# THE INTERACTING EFFECTS OF 3-INDOLEACETIC ACID, GIBBERELLIC ACID AND 6-BENZYLADENINE ON RESPIRATION, GROWTH AND NITROGEN ASSIMILATION IN EXCISED EMBRYOS OF PEA (PISUM SATIVUM L.)

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

David Cameron MacLean

1965

This is to certify that the

thesis entitled

THE INTERACTING EFFECTS OF 3-INDOLEACETIC ACID, GIBBERELLIC ACID AND 6-BENZYLADENINE ON RESPIRATION, GROWTH AND NITROGEN ASSIMILATION IN EXCISED EMBRYOS OF PEA (PISUM SATIVUM, L.)

presented by

David Cameron MacLean

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Horticulture

Samuel P. Hicks
Major professor

Date June 16, 1965

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By

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AN ABSTRACT OF A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Horticulture

1965

ABSTRACT

THE INTERACTING EFFECTS OF 3-INDOLEACETIC ACID, GIBBERELLIC ACID AND 6-BENZYLADENINE ON RESPIRATION, GROWTH AND NITROGEN ASSIMILATION IN EXCISED EMBRYOS OF PEA (PISUM SATIVUM, L)

By David Cameron MacLean

Respiration, growth and nitrogen assimilation from the cotyledons in excised pea embryos were investigated following 12 hours imbibition in treating solutions containing auxin (3-indoleacetic acid, IAA), gibberellin (gibberellic acid, GA) and a kinin (6-benzyladenine, BA) alone or in combination.

Preliminary investigations concerned the effect of these growth substances on the respiration rate of fully expanded broccoli leaf discs showed that IAA and GA ($10^{-5}$ to $10^{-3}$ M) did not affect oxygen uptake in broccoli, whereas, BA treatment ($5x10^{-5}$M) resulted in a 40 percent reduction of the respiration rate. The presence of IAA and/or GA at the same concentration did not alter the BA-induced inhibition.

Oxygen uptake, nitrogen assimilation and growth of pea embryos treated with GA or BA at $5x10^{-5}$M did not differ from the control embryos from 0 to 72 hours after imbibition. These responses were consistently inhibited by IAA treatment ($5x10^{-5}$ M) at 24 and 48 hours

after imbibition. However, growth of auxin-treated embryos was inhibited to a greater extent than nitrogen assimilation, resulting in a greater nitrogen content per unit growth at these time intervals.

A marked stimulation of respiration, nitrogen assimilation and growth was observed in pea embryos treated with $5x10^{-6}$ M IAA. A similar, though less pronounced effect was observed for BA $5x10^{-6}$ M. GA induced a consistent inhibitory effect on these parameters at $5x10^{-7}$ M.

The optimum IAA concentration for stimulation of growth, respiration and nitrogen assimilation was decreased from $5x10^{-6}$ to $5x10^{-8}$ M by including $5x10^{-5}$ M BA in the imbibing solution, indicating increased efficiency of IAA by BA. The stimulatory effect of auxin ($5x10^{-6}$ M) was blocked by GA or GA plus BA at $5x10^{-5}$ M. A marked inhibition of all parameters, equal in magnitude to the BA-induced enhancement was observed when $5x10^{-5}$ M GA was applied with auxin at $5x10^{-8}$ M. The inhibitory effect of $5x10^{-5}$ M IAA was dominant when applied alone or with BA and/or GA at various concentrations. No interaction between GA and BA was observed. Growth regulator treatment did not alter the dependency of nitrogen assimilation on respiration.

The requirement for a specific concentration

ratio of auxins, gibberellins and kinins for growth and associated phenomena was suggested from these studies. Pronounced responses to growth regulator treatments were negated by a ten-fold increase or decrease in concentration. It is postulated that specific concentration ratios of growth regulators, within physiological concentrations, might induce the production of certain nucleic acids which, in turn, may regulate protein synthesis and thereby govern plant growth and development.

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ACKNOWLEDGEMENTS

The author is indebted to Dr. David R. Dilley for his encouragement and counsel throughout these studies. The advice and assistance offered by Drs. D. H. Dewey, C. J. Pollard, H. M. Sell and S. H. Wittwer whenever requested, and during the preparation of this dissertation are greatfully appreciated. Finally, I would like to thank Dr. R. R. Dedolph for his helpful suggestions in the initial phases of these studies.

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# INTRODUCTION

Numerous chemicals have been isolated from plants which influence fundamental phases of plant growth and development. Other chemicals, some of which are structurally related to the naturally occurring substances, and which have been synthesized in the laboratory also possess plant growth regulating activity. Experimentation with these plant growth substances has had a great impact on plant science since Darwin (1880) published his book The Power of Movement in Plants. Research has led to the current agronomic use of some of these chemicals for control of flowering and fruiting, promotion or prevention of leaf and fruit abscission, production of seedless fruits, promotion of rooting on cuttings, breaking rest and dormancy of buds and seeds, weed control and other diverse phenomena.

The naturally occurring growth regulators have been classified, on the basis of biological specificity and chemical structure, into three groups; the auxins, the gibberellins and the kinins. Despite the vast accomplishments of growth regulator research, the basic biological functions of these three classes of compounds remains uncertain.

In numerous investigations, synergistic, as well

as antagonistic responses have been reported when two or more naturally occurring growth regulators were applied simultaneously. It is recognized that these opposing responses may, in some cases, be due to tissue type, stage of maturity and the relative concentration of growth regulators used. However, these factors do not fully account for the diverse responses that have been reported. Thus, it seemed desirable to further investigate the interacting effects of plant growth regulators. Experiments were conducted to ascertain the effects of auxins, gibberellins and kinins on two different plant tissues; one, broccoli leaves, incapable of further growth; and the other, pea embryos, possessing a high growth potential.

# REVIEW OF LITERATURE

Auxins, gibberellins and kinins have been the subject of several recent reviews (Miller, 1961; Galston and Purves, 1960, Phinney and West, 1960a). Therefore, only that portion of the literature concerning the basic concepts of these growth substances pertinent to this investigation is reviewed here.

## AUXINS

Charles Darwin (1880) is usually credited with initiating the concept of plant hormone research. He discovered that a stimulus, induced in the tips of Phalaris and Avena cotyledons by unilateral light, was transmitted to the lower parts where it induced a bending of the shoot towards the light. However, 50 years passed before Went identified this stimulus as auxin (van Overbeek, 1959). Currently, auxins are generally assumed to be ubiquitous in higher plants.

### Structural Requirements for Activity

The chemical structure of most auxins consists of a naphthalene, benzene or indole nucleus. The indole nucleus is characteristic of the naturally occurring auxins to which this review is limited. Although many indole compounds occur naturally and exhibit activity in auxin assays, 3-indoleacetic acid (IAA) is

regarded as the naturally occurring auxin (Kefford, 1963). The activity of the IAA analogs is due to their conversion to IAA in the assay tissues (Kefford, 1963).

To be active, auxins must possess an unsaturated ring and a side chain of certain length terminating in a carboxyl group or a functional group readily converted to a carboxyl group (Galston and Purves, 1960). In some synthetic auxins, namely the phenoxy-acids, at least one free ortho position is required for activity (Galston and Purves, 1960). The nature of these requirements for activity has resulted in the acceptance of the general concept that auxin forms a complex with a specific receptor. Foster et al. (1952) applied classical enzymic kinetics to auxin induced coleoptile growth. The auxin induced growth inhibition at high concentrations led them to conclude that there was an attachment of auxin to a receptor, and that this complex is similar to that postulated for a typical enzyme-substrate complex. Although their methods have been challenged by Lockhart (1962) and their interpretations disputed by Truelsen (1961), the concept of an auxin-receptor complex is still generally recognized.

It is suspected that the auxin receptor is a protein. Galston and Purves (1960) list as evidence for this view the low concentration of auxin necessary

for activity, suggesting its action as a cofactor or an enzyme activator, and the auxin-protein complexes that have been reported. However, the IAA-protein complex isolated by Siegel and Galston (1953) from pea roots was claimed to be an artifact of the isolation technique (Andreae and van Ysselstein, 1960). Thus, there is no conclusive evidence that auxin forms a complex with a receptor.

## Auxin and Plant Growth

The literature on the effects of auxin on growth is voluminous. Therefore, instead of duplicating what has already been reviewed (van Overbeek, 1959; Galston and Purves, 1960) only pertinent concepts will be presented.

The promotive effect of auxin on cell enlargement is well documented. From 1928 to the present time most research in this area has been carried out using Avena coleoptiles (Went and Thimann, 1937) in which auxin promotes water uptake resulting in growth by extension or cell enlargement. For this to occur the suction pressure (SP) of the coleoptile cells (or the diffusion pressure deficit) must increase. Since SP is the difference between the osmotic pressure (OP) of the cell contents and the wall pressure (WP), either an increase in OP or a decrease in WP would increase SP.

The latter is generally presumed to be the controlling factor. Tagawa and Bonner (1957) demonstrated that in coleoptiles treated with calcium or magnesium solutions, their plasticity and elasticity were markedly decreased. Potassium on the other hand, increased elasticity and IAA increased plasticity. When applied together both elasticity and plasticity were increased. An effect of auxin on cell walls was suggested. They concluded that potassium ions replaced some of the divalent cations binding adjacent pectic chains in the cell wall thereby making it more flexible. Ordin et al. (1957) suggested that auxin induced the breaking of these calcium bonds by increasing methyl esterification from methionine. Although the mechanism of auxin induced water uptake in coleoptiles is still unknown, these data strongly suggest that the chief auxin effect is a reduction in WP; possibly through an effect on the cell wall constituents.

Auxin and Respiratory Metabolism

Growth in higher plants is an energy dependent process which, for the most part, relies on the energy produced in aerobic respiration. As early as 1933, Bonner (1933) related auxin induced growth to oxidative metabolism by showing that Avena coleoptiles could not

grow under anaerobic conditions. In a later paper Bonner (1949) reported that auxin induced growth was inhibited by cyanide, and that treatment with 2,4-dinitrophenol (DNP), an uncoupling agent, also inhibits coleoptile growth, suggesting that enery-rich compounds produced in oxidative phosphorylation are necessary for growth. Similar results have since been obtained with arsenate, (Bonner, 1950) and other respiratory inhibitors in several tissue types (Galston and Purves, 1960). From these data Galston and Purves conclude that auxin promotes water uptake through an energy requiring process which increases the level of phosphate acceptors and thereby stimulates respiration indirectly. This is in agreement with the report that auxin stimulates respiration only when growth is promoted (Bonner, et al., 1953).

Auxin and Nucleic Acids

The interaction of auxin and kinetin on cell divisions in tobacco pith cultures prompted Skoog and Miller (1957) to propose that auxin exerts its effect at the nucleic acid level. Until very recently, however, this view has been, at most, speculative.

Nooden and Thimann (1963) measured the effect of IAA on growth and incorporation of $^{14}$C-leucine into protein in etiolated pea stem segments, Avena coleoptiles and artichoke tuber discs. IAA stimulated growth in

all tissues, and an increase in $^{14}C$ incorporation into protein was observed in the pea segments and tuber discs. No stimulation of protein synthesis occured in Avena coleoptiles. Using peas, they further studied the effects of specific inhibitors of protein and nucleic acid synthesis. Chloramphenicol, puromycin and Actinomycin-D all inhibited growth of pea stem sections. The progressive inhibition of elongation paralleled the inhibition of $^{14}C$-leucince incorporation at increasing chloramphenicol concentrations. They concluded that the primary locus of auxin action is on a nucleic acid that controls the synthesis of a protein necessary for growth.

Recently, Nooden and Thimann (1965) described a more thorough investigation in which they found that auxin stimulated growth and $^{14}C$-leucine incorporation into protein in all three tissue types. Addition of chloramphenicol completely blocked this effect. The growth of pea stem segments treated with the synthetic auxins, 2,4-D and NAA, were likewise inhibited by chloramphenicol. The stimulation of respiration in artichoke tissue induced by IAA was negated by chloramphenicol. Furthermore, the same concentrations of chloramphenicol that inhibit these auxin induced response also inhibit protein synthesis. From these data

they therefore concluded that protein synthesis is necessary for auxin induced growth. Thus it seems very likely that auxin exerts its effect at the nucleic acid level and induces the formation of new enzyme(s) which, in turn, affect cell wall plasticity.

## GIBBERELLINS

The gibberellins were first discovered as metabolic products of the fungus Fusarium moniliforme, Sheldon (Giberella fujikuroi) (Yabuta and Sumiki, 1938). Endogenous gibberellins have since been found in 94 species of flowering plants representing 19 families and in gymnosperms, ferns and mosses (van Overbeek, 1962). Gibberellin activity is usually found in seed extracts, and in rapidly growing vegetative parts such as vines (van Overbeek, 1962). Four different gibberellins have been isolated from higher plants and, like the auxins, they are assumed to be ubiquitous (van Overbeek, 1962; Phinney and West 1960a).

