examination of a Dowex 50-W type

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(1) Thompson (1967),
and Whatman Technical Bulletin IE2
"Advanced ion exchange celluloses".

resin was thought to be warranted. Three columns were run under the conditions described in the experimental section. Some growth inhibition was noticed in the initial column effluent. This was removed.by solvent extraction with n-butanol at pH 1.5. Aq2 was found in the initial column effluents indicating a lack of binding.

The fact that no activity could be found after the anion exchange column had been run under basic conditions had remained a disturbing factor. It was decided a further study of the anion column under basic conditions was warranted. Initially two column runs were planned, one at pH 8.5 and the other at pH 9.2 - pH7 and pH 10 being ineffective.

The activity from the column run at pH 8.5 was split into two fractions. One passed straight through the column while the other was eluted from the column with 3N formic acid. As Aq2 had been applied to the column in water rather than buffer there was some doubt as to the exact pH of absorption. Furthermore, the splitting of activity into two fractions could have indicated either Aq2 is composed of two or more fractions or, more probably, that due to a poorly favoured binding and a high flow rate the column was overloaded. A second column was run at pH 8.5, with Aq2 being applied in a weak buffer and allowed to absorb at a slower flow rate. Similar results were obtained as in the previous run. A third column was run with absorption at pH 9.2 in weak buffer. Activity was found in the initial column effluent. Only optimum binding would appear to occur in the region of pH 8.5 The method used to convert the BIO-RAD AG 1x8 resin to the formate form might be considered suspect; however even if the resin was not completely in the formate form during the initial column runs it is thought that the resin would have been almost completely converted by the repeated washing with 3N formic acid.

For several column runs Aq2 was applied in water at the desired pH, rather than in buffer. This was due to a concern regarding the ionic strength of the Aq2 solution. Appreciable amounts of hydrochloric acid and sodium hydroxide have been used to adjust the pH of the crude extract during the solvent

extraction stages and this is in addition to any indigenous ionic material present. It was thought possible that a high ionic strength may have been responsible for the lack of binding to ion exchange resins but this does not now appear to be so.

Purity of the Aq2 Extracts

The purest extract so far obtained contained 49 mg solids per GEP. Aq2 is estimated to be present in the concentration range of 1-100 µg per GEP.(1) Hence, even in the purest extract obtained so far, Aq2 composes ' 0.2% or less of the solids content of the extracts. This has severely limited the amount of Aq2 which can be spotted onto chromatostrips and has for practical purposes excluded the use of preparative paper chromatography. A further complication is that the bulk of the sugars tend to run with the same Rf zone as Aq2 in butanol:acetic acid:water solvent. The inability to bind Aq2 readily to an ion exchange resin has also been a major problem.

Solvent extraction of Aq2 has been briefly studied. Aq2 is not solvent extractable into ether (Sweet and Lewis 1971) or in n-butanol and ethyl acetate (Gallagher and Aldersley 1972). Aq2 is soluble in ethanol and methanol; however, a large proportion of the solids are also soluble in these solvents. However, aqueous ethanol solutions were found to be useful in freezing out carbohydrate material.

The Bioassay

Some degree of difficulty has been experienced with the radish cotyledon bioassay. A lack of sensitivity in addition to a toxicity problem was noted by Gallagher and Aldersley and also by this worker. The assay appeared to be very sensitive to numerous substances which appeared to influence markedly the response obtained and has been the reason for the necessity of chromatography prior to bioassaying. In almost all cases failure to run chromatography strips before

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(1) 1 GEP of Aq2 gives a response in the radish cotyledon bioassay of greater than 10 ppm kinetin. Assuming a similar potency as kinetin Aq2 could be present in the concentration of 10 µg/GEP. However the potency could feasibly be a factor of 10 greater or less than kinetin.

bioassaying caused most or all the cotyledons to die.

Micro-organism Contamination of Aq2 Extracts

The high carbohydrate content of Aq2-containing extracts appears to be an ideal source of nutrient for bacteria and fungi and the prevention of growth contamination, especially fungal growths, has been a major problem in the storage of Aq2 extracts. It has been found that the addition of chloroform inhibits but does not completely eliminate fungal growth.

Suggestions for Further Work

The fact that Aq2 appears to be split into two fractions by absorption at pH 8.5 has not been investigated further and this should be a starting point for further work. Possible avenues for exploration could be doubling the column length and/or slowing the column flow rate. Another possibility worthy of investigation would be the use of a cellulose anion exchanger which may possibly have a faster exchange rate. In any event it should be now possible to characterise the portion of Aq2 which binds to the anion column at pH 8.5 by the standard technique of paper and thin layer chromatography followed by gas liquid chromatography - mass spectrometry or mass spectrometry alone, as to date the great difficulty with characterising Aq2 has been its separation from the large amount of soluble carbohydrate and other water soluble materials.

It would now be very informative if the fraction that binds to the anion column could again be bioassayed in the more traditional cytokinin and gibberellin bioassays and to see if these results parallel those obtained by Sweet and Lewis (1971).

If further work fails to bind all of Aq2 on an anion column the use of poly-N-vinylpyrrolidone could possibly yield useful information. Glen et al. (1972) have used this material to isolate compounds from plant extracts which are capable of hydrogen bonding. If this proves unsuccessful the use of Sephadex LH-20 should be contemplated, (Armstrong, et al., 1969; Hewett and Wareing, 1973; Burrows et al., 1971; and Horgan et al., 1973).

If these techniques proved unsuccessful the techniques involved in the fractionation of sugar mixtures should be examined as it may be possible to separate Aq2 from the bulk of the carbohydrate material by this means.

The Nature of Aq2

At this stage it is not possible to draw many additional conclusions about the structure or chemical nature of Aq2. Recently Letham (1973) has described the characterisation of ten cytokinins from Zea mays, and this is the first instance, known to the author, where an anion column has been used under basic pH's, to separate cytokinins. The two cytokinins isolated in this manner were 6-(4-hydroxy-3-methylbut-trans-2-enylamino)-9-β-D-ribofuranosylpurine 5'-monophosphate, absorbed at pH 8, and 6-(1-carboxy-2-hydroxypropylamino)-9-ribofuranosylpurine, absorbed at pH 9. However, both of these compounds were also absorbed onto charcoal and bound to a cation column and hence behave quite differently to Aq2. Recent results from anion column runs 5 and 6 tend to indicate Aq2 having a somewhat higher Rf value than 0.2-0.3 as determined by Gallagher and Aldersley, and Sweet and Lewis. Greater activity appears to be present in zone 3 than zone 2 and usually the inverse applies.. This may be due to the greater degree of purity of these samples, however these results are very tentative and require further study.

In summary, Aq2 either occurs in large quantities in the pollen of P. radiata or occurs in lower quantities but is several times more potent than kinetin. It is highly soluble in water, has a low molecular weight and is non solvent-extractable. It appears to bind weakly to an anion exchange column at pH 8.5. When chromatographed in n-butanol acetic acid : water it occurs between Rf 0.2-0.4 and does not appear to resemble any of the characterised plant hormones known at the time of writing.

EXPERIMENTAL

All solvents used in this work were drum grade, and were distilled prior to use. All water used was of distilled, deionised quality. The GLC used for the introductory work on gibberellins was a Varian series 1740 instrument with flame ionisation detectors. The GLC - mass spectrum work was carried out on a AEI-MS 30-GLC mass spectrometer. Yate's Long Scarlet market strain brand of seeds were used in the bioassay.

The Bioassay

This is a slightly modified form of the radish cotyledon expansion bioassay of Letham (1968), as used by Sweet and Lewis (1971). Samples to be assayed are streaked on 5 x 57 cm strips of Whatman 3 MM chromatography paper, 2.5 cm from one end. These are developed by ascending chromatography in n-butanol: acetic acid: water (12:3:5). Each sample to be assayed is run in triplicate (to provide for the three replicates A, B, C of each sample, see below). After development the strips are dried in a fume hood overnight. The region on each developed chromatostrip between the solvent front and the origin is divided into ten equal zones. Aq2 has been found in zones 2 and 3 (Rf 0.1 to 0.3) on such chromatostrips. The chromatostrips are viewed under U.V. before being cut into zones. These zones, approximately 2 cm x 5 cm, are used in the bioassay.

A large excess of radish seeds (variety long scarlet, market strain pre-soaked for 30 minutes) are planted on a moistened sheet of Whatman 3 MM chromatography paper supported on a plastic coated wire rack. The rack is placed inside a large plastic bag, 100 cm^3 of water added, and the bag sealed using bulldog clips. This is then placed in a cabinet in the dark at 26-27^{o}C for thirty-six to forty hours.

