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SUMMARY

This thesis describes the interaction of auxins with membrane fractions prepared from shoot tissue of etiolated Zea mays seedlings.

The binding of radioactive 1-naphthylacetic acid (NAA) to these membrane fractions is investigated using a pelletting technique originally described by Hertel et al., (1972). Modifications of this technique lead to greatly improved binding data. Optimal conditions for the binding of NAA-^{14}C are established, and the possibility that binding is merely transport into membrane vesicles is eliminated.

A Scatchard analysis of the binding of NAA-^{14}C to a crude membrane pellet from coleoptiles reveals two sets of high affinity binding sites, referred to as site 1 and site 2. Estimations of dissociation constants (K) and site concentration values (n) show that for site 1, $K_1 = 1.8 \times 10^{-7}$M and $n_1 = 52$ pmole per g fresh weight, and for site 2, $K_2 = 14.5 \times 10^{-7}$M and $n_2 = 101$ pmole per g fresh weight. Two sets of binding sites are also demonstrated for 3-indolylacetic acid (IAA), and these are of somewhat lower affinity than those described for NAA.

The auxin-specificity of the NAA binding sites is investigated using a technique analogous to that used for enzyme inhibitor studies. The interaction of a selected range of auxins, auxin analogues and other phytohormones with the NAA binding sites shows that auxin-specificity resides in site 2 whereas site 1 is less specific.

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Sucrose gradient fractionation techniques are used in an attempt to characterise further the membrane pellets. A partial separation of site 1 and site 2 binding activities is achieved, with site 1 sedimenting in the lighter regions of the gradient and site 2 in the heavier bands. A study of marker enzymes and chemical composition of the gradient bands reveals that the heavier regions are enriched in plasma membranes, whereas a distinct band of Golgi activity is observed in the lighter regions. Using a phosphotungstic acid-chromic acid staining procedure, electron microscopy also demonstrates an enrichment of plasma membrane content in the heavy bands.

The binding of NAA-^{14}C to membrane fractions from mesocotyl tissue is also investigated. Early experiments suggest only one set of binding sites, kinetically equivalent to site 2 in coleoptiles. However, analogue competition and fractionation studies indicate the presence of two sets of sites with only a two-to-three-fold difference in dissociation constants.

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THE BINDING OF AUXINS TO MEMBRANE FRACTIONS FROM *ZEA MAYS*

A thesis submitted to the University of Glasgow
for the degree of Doctor of Philosophy
in the Faculty of Science

by

Susan Batt, B.Sc. (Sussex)

August, 1975

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CONTENTS

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INTRODUCTION

Of all the plant growth regulators, auxins have been known to plant physiologists for the longest time. Since their isolation, much has been written describing the effects of auxins on various physiological processes. The American Society of Plant Physiologists assigned a general definition whereby, "Auxin is a generic term for compounds characterised by their ability to influence elongation in shoot cells and their resemblance to 3-indolylacetic acid (IAA) in their physiological characteristics ..." Many plant biologists have attempted to determine the processes involved in auxin-induced stem elongation, but the fundamental question of the nature of the primary mode of action of auxin remains unanswered. Numerous theories have been proposed to answer this question and the early ideas are well documented in texts and reviews (Galston and Purves, 1960; Thimann, 1972; Audus, 1972). More recent approaches to this problem have also been summarised (Morré, 1972; Davies, 1973; Lesham, 1973; Venis, 1973), and it is one such approach that this thesis describes. A historical perspective of the theories of the primary mode of action of auxin illustrates the rationale behind the use of this approach.

Continued cell enlargement is the net result of a number of events. The initial increase in size is primarily due to an uptake of water. The cell wall increases its surface area as a result of mechanical and biochemical changes in its properties, together with new wall synthesis. Cell enlargement is an active process and requires respiratory energy, and continued RNA and protein synthesis (Cleland, 1971a). There are therefore numerous possible sites of auxin action in cell enlargement, and in fact, auxin effects on all of these events have been reported.

During the mid 1930's, many studies demonstrated that IAA stimulated respiratory metabolism in a number of elongating stem tissues, and it was thus postulated that auxin had its primary effect on these processes. However, it is now considered that these respiratory effects are the result rather than the cause of elongation (e.g. Rowan et al., 1972). Another early idea, arising from the observation that IAA induced an immediate increase in the rate of protoplasmic streaming in cells of Avena coleoptile segments, was that auxin had its effect via the "protoplasm" of these cells (Thimann and Sweeney, 1937). However, there is as yet no evidence that protoplasmic streaming is a priori involved in growth. In fact, there is some evidence that it is not, since it was found that cytochalasin B completely inhibited streaming whilst having no effect on auxin-promoted growth (Cande et al., ~~and Ray,~~ 1973). A more recent hypothesis suggested that IAA acted on elongation via oxidation in vivo to 3-methyleneoxindole (3-MO) (Moyed and Tuli, 1968; Tuli and Moyed, 1969; Basu and Tuli, 1972). However, the failure of other workers (e.g. Evans and Ray, 1973) to obtain growth stimulation with 3-MO casts severe doubt on this suggestion. Further, the hypothesis cannot account satisfactorily for the activity of non-indolic synthetic auxins.

For cell enlargement to take place, there must be increased water uptake, and it was proposed that auxins stimulate this process directly by increasing the osmotically active substances in the cytoplasm or, by stimulating active water uptake by a pump mechanism. However, there was little experimental evidence for these ideas and they are now largely discounted (see Audus, 1972). The effect of auxin on the permeability of cells to water was also widely studied and recently a

rapid, auxin-specific, promotion of influx and efflux of tritiated water in etiolated pea stem segments was reported (Kang and Burg, 1971). However, the authors stated that the effect was not causally related to growth. Furthermore, these results could not be repeated by three separate laboratories (Dowler et al., 1974), and it was suggested that the previous observations resulted from abrasion of the tissue during handling.

Much attention has been focused on auxin modifications of the mechanical and biochemical properties of plant cell walls, since a reduction in wall pressure would result in water uptake and subsequent growth. Early studies using plasmometric and bending techniques suggested that auxins had an effect on the plastic rather than on the elastic properties of cell walls. These data were later confirmed using an Instron stress-strain analyser, and it was found that pretreatment of Avena coleoptile segments with auxin resulted in a two-to-three fold increase in the plastic extensibility of the walls; this increase reached a maximum after 90-120 minutes and remained constant for 24 hours (Cleland, 1967). Some reports have also demonstrated that auxin stimulates the elastic extensibility of plant cell walls (Masuda, 1969). Similarly, recent work, using resonance frequency techniques, has shown that in pea segments and sunflower hypocotyls, IAA promotes elastic extensibility within 4 minutes (Uhrström, 1969; Burström et al., 1970). Two theories suggested that auxins induced cell wall softening via an effect on the calcium levels in the wall. Calcium is known to inhibit elongation growth and was believed to increase wall rigidity by cross-linking the carboxyl groups of galacturonic acid residues in the wall pectin. It was first suggested that auxin promoted esterification of

these carboxyl groups, thus preventing calcium bridge formation. The second theory was that auxins acted as chelating agents and removed the calcium from the walls. However, it was subsequently found that: (1) auxin-induced methyl transfer could be totally inhibited without any effect on auxin-induced growth and (2) auxin did not remove radioactive calcium, previously incorporated into cell walls (see Audus, 1972).

Attention has also been focused on the effect of auxins on degradative and synthetic processes within the cell wall. Auxin-enhanced cell wall synthesis has been observed in a number of systems. For example, in IAA-treated oat coleoptiles, incorporation of exogenous ^{14}C-glucose into all the cell wall polymers except cellulose, was increased (Ray and Baker, 1965). It was also shown that, in the presence of auxin, the pattern of wall synthesis was shifted from apposition (deposition of new wall material at the cell membrane) to intussusception (deposition of new wall material throughout the wall), and it was suggested that intussuscepted polysaccharides could cause wall loosening by forcing apart the cellulose microfibrils (Ray, 1967). Recently, it was shown that in pea stem segments, there is a highly selective pattern of cell wall turnover, and that auxin has a specific influence on a xyloglucan polymer (Labavitch and Ray, 1974a, 1974b). An auxin-induced increase in the activity of an enzyme involved in wall synthesis, β-glucan synthetase, has been demonstrated in a number of systems (Ray and Abdul-Baki, 1968; Van der Woude et al., 1972; Ray, 1973a, 1973b; Shore and MacLachlan, 1973). In pea stem segments, the increase in the particulate UDP-glucose-dependent β-glucan synthetase was shown to be a hormonally induced activation of a previously existing enzyme (Ray, 1973b), and in onion stems, the β-glucan synthetase was reported to be of plasma membrane

origin and was activated by 2,4-dichlorophenoxyacetic acid (2,4-D) both *in vivo* and *in vitro* (Van der Woude *et al.*, 1972). A number of polysaccharide hydrolases are known to exist in plant cell walls, and it has been shown that the synthesis or activity of some of these is stimulated by auxin (e.g. β-1,4-glucanase, Fan and MacLachlan, 1966; β-1,3-glucanase, β-1,6-glucanase and hemicellulase, Tanimoto and Masuda, 1968; pectinase and pectinesterase, Datko and MacLachlan, 1970). It was thus suggested that auxin has its primary effect on a hydrolase enzyme which causes wall-loosening and thus cell extension.

From the above discussion, it is apparent that auxins may act by regulating the synthesis of specific enzymes. Since enzyme synthesis is intimately dependent on nucleic acid metabolism, the suggestion that auxins may act at the gene level has received much attention. It has been found on many occasions that auxin-induced growth and cell wall extension are prevented by inhibitors of RNA and protein synthesis (Fan and MacLachlan, 1966; Noodén and Thimann, 1966; Coartney *et al.*, 1967; Masuda, 1969). Also, auxin treatment results in an increase in RNA and protein synthesis (Key, 1969).

The evidence linking the action of auxin with nucleic acid metabolism has been extensively reviewed (Trewavas, 1968; Key, 1969). The net accumulation of RNA in response to auxin left little doubt about the ability of auxin to enhance RNA synthesis in many different elongating stem segments. That this stimulation was not merely a result of growth was demonstrated by the ability of auxin to stimulate RNA synthesis even when elongation was prevented by 0.3M mannitol (Masuda, 1968). In general, the stimulation of RNA synthesis by auxin was seen only after a

lag period of 1-3 hours (Davies et al., 1968; Masuda, 1968) although a stimulation within 10 minutes has been reported (Masuda and Kamisaka, 1969). Attempts have been made to characterise the RNA species formed in response to auxin. Auxin stimulation of messenger RNA (mRNA) synthesis has been reported in soybean hypocotyls (Key and Ingle, 1968), pea stem segments (Masuda, 1968), and in lentil roots (Miassod et al., 1970). Stimulation by auxin of ribosomal RNA (rRNA) synthesis in pea stem segments (Davies et al., 1968), callus tissue of chicory root explants (Gwozdz, 1974) and lentil roots (Penon et al., 1970) has also been demonstrated.

Auxin could control RNA synthesis by an effect on the activity of the RNA polymerases. Chromatin isolated from soybean hypocotyl tissue, pretreated with 2,4-D (O'Brien et al., 1968; Guilfoyle and Hanson, 1974), and lentil root nuclei pretreated with IAA (Teissere et al., 1973) showed enhanced RNA polymerase I activity. This polymerase is of nucleolar origin α-amanitin insensitive and believed to transcribe primarily rRNA (Nagao et al., 1974). On the other hand, in etiolated maize shoots pretreated with 2,4-D, a stimulation of nucleoplasmic, α-amanitin sensitive RNA polymerase was reported (Arens and Stout, 1972). Detailed studies suggested that auxin treatment increases RNA polymerase I levels by affecting the specific activity of the enzyme, rather than by increasing template availability (O'Brien et al., 1968; Guilfoyle et al., 1975). In soybean hypocotyls the higher RNA polymerase activity was reflected primarily in the production of longer RNA chains, with only a small increase in the number of chains (Guilfoyle and Hanson, 1974).

It is probable that the effects of auxins on nucleic acid and protein synthesis are mediated by auxin receptor molecules localised in

the cytoplasm or nucleus. Several reports of auxin-binding macromolecules have appeared during the past twenty years. These included auxin-binding proteins (Siegel and Galston, 1953; Galston and Kaur, 1959; Zenk, 1964), and auxin-coupling to transfer (or soluble) RNA (tRNA) (Bendaña et al., 1965). Based on the IAA-tRNA complex, a hypothesis for auxin action was proposed whereby auxin plays an amino-acid-like role in the quantitative regulation of both RNA and protein synthesis (Armstrong, 1966). However, these early studies on IAA-conjugates were severely criticised. The complex described by Siegel and Galston appears to have been an artefact of the precipitation procedure (Zenk, 1964), the label from ^{14}C-auxins in RNA fractions largely resulted from the products of auxin catabolism (Key and Ingle, 1968) and the extraction technique used by Bendaña et al., was found to isolate both RNA and polysaccharides so that what had been previously identified as label in tRNA was actually label in a polysaccharide of similar sedimentation value (Davies and Galston, 1971). However, a later report still maintained that IAA-tRNA complexes were to be found in mung-bean hypocotyl tissue (Kobayashi and Yamaki, 1972).

More recent studies have described protein factors which, in the presence of auxins, promote RNA synthesis. A protein factor was prepared from lysates of tobacco, soybean or pea bud nuclei, which in the presence of 2,4-D, stimulated RNA synthesis in vitro when pea bud chromatin, but not purified DNA, was used as template. It was claimed that the effect was auxin-specific and that the higher rates of RNA synthesis were maintained even when exogenous E. coli polymerase was increased to saturating levels, indicating an action on template availability of the chromatin (Matthyse and Phillips, 1969). It was also claimed that the factors showed tissue-specificity, since pea bud chromatin plus pea

root factor did not result in stimulated RNA synthesis (Matthyse, 1970). Soybean hypocotyls pretreated with 2,4-D, show increased chromatin-bound RNA polymerase activity (O'Brien et al., 1968). A protein factor, isolated from soybean cotyledons, was able to stimulate polymerase activity from control, but not from 2,4-D treated hypocotyl tissue. It was proposed that the in vivo action of the hormone on the polymerase involved this protein mediator, so that the polymerase from treated tissue, being already "switched on", was incapable of further stimulation (Hardin et al., 1970). In another approach, crude extracts from pea or corn shoots were passed through an affinity column, consisting of a 2,4-D derivative coupled to agarose. Appropriate elutions yielded a protein factor, which stimulated DNA-dependent RNA synthesis, supported by E. coli polymerase and homologous DNA, by up to 200% (Venis, 1971).

The experiments so far quoted were deficient in that they did not use completely homologous systems in the RNA assays and that none showed direct evidence for auxin-binding to the protein factors. These deficiencies were absent in the work reported by Mondal et al., (1972a, 1972b). An "auxin-acceptor-protein" was prepared from the nucleoplasm derived from immature coconut endosperm which, in the presence of a completely homologous system containing RNA polymerase, initiation factor and DNA (all from coconut nuclei) stimulated RNA synthesis by two to three-fold but only in the presence of 10^{-6} M IAA. Preliminary equilibrium dialysis suggested that the protein did bind IAA-^{14}C and gel electrophoresis and hybridisation studies indicated that new species of RNA were synthesised in the stimulated reaction. The IAA-protein complex was found to interact with DNA, suggesting a template modification.

It is now necessary to consider what has been discussed so far in the light of experiments which show that auxins have rapid effects on a number of cellular processes. The development of high resolution recording techniques made it possible to monitor elongation growth over a short period of time (Ray and Ruesink, 1962; Evans and Ray, 1969; Dela Fuente and Leopold, 1970; Philipson et al., 1973). It was found that auxin promoted stem elongation in nine tissues after remarkably similar latent periods, ranging from 6 to 15 minutes (Evans, 1974a). In order for an auxin effect to qualify as the primary mediator of these rapid growth responses, it must occur within this time limit. With this in mind, it will be seen that most of the auxin effects already discussed are not detected within this period. For example, auxin-stimulation of RNA and protein synthesis generally occurred after periods of at least 1 hour. Masuda and Kamisaka (1969) claimed that auxin stimulated RNA synthesis within 10 minutes, but the statistical significance of these results ~~are~~ is questionable. Thus, while RNA and protein synthesis are crucial to long-term growth, it is unlikely that they are involved in the rapid primary response to auxins. Furthermore, tissues pretreated with inhibitors of RNA or protein synthesis still responded to auxin within the same latent period as non-treated tissues (Evans and Ray, 1969; Barkley and Evans, 1970; Cleland, 1971b; Penny, 1971; Penny et al., 1972; Pope and Black, 1972).

The study of growth promotion by nonhormonal factors is of particular interest in attempts to elucidate the mechanism of auxin action. If it can be shown that certain treatments promote growth more quickly than auxin itself, and that the observed growth has features in common with auxin-promoted growth, valuable clues as to the nature of

auxin action may be obtained. Factors which have been shown to promote rapid elongation are carbon dioxide (Rayle and Cleland, 1970; Evans et al., 1971; Barkley and Leopold, 1973), several fungal toxins (Evans, 1973; Lado et al., 1973), transient exposure to heavy water (Evans, 1973), and hydrogen ions (e.g. Rayle and Cleland, 1970).

The phenomenon of growth promotion by hydrogen ions has received much attention in the past decade, leading to yet another theory on the primary mode of auxin action - "the hydrogen ion secretion theory". When Avena coleoptiles were treated with acidic buffers, there was a latent period of less than 1 minute followed by a stimulation of elongation growth comparable to that obtained with optimum auxin concentrations. Maximal growth stimulation was obtained at pH 2.6 - 3.0, and growth rapidly ceased if the pH was changed from 3.0 to 7.0 indicating that low pH was not merely a trigger mechanism (Rayle and Cleland, 1970). The estimation of the pH optimum was later revised when it was found that the epidermis represented a significant barrier to hydrogen ions, and after removal of the epidermis, stem segments responded to buffers at pH 5.0 (Cleland, 1973; Rayle, 1973). A rapid effect of low pH has also been detected in a number of other tissues, including etiolated pea stem segments (Barkley and Leopold, 1973), Helianthus hypocotyls (Hager et al., 1971) and light grown lupin hypocotyls (Perley et al., 1975). The acid and auxin responses were found to be similar in many respects such as in their temperature dependence, promoted growth rate and dependence on the continued presence of inducing agent (Rayle and Cleland, 1970). Hydrogen ions could also induce rapid wall extensibility as shown in both an in vivo system (Rayle and Cleland, 1970) and in an in vitro system, which utilized frozen-thawed coleoptiles

(Rayle et al., 1970). Since the in vitro acid response had many characteristics in common with the in vivo response elicited by auxin and low pH, a common wall-loosening and wall-extension mechanism was proposed (Rayle and Cleland, 1972).

It was suggested that auxins might act by stimulating the release of hydrogen ions into the cell wall which would either cleave acid-labile bonds or activate enzymatic processes (Johnson et al., 1974; Evans, 1974b), thereby leading to wall-loosening (Rayle and Cleland, 1972). Using peeled Avena coleoptile segments, an auxin-induced secretion of hydrogen ions into the incubation medium was found. The effect was observed after a lag of about 20 minutes and lasted for two to three hours. It was argued that the effect was actually immediate, and that the 20 minute lag merely reflected the time taken for the hydrogen ions to reach the medium, and the slight absorption effect of the phosphate buffer. It was further demonstrated that only growth active auxins gave this response and that substances which inhibited auxin-induced growth also inhibited auxin-induced hydrogen ion secretion (Rayle, 1973; Cleland, 1973). Similar results were reported for etiolated pea stem segments (Marré et al., 1973a, 1973b) and it was also found that the stimulation of cell enlargement in leaf fragments by fusicoccin was accompanied by a rapid (i.e. within 5 minutes) decrease in the pH of the medium (Marré et al., 1974a). Furthermore, the proton extrusion effects of both fusicoccin and auxin were accompanied by a negative transmembrane potential, recognisable within a few minutes (Marré et al., 1974b).

Thus, the evidence in favour of auxin action via cell wall

acidification is (1) acid solutions stimulate growth in stem segments to the same degree as auxins, (2) the growth response to acid occurs before the response to auxins and (3) auxin induces hydrogen ion secretion. Certain experimental arguments against the proton extrusion theory have been advanced (e.g. Penny et al., 1975; Perley et al., 1975). However, in spite of these arguments the current weight of evidence lies in favour of some form of hydrogen ion excretion theory.

In a recent report, it was demonstrated that in soybean hypocotyl sections there were two separable growth responses to auxin. The first was a rapid response, occurring 12 minutes after auxin addition, and the second occurred after 30 minutes. Cytokinins were found to inhibit the second response and treatment with acid solutions resulted only in the transient, rapid response. It was proposed that the first phase of auxin promoted growth was explainable in terms of the proton secretion theory, while the second phase represents the long term response to auxin which requires protein and nucleic acid synthesis (Vanderhoef and Stahl, 1975). These results may have great significance in elucidating the overall mechanism of auxin-regulated growth.

The findings that elongation growth can be initiated after only a very short lag period and that auxins induce many other rapid responses led to the assumption that the initial interaction of exogenously applied auxin was at or near the cell surface, or, more specifically, with the plasma membrane. This idea is not new, and was implied in many of the early auxin studies. For example, during the classical "structure-activity" studies it was proposed that one criterion for auxin molecules was that they should have a high surface charge in

order to form a reversible, noncovalent attachment to the lipid constituents of the plasma membrane (see Jönsson, 1961). Other theories required specific spatial relationships within the molecules (e.g. a distance of 5.5 Å between a fractional positive charge at some point on the molecule, and the negative charge of the dissociated carboxyl group), in order that they could associate reversibly and non-covalently with an auxin-receptor (see Audus, 1972). The application of enzyme-kinetics to auxin-induced growth implied that auxins interacted reversibly with a "coleoptile receptor", although the nature of this receptor was not defined (Bonner and Foster, 1956; Housley, 1961). The existence of a specific auxin transport system (Hertel et al., 1969) also suggested physico-chemical interactions with receptor molecules in the tissue. Since the auxin-specificity of both transport and growth promotion are well correlated, it was suggested that the two processes might have a common site of action at the plasma membrane, this auxin-plasma membrane interaction being reversible and noncovalent (Hertel and Flory, 1968). Later studies supported the idea of a reversible, noncovalent interaction of auxins and auxin transport inhibitors with a plasma membrane receptor. However, the close correlation of transport and growth was questioned, since the transport inhibitor, 1-N-naphthylphthalamic acid (NPA) had no effect on short term growth either alone, or with IAA (Thomson et al., 1973).

An auxin-plasma membrane interaction is essential to the model of auxin action suggested by Hager et al., (1971). It was proposed that auxin acted cooperatively with guanosine or inosine triphosphate as an effector of a membrane-bound, ion-stimulated ATP-ase, which used respiratory energy to raise the hydrogen ion level in the cell wall

compartment, thus leading to increased activity of enzymes which soften the cell wall and allow elongation. If this model is correct, one would expect electrical balance to be maintained by either a concurrent influx of cations or an efflux of anions. IAA-enhanced potassium uptake (Lüttge et al., 1972) and auxin-inhibited chloride uptake, probably via a stimulation of efflux (Bentrup et al., 1973) have been demonstrated, although contradictory reports also exist (e.g. IAA-enhanced chloride uptake, Rubinstein and Light, 1973).

The presence of an auxin-stimulated, plasma membrane bound ATP-ase is also essential to Hager's model. Plasma membrane associated ATP-ases have been reported in a number of tissues including corn roots (Hall, 1971; Leigh et al., 1975), oat roots (Hodges et al., 1972), mung bean hypocotyls (Kasamo and Yamaki, 1974a) and in the marine diatom, Nitzschia alba (Sullivan and Volcani, 1974a). Activation by in vitro IAA treatment of a magnesium-sensitive ATP-ase from mung bean hypocotyls has also been reported (Kasamo and Yamaki, 1974b). However, this stimulation was only slight and the association of this enzyme with the plasma membrane was not rigorously proven. Clearly, this aspect of Hager's model requires further attention.

It is only recently that progress has been made in the isolation of plant plasma membranes. In general, the isolation procedure involves gentle homogenisation of the tissue, followed by a series of differential centrifugation steps and finally discontinuous sucrose gradient separation of membrane populations. In some reports, the plasma membranes were isolated using media prepared in coconut milk (Lembi et al., 1971; Hardin et al., 1972; Roland et al., 1972; Van der

Woude et al., 1974) whereas others have used defined synthetic media (Hodges and Leonard, 1974; Sullivan and Volcani, 1974). A combination of electron microscopy, and chemical and enzymatic analyses has been used to identify the plasma membranes.

Plasma membranes in both whole plant cells and in fractions recovered from sucrose gradients were found to react specifically to a phosphotungstic and chromic acid (PTA) staining procedure. This stain appeared to interact with complex carbohydrates and glycoproteins on the plasma membrane surface, and the reaction was seen in plasma membranes isolated from a wide variety of plant tissues but not with plasma membranes from rat liver or mammary gland tissue (Roland et al., 1972). Using this staining procedure, a plasma membrane-rich fraction was identified in the heavy regions (approx. 1.0M - 1.2M) of a sucrose gradient containing corn coleoptile membranes (Lembi et al., 1971).

The enzyme β-glucan synthetase has also been proposed as a marker for plant plasma membranes (Van der Woude et al., 1972). Recently, the properties of this enzyme have been further characterised, and it was shown that at high UDPG concentrations (1 mM) the synthesis of alkali-insoluble glucans by β-glucan synthetase was correlated with the abundance of plasma membranes in the 1.0M - 1.2M sucrose interface. At low UDPG concentration (1.5 μM), on the other hand, the enzyme activity is associated with dictyosomes which band at lighter regions of the gradient (0.65M - 0.80M sucrose), do not stain with PTA, and have high inosine diphosphatase activity (Van der Woude et al., 1974). Another enzyme, characteristic of oat root plasma membranes, is a monovalent ion-stimulated ATP-ase (Hodges et al., 1972). The membranes which were

enriched in this enzyme also showed high levels of glucan synthetase activity, a high sterol:phospholipid molar ratio (believed to be characteristic of plasma membranes) and, from PTA staining and a quantitative morphometric analysis, were estimated to contain in excess of 75% plasma membranes (Hodges et al., 1972). The plasma membrane preparations contained only low levels of enzyme activities associated with other cell fractions and were clearly distinguishable from Golgi, endoplasmic reticulum and mitochondrial membranes (Leonard et al., 1973; Hodges and Leonard, 1974).

A further study compared the lipid composition of plasma with total and mitochondrial membranes that plasma membranes were enriched in neutral lipids, especially triglycerides, had a high sterol:phospholipid molar ratio, showed a characteristic phospholipid distribution pattern and had high levels of palmitic acid (Keenan et al., 1973). The successful isolation has also been reported of relatively pure preparations of plasma membranes from hypotonically lysed protoplasts of the marine diatom, Nitzschia alba. From a cellular membrane pellet, membrane fractions were separated on a discontinuous sucrose gradient. The plasma membrane fraction was distinguished by a high sterol content, a high sterol:phospholipid ratio, a low carbohydrate content and high specific activities of both a Mg^{2+} dependent ATP-ase and a synergistically stimulated $Na^{+} - K^{+} - Mg^{2+}$ ATP-ase. On the basis of the latter activity, the plasma membranes were reported to be purified 20-fold (Sullivan and Volcani, 1974).

Thus, from these various reports, it seems that it is possible to isolate and identify from plant tissue, fractions considerably enriched in plasma membranes. If an auxin-plasma membrane interaction does occur,

auxin receptor molecules localised in the plasma membrane would be expected. The existence of such hormone receptors in a variety of animal tissues has already been widely established, the insulin receptor being a particularly well-studied example (Cuatrecasas, 1974).

Before presenting a detailed account of the work on plasma membrane auxin receptors, the evidence for receptors of other phytohormones will be briefly discussed. From studies on the effects of gibberellin on model membrane systems (liposomes) it was suggested that these hormones produce their physiological response through a direct effect on the physical properties of the lipid moiety of natural membranes. Further data from nuclear magnetic resonance spectroscopy studies demonstrated the formation of a complex between a phospholipid (phosphatidyl choline) and the hormone (Wood and Paleg, 1974; Wood et al., 1974). However, these effects were not specific to active gibberellins and the relevance of these artificial situations is questionable. The only other report relating to gibberellin receptors describes two protein fractions in pea epicotyl extracts which became labelled when ^{3}H-gibberellin A_1 was supplied in vivo. The protein-hormone complexes were noncovalently bound, showed some gibberellin specificity and could undergo hormone exchange with unlabelled giberellin A_1 (Stoddart et al., 1974). The subcellular localization of the protein fractions has yet to be elucidated.

A cytokinin mediator protein, which in the presence of kinetin or zeatin, promoted RNA synthesis with either chromatin or homologous DNA as template, has been isolated from crude pea chromatin (Matthyse and Abrams, 1970). Reversible low affinity binding of kinetin to plant ribosomes has been reported (Berridge et al., 1970). This work was

recently re-examined (Fox and Erion, 1975) and evidence presented for the presence of two types of binding sites in plant ribosomal fractions. The high affinity site was saturated at one cytokinin molecule per ribosome, was specific for substances with cytokinin activity and could be readily washed from the ribosomes with 0.5M KCl. The binding moiety washed from the ribosomes was shown to have characteristics of a protein and could bind cytokinins when apart from the ribosomes.

The observations that phytochrome can bind to membrane preparations both in vivo and in vitro have led to the suggestions that highly specific receptor sites for phytochrome exist in the membranes (e.g. Marmé et al., 1973; Marmé et al., 1974; Boisard et al., 1974). As yet, there appears to be no literature describing possible receptors for abscisic acid (ABA).

The search for plasma membrane auxin receptors was stimulated by the indirect evidence that auxin transport sites were localised at the plasma membrane. Since attempts with radioactive IAA were initially unsuccessful, the ability of the auxin transport inhibitor, NPA, to bind to plasma membranes in vitro was investigated. Specific NPA binding activity was found in a crude (5,000 g supernatant) fraction prepared from corn coleoptiles. Since radioactivity was lost when these fractions were pelletted through a sucrose cushion, the binding was thought to be reversible and noncovalent. When the fraction was further separated on a sucrose gradient, a direct correlation between specific NPA binding and plasma membrane content was demonstrated (Lembi et al., 1971). This binding was of high affinity with dissociation constants of the order of 10^{-8}M - 10^{-7}M (Thomson, 1972). It was also demonstrated that the

concentration dependence of NPA inhibition of auxin transport has a half-saturation point at 10^{-7}M, suggesting a correlation between binding, and transport inhibition (Thomson et al., 1973). The compounds gibberellin A_3, ABA, triiodobenzoic acid (TIBA), 2,4-D, NAA and IAA did not compete for the NPA binding sites and it was therefore proposed that the NPA receptors, although localised at the plasma membrane, were different from the auxin receptors (Thomson, 1972).

In a subsequent study, the in vitro, reversible binding of the auxins, NAA and IAA to particulate fractions from corn coleoptiles was demonstrated (Hertel et al., 1972). The technique used in these studies involved the isolation of crude membrane fractions from the coleoptiles by homogenisation and precentrifugation (e.g. at 5,000 g). Radioactive auxin (at a concentration of 10^{-7}M - 10^{-6}M) was added to aliquots of the precentrifuged supernatant and parallel samples were prepared which contained higher concentrations (e.g. 10^{-4}M) of unlabelled auxin or other competitor being studied. The samples were centrifuged at high speed (e.g. 20,000 g) and the radioactivity in the membrane pellets was determined. The difference between the counts in the samples with or without competitor addition was referred to as specific binding.

Using a Scatchard analysis (Scatchard, 1949), the kinetic parameters of IAA-^{14}C and NAA-^{14}C binding were determined. The dissociation constants were 1.2 x 10^{-6}M and 3.4 x 10^{-6}M for NAA and IAA respectively, and an auxin binding site concentration of 10^{-7}M per volume of tissue was estimated. Auxin-specificity of binding was tested by examining the ability of a range of auxins and auxin analogues to displace radioactive NAA from the membrane fractions. Among many compounds tested,

only auxins (2,4-D, IAA and NAA) and auxin analogues that interact directly with auxin in transport and/or growth (TIBA and p-chlorophenoxyisobutyric acid - PCIB) were found to interfere with NAA binding (Hertel et al., 1972). A subtle correlation (2,4,5-T > 2,3,4-T > 2,3,6-T) between the ability of the trichlorophenoxyacetic acids (T) to displace NAA-^{14}C from its binding site, and their growth properties was also demonstrated (Hertel, 1974). The crude auxin binding sites were found to be heat labile, sensitive to 0.1% sodium dodecylsulphate, practically free of DNA, starch and mitochondria and sedimented through 40% sucrose (Hertel et al., 1972). A recent preliminary report has suggested a cellular localization of auxin binding activity at the endoplasmic reticulum as well as at the plasma membrane (Hertel, 1974).

Additional evidence has suggested that the NPA and auxin receptor sites are different. NPA was not able to displace ^{14}C-NAA from the particulate fractions. Furthermore, the auxin binding sites were found to be concentrated in denser or larger particles than the NPA sites (Hertel, 1972). A comparison of the *in vitro* binding of TIBA and NPA with their effects on auxin transport suggested that TIBA interacted with the auxin receptor, which was different from the NPA site (Thomson et al., 1973). Another study demonstrated that certain morphactins and NPA analogues also interacted with the NPA binding site (Thomson and Leopold, 1974).

It has been suggested that particulate auxin receptors are important in the regulation of plant development. The release of apical dominance in the cotyledonary buds of pea was reported to be associated with a large decrease in NAA competible ^{3}H-IAA binding activity (Jablanowic

and Nooden, 1974). However, this report must be viewed with caution, since the auxin binding properties of the tissue were very poorly defined.

There thus appears to be a growing body of evidence for the presence of auxin receptors in the plasma membranes of plant cells. The interaction of auxins with these receptors may, in part, explain the many known rapid effects of auxins. It is interesting to mention here a report which sought to rationalise the rapid growth response, and delayed effects on gene expression, in terms of a common site of auxin action at the plasma membrane. It was found that plasma membranes prepared from soybean hypocotyls were able to stimulate the activity of α-amanitin-sensitive, nucleoplasmic, RNA polymerase solubilised from soybean chromatin. When the membranes were incubated with IAA or 2,4-D (at approx. 10^{-7}M) and then repelleted, the stimulatory activity was found in the supernatant. This release was not seen after incubating the membranes in buffer, or in the anti-auxin, 3,5-dichlorophenoxyacetic acid. A model was proposed whereby auxin interacted with the plasma membrane to cause the release of a regulatory factor for RNA polymerase activity (Hardin et al., 1972). Unfortunately this interesting theory has, as yet, received no further support.

The work described in this thesis attempts to characterise further the properties of auxin binding to membrane fractions from Zea mays shoot tissue. In 1949, Scatchard stated that the questions which must be answered when studying the binding of a small molecule to a macromolecule are "How many?", "How tightly?", "Where?" and "Why?". This thesis hopes to provide answers to the first three questions, and to make some suggestions regarding the fourth.

MATERIALS AND METHODS

1. PLANT MATERIAL

Seeds (fruits) of Zea mays L., cv. "Kelvedon 33" (from Hurst Gunson Cooper Taber Ltd., Witham, Essex) were soaked in tap water for 6-12 hours, sown in damp vermiculite and grown in the dark at 25 ± 1°C.

In experiments concerned with auxin binding activity, seedlings were grown for 4-5 days from planting (Coleoptile length was 25-30 mm). For rapid growth assays, 3-4 day-old seedlings were used (Coleoptile length was 15-20 mm). (See Plate 1 A and B).

2. GENERAL CHEMICALS

All general laboratory reagents were obtained from Hopkin and Williams, Chadwell Heath, Essex; or British Drug Houses (BDH), Poole, Dorset. Reagents were analytical grade.

3. HORMONES AND HORMONE ANALOGUES

(1) Sources and Purity

3-indolylacetic acid (IAA), 1-naphthylacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), α-(2-chlorophenoxy)-isobutyric acid (2-CPIBA), 6-furfurylaminopurine (kinetin), and 6-benzylaminopurine (6-benzyladenine) were all purchased from Sigma Chemical Co., London. 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) was obtained from BDH and recrystallised from water. 2,4-dichlorobenzoic acid (2,4-B) and 2,6-dichlorobenzoic acid (2,6-B) were also from BDH. Benzoic acid (BA)

was supplied by Hopkin and Williams, and 2,3,5-triiodobenzoic acid (TIBA) was from Koch-Light Labs. Ltd. Gibberellic acid (GA_3), 1-N-naphthylphthalamic acid (NPA), and cis,trans abscisic acid (ABA) were all gifts from Dr. M.A. Venis (Shell Research Laboratories, Sittingbourne, Kent). The GA_3 was recrystallised from acetone-petroleum ether (b.p. 60-80°C) and contained approximately 94% GA_3 with slight contamination by GA_4 and GA_7. The purity of the ABA was verified by gas-liquid chromatography. 2,6-dichlorophenoxyacetic acid (2,6-D), (a kind gift from Dr. V. Math, Glasgow University), was synthesised by the procedure of Koelsch (Koelsch, 1931).

The purity of all auxins and auxin analogues was verified by Dr. V. Math using mass spectrometry. (A.E.I. MS 30 Mass Spectrometer, A.E.I. Scientific Apparatus Ltd., Manchester).

(2) Solutions

Unless otherwise stated, 10^{-2}M stock solutions were prepared by dissolving the compounds in redistilled methanol. After dilution during experiments, the final methanol concentration was reduced to between 0.001% and 0.1%. Kinetin was dissolved in 50 mM potassium hydroxide. Stock solutions were stored in the dark at -20°C and were kept for no longer than one week.

4. RADIOACTIVE SOLUTIONS

1-naphthyl (acetic acid-1-^{14}C) (NAA), 3-indolyl (acetic acid-1-^{14}C) (IAA), 2,4-dichlorophenoxy (acetic acid-2-^{14}C) (2,4-D) and 2,4,5-trichlorophenoxy (acetic acid-1-^{14}C) (2,4,5-T) were obtained from the Radiochemical Centre, Amersham, Bucks., England. The specific activities of the NAA varied between 44 and 61 mCi per mmole. The specific

activity of IAA was 52 mCi per mmole, 2,4-D was 28 mCi per mmole and 2,4,5-T was 56 mCi per mmole. The organic solvents in which the samples were dissolved were dried off under a gentle stream of oxygen-free nitrogen and the samples were taken up in approximately 5 mM KOH. Stock solutions of 10 μCi per ml were stored at -20°C, in the dark.

Solutions were tested for purity by thin-layer chromatography on plastic-coated 5 x 20 cm Polygram Sil plates (Camlab, Cambridge) using two solvent systems:

(1) Isopropanol:ammonia:water, 8:1:1 (v/v).

(2) n-butanol:acetic acid:water, 62:25:15 (v/v).

Chromatograms were scanned using a Panax Radio-chromatogram Scanner. No radiochemical impurities were detected.

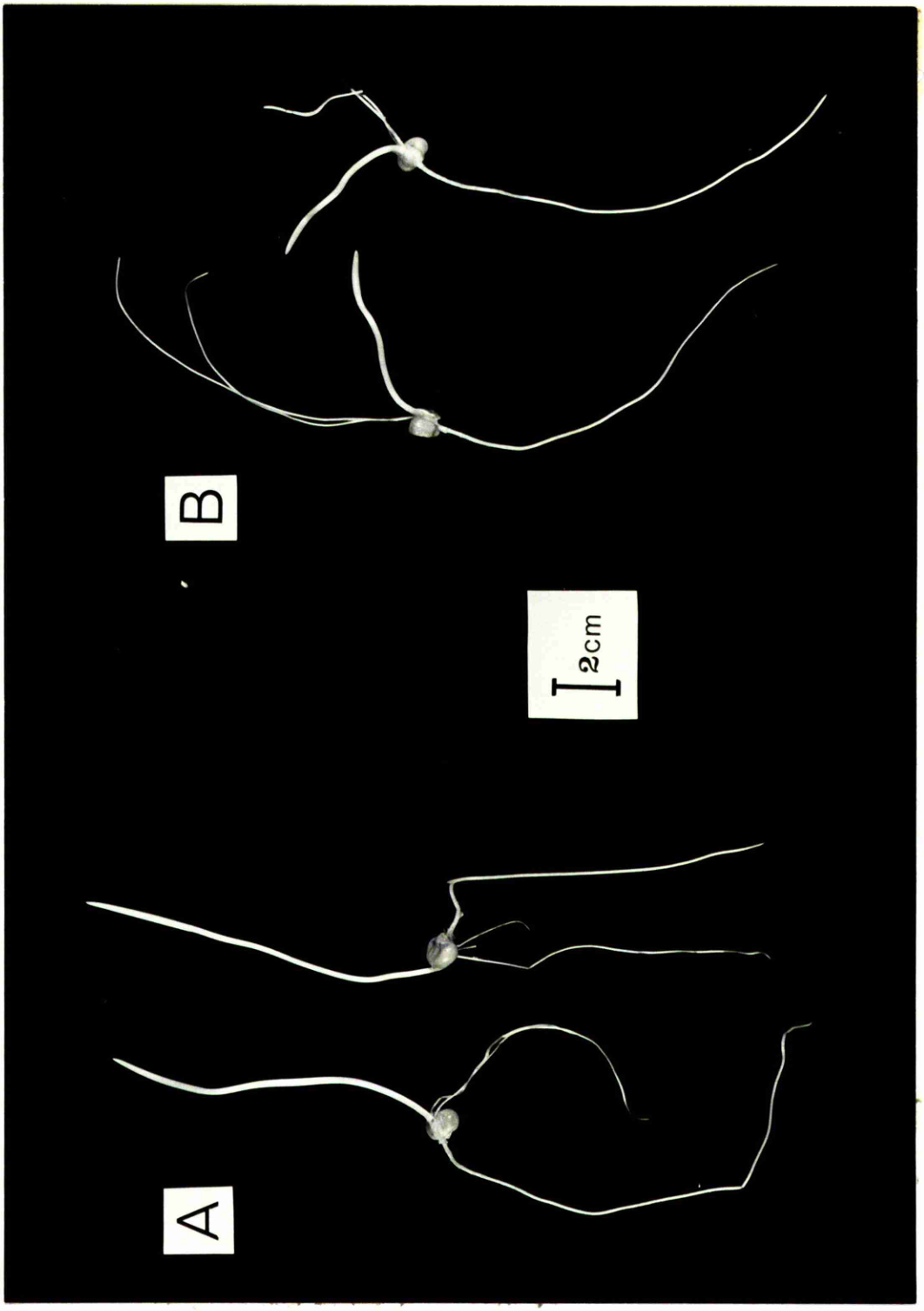
5. DETERMINATION OF RADIOACTIVITY

Radioactive samples were assayed by liquid scintillation spectrometry using a Packard Tricarb Spectrometer (model 3380). An "Automatic Activity Analyser" (model 544) gave an automatic conversion to disintegrations per minute (dpm) by reference to an external standard. A toluene-based scintillant (TBS), containing 4 g per litre of the fluor 2,5-diphenyloxazole (P.P.O., from Fisons, Loughborough, Leics., England) was used for non-aqueous samples. Aqueous samples were counted in a scintillant containing TBS and Triton-X-100 (industrial grade, Koch-Light Laboratories) in a ratio of 2:1 v/v (TXBS).

PLATE 1 : Seedlings of *Zea mays* L., cv. "Kelvedon 33"

A: 4-5 day-old seedlings.

B: 3-4 day-old seedlings.


A
B
2cm

6. PROTEIN DETERMINATION

Protein concentrations were determined by the method of Lowry (Lowry et al., 1951) using Folin and Ciocalteu's phenol reagent (BDH). This reagent was titrated with NaOH to a phenolphthalein end-point and diluted just before use to make it 1N. Usually, duplicate 50 µl samples, diluted to 0.2 ml with distilled water were used. A blank, containing 50 µl of the corresponding buffer was always carried through the same procedure as the samples. Protein values were obtained from a calibration curve using bovine serum albumin (Sigma Chemical Co.) as standard.

(1) Reagents

A. 2% Na_2CO_3 in 0.1 N NaOH.

B. Equal volumes of 1% $CuSO_4.5H_2O$ and 2% sodium potassium tartrate, mixed just before use.

C. 50 ml A + 1 ml B. Discard after 1 day.

D. Folin and Ciocalteu's phenol reagent. Dilute 1 vol reagent with 4 vols water.

(2) Procedure

To 0.2 ml sample, add 2 ml reagent C. Mix, stand 10 min. Add 0.2 ml D, rapidly with immediate mixing. Read after 30 min or longer at 750 nm on a Pye Unicam SP 500 Series 2 spectrophotometer, zeroed with water (1 cm glass cuvettes).

7. DETERMINATION OF AUXIN BINDING ACTIVITY

The methods used were similar to those described by Hertel and coworkers (Hertel et al., 1972; Hertel, 1974).

(1) Buffers

The following three buffers were routinely used:

(a) "Extraction medium":
- 0.25 M Sucrose
- 50 mM Tris (2 amino-2-(hydroxymethyl) propane-1,3-diol)
- 1 mM Na_2 EDTA (ethylenediaminetetra-acetic acid)
- 0.1 mM $MgCl_2$
- Acetic acid to pH 8.0.

(b) "Washing medium":
- 0.25 M Sucrose
- 10 mM Tri-sodium citrate
- 0.5 mM $MgCl_2$
- Acetic acid to pH 6.0.

(c) "Binding medium":
- 0.25 M Sucrose
- 10 mM Tri-sodium citrate
- 5 mM $MgSO_4$
- Acetic acid to pH 5.5.

(2) Homogenization and Preparation of the Membrane Pellet

Coleoptiles from 4-5 day-old Zea seedlings (Plate 1, A) were harvested in daylight. Leaf rolls were removed and the coleoptiles were kept on ice. Mesocotyls were similarly harvested. All subsequent operations were carried out at 0-4°C.

Coleoptiles (or mesocotyls) were cut into small pieces with a razor blade and homogenized in an equal volume of "extraction medium", using a pestle and mortar. The homogenate was squeezed through a fine mesh nylon cloth (20 μm aperture) (from Becosyn Nylon Industries, Beggan Cousland, Springfield Wireworks, Glasgow) and the residue was reground and extracted twice more. The total tissue:"extraction medium" ratio was 1:4 (w/v). Approximately 0.5 g fresh weight of coleoptile tissue or 1.0 g fresh weight of mesocotyl tissue per assay was used.

The homogenate was precentrifuged at 4,000 g (6,000 rpm) or 10,000 g (9,000 rpm) for 20 min in an 8 x 50 ml angle rotor of an M.S.E. "High Speed 18" centrifuge, and the pellets were discarded. The supernatant was respun for 30 min at either 38,000 g (18,000 rpm, "High Speed 18") or at 80,000 g (30,000 rpm) in an 8 x 50 ml angle rotor of an M.S.E. "Superspeed 50" centrifuge (referred to as 4-38Kg, 10-38Kg, 4-80Kg or 10-80Kg pellets). The supernatant was discarded and the membrane pellet was gently resuspended in "washing medium" (1:4 original tissue weight:"washing medium") using a 'teflon'-glass homogeniser. The membrane fraction was repelleted at the same speed as before.

(3) Binding Assay

In a typical binding assay, the following procedure was used. The washed membrane pellet was gently resuspended in 50 ml of "binding medium" using a 'teflon'-glass homogeniser. NAA-^{14}C was added to a final concentration of 2 x 10^{-7} M. 10 x 5 ml aliquots were pipetted into test-tubes and to each of these was added 50 μl of either water or an appropriate concentration of unlabelled NAA to give final NAA

concentrations which usually varied from 2×10^{-7} M to 10^{-6} M. Triplicate 1.5 ml samples were taken from each aliquot and pipetted into 2.5 ml polypropylene centrifuge tubes. These were spun:

either (a) at 38,000 g, 45 min in the "High Speed 18" centrifuge using adaptors custom-made by M.S.E. for the 8 x 50 ml rotor. Each adaptor held three 2.5 ml tubes

or (b) at 150,000 g, 15 min (48,000 rpm) in the "Superspeed 50" centrifuge using 2.5 ml adaptors in the 10 x 10 ml angle rotor.

The supernatant was decanted and the tubes drained for 5-10 min. The pellets were rinsed with approximately 2 ml distilled water and drained for a further 5-10 min. Pellets were recovered with Pasteur pipettes using 3 x 0.4 ml aliquots of distilled water, transferred to scintillation vials and counted in 10 ml TXBS. Total radioactivity per assay was determined by counting 0.1 ml samples from the original 5 ml aliquots.

(4) Expression of Binding Activity

In general binding activity was expressed as "total binding activity" or "specific binding activity".

Total binding activity = Δ dpm per g fresh weight of tissue.

Specific binding activity = Δ dpm per mg protein.

where Δ dpm = difference in pellet counts between the lowest and highest auxin concentration.

In some experiments binding activity was expressed as "% specific binding":

$$\% \text{ specific binding} = \frac{\Delta \text{ dpm} \times 100\%}{\text{dpm at lowest auxin concentration.}}$$

8. COMPETITION EXPERIMENTS

Several auxin analogues and other phytohormones were tested for their ability to compete for the NAA binding sites as follows.