### Gibberellins and Plant Growth

Gibberellic acid (GA) has been found to stimulate shoot elongation, promote flower formation and normal and parthenocarpic fruit set and it can replace the long day effect in several photoperiodic phenomena (Phinney and West, 1960a; van Overbeek, 1962; Wittwer and Bukovac, 1958). Thus, responses to $G_A$ application are many and varied and, in some cases, are probably dependent on the endogenous concentration of ther native growth regulating substances (see later). The responses of the various gibberellins have been thoroughly reviewed elsewhere (Wittwer and Bukovac, 1958; Bukovac and Wittwer,

1961; Phinney and West, 1960a, 1960b).

Effect of Gibberellin (GA) at the Genetic and Enzyme Level

Phinney and co-workers, in a series of studies, showed that the application of GA to any of several dwarf mutants of Zea Mays, L. resulted in plants of normal appearance (Phinney and West, 1960a, 1960b). Dwarfism in these mutants is controlled by a single gene. The ability of GA to overcome this effect suggested that the synthesis of native gibberellins in these mutants is genetically blocked, resulting in a dwarf growth habit. The growth of these genetic dwarfs is proportional to the exogenous GA concentration and is not affected by either kineten or IAA. Thus, these dwarf mutants of Z. Mays have proven useful as a quantitative bioassay for GA (Phinney and West, 1960a, 1960b).

Some of the most interesting work on the physiology of GA action is its effect on a specific protein $\alpha$-amylase. Other enzymic effects of GA, particularly on the IAA oxidase system, will be reviewed later. The effect on GA on $\alpha$-amylase activity was first reported by Hayashi (1940). Since that time much has been learned of this effect. Paleg (1960, 1961) showed conclusively that GA induces the activation of amylolytic enzymes in barley endosperm resulting in an increased

release of reducing sugars and a concomitant reduction in dry weight. Using isolated aleurone layers, the only living cells of barley endosperm, Varner (1964) and Varner and Chandra (1964) demonstrated that in GA-treated endosperm the major fraction of radioactivity incorporated from $^{14}$C-phenylalanine was recovered in $\alpha$-amylase. Thus the GA-dependent increase in $\alpha$-amylase activity is a result of increased synthesis of the enzyme. Furthermore, the duration of sensitivity of the system to Actinomycin-D and p-fluorophenylalanine suggested that GA affects the formation of a specific messenger RNA involved in the de novo synthesis of the $\alpha$-amylase molecule in barley endosperm.

The reported effects of GA at the gene, enzyme and nucleic acid level suggest a very basic GA effect. Possibly, GA induces the control of nucleic acid synthesis at the gene level thereby affecting the synthesis of specific protein which, in turn, could result in the many reported GA responses.

## KININS

### Discovery of Kinetin

A cyrstalline substance was isolated by Miller, et al. (1955) from autoclaved deoxyribonucleic acid (DNA) which markedly promoted cell division in tobacco stem segments cultured on synthetic media. Due to its ability to induce cytokinesis, the active compound, which was later shown to be 6-furfurylaminopurine (Miller, et al, 1955), was named kinetin. To include substances having similar effects on cytokinesis in tissues which were other wise unreactive, they proposed the generic name kinin.

### Structural Requirements for Kinin Activity

Following the discovery of kinetin various bio-assays were developed to determine the structural requirements for kinin activity and to find compounds more active than kinetin. They included cell division in tobacco (Rogozinska, et al. 1964) and carrot (Shantz, 1958) tissue cultures, lettuce seed germination (Skinner, et al., 1957), cell enlargement in radish leaf discs (Kuraishi, 1959), chlorophyl retention in detached Xanthium (Osborne and McCalla, 1961; Richmond and Lang 1957) and wheat leaves (Shaw and Srivastava, 1964).

With but one exception, the intact purine ring was found to be necessary for activity (Miller, 1961);

the exception, 8-azakinetin promoted cell division in soybean callus culture (Miller, 1960). The furfuryl group in kinetin, which is attached to the amino group in the 6-position, may be replaced by many substitutes and still retain activity. However, only a limited number of 6-substituted purines gave activity equal to or greater than that of kinetin. 6-Benzylaminopurine (6-benzyladenine) (Osborne and McCalla, 1961; Hamzi and Skoog, 1964; Kuraishi; 1959) and 6-(dimethylallylamino)-purine (Hamzi and Skoog, 1964; Rogozinska, et al., 1964) were more effective than kinetin.

Miller (1961) regards these active compounds as substituted adenines. Since adenine treatment gave only a slight increase of tobacco callus growth, he suggested that the marked stimulation induced by the active compounds might be due to the presence of fat-soluble substituents (the furfuryl, benzyl or dimethylallyl groups) which might permit entry into, or orientation with that portion of the cell in which growth is controlled.

Occurrence of Kinins

However, Letham (1963b) purified a substituted adenine from corn and plum fruitlets having kinetin-like activity, suggesting their natural occurrence in

plant tissues. Substances possessing kinetin-like activity in various bioassays have been obtained from corn endosperm (Miller, 1956; Letham, 1963a) solid (Shaw and Srivastava, 1964) and liquid (van Overbeek, 1962) endosperm of coconut; apple, quince, pear and plum fruitlets (Bottomley, etal., 1963, Zwar, et al., 1963); and from pea seedlings (Biswas, 1964) and pea blanching water (Skoog, 1965). Furthermore, the alkaloid tricanthine, or 6-amino-3-(γ,γ-dimethylallyl)-purine which occurs in at least three species, showed slight growth promoting activity in tobacco tissue cultures (Rozoginska, et al., 1964). However, when tricanthine was autoclaved prior to addition to the media, growth promotion was ten times greater than that induced by kinetin. Chromatographic studies of autoclaved tricanthine showed that it was probably converted to 6-(γ,γ-dimethylallylamino)-purine. They concluded that tricanthine might readily be converted to the active 6-isomer in vivo (Rozoginska, et al., 1964).

## Effects of Kinins on Plant Growth and Development

The primary effect of kinetin is on cell division. In the presence of IAA, kinetin induces mitosis and subsequent karyokinesis in tissue cultures of tobacco pith (Das, et al., 1956), pea root callus (Torrey, 1958)

and carrot root explants (Shantz, et al., 1958), however, both IAA and kinetin are without effect when supplied alone. Das, et al., (1956) suggested that a kinetin-like substance was necessary for complete cell division, and probably for DNA replication as well. Wright (1961) has correlated the time of maximum kinetin response with the period of cell division in wheat coleoptiles.

Kinins also influence cell enlargement. Kinetin stimulated expansion in etiolated bean leaf discs (Miller, 1956) and several 6-substituted purines, including kinetin and 6-benzyladenine (BA), induced the same effect in discs from light-grown radish leaves (Kuraishi, 1959). In both areas the response was due to cell enlargement. Cell elongation in pea stem segments however, was retarded by treatment with kinetin (Brian and Hemming, 1957; Katsumi, 1963). Similarly, kinetin absorbed by the roots from culture solutions supressed the growth of both roots and tops of intact dwarf pea, cucumber and tomato plants (Wittwer and Dedolph, 1963).

When applied to individual bean leaves, BA stimulates their expansion, but at the expense of non-treated leaves which very rapidly become senescent (Leopold and Kawase, 1964). It was demonstrated by Mothes and Engelbreet (1961) that treatment of one portion of an excised tobacco leaf with kinetin induces

the movement of substances in the leaf to the treated area. They proposed that endogenous kinins are perhaps the natural mobilizing agents in plants. Kinin induced transport will be discussed later in greater detail and is mentioned here only in relation to the work of Osborne and Moss (1963) on the abscission process. When these investigators applied kinetin directly to the abscission zone of bean explants, abscission was delayed. If on the other hand, kinetin was applied elsewhere on the explant, metabolite mobilization towards the site of treatment commenced and the subsequent depletion of the cells of the zone resulted in accelerated abscission. A similar delay in abscission due to kinetin treatment was observed by Chatterjee and Leopold (1964).

## Effects of Kinins on Metabolic Processes

### Enzyme Activity

Bootlby and Wright (1962) reported that starch degradation in wheat endosperm is promoted in the presence of kinetin. The rate of reducing sugar formation by starch hydrolysis is very much like that induced by GA. They therefore suggested that the effect of kinetin is on amylase activity. *In vitro* studies showed that the activity of ribonuclease and deoxyribonuclease in bean hypocotyl extracts was stimulated by kinetin

(Maciejewska-Potapczyk, 1959). However, Srivastava and Ware (1965) have since proven that the opposite occurs in vivo. The reduction of nuclease activity is in agreement with the many observations of kinin effects on senescence.

The fact that some 6-substituted purines, in the presence of xanthine, induce permanent disorganization of exposed tissues of planaria, a primative invertebrate, prompted Henderson, et al., (1962) to study the effect of kinins on xanthine metabolism. In vitro they demonstrated that kinetin is a potent inhibitor of xanthine oxidase, the enzyme that catalyzes the oxidation of xanthine to uric acid. How this relates to plant metabolism is not clear. Possibly the conversion of accumulated xanthine to purines could allow for increased nucleic acid synthesis. Steinhart, et al., (1964) have recently reported that in the roots of barley seedlings, kinetin and BA induce an increase in the rate of synthesis of tyramine methylpherase (S-adenosylmethionine: tyramine methyltransferase) which catalyzes the conversion of tyramine to N-methyltyramine. The authors conclude that kinins are involved in at least one of the steps in the synthesis of this protein.

Kinins also affect respiratory enzymes. Working with tobacco cell suspensions, Bergmann (1963) concluded

that the locus of the inhibitory effect of kinetin on respiration was in the glycolytic pathway. Tuli confirmed this using BA (1964). He further showed that in vitro, BA competes with adenosine triphosphate (ATP) and adenosine diphosphate (ADP) for the active site on hexokinase and pyruvic kinase, respectively. The absence of an effect on glutamine synthetase indicated a degree of specificity for BA on glycolytic kinases. Further, the $^{14}C$ content in phosphate esters after $^{14}CO_2$ fixation in the light was found to be much less in BA treated broccoli leaves than in the controls, indicating an inhibitory effect on phosphorylation enzymes (Tuli,1964).

Nucleic Acids

As in the case of auxins, the multiplicity of response of plant tissues to kinin treatment strongly suggests that the primary effect of kinins is at a very basic level. It was pointed out above that kinins may exert their effect on protein synthesis. Therefore it is possible that the basic effect of kinins might be at the nucleic acid level which, in turn, would affect protein synthesis. There is supporting evidence for this view.

Richmond and Lang (1957) first showed that kinetin delayed the loss of protein in detached Xanthium leaves.

The effect of kinins on protein metabolism has since received considerable attention. Thimann and Laloraya (1960) reported a stimulatory effect on kinetin on protein synthesis in isolated pea stem sections. The most thorough study in this area was performed by Osborne (1962). She found that kinetin sustained both RNA and protein synthesis in detached Xanthium leaves and leaf discs. The incorporation of $^{14}$C-orotic acid into RNA and $^{14}$C-leucine into protein was markedly higher in kinetin treated leaf discs. Further, treatment with kinetin resulted in a greater incorporation of $^{14}$C-leucine into protein perunit RNA, even though the protein/RNA ratio remained the same. Osborne (1962), therefore suggested that the kinetin induced increase in the synthesis of protein is brought about by increased RNA synthesis. That DNA synthesis was not affected by kinetin was ascribed to the absence of cell division in the tissues studies (Osborne 1962). Very recently Srivastava and Ware (1965) reported that in kinetin treated excised barley leaves the synthesis of both RNA and DNA, as measured by $^{32}$P incorporation, was maintained almost as high as in fresh leaves. A concomittant suppression in ribonuclease and deoxyribonuelease activity was also noted. These studies (Osborne, 1962; Srivastava and Ware, 1965) leave little doubt that kinetin exerts its effect on protein metabolism

through nucleis acid synthesis.

Senescence Inhibition

Senescence in most higher plant tissues is usually accompanied by chlorophyll degradation and a decline in protein and nucleic acid content. Kinins, particularly BA, can temporarily delay these catabolic processes in many detached plant tissues. Following the discovery of BA as a "senescence inhibitor" in some green leafy tissues (Bessey, 1960; Zink, 1961), many other vegetable and flower crops were screened for this effect (Dedolph, et al., 1961, 1962; MacLean and Dedolph, 1962, 1964; MacLean et al., 1963; Tuli, 1964; Wittwer, et al., 1962). Dedolph, et al., (1961) measured the rate of $CO_2$ evolution of treated and nontreated asparagus spears and concluded that the preserving effect of BA was a consequence of respiratory inhibition. The inhibitory effect of BA on glycolytic kinases in vitro (Tuli, et al., 1964) gave further support to this contention. However, Srivastava and Ware (1965) concluded that the maintenance of RNA and DNA synthesis and the supression of the activity of ribonuclease and deoxyribonuclease by kinin treatment preserves the integrity of the ribosomes. This, in turn, allows for the maintenance of protein synthesis which is necessary for the maintenance of detached plant tissues.

## INTERACTIONS

In the 195C's when auxins and gibberellins were firmly established as two distinct classes of growth regulators, experiments were conducted concerning the interactions of these substances on plant responses. Likewise, after the discovery of the kinins, they two were included in growth regulator combination studies. The more significant findings on growth regulator combinations, particularly as they pertain to the present study are herein reviewed.

