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(Signed) Ronald Merton Knight

PERMANENT ADDRESS:

General Foods Ltd.
Research Department
150 William Street
Cobourg, Ontario.

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ETHYLENE PRODUCTION AND URACIL METABOLISM IN AGEING BEAN LEAVES

BY

© RONALD MERTON KNIGHT

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Ethylene Production and Uracil Metabolism in Ageing Bean Leaves" submitted by Ronald Merton Knight in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

M. A. Spencer
Supervisor

Edwin A. Cossins

Paul Gaddie

C. T. Phan

G. Ram Chandra
External Examiner

Date December 17, 1969

ABSTRACT

Extensive research efforts have been focused on various aspects of leaf senescence. Much of the work has been directed at hormonal control of leaf senescence rather than a study of metabolic changes during leaf senescence. The purpose of this dissertation research was to investigate the possible linkage between RNA degradation in senescing wax bean leaves and ethylene production from the leaves.

Both ribonucleic acid (RNA) levels and $^{32}P_i$ incorporation into RNA decreased as leaf age increased. Likewise incorporation of both orotic acid and uracil into RNA decreased at increasing leaf ages, which was suggestive of a decreased RNA synthetic activity in the older leaves.

Evidence was obtained showing two peaks in ethylene production, a major peak at about full leaf expansion followed by a minor peak. Ethylene production also varied over a 24 h period; ethylene production was higher during the light period than during the dark. Endogenous β-alanine levels were initially high in young leaves, decreased to a minimum, and then reached a second peak that coincided with the minor ethylene peak. Labeled β-alanine was produced from labeled orotic acid or uracil during incubations with detached bean leaves. Ethylene-^{14}C was produced from β-alanine-2-^{14}C at the major ethylene peak age provided the specific activity of the labeled material was adjusted for the amount of β-alanine applied.

The activity of the enzyme dihydrouracil dehydrogenase was detected in leaves over the ages for major ethylene production. Very young and very old leaves did not show any detectable activity. A 30-45% ammonium sulfate fraction of leaf protein was used to study the uracil to β-alanine pathway. Experiments showed that dihydrouracil dehydrogenase required reduced nicotine adenine dinucleotide phosphate. Significant conversion to β-ureidopropionic acid was obtained. In vivo feedings of detached leaves with uracil, uridine, and dihydrouracil indicated that the enzyme dihydrouracil dehydrogenase was probably rate limiting in the uracil to β-alanine pathway. Another possible control lay in the fact that uracil production from orotic acid in detached leaves was low in young leaves and rapidly increased over a one to two day period. Over this same age period incorporation of orotic acid into RNA decreased, possibly indicating a change in metabolic pathways.

Linkages were thus established between RNA degradation and β-alanine and ethylene production, and information obtained on some metabolic variations with leaf age.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ci	curie
cpm	counts per minute
DNA-RNA	deoxyribonucleic-ribonucleic acid complex
dpm	disintigration per minute
ft-c	foot-candle
GC	gas chromatograph
GRC	gas radiochromatograph
h-r-RNA	heavy ribosomal RNA
l-r-RNA	light ribosomal RNA
MAK	methylated albumin kieselguhr
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
$^{32}P_i$	radioactive orthophosphate
ppm	parts per million
RNA	ribonucleic acid
s-RNA	soluble RNA
TLC	thin layer chromatography
(U)	uniformly labeled radioisotope

INTRODUCTION

A considerable volume of data has accumulated on the physiology and biochemistry of senescence in plant tissue. The general consensus of opinion is that RNA decreases accompany cellular senescence. The fate of the RNA has been the subject of numerous research efforts, but a generally accepted degradation pattern has not been elucidated. During RNA degradation a pool of low molecular weight compounds such as nucleotides, nucleosides, and free bases would probably be formed. An active uracil catabolic system producing β-alanine is present in pine callus tissue and rape seedlings, as well as in rat liver.

Previous work from this laboratory with subcellular fractions from wax bean cotyledons had shown β-alanine to be a precursor of ethylene. Research reported in this dissertation was commenced to investigate ethylene production from ageing leaves and to study the possibility that RNA degradation could give rise to the ethylene precursor, β-alanine via a catabolic system for uracil. An excised leaf system was utilized to study the metabolism of selected compounds as well as the production of ethylene at the various leaf ages. With the study the author hoped to contribute evidence linking ethylene production with known biochemical aspects of leaf senescence.

CHAPTER I

REVIEW OF LITERATURE

A. RNA Metabolism in Senescing Tissue

Recent reviews have dealt with RNA changes in ageing or senescing cotyledons, hypocotyl and leaf tissues (Woolhouse, 1967; Key, 1968; Trewavas, 1969). The consensus of opinion appears to be that as senescence progresses there is a concomitant decrease in RNA, DNA, protein and chlorophyll levels. There is considerable documentation of the alteration of RNA levels by plant hormones in plant tissue. A decreasing RNA level is generally accepted as indicative of a senescent tissue.

1. RNA Metabolism and Leaf Senescence

Kessler and Engelberg (1962) pointed out that in developing apple leaves, RNA levels increased during the first four weeks of development and then decreased with increasing age. They also studied the RNAse of the mitochondrial, microsomal, and soluble cytoplasmic fractions. Membrane bound RNAse only became active upon solubilization. The particulate fraction's RNAse had an anabolic activity while the soluble fraction's RNAse was of a degradative nature. The RNAse activity in the soluble phase was initially low and became more active as the leaf age increased.

Wood and Bradbeer (1966) studied total RNA levels in primary bean leaves induced to senesce by excising the shoot and standing it in distilled water under 16 h-long days at 21°. Their results showed a

decrease from 408 to 175 µg/g fresh weight after 3 days and to 118 µg/g fresh weight after 9 days detachment. In both attached and detached tomato leaves McHale and Dove (1968) noted that RNAse activity increased as the chlorophyll content (another senescence indicator) decreased. From results with experiments with actinomycin D as an inhibitor of protein synthesis, they suggested that the increased RNAse activity was at least partially attributed to enzyme synthesis. With excised barley leaves floated on water-agar for 6 days, Atkin and Srivastava (1968) demonstrated a decrease in every RNA component as separated by sucrose gradient centrifugation. These changes were relative to the 0 day (7 day old barley plants at time of excision). Kinetin-agar retarded these decreases. They suggested that important changes in nucleic acid metabolism occur during the early stages of senescence.

If changes in metabolism do occur these changes could be mediated by changes in RNAse activities. Udvardy et al. (1967) studied the soluble and particle-bound RNAse activities in excised Avena leaves. Incubation in the darkness resulted in an increase in both the soluble and particle-bound RNAse activities. They observed that only a part of the activity of the particulate and soluble fraction is latent. They suggested that RNAse activity is generally repressed in plant tissue and derepression is evoked by unfavourable physiological conditions, including leaf abscission.

2. RNA Metabolism in Germinating Cotyledons

Cotyledons have been the object of intensive research efforts on RNA metabolism. Studies have focused on RNA synthesis during early

stages of germination and RNA degradation during later stages.

Ledoux and Huart (1962) studied the translocation of RNA in barley seedlings. Labeled yeast RNA was shown to diffuse through the seed and into the coleoptile and roots of germinating barley. Adequate controls were taken to insure that the labeled RNA was not degraded and re-synthesized. The amount of RNA translocated undegraded decreased with the seedling age. There is no reason to believe that endogenous RNA molecules could not move the same way. Marcus and Feeley (1962), using germinating peanut cotyledons, showed an increase in the RNA and DNA contents during a 7 day germination period. The RNA synthesising activity of peanut cotyledons during the initial 8 days of germination was also illustrated by Cherry (1963). Subsequently there was a rapid loss of RNA, concomitant with a several fold RNAse activity increase in the cotyledons.