After this period the germinated radish seedlings are removed from the plastic bag and the smaller inner cotyledon of each is excised and placed on a piece of moistened filter paper in a petri dish. After sufficient cotyledons have been picked, they are sorted into nine size groups, ranging from large through medium to small cotyledons respectively, and placed on moistened

filter paper in numbered petri dishes (numbered 1-9). Each selected zone of interest from the chromatostrips described above is placed into a petri dish and moistened with between 0.7 - 1 cm^3 of water. The dishes are arranged in sets of three replicates A, B and C (i.e., three dishes for each zone being tested). Thus, if two chromatographic zones from a sample are being assayed, this requires six petri dishes. Ten cotyledons are then selected and placed on the paper zone in each dish. The cotyledons are selected in the following manner. For replicate A, they are obtained from the three dishes numbered 1-3 containing the large cotyledons. Dish 1 contributes 3 cotyledons, dish 2 contributes 3 cotyledons and dish 3 contributes 4 cotyledons. All the dishes in replicate A have their complement composed in this fashion. For replicate B they are selected in a similar manner, except dish 5 contributes 4 cotyledons with 3 each from dishes 4 and 6. In replicate C, dish 7 contributes 4 cotyledons with 3 each from dishes 8 and 9.

Lids are put on the petri dishes which are then placed on the wire racks; the racks are then placed in a plastic bag together with approximately 100 cm^3 of water, and the bags sealed. These are then placed on a bench illuminated by fluorescent lighting,(1) at 26-27°C for three days (72 hours). Additional water is added to each dish after two days.

At the end of this growth period, the cotyledons are removed from each dish, carefully blotted dry and weighed (i.e. in groups of ten). Any cotyledons obviously inconsistent with the others in a particular dish (stunted growth or leaf necrosis) are discarded and an average for that dish calculated.

The sorting is designed to give a random distribution of cotyledons at three size levels, throughout the entire bioassay - this is necessary as sensitivity to growth regulators apparently varies with cotyledon size. It is possible to apply statistics to the results (see for example Sweet and Lewis, 1971) to obtain,

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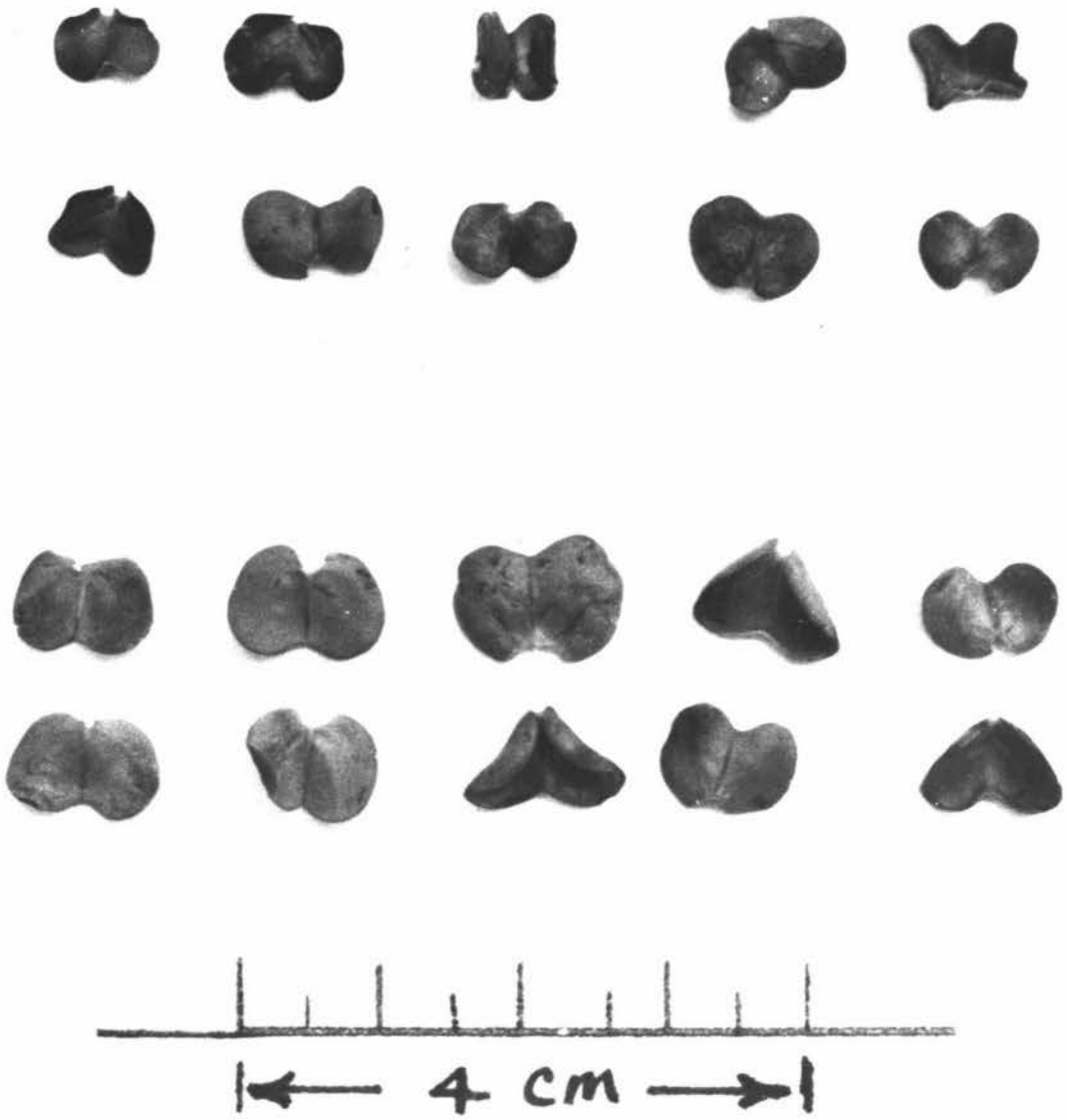
(1) The author used two Mazda 40 watt "Daylite" fluorescent tubes supported approximately 3'8" above the bench. The light intensity at dish level was found to be approximately 58 ft. candles, as measured by an Eppley thermopile coupled to a sensitive digital voltmeter. The thermopile was sensitive to light in the range 400-700 nm.



Replicates C and A for 10 ppm kinetin (direct), prior to weighing (top to bottom C - A).

for example, confidence levels; however, there are often only three replicates run per zone due to practical size restrictions on the bioassays, which often run into 400 to 600 cotyledons per bioassay. Thus such calculations are normally only carried out to establish guidelines for reliability and sensitivity; the size of a routine bioassay is often such that multiple repeat replicates would result in it becoming impractically large.

Extraction Procedure

25 g samples of pollen were washed with water by suspending the pollen in 500 cm^3 of water and shaking for 10 minutes on a Griffen flask shaker. The water in the resulting slurry was filtered off under suction, the pollen resuspended in fresh water and the process repeated two more times.

50 g of washed pollen were placed in a screw cap plastic container (2.8L capacity) with approximately 2 Kg of chilled glass balls (5 mm diameter, undrilled, BDH) and 600 cm^3 of ice-cold water. This container was vigorously shaken on a vertical-action shaking machine (400 oscillations per minute) for 10 minutes. The contents of the container were poured through a buchner funnel (without filter paper) to separate the disintegrated pollen grains and aqueous extract from the glass balls. 500 g pollen was treated in this way by repeating the procedure ten times. The yellow slurry so obtained was placed in polycarbonate centrifuge bottles (500 cm^3 capacity) and spun in a Sorvall super speed centrifuge (RC2-B, GS3 head), at 5000 r.p.m for 30 minutes.

The aqueous supernatant extract was decanted off and the yellow pollen precipitate/residue discarded. The aqueous fractions were bulked. Two-litre portions of this extract were extracted twice with equal volumes of ethyl acetate. An emulsion formed with each extraction. This emulsion was broken by centrifuging at 5000 rpm for 30 minutes in the Sorvall RC2-B. This caused lipid-like material to compress to a solid cake situated between the aqueous and the ethyl acetate layers. Ethyl acetate was drawn off and the lipid-like material separated from the aqueous extract by carefully filtering it through a plug of glass wool.