### Growth

#### Auxins and Gibberellins

The concept that auxin is necessary for all plant growth was proposed by Went and Thimann (1937). However, Kefford and Goldacre (1961) have since suggested that all tissues must be predisposed by auxin for other growth hormones to act. An excellent example of the latter suggestion was presented by Cleland (1964). He employed the antiauxin-inhibition method, using p-chlorophenoxy-iso-butyric acid (PCIB) to assess the role of endogenous auxin in the elongation of Avena leaf sections. His experiments clearly showed that these sections undergo two different types of elongation; (a) endogenous growth, which is accompanied by an increase in both cell number and cell length, and (b) GA induced growth which is due

to an increase in cell length only. The ability of auxin to overcome the PCIB-induced inhibition of endogenous growth indicates that endogenous growth requires endogenous auxin. The GA-induced elongation of these leaf sections requires both GA and auxin. GA must be applied exogenously, but the auxin requirement is satisfied by endogenous auxin (Cleland, 1964). Using the same species, Ng and Audus (1964) measured the effect of auxin and GA on the shoot elongation of both internode segments and segments including a node. The GA effect was greatly increased and the IAA effect was decreased by the inclusion of a node, suggesting that an endogenous auxin supplied by the node is necessary for the GA response. Thus, in the elongation of Avena leaf and shoot sections the theory of "predispostion" of the tissues by auxin appears to hold true.

However, this is obviously not the only way in which GA interacts with auxin. The lack of a pronounced GA effect on auxin activity in the Avena test led Galston and Warburg (1959) to suggest that GA activates a system that inhibits the oxidative destruction of IAA. They called this response the "auxin sparing effect" of GA. Halevy (1963) has since shown that the activity of the IAA oxidase system in cucumber seedlings decreases with increasing concentrations of applied GA.

Similarly, van der Kerk, et al., (1964) reported that Elema (Ph.D. Thesis, State University of Utrecht, Holland) found considerably less IAA oxidase activity in complete homogenates from GA treated etioloated pea plants than in comparable nontreated homogenates. From these studies it is evident that the "auxin sparing effect" of GA is no doubt mediated through the inactivation or inhibition of the IAA oxidase system. In this way, GA can have an indirect effect on auxin activity.

There are, however, a number of discrepencies concerning the interacting effects of IAA and GA. Synergistic responses have been observed in the growth of etiolated (Galston and Warburg, 1959) and green pea stem sections (Galston and Kaur, 1961) and in Avena (Hayashi and Murakami, 1954) and wheat leaf sections (Radley, 1958). Others have reported that the effects of IAA and GA on growth in etiolated pea sections (Kato, 1958) and in Avena coleoptile and first internode sections (Nitsch and Nitsch, 1956) were, at most simply additive.

Despite these conflicting responses, even in the same tissue and species, Galston and Purves (1960) concluded that both auxin and GA are required for growth, but the nature of their interaction is not yet understood.

Auxins and Kinins

It was mentioned above that neither IAA nor kinetin,

when supplied alone, can induce cell division in tissue cultures of tobacco pith, soybean callus (Miller, 1961) and carrot root explants (Steward, et al., 1961). However when applied together, continuous cell division and growth ensues. Hashimoto (1961) showed a similar requirement for the presence of IAA and kinetin in combination for the primary thickening of light-grown pea stem segments. Kinetin and IAA also act synergistically with respect to DNA synthesis in tobacco callus cultures (Skoog and Miller, 1957). Ballantyne (1965) demonstrated that by including the synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), the effect of BA on preserving cut flowers of daffodil was markedly enhanced. The same effect might be expected with IAA.

Not all responses are synergistic. Kinetin has been shown to inhibit IAA induced elongation in sunflower hypocotyls (de Ropp, 1956) and pea stem segments (Brian and Hemming, 1957), as well as auxin induced root growth (Raghavan, 1964). Further it has been reported that in pea stem segments (Wickson and Thimann, 1958) and intact pea plants (Sachs and Thimann, 1964) kinetin promotes the elongation of auxin inhibited buds. This, in part, explains the BA effect on the breaking of rest in dormant grape buds (Weaver, 1963). Working with pea stem sections, Katsumi (1963) found that the effect of IAA and kinetin on elongation

and fresh weight increase, when applied together, was intermediate to the stimulation and inhibition observed when IAA and kinetin were applied alone, respectively.

To resolve these differences, Fox (1964) conducted studies which showed that in the growth of tobacco and soybean tissue cultures, the antagonism between IAA and kinetin is such that the inhibitory levels of one can be overcome by providing more of the other to the medium. Thus it seems valid to conclude that auxins and kinins, either directly or indirectly, interact in plant growth.

**Gibberellins and Kinins**

Interactions between GA and kinins are limited in number. Skinner,et al., (1958) have demonstrated a GA-kinetin synergism in seed germination of three different species. The fresh weight increase of radish leaf discs treated with GA plus kinetin, relative to either alone, were only additive, and for disc expansion, less than additive (Kuraishi and Hashimoto, 1957).

In intact dwarf pea plants, Wittwer and Dedolph (1963) showed that kinetin reduces the top/root ratio and induces flowering at a lower node. GA has the opposite effect, but when applied together these responses were intermediate.

Transport

When Mothes and Engelbrect (1961) applied kinetin to localized areas of tobacco leaves, the movement of amino acids and other metabolites towards the treated region was detected. They found auxin to be ineffective in this "kinetin-induced directed transport." More recently, however, Seth and Wareing (1964) observed definite growth regulator interactions in the transport of $^{32}P$ in bean internodes. Although kinetin was not effective alone, in combination with IAA a very marked enhancement of transport occurred. A synergistic effect on transport, of even greater magnitude, was observed when IAA was applied in combination with GA, even though GA applied alone was ineffective. These observations led Seth and Wareing (1964) to conclude that these three growth regulators act through the same system and that they are in some way involved in the movement of metabolites toward growth centers. The basipetal transport of $^{14}C$-labeled BA through bean peticles is increased when IAA is also added (Osborne and Black, 1964). The lack of an IAA effect in tobacco leaves (Mothes and Engelbrect, 1961) was probably due to the presence of endogenous auxin in non-limiting amounts.

Sequential Responses to Growth Regulators

One of the most significant works to date concerning plant responses to growth regulators, is, perhaps that of Wright (1961). He treated wheat coleoptiles with IAA, GA or kinetin at 12 hour intervals from 18 to 78 hours after sowing and determined their growth at each interval. Over the same period of time, the cell number and cell volume of comparable coleoptiles were also determined. Wright found that coleoptile growth consists of two phases, a relatively short initial phase of rapid cell division, followed by a slower, almost linear, phase of cell enlargement. Growth responses showed the coleoptiles to be most sensitive to applied GA at or before 18 hours after sowing, with a steady decline thereafter. Kinetin gave an equally high response at 18 hours, but a still greater response at 30 hours, followed by a decline in activity. The GA and kinetin responses both approached the control level at 66 hours after sowing. No positive response to IAA was observed until 30-42 hours, with the maximum activity at 54 hours after sowing. In light of these data, Wright (1961) concluded that: 1) the GA effect is correlated with the period of expansion just prior to division; 2) when most cells are about to divide the coleoptile is most sensitive to kinetin; and 3) the lack of an initial IAA

response indicates that auxin is associated with the later, steady-state period of cell elongation. He further postulated that there may be an optimum concentration ratio of these three growth regulators for each stage of growth (Wright, 1961).

# MATERIALS AND METHODS

## Treatment Solutions

3-Indoleacetic acid (IAA), gibberellic acid (GA) and 6-benzyladenine (6-benzylaminopurine) (BA) were used as representative members of the auxins, gibberellins and kinins, respectively.

Stock solutions containing $1.5 \times 10^{-4}$ M IAA (Eastman Organic Chemicals), GA (K-salt of gibberellic acid; Merck and Company) or BA (Shell Chemical Company) were made up in pH 6.7 phosphate buffer. The buffer solution contained 0.067 M $KH_2PO_4$ and 0.067 M $K_2HPO_4$ (6:4 v/v) made up in distilled-deionized water. IAA and GA were first dissolved in a minimal amount of 5% (w/v) $NaHCO_3$; BA was dissolved in hot buffer solution (ca 90°C). Dilutions to obtain other concentrations were made using the buffer, or in the case of combination treatments, with another buffered stock solution. Thus, all treatment solutions were of the same pH and buffer concentrations. Treatment with the buffer alone served as the control. Solutions containing higher concentrations of the growth regulators ($5 \times 10^{-4}$ or $5 \times 10^{-3}$ M) were prepared when required. Between experiments the treatment solutions were stored at ca 5°C. Solutions used were never more than one week old.

Leaf Disc Studies

Leaf discs (10 mm diam.) were removed from interveinal portions of fully expanded broccoli (Brassica oleracea var. Italica cv. 'Spartan Early') leaves of similar physiological age. The discs were washed by gentle shaking in distilled water for two hours. This procedure insured complete randomization of discs from all leaves. The leaf discs were rinsed with distilled water, placed in 50 ml of the treating solutions and gently shaken on a wrist-action shaker for 16 hours at 20°C. Once treatment commenced the leaf tissue was maintained in the dark except for brief exposure to weak green light when necessary for tissue manipulation.

Following treatment the discs were blotted and stored at 20° C under conditions of high relative humidity to prevent drying. Thirty-two hours later (48 hours after initial contact with the treatment solutions) 0.5 gm of discs from each treatment were placed into each of four Warburg respirometers. Oxygen uptake was measured manometrically for three hours in the dark at 30° C.

All leaf disc experiments were repeated at least three times. Thus, the data presented are the averages for four Warburg flasks in each of three or more discrete trials (Tables I, II).

The initial experiments were performed to determine the effective growth regulator concentrations, and subsequent factorial experiments were conducted to study the interacting effects of these compounds on oxygen uptake.

## Pea Embryo Studies

Seeds of Alaska peas (*Pisum sativum* L., cv. Alaska), provided by the Rogers Brothers Company, Idaho Falls, Idaho (Lot No. 423), were used in all experiments. The seeds were randomly divided into replicates of 10 seeds each and soaked in treatment solutions for 12 hours (hereafter referred to as the imbibition period). The imbibed seeds were transferred to 9 cm Petri dishes (one dish per replicate) containing a Whatman No. 1 filter paper disc moistened with the treatment solution and held for 48 hours, unless otherwise specified. Imbibition and subsequent storage were carried out in the dark at 20° C.

Following the storage period, five of the original 10 seeds of a replicate having typical appearance were selected for embryo excision. The excised embryos were placed in 20 ml Warburg respirometer flasks containing water in the side arms to maintain a high relative humidity, and 0.2 ml 10% (w/v) KOH in the center well

Table I. Oxygen consumption, as percent of control, by broccoli leaf discs as affected by various concentrations of growth regulators.

| Treatment | Molar Concentration | | | | |
|---|---|---|---|---|---|
| | $10^{-5}$ | $2x10^{-5}$ | $5x10^{-5}$ | $10^{-4}$ | $10^{-3}$ |
| Kinetin | 93.2 | 75.1 | 67.9 | 50.7 | -- |
| BA | 85.8 | 60.9 | 55.3 | 47.8 | -- |
| IAA | 104.5 | 108.0 | 99.4 | 94.9 | 102.6 |
| GA | 98.7 | -- | 97.0 | 94.2 | 92.8 |

Table II. Rate of Oxygen consumption by broccoli leaf discs as affected by growth regulator treatments

| Treatment ($5 \times 10^{-5}$ M) | Oxygen uptake as percent of control |
|---|---|
| IAA | 100.9 |
| GA | 95.3 |
| BA | 58.7** |
| IAA + GA | 101.2 |
| IAA + BA | 62.3** |
| GA + BA | 57.8** |
| IAA + GA + BA | 61.1** |

**Significantly different from the control at 1% level (Duncan, 1955).

for $CO_2$ absorption. Each Warburg flask was considered a replicate. By reducing the number of embryos to five, those injured during excision could be discarded and readily replaced and oxygen uptake determinations could continue for at least three hours.

Respiration, as indexed by oxygen consumption was determined manometrically over a three hour period in the dark at 30° C. The embryos were then weighed, by replicates, to the nearest mg and their nitrogen content was determined accoding to the method of Umbreit, et al.,(1964). In some instances protein nitrogen was determined after precipitation with trichloroacetic acid.

The effect of growth regulator treatment on growth, nitrogen assimilation from the cotyledons and the respiration rates of pea embryos was thus determined. The experimental design was such that replicates were maintained separately from the commencement of imbibition, through the storage period and throughout the respiration, growth and nitrogen determinations.

Time Studies

The respiration rate, fresh weight and nitrogen content of treated embryos were determined immediately or at 24, 48, or 72 hours after the inbibition period

to ascertain the effect of growth regulator treatment with time.

Treatments in these time studies consisted of IAA, GA or BA at $5x10^{-5}$ M. Three discrete experiments were conducted in which each treatment was replicated three times.