Cherry et al. (1965), using MAK column chromatography for RNA separations from peanut cotyledons, showed a reduction in heavy ribosomal and messenger RNAs and an increase in soluble RNA after 12 days germination. They suggested that these changes in RNA were probably related to the many changes in the physiological processes that come with age.

Beevers and Guernsey (1966) estimated RNA levels in cotyledons and axis tissue of germinating pea seeds. Total cotyledon RNA decreased during germination while total axis RNA increased. This increase was greater than the decrease in cotyledon RNA, indicating a net RNA synthesis. There was no nucleotide accumulation in the cotyledons but there was in the axis. Beevers and Splittstoesser (1968) injected

labeled leucine and adenine into germinating pea cotyledons and estimated the amounts transported to the axis tissue. Twenty per cent of the labeled leucine was transported to the axis and was readily incorporated into protein. Only ten per cent of the adenine was transported to the axis and it was not readily available for nucleic acid synthesis. Radioactivity from both leucine and adenine was detected in CO_2, lipid, nucleic acids, water soluble, and water insoluble residues throughout the 11 day germination.

The RNAse levels in germinating pea seeds were investigated by Barker and Hollinshead (1967). The activity increased to a maximum at 5 days germination, passed through a minimum at 8 days and thereafter increased. Barker and Hollinshead (1964) had earlier shown that quantity of RNA in pea cotyledons falls during the first 2 weeks after germination.

It appears that the total RNA complement in senescing cotyledons decreases with age; the RNA is either translocated as such or is degraded as would be expected by increased RNAse levels. The degraded RNA could be then transported to the axis or other actively growing regions of the plant.

3. RNA Metabolism Associated with Fruit Ripening

There is less information available on nucleic acid metabolism during fruit ripening than for other storage tissue (such as cotyledons). Richmond and Biale (1967) studied RNA synthesis during the respiratory climacteric in avocado fruit. They estimated no change in total RNA during the respiratory rise period. During pre- and early climacteric

periods extensive $^{32}P_i$ labeling was observed in the heavy ribosomal and messenger RNA components as estimated by MAK column chromatography. They noted a reduction in the $^{32}P_i$ incorporation into the above mentioned fractions during the late climacteric stage. At the climacteric peak very little $^{32}P_i$ label was detected in the heavy ribosomal and messenger RNA components. They concluded that the degeneracy of the avocado is mediated through a failure to synthesize ribosomal and messenger RNA. Conflicting results were obtained by Looney and Patterson (1967) during the climacteric phase in Yellow Transparent apples. Total RNA, presumably mostly ribosomal RNA, of the cortical tissue increased as the respiratory rate increased. Looney and Patterson did not attempt to fractionate the RNA nor did they attempt a $^{32}P_i$ label to detect newly synthesized RNA. Although Looney and Patterson detected an increase in RNA, the qualitative results of Richmond and Biale are indicative of a decreased synthesis of messenger RNA and heavy ribosomal RNA, probably associated with a less active protein synthesizing system.

4. RNA Metabolism in other Plant Tissues

Cherry and Lessman (1967) compared nucleic acids from maize and pea epicotyl tissue. Using MAK column chromatography, they detected a fraction with high RNA concentration in maize shoots that was absent or in a low concentration in pea epicotyls. They suggested this to be a long lived messenger RNA. Ingle et al. (1964) studied the metabolic changes in total nucleic acids and soluble nucleotides during corn germination. Over a 120 hour period nucleic acids and soluble nucleotides increased in the whole seedling and in the axis tissue. Either a slight increase or a

static level was detected in the scutellum and the endosperm tissue.

The effect of IAA on RNA synthesis was studied by Trewavas (1968) in pea epicotyl sections. IAA increased the incorporation of labeled precursors into RNA, mostly into ribosomal RNA with lesser amounts into the messenger RNA fraction. This increased incorporation into RNA first appeared in the nuclear fraction and later in the cytoplasm. Much interest in RNA synthesis was stimulated by the demonstration by Ingle et al. (1965) of a DNA-like RNA in plant tissue. Considerable research has been initiated in many laboratories to attempt to correlate this highly labeled RNA with newly synthesized RNA and thus to the physiological phenomena such as abscission, climacteric in respiration, growth regulator responses and flowering.

5. Bacterial Contamination in Studies of Labeled RNA

A precaution in the interpretation of RNA labeling data was pointed out by Lonberg-Holm (1967) when he showed that as little as one part in 10^7 by weight of contaminating bacteria was able to alter the labeling of radish and lettuce seedling nucleic acid with $^{32}P_i$. He also found that bacterial nucleic acid was labeled in preference to those of the plant cells, probably because the bacteria were able to compete with the plant tissue for both endogenous and exogenously applied phosphate as shown by Barber (1966) with sterile and non-sterile barley plants. Contrasting with these results are those of Sobota et al. (1968). They were able to show that bacteria contributed significantly to a newly synthesized GC-rich DNA component. Bacteria did not contribute significantly to the $^{32}P_i$ incorporation into RNA of excised hypocotyl or

soybean root tips. It must be noted that the medium used in their study was a minimal medium with respect to bacterial growth and thus one would expect little RNA synthesis although DNA synthesis could occur (Neidhardt and Magasanik, 1960). This again emphasized the extreme care required in labeling studies in interpreting results in the light of experimental conditions.

6. RNA Degradation in Non-Plant Systems

RNA metabolic studies were initiated earlier and progressed more rapidly in animal systems than in plant systems, mainly because of the availability of specialized tissues (liver, heart, muscle, and blood) and the ease of extractability of the nucleic acid and the complementary enzyme systems.

Bertles and Beck (1962) showed that RNA in reticulocytes in vitro was degraded to low molecular weight products, mostly hypoxanthine, cytidine and uracil. Likewise Burka (1969) investigated RNA degradation in reticulocytes in vitro. Although all types of RNA decreased, ribosomes were degraded at a greater rate than soluble RNA. The loss of RNA from membrane bound areas was three times faster than from the cell as a whole. Eventual degradation products were free bases. Thus it appears that, during the in vitro maturation of reticulocytes, considerable RNA is degraded to the free base level.

Schutz et al. (1968) investigated the incorporation of orotic acid and $^{32}P_i$ into a rapidly labeled RNA from rat liver. Their rapidly labeled RNA was greater than 45-S in size and was resistant to DNAse,

pronase, and EDTA. Pileri et al. (1968) studied the degradation of rapidly labeled RNA in human acute leukemia cells. They noted that they contained two metabolically different RNAs, one unstable (half-life 1.5 to 3 hours) and another relatively more stable. In X-irradiated E. coli cells, degradation products appeared in the incubation medium about 30 minutes after irradiation (Pecevsky and Kucan, 1967). Mononucleotides, oligonucleotides, and some free adenine could be isolated from the incubation medium.

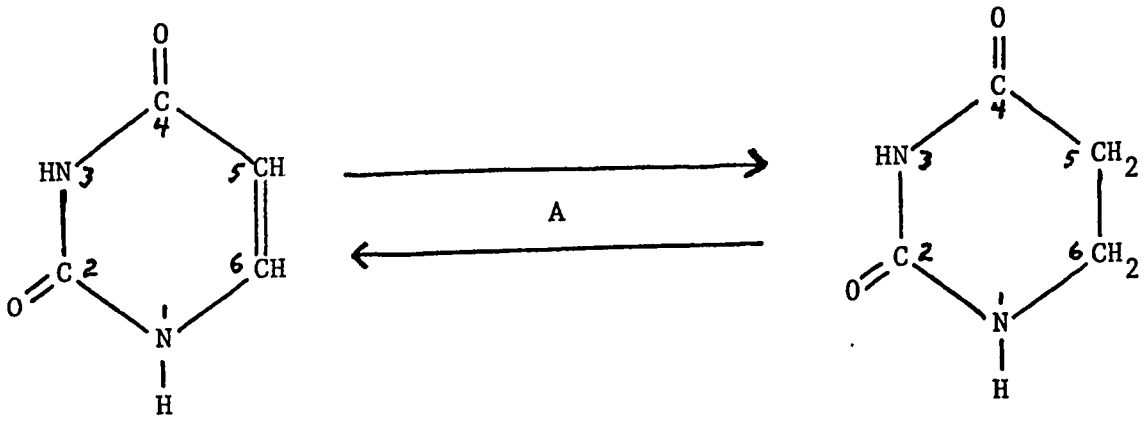
B. Metabolism of Uracil in Biological Tissue

1. Uracil Metabolism in the Animal System

Considerable research has been carried out on uracil metabolism in non-plant systems over the past fifteen years. Much of this work was done by European researchers working with rat liver.

