

REASSESSMENT OF THE RELATIONSHIP  
BETWEEN HORMONAL AND  
DEVELOPMENTAL CHANGES DURING  
ABSCISSION WITH PARTICULAR  
REFERENCE TO PEACH  
[*Prunus persica* (L.) Batsch] FRUIT

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## ABSTRACT

### REASSESSMENT OF THE RELATIONSHIP BETWEEN HORMONAL AND DEVELOPMENTAL CHANGES DURING ABSCISSION WITH PARTICULAR REFERENCE TO PEACH [Prunus persica (L.) Batsch] FRUIT

By

Franco Zucconi

Levels of extractable hormones are often correlated to physiological phenomena based on the assumption that a simple cause: effect relationship exists between the two. The presence of different and unrelated phenomena in complex organisms may, however, lead to misinterpretation of events and physiological changes. This problem is particularly serious when studying the abscission of young fruit because both persisting (actively growing) and abscising (various stages) fruits are present in the same population at the same time. Fruit growth and abscission, therefore, was used as a test system for interpreting changes in two simultaneously occurring events in relation to concurrent changes in hormone level. Peach [Prunus persica (L.) Batsch] fruit populations having a low or high abscission potential were established by either increasing or decreasing competition between fruit and shoots. Abscission probabilities were then assigned to subgroups, within each population, based on a predetermined relationship between growth and abscission. Several inhibitors, as indexed by inhibition of cress (Lepidium sativum, L.) seed germination were detected

in extracts from peach seeds. The biological activity of two inhibitors was particularly prominent. One (Inhibitor II) was identified as abscisic acid (ABA) by gas liquid chromatography and gas chromatography-mass spectroscopy. ABA levels, in seeds from fruit separated according to their abscission probability, were inversely related to abscission, and positively related to fruit size, growth rate and persistence. The levels of a second inhibitor (Inhibitor III) differed markedly between fruit with a high and low abscission potential. The existence of different patterns of ABA and Inhibitor III content in fruit selected according to their potential to persist or to abscise, in comparison to those randomly collected, stresses the importance of sampling when relating changes in hormone level to physiological events under study. The ABA with growth association found in this study would have been missed, and an ABA with abscission relationship would have been implied, if fruit samples were not classified according to abscission potential. Further, the existence of opposite trends of Inhibitor III in persisting and abscising fruit would have not been detected in randomly collected samples. Commonly used approaches for relating changes in hormone level to physiological processes are dependent on the hypothesis that the two events have a cause:effect relationship. Unfortunately such a hypothesis is neither adequately tested nor confirmed by experiments confined to the analysis of hormonal levels and morphological changes. Arguments are presented to show that the hypothesis itself (parallel variations between hormone and event) may be inadequate for an understanding of the role of hormones. Suppression or substitution of the hormone source may provide more critical data.



Yet the complexity of factors and interactions common to biological processes are probably not adequately represented in experimental approaches dependent on a single system or model. The value of a multiple experimental approach, as a working model in relating the role of hormones to the regulation of physiological events is discussed.

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WITH PARTICULAR REFERENCE TO PEACH  
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## INTRODUCTION

Abscission studies in this century have gradually evolved from the early investigation of the natural control of longevity, to the present emphasis on chemical control of abscission. The economic needs underlying such a shift are understandable, since the benefits of controlling abscission are numerous. Yet, results thus far achieved are unsatisfactory. Meanwhile, fruit and leaf abscission control in agriculture remains confined to a few, specific cases.

This conclusion should not come as a surprise. The modest technical achievements reflect the fragmentary of physiological knowledge of the phenomenon itself. Unfortunately, research has not been pursued sufficiently in this area so as to give a thorough understanding of natural mechanisms which control abscission.

A deeper physiological understanding is needed which, in turn, will also make available better technical tools. Research in the last 20-30 years, however, has been more successful in defining the morphological changes associated with abscission than in defining the nature of its regulation. Meanwhile in an attempt to solve immediate problems, created by the practical need of controlling abscission, scientists placed a greater emphasis on screening chemical substances.

One might ask at this point, how can we improve the study of regulation of abscission? A partial answer is implicit in the continuing effort toward perfecting methods and in refining analysis.

More attention, however, should also be given to evaluating the available models and working hypotheses. The evolution of the research from the study of longevity to external manipulation of abscission implies not only a change in interest, but also a shift from a general to narrow approach. Unfortunately, the more specific the research becomes, the less attention is devoted to the study of general models necessary for the formulation of new concepts. It is rather symptomatic that most of today's working hypotheses have remained unchanged for decades in spite of the limited support they appear to receive from the experimental data. For example, research on abscission has yet to propose an alternative to the use of simplified or reduced (deblated) systems which, adequate to the analysis of some morphological changes in an abscission layer, remain of questionable value in the study of the physiological control of abscission. Also, the attention devoted to levels of natural hormones, in explaining abscission or its inhibition, appears excessive. It is doubtful that a purely quantitative approach will elucidate the role of hormones in the regulation of a complex, integrated phenomenon, such as abscission.

In this work, an attempt was made to critically evaluate the available knowledge on the natural control of abscission and to analyze the possibility of improving models and hypotheses needed for its study. Emphasis was placed on using intact systems. In so doing, we stressed the separation of abscission from concurrent events to allow for a better interpretation of chemical changes associated with this event. The problem is serious because complex systems always imply the presence of a number of events unrelated to the one being studied,

and often not easily distinguished. Selection and sampling of the biological material used for extraction and the parameters used to represent the event being studied may influence the mathematical associations which may be established.

The main postulate, however, that a hormone-event relationship exists which may be viewed as a simple cause-effect model, is neither implied nor tested by this type of research. A theoretical study was therefore pursued to establish the assumptions needed for this hypothesis, and to observe how well experimental data support such a hypothesis.

## LITERATURE REVIEW

Abscission is the process by which an organ, or part of an organ, separates from the main body of the plant. In fruits and leaves an abscission zone can be identified at the base of the organ or its supporting stalk characterized by smaller, less lignified cells. An abscission layer usually develops through this zone, bringing about separation. Abscission occurs at defined stages in the life of a deciduous organ or following traumatic or accidental interruptions of its development.

The existence of an induction phase is generally accepted to explain the transition from a nonabscising to an abscising stage. Often the demarcation between the two stages is not well defined. Also, it is usually assumed that some chemical message, sent to the abscission zone, is responsible for the regulation of abscission layer formation. Physiologists have been searching for a messenger role for plant hormones because these substances are claimed to have similar regulatory functions in other phenomena. It is finally hypothesized that these messengers are synthesized outside of the abscission zone itself.

The relevance of the above hypotheses and the data generated in testing them experimentally will be briefly discussed here. Since many reviews have presented a comprehensive survey of literature on abscission (6,7,8,31,65,68,117), this review will be limited to that

research which has been most relevant to the development of hypotheses on the regulation of abscission. Auxin studies will be discussed in detail since they are associated with some of the most interesting and imaginative work done thus far. Less emphasis will be given to other hormones because of their lesser impact in this field of study.

### Internal Factors Which Control Abscission in Leaves

#### Auxins

Extensive research has been done to clarify the regulatory role of plant hormones in abscission. Observation of a chemical correlation in the control of longevity (Kuster, 1916; from Jacobs:65) antedates verification of the existence of hormones in plants. However, it was only after Went's discovery of auxin, indoleacetic acid (IAA) that we witness the greatest advancement in studies of abscission.

During the period 1933 to 1940, auxin studies were further stimulated by providing convincing evidence for a role of IAA in abscission (79,80,95,99,100) and by introducing sensitive plant systems and research methods, many still in use today. However, it was only after this stage that IAA was proved to be a common plant hormone and not just an hetero-auxin with auxin-like activity in vascular plants.

The work which followed generated the main body of hypotheses on endogenous control of abscission that we possess today.

Coleus.--Abscission in coleus (Coleus blumei, Benth.) has been extensively studied by Myers (99,100,101) and Jacobs (64 to 73,85,120, 132), and the most significant findings are summarized below. Coleus

leaves are short lived and abscise at intervals that are inversely proportional to their age. It is claimed that diffusable auxin is produced in the leaf blade in amounts which significantly correlate with the natural longevity of the leaf (100,132). Although no positive identification has been made, data suggest that the leaf-produced auxin is IAA (74,120). The regulatory role of the leaf blade is also claimed based on the observation that deblading causes a rapid abscission (1-7 days) of all petioles irrespective of their age (78, 95,100,132).

The role of IAA in inhibiting abscission is also, according to Jacobs, established since IAA applied to debladed petioles restores the longevity observed in the intact leaf (74,120). This inhibition appears rather specific since other growth regulators were found to be inactive (48).

Natural regulation of ~~abscission~~ includes not only means by which this event can be inhibited, but also means by which it can be stimulated. Correlation effects with other organs were suggested to account for such a stimulation. Apices and axillary shoots, in particular, were found to be responsible for the stimulation of natural leaf abscission. Externally supplied IAA substituted for the apex. Leaves (young and old) also stimulate abscission of other leaves or debladed petioles but IAA can substitute effectively only for apical leaf pairs 1 and 2.

In 1955, Jacobs proposed (64) that leaf abscission was indeed regulated by the competition between the retarding effect of the auxin coming from the blade and the stimulating effect of the auxin coming



from apices, other leaves (mostly young ones) and axillary shoots.

This hypothesis was named as the auxin-auxin balance theory.

Unfortunately, this hypothesis fails to account for the action of leaf pair 1 (65). Also, Myers observed (100) that maximum longevity characterized winter leaves which had a low auxin level and, vice versa, spring leaves showed maximum auxin level but were short lived. This observation places some doubt as to the validity of a purely quantitative approach to the interpretation of the role of auxin (100).

A peculiarity of the coleus as a plant model is the role of cytokinesis in abscission. Cell division (starts in leaf pairs 2-3 and is complete in leaf pairs no. 5-7) does not show a clear relationship to deblading (100,119,132) as it does to longevity (i.e., the life span of the leaf). Deblading hastens cell division in leaf pair 2 but inconsistently in 3 and 4. Leaf pairs 5-7 are seldom affected.

These different responses should be interpreted as evidence for the presence of two distinct abscission processes. The first process is typified by cytokinesis preceding abscission and characterizes entire leaves and debladed leaf pairs 1 and 2. The second process dominates leaf pairs 3 to 7 in which blade excision suppresses cytokinesis, but not abscission. IAA applied to petiolar stumps restores cell division in these leaves, therefore causing a switch to the previous pattern. Finally, a distinct feature of coleus leaves is indeterminate growth which ceases shortly (about a week) before abscission. Deblading stops petiole growth, while applied IAA restores it (100). By contrast, leaves of other species generally show a determinate growth and abscise long after growth ceases. Coleus may

represent, in this respect, a rather peculiar system, and thus a questionable model for broad generalization.

Other plants.--The work on abscission, after coleus, became highly specialized due to the introduction of explants (i.e., portions of plant containing the abscission zone) as working systems.

Unfortunately detailed comparisons with intact plants are too scarce in these studies (21) to allow generalization. In addition, significant differences often exist between intact systems and explants. For instance, explants abscise readily, at essentially all stages of development, as if the inductive phase was lacking and the development of the abscission layer entrained (synchronized) by deblading. Another difference is that explants from mid-aged leaves require the longest time to abscise (106).

Results obtained with coleus are frequently inconsistent with those obtained with other plants, mainly bean (Phaseolus vulgaris, L.), cotton (Gossipium hirsutum, L.) etc. Auxin extracted from bean leaves (121) does not correlate with the longevity of the leaves. More important, in plants other than coleus, auxins only partially substitutes for the blade (65,89,95) and the presence of the blade is not an absolute requirement for the persistence of the petiole (20, 89,95).

Despite these limitations, studies on bean and cotton explants have helped greatly in clarifying the dual response to hormones. As a matter of fact, the possibility of applying hormone on either side of the abscission zone in explant systems facilitated the study of the

competitive action of auxin placed distally or proximally. This work generated the auxin-gradient hypothesis according to which longevity is favored by higher levels of auxin in the leaf than in the stem. This hypothesis was proposed by Addicott, Lynch and Carns (12) in 1955; therefore it is contemporary to the auxin-auxin balance hypothesis, and, even if less well documented, is consistent with it.

The auxin-gradient theory does not apply to all ranges of hormone concentrations. High doses delay abscission irrespective of the site of application (distal or proximal) (17,18,49,118). A regulatory role of the concentration was therefore proposed by Gaur and Leopold in 1955 (49). This response closely resembles the diphasic growth-response curves of Thimann (124). It differs, however, in that delayed applications of auxin do not reverse the growth response as they do on abscission. Further, Rubinstein and Leopold (117) found that by delaying the application of a retarding dose of auxin, to a debladed petiole, the hormone accelerated abscission. They postulated, therefore, the existence of two stages, in abscission, differing in their response to auxin.

The conclusions of Rubinstein and Leopold are consistent with previous observations (Barlow:15) relative to the existence of an auxin-sensitive period in apple pedicels. Yamaguchi (136), too, had proposed the existence of an initial phase, following deblading, characterized by a progressive cessation of the inhibition imposed by the subtending leaf. An interesting corollary to the two stages theory is the observation that all synthetic auxins delay abscission during the first

stage, but that only auxins that cause growth hasten abscission in stage II (35).

The two stage concept has received wide acceptance, and it has been interpreted that phase I represents a period when induction is believed to take place. The full impact of this concept cannot be appreciated yet. Only few biochemical changes have been associated thus far with stage I (5,37,40). Existence of stages in abscission has seldom been found outside bean (15). The two stage response is not apparent in most plants (8,15,78). More important, no information exists as to the applicability of this concept to intact systems.

#### Other Hormones

Ethylene.--Ethylene has been a long-standing tool for hastening abscission. For a long time, however, it was considered to be merely an interesting and remarkable curiosity. In the last 15 years, however, evidence has implicated this gas as a true phytohormone, involved in the regulation of growth (28,29,34,50,51,74), fruit ripening (25,26), reproductive activities (27,30) and abscission. Ethylene is produced during abscission (63,114) and its accumulation strongly influences this event as shown with withdrawal experiments (3,8,72). Also, it hastens abscission when given directly on shedding organs. Further, chemicals which accelerate abscission stimulate the production of ethylene (1,114).

Experiments with bean explants disclosed the existence of two stages of response to ethylene (4,63,136). During the first stage explants are insensitive although there is some evidence that ethylene

itself shortens this stage (63). It is during the second stage that an ethylene hastening-effect becomes evident. Abeles (1) suggested that the auxin effect in the second of the two stages of abscission (116) is derived from the ethylene produced as a result of auxin treatment. He also proposed that the speeding action of proximal auxin treatment is ethylene mediated. In such a case IAA would diffuse against polarity and, therefore, more slowly than ethylene. This hypothesis is not sufficiently substantiated and studies with other synthetic auxins (72,116) do not support it.

There is some evidence that ethylene is not always associated with abscission. No correlation was found between ethylene produced by several chemicals and their abscission-accelerating effect. On citrus explants, GA and ABA caused abscission but detectable increases in ethylene were noted only after separation (110). No ethylene accompanied GA and ABA accelerated abscission of citrus fruit (87). Abeles noticed that GA, which is a weak accelerant of bean abscission, caused a many-fold increase in ethylene production compared to ABA, which is a stronger accelerant (1). A further example of this ambiguity is the higher production of ethylene in explants from young than from old leaves (24,58).

It is impossible for the moment to establish if ethylene is a general requirement for abscission. Possibly, as seen in the case of auxin, quantitative measurements are not the best approach to the understanding of the physiological role of this hormone.

Abscisic acid (ABA).---The recent discovery of ABA (9) has given a new impetus to abscission studies, but this hormone is turning

out to be rather enigmatic. ABA was found in cotton fruit and related to senescing factors previously found in leaves (16,57,73, 104,105,115). The nomenclature is misleading, however, since convincing evidence for the regulatory role of this hormone in abscission is still lacking. High levels of ABA were observed in young nonabscising leaves, whereas the levels were lower in old and abscising leaves (108,125). Also, most of the ABA accelerating effect on abscission is confined to the response of explants (1,9,13,40,47,110,122). In contrast, intact plants react only to very high concentrations or to repeated applications (38,45,47,60,122).