The aqueous extract was then reduced in volume by one fifth,

using a Buchi Rotavapor R; the temperature of the waterbath was kept below 40°C. The pH was then adjusted to pH 6.8, and 400 cm^3 portions of this solution were shaken twice with activated charcoal (2 x 10 g, Darco G-60) for 30 minutes. The resulting solution was reduced in volume by 50%, the pH adjusted to pH 2.5 with dilute hydrochloric acid, and extracted with an equal volume of water-saturated n-butanol followed by an equal volume of n-butanol. The pH of the aqueous phase was adjusted to pH 6.8 and the solution again extracted with an equal volume of water-saturated n-butanol followed by an equal volume of n-butanol. The resulting aqueous solution will be referred to as the crude extract.

Column Chromatography

Anion Exchange Chromatography

A 30 cm x 1.9 cm glass column was packed with BIO-RAD A.G. 1 x 8 200-400 mesh, chloride form anion exchange resin, the resin having first been de-fined several times in distilled water (until the resin settled relatively rapidly). After packing, the column was then back-washed five times.

The formate form of the resin was then formed. The recommended conversion procedure, which was initially followed, was:

$Cl^- \longrightarrow OH^-$, 20 bed-volumes 1N NaOH;

$OH^- \longrightarrow$ formate, 2 bed-volumes 1N formic acid.

In fact, excess of 30 bed-volumes of 1N NaOH was passed through the column and a strong chloride precipitation with $AgNO_3$ [2] was still obtained. As bicarbonate is exchanged preferentially it [3] was decided to displace chloride with bicarbonate and then to displace bicarbonate with formate; removing the CO_2 under vacuum.

The resin was removed from the column and 2N formic acid added until effervescence ceased. The resin was filtered off and

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(2) Test for completeness of conversion to OH^- form:- acidify a portion of effluent with conc HNO_3 and add 1% $AgNO_3$.

(3) AG 1-x8 Order of Selectivity
I phenolate HSO_4 ClO_3 NO_3 Br CN HSO_3 NO_2 C
HCO_3 IO_3 H_2COO Acetate OH F
BIO-RAD Laboratories Price List W. June 1971.

fresh 2N formic acid added and the mixture was placed under vacuum for 3 hours. This was repeated two more times. The column was repacked and backwashed.

The anion column was run seven times in all. After the first run the column was reconverted to the chloride form by eluting with 2N NaCl (20 cm^3 portions of effluent were titrated after adding 20 cm^3 of 1N NaOH - complete conversion was established after the last traces of sodium formate-formic acid buffering ceased. An additional litre of 2N NaCl was run through the column to ensure complete conversion). The column was then converted to the formate form using $KHCO_3$ solution as described above. After the second column run, the column was washed with 2N formic acid followed by water and re-used.

Run 1:

The column was washed with water until pH 5 was reached. 50 gram equivalents of pollen (GEP)[4] extract was placed on the column in 35 cm^3 of water. The column was then eluted with water and 80 cm^3 fractions collected. A drop of chloroform was added to each fraction and these were stored under refrigeration until required, when they were reduced in volume to 4 mls on a Rotavapor R. Seven 80 cm^3 fractions were collected in all; F_1 - F_7. An additional 500 cm^3 fraction, fraction W, was also collected. This was reduced in volume to 25 cm^3 before being applied to chromatostrips. The pH of all fractions were measured and found to be between pH 4.5 and pH 6.5.

Activity of the fractions was tested for using the radish cotyledon bioassay. All fractions caused growth inhibition.

Concentrated F_1 appeared to contain a large quantity of dissolved material - it was very syrupy. The concentrate was diluted to approximately 50 cm^3 with water giving solution A_1. Fractions F_2 - F_6 were combined giving solution A_2. Solutions A_1 and A_2 were adjusted to pH 2.5 with dilute HCl and were both extracted three times with equal volumes of ethyl acetate followed by three equal volumes of water-saturated n-butanol. The pH was readjusted to pH 7 and the solutions again extracted with ethyl acetate followed by water-saturated n-butanol as above. Both A_1

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(4) The extract obtained from 50 grams of pollen.

and A_2 were reduced in volume to 25 cm^3. These solutions were assayed for activity in the radish cotyledon bioassay; activity was found only in solution A_1.

Run 2:

50 GEP extract was placed on the column in 50 cm^3 of water. The column was then eluted with water and 120 cm^3 of effluent was collected as one fraction. This was reduced in volume to 50 cm^3 and a 2 cm^3 sample C.E. was taken for bioassay. The pH of the remaining 48 cm^3 was reduced to pH 2.5 with dilute HCl and extracted twice with equal volumes of water-saturated n-butanol. A 2 cm^3 sample B.A.E. of this was taken for bioassay. The pH was readjusted to pH 8.5 with dilute NaOH and the solution extracted twice with equal volumes of water-saturated n-butanol; the resulting solution was designated B.B.E. Solutions of pH 2.5 were adjusted to approximately pH 7 before being bioassayed. Activity was found in B.A.E. and B.B.E. while the cotyledons from fraction C.E. died.

Run 3:

An ammonium formate - formic acid buffer, pH 11 was prepared, being 0.01 M in formate. 50 GEP extract was reduced in volume to approximately 10 cm^3, and 40 cm^3 of buffer added. A white precipitate P_1 formed which was filtered off. A 5 cm^3 aliquot (F_1) of the filtrate was taken immediately after filtration and neutralised. The remaining filtrate (B_{24}) was allowed to stand at room temperature for 24 hours.

The solid P_1 when examined under a microscope appeared to be crystalline, had a melting point in excess of 230°C, gave a residue in an ash test, and appeared to be inorganic in nature. The remaining solid was transferred to a 10 cm^3 volumetric flask and water added to make up the volume. Very little if any of the solid P_1 appeared to dissolve. However the aqueous supernatant was assayed for activity. The supernatant from P_1 was found to be inactive, while F_1 and B_{24} were comparably active, thus establishing Aq2's stability in the buffer used.

50 GEP extract was reduced in volume and made up to 50 cm^3 with buffer, pH 11, the precipitate being filtered off and discarded. This solution was applied to the column. After absorption the column was eluted with formate buffer pH 10.2.

The first 50 cm^3 from the column was collected as a single fraction, and thereafter seven 100 cm^3 fractions were collected. An additional 250 cm^3 of buffer was passed through the column. The column was then eluted with a 0.01 M formate buffer, pH 2.5, and approximately 2 L (acid fraction) of this solution passed through the column. No activity was found in any of these fractions; however, some fractions had remained in the fridge for several weeks before being bioassayed.

At this stage additional pollen extract had to be prepared. A total of 1 Kg of pollen was extracted, extract C_2. Assays of this extract indicated that it was not as active as previous extracts, but still appeared to contain activity due to Aq2.

Run 4:

The anion column was washed with 0.01 M formic acid followed by water and then equilibrated with buffer, pH 11. 25 GEP extract (C_2) was reduced in volume to approximately 10 cm^3 and made up to 50 cm^3 with buffer, pH 11. The white precipitate was filtered off, the filtrate being applied to the column. The column was eluted with 150 cm^3 buffer (F_1) pH 11 followed by a 0.01 M formate buffer, pH 4 and the following fractions collected.

F_2	570 cm^3	pH 11	pH 7
F_3	300 cm^3	pH 7	rapidly dropping to pH 4
F_4	500 cm^3		
F_5	280 cm^3		

Following the collection of F_5, 1 L 2N formic acid was used to elute the column, and this was collected as one fraction, F_6.

All fractions were taken to dryness on the Rotavapor R and and extracted with methanol,(5) by shaking for two hours, and this extract tested in the bioassay. The results from this assay were not conclusive - there may have been weak activity in F_1-F_4 and F_6, however, these may have been high background fluctuations. The residues from F_2 - F_6 were dissolved in water and combined. The combined aqueous solution was freeze dried and this was extracted with the combined methanol solution obtained from extracting fractions F_2 - F_6, for four hours. This methanolic solution was placed in the freezer for two hours and then

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(5) Preliminary studies suggested that Aq2 is soluble in methanol and ethanol.

rapidly filtered. No activity was found in this solution. The solid material from F_1 and the combined solids from F_2 - F_6 were dissolved in water and assayed - a possible weak response was obtained from the combined fractions with little or no response from F_1.

Run 5:

Following run 4 the column was washed with water until the pH reached pH 4. The column was then equilibrated with 0.01 M ammonium formate buffer pH 8.5. When the column had reached this pH, it was then washed with 1 column-volume of water. 40 GEP of crude extract E_{45} (6) was adjusted to pH 8.5 with dilute NaOH. This was applied to the column in approximately 60 cm^3 of solution. A further column-volume of water at pH 8.5 was used to wash the column. 120 cm^3 of effluent, F_1, was collected. The column was then eluted with 3N formic acid and approximately 1 L collected, F_2.