The membrane pellet was prepared as described and resuspended in 100 ml "binding medium". NAA-^{14}C was added to a concentration of 2×10^{-7}M. The required concentration of competitor was added to 50 ml (treatment), and the same volume of solvent added to the other 50 ml (control). 50 µl from a range of unlabelled NAA solutions of varying concentrations were added to 10 x 5 ml aliquots from both treatment and control, and the binding assay continued as previously described.

9. BIOASSAYS

(1) Corn Coleoptile, Straight Growth

Coleoptiles from 3-4 day-old *Zea mays* were used. All operations were carried out in dim green light at 25 ± 1°C. 5 mm segments, 2 mm behind the tip were excised using a cutting device with parallel blades, and placed in distilled water for 30-60 min. Test compounds were made up in redistilled methanol to a concentration of 10^{-2} M, and diluted to the appropriate concentration with 10 mM phosphate buffer, pH 6.0. Each assay consisted of 10 segments in 5 ml of

buffered test solution. Buffer controls contained an equivalent volume of methanol. After 24 hr the segments were measured using a shadowgraph technique, as described below.

(2) Oat Coleoptile, Straight Growth

4 day-old Avena sativa L., cv. "Victory" were grown as described for Zea mays. 5 mm coleoptile segments, 2 mm behind the tip were used. The assay procedure was as described abcve.

(3) Pea internode, Straight Growth

8 day-old Pisum sativum L., cv. "Alaska" were grown in moist vermiculite as previously described. The seedlings were at the 3rd internode stage (internode approximately 2-5 cm in length), with the hook fully curved. The hook was removed and 10 mm segments, 2 mm below the tip were excised. The assay was performed in 5 cm plastic petri dishes containing 10 ml buffered test solution and measurements made as in the corn coleoptile test.

10. SHADOWGRAPH TECHNIQUE

Segments from the bioassays were arranged in rows on a glass plate. This was placed in a photographic enlarger, and the images, (enlarged five-fold), were projected onto photographic paper which was subsequently exposed, developed, fixed and glazed. The lengths of the segments were determined from the shadowgraph.

11. RAPID GROWTH MEASUREMENTS

The technique used was a refinement of that described by Philipson et al. (1973) which employed a linear displacement transducer (Type D2/2.00 mm, Sangamo Weston Controls Ltd.).

(1) Apparatus

Coleoptile segments were threaded on to a nylon line of 0.4 mm diameter, ("Racine Torture" Nylon fishing line, 15 lb or 16 lb), and placed in a 25 ml cylindrical reservoir which was supported between 'teflon' brackets and held on a brass back plate. The apparatus was designed such that the lever, reservoir and transducer could be easily positioned relative to each other (see Plate 2, A and B). Polythene tubing was threaded above and below the coleoptile segments to provide contact with the brass lever. This lever was counter-balanced with a small weight. Elongation of the segments resulted in movement of the lever which displaced the central armature of the transducer. The transducer operated on a stabilized 12 volt DC supply and generated a DC output signal proportional to the displacement of the armature. This signal was connected to a potentiometric chart recorder and elongation was monitored continuously. The recorder was set at a sensitivity of 5 volts and chart speed of 5 mm per min. The apparatus was calibrated by displacing the lever at the point where the coleoptiles act. The distances displaced were measured by a micrometer screw and the corresponding chart distances were recorded. A chart displacement of 1 mm was equivalent to 14 μm transducer displacement. Chart displacement was linearly proportional to lever displacement over the total lever displacement of 2 mm.

PLATE 2. A: Apparatus used for rapid growth measurements.

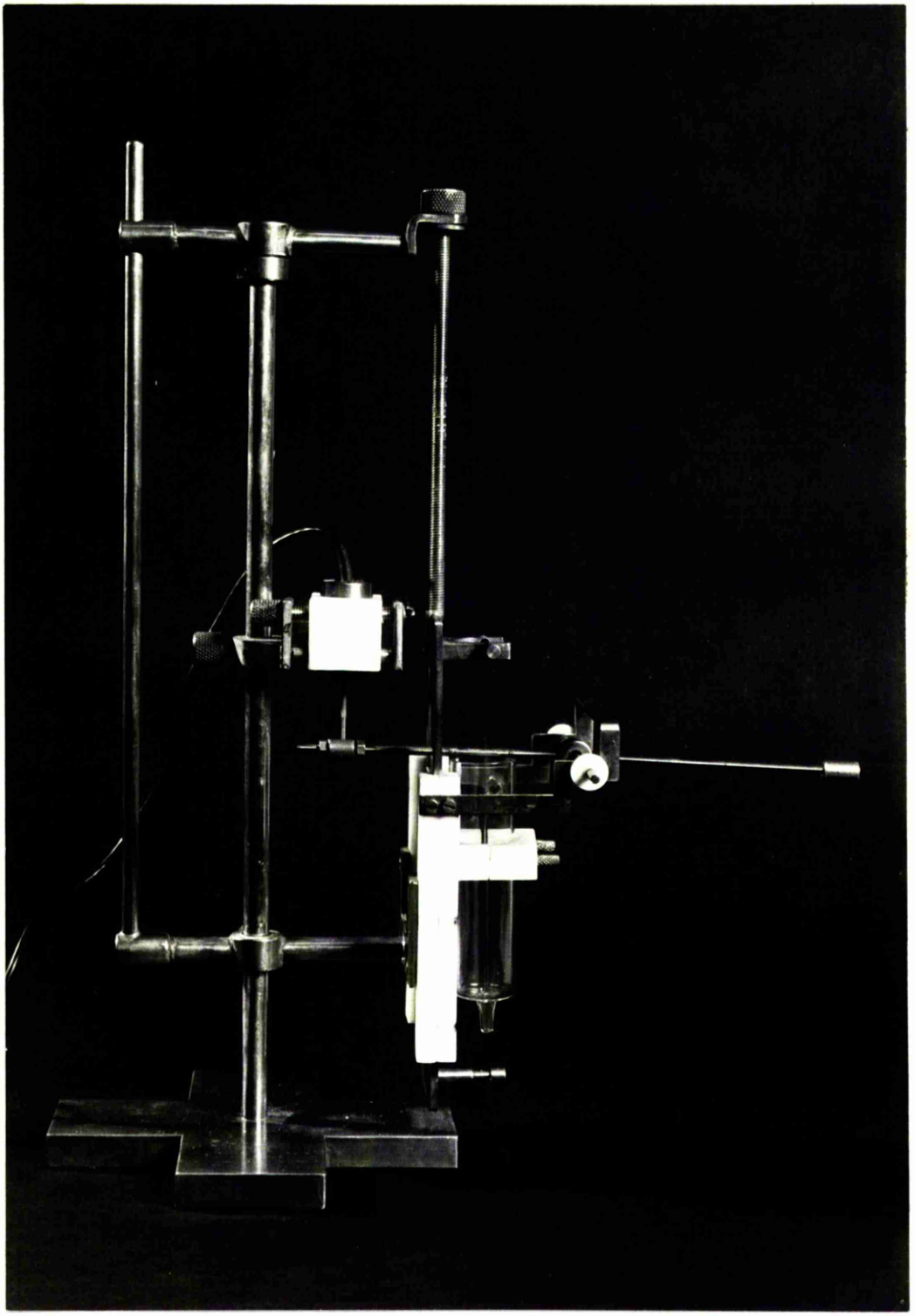
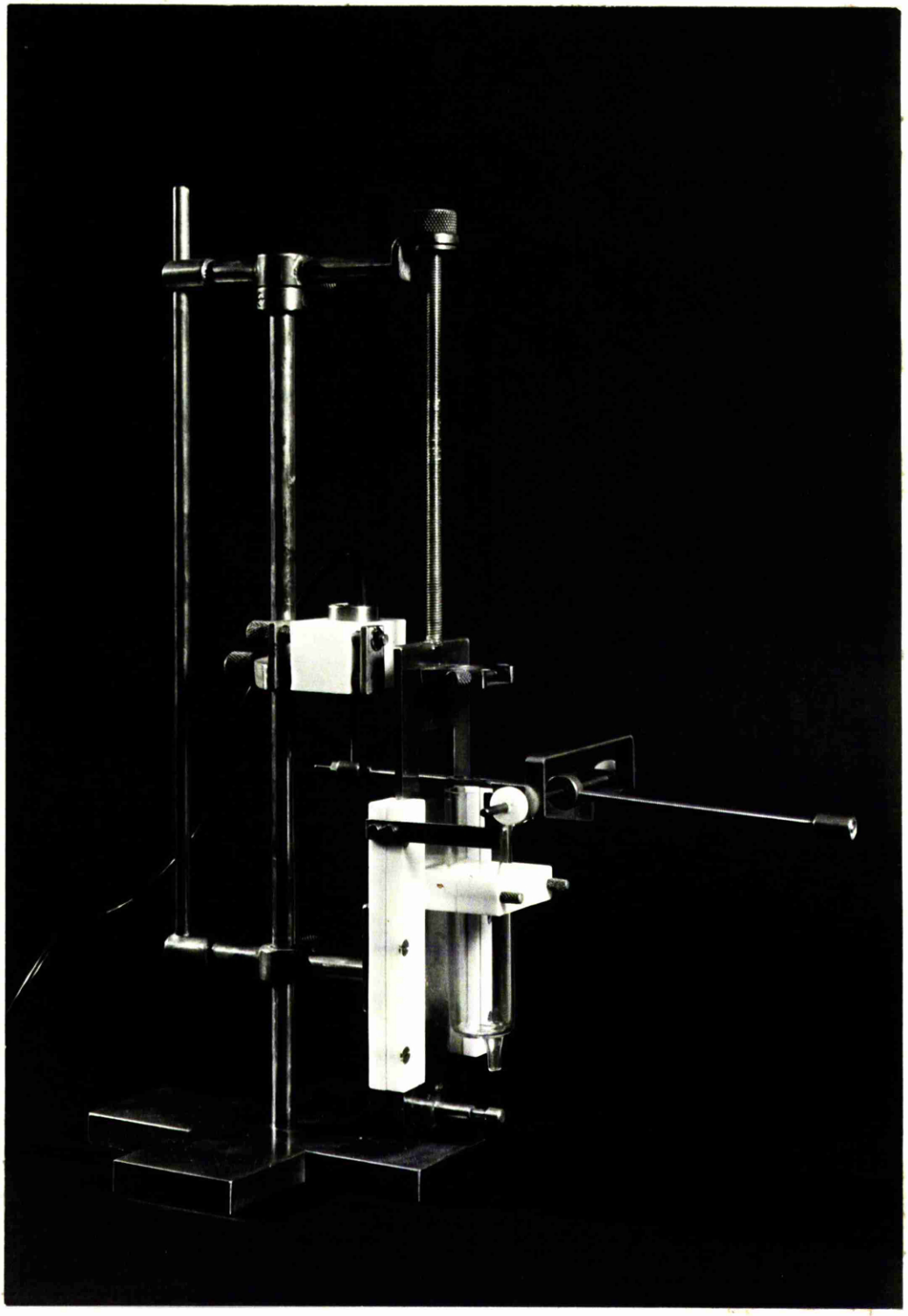


PLATE 2. B: Apparatus used for rapid growth measurements.



(2) Experimental

All operations were carried out in dim green light at 25 ± 1°C. Segments, 5 mm in length, were excised 2 mm below the tip of 3-4 day-old Zea seedlings (Plate 1, B), using a cutting device with parallel blades. Segments were placed in 10 mm phosphate buffer, pH 6.0 (10 mM KH_2PO_4, adjusted to pH 6.0 with KOH) and used immediately. Ten segments were used in each assay and were arranged in an "apex-to-apex" - "base-to-base" fashion on the nylon thread. Solutions were indirectly aerated and maintained at 25°C. They were passed continuously through the reservoir via polythene tubing and Pasteur pipettes, at a flow rate of approximately 10 ml per min. The flow rate was increased whilst solutions were changed, and changeover was achieved in approximately 45 seconds. The compounds to be tested were dissolved in redistilled methanol as 10^{-1} M or 10^{-2} M stock solutions. Working solutions of 10^{-4} M or 10^{-5} M were prepared by dilution in 10 mM phosphate buffer, pH 6.0. Buffer controls contained an equivalent volume of methanol. Segments were placed in the apparatus and growth in buffer alone was recorded for approximately 30 min before treatment was begun at time zero. Growth was then monitored for approximately 1 hour.

12. SUCROSE GRADIENT FRACTIONATION OF THE MEMBRANE PELLET

The sucrose gradients used were first described by Hodges et al., (1972) for the separation of membranes from oat roots.

Two types of discontinuous sucrose gradients were used:

(1) 'Complex'

(2) 'Simple'.

The 'complex' gradients consisted of the following sucrose solutions: 2 ml of 45% w/w (1.58 M) and 3 ml each of 38% (1.29 M), 34% (1.14 M), 30% (0.99 M) and 25% (0.81 M). All sucrose solutions contained 50 mM Tris, 0.1 mM $MgCl_2$ and were adjusted to pH 8.0 with acetic acid (Fig. 1, A).

The 'simple' gradients consisted of 6 ml of the 45% sucrose solution and 8 ml of either 34% or 30% solution (Fig. 1, B).

A 4-38Kg membrane pellet was prepared as previously described and resuspended in 18% w/w (0.56 M) sucrose solution. Approximately 5 ml (containing 10-20 mg protein) were layered onto the gradient. Loaded gradients were centrifuged for 1.5 hr at 100,000 g at 4°C (29,000 rpm in 3 x 20 ml swing-out rotor, "Superspeed 50" centrifuge).

Visible bands occurred at the sucrose interfaces and were removed with a Pasteur pipette. They were diluted to 35 ml with cold (4°C) "extraction medium" and repelleted at 80,000 g for 30 min. These pellets were assayed for binding or enzyme activity as described.

13. SUCROSE GRADIENT FRACTIONATION OF "MITOCHONDRIAL PELLET"

A discontinuous sucrose gradient based on that described by Greenawalt et al., (1967) was used to obtain an enriched mitochondrial fraction from a low speed pellet prepared from mesocotyls.

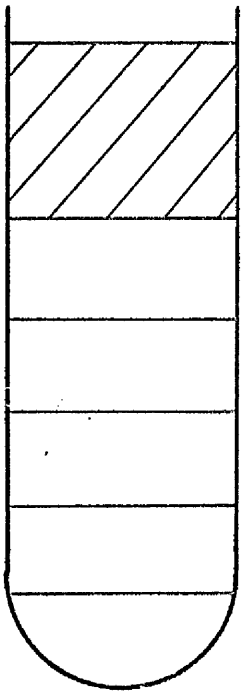
The gradient consisted of the following sucrose solutions: 2 ml of 1.95 M and 3 ml each of 1.65 M, 1.50 M, 1.35 M, 1.20 M and 0.90 M. All sucrose solutions contained 50 mM Tris, 0.1 mM $MgCl_2$ and were adjusted to pH 8.0 with acetic acid.

Fig. 1 Discontinuous sucrose gradients for membrane fractionation.

A : "Complex" gradient

B : "Simple" gradient

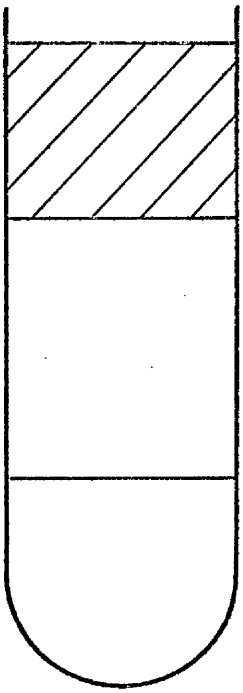
A



SUCROSE SOLUTIONS

% (W/W)	CONC. (M)	VOL. (ml)
18+ SAMPLE	0·56	5
25	0·81	3
30	0·99	3
34	1·14	3
38	1·29	3
45	1·58	2

B



18+ SAMPLE	0·56	5
30 or 34	0·99 or 1·14	8
45	1·58	6

A mesocotyl homogenate was prepared as previously described and spun at 1,000 g (3,500 rpm, "High Speed 18") for 10 min and 10,000 g for 20 min. This 1-10Kg pellet was resuspended in 0.56 M sucrose solution and 3 ml were layered onto each gradient. Gradients were centrifuged for 1.5 hr at 100,000 g. Bands were removed and repelleted as described above. Pellets were assayed for binding activity, and succinate dehydrogenase activity as described later.

14. ELECTRON MICROSCOPY

Pellets were obtained from gradient bands and cut into small pieces. They were fixed for 2 hr at room temperature in 0.1 M phosphate buffer, pH 7.4 containing 3% glutaraldehyde, and washed overnight at 4°C in 0.1 M phosphate buffer, pH 7.4, with 10% sucrose as an osmoticum. They were then postfixed in 1% OsO_4 (in phosphate buffer) for 2 hr at room temperature and washed in two changes (10 min each) of 0.1 M phosphate buffer (standard fixation procedure, Sabatini, Bensch & Barrnett, 1963).

Samples were dehydrated through a graded series of alcohol concentrations (30%, 50%, 70%, 90%) and finally in three changes of 100% alcohol.

They were then embedded in Spurr resin using the "standard mixture" (Spurr, 1969). Samples were left in 1:1 Spurr:alcohol for at least 1 hr, 3:1 Spurr:alcohol for at least 1 hr and then overnight in 100% Spurr. The resin was polymerised at 60°C overnight.

Sections were prepared using an LKB Ultramicrotome 3 (LKB Instruments, Croydon, Surrey) and stained either by a standard alkaline lead citrate procedure (Reynolds, 1963) or by a phosphotungstic

acid (PTA) - chromic acid procedure modified as follows from that described by Roland et al., (1972). Sections were placed on gold grids and floated on water (section side down) for 5 min, at 25°C. Using 'teflon'-coated tweezers, the grids were transferred and floated on 1% periodic acid for 30 min at 38°C in a humid enclosure. The grids and tweezer tips were gently rinsed with water for 20 sec. The grids were then floated on a solution containing 1% PTA and 10% chromic acid for 2 min at 38°C in a humid enclosure. Grids were finally rinsed with distilled water for 20 sec.

Samples were viewed with a Philips 300 electron microscope.

15. ENZYME ASSAYS

(1) ATP-ase (ATP-phosphohydrolase) (E.C. No. 3.6.1.3)

Phosphate release by ATP-ase was assayed by a modified procedure of Lowry and Lopez (1946).

The assay was performed in a 1 ml reaction mixture containing 20 mM Tris-MES, pH 6.0 (2(N-Morpholino)ethanesulphonic acid, from Sigma Chemical Co.), 5 mM ATP* (disodium ATP from Sigma Chemical Co.), 17 mM Na^+ (contributed by the ATP), 32 mM KCl, 5 mM $MgCl_2$ and 0.2 ml enzyme sample, (dissolved in Tris-MES, pH 6.0). Tubes were incubated at 30°C for 15 min and the reaction stopped by the addition of 0.5 ml of 10% TCA (trichloroacetic acid). To the samples was added 4 ml acetate buffer (0.1 M acetic acid + 25 mM sodium acetate, pH 4.0), 1 ml ammonium molybdate (obtained from BDH as a 1% solution in 2 N H_2SO_4), and 0.5 ml of 10% $FeSO_4$ containing one drop of concentrated H_2SO_4 (made up fresh daily). After Vortex mixing, samples were centrifuged at top-speed in

*ATP titrated to pH 6.0 with NaOH before use.

a bench centrifuge for 7-8 min. Absorbance of the supernatant was read at 660 nm. Phosphate release was calculated from a calibration curve using potassium phosphate as standard.

(2) Succinate Dehydrogenase (SDH) (E.C. No. 1.3.99.1)

SDH activity was determined by the method described by Morré (1971).

The assay was performed in a 1 ml reaction mixture containing 0.2 ml of 0.2 M phosphate buffer, pH 7.4, containing 5 mg per ml bovine serum albumin, 0.4 ml of 0.125 M sodium succinate, pH 7.4, and 0.2 ml of enzyme sample. This mixture was preincubated at 37°C for 5 min and the reaction started by the addition of 0.2 ml of 0.5% INT in 1 mM EDTA (INT = 2-(p-indophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium, obtained from BDH). After 15 min the reaction was stopped by the addition of 1 ml of 10% TCA. The formazan colour was extracted into 4 ml ethyl acetate and after brief centrifugation, the absorbance was read at 490 nm. Enzyme activity was calculated using the relationship that 1 nmole formazan per ml = 20.1 absorbance units.

(3) Glucan Synthetase

Glucan synthetase activity was determined by the procedure described by Van der Woude et al., (1974).

Pellets were recovered from gradient bands as already described, and resuspended in 1 ml of 0.1 M Tris-HCl, pH 8.0, 4 mM K_2-EDTA and 1 mM dithiothreitol (DTT). Assays were performed in a 200 µl reaction mixture containing 10 µmoles Tris-HCl, pH 8.0, 0.4 µmoles EDTA, 0.1 µmole DTT (all contributed by 100 µl of enzyme sample),

4 μmoles $MgCl_2$ (40 μl of 0.1 M $MgCl_2$) 2 μmoles cellobiose (20 μl of a 100 μmole per ml solution), 0.27 nmole UDP-D-(^{14}C)-glucose (20 μl of a 13.5 nmole per ml solution) and 0.2 μmole UDP-D-(^{12}C)-glucose (20 μl of a 10 μmole per ml solution). (Cellobiose and UDP-D-(^{12}C)-glucose from Sigma; UDP-D-(^{14}C)-glucose from the Radiochemical Centre, Amersham, specific activity 284 mCi per mmole).

Reaction mixtures, in 3 ml polypropylene tubes were incubated at 30°C for 15 min, and the reaction was terminated by heating the tubes in a boiling water bath for 5 min. The assay mixture was then fractionated into products of varying solubility as follows:

(a) Hot water soluble

1-2 mg of Whatman cellulose were added to each tube and the mixtures extracted three times with 1 ml of water at 100°C for 5 min. The insoluble residue was recovered each time by centrifugation at 38,000 g for 10 min. The hot water soluble products were recovered from the hot water extracts by the addition of 0.5mM of 20 mg per ml Ficoll (mol. wt. = 400,000, Pharmacia, Uppsala) followed by precipitation from 70% ethanol overnight at 4°C, and centrifugation at 38,000 g for 20 min. Precipitates were washed once with 70% ethanol and solubilized in 0.5 ml hot water (100°C, 5 min). The solubilized product and 2 x 0.5 ml water washes were transferred to scintillation vials, and radioactivity determined as already described.

(b) Lipid soluble

The hot water insoluble residue was extracted once with 1 ml of methanol:chloroform (2:1 v/v) and once with 1 ml of absolute methanol. The extracts were transferred to scintillation vials, dried down, dissolved in 1 ml of methanol and radioactivity determined.

(c) Insoluble

The remaining pellet was resuspended in 3 x 0.5 ml aliquots of water and these transferred to scintillation vials. Radioactivity was determined as already described.

(4) IDP-ase (inosine diphosphatase)(E.C. No. 3.6.1.6)

IDP-ase activity was determined by the method of Ray et al., (1969). The latent enzyme was activated by the detergent, sodium deoxycholate (Powell and Brew, 1974).

The assay was performed in a 1 ml reaction mixture containing 0.78 ml of 50 mM Tris-HCl, pH 7.5, 20 µl of 0.1 M $MgCl_2$, 0.1 ml of 30 mM IDP-Na_2 (from Sigma Chemical Co., titrated to pH 7.0 with NaOH), 0.1 ml of enzyme solution containing 1% sodium deoxycholate.

Tubes were incubated for 15 min at 30°C and the reaction stopped by the addition of 0.5 ml of 10% TCA. Phosphate release was determined as already described for ATP-ase.

16. STEROL AND PHOSPHOLIPID DETERMINATION

(1) Lipid Extraction for Phosphorus Analysis

Lipids were extracted by the general method of Folch et al., (1957).

Pellets from the gradient bands were obtained as previously described, and suspended in 1 ml of water. A 0.4 ml aliquot was added to 8 ml of chloroform:methanol (2:1 v/v) and left at room temperature, in the dark, overnight. The mixture was spun for 7 min at top speed

in a bench centrifuge, the supernatant decanted into a fresh tube and 0.2 volumes (1.6 ml) of 0.04% $MgCl_2$ were added. The samples were mixed and spun at top speed in a bench centrifuge for 2 min giving a biphasic system. The upper phase was discarded and the lower phase was washed twice with small volumes of upper phase and the washes discarded. Methanol was then added to give a single phase and the extract was divided into two equal portions for phosphorus analysis.

(2) Digestion and Phosphorus Analysis

This procedure used the method of Rouser et al., (1966). The duplicate samples from above were dried down in Kjeldahl flasks and exactly 0.9 ml of 72% perchloric acid was added, with one anti-bumping granule. This mixture was refluxed for 20 min. After cooling, 5 ml of water were added and the sample was mixed. 1 ml of 2.5% aqueous ammonium molybdate and 1 ml of fresh 10% ascorbic acid were added and after mixing, the sample was heated for 5 min at 60°C in a water bath. After cooling, the absorbance at 820 nm was read. Values are converted to µg phosphorus by reference to a calibration curve using potassium phosphate as standard.

(3) Sterol Estimation

Sterols were determined by a modification of the method described by Stadtman (1957).

5 ml of reagent (a filtered mixture of 640 ml of acetic anhydride, 360 ml of glacial acetic acid and 6 g of p-toluene sulphonic acid) were added, with mixing, to each of two 0.2 ml samples of the membrane suspension. After standing for 5 min, the samples were clear

and 0.5 ml of concentrated H_2SO_4 was added, dropwise with cooling. Samples were then maintained at 25°C for 15 min after which absorbance at 625 nm was read. Sterol concentration was calculated by reference to a calibration curve using sitosterol (Sigma, containing 60% sitosterol and 40% campesterol) as standard and an average sterol molecular weight of 400.

17. EFFECT OF ENZYME TREATMENTS ON BINDING ACTIVITY

The following enzymes were purchased from Sigma Chemical Company.

(1) Trypsin (E.C. No. 3.4.4.4) from bovine pancreas. 12,500 BAEE units per mg protein (1 BAEE unit (α-N-Benzoyl-L-arginine ethyl ester HCl) = OD_{253} of 0.001 per minute in 3.2 ml at pH 7.6, at 25°C).

(2) Phospholipase - A (E.C. No. 3.1.1.4) from Vipera russelli, 5 units per mg protein; or from bee venom, 1,550 units per mg protein. (1 unit hydrolyses 1.0 μmole of L-α-lecithin to lysolecithin and a fatty acid per min at pH 6.5 (V. russelli) or pH 8.5 (bee venom) at 37°C).

(3) Phospholipase - C (E.C. No. 3.1.4.3) from Clostridium welchii, 5 units per mg protein (1 unit liberates 1.0 μmole of water-soluble organic phosphorus from egg yolk lecithin per minute at pH 7.3 at 37°C).

(4) Phospholipase - D (E.C. No. 3.1.4.4) from cabbage, 20 - 30 units per mg protein (1 unit liberates 1 μmole choline from lecithin per hour at pH 5.6 at 26°C).

An unwashed 4-80Kgmembrane pellet was prepared as previously described and resuspended in a sucrose (0.25 M), Tris (50 mM), acetate buffer. For trypsin, phospholipase-A and phospholipase-C treatments, the pH was 7.4. For phospholipase-D the pH was 6.0. Phospholipase-C is a calcium-requiring enzyme and thus 10 mM $CaCl_2$ was also included in the buffer.

Enzymes were dissolved in buffer (at either 0.1 mg per ml or 1 mg per ml) and added to 10 ml aliquots of resuspended membranes to give a range of enzyme concentrations. These aliquots were incubated at 25°C for 20 min in a shaking water bath. They were then transferred to ice cold centrifuge tubes and diluted with 20 ml of cold "washing medium". The enzyme-treated membranes were repelleted at 80,000 *g* for 30 min and the pellets rinsed with distilled water. Each pellet was resuspended in 15 ml "binding medium" and NAA-^{14}C added to a concentration of 2×10^{-7} M. 3 x 5 ml aliquots were taken and 50 µl of unlabelled NAA was added to give final NAA concentrations of (1) 2×10^{-7} M, (2) 4×10^{-7} M, (3) 10^{-6}M. The binding assay was then continued as previously described.

18. EFFECT OF TEMPERATURE ON BINDING ACTIVITY

An unwashed 4-80Kgmembrane pellet was prepared as previously described and resuspended in "washing medium". 10 ml aliquots were incubated at temperatures between 0°C and 60°C for 15 min. The aliquots were transferred to ice-cold centrifuge tubes, diluted with 20 ml "washing medium" and repelleted at 80,000 g for 30 min. Each pellet was resuspended in 15 ml "binding medium" and the binding assay performed as described above.

19. EQUILIBRIUM DIALYSIS OF A TRITON-X-100-SOLUBILISED MEMBRANE FRACTION

A 4-38Kg pellet was prepared as previously described. A portion of this pellet was resuspended in "binding medium" and used in the equilibrium dialysis assay as described below. (Referred to as 'crude' sample). Another portion of the pellet was resuspended in 50 mM Tris-acetate buffer, pH 7.6, containing 0.2% (w/v) Triton-X-100 (scintillation grade, from BDH). (Referred to as 'Triton' sample). This sample was kept on ice for 2 hours, with occasional homogenisation, and after 20 min, it appeared to be clear. The 'Triton' sample was centrifuged at 120,000 g for 1 hour (40,000 rpm in an 8 x 25 ml angle rotor, "Superspeed 50" centrifuge). The supernatant was passed through a 10 cm x 1.5 cm Sephadex G25 (medium) column, equilibrated with "binding medium". The absorbance of the eluate (1 ml fractions) was determined at 280 nm, and the peak five fractions eluting in the excluded volume were combined. (Referred to as 'Triton extract').

Aliquots (1 ml) from the 'crude' sample and the 'Triton extract' were each dialysed against:

(a) 10 ml "binding medium", containing 2.2×10^{-7} M NAA-^{14}C

and (b) 10 ml "binding medium", containing 2.2×10^{-7} M NAA-^{14}C and 5×10^{-6} M unlabelled NAA.

Samples were equilibrated on a rotary turntable at 4°C for 23 hours. Duplicate 100 µl samples were taken from 'inside' the dialysis tubing for protein determination. The radioactivity in duplicate 250 µl samples from 'inside' and 'outside' the dialysis tubing was also assayed.

Binding activity is calculated as follows:

Δdpm_1 = dpm inside - dpm outside (at 2.2×10^{-7} M NAA)

Δdpm_2 = dpm inside - dpm outside (at 5×10^{-6} M NAA)

Binding activity = $\Delta dpm_1 - \Delta dpm_2$.

20. STATISTICAL ANALYSES

Statistical analyses were performed using an Olivetti Programma 101 desk-top computer.

(1) Mean and Standard Error of Mean

Mean and Standard Error of the mean were calculated from the following formulae:

$$M = \frac{\Sigma x}{N}$$

$$\sigma = \sqrt{\frac{\Sigma (x - M)^2}{N - 1}}$$

$$S.E. = \frac{\sigma}{\sqrt{N}}$$

where M = mean of population

σ = Standard deviation

S.E. = Standard error of mean

N = number of observations.

Standard errors are shown on graphs as vertical bars drawn symmetrically about the mean.

(2) "Student's" t-test

This t-test was used to ascertain whether the mean values of two samples differed significantly. The computation used was as follows:

$$\sigma = \sqrt{\frac{(N_1 - 1)S_1^2 + (N_2 - 1)S_2^2}{N_1 + N_2 - 2}}$$

$$t = \frac{M_1 - M_2}{\sigma\sqrt{\frac{1}{N_1} + \frac{1}{N_2}}}$$

Where M_1 and M_2 = mean of 1st and 2nd samples respectively

S_1^2 and S_2^2 = variance of 1st and 2nd samples respectively

N_1 and N_2 = number of observations in 1st and 2nd samples respectively.

The significance levels were obtained from the "Student's" t distribution (Fisher and Yates, Statistical Tables, 1963) with $N_1 + N_2 - 2$ degrees of freedom. The following notation was used, where P = probability value:

$P > 0.05$: N.S. (not significant)

$P \leqslant 0.05$: *

$P \leqslant 0.01$: **

$P \leqslant 0.001$: ***

(3) Regression Analysis

This analysis was used to draw the regression line between the two variables x and y. The parameters, a and b of the regression equation:

$$y = a + bx$$

were computed using the formulae:

$$b = \frac{N \Sigma xy - (\Sigma x)(\Sigma y)}{N \Sigma x^2 - (\Sigma x)^2}$$

$$a = \frac{\Sigma y - b \Sigma x}{N}$$

(4) Weighted Regression Analysis

For the competition experiments, the weighted regression analysis of Wilkinson (1961) was used. Analyses were carried out with the aid of a Univac 1108 computer, using a BASIC programme (by courtesy of Shell Research Laboratories, Sittingbourne, Kent). Values for $K \pm$ S.E. and $n \pm$ S.E. were obtained (these values are defined below).

21. KINETIC ANALYSES

In the binding assay, the interaction of a small molecule, (auxin), with a macromolecule is observed. The equilibrium aspect of such an interaction has been correlated quantitatively by equation (1):

$$B = \frac{n\,k\,F}{1 + kF} \qquad (1)$$ (see Edsall and Wyman, 1958)

where B = moles of bound auxin

n = number of binding sites

k = intrinsic site binding constant (an association constant)

F = molar concentration of nonbound auxin = 'free' auxin

K is defined as the dissociation constant = $\frac{1}{k}$, and is equal to F when $B = \frac{n}{2}$.

(The derivation of equation (1) may be found in Appendix I).

(1) Scatchard Plot

Scatchard (1949) suggested the following linear transformation of equation (1):

$$\frac{B}{F} = kn - kB \qquad (2)$$ (Scatchard, 1949)

For a homogeneous population of binding sites, a graph of $\frac{B}{F}$ against B yields a straight line which may be computed by a standard regression analysis.

Calculation of Data

(a) $B = \frac{\text{dpm in pellet}}{\text{dpm per assay}} \times \text{Auxin concentration} \times 1.5 \times 10^{-3}$ (moles bound per assay)

(b) F = molar auxin concentration

(c) n = intercept on B-axis

(d) $K = \frac{1}{k} = \frac{-1}{\text{slope}}$

(2) Double-Reciprocal Plot

Equation (1) can also be transformed as follows:

$$\frac{1}{B} = \frac{1}{F} \cdot \frac{1}{nk} + \frac{1}{n} \qquad (3)$$

A graph of $\frac{1}{B}$ against $\frac{1}{F}$ yields a straight line which may be computed by the weighted regression analysis of Wilkinson (1961). From this graph:

$$K = \frac{1}{k} = \frac{-1}{\text{intercept on } \frac{1}{F} \text{ axis}}$$

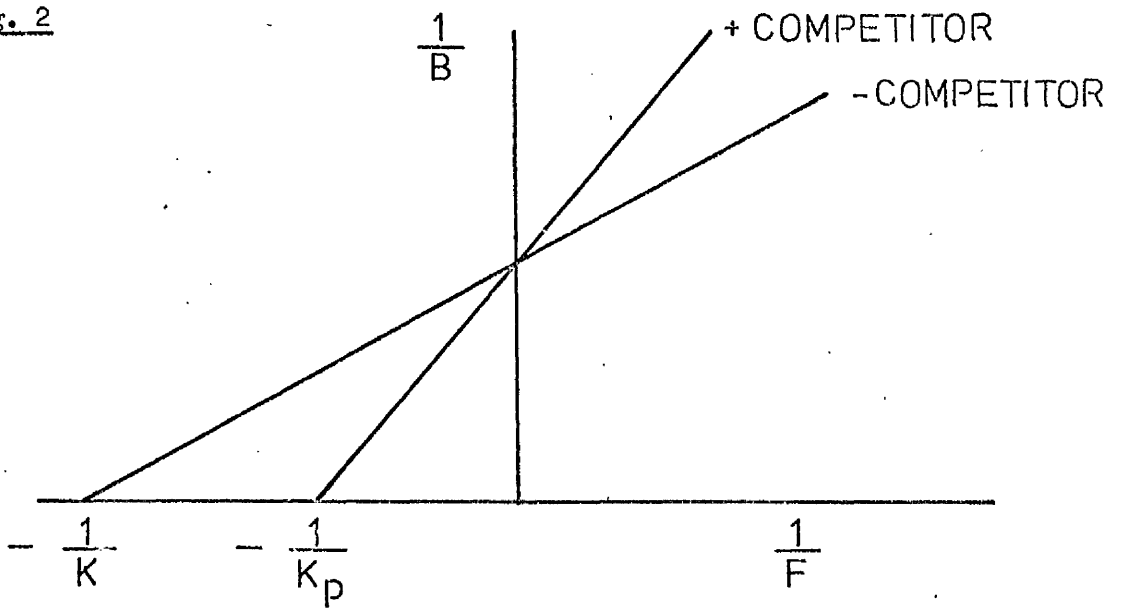
$$n = \frac{1}{\text{intercept on } \frac{1}{B} \text{ axis}}$$

Application in Competition Experiments

In the competition experiments, the double-reciprocal plot was used to illustrate whether or not a compound was having a competitive effect on the binding of NAA. Two lines were computed, one

in the absence of competitor and one in its presence. Fig. 2 shows the result if the compound is having a purely competitive effect:

Fig. 2



The two lines have a common intercept on the $\frac{1}{B}$ axis, i.e. they have a common n value. The inhibitor constant, Ki, may be calculated from the equation:

$$K_i = \frac{i}{\frac{K_p}{K} - 1} \qquad (4) \quad \text{(Dixon and Webb, 1958)}$$

where i = concentration of competitor

K = dissociation constant in the absence of competitor

K_p = dissociation constant in the presence of competitor at concentration, i.

EXPERIMENTS AND RESULTS

1. PRELIMINARY CHARACTERISATION OF THE BINDING OF NAA-^{14}C TO A MEMBRANE FRACTION ISOLATED FROM ZEA COLEOPTILES

The first experiments show the development, improvement and preliminary characterization of the binding assay.

(1) Original Experiments

The original experiments followed closely the procedure outlined by Hertel et al., (1972). Coleoptiles were homogenised using a Sorvall "Omni-mixer", at top-speed for 45 sec, in a buffer containing 0.6 M sucrose, 80 mM Tris, 14 mM 2-mercaptoethanol, 25 mM MES, 10 mM $MgCl_2$, 0.5 mM EDTA, and adjusted with acetic acid to pH 7.5. A 4:1 (v/w) buffer:tissue ratio was used. The homogenate was filtered through nylon cloth and precentrifuged at 10,000 g for 20 minutes. The pH of the 10Kg supernatant was adjusted to pH 5.5 with acetic acid, and NAA-^{14}C was added to a final concentration of 2×10^{-7}M. To aliquots of 5 ml, 50 µl of either water or unlabelled NAA were added to give final concentrations ranging up to 5×10^{-4} M. Three 1.5 ml samples were taken from each aliquot and centrifuged at 38,000 g for 30 minutes. Pellets were recovered and counted as previously described. The results of one of these early experiments are shown in Fig. 3 and Table 1. The data are consistent with those of Hertel et al., (1972) and demonstrate that the radioactivity retained in the pellet decreases with increasing concentration of unlabelled NAA, indicating saturation kinetics.

Fig. 3 Early experiment showing the binding of NAA-^{14}C to a membrane fraction from *Zea mays* coleoptiles. Each point is the mean of three replicates. Standard errors of mean values are shown as vertical bars drawn symmetrically about the mean. Each bar is twice the S.E.

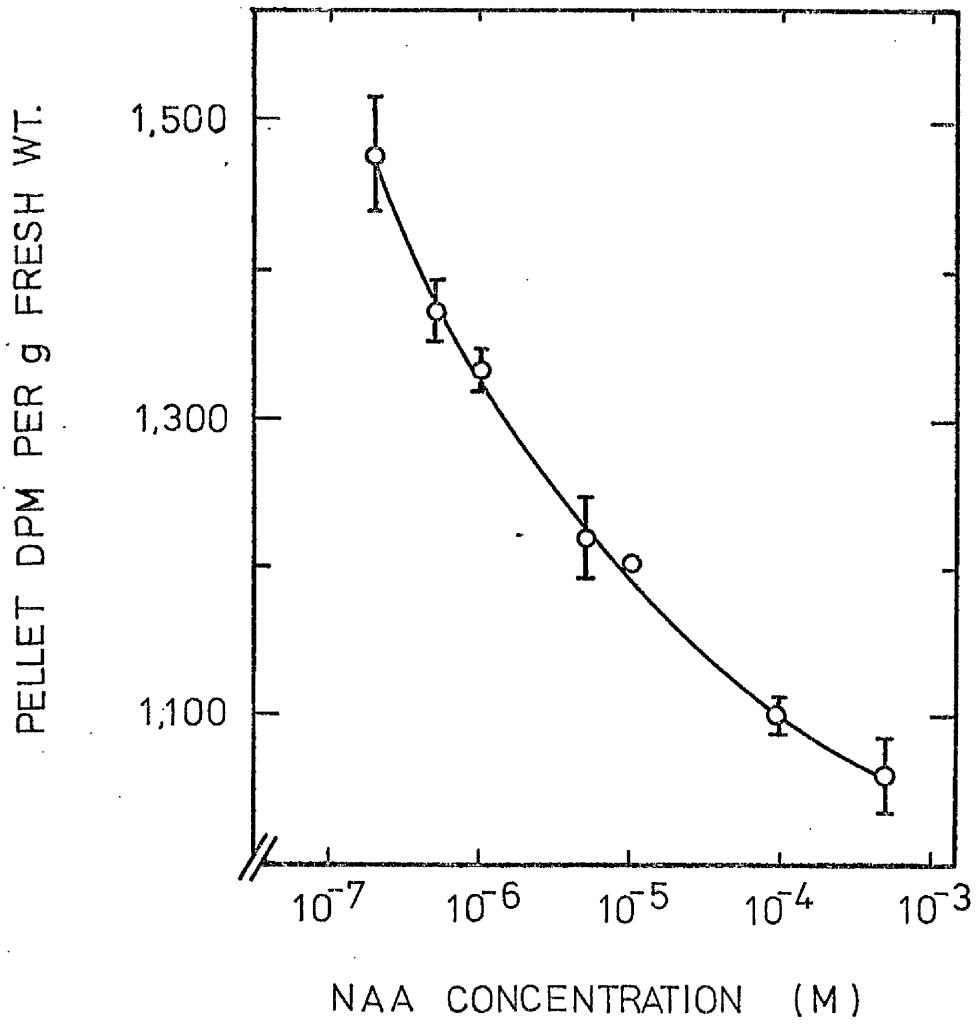

PELLET DPM PER g FRESH WT.
1,500
1,300
1,100
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
NAA CONCENTRATION (M)

Table 1 Comparison of data from Fig. 3 with data from Hertel et al., (1972). The range of NAA concentrations was 2×10^{-7} M to 5×10^{-4} M. (*Comparison on a cpm basis must assume the same specific activity of NAA-^{14}C, and the same counting efficiency).

Table 2 Improvement of binding data by modified buffers (Hertel, 1974) and pestle and mortar homogenisation. Binding activity is assayed between 2×10^{-7} M and 5×10^{-4} M.

Table 1

BINDING ACTIVITY	FROM FIG. 3	FROM HERTEL et al., 1972
% Specific binding	28	24
*Δ cpm per g fresh wt.	376	310

Table 2

CONDITIONS	ORIGINAL BUFFERS 10Kg SUPERNATANT MECHANICAL HOMOGENISATION	MODIFIED BUFFERS 10-38Kg PELLET MECHANICAL HOMOGENISATION	MODIFIED BUFFERS 10-38Kg PELLET PESTLE & MORTAR HOMOGENISATION
Δ dpm per g fresh wt.	418	563	769

(2) Improved Experiments

The procedure outlined above differs from that described in the Materials and Methods section in four respects:

(a) Method of homogenisation.

(b) Buffer composition.

(c) Membrane-fraction preparation.

(d) Range of unlabelled NAA concentrations.

Using buffers of the composition described in the Materials and Methods section (Hertel, 1974), a 10-38Kg pellet (unwashed) and pestle and mortar homogenisation, a marked improvement in binding activity was found (Table 2). Pestle and mortar homogenisation, modified buffers, and isolated membrane pellets were therefore used in subsequent experiments.

Hertel (1974), reported that binding activity was improved if a washed 10-38Kg pellet (as described in Materials and Methods) was used in the binding assay. The effect of pellet washing was tested on four occasions, the first two being in the early stages of this work and the second two being approximately one year later. (Tables 3 and 4).

The data in Table 3 indicate that washing the pellet has very little effect on % specific binding and actually reduces the total specific binding (Δdpm). The data in Table 4, however, show that the pellet wash improves binding activity. The major difference between the two sets of experiments is that they were performed with different batches of seed. In experiments performed with the early seed batch, a pellet-wash step was not included. Experiments using the later seed batch, however, did include the pellet-wash.

Table 3 Effect of washing the 10-38Kg pellet on binding activity. Original batch of seed. The range of NAA concentrations was 2×10^{-7} M to 10^{-4} M.

* The lower binding in this experiment is the result of mechanical homogenisation.

Table 4 Effect of washing the 10-38Kg pellet on binding activity. Later batch of seed. The range of NAA concentrations was 2×10^{-7} M to 10^{-6} M.

Table 3

BINDING ACTIVITY	* EXPT. 1		EXPT. 2	
	- WASH	+ WASH	- WASH	+ WASH
Δ dpm per g fresh wt.	566	493	978	690
% specific binding	47	46	74	70

Table 4

BINDING ACTIVITY	EXPT. 1		EXPT. 2	
	- WASH	+ WASH	- WASH	+ WASH
Δ dpm per g fresh wt.	628	838	612	944
% specific binding	33	44	29	40

In the experiments so far described, a wide range of unlabelled NAA concentrations was used (from 2×10^{-7} M to 5×10^{-4} M). A Scatchard analysis of this data (Figs. 4 and 5) indicates a homogeneous population of binding sites over the NAA concentration range 2×10^{-7} M - 10^{-6} M, with a K value of 1.3×10^{-6} M. The concentration of binding sites, n, is estimated to be 72 pmole per g fresh weight of tissue. Above 10^{-6} M NAA, binding saturates very slowly with increasing NAA concentration, indicating low affinity, possibly non-specific binding, at a large number of sites. Therefore, in order to investigate the parameters of the high affinity sites, binding over the concentration range 2×10^{-7} M to 10^{-6} M NAA was investigated in greater detail.

Figs. 6 and 7 show the results of an experiment using the modifications described above. Also, in this experiment, the initial pellet preparation and final pelleting step were performed at 80Kg and 150Kg respectively. The combination of all these factors results in the mean total binding activity from a number of experiments being improved to $1,369 \pm 68$ dpm per g fresh weight. From Fig. 6, it can be seen, as before, that as the NAA concentration increases, pellet radioactivity decreases, indicating saturation of the NAA binding sites. The pellet radioactivity never decreases to zero over this concentration range, since there is always residual low affinity binding together with radioactivity trapped in the small volume of aqueous phase in the pellet. The Scatchard analysis (Fig. 7) gives a biphasic plot, indicating that within the membrane preparation there are at least two sets of high affinity binding sites for NAA. The binding parameters of the two sites

can be calculated directly from the intercepts and slopes of the two lines, and are as follows:

Higher affinity site, (site 1), $K_1 = 1.5 \times 10^{-7}$ M

$n_1 = 38$ pmole per g fresh weight

Lower affinity site, (site 2), $K_2 = 16.1 \times 10^{-7}$ M

$n_2 = 96$ pmole per g fresh weight

K and n-values were found to vary between experiments. A two-to-three fold variation in K was observed.

The technique for evaluating the binding parameters from a Scatchard plot indicating two classes of independent sites, has recently been refined by Hunston (1975). Table 5 shows a comparison of the K and n-values as determined by the Hunston analysis and the intercept-slope analysis. As can be seen, the K values do not differ appreciably, but the analysis results in a different distribution of binding sites between n_1 and n_2. Since interest was primarily in the values of the dissociation constants, the simpler intercept-slope evaluation of K and n was used.

(3) The Effect of pH on Binding of NAA-^{14}C

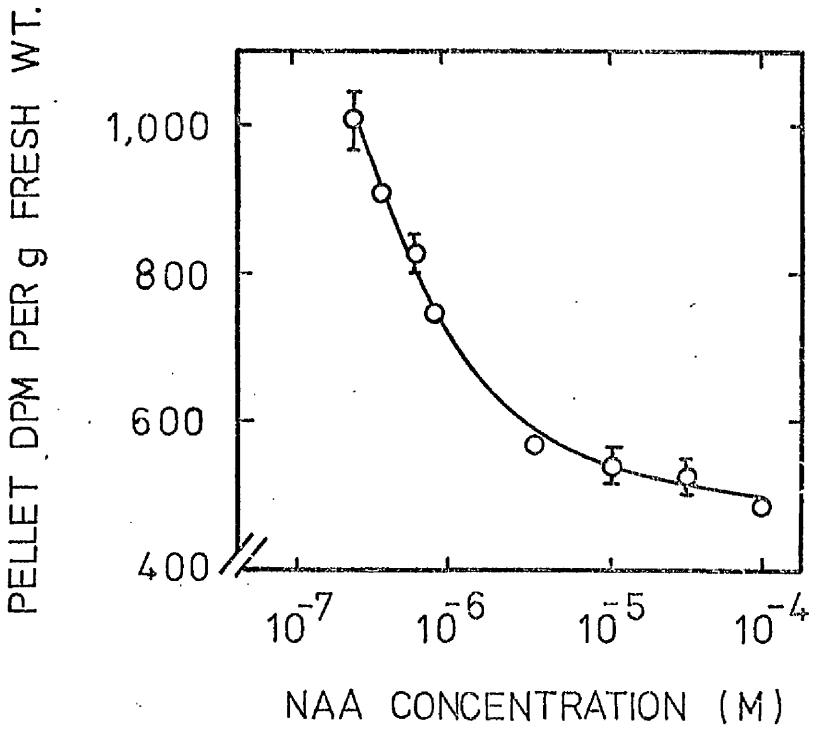
The effect of pH on total binding activity is shown in Fig. 8 . Very low activity is observed at pH $\leq$ 5.0 or pH $\geq$ 7.0. A distinct optimum is seen at pH 5.5, and reasonable activity is still apparent at pH 6.0 and pH 6.5.