Gibberellins.--GA<sub>3</sub> is active both in accelerating (high concentration:16,32,36,93) and in retarding abscission (low concentration: 36,93). These results, however, may not be generalized. GA remains a relatively weak accelerator, and its activity declines with increased age of the organ. This suggests that we may be confronted by a type of juvenile response (68,92,93). GA may play a role by interfering with correlative competition. GA applied on the stem apex causes an increase in IAA production (a growth response?); and this, more than GA itself, may be responsible for accelerating abscission (98).

Cytokinins.--Cytokinins, too, have been tested for a possible regulatory role in abscission, since they are known to retard senescence (14,86,97,107,123), to stimulate parthenocarpic fruit growth (41,42,86), and to mobilize nutrients to the site of application (41,84,85,97).

Responses of explants to cytokinins are, however, confusing. Distal application delayed abscission in coleus (53) and hastened

abscission in bean (109). Both responses were observed in lupine (*Lupinus luteus*, L.) depending on the site of application (33). Dual responses with respect to concentration and time of application were also found (36). A drop in natural cytokinin activity associated with abscission has been reported by Bornman (19). Treatments with cytokinins before explant excision lengthened stage I of abscission (36).

#### Internal Factors Which Control Abscission in Fruit

The fundamental work of Luckwill (91) and Wright (135) showed parallel changes in levels of extractable hormone (auxin?) and fruit abscission. A general criticism of these studies is that the nature of hormones analysed remains uncertain. A better definition of the nature of the extracts exists in other studies (102,111,112), but Powell and Pratt (111) concluded that it is improbable that extracted auxins directly relate to abscission.

Van Steveninck investigated some correlative factors controlling abscission in lupine. He showed (126,127,128,129) that the first pods formed promoted abscission of the remaining flowers on the cluster. Auxin could substitute for the effect of the pod, naphthaleneacetic acid (NAA) and 2,3,5 triiodobenzoic acid (TIBA) were the most effective. He concluded (128) that endogenous controlling factors may be an "antiauxin" (!).

A search for abscission accelerating factors in lupine (130) revealed the presence of an auxin-like material which delayed abscission of pods, but promoted abscission of flowers. An inhibitor of coleoptile growth was also found which accelerated abscission of explants independent of the site of application.

Studies of auxin applications to fruit are inconclusive. Synthetic auxins may increase abscission (flower, young fruit and ripe fruit) or increase persistence (mature fruit and seedless fruit).

Hartmann proposed (59) that if auxin increases persistence antiauxins should promote abscission. Application of antiauxins to mature olives apparently confirmed this suggestion. However, when auxins were used (not explored by Hartmann) fruit abscission was also induced (44). Similarly, an abscission accelerating effect of both auxins and "antiauxins" was shown in lupine flowers (128).

Because of complexity of the fruit, the suggestion has been made that auxins alone are insufficient to account for fruit abscission (65). It is not surprising, therefore, that growth inhibitors have been associated with fruit abscission (130). These studies culminated with the identification of ABA in cotton fruit (103).

The association of ABA and abscission in cotton reported by Addicott and collaborators, however, seems open to question. In a recent paper (43), Davis and Addicott relate levels of ABA to total fruit abscission in cotton. However, if compared to the rate of abscission (Section I) no correlation can be observed. On the other hand, ABA correlates with fruit growth rate calculated from the same paper (Section I). Further, immature peach fruit undergoing June drop show a greater content of ABA in persisting (growing) than in abscising fruit (Section I). Finally, application of synthetic ABA to fruit has little or no control over their abscission (60,137).



A far more interesting association with the regulation of abscission in fruit is shown by ethylene. This hormone appears to have a natural regulatory role (88,134) and it is widely used to accelerate abscission (22,23,40,46,55,61,72,88). As in leaves, however, ethylene is active only when fruit already show some tendency to abscise.

It is apparent from the above discussion that aims and research approaches in fruit abscission studies are similar to those found in leaf studies. As a matter of fact it is postulated that the abscission is similarly regulated in the two organs (65). Two arguments are given to support such a hypothesis. The first is based on the presumed homology of the two organs (65). The second is based on analogies which may be shown in the behavior of the two organs. Hormones are produced in the fruit as in the leaf blade. Also, a reduction in fruit integrity may result in rapid abscission, analogous to that observed on deblading.

The overall subject, however, deserves more attention. Analyses of hormone levels and response to growth regulators suggested that a simple, direct mechanism for the control of fruit abscission is hard to imagine (111). On the other hand, this should not be surprising in view of the complexity of the fruit. Unlike leaves, fruit enclose megasporangia which have a complex development of their own. Also, they are heterotrophic and therefore more prone to correlative control (65).

The phylogenetic explanation, too, is far from satisfying. Homology does not imply analogy. Furthermore, the fruit is homologous

not to a leaf but to a shoot. In a true sense, fruit abscission could represent the most conspicuous example of shoot abscission in nature.

In contrast with the proposed analogy between leaf and fruit abscission, a number of differences may be shown in the behavior of these two organs. For example, active growth and young age are specifically associated with persistence in leaves. By contrast, abscission in fruit is predominantly linked with young and actively growing organs. In some instances (avocado, tomato, etc.), abscission occurs only in young fruit, while mature fruit do not develop a complete abscission layer and may not physiologically separate from the plant.

The regulation of abscission in young fruit (June drop) is in itself intriguing, since it involves only part of the fruit population and does not extend to the leaves. The site of development of abscission, in fruit, may also present a unique situation. Tomato flowers and immature fruit abscise between peduncle and stem while mature fruit, in varieties which shed their fruit, abscise between fruit and peduncle. In peach, the abscission occurs between the peduncle and the shoot in early drops (bud, flowers and set fruit) while the fruit, but not the peduncle, abscises during June drop. Finally the peduncle abscises again at maturity. Known ubiquitous hormones could hardly account for such a specific response in often adjacent abscission zones (olive, peach, etc.). Some other activation-deactivation mechanism must exert a permissive role on the hormone regulation of abscission layer development.

Mode of Action of HormonesAuxin Transport

Auxin transport has received much attention as a potential factor in abscission control. Since the abscission zone does not form a barrier to transport (54,66,72), attention has been focused on polarity of transport. In bean sections polar (basipetal) transport declines with increasing age of the leaf. The reduced basipetal movement is compensated by increased acropetal transport (96). Coleus, however, shows no acropetal transport (67,95). The decline in polar transport due to aging in this species supposedly implies a different mechanism. Jacobs attached great importance to differences in auxin transport in coleus leaves of different age (65,68). He suggested (68) that this may explain why constant auxin doses give different responses on petioles of different ages (i.e., lesser growth and faster abscission with petioles of increasingly older leaves). It is difficult, however, to share Jacobs' point of view. Available data show both a reduced transport and a reduced amount of diffusible auxin in older leaves. This contrasts with the fact that, in debladed petioles, constant amounts of auxin are necessary to reproduce natural longevity. The matter is rendered more confusing if we observe that data of other researchers (83,95) are not consistent with those of Jacobs (73). The claim that these differences may depend on the methodological approaches used (68) further suggests that the subject is neither clear nor concluded.

### Locus of Action

A considerable amount of work has been done to establish the locus of action of auxin. An early theory (79,89,90,95) stated that auxin primarily causes growth and thereby indirectly prevents abscission.

Experiments on coleus support this concept (66,71,76,77). In intact coleus plants, petioles elongate until a few days before abscission (71). Also a compensatory growth response exists in leaves, which shows correlation competitions between growth and abscission (68,70). Excising the leaf blade (presumably removing the source of IAA) causes uniform abscission at all nodes coupled with a consistent absence of petiole elongation (71,101). IAA substitutes for the leaf blade for both growth and longevity stimuli (71,79,95,100,101,132). Interestingly, the IAA effect on longevity was progressively greater on longer petioles (47,95). If the primary IAA effect was on the abscission zone, the opposite would have been expected.

As we can see there are strong suggestions to justify reinterpreting the auxin-abscission relationship as a growth-abscission relationship. Yet not all data support this assumption. For example, auxin's two phase action (18) and the auxin gradient theory (12) imply a direct action of this hormone on abscission.

A direct auxin action on abscission has also been proposed based on the stimulatory effect that IAA has on abscission-regulating enzymes. The matter, however, seems ambiguous. Cellulase activity is increased by IAA in growing tissues (94) and inhibited in the abscission

zone (2,62,112). The opposite may be observed when ethylene is used (2,62,112,113). Addicott (1970) interprets these results as suggesting that neither hormone has a primary or direct role on the enzyme activity.

A growth-dependent auxin-regulatory role has been proposed also for fruit abscission (11). This conclusion was mostly based on the observation that growth regulators may stimulate growth and longevity of seedless (parthenocarpic) fruit. The matter, however, seems not so simple and examples exist which tend to deny such an association. So, auxins may cause fruit drop in early developmental stages, when growth is very rapid, and may inhibit abscission in mature, nongrowing fruit.

#### Reevaluation of Concepts Related to Abscission Regulation

Based on the available information, no cohesive model of the natural control of abscission is currently available. No abscission-controlling factor has been isolated which can be conclusively related to inhibition or stimulation of abscission under natural conditions. That the endogenous abscission-retarding substance is solely IAA, as proposed by Jacobs (68), is questionable. First, this conclusion would be limited to coleus, which represents an atypical plant. Secondly, it would apply to some, but not all, of the leaves of coleus (65) and to the diffusible but not extractable IAA (68,120). Thirdly, in no plant system has it been established that abscission itself (and not the cytokinesis, growth, etc.) is directly controlled by extractable, diffusible or externally supplied growth regulators.

Are We Adopting Sound  
Abscission Concepts?

The best available evidence for hormonal regulation of abscission remains indirect, based primarily on the response of debladed petioles to exogenously supplied growth regulators. The use of debladed petioles as a working model should be carefully evaluated. The homogeneity of such a model with an intact system still remains to be determined. This is not to say that the morphological development of an abscission layer may not be comparable in excised and intact systems (although most often different developments characterize the two systems: 21,100,119) but rather that abscission, per se, may be differently controlled depending on the amount of tissues or organs attached to the abscission zone in the system used.

An obvious difference between intact and excised systems is that the former is characterized by an induction phase. A comparable induction phase, however, has not been unequivocally identified in debladed systems. Further, intact leaves are not induced to abscise by supplying known phytohormones, and debladed petioles may not be induced to persist the way entire leaves do (coleus is not an exception, as will be discussed later). Therefore intact and debladed systems are hardly comparable since intact systems lack the responsiveness of the debladed ones, and the latter the possibility of retaining their petioles.

The assumption that deblading suppresses the flow of an anti-abscission hormone remains fundamentally unsubstantiated. Also, it would seem rather unrealistic to ignore other chemical changes, including active (1) or inactive substances, and functional changes

which may result as a consequence of deblading. Physical changes may also play a role. Tissue turgor and vascular flow characterize entire systems but are lacking or substantially reduced in debladed petioles. Unfortunately, no effort has been directed to establish if these chemical and physical changes have an impact at the regulatory level.

The inevitability of abscission of the debladed petioles raises the question as to whether or not it could be more simply interpreted as showing some type of specific response to the ablation of the blade. This interpretation would appear in contrast with the dynamic inhibition and stimulation control achieved by growth regulators on debladed systems. We must recognize, however, that the so-called inhibition or stimulation via growth regulators in systems which are in an induced stage (coleus, debladed petioles) only implies a difference in time to separation. In all cases (i.e., control and treated) abscission remains the sole, ultimate response observed in explants. Why, then, should quantitative differences in time to separation imply stimulation or inhibition and not simply the existence of different pathways to final separation? Every study of kinetics of abscission development would support, or at least could not refute, this hypothesis. In coleus, the cytokinesis-dependent abscission may lose this dependence following deblading and some growth regulator may restore it (25,65,100). Anatomical differences in the development of the abscission layer are known for bean too (21).

One might claim that IAA can maintain longevity of debladed petioles in coleus comparable to that of intact systems as an indication that this hormone truly prevents abscission. This needs not to be

viewed as a conclusive evidence, since coleus leaves are unusually short-lived and show a continuous evolution of the abscission layer during leaf development, leading to separation of the leaves as they age. A quiescent abscission zone characterizes the leaves of most species, which again raises a question on the value of coleus as a model system. Abscission in coleus may be viewed as a continuous process during leaf development. At any time, in both intact and debladed petioles, abscission is underway differing only in the pattern of development. Shifts caused by IAA in debladed petioles from a rapid to slow abscission and from cytokinesis-dependent to nondependent remain consistent with the assumption that different pathways exist which may lead to the same end. It also suggests that the role of IAA in abscission must be indirect. Separation will ultimately occur by cytolysis, independent of the presence of natural (?) or applied IAA.

It may be expected that some systems will present homogenous development of IAA-treated and untreated debladed petioles. Some biochemical processes occurring in stage I appear delayed but not prevented by IAA (37,40). It would be necessary to explain, however, why the delay obtained in this condition is only a temporary one. Also, the effectiveness of IAA decreases with the time elapsed from deblading to treatment, or with the age of the leaf. Thus, it appears that this hormone acts within the limit imposed by the development of the abscission process itself and by no means reverses or inhibits such a development. But then the possibility exists that we are confronted by the generic inhibition of some process, which could be



equally well obtained with other inhibitors (37,40) and not by a specific regulation of the abscission process.

#### What Do Our Biomodels Represent?

The above discussion points to the inadequacy of biomodels commonly used in studying the regulation of abscission. Further reasons may be given which show that this inadequacy is not accidental.

First, the attempt made to create simpler systems through a progressive reduction of organs or tissue adjacent to the abscission zone (deblading, excision) did not succeed in providing a direct control over the cytolytic phase of separation. Thus, in spite of our efforts we are far from the needed simplification, and the system remains under the influence of some elusive regulatory mechanism.

Secondly, the commonly used biomodels are also inadequate because they relegate us to the study of abscission after induction has taken place, and, therefore, they are useless for elucidating the early, nonreversible events associated with abscission. Induction of abscission remains the most challenging problem. Our failure to control abscission for agricultural purposes results mainly from our lack of understanding of the mechanism of inducing abscission.

The search for simplified systems is valid where it leads to a more direct control over cytolysis of the abscission layer. Systems of this type would provide a powerful tool for the study of conditions external to the abscission zone which are necessary for abscission to take place. At the same time, however, such systems would be of limited value in determining natural factors associated with abscission.

The epigenetic control of biological events may hardly be analyzed when components are separated from the overall development of the organism. Therefore, the utility of simple systems remains confined to testing hypotheses relative to regulatory mechanism. The hypotheses themselves, however, would still need to come from the study of integrated intact systems.

Unfortunately, attempts to study intact organisms remains confined to *Coleus* (leaves) and *Lupinus* (fruit) and even here they have not been pursued in sufficient depth. At the same time, attempts to understand regulation of intact systems by analyzing hormone levels and relating them to changes in development deserves some consideration. These analyses would be meaningful only if related to truly representative plant systems (see Sections I and III) and if the assumption of simple dose:response relation in the hormonal regulation of development was valid. These assumptions, however, need further testing.

SECTION I: RELATIONSHIP BETWEEN CHANGES IN SEED ABSCISIC ACID  
LEVELS AND FRUIT GROWTH AND ABSCISSION IN PEACH [Prunus  
persica (L.) Batsch] AS INFLUENCED BY  
FRUIT SAMPLING TECHNIQUE

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ACID LEVELS AND FRUIT GROWTH AND ABSCISSION IN PEACH  
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Abstract. Growth and abscission of immature peach [Prunus persica (L.) Batsch] fruit were studied and related to changes in levels of endogenous abscisic acid (ABA). Fruit populations having a low or high abscission potential were established by either increasing or decreasing competition between fruit and shoots. Abscission probabilities were then assigned to subgroups, within each population, based on a predetermined relationship between growth and abscission. ABA was extracted from seeds of fruit with a known abscission probability and growth rate. Levels of ABA were negatively correlated with abscission. A strong positive association was found between ABA levels and fruit growth. The importance of isolating concurrent physiological processes when attempting to correlate such events with levels of endogenous hormones is discussed.

