THE INTERACTION OF PLANT GROWTH REGULATORS WITH CELL MEMBRANE CONSTITUENTS

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DECLARATION

During the period of registration as a candidate for the degree of Ph. D., the author has not been registered for any other award of C.N.A.A. or any other university.

Material contained in this thesis has not been used in any other submission for an academic award.

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FOR MY PARENTS

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Norman P. Sheridan

ABSTRACT

The thesis describes the interaction of auxins with membrane fractions prepared from etiolated epicotyl tissue of Pisum Sativum seedling. The interaction of auxins with phospholipids was also examined.

Two classes of high affinity binding tissue sites were found in the growing region of the epicotyl tissue. Kinetic analysis of the data resulted in dissociation constant values of: $K_1 = 2.2 \times 10^{-7}$ M, $n_1 = 1.8 \times 10^{-10}$ moles/g fresh wt; $K_2 = 11 \times 10^{-7}$ M, $n_2 = 3 \times 10^{-10}$ moles/g fresh wt. These sites were not found in the non-growing region of the pea epicotyl suggesting that they may be involved in the growth process.

From the competition studies reported here, it would appear that site 2 showed greater auxin specificity than site 1 and this could be considered a candidate as an auxin receptor.

Sucrose gradient fractionation techniques were employed to further separate the two binding sites and it was shown that site 2 binding was associated with fractions rich in plasma membrane while site 1 was associated with the endoplasmic reticulum.

Separation of the solubilized sites by gel permeation methods indicated an apparent molecular weight of 42,000 daltons.

IAA was shown to complex with the polar head group region of phospholipids, in $CDCl_3$, although the strength of the complex was rather low ($Kd = 1.9 \times 10^{-2}$ Molal).

The strength of binding was influenced by the polar head groups of the phospholipids, but did not appear to be affected by the fatty acyl chain length.

The physiological significances of such interactions are discussed.

CONTENTS

INTRODUCTION

1. HISTORICAL BACKGROUND

Plant growth is controlled in a variety of ways and one of the more important control systems is provided by the plant growth substances - the so-called plant hormones. Many definitions of plant hormones have been formulated, such as; "A plant hormone is an organic substance which is produced within a plant and which will, at low concentrations, promote, inhibit, or qualitatively modify growth, usually at a site other than its place of origin. Its effects do not depend upon its calorific content or its content of essential elements." (Hill, 1980).

The plant growth regulators can be subdivided into five groups; the auxins, gibberellins, cytokinins, inhibitors and ethene; a division dependent mainly on their chemical structure, and their physiological effects.

Many auxins are related to indole acetic acid (IAA), and indeed IAA appears to be the principle auxin in most plants. Other indole compounds are found in plants but it is thought that their auxin-like activity is due to conversion to IAA (e.g. Leopold, 1955).

Attempts have been made to elucidate the mechanisms involved in auxin induced growth, but since the discovery

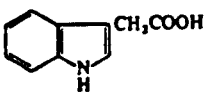
of IAA about 50 years ago little progress has been made in determining its primary mode of action.

One of the problems facing research workers is the variety of responses that auxins can evoke. The auxins can affect root growth, fruit formation, leaf fall, inhibition of bud formation, sex expression and growth. The nature of the response depends on the kind of tissue and its developmental stage, a phenomenon that is unique to plants (Trewavas, 1981).

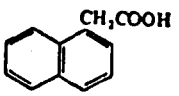
There have been many attempts to determine a unified structure -activity relationship for the group of chemicals exhibiting auxin activity (fig 1). Veldstra (1944a; 1944b) proposed two basic tenets for auxin activity - interaction at a membrane site and that this interaction should be non -covalent.

Many theories have been advanced over the years but none have been totally satisfactory. The charge -separation theory of Porter and Thimann (1959; 1965) appears to hold good for most classes of auxins. The theory states that active auxin molecules are characterized by the presence of a fractional positive charge situated 0.55 nm from the negatively charged carboxyl group. Farrimond et al. (1978) agreed with this theory but suggested that the critical separation distance was 0.50 nm rather than 0.55 nm.

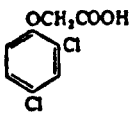
The main conclusion from the literature is that plant



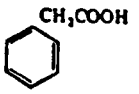
IAA
Indole-3-acetic acid



NAA
Naphthalene-1-acetic acid



2,4-D
2,4-Dichlorophenoxy-acetic acid



PAA
Phenylacetic acid

Fig. 1

Chemical structures of some of the more common auxins.

cells should have specific recognition sites for auxins; ie they should contain receptors (for review see Venis, 1985).

Over the years there have been many theories as to how auxins stimulate growth, or more specifically, induce stem elongation. Most of the theories have fitted the current trend in biochemical research at the time. This is not altogether surprising since cell enlargement is the net result of a number of events. The initial size increase is due to water uptake and the cell wall increases its surface area as a result of biochemical/biophysical changes in its structure. Enhanced ribonucleic acid (RNA) and protein synthesis also occurs which requires an increase in respiratory energy (Cleland, 1971a). Auxins could influence cell enlargement at any of these points.

Early studies indicated that IAA stimulated respiratory metabolism in elongating stem tissue and it was postulated that the primary effect of auxins was on this process. The stimulated respiratory metabolism is now thought to be the result rather than the cause of elongation (Rowan et al., 1972).

It had also been postulated that IAA had its primary effect on protoplasmic streaming in cells (Thimann and Sweeny, 1937), since the rate of streaming was immediately increased by the addition of IAA. Cande et al. (1973) found that cytochalasin B inhibited streaming but had no effect on auxin-induced growth, suggesting that streaming was not

necessarily involved in growth.

In view of IAA's small molecular size and its occurrance in plants in very low concentrations, it was, at one stage, considered to act as a co-enzyme. In the early forties new improved protein separation techniques were used to try and isolate a protein fraction that contained auxin (Bonner and Wildman, 1946). It was then hoped that this fraction would be associated with some enzyme important in the development of the plant. These research workers reported isolation of a protein fraction from spinach leaves which contained a high auxin level and was associated with a phosphatase enzyme. It was later demonstrated that the IAA was formed by the reaction of hot alkali (part of the extraction procedure) on tryptophan in the protein (Schoken, 1949).

At a later date it was thought that auxins were involved in nucleic acid metabolism (Trewavas, 1968; for review). The basis of this theory was that it would explain the multiple effects of IAA and its ability to be active in such low concentrations (Jacobs, 1979). Bendana et al., (1965) reported that IAA stabilized soluble RNA (tRNA) thus giving it a longer half-life, but their separation techniques were later found to be faulty (Davies and Galston, 1971). However a later report (Kobayashi and Yamaki, 1972) maintained that tRNA-IAA complexes were found in mung-bean hypocotyl tissue. The RNA was characterized by column chromatography and ion -exchange columns, and

tested for binding to radioactive IAA. The radioactive peak overlapped the tRNA peak on a methylated albumin-kieselguhr column. Similar experiments were carried out on pea stems (Davies, 1971), but no peak of radioactivity was found associated with RNA.

Research carried out by Fellenberg (1969) suggested that the auxins may act by direct interaction with the genome. IAA, ∝-napthylacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) lowered the melting point (Tm) of pea epicotyl nucleoprotein. IAA also had the same effect on deoxyribonucleic acid (DNA). This effect was not specific to auxin-receptive tissue since salmon-sperm DNA was also affected by IAA and NAA (Fellenberg, 1971). Some of these, and other, experiments were carried out by Penner and Early (1972), and they found that all the auxin-induced changes in the DNA Tm were, in fact, due to changes in the pH of the solutions. When the pH was maintained at pH 7, there were no significant changes in the Tm values. Pea histone samples bound auxins, but the affinities observed were too low to be physiologically significant (Venis, 1968). Ebright and Wong (1981) suggested that IAA acted in a similar manner to adenosine 3', 5' cyclic phosphoric acid (cAMP) in that it could enter the DNA complex by taking the place of adenine. This would then destabilize the complex and thus enhance transcription.

Relatively recent studies have described how IAA could

affect RNA synthesis and RNA polymerase activity. Hypocotyls pretreated with 2,4-D showed a concentration dependent increase in RNA synthesis using isolated chromatin (O'Brien et al., 1968). A factor isolated from soybean cotyledons enhanced RNA polymerase synthesis in chromatin from control plants, but not from 2,4-D treated plants (Hardin et al., 1970). The in vivo action of the IAA on the polymerase was via this factor, so polymerase from treated plants was already active and could not be stimulated further. This stimulation was small and the factor also stimulated polymerase from E.coli..

Venis (1971) studied this effect by quite a different method. The E-lysyl derivatives of IAA and 2,4-D were linked to Sepharose and crude extracts of pea and corn were passed through the column. Material bound to the column could be eluted successively with 1M NaCl and 2mM KOH (pH 11.2) and the material eluted by the 2mM KOH stimulated RNA synthesis, using DNA as a template, by up to 200% . However auxin was not needed for stimulated activity and it was not possible to detect auxin binding to the eluted fraction. In view of the severe elution conditions needed, it is possible that the retained fraction did not contain real receptors for auxins. Both Venis (1973) and Cherry (1973) suggested that auxins interacted with the plasma membrane causing the release of some factor which could affect a specific RNA polymerase leading to an altered transcriptional pattern.

Equipment designed to measure elongation over short periods of time renewed interest in rapid responses to auxins. Evans (1974) found a time lag of 6-15 minutes after addition of auxin before elongation commenced. So for an auxin effect to qualify as the primary response, it must occur within this time limit. Masuda and Kamisaka (1969) claimed that auxin stimulated RNA synthesis within 10 minutes. This suggested that the primary effect of auxin was to affect RNA synthesis. Use of a higher temperature, higher IAA concentrations and faster flow rates of the IAA solutions resulted in the disappearance of the time lag before elongation (Evans, 1974). Neither actinomycin D nor cycloheximide had any effect on the time lag before elongation occurred, as would be expected if the rapid elongation was directly dependent on de novo synthesis of RNA or protein (Evans and Ray, 1969; Cleland, 1971b).

More recent studies (Sakurai and Masuda, 1979) showed that cycloheximide and cordycepin both depressed auxin-induced β-glucan degradation of cell wall, elongation and cell wall loosening. In work carried out on E.coli. (Drobysheva and Drobysheva, 1979) IAA at low levels was able to stimulate biosynthesis, but was inhibitory at high levels.

The hydrogen ion secretion theory was formulated by Rayle and Cleland (1970) to explain pH dependance of cell elongation. After treatment with acidic buffers, Avena

coleoptiles showed growth rates similar to those obtained with optimal auxin concentrations. Maximal growth was obtained at pH 2.3-3.0, and growth inhibited if the pH was increased to pH 7, indicating that the low pH was more than just an initiation process.

It was found that the cuticle was a significant barrier to hydrogen ions (Rayle, 1973); after its removal stem segments showed maximal growth at pH 5. Hydrogen ions induced wall extensibility both in vivo (Rayle and Cleland, 1972), and in vitro (Rayle et al., 1970). In view of the similarities between the in vitro acid response and the in vivo responses to low pH and auxin, a wall-acidification theory has been proposed independently by Hager et al. (1971) and Cleland (1971a; 1971b). This proposed that auxin regulated wall loosening by causing a drop in pH in the cell wall, and Hager et al., (1971) suggested that this was accomplished by auxin stimulating a plasma membrane adenosine triphosphatase (ATPase) which pumped protons into the cell wall from the cytoplasm. This would lead to an increase in wall plasticity by stimulating a cell wall hydrolase with an acidic optimum. Other workers have suggested a more direct action such as the breaking of acid-labile covalent bonds (Rayle and Cleland, 1970), or the breaking of hydrogen bonds in the wall (Keegstra et al., 1973).

No pH optimum for acid-induced extension was found (Rayle and Cleland, 1972), a result which tended to remove

support for the cell wall hydrolase theory. A very high negative free energy of hydrolysis was required for acid lability at a pH as high as pH 5, making the concept of acid-labile covalent bonds unlikely (Ray, 1974). Rayle and Cleland (1972) found that acid treatment of walls in the absence of tensile stress did not make the walls capable of extension when they were later stressed at neutral pH. This suggested that the bonds reformed as quickly as they were being broken. Ray (1974) suggested that the acidic pH alters the ionic interactions or hydrogen bonds between polymers that are not covalently linked, allowing them to slide past each other when under stress. Keegstra et al. (1973) have postulated that low pH may weaken hydrogen bonding between cellulose microfibrils and the xyloglucan component of the wall matrix, allowing the microfibrils to slip through the matrix.

Jacobs and Ray (1976) found that auxin induced a decrease in free space pH in peas and maize segments. This drop in pH began to occur before increased elongation in response to IAA. p-Chlorophenoxyisobutyric acid (PCIB) and phenylacetic acid (PAA) did not cause significant elongation stimulation nor any substantial change in free space pH. Since both were chemically similar to IAA, and as strongly acidic, these results suggest that the pH decrease seen with IAA was not due to diffusion of undissociated IAA into the free space. Penny et al. (1975) in a similar experiment did not detect any marked pH drop in response to IAA, but the microelectrodes used were not

pushed far enough into the xylem vessel so no changes would have been detected (Philip, 1978).

Cleland's results (1976b) agreed with these findings. He also found that potassium uptake equalled proton extrusion, and that both processes were stimulated equally by the phytotoxin fusicoccin (FC) and IAA over 3-4 hours. It was thought that they may activate an electro-neutral K^+-H^+ anti-port system which required ATP. Evidence for coupling proton extrusion to potassium uptake has also been forwaded by Marré et al. (1974), but some authors believe that the acidification of the cell wall does not necessarily mean that auxins activate a proton pump. Sloane and Sadava (1975) claimed that acidificaton was due to carbon dioxide (CO_2), and Haschke and Luttge (1975) suggested that retention of respiratory CO_2 contributed to the free space pH drop either directly or through formation of organic acids.

There have been opponents to the acid growth theory. Parrish and Davies (1977) and Vanderhoef et al. (1977), working on pea stems and soybean hypocotyls respectively found that the segments secreted protons in the presence and absence of IAA, and that IAA did not stimulate this effect. Perley et al. (1975) argued that IAA and exogenously applied protons promoted growth via different mechanisms. Pope (1977) separated acid-induced growth from IAA-induced growth by a pH3/pH7 pretreatment which inhibited only the acid-induced growth. Pope (1978)

proposed that IAA promoted the breaking of specific covalent links in the non-cellulose cell wall matrix while protons affected the non-covalent ones, via two separate processes and Lado et al. (1977) have suggested that auxins influence proton secretion by indirect methods.

The whole subject received some clarification with the discovery that there are two separable growth responses to auxin (Vanderhoef and Stahl, 1975; Dute and Vanderhoef, 1979); an early burst of growth and a later phase associated with long term, steady state growth. Acid-induced growth mimicked the auxin-induced fast response which was associated with cell wall loosening (Vanderhoef et al., 1977). A further difference between the two responses was that actinomycin D had no effect on the initial response, but the second response was strongly affected by it (Vanderhoef, 1979) indicating that gene expression may be involved. Vanderhoef and Dute (1981) suggested that the second response may be due to auxin-induced supply of wall material in its regulation of elongation.

It has been suggested that the primary stage in growth extention involved the cell wall becoming more ductile (Heyn, 1981). A dextranase was found to break down some cell wall components to give arabinose and glucose. The activity of this dextranase was dependent on auxin and the breaking of these cross-linkages would impart the necessary plasticity to the wall for extension to occur. Nishitani

and Masuda (1981) indicated that IAA caused cell loosening 15 mins after application. This was accompanied by a decrease in the arabinose and galactose content in the hemicellulose, and an increase in the viscosity of pectin. It was suggested that it was via these processes that IAA brought about cell loosening and therefore the extension of the cells.

Indirect evidence in favour of the acid secretion theory came from Evans et al. (1980) and Moloney et al. (1981) who found that auxin-induced modification of cell wall pH played a part in elongation control in roots. Thus it would appear that the current weight of evidence lies in favour of the acid secretion theory.

2. RECEPTOR THEORIES

The rapid responses to auxins led to the assumption that the initial interaction with auxin was at the cell surface, or more specifically, on the plasma membrane. Although gaining support, it is not a new idea. In the early structure-activity studies it was proposed that auxin molecules should have a high surface charge in order to form a reversible non-covalent attachment with the plasma membrane lipids (Veldstra, 1944b).

Application of procedures used in enzyme kinetic

studies to auxin-induced growth in coleoptiles implied that auxins interacted with a receptor (Bonner and Foster, 1956; Housely, 1961) although no attempt was made to define this receptor. The existence of an auxin-specific transport system also lent support to the idea that there were physio-chemical interactions with receptor molecules in the tissue (Hertel et al., 1969). Organic acids similar to IAA but lacking auxin activity in stimulating growth, e.g. benzoic acid (BA), were not transported, and auxins with lesser growth promoting activity were less efficiently transported, e.g. L(-) 3-indole-2-methyl acetic acid as compared to D(+) 3-indole-2-methyl acetic acid.

The similarities between auxin specific transport and growth promotion suggested a common site of action at the plasma membrane. Auxin binding at the proposed site was thought to be reversible and non-covalent (Hertel and Flory, 1968). Hertel et al. (1969) proposed that the primary site of auxin action may be the specific stage in transport. Later studies supported the idea of a reversible, non-covalent interaction of auxins and auxin transport inhibitors with a plasma membrane receptor, but there was no close correlation between transport and growth since the transport inhibitor 1-N-naphthylphthalamic acid (NPA) had no effect on growth either alone or with IAA (Thomson et al., 1973).

In many other systems proton transport was known to result from the activity of membrane bound ATPases (e.g.

Bowman and Slayman, 1977; Scarborough, 1976), and it has been suggested that auxins activate an ATPase-proton pump (Hager et al., 1971; Cleland, 1976b). The presence of plasma membrane associated ATPases has been reported in a variety of tissues including corn roots (Hall, 1971), oat roots (Hodges et al., 1972) and in mung bean hypocotyls (Kasamo and Yamaki, 1974a). Activation of a magnesium-dependent ATPase by in vitro treatment with IAA has been reported in mung bean hypocotyls (Kasamo and Yamaki, 1974b). This activation was small and the association of the enzyme with the membrane was not vigorously tested. Cross et al. (1978a) were able to solubilize auxin receptors with Triton-X 100 while the majority of ATPase activity remained pelletable. Fractionation by gel exclusion chromatography indicated that the receptors did not have ATPase activity, suggesting that auxin did not interact directly with an ATPase-proton pump.

Some animal homone effects are mediated indirectly by cAMP, and it is known that the hormone receptors are distinct from adenylcyclase (Sahyoun et al., 1977), an observation which led to the mobile receptor concept of hormone action. An analogous situation could apply in plants, where the auxin-receptor complex may interact with and stimulate, possibly indirectly, a separate ATPase pump (Cross et al., 1978a). Venis (1977c) also questioned Kasamo and Yamaki's results. Using data on a purified ATPase from Schwartz et al. (1975) with results from Kasamo and Yamaki

(1974b), he calculated that there was one IAA molecule for every 250 ATPase molecules at 10^{-13} M IAA - the highest concentration at which IAA still stimulated the ATPase. He concluded that this ratio could not be reconciled with maximal enzyme activation or indeed with any activation at all. Thompson et al. (1983) have reported the activation of an ATPase pump by auxins. Use was made of a lipid bilayer membrane and changes in the ion flux across the membrane were recorded. Only when the receptor, NAA and ATP were present together was there a substantial increase in current. It was suggested that the auxin receptor and the ATPase were loosely associated and functionally dependent.

Recently an auxin-stimulated ATPase was found in membrane fractions prepared on linear sucrose gradients (Scherer, 1981). The ATPase was shown to be associated with the plasma membrane, and was auxin specific in that it was stimulated by the active auxins IAA and 2,4-D, but not by its inactive analogues 2,3-D or 3,5-D.

Kubowicz et al. (1982) reported the presence of an ATP-dependent Ca^{2+} pump on the plasma membrane. IAA treatment led to a 100% increase in Ca^{2+} transport activity, and pretreatment with cytokinin was inhibitory. It was suggested that growth regulation may result from changes in the efflux pumping of Ca^{2+}. The importance of Ca^{2+} was also shown by Buckhout et al. (1980), who indicated that Ca^{2+} had the opposite effect to auxins in many responses. There was also evidence that auxins

promoted release of Ca^{2+} from membranes. This divalent ion release was not ion specific, in that Mn^{2+} was released in the same way, but auxin specific (Buckhout et al., 1981). Changes in the ultrastructure of plasma membranes were reportedly caused by auxins and Ca^{2+} (Morre and Bracker, 1976). IAA caused the membrane to become 10-15% thinner than controls and Ca^{2+} caused a thickening of the membranes. How important this was is not clear since the concentrations of Ca^{2+} used were much higher that physiological levels.

The protein calmodulin is thought to be involved in the action of plant growth regulators (Kelly, 1983; for review). The Ca^{2+} - calmodulin complex has been linked to the events that occur between the primary action of the growth regulator and the morphological expression of its effects. Inhibitors of calmodulin action were shown to retard auxin induced elongation of corn and oat coleoptiles (Raghothama et al., 1983). Two classes of calmodulin inhibitors at low concentrations stimulated, and at higher concentrations inhibited the responses to auxins, gibberellin and cytokinin (Elliott et al., 1983), suggesting calcium involvement in the action of plant growth regulators and supporting a unifying theory of action.

Hertel (1983) tried to correlate the auxin transport system with auxin action. According to the chemiosmotic theory of transport (Rubery and Sheldrake, 1974), transport

consists of two processes - an accumulation into the cytoplasm by a pH gradient across the plasma membrane, and an efflux of auxin through polar channels in the plasmalemma. This has been supported by uptake experiments in coleptile segments (Sussman and Goldsmith, 1981a and 1981b; Edwards and Goldsmith , 1980). Hertel suggested that the IAA exit carrier also acted as a carrier for Ca^{2+} influx. As a result of this carrier Ca^{2+} would leak into the cytoplasm where Ca^{2+} enhances the flow of Golgi vesicles and thus cell elongation (Hertel, 1983).

The search for binding sites on membranes or cell organelles has been a more recent area of interest for those trying to elucidate the biochemical basis of auxin action.

An estimation of the maximal affinity of auxin for its binding site could be calculated from the saturation ~~of~~ kinetics of a physiological auxin effect such as growth or transport (Hertel et al., 1972). The Km of the in vitro binding should not be much lower than the upper auxin levels where the physiological effect still responded to a concentration change. The in vitro reversible binding of IAA and NAA to particulate fractions from corn coleoptiles was demonstrated (Hertel et al., 1972). Crude membrane fractions were prepared to which were added radioactive auxin at concentrations of 10^{-7} -10^{-6} M. To some of these samples were added unlabelled auxin or other competitors at higher concentrations. The samples were centrifuged at

high speed and the radioactivity in the membrane pellets determined. The kinetic parameters of IAA and NAA were determined using Scatchard analysis (Scatchard, 1949) and dissociation constants of 1.2×10^{-6} M and 3.4×10^{-6} M for NAA and IAA respectively were calculated.

Auxin specificity of binding was tested using a range of auxins and auxin analogues, and their ability to displace ^{14}C-NAA from the membrane fractions was examined. Of the compounds tested, only auxins (2,4-D, NAA and IAA) and auxin analogues that affect transport (triiodobenozoic acid - TIBA- and PCIB), were found to compete with NAA binding (Hertel et al., 1972). These crude binding sites were shown to be heat labile, free of DNA, starch and mitochondria, and sedimented through 40% sucrose.

Both TIBA and NPA are effective inhibitors of auxin transport (Morgan and Soding, 1958) and since transport processes would be expected to take place at the cell surface, an interaction between these two transport inhibitors and the plasma membrane would appear likely.

Specific NPA binding to a crude membrane fraction prepared from corn coleoptiles was found (Lembi et al., 1971). The binding was reversible and covalent. A direct correlation between specific NPA binding and plasma membrane content was shown to exist as defined by specific staining in electron microscopy. NPA binding was of high affinity - an apparent dissociation constant of 10^{-7}M was

found for a majority of the binding sites, while a small population of the binding sites had a higher affinity (Km = 10^{-8}M). The Km values of *in vitro* NPA binding were in good agreement with the saturation point for the NPA effect on auxin transport, suggesting a correlation between binding and transport inhibition (Thomson *et al.*, 1973). However auxins did not compete for the NPA binding sites (Thomson, 1972) and it was suggested that NPA inhibited auxin transport via different, perhaps neighbouring sites at the plasma membrane (Thomson *et al.*, 1973).

The NPA binding sites have been localized immunochemically (Jacobs and Gibert, 1983). Studies on pea stem tissue indicated that the presumptive auxin transport carrier was localized on the plasma membrane at the basal ends of parenchyma cells sheathing the vascular bundles.

TIBA was also shown to bind specifically to the plasma membrane of corn coleoptiles (Hertel *et al.*, 1972) and in contrast to NPA, did inhibit auxin binding, suggeting that the transport inhibitors act via different primary sites.

In the auxin binding studies carried out by Hertel *et al.* (1972), binding was examined over a wide auxin concentration range and results indicted a single class of auxin binding sites in the corn coleoptiles. Batt *et al.* (1976) examined this auxin binding in more detail, and Scatchard amalysis of the data indicated the presence of at least two classes of high affinity NAA binding sites, with

dissociation constants of 1.8×10^{-7} M and 14.5×10^{-7} M. The higher affinity site has been termed site 1 and the lower affinity site as site 2. IAA was also shown to bind to two sets of binding sites, with somewhat lower affinities than NAA. Competition data suggeted that the two auxins bound to the same sites. The binding reported by Hertel et al. (1972) appeared to be predominantly site 2 binding (Batt et al., 1976).

Site 2 showed specific binding compatible with that expected of an auxin receptor site (as defined by Hertel et al., 1972) in that only active auxins, anti-auxins or transport inhibitors were able to compete with NAA binding. Site 1 was shown to be less specific in that inactive analogues (BA, 2,4-dichlorobenzoic acid (2,4-B) and 2,4-D) were also able to compete with NAA for binding (Batt et al., 1976).

Further characterization of these two binding sites was carried out (Batt and Venis, 1976) wtih extensive use of centrifugation techniques and the use of enzyme markers to try and chacraterize the membrane fractions involved. Differential centrifugation was used to separate site 2 binding from site 1, and this fraction was shown to be rich in mitochondria. However site 2 binding was separated from succinic dehydrogenase (SDH) activity (considered a mitochondrial marker) by use of sucrose gradients. Site 1 binding and site 2 binding could also be separated by use of discontinuous sucrose gradients (Batt and Venis, 1976).

By sterol : phospholipid ratio determinations, and by specifically staining sections for electron microscopy, the gradient bands containing site 2 binding appeared to be rich in plasma membrane. The lighter gradient bands containing site 1 were enriched in Golgi membranes and endoplasmic reticulum.

Ray et al. (1977a), also working on corn coleoptile tissue, found evidence for only one class of NAA binding sites. At high concentrations of unlabelled NAA there was still an appreciable amount of ^{14}C-NAA bound to the membrane fractions which was not concentration dependent. This binding has been termed "non-specific" binding. The non-specific component of binding must be subtracted from all results, otherwise any kinetically simple binding process would give a curved plot with Scatchard analysis of the data (Klotz and Hudson, 1971). Thus it was suggested that the results obtained by Batt et al. (1976) indicated two classes of binding sites only because they did not allow for non-specific binding (Ray et al., 1977a). The single class of binding sites obtained had a dissociation constant of $5\text{-}7\times10^{-7}$ M, and binding was insensitive to monovalent salts indicating that binding was not primarily ionic. Specific binding was reversibly inactivated by reducing agents such as dithioerythritol suggesting a reducible group, possibly a disulphide group, was located at the binding site and was required for its functioning. Specific binding was also shown to be affected by a heat-stable, organic factor, termed "supernatant factor"

(SF), which was found in the corn tissue (Ray et al., 1977a).

Computer fitting of the NAA binding data for unfractionated maize coleoptile membranes was carried out (Murphy, 1980a) using models with one or more binding sites. The results indicated that only site 1 binding was required for a good fit.

This single NAA binding site was shown to be localized on the endoplasmic reticulum, (Ray, 1977) and the sites were associated with the membrane and not the attached ribosomes. Results also indicated that a small minority of the NAA binding sites were associated with the Golgi and/or plasma membranes.

The localization of the major auxin binding site on the endoplasmic reticulum (ER) would appear to conflict with the suggestions that the primary auxin action should take place on the plasma membrane, and Ray forwarded a hypothesis to explain how the ER localization (Ray, 1977) could bring about the proton excretion thought to be involved in cell extension (Cleland, 1975; Jacobs and Ray, 1976). Binding of the auxin to the ER binding site could induce proton transport from the cytoplasm into the ER cisternal space. These protons, along with secretory proteins, would be transported, possibly in the Golgi system, to the cell exterior - the cell wall space. Transport to the cell exterior via the Golgi appartus would

take 10-20 minutes (Bowles and Northcote, 1974), which was the lag associated with auxin action on elongation and acid excretion (Jacobs and Ray, 1976). The hypothesis also suggested a link between the rapid and long term effects of auxin which were thought to be two separate responses (Vanderhoef et al., 1976). The long term auxin effects that involve protein synthesis stimulation may stem from better delivery of secretory proteins into the ER lumen (Ray, 1977).

As a further test of the suggestion that this ER auxin binding site was a receptor site, its affinity for auxin analogues and related ring compounds was examined (Ray et al., 1977b). Over forty compounds were tested for competitive displacement of ^{14}C-NAA from the binding site. All the active auxins tested bound to the site, as did analogues that acted as competitive antagonists of auxin action. These compounds were also tested for their ability to elicit a cell elongation response in maize coleoptile segments. These activities generally paralleled the affinities of the auxin binding site from the same compounds. This was especially true when experiments were carried out in the presence of SF (Ray et al., 1977b), and the results would tend to indicate that the sites examined could be physiological receptor sites.

Venis (1977a) was able to solubilize the two classes of binding sites already described (Batt and Venis, 1976) by precipitation with acetone. The dissociation constants of

the solubilized binding sites were in good agreement with those already deduced from membrane experiments (Batt et al., 1976), suggesting that the sites were not affected by the solubilization procedure. Partial purification of the sites was achieved by gel filtration and ion exchange, and separation on Sephadex G-100 resulted in an asymmetric peak. The apparent molecular weight at the shoulder was 47,300 daltons, and at the peak was 40,300 daltons (Venis, 1977a). It was suggested that these two values represented the molecular weights of the two binding sites, which were partially separated on the column.