F_1 was reduced to 20 cm^3. F_2 was reduced to dryness and washed several times with water in an effort to remove formic acid. This fraction was then adjusted to approximately pH 6 and made up to 20 cm^3 before being bioassayed.

F_1 caused the cotyledons to die (toxic effect?). F_2 allowed growth (blank level only), but no activity was detectable.

Both fractions were then adjusted to pH 2.1 and extracted twice with equal volumes of n-butanol and their pH's readjusted to approximately pH 6 giving fractions F_1A and F_2A.

Activity was found in both fractions; slightly more activity being found in fraction F_1A.

Run 6:

The 3N formic acid used to elute the column in run 5 had slightly disrupted the resin bed. The column was backwashed five times to resettle the packing prior to washing with water. The column was then equilibrated to pH 8.5 using a 0.01 M ammonium formate buffer.

Fractions F_1A, F_2A from run 5 and the remaining crude extract E_{45} were bulked together to give 37 GEP extract Y. 7 GEP extract Y were removed for bioassaying. The remaining 30 GEP extract Y were reduced in volume and then diluted with

(6) E_{45} : combination of extracts E_4, 100 GEP and E_5 200 GEP, prepared in April, 1973.

0.01 M formate buffer pH 8.5. Additional dilute NH_4OH had to be added to maintain this pH. This extract was then applied to the column in 60 cm^3 of solution. 2 column-volumes of 0.01 M formate buffer pH 8.5 were used to wash the column; approximately 200 cm^3 being collected, F_1.

The column was then eluted with 3N formic acid, approximately 1.2L being collected, F_2. F_1 and F_2 were both reduced to 15 cm^3 volume and adjusted to pH 2. They were then extracted twice with equal volumes of n-butanol, their pH's adjusted to approximately pH 6, giving fractions F_1E and F_2E. These were then bioassayed. Fractions F_1E and F_2E and fraction Y all showed good activity.

Run 7:

The column was washed with a further 1.5L of 3N formic acid followed by 2L of water and then equilibrated with 0.01 M ammonium formate buffer pH 9.2. 36 GEP extract C_2 were reduced in volume and diluted with formate buffer pH 9.2. Additional dilute NH_4OH was required to maintain pH 9.2. 30 GEP of this extract were applied to the column in 60 cm^3 of solution. The column was then washed with 2 column-volumes of formate buffer pH 9.2, giving fraction F_1.

The column was then eluted with 1.5N formic acid; approximately 500 cm^3 was collected, giving fraction F_2. 3N formic was then used for elution and approximately one litre collected, giving fraction F_3.

Fractions F_1, F_2, and F_3 were reduced in volume to 15 cm^3 and their pH's reduced to pH 2. They were then extracted twice with equal volumes of n-butanol. The pH of these solutions was then adjusted to approximately pH 6 giving fractions F_1A, F_2A, and F_3A. These were then bioassayed, along with a sample of extract C_2.

C_2, F_1A and F_2A all showed activity. The cotyledons in F_3A died (toxic effect?). F_1A showed much greater activity than F_2A.

Cellulose Phosphate Cation Exchange Chromatography

A glass column 30 cm x 1.5 cm was packed with a slurry of Whatman P.11 cellulose phosphate powder which had been de-fined. The packed column was conditioned in the following manner:

(1) Washed with 0.5 N HCl until effluent pH 1, then washed with a further 3 column-volumes of 0.5 N HCl .
(2) Washed with water until pH 4.
(3) Washed with 0.5 N NaOH until the pH reached pH 11, then washed with an additional 5 column-volumes.
(4) Washed with water until approximately pH 9.
(5) Equilibrated with 0.2 M ammonium acetate buffer, pH 3.1, until the effluent reached pH 3.1.
(6) Washed with two column-volumes of water.

Run 1:

After the washing procedure outlined above, the cellulose phosphate was considerably whiter than before washing. Approximately 1.3L of ammonium acetate buffer, pH 3.1, was required to equilibrate the column. 25 GEP extract C_2 was reduced in volume and made up to 25 cm^3 with buffer, pH 3.1. This was placed on the column and washed with an additional 100 cm^3 of buffer, pH 3.1. The column was then eluted with 0.3 M ammonium hydroxide and the following fractions collected.

F_1	130 cm^3	from column - includes effluent
F_2	40 cm^3	after changing to 0.3 M NH_4OH
F_3	160 cm^3	0.3 M NH_4OH
F_4	220 cm^3	0.3 M NH_4OH

All four fractions were assayed for activity. Activity was found in fraction F_3.

Run 2:

The column was equilibrated with 0.2 M ammonium acetate buffer, pH 3.1 and then washed with 3 column-volumes of water. 40 GEP extract (ethanol treated extract) was applied in 20 cm^3 at pH 3.1, dil. HCl being used to lower the pH of the extract. Dilute HCl , pH 3.1, was used to wash the column. The pH of the effluent after 1 column-volume dropped to pH 2. A total of 150 cm^3 of effluent was collected as one fraction. The column was then eluted with 0.3 M ammonium hydroxide - a total of 500 cm^3 being collected as one fraction. Both of these fractions were reduced to 20 cm^3 in volume, fraction 1 (effluent) having first been adjusted to pH 5.8. Good activity was detected in fraction 1 indicating Aq2 had not bound to the column.

Cation Exchange Chromatography

Run 1:

A 30 cm x 1.9 cm glass column was packed with BIO-RAD AG 50-W x 4 100-200 mesh H^+ form, cation exchange resin after it had been de-fined. The column was then backwashed five times and then exhaustively washed by eluting with 4L of water.

100 GEP extract was applied to the column at pH 6.2 and the column eluted with water. Two 500 cm^3 fractions F_1 and F_2 were collected. The column was then eluted with 5N ammonium hydroxide solution, two 500 cm^3 fractions F_3 and F_4 being collected. These fractions were reduced to dryness and made up to 50 cm^3 volume. The ammonium hydroxide fractions were taken to dryness, washed and taken to dryness again before being made up to volume. These fractions were assayed for activity. A growth inhibitory effect was obtained from fraction F_1 while no activity could be found in fractions F_2 - F_4.

The four fractions were combined and assayed to test for a synergistic effect - no activity was detected.

Run 2:

A second cation exchange column (BIO-RAD AG 50-W x 4 100-200 mesh H^+ form) was prepared. 50 GEP extract was applied at pH 6.5 and eluted with water, three fractions being collected: F_1 200 cm^3; F_2 200 cm^3; F_3 280 cm^3, The column was then eluted with 100 cm^3 of 6N ammonium hydroxide.

Fractions F_1 - F_3 were reduced to dryness and then made up to 25 cm^3 and aliquots taken for bioassaying. These fractions were then diluted to 100 cm^3 and twice extracted with water-saturated n-butanol at acidic pH; F_1 at pH 1.5, F_2 at pH 3.5, F_3 at pH 3.6. Aliquots were removed for bioassaying (F_1A, F_2A, F_3A). The pH's were then adjusted to pH 7 using dilute sodium hydroxide and each fraction again extracted twice with equal volumes of water-saturated n-butanol; samples were again removed for bioassaying (F_1B, F_2B, F_3B). F_1 produced growth inhibition while no activity was detected in F_2 and F_3. F_1A showed weak activity with no activity in F_2A or F_3A. Good activity was obtained in F_1B with no response from F_2B or F_3B.

Run 3:

The column was regenerated by washing with 3L IN HCl

Run 3.

The column was conditioned as previously described. 40 GEP extract (ethanol treated extract) was applied in 200 cm^3 of distilled water adjusted to pH 3.1 with diluted HCl. The column was washed with 200 cm^3 of dilute HCl, pH 3.1. All the column effluent to this point was collected as fraction F_1. The column was then eluted with 0.3 M ammonium hydroxide, a total of 500 cm^3 being collected as fraction F_2.

Fraction F_1 was adjusted to near neutrality with dilute NaOH and then reduced to 20 cm^3. Fraction F_2 was reduced to almost dryness several times to remove the bulk of the ammonia, neutralised with dilute HCl and then made up to 20 cm^3.