In studies concerning the regulatory role of hormones, developmental events are often related to changes in levels of naturally occurring growth substances. However, a number of physiological events may occur simultaneously in a living organism which may lead to a misinterpretation of the relationship of the event and changes in hormone levels (see Section III).

This problem is particularly serious when studying the abscission of young fruit because both persisting (actively growing) and abscising (various stages) fruit are present in the same population and at the same time (15,24). Fruit growth and abscission, therefore, appeared as an ideal test system for interpreting changes in 2 simultaneously occurring events in relation to concurrent changes in hormone level.

A prerequisite for such a study is to be able to recognize and separate persisting and abscising fruit. Earlier attempts to do so were based on visual signs (fruit size, color change or loosening) of abscission (6,15,18). Such indices, however, may only adequately identify fruit in the terminal stage of abscission (24), i.e., well after induction and other critical changes have taken place.

In this work, growth and abscission were jointly studied in peach fruit undergoing June drop. The separation of the 2 events early in development was sought both in a physical and parametric sense. We recognize that such an approach may adequately separate growth and abscission from each other, but possibly not from other simultaneously occurring events. This limitation is not critical, however, since we focus on the adequacy of correlative analyses in interpreting hormone studies, and this may be viewed independently from any specific phenomenon. Thus, growth could represent any event which is specifically associated with persisting fruit and which is parametrically equivalent to (i.e., parallels) growth. The reverse holds for abscission. Whatever the names, the prerequisites for testing our hypothesis are the existence of distinct phenomena localized

in different fruits, and an adequate separation of such fruit into corresponding subpopulations. The first prerequisite is fulfilled in our study by the selection of the experimental system. The second is the object of a specific analysis reported herein.

A similar comment could be made regarding techniques employed in hormone analysis. The simplest methods were used because we focused not on a specific hormone but on the influence that the choice of parameters and sampling methods may have on the interpretation of hormone data.

In this paper we report on the characterization of growing and abscising fruit, on their separation into subpopulations of differing abscission potential, and on the hormone analysis of the designated subpopulations. We also discuss the relationship of endogenous ABA with abscission when related to fruit either selectively grouped into subpopulations or randomly sampled.

#### Materials and Methods

Characterization of fruit growth and abscission. Fruit growth (elongation) and abscission were monitored at 4-10 day intervals during Stage I and II of development (May 14 to July 16). Eight representative branches, containing 10-20 fruit each, were selected for monitoring fruit length from each of 4 mature peach (Prunus persica, cv. Redhaven) trees. It has been previously observed (15) that peach fruit undergoing June drop are often smaller than the persisting fruit. Based on this observation, the data were kept separate for each individual fruit throughout the entire period. Therefore it was possible, by an

a posteriori analysis, to trace back to the first significant difference in length between persisting fruit and those which were known to have abscised later. The same 8 branches plus 8 additional ones were used to monitor fruit drop on the same trees.

Detailed measurements on fruit length and fresh wt. were made on fruit sampled (600 to 200 fruit, going from earlier to later dates, per each of the 4 peach trees) at 4-day intervals from May 28 to June 22 from comparable branches to those used for monitoring growth and abscission. The fruit were quick frozen in the field and held at  $-20^{\circ}\text{C}$  until used for hormone analysis.

Sampling for hormone analysis differed by 0 to 3 days from the time of morphological measurements in the field. Data reported in this study, however, were calculated as running averages to coincide with the time of fruit sampling.

The time of half-maximum expression (26) of growth and abscission ( $1/2G_{\text{max}}$  and  $1/2A_{\text{max}}$ , respectively) is used to refer to the position of the mode of curves expressing the rates of the 2 events.

Separation of abscising from persisting fruit. A primary objective in our study was to separate a fruit population into subpopulations having distinct differences in abscission potential. Two approaches were used: (a) modification of fruit abscission potential in vivo, and (b) separation of fruit into classes based on differences in fruit growth.

a) Modification of the fruit abscission potential in vivo.

Fruit, young leaves and apices compete with each other (1,14,20,25) for

available metabolites during growth and development. Consequently, the degree of immature fruit abscission is affected by fruit load, leaf area and competing growing points (3,4,9,10,16). In this study, fruit populations with different abscission potential were established by creating a favorable or unfavorable balance between fruit load and the 2 other factors.

Four mature Redhaven trees, each with 6-8 uniform scaffolds, were selected. Alternate scaffolds on each tree were treated so as to develop a fruit population with a high or a low abscission potential (HAP and LAP, respectively). The LAP-designated scaffolds were girdled and competition between developing organs was minimized by removing two-thirds of the 1-yr-old branches and one-third of 2- and 3-yr-old branches before anthesis (Fig. 1). In addition, two-thirds of the fruits set were thinned 2 wk after bloom. In contrast, maximum competition was maintained in the scaffolds designated HAP by not reducing the number of fruits or shoots.

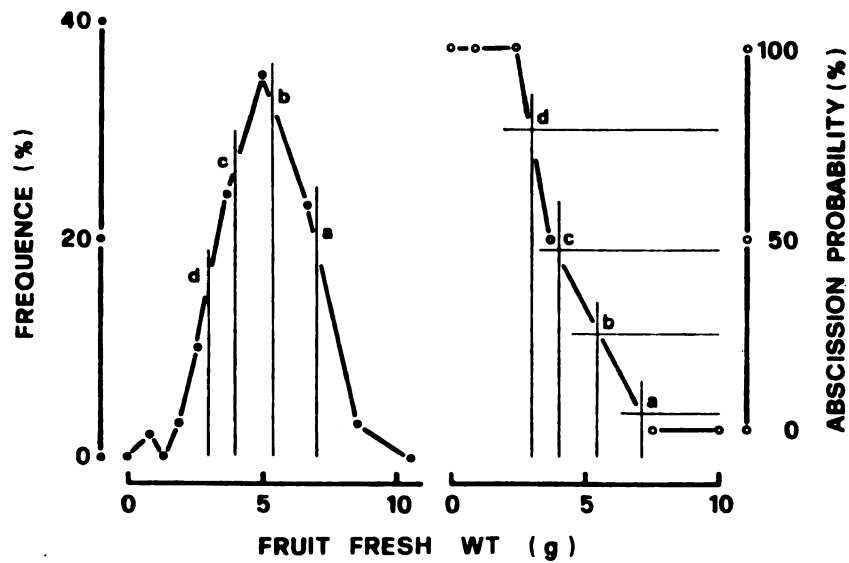
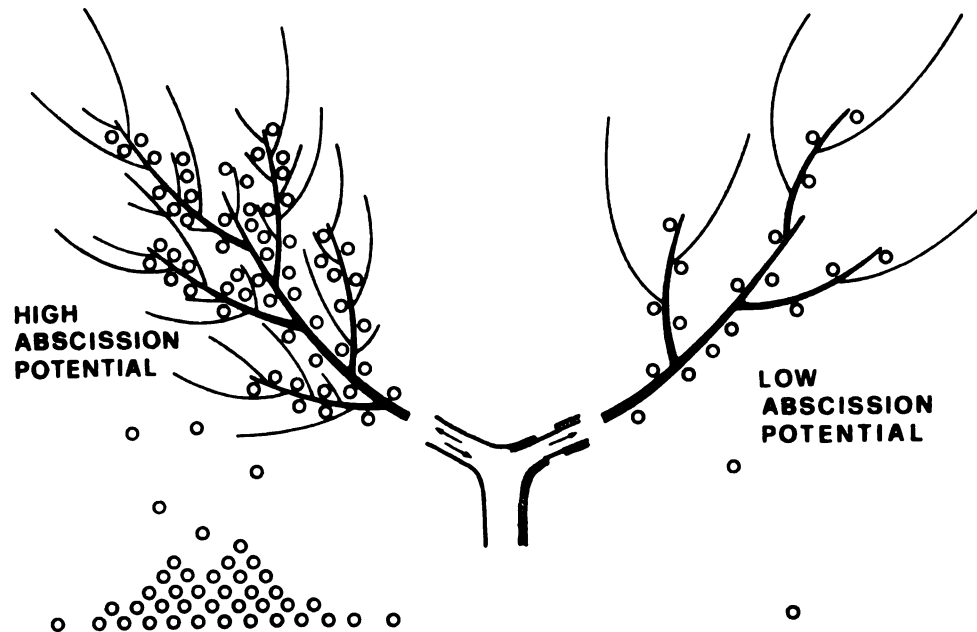
Fruit growth (length) and abscission (drop) were recorded at 4-10 day intervals during Stage I and II of development (May 14 to July 16) on 4 (growth) or 8 (abscission) branches for each of the HAP and LAP populations. Branches for record purposes contained 14-25 fruit each for the HAP and 5-10 fruit (after thinning) for the LAP population.

b) Establishing classes of abscission probability in relation to the growth of the fruit. A second approach was devised to separate fruits with different abscission potentials, in which the fruit of each population were segregated into subpopulations with different abscission



Fig. 1. Diagrammatic representation of treatments imposed to establish high and low abscission potential fruit population. The number of shoots and fruit was reduced, and branches were girdled, to obtain a population characterized by a low abscission potential (LAP). The organs were left intact in the high abscission potential (HAP) population.

Fig. 2. Illustration of the size distribution and abscission probability curves used in selecting subgroups of fruit (letters) with an estimated abscission probability for hormone analysis. These curves are representative of fruit collected June 14 from the low abscission potential treatment of tree no. 4.



potential. This was based on an a posteriori analysis of the growth tendencies associated with persisting and abscising fruit. It was previously found that abscising fruit are smaller than persisting ones (15). Further observations made in this work show that differences in size appear 2-3 weeks before drop (see Results and Discussion, Fig. 3) and increases with time. This finding, in particular, led to the conclusion that there was an inverse relationship between fruit size and abscission probability.

Based on this premise, data on fruit length recorded for individual fruits for each tree, population and time were grouped into classes differing by 2 mm. An abscission probability was then assigned to each class based on the amount (%) of fruit present in that particular class, and at that particular time, and which was observed to have later abscised.

Results on the relationship of size to abscission probability will be reported in this paper as indexed by fruit fresh wt instead of fruit length. The conversion was based on a factor calculated from measurements made in the laboratory on fruit sampled at frequent intervals for hormone analyses (see previous "Characterization of fruit growth and abscission").

#### Hormone measurement in relation to abscission.

a) Plant material. All of the fruit used for hormone analyses was collected from 1 tree in which the LAP branches had a fruit drop of 31% and the HAP branches 93%. Samples of 600 to 200 fruit, going from earlier to later dates, were collected from each population at 4-day

intervals from May 28 to June 22. Branches comparable to those selected for monitoring fruit growth and abscission were used. The fruits were quick frozen in the field and stored at  $-20^{\circ}\text{C}$ . Only samples harvested June 6, 10, 14 and 18 were used for hormone analyses. These samples were assigned, based on size (fresh wt), to subgroups differing by 0.50 g for fruit harvested on June 6 and 10, and 1.0 g for those harvested on June 14 and 18. An abscission potential was then assigned to each subgroup based on the relationship between size and abscission probability determined experimentally from comparable fruits monitored in vivo throughout their development.

Three or 4 subgroups per each sample (population and date) were selected for hormone analyses. The subgroups represented fruit above, near and below the mode of fruit size distribution of each sample.

The abscission probability of fruit near the mode remained almost constant between samples harvested at different times ranging between 10 and 25% for the LAP and 80 to 90% for the HAP population. This near constancy occurred most likely because, within the time period explored, fruit drop was balanced by the continuous appearance of new abscising fruit, as indexed by growth (see Fig. 3). Subgroups above and below the mode were selected to provide fruit samples of comparable abscission probability for the different sampling times. Fruit above the mode ranged between 0-10% for the LAP or 65-70% for the HAP populations. Fruits below the mode ranged between 45-55% and 75-80% (two different groups) for the LAP population and 95-100% for the HAP population (Table 1).

Table 1. Sample identification, fresh wt. and assigned abscission probability of peach fruit subpopulations selected for ABA analysis. Fruit were collected from tree 4 (see Fig. 6).

| Sampling time | Low abscission potential population |                     |                       |                            | High abscission potential population |                     |                       |                            |
|---------------|-------------------------------------|---------------------|-----------------------|----------------------------|--------------------------------------|---------------------|-----------------------|----------------------------|
|               | Sample <sup>z</sup>                 | Fruit fresh wt. (g) | Position <sup>y</sup> | Abscission probability (%) | Sample <sup>z</sup>                  | Fruit fresh wt. (g) | Position <sup>y</sup> | Abscission probability (%) |
| 6/6           | a 1                                 | 1.5                 | above                 | 10                         | e 1                                  | 1.3                 | above                 | 70                         |
|               | b 1                                 | 1.2                 | near                  | 25                         | f 1                                  | 0.9                 | near                  | 80                         |
|               | c 1                                 | 0.9                 | below                 | 55                         |                                      |                     |                       |                            |
| 6/10          | a 2                                 | 2.6                 | above                 | 5                          | e 2                                  | 2.0                 | above                 | 70                         |
|               | b 2                                 | 2.0                 | near                  | 15                         | f 2                                  | 1.5                 | near                  | 80                         |
|               | c 2                                 | 1.4                 | below                 | 55                         | g 2                                  | 1.0                 | below                 | 95                         |
|               | d 2                                 | 1.0                 | below                 | 85                         |                                      |                     |                       |                            |
| 6/14          | a 3                                 | 7.0                 | above                 | 5                          | e 3                                  | 3.5                 | above                 | 65                         |
|               | b 3                                 | 5.3                 | near                  | 20                         | f 3                                  | 2.3                 | near                  | 90                         |
|               | c 3                                 | 4.1                 | below                 | 45                         | g 3                                  | 1.6                 | below                 | 100                        |
|               | d 3                                 | 3.0                 | below                 | 80                         |                                      |                     |                       |                            |
| 6/18          | a 4                                 | 11.0                | above                 | 0                          | x 4                                  | 8.2                 | above                 | 10                         |
|               | b 4                                 | 9.5                 | near                  | 10                         | y 4                                  | 6.5                 | near                  | 35                         |
|               | d 4                                 | 5.5                 | below                 | 75                         | z 4                                  | 5.0                 | below                 | 70                         |

<sup>z</sup>Letters represent different position on the fruit size distribution curve (see Fig. 8); numbers refer to time of sampling.

<sup>y</sup>Position relative to the mode of the fruit size distribution curve.

One exception to the near constant abscission probability of fruit near the modal size existed in the HAP sample harvested on June 18, due to the abundant abscission (55% of the original fruit population) that occurred prior to the sampling date. Subgroups from this sample have been indicated by letters x, y and z to distinguish them from the other subgroups which are indicated by letters a to g.

b) Hormone measurements. Hormone analyses were limited to inhibitors contained in the seed because of the prominent role of the seed in peach fruit growth and abscission (5,7,11,12,17,19,21,24), and the reported (2,6,18) role of inhibitors, like ABA, in fruit abscission.

Ten to 40 seeds (total of 1-4 g fresh wt) were lyophylized, homogenized with 50 ml of 80% methanol and extracted for 36 hr at 4°C with 2 changes of solvent. The combined supernatant was filtered and evaporated to dryness. The residue was dissolved in methanol and streaked on silica gel G plates (Eastmann Chromogram 6060) and developed with isopropanol, ammonia, water (10 : 1 : 1 v/v). Transverse sections (0.067  $R_f$  units) were cut from the plates and eluted with 6 ml methanol by shaking for 4 hr. Each eluate was added to a plastic Petri dish (2.5 cm diam.) containing filter paper (Whatman 3MM) discs and evaporated under partial vacuum. At dryness, 0.5 ml of water was added and allowed to equilibrate for 30 min.

Cress (Lepidium sativum, L.) seed germination was used to assay inhibitors. In preliminary experiments (Appendix 1, page 111) it was shown that this assay was sensitive to ABA, and that  $GA_3$  did not overcome the inhibition imposed by ABA. Also, kinetin was neither

active directly, nor had a permissive effect (13) in overcoming the inhibition imposed by ABA. Although IAA inhibits cress seed germination and interacts with ABA (Appendix 1, page 111), the above chromatographic procedure would favor separation of the two components.