Cross and Briggs (1978) also solubilized the auxin binding sites from corn coleoptiles by acetone precipitation and by use of Triton X-100. There was no difference in the activities of the binding sites solubiized by the two methods, but results did differ from those obtained by Venis (1977a). Only one auxin binding site was obtained as determined from kinetic experiments, and only one chromatographically distinct binding site was partially isolated. The molecular weight of the binding site was 80,000 daltons, and aggregation of the molecules occurred when the ionic strength of the buffer was lowered (Cross and Briggs, 1978). It was suggested that Venis (1977a) had obtained a lower molecular weight for the binding species because he did not protect against proteolytic attack. No explanation was offered for the difference in the numbers of binding species.

Venis (1980) made a detailed study on the molecular weights of the binding sites in an attempt to clarify some of these discrepancies. No auxin binding protein had a molecular weight greater than 40,000-45,000 daltons, even in the presence of a protease inhibitor, nor did any aggregation occur at low ionic strength. It was suggested that the 80,000 mol. wt. protein found by Cross and Briggs (1978) was itself an aggregate. Acrylamide gel patterns (Venis, 1980) suggested the presence of two auxin binding proteins and this was supported by isoelectric focusing and ion-exchange chromatography. The auxin binding proteins did not associate with ATPase, IAA-oxidase or auxin conjugating enzymes. Murphy (1980b) also found a mol. wt. of about 40,000 daltons for acetone prepared membrane binding sites. There was agreement on one point however, Cross and Briggs (1978) showed that a free sulphydryl group was required for binding. These results substantiated those of Venis (1977b).

The auxin binding site examined by Cross and Briggs (1978) was later shown to be associated with the endoplasmic reticulum (Cross and Briggs, 1979). This membrane fraction contained hydroxylases which act specifically on aromatic molecules (Beveniste et al., 1978) and the coupling of these two facts led Cross and Briggs (1979) to suggest that the auxin binding site may be directly involved in an electron transport chain which could be involved in either the degradation of IAA or form the basis for auxin action via a proton pump.

Results also showed that the supernatant factor which competitively inhibited auxin binding to the membrane also bound competitively to the solubilized protein (Cross and Briggs, 1979). Venis and Watson (1978) were able to isolate naturally occuring SF from corn tissue and identified it as a mixture of 6-methoxy-2-benzoxazolinone (MBOA) and 6,7-dimethoxy-2-benzoxazolinone (DMBOA). The DMBOA was fifty times more active than MBOA in inhibiting NAA binding.

Jacobs and Hertel (1978) have partially characterized a single class of binding sites from Cucurbita hypocotyls C/ that was localized on the plasma membrane and had a Km of 1-2 $x10^{-6}$M for IAA at pH 5. The binding specificity of the site was measured with several auxins and anti -auxins and paralleled their activities as inhibitors of auxin transport. Therefore it was suggested that this site was involved in polar transport of auxins. This site was described as site III to distinguish it from sites I and II previously described. Goldsmith (1982) suggested that site III binding may in fact be uptake into vesicles, again implying a role in auxin transport. Hertel et al. (1983) gave further indications that site III was associated with auxin transport. Accumulation of auxin by membrane vesicles was saturable and required a pH gradient across the membrane, with a higher pH inside the vesicles. There was also good agreement between the in vivo and in vitro effects of various analogues of NPA.

Dostrom et al. (1980) reported details of a soluble receptor site from tobacco callus tissue. The binding of IAA to this cytoplasmic receptor was very low compared to other binding sites, had a much higher pH optimum of pH 7.5-7.8, but was very specific. The concentration of the sites changed dramatically during each culture period of the callus tissue suggesting a positive role in development. Murphy (1980b) reported the presence of a cytosol NAA binding site in maize coleoptile tissue with a molecular weight of 38,700 daltons. Synthetic auxins were tested for their ability to inhibit NAA binding and better correlation between their growth promoting effects and their ability to inhibit NAA binding was found with this soluble site than with membrane sites.

3. AUXIN INTERACTION WITH PHOSPHOLIPIDS

All cells are isolated from the enviroment by a membrane, which allows passage of some molecules but not others. Lipids form 20-40% of the total dry weight of membranes and of this the main components are the phospholipids. The phospholipids are amphipathic; the hydrocarbon chains are hydrophobic and the polar head groups are hydrophilic. On addition to water they do not form simple solutions but aggregate into a variety of spherical and rod shaped particles. The hydrophobic chains of the molecules cluster together to the exclusion of water, while the polar head groups project into the aqueous

phase. These particles can take on a variety of forms depending on the method of preparation (Szuka and Papahadjopoulos, 1980; for review). They can consist of spherical phospholipid bilayers called vesicles, or larger multi lamellar particles called liposomes. Natural membranes tend to take on the bilayer structure.

It is thought that lipids can form specific sites through the variety of polar head groups on the membrane surface and could play a role in membrane organization and function (Abramson and Katzman, 1968).

Initial experiments carried out by Wiegl (1969b) suggested that there was some specificity in the interaction between auxin and phospholipid. In these experiments ^{14}C-IAA or ^{14}C-2,4-D partitioned out in carbon tetrachloride (CCl_4) if the apolar solvent contained lecithin (phosphatidyl choline). Of the compounds tested, urea, glucose, tryptophan and glycerin did not partition in the same manner. Specificity towards the lipid used was also noted. Cephalin and phosphatidyl serine caused partitioning of the auxin into the CCl_4 phase, although not as efficiently as lecithin, while a variety of other lipids tested had no effect. The partitioning exhibited saturation characteristics; an increase in IAA concentration resulted in a decrease in the percentage of IAA in the lipid phase. Non -radioactive IAA or indolebutyric acid reduced the amount of ^{14}C-IAA in the lipid phase but BA, phenoxyacetic acid and phenylacetic acid did not thus showing specific

competition. IAA did not, however, affect the amount of ^{14}C-2,4-D in the lecithin phase.

On the basis of this work Weigl (1969a and b) proposed an orientation of the IAA to the lecithin molecule to form a complex by hydrogen bonding of the amine proton on the indole ring to the oxygen of the phosphate group.

Veen (1974) tried to correlate these effects with the physiological effects of the auxins. Partitioning of certain auxins between water and lecithin -petroleum ether was tested; IAA and NAA partitioned into the lecithin phase but so did the inactive analogue β-NAA. The inactive compound α-decalylaceetic acid was also found associated with the lecithin phase and it was concluded from these results that the observed interaction of IAA and lecithin was not related to the physiological activity of the auxins and reflected the general solubility of weak acids in organic solvents.

A new model membrane system was developed (Kennedy, 1971) which simulated, in many ways, the behaviour of a plant cell membrane. It consisted of a Millipore filter impregnated with egg yolk lecithin, n-octanol and n-tetradecane and was used in studying the penetration and associated effects of plant growth substances. The auxin analogue 2,4-D (10^{-3}M) caused a marked drop in conductivity across the membrane at pH 3, but not at pH 5.5, suggesting

that it was the non -ionized form that was causing the effect. A water molecule or hydrated ion passing between the phosphatidyl choline head groups would bind to them causing them to tilt and be drawn into the membrane, tending to create an aqueous channel. This mechanism was similar to that proposed by Woolman (1970) to account for the transport of hydrophilic molecules through temporary aqueous channels in the membrane. Substances that tended to hold the phospholipids in their normal position in the membrane would mitigate against the formation of these channels and thus decrease the ion flux through the membrane. The interaction of the 2,4-D molecule with the hydrophilic head groups of lecithin may facilitate transport of the molecule into the body of the membrane.

Kennedy and Harvey (1972) showed that the non -ionized form of auxins (IAA, 2,4-D, 2,4,5-trichlorophenoxy acetic acid and 2,6-dichlorophenoxyacetic acid) bound more strongly to lecithin vesicles than did the ionized forms. Binding of the non -ionized forms also caused an increase in chloride ion flux across the lipid bilayer which did not happen in the presence of the ionized forms.

Lipid vesicles subjected to a pH gradient accumulated weak acids and bases (Crammer and Prestegard, 1977; Nichols and Deaner, 1976) giving further evidence that protein carriers need not be required for auxin accumulation. Rubery (1979) has produced a model for auxin accumulation based on the greater permability of the membrane to the hydrophobic

IAA molecule.

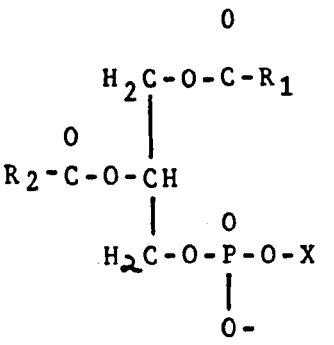
Nuclear magnetic resonance (NMR) studies indicated a strong interaction between indole and phosphatidyl choline (PC) in membranes (Bray et al., 1974) where the indole was thought to intercalate between the PC fatty acid chains near the polar head. The amine proton of the indole ring may form a hydrogen bond with the oxygen on the phosphate group. This was also suggested by Wiegl (1969b). The importance of this bond was indicated by the fact that indole interacted with PC in deuterated chloroform ($CDCl_3$) whilst 1-methyl indole did not. Large upfield shifts of the N-trimethyl group ($N(CH_3)_3$) implied close proximity of this group to the top of the aromatic ring in the indole, and shielding by it. Results also suggested that the indole may affect water structure at the polar head of the PC molecule and may influence water transport across the water -polar head interface.

Paleg et al. (1973) reported the first use of NMR to measure interactions of plant growth substances with PC in $CDCl_3$. On addition of IAA the only PC resonance peak to be affected was that of the N-trimethyl group. This can be compared to pronounced magnetic shielding and deshielding effects associated with an aromatic ring system (Laszlo, 1967). There was no peak broadening with IAA, unlike gibberellic acid, supporting the interpretation of aromatic ring diamagnetic shielding effects. The results also suggested a complex formation between two moles of IAA to

one mole of PC, a complex in which the carboxyl group on the IAA did not play a part; an orientation different to that postulated by Weigl (1969a and b).

Further work by Paleg and his co-workers (Marker et al., 1977) has questioned the validity of the 2:1 complex and suggested a 1:1 complex would fit the results better. The use of three probes (^{1}H, ^{13}C, ^{31}P) meant that the effect of interaction could be determined for most of the nuclear species present in the two molecules. Most of the interactions were not particularly strong (Kd $=10^{-2}$ Molal), even though large chemical shift changes were noted. The chemical shifts suggested that the indole ring was associated with the quaternary nitrogen (as had been reportd by Paleg et al., 1973). The results also suggested that the carboxyl group was projected downwards into the lipids and possibly hydrogen bonded to an oxygen of the phosphate group. This would explain the extent of the IAA effect down into the glycerol region of the lipid.

Weigl (1969a) had suggested that differences in interaction of IAA with different phospholipids (fig 2) were due to changes in their polar head groups. Work on plant PC and dipalmitoyl PC indicated differences in interaction with IAA which were ascribed to changes in the fatty acid composition - one of the most variable characteristics of membrane phospholipids (Marker et al., 1977). There may have also been a measure of specificity in response with different head groups. Phosphatidyl



Where R_1 and R_2 are fatty acyl chains

$R_1 = R_2 = (CH_2)_{13}\ COOH$ dimyristoyl PL

$R_1 = R_2 = (CH_2)_{15}\ COOH$ dipalmitoyl PL

Add X

= H phosphatidic acid

= $CH_2\text{-}\underset{\underset{OH}{|}}{CH}\text{-}CH_2OH$ phosphatidyl glycerol

= $CH_2\text{-}CH_2\text{-}\overset{+}{N}(CH_3)_3$ phosphatidyl choline

= $CH_2\text{-}CH_2\text{-}\overset{+}{N}H_3$ phosphatidyl ethanolamine.

Fig. 2

Chemical structures of phospholipids.

ethanolamine (PE) gave a lower dissociation constant than PC although it was not clear if the fatty acid chains were the same in the two molecules.

Continuation of this work (Marker et al., 1978) again in $CDCl_3$, determined the dissociation constants with different phospholipids. The similarities of the Kd values for PE and PC suggested that the phosphate group was important for binding and that charge-induced dipole interactions between the aromatic ring of IAA and the N-trimethyl group and amino group provided stability to the complex.

Recently further work by Paleg and co-workers has been carried out under more physiologically correct conditions. The interaction of auxins with lecithin liposomes dispersed in deuterium oxide (D_2O) was examined. The auxin -induced changes in chemical shifts of the polar head groups were less than in $CDCl_3$ and the stoichiometry appeared to be 1:1 with a Kd of 4.7 mM at pH 4. The interaction was pH dependent, increasing at lower pH due to protonation of the IAA carboxyl groups (Jones et al., 1984). The interactions were ligand specific in that auxins related to IAA induced different chemical shift changes and gave rise to different dissociation constants (Jones and Paleg, 1984a). Apart from 2,4-D the interactions showed a general correlation with physiological activity. It was thought that the weak interaction with 2,4-D was due to its inability to penetrate deep into the bilayer because of either charge or

geometrical factors.

The behavior of ionic compounds in $CDCl_3$ is quite likely to be different to their behavior in aqueous systems, but interactions with phospholipids would correlate well to their behavior in the hydrophobic interior of the lipid bilayer. Thus some important information on the nature of the interaction could be obtained from these experiments.

The interaction of plant growth substances with phospholipids has also been studied using differential scanning calorimetry (DSC) and electron spin resonance. Pauls et al. (1982) utilized both these methods in a study of the interaction of phospholipids with gibberellins. DSC of phospholipids can be characterized by two temperature transitions - a major peak associated with the "melting" of the crystalline like structure of the bilayer and a lower temperature transition thought to be associated with the "melting" of the polar head group regions of the bilayer (Hinz and Sturtevant, 1972).

With the addition of gibberellic acid to a sample of liposomes the pretransition peak was eliminated and the onset temperature of the major peak was lowered (Pauls et al., 1982). There was not a concomitent decrease in enthalpy suggesting that the gibberellins perturbed the membrane but did not complex to it. Electron spin resonance results confirmed this and also suggested that the

gibberellins associated with the surface of the membrane rather than penetrated into the interior of the bilayer (Pauls et al., 1982).

Since the phospholipid composition in membranes from different tissues, and indeed within cells, is extremely variable, it is possible that complex formation between IAA and membranes from different tissues could produce different effects. Such a mechanism could operate in addition to a protein receptor site.

This thesis confronts some of the unresolved issues raised in the introduction with particular regard to the binding of auxins to cell membranes. It attempts to determine which membrane fractions the auxin binding sites are associated with and whether these binding sites are receptor sites involved in the growth process. It further investigates the characteristics of these auxin binding sites to try and determine their physiological roles. The binding of auxins to phospholipids is also examined to try and elucidate any possible physiological functions from any such interaction.

MATERIALS AND METHODS

1. CHEMICALS

PLANT MATERIAL

Pea seeds (Pisum sativum, Kelvedon Wonder, Griffin and George) were soaked for four hours in tap water, planted in moist vermiculite and grown for one week in darkness at room temperature. Some of the later experiments (gel permeation studies) were carried out on the Lincon variety due to difficulty in obtaining Kelvedon Wonder.

Pea epicotyl sections 3-4 cm long, begining just below the apical node, were harvested on ice in daylight.

AUXINS AND AUXIN ANALOGUES

Radioactive IAA - indole-3-acetic acid-^{14}C - was obtained from the Radiochemical Centre, Amersham, Berks., U.K.. The specific activity of the ^{14}C-IAA was 52 mCi per mmole and the purity of the solution was tested by thin layer chromatography.

IAA, α-naphthylacetic acid (NAA) and β-NAA were obtained from Sigma Chemical Co..

PHOSPHOLIPIDS

Egg yolk lecithin, dipalmitoyl phosphatidylcholine (DPPC), dimyristoyl phosphatidylcholine (DMPC) and dipalmitoyl phosphatidylethanolamine (DPPE) were all purchased from Sigma Chemical Co.. Also obtained from Sigma were; choline, phosphoryl choline, glycero phosphoethanolamine and dicetyl phosphate.

CHEMICALS

Radioactive uridine diphospho-D-glucose (UDP-glucose ^{14}C) (260 Ci/mol.) was obtained from The Radiochemical Centre, Amersham. Deuterium oxide (D_2O) and deutero chloroform ($CDCl_3$) were obtained from Merk. Sephacryl S-200, Sephadex G-25, DEAE 50 and Lentil lectin Sepharose 4B were purchased from Pharmacia and DE-32 from Whatmans. All general chemicals were purchased from either Sigma or BDH and were of analytical grade.

2. MEMBRANE PREPARATION

PARTICAL PREPARATION

All procedures were carried out at 4 °C. Epicotyl sections were chopped up in an equal volume of grinding buffer (0.25M sucrose, 50mM Tris-acetate, pH 8.0, 0.1mM magnesium chloride, 1mM EDTA and 0.5mM

phenylmethylsulphonylfluoride <PMSF>). The PMSF was added as a protease inhibitor (Gardner et al., 1971). This grinding buffer was used as specified except in certain experiments noted below in which extra magnesium was added.

The tissue was homogenized in a Waring blender for 10 secs., the fluid squeezed through muslin and collected. The remaining material was washed in an equal volume of grinding buffer and again squeezed through muslin. The filtrates were combined.

The crude filtrate was precentrifuged at 4,000xg for 30 mins (M.S.E. Superspeed 50) (Batt and Venis, 1976) to remove unfractionated cells and debris and the supernatant used further. Membrane fractions were prepared by differential centrifugation into the following fractions 4,000 -38,000xg fraction prepared by centrifugation at 38,000xg for 30 mins., 38,000 -80,000xg fraction prepared by centrifugation of the supernatant of the previous spin at 80,000xg for 30 mins. (Gurner, 1980). In some cases the initial fraction was further split into the 4,000 -10,000xg and 10,000 -38,000xg fractions.

DISCONTINUOUS SUCROSE GRADIENTS

Membrane pellets were initially obtained by differential centrifugation to give the 4,000 -38,000xg fraction which was then resuspended in 18% (w/w) sucrose in grinding buffer. This was then layered on gradients as described by Hodges et al. (1972). "Complex" gradients

consisted of 2ml of 45% and 3ml each of 38%, 34%, 30% and 25% (w/w) sucrose all in grinding buffer (Batt and Venis, 1976). The % sucrose was the total sucrose concentration in the buffer.

"Simple" gradients were also prepared as described by Batt and Venis (1976) and consisted of 6ml of 45% and 8ml of 30% sucrose.

In experiments involving high magnesium concentrations, the grinding buffer used for homogenization contained 4mM $MgCl_2$ and all the density gradients contained 3mM $MgCl_2$ (Ray, 1977).

Gradients were centrifuged at 100,000xg for 90 mins. at 4°C in a 3 x25ml swing-out rotor. The visible bands at the sucrose interfaces were collected using a Pasteur pipette, diluted with cold grinding buffer and repelleted at 100,000xg for 30 mins. The pellets were then resuspended in binding buffer (0.25M sucrose, 10mM 3-(N-morpholino)propane sulphonic acid <MOPS>, 1mM $MgCl_2$, pH 5.5, 0.5mM PMSF), using a glass-teflon homogenizer, ready for further use.

3. BINDING STUDIES

BINDING ASSAY

The membrane pellets were resuspended in binding buffer and made up to a known volume (usually 25ml). Radioactive

IAA was present usually at a concentration of 2×10^{-7}M. To 4ml portions of the labelled suspension was added 1ml of either binding buffer or an appropriate concentration of unlabelled IAA to give final concentrations up to 10^{-3}M. Triplicate 1.5ml aliquots from each sample were centrifuged at either 100,000xg for 30 mins or 40,000xg for 40 mins.

The supernatants were removed and the pellets drained at 4°C. The tubes and the pellets were rinsed twice with ice-cold binding buffer, left to drain again and the excess fluid on the tubes removed with tissue paper. The pellets were solubilized using 0.5ml of NCS (Amersham) and left overnight at 4°C for digestion to occur. The resultant mixture was transfered to scintillation vials using 3 x3ml of scintillation fluid (Koch Light Scintimix 2). Total radioactivity per assay was determined by counting 100µl samples from the original 5ml aliquots. Samples were counted for 15 mins each in a scintillation counter (Packard Tri-carb 300 CD). This counting time was determined to reduce counting error to less than 5%.

DETERMINATION OF BINDING ACTIVITY

The binding of auxin to a receptor site was assumed to have similar kinetic characteristics to enzyme -substrate interactions and it was also assumed that one ligand molecule bound to one receptor site. Thus they are thought to have reversible binding reactions of the type:

$$A + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AR \qquad \text{(eqn 1)}$$

in which A is the auxin, R the receptor site and AR the complex formed. k_1 and k_{-1} are the rate constants for, respectively, the association and dissociation of the complex.

The reaction is an example of a second order chemical reaction, thus an equilibrium is reached between association and dissociation so that:

$$K_A = (AR) \,/\, (A)\ (R) \qquad \text{(eqn 2)}$$

K_A is the affinity constant for the interaction and is characteristic for A and R. K_A is the reciprocal of the free auxin concentration when the receptor sites are half -saturated ((AR)= (R)). Therefore its value is assumed to correspond to the reciprocal of the auxin concentration at which physiological effects occur - if the receptor has a physiological function.

These parameters can be determined by the method devised by Scatchard (1949) for the binding of small molecules to macromolecules. If in eqn 2 (AR) is replaced by the concentration of bound auxin B, (A) by the concentration of free auxin F and the concentration of receptor sites as n, then eqn 2 can be rearranged as:

$$K_A = B \,/\, F\ (n-B) \qquad \text{(eqn 3)}$$

or as:

$$B / F = Kn - KB \qquad \text{(eqn 4)}$$

A further rearrangement gives:

$$1/B = (K_D + F) / nF \qquad \text{(eqn 5)}$$

or as:

$$1/B = K_D/n \; 1/F + 1/n \qquad \text{(eqn 6)}$$

Thus in the analysis for a homogenous population of binding sites a double reciprocal graph of 1/B against 1/F gives a straight line. The dissociation constant is determined as the negative reciprocal of the abscissa intercept and n is equal to the reciprocal of the intercept on the 1/B axis.

EFFECTIVENESS OF PELLET COUNTING

A variety of methods are available for the transfer of radioactive auxin in the membrane pellet to the scintillation vial. These methods were tested to find the most effective.

Membranes equilibrated with ^{14}C-IAA were pelleted in the normal manner. To some were added 1ml methanol (Ray et

al.,1977a), the tubes shaken for 15 mins and the methanol transfered to the vials.

Batt (1975) transfered pellets using a Pasteur pipette and distilled water, and this method was also tested. Some pellets were digested with 0.5ml NCS - a tissue solubilizer - and the contents transfered to scintillation vials. Other pellets were shaken in scintillation fluid and then transfered to the scintillation vials.

Scintillation fluid was added to a final concentration of 9ml, and vials counted in the usual way, after dark adaptation to mimimize chemiluminescence.

IDENTIFICATION OF RADIOACTIVITY IN THE MEMBRANE PELLET

Since the epicotyl tissue could contain enzymes capable of metabolizing IAA, it was possible that the radioactivity measured was no longer of ^{14}C-IAA, but a radioactive metabolite. A membrane sample equilibrated with ^{14}C-IAA was repelleted and the pellet washed with distilled water. The pellet was then resuspended in a small volume of ethanol. After centrifugation to remove particulate material, some of the ethanol extract was loaded onto a thin-layer silica gel plate (F256 impregnated). Also loaded onto the plate were a sample of stock ^{14}C-IAA solution and some unlabelled IAA solution. The plates were run using 8% NaCl solution as the mobiile phase and after drying were visualized under U.V. light. A travelling

Geiger-Muller tube was used to count the radioactivity along the plate.

EFFECTS OF PH ON AUXIN BINDING

Aliquots of membrane suspension (4,000 -38,000xg fraction) were centrifuged at 100,000xg for 40 mins. Solutions of binding buffer were prepared over the pH range 3-8, containing ^{14}C-IAA at 10^{-7} M with or without IAA 10^{-3} M. Membrane pellets were washed, then suspended in buffer of a particular pH. After equilibrium, the samples were repelleted and the radioactivity of the pellets determined. This procedure was repeated using the 4,000- 10,000xg and the 38,000- 80,000xg membrane fractions.

SOLUBILIZATION OF MEMBRANE PROTEINS

The method followed for the solubilization of proteins from the membrane samples was that of Venis (1977a). Resuspended membrane samples were added dropwise into 20 times their volume of ice-cold acetone, with stirring, and the precipitate pelleted. The precipitate was washed twice in ice-cold acetone before being dried in a nitrogen stream and stored under liquid nitrogen.

EQUILIBRIUM DIALYSIS

Visking tubing was soaked in distilled water for 15 mins, then a suitable length placed beween the two halves

of a dialysis cell. Binding buffer (1ml) containing 14C-IAA was added to one side of the cell and to the other side was added 1ml of the solution to be tested for IAA binding. The cell was sealed and shaken overnight at 4°C. 100µl aliquots were removed from each side of the cell and added to 3ml of scintillation fluid and radioactivity determined.

Binding activity was measured as the difference in radioactivity between the two half-cells.

EFFECT OF TEMPERATURE ON BINDING ACTIVITY

A 4,000- 38,000xg membrane fraction was prepared as previously described and resuspended in binding buffer. Aliquots were incubated at temperatures between 4°C and 100°C for 6 mins, then repelleted at 40,000xg for 30 mins at 4°C. Binding assays were performed on the pellets as described above.

The effect of temperature on the binding activity of the acetone precipitated proteins was also tested. The material was dissolved in binding buffer and aliquots incubated at the required temperature for 6 minutes before being rapidly cooled to 4°C. Binding was then determined by equilibrium dialysis as previously described.

RAPIDITY AND REVERSIBILITY OF IAA BINDING

A Sephadex G-25 column was prepared in a Pasteur pipette (6 x 0.5 cm) and equilibrated with binding buffer containing 10^{-8}M ^{14}C-IAA (Dohrmann and Ray, 1976). Solubilized acetone precipitate was prepared either in binding buffer alone or binding buffer containing 2 x 10^{-7}M and left for several minutes prior to being added to the column. The void volume of the column was collected and assayed for radioactivity.

On a G-25 Sephadex column protein and other large molecules of the sample should be excluded from the column and emerge in the void volume. If they do bind auxin then the measured radioactivity of the void volume should be increased above the basal level. Excess free IAA added to the column should be partially retained by the column and eluted at a later stage.

This could be easily tested by the elution of the sample of binding buffer containing non-radioactive IAA. If this was eluted in the void volume than a decrease in radioactivity would be measured.

As a test for reversibility of binding, two solutions of precipitated proteins were prepared: one contained ^{14}C-IAA (2 x 10^{-7}M) and the other non-radioactive IAA at the same concentration. The samples were left for 15 mins for equilibrium to be reached. To the sample containing ^{14}C-IAA was added non-radioactive IAA and the sample added to the column. To the sample containing IAA was added

^{14}C-IAA and the sample added to the column. The void volumes were collected and radioactivity determined.

4. LOCATION OF IAA BINDING SITES

ASSAY OF ENZYME MARKERS

The activity of the mitochondrial marker enzyme cytochrome c oxidase was determined by the method of Appelmans *et al.* (1955). Membrane suspension was added to 1ml of 0.03M phosphate buffer, pH 7.5, containing 1.7 x 10^{-5}M reduced cytochrome c, in a 1ml cuvette. The decrease in absorbance at 550 nm was measured for 3-6 mins.

NADH-cytochrome c reductase was assayed as described by Lord *et al.* (1973). Membrane suspension was added to 1ml of 50mM Tris acetate buffer, pH 7.4, containing 10^{-3}M KCN, 10^{-4}M NADH and 0.53 mg/ml cytochrome c. Reduction of cytochrome c was measured at 550 nm for 3-6 mins.

The tonoplast marker - acid phosphatase - was assayed by the hydrolysis of p-nitrophenylphosphate. Membrane suspension and 50µl of 0.06M acetate buffer pH 5, containing 1.2% Triton X-100 and 1.5 mg/ml p-nitrophenyl phosphate were incubated together for 20 mins at 35°C. The reaction was terminated by the addition of 60µl 1M $HClO_4$ and 0.6ml 0.2M Na_2CO_3. Absorbance was measured at 400 nm (Semadeni, 1967, modified by Dohrmann *et al.*, 1978).

ATPase was assayed by the method of Lowry and Lopez (1946), modified by Batt and Venis (1976). Membrane suspension was added to 1ml of 20mM Tris buffer, pH 6, containing 5mM ATP (disodium salt), 33mM KCl and 5mM $MgCl_2$. Solutions were incubated at 30°C for 15 mins, then the reaction stopped with 0.5ml of 10% (w/v) trichloroacetic acid, followed by 4ml acetate buffer (0.1M acetic acid, 25mM sodium acetate, pH 4), 1ml ammonium molybedenate (1% soln, in 2N H_2SO_4) and 0.5ml of 10% (w/v) $FeSO_4$ containing one drop of concentrated sulphuric acid. The mixure was shaken, centrifuged on a bench centrifuge and the absorbance of the supernatant determined at 660 nm.

The activities of the glucan synthetases were determined by the method of Ray (1977), and modified from Van der Woude et al., (1974). To 40µl of 50mM Tris buffer, pH 8, containing 17.5 nCi of UDP- ^{14}C-glucose was added 100µl membrane fraction. For glucan synthetase 1 the assay medium also contained 58mM $MgCl_2$, and for glucan synthetase 2 the assay contained 0.5mM unlabelled UDP-glucose and no magnesium. Samples were incubated at 25°C for 15 mins then 1ml of 70% ethanol and 50µl of 50mM $MgCl_2$ were added. This was boiled for 1 min and left at 4°C overnight. The mixture was centrifuged for 5 mins at 1000 xg and the pellet washed four times with 70% ethanol to remove all unreacted radioactive UDP-glucose and ethanol soluble by-products. The pellets were drained then resuspended in scintillation fluid and radioactivity determined.

PROTEIN DETERMINAION

Protein content was determined by the BS7 method of Ohnishi and Barr (1978). The BS7 solution was prepared by diluting Biuret reagent (3g copper sulphate and 9g sodium potassium tartrate were dissolved in 500ml of 0.2M sodium hydroxide. 5g potassium iodide was added and the volume made up to 1L with 0.2M sodium hydroxide) 1:7 with 2.3% sodium carbonate solution.

To 2ml of this was added 0.5ml of suitably diluted protein soluion, mixed and allowed to stand for 10 mins. Then 0.1ml of Folin-Ciocalteau reagent was added, the mixture shaken and the absorbance at 750 nm read. The protein concentration was calculated from a calibration curve determined with bovine serum albumin (0.5 - 5 mg/ml).