Interaction Studies

After the effect of concentration from $5x10^{-8}$ through $5x10^{-5}$ M of each growth regulator was determined, treatment with various growth regulator combinations was applied. In these experiments the effect of an increasing concentration of one growth regulator, in the presence of the other two, applied either separately or together, at $5x10^{-5}$ M was tested. For example, if IAA was applied in increasing concentrations ($5x10^{-8}$ to $5x10^{-4}$ M), then GA, BA and GA plus BA were also applied at $5x10^{-5}$ M. Thus in all experiments, when the concentration of one growth regulator was varied the other two, alone and in combination, were held constant at $5x10^{-5}$ M. Growth, nitrogen content and the respiration rate of each replicate was determined as in the time studies.

Manometer limitations necessitated replication by days or runs. A control was included each time, and due to the nature of the experimental design, certain

treatments overlapped. As a result, the data for the controls were based on 21 observations; six observations were obtained for each treatment in which IAA, GA and BA were applied alone or in combination at $5x10^{-5}$ M. Data for the remaining combinations were based on at least three observations each.

## RESULTS AND DISCUSSION

### BROCCOLI LEAF DISC STUDIES

The effects of various concentrations of IAA, GA, kinetin and BA on oxygen uptake by broccoli leaf discs are presented in Table I. Treatment with kinetin and BA resulted in increasing inhibition of oxygen consumption with increasing concentration. BA has been shown to be more active than kinetin in several different bioassays (Kuraishi, 1959; Osborne and McCalla, 1961; Strong, 1958; Wittwer and Dedolph, 1963). Similarly, these studies showed that inhibition by BA at a given concentration was consistently greater than the kinetin-induced inhibition. Therefore, in all further experiments, BA rather than kinetin was used for kinin treatments. Neither GA nor IAA appreciably affected oxygen consumption in broccoli leaf discs at the concentrations used ($10^{-5}$ to $10^{-3}$ M).

The results of factorial design treatments with the three growth regulators at $5 \times 10^{-5}$ M are presented in Table II. The absence of an interacting effect was clearly demonstrated. Oxygen uptake as compared to the controls was consistently suppressed by treatment with BA. This BA-induced respiration inhibition was previously observed in broccoli (MacLean and Dedolph,

1964; MacLean, et al., 1963; Tuli, 1964). IAA and GA, on the other hand, did not significantly affect overall respiration when applied either singly or in combination with each other. Furthermore, the inhibitory effect of BA was not altered by treatment with either IAA, GA or IAA plus GA.

The data for oxygen uptake (Table II) were not confounded by tissue changes. Fully expanded leaves were used, and the number of 10 mm discs per gram fresh weight was constant for each experiment. Oxygen uptake measurements were taken 48 hours after treatment application. The only noticable tissue difference were in the BA treated discs which were somewhat greener than the controls or other treated tissue at the time of oxygen uptake determinations.

The absence of a GA-effect on oxygen uptake is in agreement with the data of Weller, et al., (1957) on beans. They found that although oxygen uptake was increased by GA on a plant part basis, there was but little effect when expressed on a fresh weight basis. The increased respiratory activity was evidently a function of growth.

Auxin-induced respiration increases have been correlated with growth (French and Beevers, 1953), and it has been suggested (Bonner and Bandurski, 1952) that

auxin may couple the respiratory and growth processes by making the energy formed in respiration available only to those tissues capable of growth. Thus, the absence of an appreciable effect of auxin on oxygen consumption in broccoli leaf discs may be, as in the case of GA, due to the fact that the tissues were fully expanded and incapable of further growth.

## PEA EMBRYO STUDIES

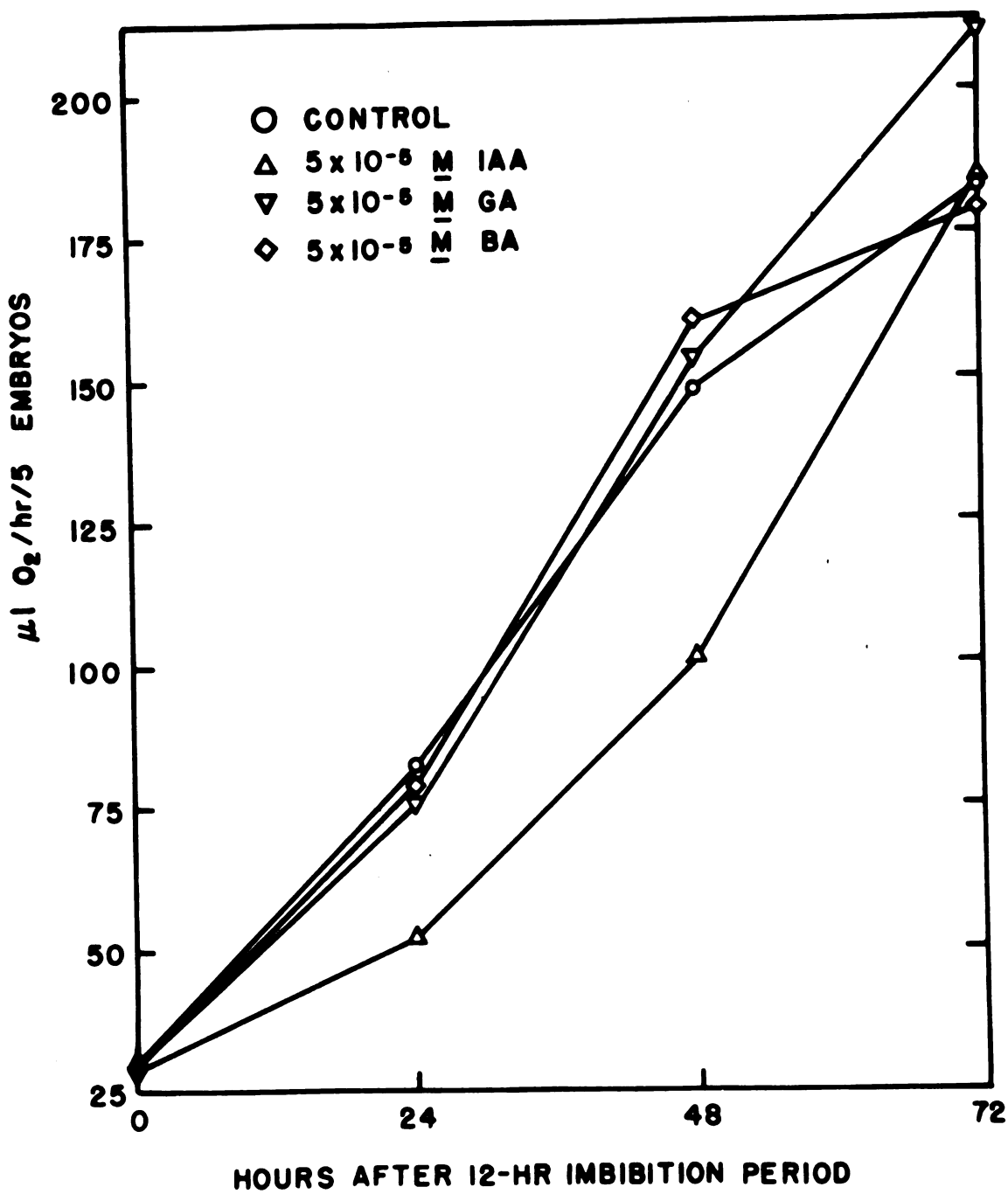
### Time Studies

The effect of treatment with $5 \times 10^{-5}$ M IAA, GA and BA on the rate of oxygen consumption by excised pea embryos during a 72-hour period following imbibition in the treating solution is presented in Figure 1. There was no effect of BA throughout the 72 hour period. GA did not influence oxygen uptake until after 48 hours, whereupon the respiration rate of GA treated embryos continued to increase in a linear manner, while the rates for the controls and BA treated embryos leveled off. The auxin treated tissue respired at a reduced rate throughout the first 48 hours. At 72 hours, however, their respiration rate was as high as the controls (fig. 1).

The non-treated embryos grew in a linear manner after the first 24 hours (fig. 2). (The radicle generally emerged through the testa 18-24 hours after

Figure 1

The respiration rate of control and IAA-, GA- and BA-treated pea embryos as a functicn of time.


CONTROL
5 x 10-5 M IAA
5 x 10-5 M GA
5 x 10-5 M BA
200
175
150
125
100
75
50
25
0
24
48
72
µl O2/hr/5 EMBRYOS
HOURS AFTER 12-HR IMBIBITION PERIOD

the imbibition period.) Neither GA nor BA affected embryo growth, IAA treatment, on the other hand, resulted in suppressed growth for 48 hours.

Although the general response patterns were the same for both oxygen uptake (fig. 1) and growth (fig. 2), differences were apparent. Both oxygen uptake and fresh weight determinations were made on the same embryos. Metabolic changes would necessarily preceed visible differences. Thus, a lag period between altered respiratory metabolism and subsequent effects on growth could account for the lack of the IAA and GA induced differences in oxygen uptake at 72 hours to be reflected in growth.

The increase with time in nitrogen content of the growth regulator treated embryos was generally similar to that of growth and oxygen uptake when expressed on a per embryo basis (fig. 3). The nitrogen content of IAA-treated embryos was significantly less than the control embryos at 72 hours. Otherwise, all treatments at all times, were not substantially different than the controls. However, when the nitrogen content was expressed on a per unit growth basis (mg N/gm fresh weight) a different response was observed (fig. 3). Although the GA and BA treated embryos were not different than the controls, the IAA treated embryos were.

Initially, the nitrogen content of the auxin treated embryos was less than the others, but at 24 and 48 hours after imbibition it was greater. These data suggest that at zero-time the exogenous IAA coupled with endogenous auxin results in a superoptimal concentration which impairs nitrogen assimilation from the cotyledons before growth commences. However, at 24 hours, when growth was evident, this was overcome and the nitrogen content per unit growth of the IAA-treated embryos was greater than that of the others. At 24 and 48 hours after imbibition, nitrogen assimilation in auxin treated embryos was proportionately greater than growth (fig. 3). Thus other factors than nitrogen were limiting growth.

In seedling growth, the nitrogen content generally parallels fresh weight increases resulting in a relatively constant ratio of nitrogen per unit growth. These data (fig. 3) indicate that GA and BA had no effect on this ratio, which became constant within 48 hours. Treatment with IAA delayed the time at which this constant ratio was reached by 24 hours.

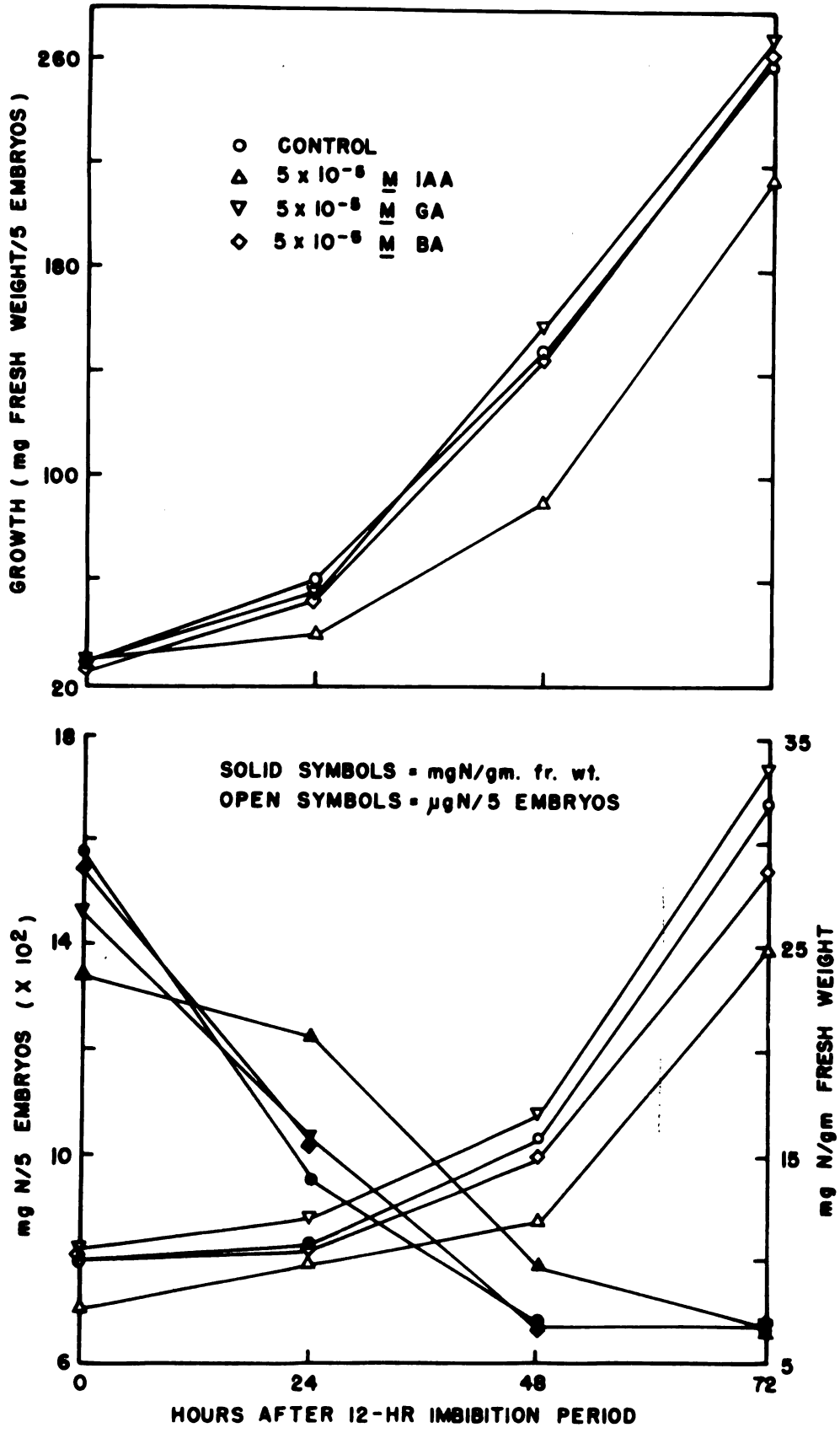
Throughout these time studies the only consistent marked deviation from the controls was that induced by IAA. If it is assumed, as Wright (1961) suggested, that specific concentration ratios of auxins, gibberellins and kinins are required for the growth and development

Figure 2

Growth of control and IAA-, GA- and BA- treated pea embryos as a function of time.