The insensitivity of cress seeds to GA and kinetin enabled us to reduce the hormone isolation procedures to a simple chromatography (see above), thus avoiding losses of hormone often experienced with purification technics (8). Ten seeds per Petri dish and 3 dishes per eluate were used. Seeds were incubated for 36 hr in the dark at 25°C, and both percent germination and root length were recorded.

Preliminary hormone analyses of peach fruit revealed the presence of 4 inhibitory zones ( $R_{fs}$ : 0.17, 0.53, 0.77, 0.95). Inhibitors II ( $R_f$  0.53) and III ( $R_f$  0.77) showed prominent activity. The identification and levels of Inhibitor II, which ran at the same  $R_f$  as ABA, will be stressed in this paper. Subsequent analyses on Inhibitor II levels in fruit with different abscission probabilities were confined to the chromatographic band corresponding to an ABA standard spotted on both edges of each chromatogram and localized with UV light. Results were quantified relatively to a range of concn of authentic cis-trans trans-trans ABA and expressed as ABA-equivalents per seed or per gram of seed.

For identification of inhibitor II, 5 g of seed tissue were extracted and chromatographed on TLC according to the above procedure. The eluate from the band corresponding to authentic ABA was methylated with diazomethane (27), dried, redissolved in ethylacetate, and injected into a Packard 7300 gas-liquid chromatograph. This instrument was

equipped with a hydrogen flame ionization detector, and 2 mm (i.d.) x 1.83 m column containing 3% SE-30 on Supelcoport 60/80 mesh. The carrier gas was nitrogen (40ml/min). The inlet, column and detector temperature was maintained at 230, 180 and 240 C, respectively.

A subsample of the above extract was subjected to GC-MS using a LKB-9000 instrument interfaced with PDP 8/1 computer equipped with an analogous column to that used for GLC. The carrier gas was Helium (30 ml/min). Temperature was 180, 230, 220 and 230 C, respectively, for column, inlet, source and molecular separator. Entrance and exit slits were 0.08 and 0.3 mm, respectively. The ion source was operated at 70 eV.

## Results and Discussion

Characterization of fruit growth and abscission. After an initial wave of abscission following anthesis, the rate of fruit growth paralleled the rate of fruit abscission during Stage I and II (Fig. 3). The  $1/2_{max}$  expression of growth and abscission differed by 1 day.

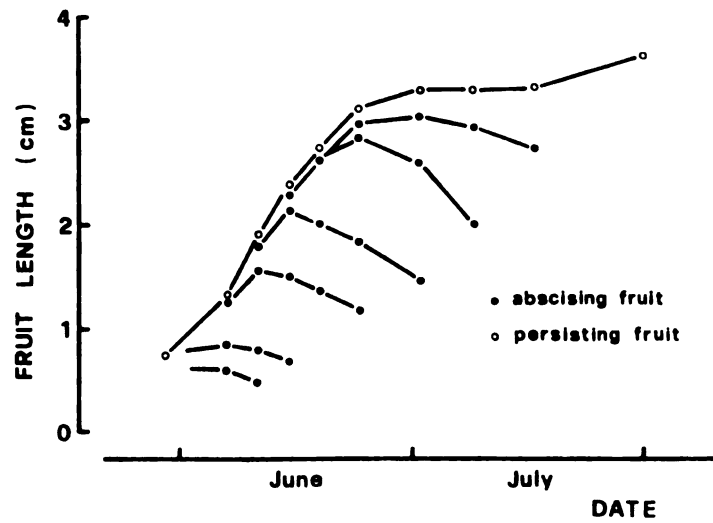
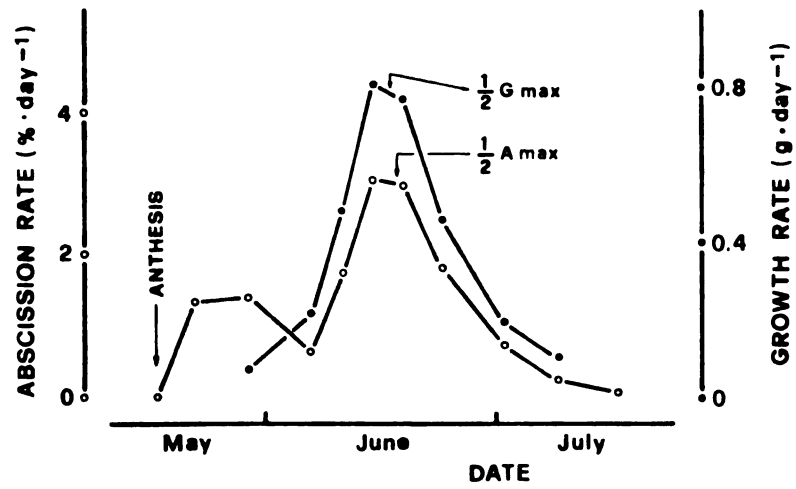
Fruit size measurement on the tree revealed that in the early stage of fruit development the growth (indexed by length) of persisting fruit did not differ from that of fruit which subsequently abscised. However, the growth rate of abscising fruit began to decrease, and was distinctly smaller, 1 to 3 weeks before separation from the tree (Fig. 4). Separation occurred at any time after the fruit had attained maximum size.





Fig. 3. Relationship between abscission and growth rates of peach fruit during Stage I. The two phenomena paralleled each other during June drop (their 1/2 max expression differed by 1 day).

Fig. 4. Growth curves of abscising and persisting fruit. Abscising fruit were distinctly smaller than persisting fruit as early as 2-3 wk before separation.



Based on this observation, the first change in fruit growth (i.e., the first measurable difference from the mean of persisting fruits) could be used as an early index of abscission. An abscission curve indexed by a change in growth is compared in Fig. 5 to one indexed by fruit drop, a parameter often used in physiological studies. The physiological significance of abscission indexed by growth becomes more apparent when one considers that fruit drop is often a reflection of nonphysiological events, i.e., rain, wind, etc., occurring sometime after the development of the separation layer. Thus, the drop parameter indexes abscission post factum and, therefore, is of limited value in the study of fruit abscission. Further, abscission indexed by growth may prove useful in this work since it helps to differentiate abscission from growth parameters (compare Fig. 3 and 5).

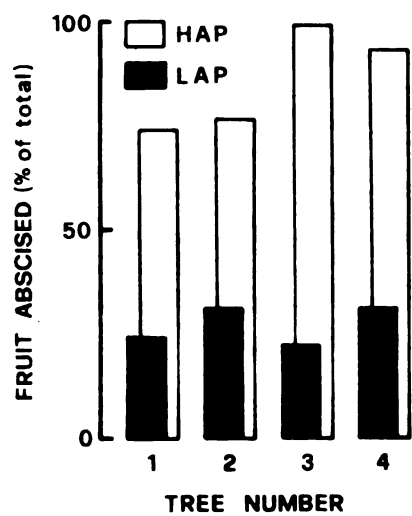
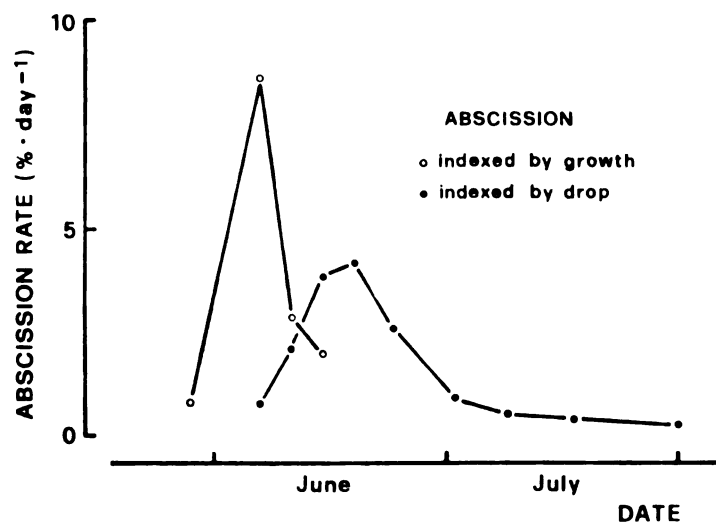
#### Separation of abscising from persisting fruit.

a) Modification of the fruit abscission potential in vivo. The total abscission of the LAP and HAP designated populations was markedly different (Fig. 6). Only 23 to 31% of the fruit abscised in the LAP compared to 74 to 99% in the HAP population. The difference was quantitative, however, since curves representing rate of abscission were quite coincident in the two populations (Fig. 7A). Fruit growth was lower in the HAP than in the LAP population (Fig. 7B). Like abscission, however, only quantitative differences were observed between the two populations.

b) Fruit abscission potential in relation to growth. In the LAP population the fruit size distribution at 4 different sampling dates

Fig. 5. A comparison of peach fruit abscission as indexed by a change in growth or by separation of the fruit. The growth index represents the first evidence of a difference in fruit elongation between persisting and abscising fruit (see Fig. 4).

Fig. 6. A comparison of abscission in fruit populations established as high (HAP) and low (LAP) abscission potential.



reflected a complete range of abscission potentials from 100% for the smallest fruit to 0% for the largest ones (Fig. 8). However, in the HAP population 0% abscission probability could be associated with the largest fruit only at the later sampling dates (June 14, 18) when approximately one-third of the potentially abscising fruit had already dropped.

These data show that populations with different abscission potential can be established by altering the competition among and between organs. Also, abscission potential may be predicted for a given fruit population at a given time on the basis of fruit size alone. The two procedures provide independent approaches to separating abscission and growth for experimental purposes.

#### Hormone measurements in relation to abscission.

a) Evidence for ABA. GLC traces of methylated Inhibitor II showed the same retention time (6.2 min) of, and cochromatographed with, authentic MeABA. The mass spectrum of the methylated Inhibitor II (Fig. 9) was identical to that obtained with authentic MeABA. Thus, based on chromatographic and GC-MS similarities, we concluded that Inhibitor II is ABA.

#### b) Levels of ABA in fruit with different abscission potential.

Changes in ABA content in subgroups of fruit with a constant abscission probability are shown in Fig. 10. A continuous increase in the level of ABA is apparent for samples harvested between June 6 and 14, in both LAP and HAP populations. In the LAP population, a sharp decline in the level of ABA can be observed in the fruit sampled on June 18.

Fig. 7. A comparison of abscission (A), indexed by growth or drop, and growth (B) of fruit from high and low abscission potential populations (respectively HAP and LAP).

Fig. 8. Relationship between abscission probability and fruit size at 4 different times during June drop in populations of fruit characterized by a high (HAP) or low (LAP) abscission potential.



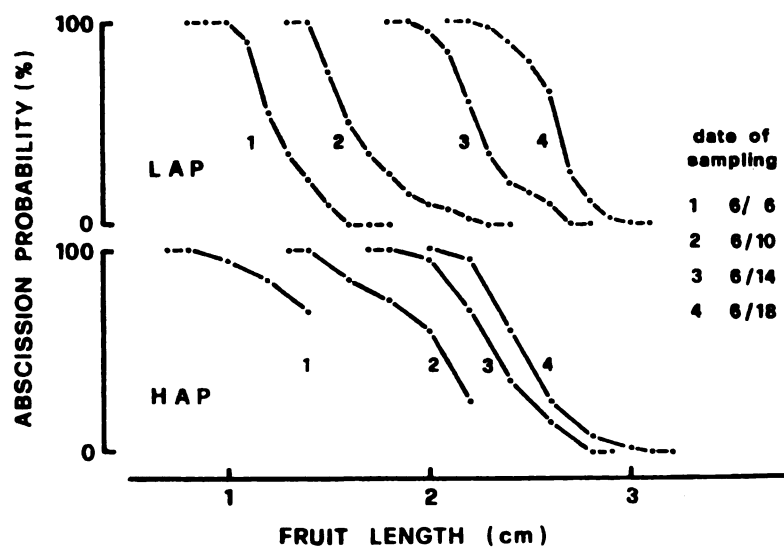
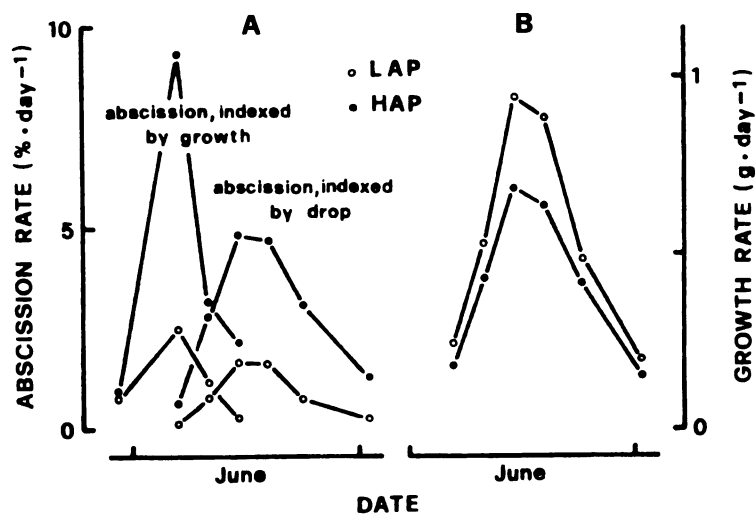


Fig. 9. Mass spectrum of methylated Inhibitor II extracted from peach seeds. This MS is identical to that of authentic MeABA.

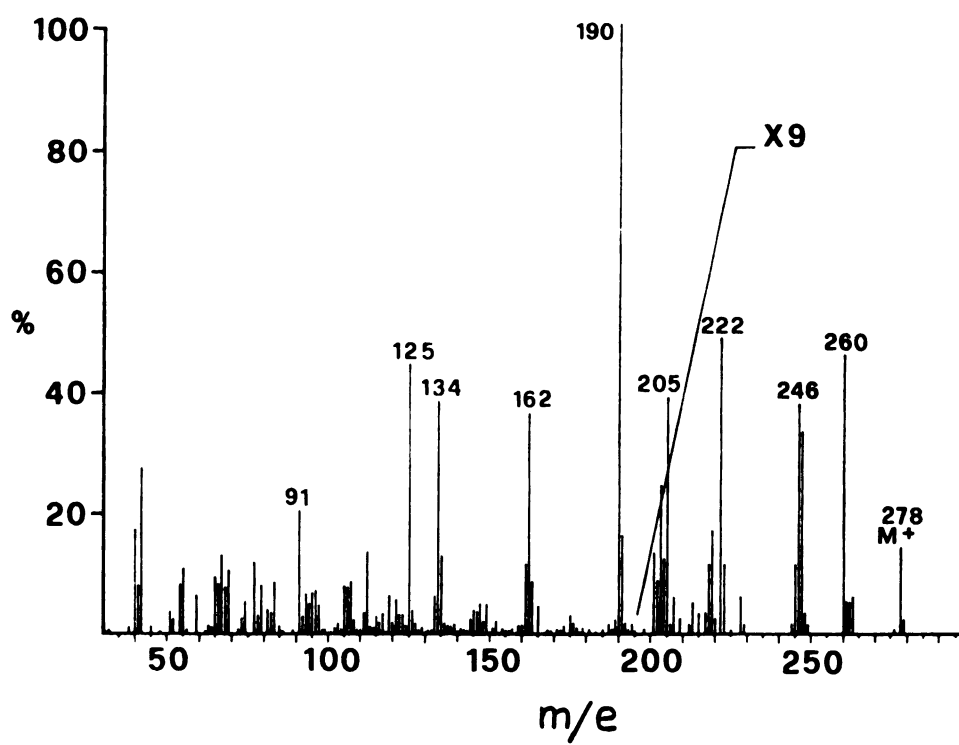
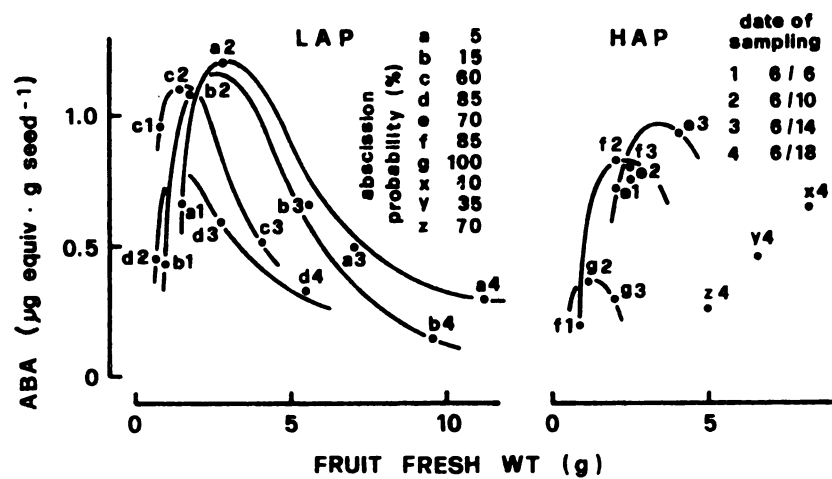
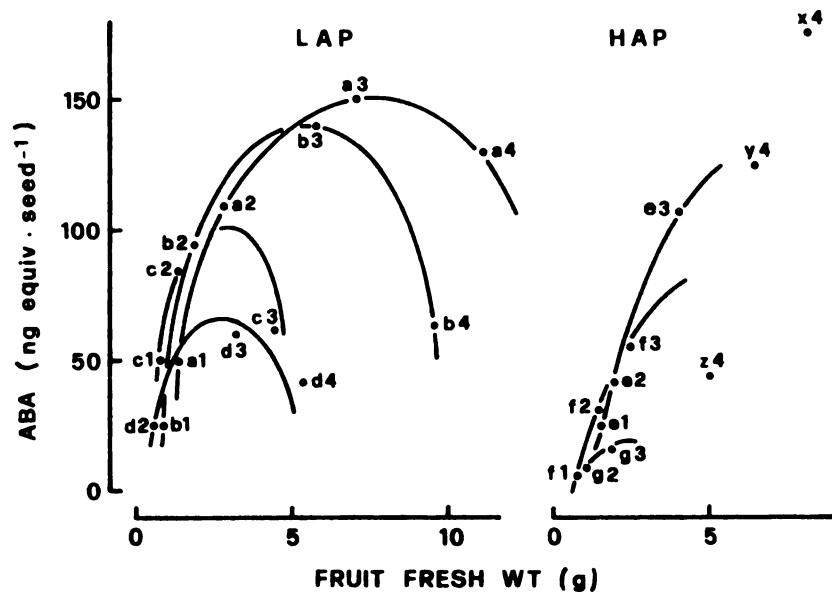