ELECTRON MICROSCOPY

Aliquots were taken from the sucrose gradient bands, glutaraldehyde added, as a fixative, to a final concentration of 4% and left for 1 hr. The samples were then centrifuged at 40,000 xg for 40 mins at 4°C, and the pellets left in grinding buffer overnight to leach out any excess glutaraldehyde. The pellets were removed using a glass point, cut into pie-segments, and post-fixed in 2.5% OsO_4 (in 50mM phosphate buffer pH 8, containing 0.25M sucrose) for 2 hrs (modified from Sabatini et al., 1963).

Samples were dehydrated through a graded series of acetone concentrations (30%, 50%, 70% and 90%) for 20 mins each. They were then washed three times in 100% acetone (dried over anhydrous copper sulphate) and three times in propylene oxide. Samples were transferred to a 3:1 mixture of Epon mix : propylene oxide overnight, then two overnight changes into fresh resin, and embedded in fresh polymeriser overnight at 60°C. The embedding resin was that of Luft (1961) - a 1:1 Epon mixture A:B containing 2% DMP30 accelerator (2,4,6,- tri dimethylaminomethyl phenol). Mixture A consisted of Epon (Epikote) 812 (62ml) and dodecyl succinic anhydride (100ml), and mixture B was Epon 812 (100ml) and methyl nadic anhydride (89ml).

Sections were prepared using a Huxley Mark 2 ultramicrotome, carbon coated and viewed under a Philips 30 electron microscope.

COMPETITION STUDIES

Auxin analogues were tested for their ability to compete for the IAA binding sites.

Membrane pellets were prepared as before and resuspended in binding buffer containing 10^{-7}M ^{14}C-IAA. To half of this was added the required concentration of auxin analogue being tested. To 4ml aliquots of either control or treatment samples were added 1ml aliquots of a range of unlabelled IAA solutions of varying

concentrations. The binding assay was then continued as before.

CHROMATOGRAPHY ON SEPHACRYL S-200

A column (50 x 2.6 cm) was packed with Sephacryl S-200 (Pharmacia) and equilibrated with binding buffer containing 10^{-7}M ^{14}C-IAA. Proteins prepared by the acetone precipitation method were dissolved in the same buffer and layered onto the column. Elution was carried out at 20 ml/hr and 2ml eluant fractions were collected. Fractions were monitored for proteins by absorbance at 280 nm. Aliquots (0.1ml) were added to 3ml scintillation fluid and radioactivity determined. To determine the effect of high ionic strength on the elution from the column, experiments were carried out with binding buffer containing 0.1M NaCl.

ION EXCHANGE CHROMATOGRAPHY

DEAE-cellulose (Whatmans DE32) was swollen in 0.5M HCl then washed with distilled water until the pH of the eluant was at pH 4. It was then washed in 0.5M NaOH and washed until the eluant reached pH 7. The ion-exchanger was then washed in a five-fold dilution of binding buffer (Venis, 1977a) until the eluant had the same pH and conductivity as the starting buffer.

A column (4 x 1 cm) was packed with the gel and equilibrated with the buffer. Acetone precipitated protein

was dissolved in the buffer, added to the column and eluted with the buffer until the absorbance of the eluant at 280 nm approached zero. Elution was continued step-wise with binding buffer and binding buffer containing 0.5M NaCl. 2ml fractions were collected and 1ml aliquots were assayed for IAA binding by equilibrium dialysis.

In some experiments Sephadex DEAE A-50 was used as this was expected to give better resolution.

AFFINITY CHROMATOGRAPHY

A 5 x 1 cm column was packed with lentil-lectin Sepharose 4B (Pharmacia) and equilibrated with binding buffer. Acetone precipitated proteins were dissolved in buffer, added to the column and eluted with buffer until the absorbance of the eluant at 280 nm reached zero. The column was sequentially washed with N-acetylglucosamine (0.2M), methyl α-D-glucoside (0.2M), methyl α-D-mannoside (0.2M), and finally 1% sodium deoxycholate. Fractions collected were tested for IAA binding by equilibrium dialysis.

5. INTERACTION OF PHOSPHLIPIDS WITH IAA

Proton spectra were run on a Perkin Elmer R32 90 MHz spectrometer or on a Brucker 80 MHz FT spectrometer, using a sweep width of 10 ppm. For the Perkin Elmer spectrometer

all $CDCl_3$ solutions were locked onto tetrametyl silane (TMS) while for the Brucker spectrometer the deuterium signal was used for locking purposes.

A known concentration of phospholipid in $CDCl_3$ was prepared (approx. 1.5×10^{-2}Molal) and to half of this solution was added IAA to a known final concentration. These two solutions were then mixed in varying proportions thus giving a series of solutions with a constant phospholipid concentration but varying IAA concentrations. NMR spectra of the samples were recorded. The interactions of other auxins and lipid analogues were similarily tested.

Lipid vesicles were prepared by a modification of the method of Szoka and Paphadjopoulos (1980). A known concentration of phospholipid was dissolved in chloroform and added to a round bottomed flask. The chloroform was slowly removed by rotary evaporation so that the phospholipid formed a thin layer over the surface of the flask. Deuterated water was then added and vesicles formed by vigorous shaking over nitrogen.

ANALYSIS OF NMR DATA

The strength of the complex may be deduced from the chemical shifts of resonance peaks resulting from the complex (Nicholson and Spotwood, 1973). Assuming a 1:1 complex governed by an affinity constant K, then:

$$A + D \rightleftharpoons CX \qquad \text{(eqn 7)}$$

Then

$$K = (CX) / (A)\ (D) \qquad \text{(eqn 8)}$$

or

$$K = CX / (A - CX)\ (D - CX) \qquad \text{(eqn 9)}$$

This can be arranged further to give a quadratic equation in CX.

The observed chemical shift can be related to the strength of the complex by the equation:

$$\delta = \frac{\delta_A (A_o - CX)}{A_o} + \frac{\delta_{CX} (CX)}{A_o} \qquad \text{(eqn 10)}$$

$$\delta(A_o) = \delta_A (A_o - CX) + \delta_{CX} (CX) \qquad \text{(eqn 11)}$$

$$(\delta - \delta_A) A_o = (\delta_{CX} - \delta_A) CX \qquad \text{(eqn 12)}$$

$$\therefore \Delta = (CX/A_o)\Delta_o = (\Delta_o/A_o)CX \qquad \text{(eqn 13)}$$

where : δ = observed chemical shift

δ_A = chemical shift of PL molecule

δ_{cx} = chemical shift of the complex

Δ = observed chemical shift from δ_A

Δ_o = chemical shift change in the complex

A_o = phospholipid concentration

D_o = auxin concentration

CX = complex concentration

A computer programme was devised (J. Wyer) to determine the 'best fit' value of the equilibrium constant K. On the basis of experimental A_o and D_o values and a guessed estimate of K, eqn 7 was solved to give CX/A_o values between 0 and A_o, using the Newton-Raphson method. The experimental values of Δ were regressed against the calculated CX/A_o values for a particular guess of K, and the regression coefficient obtained. Depending on this value, the present K value and the nature of the change in the regression coefficient, the value of K was modified and the regression analysis carried out with the new K value. This was repeated until two conditions were satisfied: the regression coefficient was greater than 0.98, and RC-RK was less than 1×10^{-7}, where RC-RK was the difference between

successive values of the regression coeffeicient.

THERMAL TRANSITIONS IN PHOSPHATIDYL CHOLINE

Pure powdered phosphatidyl choline was weighed into an aluminium pan and water added which appeared to uniformly wet the powder. In some experiments IAA was also added. The pan was sealed and placed in the differential scanning calorimeter (Perkin Elmer DSC 4). A similar amount of water was sealed in another pan and placed in the reference sample holder. The temperature was raised to 50°C - a temperature above all transitions of interest - to facilitate water penetration into the largely hydrophobic lipid structure (Cassel, 1973). The pans were then slowly cooled to below the transition temperatures and thermograms run, with heating or cooling rates of 10°C/min.

RESULTS

1. ORIGINAL EXPERIMENTS

Initial experiments were carried out to determine the optimum conditions for IAA binding to pea epicotyl membranes.

EFFECT OF WASHING ON IAA BINDING TO MEMBRANE FRACTIONS

Several workers have published results indicating that a supernatant factor was present in maize coleoptiles that was capable of lowering NAA binding to membrane fractions (e.g. Ray *et al.*, 1977a).

Results here (table 1) indicate that some sort of inhibitor was present in the pea membrane fractions, that it appeared to be water soluble and could be removed by twice washing the membrane fractions. Thus a washing step was routinely employed in further membrane preparations.

EFFICIENCY OF VARIOUS METHODS OF PELLET COUNTING

Use of a glass pipette to transfer pellets to the scintillation vials resulted in a certain amount of material sticking to the glass, thus leading to large errors in counting. While the use of methanol may be possible in the extraction of NAA from membrane pellets (Ray *et al.*, 1977a), it was not the most effective method

Table 1.

The effect of pellet washing on binding of ^{14}C-IAA.

Results are from a typical experiment.

TREATMENT	PELLET d.p.m./ASSAY
ORIGINAL PELLET	558
FIRST WASH	583
SECOND WASH	745
THIRD WASH	634

Statistics?

Table 2.

Efficiency of pellet transfer and ^{14}C-IAA extraction.

Results are from a typical experiment.

TYPE OF EXTRACTION	d.p.m./ASSAY (a)
NCS	2139
Scintillation fluid	678
Methanol	648
Pasteur pipette	598

(a) Background counts have been subtracted from these results.

for IAA (table 2). Total digestion of the membrane pellet by NCS prior to its transfer to the vials gave the highest counts of all the methods tested.

It was true that use of NCS lead to a small increase in the background counts probably due to chemoluminescence. This could be reduced by dark adapting the vials for 24 hours prior to their counting.

TLC OF IAA SOLUTIONS

Under U.V. light one spot was detected for the unlabelled IAA sample with a Rf value of 0.6. Examination of the radioactive counts along the plate (fig 3) revealed that both the stock ^{14}C-IAA solution and the sample extracted from the membrane pellet resulted in single spots also with Rf values of 0.6. These results were comparable to published values (McDougal and Hillman, 1978). Thus it would appear that no metabolic breakdown of the ^{14}C-IAA took place and that the stock solution of ^{14}C-IAA was shown to be pure.

PH OPTIMA OF THE BINDING SITES

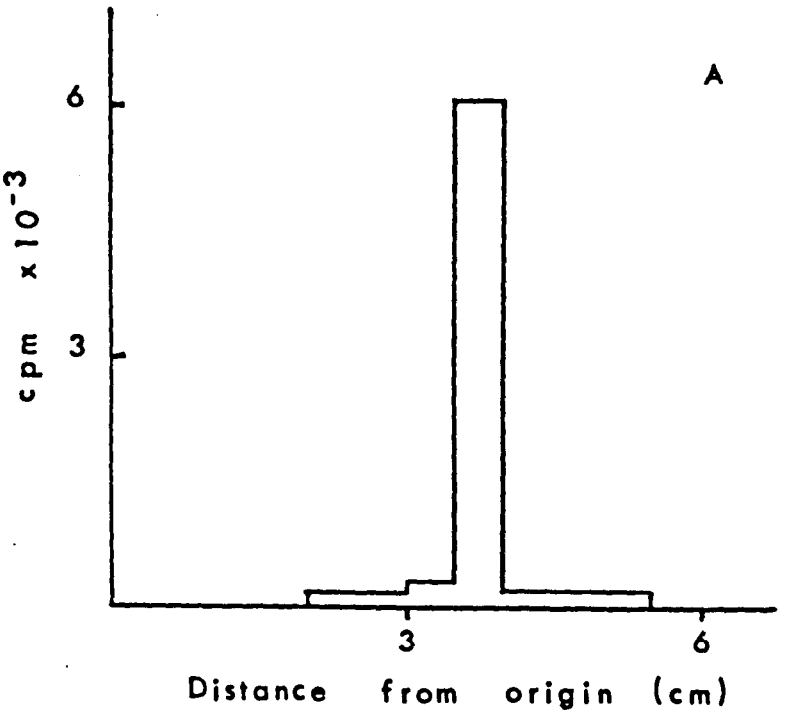
Initial experiments were carried out at pH 5.5, the pH for optimum auxin binding in maize coleoptiles (Batt *et al.*, 1976). Experiments were now conducted to determine the pH optima of the IAA binding sites in pea epicotyl tissue.

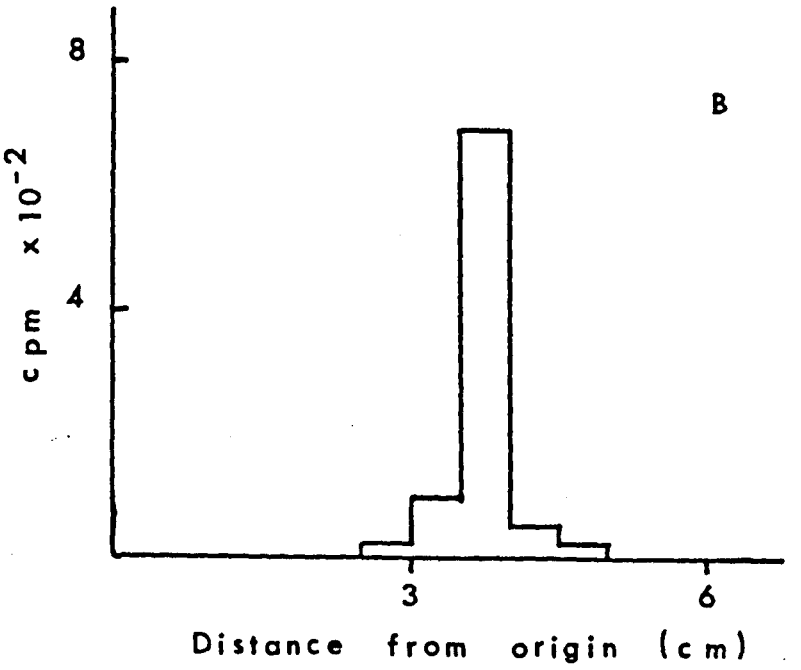
Fig. 3

Thin layer chromatography on silica gel plates using 8% NaCl as the mobile phase.

A = stock ^{14}C-IAA solution.

B = Methanol extract from a radioactive pellet.


A
6
3
cpm x10⁻³
3
6
Distance from origin (cm)


B
8
4
cpm x10⁻²
3
6
Distance from origin (cm)

Results obtained for the 4,000 -38,000 xg membrane fraction indicated two discrete pH optima (fig 4). There was a peak of auxin binding at pH 5 and another at pH 6. Analysis of the 38,000 -80,000 xg fraction indicated a sole pH optimum peak at pH 6 (fig 5). In experiments carried out on the 4,000 -10,000 xg membrane fraction (fig 6) only one binding peak was observed and this was at pH 5. Non-specific IAA binding was shown to decrease with increasing pH (fig 7).

RAPIDITY AND REVERSIBILITY OF IAA BINDING

Binding of ^{14}C-IAA to solubilized acetone precipitated membrane extracts was measured by gel filtration chromatography on a Sephadex G-25 column.

The addition of 2×10^{-7}M ^{14}C-IAA to the extract several minutes before addition to the column did not significantly affect the measured binding as compared to a sample prepared in binding buffer alone (table 3). This suggests that the time taken for the sample to pass through the column was sufficient for equilibrium to be attained.

It is important to note that the amount of labelled IAA added to the sample was greater than the concentration in the column, and that the excess IAA did not appear in the fractions taken for radioactivity measurements (the void volume). This could be further proved by the elution of a sample of binding buffer containing neither solubilized

Fig. 4

The effect of pH on specific IAA binding to the 4,000 - 38,000 xg membrane fraction.

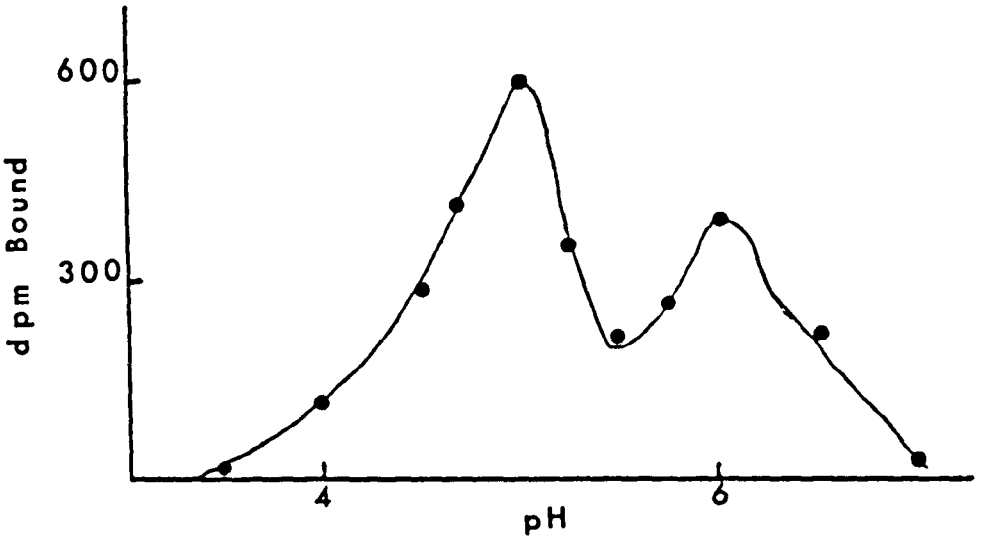
Each point is the mean of three replicates.

Non-specific binding has been subtracted from these results.

Fig. 5

The effect of pH on specific IAA binding to the 38,000 - 80,000 xg membrane fraction.

Non-specific binding has been subtracted from these results.



Joining of points
Buffers. Strength?
Statistics?

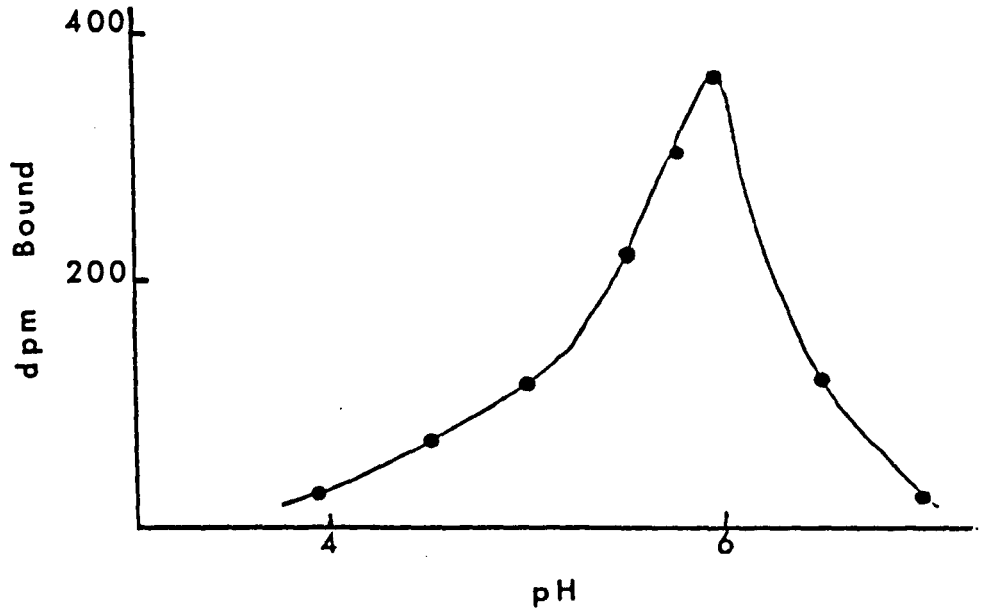


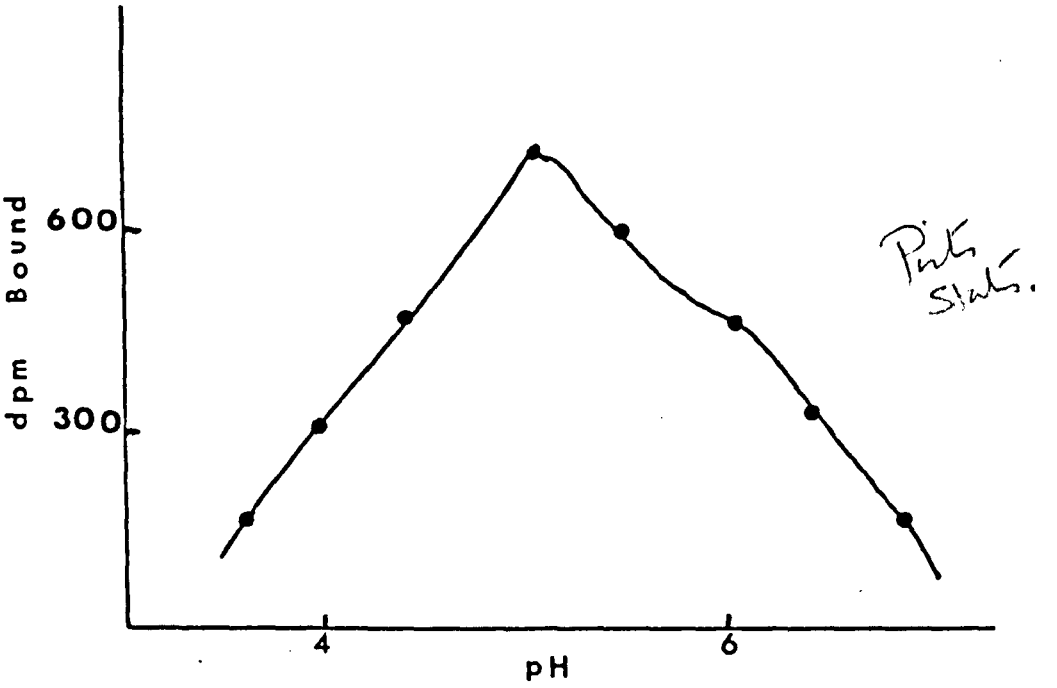
Fig. 6

The effect of pH on specific IAA binding to the 4,000 - 10,000 xg membrane fraction.

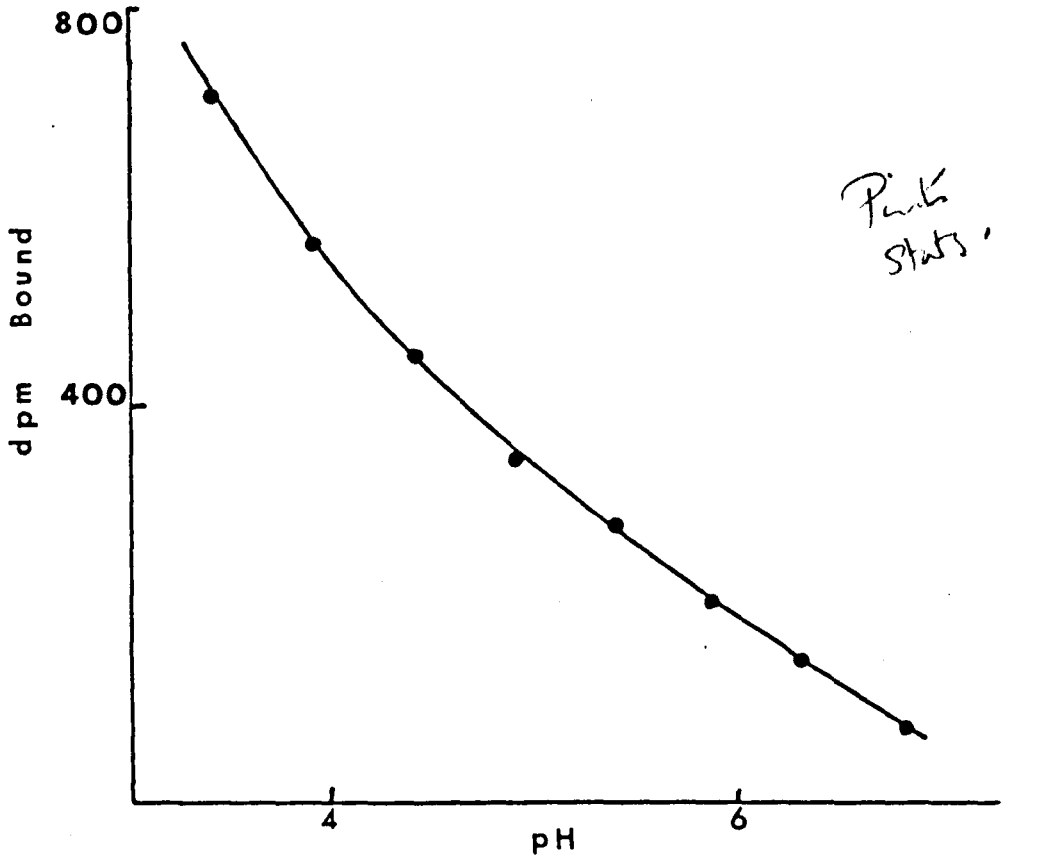
Non-specific binding has been subtracted from these results.

Fig. 7

The effect of pH on non-specific IAA binding to the 4,000 - 38,000 xg membrane fraction.



Pink Slats.



Pink Slats.

Table 3.

Rapidity of IAA binding to a solubilized acetone-precipitated membrane fraction (4,000 - 38,000 xg).

Samples were eluted on a Sephadex G-25 column with binding buffer containing 10^{-8} M ^{14}C-IAA.

The void volume was collected and assayed.

SAMPLES PREPARED IN:		d.p.m.
Binding buffer	(a)	2146 ± 252
Binding buffer + ^{14}C-IAA (2×10^{-7}M)		2208 ± 173
Buffer alone through column	(b)	4026 ± 89
Buffer + ^{14}C-IAA (2×10^{-7} M)		3989 ± 127

Results are the mean of three experiments.

(a) d.p.m. is the excess d.p.m./unit volume above the base line d.p.m./unit volume.

(b) d.p.m. is the total d.p.m. measured/unit volume.

extract nor ^{14}C-IAA (table 3). In this case the fraction representing the void volume did not show a reduction in radioactivity as compared to an equal volume of column buffer. This shows the lack of significant overlap of the void volume fraction and the fractions containing excess free IAA.

The reversibility of IAA binding could be easily demonstrated by the prior equilibration of the solubilized sample with unlabelled IAA (2×10^{-7}M). To this sample was added an equal volume of equimolar ^{14}C-IAA and the sample immediately added to the column. The void volume was collected and radioactivity measured. The amount of binding was comparable to the experiment in which the IAA solutions were added in reverse order (table 4).

EFFECT OF TEMPERATURE ON IAA BINDING

As can be seen from the graph (fig 8) the auxin binding sites were very temperature sensitive. Experiments with the membrane pellet fractions showed 50% loss of activity by pre -incubation at 20°C for six minutes and 85% loss at 60°C. All specific binding appeared to be lost by boiling the samples.

The acetone precipitate was more temperature sensitive with 70% loss of activity by pre -heating at 20°C. Non specific binding did not appear to be affected by heating.

EFFECT OF MONOVALENT IONS ON IAA BINDING

Table 4.

Reversible nature of IAA binding to a solubilized acetone precipitated membrane fraction (4,000 - 38,000 xg).

Samples were dissolved either in binding buffer + ^{14}C-IAA (2×10^{-7} M) or binding buffer + IAA (2×10^{-7} M). After allowing equilibrium to be attained, equal volumes of the other buffer were added and the sample added to a Sephadex G-25 column. Samples were eluted with binding buffer + ^{14}C-IAA (10^{-8} M) and the void volume collected and assayed.

ADDITION TO BINDING BUFFER		d.p.m.
1°	2°	(a)
^{14}C-IAA (2×10^{-7} M)	IAA (2×10^{-7} M)	1863 ± 94
IAA (2×10^{-7} M)	^{14}C-IAA (2×10^{-7} M)	1795 ± 113

Results are the mean of four experiments.

(a) Amount bound is the excess d.p.m./unit volume above the baseline d.p.m./unit volume.

Fig.8

The effect of temperature on specific IAA binding to the 4,000 - 38,000 xg membrane fraction.

●----● Membrane pellet

Binding was measured by the centrifugation method.

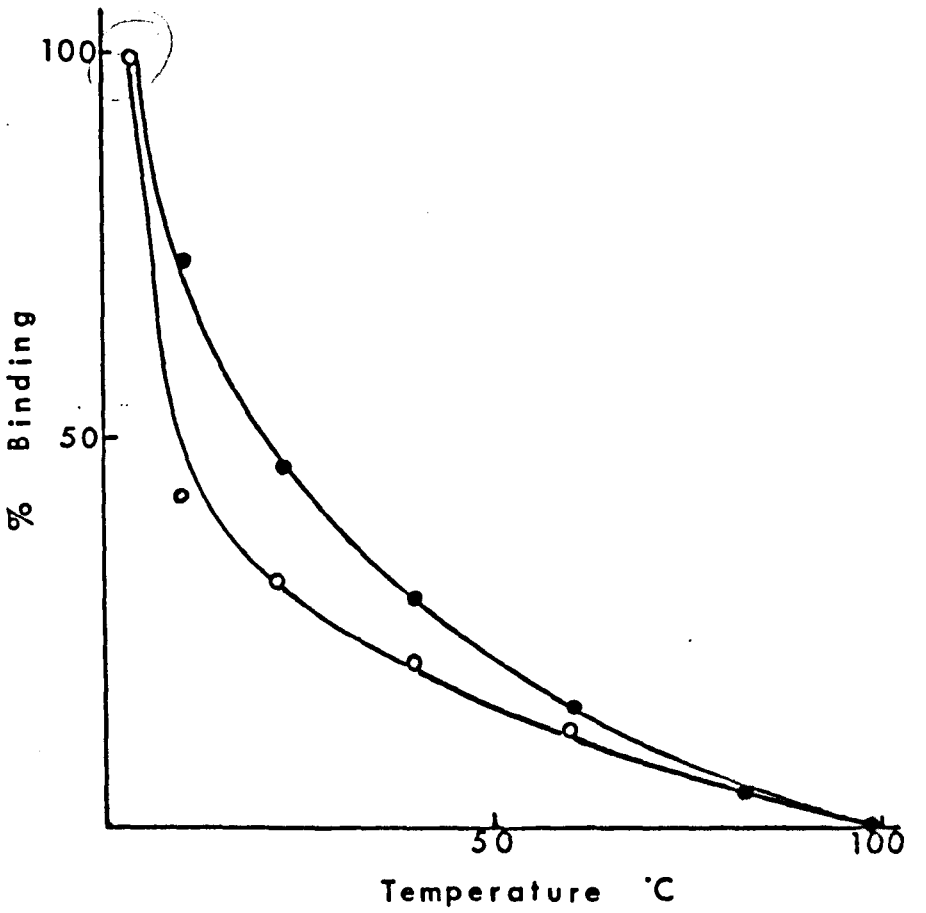
O----O Solubilized membrane by the acetone precipitation method.

Binding was measured by the equilibrium dialysis.

100% binding was determined as the binding obtained at 4°C.

Results are the average of two experiments.