Fig. 4 Binding of NAA-^{14}C to a 4-38 Kg, washed membrane pellet from Zea coleoptiles, over a wide range of NAA concentrations. Each point is the mean of three replicates.

Fig. 5 Scatchard analysis of data from Fig. 4. Each point is the mean of three replicates, and lines were computed using standard regression analyses.

$$K = \frac{1}{\text{slope}}$$

n = intercept on x-axis.


PELLET DPM PER g FRESH WT.
1,000
800
600
400
10^{-7}
10^{-6}
10^{-5}
10^{-4}
NAA CONCENTRATION (M)

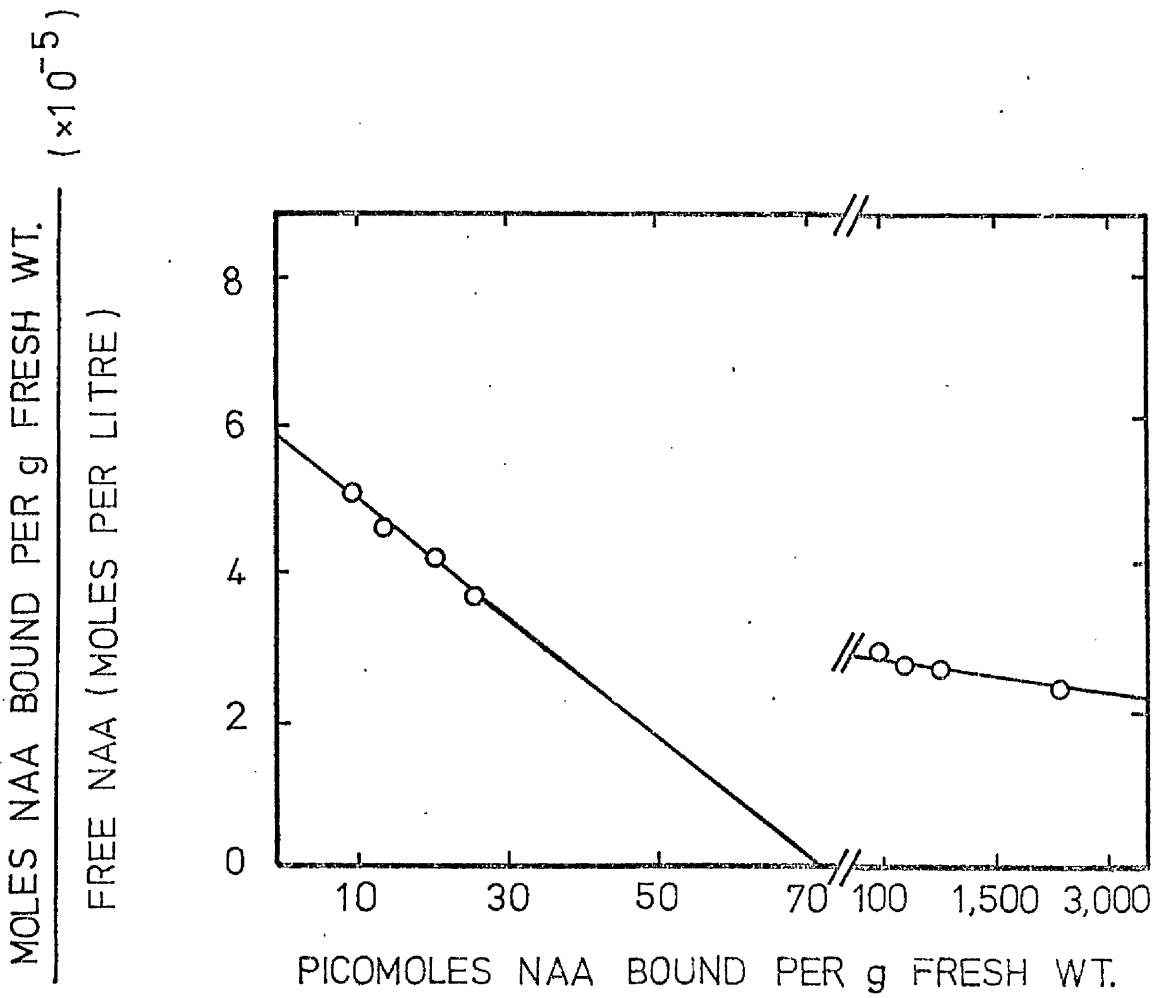

MOLES NAA BOUND PER g FRESH WT. / FREE NAA (MOLES PER LITRE) ($\times 10^{-5}$)
8
6
4
2
0
10
30
50
70
100
1,500
3,000
PICOMOLES NAA BOUND PER g FRESH WT.

Fig. 6 Binding of NAA-^{14}C to membrane fractions prepared from Zea coleoptiles. Pestle and mortar homogenisation, modified buffer conditions, 4-80Kg washed pellet and a range of NAA concentrations of 2×10^{-7} M to 10^{-6} M were used. Each point is the mean of three replicates.

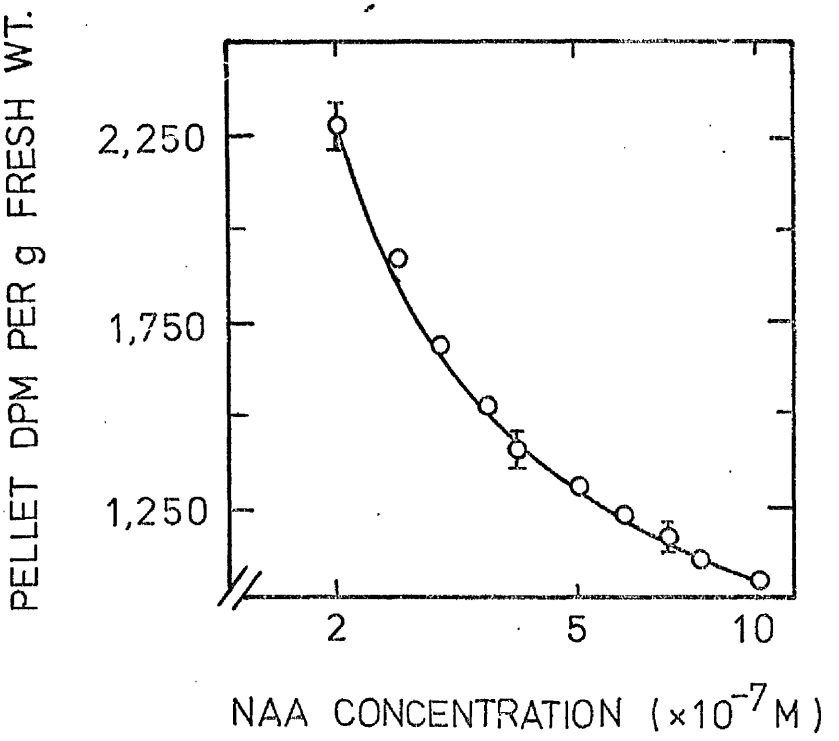
Fig. 7 Scatchard analysis of the data from Fig. 6. Each point is the mean of three replicates, and the lines are drawn by standard regression analyses.

$$K_1 = \frac{1}{\text{slope of line through first five points}}$$

$$K_2 = \frac{1}{\text{slope of line through last five points}}$$

n_1 = intercept of first line on x-axis

$n_1 + n_2$ = intercept of second line on x-axis


PELLET DPM PER g FRESH WT.
2,250
1,750
1,250
2
5
10
NAA CONCENTRATION (×10⁻⁷ M)

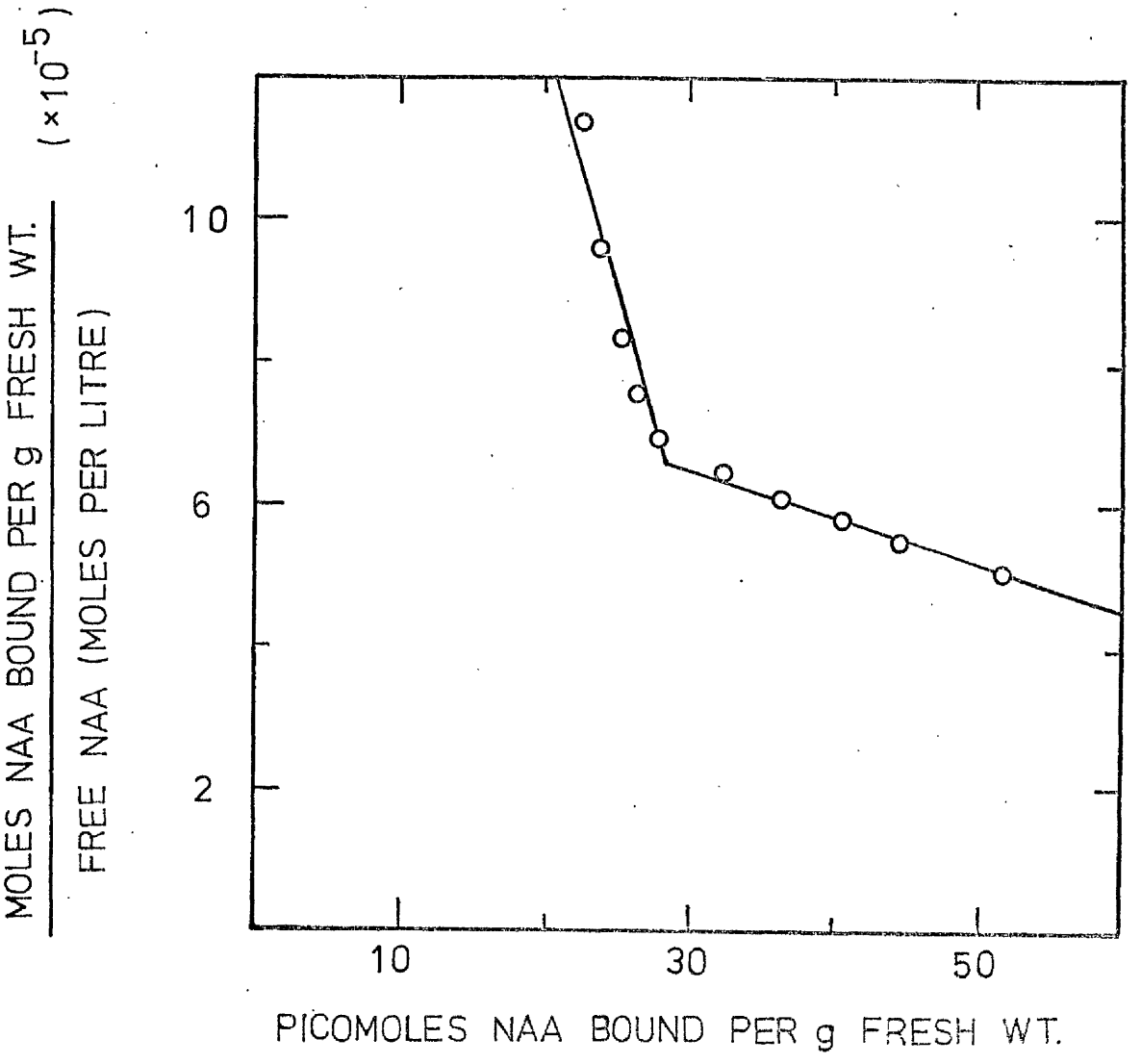
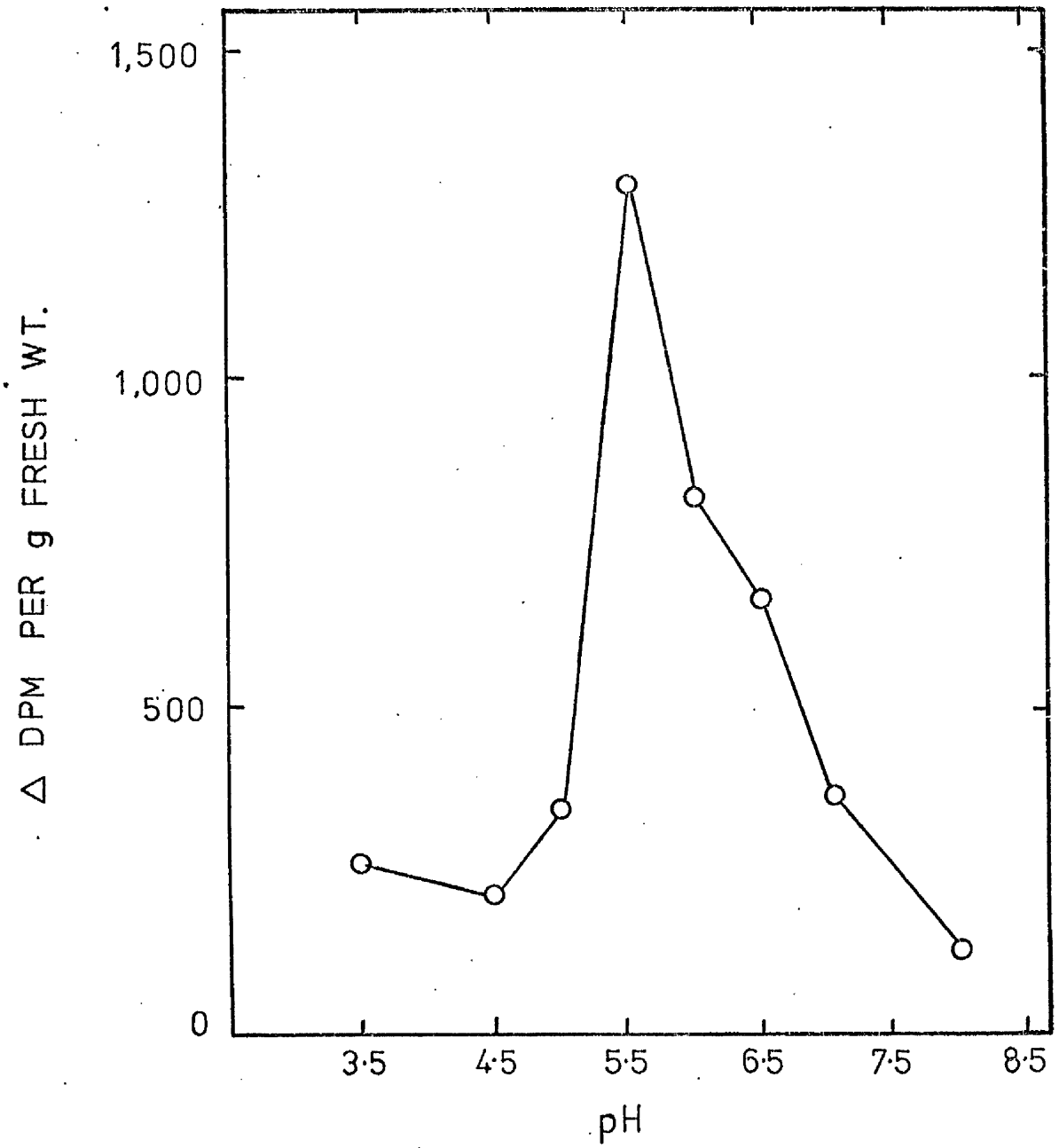

MOLES NAA BOUND PER g FRESH WT. / FREE NAA (MOLES PER LITRE) (×10⁻⁵)
10
6
2
10
30
50
PICOMOLES NAA BOUND PER g FRESH WT.

Table 5 A comparison of the binding parameters for site 1 and site 2 as determined by the Hunston analysis and the intercept-slope analysis. The data represent the mean values from five experiments in which a 4-80Kg membrane pellet and a range of NAA concentrations of 2×10^{-7} M to 10^{-6} M were used.

Table 5

PARAMETER	HUNSTON ANALYSIS	INTERCEPT-SLOPE ANALYSIS
K_1 (M)	1.4×10^{-7}	1.8×10^{-7}
n_1 (pmole per g fresh wt.)	31	52
K_2 (M)	16.7×10^{-7}	14.5×10^{-7}
n_2 (pmole per g fresh wt.)	122	101

Fig. 8 Effect of pH on total binding activity. The range of NAA concentrations was 2×10^{-7} M to 5×10^{-4} M.


1,500
1,000
500
0
Δ DPM PER g FRESH WT.
3·5
4·5
5·5
6·5
7·5
8·5
pH

(4) Identification of Radioactivity in the Membrane Pellet as NAA-^{14}C

It is possible that a coleoptile homogenate has the capacity to metabolise exogenous NAA so that the radioactivity in the pellet is no longer present as NAA-^{14}C, but as a radioactive metabolite. Since experiments are performed at 0-4^{o}C, such metabolic changes are unlikely. However, in order to examine this possibility, a membrane fraction was prepared as normal and NAA-^{14}C added. The fraction was recentrifuged, the radioactive pellet rinsed with distilled water, and resuspended in a minimum volume of methanol. After centrifugation, the methanol extract was loaded onto a thin-layer plate. A similar plate, with NAA-^{14}C standard, was also prepared. The plates were developed in n-butanol:acetic acid:water (62:25:15, v/v) and after drying, they were cut up into R_f zones and counted in 10 ml TBS. The results are shown in Fig. 9 and it can be seen that the radioactivity from the membrane pellet migrates as a single peak corresponding to the NAA-^{14}C standard at R_f 0.8. Thus it appears that under the experimental conditions described, no metabolism of NAA-^{14}C is taking place.

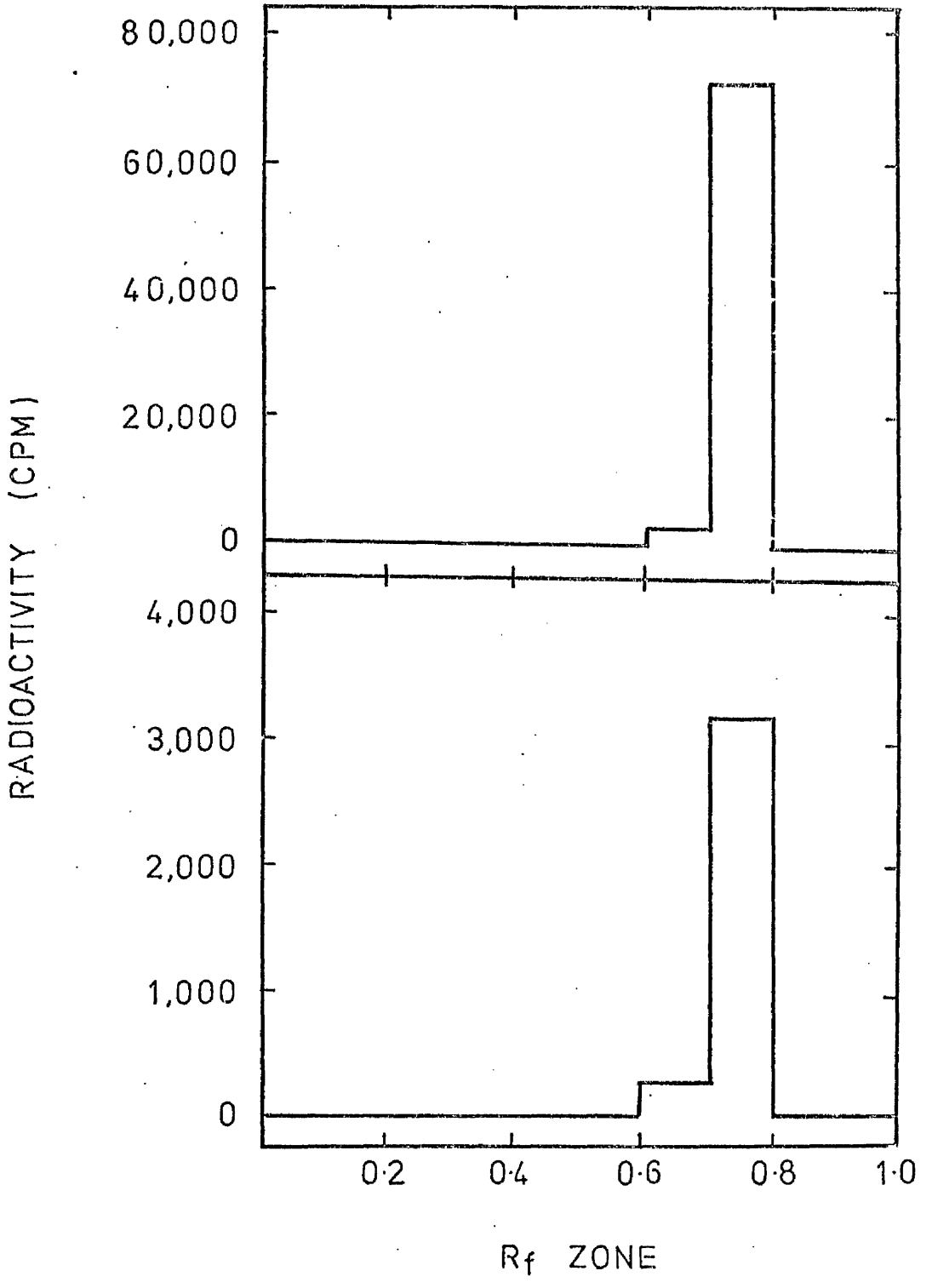
(5) Solubilisation of Binding Activity

Under the extraction conditions used, the membrane material forms closed vesicles (see Plates 5-9). Thus, it is possible that the pelletable binding activity does not reflect the binding of NAA-^{14}C to a receptor on the membrane surface, but rather that it demonstrates the concentration-dependent transport of NAA-^{14}C into the vesicles. To test this possibility, the membranes were solubilized using the detergent, Triton-X-100, and binding activity assayed using equilibrium dialysis.

Fig. 9 Thin-layer chromatography on plastic-backed silica plates in n-butanol:acetic acid:water, (62:25:15, v/v).

A : NAA-^{14}C Standard

B : Methanol extract from a radioactive membrane pellet.


80,000
60,000
40,000
20,000
0
4,000
3,000
2,000
1,000
0
RADIOACTIVITY (CPM)
0·2
0·4
0·6
0·8
1·0
Rf ZONE

(See Materials and Methods). If binding activity represents transport into vesicles, a large proportion of the activity should be lost after this treatment.

From the results shown in Table 6 it can be seen that, on a total activity basis (Δ dpm per ml per g tissue), the Triton-solubilized preparation retains 87% of the binding activity of the 'crude' control. On a specific activity basis (Δ dpm per ml per mg protein) there is over a two-fold enrichment in binding activity. Thus, it seems that NAA-^{14}C binding is not a reflection of transport into membrane vesicles.

(6) The Binding of IAA-^{14}C to a Membrane Pellet from Zea coleoptiles

Since IAA is an endogenous auxin of Zea mays coleoptiles (Greenwood et al., 1972), the binding activity of IAA-^{14}C to the membrane preparations was examined (Fig. 10). As previously described for NAA-^{14}C binding, there appears to be two sets of binding sites (Fig. 11) with K values for IAA of 1.7×10^{-6} M and 5.8×10^{-6} M, and n values of 51 and 100 pmole per g fresh weight respectively. The binding affinities are 5 to 10 fold lower than those calculated for NAA, and consequently the observed Δ dpm is considerably reduced. The numbers of binding sites are in remarkable agreement with those obtained for NAA.

(7) A Comparison of the Binding of Other Radioactive Auxins with NAA-^{14}C Binding

Table 7 shows a comparison of total binding activity obtained with NAA-^{14}C, IAA-^{14}C, 2,4-D-^{14}C and 2,4,5-T-^{14}C over a concentration range of unlabelled auxin between approximately 2×10^{-7} M and 10^{-6} M.

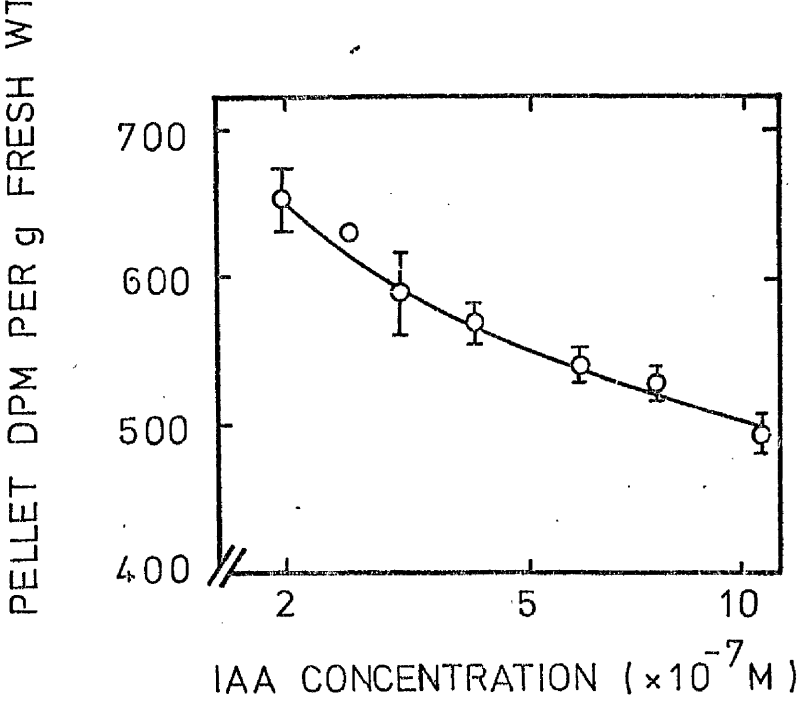
Table 6 Equilibrium dialysis of a 'crude' and a Triton-X-100 solubilized 4-38Kg membrane and sample.

Table 6

SAMPLE	PROTEINS (mg/ml)	FRESH WT. (g/ml)	(dpm per 250 µl)						BINDING ACTIVITY		
			(2.2×10^{-7}M)			(5×10^{-6}M)					
			INSIDE	OUTSIDE	Δdpm_1	INSIDE	OUTSIDE	Δdpm_2	$\Delta dpm_1 - \Delta dpm_2$	Δdpm/ml/g tissue	Δdpm/ml/mg protein
'Crude	1.40	0.8	5601±10	5139±7	462	5253±18	5109±12	144	318	1595	911
'Triton'	0.76	1.18	5758±12	5166±10	592	5249±20	5064±4	185	407	1380	2142

Fig. 10 Binding of IAA-^{14}C to a membrane pellet from Zea coleoptiles. Each point is the mean of three replicates.

Fig. 11 Scatchard analysis of the data from Fig. 10. Each point is the mean of three replicates and the lines are drawn by standard regression analyses.


PELLET DPM PER g FRESH WT
700
600
500
400
2
5
10
IAA CONCENTRATION (×10⁻⁷M)

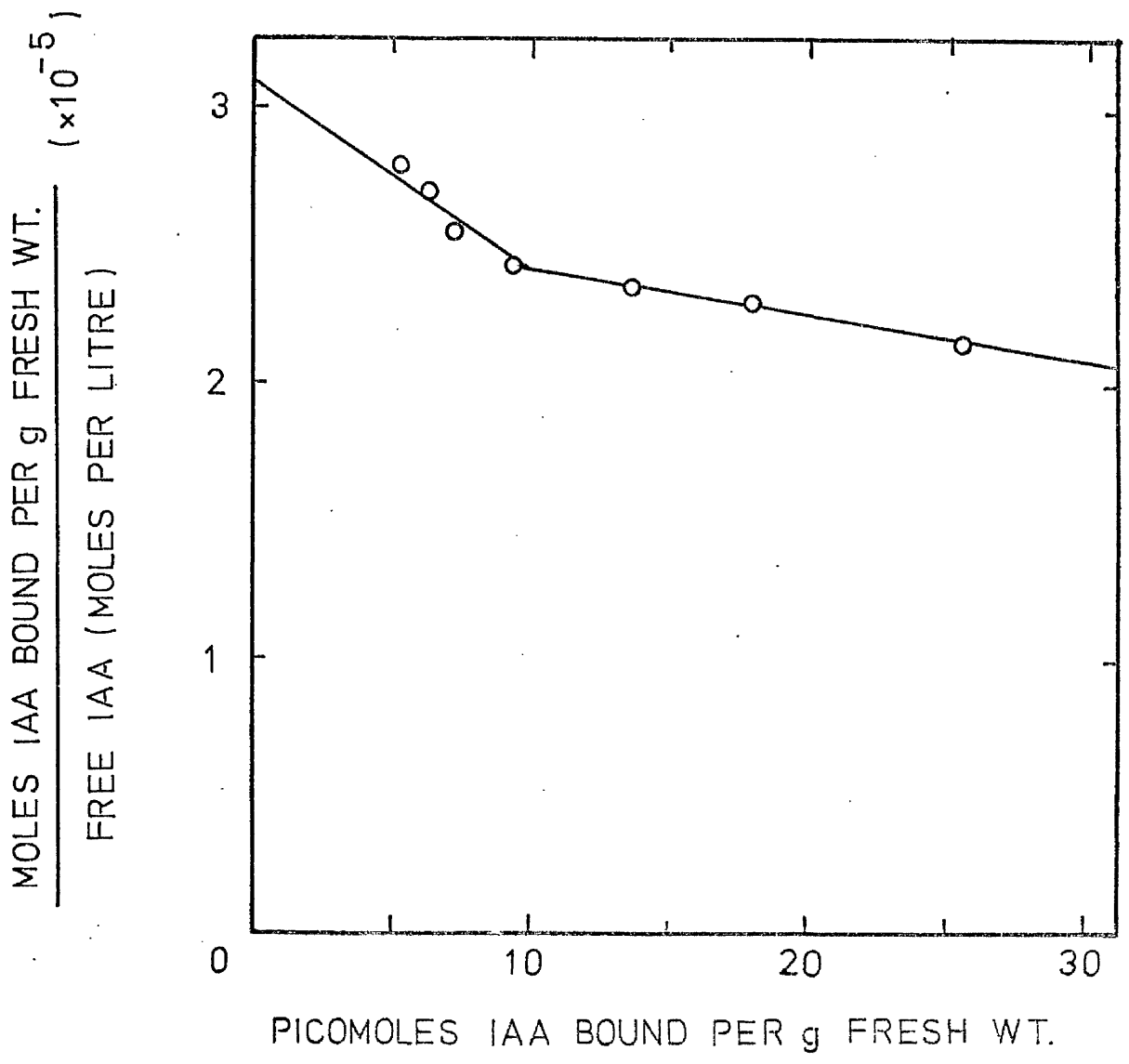

MOLES IAA BOUND PER g FRESH WT. / FREE IAA (MOLES PER LITRE) (×10⁻⁵)
3
2
1
0
10
20
30
PICOMOLES IAA BOUND PER g FRESH WT.

Table 7 Comparison of total binding activities of a 4-38Kg pellet, obtained with NAA-^{14}C, IAA-^{14}C, 2,4-D-^{14}C and 2,4,5-T-^{14}C. The range of concentration of auxin was 2×10^{-7} M to 10^{-6} M.

Table 7

COMPOUND	NAA	IAA	2,4-D	2,4,5-T
BINDING ACTIVITY (Δ dpm/g fresh wt.)	909±42	162±5	105±16	475±23

These data show that, in terms of Δ dpm per g fresh weight, NAA-^{14}C gives much better results than the other auxins. The low value for 2,4-D can in part be explained by its lower specific activity (28 mCi per mmole), but the specific activities of the other compounds are of the same orders as that of NAA. It is possible to estimate K and n values with these low levels of binding (Figs. 10 and 11) but the errors are often so great as to render the data of doubtful value. It was therefore decided to use NAA-^{14}C in all subsequent studies.

2. THE AUXIN-SPECIFICITY OF THE NAA-^{14}C BINDING SITES

If the binding of NAA-^{14}C has relevance to the physiological activity of auxins, the binding sites should exhibit some measure of specificity. In order to examine this important question, several auxin analogues were tested for their ability to interact with the two sets of NAA binding sites.

(1) Determination of the Growth Activity of Auxins and Analogues

Most of the published information on the physiological auxin activity of synthetic auxin analogues, derives from the 1950's when the chemical purity of the compounds tested was often not rigorously established. It was therefore decided to retest the selected compounds for auxin activity and to include tests on the species (Zea) being studied. This was done by:

(a) Standard bioassay techniques.

(b) Rapid growth measurements.

The standard bioassays measure the net effect, after 24 hours, of a number of processes resulting in stem elongation. In the rapid growth measurements, coleoptile elongation can be detected within 10 min (Ray and Huesink, 1962) and it might be argued that this assay is more closely related to the primary response to an auxin.

The auxins and auxin analogues used in the experiments are listed in Table 8, together with their activities as already quoted in the literature.

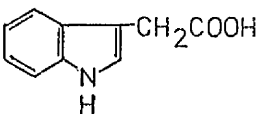
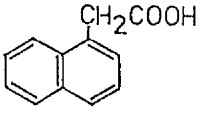
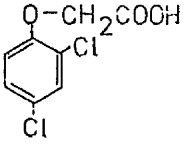
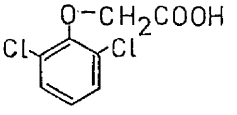
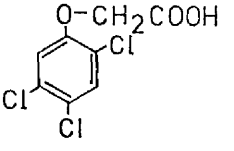
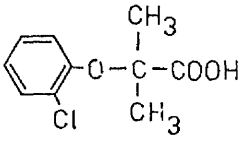
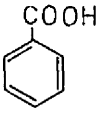
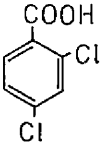
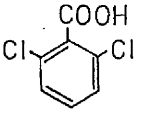
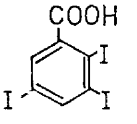
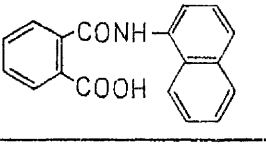
Table 8 List of auxins and auxin analogues used in auxin-specificity experiments.

(1) Osborne et al., (1954)

(2) Niedergang-Kamien and Skoog, (1956)

(3) Thomson et al., (1973)

(4) Morgan and Söding, (1958).

COMPOUND	ABBREV.	STRUCTURE	REPORTED AUXIN ACTIVITY*
3-indolylacetic acid	IAA		+++, very active
1-naphthylacetic acid	NAA		+++, very active
2,4-dichloro-phenoxyacetic acid	2,4-D		+++, very active
2,6-dichloro-phenoxyacetic acid	2,6-D		Inactive; antiauxin; slight activity after 6 hr but not after 24 hr (1).
2,4,5-trichloro-phenoxyacetic acid	2,4,5-T		+++, very active
α-(2-chloro-phenoxy)-isobutyric acid	2-CPIBA		Strong antiauxin
Benzoic acid	BA		Inactive
2,4-dichloro-benzoic acid	2,4-B		Inactive
2,6-dichloro-benzoic acid	2,6-B		+ slightly active
2,3,5-triiodo-benzoic acid	TIBA		Weak auxin; strong antiauxin; inhibitor of polar auxin transport (2), (3).
1-N-naphthyl-phthalamic acid	NPA		Inhibitor of polar auxin transport (3), (4).

*As quoted by Jönsson (1961) for the oat coleoptile cylinder test.

(a) Standard Bioassays

The results of the corn coleoptile, oat coleoptile and pea internode straight growth tests are shown in Table 9. IAA, NAA, 2,4-D and 2,4,5-T show highly significant growth activity at 10^{-5} M in all three tests. BA and 2,4-B show no growth activity. 2,6-B, 2-CPIBA and 2,6-D are inactive in the oat and corn coleoptile tests but in the pea internode test, 2,6-D shows significant activity at 10^{-4} M, and 2,6-B is also slightly active at this concentration; 2-CPIBA at 10^{-5} M shows very slight activity in the pea test. These results are, in general, consistent with those quoted in Table 8 apart from the lack of activity of 2,6-B in the oat test. No attempt was made to measure anti-auxin activity.

(b) Rapid Growth Measurements

Activity of the auxins and auxin analogues (at a concentration of 10^{-5} M) in inducing rapid elongation responses in corn coleoptile segments is illustrated in Fig. 12 and Table 10. The terms "latent period" and "promoted rate of growth" will first be explained.

Fig. 13

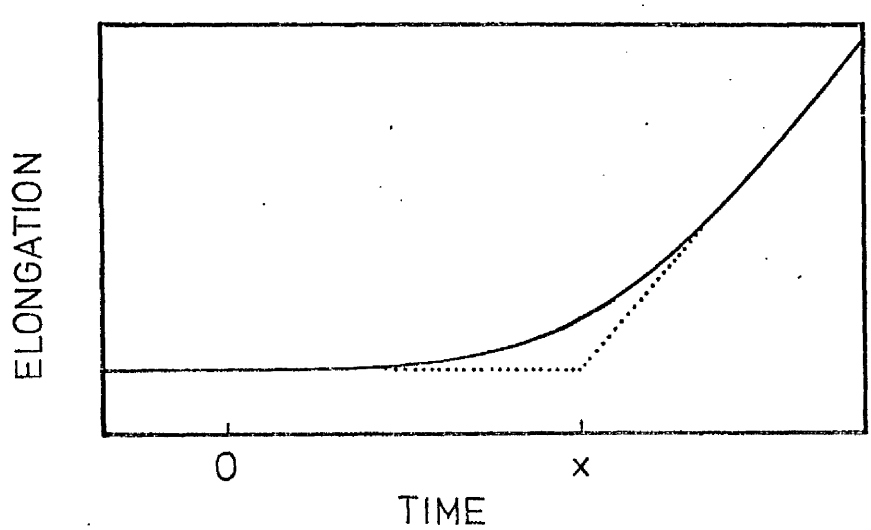


Table 9 Standard bioassay data. All compounds were tested at 10^{-5} M. Compounds inactive at 10^{-5} M were also tested at 10^{-4} M. Results obtained at the higher concentration are indicated †. Results show the mean ± S.E. of 10 segments. Significance values are from a "Student's" t-test, using the notation already described.

$$\% \text{ change above control} = -\left(\frac{C-O}{O} \times 100\%\right) + \left(\frac{F-O}{O} \times 100\%\right)$$

where C = buffer control length

O = original segment length (5 mm for oat and corn segments; 10 mm for pea segments).

F = final length with compound.

Values are corrected to the nearest %.

Table 9

COMPOUND	OAT COLEOPTILE			CORN COLEOPTILE			PEA INTERNODE		
	Mean final length ±S.E. (mm)	% Change above control	Significance	Mean final length ±S.E. (mm)	% Change above control	Significance	Mean final length ±S.E. (mm)	% Change above control	Significance
Buffer control	6.14±0.06	-	-	7.06±0.19	-	-	12.16±0.16	-	-
IAA	8.47±0.13	47	***	8.16±0.16	22	***	14.34±0.11	22	***
NAA	7.84±0.10	34	***	8.58±0.23	31	***	14.76±0.11	26	***
2,4-D	8.16±0.15	40	***	9.24±0.23	44	***	15.22±0.13	31	***
2,6-D	6.02±0.14	-2	N.S.	7.20±0.30	3	N.S.	†13.33±0.11	12	***
2,4,5-T	8.00±0.12	37	***	8.66±0.28	32	***	14.72±0.18	26	***
2-CPIBA	6.18±0.06	1	N.S.	7.26±0.20	4	N.S.	12.66±0.15	5	*
BA	6.22±0.09	2	N.S.	6.88±0.21	-3	N.S.	12.56±0.06	4	N.S.
2,4-B	6.00±0.07	-3	N.S.	6.64±0.17	-8	N.S.	12.65±0.30	5	N.S.
2,6-B	6.33±0.12	4	N.S.	6.74±0.12	-6	N.S.	†13.12±0.18	10	*

Fig. 12 Traces from rapid growth experiments.

A : 10 mM Phosphate buffer, pH 6.0, 25°C.

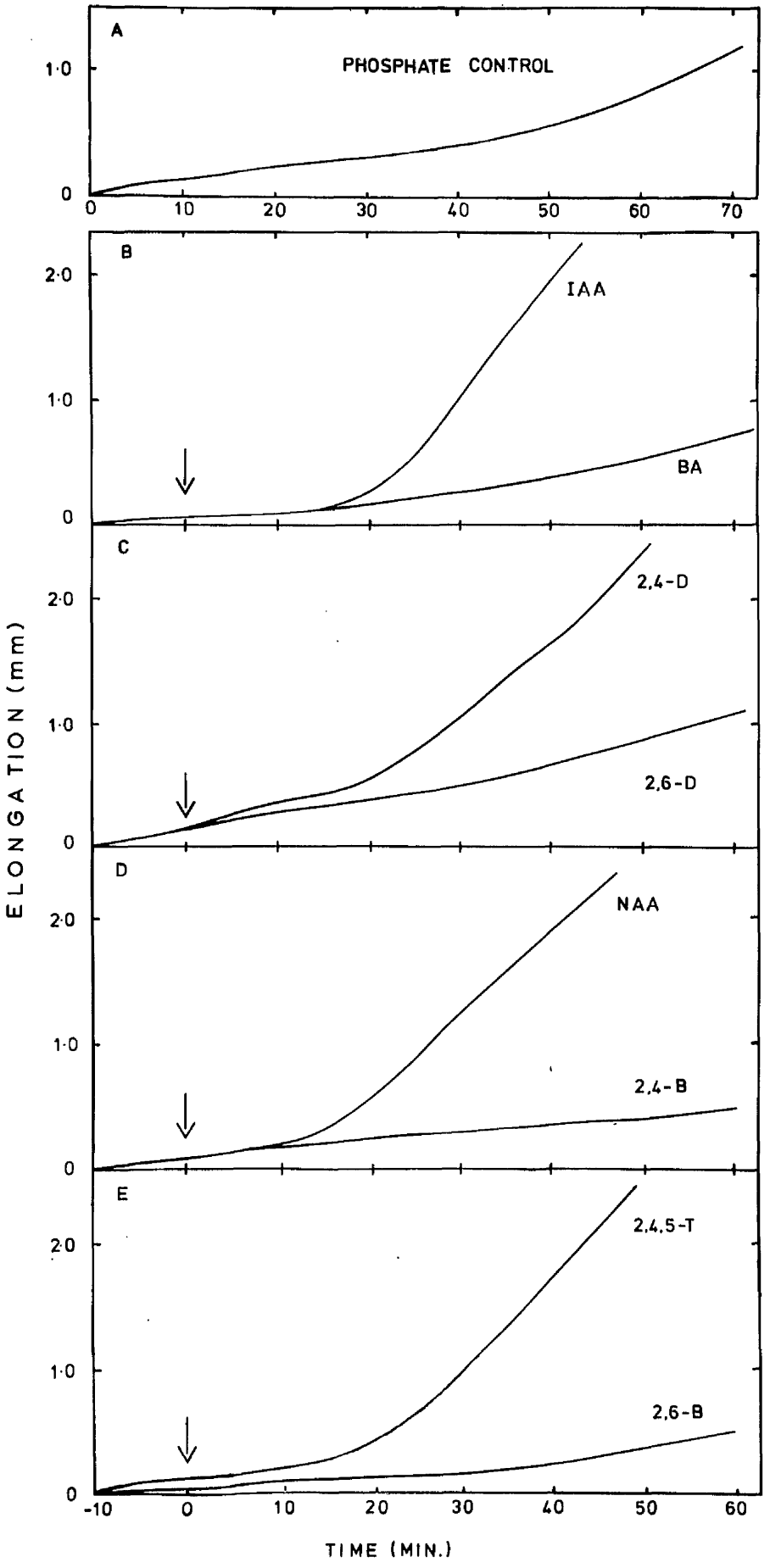
B : 10^{-5} M IAA and 10^{-5} M BA.

C : 10^{-5} M 2,4-D and 10^{-5} M 2,6-D.

D : 10^{-5} M NAA and 10^{-5} M 2,6-B.

E : 10^{-5} M 2,4,5-T and 10^{-5} M 2,4-B.

Arrows indicate time of commencement of analogue treatment.


A
PHOSPHATE CONTROL
B
IAA
BA
C
2,4-D
2,6-D
D
NAA
2,4-B
E
2,4,5-T
2,6-B
ELONGATION (mm)
TIME (MIN.)
0
1·0
2·0
-10
10
20
30
40
50
60
70

An endogenous rate of growth is first measured with the segments bathed in buffer. After application of an active test compound, this endogenous rate is maintained for some time and then an increased growth rate is observed, which gradually increases to a maximum. The lines of the endogenous growth and the promoted growth are extrapolated to meet at the point, x. The time between t = 0 and t = x is known as the "latent period". "Promoted rate of growth" is the maximum growth rate obtained, minus the endogenous rate.

When the coleoptile segments are left in 10 mM phosphate buffer, pH 6.0 at 25°C, an endogenous rate of growth which varies between experiments from approximately 6 μm per min to 20 μm per min, is observed (mean endogenous rate = 14.8 ± 0.8 μm per min). After about 55 min, the growth rate in phosphate buffer increases to approximately 40 μm per min (Fig. 12A). This may represent regeneration of the physiological tips of the coleoptile segments (Evans, 1973). In order to avoid this, segments were not preincubated for longer than necessary, and experiments were completed within 1 hour.

Fig. 12 B,C,D and E , show that IAA, NAA, 2,4-D, and 2,4,5-T all promote rapid elongation of the coleoptile segments. BA, 2,6-D, 2,4-B and 2,6-B are without effect, although the "phosphate effect" (Fig.12A) is usually detectable. 2,6-B was also tested at a concentration of 10^{-4} M and still showed no activity. From Table 10 , it can be seen that IAA gives the largest promoted rate of growth of 81 μm per min, with a latent period of 14.5 min. These data are consistent with published results for oat coleoptiles (Ray and Ruesink, 1962; Philipson et al., 1973) and are also in general agreement with the

Table 10 Results of rapid growth experiments.

Numbers in brackets represent the number of experiments performed, and the data show the mean $\pm$ S.E. of those experiments. Compounds were tested at 10^{-5} M.

† 2,6-B was also tested at 10^{-4} M, the same result being obtained.

Table 10

COMPOUND	LATENT PERIOD (min)	PROMOTED RATE OF GROWTH (μm per min)
IAA (8)	14.5 ± 1.5	80.8 ± 6.7
NAA (9)	16.6 ± 0.7	58.3 ± 8.3
2,4-D (4)	20.0 ± 1.5	51.7 ± 2.3
2,6-D (5)	NO ACTIVITY	NO ACTIVITY
2,4,5-T (5)	20.6 ± 2.2	57.9 ± 3.3
BA (3)	NO ACTIVITY	NO ACTIVITY
2,4-B (4)	NO ACTIVITY	NO ACTIVITY
2,6-B† (5)	NO ACTIVITY	NO ACTIVITY

previous determinations of the compounds as 'active' or 'inactive' (Table 8). 2,6-B is reported to have slight auxin activity (Jönsson, 1961) but, in the assays used so far, has given a positive result only in the pea internode test.

(2) Competition Experiments - The Ability of Auxins and Auxin Analogues to Compete for the NAA Binding Sites

Since radioactive auxins other than NAA show poor binding activity (Table 7), and only a limited range of radioactive analogues is available, it was not possible to test the auxin-specificity of binding directly. An indirect method was chosen which used the principles, developed in enzyme-kinetics studies, for examining the effect of enzyme inhibitors (Dixon and Webb, 1958). The procedure was to analyse the binding of NAA-^{14}C (over the usual concentration range) to a membrane fraction, in the presence and absence of a fixed concentration of the auxin analogue under investigation. The data were calculated from a double-reciprocal plot ($\frac{1}{B}$ vs. $\frac{1}{F}$), computed by a weighted regression analysis (Wilkinson, 1961).

In the case of enzyme-inhibitor interactions, a range of interpretations of the data has been defined (Dixon and Webb, 1958). However, in the present study, only three situations have been distinguished:

(a) "Competitive"

(b) "Not-competitive"

(c) "No competition".

The graphical equivalents of these interactions are demonstrated in Fig. 14.

Fig. 14 Graphical representation of the possible interactions between NAA and an auxin analogue. Graphs are simplified by considering one binding site only.

A : Competitive interaction.

B : Not-competitive interaction.

C : No competition.

——— - auxin analogue.

------- + auxin analogue.