Figure 3

Nitrogen content of control and IAA-, GA- and BA-treated pea embryos as a function of time. Nitrogen content expressed on a plant part basis (N/5 embryos) and on a per unit growth basis (N/ unit fresh weight).


GROWTH ( mg FRESH WEIGHT/5 EMBRYOS )
260
180
100
20
CONTROL
5 x 10⁻⁵ M IAA
5 x 10⁻⁵ M GA
5 x 10⁻⁵ M BA
SOLID SYMBOLS = mgN/gm. fr. wt.
OPEN SYMBOLS = µgN/5 EMBRYOS
mg N/5 EMBRYOS ( X 10² )
18
14
10
6
35
25
15
5
mg N/gm FRESH WEIGHT
0
24
48
72
HOURS AFTER 12-HR IMBIBITION PERIOD

of plant tissues, then the IAA induced inhibition of growth and respiration in these time studies would suggest that auxin was the predominate endogenous growth regulator during the first few days of embryo growth.

On the other hand, the absence of a pronounced effect of GA and BA on pea embryos could suggest that the endogenous concentration is so low that even with exogenous applications of $5 \times 10^{-5}$ M the total gibberellin or kinin concentration was still below the threshold level for activity. The validity of this is doubtful since both GA (Phinney and West, 1960b) and BA (Miller, 1961) are active at very low concentrations in several *in vivo* systems.

Another possibility suggested by the lack of a GA or BA effect is that neither is required during these early stages of growth. However, if the efficiency of plant systems is considered, and if it is realized that germinated seeds are a rich souce of endogenous gibberellins (van Overbeek, 1962) and kinins (Biswas, 1964; van der Kerk, et al., 1964), then such a supposition is very unlikely.

The data from these time studies seem best explained in terms of the ratio of these three groups of plant growth regulators. Raghavan and Torrey (1964) suggested a rapid sequence of changing hormone interactions during the early growth of *Capsella* embryos.

Thus, in these time studies, addition of auxin may have resulted in a ratio within the embryos, in which auxin was exceedingly dominant, not necessarily in terms of actual concentration, but in effective concentration. The resultant superoptimal auxin concentration therefore inhibited growth and respiration. Furthermore, treatment with GA and BA during this period when endogenous auxin was dominant, had no effect. Even though the actual relative concentration of GA or BA was increased by application of $5 \times 10^{-5}$ M, the relative effective concentration was not similarly altered and the effect of endogenous auxin was predominate.

Protein Determinations

To better interpret the nitrogen content data, the total and protein nitrogen content of comparable embryos as affected by growth regulator treatment at $5 \times 10^{-5}$ M was determined 48 hours after the imbibition period (Table III). Treatment differences were not statistically significant regardless of the basis used to express the data. However, these data indicate that the nitrogen content of excised pea embryos is a reasonably valid estimate of protein content since the majority of nitrogen is in the form of protein nitrogen (64-80%).

Table III. Relationship between protein nitrogen and total nitrogen in excised pea embryos 48 hours after a 12-hour imbibition period. (IAA, GA and BA at $5 \times 10^{-5}$ M).

| | Chemical Treatment | | | |
|---|---|---|---|---|
| | Control | IAA | GA | BA |
| µg N/5 embryos | | | | |
| Total | 1003 | 977 | 845 | 907 |
| Protein | 664 | 694 | 646 | 722 |
| mg N/gm fr wt | | | | |
| Total | 7.99 | 8.80 | 7.48 | 8.89 |
| Protein | 5.15 | 6.60 | 5.73 | 7.17 |
| Protein N as % of total | | | | |
| per 5 embryos | 66.2 | 71.0 | 76.4 | 79.6 |
| per gm fr wt | 64.4 | 75.0 | 76.6 | 80.6 |

Effect of Growth Regulator Concentrations

Prior to conducting studies on the interacting effects of IAA, GA and BA it was necessary to ascertain the effect of these growth regulators when applied alone. The pattern of response to concentration for nitrogen content, fresh weight increase and respiration rate of excised pea embryos was similar for each growth regulator (figs. 4, 5, 6). A marked stimulation of these three parameters occurred at $5x10^{-6}$ M IAA (fig. 4). A ten-fold increase in concentration induced a drastic reduction of these responses, indicating that at $5x10^{-5}$ M, the total auxin concentration (exogenous plus endogenous) was superoptimal for growth, nitrogen assimilation from the cotyledons and normal respiration. Responses to IAA concentrations below $5x10^{-6}$ M were lower, but not necessarily inhibitory.

GA treated embryos exhibited no pronounced responses to concentrations from $5x10^{-8}$ to $5x10^{-5}$ M (fig. 5). Nevertheless, $5x10^{-7}$ M GA did result in a consistent reduction of growth, nitrogen content and respiration rate.

The responses to BA at $5x10^{-6}$ M were slightly stimulatory relative to the response induced by a ten-fold higher or lower concentration (fig. 6). However, at $5x10^{-6}$ M BA, none of these responses were signifi-cant.

Figure 4

Effect of IAA concentration on oxygen uptake, nitrogen content and fresh weight of excised pea embryos 48 hours after a 12-hour imbibition period. Points are the averages of three experiments, each of which consisted of three replicates. Control values were; 189.0 µl, 1343 µg, and 177.2 mg for oxygen uptake, nitrogen assimilation and fresh weight, respectively.

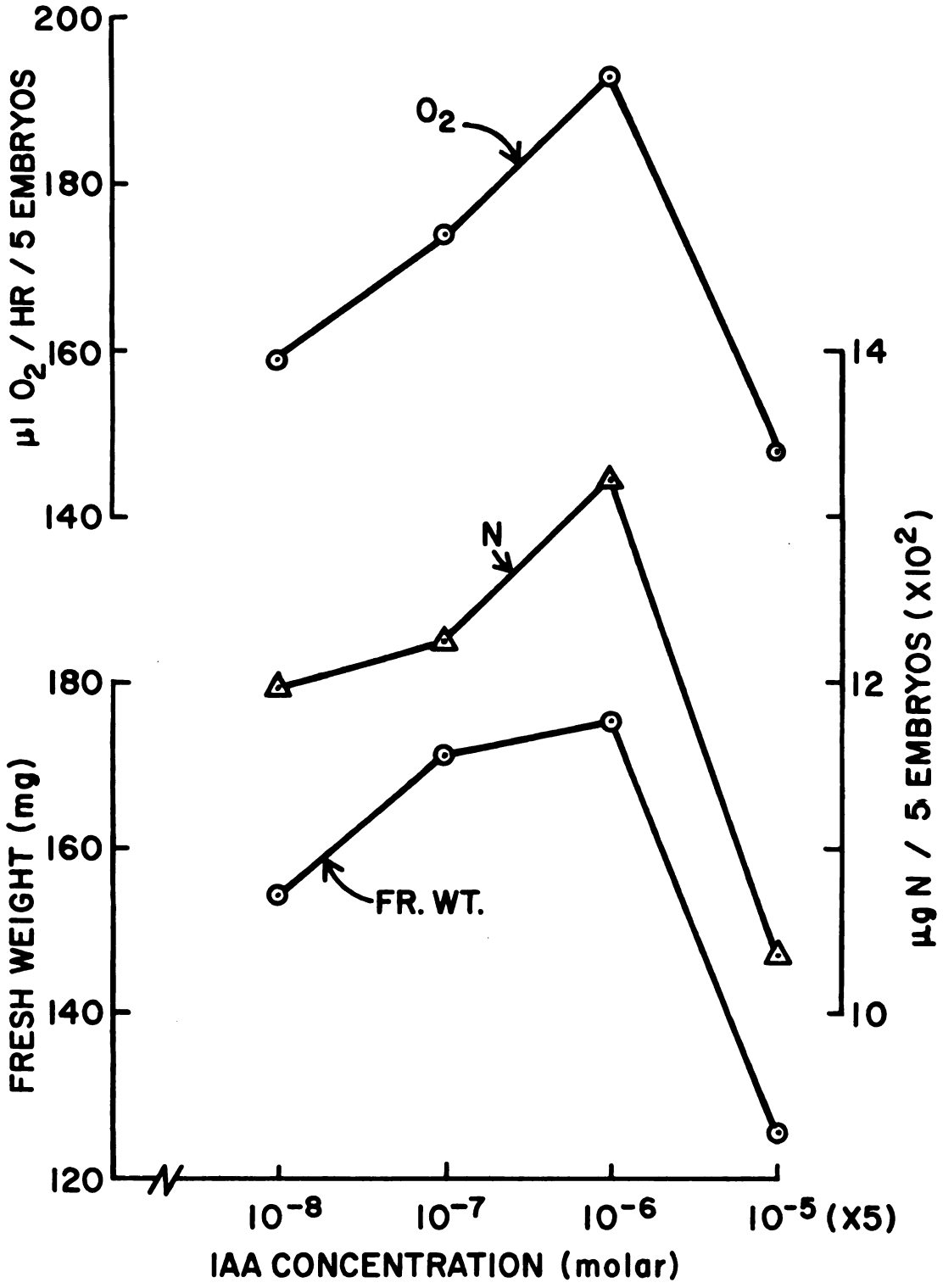

200
180
160
140
µl O2 / HR / 5 EMBRYOS
O2
N
FR. WT.
180
160
140
120
FRESH WEIGHT (mg)
14
12
10
µg N / 5 EMBRYOS (X10²)
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵ (X5)
IAA CONCENTRATION (molar)

Figure 5

Effect of GA concentration on oxygen uptake, nitrogen content and fresh weight of excised pea embryos 48 hours after a 12 hour imbibition period. Points are the averages of three experiments, each of which consisted of three replicates. Control values were; 189.0 µl, 1343 µg, and 177.2 mg for oxygen uptake, nitrogen assimilation and fresh weight, respectively.