Same binding in
Dilt. Actual figures


100
50
% Binding
50
100
Temperature °C

Both sodium and potassium had an inhibitory effect on specific IAA binding to the crude membrane pellets. Sodium was only slightly less inhibitory than potassium (fig 9) with 50% loss of activity at 0.025M. It was not sure if this ion effect was acting directly at the binding site or more indirectly on the protein molecule.

EFFECT OF DIVALENT IONS ON IAA BINDING

Magnesium and calcium were both capable of stimulating IAA binding to the membrane fractions but to varying levels, as can be seen from fig 10. Magnesium showed maximal stimulation at 10^{-3}M. Calcium was only weakly stimulatory with maximal stimulation at 10^{-2}M. Thus 1.0mM $MgCl_2$ was routinely used in the binding buffers for all experiments.

DIFFERENTIAL CENTRIFUGATION

The results from the pH optima experiments suggested that there were two distinct binding sites associated with the 4,000 -38,000xg membrane fraction, but only one associated with the 38,000 -80,000xg fraction.

The amount of specific binding to each fraction prepared by differential centrifugation was determined and the results shown in table 5.

Fig. 9

The effect of monovalent ions on the specific binding of IAA to the 4,000 - 38,000 xg membrane fraction.

●----● Sodium

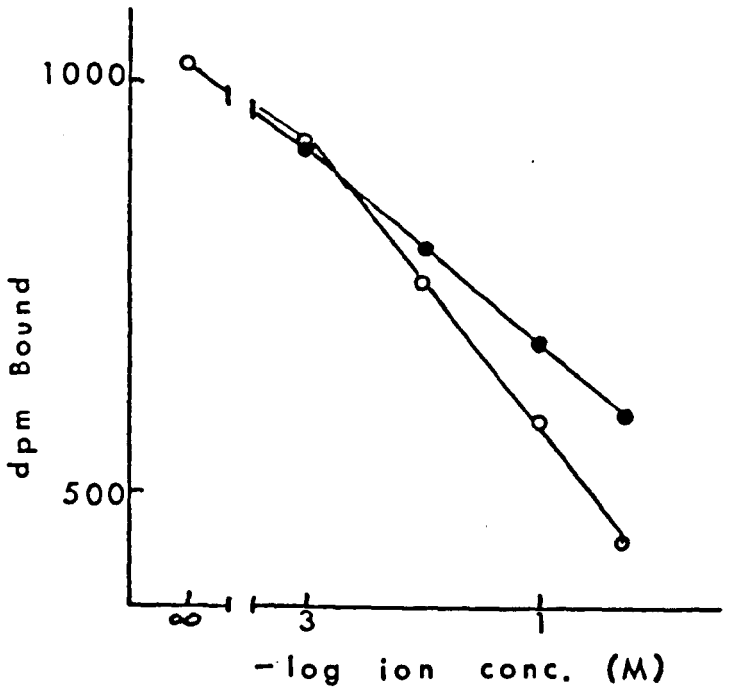
O----O Potassium

Fig. 10

The effect of divalent ions on the specific binding of IAA to the 4,000 - 38,000 xg membrane fraction.

●----● Magnesium

O----O Calcium


1000
500
dpm Bound
∞
3
1
-log ion conc. (M)

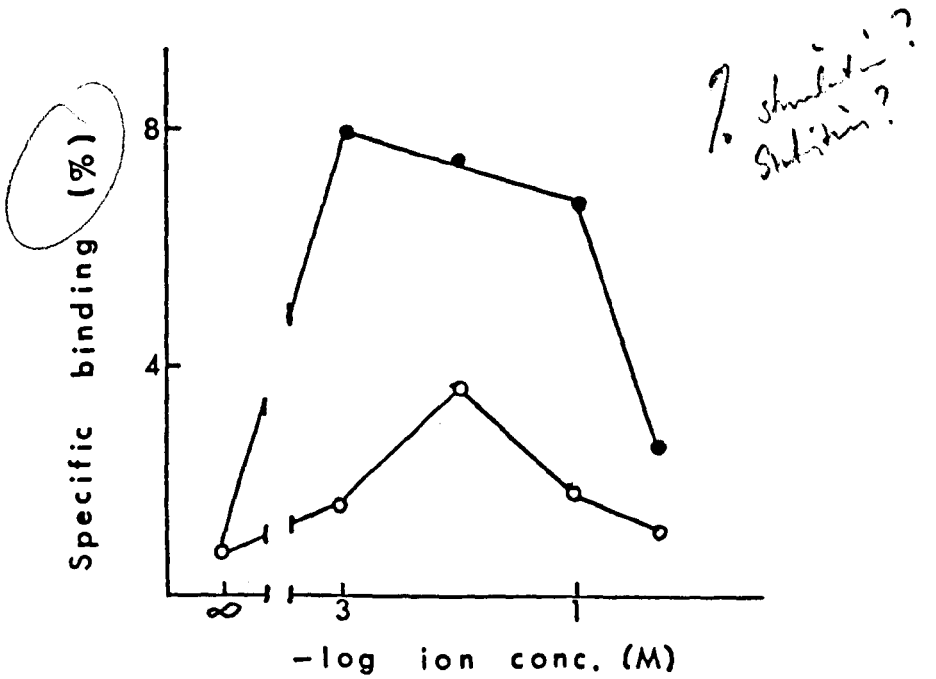

Specific binding (%)
8
4
∞
3
1
-log ion conc. (M)

Table 5.

Specific IAA binding activities of membrane fractions prepared by differential centrifugation.

Experiments were carried out with ^{14}C-IAA at a concentration of 10^{-7} M.

Non-specific binding was determined as the binding obtained in the presence of 10^{-3} M IAA and was subtracted from total binding to give specific binding.

Results are the mean of three experiments.

FRACTIONS	d.p.m./g fresh wt.
0 - 4,000 xg	12 ± 16
4,000 - 38,000 xg	475 ± 98
38,000 - 80,000 xg	245 ± 72
4,000 - 80,000 xg	614 ± 150

The 4,000 -38,000xg membrane fraction made up a substantial proportion of the total specific binding present in the tissue and this fraction was routinely used for experiments.

2. BINDING KINETICS

Using the improved methods and techniques the kinetics of IAA binding to the 4,000 -38,000 xg membrane fraction was determined. As can be seen in fig 11 the ^{14}C-IAA bound to the membrane pellet decreased with increasing unlabelled IAA concentrations. Any ^{14}C-IAA bound at high IAA concentrations (10^{-3}M) was deemed to be of a non -specific nature and subtracted from total binding before kinetic analysis. Kinetic analysis (fig 12) indicated the presence of two classes of high affinity IAA binding sites. The dissociation constants were:

Site 1 $K_1 = 2.2 \times 10^{-7}$M

$n_1 = 1.8 \times 10^{-10}$moles/ g fresh wt

Site 2 $K_2 = 11 \times 10^{-7}$M

$n_2 = 3.0 \times 10^{-10}$moles/ g fresh wt

KINETICS ON SOLUBILIZED BINDING SITES

Solubilized auxin binding sites were prepared by dissolving in binding buffer the powder prepared by acetone precipitation of the membranes. The kinetics of the

Fig. 11

Total binding of ^{14}C -IAA to a 4,000 - 38,000 xg membrane fraction at different concentrations of IAA.

Each point is the mean of three replicates.

Both?

Fig. 12

Double reciprocal plots of the data from fig. 11.

Non-specific binding was subtracted from total binding for the plots.

Binding was per g fresh wt. of material.

A = site 1

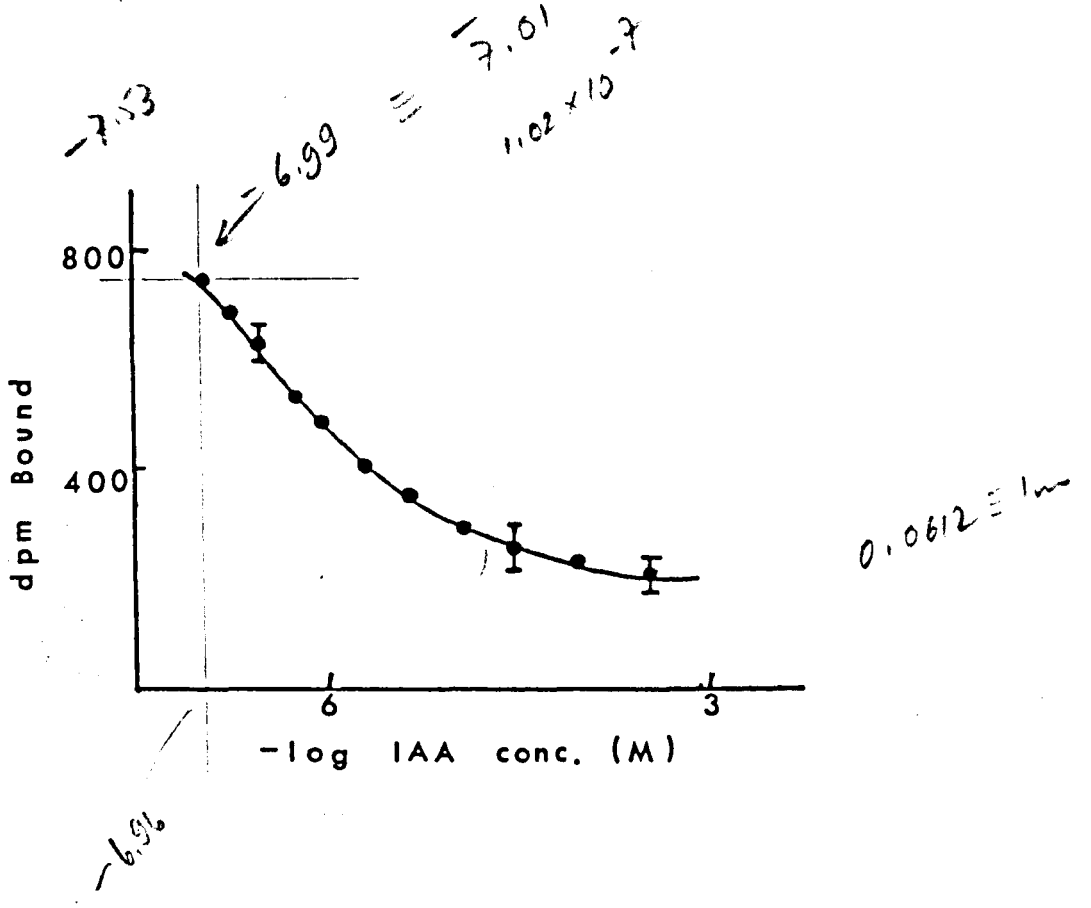
$K_1 = 2.2 \times 10^{-7}$ M

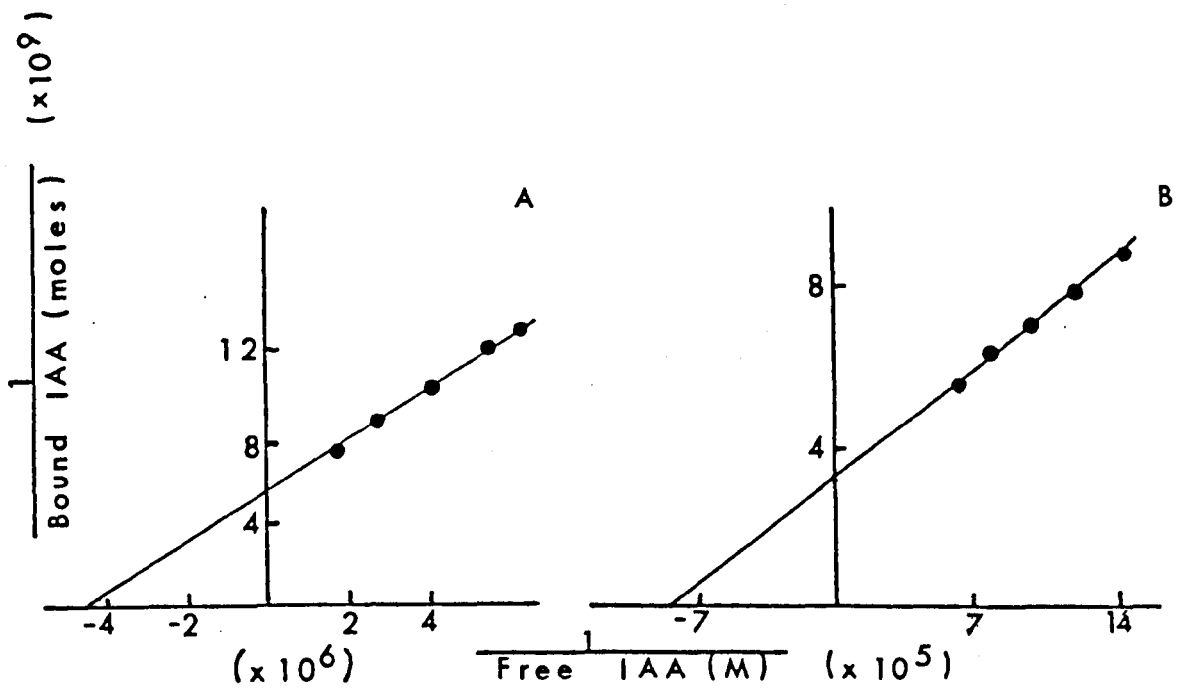
$n_1 = 1.75 \times 10^{-10}$ moles/g fresh wt

B = site 2

$K_2 = 11 \times 10^{-7}$ M

$n_2 = 3 \times 10^{-10}$ moles/g fresh wt


dpm Bound
800
400
6
3
-log IAA conc. (M)


1/Bound IAA (moles) (x10^9)
A
12
8
4
-4
-2
2
4
(x10^6)
B
8
4
-7
7
14
1/Free IAA (M) (x10^5)

solubilized binding sites were determined to ensure that the solubilization process had not destroyed the binding properties of the sites. Binding was determined by equilibration dialysis and the results shown in fig 13. Double reciprocal plots of the data (fig 14) indicate two classes of high affinity IAA binding sites with binding parameters of:

100% recovery?

Site 1: $K_1 = 4.1 \times 10^{-7}$M

$n_1 = 2.4 \times 10^{-10}$moles/ g fresh wt

Site 2: $K_2 = 11.9 \times 10^{-7}$M

$n_2 = 2.9 \times 10^{-10}$moles/ g fresh wt

The results are similar to those obtained for the intact membrane suggesting that the solubilization method did not appear to affect the binding abilities of the two classes of IAA binding sites.

3. LOCALIZATION OF THE AUXIN BINDING SITES

FRACTIONATION ON COMPLEX SUCROSE GRADIENTS

Table 6 indicates that the mitochondrial marker - cytochrome c oxidase - showed maximal activity in the pellet obtained after centrifugation on the sucrose gradient. The activity of cytochrome c reductase, which is indicative of the endoplasmic reticulum, peaked at the 25-30% sucrose interface with some activity in the 18-25% fraction (table 6).

Fig. 13

Total binding of ^{14}C-IAA to a solubilized 4,000 - 38,000 xg membrane fraction.

Solubilization was achieved by buffer extraction of the acetone precipitated membranes. Binding was measured by equilibrium dialysis.

Each point is the mean of three replicates.

Fig. 14

Double reciprocal plots of the data from fig. 13. Non-specific binding was subtracted and binding expressed as binding per g fresh wt. of material.

A site 1

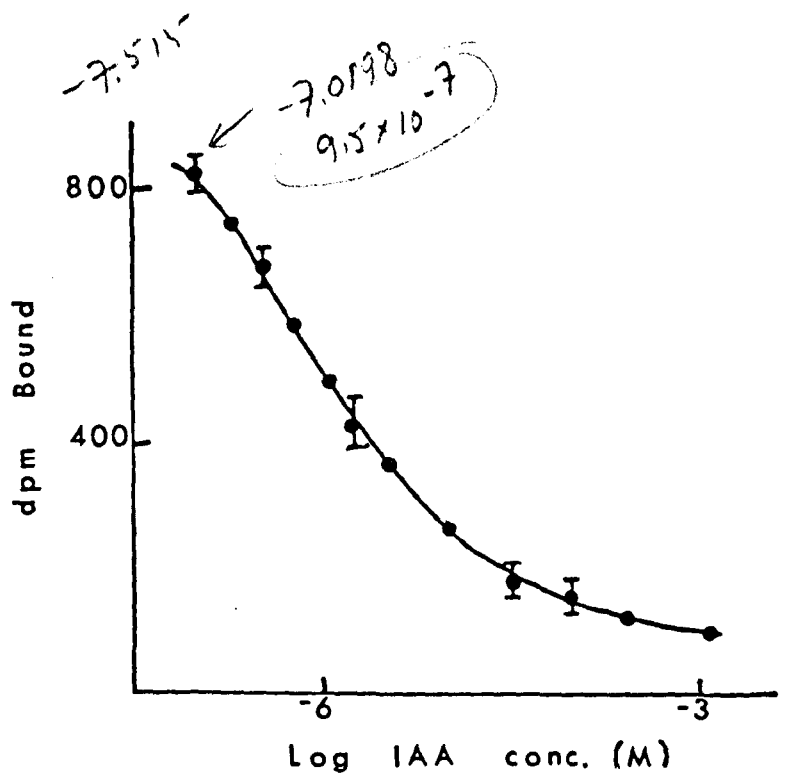
$K_1 = 4.1 \times 10^{-7}$ M

$n_1 = 2.4 \times 10^{-10}$ moles /g fresh wt

B site 2

$K_2 = 11.9 \times 10^{-7}$ M

$n_2 = 2.6 \times 10^{-10}$ moles /g fresh wt


dpm Bound
800
400
-6
-3
Log IAA conc. (M)

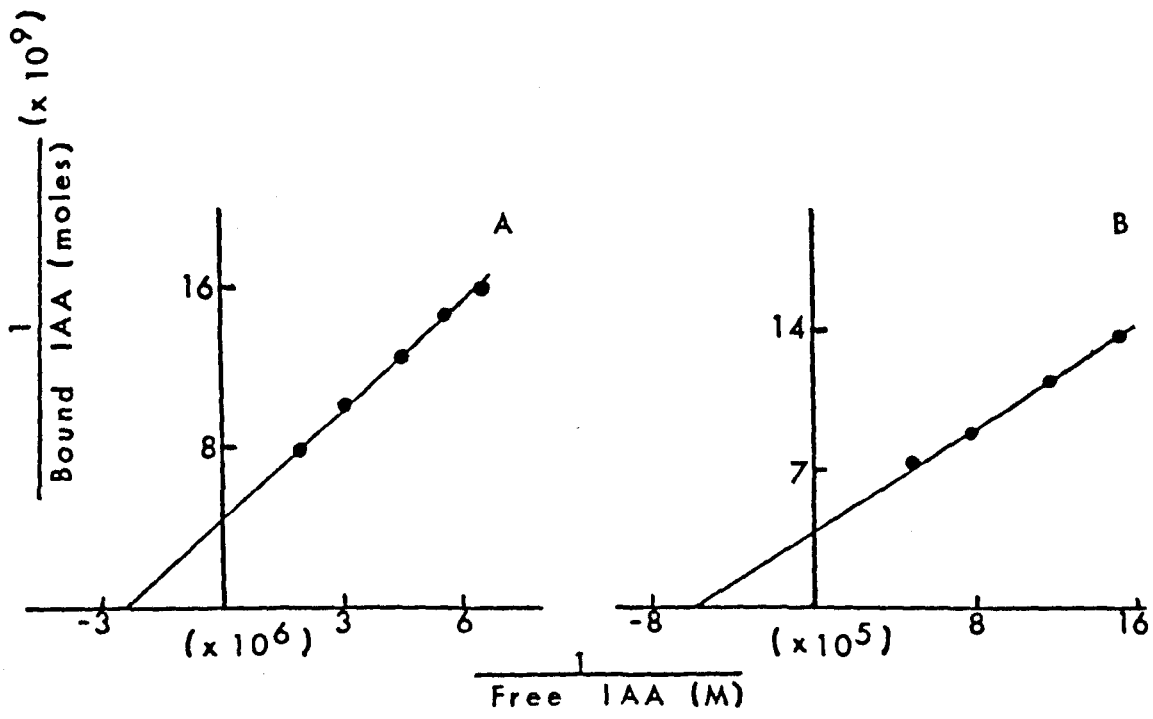

1 / Bound IAA (moles) (x 10^9)
A
16
8
-3
(x 10^6)
3
6
B
14
7
-8
(x 10^5)
8
16
1 / Free IAA (M)

Table 6.

Distribution of the enzyme activities of cytochrome c oxidase (CCO) and cytochrome c reductase (CCR) in a 4,000 - 38,000 xg membrane fraction separated on a complex sucrose gradient.

INTERFACE SUCROSE %	ACTIVITY [a] / mg PROTEIN			
	CCO		CCR	
18 -25	0.000	0	0.043	0.037
25 - 30	0.001	0.001	0.056	0.056
30 - 34	0.002	0.024	0.013	0.016
34 - 38	0.009	0.004	0.023	0.011
38 - 45	0.018	0.375	0.022	0.039 Strs.
PELLET	0.125	0.027	0.020	0.004

(a) Activity was expressed as the change in absorbance at 550nm per minute.

Maximal activity of glucan synthetase 1, considered a marker for Golgi membranes, occured at the 30-34% sucrose interface. A large amount of the enzyme was also found at the 25-30% sucrose interface (table 8).

ATPase is thought to be a marker enzyme for the plasma membrane and this enzyme showed maximal activity in the 34-38% sucrose interface fraction (table 9). Glucan synthetase 2 also had maximal activity in this fraction and is also considered a marker enzyme for the plasma membrane (table 8).

The tonoplast marker enzyme - acid phosphatase - had maximal activity in the 25-30% sucrose fraction (table 9).

IAA binding to the fractions obtained from the sucrose gradients showed two distinct binding peaks (representative results are shown in table 7). One was associated with the 25-30% sucrose interface fraction and the other in the 34-38% sucrose fraction. The more dense fraction was apparently associated with the plasma membrane. No clear localization for the other auxin binding peak could be made however since a number of enzymes had high activities in the 25-30% fraction. Thus this auxin binding peak could be associated with the endoplasmic reticulum, the Golgi membranes or the tonoplast. Further characterization of this fraction would be necessary to determine its location.

Table 7.

Distribution of protein and IAA binding in a 4,000 - 38,000 xg membrane fraction separated on a complex sucrose gradient.

INTERFACE	PROTEIN	IAA BINDING	
SUCROSE %	mg/ml	d.p.m./mg protein	
18 - 25	0.85	35	23.8
25 - 30	1.00	180	180
30 - 34	1.24	153	185.7
34 - 38	0.47	383	180.0
38 - 45	1.75	142	248.5
PELLET	0.20	26	5

Results shown are those from a typical experiment.

Stats?

Table 8.

Distribution of glucan synthetase 1 and glucan synthetase 11 in a 4,000 - 38,000 xg membrane fraction separated on a complex sucrose gradient.

INTERFACE SUCROSE%	GLUCAN SYNTHETASE 1 d.p.m./mg protein		GLUCAN SYNTHETASE 11 d.p.m./mg protein	
18 - 25	2360	2006	58	49
25 - 30	6025	6025	109	109
30 - 34	6500	8060	493	611
34 - 38	2480	1166	1560	733
38 - 45	1270	254	724	145

Table 9.

Distribution of ATPase and acid phosphatase activities in a 4,000 - 38,000 xg membrane fraction separated on a complex gradient.

INTERFACE SUCROSE %	ACTIVITY / mg PROTEIN			
	ATPase (a)		Acid phosphatase (b)	
18 - 25	0.000	0	0.825	0.7
25 - 30	0.015	0.015	0.837	0.837
30 - 34	0.134	0.165	0.772	0.801
34 - 38	0.192	0.090	0.578	0.272
38 - 45	0.035	0.06	0.356	0.07

(a) Activity was measured as the change in absorbance at 660 nm.

(b) Activity was measured as the change in absorbance at 440 nm.

DISTRIBUTION OF AUXIN BINDING SITES ON SUCROSE GRADIENTS CONTAINING HIGH Mg^{2+} CONCENTRATIONS

In experiments employing use of high magnesium concentrations in the buffers, there was a shift in the activity peak of the endoplasmic reticulum marker enzyme (table 10). Cytochrome c reductase activity peaked at the 34-38% sucrose interface, as compared to the peak at the 25-30% sucrose interface found in experiments with low magnesium concentrations. The high magnesium concentration did not appear to have any effect on the localization of any of the other marker enzymes (table 10), suggesting that the ER was the only membrane fraction affected.

A comparison of IAA binding in the fractions prepared on a normal sucrose gradient and on a gradient formed in the presence of high Mg^{2+} concentration is shown in table 11. The IAA binding in the high Mg^{2+} concentration showed only one binding peak, at the 34-38% sucrose interface with the loss of the binding peak at the 25-30% sucrose interface. It was suggested that this single binding peak consisted of the plasma membrane binding sites and binding sites associated with ER membranes, the enzyme marker of which was also shifted to this fraction.

To confirm that this single IAA binding peak consisted of binding sites from both the ER and the plasma membrane, a further test was carried out. Membranes were separated out on the high magnesium concentration sucrose gradient

Table 10.

Distribution of marker enzymes in a 4,000 - 38,000 xg membrane fraction on a complex sucrose gradient prepared with a high magnesium concentration.

(a) d.p.m.

(b) Change in absorbance at 550 nm per minute.

(c) Change in absorbance at 660 nm.

(d) Change in absorbance at 440 nm.

INTERFACE SUCROSE %	GLUCAN SYNTHETASE 1 (a)		GLUCAN SYNTHETASE 11 (a)		CYTOCHROME C OXIDASE (b)		CYTOCHROME C reductase (b)		ATPase (c)		ACID PHOSPHATASE (d)	
18 - 25	1670	2006	---	69	---	0	---	0.037	0.001	0	0.736	0.7
25 - 30	5020	6025	82	109	---	0.007	0.008	0.036	0.004	0015	0.742	0.823
30 - 34	5800	8268	306	611	0.001	0.024	0.022	0.016	0.098	0.156	0.655	0.581
34 - 38	1870	1166	1268	733	0.035	0.004	0.218	0.011	0.155	0.090	0.431	0.242
38 - 48	910	254	524	1041	0.122	0.375	0.041	0.022	0.031	0.060	0.154	0.07

Diffuse fgmi to 8/9

Table 11.

Effect of magnesium concentration on distribution of IAA binding to a 4,000 - 38,000 xg membrane fraction separated on a complex sucrose gradient.

INTERFACE	IAA BINDING		
SUCROSE %	d.p.m. / mg protein		
	A	B	C
18 - 25	13	0	10
25 - 30	170	40	94
30 - 34	150	120	63
34 - 38	275	432	187
38 - 45	36	164	33

A - Sucrose gradient prepared with a low magnesium concentration.

B - Sucrose gradient prepared with a high magnesium concentration.

C - 'Stripping experiment'. The 34 - 38% sucrose fraction from B was diluted with EDTA and refractionated on a sucrose gradient prepared with a low magnesium concentration.

and the 34-38% sucrose fraction removed. This was diluted with grinding buffer and EDTA addded to a final concentration of 3mM. This was then loaded onto a normal sucrose gradient and recentrifuged. IAA binding on the interfaces from this spin showed the reappearance of the binding peak at the 25-30% sucrose interface (table 11). It is assumed that the EDTA dissociated the ribosomes from the rough ER and thus the ER fractionated at the lower sucrose density. If so then these results suggest that the IAA binding peak at the lower sucrose density was associated with the endoplasmic reticulum.

ELECTRON MICROSCOPY

Of special interest was the comparison of the electron micrographs of the 34-38% sucrose gradient interfaces prepared in high and low magnesium concentrations. Few mitochondria were observed (fig 15) confirming the activity profile of cytochrome c oxidase. The nature of the membranes present in the fraction prepared with a low magnesium concentration could not be determined by their morphology.

In the micrograph of the fraction prepared with a high magnesium concentration a large portion of membrane present had the typical characteristics associated with rough endoplasmic reticulum. This would suggest that magnesium prevents stripping of the ribosomes from the ER so that the ER would partition in the heavier sucrose fraction. This

Fig. 15

Electron micrograms of fractions obtained from sucrose gradient separation of a 4,000 - 38,000 xg membrane fraction.

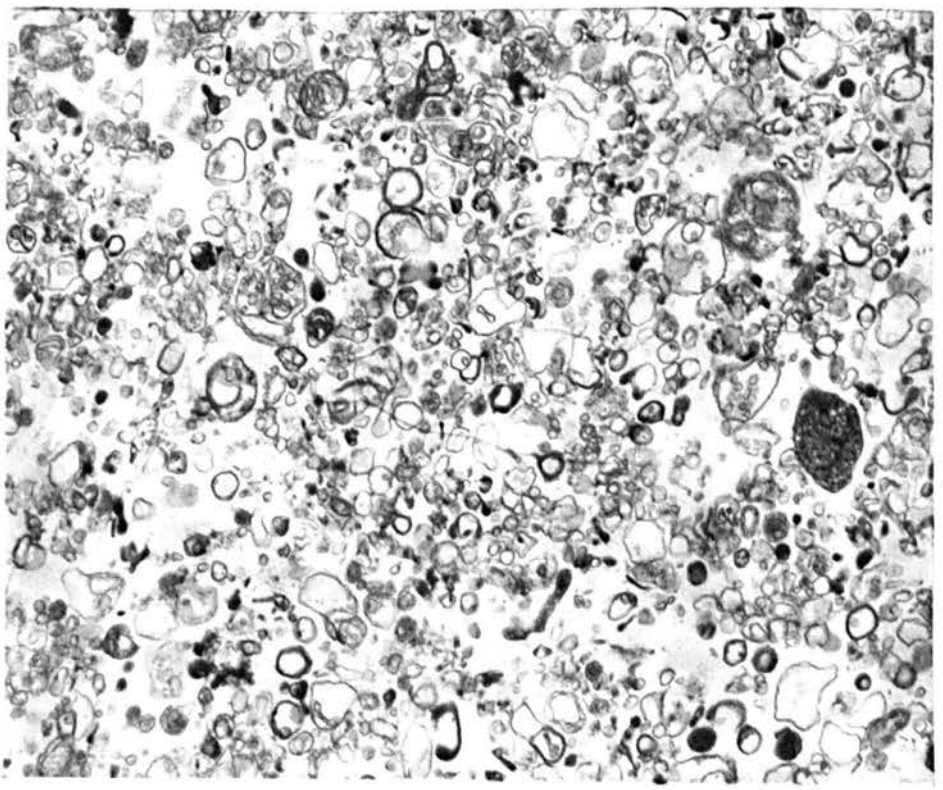
A A sample from the 34 - 38% sucrose interface fraction prepared with a low magnesium concentration.