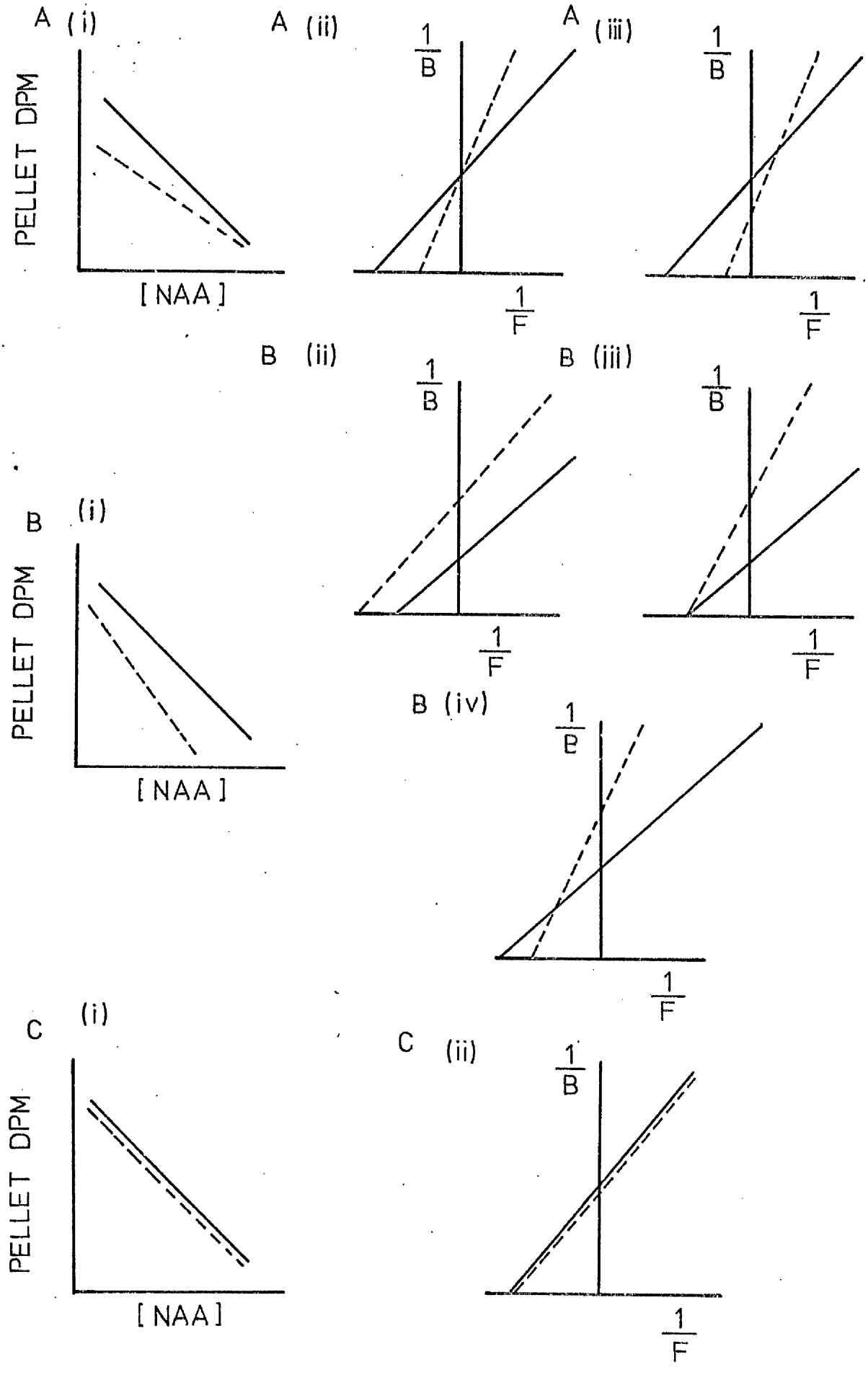

A (i)
PELLET DPM
[NAA]
A (ii)
1/B
1/F
A (iii)
1/B
1/F
B (ii)
1/B
1/F
B (iii)
1/B
1/F
B (i)
PELLET DPM
[NAA]
B (iv)
1/B
1/F
C (i)
PELLET DPM
[NAA]
C (ii)
1/B
1/F

Fig. 14, A (ii) shows the double-reciprocal plot for a purely competitive interaction. The two lines, in the presence and absence of the compound, cross the $\frac{1}{B}$ -axis at exactly the same point yielding a common n-value. The result illustrated in Fig. 14, A (iii) is often obtained (referred to as the "crossover" effect), where the two lines intersect in the positive quadrant of the graph, rather than on the $\frac{1}{B}$ -axis. There is no kinetic explanation other than competitive for this situation, which probably arises because non-specific binding is not subtracted from the data. When this is done, the "crossover" (C.O.) effect is reduced. However, this procedure was considered undesirable since it appears to distort the kinetic analysis (particularly in mesocotyl experiments described later). Furthermore, no genuine plateau value is ever achieved at practical concentration limits and hence the selection of a non-specific binding level becomes an arbitrary process.

Fig. 14,B shows the situation described as "not-competitive". An interaction in the binding process does occur, but it is not competitive. The double-reciprocal plot may have a variety of forms (ii - iv), in all of which the n-values in the presence and absence of analogue, should be significantly different. In the "no competition" situation (Fig. 14, C), the lines in the presence and absence of the auxin analogue are superimposable.

Figs. 15-24 show representative examples of the interactions which each of the selected compounds has with the NAA binding sites. The figures present the ordinary binding curve (A) and the double-reciprocal plots (B and C) for the high and lower affinity sites (referred to as site 1

and site 2 respectively). For interactions which show "no competition", double-reciprocal plots were not performed since the result is obvious from inspection of the ordinary binding curve. For competitive interactions, Ki values were calculated.

It is possible to obtain statistical evidence that an interaction is "competitive" by performing a 't'-test on the n-values obtained from the weighted regression analysis. These 't'-tests are shown on the corresponding figure legends. In the "competitive" case, the n-values should be not significantly different. (An exception to this is where there is a marked "crossover" effect, (see Fig. 19, B) where, although the interaction is obviously competitive, the n-values are significantly different).

Table 11 summarises the results from a number of experiments performed with each compound. For site 1, IAA (Fig. 15), 2,6-D (Fig. 17), 2,4,5-T (Fig. 18), 2-CPIBA (Fig. 19), BA (Fig. 20), 2,6-B (Fig. 22), TIBA (Fig. 23) and NPA (Fig. 24) all have unanimously "competitive" interactions. 2,4-D (Fig. 16) and 2,4-B (Fig. 22) show some evidence for "competitive" and some evidence for "not-competitive" interactions. For site 2, however, 2,6-D, BA and 2,4-B show either "no competition" or "not-competitive" interactions whereas IAA, 2,4-D, 2,4,5-T, 2-CPIBA, TIBA and NPA are all competitive.

Thus, it seems that the compounds generally believed to be inactive (2,6-D, BA and 2,4-B), are able to compete for site 1 but not for site 2. The active auxins (IAA, 2,4-D and 2,4,5-T), the antiauxin (2-CPIBA), and the auxin-transport inhibitors (TIBA and NPA), all compete for site 2 and generally for site 1 also (the only exception being the

variable behaviour of 2,4-D). 2,6-B gives variable results for site 2 but it does show some evidence for competitive behaviour, whereas 2,4-B shows no such evidence. It is important to note here that the Ki-values obtained for the IAA interaction (2.5×10^{-6} M for site 1 and 7.3×10^{-6} M for site 2) are of the same order as the K-values obtained from direct binding studies (1.7×10^{-6} M for site 1 and 5.8×10^{-6} M for site 2, Fig. 10).

In conclusion, it appears that site 2 shows the binding specificity compatible with the expected properties of an auxin-receptor site, in that only active auxins, auxin antagonists or auxin transport inhibitors interact competitively. Binding to site 1, on the other hand, is less specific.

Fig. 15 IAA competition for NAA-^{14}C binding sites in a membrane fraction from Zea coleoptiles.

A. Binding data.

Each point represents the mean of three replicates.

B. Double-reciprocal plot of site 1 data.

Each point represents the mean of three replicates, and the lines are computed by a weighted regression analysis through all the data.

Interaction = competitive, Ki = 4.9×10^{-6}M.

C. Double-reciprocal plot of site 2 data.

Calculated as for site 1.

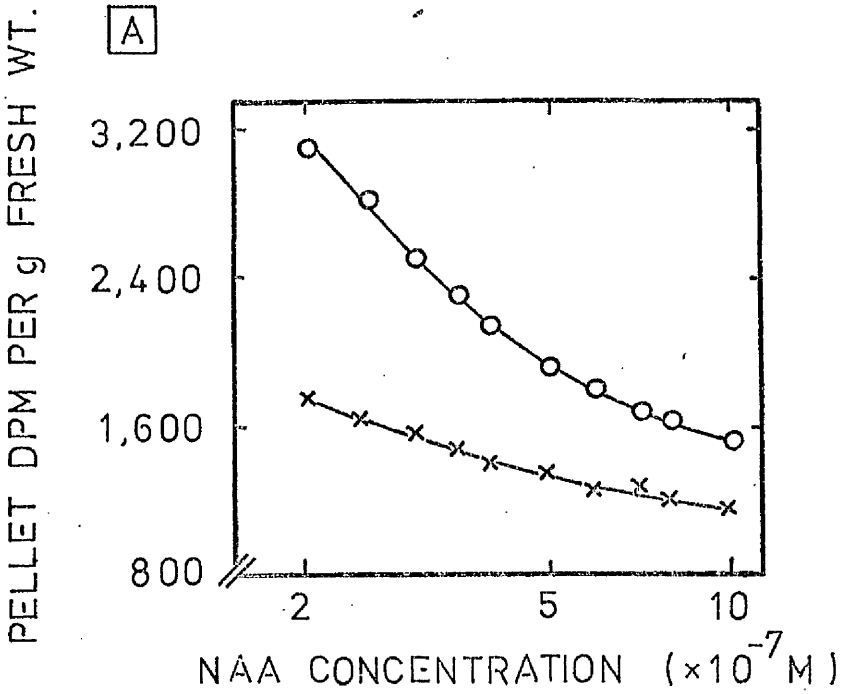
Interaction = competitive, Ki = 16.1×10^{-6}M.

O——O NAA only

×——× + 8×10^{-6} M IAA.

Statistical Analysis $n \pm$ S.E. ($\times 10^{-11}$ moles per assay) is calculated by the weighted regression analysis.

SITE 1				SITE 2			
n(NAA)	n(IAA)	t	Signif.	n(NAA)	n(IAA)	t	Signif.
3.833 ± 0.126	4.093 ± 0.402	0.616	N.S.	10.690 ± 0.779	10.430 ± 1.927	0.125	N.S.


A
PELLET DPM PER g FRESH WT.
3,200
2,400
1,600
800
2
5
10
NAA CONCENTRATION (×10⁻⁷M)

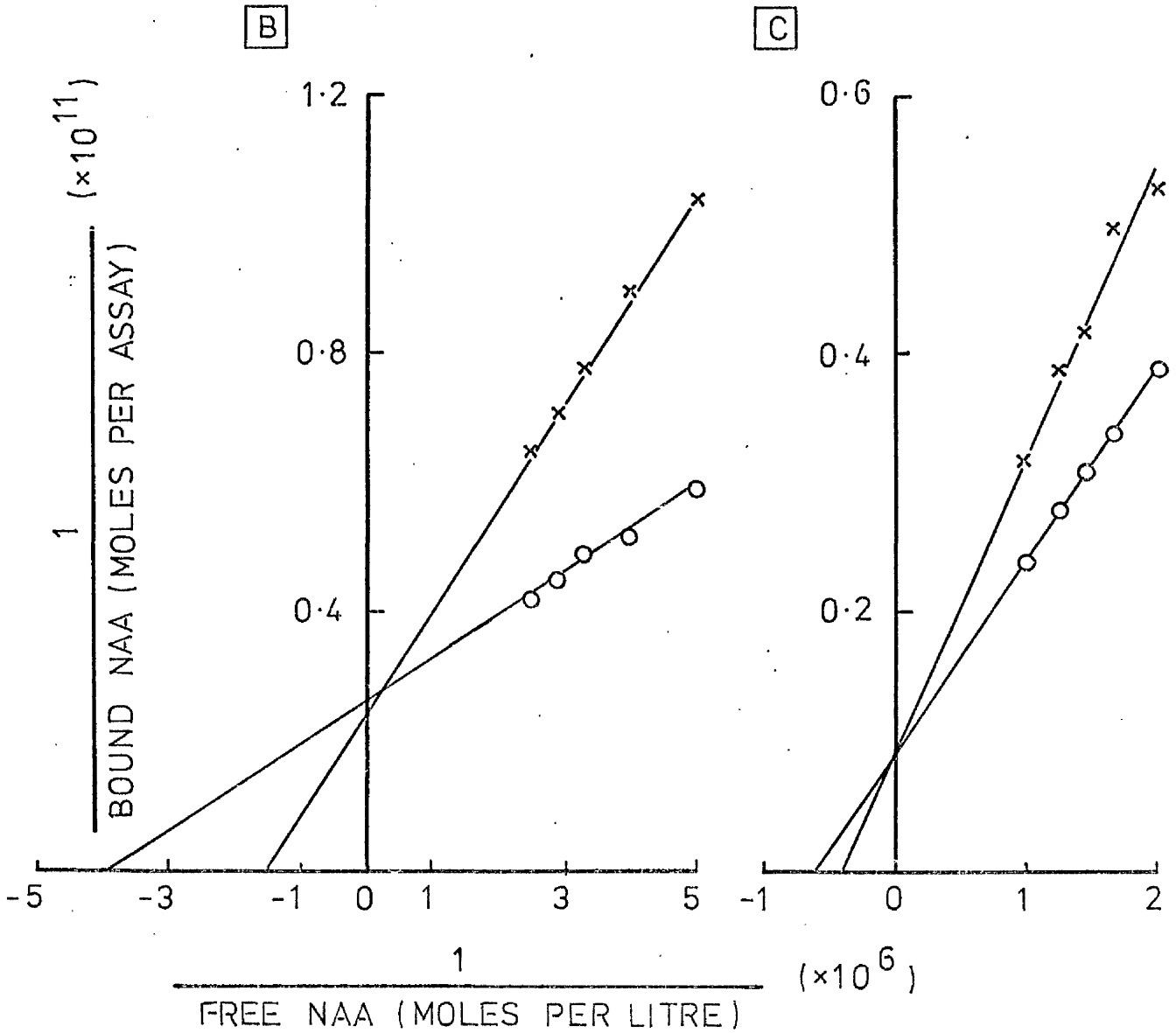

B
C
1 / BOUND NAA (MOLES PER ASSAY) (×10¹¹)
1·2
0·8
0·4
0·6
0·4
0·2
-5
-3
-1
0
1
3
5
-1
0
1
2
1 / FREE NAA (MOLES PER LITRE) (×10⁶)

Fig. 16 2,4-D competition for NAA-^{14}C binding sites in a membrane fraction from Zea coleoptiles.

A. Binding data.

Each point represents the mean of three replicates.

B. Double-reciprocal plot of site 1 data.

Each point represents the mean of three replicates and the lines are computed by a weighted regression analysis through all the data.

Interaction = Not-competitive.

C. Double-reciprocal plot of site 2 data.

Calculated as for site 1.

Interaction = competitive, Ki = 2.5×10^{-5}M.

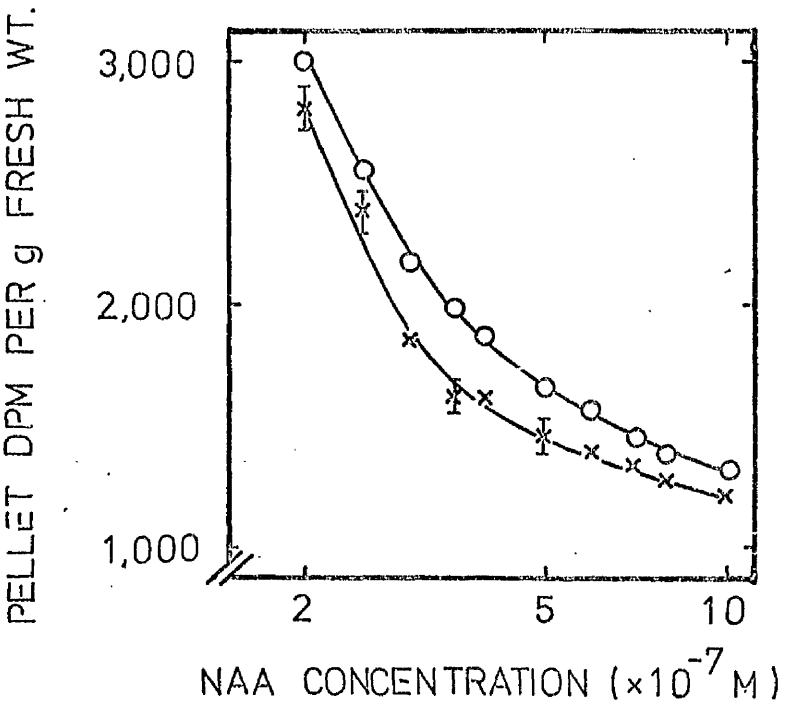
O———O NAA only

×———× + 10^{-5} M 2,4-D.

Statistical Analysis $n \pm$ S.E. (x 10^{-11} moles per assay) is calculated by the weighted regression analysis.

SITE 1				SITE 2			
n(NAA)	n(2,4-D)	t	Signif.	n(NAA)	n(2,4-D)	t	Signif.
2.014 ± 0.112	1.281 ± 0.106	4.729	**	7.458 ± 0.910	8.746 ± 1.107	0.899	N.S.

A


PELLET DPM PER g FRESH WT.
3,000
2,000
1,000
2
5
10
NAA CONCENTRATION (×10⁻⁷ M)

B

C

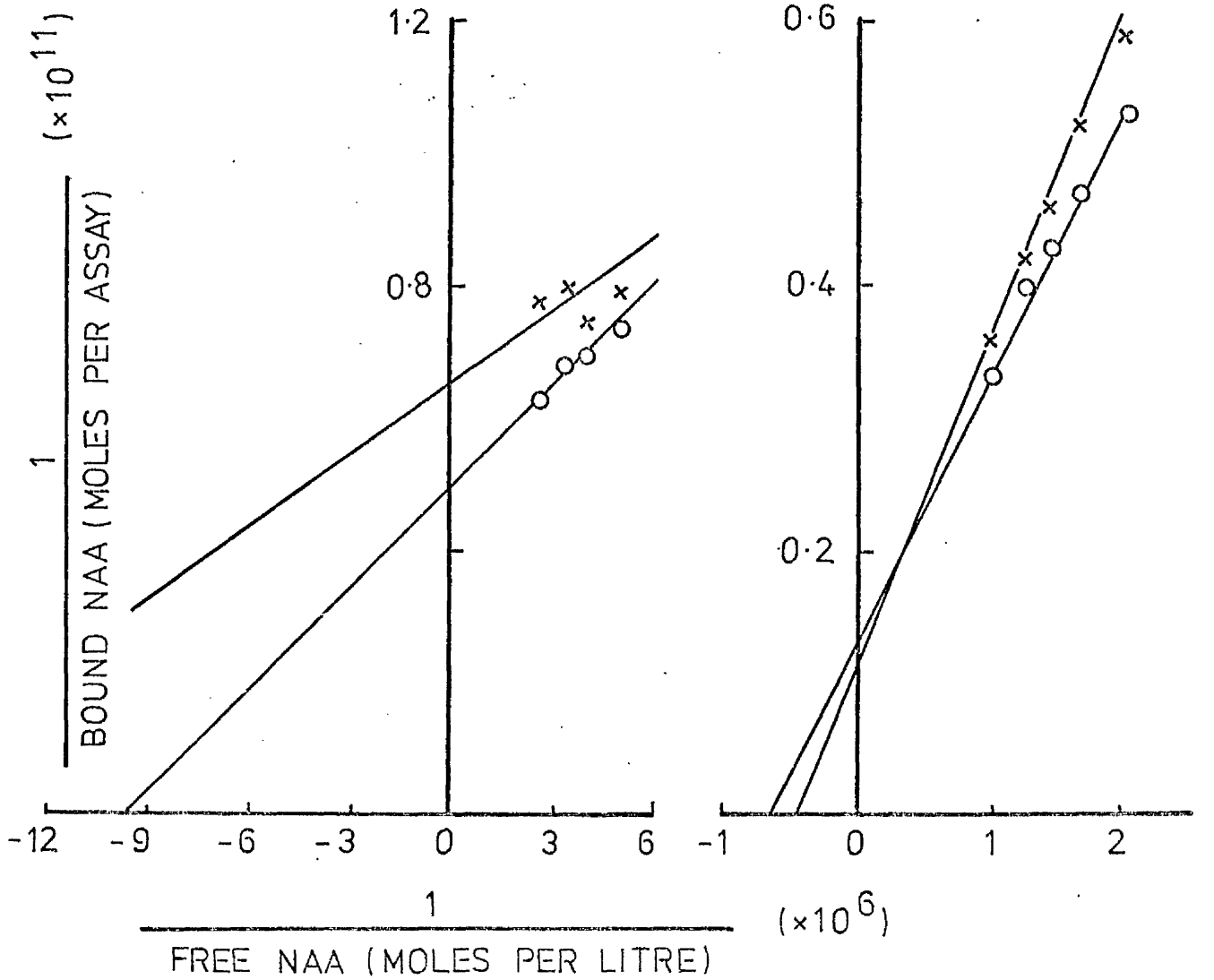

1/BOUND NAA (MOLES PER ASSAY) (×10¹¹)
1·2
0·8
-12
-9
-6
-3
0
3
6
0·6
0·4
0·2
-1
0
1
2
1/FREE NAA (MOLES PER LITRE) (×10⁶)

Fig. 17 2,6-D competition for NAA-^{14}C binding sites in a membrane fraction from Zea coleoptiles.

A. Binding data.

Each point represents the mean of three replicates.

Binding data shows for site 2,

Interaction = No competition.

B. Double-reciprocal plot of site 1 data.

Each point represents the mean of three replicates and the lines are computed by a weighted regression analysis through all the data.

Interaction = competitive, Ki = 1.1 x 10^{-5}M.

O——O NAA only

×——× + 10^{-5} M 2,6-D.

Statistical Analysis n ± S.E. (x 10^{-11} moles per assay) is calculated by the weighted regression analysis.

SITE 1			
n(NAA)	n(2,6-D)	t	Signif.
1.023±0.108	1.136±0.123	0.689	N.S.

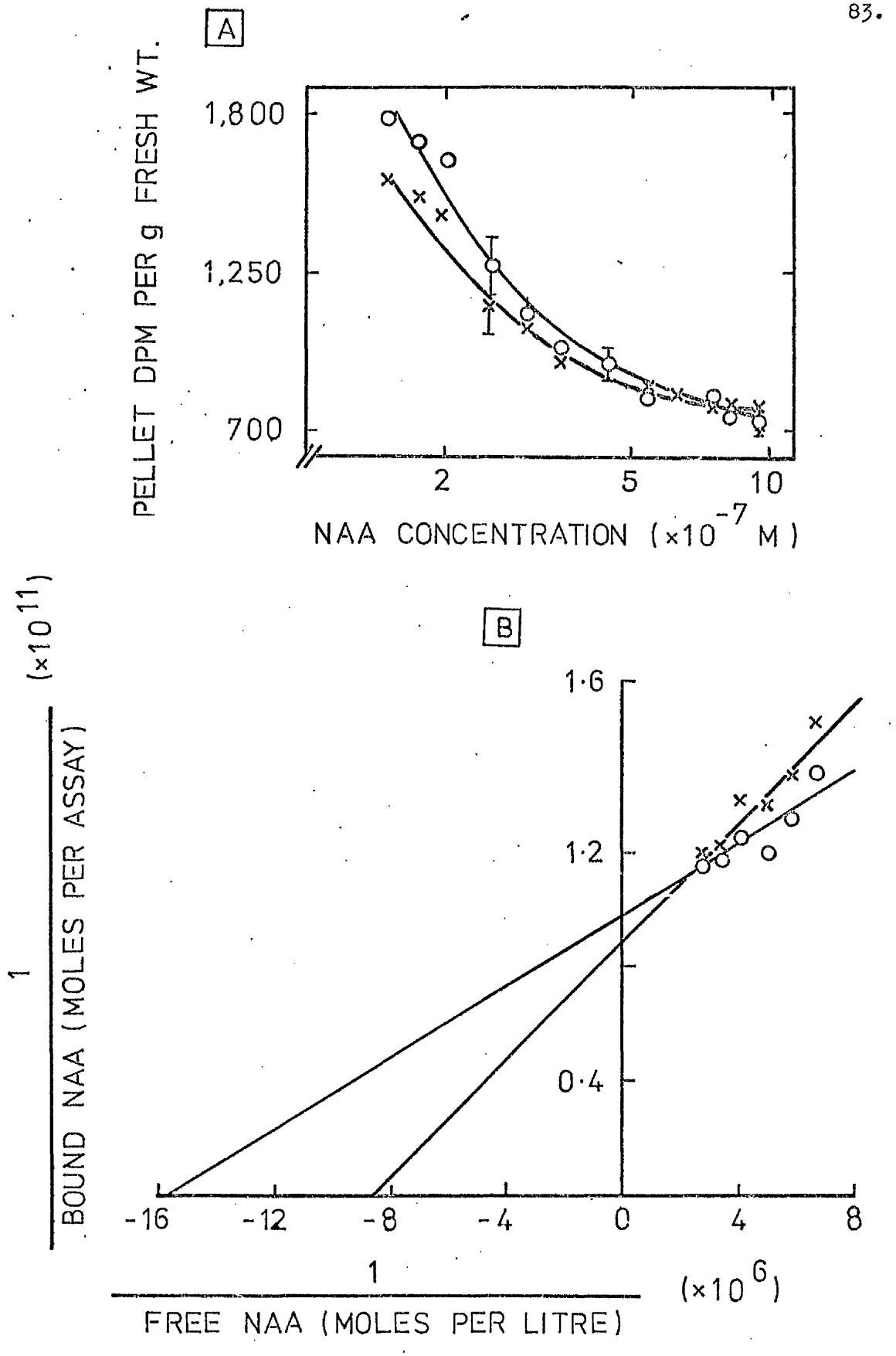

A
PELLET DPM PER g FRESH WT.
1,800
1,250
700
2
5
10
NAA CONCENTRATION (×10⁻⁷ M)
B
1 / BOUND NAA (MOLES PER ASSAY) (×10¹¹)
1·6
1·2
0·4
-16
-12
-8
-4
0
4
8
1 / FREE NAA (MOLES PER LITRE) (×10⁶)

Fig. 18 2,4,5-T competition for NAA-^{14}C binding sites in a membrane fraction from Zea coleoptiles.

A. Binding data.

Each point is the mean of three replicates.

B. Double-reciprocal plot of site 1 data.

Each point represents the mean of three replicates and the lines are computed by a weighted regression analysis through all the data.

Interaction = competitive, Ki = 2.5×10^{-6} M.

C. Double-reciprocal plot of site 2 data.

Calculated as for site 1.

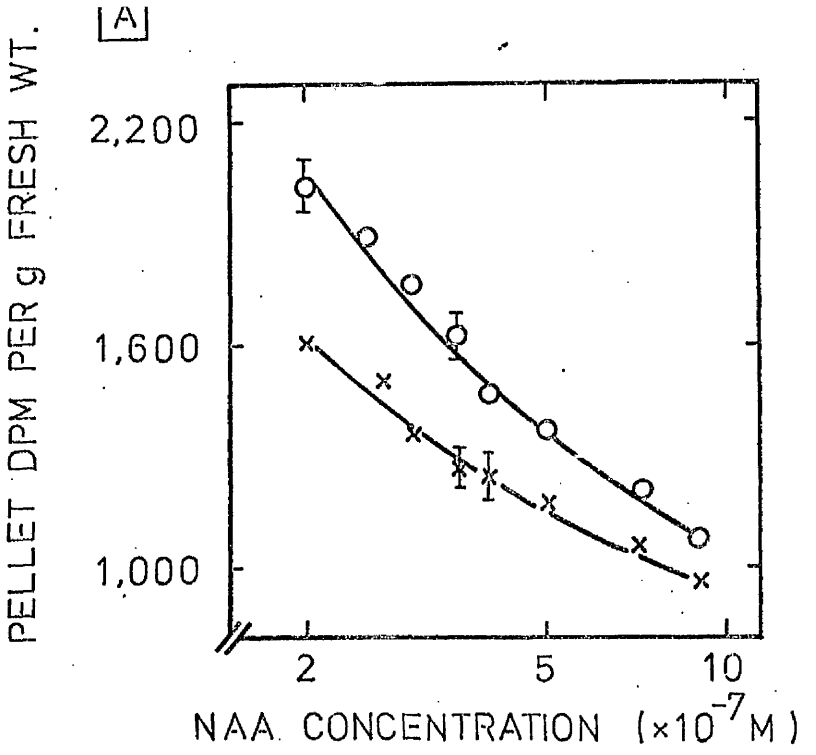
Interaction = competitive, Ki = 9.1×10^{-6} M.

O——O NAA only

×——× + 5×10^{-6} M 2,4,5-T.

Statistical Analysis $n \pm$ S.E. ($\times 10^{-11}$ moles per assay) is calculated by the weighted regression analysis.

SITE 1				SITE 2			
n(NAA)	n(2,4,5-T)	t	Signif.	n(NAA)	n(2,4,5-T)	t	Signif.
3.035 ± 0.198	4.292 ± 1.237	1.003	N.S.	5.248 ± 0.287	5.481 ± 1.015	0.221	N.S.


A
PELLET DPM PER g FRESH WT.
2,200
1,600
1,000
2
5
10
NAA CONCENTRATION (×10⁻⁷M)

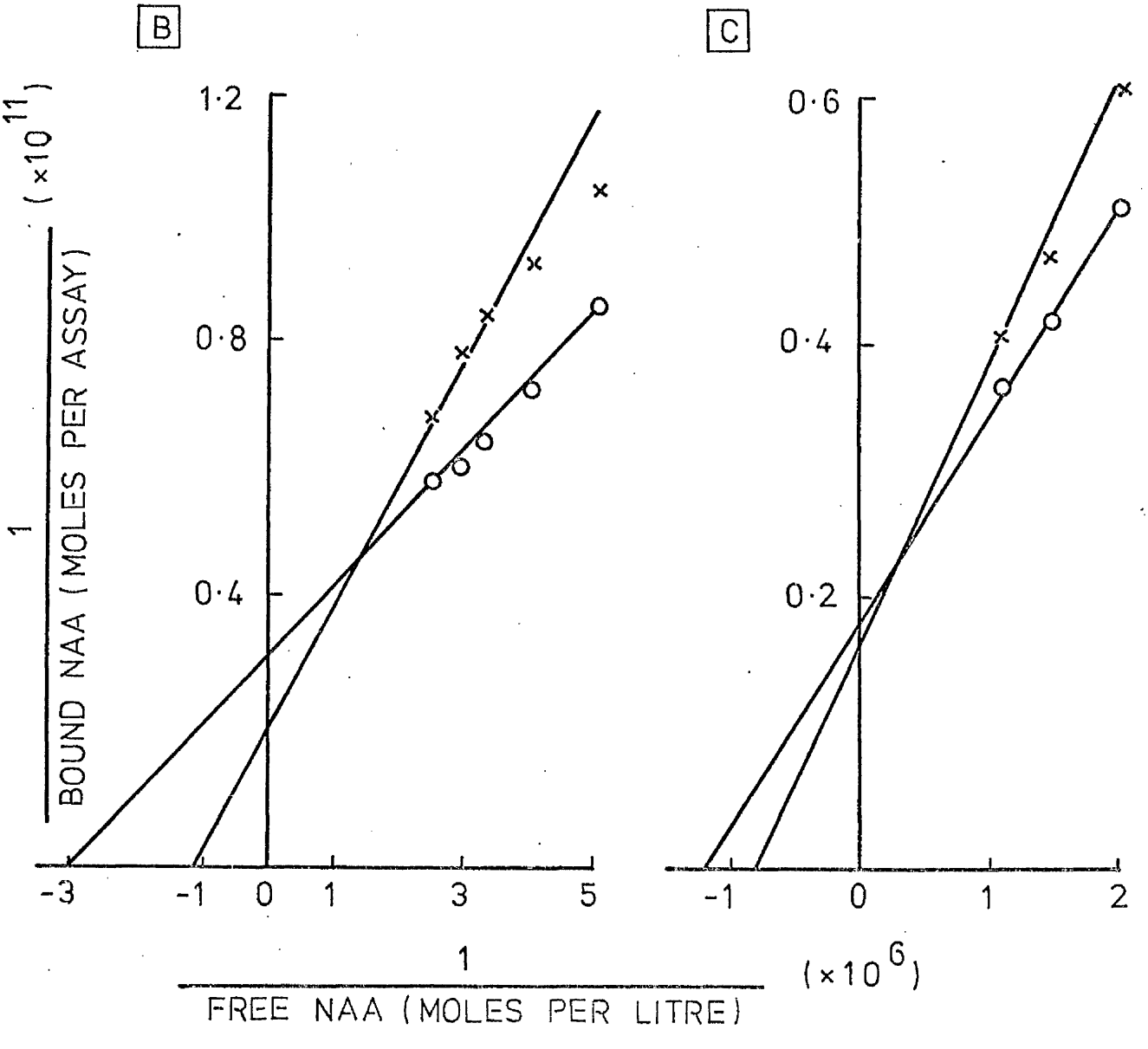

B
C
1·2
0·8
0·4
0·6
0·4
0·2
-3
-1
0
1
3
5
-1
0
1
2
(×10¹¹)
1
BOUND NAA (MOLES PER ASSAY)
(×10⁶)
1
FREE NAA (MOLES PER LITRE)

Fig. 19 2-CPIBA competition for NAA-^{14}C binding sites in a membrane fraction from Zea coleoptiles.

A. Binding data.

Each point represents the mean of three replicates.

B. Double-reciprocal plot of site 1 data.

Each point represents the mean of three replicates, and the lines are computed by a weighted regression analysis through all the data.

Interaction = competitive, Ki = 2.6×10^{-6}M.

C. Double-reciprocal plot of site 2 data.

Calculated as for site 1.

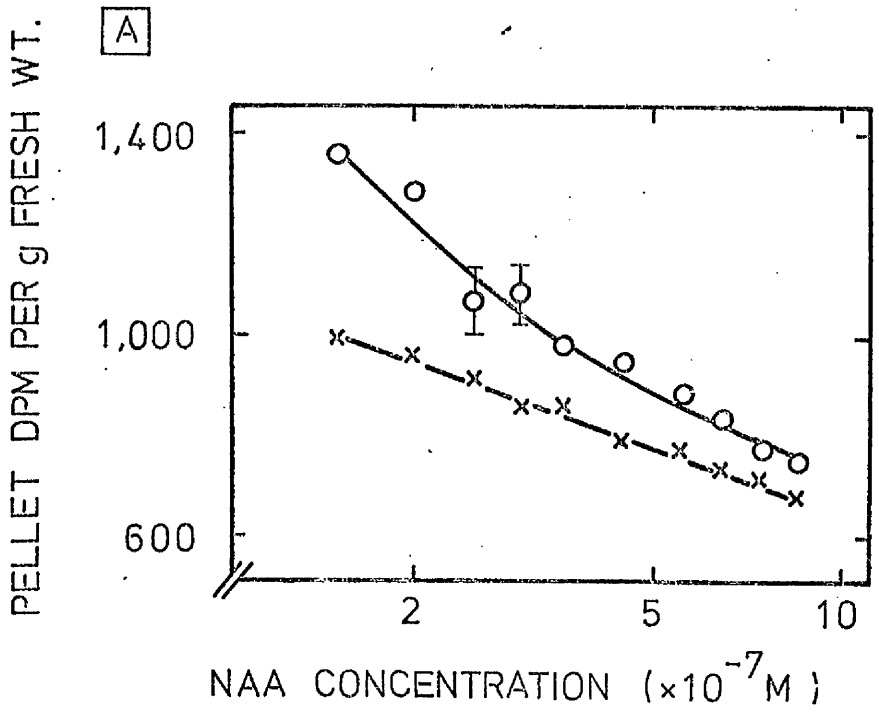
Interaction = competitive, Ki = 6.5×10^{-6}M.

O———O NAA only

×———× + 5×10^{-6} M 2-CPIBA.

Statistical Analysis $n \pm$ S.E. ($\times 10^{-11}$ moles per assay) is calculated by the weighted regression analysis.

SITE 1				SITE 2			
n(NAA)	n(2-CPIBA)	t	Signif.	n(NAA)	n(2-CPIBA)	t	Signif.
2.232 ± 0.299	3.769 ± 0.483	2.706	*(C.C.)	4.397 ± 0.511	5.677 ± 0.720	1.450	N.S.


A
PELLET DPM PER g FRESH WT.
1,400
1,000
600
2
5
10
NAA CONCENTRATION (×10⁻⁷M)

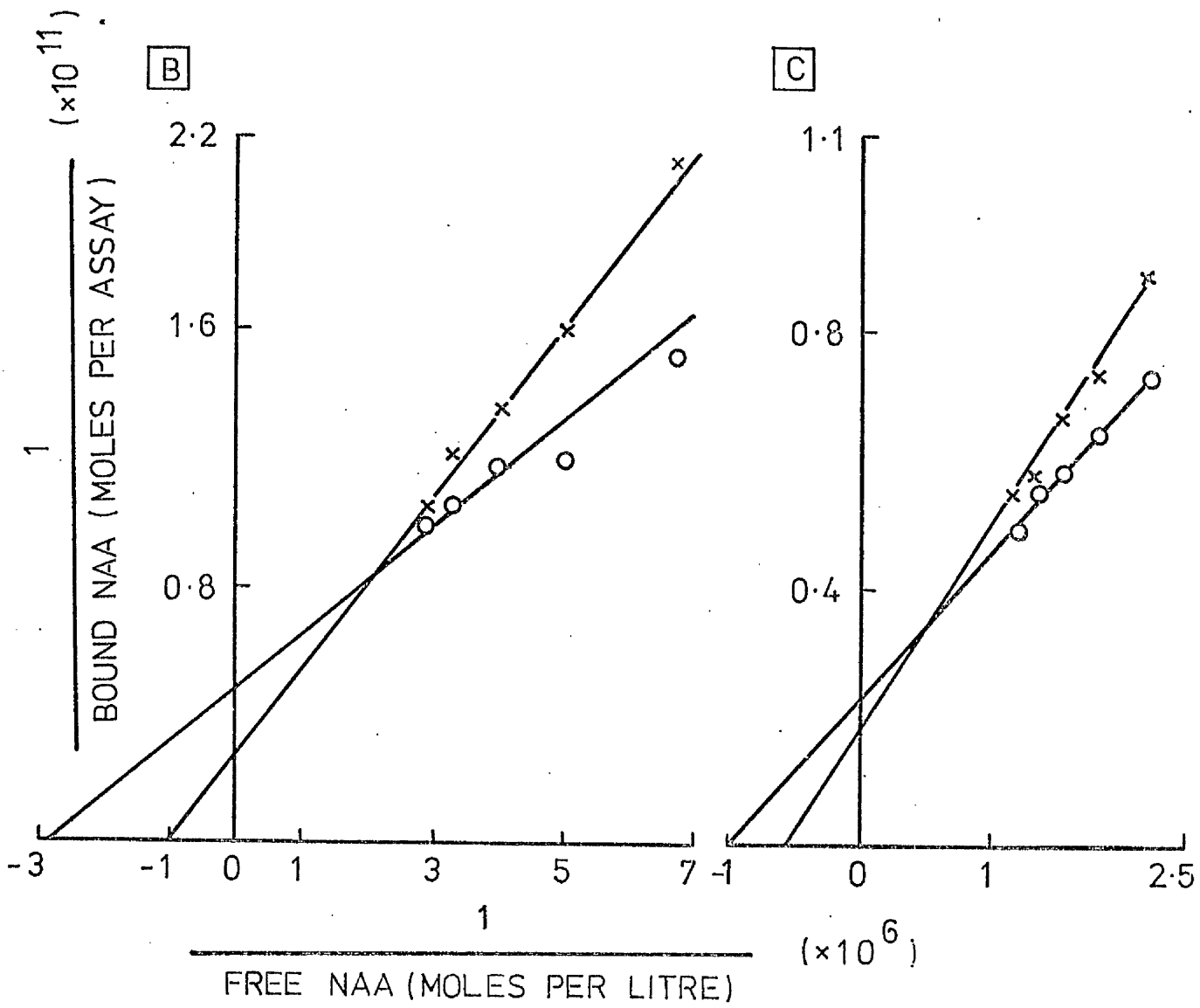

B
C
1 / BOUND NAA (MOLES PER ASSAY) (×10¹¹)
2·2
1·6
0·8
-3
-1
0
1
3
5
7
1·1
0·8
0·4
-1
0
1
2·5
1 / FREE NAA (MOLES PER LITRE) (×10⁶)

Fig. 20 BA competition for NAA-^{14}C binding sites in a membrane fraction from Zea coleoptiles.

A. Binding data.

Each point represents the mean of three replicates.

Binding data shows, for site 2 interaction = No competition.

B. Double-reciprocal plot of site 1 data.

Each point is the mean of three replicates and the lines are computed by a weighted regression analysis through all the data.

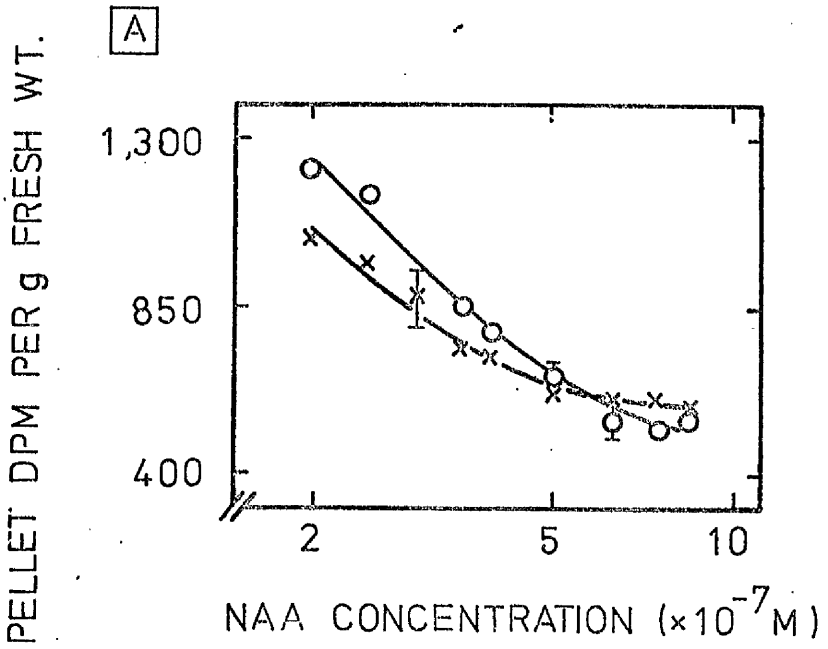
Interaction = competitive, Ki = 6.0×10^{-6}M.

O———O NAA only

×———× + 5×10^{-6} M BA.

Statistical Analysis $n \pm$ S.E. ($\times 10^{-11}$ moles per assay) is calculated by the weighted regression analysis.

SITE 1			
n(NAA)	n(BA)	t	Signif.
1.225 ± 0.086	1.322 ± 0.182	0.485	N.S.


A
PELLET DPM PER g FRESH WT.
1,300
850
400
2
5
10
NAA CONCENTRATION (×10⁻⁷M)

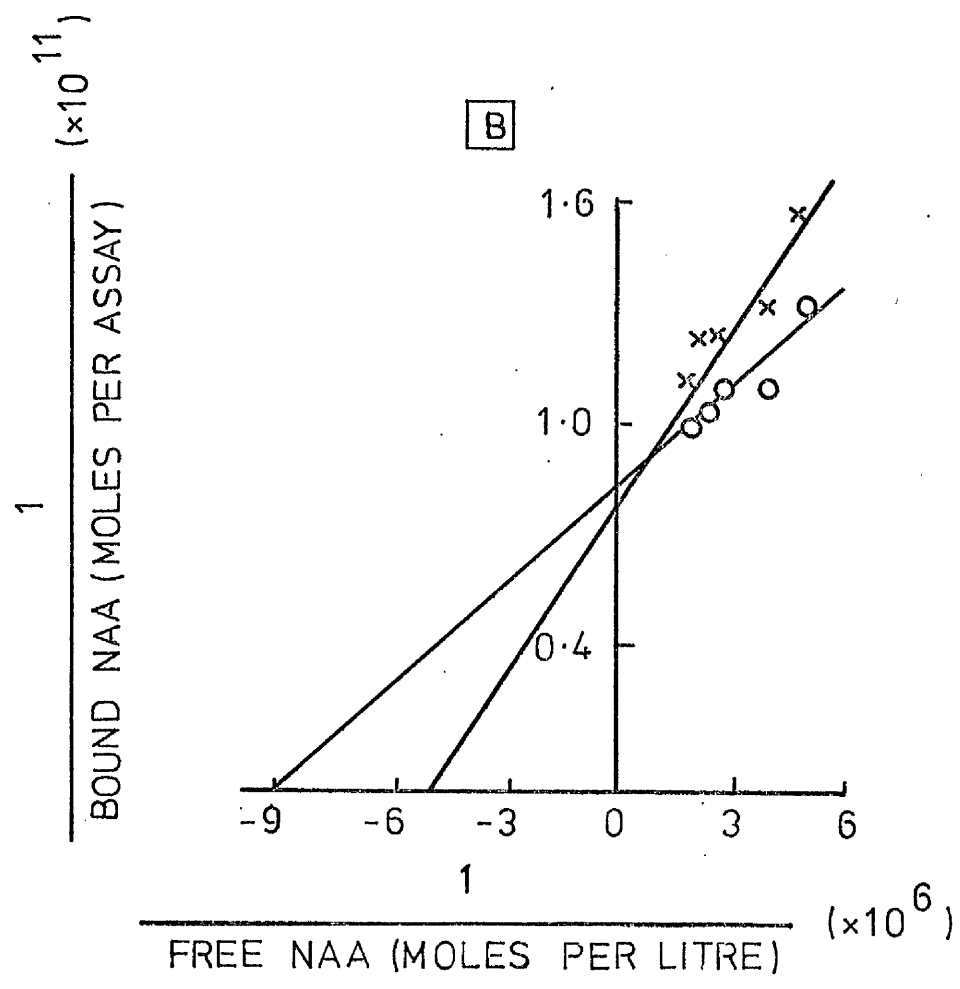

B
1 / BOUND NAA (MOLES PER ASSAY) (×10¹¹)
1·6
1·0
0·4
-9
-6
-3
0
3
6
1 / FREE NAA (MOLES PER LITRE) (×10⁶)

Fig. 21 2,4-B competition for NAA-^{14}C binding site in a membrane fraction from Zea coleoptiles.

A. Binding data.

Each point represents the mean of three replicates.

B. Double-reciprocal plot of site 1 data.

Each point is the mean of three replicates and the lines are computed by a weighted regression analysis through all the data.

Interaction = Not-competitive.

C. Double-reciprocal plot of site 2 data.

Calculated as for site 1.

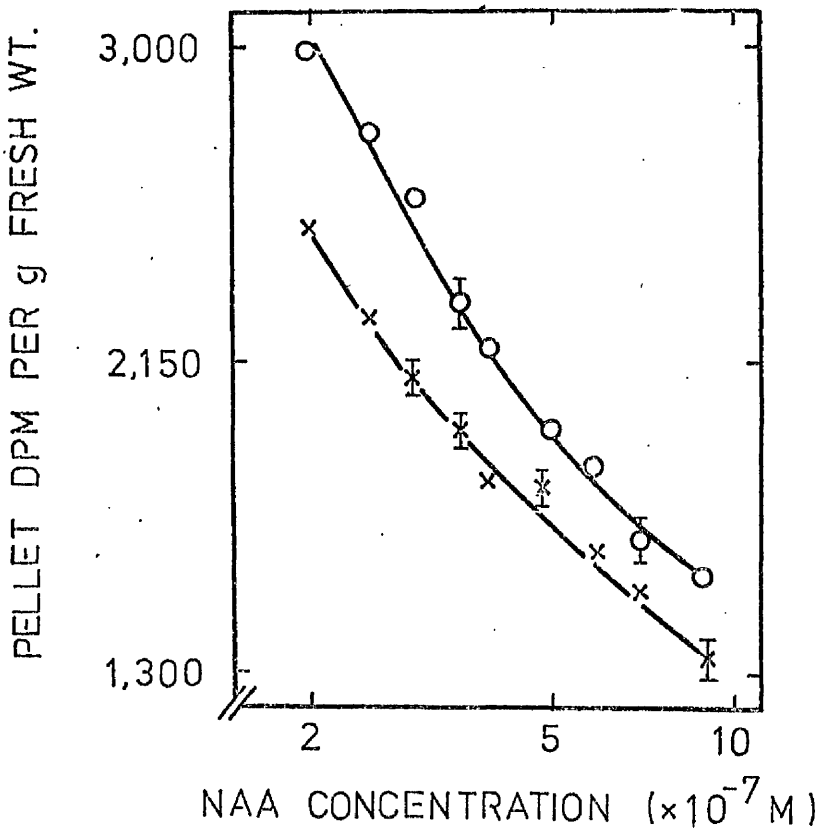
Interaction = Not-competitive.

O——O NAA only

x——x + 5 x 10^{-6} M 2,4-B.

Statistical Analysis $n \pm$ S.E. (x 10^{-11} moles per assay) is calculated by the weighted regression analysis.