10 cm^3 of fractions F_1 and F_2 were combined and this solution then reduced to 10 cm^3, giving fraction Com. One and two GEP of fractions F_1, F_2 and Com were bioassayed and R_f zones 1, 2 and 3 examined. No conclusive results were obtained.

followed by water until the pH rose to pH 5. 50 GEP extract (ethanol treated extract) was diluted to 1L and applied to the column at pH 2 (dilute HCl being used for acidification) and washed with water at pH 2. The column was then eluted with 5N ammonium hydroxide. Two fractions were collected, the effluent (F_1) 1.5L and the ammonium hydroxide eluate (F_2) 1.4L . These fractions were reduced in volume and made up to 25 cm^3. 10 cm^3 of each were combined and this was reduced in volume and made up to 10 cm^3, FC. Samples of each fraction were bioassayed. Activity was found in F_1 and FC, with no activity detectable in F_2.

Freeze Drying

This appeared to have little effect on the activity of the crude extract and has proved a useful method for estimating the extent of purification obtained, on a weight for weight comparison.

Purification of Crude Extract by Alcohol Treatment

The crude extract C_2 was found to be supporting bacterial or fungal growth in the form of a white film lying over the surface. This was filtered using glass wool followed by a sintered glass funnel - the ensuing extract was crystal clear. Absolute alcohol was added as a preservative and the extract stored at $4^{\circ}C$ for two days. At the end of this period a small amount of crystalline material had formed which was filtered off. It thus appeared feasible to try and freeze out some of the soluble sugars. 200 GEP extract was taken and reduced to a thick syrup, approximately 10 cm^3 in volume. This was made up to 30 cm^3 with water and 70 cm^3 of absolute alcohol were added - a thick syrupy mass formed below an alcohol layer. Addition of 20 cm^3 of water with vigorous stirring produced a white precipitate. The mixture was placed in the freezer overnight and the precipitate filtered off the following day, approximately 1 g of material being obtained. The solution was again reduced in volume to 30 cm^3 and absolute alcohol added with stirring - a white precipitate formed after adding approximately 30 cm^3 (50% solution) and precipitation continued until approximately 70 cm^3 (70% solution) had been added. When stirring was stopped, this apparent white precipitate formed a brown syrup as a second phase. The mixture was placed

in the freezer for 1½ hours and the upper layer decanted off. The lower syrupy layer was extracted with 50 cm^3 of 70% ethanol. This was placed in the freezer for 1½ hours and the supernatant decanted off. Additional alcohol was added to the supernatant until precipitation appeared to stop; this mixture was then placed in the freezer overnight. The following day the supernatant was decanted off and the alcohol supernatants combined, the alcohol removed under vacuo and the residue redissolved in water.

A sample of this extract and a sample of the crude extract were freeze dried and the solids content of each determined. C_2 contained 112 mg/GEP extract while the ethanol treated extract (obtained above) contained 49 mg/GEP extract. The ethanol precipitation thus reduced the solids content by 56%. Bioassays of both extracts showed comparable activity.

Preliminary Investigation of a GLC Method of Analysis of Gibberellins

The method and conditions outlined by Carvell et al. (1967) were used as a general guide. (Chromatography was carried out on a Varian 1740 series GLC). A five foot ¼" diam. stainless steel column was packed with 2% SE33 (GP90 silicone gum rubber. ANALABS, Connecticut), on chromasorb W, acid washed DCMS treated, 80 mesh. Gibberellic acid (Koch Light Laboratories, Puriss) was methylated using ethereal solutions of diazomethane. After one minute, excess diazomethane was removed and the sample reduced to dryness. This was taken up in a small amount of redistilled methanol and injected onto the SE33 column (70 cm^3/min. N_2 carrier gas, column 212°C). Two major overlapping peaks were obtained.

By repacking the SE33 column with fresh support and liquid phase (prepared at the same time as the original packing) and rechromatographing the same sample of methyl ester, a single peak was obtained.

The trimethyl silyl ethers of the methyl ester GA_3 was prepared using TRI-SIL (Reirce Chemical Co.) at room temperature for one hour. GLC chromatography (Varian 1740) of this solution yielded one major peak and two very minor peaks. This sample

was also examined by combined GLC-MS. The GLC trace (Pye 104, 3% SE30 column, 40 cm^3 helium carrier gas, isothermal at 225°C) indicated the presence of two peaks; the minor peak having the shorter retention time. Mass spectra (AEI MS 30, 70 ev source, 4kV accelerating voltage, resolution 1000, scan rate 10 sec/decade) were taken from the leading and trailing zones of the minor peak and at the leading, middle and trailing zones of the major peak.

APPENDIX

Detailed Results of Bioassays and Histograms

ANION EXCHANGE COLUMN RUN 1

Sample	Rf zone	Replicate
		A
F_1	2	N.G.
	3	N.G.
F_2	2	94
	3	102
F_3	2	111
	3	109
F_4	2	92
	3	86
F_5	2	100
	3	104
F_6	2	96
	3	97
F_7	2	92
	3	98
Blank		170
10 ppm Kinetin		255

Key to table of bioassay results

N.G. No growth, or death of cotyledons.

($\bar{x}$) Weight averaged for 10 cotyledons from x cotyledons.

ANION EXCHANGE COLUMN RUN 1
SOLVENT EXTRACTION OF EFFLUENT

Sample	Rf zone	Replicates			
		A	B	C	$\bar{X}$
A_1	2	233($\bar{9}$)	187	166	195
	3	165	129	112	136
A_2	2	103	118	86	103
	3	161	123	110	131
Blank		144	100	92($\bar{9}$)	112

ANION COLUMN RUN 2
SOLVENT EXTRACTION OF EFFLUENT

Sample	Rf zone	Replicates		
		A	B	$\bar{X}$
Column Effluent		N.G.	N.G.	N.G.
B.A.E.	2	156($\bar{9}$)	N.G.	156
	3	198	N.G.	198
B.B.E.	2	184($\bar{9}$)	0.192	188
	3	N.G.	N.G.	
Extract E_2	2	146	254	201
	3	N.G.	N.G.	N.G.
E_2 Direct(1)	2	N.G.	100	100
	3	N.G.	N.G.	N.G.

(1) Extract E_2 applied direct to bioassay: not chromographed on Whatman 3 MM

ANION EXCHANGE COLUMN RUN 2

STABILITY OF Aq2 IN BASE

Sample	Rf zone	Replicates			
		A	B	C	$\bar{X}$
P_1	2	170	146	110	142
Filtrate	2	253	244($\bar{7}$)	130($\bar{4}$)	209
	3	N.G.	N.G.	N.G.	N.G.
2 GEP Filtrate	2	N.G.	N.G.	N.G.	N.G.
B_{24}	2	335($\bar{7}$)	N.G.	179($\bar{7}$)	257
	3	254($\bar{4}$)	N.G.	240($\bar{4}$)	247
	4	168	121	155	148
	5	198	140	113	150
	6	170	127	98	132
10 ppm Kinetin		219	169	150	189

ANION EXCHANGE COLUMN RUN 3

pH 10.2

Sample	Rf zone	Replicates A	B	C	$\bar{X}$
Adsorbate	2	134($\bar{9}$)	85	94($\bar{8}$)	105
	3	N.G.	N.G.	N.G.	N.G.
F_1	2	175	N.G.	74($\bar{7}$)	125
	3	93($\bar{9}$)	91($\bar{9}$)	72($\bar{9}$)	85
F_2	2	158	117	110	129
	3	174	105	112	130
	4	192	118	106	139
F_3	2	168	125	160	131
	3	158	124	135	139
F_4	2	188	117	N.G.	150
	3	161	113	N.G.	137
F_5	2	187	116	42($\bar{8}$)	132
	3	194	126	110	143
F_6	2	153	114	121($\bar{9}$)	129
	3	186	113	120	139
F_7	2	215	124	97	144
	3	202	125	99	142
10 ppm Kinetin		230	192	168	197
Blank		162	115	116($\bar{9}$)	131

ANION EXCHANGE COLUMN RUN 4

pH 11

Sample	Rf zone	Replicates			
		A	B	C	$\bar{X}$
F_1	2	181	142($\bar{8}$)	128	150
	3	166	131($\bar{7}$)	108($\bar{9}$)	135
F_2	2	158	106	117	127
	3	143	128	133	135
F_3	2	156	137($\bar{9}$)	131	142
	3	161	130	137($\bar{9}$)	143
F_4	2	155	125	122	134
	3	164	137($\bar{9}$)	119	140
F_5	2	152	126	109($\bar{9}$)	129
	3	157	133	110	133
A	2	144	138	104	129
A	3	175	151	136	154
10 ppm Kinetin Direct		232	203	176	204
10 ppm Kinetin (New) Direct		235	221	146	209
Blank		133	150	101	
Blank		134	136	118	129
2 GEP com.	1	147($\bar{9}$)	153	108(9)	136
	2	150	134($\bar{8}$)	101($\bar{6}$)	128
	3	151	117	102	123
2 GEP F.1	1	149	122	104	125
	2	151	130($\bar{6}$)	92(7)	119
	3	111	135($\bar{9}$)	89($\bar{3}$)	112