12.4 mm = 1μm

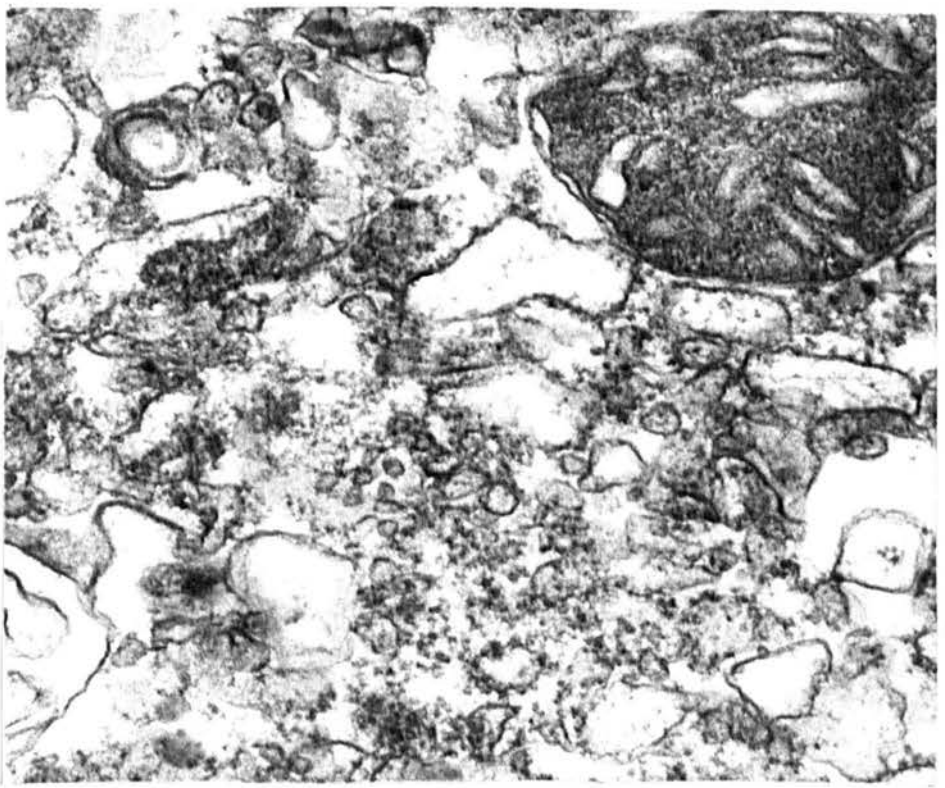
B A sample from the 34 - 38% sucrose interface fraction prepared with a high magnesium concentration.

5.5 mm = 100nm.

A



B



would support results obtained with the activity profiles of cytochrome c reductase.

INVESTIGATIONS ON IAA BINDING SITES IN THE NON-GROWING REGION OF THE PEA EPICOTYL

As supportive evidence that the two binding sites already observed were involved in the growth process, a search for these binding sites was conducted on the non -growing region of the epicotyl.

Kinetic studies carried out on this region of the epicotyl gave no indication of IAA specific binding sites. Analysis of the data using the double reciprocal plot gave a single straight line with a dissociation constant approaching infinity (fig 16). This suggested that the only IAA binding observed was of a non -specific nature with very low affinity.

In a further search for auxin binding sites in this region, a pH profile of the 4,000 -38,000 xg membrane fraction was prepared. No pH dependent specific binding peak was observed (fig 17) and the results were similar to those obtained for non specific binding (fig 7).

PH PROFILES OF AUXIN BINDING IN THE LIGHT AND HEAVY MEMBRANE BANDS

From the results obtained with the crude membrane

Fig. 16

Double reciprocal plot of the binding data of ^{14}C-IAA to a 4,000 - 38,000 xg membrane fraction from the non-growing region (1st internode) of the pea epicotyl.

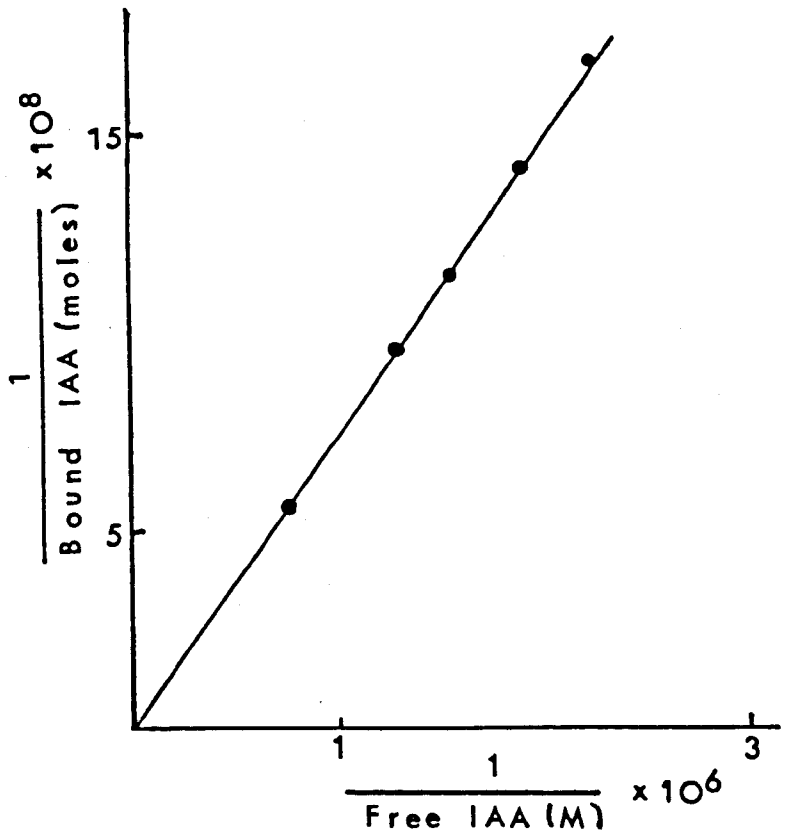
$K \longrightarrow \infty$

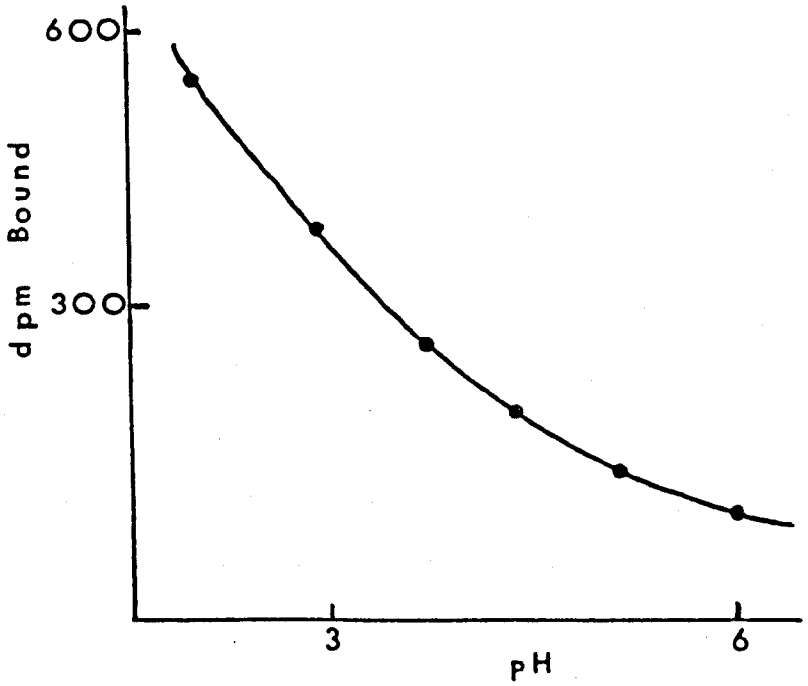
No apparent high affinity binding sites present.

The results are the average of two experiments.

Fig. 17

The effect of pH on IAA binding to a 4,000 - 38,000 xg membrane fraction prepared from the non-growing region of the pea epicotyl.


1 / Bound IAA (moles) x10^8
15
5
1
3
1 / Free IAA (M) x10^6


600
dpm Bound
300
3
6
pH

fractions it appeared posible that the two pH optima resulted from binding sites located on different membranes. With the separation of the two classes of auxin binding sites this theory was examined.

Results obtained with the light membrane fraction (fig 18) indicated one IAA binding peak at pH 6. Analysis of the data obtained with the heavy membrane fraction (fig 19) suggested that this class of binding sites had a pH optimum of pH 5. Thus in further experiments on these fractions the binding buffer was adjusted accordingly.

KINETICS OF IAA BINDING TO THE LIGHT AND HEAVY MEMBRANE FRACTIONS

If the two IAA binding sites separated by simple sucrose gradient centrifugation represented the two kinetically distinct classes of binding sites already reported, then it should be possible to demonstrate this.

The binding activity and the kinetic analysis for the light membrane fraction are shown in fig 20, while those of the heavy membrane band are shown in fig 21. The kinetic analyses suggests that there is only one class of distinct IAA binding sites located in each fraction. The binding parameters of the two sites were:

Light band:

Fig. 18

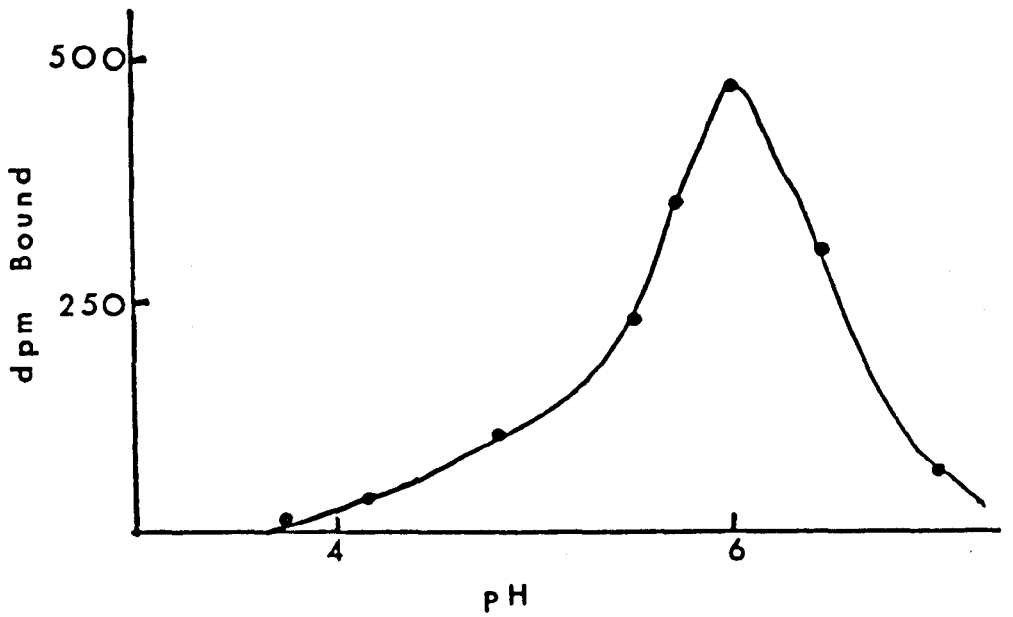
The effect of pH on specific IAA binding to the light membrane fraction prepared on a simple sucrose gradient.

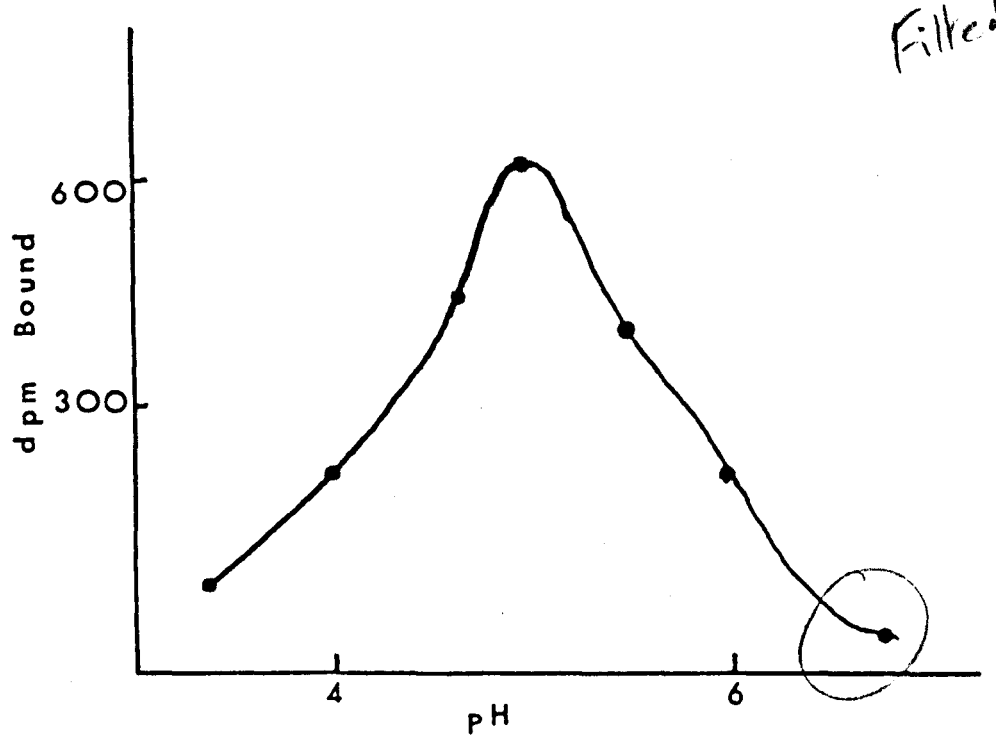
Each point is the mean of three replicates.

Fig. 19

Effect of pH on specific IAA binding to the heavy membrane fraction prepared on a simple sucrose gradient.

Each point is the mean of three replicates.





Filled?

Fig. 20

Binding of IAA to the light membrane fraction prepared by separation of a 4,000 - 38,000 xg membrane fraction on a simple sucrose gradient.

A Specific binding data.

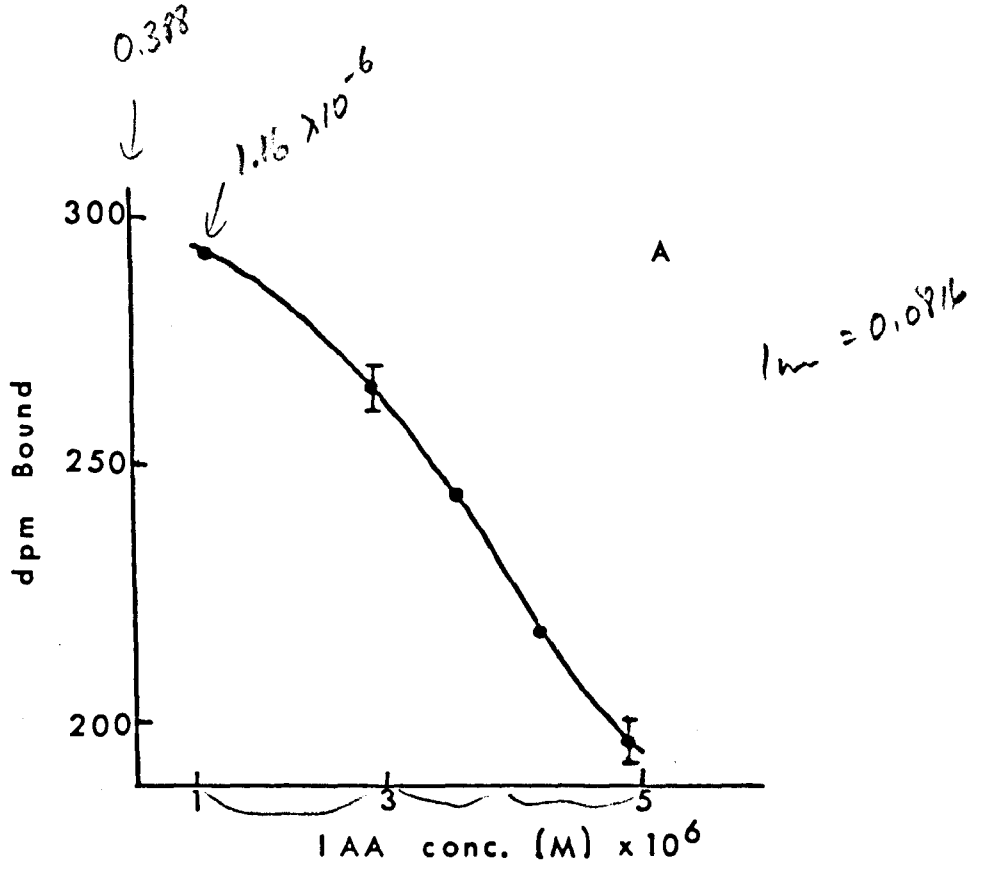
Results are the mean of three replicates.

B Double reciprocal plot of the data from A. Data was analysed as binding per g fresh wt. of material.

$K_1 = 1.8 \times 10^{-7}$ M

$n_1 = 1.6 \times 10^{-10}$ moles/ g fresh wt

Results suggest that this represents site 1 binding.


0.388
1.16 x10-6
A
1m = 0.0816
300
250
200
dpm Bound
1
3
5
IAA conc. (M) x 10^6

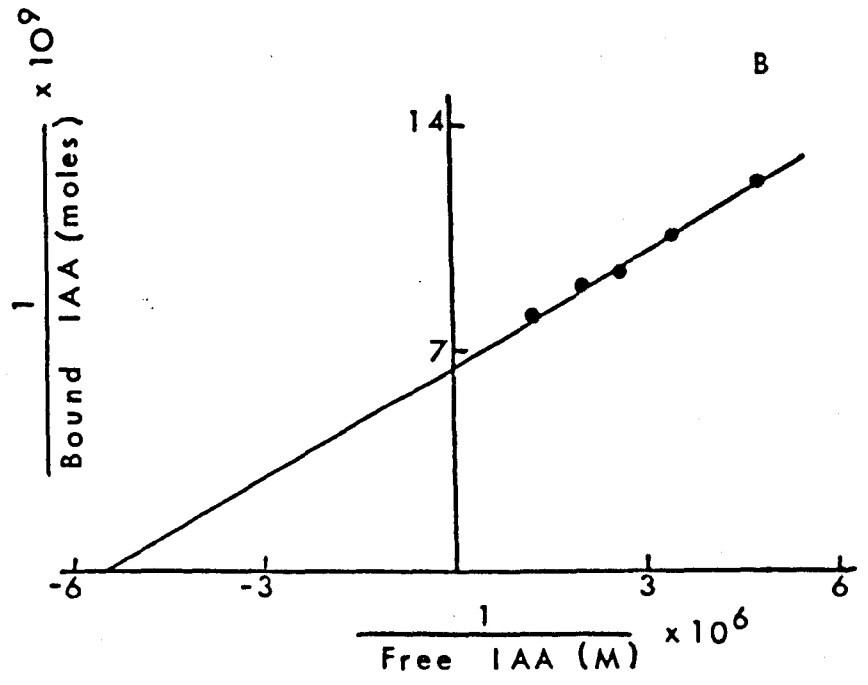

B
1 / Bound IAA (moles) x 10^9
14
7
-6
-3
3
6
1 / Free IAA (M) x 10^6

Fig. 21

Binding of IAA to the heavy membrane fraction prepared by separation of a 4,000 - 38,000 xg membrane fraction on a simple sucrose gradient.

A Specific binding data.

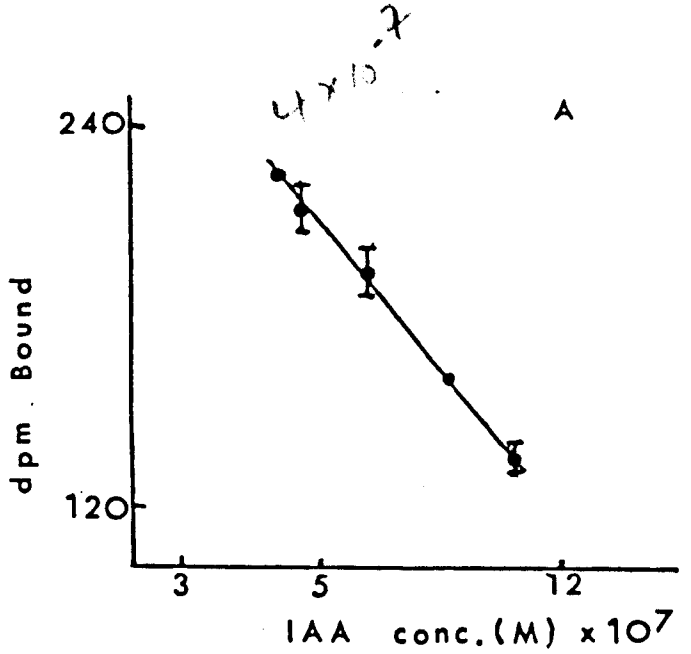
Results are the mean of three replicates.

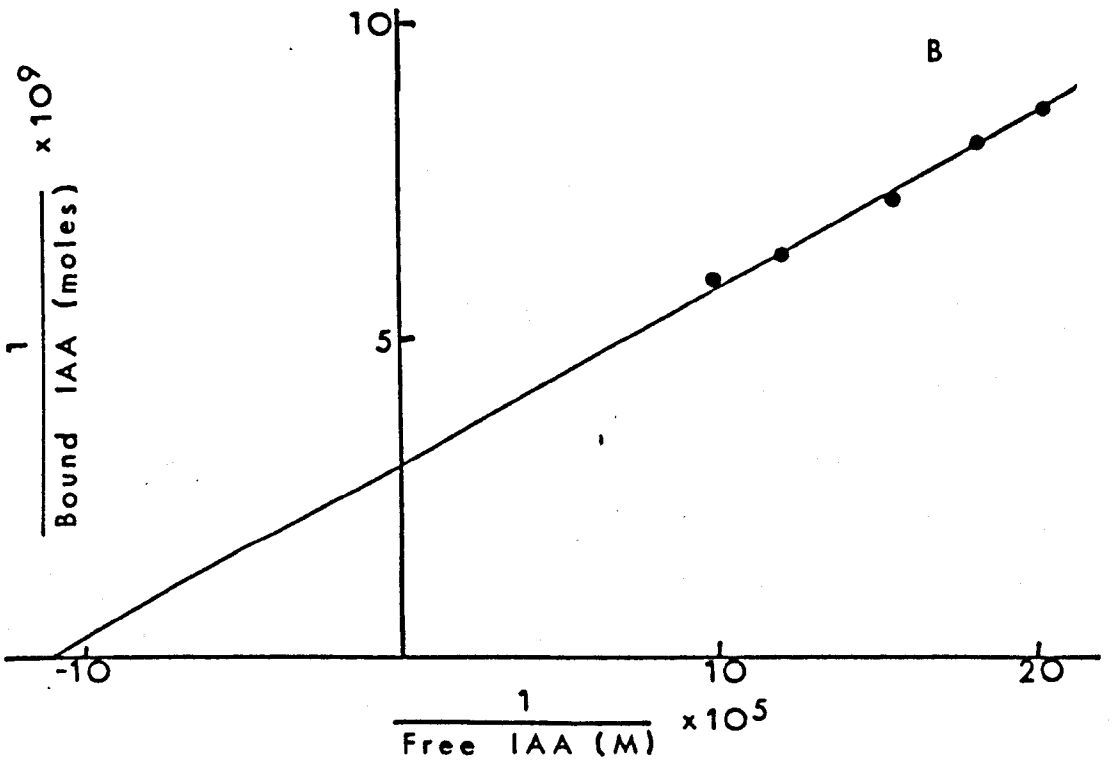
B Double reciprocal plot of the data from A. Data was analysed as binding per g fresh wt. of material.

$K_2 = 8.9 \times 10^{-7}$ M

$n_2 = 3.3 \times 10^{-10}$ moles / g fresh wt

Results suggest that this represents site 2 binding.


A
240
120
dpm . Bound
3
5
12
IAA conc.(M) x 10^7


B
10
5
-10
10
20
1 / Bound IAA (moles) x 10^9
1 / Free IAA (M) x 10^5

$K_1 = 1.8 \times 10^{-7}$ M

$n_1 = 1.6 \times 10^{-10}$ moles/ g fresh wt

Heavy band:

$K_2 = 8.9 \times 10^{-7}$ M

$n_2 = 3.3 \times 10^{-10}$ moles/ g fresh wt

These results for the light and heavy bands were similar with the values previously determined for site 1 and site 2 respectively in the crude membrane pellets. Site 1 binding was found in the light band and site 2 in the heavy band. The results also suggest that good separation of the two classes of binding sites could be achieved by use of the simple sucrose gradient.

4. SPECIFICITY OF THE IAA BINDING SITES

It was thought possible to determine the nature of the interaction of certain auxin analogues with the IAA binding sites. This was done indirectly using the priciples developed for enzyme inhibitor studies (Dixon and Webb, 1958). The procedure involved analysing the binding of ^{14}C-IAA (over the usual concentration range) to the membrane fractions, in the presence or absence of a fixed concentration of the auxin analogue. The binding parameters were calculated from the double reciprocal plot of the data. The experiments were carried out on the membrane fractions prepared on the simple sucrose gradients.

The ability of NAA and β-NAA to compete with IAA for the two binding sites was examined. The binding data and the corresponding double reciprocal plots are shown in figs 22 and 23 for site 1 and figs 24 and 25 for site 2. NAA showed competition for both sites, although in both cases a small cross over effect was observed. The cross over effect occurs when the two lines intersect in the positive quadrant of the graph (in the ideal experiment the lines would intersect on the 1/B axis). This cross over may occur because not all the non specific binding was subtracted from the results, or could be within the limitations of the experiment.

β-NAA exhibited competition for the binding sites found in the light sucrose band (site 1), but showed no competition for site 2. No double reciprocal plot was performed on the data from site 2 since this result was obvious from the binding curve.

5. SEPARATION OF THE AUXIN BINDING SITES

ION EXCHANGE CHROMATOGRAPHY

Sephadex DEAE 50 was found to shrink in the column when salt was present in the elution buffer so future experiments were carried out using DE 32. However, with the DEAE 50 the auxin binding proteins were eluted with the

Fig. 22

NAA competition for IAA binding sites in a light membrane fraction prepared from a 4,000 - 38,000 xg membrane fraction separated on a simple sucrose gradient.

A Specific binding data.

Each point is the mean of three replicates.

B Double reciprocal plot for the data from A. Data is presented as binding per g fresh wt. of material.

●----● IAA only

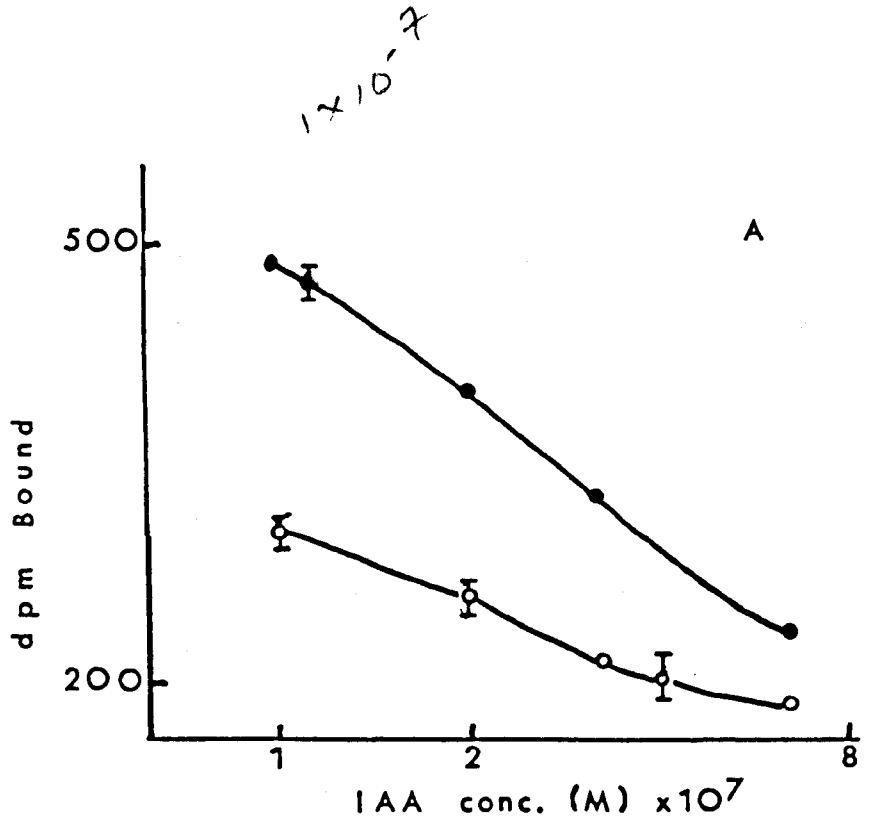
$K_1 = 2.1 \times 10^{-7}$ M

$n_1 = 1.7 \times 10^{-10}$ moles / g fresh wt

O----O IAA + 10^{-6} M NAA

$K_I = 6.3 \times 10^{-7}$ M

Interaction is competitive.


1 x 10-7
A
500
200
dpm Bound
1
2
8
IAA conc. (M) x10^7

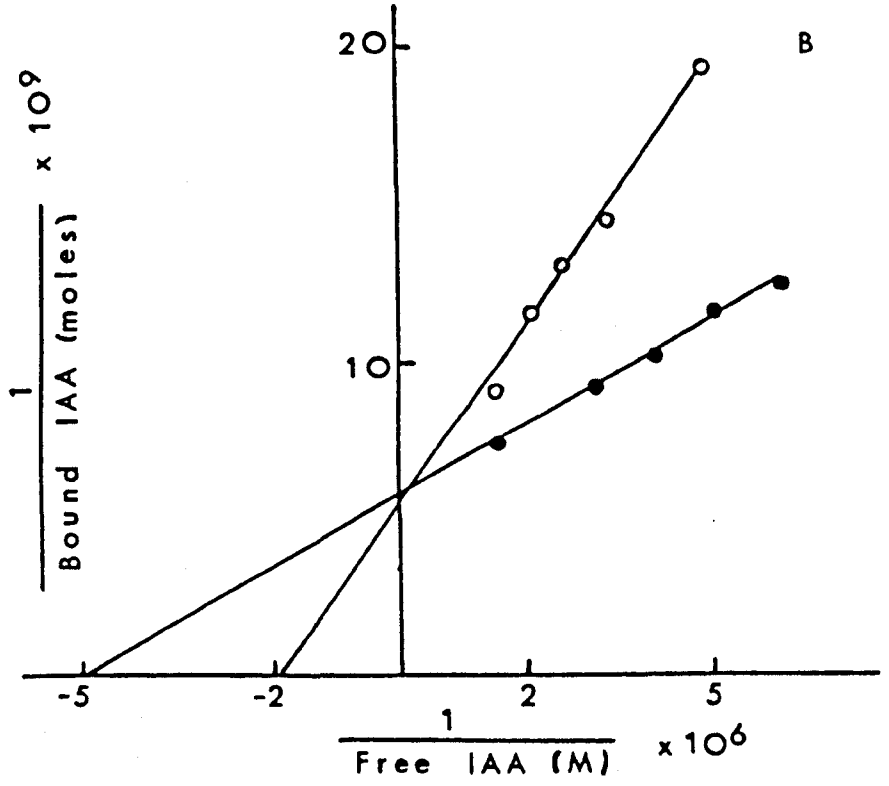

B
20
10
1 / Bound IAA (moles) x 10^9
-5
-2
2
5
1 / Free IAA (M) x 10^6

Fig. 23

β-NAA competition for IAA binding sites on a light membrane fraction prepared by separation of a 4,000 - 38,000 xg membrane fraction on a simple sucrose gradient.