SITE 1				SITE 2			
n(NAA)	n(2,4-B)	t	Signif.	n(NAA)	n(2,4-B)	t	Signif.
4.129 ± 0.184	3.401 ± 0.213	2.591	*	7.488 ± 0.472	5.532 ± 0.477	4.337	**


PELLET DPM PER g FRESH WT.
3,000
2,150
1,300
2
5
10
NAA CONCENTRATION (×10⁻⁷ M)

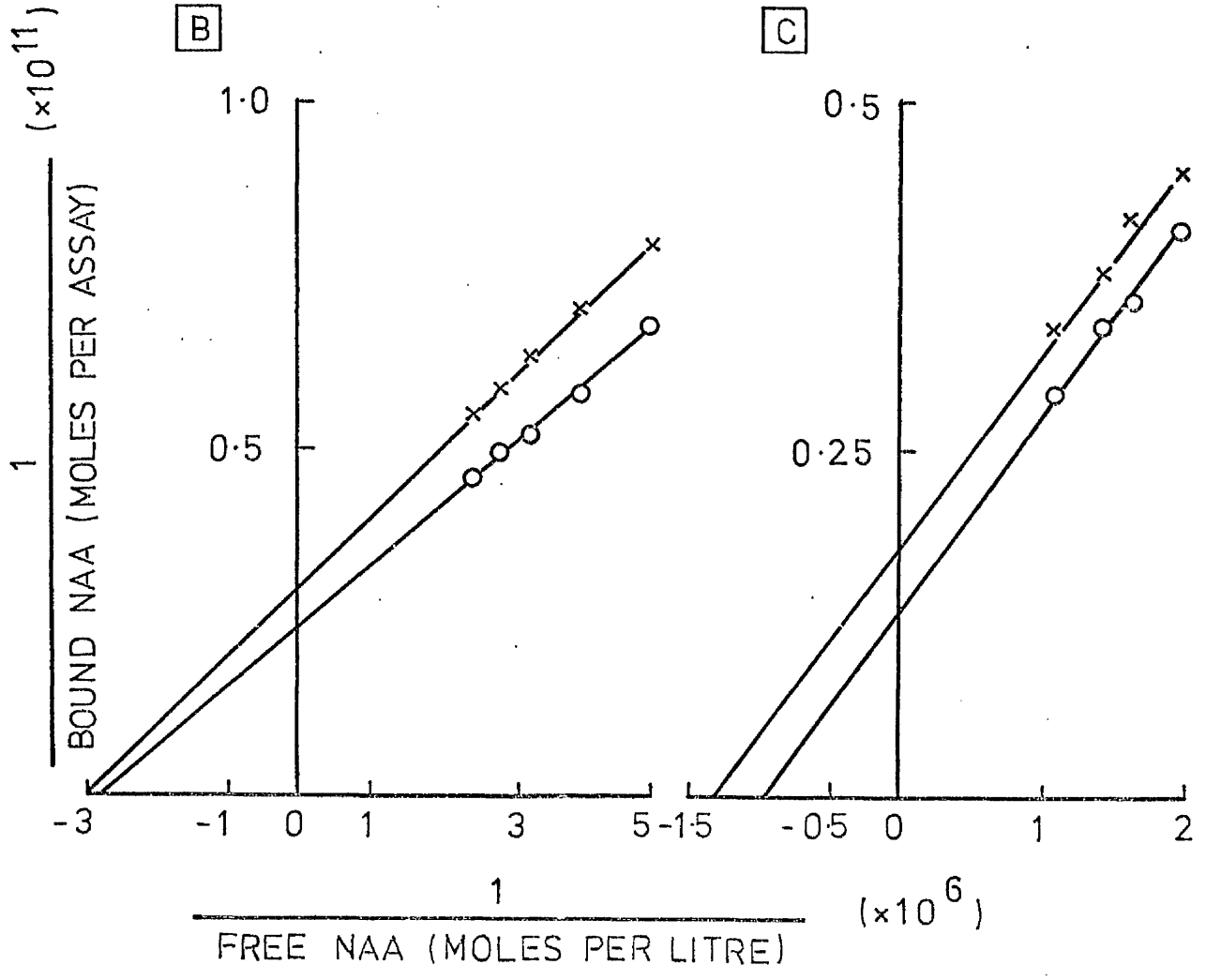

B
C
1/BOUND NAA (MOLES PER ASSAY) (×10¹¹)
1·0
0·5
0·5
0·25
-3
-1
0
1
3
5
-1·5
-0·5
0
1
2
1/FREE NAA (MOLES PER LITRE) (×10⁶)

Fig. 22 2,6-B competition for NAA-^{14}C binding sites in a membrane fraction from Zea coleoptiles.

A. Binding data.

Each point represents the mean of three replicates.

B. Double-reciprocal plot of site 1 data.

Each point is the mean of three replicates and the lines are computed by a weighted regression analysis through all the data.

Interaction = competitive, Ki = 7.9×10^{-5}M.

C. Double-reciprocal plot of site 2 data.

Calculated as for site 1.

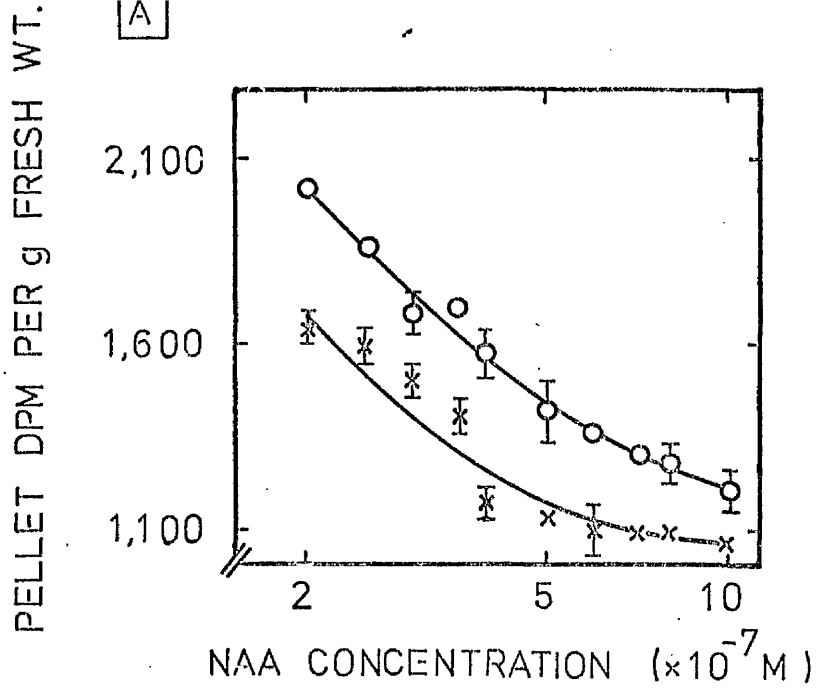
Interaction = competitive, Ki = 7.1×10^{-6}M.

O———O NAA only

x———x + 10^{-5} M 2,6-B.

Statistical Analysis n ± S.E. (x 10^{-11} moles per assay) is calculated by the weighted regression analysis.

SITE 1				SITE 2			
n(NAA)	n(2,6-B)	t	Signif.	n(NAA)	n(2,6-B)	t	Signif.
3.689 ± 0.550	3.395 ± 0.695	1.050	N.S.	9.762 ± 2.354	17.184 ± 4.875	1.371	N.S.


A
PELLET DPM PER g FRESH WT.
2,100
1,600
1,100
2
5
10
NAA CONCENTRATION (×10⁻⁷M)

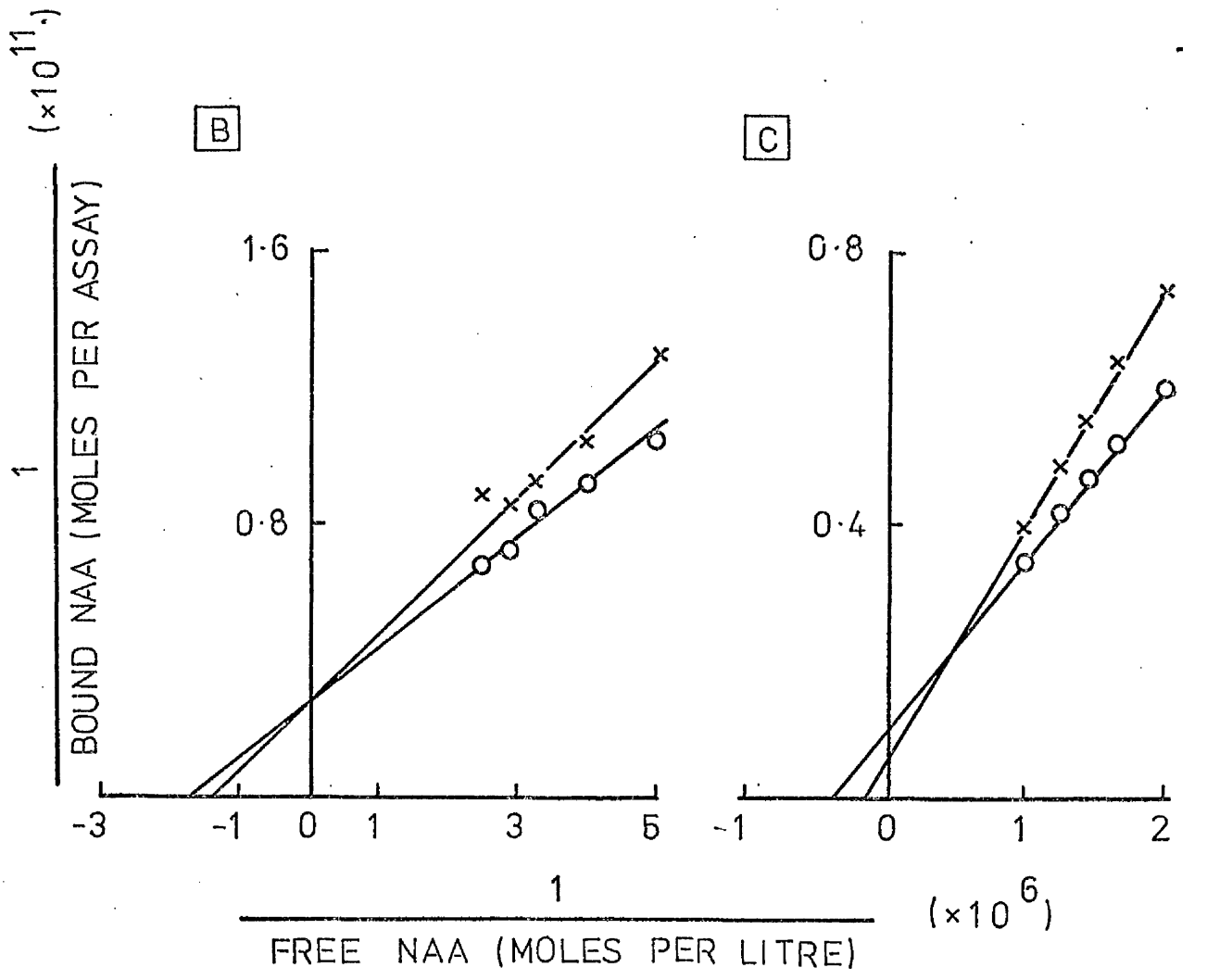

B
C
1 / BOUND NAA (MOLES PER ASSAY) (×10¹¹)
1·6
0·8
-3
-1
0
1
3
5
0·8
0·4
-1
0
1
2
1 / FREE NAA (MOLES PER LITRE) (×10⁶)

Fig. 23 TIBA competition for NAA-^{14}C binding sites in a membrane fraction from Zea coleoptiles.

A. Binding data.

Each point is the mean of three replicates.

B. Double-reciprocal plot of site 1 data.

Each point represents the mean of three replicates and the lines are computed by a weighted regression analysis through all the data.

Interaction = competitive, Ki = 2.8×10^{-6}M.

C. Double-reciprocal plot of site 2 data.

Calculated as for site 1.

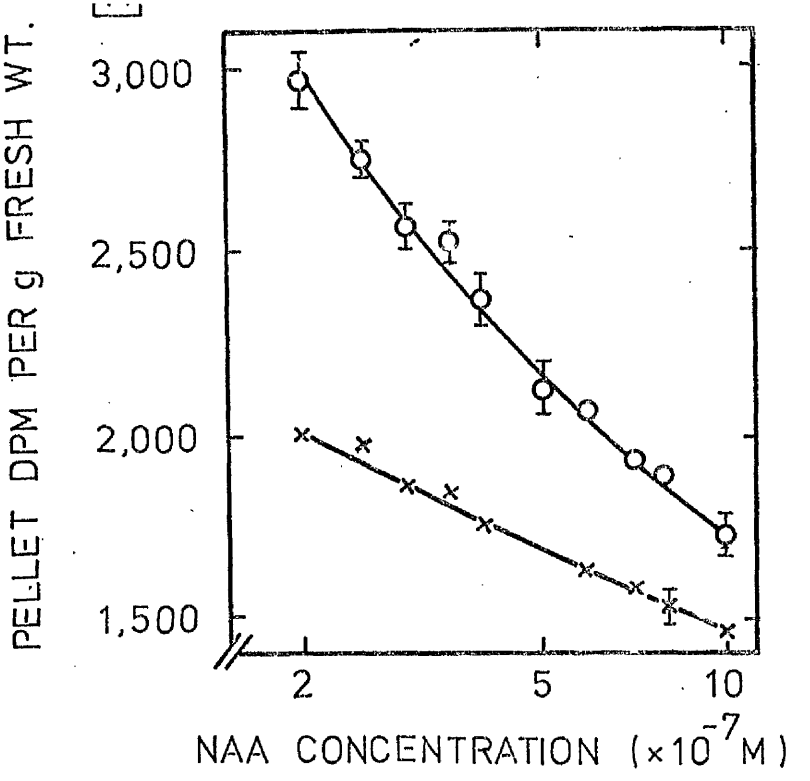
Interaction = competitive, Ki = 3.0×10^{-6}M.

O——O NAA only

×——× + 2×10^{-6} M TIBA.

Statistical Analysis $n \pm$ S.E. ($\times 10^{-11}$ moles per assay) is calculated by the weighted regression analysis.

SITE 1				SITE 2			
n(NAA)	n(TIBA)	t	Signif.	n(NAA)	n(TIBA)	t	Signif.
5.574 ± 0.707	5.932 ± 0.629	0.378	N.S.	11.897 ± 1.354	14.334 ± 2.587	0.833	N.S.


A
PELLET DPM PER g FRESH WT.
3,000
2,500
2,000
1,500
2
5
10
NAA CONCENTRATION (×10⁻⁷M)

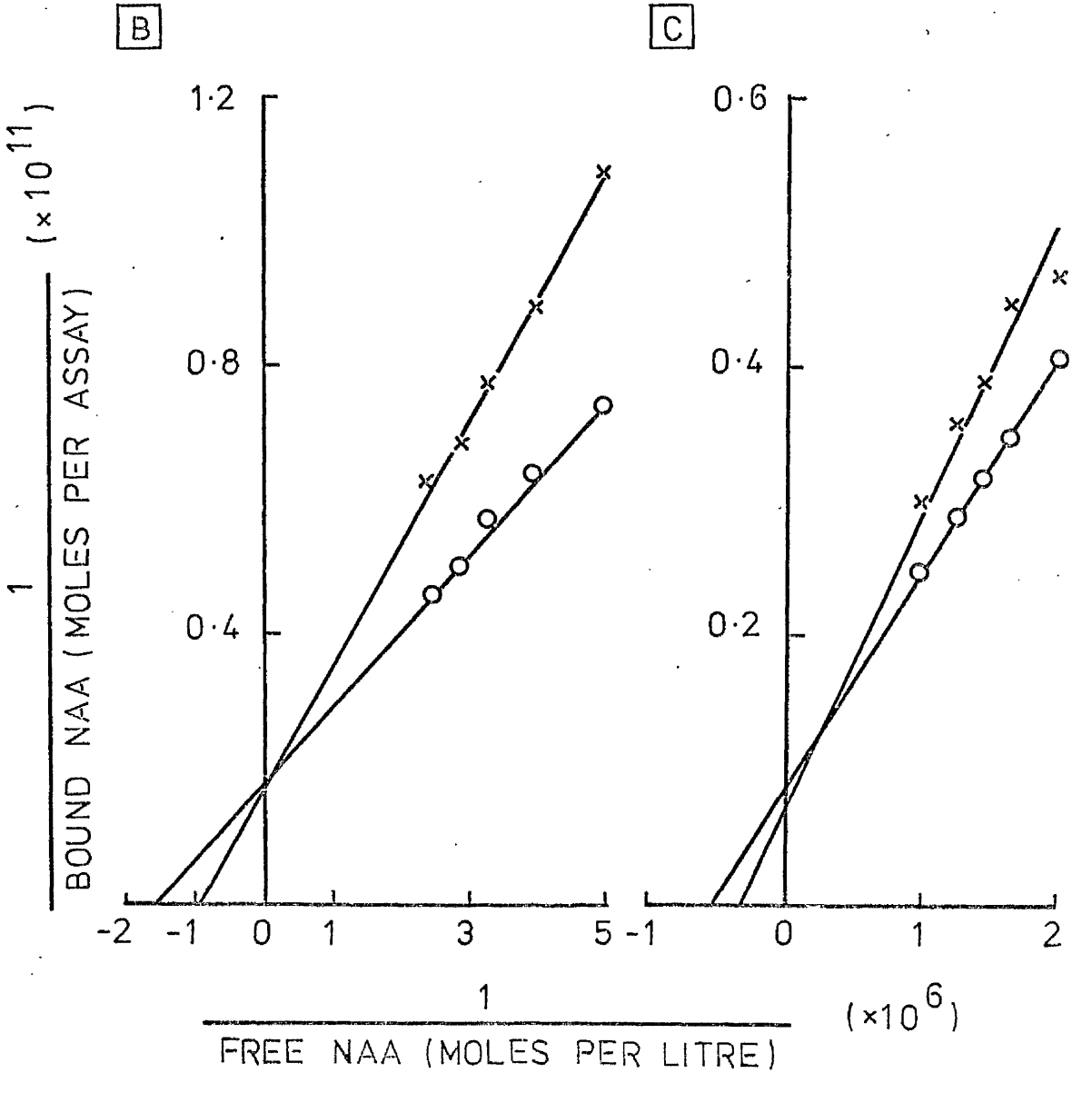

B
C
1·2
0·8
0·4
0·6
0·4
0·2
1 / BOUND NAA (MOLES PER ASSAY) (×10¹¹)
-2
-1
0
1
3
5
-1
0
1
2
1 / FREE NAA (MOLES PER LITRE) (×10⁶)

Fig. 24 NPA competition for NAA-^{14}C binding sites in a membrane fraction from Zea coleoptiles.

A. Binding data.

Each point represents the mean of three replicates.

B. Double-reciprocal plot of site 1 data.

Each point represents the mean of three replicates, and the lines are computed by a weighted regression analysis.

Interaction = competitive, Ki = 3.0×10^{-6}M.

C. Double-reciprocal plot of site 2 data.

Calculated as for site 1.

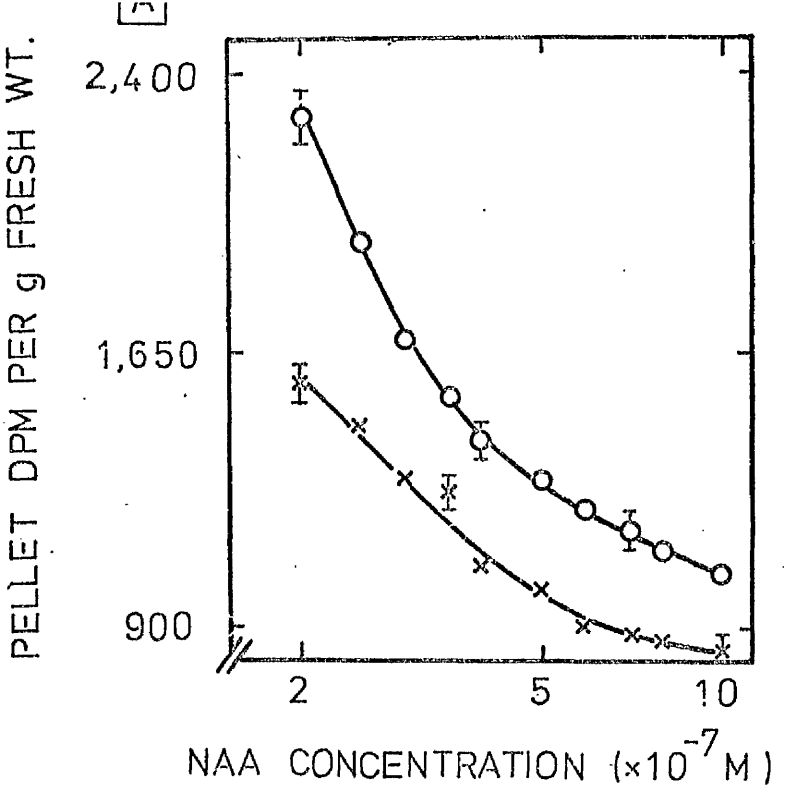
Interaction = competitive, Ki = 2.3×10^{-5}M.

O——O NAA only

x——x + 8.3×10^{-6}M NPA.

Statistical Analysis $n \pm$ S.E. ($\times 10^{-11}$ moles per assay) is calculated by the weighted regression analysis.

SITE 1				SITE 2			
n(NAA)	n(NPA)	t	Signif.	n(NAA)	n(NPA)	t	Signif.
2.223 ± 0.114	3.092 ± 0.416	2.016	N.S.	7.976 ± 0.782	7.692 ± 1.366	0.180	N.S.


A
PELLET DPM PER g FRESH WT.
2,400
1,650
900
2
5
10
NAA CONCENTRATION (×10⁻⁷ M)

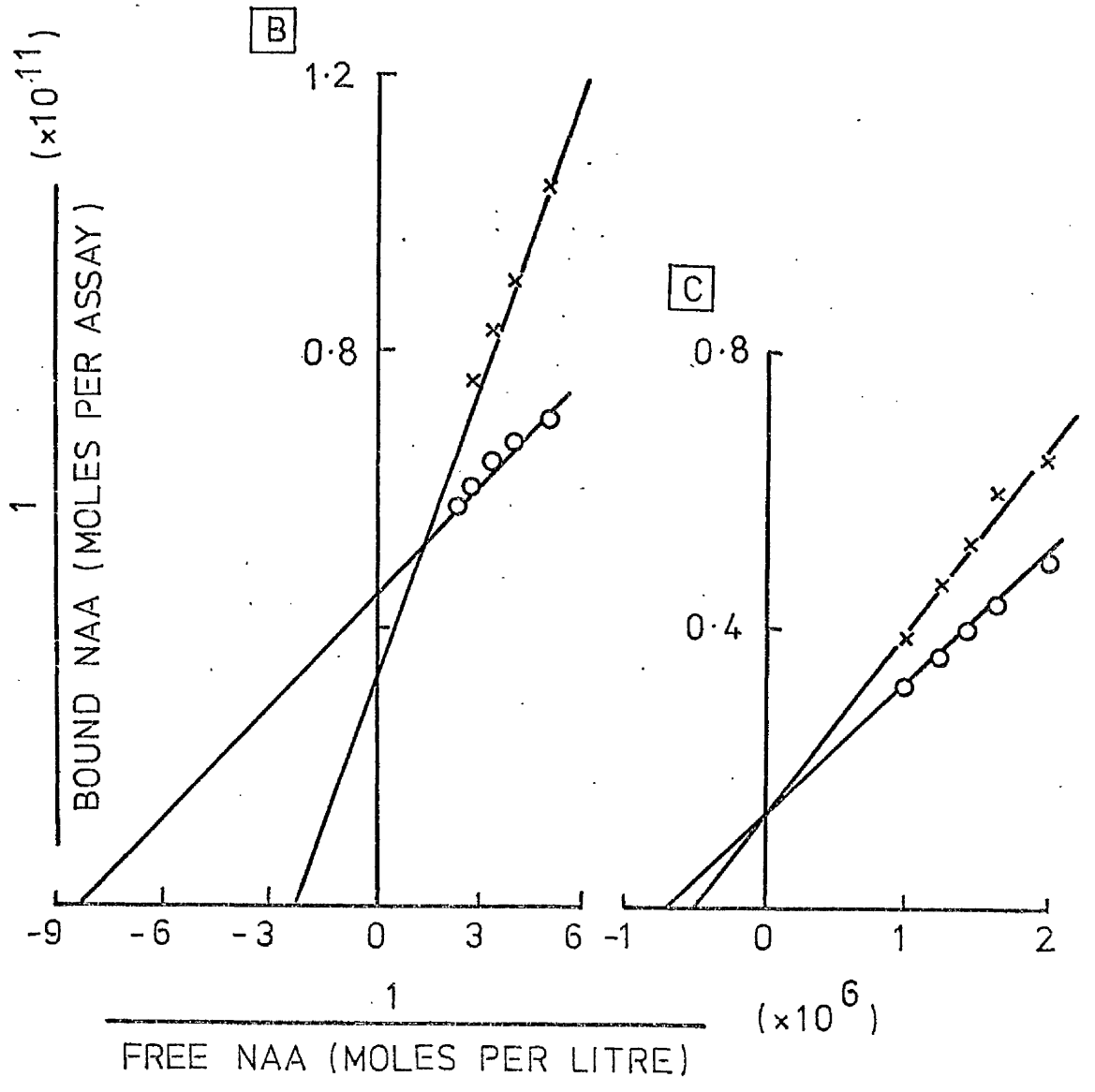

B
C
1·2
0·8
0·8
0·4
1 / BOUND NAA (MOLES PER ASSAY) (×10⁻¹¹)
-9
-6
-3
0
3
6
-1
0
1
2
1 / FREE NAA (MOLES PER LITRE) (×10⁶)

Table 11 Summary of the results of the competition experiments. Mean Ki values are calculated for competitive interactions. The numbers in the "competitive", "not-competitive" and "no competition" columns represent the number of times that particular interaction occurred.

Table 11

COMPOUND	SITE 1					SITE 2				
	NO. OF EXPTS.	MEAN Ki (M)	COMPETITIVE	NOT COMPETITIVE	NO COMPETITION	NO. OF EXPTS.	MEAN Ki (M)	COMPETITIVE	NOT COMPETITIVE	NO COMPETITION
IAA	4	2.5×10^{-6}	4	0	0	6	7.3×10^{-6}	6	0	0
2,4-D	5	5.4×10^{-6}	2	3	0	5	1.1×10^{-5}	5	0	0
2,6-D	4	1.4×10^{-5}	4	0	0	6	-	0	3	3
2,4,5-T	4	4.4×10^{-6}	4	0	0	3	8.5×10^{-6}	3	0	0
2-CPIBA	4	2.7×10^{-6}	4	0	0	4	4.2×10^{-6}	4	0	0
BA	2	3.6×10^{-6}	2	0	0	3	-	0	0	3
2,4-B	4	4.7×10^{-5}	2	2	0	4	-	0	2	2
2,6-B	3	2.9×10^{-6}	3	0	0	5	4.9×10^{-6}	3	0	2
TIBA	4	1.9×10^{-6}	4	0	0	4	2.4×10^{-6}	4	0	0
NPA	3	6.6×10^{-6}	3	0	0	3	1.6×10^{-5}	3	0	0

3. THE INTERACTION OF OTHER PHYTOHORMONES WITH THE NAA BINDING SITES

There are many reports of exogenously applied plant hormones interacting in the physiological effects produced by other exogenously applied plant hormones (e.g. Galston and Davies, 1969; Wareing et al., 1968; Tanada, 1972). One hormone is said to "modify", "decrease", "overcome", "reverse", "counteract", "inhibit", "potentiate", etc. the action of the other. In this study, the ability of the hormones GA_3, ABA, benzyladenine and kinetin to compete for the NAA binding sites was tested. The experiments were performed as described for the "competition" experiments in the previous section.

The results are shown in Figs. 25-27 and Table 12. ABA (Fig. 25) appears not to interact competitively with either site 1 or site 2. Benzyladenine (Fig. 26) is competitive for site 2 and either not-competitive or very weakly competitive for site 1. Kinetin (not illustrated) is also only weakly competitive for site 1 and gives varied results for site 2. When the interaction is competitive, the site 2 Ki agrees with that obtained for benzyladenine. GA_3 (Fig. 27) competes for site 1 but not for site 2.

It thus appears that ABA does not interact with either site 1 or site 2, GA_3 is competitive for site 1 but not site 2 and, in general, the cytokinins tested interact competitively with site 2 and weakly with site 1 also.

Fig. 25 ABA competition for NAA-^{14}C binding sites in a membrane fraction from Zea coleoptiles.

A. Binding data.

Each point represents the mean of three replicates.

Binding data shows that interaction = no competition.

O——O NAA only

×——× + 6 x 10^{-6} M ABA.

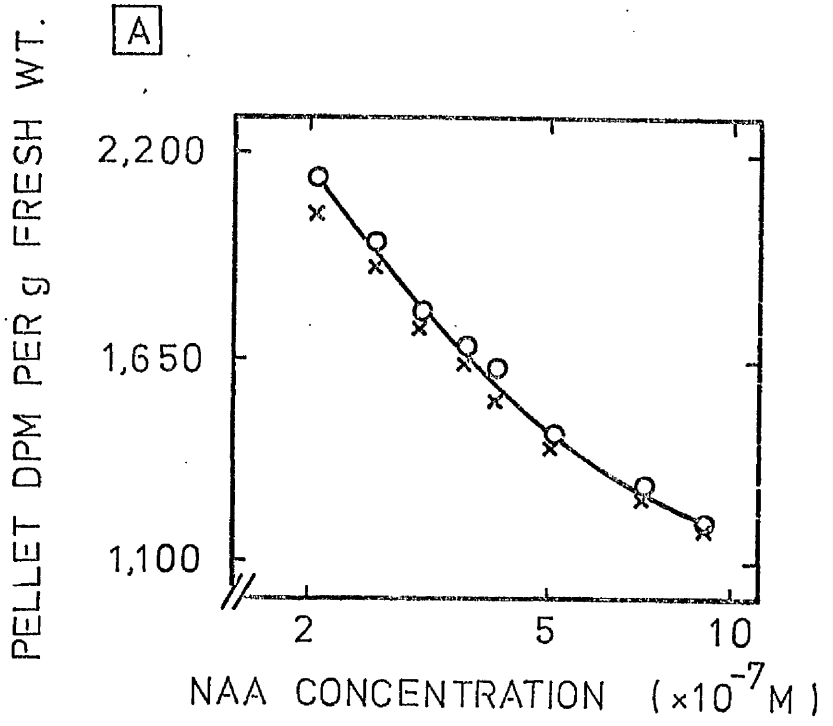

A
PELLET DPM PER g FRESH WT.
2,200
1,650
1,100
2
5
10
NAA CONCENTRATION (×10⁻⁷M)

Fig. 26 Benzyladenine competition for NAA-^{14}C binding sites in a membrane fraction from Zea coleoptiles.

A. Binding data.

Each point represents the mean of three replicates.

B. Double-reciprocal plot of site 1 data.

Each point represents the mean of three replicates and the lines are calculated by a weighted regression analysis through all the data.

Interaction = not-competitive.

C. Double reciprocal plot of site 2 data.

Calculated as for site 1.

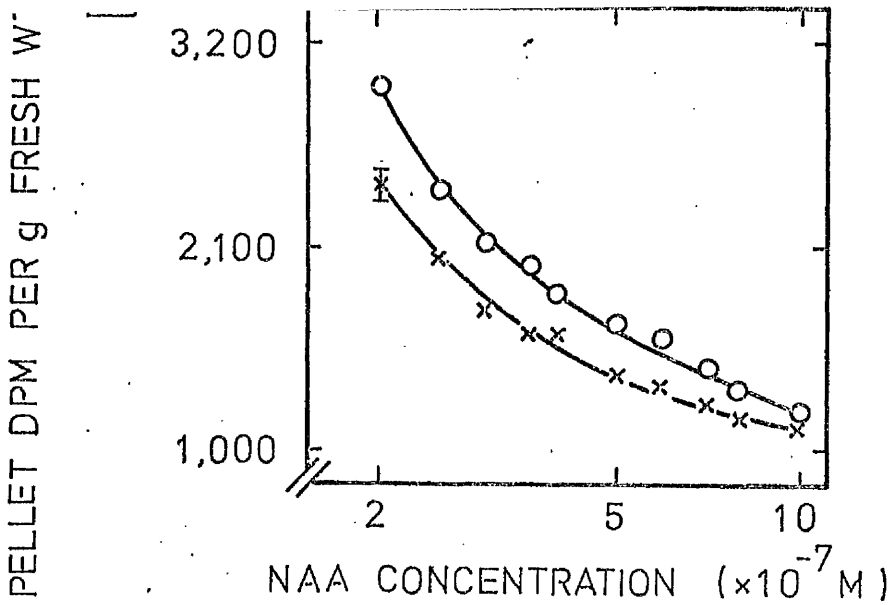
Interaction = competitive, Ki = 6.6×10^{-6}M.

O——O NAA only

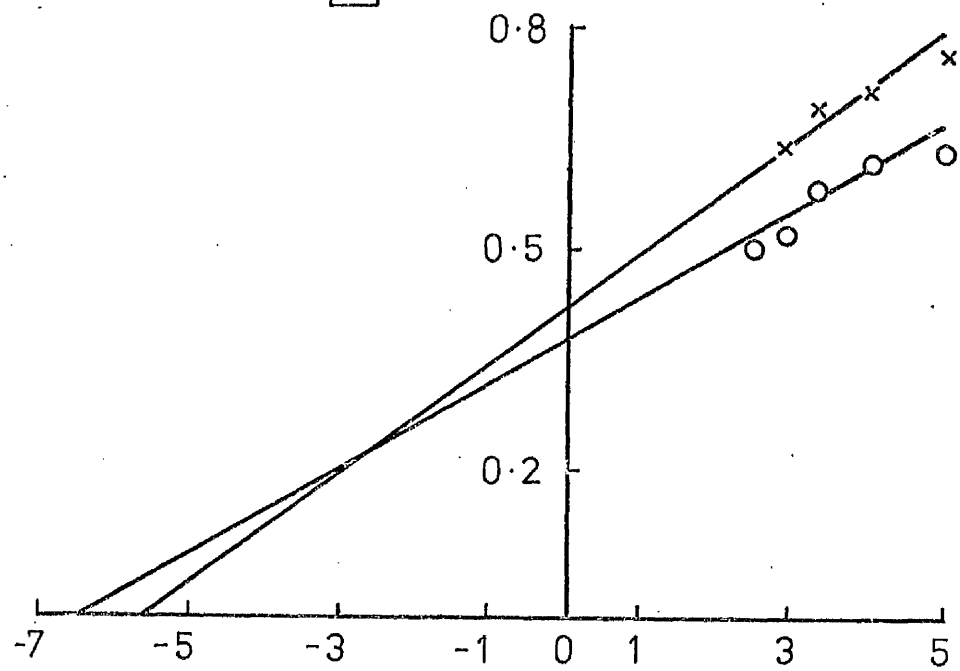
×——× + 7×10^{-6}M Benzyladenine

Statistical Analysis $n \pm$ S.E. ($\times 10^{-11}$ moles per assay) calculated by the weighted regression analysis.

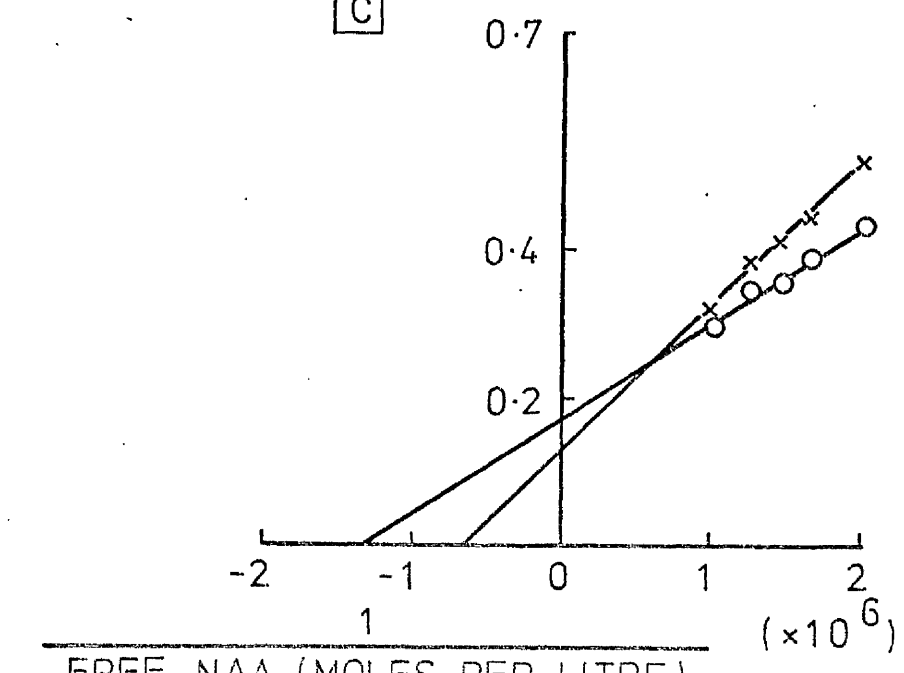
SITE 1				SITE 2			
n(NAA)	n(Ben.Ad.)	t	Signif.	n(NAA)	n(Ben.Ad.)	t	Signif.
2.733 ± 0.168	2.633 ± 0.290	0.299	N.S.	5.764 ± 0.638	7.898 ± 1.150	1.857	N.S.



B



C



$$\frac{1}{\text{BOUND NAA (MOLES PER ASSAY)}} \quad (\times 10^{11})$$

$$\frac{1}{\text{FREE NAA (MOLES PER LITRE)}}$$

Fig. 27 GA_3 competition for NAA-^{14}C binding sites in a membrane fraction from Zea coleoptile.

A. Binding data.

Each point represents the mean of three replicates.

B. Double reciprocal plot of site 1 data.

Each point represents the mean of three replicates and the lines are computed by a weighted regression analysis through all the data.

Interaction = competitive, Ki = 3.2×10^{-6}M.

C. Double reciprocal plot of site 2 data.

Calculated as for site 1.

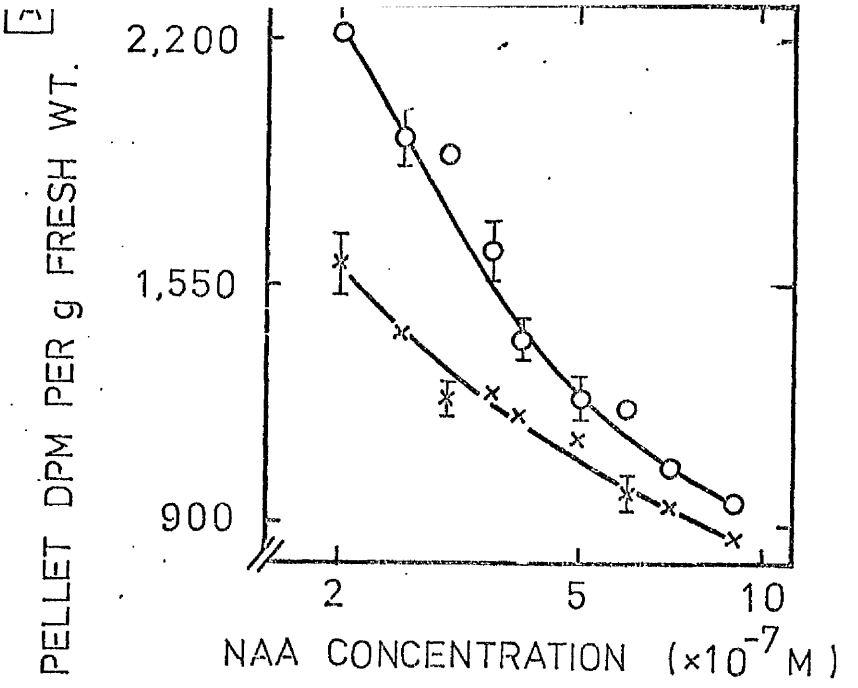
Interaction = not-competitive.

O——O NAA only

×——× + 6×10^{-6} M GA_3.

Statistical Analysis n ± S.E. (x 10^{-11} moles per assay) calculated by the weighted regression analysis.

SITE 1				SITE 2			
n(NAA)	n(GA_3)	t	Signif.	n(NAA)	n(GA_3)	t	Signif.
1.943±0.238	2.385±0.300	1.154	N.S.	3.916±0.534	3.592±0.568	0.415	N.S.


A
2,200
1,550
900
PELLET DPM PER g FRESH WT.
2
5
10
NAA CONCENTRATION (×10⁻⁷ M)

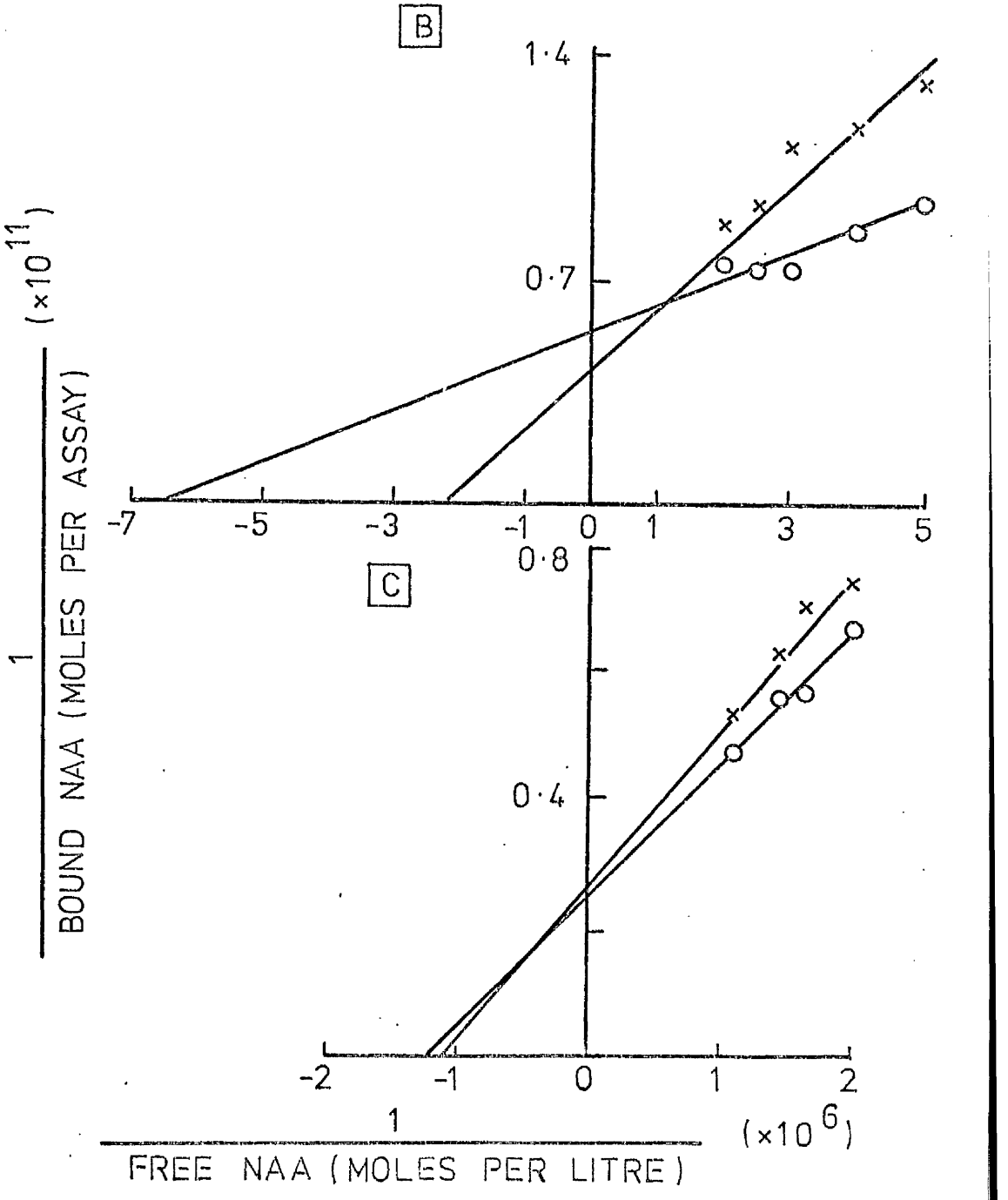

B
1·4
0·7
-7
-5
-3
-1
0
1
3
5
C
0·8
0·4
-2
-1
0
1
2
1 / BOUND NAA (MOLES PER ASSAY) (×10¹¹)
1 / FREE NAA (MOLES PER LITRE) (×10⁶)

Table 12 Summary of the results of the experiments considering the interactions of other phytohormones with the NAA binding sites.

Mean Ki values are calculated for competitive interactions.

The numbers in the "competitive", "not-competitive" and "no-competition" columns represent the number of times that particular interaction occurred.

Table 12

COMPOUND	SITE 1					SITE 2				
	NO. OF EXPTS.	MEAN Ki (M)	COMPETITIVE	NOT COMPETITIVE	NO COMPETITION	NO. OF EXPTS.	MEAN Ki (M)	COMPETITIVE	NOT COMPETITIVE	NO COMPETITION
ABA	6	-	0	1	5	6	-	0	1	5
Benzyl-adenine	4	2.8×10^{-5}	2	1	1	4	1.2×10^{-5}	4	0	0
Kinetin	4	7×10^{-5}	2	2	0	4	1.1×10^{-5}	2	1	1
GA_3	4	5.5×10^{-6}	4	0	0	4	-	0	3	1

4. INTRACELLULAR LOCALIZATION OF THE AUXIN BINDING SITES

The binding of radioactive NAA to a preparation referred to as a "crude membrane preparation" has been demonstrated. Some of the questions which suggest themselves are (i) what is the nature of this membrane fraction? and (ii) are the binding sites localized specifically in particular types of membranes?

(1) Differential Centrifugation of the Coleoptile Homogenate

The first approach to the problem of the cellular localization of binding activity was to examine binding in a variety of fractions, obtained by differential centrifugation of the coleoptile homogenate. The total binding activities in the 0-1 Kg, 1-4 Kg, 4-10 Kg, 1-10 Kg, 4-38 Kg, 10-38 Kg and 4-80 Kg pellets are shown in Table 13.

Greatest binding activity is obtained with a 4-80 Kg pellet. A substantial proportion of this activity (67% of the 4-80 Kg value) is recovered in the 4-38 Kg pellet, and this fraction was most frequently used since it was operationally convenient to prepare. The 10-38 Kg pellet contains 45% of the 4-80 Kg binding activity. This pellet will be depleted of mitochondria relative to the 4-38 Kg fraction. However, a substantial amount of binding activity is lost in the 4-10 Kg pellet and consequently, a 10-38 Kg fraction was not often used. Significant amounts of binding activity are found in the 1-4 Kg, 4-10 Kg and 1-10 Kg pellets, which are believed to be enriched in mitochondria. Very little binding activity is found in the 0-1 Kg pellet which contains nuclear material, starch and cell wall fragments.

Table 13 Total binding activities in pellets obtained by differential centrifugation of a coleoptile homogenate. The range of NAA concentration was 2×10^{-7}M to 10^{-6}M. Figures represent the mean $\pm$ S.E. of at least three experiments.

0-1 Kg = Homogenate centrifuged at 1,000 g, 10 min.

1-4 Kg = 1,000 g supernatant centrifuged at 4,000 g, 20 min.

4-10 Kg = 4,000 g supernatant centrifuged at 10,000 g, 20 min.

1-10 Kg = 1,000 g supernatant centrifuged at 10,000 g, 20 min.

4-38 Kg = 4,000 g supernatant centrifuged at 38,000 g, 30 min.

10-38 Kg = 10,000 g supernatant centrifuged at 38,000 g, 30 min.

4-80 Kg = 4,000 g supernatant centrifuged at 80,000 g, 30 min.

Table 13

BINDING ACTIVITY	FRACTION						
	0-1 Kg	1-4 Kg	4-10 Kg	1-10 Kg	4-38 Kg	10-38 Kg	4-80 Kg
Δ dpm per g fresh weight	26±6	180±30	233±12	285±31	909±42	631±45	1,369±68

(2) Binding Activity in Mitochondrial Fractions

Several lines of evidence are available to show that binding of NAA-^{14}C to mitochondria does not contribute significantly to the total binding activity.

Since the low speed pellets are enriched in mitochondria, the nature of the binding activity detected in these pellets was first considered. Figs. 28 and 29 show that the binding of a 4-10 Kg pellet has the kinetic properties of the lower affinity (site 2) binding site, with no indication of site 1 activity. Similar results are obtained with 1-4 Kg and 1-10 Kg pellets. The possibility that site 2 binding represents binding of NAA-^{14}C to mitochondria is rendered improbable by the finding that a 10-38 Kg pellet, which is relatively poor in mitochondria, possesses substantial site 2 activity (Figs. 28 and 29).

The activity of a mitochondrial marker enzyme, succinate dehydrogenase (SDH), in differentially centrifuged pellets was examined, together with the binding activities of these pellets (Table 14). It can be seen that most of the SDH activity is found in the 4-10 Kg pellet, whereas most of the binding activity is in the 10-38 Kg pellet. A similar result is obtained with pellets prepared from mesocotyls (Table 15). (The binding activity of mesocotyl membrane fractions is fully described later). In both Tables 14 and 15, the same pattern of results is seen whether specific activities (i.e. on a mg protein basis) or total activities (on a g fresh weight basis) are considered. The binding activities in Table 14 represent predominantly site 2 binding (because of the NAA concentration range selected), and illustrate further the lack of correlation between site 2 binding, and mitochondrial activity.