Fig. 10. ABA levels in peach seeds in relation to fruit size and expressed on per seed (top) or concentration (bottom) basis. Fruit samples were harvested at 4 different times (1 through 4) from the LAP (left) and HAP (right) populations. Subpopulations with different abscission probability (letters) were selected for analysis as shown in Fig. 2 and Table 1.



A similar decline was not apparent in the HAP population. Here, however, fruit samples with a comparable abscission potential with previous samples were not available.

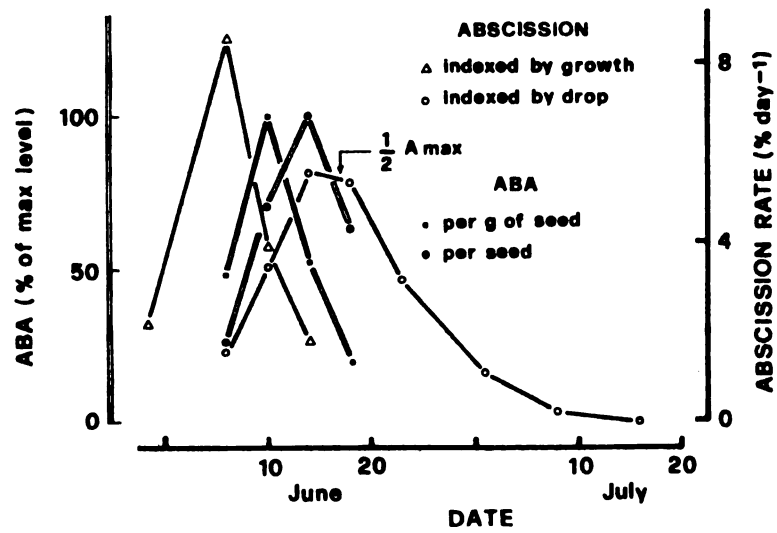
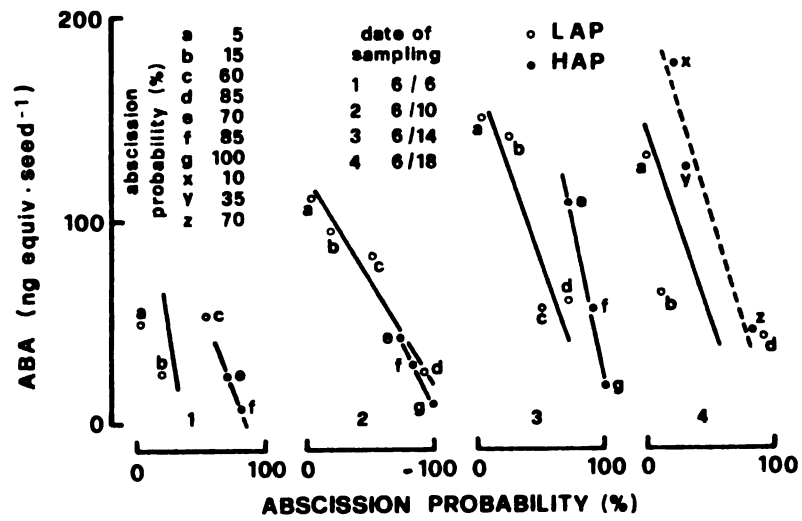
Similar patterns but different levels of ABA are evident between large and small fruit (characterized by a lower and higher abscission potential, respectively) within each population. Further, ABA patterns are similar if the level of this hormone is expressed on a per seed or per gram fresh wt basis.

When the ABA content of subgroups with different abscission potentials is plotted against abscission probability, a strong inverse relationship between ABA and abscission is apparent (Fig. 11) for all sampling times and populations. A comparison between rate of abscission and change in ABA levels with time shows that the increase in ABA does not precede abscission, when indexed by growth, as would be expected if ABA was associated with early events of fruit abscission (Fig. 12). However, if abscission is indexed by fruit drop, then a close association between ABA and abscission is apparent. The latter relationship is consistent with previous observations in the literature. However, as pointed out earlier, indexing abscission by fruit drop is of doubtful significance since the drop itself represents the terminal phase of abscission and it is frequently brought about by nonphysiological factors. Further, the ABA with drop association is inconsistent with our observation of declining ABA levels in fruit which are developing an abscission layer (Fig. 11).

Our data show that changes in ABA levels are more closely correlated with fruit growth than with fruit abscission. The above is

Fig. 11. The relationship between ABA levels and abscission probability in fruit of different subpopulations (letters) harvested at 4 dates (numbers) from HAP and LAP populations.

Fig. 12. Relationship between levels of ABA (double line) extracted from abscising fruit (groups c,d,e,f,g,z in Fig. 10 and Table 1) and abscission as indexed by growth and drop parameters.





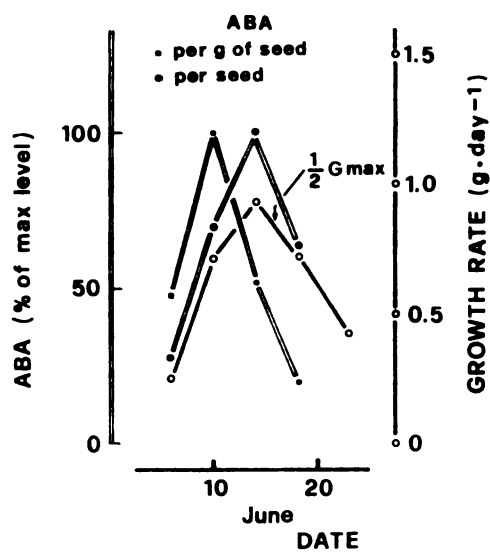
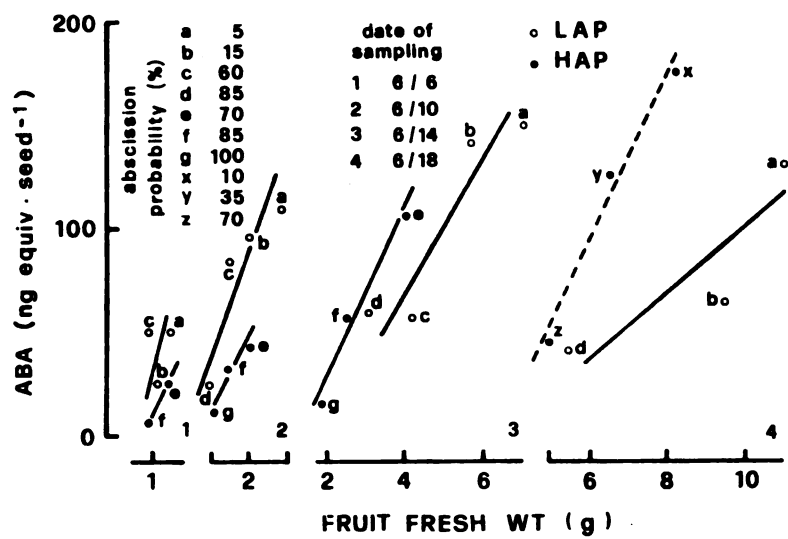
evident because: (a) ABA levels are closely correlated to fruit size for all sampling dates and populations (Fig. 13), (b) ABA levels are generally higher in fruits from LAP than in HAP population (Figs. 10, 11, 13) and (c) ABA levels in seed of fruit having a high probability to persist parallel their growth rate as illustrated in Fig. 14.

Thus, most of the extracted ABA appears to occur in growing rather than in abscising fruit. This conclusion is in contrast with that of Martin and Nishijama (18) for peach and that of Davis and Addicott for cotton (6). Although in the work by Martin and Nishijama, ABA levels were not studied in relation to fruit growth, these authors reported greater levels of ABA in abscising (i.e., loose) than in persisting (i.e., tight) fruits. Loose fruit represent, however, the very last stage of evolution of the abscission layer and may not be compared with fruit used in this study which represent earlier stages. Also, high ABA levels on loose fruit could be a consequence of moisture stress occurring prior to abscission, when vascular connection with the plant ends. We have observed (unpublished) a marked increase in ABA within a few hours after potential persisting fruit were removed from the tree.

The role of ABA in abscission of cotton fruit is of particular interest in reference to our studies because this was one of the first plant organs from which ABA was isolated and considered to be an abscission-accelerating hormone (2,6,22,23). Further, Addicott and co-workers have developed detailed data on ABA levels in relation to fruit growth and development. Davis and Addicott (6) reported a strong correlation between ABA content and abscission. However,

Fig. 13. Relationship between ABA levels and fruit size as measured in different subpopulations of fruit (letters) harvested at 4 dates (numbers) and from HAP and LAP populations.

Fig. 14. Relationship between ABA levels and growth rate of fruit with a high probability to persist (groups a and b from Fig. 10 and Table 1).



comparison of ABA levels to cumulative abscission used in these studies may be misleading. A comparison of their ABA data with rates of abscission and growth shows that these results parallel our observations on peach. As a matter of fact, maximum ABA levels in cotton follow rather than precede abscission (Fig. 15A) and closely correlate with the growth rate of the fruit (Fig. 15B).

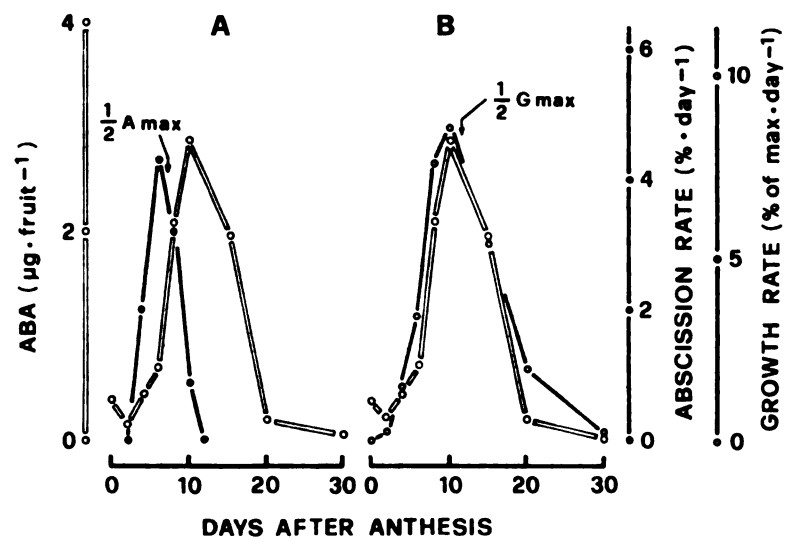
Two further observations may support our reinterpretation of cotton data. One is that most of the ABA (about two-thirds including the peak) is present in cotton after fruit abscission has ended (Fig. 15 and Fig. 6, Ref. 6). This ABA was extracted from growing fruits. Secondly, the abscission potential was always low in cotton (approx. 22% of total fruit; Figs. 2 and 6, Ref. 6). Thus, the ABA contribution from abscising fruit must have been rather small in cotton and the ABA extracted may be reasonably assumed to have been associated with the persisting fruit.

### Summary and Conclusions

Investigators frequently seek to relate levels of extractable hormones to physiological phenomena based on the assumption that a simple cause-effect relationship exists between the 2. The presence of different and unrelated phenomena in complex systems may, however, complicate such a study and experimental approaches should be devised so as to discriminate between concurrent phenomena.

Abscission and growth of immature peach fruit were utilized in this work as a test system to establish the effect of concurrent, parallel physiological events on the interpretation of a physiological

Fig. 15. Abscission (A) and growth (B) rate versus ABA levels in cotton fruit (a recalculation of data from Davis and Addicott: 1972).



study. Fruit growth and abscission phenomena frequently overlap (cotton, tomato, apple, etc.) or may occur simultaneously as in peach (Fig. 5). Such phenomena should be considered as physiologically distinct and very significant events. Abscission during June drop may involve 60-80% of the fruit population in peach, and the persisting fruit may increase dramatically (20 times) in fresh wt. during the same period.

Random sampling was considered as inadequate for discriminating between different events coevolving in a mixed population. Also, the ratio between persisting and abscising fruit changes continuously during this abscission phase (June drop) and this, in itself, may lead to an artificial variation in the levels of extractable hormones from a random sample (see section 3). Therefore, fruit populations were established having a high and low abscission potential by either increasing or decreasing correlative competition between fruit and other organs. An abscission probability was assigned to subgroups, within each population, based on a predetermined relationship between fruit size and abscission potential.

Analysis of ABA levels in seeds from fruit thus differentiated indicated an inverse relationship with abscission (Fig. 11), but a positive relationship with fruit size (Fig. 13) and growth rate (Fig. 14). Published data on cotton fruit abscission (6), when analyzed in a similar manner, agree with our findings (Fig. 15). Thus, both in peach and cotton most of the extracted ABA was probably derived from persisting and actively growing fruit.

Comparison of ABA levels in abscising (generally collected when fruit is visually senescing or loose) versus persisting fruit shows a higher level in the former group (6,18) but not consistently so (6). However, as stated earlier, sampling at this time is representative of the terminal stage in the abscission process and is probably of limited physiological significance. Further, the high ABA levels in abscising fruit, when selected on the basis of senescence or drop, may be a consequence of moisture stress which occurs prior to abscission.

It is significant that the ABA vs. growth association found in this study would have been missed, and an ABA vs. abscission association would have appeared (Fig. 12), had the above distinction between fruits been ignored and ABA related only to abscission (drop). This ambivalence may not necessarily be interpreted, however, as supporting a different, alternative interpretation. First, data from both peach and cotton suggest that ABA is present in growing fruit and tends to decline in abscising fruit. Secondly, growth and abscission are mutually excluding events in this stage of fruit development and are characteristic of different subpopulations of fruit. Therefore any hormonal factor could be meaningfully associated to either growth or abscission but not to both events; to do so would be paradoxical. Thirdly, to consider different interpretations to a single set of data depending on hypotheses or parameters we adopt would be scientifically unsound.