A Specific binding data.

Each point is the mean of three replicates.

B Double reciprocal plot of the data from A. Binding was per g fresh wt. of material.

●----● IAA only

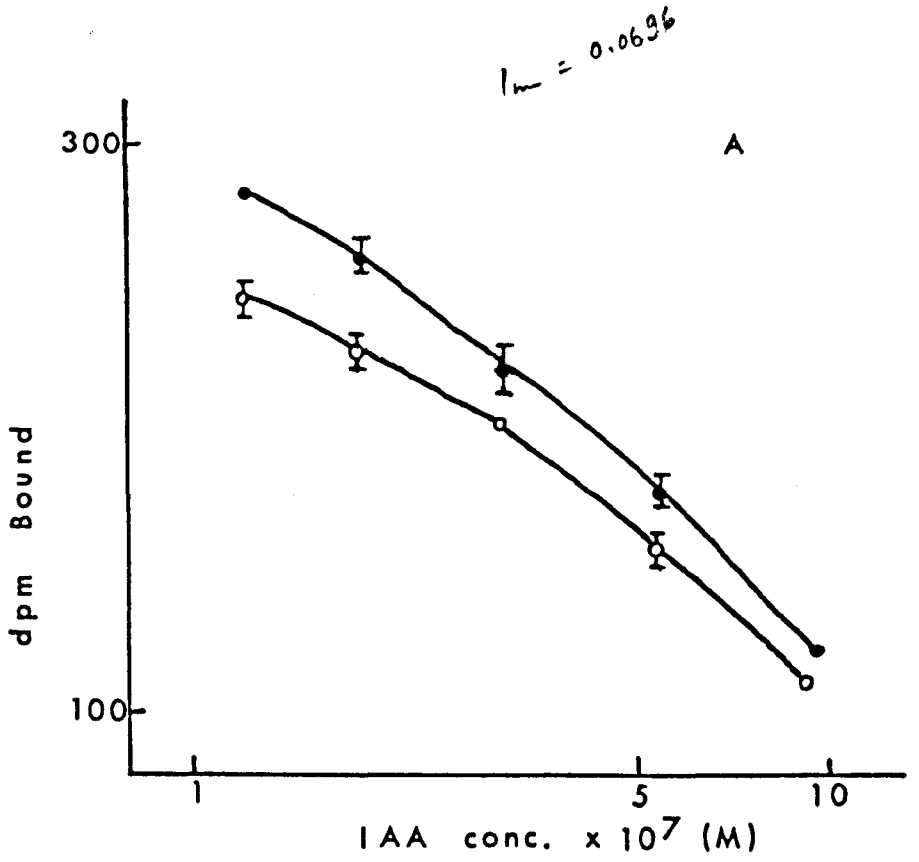
$K_1 = 2.0 \times 10^{-7}$ M

$n_1 = 1.8 \times 10^{-10}$ moles/ g fresh wt

O----O IAA + 10^{-6} M β-NAA

$K_I = 2.6 \times 10^{-6}$ M

Interaction is competitive.


I_m = 0.0696
A
300
100
dpm Bound
1
5
10
IAA conc. x 10^7 (M)

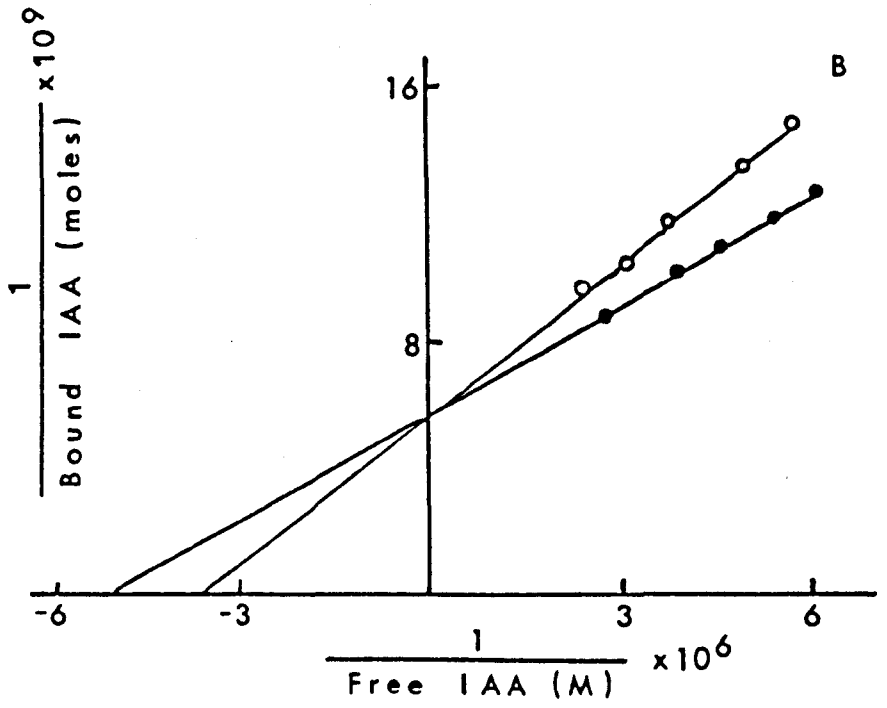

B
16
8
-6
-3
3
6
1 / Bound IAA (moles) x 10^9
1 / Free IAA (M) x 10^6

Fig. 24

NAA competition for IAA binding sites on a heavy membrane fraction prepared by separation of a 4,000 - 38,000 xg membrane fraction on a simple sucrose gradient.

A Specific binding data.

Each point is the mean of three replicates.

B Double reciprocal plot of the data from A. Results are expressed as binding per g fresh wt. of material.

●----● IAA only.

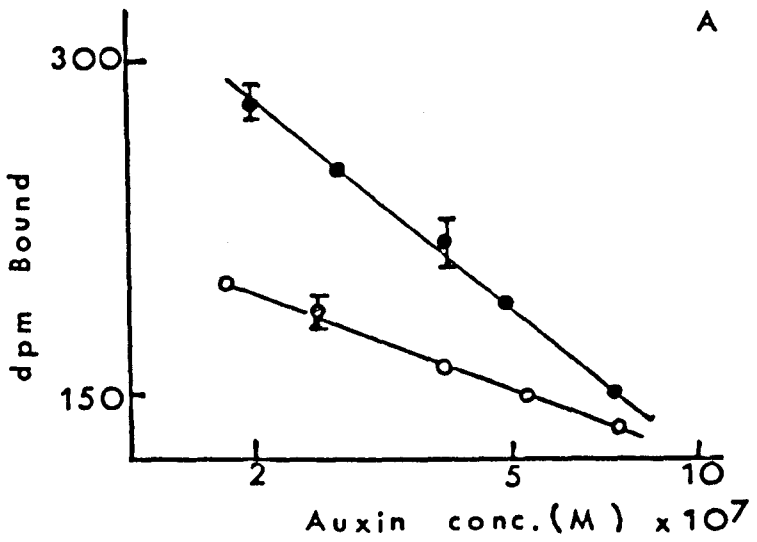
$K_2 = 9.7 \times 10^{-7}$ M

$n_2 = 5.7 \times 10^{-10}$ moles / g fresh wt

O----O IAA + 10^{-6} M NAA

$K_I = 9.8 \times 10^{-7}$ M

Interaction appears to be competitive.


A
300
150
dpm Bound
2
5
10
Auxin conc.(M) x10⁷

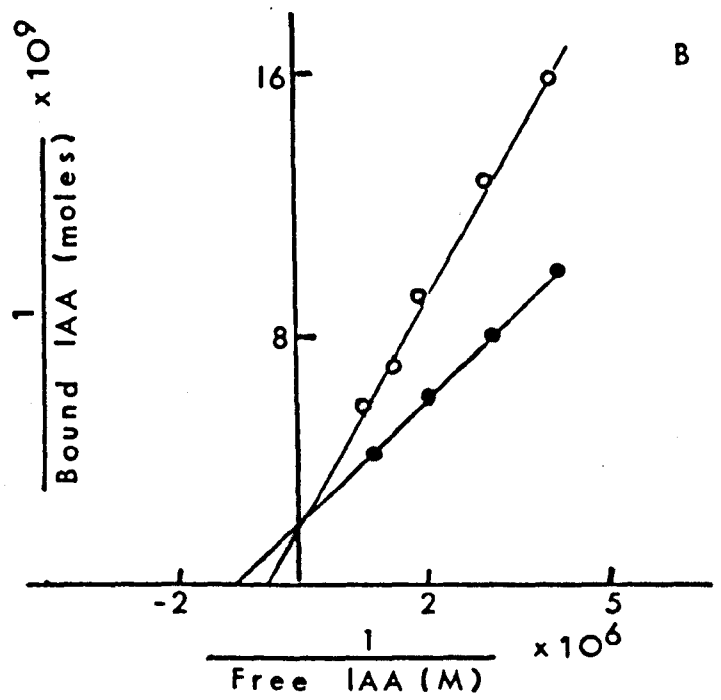

B
16
8
1/Bound IAA (moles) x10⁹
-2
2
5
1/Free IAA (M) x10⁶

Fig. 25

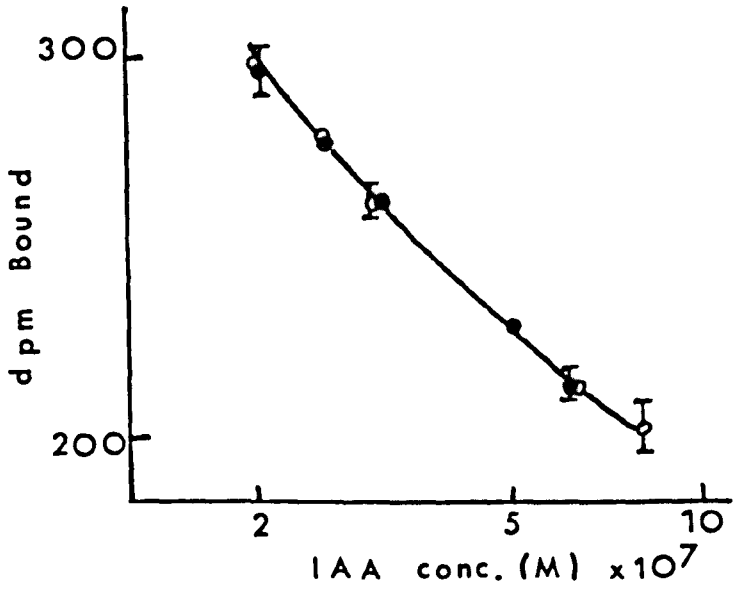
β-NAA competition for IAA binding sites on a heavy membrane fraction prepared by separation of a 4,000 - 38,000 xg membrane fraction on a simple sucrose gradient.

●----● IAA only.

O----O IAA + 10^{-6} M β-NAA

Results are the average of two experiments.

Interaction = no competition.


300
200
dpm Bound
2
5
10
IAA conc. (M) x10^7

dilute binding buffer (fig 26) along with a majority of other proteins.

Use of the DE 32 column yielded good separation of the proteins and the results from a typical run are shown in fig 27. The auxin binding proteins, as determined by equilibrium dialysis, were associated with the protein peak eluted with binding buffer containing 0.2 M NaCl.

No separation of the two classes of auxin binding sites was achieved by the stepwise elution from the ion exchange column. Not enough material was recovered from the column for kinetic analysis to be carried out so it is unclear if the IAA binding resulted from the presence of one or both of the two classes of auxin binding sites.

GEL FILTRATIION

It should be noted that these experiments were carried out using the Lincon variety of Pisum sativum and not the Kelvedon wonder variety used for other experiments.

Elution of the solubilized (acetone precipitated) proteins from the 4,000 -38,000 xg membrane fraction was achieved using a Sephacryl S-200 column. Analysis of the eluted fractions (fig 28) indicated the presence of one IAA binding peak. By comparison to the elution volumes of standard proteins previously separated on the column it was possible to determine the apparent molecular weight of the

Fig. 26

Ion exchange separation on a Sephadex DEAE 50 column of solubilized material from a 4,000 - 38,000 xg membrane fraction prepared by acetone precipitation.

A Five fold dilution of binding buffer.

B Binding buffer.

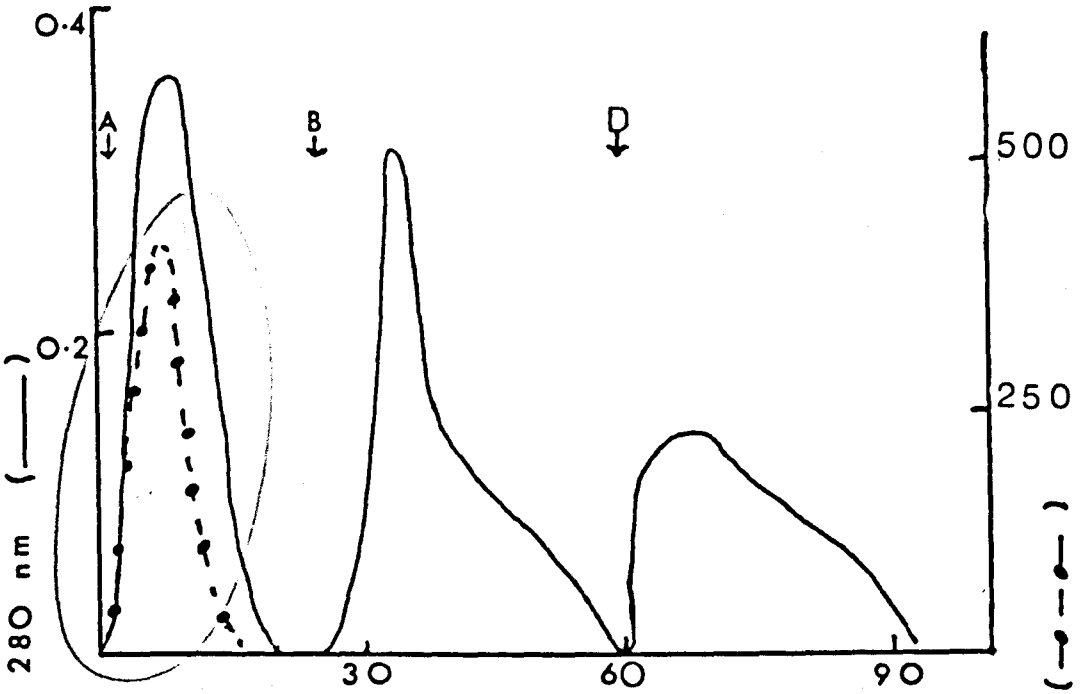
D Binding buffer + 0.5 M NaCl.

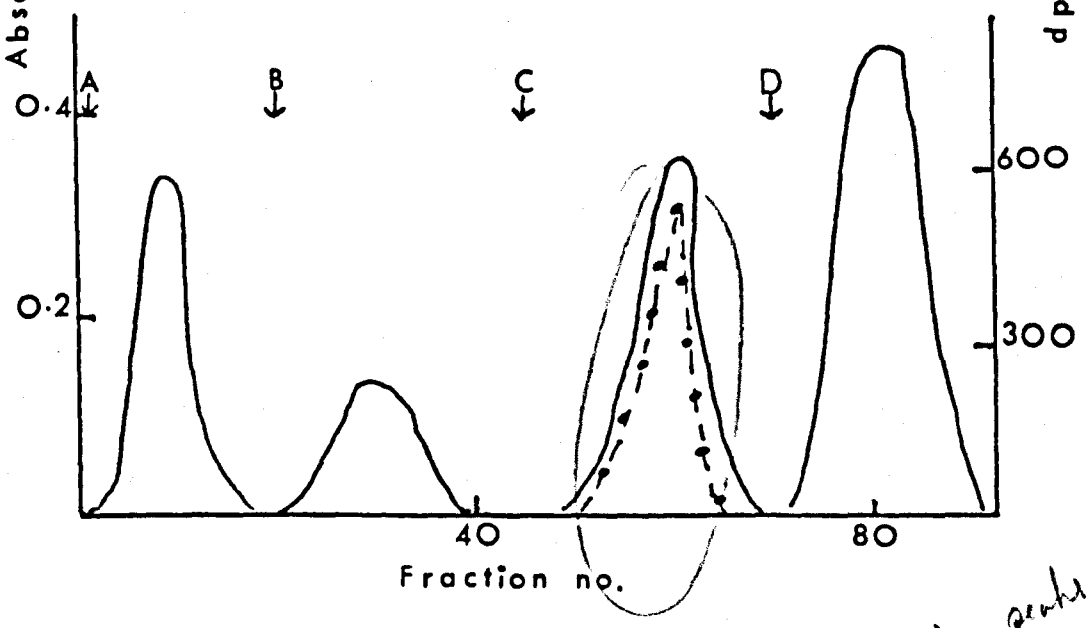
Fig. 27

Ion exchange separation on a Whatmans DE32 column of solubilized material from a 4,000 - 38,000 xg membrane fraction prepared by acetone precipitation.

Elution buffers similar to fig. 26 with the inclusion of:

C Binding buffer + 0.2 M NaCl.


0·4
0·2
A
B
D
500
250
30
60
90
Absorbance at 280 nm (——)
dpm Bound (—•—)
Fraction no.


0·4
0·2
A
B
C
D
600
300
40
80
Absorbance
dpm Bound
Fraction no.

IAA binding peak. This was calculated to be 42,400 ± 5,000 daltons.

The inclusion of 0.1M NaCl to the buffer to increase the ionic strength had no apparent effect on the IAA binding peak (fig 29). The IAA binding peak was eluted with an apparent molecular weight of 38,600 ± 3,000 daltons.

AFFINITY CHROMATOGRAPHY

To try and ascertain if the auxin binding proteins were glycoproteins, samples were eluted on a lentil lectin column. Proteins bound to the column were sucessively eluted with NAc-glc., Met-glc. and Met-man. as shown in fig 30. Auxin binding to these fractions was determined by equilibrium dialysis. As the results show, no auxin binding peaks were observed. Sodium deoxycholate was added to the column in an attempt to elute off any remaining proteins and this fraction too failed to show an auxin binding peak. Therefore it could not be determined if the auxin binding proteins had lost their activity during their passage through the column, or whether they were still bound, possiblly non specifically, to the lentil lectin.

6. INTERACTION OF AUXINS WITH PHOSPHOLIPIDS

The proton magnetic resonance spectrum of lecithin (1.4 x 10^{-2} Molal) in $CDCl_3$ showed (fig 31) discrete identifiable

Fig. 28

Separation of acetone precipitated material from a 4,000 - 38,000 xg membrane fraction of Lincon variety pea epicotyl tissue on a Sephacryl S200 column.

Buffer = binding buffer, pH 5.5, 10^{-7} M ^{14}C-IAA. Flow rate 20ml/hr, 2 ml samples collected. IAA binding was measured as the increase in radioactivity per fraction above the buffer levels.

Fig. 29

Separation of acetone precipitated material from a 4,000 -38,000 xg membrane fraction of Lincon variety pea epicotyl tissue on a Sephadex S200 column with buffer of high ionic strength.

Buffer = binding buffer, pH 5.5, 10^{-7} M ^{14}C-IAA, 0.1 M NaCl.

Experimental conditions were otherwise similar to those of fig. 28.

Results are from a typical experiment.

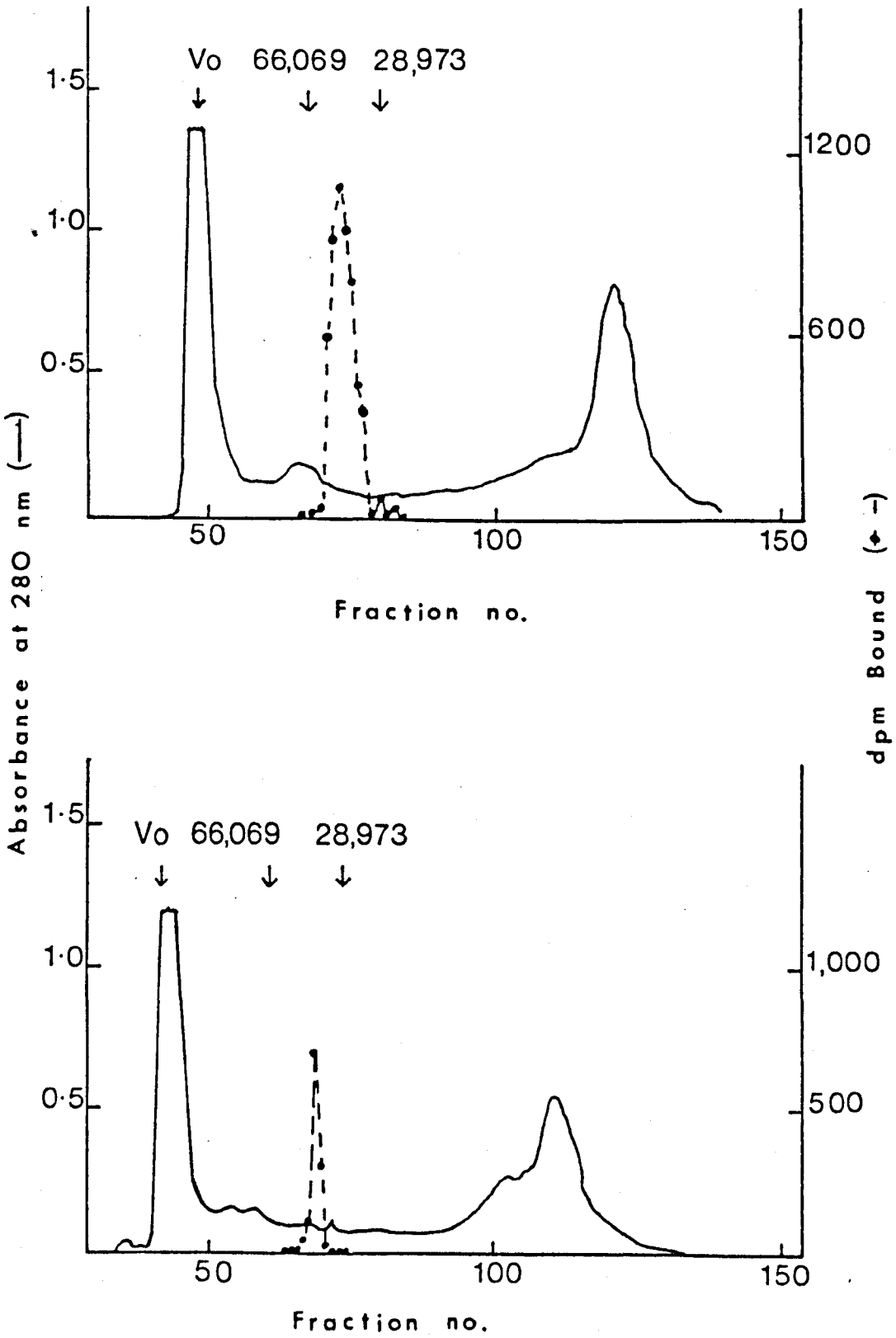

Vo
66,069
28,973
1·5
1·0
0·5
1200
600
50
100
150
Fraction no.
Absorbance at 280 nm (—)
dpm Bound (•—•)
Vo
66,069
28,973
1·5
1·0
0·5
1,000
500
50
100
150
Fraction no.

Fig. 30

Separation of acetone precipitated material from a 4,000 - 38,000 xg membrane fraction on a Sepharose 4B lentil lectin column.

Separation was achieved by stepwise elution with:

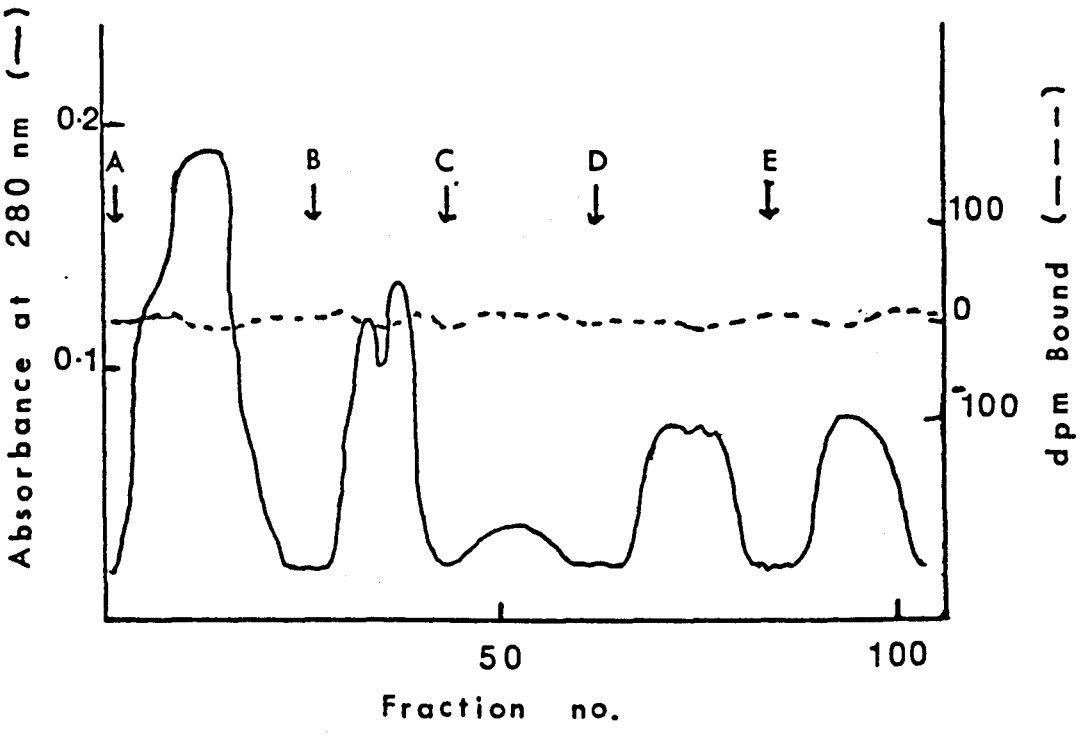
A Binding buffer

B Binding buffer + 0.2 M N-acetylglucosamine

C Binding buffer + 0.2 M methyl-α-D-glucoside

D Binding buffer + 0.2 M methyl-α-D-mannoside

E 1% sodium deoxycholate


Absorbance at 280 nm (—)
0·2
0·1
A
B
C
D
E
100
0
100
dpm Bound (— — —)
50
100
Fraction no.

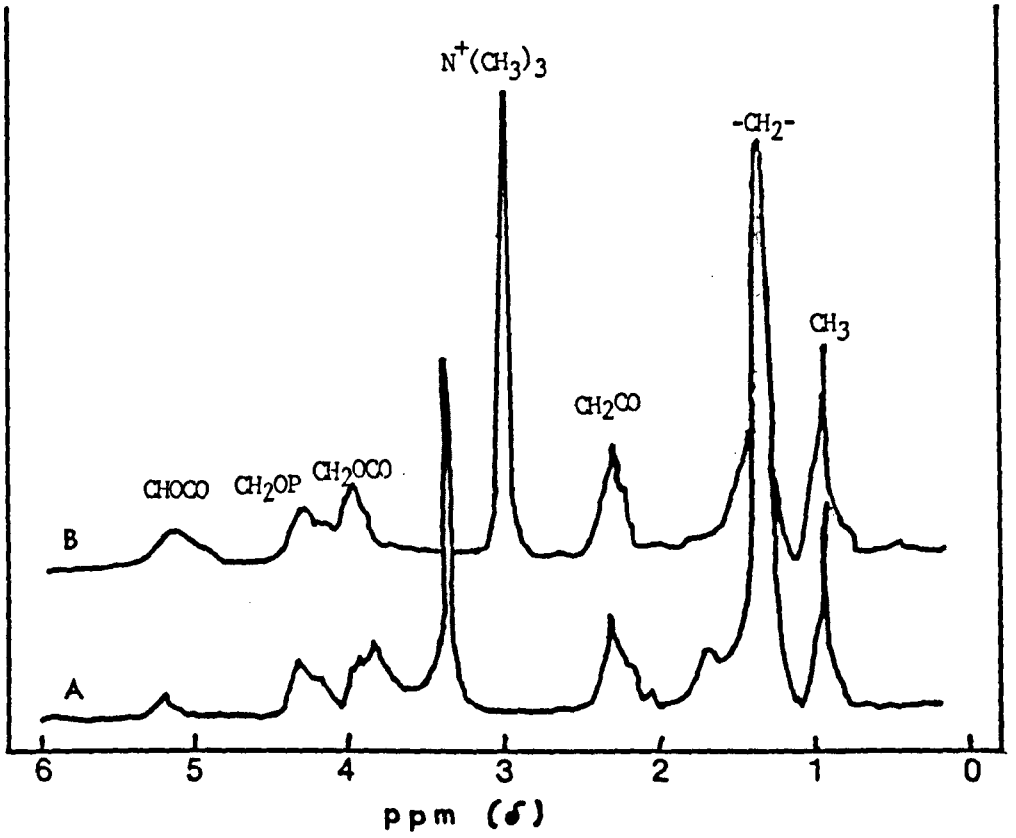
points?

Fig. 31

90 MH_z spectrum of lecithin dissolved in $CDCl_3$ in the presence and absence of added IAA.

A Lecithin alone (1.4×10^{-2} Molal)

B Lecithin + IAA (6×10^{-3} Molal)


N+(CH3)3
-CH2-
CH3
CH2CO
CHOCO
CH2OP
CH2OCO
B
A
6
5
4
3
2
1
0
ppm (δ)

resonances for the N-trimethylamino group (singlet at 3.37 ppm), the $-CH_2-$ group (1.26 ppm), the methyl group (0.88 ppm), the CH_2OCO group at 3.9 ppm, the CH_2OP group (4.2 ppm) and the CH_2CO group at 2.3 ppm.

IAA was added up to a final concentration of 6×10^{-3} Molal and the effects, if any, on the resonance peaks were observed (table 13). From this it can be sen that only the N-trimethyl group showed any significant chemical shift changes upon addition of IAA. There was a small downfield shift change associated with the CH_2OCO group but to no significant extent.