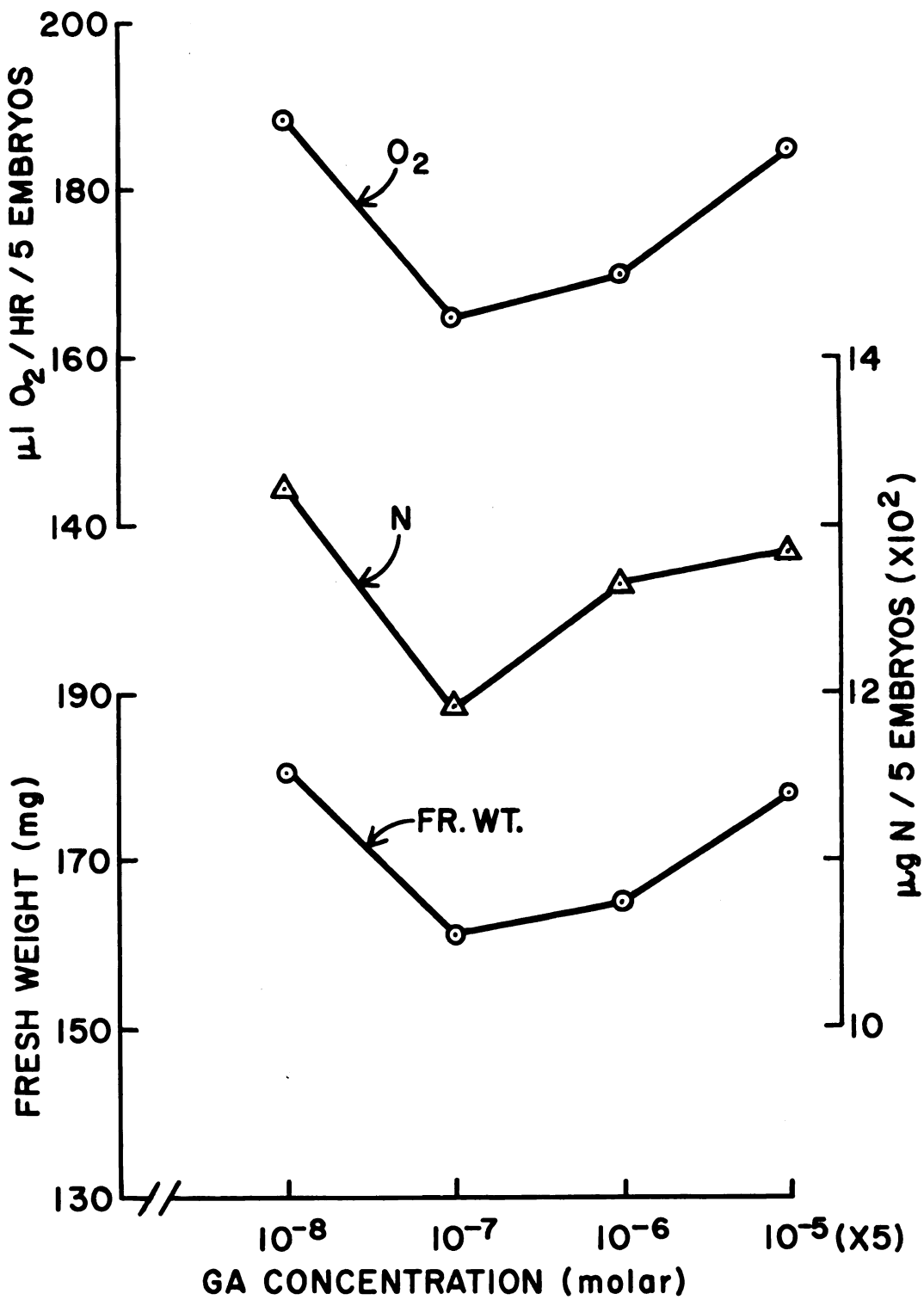
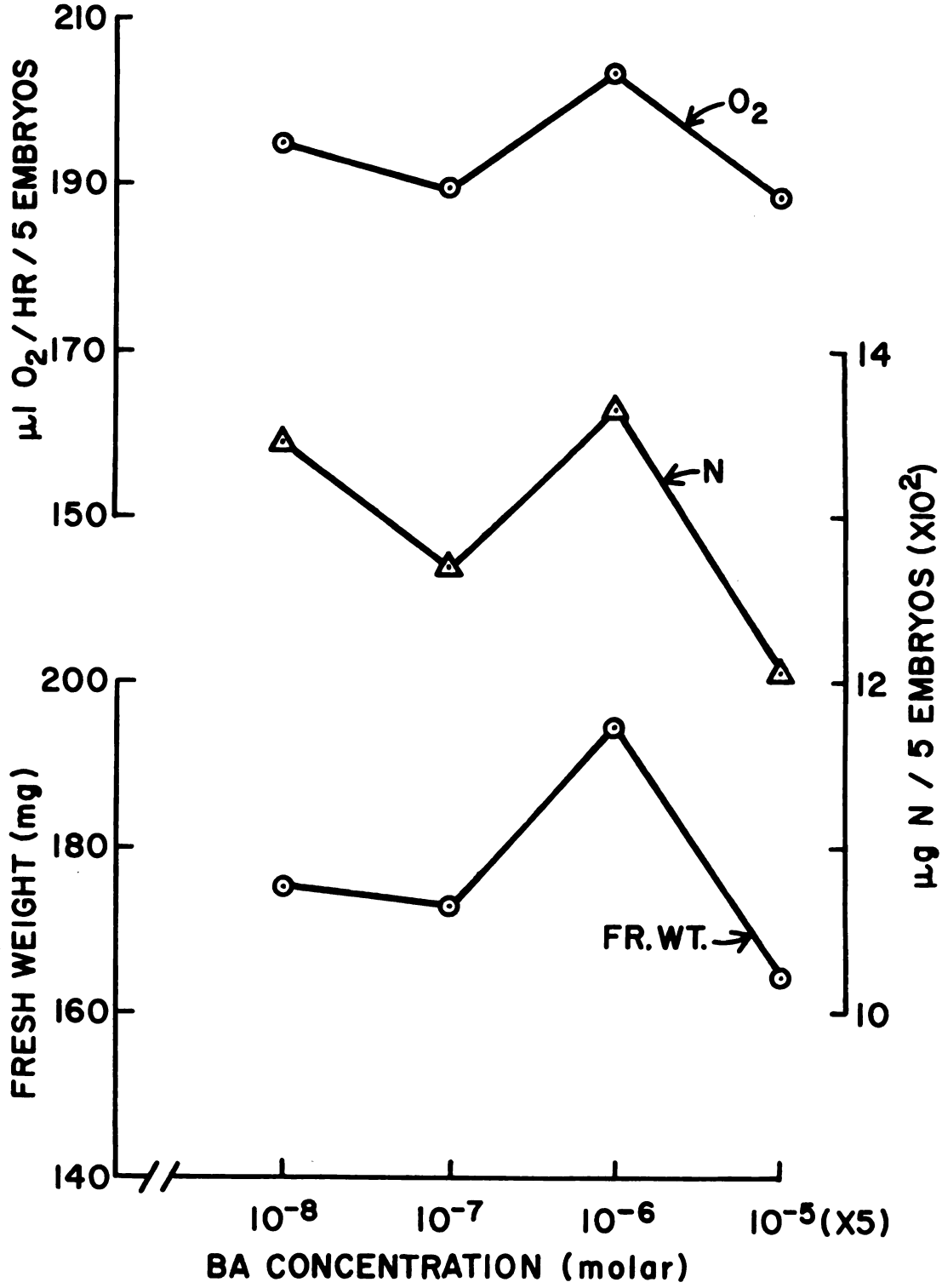

200
180
160
140
190
170
150
130
μl O2 / HR / 5 EMBRYOS
FRESH WEIGHT (mg)
O2
N
FR. WT.
14
12
10
μg N / 5 EMBRYOS (X10²)
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵ (X5)
GA CONCENTRATION (molar)

Figure 6

Effect of BA concentration on oxygen uptake, nitrogen content and fresh weight of excised pea embryos 48 hours after a 12 hour imbibition period. Points are the averages of three experiments, each of which consisted of three replicates. Control values were; 189-0 µl, 1343 µg, and 177.2 mg for oxygen uptake, nitrogen assimilation and fresh weight, respectively.


210
190
170
150
µl O2 / HR / 5 EMBRYOS
O2
N
14
12
10
µg N / 5 EMBRYOS (X10²)
200
180
160
140
FRESH WEIGHT (mg)
FR. WT.
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵(X5)
BA CONCENTRATION (molar)

Interaction Studies

Figure 7 depicts the interacting effects of GA, BA and GA plus BA, all at $5x10^{-5}$ M, with increasing IAA concentrations on respiration and nitrogen assimilation. For instance, the BA labeled curve describes the response observed when embryo were treated with $5x10^{-5}$ M BA alone (on the ordinate) and in combination with IAA concentrations from $5x10^{-8}$ to $5x10^{-4}$ M. Similar curves are presented for $5x10^{-5}$ M GA and GA plus BA over the same auxin concentrations. The most pronounced responses in these experiments where the IAA concentration was varied occurred at the low auxin concentration ($5x10^{-8}$ M). The rate of oxygen uptake and nitrogen assimilation were markedly stimulated by BA and inhibited by GA at the low auxin concentration. The stimulation and inhibition were of the same magnitude. Obviously, exogenous IAA at $5x10^{-8}$ M did not result in a superoptimal auxin concentration. The optimal IAA concentration in the absence of added BA or GA was $5x10^{-6}$ M (fig. 4). Furthermore, in the absence of exogenous IAA or when the IAA concentration was increased ten-fold to $5x10^{-7}$ M, these stimulatory and inhibitory effects were absent, indicating a requirement for a specific concentration ratio of these growth regulators for optimum growth. When embryos were subjected to a combination of GA plus

BA over increasing IAA concentrations the response paralleled that obtained with GA alone, suggesting that at low auxin concentrations the endogenous effective concentration of GA was greater than that of BA. At $5x10^{-7}$ M, IAA apparently became predominant and increasing auxin concentration progressively inhibited oxygen uptake and nitrogen assimilation. The almost toxic effect of $5x10^{-4}$ M IAA on oxygen uptake was not as pronounced for nitrogen content. This was probably due to the relatively high nitrogen content of the embryos prior to treatment.

The data for oxygen uptake (figs. 7, 10, 11) were expressed on a per 5 embryo basis (5 embryos per treatment replicate) throughout the interaction studies. This was justified by the highly significant correlation ($r = 0.864$) between oxygen uptake on a per 5 embryo versus oxygen uptake on a per mg nitrogen basis for all combinations (fig. 8).

The growth response of embryos to combination growth regulator applications very closely paralleled the respiratory response. Significant correlations were observed between growth and oxygen uptake regardless of the units used (fig. 9). Thus in all growth regulator interactions, the general patterns of response for fresh weight increase, nitrogen assimilation and

Figure 7

Effect of increasing IAA concentrations in the presence of $5 \times 10^{-5}$ M GA, BA and GA plus BA on the rate of oxygen consumption (top) and nitrogen content (bottom) of excised pea embryos.

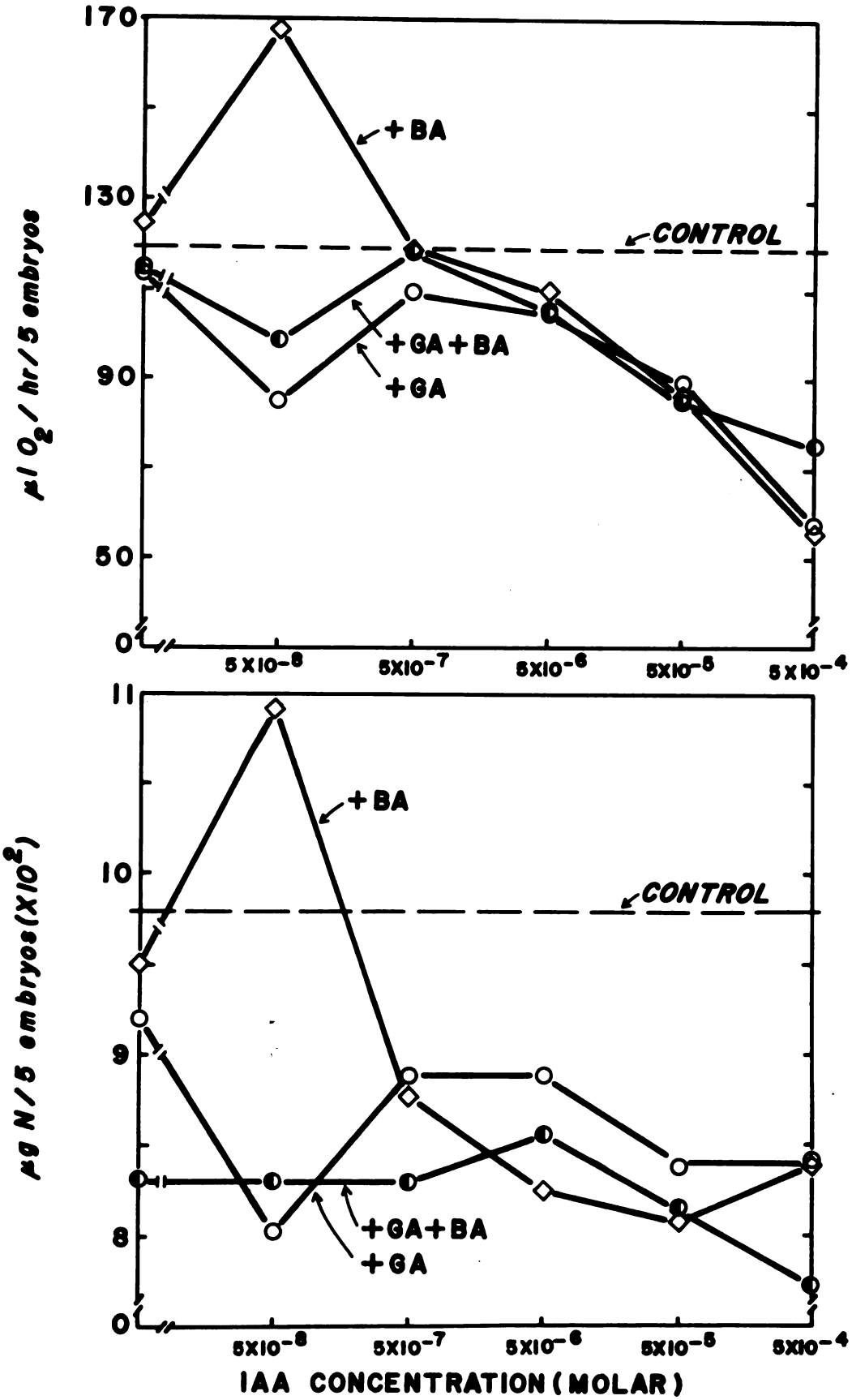
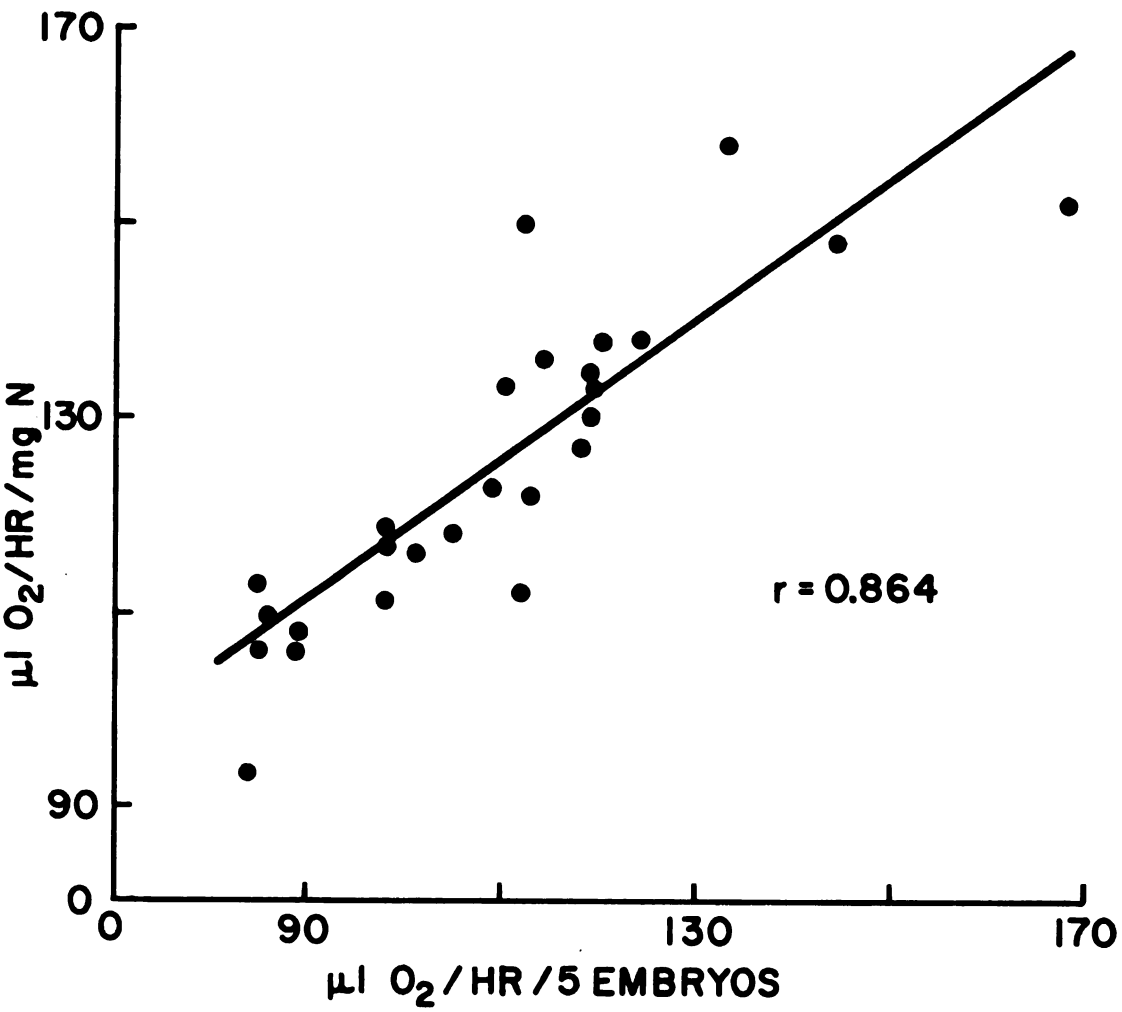