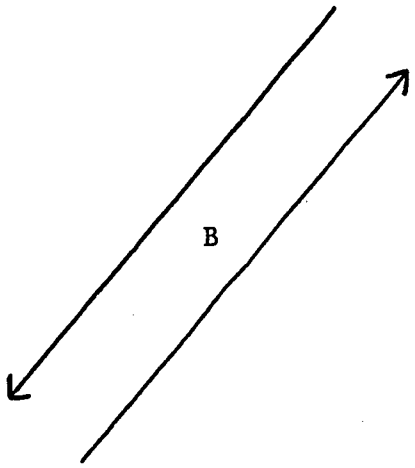
Fritzson (1957) reported the catabolism of uracil to β-alanine in rat liver slices with intermediates according to the scheme outlined in Figure 1. The β-ureidopropionic acid to β-alanine step was found to be the only irreversible step in the overall process. Grisolia and Wallach (1955) studied an enzyme dihydrouracil hydrase (EC 3.5.2.2), from a beef liver preparation that catalyzed the reversible conversions between dihydrouracil and β-ureidopropionic acid. The pH optimum for ring cleavage was 10.0. Ring formation was a maximum at pH 5.0 and was negligible at pH 6.5. Canellakis (1956) worked with a powdered preparation from rat liver that degraded uracil-2-^{14}C to β-alanine and $^{14}CO_2$. He detected intermediates of dihydrouracil and β-ureidopropionic acid

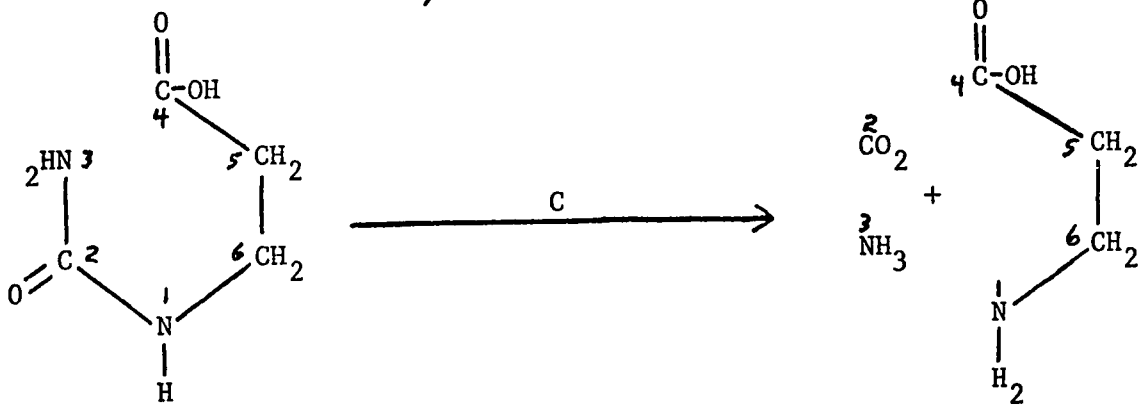
Figure 1. The uracil to β-alanine pathway. The following enzymes are involved in the pathway: "A", dihydrouracil dehydrogenase (4,5-dihydrouracil: NADP oxidoreductase, EC 1.3.1.2); "B", dihydrouracil hydrase (4,5-dihydropyrimidine amidohydrolase, EC 3.5.2.2); and "C", β-ureidopropionic acid decarbamylase (N-carbamoyl- β-alanine amidohydrolase, EC 3.5.1.6). Known cofactors for "A" are NADH (bacterial system) and NADPH (animal system). Salicylate acts as an activator for "A" while Mg^{++} and Mn^{++} are activators of "B" in the ring opening reaction.



Uracil

Dihydrouracil





β-ureidopropionic acid

β-alanine

and noted the requirement of NADPH for the reduction of uracil. After feeding intact rats with ^{14}C-labeled uracil Fritzson and Pihl (1957) recovered $^{14}CO_2$ and detected labeled dihydrouracil, β-ureidopropionic acid and β-alanine in the urine. These results gave ample proof for a novel catabolic system operating in animal tissue. Canellakis (1957) took the phenomenon one step further. He showed an inverse relationship between the capacity to degrade uracil and the capacity to incorporate uracil into RNA. He felt that there could be a homostatic mechanism which, in combination with other factors, regulates the rate of RNA synthesis. Thus during active RNA synthesis, uracil would be incorporated into RNA rather than being degraded.

As is common in enzyme research, detailed studies of the pathway began with partial enzyme purifications. Fritzson (1959) used an ammonium sulfate fractionation technique to purify dihydrouracil dehydrogenase (4,5-dihydrouracil:NADP oxidoreductase, EC 1.3.1.2) from the soluble cellular fraction. He concluded that this enzyme, requiring NADPH, was the rate limiting step in the overall uracil to β-alanine pathway. The relationship between the uracil catabolyzing enzymes and the rate of rat liver regeneration after partial hepatectomy was studied by Fritzson and Spaeren (1962). They found a parallel relationship between dihydrouracil dehydrogenase and the growth rate. β-ureidopropionic acid decarbamylase also showed a similar relationship to the growth rate but dihydrouracil hydrase showed no similarities.

Peret et al. (1964) showed that the yeast Torulopsis utilis grown on uracil was adaptable to the utilization of dihydrouracil and β-ureidopropionic acid. This further suggested the widespread nature of

the uracil catabolic pathway.

Fritzson (1964) showed that the liver growth rate and/or increased nucleic acid synthesis was associated with a decrease in the uracil degrading enzymes. A further correlation between nucleic acids and the uracil catabolic system was documented by Fritzson (1967). He pointed out that the activity of 5'-nucleotidase (a dephosphorylating enzyme of pyrimidine nucleotides) followed a similar pattern to the activity of dihydrouracil dehydrogenase and dihydrouracil hydrase. Hardonk and Koudstaal (1968) pointed out the significance of 5'-nucleotidase in the conversion of uridine mononucleotide to uracil as well as cytosine mononucleotide to cytosine. Thus if RNA is degraded to nucleotides, it is conceivable from the above studies that the 5'-nucleotidase is capable of generating a pool of uracil or direct precursors of uracil.

2. Inhibitors in Uracil Metabolism

Considerable research has also been conducted into the inhibitors of the uracil catabolic system. Sebesta (1961) reported the thymine analogues, 5-bromouracil and 5-nitrouracil, as acting as competitive inhibitors of uracil degradation. Newmark et al. (1962) worked with halogenated uracils to estimate their capacity to act as inhibitors of the system. One of these was 5-fluorouracil which was reduced by, and was a very effective inhibitor of dihydrouracil dehydrogenase. The inhibitory capacity followed the F>Cl>Br>I halogen series. The compounds 5-hydroxyuracil and 5-alkyl-uracil were not reduced but were effective inhibitors of uracil degradation.