There was a small degree of line broadening of the $N(CH_3)_3$ renonance peak dependent on IAA concentration (table 14 and fig 32) but not enough to suggest that the interaction between IAA and lecithin was ionic in nature. The minimal line broadening and the large upfield shifts of the $N(CH_3)_3$ peak suggested that the interaction involved aromatic ring diamagnetic shielding.

These results suggested that the polar head groups of the phospholipid were the major sites affected and were probably the sites of action for complexing with IAA. In contrast, the resonance peaks associated with the fatty acid chains showed no observable changes.

It was possible to achieve saturation of the interaction although a large excess of IAA was required

Table 13.

Chemical shifts of some of the groups of egg yolk lecithin as a function of IAA concentration in $CDCl_3$.

Lecithin concentration = 1.4×10^{-2} Molal.

IAA CONCENTRATION	CHEMICAL SHIFTS					
x 10^3 Molal.	ppm (δ)					
	CH_2CO	$-CH_3$	$-CH_2-$	$-\overset{+}{N}(CH_3)_3$	CH_2OCO	CH_2OP
0	2.2983	0.8800	1.2575	3.3094	3.9396	4.2182
0.4801	2.2915	0.8925	1.2582	3.2715	3.9658	4.2176
0.9046	-	0.9019	1.2522	3.2467	3.9827	4.0773
1.0186	2.2999	0.8801	1.2573	3.2376	3.9869	4.2205
1.9395	2.2995	0.8799	1.2569	3.1790	3.9899	4.2205
2.6193	2.3012	0.8801	1.2572	3.1618	3.9845	4.2704
4.1986	2.2992	0.8798	1.2566	3.0739	3.9868	4.2199
4.6085	2.2983	0.8812	1.2576	3.0471	3.9880	4.2180
4.8788	2.3027	0.8803	1.2571	3.0367	3.9857	4.2200
5.7860	2.2994	0.8795	1.2561	2.9226	3.9854	4.2180

Measurements were made on a Perkin-Elmer R32 90 MH_z spectrometer.

Table 14.

Effect of IAA concentration in $CDCl_3$ on the N-trimethyl resonance peak position and its half-width.

Lecithin concentration = 1.45×10^{-2} Molal.

IAA CONCENTRATION $\times 10^2$ Molal	N-trimethyl resonance peak	
	Location (H_z)	Half-width (H_z)
0	301	2.77
0.6	293	2.77
0.8	289	2.89
1.1	284	2.89
1.7	276	3.00

Measurements were made on a 90 MH_z spectrometer.

Fig. 32

The location of the N-trimethyl resonance peak of lecithin, on an expanded scale, in the presence of increasing amounts of IAA.

Spectra were run on a 90 MH_z spectrometer.

Lecithin concentration = 1.45×10^{-2} Molal.

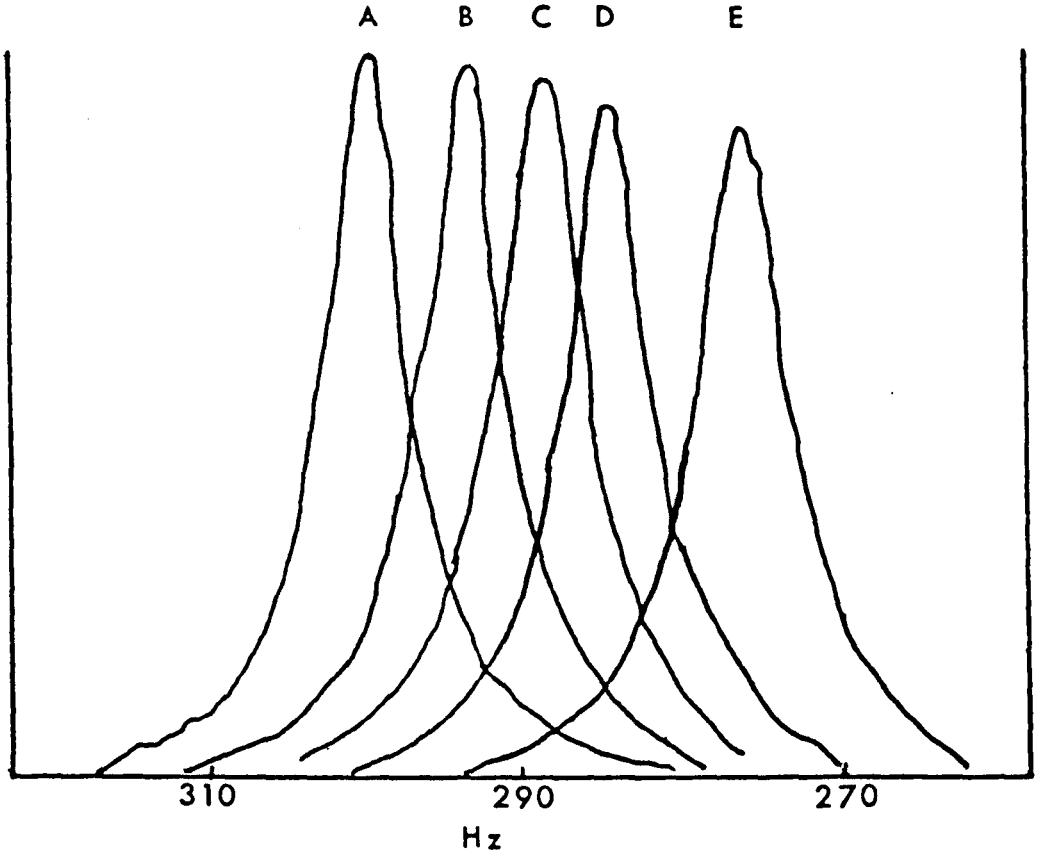
A Lecithin alone

B Lecithin + 0.6×10^{-2} Molal IAA

C Lecithin + 0.8×10^{-2} Molal IAA

D Lecithin + 1.1×10^{-2} Molal IAA

E Lecithin + 1.7×10^{-2} Molal IAA


A
B
C
D
E
310
290
270
Hz

(fig 33). Computation of the results gave a value for the dissociation constant of 1.9×10^{-2} Molal.

INTERACTION OF IAA WITH CONSTITUENT PARTS OF THE PHOSPHOLIPID MOLECULE

To acertain which parts of the phospholipid molecule were essential for the interaction with IAA in $CDCl_3$, constituent parts were dissolved in $CDCl_3$ either with or without IAA and spectra run.

Since the greatest effect of IAA was on the polar head group, free choline was first tested. Spectra of free choline in $CDCl_3$ were run then IAA added to a final concentration of 2 mg/ml - a concentration similar to that which would cause significant changes in the chemical shifts of the phospholipid molecule. Analysis of the spectra (fig 34) indicated that IAA had no observable effect on free choline.

Spectra of phosphoryl choline in the presence and absence of IAA (fig 35) indicated that some interaction was taking place. The major resonance peak at 1.55 ppm showed a downfield shift with the addition of IAA. Two major resonance peaks were observed for dicetyl phosphate (fig 36), one at 1.35 ppm and the other at 3.37 ppm. Only this later peak was affected by the addition of IAA causing a small upfield shift similar to that obtained with the phospholipid molecules. The interaction of glycerophosphate

Fig. 33

The relationship between the shift of location of the N-trimethyl resonance peak of lecithin in $CDCl_3$ and the amount of IAA added.

Results were measured as a function of the ratio of IAA concentration/lecithin concentration.

$Kd = 1.9 \times 10^{-2}$ Molal.

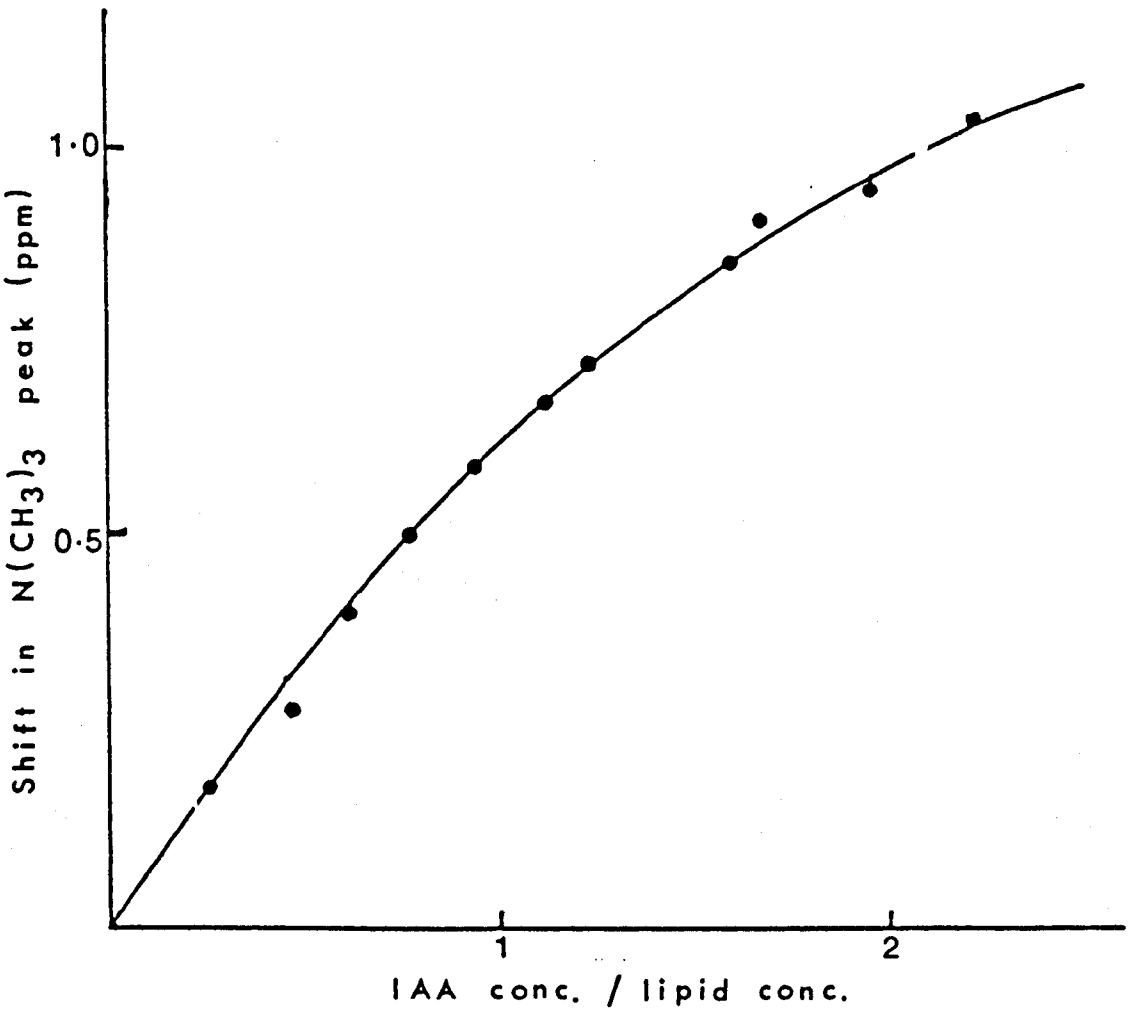
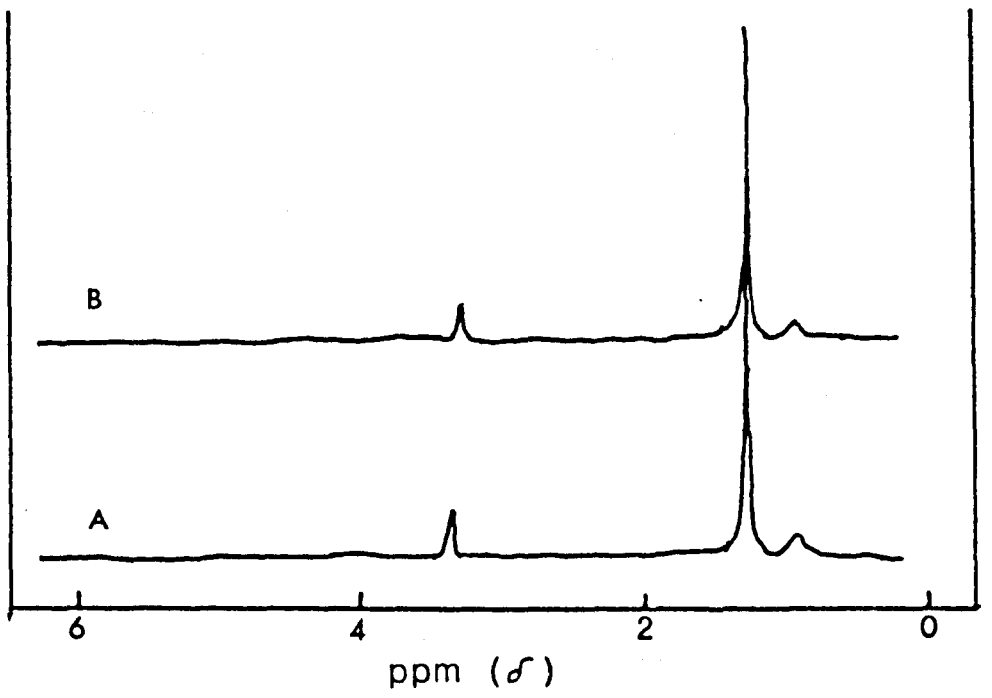

1·0
0·5
Shift in $N(CH_3)_3$ peak (ppm)
1
2
IAA conc. / lipid conc.

Fig. 34

80 MH_z spectrum of choline dissolved in $CDCl_3$ in the absence (A) and presence (B) of IAA.

Fig. 35

80 MH_z spectrum of phosphoryl choline dissolved in $CDCl_3$ in the absence (A) and presence (B) of IAA.


B
A
6
4
2
0
ppm (δ)

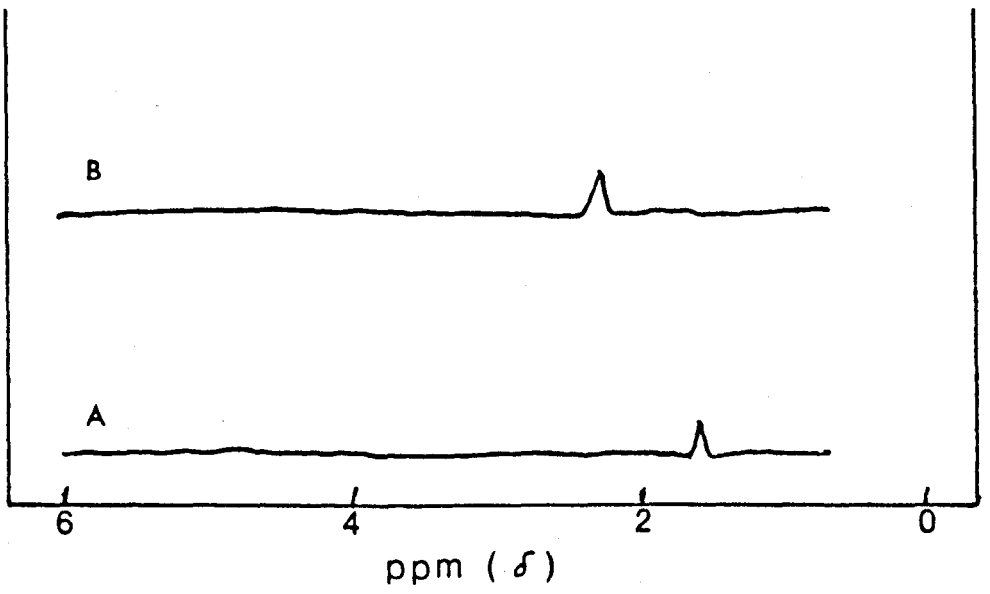
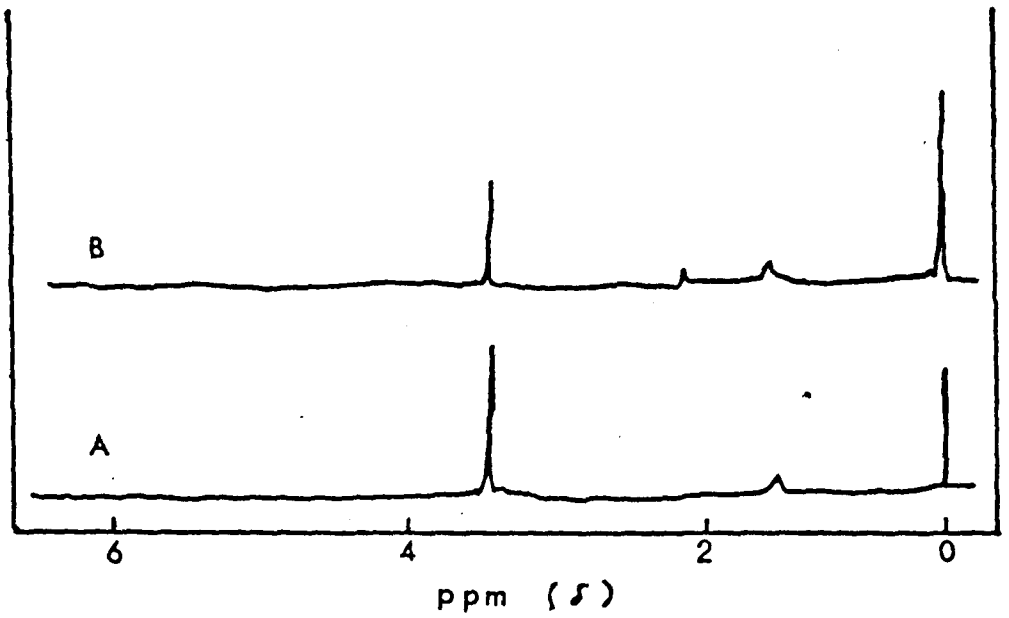

B
A
6
4
2
0
ppm (δ)

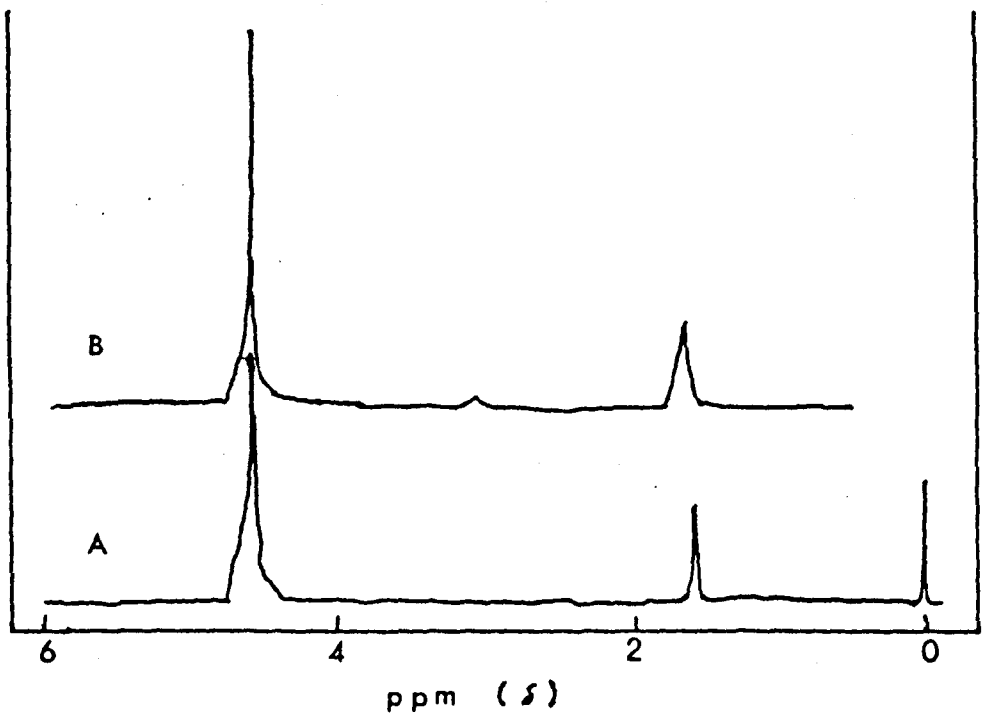
Fig. 36

80 MH_z spectrum of dicetyl phosphate dissolved in $CDCl_3$ in the absence (A) and presence (B) of IAA.

Fig. 37

80 MH_z spectrum of glycerophosphate in $CDCl_3$ in the absence (A) and presence (B) of IAA.


B
A
6
4
2
0
ppm (δ)


B
A
6
4
2
0
ppm (δ)

with IAA resulted in a large downfield shift change of the peak at 1.55 ppm (fig 37).

EFFECT OF FATTY ACYL CHAIN LENGTH ON INTERACTION WITH IAA

The interaction of IAA with dipalmitoyl PC was compared to the interaction with dimyristoyl PC. Results (fig 38) indicated that there was little difference in the specificity of interaction in $CDCl_3$ as measured by the chemical shift changes of the choline head groups.

EFFECT OF DIFFERENT POLAR HEAD GROUPS ON THE INTERACTION WITH IAA

Since IAA appeared to primarily affect the chemical shifts of the polar head group of the PC molecules tested, a comparison was made between two phospholipids differing only in their polar head groups. Analysis of the data (fig 39) indicated that interaction with dipalmitoyl phosphatidyl ethanolamine resulted in greater chemical shift changes for the ethanolamine head group than with the choline head group of dipalmitoyl phosphatidyl choline.

SPECIFICITY OF AUXIN BINDING TO LECITHIN IN $CDCl_3$

Auxin analogues were tested for their interaction with egg yolk lecithin dissolved in $CDCl_3$. As the results show (fig 40) there was no discernible difference between the

Fig. 38

The effect of fatty acyl chain length on phosphatidyl choline interaction with IAA in $CDCl_3$.

●----● Dipalmitoyl phosphatidyl choline.

O----O Dimyristoyl phosphatidyl choline.

Fig. 39

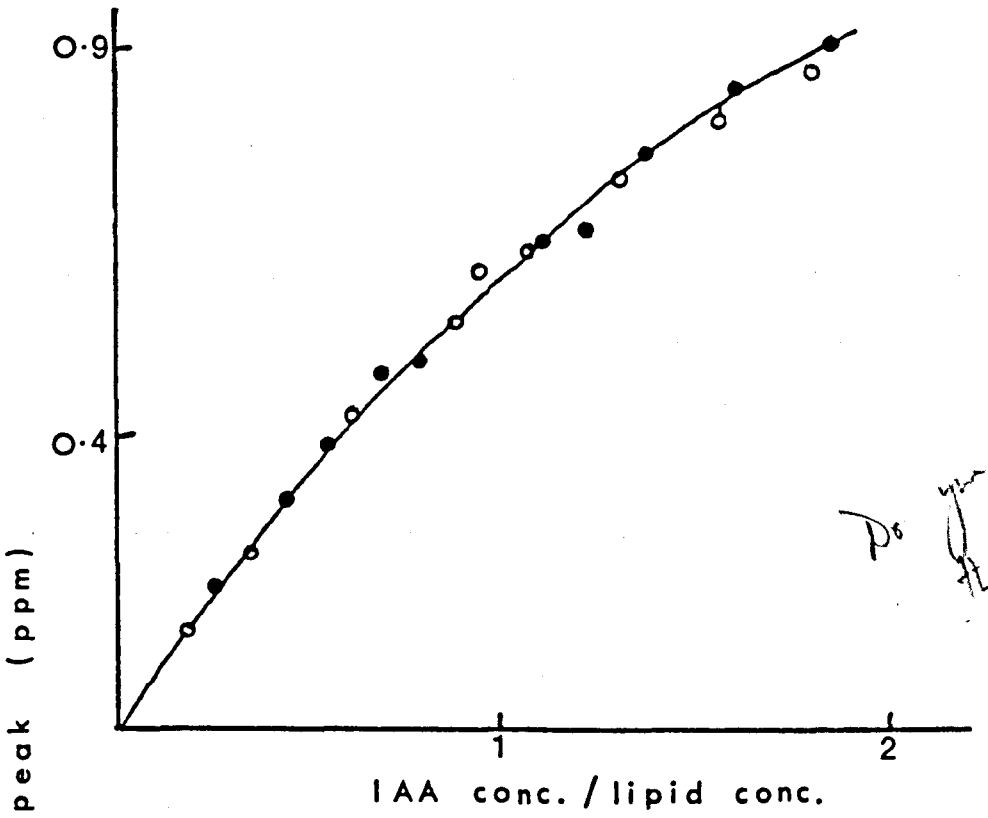
The effect of different phospholipid polar head group regions on their interaction with IAA in $CDCl_3$.

●----● Dipalmitoyl phosphatidyl ethanolamine.

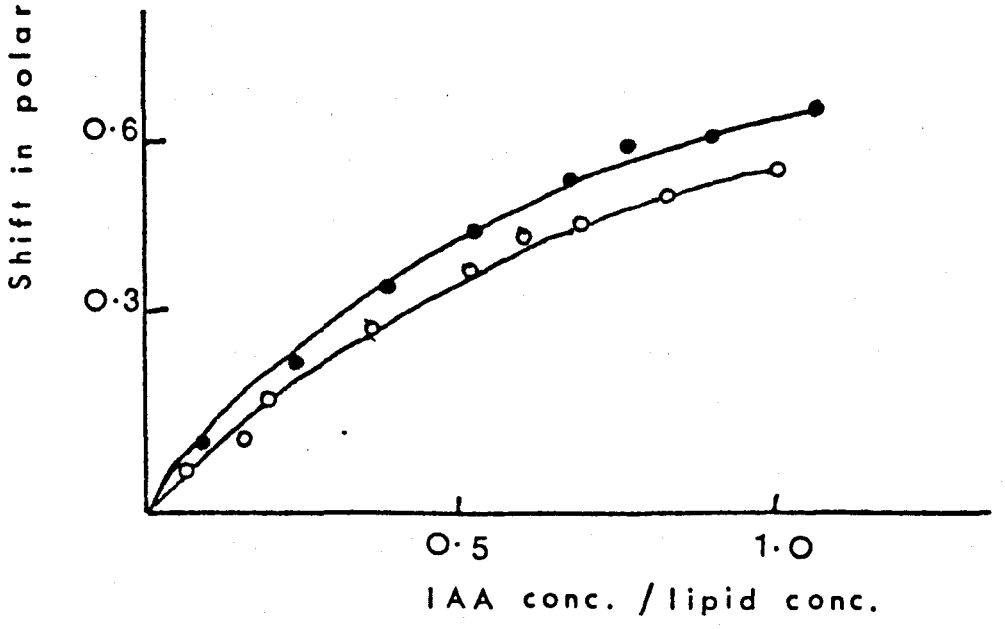
O----O Dipalmitoyl phosphatidyl choline.

For both these experiments the lipid concentration was held constant at approximately 1.5×10^{-2} Molal and the IAA concentration varied.

The change in shift of the polar head group region of the lipid was measured on a 80 MH_z spectrometer.



Do you have TCV
Flow curves
of this shape?



specificity of IAA, NAA and β-NAA as measured by their effect on the head group of the phospholipid.

SPECIFICITY OF AUXIN BINDING TO LECITHIN IN D_2O

The binding of auxin analogues to lipid vesicles was tested for specificity as a comparison with the results obtained in $CDCl_3$. As can be seen from fig 41 both NAA and β-NAA were much less effective than IAA in causing chemical shift changes of the polar head groups of the lipid. NAA had a similar affinity for the lipid as β-NAA and significant changes in the chemical shift of the $N(CH_3)_3$ group were observed only at high concentrations of the auxin. It was also noted that a significant concentration of auxin was required before any chemical shift changes were measured.

THERMAL TRANSITIONS IN PHOSPHATIDYL CHOLINE

Heating curves for DMPC alone and with the addition of IAA (1:1 molar ratio) can be seen in fig 42. The transition temperature was taken at the onset of the heat absorption peak and for DMPC was 27°C. The pretransition peak is also peculiar to each phospholipid type and for DMPC occured at 18°C, although this peak was rather broad and difficult to measure.

The addition of IAA produced alterations in both the main transition and pretransition peaks. The main

Fig. 40

The interaction of various auxin analogues with dipalmitoyl phosphatidyl choline in $CDCl_3$.

●----● IAA

O----O NAA

▲----▲ β-NAA

Fig. 41

The interaction of various auxin analogues with dipalmitoyl phosphatidyl choline vesicles in D_2O.

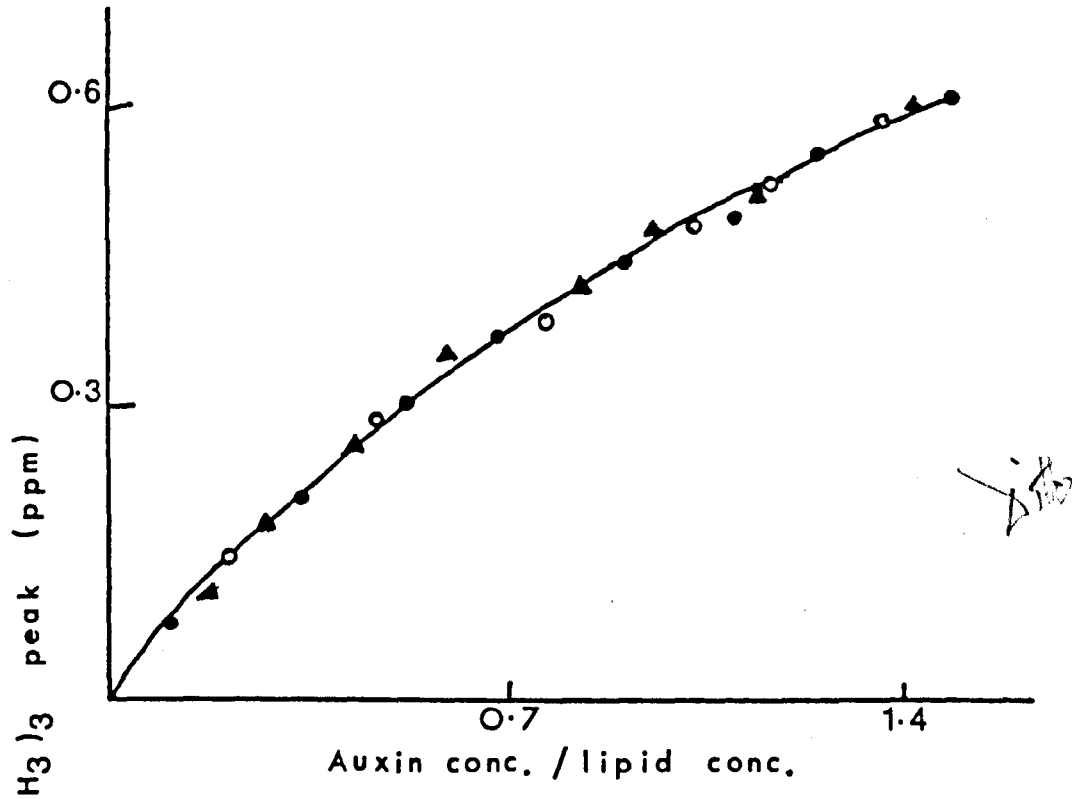
●----● IAA

O----O NAA

▲----▲ β-NAA

For both these experiments the lipid concentration was constant at approximately 1.5×10^{-2} Molal and the auxin concentration varied.