170
130
90
50
0
+BA
CONTROL
+GA+BA
+GA
μl O2 / hr / 5 embryos
5X10-8
5X10-7
5X10-6
5X10-5
5X10-4
11
10
9
8
0
+BA
CONTROL
+GA+BA
+GA
μg N / 5 embryos (X10²)
5X10-8
5X10-7
5X10-6
5X10-5
5X10-4
IAA CONCENTRATION (MOLAR)

Figure 8

Correlation between the rate of oxygen consumption per unit nitrogen and the oxygen uptake rate on a per plant basis. ($Y = 45.4 + 0.726\ x$)


170
130
90
0
0
90
130
170
µl O2/HR/mg N
µl O2/HR/5 EMBRYOS
r = 0.864

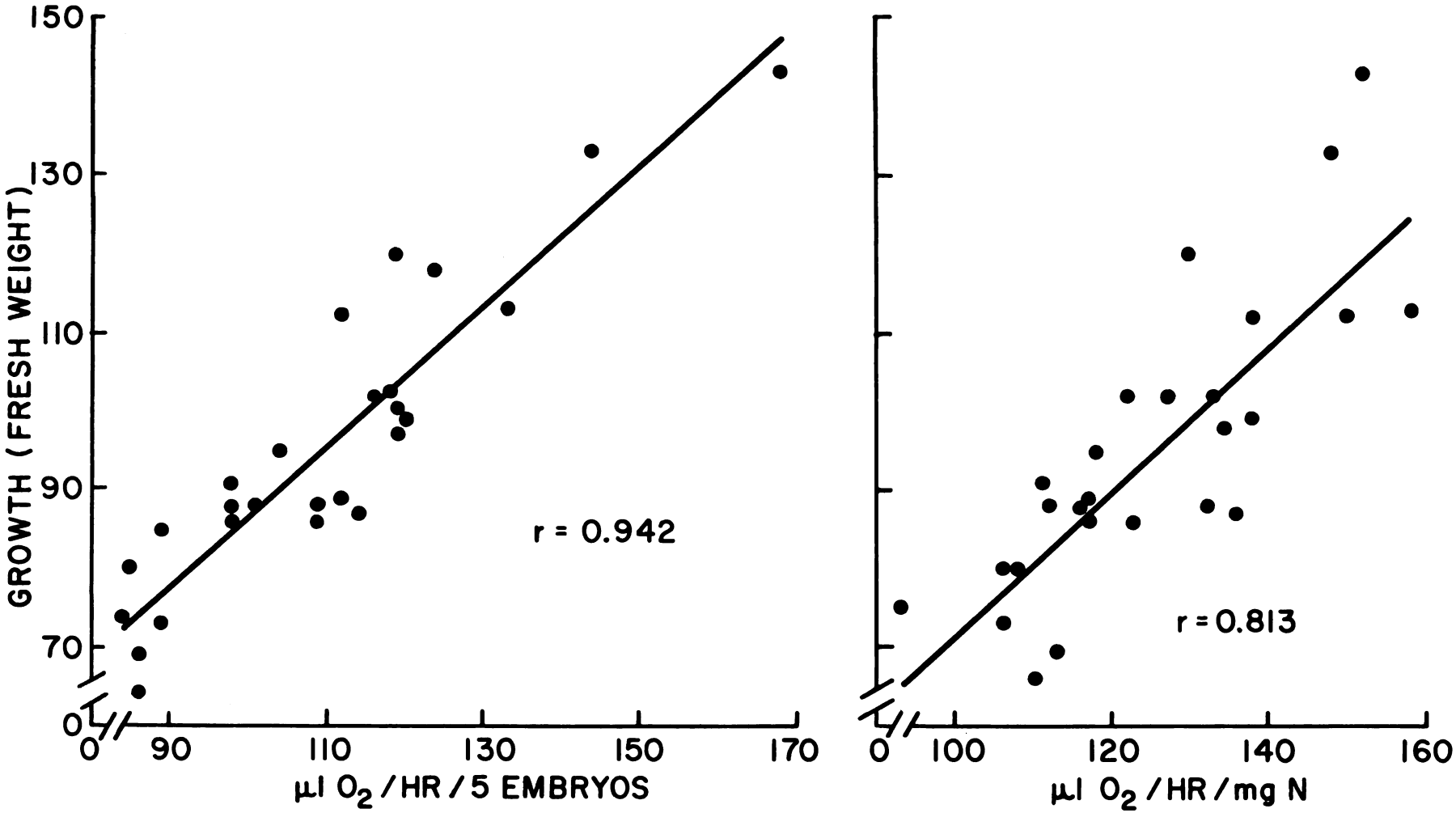
oxygen uptake, for a given treatment combination, were similar.

The data for experiments in which the GA concentration varied and the concentration of IAA, BA and IAA plus BA were held constant at $5x10^{-5}$ M are presented in figure 10. The concentration of IAA in combination treatments was $5x10^{-5}$ M. This concentration was based on preliminary determinations of oxygen uptake of embryos in the treating solutions. Later, when determinations were made in a moist chamber, it was shown that $5x10^{-6}$ M rather than $5x10^{-5}$ M IAA was most effective. There was no marked GA effect in the range of concentrations tested (fig. 10). Nitrogen assimilation was slightly stimulated in the IAA and IAA plus BA treated embryos in the presence of a low GA concentration ($5x10^{-8}$ M). However, this increase was still well below the control level. The effect of IAA at $5x10^{-5}$ M was dominant over the BA effect and the effect of increasing GA concentrations (fig. 10).

However, when BA was the variable (fig. 11), a marked increased in oxygen uptake and nitrogen assimilation occurred at $5x10^{-6}$ M in the presence of GA at $5x10^{-5}$ M. At BA concentrations above or below $5x10^{-6}$ M, the stimulatory effect was absent. A stimulatory response was observed at $5x10^{-6}$ M BA in the absence

Figure 9

Correlations between growth and the rate of oxygen consumption when expressed on a plant part basis (left; Y = 2.10 + 0.887x) or on a per unit nitrogen basis (right; Y = 22.67 + 0.929x).


150
130
110
90
70
0
GROWTH (FRESH WEIGHT)
r = 0.942
0
90
110
130
150
170
µl O2 / HR / 5 EMBRYOS
r = 0.813
0
100
120
140
160
µl O2 / HR / mg N

of added GA (fig. 6) which suggests that GA was not interacting with BA in promoting this stimulation (fig. 11). The curve for IAA plus GA, over increasing BA concentrations very closely followed the curve for IAA (fig. 11), demonstrating once more the dominant, inhibitory effect of $5x10^{-5}$ M IAA on excised pea embryos.

The dominant features of the interaction studies were: 1) The downward shift observed in the optimal IAA concentration induced by BA. In the absence of BA the optimum IAA concentration was $5x10^{-6}$ M, while in the presence of $5x10^{-5}$ M BA the optimum IAA concentration was reduced to $5x10^{-8}$ M. 2) The dominant effect of IAA when applied alone or in combination with GA or BA in the presence of increasing GA or BA concentrations. 3) The dominant effect of BA in the presence of GA. 4) The inability of various growth regulator combinations to alter the dependency of nitrogen assimilation on respiration.

An explanation of some of these dominant features will be attempted here. The significant effect of BA on reducing the effective concentration of IAA (figs. 4, 7) suggested that the presence of BA either increased the sensitivity of the embryos to IAA or enhanced the activity of IAA per se. The former

Figure 10

Effect of increasing GA concentrations in the presence of $5 \times 10^{-5}$ M IAA, BA and IAA plus BA on the rate of oxygen consumption (top) and nitrogen content (bottom) of excised pea embryos.