Using a rat liver powder preparation Pithova and Sorm (1963) showed that uracil degradation was inhibited by compounds possessing a -NH-CO-NH- grouping in a 6-membered ring. Their inhibitory ability disappeared upon methylation of the nitrogen atoms in this grouping. Xanthine, uric acid, orotic acid, 2-pyridone, and 5-bromo-6-aza-uracil all inhibited the first step of uracil degradation.

The inhibition by 5-substituted uracil derivatives is competitive. The effect of the carcinogen, 2-acetylamino-fluorine, on the uracil degrading enzyme in rat liver was studied by Fritzson and Efskind (1965). The carcinogen caused a decrease in all enzyme activities of the uracil catabolic system. The removal of the carcinogen from the incubation medium was followed by a recovery of the enzyme activities. Dorsett et al. (1969) reported the in vitro effects of 5-cyanouracil on dihydrouracil dehydrogenase from rat and hamster livers. In rat liver, 5-cyanouracil blocked uracil reduction. In hamster livers, the chemical did not effect the rate of the reaction with uracil. They conclude that the possibility exists for separate enzymes for the respective reductions.

To date work has concentrated on inhibitors of the rate limiting enzyme in the uracil to β-alanine pathway. Major research efforts were focused on medical and pharmacological applications.

3. Uracil Metabolism in Plant Systems

The state of knowledge of the plant uracil catabolic system is not nearly as advanced as for the animal systems. Much of the early work involved incubations with radioactive metabolites, followed by their

extraction and separation. Demonstration of the existence of a pathway depended upon the detection of metabolites of the proposed pathway.

Evans and Axelrod (1961) reported the metabolism of uracil-2-^{14}C to $^{14}CO_2$, β-ureidopropionic acid, and dihydrouracil in germinating rape seeds. Longer germination times gave higher levels of β-ureidopropionic acid. They estimated that only about 1 per cent of the radioactivity from uracil-2-^{14}C appeared in nucleic acids during a 24 h incubation of 3 day old rape seedlings. Barnes and Naylor (1962), working on pine tissue, reported the formation of β-alanine from uracil and orotic acid as well as detecting intermediates of their metabolism. Tsai and Axelrod (1965) were able to show β-alanine formation from uracil in rape seedlings. These reports confirmed the operation of a similar pathway in plants to that proposed for animals by Canellakis (1956) and Fink et al. (1956).

Efforts were made to determine the relative metabolism of uracil into RNA or degradation to other metabolites. Buchowicz and Reifer (1964) studied the anabolism of uracil-2-^{14}C in winter wheat seedlings. Three g fresh weight of excised 5 day old wheat seedlings were fed with 1.0 ml of solution containing the label and varying amounts of β-alanine for 4 h at 30° under direct sunlight. They found that β-alanine (100 μmoles) was able to cause a 22 per cent inhibition of the uracil-2-^{14}C incorporation into polynucleotides. β-alanine also caused an 80 per cent inhibition of uracil conversion to uridine mononucleotides. They proposed that the role of the degradative enzymes is in the regulation of the production of β-alanine rather than in the exhaustion of the available uracil. This is in contrast to the idea

held in the animal field where workers assume that the low intensity of pyrimidine anabolism *in vivo* results from high activity of degradative enzymes which rapidly exhaust any substrates introduced from outside. The metabolism of uracil-2-^{14}C and uracil-6-^{14}C in cucumber seedlings was investigated by Bauerova et al. (1964a). With uracil-2-^{14}C, they found a peak in $^{14}CO_2$ evolution after 1 hour. $^{14}CO_2$ is produced from C2 during the β-ureidopropionic acid to β-alanine conversion. With uracil-6-^{14}C, a maximum $^{14}CO_2$ peak was noted 3 hours after commencing the incubation. $^{14}CO_2$ from U-6-^{14}C arises from metabolism of β-alanine-^{14}C. They suggest a transamination reaction (as later shown by Stinson and Spencer, 1969) with β-alanine, giving rise to malonic semi-aldehyde and further degradation of the two carbon unit formed from it by decarboxylation. Tsai and Axelrod (1965) illustrated that radio-activity from uracil-5, 6-^{3}H appeared in malate, lipids and organic acids in rape seedlings. Label from β-alanine-^{14}C was present in fatty acids and steroids; β-alanine-2-^{14}C was a better precursor for labeling than β-alanine-1-^{14}C. This was suggestive that radioactivity entered these compounds as acetate. Bauerova et al. (1964a) also studied known inhibitors of dihydrouracil dehydrogenase. Using 5-bromouracil as an inhibitor, they noted that the radioactivity in the acid soluble and RNA fraction rose sharply after a uracil-2-^{14}C incubation. In an identical experiment with uracil-6-^{14}C no increase in activity was observed. When they used uracil-2-^{14}C, $^{14}CO_2$ was reused and increased incorpora-tions in the acid soluble and RNA fractions were attributed to the sugar moiety of the nucleotides rather than the original uracil-2-^{14}C incorporation (Bauerova et al.,1964b). Older cucumber seedlings de-graded uracil more rapidly than young tissue while uracil incorporation

into RNA was most prominent in the young tissue. They concluded that uracil is used as an RNA precursor only when it is at a "significantly high" concentration in the tissue and this concentration is determined by the activity of degradative enzymes.

Robern et al. (1965) estimated the free nucleotides present in wheat embryos incubated with $^{32}P_i$. They found up to 30 per cent of the label present in purine and pyrimidines thus indicating a pool of free nucleotides in wheat embryos, probably resulting from degradation of nucleic acids.

Brown (1963) was unable to detect uracil or other free bases by anion exchange chromatography of pea seed extracts. Grzelczak and Buchowicz (1967) estimated levels of uracil and uridine in wheat plants. For both uracil and uridine, levels in the whole seed were lower than in the green parts either on a per g fresh weight or a per plant basis. Considering 6,8,10, and 14 day old green parts of the wheat plant both uracil and uridine levels were high on 6,8, and 14 days and low on day 10. They did not propose any explanation for the altering levels.

4. Enzyme Purifications for the Plant System

Mazus and Buchowicz (1967) were able to separate the dihydroorotase activity from the dihydropyrimidinase activities from a crude preparation from pea plants, by use of differential heat-tolerance properties of the respective enzymes. Mazus and Buchowicz (1968) isolated and characterized dihydropyrimidinase from pea plants. The enzyme catalyzed the ring opening of both dihydrouracil and dihydrothymine, had no cofactor requirements and was stable at a pH

9 to 10. This is in contrast to the animal preparation that showed a cofactor requirement of either Mg^{++} or Mn^{++}.

Up to the present time this has been the only enzyme of the uracil to β-alanine pathway isolated and purified from a plant source.

C. Orotic Acid Synthesis and General Metabolism

The synthesis of orotic acid in animal systems from the precursors carbamyl phosphate and aspartic acid has been adequately reviewed by Reichard (1959), Crosbie (1960), and Mahler and Cordes (1966). Orotic acid is synthesized and is the precursor for both the uracil and cytosine nucleotides. Once formed orotic acid is then enzymically attached to ribose-5'-phosphate forming orotidine-5'-phosphate. The final step in uridylic acid (UMP) formation is the irreversible decarboxylation of orotidine-5'-phosphate. Cytidine nucleotides are formed by the amination of UTP. Thus orotic acid plays an important role in pyrimidine biosynthesis and hence is a very good precursor for labeling RNA.

Considerable work has been directed towards the purification and characterization of the various enzymes in the biosynthesis of orotic acid and its subsequent metabolism to UMP. Some emphasis has also been given to the study of various inhibitors of the enzymes in the sequence. A detailed description of the enzymes involved and the inhibitors studied is not central to the study undertaken in this thesis and will not be included in this review.