The change in shift of the polar head group region of the lipid was measured on a 80 MH_z spectrometer.


0·6
0·3
0·7
1·4
Shift in $N(CH_3)_3$ peak (ppm)
Auxin conc. / lipid conc.

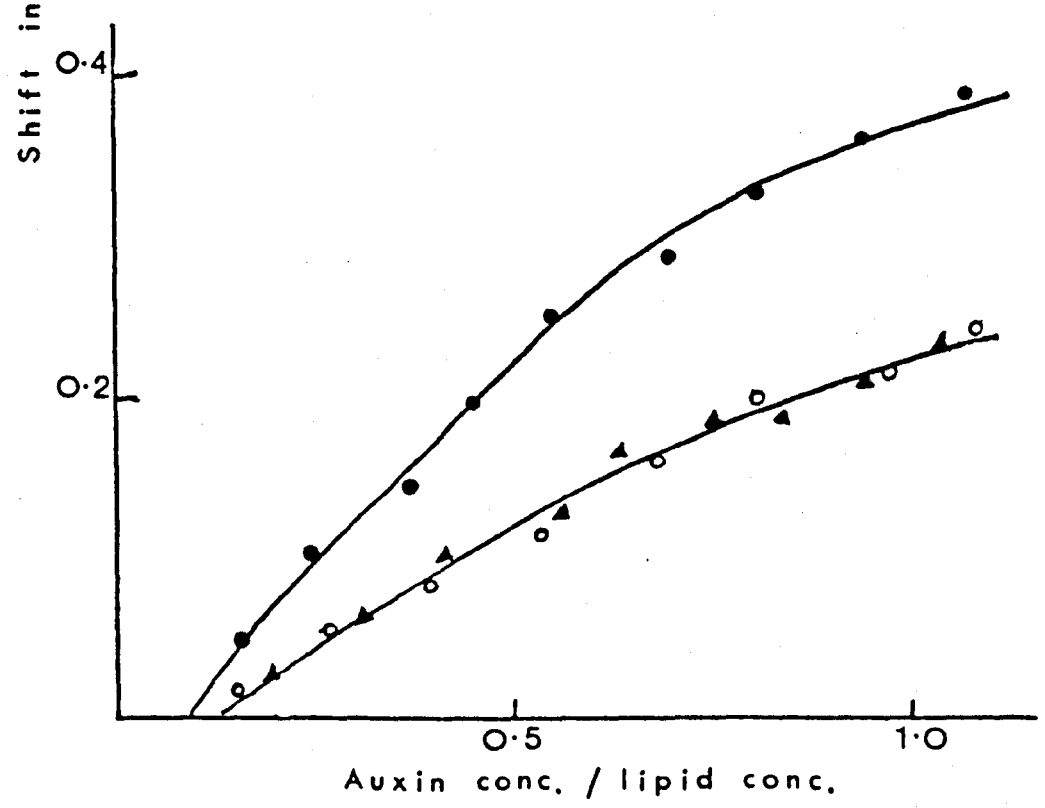

0·4
0·2
0·5
1·0
Shift in
Auxin conc. / lipid conc.

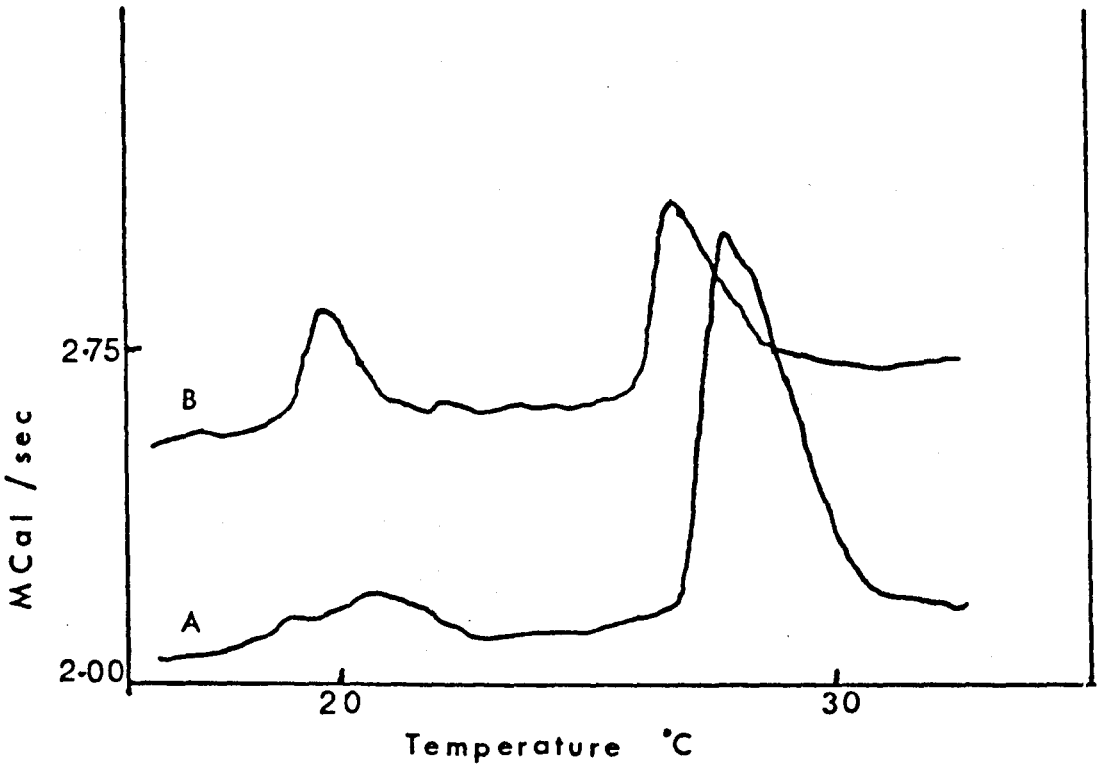
Fig. 42

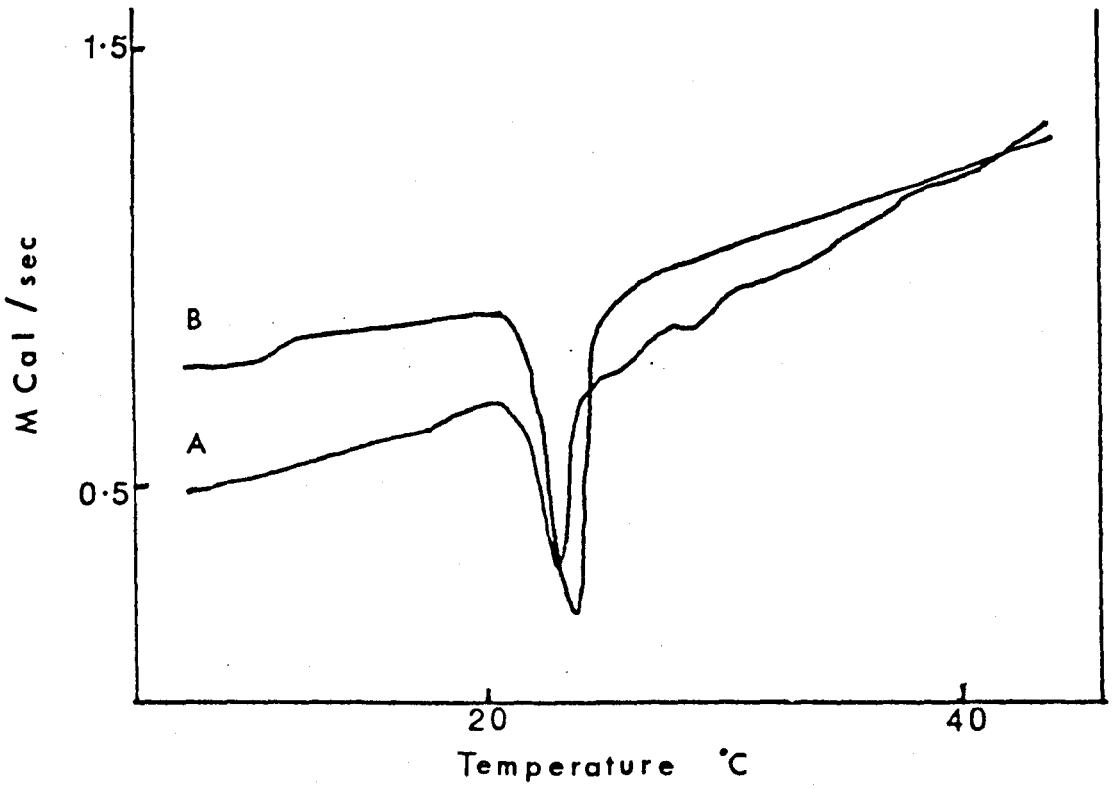
Heating curves of dimyristoyl phosphatidyl choline vesicles either alone (A) or in the presence of IAA (B).

Fig. 43

Cooling curves of dimyristoyl phosphatidyl choline vesicles either alone (A) or in the presence of IAA (B).

Experiments were performed on a Perkin-Elmer DSC 4 differential scanning calorimeter.


2·75
2·00
M Cal / sec
B
A
20
30
Temperature °C


1·5
0·5
M Cal / sec
B
A
20
40
Temperature °C

transition was reduced by 1.5°C to 25.5°C and was considerably broadened. Onset of the pretransition peak was unaffected by the addition of IAA but the peak was more pronounced. The enthalpy of the transition was greatly increased by the addition of IAA (table 15).

The cooling curves of the samples (fig 43) showed a loss of the pretransition peak possibly due to supercooling effects. The transition temperature of the sample containing IAA was 1°C lower than that of lipid alone. IAA also increased the enthalpy of cooling by a significant amount (table 15).

Table 15.

The enthalpies of the main transition peak of DMPC vesicles in the absence and presence of IAA.

The results were calculated from figs. 42 and 43.

A	HEATING CURVE
SAMPLE	ENTHALPY KJ/MOL
DMPC	2.0
DMPC + IAA	14.9

B	COOLING CURVE
SAMPLE	ENTHALPY KJ/MOL
DMPC	3.4
DMPC + IAA	11.9

DISCUSSION

Initial experiments were carried out to determine the optimum conditions for IAA binding to pea epicotyl membrane fractions.

Many membrane isolation techniques employed the mortar and pestle method for grinding up the plant material (e.g. Hodges and Leonard, 1974), but this method was not used in these studies because of the large amounts of material required and the time needed for the process. Ray et al. (1977a) employed a mechanical homogenizer which did not appear to affect results and a similar process was used here.

The inclusion of a wash step increased binding of IAA to the crude membrane fractions (table 1) and it was thought that this was due to removal of a soluble "supernatant factor", although no experiments were conducted to test this. (Hertel, 1974) first reported the presence of this SF and it has been suggested that its function was to modify auxin activity within the cell. Batt (1975) indicated that SF was not always present in corn coleoptile tissue. For one batch of corn a wash step did not increase NAA binding to any significant extent, but in a later batch a wash step was necessary. The physiological significance of SF has yet to be determined.

For many of the experiments the membrane pellet was

prepared by differential centrifugation which led to the loss of some binding activity both in the precentrifugation pellet and in the final supernatant. The 4,000 -38,000 xg membrane fraction was used extensively for experiments since it appeared to contain both classes of binding sites.

In experiments carried out on the membrane fractions a certain amount of binding was termed non -specific binding. It was defined as the amount of radioactive IAA still bound to the membranes in the presence of a large excess of unlabelled IAA and was thought to include IAA trapped inside membrane vesicles and IAA bound to very low affinity sites. This non -specific binding was always subtracted from total IAA binding before any kinetic analysis was carried out since it could seriously affect the results. It has been reported (e.g. Klotz and Hudson, 1971) that non -specific binding can cause curvature of both the Scatchard plot and the double reciprocal plot which can cause miscalculation of binding data. This was a criticism made by Ray et al. (1977a) against Batt et al. (1976) who published results indicating, for the first time, the presence of two classes of auxin binding sites in corn coleoptiles. Other workers (Hertel et al., 1972; Ray et al., 1977a) had only found evidence for one class of auxin binding sites - although there was disagreement as to its location. It was suggested that the two classes of binding sites reported by Batt et al. (1976) resulted from an artefact due to inclusion of non -specific binding in analysis of the data. Results here show that with the

subtraction of non -specific binding there appears to be two discrete classes of IAA binding sites in pea epicotyl tissue (fig 12).

The subtraction of non -specific binding from the results of Batt et al. (1976) did not result in linear Scatchard plots (Rubery, 1981) indicating that there was indeed site heterogenity. Dohrmann et al. (1978) included corrections for non -specific binding in their calculations and found three kinetically distinct sites in maize coleoptiles.

Ray et al. (1977a) and Hertel (1974) conducted their experiments over a wide range of NAA concentrations, while Batt and Venis (1976) concentrated on a more narrow range. The kinetic parameters of the site found by Hertel (1974) were similar to site 2 binding found by Batt and Venis (1976) and it was suggested that these other workers missed site 1 binding because of the NAA concentrations used.

Many of the hypotheses of auxin induced growth assume that there is an interaction with or at the plasma membrane (e.g. Hertel et al., 1972) and that in vitro auxin binding is the result of this interaction. Some research workers have suggested that the single class of auxin binding sites was located on the ER (Ray, 1977) which would question these early assumptions. Others (Batt and Venis, 1976) have indicated the presence of a plasma membrane associated binding site and another site located on either the ER or

the Golgi apparatus.

Initial experiments, e.g. the results obtained for the pH optima of the binding sites (figs 4-7), suggested that the two classes of binding sites were localized on different membranes.

The main characterization of membranes depend on their biochemical properties rather than on their physical characteristics. It has been known for some time that certain enzymes are located solely on a particular membrane and use was made of some of these. Mitochondria were characterized by cytochrome c oxidase activity (Appelmann et al., 1955) while cytochrome c reductase was associated with the endoplasmic reticulum (Hodges and Leonard, 1974). The tonoplast could be identified by the presence of acid phosphatase (Semandi, 1967) and the Golgi bodies by glucan synthetase 1 (Van der Woude et al., 1974). Both ATPase (Lowry and Lopez, 1946) and glucan synthetase 2 (Van der Woude et al., 1974) were thought to be associated with the plasma membrane.

From the results obtained with the discontinuous sucrose gradients it appeared that the two classes of IAA binding sites were associated with different membrane fractions (table 7). One peak of ^{14}C-IAA binding was associated with light membranes - sucrose concentration 25-30% - and the other peak associated with membranes found at the 34-38% sucrose interface. These results were

in good accord with those reported by Batt and Venis (1976) working on corn coleoptiles. The heavier membrane fraction consisted mainly of plasma membrane as determined by enzyme marker assays. The distribution of plasma membrane in the sucrose gradients correlated well with results obtained by others working on different tisues; oat roots (Hodges et al., 1972), soybean hypocotyls (Hardin et al., 1972) and corn coleoptiles (Batt and Venis).

It was less easy to assign a localization for the other IAA binding site found in the lighter membrane fraction since that fraction was rich in endoplasmic reticulum and Golgi bodies. Batt et al. (1976) tentatively suggested that this class of binding sites was localized on the Golgi bodies while Ray (1977) reported the presence of an endoplasmic reticulum associated binding site. Further experimentation was carried out to determine its location. Use was made of the fact that high magnesium concentrations prevent the stripping of ribosomes from the endoplasmic reticulum (Quail, 1975). Under these conditions the rough ER fractionated at a higher sucrose concentration. Under the experimental conditions used here it appeared to fractionate in the same fraction as that of the plasma membrane (table 11).

No other membrane fraction appeared to be affected by the high magnesium concentration as determined by localization of the various enzyme markers (table 10). Examination of EM micrographs (fig 15) of the fraction that

contained the cytochrome c reductase activity clearly showed the typical structure of the rough endoplasmic reticulum. The IAA binding peak was associated with this fraction (table 11) suggesting that the binding was associated with the ER and not the Golgi bodies. To confirm this, the fraction containing the ER and the plasma membrane was treated with EDTA which resulted in the dissociation of the ribosomes from the endoplasmic reticulum. Refractionation of this sample resulted in the reappearance of an IAA binding peak at the 25-30% sucrose interface. Not only did this suggest that one class of IAA binding sites in pea epicotyl tissue was associated with the ER but it also suggested that it was associated with the membrane itself and not the ribosomes. Ray (1977) used similar techniques to show that the NAA binding site in maize coleoptiles was localized on the ER and a similar conclusion was reached by Normand et al. (1975) also working on maize tissue.

Because of the small amount of material found in each fraction it was impossible to carry out kinetic analysis on the membranes separated on the complex sucrose gradient. Use was made of a simple discontinuous sucrose gradient (Batt and Venis, 1976) to fractionate the crude membrane pellet into light and heavy membrane bands. Kinetic analysis of these two fractions was carried out and the results indicated that site 1 activity was associated with the light membrane band (fig 20) and site 2 activity was associated with the heavy band (fig 21). The dissociation

constants obtained for both sites were in good agreement with the data obtained from analysis of the crude membrane pellet suggesting that there was little cross contamination between the two fractions.

The pH profiles of the two membrane fractions obtained from the simple sucrose gradient (figs 18 and 19) indicated that site 1 binding had a pH optimum of pH 6 whereas binding to site 2 showed maximal activity at pH 5. Plant cells typically have a greater pH inside the cell than the surrounding extracellular fluid (approx. pH 7 inside, approx. pH 5 outside). It would make biological sense for a receptor to have a pH optimum similar to the pH of the medium with which it comes in contact. Thus it would make sense for an auxin binding site located on the plasma membrane and in contact with the extracellular fluid to have a lower pH optimum than a binding site on the ER and in contact with the more alkaline cytosol.

It would appear that good separation of the two classes of IAA binding sites could be achieved by the use of the simple sucrose gradient and that the two sites were associated with different membrane fractions.

Having determined the kinetics of binding and the location of the two IAA binding sites, attention was turned to the specificity of auxin binding. As has been stated above, Hertel *et al.* (1972) suggested that the *in vitro* binding site should be specific in that only growth active

auxins, competitive anti -auxins or transport inhibitors should interact with the receptor site and that there should be no interaction with inactive auxins.

Competition studies were carried out using the principles of enzyme -inhibitor studies (Dixon and Webb, 1958). This method was used by Thomson and Leopold (1974) in studies on the effects of morphactins on NPA binding in corn coleoptile tissue and by Batt et al. (1976) in studies on the specificity of auxin binding to corn coleoptile membranes.

The results obtained here (figs 22-25) indicate that NAA and β-NAA both showed competition for site 1 while β-NAA had no discernable effect on IAA binding to site 2. Although only two auxin analogues were tested (one active and the other inactive) the results suggest that site 2 was the more specific auxin binding site since the inactive β-NAA did not compete for binding. However many more auxins would need to be tested before it could be said that site 2 was an auxin specific binding site . These results were in accord with those of Batt et al. (1976) who studied the specificity of the auxin binding sites in corn coleoptiles in much greater detail.

The binding kinetics of the binding sites solubilized by the acetone precipitation method (Venis, 1977a) were determined by equilibrium dialysis. The kinetics of the two sites (fig 14) were similar to the binding data obtained

from experiments on the crude membrane pellets suggesting that the solubilization method did not appear to affect the binding sites. Similar results were obtained by Venis (1977a) and Cross and Briggs (1978) working on corn coleoptile tissue.

Neither of the two classes of auxin binding sites were found in the lower, non -growing region of the pea epicotyl (fig 16) and it was suggested that this provides circumstantial evidence that one or both of the binding sites may be involved in the growth process. Working on maize coleoptiles, Kearns (1982) showed that as the coleoptile size increased, the auxin binding and auxin responsivness decreased, again suggesting a receptor role for the auxin binding sites. Taking this in consideration with the effect of temperature on IAA binding (fig 8) it can be argued that the binding sites are very temperature sensitive so are possibly unstable and degrade with age therefore loosing their binding capacity.

Separation of the solubilized proteins from the crude membrane fraction by column chromatography (figs 28 and 29) resulted in a single auxin binding peak being observed. In buffer of low ionic strength it had an apparent molecular weight of 42,400 and in buffer of high ionic strength a molecular weight of 38,600 was seen. There was no significant difference in these values. Venis (1977) showed the presence of two auxin binding peaks in work on corn coleoptiles, although the results were of a binding

peak with a "shoulder". The molecular weights were 47,300 and 40,300. Cross and Briggs (1978) also working on corn coleoptiles only found evidence of a single auxin binding peak with a molecular weight of 80,000 daltons. It was thought that differences between these two results were due to proteolysis in the fractions prepared by Venis (1977). Later results by Venis (1980) discounted this and it was thought unlikely that proteolysis occured here since PMSF was present at all stages of the preparation. It was unclear if the results shown here represent one or both of the auxin binding proteins in pea epicotyl tisue since kinetic analysis on the fractions was not carried out.

The presence or absence of any IAA -phospholipid interaction was also investigated as were some of the characteristics of any such interaction. Initial NMR results carried out in deuterochloroform confirmed earlier studies (Marker et al., 1978) that the region of the phospholipid molecule most affected was the polar head group region. Large upfield changes in the chemical shift of the N-trimethyl group of PC were observed (fig 31) and were shown to be dependent on the concentration of IAA added (table 13).

It is thought that the lecithin molecules must be in an organised multimolecular structure for any interaction to take place (Marker et al., 1978). Lecithin forms inverted micelles in chloroform (Levine et al., 1972) and can be induced to form unilamellar vesicles in water (Szoka and

Papahadjopoulos, 1980).

While the N-trimethyl head group appeared to be the most affected group with the addition of IAA, there was a small downfield shift associated with the carbonyl groups of the fatty acids. The dissociation constant for the interaction between IAA and PC was 1.9×10^{-2} Molal, assuming a 1:1 complex. This was not a very strong interaction but could be of physiological significance since the strength of some enzyme-inhibitor complexes have been shown to be of a similar order of magnitude (Nicholson and Spotswood, 1973; for review).

As studied here, the length of the fatty acid chains did not appear to have any effect on the strength of the complex formed with IAA in $CDCl_3$ (fig 38), although no experiments were carried out with short chain fatty acids or with unsaturated fatty acids. It is pertinent to note that recent studies (Jones and Paleg, 1984b) carried out in D_2O indicated that a minimum chain length of eight carbon atoms was required for interaction with IAA to be observed. With the amphiphiles tested a minimum chain length of eight carbon atoms was also required for micelle formation and it was suggested that it was not the total amount of lipid present that was important but rather the amount of lipid present in micelle form.

Differences in the polar head groups of the phospholipids appeared to be of greater importance than the

fatty acyl chain length (fig 39). Small differences in the interactions with IAA were observed with phosphatidyl choline and phosphatidyl ethanolamine. The results would suggest that the polar head group region plays an important role in the interaction with IAA. These two phosphoglyceride structures were examined because they are considered to be the most common in higher plants. It would have been interesting to investigate the interaction of IAA with other polar head group regions such as phosphatidyl serine which contains an acid group or phosphatidyl inositol which contains an alcohol.

The interaction between IAA and phospholipid did not appear to be simply a complex formation with the positively charged polar head group. No interaction between IAA and choline in $CDCl_3$ was observed (fig 34), while compounds containing the phosphate and choline moieties interacted weakly with IAA (fig 35). This confirmed earlier reports (Weigl, 1969b; Marker *et al.*, 1978) that the phosphate group appeared to be a requirement for complexing to occur.

Of special interest has been whether interaction in $CDCl_3$ could be correlated to interaction in D_2O. Auxin specificity of the interaction in the two systems was examined and fundamental differences were apparent. In $CDCl_3$, IAA, NAA and β-NAA all caused similar changes in the chemical shift of the N-trimethyl group of DPPC with increasing concentrations (fig 40). The interactions between auxins and DPPC in aqueous media were somewhat

different. Some specificity was noted in that IAA had a stronger effect than did NAA, but NAA and β-NAA caused similar effects. Jones and Paleg (1984a) found, however, that NAA complexed with a higher affinity than β-NAA. The reasons for this disparity are unclear.

The regions of the phospholipid molecules involved in complex formation included the polar head group and possibly the phosphate group and the fatty acid carbonyl groups. One possible orientation would for the aromatic ring system of IAA to be orientated adjacent to the polar head group and for either the proton on the indole ring nitrogen, or the carboxyl group proton to hydrogen bond to an oxygen of the phosphate group (Marker et al., 1978).

Bray et al. (1974) indicated the importance of the N-H...O-P hydrogen bond while working on a similar system. If this hydrogen bonding did occur in this system it is possible that hydrogen bonds are also formed between the carbonyl group of the fatty acids and the indole carboxyl group proton. This would further stabilize the complex. This structure would be rather different from the one proposed by Weigl (1969b) in which it was suggested that the indole carboxyl group bound to the N-trimethyl group and the proton on the indole nitrogen hydrogen bonded to an oxygen from the phosphate group.

The indole ring has been reported to be a strong electron donor in charge -transfer complexes (Deranleau and

Schwzer, 1970) and thus could act as a site for ion pairing with the positively charged N-trimethyl group. The resultant electron distribution could account for the upfield shifts in the proton NMR resonances.

Vesicles, by their very nature, contain phospholipid head groups on both their interior and exterior surfaces. If IAA were to complex to only one set of head groups, a splitting of the N-trimethyl resonance peak would be expected. No splitting was observed suggesting that both the inside and outside polar head groups were affected equally. This would indicate that IAA could quickly traverse the lipid layer emphasizing the lipid -soluble nature of the weak acid.

Results obtained with DSC (figs 42 and 43) corroborate those obtained with NMR experiments in that IAA appears to affect mainly the polar head group region of the lipid bilayer. The results were quite different to those obtained with gibberellic acid (Pauls et al., 1982) in that the pretransition peak was enhanced rather than eliminated. This would suggest that the IAA molecules complex with the phospholipid molecules in the bilayer, possibly enhancing orderliness, rather than simply perturb it - as has been suggested for gibberellic acid (Pauls et al., 1982).

Certain lipid -soluble weak acids have been shown to act as uncouplers of oxidative phosphorylation in mitochondria and facilitate proton transfer through lipid

bilayers (Kessler et al., 1976). The weak acids would act as proton carriers forming electrically neutral carrier -ion complexes which could easily penetrate the hydrophobic core of the membrane. Proton exchange could also occur with the membrane lipids via complex formation. It is unclear whether this process plays a role in proton transport, but it should be given some consideration since it has been well documented that the primary growth process can be mimicked by acid treatment (e.g. Rayle and Cleland, 1970).

The physiological roles of the auxin binding sites 1 and 2 have not yet been fully defined. The current weight of evidence for cell growth favours the acid growth theory (Rayle, 1973; Cleland, 1973) in which auxins are thought to promote rapid elongation by a proton secretion mechanism.

Vanderhoef and Dute (1981) provided evidence - on soy bean seedlings - for auxin regulated wall loosening as well as the sustained growth that follows. The initial rapid response to the auxin is primarily due to wall loosening. The second response that follows results in steady state or sustained growth. Acid mimics the first response but does not induce a steady state elongation. Because a proton secretory mechanism is required for the acid growth theory an interaction with the plasma membrane was assumed. This proton extrusion was also thought to be ATP-dependent (Kasamo and Yamaki, 1976). Cross et al. (1978b) showed that ATPase activity was not covalently linked to the membrane bound auxin binding site. This does not however mean that

ATPase activity can not be modulated by an auxin receptor. The mobile receptor theory of Jacobs and Cuatrecusas (1977) proposes that hormone receptors and their target sites are physically separate and diffuse independently in the membrane matrix. Recent work by Thompson *et al.* (1983) has suggested the activation of an ATPase pump by auxins plus receptor.

These results make it possible to tentatively assign to the site 2 binding site on plasma membranes a part in the auxin induced ATP-dependent proton pump.

Ray (1977) suggested that the ER bound auxin receptor was involved in proton extrusion - the protons being excreted via the Golgi apparatus. Venis (1977c) proposed that the ER associated auxin binding site was a pro-receptor which, after modification, became a specific functional receptor, presumably at the plasma membrane.

The secondary stage of auxin induced growth involves protein and nucleic acid synthesis. There is no evidence to link these processes to the proton secretion that occurs probaby at the plasma membrane and the two processes were shown to be quite separate (Vanderhoef and Stahl, 1975). Data in this thesis shows that site 1 binding was associated with the endoplasmic reticulum - an organelle that plays an essential role in protein synthesis. It would be interesting to speculate that auxin binding to site 1 in some way initiates the synthesis of proteins essential for

the second phase of auxin induced growth.

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APPENDIX 1.

ABBREVIATIONS

IAA	indole-3-acetic acid
NAA	α-napthylacetic acid
β-NAA	β-napthylacetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
2,4-B	2,4-dichlorobenzoic acid
BA	benzoic acid
PAA	phenylacetic acid
PCIB	p-chlorophenoxyisobutyric acid
NPA	I-N-naphthylphthalamic acid
TIBA	triiodobenzoic acid
SF	supernatant factor
FC	fusicoccin
PC	phosphatidyl choline
PE	phosphatidyl ethanolamine
DPPC	dipalmitoyl phosphatidyl choline
DMPC	dimyristoyl phosphatidyl choline
DPPE	dipalmitoyl phosphatidyl ethanolamine
NMR	muclear magnetic resonance
$CDCl_3$	deuterated chloroform
D_2O	deuterium oxide
$N(CH_3)_3$	N-trimethly group
DSC	differential scanning calorimetry
EM	electron microscopy
PMSF	phenyl methyl sulphonyl fluoride

MOPS	3-(N-morpholino) propane sulphonic acid
UDP-glucose	uridine diphospho-D-glucose
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
cAMP	adenosine 3', 5' cyclic phosphoric acid
Tm	melting point
NAc - glc	N-acetyl glucosamine
Met - glc	methyl-α-D-glucoside
Met - man	methyl-α-D-mannoside

APPENDIX 2.

COURSES ATTENDED

Photoperception by plants. The Royal Society. 9/3/83 - 10/3/83.

Gel filtration and electrophoresis. Loughborough University. 29/3/82 - 2/4/82.

The sixth international meeting of NMR spectroscopy. Edinburgh University. 10/7/83 - 15/7/83.

Regular research seminars were held at the Polytechnic and at one of these I gave a seminar on the work described in this thesis.

Frequent discussions were held with my supervisor, Dr. P. Bligh and Dr. J. Wyer and there have been discussions and correspondence with my external advisor, Dr. M. Venis.