In conclusion, our study demonstrates the need for a better definition and separation of physiological events if we are to achieve



a meaningful understanding of physiological processes in general and of the abscission of fruit in particular.

This study, however, and its conclusions are dependent on the hypothesis that hormones and events may be directly related. Yet this hypothesis, and the conditions necessary for it, are not directly tested in experiments which analyze hormone levels. It follows that the ABA with growth association previously reported will have physiological relevance if, and only if, one proves that the active hormone was quantitatively extracted and that it was directly, proportionally and singularly related to the regulation of the event in question (see section III). We would also need to establish that the parameters used to express a specific event do not also represent other phenomena as well. Alternative hypotheses are possible, however, which lead to different mathematical associations and physiological conclusions.

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SECTION II: CHANGES IN LEVELS OF AN ACIDIC INHIBITOR  
IN GROWING AND ABSCISING PEACH [Prunus persica (L.)  
Batsch] FRUIT

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Abstract. Immature peach [Prunus persica (L.) Batsch] fruits were segregated into subpopulations based on their growth and abscission potential. Changes in levels of an acidic inhibitor, analyzed in this work, showed marked differences between fruit with a low and a high abscission potential. The importance of isolating events in physiological studies is apparent in this study where different conclusions may be drawn depending on whether the inhibitor was extracted from a random sample or from fruit isolated according to their abscission tendency.

Levels of extractable hormones are often correlated to physiological phenomena, based on the assumption that a simple cause: effect relationship exists between the 2. The presence of different and unrelated phenomena in complex organisms may, however, lead to misinterpretation of events and physiological changes (see Section III).

In a previous study (Section I) growth and abscission of immature peach (cv Redhaven) fruit were studied to establish the effect of 2 concurrent events on the interpretation of the role of hormones in abscission. Fruit populations with different abscission potentials were established by varying competition between growing organs. Further, an abscission probability was assigned to subpopulations,



within each population, based on a predetermined relationship between fruit size and abscission.

It is recognized that the terms growth and abscission, in this study, only refer to the most relevant changes occurring in these different groups of fruit. The groups thus formed, and the chemical changes associated with them, could represent other events occurring concurrently. This limitation is not critical, however, since we focus on the adequacy of correlative analysis in the interpretation of the role of hormones and this independently from any specific event.

Growth inhibitors have been frequently viewed as potential abscission-regulation hormones (1,2,3,5,8,9). Several inhibitors, as indexed by inhibition of cress (Lepidium sativum, L.) seed germination have been detected in peach fruitlets (see Section I). The biological activity of two inhibitors was particularly prominent. One has been identified as ABA, but no direct correlation was found between this hormone and abscission (Section I). The relationship of the other inhibitor (Inhibitor III) with persisting and abscising fruit is the subject of this report.

#### Material and Methods

In a previous study (Section I) peach fruit populations differing in their potential to abscise during June drop were established within a given tree by creating a favorable or unfavorable balance between fruit load, leaf area and competing growing points. Total abscission was 23-31% and 76-99% in the low (LAP) and in the high (HAP) abscission potential designated populations, respectively. It

was also observed that abscising fruit may be recognized some time before detachment due to a characteristic reduction in their growth rate, when compared to persisting fruit. Such a difference appears 2-3 wk before drop and increases with time. This observation, in particular, permitted us to establish a direct relationship between fruit size and abscission potential. Abscission probability levels were estimated for single classes of fruit, differing in size, within each population and sampling time (see Section I).

Based on the above, subgroups of fruit with a predetermined growth and abscission potential were selected for chemical analysis. Samples of 200-600 fruit were harvested on June 6, 10, 14 and 18, from scaffolds representing each of the above populations, and quick-frozen in the field. These samples were divided in laboratory into subpopulations. The earlier harvests were grouped into 0.5 g classes and the latter harvests into 1 g classes. An estimated abscission potential was then assigned to each size class based on a previously determined relationship between fruit size and abscission probability (Section I). In particular, subgroups of fruit from each population were chosen, for inhibitor analysis, so as to represent a constant abscission probability in samples harvested at different times. These fruits also held a constant position relative to the mode of fruit size distribution of samples representing each population and sampling time (Section I). The fruit representing the modal size, for the samples harvested at different times, had a 10-25% or an 80-90% abscission probability, respectively, for the LAP and HAP populations. Fruit samples selected above the mode ranged between 0-10% (LAP) and 60-70% (HAP) abscission

probability. Samples below the mode ranged between 45-55% and 75-85% (2 samples) in the LAP population, and 95-100% in the HAP population (Tab 1).

One exception to this constant size-abscission relationship existed in the HAP sample harvested on June 18. Subgroups from this sample have been indicated with letters x, y, z to distinguish them from the other subgroups indicated with letters a to g.

Lyophilized seeds (10-40, according to size) from each of the above subgroups were homogenized with 50 ml of 80% methanol and extracted for 36 hr at 4°C with 2 changes of solvent. The combined supernatant was filtered and evaporated to dryness. The residue was dissolved in methanol, streaked on TLC plates (Eastman Chromogram 6060) and developed with isopropanol-ammonia-water (10-1-1, v/v).

Preliminary hormone analysis revealed the presence of 4 inhibitory zones ( $R_{fs}$  0.17, 0.53, 0.77, 0.95) when an acidic fraction (partitioned in ether at pH 5) was chromatographed. Subsequent analysis of Inhibitor III ( $R_f$  0.77) was confined to inspection of the chromatographic band corresponding to this inhibitor. To help localize this band, authentic (cis-trans, trans-trans) ABA was spotted on either side of the extract to serve as a marker. After development, a band (its position relative to ABA was:  $R_{ABA} = 1.25-1.55$ ) containing Inhibitor III was removed, based on UV localization of ABA, and eluted with pure methanol by shaking for 4 hr. Results were quantified relative to a range of concn of ABA, and expressed as ABA-equivalents per seed or per g of seed.

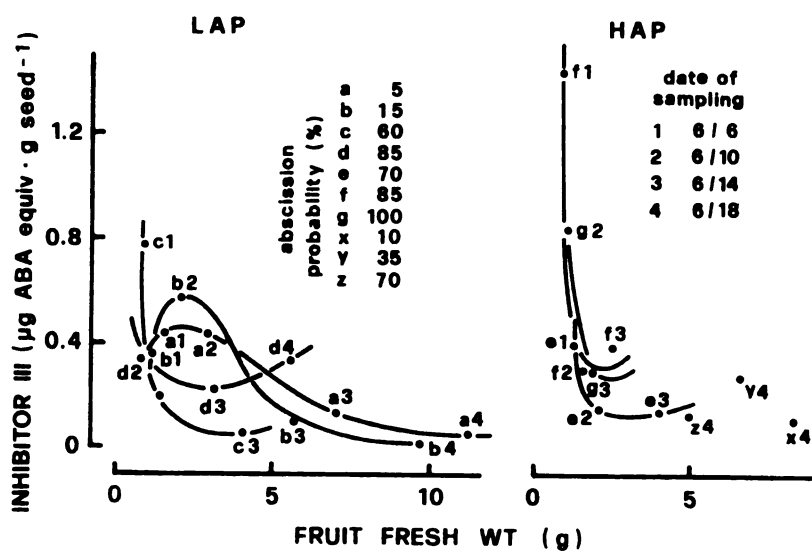
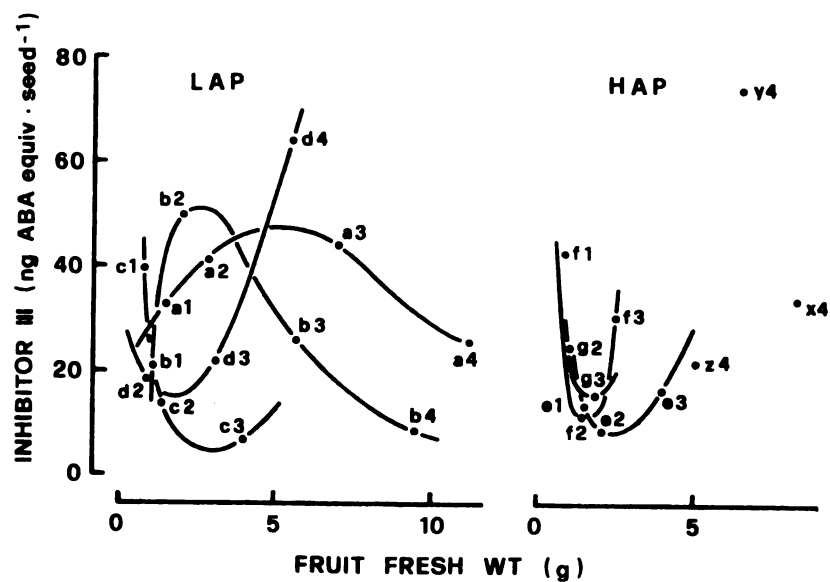
Eluates of the chromatogram band were transferred to Petri dishes (2.5 cm, i.d.), containing a Watman 3 MM paper disk, and dried under vacuum. At dryness, 0.5 ml of water was added and allowed to equilibrate for 30 min.

Cress seed germination was used as an assay. In preliminary experiments (Appendix 1, page 111) it was shown that this assay was sensitive to ABA and that GA and kinetin could not overcome, neither alone nor in combination (6), the inhibition imposed by ABA. This insensitivity to GA and kinetin enabled us to reduce hormone isolation procedures to a simple chromatography, thus avoiding losses associated with purification techniques (4). Ten seeds per dish and 3 dishes per eluate were used. Seeds were incubated for 36 hr at 25°C. Germination was evaluated by combining indexes of both number and length of roots present. This was done by multiplying % germination and root elongation, expressed as % of the elongation of the control.

## Results

Levels of Inhibitor III in the seeds show changes which appear specifically associated to the different fruit subpopulations segregated according to the abscission potential. In particular, Inhibitor III in fruit with a low (5%) abscission probability increased and declined (Fig. 1, subpopulation a) with a peak intermediate between samples a<sub>2</sub> and a<sub>3</sub> (harvested June 10 and 14, respectively). Fruits with a 15% abscission probability (b) showed a narrower and earlier peak, while fruit with the highest abscission probability (c and d; 60 and 80%, respectively) showed levels of Inhibitor III opposite to those observed in fruit with a low abscission probability (a and b).

Fig. 1. Levels of Inhibitor III in seed from fruit subpopulations (letters) representing different abscission probability, isolated from samples harvested at 4 different times (numbers) from 2 populations having a high (HAP) and a low (LAP) abscission potential. Levels of Inhibitor III are expressed as ABA equivalents per seed or per g fresh wt.



The distribution of Inhibitor III in fruit of the HAP population (70-100% abscission probability) was similar to that in fruit of the LAP population having the highest abscission probability (c and d). These results were similar when levels of Inhibitor III are expressed on a per seed basis or per gram fresh wt (see Fig. 1) basis.

#### Discussion

Differences in Inhibitor III levels with fruit subpopulations in this study showed a consistent relationship with the abscission potential associated with such subpopulations. This association was apparent as opposite trends in fruit having a high and low abscission probability from the LAP population, and by a similar pattern in abscising fruit (c,d,e,f,g) regardless of the parent population (Fig. 1).

Since the division into populations (HAP and LAP) and subpopulations represents two independent approaches in differentiating persisting and abscising fruits, the above association is of special interest and we view it as particularly meaningful. Further, since differences in abscission potential between LAP and HAP populations were induced by increasing competition, the above findings may be interpreted to mean that hormone variations are independent of the method used to establish such distinct groups.

The existence of different patterns of Inhibitor III, in fruit selected according to their potential to persist or to abscise, stresses the importance of sampling procedures as a means of better

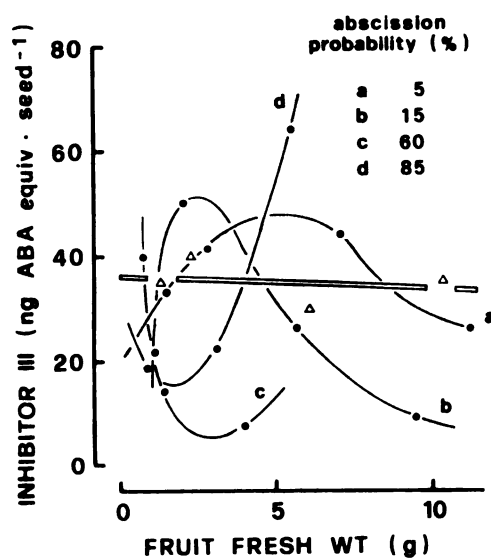
isolating and representing physiological events under study. The existence of opposite patterns in persisting and abscising fruits of the LAP population would have been missed, and no difference would have been observed (Fig. 2), had no attempt been made to group fruit according to abscission potential, but rather extractions made on randomly collected samples. These data provide further proof of the ambiguity (see also Section I) possible when concurrent events are not adequately separated in physiological studies.

The marked association observed between Inhibitor III and subpopulations of fruit representing different abscission potential should not be interpreted in a quantitative sense. This is not critical, however, since the eventual dependence of a physiological response on a hormone does not require that the two vary in parallel (see Section III). It is also important to note that the trend of Inhibitor III in abscising fruit does not represent a simple quantitative variation of the pattern observed in persisting fruit. On the contrary, distinct opposite patterns characterize the 2 groups with a coincidence of a maximum inhibitor level of 1 group corresponding to a minimum level in the other group, and vice versa.

We lack any support from the literature for interpreting the physiological role of this hormone. The presence of an inhibitory compound with similar chromatographic characteristics was shown in olive (10,11). No evaluation in relation to fruit development was made in such studies. Data from peach in this study suggest, however, that a significant and consistent difference in the pattern of



Fig. 2. Levels of Inhibitor III in subpopulation of LAP fruits, representing different levels of abscission probability (letters a to d) and levels of Inhibitor III if extracted from random samples (double line). Data representing random samples were calculated by averaging the levels of Inhibitor, extracted from the various subpopulations, proportionally to the relative frequency that characterized such subpopulations in the population size-distribution:  $(2 \cdot \underline{a} + 3 \cdot \underline{b} + 2 \cdot \underline{c} + \underline{d})/8$ .



Inhibitor III exists between persisting and abscising fruits and that this substance deserves further attention.

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SECTION III: A REASSESSMENT OF THE STUDY OF THE  
REGULATORY ROLE OF HORMONES

### SECTION III: A REASSESSMENT OF THE STUDY OF REGULATORY ROLE OF HORMONES

Abstract. The problem of assessing dependent relationships between hormone and hormone-regulated biological processes is analyzed in this study. The hypothesis that changes in hormone level may show parallel variations with the development of a physiological event appears tenuous, given the existence of artificial, biologically unsound conditions needed to support the hypothesis itself. Different methods, such as the suppression or substitution of the hormone source, seem more appropriate and yet they require specific conditions to become applicable. The complexity of factors and interactions existing in the integrated regulation of biological processes may hardly be studied by the use of single systems, models or methods and requires a number of different approaches. The use of multiple experimental approaches, as a working method, is discussed.

The understanding of the natural control of the abscission process remains extremely limited after decades of study. The hormonal hypothesis itself, used to justify the regulation of abscission, remains to be proved. Also the systems and models so far devised to represent this process appear inadequate (see Literature Review pag. 19-24). An attempt was made in this study to discriminate and isolate abscission from concurrent events, so as to provide for a more

critical interpretation of physiological changes associated with abscission. Unfortunately, studies of this type are, at best, confined to the factual observation of events, but remain, per se, inconclusive in confirming the assumption--can hormones and physiological changes be meaningfully associated.

A debate over this assumption requires a discussion as to what the epigenetic regulation is, what position hormones may hold in such a regulation and how experiments may be devised to test the conclusion reached. This discussion goes beyond abscission, per se, and becomes applicable to the study of the regulation of physiological phenomena in general. However, when a specific study, such as that conducted on abscission in this century, fails to reach a conclusive explanation of the process analyzed, the adequacy of the study should be reevaluated eventually starting from its theoretical basis.

#### Statement of the Problem

A well-established approach for studying the regulatory role of plant hormones is to relate their levels to the development of physiological phenomena. Such an approach is based on the assumption that the two events have a cause:effect relationship and that changes in development parallel changes in hormone levels.