The reactions for the formation of orotic acid from carbamyl

phosphate and aspartic acid are reversible and thus excess orotic acid could be metabolized by reversing its synthesis. The reversal probably is not necessary. It is well known that aspartate transcarbamylase is a rate controlling enzyme in pyrimidine biosynthesis, exhibiting feedback inhibition by CTP, UTP, and/or UMP depending upon the tissue studied. Once UMP is formed, the pathway cannot be reversed. If UMP is not incorporated into RNA, it can be converted to the free base uracil and undergo the metabolism outlined in other sections of this chapter.

1. Metabolism of Orotic Acid to Uracil

As mentioned above, uracil could be formed by the loss of a pentose phosphate grouping from UMP. Enzymes for these transformations have not been extensively documented. An enzyme is known in microorganisms that will reversibly catalyze the formation of UMP from uracil and 5'-phosphoribosyl-1-pyrophosphate. Thus excess UMP could be converted to a uracil via this type of reaction.

A novel reaction for uracil formation from orotic acid in E. coli was illustrated by Kulhanek et al. (1965). This reaction involved a direct decarboxylation of orotic acid to uracil thus by-passing the intermediates orotidine-5'-phosphate and uridine-5'-phosphate.

2. Orotic Acid Biosynthesis in Plant Tissue

The biosynthesis of orotic acid in plant tissues has been studied in some depth in the last decade. Generally similar enzymes are involved in the biosynthesis of orotic acid in plant systems as

are active in the animal system. However, some variations have been observed. Rybicka et al. (1967) showed that the carbamyl moiety of citrulline was incorporated into newly synthesized pyrimidine bases of wheat seedlings more rapidly than carbamyl phosphate, although the latter was incorporated. This is in contrast to the animal system where carbamyl phosphate is the primary precursor.

3. Orotic Acid Metabolism in Plant Systems

Buchowicz and Reifer (1961) were able to isolate UMP, CMP, uridine and uracil from orotic acid feedings of wheat seedlings and young wheat leaves. The presence of uridine was unique in that uridine is not detected in orotic acid metabolism in the animal system. They postulated from their product formations that orotic acid was metabolized to uracil, by-passing the UMP formation step. Thus uracil and uridine were intermediates in UMP formation. Therefore uracil formation was by a direct decarboxylation of orotic acid similar to that proposed for the *E. coli* system by Kulhanek et al. (1965).

In contrast to the findings of Rybicka et al. (1967), Kapoor and Waygood (1965) isolated and purified orotidine-5'-phosphate pyrophosphorylase from wheat leaves. This enzyme forms OMP from orotic acid and 5'-phosphoribosyl-1-pyrophosphate. Kapoor and Waygood found no evidence of an orotic acid decarboxylase. Wolcott and Ross (1967) studied the enzyme orotidine-5'-phosphate decarboxylase in bean leaves and concluded that this enzyme was involved in the normal pathway from orotic acid to UMP via OMP formation and decarboxylation.

Barnes and Naylor (1962) fed orotic acid-6-^{14}C to pine tissues and were able to isolate radioactive uridine, uracil, dihydrouracil, β-ureidopropionic acid, and β-alanine. This indicated the presence of systems capable of metabolizing UMP to uridine and uracil, as well as the normal system involving the phosphorylation of UMP to UTP and its incorporation into RNA. The detection of label in dihydrouracil, β-ureidopropionic acid, and β-alanine indicated enzyme systems present capable of metabolizing uracil via a pathway proposed by Canellakis (1956) and Fink et al. (1956).

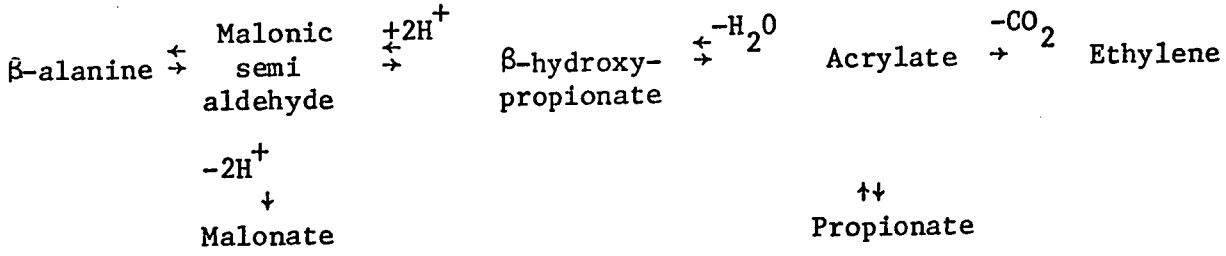
D. β-alanine as a Precursor in Ethylene Evolution

Considerable research has been directed towards the biogenesis and the effects of ethylene in plant tissue. That ethylene is a natural hormone is now generally accepted. Knowledge of the biogenesis of ethylene is incomplete. Many precursors of ethylene have been proposed for a wide variety of tissues. It is highly probable that more than one pathway is operative even in the same tissue. Recent theses have reviewed the early work in the ethylene biogenesis area (Meheriuk, 1965; Thompson, 1966; and Stinson, 1968).

Most of the studies involving β-alanine as a precursor have been on subcellular preparations. When whole tissues are used the additional problem of precursor uptake arises. The actual β-alanine levels *in vivo* in plant systems are unknown. No evidence is available on changes in β-alanine levels at various physiological ages of plant tissue. β-alanine, as a constituent of coenzyme A, plays a very important role in plant metabolism. Other roles for β-alanine are certainly possible.

1. The β-alanine to Ethylene Pathway

Studies using a subcellular enzyme system for bean cotyledons led Thompson and Spencer (1966) to propose the following pathway for the conversion of β-alanine to ethylene.



With a crude powder, β-alanine stimulated ethylene production by 35 per cent. In the presence of appropriate cofactors for the proposed biochemical conversions, further increases in ethylene production were noted. By adding malonate, Thompson (1966) noted a further 83 per cent stimulation in ethylene production. That malonate was not a precursor of ethylene was shown by Stinson (1968) with a solubilized enzyme preparation from a subcellular fraction from bean cotyledons. He failed to detect any ethylene-^{14}C with malonate-2-^{14}C in the incubation medium and also noted no radioactivity in malonic semialdehyde. He concluded that malonate was not an ethylene precursor and its function in promoting ethylene biosynthesis was one of preventing the loss of carbons from the proposed pathway (Thompson and Spencer, 1966). The absolute requirement for adenosine triphosphate for the conversion of β-alanine to ethylene was also noted.

With these preparations radioactive ethylene was obtained enzymically from β-alanine -2-^{14}C. A dilution factor of 0.57 indicated that the conversion was fairly direct. Radioactivity was also detected

in the proposed intermediates. Ku et al. (1967) suggested that the low conversion of β-alanine to ethylene (0.001 per cent) obtained by Thompson and Spencer (1967) was evidence that β-alanine was not an immediate precursor. Burg and Clagett (1967) failed to obtain a stimulation of ethylene production when β-alanine was added to apple and banana slices. They also concluded from these results that β-alanine was not a precursor in these tissues, but no details were given as to the concentration used or other conditions of the experiment. β-alanine was a very effective precursor of ethylene in Penicillium digitatum as shown by Wang et al. (1964) and Jacobsen and Wang (1968).

2. Enzymes of the β-alanine Pathway

The known enzymes for the conversion of β-alanine to acrylate for animal and microbial systems have been described in a recent thesis (Stinson, 1968). There is no evidence in any tissue for an enzyme that decarboxylates acrylic acid to ethylene. Jacobsen and Wang (1965, 1968) showed a rapid labeling of ethylene when Penicillium digitatum was incubated with acrylate -^{14}C. Very little evidence is available for these enzyme systems in plant tissue.

The first enzyme in the pathway as proposed by Thompson and Spencer (1966), β-alanine aminotransferase, has been isolated from bean cotyledons and characterized by Stinson and Spencer (1969). The enzyme utilized oxaloacetate and pyruvate as amino acceptors but not α- ketoglutarate. The enzyme also required fairly high β-alanine levels for the reaction to proceed. Investigations to date with animal systems have used α-ketoglutarate as an amino acceptor (brain and liver, Roberts and Bregoff, 1953; pig kidney, Kupiecki and Coon, 1957).