ANION EXCHANGE COLUMN RUN 5

pH 8.5

Sample	Rf zone	Replicates			
		A	B	C	$\bar{X}$
F_1	2	152($\bar{8}$)	102($\bar{8}$)	N.G.	-
	3	N.G.	N.G.	N.G.	N.G.
F_2	2	184	136	118	146
	3	181	160($\bar{9}$)	123	155
E_{45}	2	275($\bar{9}$)	323($\bar{8}$)	232($\bar{6}$)	277
	3	219($\bar{5}$)	220($\bar{8}$)	144($\bar{6}$)	194
F_1A	2	347($\bar{8}$)	290($\bar{9}$)	226($\bar{8}$)	288
	3	196($\bar{5}$)	N.G.	149($\bar{6}$)	172
F_2A	2	285	236	220	247
	3	181	168	147	165
10 ppm Kinetin		239	179($\bar{9}$)	163($\bar{9}$)	194
Blank		160	125	119	
					138
Blank		184	125	117	

ANION EXCHANGE COLUMN RUN 6

Sample	Rf zone	Replicates			
		A	B	C	$\bar{X}$
Combined Crude	2	279($\bar{6}$)	326	175	260
	3	157($\bar{6}$)	206($\bar{3}$)	127($\bar{1}$)	163
F_1E	2	238($\bar{3}$)	289($\bar{7}$)	206	244
	3	277($\bar{1}$)	192($\bar{1}$)	N.G.	N.G.
F_2E	2	246	239($\bar{9}$)	139	208
	3	208	196	143	182
Kinetin Direct		268	187	137	197
Blank		132	155	100	147
		164	137	191	

ANION EXCHANGE COLUMN RUN 7

Sample	Rf zone	Replicates			
		A	B	C	$\bar{X}$
Extract C_2	2	184	145	161	163
	3	194	174	146($\bar{9}$)	171
F_1A	2	199	184($\bar{9}$)	171	185
	3	216	177($\bar{8}$)	171($\bar{9}$)	188
F_2A	2	167	133	142	147
	3	181	151($\bar{9}$)	154	162
F_3A	2	N.G.	N.G.	N.G.	N.G.
	3	N.G.	N.G.	N.G.	N.G.
Kinetin		232	207($\bar{9}$)	192($\bar{8}$)	210
Blank		136	131	126	131

CELLULOSE PHOSPHATE COLUMN RUN 1

Sample	Rf zone	Replicates			
		A	B	C	$\bar{X}$
F_1	2	183($\bar{6}$)	128($\bar{3}$)	76($\bar{3}$)	129
	3	N.G.	N.G.	N.G.	N.G.
F_2	2	167	149	84	133
	3	174	169	109	151
F_3	2	191	191	93	159
	3	159	136	108	134
F_4	2	152	142	87	127
	3	154	132	99	128
10 ppm Kinetin Direct		277	239	154	223
Blank		153	135	82	124

CELLULOSE PHOSPHATE COLUMN RUN 2

Sample	Rf zone	Replicates			
		A	B	C	$\bar{X}$
1 GEP F_1	1	154	146	121($\bar{8}$)	140
	2	171($\bar{8}$)	155	127($\bar{7}$)	151
	3	148($\bar{5}$)	117($\bar{5}$)	110($\bar{8}$)	129
2 GEP F_1	2	162($\bar{9}$)	159($\bar{9}$)	143($\bar{7}$)	155
	3	145($\bar{4}$)	136($\bar{3}$)	N.G.	141
1 GEP F_2	1	134	96	85($\bar{9}$)	105
	2	151	129	97	126
	3	165	131	107	134
1 GEP F_3	1	144	129	87	120
	2	130	132	101	121
	3	131	128	114	124
1 GEP F_4	1	125($\bar{9}$)	122	70	106
	2	158	142	93	131
	3	152	148	100	133
10 ppm Kinetin		221	217	155	198
Blank		115	115	105	112

CELLULOSE PHOSPHATE COLUMN RUN 3

Sample	Rf zone	Replicates			
		A	B	C	$\bar{X}$
1 GEP Com.	1	157	121	114	131
	2	201	150($\bar{8}$)	122($\bar{9}$)	158
	3	168	138	128($\bar{9}$)	145
1 GEP F_1	1	128	137	114	126
	2	173	140($\bar{8}$)	111($\bar{9}$)	141
	3	170	144	107	140
1 GEP F_2	1	134	125	87	115
	2	151	124	102	126
	3	149	146	95	130
2 GEP Com.	1	179	145	112	146
	2	160	164	116	169
	3	126	163($\bar{4}$)	93($\bar{1}$)	156
2 GEP F_1	1	163	140	162	135
	2	169	147($\bar{8}$)	93($\bar{8}$)	138
	3	173($\bar{8}$)	119($\bar{5}$)	102($\bar{6}$)	131
2 GEP F_2	1	147	122	96	119
	2	134	132	90	119
	3	145	145	94	128
10 ppm Kinetin		183	205	144	178
Blank		140	137	85	120

CATION EXCHANGE COLUMN RUN 1

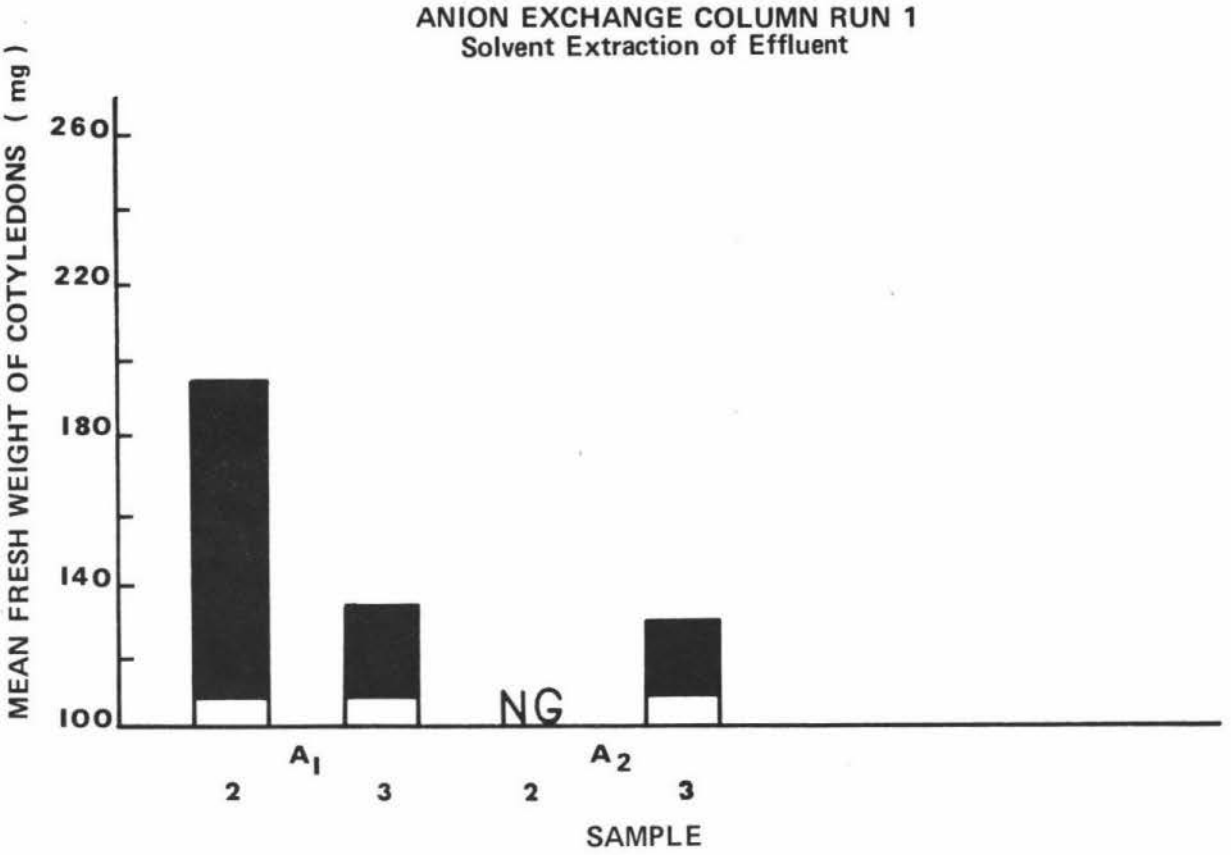
Sample	Rf zone	Replicates			
		A	B	C	$\bar{X}$
F_1	2	N,G.	N.G.	N.G.	N.G.
	3	N.G.	N.G.	N.G.	N.G.
F_2	2	183	147	138	156
	3	173	156	130	153
F_3	2	167	149	153	157
	3	184	155	148	162
F_4	2	178	137	132	149
	3	185	152	137	158
10 ppm Kinetin		267	201	272	213
10 ppm Kinetin Direct		272	240	214	242
Blank		175	151	132	156
Blank		185	152	142	