Fig. 28 The binding of NAA-^{14}C by :

(a) O———O a 10-38 Kg membrane pellet from Zea coleoptiles.

(b) ×———× a 4-10 Kg membrane pellet from Zea coleoptiles.

Each point represents the mean of three replicates.

Fig. 29 Scatchard plot of data from Fig. 28

O———O 10-38 Kg pellet

×———× 4-10 Kg pellet

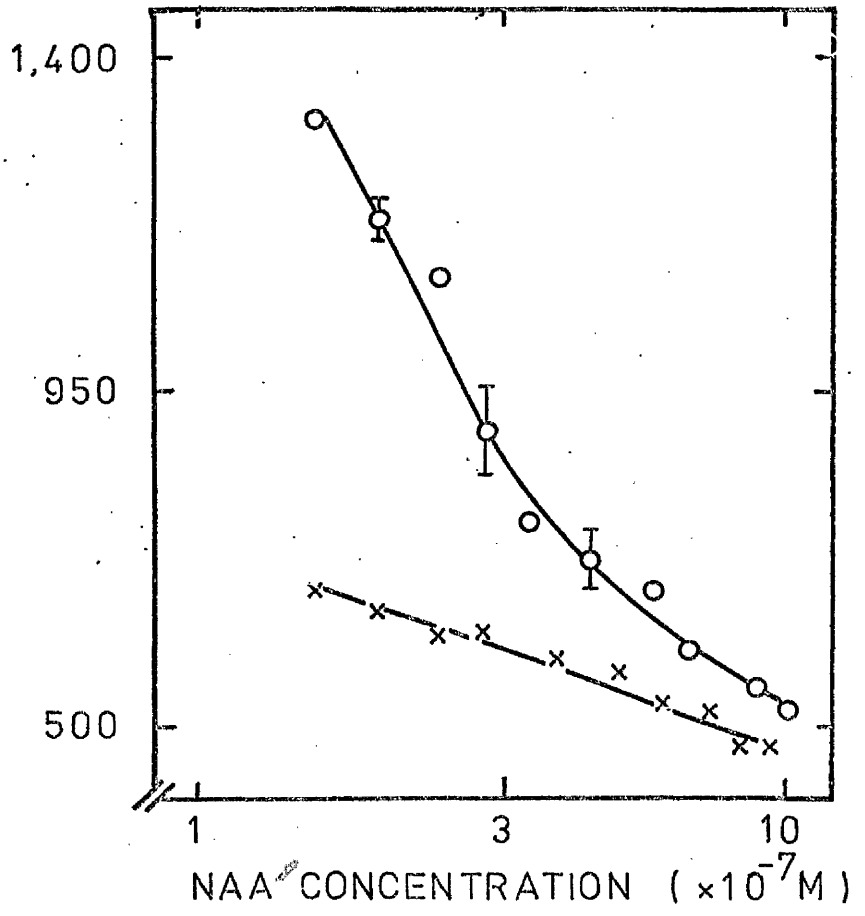
Each point represents the mean of three replicates and the lines were computed by standard regression analyses.

K-values

For the 10-38 Kg pellet, $K_1 = 2.9 \times 10^{-7}$M

$K_2 = 10.2 \times 10^{-7}$M.

For the 4-10 Kg pellet, $K = 15.7 \times 10^{-7}$M.


PELLET DPM PER g FRESH WT.
1,400
950
500
1
3
10
NAA CONCENTRATION (×10⁻⁷M)

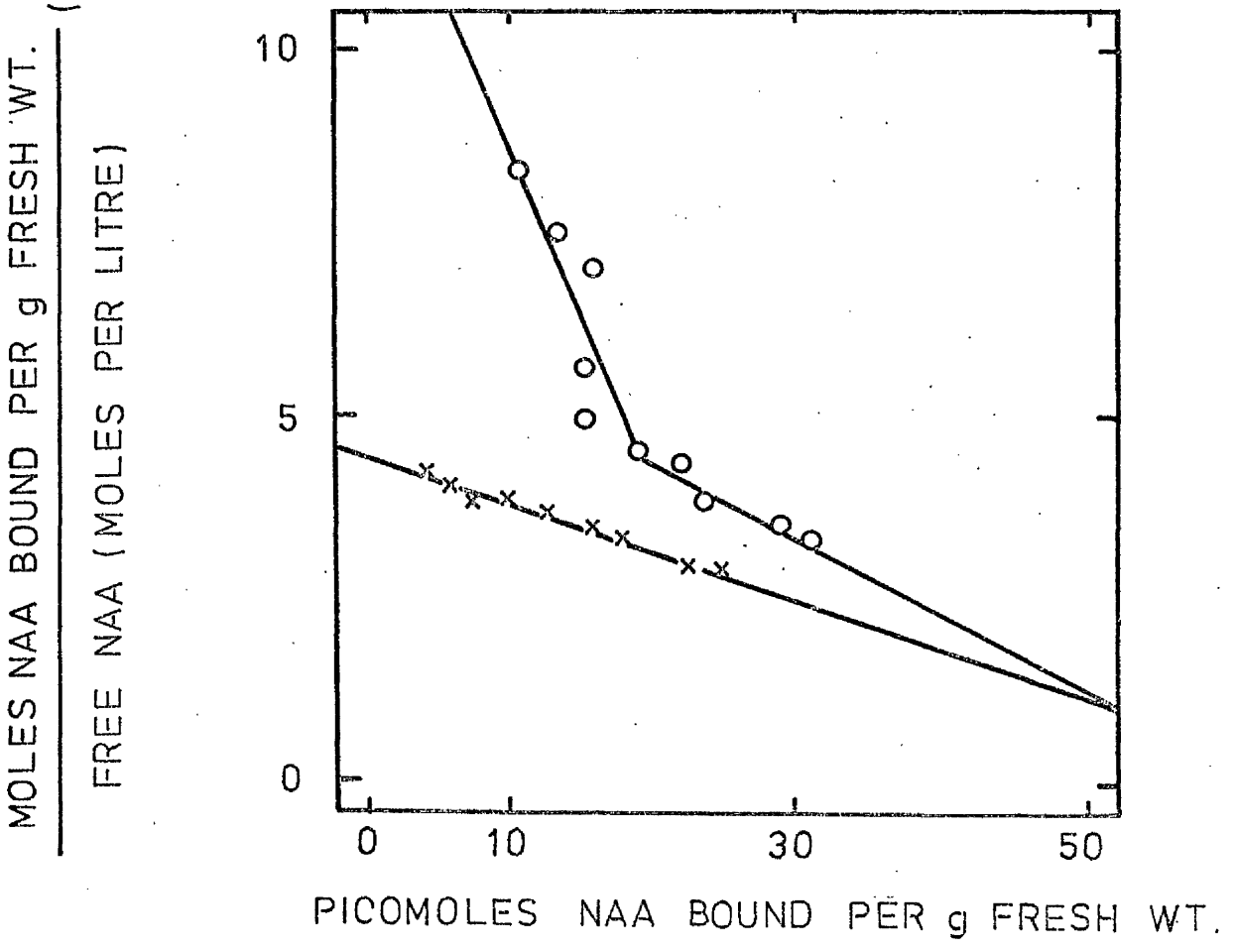

MOLES NAA BOUND PER g FRESH WT. / FREE NAA (MOLES PER LITRE) (×10⁻⁵)
10
5
0
0
10
30
50
PICOMOLES NAA BOUND PER g FRESH WT.

Table 14 A comparison of binding activity and SDH activity in 4-10 Kg and 10-38 Kg pellets from *Zea* coleoptiles. Binding is examined between 4×10^{-7} M NAA and 14×10^{-7} M NAA.

Table 14

PELLET	SPECIFIC ACTIVITIES		TOTAL ACTIVITIES	
	Binding Activity (Δdpm/mg protein)	SDH Activity (nmoles formazan/ min/mg protein)	Binding Activity (Δdpm/g fresh wt)	SDH Activity (nmoles formazan/ min/g fresh wt.)
4-10 Kg	452	16.0	244	7.4
10-38 Kg	574	5.4	608	4.3

Table 15 A comparison of binding activity and SDH activity in 1-10 Kg and 10-38 Kg pellets from Zea mesocotyls. Binding is examined between 3×10^{-7} M NAA and 10^{-6} M NAA.

Table 15

PELLET	SPECIFIC ACTIVITIES		TOTAL ACTIVITIES	
	Binding Activity (Δdpm/mg protein)	SDH Activity (nmoles formazan/ min/mg protein)	Binding Activity (Δdpm/g fresh wt.)	SDH Activity (nmoles formazan/ min/g fresh wt.)
1-10 Kg	22	22.4	21	9.0
10-38 Kg	156	2.3	178	1.2

A 1-10 Kg pellet prepared from mesocotyls has been further fractionated on a discontinuous sucrose gradient ("mitochondrial gradient"), in which mitochondria are reported to sediment at the 1.20M - 1.35M sucrose interface. The bands were recovered from the interfaces of the gradient and assayed for SDH and binding activities (Table 16). Total SDH activity peaks at the 1.20M - 1.35M interface, and although this fraction also possesses considerable binding activity, there is clearly no correlation between SDH and binding activities in the other fractions (particularly the 0.56M - 0.90M, and 0.90M - 1.20M fractions).

It therefore seems that the presence of mitochondria in centrifugal fractions displaying binding activity is no more than coincidental.

(3) Binding Activity in Membrane Pellets from Zea Coleoptiles Fractionated on Sucrose Gradients

It has now been established that most of the binding activity of the coleoptile homogenate is localised in the high-speed (4-38 Kg, 10-38 Kg, or 4-80 Kg) pellets. The fractionation by discontinuous sucrose-gradient centrifugation of a similar pellet prepared from oat roots has been described by Hodges et al., (1972). Using this method, the distribution of the binding activities in a fractionated coleoptile pellet was determined.

The distribution of material from a 4-38 Kg pellet, fractionated on a "complex" gradient is shown in Plate 3, A. Visible bands are seen at all the sucrose interfaces. Fig. 30 shows the

Table 16 A comparison of binding activity and SDH activity of a gradient-fractionated 1-10 Kg pellet from Zea mesocotyls. Binding is examined between 3×10^{-7} M NAA and 3×10^{-6} M NAA.

Table 16

INTERFACE (M Sucrose)	SPECIFIC ACTIVITIES		TOTAL ACTIVITIES	
	Binding Activity (Δdpm/mg protein)	SDH Activity (nmoles formazan/ min/mg protein)	Binding Activity (Δdpm/ assay)	SDH Activity (nmoles formazan/ min/assay)
0.56 - 0.90	1004	3.3	69	0.05
0.90 - 1.20	917	4.1	251	0.27
1.20 - 1.35	199	36.0	239	10.08
1.35 - 1.50	183	25.7	38	1.23
1.50 - 1.95	81	10.8	6	0.17

Plate 3 Distribution of bands in discontinuous sucrose gradients.

A: "Complex" gradient

B: "Simple" gradient.

A

BAND

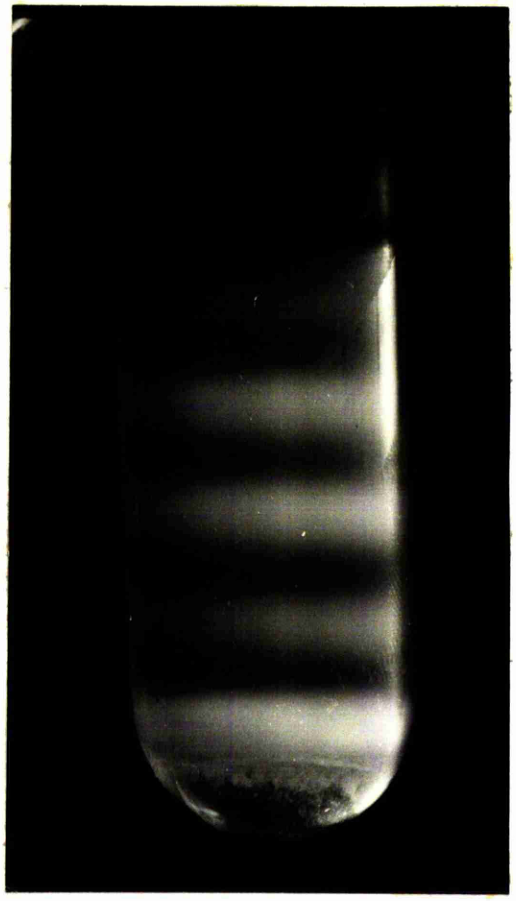
0·56M – 0·81M

0·81M – 0·99M

0·99M – 1·14M

1·14M – 1·29M

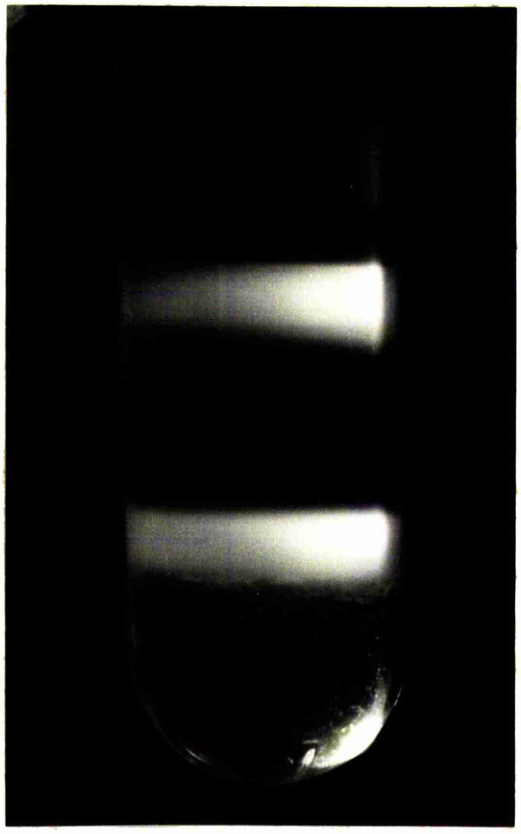
1·29M – 1·58M



B

0·56M – 0·99M

0·99M – 1·58M



distribution of total binding activity in the bands recovered from the gradients. Approximately 60% of the activity is found in the light fractions (0.56M - 0.81M and 0.81M - 0.99M), of the gradients. Table 17 shows a comparison of both specific and total binding activities in the gradient-bands. Specific activity is also at a maximum in the two light bands.

Unfortunately, it is not possible to recover sufficient material from the bands of the "complex" gradient to perform a full kinetic analysis on each band. However, Hodges et al., (1972) also suggested a simplified gradient from which only two bands are recovered. These bands represent the combination of the two light bands (0.56M - 0.81M and 0.81M - 0.99M) and the three heavier bands (0.99M - 1.14M, 1.14M - 1.29M and 1.29M - 1.58M) of the "complex" gradient (Plate 3, B). Using this "simple" gradient, sufficient material can be easily obtained for a full kinetic analysis of the two bands. Fig. 31 shows the binding activity in the two bands isolated from a "simple" gradient. As expected, more activity is found in the light (0.56M - 0.99M) band, (approximately 70% of the total activity recovered from the gradient). The Scatchard analysis (Fig. 32), suggests that there is only one distinct set of sites in each band. The mean K and n-values (from 8 experiments) for these bands are as follows:

0.56M - 0.99M : $K = 3.9 \pm 0.4 \times 10^{-7}$ M

$n = 24 \pm 3$ picomoles per g fresh wt.

0.99M - 1.58M : $K = 11.6 \pm 1.2 \times 10^{-7}$ M

$n = 32 \pm 7$ picomoles per g fresh wt.

Fig.30 Distribution of binding activity in a 4-38 Kg membrane fraction from Zea coleoptiles, fractionated on a "complex" gradient. Binding is investigated between 2 x 10^{-7}M and 7 x 10^{-7}M NAA.

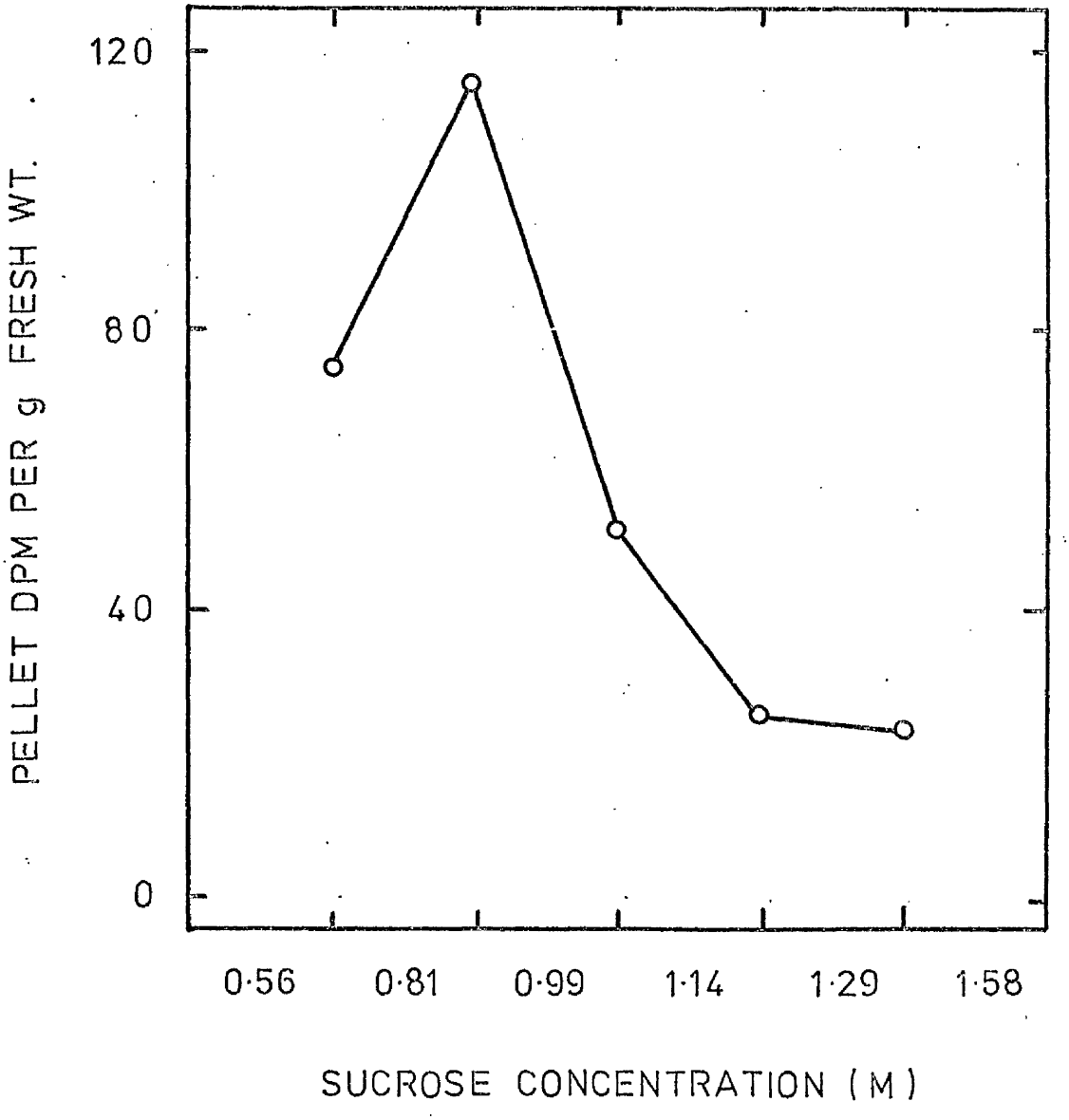

120
80
40
0
PELLET DPM PER g FRESH WT.
0·56
0·81
0·99
1·14
1·29
1·58
SUCROSE CONCENTRATION (M)

Table 17 Binding activity in a 4-38 Kg pellet from Zea coleoptiles, fractionated on a "complex" gradient, expressed as "total" and "specific" activity.

Table 17

INTERFACE (M Sucrose)	SPECIFIC ACTIVITY (Δ dpm per mg protein)	TOTAL ACTIVITY (Δ dpm per g fresh wt.)
0.56 - 0.81	2037	80
0.81 - 0.99	1515	120
0.99 - 1.14	727	57
1.14 - 1.29	218	30
1.29 - 1.58	173	28

Fig.31 Binding activity of a 4-38 Kg membrane pellet from Zea coleoptiles fractionated on a "simple" gradient. Each point represents the mean of three replicates.

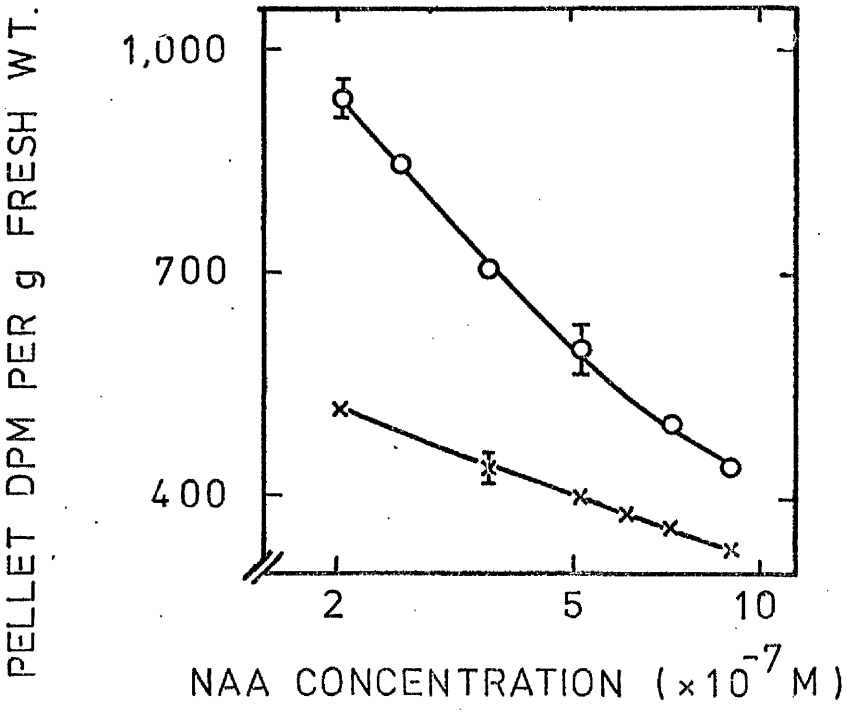
O——O 0.56M - 0.99M band

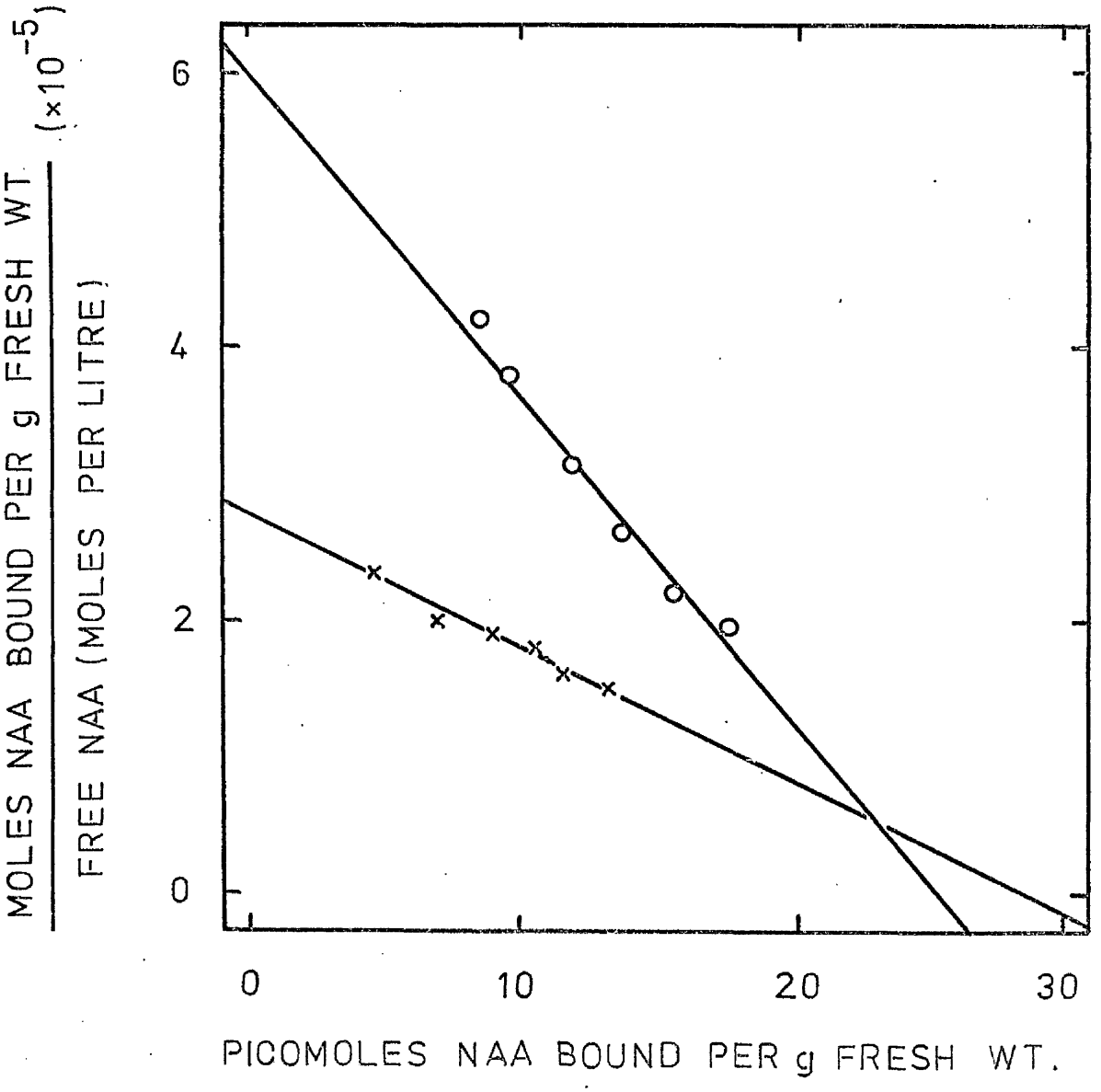
×——× 0.99M - 1.58M band

Fig.32 Scatchard plot of data from Fig. 31. Each point represents the mean of three replicates and the lines are computed by standard regression analyses.

O——O 0.56M - 0.99M band

×——× 0.99M - 1.58M band


PELLET DPM PER g FRESH WT.
1,000
700
400
2
5
10
NAA CONCENTRATION (×10⁻⁷ M)


MOLES NAA BOUND PER g FRESH WT. (×10⁻⁵)
FREE NAA (MOLES PER LITRE)
6
4
2
0
0
10
20
30
PICOMOLES NAA BOUND PER g FRESH WT.

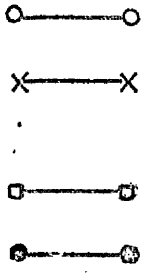
The K values for the light and heavy bands are of the same order as those previously determined for site 1 and site 2 respectively in unfractionated 4-38 Kg pellets. It would appear, therefore, that the "simple" gradient provides a means of separating the two binding sites. If this is indeed the case, the n-values indicate that there is approximately 50% recovery of site 1 activity and 30% recovery of site 2 activity.

(4) Auxin-Specificity of Binding to Light and Heavy Membrane Bands

If the two bands recovered from the "simple" gradient truly represent the two sites found in an unfractionated pellet, then the auxin-specificity as determined for the unfractionated sites should be retained. This was tested by examining the ability of three selected compounds, IAA, BA and TIBA, to compete for the NAA binding sites which are recovered from the two bands of the "simple" gradient. The binding data and the corresponding double-reciprocal plots are shown in Figs. 33 and 34. IAA and TIBA compete for the binding sites recovered in both bands while BA competes for the binding site in the light band, but shows no competition for the site in the heavy band. Table 18 compares the Ki values determined for the compounds tested, in unfractionated and "simple"-gradient-fractionated pellets. It is evident that the binding specificities of the light and heavy gradient bands correspond to those of site 1 and site 2 respectively.

It therefore appears that by using the "simple" gradient technique it is possible to separate, at least partially, the two NAA binding sites which are found in an unfractionated pellet.

Fig. 33 Binding data from competition experiments, in a 4-38 Kg pellet from Zea coleoptiles, fractionated on a "simple" gradient. Each point represents the mean of three replicates.



o——o NAA only
x——x + competitor
} in the 0.56M - 0.99M band

□——□ NAA only
■——■ + competitor
} in the 0.99M - 1.58M band

A: + IAA (5×10^{-6}M)

B: + BA (5×10^{-6}M)

C: + TIBA (4×10^{-6}M)

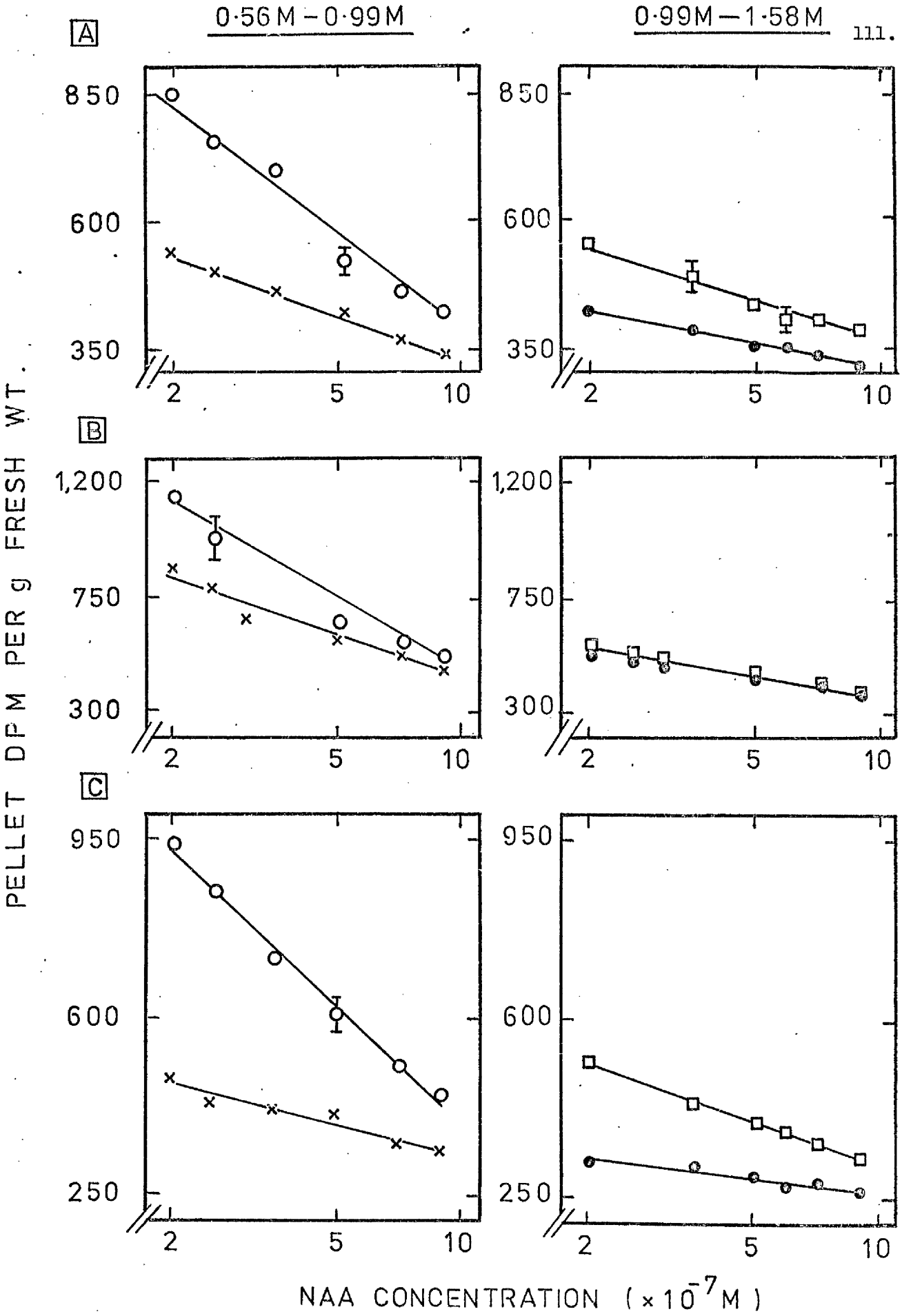

A
0·56M – 0·99M
0·99M – 1·58M
850
600
350
B
1,200
750
300
C
950
600
250
2
5
10
PELLET DPM PER g FRESH WT.
NAA CONCENTRATION (×10⁻⁷M)

Fig. 34 Double-reciprocal plots of data from Fig. 33. Each point represents the mean of three replicates, and lines are computed by weighted regression analyses.

o——o NAA only
x——x + competitor
} in the 0.56M - 0.99M band

□——□ NAA only
●——● + competitor
} in the 0.99M - 1.58M band

A: + IAA (5×10^{-6}M)

B: + BA (5×10^{-6}M)

C: + TIBA (4×10^{-6}M)

Statistical Analysis $n \pm$ S.E. ($\times 10^{-11}$ moles per assay) is calculated by the weighted regression analysis.

COMPOUND	0.56M - 0.99M				0.99M - 1.58M			
	n(NAA)	n(comp.)	t	Signif.	n(NAA)	n(comp.)	t	Signif.
IAA	4.448 ± 0.262	4.392 ± 0.315	0.141	N.S.	6.128 ± 1.579	6.803 ± 1.109	0.350	N.S.
BA	6.209 ± 0.511	6.590 ± 0.458	0.556	N.S.	-	-	-	-
TIBA	4.827-0.201	7.784-0.687	4.130	** (C.O.)	5.553 ± 0.360	9.821 ± 1.627	2.560	* C.O.

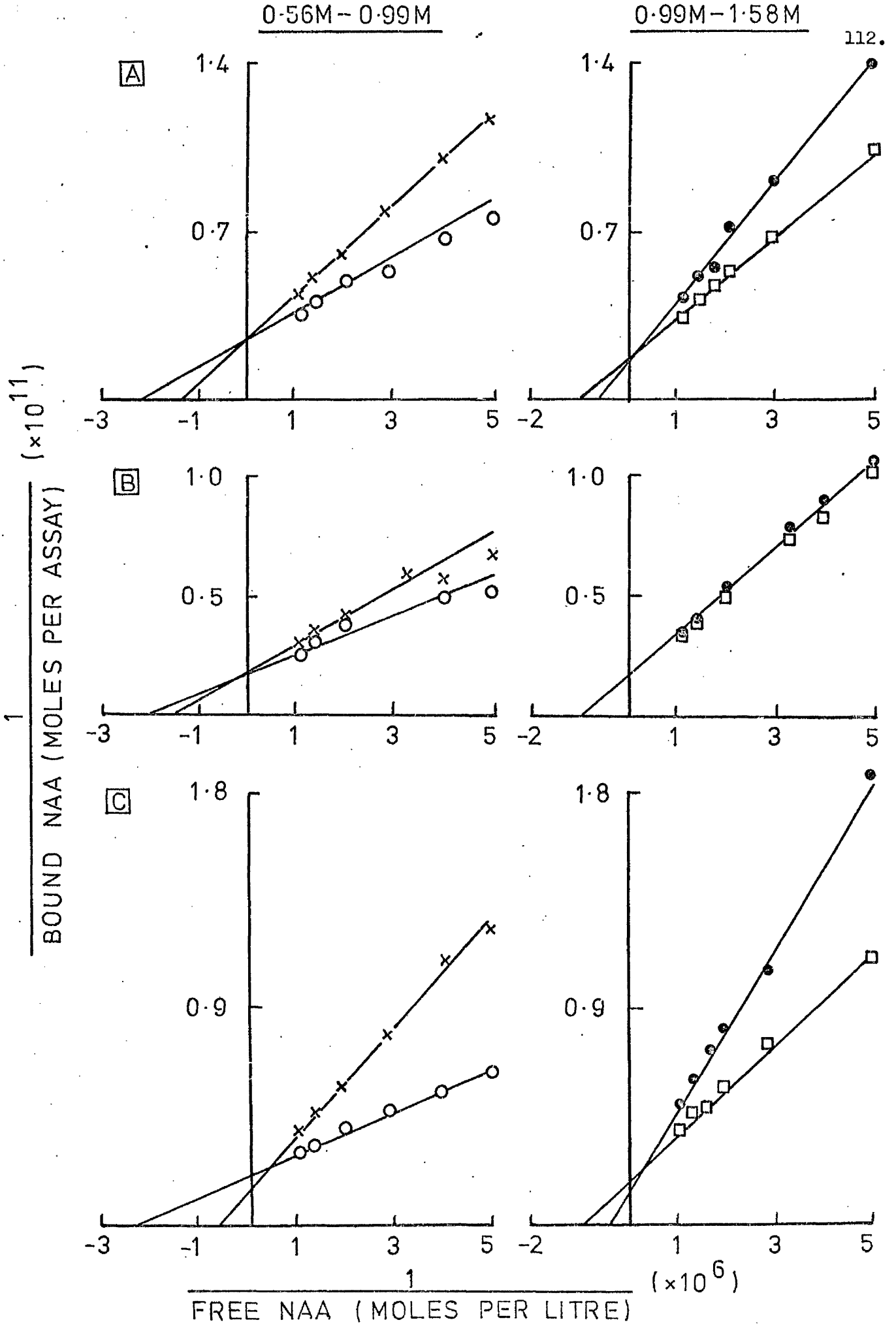

0·56M – 0·99M
0·99M – 1·58M
A
B
C
1·4
0·7
1·0
0·5
1·8
0·9
-3
-2
-1
1
3
5
1 / BOUND NAA (MOLES PER ASSAY) (×10^11)
1 / FREE NAA (MOLES PER LITRE) (×10^6)

Table 18 A comparison of Ki values, as calculated for site 1 and site 2 in an unfractionated 4-38 Kg membrane pellet from Zea coleoptiles, and for the sites recovered in the two bands of a "simple" gradient.

Table 18

COMPOUND	UNFRACTIONATED Ki (M)		FRACTIONATED Ki (M)	
	Site 1	Site 2	0.56M - 0.99M	0.99M - 1.58M
IAA	2.5×10^{-6}	7.3×10^{-6}	7.9×10^{-6}	9.1×10^{-6}
BA	3.6×10^{-6}	No competition	12×10^{-6}	No competition
TIBA	1.9×10^{-6}	2.4×10^{-6}	1.3×10^{-6}	1.9×10^{-6}

(5) Enzymatic and Chemical Characterization of the "Complex" Gradient Bands

Evidence just presented indicates that site 1 binding activity is localized in a light membrane fraction, whereas site 2 activity is found in a heavier fraction. The nature of these fractions was further investigated with respect to enzymic activity and chemical composition. Marker enzymes for different membrane fractions of animal tissues have been well defined, but the same patterns of activity are not always found in plant systems. However, several enzymatic markers for plant membranes have been reported and the distribution of these after fractionation of membrane pellets on a "complex"-gradient was studied. The molar ratio of sterol:phospholipid can also be used to distinguish between membrane populations, and this was studied.

The bands from a fractionated 10-38 Kg coleoptile pellet were each resuspended in the same volume of appropriate buffer and analysed for:

(1) ATP-ase activity (at pH 6.0)

(2) Glucan synthetase activity (at high (1 mM) UDPG-concentration considering the hot water insoluble product).

(3) IDP-ase activity (activated by 0.1% sodium deoxycholate).

(4) Sterol:phospholipid molar ratio.

Table 19 shows the results of these assays, expressed as "specific" and "total" activities. Specific activities of acidic ATP-ase and glucan synthetase are highest in the heavier regions of the gradient, while IDP-ase activity peaks in the 0.81M - 0.99M band. The sterol:phospholipid molar ratios show the highest values in the heavier regions of the

Table 19 Distribution of enzyme activities and sterol: phospholipid ratios in a 10-38 Kg pellet from Zea coleoptiles, fractionated on a "complex" gradient.

A: Specific activities, expressed in nmoles per min per mg protein.

B: Total activities, expressed in nmoles per min per g fresh weight.

Table 19

A.

SUCROSE (M)	ATP-ase (pH 6.0)	Glucan Synthetase (hot H_2O insoluble, 1 mM UDPG)	IDP-ase (0.1% deoxy-cholate)	Sterol:Phospholipid (molar ratio)
0.56-0.81	52	5.1	148	0.36
0.81-0.99	88	19.5	307	0.35
0.99-1.14	174	43.3	141	0.47
1.14-1.29	167	36.1	80	0.64
1.29-1.58	183	41.3	38	0.79

B.

SUCROSE (M)	ATP-ase (pH 6.0)	Glucan Synthetase (hot H_2O insoluble, 1 mM UDPG)	IDP-ase (0.1% deoxy-cholate)	Sterol:Phospholipid (molar ratio)
0.56-0.81	2.4	0.20	6.8	0.36
0.81-0.99	8.8	2.02	30.7	0.35
0.99-1.14	11.1	1.95	9.0	0.47
1.14-1.29	12.4	1.95	5.9	0.64
1.29-1.58	12.5	1.55	2.6	0.79

Table 20 Distribution of enzyme activities and sterol: phospholipid ratio, in a 10-38 Kg membrane pellet from Zea mesocotyls, fractionated on a complex gradient.

A: Specific activities, expressed in nmoles per min per mg protein.

B: Total activities, expressed in nmoles per min per g fresh weight.

Table 20

A.

SUCROSE (M)	ATP-ase (pH 6.0)	Glucan Synthetase (hot H_2O insoluble, 1 mM UDPG)	IDP-ase (0.1% deoxy-cholate	Sterol:Phospholipid (molar ratio)
0.56-0.81	39	5.1	197	0.54
0.81-0.99	43	22.7	243	0.31
0.99-1.14	66	31.1	135	0.41
1.14-1.29	63	30.1	79	0.54
1.29-1.58	57	44.0	19	0.75

B.

SUCROSE (M)	ATP-ase (pH 6.0)	Glucan Synthetase (hot H_2O insoluble, 1 mM UDPG)	IDP-ase (0.1% deoxy-cholate)	Sterol:Phospholipid (molar ratio)
0.56-0.81	1.14	0.06	7.4	0.54
0.81-0.99	3.07	0.85	18.2	0.31
0.99-1.14	4.39	1.32	6.8	0.41
1.14-1.29	3.31	1.39	3.8	0.54
1.29-1.58	2.30	1.05	0.4	0.75

gradient. A generally similar distribution of ATP-ase, glucan synthetase, IDP-ase and sterol:phospholipid ratio is seen in a fractionated 10-38 Kg pellet from mesocotyls (Table 20).

Using the assay conditions described, ATP-ase and glucan synthetase are reported to be markers for plant plasma membranes (Hodges et al., 1972; Van der Woude et al., 1974). A high sterol:phospholipid ratio is also reported to be characteristic of plant plasma membranes (Hodges et al., 1972; Sullivan and Volcani, 1974). IDP-ase is reported to be a Golgi membrane marker (Ray et al., 1969). Thus, it seems that the heavy regions of the gradient are enriched in plasma membranes relative to the lighter bands, which show a Golgi-enriched region.

6. Electron Microscopy of the "Complex" Gradient Bands

The bands recovered from the "complex" gradient were prepared for electron microscopy as described in the Materials and Methods. Sections prepared from each band were stained both by the standard alkaline lead citrate procedure, (Reynolds, 1963, referred to as lead strain), or by the PTA-chromic acid procedure (referred to as specific stain) described by Roland et al., (1972). The alkaline lead citrate is a general stain for cellular membranes whereas the PTA-chromic acid stain is reported to be specific for plant plasma membranes.

Plate 4 shows a specifically-stained section of a root tip of wild oat (Avena fatua). Apart from the cell wall, the plasma membrane is the only cellular component which stains densely. Plates 5-9 show electron micrographs of the membrane fractions recovered from the "complex" gradient. In all the fractions, there is, in general, a

homogeneous distribution of the lead stain throughout the sections. The 0.56M - 0.81M, 0.81M - 0.99M and 0.99M - 1.14M fractions show very little evidence of specifically-stained material (Plates 5B, 6B and 7B). In the 1.14M - 1.29M band, the lead-stained sections show the presence of a very high proportion of mitochondria (Plate 8A). This is to be expected, since a 4-38 Kg pellet was used for the gradients. In the 1.14M - 1.29M fraction, there is much more specifically stained material than in the lighter bands (Plate 8B). This is not apparent on a first inspection because of the high mitochondrial content of the sections. However, most of the non-mitochondrial material does stain specifically. The lead-stained section of the 1.29M- 1.58M band shows considerable heterogeneity in the membrane material (Plate 9A). However, this fraction has the highest proportion of specifically stained material (Plate 9B).

It therefore seems that the membrane material which sediments in the heavier regions of the gradient is enriched in plasma membrane vesicles. This observation is in agreement with the enzyme marker results previously presented. In the light fractions of the gradient many lead-stained membrane vesicles, but very few specifically-stained vesicles are seen. There are no obvious stacks of Golgi cisternae in the light fraction, although the vesicles themselves could be partly of Golgi origin since glutaraldehyde was not induced during the isolation procedure.

Plate 4. Section of a root-tip of wild oat (Avena fatua), stained by the PTA-chromic acid procedure.
X 28,700.

CW = Cell wall.

PM = Plasma membrane.

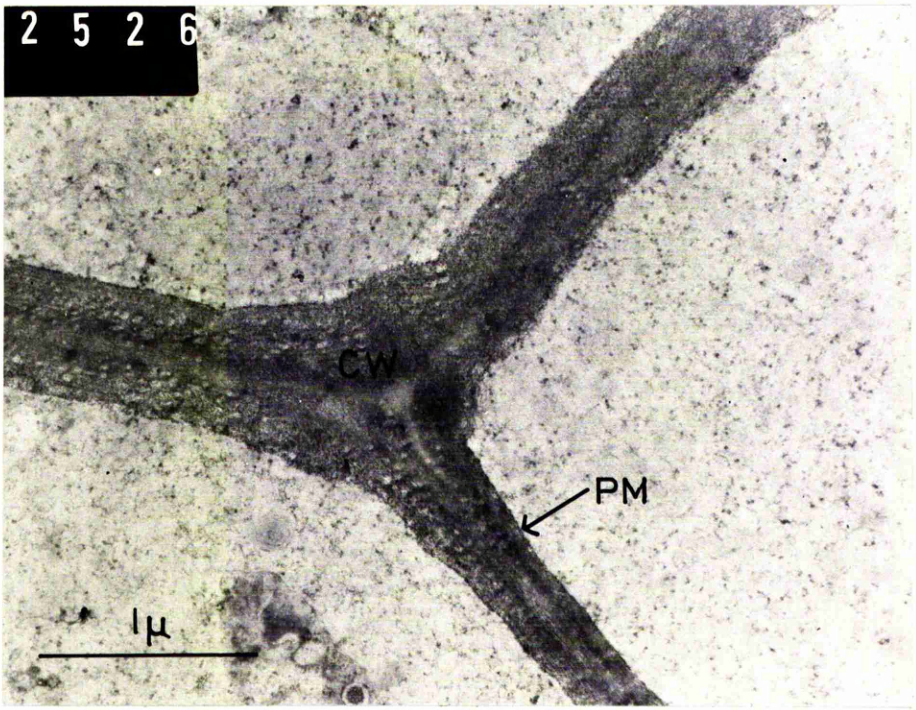

2 5 2 6
CW
PM
1μ

Plate 5. Electron micrograph of the 0.56M - 0.81M band.

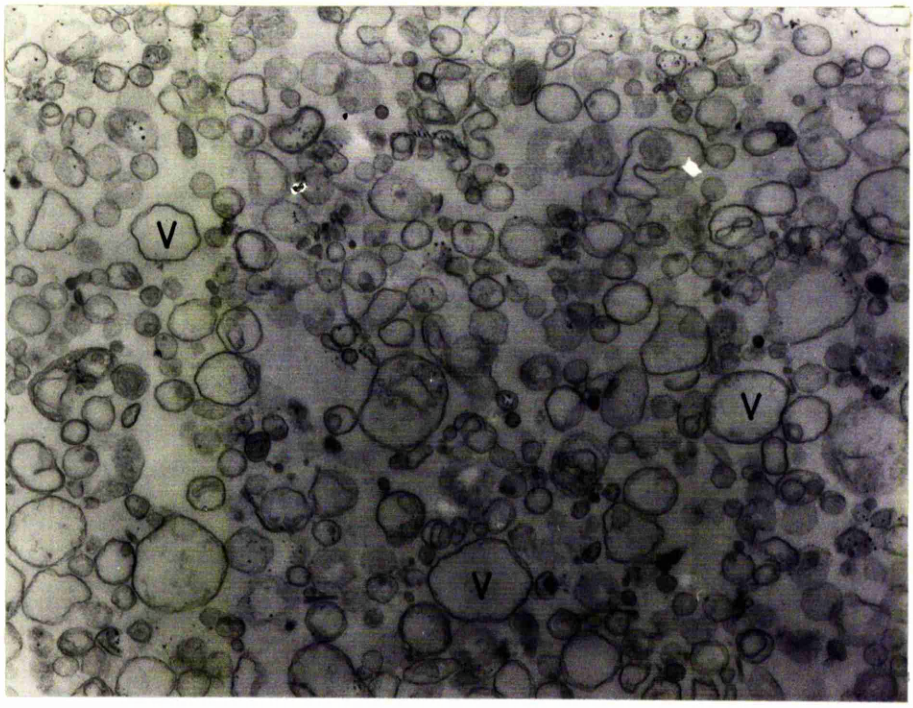
X 28,700.