3. Formation of β-alanine in Biological Systems

Various metabolites have been linked with the formation of β-alanine. Although the formation of β-alanine is known, factors controlling its formation, and endogenous concentrations, have still to be illustrated. A review of possible precursors follows:

1. Aspartic acid. Virtanen et al. (1938) showed that a Rhizobium leguminosarum culture was able to decarboxylate aspartic acid and form β-alanine and CO_2. Rogers (1955), using a squash preparation, was able to show the decarboxylation of aspartic acid, but only identified CO_2 as a product. Naylor et al. (1958) studied the utilization of aspartic acid-(U)-^{14}C in root, stem, and leaf tissues of 12 species of seed plants. They were unable to detect any evidence for β-alanine formation from aspartic acid over a 3 to 6 hour period in young rapidly growing tissue.

2. Propionate. Stadtman (1956) studied the oxidative metabolism of propionate-1-^{14}C in cell free extracts of Clostridium propionicum and noted β-alanine-^{14}C formation. Ammonia and catalytic amounts of acetyl-phosphate were required for the proposed reactions.

3. Uracil. Di Carlo et al. (1952) suggested a reductive pathway for β-alanine formation from uracil was operating in yeast. This idea was later supported by work with slices and cell free preparations from rat liver (Canellakis, 1956; and Fink et al., 1956). They had evidence for a pathway from uracil to β-alanine with intermediates of dihydrouracil and β-ureidopropionic acid.

Barnes and Naylor (1962) used pine tissue and noted β-alanine formation from both orotic acid and uracil. They were able to also detect the intermediates proposed by Canellakis (1956) and Fink et al. (1956).

4. Roles of β-alanine in Plant Metabolism

The role of coenzyme A in respiration and fatty acid metabolism is common knowledge. One molecule of β-alanine is present in each pantothenate molecule. Pantothenic acid is a constituent part of the coenzyme A molecule. Sauer et al. (1964) characterized the heat-stable acyl carrier protein (ACP) of fatty acid biosynthesis. All acyl carrier preparations contained one residue each of 2-mercaptoethylamine and β-alanine. They also were able to isolate a radioactive peptide from a peptic digest of enzymically prepared acetoacetyl-^{14}C-ACP. This peptide contained acetoacetyl-^{14}C, 9 amino acid residues and stoichiometric amounts of 2-mercaptoethylamine and β-alanine. The exact linkages have not been illucidated but certainly β-alanine plays an important role in fatty acid biosynthesis.

Rubinstein and Leopold (1962) showed that the application of 5 mM β-alanine in the agar support medium for bean leaf explants was able to shorten the abscission time of the explants from 100 h to 34 h. Rubinstein and Abeles (1965) showed that amino acids that were able to stimulate ethylene production would also stimulate abscission. Both L-alanine and L-glutamate had stimulatory roles while L-leucine and L-valine had no effect on the two processes. Chatterjee and Leopold (1965) studied the effect of β-alanine on the abscission process in bean leaf explants. Leaf abscission was divided into two

separate processes, the first of which was found to be inhibited by auxin, with young leaves showing the highest inhibition. The second stage of leaf abscission was promoted by β-alanine with older leaves showing more response to β-alanine than did young leaves. β-alanine had no effect on the first stage and auxin promoted the second stage of leaf abscission. This second stage usually brings about separation and leaf fall. The promotion by β-alanine of leaf abscission is similar to abscission acceleration by indole acetic acid (Andreae et al., 1968), 2,4-dichlorophenoxyacetic acid (Holm and Abeles, 1968), 2,4,5-trichlorophenoxyacetic acid (Maxie and Crane, 1967), and other regulators stimulating leaf abscission. It is interesting to note that regulators accelerating leaf abscission also stimulated ethylene production. It is entirely possible that the effect of β-alanine on leaf abscission may be mediated by ethylene produced from β-alanine.

E. The Biogenesis of Ethylene

The physiology of ethylene as well as its biogenesis has been the subject of recent reviews (Burg, 1962; Jansen, 1965; Phan, 1969; Pratt and Goeschl, 1969; and Spencer, 1969). Ethylene has been shown to be produced from a wide variety of compounds both enzymically and non-enzymically. Some of the compounds recently linked with ethylene biogenesis are: α-keto-γ-methylmercaptopropionic aldehyde, and methylthiobutyrate, Ku et al. (1969); linolenic acid, Abeles (1966); β-hydroxyethylhydrazine, Palmer et al. (1967); pyruvate, Shimokawa and Kasai(1967a); propanal, Lieberman and Kunishi(1967);ethionine, Shimokawa and Kasai (1967b);methionine, Lieberman et al. (1966) and Burg and Clagett (1967); Methional, Mapson et al. (1969); dicarboxylic acids, Jacobsen and Wang

(1968); acetate, Ketring et al. (1968); ethanol, Phan (1962); and β-alanine, Thompson and Spencer (1967) and Stinson (1968). The above list of compounds producing ethylene was tabulated from a wide variety of tissues including fruit, vegetables and microorganisms.

1. Biological Production, and Detection, of Ethylene

Most ethylene biogenesis work has been attempted using radioactive precursors. A major criticism of these studies is the very low conversions to ethylene, generally about 0.002 per cent. Although this is low in terms of accepted criteria for enzyme reactions, one must recall that for physiological responses to ethylene only low levels are required. In pea seedlings, the gas in a concentration as low as 0.025 ppm will cause a three-fold effect-leaf epinasty, stem swelling and inhibition of extension growth (Denny and Miller, 1935). The 0.025 ppm is the concentration in the atmosphere surrounding the tissue. It is very difficult to measure the in situ ethylene concentration because of technical limitations. The internal ethylene concentration of a plant, organ to organ and even cell organelle to organelle, may be quite different from that measured in the surrounding atmosphere.

2. Physiological Effects of Ethylene

The physiological effects of ethylene have been the subject in part or in total of recent reviews (Pratt and Goeschl, 1969 and Spencer, 1969). Some of the plant responses to ethylene will be mentioned here for illustrative purposes with no effort being made to include a comprehensive review.

Physiological responses to minute quantities of ethylene are the

bases of a sensitive bioassay of the gas (Denny and Miller, 1935). The formation of isocoumarin in carrot root tissue is induced by ethylene (Chalutz et al., 1969). Valdovinos et al. (1967) pointed out that ethylene is able to decrease levels of diffusible auxin in pea seedling tissue by affecting auxin synthesis. The abscission of primary leaves of Phaseolus is mediated by ethylene (Curtis, 1968). Rhizome and shoot growth in low bush cranberry is stimulated by ethylene (Kender et al., 1969). Holm and Abeles (1968) showed that the application of 1 ppm ethylene for 24 h inhibited the hypocotyl growth of etiolated soybeans and caused an increase in the RNA, DNA, and protein content of the hypocotyls. Ketring and Morgan (1969) illustrated ethylene's effect in the breaking of dormancy in peanut seeds. Catchpole and Hillman (1969) showed that ethylene was able to cause tuber swelling in potato stolons. Their results show that ethylene may play a role in the early stages of tuber initiation.

3. Regulators of Ethylene Metabolism

A variety of chemicals have been shown to have physiological effects on plants. Many of these effects are traced back to either a stimulated ethylene production by the chemical or a direct production of the gas from the applied chemical. Either way the physiological effect is mediated by ethylene. Some compounds exhibiting these characteristics are: 2-chloroethanephosphonic acid and Ethrel (about 90% 2-chloroethanephosphonic acid and 10% of its anhydride and ester), Morgan (1969), Kender et al. (1969), and Russo et al. (1968); malformin B, Curtis (1968); 2,4-dichlorophenoxyacetic acid, Maxie and Crane (1967); picloram, Baur and Morgan (1969); indole acetic acid, Burg and

Burg (1967); and kinetin, Fuchs and Lieberman (1968).