Although all developmental processes are most likely influenced by bioregulators, the hypothesis of parallel variation of chemical and morphological changes has neither been proved a priori nor is it confirmed by the experimental observation of occasional parallelisms. The best evidence for such a hypothesis remains indirect, derived from



observations of dose:response relationships in some endocrine processes or in certain simplified plant systems under prescribed conditions.

It would appear inappropriate, however, to interpret the hormone regulatory role based on responses obtained in simplified plant systems, which are frequently selected sites of response and represent only a single component part of reactions of a complex physiological process. The in equilibrium condition which characterizes these reaction-type models may hardly be equated to the endogenous situation existing in complex systems. Also, the dose:response association found in specialized assays may not be specific for hormones acting in integrated systems, common to plants, where many processes occur simultaneously and with a high degree of interaction. The question, therefore, remains how reasonable is it to expect parallel variations between hormones and complex physiological processes?

In the following discussion an attempt is made to re-evaluate the conditions necessary to explain causal dependence as related to the regulation of complex plant systems. Hormone:event parallel variations, suppression or substitution of a hormone source and the use of multiple experimental approaches will be discussed.

#### Prerequisites for the Hormone:Event Parallel Variation: A Conditional Inductive Analysis

The aim of scientific research is to propose reasonable, alternative hypotheses that may account for an event been studied, and to devise critical experiments that will exclude one or more of such hypotheses (7). The application of this method to our problem

requires, (a) the statement of which conditions are necessary for the existence of hormone:event parallel modulations, and (b) the observation of how critically our experimental approaches may probe them.

A necessary prerequisite for the hypothesis of hormone:event parallel variation is the existence of proportional, single and direct effect of the causal agent. The first assumption, a proportional action of hormones, is probably applicable to many systems and consistent with the catalytic role of most bioregulators. Yet, threshold effects also exist which escape simple quantitative relationships. Certain thermo- or photosensitive reactions fit this category. The observation of dual hormone action (i.e., promotion or inhibition of the same event depending on conditions) also opposes direct, quantitative comparison. Permissive role or entraining (synchronizing) action of hormones can also be suggested which may have the same qualitative impact on regulation.

The second assumption, control by a single regulator, entails a coincidence of regulator and regulation of a process. This model fits rather well the formative function attributed to the hypothetical calines. Yet this very concept appears overly simplified. No event (physical or biological) is regulated by a sole factor. Then, the limiting role observed for hormones acting on specialized assay systems may not be confused with the integration of regulatory functions existing in complex systems, which implies the interaction of a number of factors both endogenous and exogenous. But then, if hormones play only a part in the regulation of physiological events, linearity of response in a hormone:event relationship may be expected only in very



limited, specific situations even when the hormone acts in a proportional manner.

An example of responses to be expected when more than one regulator is involved in the control of an event is given in Fig. 1. Here, a response (R) is generated by the interaction of 2 factors (A and B). Models of this type show that the complexity of response may be great and unpredictable depending on the presence and interaction of different factors.

This is not to deny the possibility of occasional findings of parallel variations between chemical and morphological changes, as much as to question the relevance of such findings. Cases of parallelism may exist, for example, where the association between hormone and event would be hard to reach. Let us use again the above model ( $R = A \cdot B$ ) and assume that the response is regulated in this case by the interaction of a factor A which rises and declines, and a factor B which continuously declines within a given interval of time. The response too would then show a rise and decline parallel to, and yet seemingly preceding, factor A (Fig. 2). The apparent anteposition of the effect to the cause in this case does not disprove their association.

A further assumption required by the parallel variation hypothesis is that hormone and event are directly related. However, physical processes such as the rate of diffusion, compartmentalization, conjugation, binding, etc. may alter the significance of hormone concn. Also, any chain of reactions initiated by a regulator may require considerable time for expression, thus making direct correlations difficult. Finally, the development of a process may be carried on

Fig. 1. Hypothetical result (R) of the interaction (product) of 2 factors schematically represented by regression lines A and B.

Fig. 2. Hypothetical result (R) of the interaction (product) of factors A and B.

by sequential regulatory events in the absence of the initiating factor.

The list of prerequisites does not end here nor is it confined to the biological characterization of the event itself. Experimental methodology may add further problems. For example, a number of events may coexist in biological organisms. Parameters expressing such events often become equivalent (interchangeable) when compared to chemical analyses. Unfortunately, the mathematical associations which may be found by indiscriminately correlating chemical and morphological data, in this case, will greatly outnumber the physiological associations, thus complicating rather than clarifying the understanding of physiological relationship. An obvious consequence created by this condition is that an inadequate separation of coevolving events may lead to different interpretation of physiological studies depending on the parameters chosen.

An abnormal situation may also exist when coevolving events characterize separate groups of the same population. Flower bud differentiation, or growth and abscission of immature fruit, may be chosen as examples. In a previous study (Section I) it was shown that abscisic acid (ABA) from immature peach fruit may be correlated to both fruit growth and abscission. Since the two events exclude one another, the above correlations may not be accepted as the same data would support different, alternative interpretations. However, when fruits were separated according to their potential to persist or to abscise, ABA could be associated to the persisting but not to the abscising fruit.

The presence of separate events in a given population may further induce artificial changes of extractable hormones. The above example of potential persisting and abscising fruit, in a same mixed population, may help to clarify this point. When random samples from such a population are extracted, the physiological distinction between the different fruits is lost and their hormone blended. In this condition, potentially abscising fruit, for example, would "dilute" hormones associated to persisting fruit, proportionally to the ratio between the two groups of fruits. The result may be that continuous change in the level of the hypothetical hormone, parallel to the change of such a ratio, as illustrated in Fig. 3.

An example of the significant role that sampling may play in avoiding misinterpretations is illustrated in Fig. 4. The dynamic differences of the levels of an acidic inhibitor extracted in growing and abscising fruit (Section II) would be missed in extracts from fruit randomly sampled.

The arguments presented show that the assumption of proportional, single and direct hormone action does not justify the hypothesis of parallel development of hormones and physiological events.

It would seem artificial, however, to attempt to correct such indeterminedness by additional assumptions since the analysis has already shown serious inconsistencies (Fig. 5). First, the condition of proportional, single and direct hormone action, necessary for the hypothesis of parallel variation, is probably not met in most complex systems. Consequently a series of alternative situations may exist in which causal relationships do not imply parallel variations.

Fig. 3. Abscising fruit [induced but not separated (A)] may dilute a hormone specifically associated with the persisting fruit existing in the same population (B) when random samples are used for chemical analysis.

Fig. 4. Differences in the levels of an acidic inhibitor isolated from fruit separated into subpopulations according to their abscission probability (letters a to d) and from a random fruit sample (double line).



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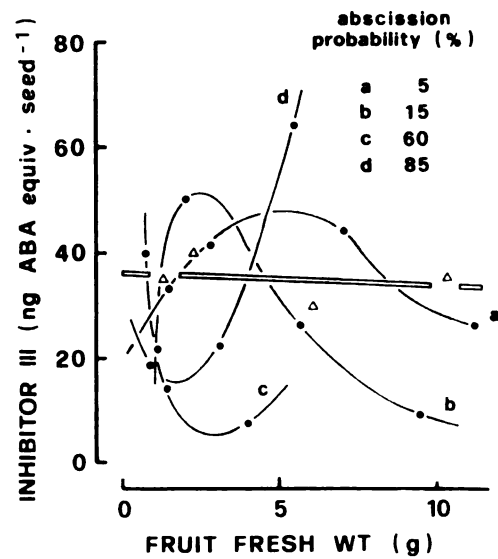
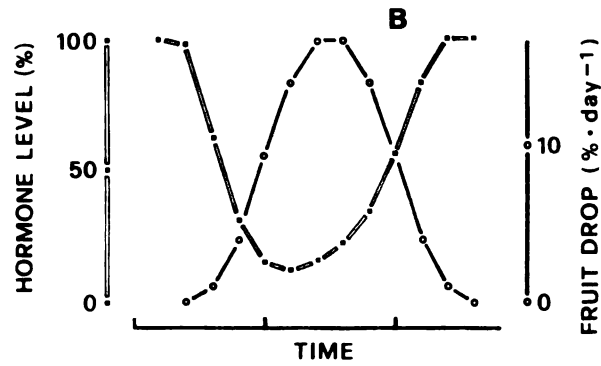
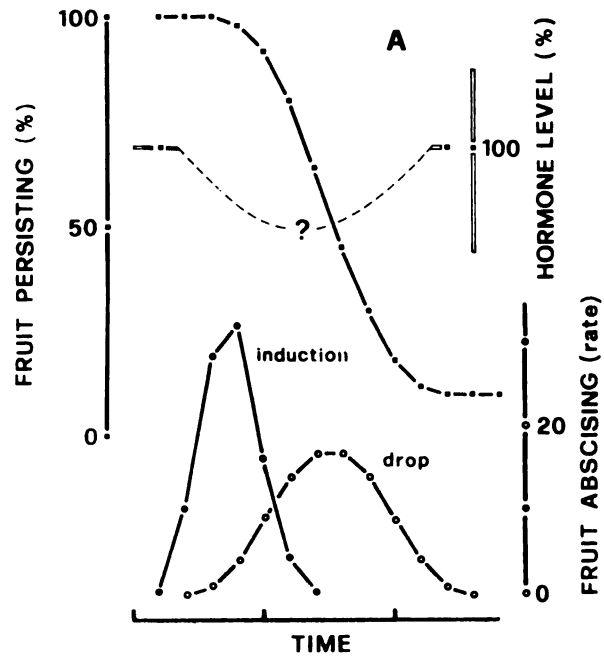
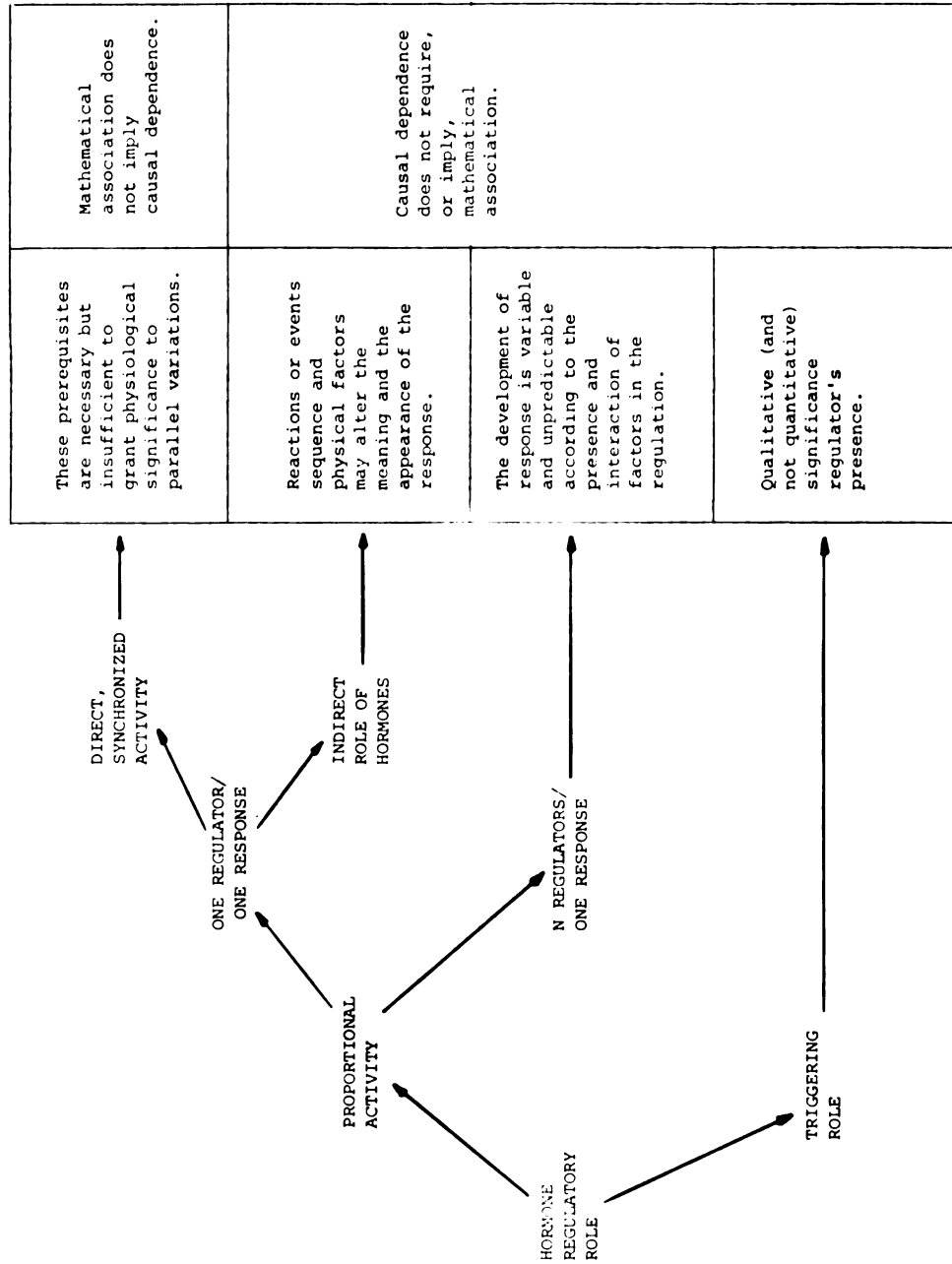


Fig. 5. Diagrammatic representation of the assumptions necessary to postulate the existence of a hormone:event parallel variation in the regulation of a biological process.



Secondly, such a condition, if met, would not justify the presumption that mathematical and physiological associations would coincide.

In conclusion the search for parallel trends would neither deny physiological dependence when lacking mathematical association nor imply such a dependence when mathematical association was observed.

In a sense, these conclusions should have been foreseen. Any research program which does not account for conditions it may not exclude (such as nonproportional, integrated and indirect hormone activity, in this case) is, at best, tentative. Also, the discriminating role attributed to mathematical association in this method seems excessive. The researcher must set the condition for homogeneous comparison and not ask correlations to reveal what he himself cannot discern. "Equation and measurement are useful when, and only when, they are related to a proof, but proof and disproof come first. . . ." (7).

#### Other Potential Approaches to the Study of Bioregulators

It seems fair to conclude from the preceding discussion that the observation of parallel variations is of limited significance in studying the regulation of complex processes and should be confined to the study of specific well-defined systems. The problem remains--how to determine the hormone regulatory role in the development of plants, independent from the requirements of proportional, single and direct action?

Jacobs (5) suggested that the study of the relationship between hormone and physiological events could be made more critical if the source of the hormone was suppressed and then appropriately substitute with the postulated bioregulator in pure form.

This suggestion appears reasonable and in fact consistent with similar approaches frequently used in endocrine studies. Yet some problems may be proposed when this approach is used for studying plant systems.

The suppression of a hormone source is frequently achieved by excision of the putative source organ. This approach is often imprecise in plants where organs have multiple functions. Thus, the hypothesis that antiabscission hormones are produced in the leaf blade may hardly be confirmed by observing the abscission response after deblading. The role of the blade itself, and its interrelation with the petiolar abscission zone, may not be limited only to the production of such regulators. Thus, deblading could be viewed as being comparable to the removal of the stomach while attempting to elucidate the role of gastrointestinal hormones.

The use of genetic mutants or specific inhibitors of hormone synthesis or action was proposed (5) as an alternative to excision. Each of these approaches involves a number of assumptions and conditions which must be carefully established to avoid misinterpretation. Also, processes are generally controlled by multiple interacting factors so that changes derived from the suppression of a specific hormone may be due totally or in part to the lack of interaction rather than to the absence of the hormone.

The concept of substituting the natural source of the hormone by an external supply of the pure chemical was also proposed (5). Does such an approach provide conclusive evidence? Penetration barriers, alternative transport routes, rate of diffusion, inactivation mechanisms, etc. may markedly alter the response from the applied compared to the endogenous regulators in plants. Also, growth regulators tend to give different and even multiple responses when different plants or organs are treated at different stages of development, thus suggesting that factors other than hormones may play a fundamental role in determining physiological responses.