CATION EXCHANGE COLUMN RUN 2

Sample	Rf zone	Replicates			
		A	B	C	$\overline{X}$
F_1	2	123	N.G.	N.G.	-
F_2	2	121	141	100	151
F_3	2	159	134	96	130
F_1A	2	178	152	113	148
F_2A	2	170	146	95	137
F_3A	2	165	156	107	142
F_1B	2	244	185	115	181
F_2B	2	175	130	94	133
F_3B	2	173	138	93	135
10 ppm Kinetin Direct		283	195	171	217
Blank		170	104	107	131
Blank		168	126	109	

CATION EXCHANGE COLUMN RUN 3

Sample	Rf zone	Replicates			
		A	B	C	$\bar{X}$
1 GEP F_1	2	221	192($\bar{9}$)	214	209
	3	165	184($\bar{8}$)	169($\bar{3}$)	173
2 GEP F_1	2	253($\bar{6}$)	225($\bar{2}$)	N.G.	239
	3	N.G.	N.G.	N.G.	N.G.
1 GEP F_2	2	128	121	110	119
	3	151	113	122	129
2 GEP F_2	2	163	119($\bar{9}$)	97	127
	3	161	128	102	130
1 GEP Com.	2	239($\bar{8}$)	249($\bar{9}$)	161($\bar{9}$)	216
	3	202	239($\bar{8}$)	173($\bar{6}$)	205
2 GEP Com.	2	269($\bar{5}$)	223($\bar{1}$)	192($\bar{4}$)	228
	3	185($\bar{5}$)	225($\bar{1}$)	164($\bar{2}$)	191
10 ppm Kinetin		228	202	192($\bar{9}$)	207
Blank		149	123	75	116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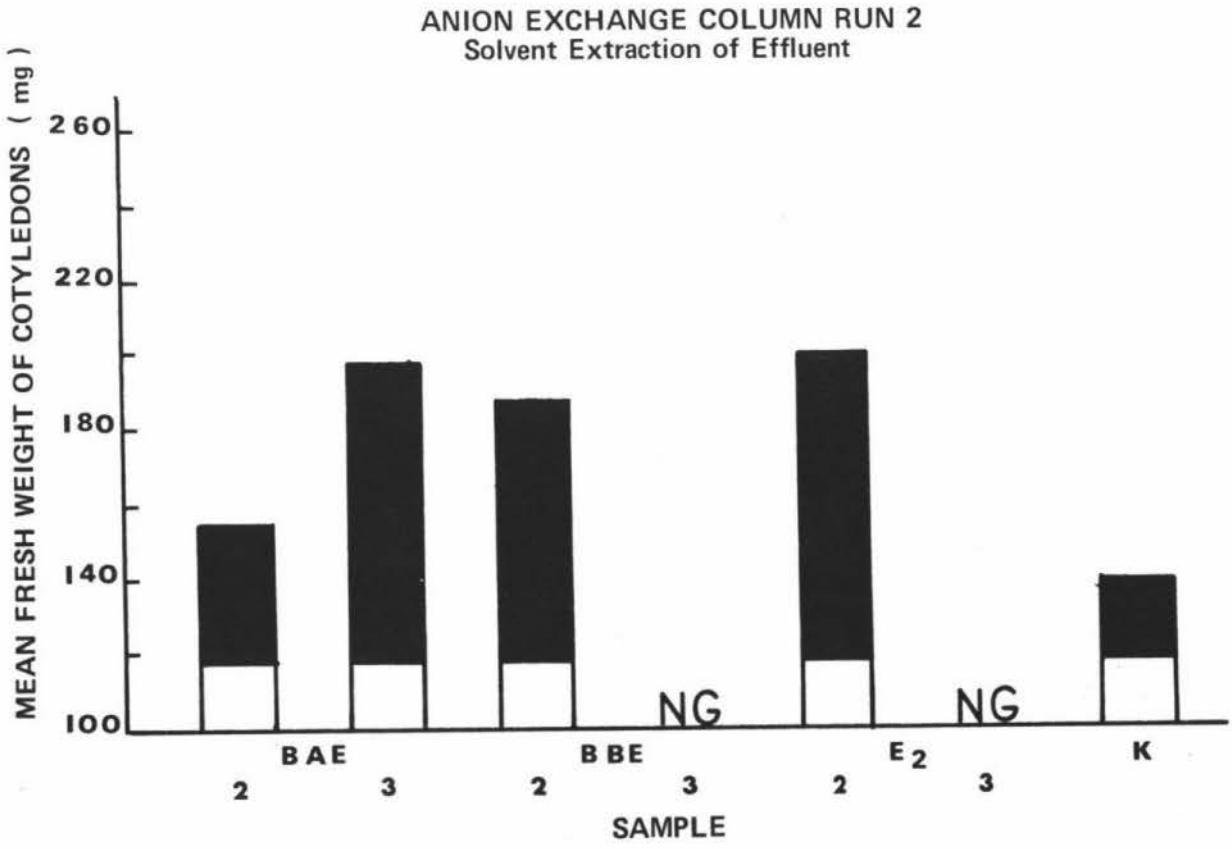
220
180
140
100
NG
A1
A2
2
3
2
3
SAMPLE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Solvent Extraction of Effluent
MEAN FRESH WEIGHT OF COTYLEDONS (mg)
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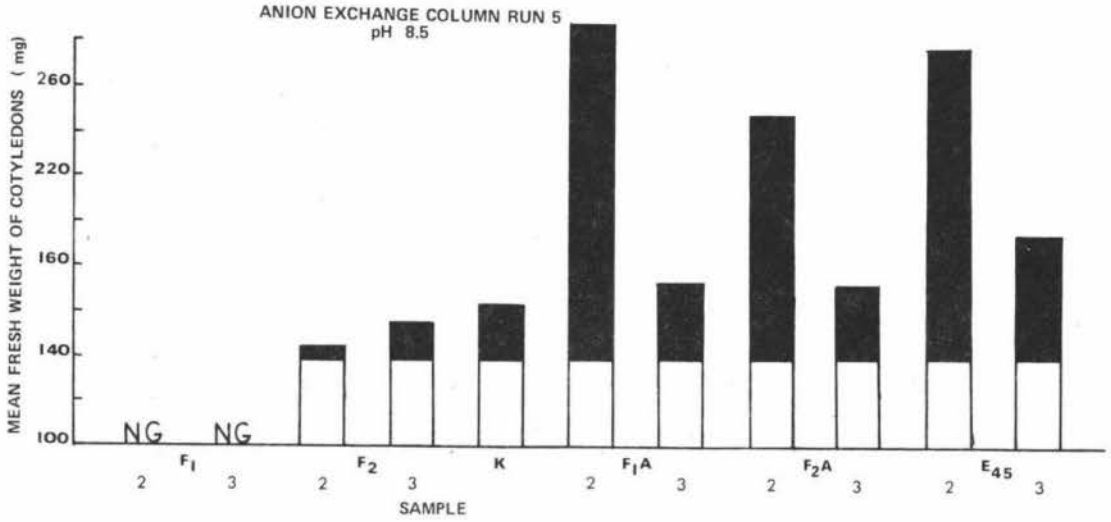
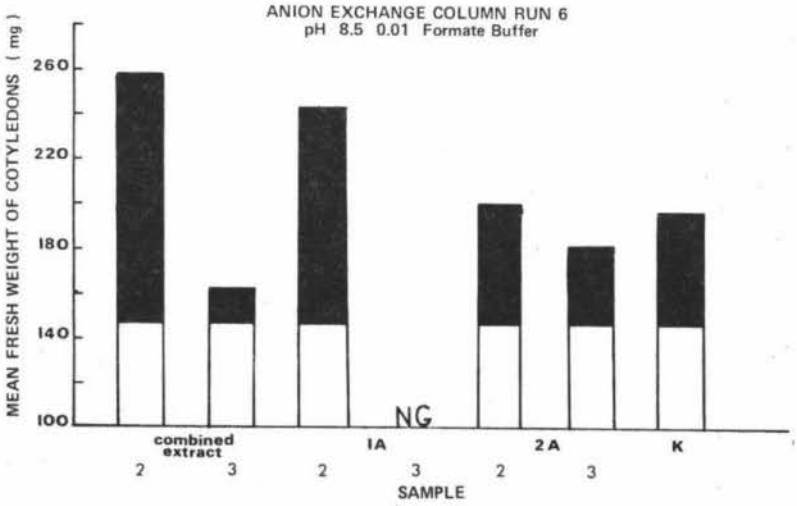
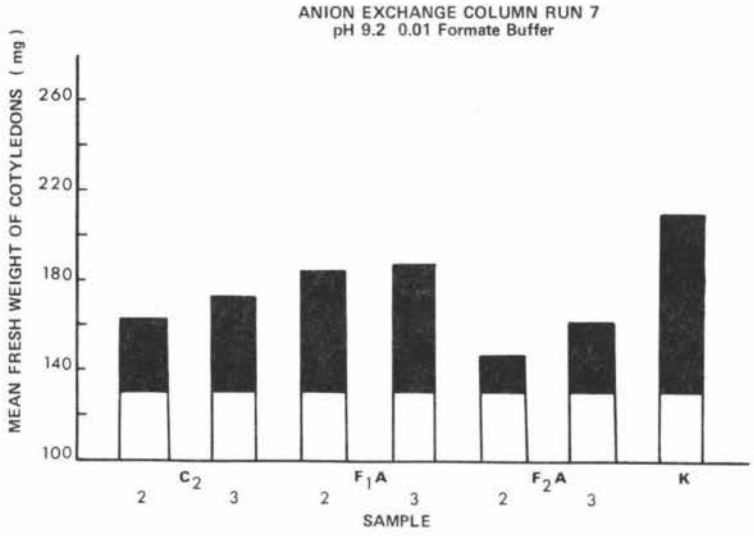
220
180
140
100
NG
NG
BAE
BBE
E2
K
2
3
2
3
2
3
SAMPLE