A : Lead-stain.

B : Specific (PTA-chromic acid) - stain.

V = Vesicles.

A



B

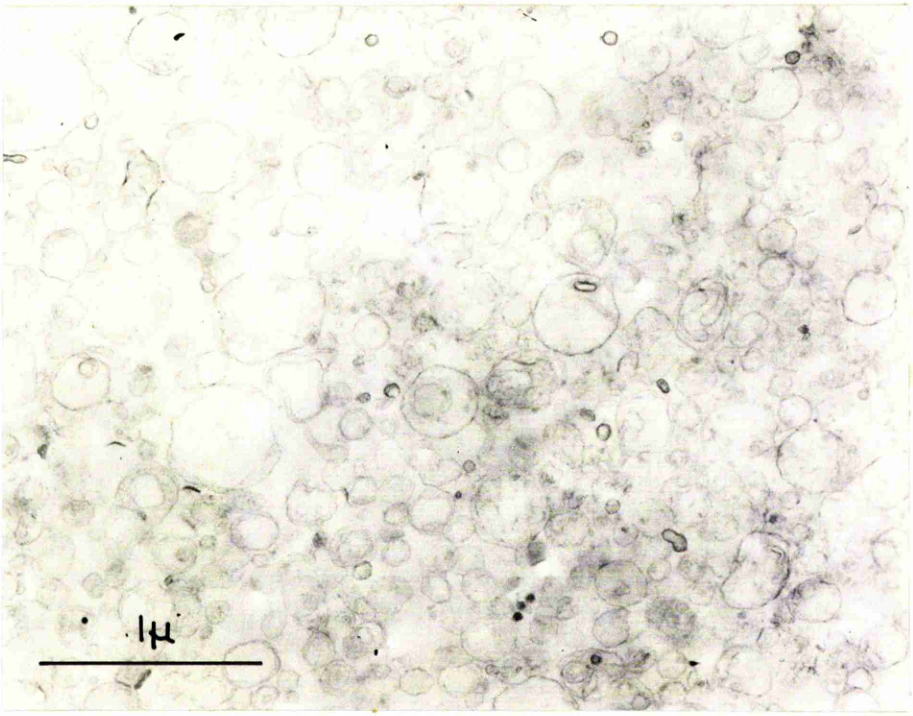


Plate 6. Electron micrograph of the 0.81M - 0.99M band.

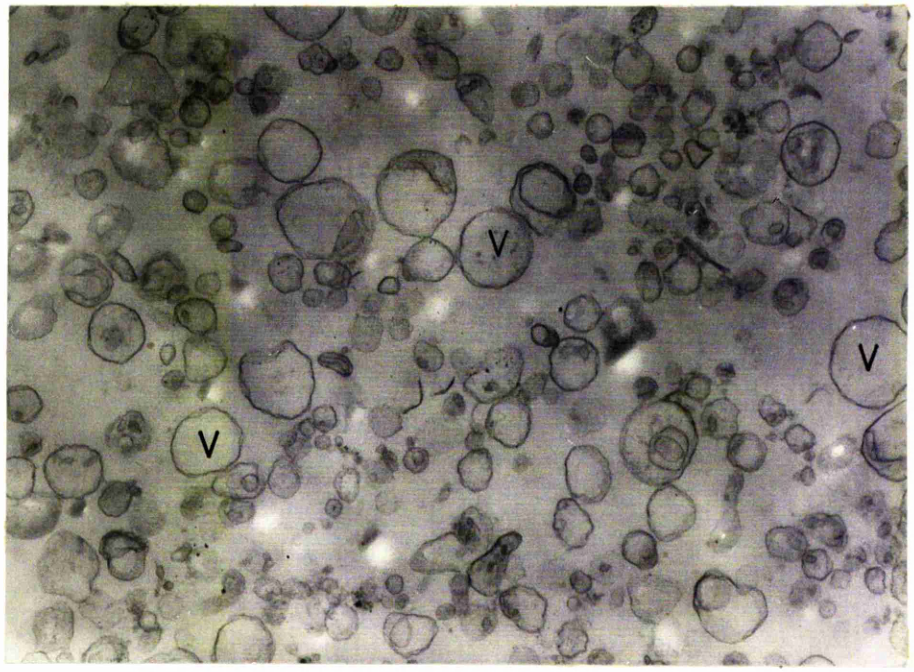
X 28,700.

A : Lead-stain.

B : Specific-stain.

V = Vesicle.

A



B

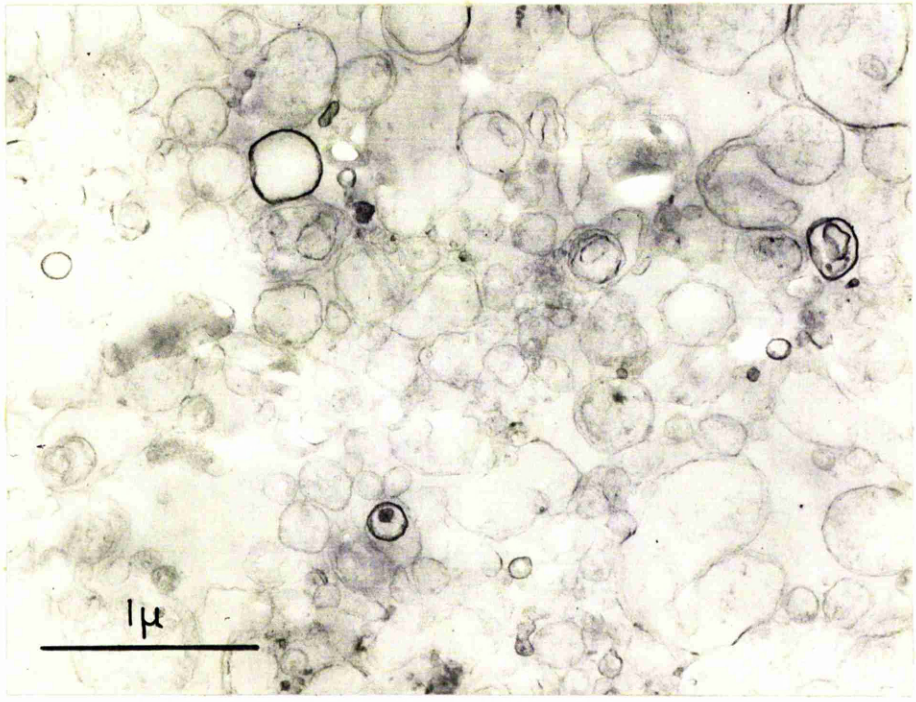


Plate 7. Electron micrograph of the 0.99M - 1.14M band.

X 28,700.

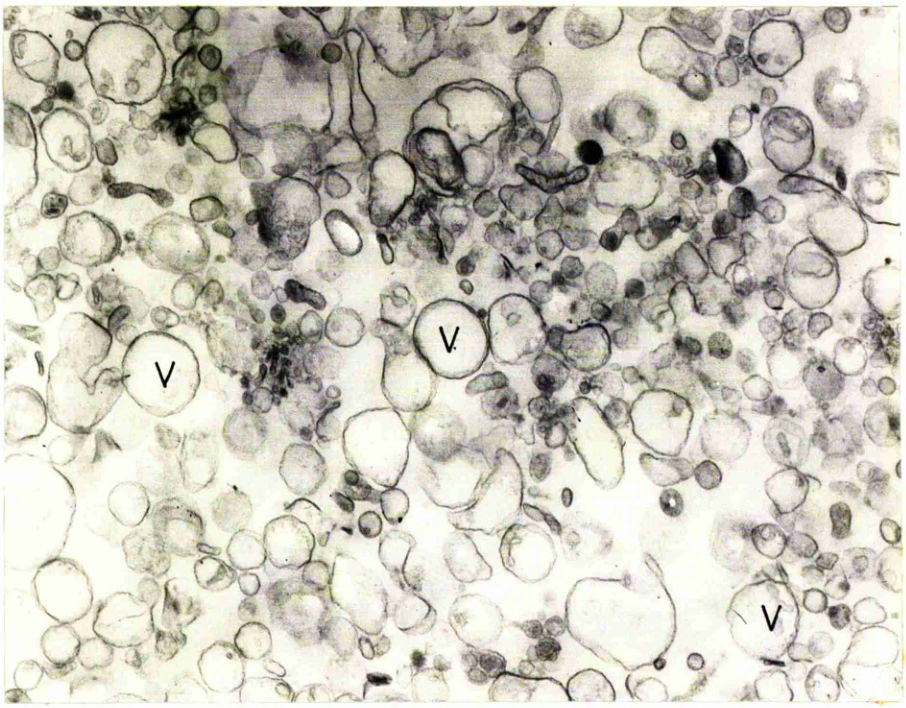
A : Lead-stain.

B : Specific-stain.

V = Vesicle.

PM = Plasma Membrane.

A



B

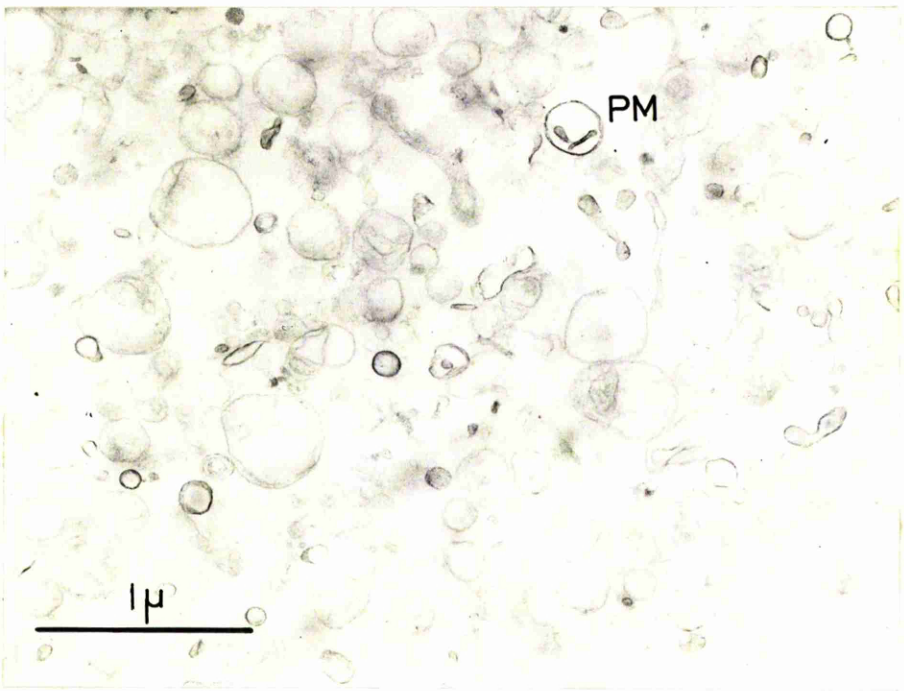


Plate 8. Electron micrograph of the 1.14M - 1.29M band.

X 28,700.

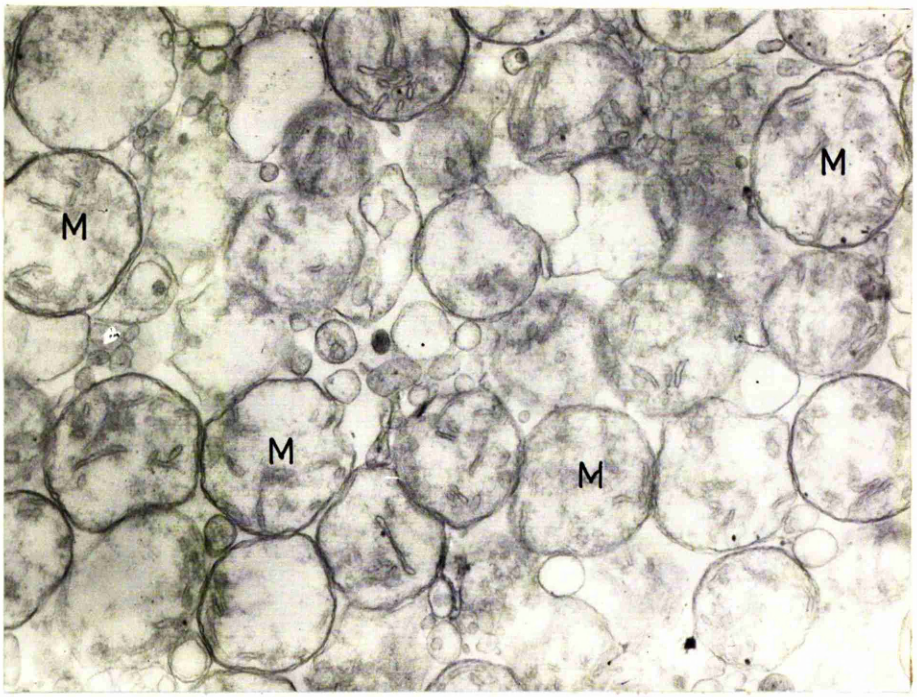
A : Lead-stain.

B : Specific-stain.

M = Mitochondrion.

PM = Plasma membrane.

A



B

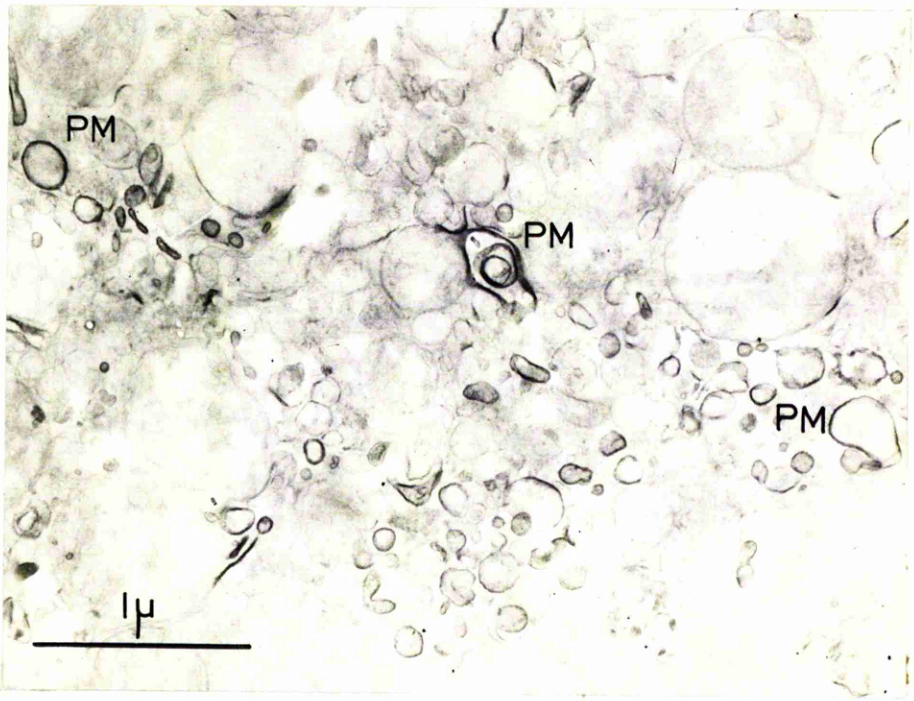


Plate 9. Electron micrograph of the 1.29M - 1.58M band.

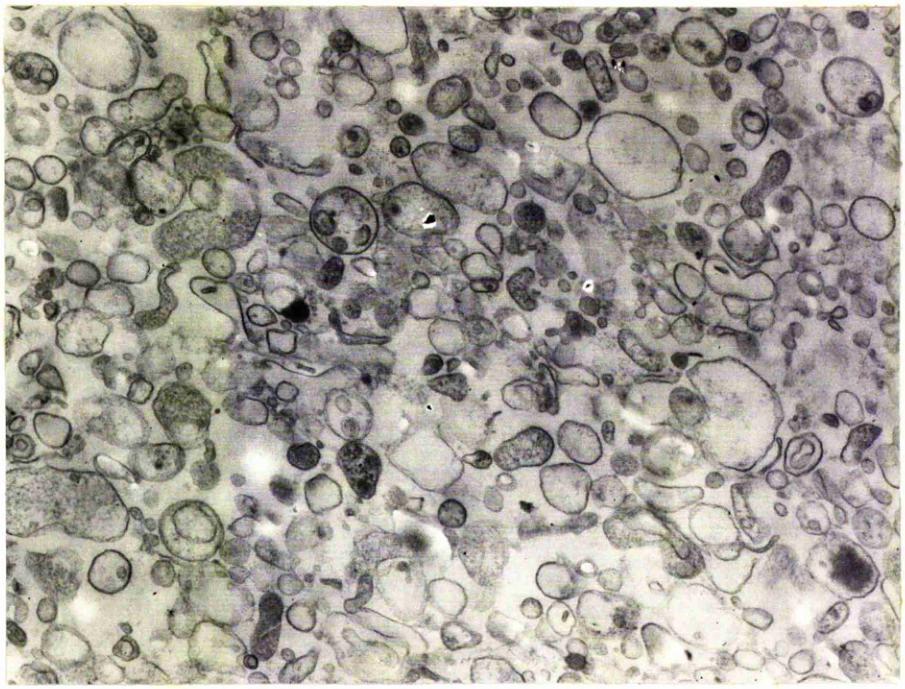
X 28,700.

A : Lead-stain.

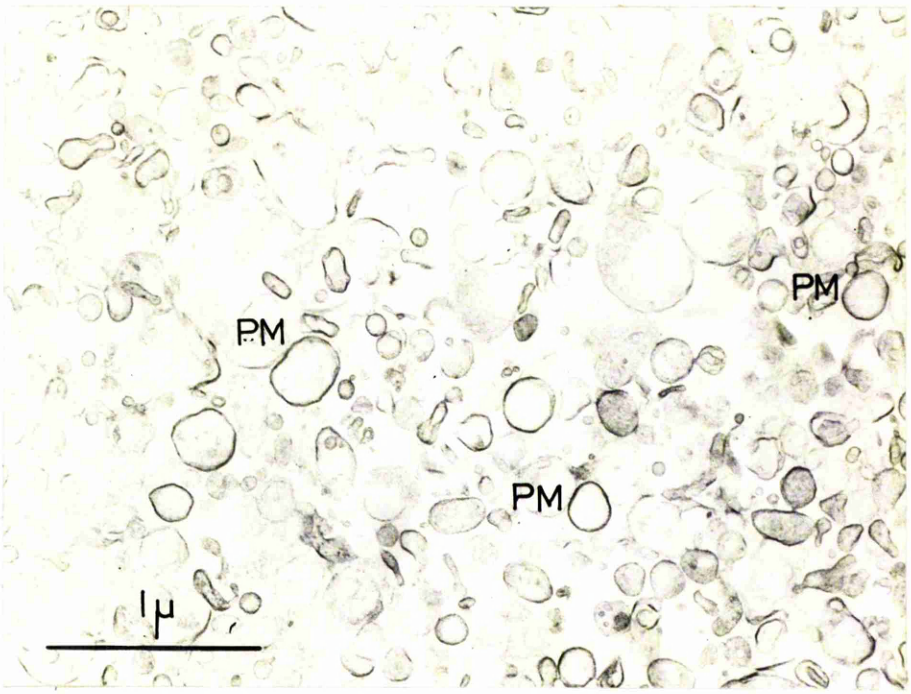
B : Specific-stain.

PM = Plasma membrane.

A



B

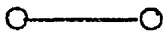


5. EFFECT OF TEMPERATURE ON BINDING ACTIVITY

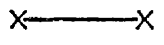
The effect of temperature on total binding activity, and on site 1 and site 2 activities in a crude pellet was examined. Site 1 activity was assayed over a range of NAA concentrations from 2×10^{-7}M to 4×10^{-7}M, and site 2 over a range of 4×10^{-7}M to 10^{-6}M. The results are shown in Fig. 35 and Table 21. After incubation at 30°C, approximately 20% of total activity is lost, and at 60°C approximately 80% is lost. Site 1 appears to be more temperature-sensitive than site 2. For example, at 30°C, 30% of site 1 activity is no longer detectable whereas only 7% of site 2 activity is lost. Site 2 becomes more labile at 40°C, when 19% of activity is lost. At 60°C 87% of site 1 activity and 62% of site 2 activity cannot be detected.

These results thus indicate that total pelletable binding activity is significantly decreased at temperatures $\geqslant$ 30°C. Site 1 and site 2 activities are different in their sensitivity to temperature, site 1 being sensitive at temperatures $\geqslant$ 20°C, and site 2 activity not decreasing until approximately 40°C.

Fig. 35 Effect of temperature on NAA-^{14}C binding activity in a 4-80 Kg membrane fraction from Zea coleoptiles.



Total binding activity, assayed between 2 x 10^{-7}M NAA and 10^{-6}M NAA



Site 1 activity, assayed between 2 x 10^{-7}M NAA and 4 x 10^{-7}M NAA

Site 2 activity, assayed between 4 x 10^{-7}M NAA and 10^{-6}M NAA.

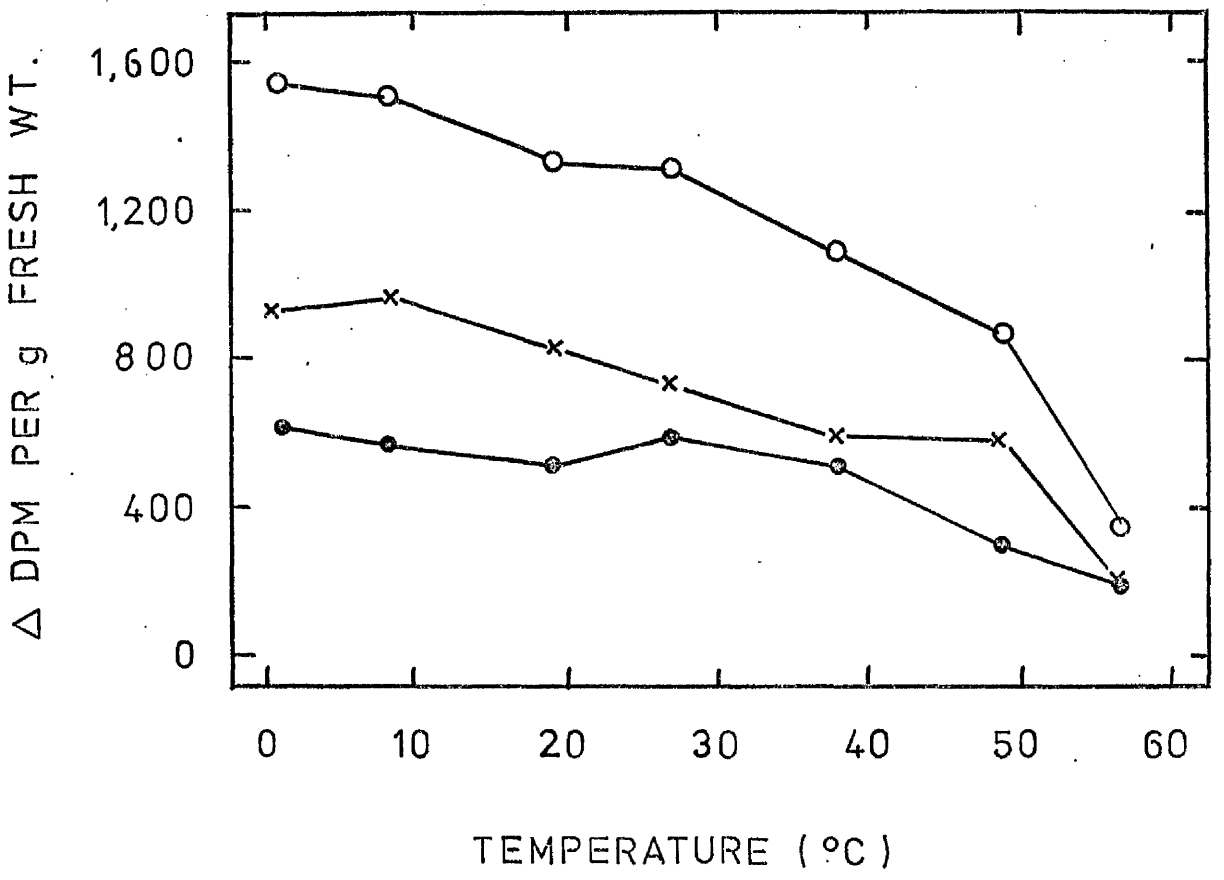

Δ DPM PER g FRESH WT.
1,600
1,200
800
400
0
0
10
20
30
40
50
60
TEMPERATURE (°C)

Table 21 Effect of temperature on NAA-^{14}C binding activity of a 4-80 Kg pellet from Zea coleoptiles.

Binding activity is expressed as % of that obtained at $0^{\circ}C$.

Data represent the mean $\pm$ S.E. from three experiments.

Table 21

TEMPERATURE (°C)	BINDING ACTIVITY AS % OF BINDING AT 0°C		
	TOTAL	SITE 1	SITE 2
0	100	100	100
10	95 ± 4	92 ± 12	104 ± 12
20	93 ± 6	87 ± 4	108 ± 13
30	79 ± 6	70 ± 6	93 ± 8
40	66 ± 3	58 ± 5	81 ± 4
50	52 ± 6	48 ± 15	60 ± 13
60	21 ± 1	13 ± 4	38 ± 7

6. EFFECT OF ENZYME TREATMENTS ON BINDING ACTIVITY

In several animal hormone-receptor systems, the effect of enzyme treatments on the binding activity of polypeptide hormones and other small molecules has revealed information concerning the nature of the receptor molecules (e.g. Cuatrecasas, 1971a, 1971b; Pasternak and Snyder, 1974). The effect of trypsin digestion and phospholipase treatment of crude membrane fractions from Zea coleoptiles was therefore examined.

(1) Trypsin Digestion

The effect of trypsin digestion on binding activity in a 4-80 Kg membrane preparation is shown in Fig. 36. There is very little effect on total binding activity at trypsin concentrations up to 20 μg per ml. At a trypsin concentration of 40 μg per ml, 25% of the total binding activity is no longer detectable while increasing the trypsin concentration to 100 μg per ml results in a further 5% loss of total binding activity. At 100 μg per ml trypsin, both site 1 and site 2 show a 30% loss in binding activity. Site 1 is somewhat more sensitive to lower concentrations of trypsin than is site 2 (e.g. 25% of site 1 activity, but only 5% of site 2 activity is abolished at 30 μg per ml trypsin).

(2) Phospholipase Treatment

Typical experiments showing the effect of phospholipase treatment on the binding activity of a 4-80 Kg membrane preparation, are shown in Figs. 37 and 38. Phospholipase-A (from both Vipera russelli and bee venom) has a dramatic effect on binding activity (Fig. 37). Virtually

Fig. 36 Effect of trypsin digestion on NAA-^{14}C binding activity in a 4-80 Kg membrane fraction from Zea coleoptiles.

Total binding activity assayed between 2×10^{-7}M NAA and 10^{-6}M NAA.

Site 1 activity, assayed between 2×10^{-7}M NAA and 4×10^{-7}M NAA.

Site 2 activity, assayed between 4×10^{-7}M NAA and 10^{-6}M NAA.

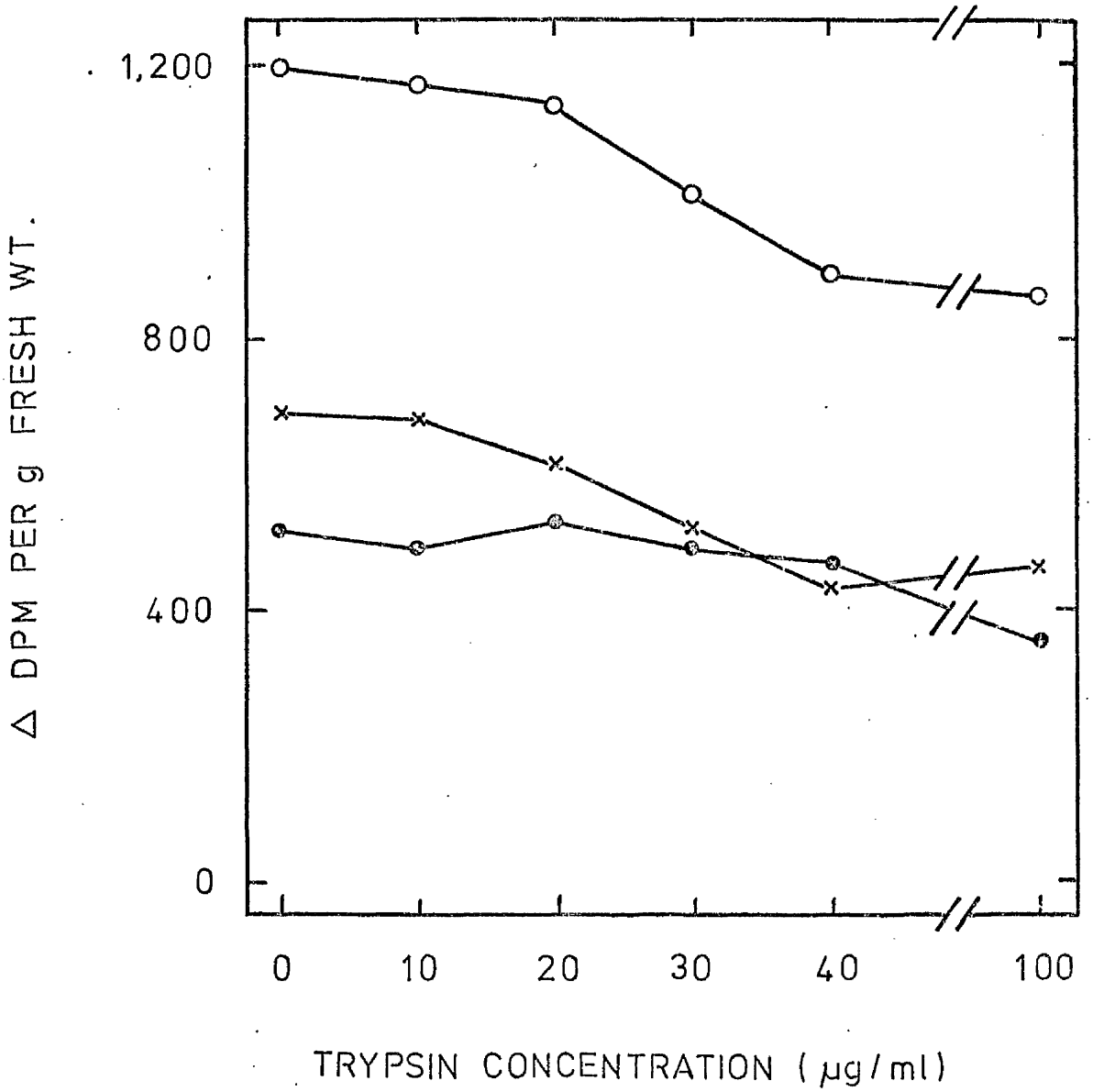

1,200
800
400
0
Δ DPM PER g FRESH WT.
0
10
20
30
40
100
TRYPSIN CONCENTRATION (µg / ml)

Fig. 37 Effect of phospholipase treatment on NAA-^{14}C binding activity in a 4-80 Kg membrane fraction from Zea coleoptiles.

A: Phospholipase-A from Vipera russelli (activity, 5 units per mg protein).

B: Phospholipase-A from Bee venom (activity 1,550 units per mg protein).

O——O Total binding activity, between NAA concentrations of 2 x 10^{-7}M to 10^{-6}M.

X——X Site 1 binding activity, between NAA concentrations of 2 x 10^{-7}M to 4 x 10^{-7}M.

⊗——⊗ Site 2 binding activity, between NAA concentrations of 4 x 10^{-7}M to 10^{-6}M.

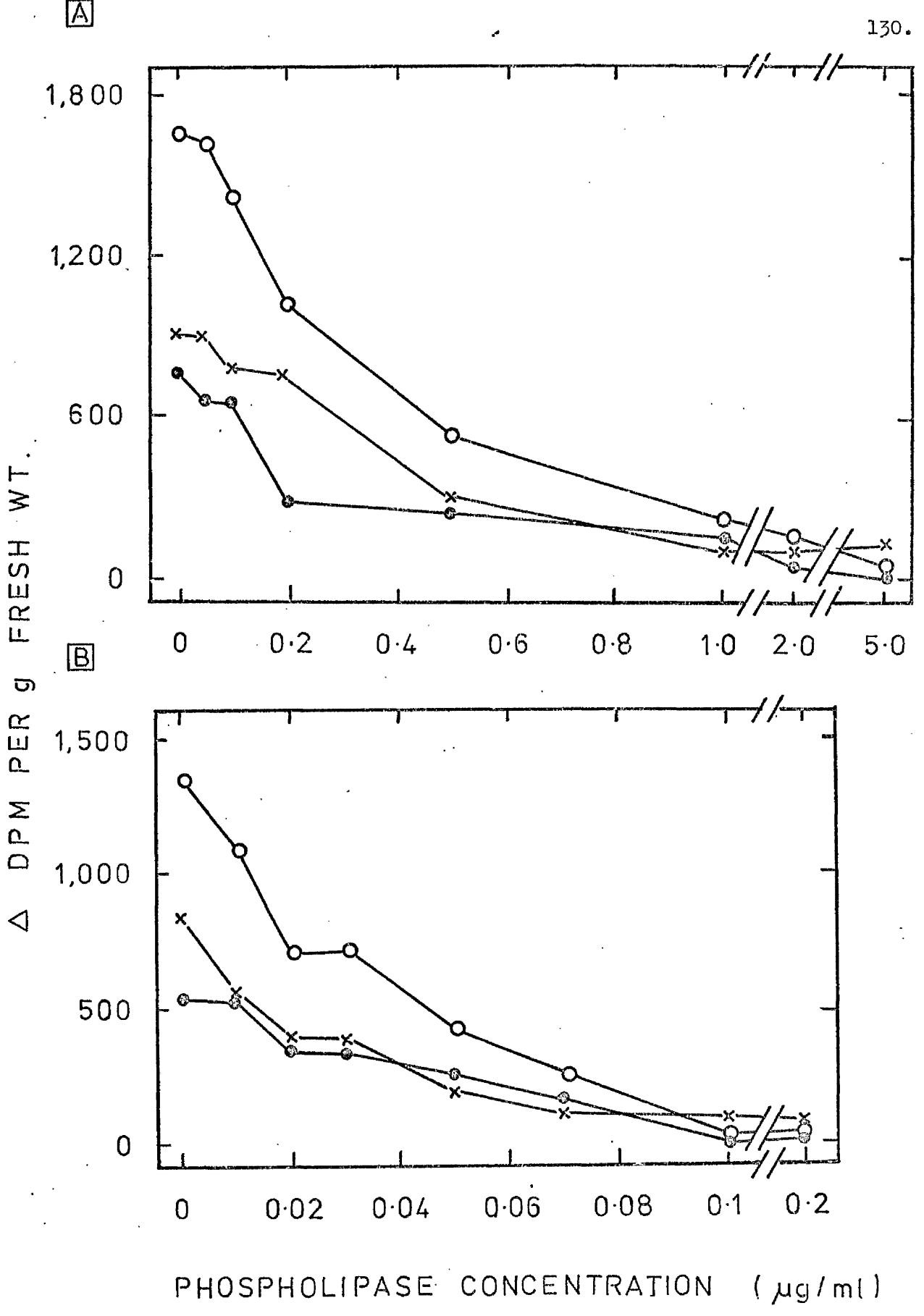

A
1,800
1,200
600
0
0
0·2
0·4
0·6
0·8
1·0
2·0
5·0
B
1,500
1,000
500
0
0
0·02
0·04
0·06
0·08
0·1
0·2
Δ DPM PER g FRESH WT.
PHOSPHOLIPASE CONCENTRATION (μg/ml)

Fig. 38 Effect of phospholipase treatment on NAA-^{14}C binding activity in a 4-80 Kg membrane fraction from Zea coleoptiles.

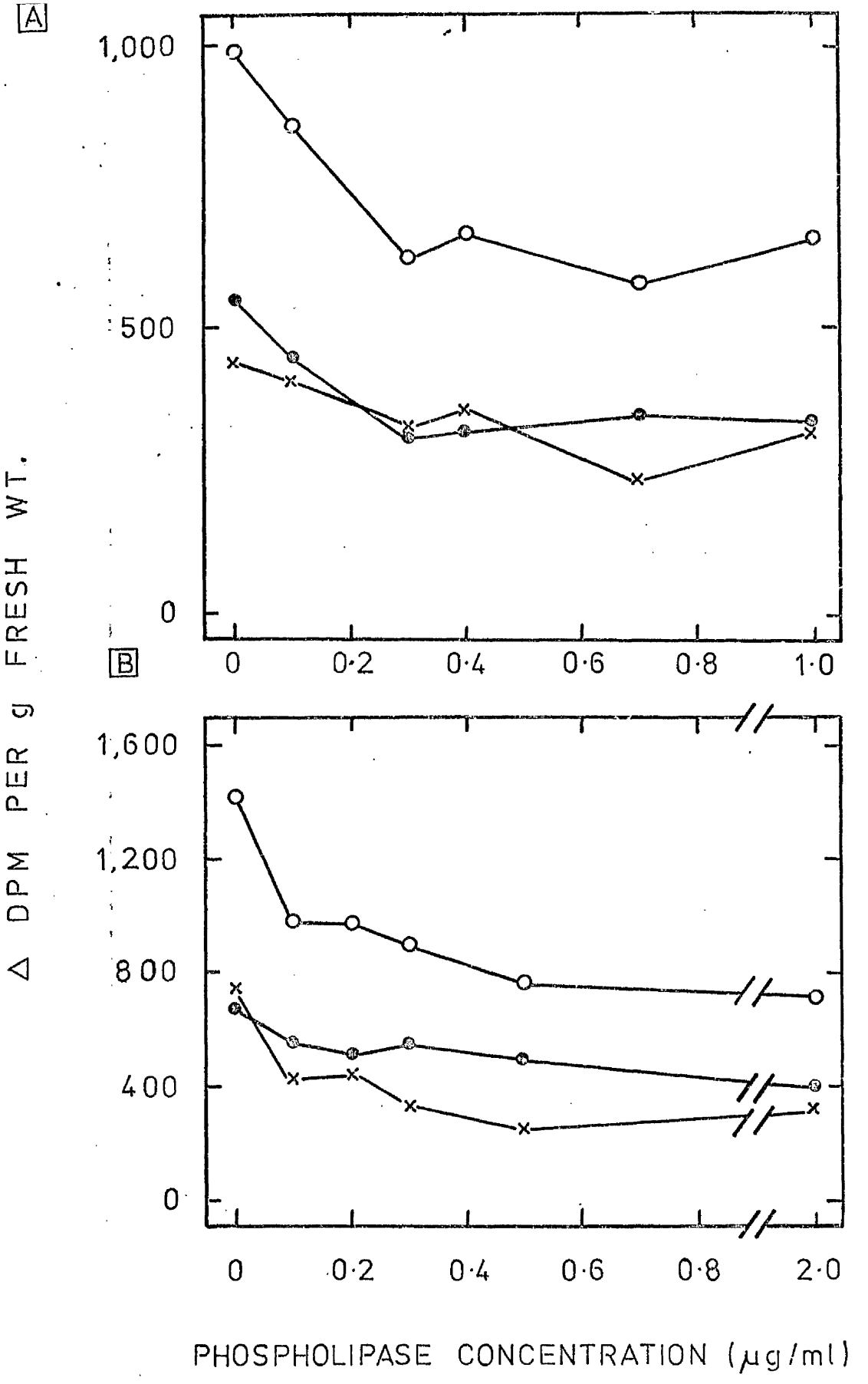
A: Phospholipase-C from Clostridium welchii (activity, 5 units per mg protein).

B: Phospholipase-D from cabbage (activity, 20-30 units per mg protein).

O——O Total binding activity, between NAA concentrations of 2×10^{-7}M to 10^{-6}M.

X——X Site 1 binding activity, between NAA concentrations of 2×10^{-7}M to 4×10^{-7}M.

⊗——⊗ Site 2 binding activity between NAA concentrations of 4×10^{-7}M to 10^{-6}M.


A
1,000
500
0
0
0·2
0·4
0·6
0·8
1·0
B
1,600
1,200
800
400
0
0
0·2
0·4
0·6
0·8
2·0
Δ DPM PER g FRESH WT.
PHOSPHOLIPASE CONCENTRATION (μg/ml)

all the binding activity is lost at enzyme concentrations of 2-5 μg per ml (Vipera russelli) and 0.1 μg per ml (bee venom). These differences in sensitivities to the two enzymes are reflections of the differing specific activities of these enzymes. It seems that both sites 1 and 2 are similarly sensitive to phospholipase-A.

Phospholipase-C and phospholipase-D (Fig. 38) do not reduce the binding activity as dramatically as phospholipase-A. Approximately 40-50% of binding activity is sensitive to phospholipase-C and phospholipase-D treatments at enzyme concentrations of 0.5 - 1.0 μg per ml. Again, both site 1 and site 2 are sensitive to these enzyme treatments.

7. NAA-^{14}C BINDING ACTIVITY IN MEMBRANE FRACTIONS PREPARED FROM ZEA MESOCOTYLS

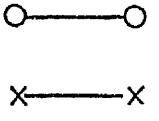
Since the harvesting of coleoptile tissue is a time-consuming and tedious process, an alternative source of membrane material was investigated. Mesocotyl tissue is responsive to auxins (Nitsch, 1956) and therefore binding activity in membrane fractions from this tissue was examined.

(1) Binding Activity in a 4-80 Kg Membrane Fraction from Mesocotyls

The binding of NAA-^{14}C to identically prepared membrane fractions from Zea coleoptiles and mesocotyls is illustrated in Fig. 39 and 40. As already noted for coleoptiles, radioactivity in the mesocotyl membrane pellet decreases with increasing NAA concentration, indicating saturation kinetics (Fig. 39). However, total binding activity of the 4-80 Kg mesocotyl fraction (on a Δdpm basis) is much lower, (Δdpm per g fresh weight = 336), than that observed in coleoptiles, (Δdpm per g fresh weight = 1,255). The Scatchard analysis (Fig. 40) suggests that there is a homogeneous population of binding sites in the mesocotyl membranes, with a dissociation constant, K, of 14.0×10^{-7}M and n-value of 67 pmole per g fresh weight.

Thus, it seems that the 4-80 Kg mesocotyl membrane fraction has only one set of binding sites with a K-value equivalent to that determined for site 2 in coleoptiles and n-value intermediate between n_1 and n_2 as determined for coleoptiles.

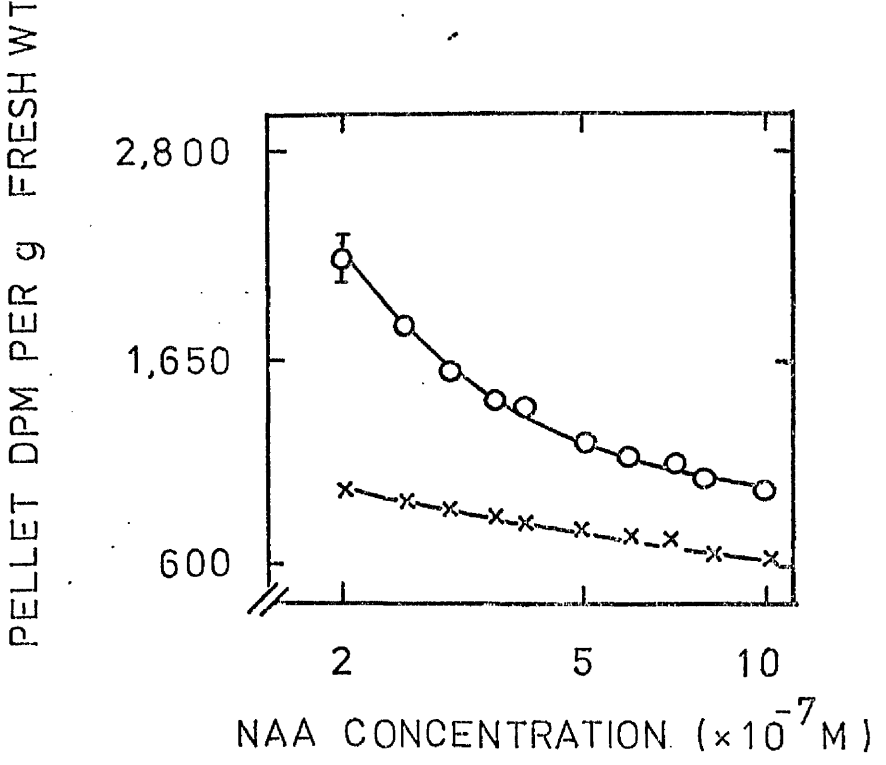
Fig. 39 Comparison of binding activities in washed 4-80 Kg membrane fractions prepared from Zea coleoptiles and mesocotyls. Each point represents the mean of three replicates.

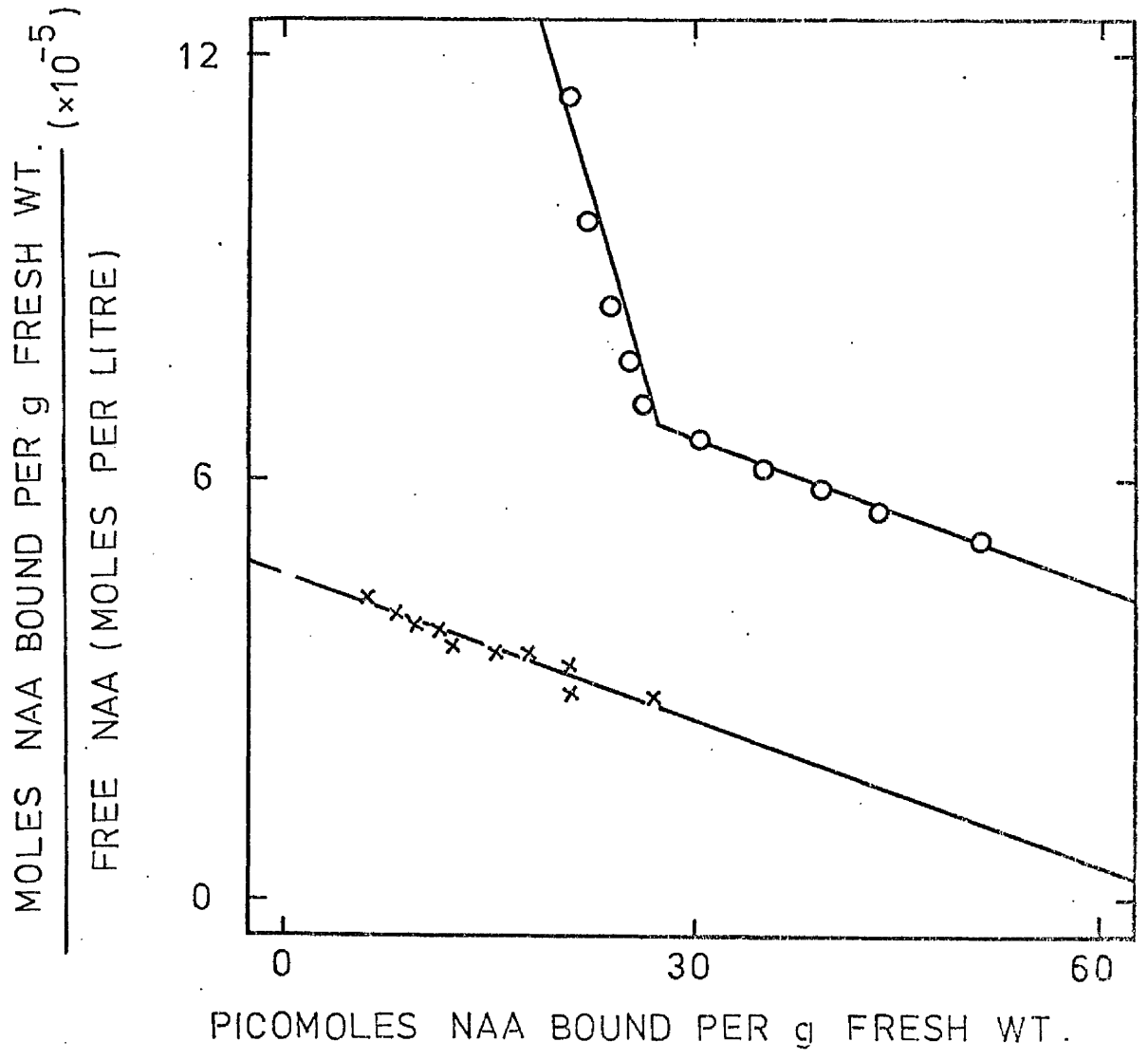


O———O coleoptiles

X———X mesocotyls

Fig. 40 Scatchard analyses of data from Fig. 39. Each point represents the mean of three replicates and lines are drawn by standard regression analyses.


PELLET DPM PER g FRESH WT
2,800
1,650
600
2
5
10
NAA CONCENTRATION (×10⁻⁷M)


MOLES NAA BOUND PER g FRESH WT. / FREE NAA (MOLES PER LITRE) (×10⁻⁵)
12
6
0
0
30
60
PICOMOLES NAA BOUND PER g FRESH WT.

(2) The Auxin-Specificity of Binding in Mesocotyl Membranes

The data of Figs. 39 and 40 indicate that the 4-80 Kg mesocotyl membrane fraction possesses binding activity which is kinetically equivalent to site 2 activity of coleoptiles. It has already been demonstrated that in coleoptiles, site 2 activity is auxin-specific (Table 11). If binding activity observed in mesocotyls is truly equivalent to the site 2 activity of coleoptiles, it should also be auxin-specific.