The difficulties arising in the attempt to interpret changes in hormone levels or responses subsequent to suppression and substitution of a hormone source have been recognized (3,5,10). Nevertheless, the point has been made (3,4) that to satisfy a number of these requirements may increase the probability of arriving at the proper conclusions with respect to the physiological dependence of developmental processes on hormones. It should be pointed out, however, that sequential research may reduce the unknown only when each experiment is capable of conclusively discriminating between its own preexisting hypotheses. On the other hand, uncertainty would persist and may be broadened by uncritical sequential research.

The difficulty with the above approaches is that they would become useful when, and only when, the conditions in which they are applied are critically controlled. To overlook this point may cause one to lose perspective of the problem of determining the role of hormones and to become method oriented. It is difficult to conceive an

ultimate method (7,8), but only a series of methods each capable of scrutinizing certain hypotheses, and each valid under certain conditions which the researcher must carefully verify. When the hypotheses are poorly formulated and conditions inadequately controlled, no conclusion can be reached in so far as the assumptions needed to accept or refute the hypothesis remain unestablished.

#### The Use of Multiple Approaches to Hormone Studies

The sense of perspective that the researcher must maintain in relation to the scientific method may be sharpened by the use of "conditional inductive analysis" (1,2,6,7) of hypothesis and preexisting knowledge. Such a method, as Bacon suggested (1), facilitates the design of better as well as fewer experiments. A similar approach is also implicit in Chamberlin's (2) "multiple hypotheses" method. To ensure that a hastily conjured explanation does not become a "controlling idea," Chamberlin suggests adoption of multiple alternative hypotheses. This may reduce the danger of following insignificant or even incorrect paths. It also is a deterrent against the concept that single explanatory hypotheses or theories are sufficient to account for complex phenomena. An "adequate explanation often involves the coordination of several causes. This is especially true when research deals with a class of complicated phenomena naturally associated, but not necessarily of the same origin and nature" (2). Chamberlin's conclusions are rather suggestive particularly in attempting to investigate the field of epigenetic regulation of integrated plant systems.

The emphasis on phytohormones' regulatory role in this regulation should be reconsidered. These compounds may enhance but not induce a

physiological process (6,9,10). Also, their role is probably dependent on interaction with other substances and processes. These attributes, then, are rather aspecific in regulatory processes where many factors (including enzymes, substrate, reaction products, energy carriers and also physical processes) may intermittently play the role of primary or secondary regulators. The wide spectrum of activity of phytohormones and the minute amount needed to elicit a response suggests that these compounds may exert their effect early in the chain of sequential regulatory steps needed for the integration of development. At the same time they do not represent the key step as suggested by the requirement of other (unknown) conditions for them to become active and specifically operative.

The experimental approach to the study of epigenetic control also presents particular problems. Given the complexity of biological organisms, hypotheses made to account for the regulation of development may hardly be proved in single experiments but require to be continuously recycled in sequential experiments. The problem, however, is rendered more complex by the need to define, in epigenetic control, both the multiple factors responsible for a process and their interaction. Attempts to isolate factors and events (excised systems) of a process may help to recognize and facilitate their study; however, we must recognize that integration is often lost in isolated systems and that the regulatory factors may acquire new and different functions than when a part of the whole.

It becomes apparent that no single system or model, no matter how perfect, may, per se, allow for the understanding of the many factors



involved in the regulation of complex processes. The observation of different systems is necessary to avoid the danger of limiting our analysis to single events of a regulatory process or to related limiting factors. The problem is rendered even more complex by the fact that in biological systems we are always confronted by the presence of different coevolving phenomena. The problem, therefore, is not only to discover the multiple factors associated with the process under study, but also to distinguish them from factors responsible for different, unrelated processes.

The solution of such a problem requires the simultaneous use of different approaches. Analogous to the multiple hypothesis approach, a multiple experimental approach may offer certain advantages. This method requires the use of a number of systems each differing in some of the conditions associated to the one being studied. For example given x, y, w events or conditions which may coexist with z condition or event to be studied, better associations may be found by performing serial experiments (A+B+C etc.) of the type:

$$\begin{array}{ccccccc} A & + & B & + & C & + & \text{etc.} & (I) \\ \hline \underline{x+y+w} & & \underline{x+y+z} & & \underline{w+y+z} & & & \end{array}$$

For example, a change in the level of a specific compound, in fruit which develop an abscission layer, may be associated to abscission as well as to changes in fruit growth, seed development or perhaps other events in immature fruit, or to ripening, onset of seed dormancy (and others), in mature fruit. The simultaneous use of both systems may help to establish common denominators. This study could also be made more critical by additional use of seedless fruit (parthenocarpic

or induced seedlessness) or by the imposition of different regimes of correlative competition with other organs. Further, a dynamic quality may be added by the use of inhibition of synthesis or substitution for the compound studied, as suggested by Jacobs (5). The point remains, however, that no one of these methods would establish, per se, causal dependence when studied in isolated experiments. Multiple approaches must be applied to the use of substitution or suppression of hormone sources. Let us use again the example of the fruit. If we wish to establish the role of the seed in the regulation of abscission there would be some merit to use seedless fruit. It would be uncertain, however, if parthenocarpic fruit could be taken in this case to represent seedlessness or if they would represent a specific physiological condition. The use of fruit in which the seed was suppressed (destroyed) at different stages of fruit development would help overcoming this doubt. But then not every approach to suppression of the seed is acceptable. For example, killing the seed by wounding (11) or cold (4) would have a different impact on abscission of the injured fruit. This consideration points to the existence of a second complexing factor. In a true sense, the transfer of a problem from the theoretical to the experimental expands the number of hypotheses and assumptions beyond those needed to account for the specific event being studied. If we look back to the above model of multiple experimental approaches (I) this should then include a set of new, method-dependent (s,t,u etc.) variables in addition to those associated with the event being studied. The new variables must be

evaluated carefully to avoid increasing experimental complexity or introducing ambiguity.

The method of multiple experimental approaches, as described in this discussion, may appear fundamentally similar to the method of recycling hypotheses in sequential experiments, particularly with respect to the possibility of reducing misinterpretations. Yet, to paraphrase Chamberlin (2) events which require the coordination of several factors may neither be contained in single hypothesis nor represented by a single model or system. In this sense, the multiple experimental approach may contribute to the experimental work what the multiple hypotheses method does for theoretical analysis.

### Conclusions

The dilemma of understanding causative association has no simple solution and even less in attempting to approach a problem as complex as the epigenetic regulation of a biological organism. Yet, an effort must be made to generate reasonable hypotheses, find acceptable methods and devise critical experiments if progress is to be made. This does not imply that a hypothesis, model or experiment to be accepted must also be "free of errors." The best experiments may only reduce the number of preexisting hypotheses, and the most perfect conclusions will remain tentative in proportion to the number of unestablished conditions still existing. But then such an approximation is not in itself an obstacle to scientific progress as far as an effort is made to recycle the experimental process and to establish that each time the hypotheses, the outcome and the conclusions

are related in a rigorous syllogism (6). However, when conditions are not critically established, hypotheses not sufficiently explained and methods or models indiscriminately accepted, the whole experimental process would be based on elliptic (incomplete) reasoning and not error but confusion will follow.

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## SUMMARY AND CONCLUSIONS

The dilemma of understanding causative associations has no simple solution and even less so in attempting to approach a problem as complex as the epigenetic regulation of the abscission process. Yet, an effort must be made to generate reasonable hypotheses, find acceptable methods and devise critical experiments, if progress is to be made.

In this work, an attempt was made to critically evaluate the available knowledge on the natural control of abscission and to analyze the possibility of improving models and hypotheses needed for its study. Emphasis was placed on using intact systems. In so doing, we stressed the separation of abscission from concurrent events to allow for a more critical interpretation of chemical changes associated with this physiological event.

Levels of extractable hormones are often correlated to physiological phenomena based on the assumption that a simple cause: effect relationship exists between the two. The presence of different and unrelated phenomena in complex organisms may, however, lead to misinterpretation of events and physiological changes. This problem is particularly serious when studying the abscission of young fruit because both persisting (actively growing) and abscising (various stages) fruits are present in the same population at the same time. Fruit growth and abscission, therefore, was used as a test system for

for interpreting changes in two simultaneously occurring events in relation to concurrent changes in hormone level.

Peach fruit populations having a low or high abscission potential were established by either increasing or decreasing competition between fruit and shoots. Abscission probabilities were then assigned to subgroups, within each population, based on a predetermined relationship between growth and abscission.

It is recognized that the terms growth and abscission only refer, in this work, to the most relevant changes occurring in these different groups of fruit. The groups thus formed, however, could represent other events occurring concurrently. This limitation is not critical, however, since we focus on the adequacy of correlative analysis in the interpretation of the role of hormones and this independently from any specific phenomenon.

Several inhibitors, as indexed by the inhibition of cress (Lepidium sativum L.) seed germination were detected in peach seeds. The biological activity of 2 such inhibitors was particularly prominent. One (Inhibitor II) was identified as ABA by GLC and GC-MS. ABA levels, in seeds from fruit separated according to their abscission potential, were found to be inversely related with abscission but positively related to fruit size, growth rate and persistence. Data on cotton fruit abscission (43), when analyzed in a similar manner, agree with those obtained for peach, that most of the extractable ABA was derived from persisting and actively growing fruit.

The levels of a second Inhibitor (Inhibitor III) were also analyzed and compared to abscission potential of the fruit. The



distribution of Inhibitor III consistently differed between fruit with a high or low abscission potential both when populations and subpopulations were compared.

Since the classification into populations and subpopulations represents two independent approaches in differentiating persisting and abscising fruits, the above association is of special interest and we view it as particularly meaningful. Further, since differences in abscission potential between populations were induced by increasing competition, the above findings may be interpreted to mean that hormone variations are independent of the method used to establish such distinct groups.

The existence of different patterns of ABA and Inhibitor III content in fruit selected according to their potential to persist or to abscise, in comparison to those randomly collected, stresses the importance of sampling when relating changes in hormone level to physiological events under study. The ABA with growth association found in this study would not have been identified, and an ABA with abscission relationship would have been implied, if fruit samples were not classified according to abscission potential. Further, the existence of opposite trends of Inhibitor III in persisting and abscising fruit would have not been detected in randomly collected samples.

In general, the approach used in this and many other studies is based on the hypothesis that a physiological meaning may be attached to the observation of parallel changes of hormone levels and the rate of development of biological processes. Unfortunately such a hypothesis is neither adequately tested nor confirmed by experiments

confined to the analysis of hormonal levels and morphological changes. Arguments are presented to show that the hypothesis itself (parallel variations between hormone level and event) may be inadequate for an understanding of the role of hormones. Suppression or substitution of the hormone source may provide more critical data. Yet, the complexity of factors and interactions common to biological processes are probably not adequately represented in experimental approaches dependent on a single system or model. Observations from different systems are necessary to avoid the danger of limiting our analysis to single events in a regulation or to contingently limiting factors. The problem, therefore, is not only to discover the multiple factors associated with the process under study, but also to distinguish them from factors responsible for different, unrelated processes.

The solution of how to approach the study of multiple interacting factors in the regulation of a process and to distinguish them from other, unrelated factors or events, requires the simultaneous use of a plurality of approaches. This method requires the use of a number of systems each differing for some of the conditions coexisting or associated to the one being studied. The value of a multiple experimental approach, as a working model in relating the role of hormones to the regulation of physiological events, is discussed.

## APPENDIX

SUPPORTING EVIDENCE FOR THE SPECIFICITY OF  
CRESS (Lepidium sativum, L.) SEED BIOASSAY

## APPENDIX

### SUPPORTING EVIDENCE FOR THE SPECIFICITY OF CRESS (Lepidium sativum, L.) SEED BIOASSAY

An important feature of a bioassay is the specificity of its response to single hormones. This could reduce hormone-isolating procedures, thus avoiding losses of hormones always experienced with purification technics.

The work reported here deals with the definition of cress seed response to IAA, GA, kinetin and ABA. Seeds were incubated in Petri dishes (2.5 cm i.d.) containing filter paper (Watman 3 MM) saturated with 0.5 ml of active solution. Germination was measured after 36 hr incubation at 25°C. Indices of number and length of roots present were combined by multiplying % of germinated seeds and root elongation, expressed as % of the elongation in the control.

GA<sub>3</sub> ( $10^{-7}$  to  $10^{-3}$  M) did not overcome the inhibition of germination imposed by ABA ( $10^{-6}$  and  $10^{-5}$  M), as shown in Fig. 1. Also, kinetin ( $10^{-9}$  to  $10^{-5}$  M) was neither directly active nor showed a permissive action on GA in overcoming the inhibition imposed by ABA (Fig. 2). IAA ( $10^{-7}$  to  $10^{-3}$  M) was inhibitory and interacted with ABA by causing an even greater inhibition (Fig. 3).

A satisfactory specificity of the cress seed germination test to ABA is indicated in these data. The lack of response to GA and kinetin is sharper than in wheat coleoptile straight growth test. Although IAA was inhibitory and interacted with ABA, chromatographic

procedures used in Sections I and II of this thesis would favor separation of IAA from both ABA and Inhibitor III.

Fig. 1.--Cress seed germination in the presence of ABA and GA<sub>3</sub>.

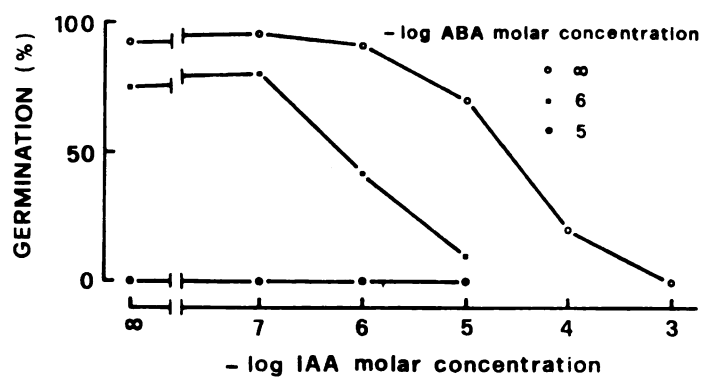
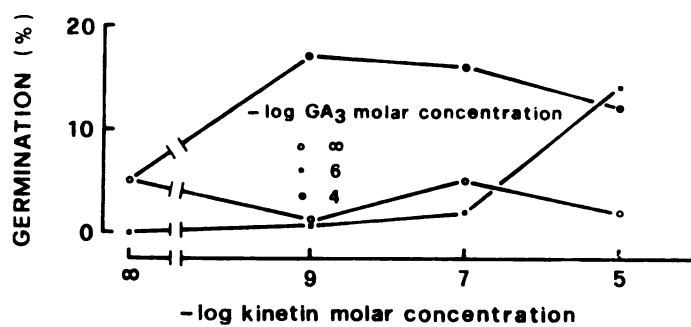
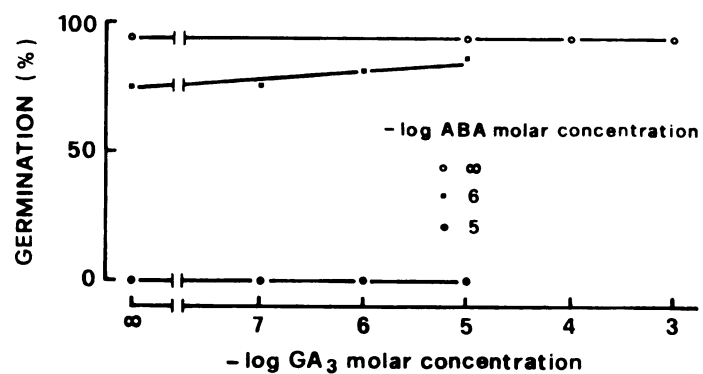
GA<sub>3</sub> does not reverse the inhibition imposed by ABA.

Fig. 2.--Cress seed germination in the presence of ABA, GA and

kinetin. Kinetin does not significantly increase germination either alone or in the presence of GA<sub>3</sub>.

Fig. 3.--Cress seed germination in the presence of IAA and ABA.

The two regulators interact by causing a strong inhibition.



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