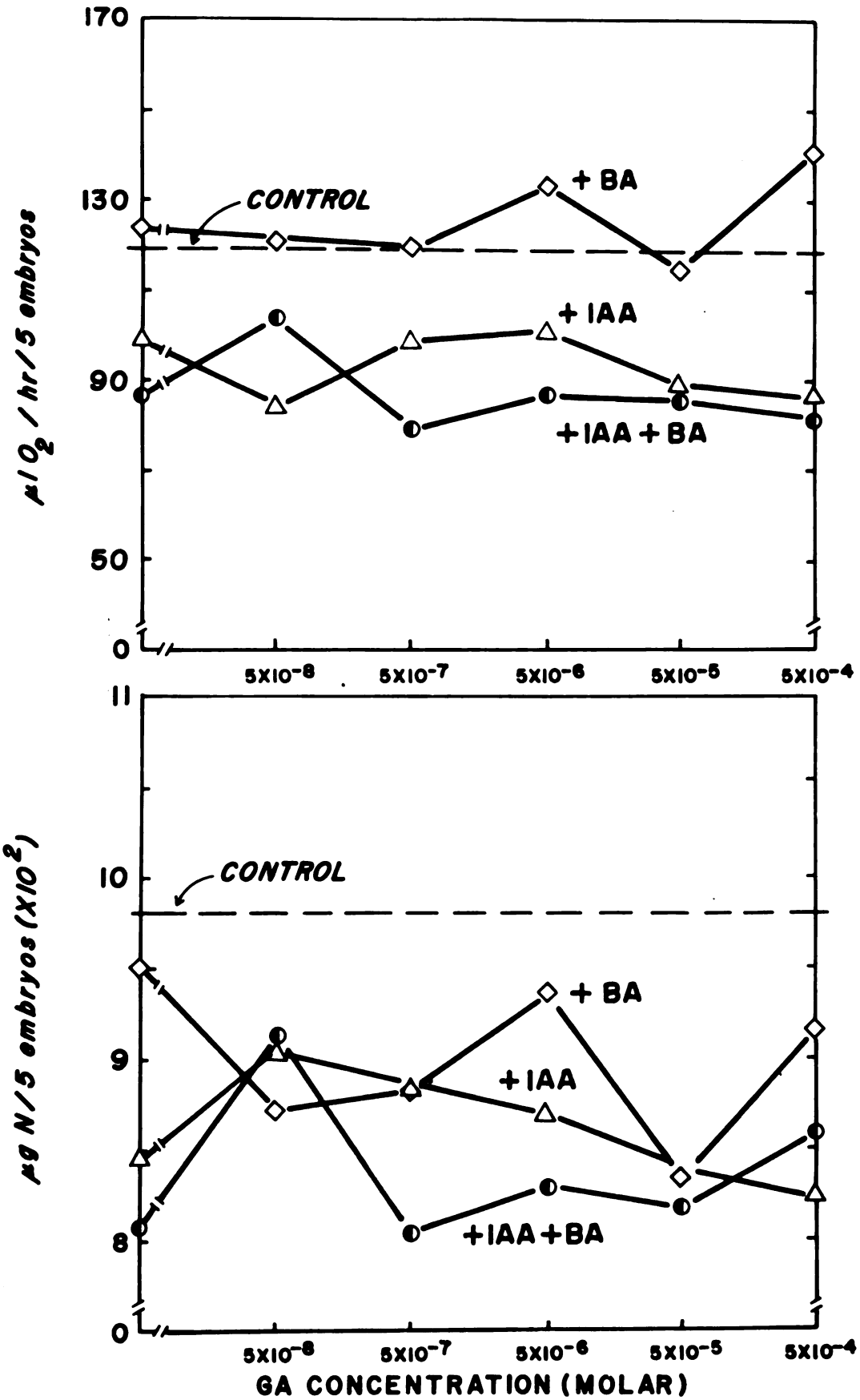
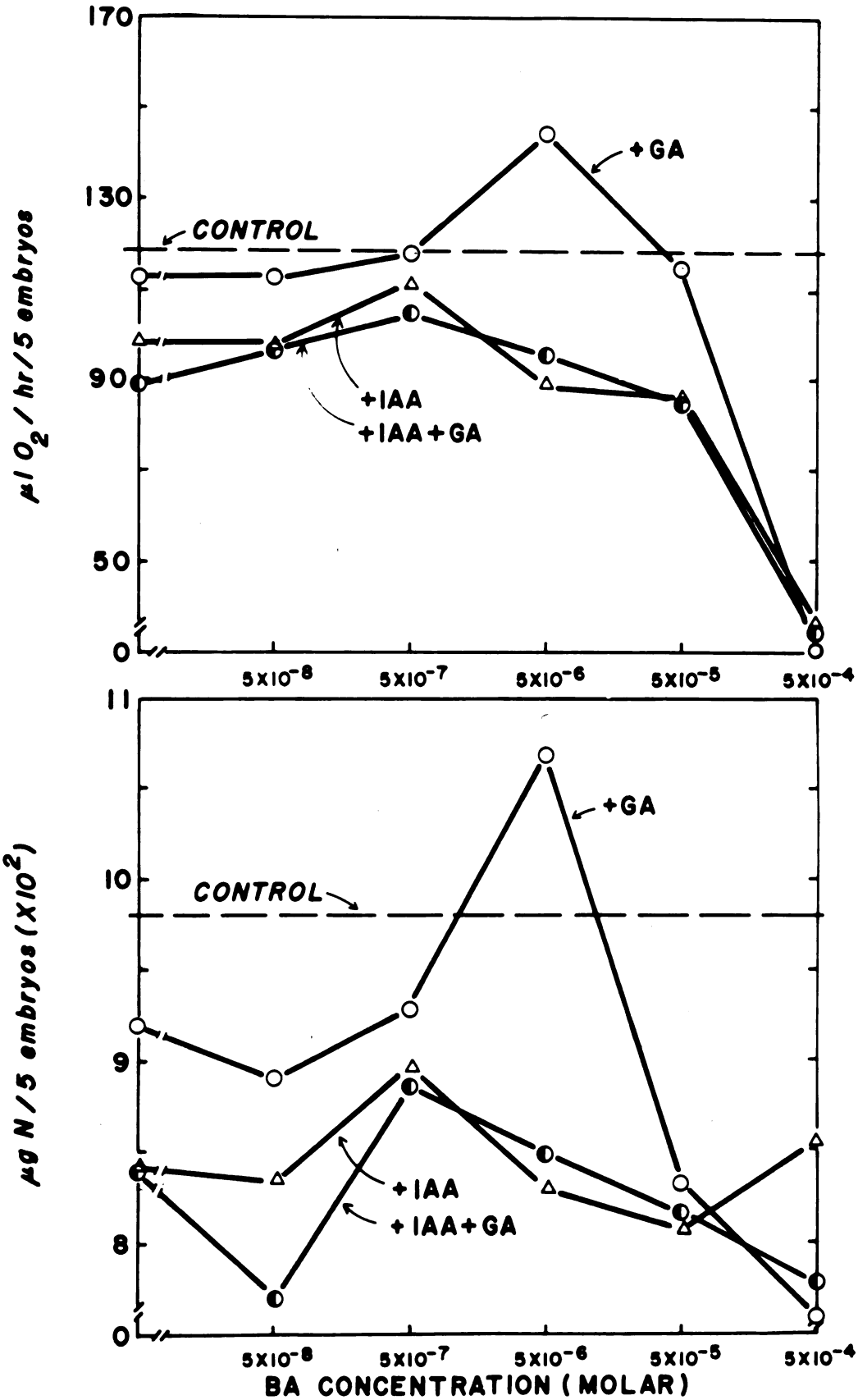

170
130
90
50
0
CONTROL
+ BA
+ IAA
+IAA + BA
µl O2 / hr / 5 embryos
5X10-8
5X10-7
5X10-6
5X10-5
5X10-4
11
10
9
8
0
CONTROL
+ BA
+ IAA
+IAA+BA
µg N / 5 embryos (X10²)
GA CONCENTRATION (MOLAR)

Figure 11

Effect of increasing BA concentrations in the presence of $5 \times 10^{-5}$ M IAA, GA and IAA plus GA on the rate of oxygen consumption (top) and nitrogen content (bottom) of excised pea embryos.


170
130
90
50
0
CONTROL
+GA
+IAA
+IAA+GA
µl O2 / hr / 5 embryos
5X10-8
5X10-7
5X10-6
5X10-5
5X10-4
11
10
9
8
0
CONTROL
+GA
+IAA
+IAA+GA
µg N / 5 embryos (X10²)
5X10-8
5X10-7
5X10-6
5X10-5
5X10-4
BA CONCENTRATION (MOLAR)

possibility appears to be the most likely. Nooden and Thimann (1965) demonstrated that continued protein synthesis was necessary for growth. Further, Srivastava and Ware (1965) showed the kinins to be involved in sustaining nucleic acid synthesis. It is possible therefore, that the increased sensitivity of the embryos to IAA by BA was a consequence of BA-induced reduction of nuclease activity and/or increased protein synthesis.

Conversely, GA had an inhibitory effect on IAA activity. At the low auxin concentration ($5x10^{-8}$ M), GA induced an inhibitory response equal in magnitude to the BA-induced stimulation, indicating that in some manner GA interferes with auxin activity. Ng and Audus (1964) studied growth regulator interactions in Avena and concluded that GA and IAA probably act at the same growth promoting centers and possibly compete for them. The possibility of this occurring in pea embryos is not overruled. However, since the $5x10^{-5}$ M GA plus $5x10^{-8}$ M IAA response (fig. 7) was less than that which occurred when each was applied alone at these same concentrations, the observed GA-IAA interaction in pea embryos suggests more than a simple competition for active sites.

The absence of a pronounced response to GA concentrations, either alone (fig. 5) or in combination with

IAA, BA or both (fig. 10) is difficult to interpret. During the first few days after germination, embryo growth is chiefly a result of cell enlargement, a process to which GA has been closely linked (Phinney and West, 1960b). However, since germinated seeds are a rich source of endogenous gibberellins (van Overbeek, 1962), the possibility exists that any exogenous GA results in a supereptimal concentration which, in turn, inhibits growth.

The maximum concentration response to treatment with BA occurred at $5x10^{-6}$ M (fig. 6). In the presence of $5x10^{-5}$ M GA this concentration response was not altered, indicating that GA did not influence the BA induced stimulation at this concentration. However, in the presence of IAA or IAA plus GA at $5x10^{-5}$ M the BA response was absent. Thus, when the auxin concentration was low relative to BA, during the first few days of embryo growth after imbibition, it appears that growth and associated phenomena were stimulated. Whereas when the IAA concentration was low relative to GA the opposite response occurred. Whenever IAA at $5x10^{-5}$ M or BA at $5x10^{-4}$ M was applied, inhibition occurred regardless of other combinations. Exogenous IAA or BA at these concentrations probably results in a total auxin or kinin concentration which is superoptimal.

The various responses to different growth regulator combinations and concentrations herein reported, are probably not a consequence of differential uptake. By imbibing dry pea seeds in the treating solutions for 12 hours it is very probably that all cells received chemical treatment. Further, the treating solutions were the only souce of moisture after the imbibition period. In broccoli leaf discs the duration of exposure to the growth regulators (16 hours) probably assured their uptake.

# SUMMARY AND CONCLUSIONS

In the preliminary studies on fully expanded broccoli leaf discs IAA and GA were ineffective on oxygen uptake. The only response to growth regulator treatment occurred when BA was included in the treatment solution. The inhibitory effect of BA either alone or in combination with IAA and/or GA, on oxygen uptake was probably the result of delayed senescence through maintenance of RNA and DNA synthesis, as suggested by Srivastava and Ware (1965).

The effects of IAA, GA and BA treatments on excised pea embryos were assessed using three parameters: 1) growth, as fresh weight increase; 2) nitrogen assimilation from the cotyledons; and 3) the respiration rate, as indexed by oxygen uptake.

In all treatments these three parameters were similarly affected by any given treatment. Significant correlations between growth and respiration were obtained for embryos receiving combination growth regulator treatments. Further, various growth regulator treatment combinations did not dissociate the similarity between growth, nitrogen assimilation and respiration.

Interactions between IAA and BA and between IAA and GA were observed. However, no such interaction between GA and BA could be detected. The IAA concentra-

tion which induced maximum stimulation was reduced from $5x10^{-6}$ M to $5x10^{-8}$ M by $5x10^{-5}$ M BA. The IAA response at $5x10^{-6}$ M was blocked when $5x10^{-5}$ M GA was included in the treatment. Further, this GA concentration resulted in a marked inhibition of growth, nitrogen assimilation and oxygen uptake when applied in combination with $5x10^{-8}$ M IAA. The stimulatory effect of BA at $5x10^{-6}$ M was not appreciably affected by $5x10^{-5}$ M GA. A ten-fold increase or decrease in growth regulator concentration usually removes these responses, suggesting the requirement for a specific concentration ratio of these three groups of plant growth regulators, and perhaps others as yet undiscovered, for plant growth and development.

A possible mechanism for the hormonal control of plant growth and development can be formulated from the results presented in this dissertation and the data of other investigators. Wright (1961) inferred that during the early growth of wheat coleoptiles, different growth regulator ratios occur. A sequential shift in the ratio occurs with growth in which gibberellin, kinetin and auxin dominate the ratio in that order. It was also demonstrated that the inhibitory effects of one growth regulator could be overcome by increasing the concentration of another (Fox, 1964). Thus, there is precedent for the governing effect of specific growth

regulator ratios.

The multiplicity of effects induced by IAA, GA or BA treatments suggest that their primary effect is very basic. The manner in which these basic effects are expressed may be influenced by the tissue type and environmental factors, resulting in a great variety of responses. The basic effect may be at the nucleic acid level. The evidence for auxin (Nooden and Thimann, 1963, 1964) gibberellin (Phinney and West, 1960b; Varner, 1964; Varner and Chandra, 1964) and kinin (Osborne, 1962; Srivastava and Ware, 1964) effects at the nucleic acid level have already been presented.

An intricate balance between these three growth regulator groups probably exists. The balance may be such that the addition of a reasonable concentration of one growth regulator would affect the relative effective concentration of all three. The number of possible concentration combinations is almost limitless

The response to GA concentrations was minor relative to IAA and BA concentrations. Further, the absence of the pronounced inhibition at $5 \times 10^{-4}$ M GA that occurred in the presence of IAA of BA at $5 \times 10^{-4}$ M suggests that, if such a balance exists, GA must be less effective on the balance than corresponding additions of BA or IAA. Further, superoptimal IAA or BA concentrations

($5 \times 10^{-4}$ M) probably disrupt the entire balance, whereas the inhibitory effect of GA at the same concentration is much less.

Therefore, if the action of these growth regulators is assumed to be at the nucleic acid level, one ratio may induce the synthesis of enzymes governing different responses. The type of tissue, stage of maturity and the environmental conditions prevailing at the time a specific concentration ratio occurs could further influence the responses to the basic effect.

With such a scheme, the many responses ascribed to the hormonal control of plant growth and development can be rationalized. However, this hypothesis can be tested only after a tissue is found that can be starved of endogenous growth regulators and still retain the capacity for growth when they are added back.

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