4. Effects of CO_2 on Ethylene Metabolism

Since 1942, carbon dioxide has been known to be an inhibitor of ethylene production (Hansen, 1942). Abeles and Gahagan III (1968) and Burg and Burg (1967) presented evidence for the role of CO_2 both as a competitive inhibitor of ethylene action and an agent that limits ethylene production. Carbon dioxide's role in inhibition of ethylene production has commercial application in controlled atmosphere storage of fruit. Carbon dioxide also has other effects on fruit storage.

5. Ethylene and Abscission

Many of the above chemical stimulators of ethylene production lead to the abscission of leaves. Recent work has revealed some of the possible underlying actions of these chemicals. Horton and Osborne (1967) and Abeles (1969) described experiments that showed the cellulase activity in abscission zones of Phaseolus was enhanced by ethylene treatments. Abeles (1967) illustrated that an application of as little as 10^{-1} mμl ethylene per ml of air was able to accelerate leaf abscission in cotton and coleus explants. This abscission process stimulated by ethylene was accompanied by an enhanced incorporation of $^{32}P_i$ into r-RNA and m-RNA fraction in the abscission zone of bean explants (Holm and Abeles, 1967). The 6 hour control explants gave a similar labeling pattern to 3 hour ethylene-treated explants.

6. Mechanism of Action

The effects of ethylene on plant systems are marked and variable. The mode of action is not readily understood. Some possible mechanisms of action are reviewed by Spencer (1969). These include alteration of substrate and cofactor concentrations as well as the presence of competitive and non-competitive inhibitors or activators, feedback regulators, and repressors or inducers of enzyme activity.

7. Ethylene Production from Plant Systems

Extensive literature is available on ethylene production by a large number of microorganisms and animal tissue. All plants thus far examined also produce ethylene to varying extents. This includes intact plants, Morgan and Hall (1964); detached leaves, Pratt (1954) and Hall et al. (1957); cotyledons, Thompson and Spencer (1967); flowers, Fischer (1950) and Phan (1965); roots and tubers, Burg (1962) and Goeschl et al. (1967); and germinating seeds and seedlings, Meheriuk and Spencer (1964), Spencer and Olson (1965), and Goeschl et al. (1967).

Ethylene production is associated with the climacteric rise in the ripening of many fruits. In some fruit ethylene production has been reported to follow the climacteric rise in respiration (tomatoes, Spencer (1956); tomatoes, Workman and Pratt (1957). In others it precedes (bananas, Mapson and Robinson, (1966) or coincides exactly with the respiratory peak of the climacteric rise (Hansen, 1942). There appears to be a dependence of ethylene biosynthesis on energy derived from respiration (Burg and Thimann, 1961).

Forsyth and Hall (1967), working with developing cranberry fruit, showed ethylene levels to increase to a peak at fruit maturation and then decrease. Galliard et al. (1968), with apple peel disks, indicated the requirement of an ageing period for the development of the ethylene producing system. Thus the state of the tissue is a very important factor for the ethylene producing system.

Very little work has been reported on ethylene production from attached or detached ageing leaves. Pratt (1954) showed that detached leaves from milk thistle produced ethylene. He estimated a production of 10.8 ml of ethylene from 13.5 kg of thistle leaves in 4 days. His determination was made in a static collection system over a long period of time. He noted no visible bacterial contamination over the collection period. Hall et al. (1957) studied ethylene production from cotton plant leaves and noted no detectable ethylene during the vegetative stage. Once the reproductive stage was reached, he estimated the ethylene production at about 1.96 ml per kg fresh weight over about 100 hours. A moving air stream was used in his collection system. Detached mature healthy leaves produced about 0.13 ml ethylene per kg fresh weight per 100 hour collection period. Senescent leaves did not produce measureable quantities of ethylene. A recent report by Rogers (1969) showed that Valencia orange leaves produced more ethylene than did either mature or green rind tissue. He also noted that leaves produced more ethylene in the light than in the dark.

CHAPTER II

MATERIALS AND METHODS

A. Sources of Chemical Materials

Chemicals were generally of the highest purity available and from the following sources: β-alanine-2-^{14}C, uracil-2-^{14}C, uracil-6-^{3}H, and carrier-free $H_3{}^{32}PO_4$ from the New England Nuclear Corporation; uracil and orotic acid -6-^{14}C from Calbiochem; ethylene-^{14}C (U, > 98%) from the Nuclear Chicago Corporation; PPO (2, 5-diphenyloxazole) and POPOP (p-bis2-(5-phenyloxazoyl)-benzene) from either the Nuclear Chicago Corporation or Fraser Medical Supplies, Vancouver; uridine, dihydrouracil, NADPH, alcohol dehydrogenase, human serum albumin (Grade III), and β-ureidopropionic acid from the Sigma Chemical Company; ammonium sulphate (special enzyme grade) from Mann Research Laboratories; ninhydrin from Pierce Chemical Company, Rockford, Illinois; β-alanine and acetaldehyde from Eastman Organic Chemicals; albumin as Bovine Albumin Fraction V Protein from Nutritional Biochemicals Corporation. All other chemicals were obtained as reagent grade from Fisher Scientific Co. Ltd. The following materials were obtained from various suppliers - high purity methane, the 98.7% helium, 1.3% butane mixture, and the CO_2 standards from Matheson of Canada Ltd., Whitby, Ontario; ethylene (USP 99%) from Ohio Chemical and Manufacturing Company; Sephadex G-25 from Pharmacia; Cab-O-Sil (#M-5) from Cabot Corporation, Boston; Beem capsules from Ladd Research Industries Inc., Burlington, Vermont; Amberlite IR-120 from Mallinckrodt Chemical Works, Montreal and MN-cellulose powder 300 from Canadian Laboratory Supplies Incorporated.

All water used was double distilled deionized and contained <2 ppm ionizable impurities.

B. Growth of, and Feeding Conditions for, Bean Leaves

1. Growth Conditions

Seeds of Phaseolus vulgaris L var Kinghorn wax were grown in sterilized wooden flats in a sterile soil-peat-sand (3:2:1) mixture. The flats were grown under two different sets of conditions.

Growth Conditions #1. Plant material for early experiments was grown in a growth room used extensively by other University Departments. Flats were placed in the growth room at 65°F with a 16 h day and a light intensity of 1200 ft-c. The light intensity was variable depending upon where the bench space was available. No humidity control was present. The growth conditions were not optimum for bean growth but were the closest available at the time to the desired conditions.

Growth Conditions #2. Once a growth cabinet became available, it was used exclusively for the growth of bean plants. This allowed for a more favourable temperature as well as a constant humidity (50%). Flats were placed in the cabinet adjusted to 77°F with a 14 h day and a light intensity of 1800 ft-c. Temperature fluctuations from 74°F to 78°F were observed. These were attributed to slight alterations in the temperature control when the temperature chart paper was changed. These fluctuations resulted in differences in the growth rate of the

bean plants. The ethylene production system gave a good indication of the growth rate. Plants grown at the lower temperatures had an ethylene evolution peak at about day 8 or 9 days after planting while the 77°F growth rate resulted in an ethylene production peak at 7 days after planting (Chapter III). The ethylene production from the leaves was periodically determined to accurately determine the leaf age relative to the ethylene production peak.

2. Conditions for Handling Tissue

Plants were cut in the growth chamber at soil level and transported to the laboratory in a plastic bag. Petioles were then cut under water and leaves were placed in a 2500 ml culture flask with petioles immersed in a beaker of water. In feeding experiments petioles were placed in Beem capsules containing the labeled material. The culture flask was covered with aluminum foil to negate any effects of laboratory light. Ethylene collections commenced within 10 min of stem cutting in the growth chamber.