Competition experiments, as previously described for coleoptiles, were performed using mesocotyl membrane preparations. The results of these experiments are summarised in Table 22. The mesocotyl membranes do not show the auxin-specificity demonstrated by site 2 in coleoptiles. Compounds which had a competitive interaction for both sites 1 and 2 in coleoptiles (IAA, 2,4,5-T, 2-CPIBA and TIBA) also have a unanimous competitive interaction in mesocotyls. The Ki values in general are intermediate between the site 1 and site 2 values obtained for coleoptiles. Those compounds, however, that were "competitive" for one of the sites in coleoptiles, but showed "no competition" or "not-competitive" behaviour for the other site (2,4-D, 2,6-D, BA, 2,4-B and 2,6-B), give contradictory results in the mesocotyl experiments. Evidently, there is a lack of well-defined auxin-specificity; this can be clearly seen in the ability of the inactive compound, 2,4-B, to interact competitively in three out of five experiments. It therefore seems that the idea that mesocotyl membranes only possess site 2 activity is an oversimplification and the above data suggest the presence of a mixture of auxin-specific and auxin non-specific sites.

Table 22 Summary of the results of the competition experiments using membrane preparations from Zea mesocotyls. Mean Ki values are calculated for competitive interactions.
The numbers in the "competitive", "not competitive" and "no competition" columns represent the number of times that particular interaction occurred.

Table 22

COMPOUND	NO. OF EXPTS.	MEAN Ki (M)	COMPETITIVE	NOT COMPETITIVE	NO COMPETITION
IAA	2	6.4×10^{-6}	2	0	0
2,4-D	4	2.5×10^{-5}	2	2	0
2,6-D	3	1.6×10^{-5}	1	0	2
2,4,5-T	4	8.8×10^{-6}	4	0	0
2-CPIBA	3	3.4×10^{-6}	3	0	0
BA	3	1.9×10^{-6}	1	0	2
2,4-B	5	1.3×10^{-5}	3	0	2
2,6-B	4	2.0×10^{-5}	3	1	0
TIBA	2	2.5×10^{-6}	2	0	0

(3) Gradient Fractionation of the Mesocotyl Membranes

The results from mesocotyl experiments so far described indicate that more than one population of binding sites may be present in the high speed (4-38 Kg, 10-38 Kg or 4-80 Kg), membrane pellet but that these sites cannot be distinguished kinetically in the crude pellet. Thus, sucrose-gradient-fractionation techniques, as already described for coleoptiles, were used in an attempt to determine if more than one population of binding sites was present.

As already seen in Table 15, very little binding activity, but most of the SDH activity, is lost in the low speed (1-10 Kg) mesocotyl pellet. Also, from Table 19, it appears that the high-speed pellet, fractionated on a "complex" gradient, has a similar distribution of marker enzymes to coleoptiles. These data suggest that the membrane fraction prepared from mesocotyls is equivalent in relative membrane composition, at least, to the fraction obtained from coleoptiles.

Fig. 41 shows the distribution of binding activity in a mesocotyl membrane preparation, fractionated on a "complex" gradient. As seen with coleoptiles, most of the binding activity (66%), is in the light bands (0.56M - 0.81M and 0.81M - 0.99M) of the gradient.

The binding activity in a mesocotyl preparation, fractionated on a "simple" gradient is shown in Fig. 42. Again, more binding activity is found in the light band than in the heavy band. The Scatchard analysis (Fig. 43), however, demonstrates that two sets of sites, which are kinetically different, are separated on the "simple" gradient.

Fig.41 Distribution of binding activity in a 4-38 Kg membrane fraction from Zea mesocotyls, fractionated on a "complex" gradient. The range of NAA concentrations was 3×10^{-7}M to 8×10^{-7}M.

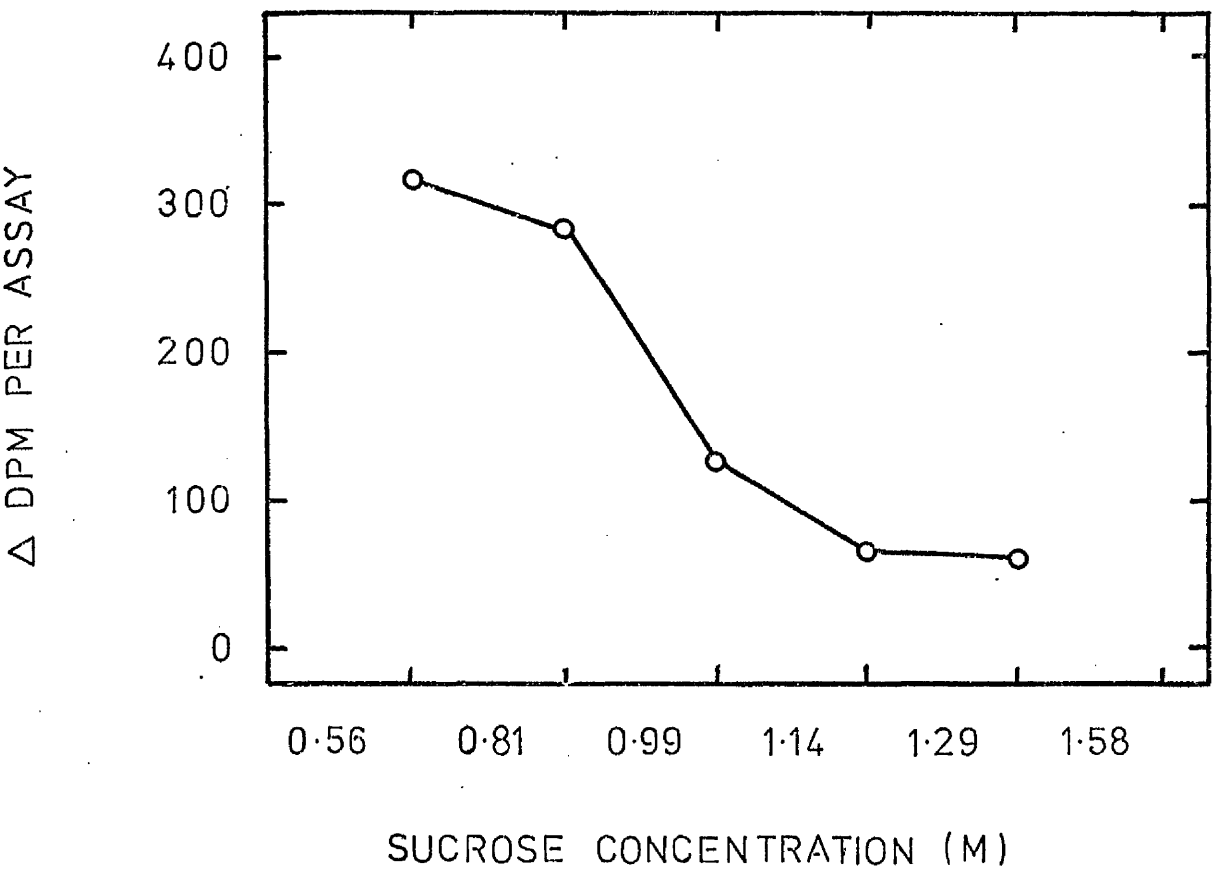

400
300
200
100
0
Δ DPM PER ASSAY
0·56
0·81
0·99
1·14
1·29
1·58
SUCROSE CONCENTRATION (M)

Fig. 42 Binding activity of a 4-38 Kg pellet from Zea mesocotyls, fractionated on a "simple" gradient. Each point represents the mean of three replicates.

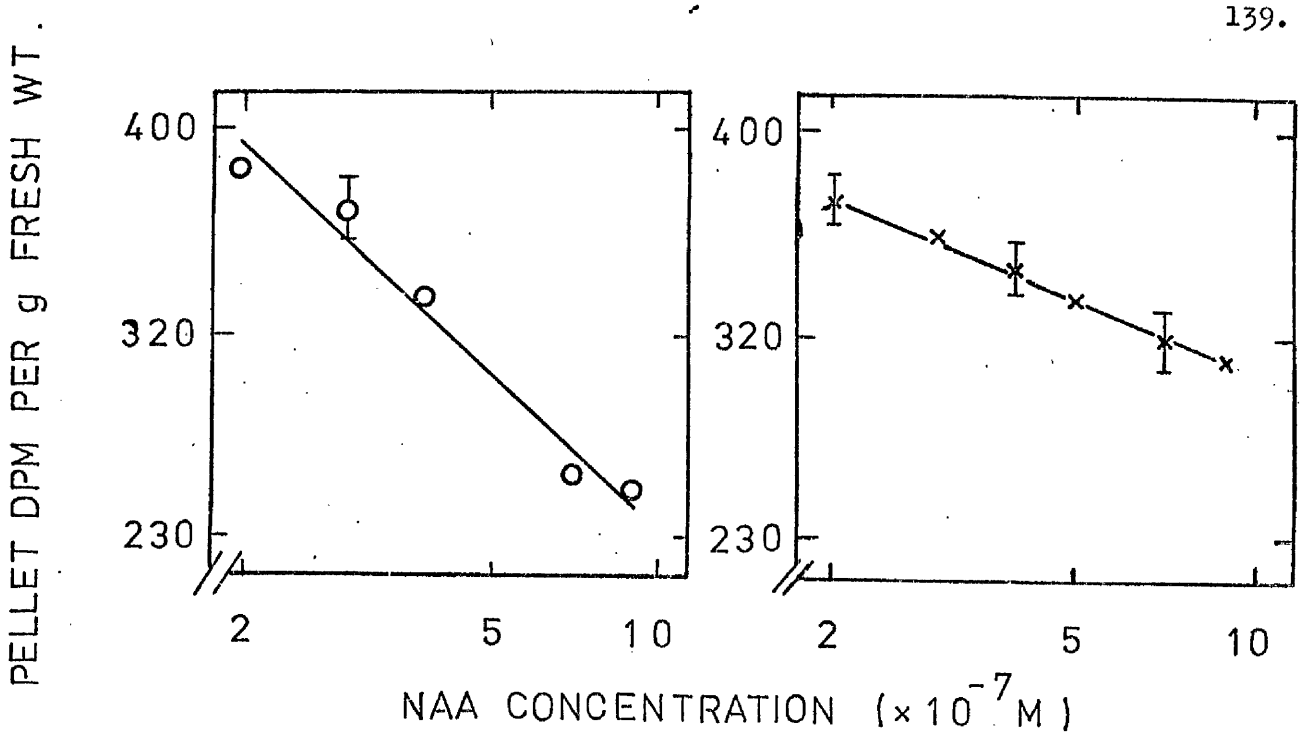
O——O 0.56M - 0.99M band

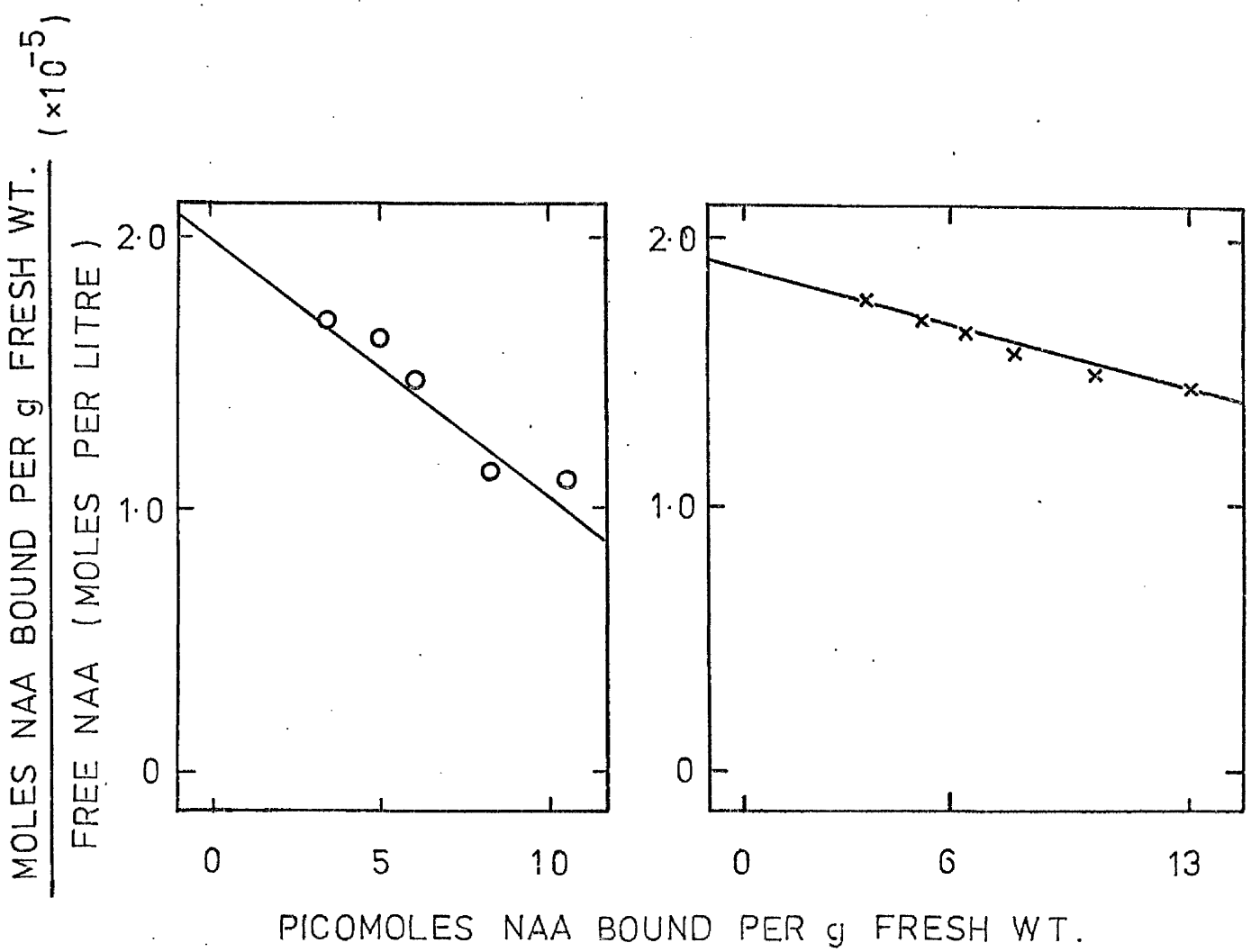
X——X 0.99M - 1.58M band

Fig. 43 Scatchard analyses of data from Fig. 42. Each point represents the mean of three replicates and the lines are computed by standard regression analyses.

O——O 0.56M - 0.99M band

X——X 0.99M - 1.58M band


PELLET DPM PER g FRESH WT.
400
320
230
2
5
10
NAA CONCENTRATION (×10⁻⁷M)


MOLES NAA BOUND PER g FRESH WT. / FREE NAA (MOLES PER LITRE) (×10⁻⁵)
2·0
1·0
0
0
5
10
6
13
PICOMOLES NAA BOUND PER g FRESH WT.

The K-values are as follows:

$\underline{0.56M - 0.99M}$: $K = 10.4 \times 10^{-7}$ M

$\underline{0.99M - 1.58M}$: $K = 30 \times 10^{-7}$ M

There is thus only a three-fold difference between the dissociation constants, and in a crude membrane fraction it is difficult to distinguish two sets of sites when the binding affinities are this similar. If, in fact, the data in Figs. 39 and 40 are analysed as two lines the following values are obtained, giving a two-fold difference in dissociation constant:

First five points : 'K_1' = 9.4×10^{-7} M

Last five points : 'K_2' = 17.4×10^{-7} M

It therefore seems that there are two sets of binding sites in the mesocotyl membrane fraction, with affinities differing only two to three-fold. Whether these fractionated sites show differences in their auxin-specificity and are equivalent to the sites in coleoptiles has not been demonstrated.

DISCUSSION

Since exogenously applied auxins have a rapid effect on elongation growth and on a number of other physiological processes (Evans, 1974a), it was suggested that the initial interaction of auxins was with the plasma membrane (e.g. Morré, 1972; Venis, 1973). A direct study of the interaction of auxins with membranes prepared from Zea mays coleoptile tissue was first reported by Hertel et al., (1972). A more complete investigation of the properties of this interaction is described in this thesis.

It was found initially (Table 1) that NAA-^{14}C binds to crude membrane preparations from Zea mays coleoptiles, with results entirely consistent with those of Hertel et al., (1972). However, since the data showed a very low "signal:noise" ratio (e.g. 500 dpm specifically bound over a range of NAA concentrations from 2×10^{-7}M to 10^{-4}M compared with a non-specific level of 1,000 dpm, Fig. 3), it was necessary to modify the experimental procedure, in order to improve this ratio. Pestle and mortar homogenisation, modified buffers and washed membrane pellets isolated at high speed, resulted in a three to four fold improvement in the binding activity (Fig. 6), and this over a restricted NAA concentration range (2×10^{-7} M - 10^{-6} M). It is probable that mechanical homogenisation disrupts the membrane material to such an extent that small fragments, containing NAA binding sites, remain in the supernatant after centrifugation. In fact, most plant membrane isolation procedures utilise either pestle and mortar homogenisation (Hodges and Leonard, 1974) or a low-shear mechanical technique (Lembi et al., 1971), and in this study, pestle and mortar homogenisation appeared to give improved

pelletable binding activity. Hertel (1974) reported inhibition of binding activity on adding back the supernatant derived from the washing step. It is thus possible that the pellet washing procedure removes an inhibitor of binding and that coleoptiles from the original seed batch contained insignificant levels of this substance since initial studies revealed no improvement of binding activity upon washing (Table 3). Presumably, Hertel's seed was similar, in this respect, to that used in the later stages of the work described here, where a wash step was essential to obtain satisfactory binding activity (Table 4). For many experiments, a crude membrane pellet was isolated after a series of differential centrifugation steps. Since this procedure does not result in a clean separation of membrane populations, it is inevitable that some binding activity is lost both in the precentrifuged pellet and in the final supernatant. A 4 - 38 Kg membrane pellet was found to be operationally convenient to prepare and this pellet shows levels of total binding activity which are sufficient to demonstrate two sets of NAA binding sites. Later experiments, however, demonstrated improved binding activity when a higher speed (4 - 80 Kg) pellet is used and when the final pelletting step is also at high speed (150 Kg) (Fig. 6). This suggests that significant binding activity is associated with relatively light membranes and this observation is supported by the sucrose gradient fractionation experiments. In some binding experiments, particularly with the later seed batch, the 4 - 38 Kg pellet was lacking in site 1 activity and under these circumstances it was essential to use high speed pellets.

The analysis widely used for determining the binding parameters of a ligand-macromolecule interaction is that described by

Scatchard, (1949). The equation suggested is a linear transformation of the binding equilibrium equation. It was proposed to be more accurate than a double-reciprocal transformation, since the extrapolation necessary to determine the binding parameters is far easier, especially if several different classes of binding sites are present (Scatchard, 1949). A Scatchard analysis of the binding of NAA-^{14}C over a restricted concentration range ($2 \times 10^{-7}M$ to $10^{-6}M$), to a crude coleoptile membrane pellet yielded a biphasic plot, indicating the presence of two sets of high affinity binding sites for NAA (Fig. 7). From direct extrapolations, dissociation constants (K) of $1.8 \times 10^{-7}M$ and $14.5 \times 10^{-7}M$, and site concentration values (n) of 52 and 101 pmole per g fresh weight of tissue, were obtained for site 1 and site 2 respectively. At higher NAA concentrations (Fig. 5), a low affinity ($K \simeq 10^{-5} - 10^{-4}M$), possibly nonspecific binding at a large number of sites, was detected. Since it would have been necessary to use nonphysiological levels of NAA, the properties of this binding activity were not further investigated. The values of K and n when a wide range of NAA concentrations ($2 \times 10^{-7}M - 10^{-4}M$) was used (Hertel et al., 1972; Hertel, 1974; Fig. 5) are either intermediate between site 1 and site 2 values or almost equal to the site 2 parameters. It was demonstrated (Fig. 7) that the range of NAA concentrations necessary to determine accurately the parameters for site 1 and site 2 is $10^{-7}M$ to $10^{-6}M$. Thus, the original data were unable to distinguish the two sets of sites. In fact, in the published experiments (Hertel et al., 1972; Hertel, 1974) there were usually only two or three points within the concentration range, $10^{-7}M - 10^{-6}M$, and these were often selected such that site 1 was completely missed and only a single, homogeneous population of binding sites was apparent.

From the saturation kinetics of physiological auxin effects, such as growth promotion or polar transport, it is possible to make rough estimates of the maximum affinity of auxin for its binding site. For example, the "K" determined from transport data is approximately 5×10^{-6}M (Hertel and Leopold, 1963) and that determined from growth studies is 10^{-7}M. - 10^{-6}M (see Housley, 1961). These estimates are of the same order as the disscciation constants determined for the *in vitro* binding of auxins and this correlation lends support to the claim that the observed *in vitro* interaction has physiological significance and may be associated with the primary auxin receptor(s) of the transport and growth systems.

Since IAA has been proved to be an endogenous auxin of *Zea mays* coleoptiles (Greenwood *et al.*, 1972) it would have been ideal to use this auxin in the *in vitro* binding studies. The *in vitro* binding of IAA tc crude coleoptile membrane fractions could be demonstrated (Fig. 10) but the total binding activity is five to ten fold lower than that obtained using NAA (Table 7). Scatchard plots (Fig. 11) revealed that two sets of binding sites are present and that the lower binding activity is reflected by lower binding affinities while the site concentration values are the same as those obtained for NAA. Similar results were reported by Hertel *et al.*, (1972). Other radioactive auxins which were tested for *in vitro* binding activity also gave poor results when compared to NAA (Table 7). Since it is very difficult to estimate kinetic parameters with any degree of accuracy when the total binding activity is so low, it was decided to use NAA-^{14}C in all subsequent experiments.

In the original auxin-binding study (Hertel et al., 1972), it was stated that "the in vitro binding site should be specific in that only growth-active transportable auxins and competitive antiauxins or transport inhibitors, but no inactive analogues should interact with the receptor". The question of the auxin-specificity of the NAA binding sites has been considered in detail in this study. The auxins and analogues were selected to include a range of reportedly growth active and inactive compounds with only slight differences in their structure (e.g. 2,4-D and 2,6-D; 2,4-B and 2,6-B), an antiauxin (2-CPIBA) and inhibitors of polar auxin transport (TIBA, NPA). In general, the growth activity of the compounds, as determined from standard bioassays, agrees with previously published data (Jönsson, 1961), the principal exception being 2,6-B which only shows growth activity in the pea internode bioassay at a concentration of 10^{-4}M (Table 9). During the early studies on the structural requirements which a molecule must satisfy in order that it have physiological auxin activity, it was demonstrated that while benzoic acid itself was inactive, ortho-substitution by chlorine atoms conferred auxin activity on the molecule. Activity was reported to increase after 2-chloro-, 2,6-dichloro- and 2,3,6-trichloro- substitutions (Jönsson, 1961; Audus, 1972). However, these studies often utilised chemicals whose purity was not rigorously established and the discrepancy in the published data for 2,6-B and the results from the corn and oat coleoptile tests reported here, might be explained in terms of contaminated chemicals in the original experiments. The compounds which show growth activity in the corn and oat coleoptile straight growth tests are also active in the rapid growth assays. The ability of IAA, NAA and 2,4-D to promote rapid elongation in corn coleoptiles has been previously reported

(Hertel et al., 1969). These workers also demonstrated the antiauxin activity of 4-chlorophenoxyisobutyric acid (4-CPIBA), a compound closely related to 2-CPIBA. The ability of NPA and TIBA to inhibit polar auxin transport in corn coleoptiles and the inability of NPA to affect rapid elongation has also been established (Thomson et al., 1973). Because of the variability between the four assay procedures, no attempt is made to assign an order of activity to the compounds.

The limited availability of radioactive auxins and analogues and the low binding activity of those which are obtainable, made a direct analysis of the auxin-specificity of in vitro binding difficult. An indirect approach, using principles of enzyme - inhibitor studies (Dixon and Webb, 1958) has therefore been used. This approach has been successfully utilised elsewhere, for example in studies of the effect of morphactins on NPA binding in corn coleoptile membranes (Thomson and Leopold, 1974) and the effect of acetylcholine on ^{3}H-nicotine binding to fractions prepared from housefly heads, or the nerve cord of the Madagascan cockroach (Aziz and Eldefrawi, 1973). Hertel et al., (1972) investigated auxin-specificity by adding increasing concentrations (up to 5×10^{-4} M) of the unlabelled analogue and observing whether NAA-^{14}C was displaced from the crude membrane pellets. Several arguments against this approach are apparent. Firstly, it now seems that either a mixture of site 1 and site 2 binding activities, or site 2 activity alone was detected in the early studies and secondly, the different sites do not necessarily show the same auxin-specificity. Also, the various analogues, at high concentrations, may have spurious effects on the membranes, which are unrelated to the NAA binding sites. In the competition experiments described in this study (Figs. 15 - 24), the effect of the analogues (at

concentrations $\leq 10^{-5}$ M) on the displacement of NAA-^{14}C by unlabelled NAA represents a direct effect on the in vitro binding reaction which is known to be taking place.

Another advantage of the approach used here is that it is possible to estimate dissociation constants (or, more precisely, inhibitor constants) for the various compounds. These were calculated from a weighted regression analysis (Wilkinson, 1961) of the data which is presented on double-reciprocal plots. The use of these plots for determining binding parameters has been criticised (e.g. Scatchard, 1949) since nonlinearity (which is apparent when the simple situation of a homogenous class of independent binding sites does not apply) may be overlooked. In this study, it was clear from either Scatchard, or double reciprocal plots, that two sets of binding sites exist in crude coleoptile membrane fractions. Each binding site may be analysed independently, since it is relatively easy, even though rather subjective, to assign the points of the total curve to site 1 or site 2. The graphical representation of the data on double-reciprocal plots has been discussed in the results section.

Using the enzyme inhibitor approach, and the analysis discussed, it became evident (Figs. 15 - 24) that site 2 shows the binding specificity compatible with the expected properties of an auxin-receptor as defined previously by Hertel et al., (1972). Site 1, on the other hand, was less specific, this being particularly demonstrated by the ability of the inactive compounds, 2,6-D and BA to interact competitively for the binding sites, and by the variable behaviour of 2,4-D in its site 1 interaction (Table 11). The similarity between the site 1 and site 2 K_i values for

IAA determined from competition studies (Fig. 15) and the dissociation constantsdetermined from direct binding experiments (Fig. 11) is important since it supports the claim that the NAA and IAA binding sites are truly equivalent and represent a receptor for all molecules with physiological auxin activity. The auxin-specificity of the binding sites described by Hertel et al., (1972) corresponds to the properties established for site 2 in the present study. On the other hand, since the previous work of Hertel et al., (1972) failed to distinguish two sets of binding sites (for reasons already discussed), the ability of inactive compounds such as BA and 2,6-D, to compete for site 1 binding was overlooked.

The observation that NPA interacts competitively with both site 1 and site 2 (Fig. 24) conflicts with published data which failed to demonstrate any displacement of NAA-^{14}C by NPA (Lembi et al., 1971; Thomson, 1972; Hertel et al., 1972). Again, the inability to demonstrate the competition by NPA due to site 1 interaction is not surprising since site 1 activity was not detected in the earlier studies. However, the interaction with the auxin-specific binding site (site 2) also seems to have been missed. It should be noted that the NPA binding interactions demonstrated here (Fig. 24) are relatively weak (K_i values of 6.6×10^{-6}M and 1.6×10^{-5}M for site 1 and site 2 respectively), while studies on direct binding of NPA (Thomson, 1972) showed that the compound binds with high affinity ($K = 10^{-8}$M - 10^{-7}M) to plasma membrane sites. This would suggest that there are NPA binding sites distinct from the two sets of auxin binding sites.

The interactionsof exogenously applied phytohormones in physiological systems have received much attention (e.g. Wareing et al.,

1968; Galston and Davies, 1969). However, the results obtained are very difficult to interpret, since the simple observation that two growth substances have synergistic, or antagonistic effects in a given physiological system, does not necessarily indicate that they are acting at the same point in that system. The demonstration that different growth substances interact with the same receptor molecule would be a rather more direct indication of the antagonistic or synergistic action of those substances. However, it may not be necessary that the growth substances interact with the same receptor molecules in order that one should modify the effect of the other. Perhaps the receptors for two different growth regulators are distinct but connected by, for example, being localised on the same membrane system. In this case, the binding of one of the substances to its receptor could modify, either positively or negatively, the binding of another substance to its respective receptor.

IAA and ABA antagonism on the phytochrome-mediated attachment of mung bean root tips to glass has been reported (Tanada, 1973a, 1973b). Low concentrations (10^{-9}M) of IAA and ABA have a rapid effect on this phenomenon, IAA inducing detachment and ABA inducing attachment of the root tips. The opposite effect of the two hormones is observed with barley root tips. It was postulated that IAA and ABA both act on the plasma membrane to bring about opposing changes in the surface electric charge of plant cells. In this study (Fig. 25), it was demonstrated that ABA does not interact competitively with either of the membrane-associated NAA binding sites. This result was previously reported for both the auxin (Hertel et al., 1972) and the NPA binding sites (Thomson, 1972). Thus it appears that the antagonistic effects of IAA and ABA at the plasma membrane are probably not mediated by the auxin receptor molecules described here.

In general, the cytokinins, benzyladenine and kinetin interact in a weakly competitive manner for both site 1 and site 2 (Table 12). The only other study on cytokinin receptor molecules describes the specific binding of benzyladenine to plant ribosomal fractions (Fox and Erion, 1975). Since site 2 binding activity is thought to be localised in plasma membrane rich fractions, it is unlikely that the competition of cytokinins for site 2 and the binding of cytokinins to ribosomes are in any way related. Thus the significance of the site 2 cytokinin competition is obscure, especially since cytokinins are known to inhibit the long-term growth response to auxin (Vanderhoef and Stahl, 1975) rather than the rapid, possibly acid-mediated growth response which is suggested to stem from an auxin-plasma membrane interaction (Hager et al., 1971). One cautionary note is that the cytokinin competition studies give somewhat variable results and the statistical significance is questionable in some instances (e.g. Fig. 26, site 1).

Since the function of site 1 binding activity is unknown, it is difficult to determine the relevance of the competitive interaction of GA_3 for site 1 (Fig. 27). Very little other literature is available on gibberellin receptor molecules, the only significant study being the isolation of protein fractions from pea stems which have high affinity for radioactive gibberellin A_1 (Stoddart et al., 1974). The subcellular origin of these proteins was not determined and thus no parallels can be drawn between this study and the observations on gibberellin competition. GA_3 did not interact with either the auxin, or the NPA binding sites in other studies (Hertel et al., 1972; Thomson, 1972), and this is consistent with the inability of GA_3 to interact competitively for site 2 in this

study (Fig. 27). Again, the statistical significance of the gibberellin results for site 2 is not always conclusive (e.g. Fig. 27, site 2).

If, as has been suggested, an auxin-plasma membrane interaction is the primary process in auxin-induced growth (Hager et al., 1971), and that in vitro auxin binding is the manifestation of this interaction, it is essential to demonstrate that the auxin-specific binding is to plasma membranes, as opposed to some other membrane fraction. Very little evidence for auxin binding specifically to plasma membranes was presented in the early reports (Hertel et al., 1972), and so this question has been studied in some detail here. Another question which suggested itself was whether site 1 and site 2 are associated with the same or different membrane fractions.

The total binding activity detected in the very low speed pellet (0 - 1 Kg) is insignificant (Table 13) and since this pellet consists mainly of starch, unbroken cells, cell wall fragments and DNA (Hertel et al., 1972) it is unlikely that binding to nuclei or cell walls contributes to auxin-specific binding. The pellets obtained from the precentrifugation step of the crude membrane preparation procedure contain 10 - 20% of the total binding activity of the higher speed pellets (Table 13), and this activity has the kinetic properties of site 2 (Fig. 29). These pellets also contain 60 - 70% of the specific SDH activity (Table 14), a marker enzyme for mitochondria (Morré, 1971). However, site 2 activity is not specifically associated with mitochondrial membranes since there is no enrichment of site 2 binding activity in SDH-enriched crude pellets (Table 14) or in SDH-enriched bands from sucrose gradient fractionated mitochondrial pellets (Table 16). Also, 10 - 38 Kg pellets which are substantially depleted in mitochondria still show

significant site 2 activity (Fig. 29). The site 2 binding activity detected in the low speed precentrifugation pellets probably reflects binding to heavier members of the membrane population found in the high speed pellets. Auxin binding activity was also detected in fractions virtually free from mitochondria as assayed by cytochrome-c oxidase activity (Hertel et al., 1972).

Further fractionation of the crude membrane pellet on 'complex' sucrose gradients revealed that approximately 60% of the binding activity is in the light regions of the gradient and 40% in the heavier bands (Fig. 30). A kinetic analysis is facilitated if the complex gradient bands are condensed, as in the 'simple' gradient. Such a kinetic analysis demonstrated that site 1 activity is associated with the light bands and site 2 with the heavier bands of the gradient (Fig. 32). That the fractionated bands represent site 1 and site 2 was further demonstrated since the light and heavy bands retain the binding-specificity determined for site 1 and site 2 respectively in crude membrane fractions (Figs. 33 and 34). The somewhat higher K and K_i values determined for the light band (generally, two-to-three fold higher than site 1 in the crude pellet) may indicate that the sites are not completely separated and that there is some contamination in the light fraction with site 2. Thus it appears that at least a partial separation of site 1 and site 2 activities can be achieved and that the two sites are associated with membrane fractions which band at different regions of the sucrose gradient.

It is only recently that the characterisation of plant membrane populations has received much attention. This contrasts with the situation in animal cells where membrane fractions, especially plasma

membranes, are readily identified on the basis of distinctive morphological and biochemical characteristics (Emmelot et al., 1974). However, some advances in plant membrane characterisation have been made, and the following properties assigned to the various fractions: (1) Golgi membranes are characterised by latent IDP-ase activity and β-glucan synthetase activity at low (1.5 μM) UDPG levels (Ray et al., 1969; Van der Woude et al., 1974) (2) NADH cytochrome-c reductase is associated with endoplasmic reticulum (Hodges and Leonard, 1974) (3) plasma membranes are identified by the presence of relatively high activities of β-glucan synthetase at high (1 mM) UDPG levels (Van der Woude et al., 1974); monovalent cation stimulated ATP-ase at pH 6.0 (Hodges and Leonard, 1974); high sterol:phospholipid ratio (Keenan et al., 1973); and vesicles which react specifically to a PTA-chromic acid staining procedure (Roland et al., 1972).

Some of these properties were used in the present study to determine the nature of the membranes which contain site 1 or site 2 binding activity. The distributions of latent IDP-ase, β-glucan synthetase (1mM UDPG), ATP-ase (pH 6.0) and the sterol:phospholipid ratio, in complex gradient fractionated coleoptile and mesocotyl membranes shown here (Tables 19 and 20), agree remarkably well with those reported for oat roots (Hodges et al., 1972), onion stems (Van der Woude et al., 1974) and soybean hypocotyls (Hardin et al., 1972). When lead citrate is used as a section stain for plant membrane fractions, it is impossible to distinguish accurately and consistently membrane populations on the basis of staining quality (Roland et al., 1972). However, the use of the PTA stain to identify plant plasma membrane fractions has been reported in corn coleoptiles (Lembi et al., 1971), oat roots (Hodges et al.,

1972), maize roots (Leigh et al., 1975), soybean hypocotyls (Hardin et al., 1972) and onion stems (Van der Woude et al., 1974). In this study, vesicles which react with the PTA stain were found to be enriched in the 1.14 M - 1.29 M and the 1.29 M - 1.58 M sucrose interfaces, with a few vesicles in the 0.99 M - 1.14 M band. In several other studies, attempts have been made to estimate the plasma membrane content of various membrane fractions by techniques of quantitative electron microscope morphometry (Lembi et al., 1971; Hodges et al., 1972; Hardin et al., 1972; Van der Woude et al., 1974). However, the technique is very subjective, since it is not always absolutely clear whether a particular membrane vesicle is specifically stained. In this study, therefore, the plasma membrane vesicles were distinguished only by their qualitative differences. Current attempts to develop a specific colorimetric technique for plasma membranes (Hodges, personal communication) will be of considerable value in the quantitative analysis of these fractions. Due to tissue variations and slight differences in gradient composition in the reported studies, it was not possible to assign membrane fractions to absolute sucrose density values. However, a general observation was that plasma membrane enriched fractions are found in the heavy regions, of sucrose concentration ranging from 1.1 M - 1.6 M, of discontinuous sucrose gradients. The lighter regions are less well characterised, although they do appear to contain a Golgi-enriched zone and there is some evidence for high endoplasmic reticulum content (Dr. M.A. Venis, personal communication).

It has been demonstrated that site 1 and site 2 activities are localised in the light and heavy regions respectively of the sucrose gradients (Fig. 32). Thus, within the present limitations of plant

membrane identification, it may be stated that the auxin-specific (site 2) *in vitro* binding activity of coleoptiles is associated with plasma membrane-enriched fractions. This has not been demonstrated in any previous study, although the association of the NPA binding sites with plasma membranes has been reported (Lembi *et al.*, 1971). Site 1 activity appears to be associated with a membrane fraction containing Golgi and possibly endoplasmic reticulum membranes. A preliminary demonstration of auxin binding activity in endoplasmic reticulum fractions has also been reported (Hertel, 1974).

There is very little evidence available as to the nature of the auxin receptor molecules. Membranes are known to consist of three major components - proteins, lipids and oligosaccharides (e.g. Singer and Nicolson, 1972), any of which could have the necessary receptor properties, either alone or, more likely, in combination with one of the other components. In many of the studies on animal hormone receptors, this question has been approached using various techniques such as detergent solubilisation of the receptors from membrane fractions (Cuatrecasas, 1974), examination of the binding properties in the presence of specific modifying reagents such as iodoacetamide and p-chloromercuribenzoate (Wilson *et al.*, 1975) and examination of the sensitivity of the binding reaction to temperature and various enzymatic treatments (Cuatrecasas, 1971a, 1971b; Pasternak and Snyder, 1974).

In this study, it was demonstrated that 0.2% Triton-X-100 solubilises 87% of the total binding activity of crude membrane fractions (Table 6). However, since a convenient, sensitive assay for the solubilised binding activity could not be found, no further character-

isation of the activity was possible. The effect of temperature, trypsin digestion and phospholipase treatment on site 1 and site 2 activity in crude membrane fractions was also studied. At 60°C, 80% of the total binding activity is no longer detectable and site 1 appears to be more temperature sensitive than site 2 (Table 21). Both site 1 and site 2 are relatively insensitive to trypsin digestion with only 30% lost at a trypsin concentration of 100 µg per ml (Fig. 36). Treatment with low concentrations of phospholipase-A abolishes site 1 and 2 binding activities whereas only 40 - 50% is sensitive to phospholipases-C and D (Figs. 37 and 38). Whether these enzyme effects are on the total number of receptor molecules or on their affinity has not been investigated. These data give preliminary evidence on the nature of the receptor molecules and suggest that the receptors for site 1 and site 2 binding are within a membrane complex and require the integrity of both phospholipids and proteins for activity.

The experiments on mesocotyl tissue were originally performed merely in an attempt to facilitate experimental procedure. The initial observation was that only one set of sites, kinetically equivalent to site 2 in coleoptiles, is present in the crude mesocotyl preparations (Fig. 40). This was encouraging since, if it could be shown that this binding was truly equivalent to the auxin-specific, site 2 activity of coleoptiles, it would represent a convenient method of preparing membrane fractions free from site 1 activity. However, the competition experiments showed that this is not the case (Table 22), and that the crude mesocotyl fractions contain a mixture of auxin specific and nonspecific sites. Sucrose gradient fractionation demonstrated that there are two sites which can be distinguished kinetically but the

dissociation constants differ by only two to three fold (Fig. 43). Although, from marker enzyme studies (Table 20), it seemed that the mesocotyl membranes are similar in composition to the equivalent coleoptile preparation, it has not been demonstrated that the mesocotyl membrane binding sites in the light ($K \approx 10^{-6}M$) and heavy ($K \approx 2\text{-}3 \times 10^{-6}M$) bands are equivalent to site 1 and site 2 respectively of coleoptiles. An investigation of the auxin specificity of gradient-fractionated mesocotyl membranes should provide this information.

The data described in this thesis therefore provide convincing evidence for the *in vitro* binding of auxins to membrane fractions from *Zea mays* shoot tissue. The possible relevance of the auxin-specific, plasma membrane associated site 2 binding activity of coleoptiles to the theories on the mode of auxin action in elongation growth will be discussed below. However, the physiological importance of site 1 binding activity is less obvious. Since it is not auxin-specific, the role of site 1 binding in auxin action is questionable. Yet, it is also difficult to believe that a high affinity binding site of no physiological value would be evolved. The data presented suggest that site 1 activity may be associated with Golgi membranes, although this is only based on the activity of one enzyme. One study has demonstrated that IAA produces significant changes in the dictyosomes of expanding oat coleoptile cells and it was suggested that auxin-induced growth is mediated through the Golgi apparatus (Gawlik and Shen-Miller, 1974). Since Golgi membranes are known to be intimately connected with cell wall synthesis (Northcote, 1972), these observations merit further study.

The current weight of evidence favours the hypothesis that auxins promote rapid elongation growth via a hydrogen ion secretion mechanism (Rayle, 1973; Cleland, 1973). Because of the rapidity of this effect, an interaction of auxin with the plasma membrane has been assumed. The data presented here suggest that site 2 binding activity is an *in vitro* manifestation of such an interaction. However, it has not yet been demonstrated that the binding phenomenon *per se* produces a physiological response. Thus, further evidence is required to complete the story of auxin → plasma membrane → ? → release of H^+ → cell wall loosening → rapid elongation growth. An auxin-activated ATP-ase or proton pump has been suggested as the mediator of hydrogen ion secretion (Hager *et al.*, 1971) but direct evidence for such a pump has not been forthcoming. There is also very little evidence concerning the mechanism by which the released hydrogen ions induce cell wall loosening.

Although an interaction of auxin with the plasma membrane may be the primary response in rapid elongation growth, its relevance to long-term growth which requires protein and nucleic acid synthesis is less well-defined. There are many reports of auxin effects on protein and nucleic acid metabolism (Key, 1969) and also a growing body of evidence for cytoplasmic and nuclear receptors (O'Brien *et al.*, 1968; Matthyse and Phillips, 1969; Mondal *et al.*, 1972a). The report of Hardin *et al.*, (1972) was an ambitious attempt at reconciling rapid membrane-associated effects of auxin with longer term changes in nucleic acid metabolism. This still remains a most interesting idea, and further experimental evidence would be welcome. However, if the rapid and long-term growth responses are distinct, a reconciliatory theory may

not be necessary. In fact, a separation of the rapid acid-mediated growth response to auxin from the longer-term, cytokinin-inhibited growth response has been recently reported (Vanderhoef and Stahl, 1975). Perhaps plasma membrane receptors are necessary to the first phase of growth and the nuclear and cytoplasmic receptors important for the second phase.

Thus, this thesis provides evidence for plasma-membrane associated auxin receptors, and the importance of these receptors to rapid growth promotion by auxins is discussed. The main area where these data can be improved is in the more rigorous identification and localization of the receptor molecules. Even gradient fractionated membrane populations are heterogenous (Plates 5 - 9), and improved separation techniques would be of great value. Techniques of solubilisation and affinity chromatography have been successfully employed in the isolation of animal membrane-associated hormone receptors (Cuatrecasas, 1974) and if these can be applied to plant tissues, perhaps an auxin receptor molecule will be finally isolated and identified.

APPENDIX I

1. DERIVATION OF BINDING EQUILIBRIUM EQUATION

(after Van Holde, 1971)

Consider a macromolecule, P, with n binding sites and which reacts with a small molecule, F.

The reactions are as follows:

Reaction	Association constant
$P + F \rightleftharpoons PF$	$k_1 = \frac{[PF]}{[P][F]}$
$PF + F \rightleftharpoons PF_2$	$k_2 = \frac{[PF_2]}{[PF][F]}$
⋮	⋮
$PF_{n-2} + F \rightleftharpoons PF_{n-1}$	$k_{n-1} = \frac{[PF_{n-1}]}{[PF_{n-2}][F]}$
$PF_{n-1} + F \rightleftharpoons PF_n$	$k_n = \frac{[PF_n]}{[PF_{n-1}][F]}$

1.1

The binding experiment yields the number of moles of F that are bound per macromolecule, and since the population is heterogeneous, this is an average quantity, B.

$$\text{Thus} \qquad B = \frac{[F]\ \text{bound}}{[P]\ \text{total}} \qquad 1.2$$

From 1.1. $[F]$ bound = $[PF] + 2[PF_2] + 3[PF_3] + \ldots\ldots$ 1.3.

Since PF_2 binds 2 molecules of F etc.

and $[P]$ total = $[P] + [PF] + [PF_2] + \ldots\ldots\ldots$ 1.4.

Also, from 1.1. $[PF] = k_1[P][F]$, $[PF_2] = k_1k_2[P][F]^2$, 1.5.

From 1.3. - 1.5. a general expression for B is obtained:

$$B = \frac{k_1[F] + 2k_1k_2[F]^2 + \ldots + n(k_1k_2..k_n)[F]^n}{1 + k_1[F] + k_1k_2[F]^2 + \ldots + (k_1k_2\ldots k_n)[F]^n} \qquad 1.6.$$

To simplify this equation, it is assumed that all the sites are the same, and that the binding at any one site is independent of the occupation of other sites, such that k measures the binding of F by any one site. However, one must also consider that there will be many different forms of a molecule of P with i out of n sites occupied:

$$\text{No. of forms of P} = \frac{n!}{(n-i)!\,i!} \qquad 1.7.$$

If $[PF_i]$ represents the sum of these forms, then for each '$[PF]$' value

$$[PF_i] = k^i [P] [F]^i \qquad 1.8.$$

Equation 1.6. is now written

$$B = \frac{\sum_{i=1}^{n} \frac{i\,n!}{(n-i)!\,i!} k^i [F]^i}{1 + \sum_{i=1}^{n} \frac{n!}{(n-i)!\,i!} k^i [F]^i} \qquad 1.9.$$

From the Binomial Expression:

Denominator of 1.9. = $(1 + k[F])^n$

Numerator of 1.9. = $nk[F](1 + k[F])^{n-1}$

$$\therefore \quad B = \frac{nk[F](1 + k[F])^{n-1}}{(1 + k[F])^n}$$

$$\therefore \quad B = \frac{nk[F]}{1 + k[F]} \qquad 1.10.$$

Scatchard transformation of 1.10:

$$\frac{B}{F} = kn - kB \qquad 1.11$$

Double-Reciprocal Transformation of 1.10:

$$\frac{1}{B} = \frac{1}{F} \cdot \frac{1}{nk} + \frac{1}{n} \qquad 1.12$$

ABBREVIATIONS

IAA	3-indolylacetic acid
NAA	1-naphthylacetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
2,6-D	2,6-dichlorophenoxyacetic acid
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
2-CPIBA	2-chlorophenoxyisobutyric acid
BA	Benzoic acid
2,4-B	2,4-dichlorobenzoic acid
2,6-B	2,6-dichlorobenzoic acid
TIBA	2,3,5-triiodobenzoic acid
NPA	1-N-naphthylphthalamic acid
ABA	Abscisic acid
GA	Gibberellic acid
Ben. Ad.	Benzyladenine
ATP	Adenosine triphosphate
BDH	British Drug Houses
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
INT	2-(p-indophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium
MES	2(N-morpholino)ethanesulphonic acid
P.P.O.	2,5-diphenyloxazole
PTA	Phosphotungstic acid
TCA	Trichloroacetic acid
Tris	2 amino-2-(hydroxymethyl)propane-1,3-diol
TBS	Toluene based scintillant, containing 4 g P.P.O. per litre

TXBS	Triton-X-100 based scintillant, containing TBS and Triton-X-100 in a ratio of 2:1 (v/v)
UDPG	Uridine diphosphate-glucose
v/v	Volume for volume
w/v	Weight for volume
w/w	Weight for weight
ATP-ase	ATP-phosphohydrolase
IDP-ase	Inosine diphosphatase
SDH	Succinate dehydrogenase
µg	Microgram(s)
mg	Milligram(s)
g	Gram(s)
µl	Microlitre(s)
ml	Millilitre(s)
l	Litre(s)
nm	Nanometre(s)
µm	Micrometre(s)
mm	Millimetre(s)
pmole	Picomole(s)
nmole	Nanomole(s)
µmole	Micromole(s)
mmole	Millimole(s)
M	Molar

cpm	Counts per minute
dpm	Disintegrations per minute
mCi per mmole	Milli-curies per millimole
Fig(s).	Figure(s)
Kg	X 1,000 g = X 1,000 relative centrifugal force
rpm	Revolutions per minute
min	Minute(s)
^{o}C	Degrees Centigrade
wt	Weight
R_f	$\frac{\text{distance from origin}}{\text{distance of solvent from origin}}$ (chromatograms)
S.E.	Standard Error
P	Probability
N.S.	Not significant
K	Dissociation constant
k	Intrinsic site binding constant (an association constant)
B	Moles of bound auxin
n	Number of binding sites
F	Molar concentration of nonbound auxin = 'free' auxin
K_i	Inhibitor constant
C.O.	Crossover effect

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