ANION EXCHANGE COLUMN RUN 5
pH 8.5
MEAN FRESH WEIGHT OF COTYLEDONS (mg)
260
220
180
140
100
NG
NG
F1
F2
K
F1A
F2A
E45
2
3
2
3
2
3
2
3
2
3
2
3
SAMPLE


ANION EXCHANGE COLUMN RUN 6
pH 8.5 0.01 Formate Buffer
MEAN FRESH WEIGHT OF COTYLEDONS (mg)
260
220
180
140
100
combined extract
NG
1A
2A
K
2
3
2
3
2
3
SAMPLE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pH 9.2 0.01 Formate Buffer
MEAN FRESH WEIGHT OF COTYLEDONS (mg)
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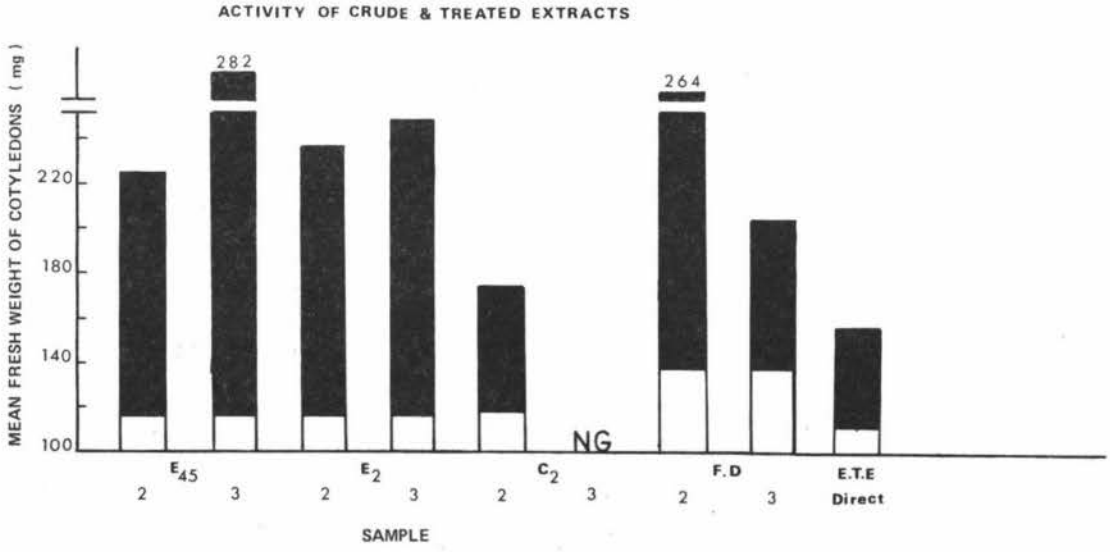
180
140
100
C2
F1A
F2A
K
2
3
2
3
2
3
SAMPLE


ACTIVITY OF CRUDE & TREATED EXTRACTS
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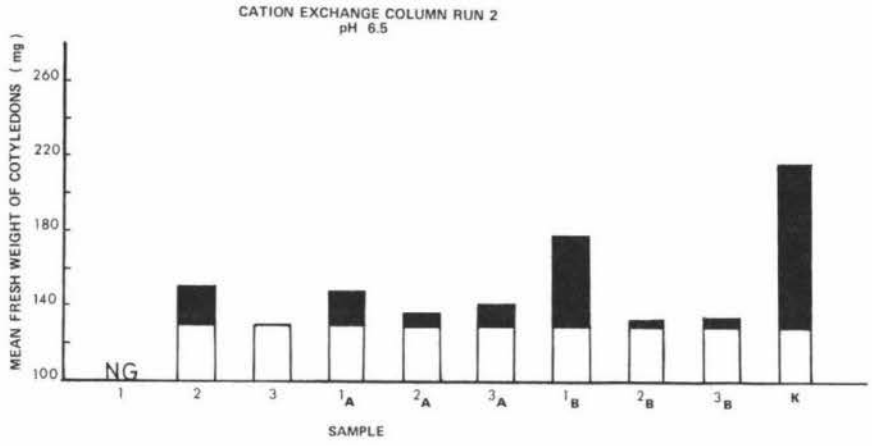
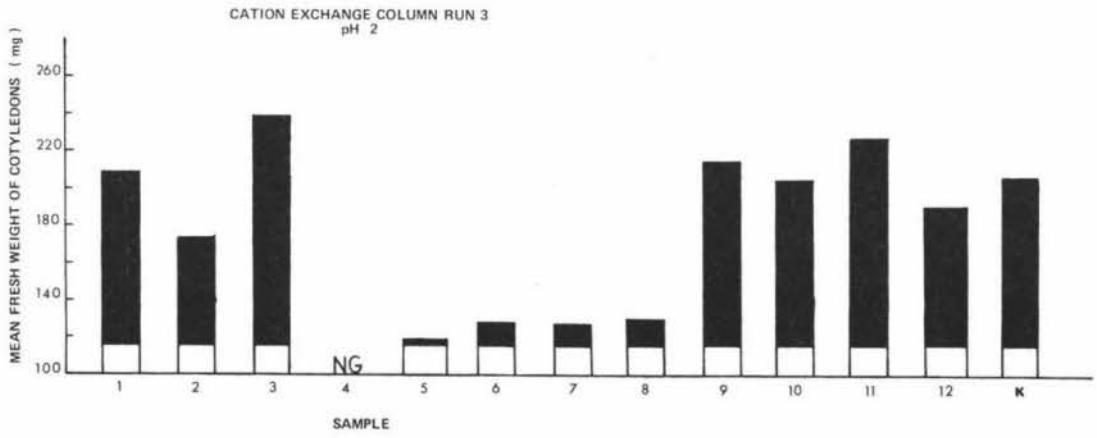
282
264
220
180
140
100
NG
E45
E2
C2
F.D
E.T.E
Direct
2
3
2
3
2
3
2
3
SAMPLE


CATION EXCHANGE COLUMN RUN 2
pH 6.5
MEAN FRESH WEIGHT OF COTYLEDONS (mg)
260
220
180
140
100
NG
1
2
3
1A
2A
3A
1B
2B
3B
K
SAMPLE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pH 2
MEAN FRESH WEIGHT OF COTYLEDONS (mg)
260
220
180
140
100
NG
1
2
3
4
5
6
7
8
9
10
11
12
K
SAMPLE

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