C. $^{32}P_i$ Incorporation, Isolation, and Purification of RNA

1. $^{32}P_i$ Feeding of Bean Leaves

Leaves grown under growth conditions #1 were harvested at various ages and fed with carrier-free $H_3{}^{32}PO_4$ by placing the petioles in Beem capsules containing the label. Feeding time was 4 h in the fume hood in diffuse light (less than 150 ft-c). The initial label was

followed with small feedings of water. RNA was then extracted as outlined in Section C.2. and characterized by MAK column chromatography (Section C.3.).

2. Isolation and Purification of RNA

The phenol - detergent method of Cherry (1964) was adopted, with minor modifications in detergent concentration and bentonite purification (Knight, 1966). The ethanol precipitated RNA was collected by centrifugation at 20,000 x g for 30 min at 0°. The precipitate was dissolved in 0.3 M NaCl, in 0.05 M phosphate buffer at pH 6.7, and placed in 1 inch dialysis tubing. The solution was dialyzed for at least 2 days against twice daily changed 0.3 M NaCl, 0.05 M phosphate buffer at pH 6.7. The dialyzed RNA was applied to the MAK column.

3. Methylated Albumin Kieselguhr (MAK) Column

The methylated albumin was prepared as outlined by Hayaski et al. (1963). The column dimensions and packing were identical to those described by Mandell and Hershey (1960). A linear gradient of NaCl (0.3 M to 1.4 M) was pumped through the column at a flow rate of 50 ml/h. Five ml fractions were collected in an LKB fraction collector (Ultrarac type 7000).

4. RNA and RNA-^{32}P Estimations from the MAK Column Separations

Absorbance measurements were made on each fraction in a Beckman DU-2 spectrophotometer at 260 mμ. For an elution profile, absorbance at 260 mμ was plotted against fraction number.

^{32}P estimations were made by pipetting 0.5 ml from selected fractions into 10 ml of the dioxane liquid scintillation fluor (Section C.5.).

5. Liquid Scintillation Counting

All radioisotopes were counted in a 1,4- dioxane fluor (7.0 g PPO (2,5 - diphenyloxazole), 0.3 g POPOP (p-bis-2- (5-phenyl-oxazaloyl)-benzene), 100 g naphthalene, and 1 litre of 1,4-dioxane. Ten ml of fluor was added per vial. Radioactivity was determined with a Nuclear Chicago Unilux II liquid scintillation system, optimized for the respective isotopes according to the operations manual. Appropriate quench curves were constructed (Unilux II operations manual, 1967) and can be found in the Appendix (Figure A-1 and A-2 for ^{14}C and ^{3}H respectively).

D. Ethylene Production from Bean Leaves

1. Ethylene Collection System

The ethylene collection apparatus was essentially the same as used by Stinson (1968). Residual ethylene was removed from the compressed breathing air (Canadian Liquid Air Co.) by passing it through a U-tube (1.4 cm ID) containing silica gel (28-200 mesh) coated with mercuric perchlorate and maintained at ice-water temperatures. A manifold system was used to distribute the air to the collection apparatus. A flowmeter monitored the air (60 ml/min) to a 2500 ml culture flask in which the leaves were placed. The air then passed from the flask through two U-tubes containing drierite and lithasorb respectively and

then to a cold trap and collection tube maintained at dry ice-acetone temperatures. The collection tube (3.5 mm ID) contained 0.5 g silica gel (Davison, grade 15,35-60 mesh). The collection period was generally 0 to 2 h and 2 to 4 h. For some experiments a 0 to 4 h and a 4 to 8 h collection regime was used.

Immediately prior to collecting ethylene, the U-tube containing silica gel was continuously flushed with purified air and boiled in water for at least 15 min to rid it of residual adsorbed material. After the collection period, the U-tubes were sealed with rubber tubing and stored in dry ice-acetone until GC measurements were made.

2. Ethylene Analysis

The collection tube was maintained at dry ice-acetone temperature and connected to a two-way valve inserted into the helium line of a Perkin Elmer Model 811 flame ionization gas chromatograph. The valve was opened and the U-tube initially flushed with helium for 50 sec with one end of the U-tube open to the atmosphere and then 10 sec with the U-tube reconnected to the helium input line into the gas chromatograph. This procedure prevented the air in the U-tube from blowing out the flame of the gas chromatograph.

With the valve closed, the U-tube was heated to 40^{o}, thereby releasing the ethylene. The two way valve was then opened, the contents of the tube flushed onto a 50 cm x 6 mm ID column that contained activated alumina with 2-1/2% silicone 550. Column temperature was 20^{o} and detector temperature was 125^{o}.

A standard curve was obtained by injection into the gas chromatograph, with a gas tight syringe, known amounts of ethylene. Peak height vs. mμl ethylene was plotted (Appendix, Figure A-3).

E. Carbon Dioxide Production from Bean Leaves

1. Carbon Dioxide Estimation from Excised Leaves

A Beckman Infrared analyzer (Model 215A) and a 10 mv potentiometric recorder were used to monitor CO_2 production from excised leaves at various ages. The instrument was standardized with purified N_2 (Liquid Carbonic Corporation) as reference and 560 ppm CO_2 in N_2 as standard. Compressed breathing air (Canadian Liquid Air Company) contained no detectable CO_2 and was used as reference gas in the experiments. Compressed breathing air was also used to flush the 2500 ml culture flask containing leaves. Appropriate flowmeters and drierite-drying tubes were placed before the analyzer and after the culture flask.

Variation in flow rates in the range 0-800 cc/min had no effect on the sensitivity of the instrument. After placing the leaves to be analyzed (generally three excised leaves with their petioles in water) in the culture flask and closing it with a stopper with inlet and outlet glass tubing, the flow rate to sample chamber was reduced to about 20 cc/min for five min. This allowed for a rapid buildup of CO_2 in the 2500 ml chamber. The flow rate was then increased or decreased in small increments until a zero slope was obtained on the recorder. At this point it was assumed that the CO_2 output from the leaves was at a

constant concentration and rate with the air sweeping the flask. Calculations were based on flow rate measurements and CO_2 concentrations from a calibration curve (Beckman IR-215A Operations Manual, 1968).

2. Carbon Dioxide Estimation in the Growth Cabinet

The infrared analyzer, recorder and accompanying flowmeters and drierite drying tubes were arranged outside the growth cabinet. The infrared analyzer was standardized with 560 ppm CO_2 in N_2 against a N_2 flow in the reference.

An aquarium air pump was used to move the growth cabinet air (100 cc/min) through the drierite tube and flowmeters before entering the analyzer. Compressed breathing air was used as reference gas. The 10 mv potentiometric recorder gave a record of the CO_2 levels in the growth cabinet. The 10 mv scale allowed for CO_2 estimations from 0 to 600 ppm. If the pen response moved upscale above 600 ppm, the recorder was switched to the 100 mv scale, thus giving 600 ppm as a chart paper reading of 10 (100 mv scale) compared with 100 (10 mv scale). Readings above 10 on the 100 mv scale could not be estimated on the calibration curve supplied with the instrument.

F. Conversion of Uracil to β-alanine

1. Preparation of a Partially Purified Enzyme System

(a) Preparation of Supernatant Fraction

Leaf tissue was suspended in 0.10 M potassium phosphate buffer, pH 7.4, containing 1 mM glutathione, and 0.1 mM EDTA (20 ml homogenizing

buffer /10 g tissue). The suspension was homogenized in a Waring Blendor for 2 min at full speed. The homogenate was squeezed through 4 layers of cheesecloth. The filtrate was centrifuged at 6000 x g for 10 min. The supernatant layer was immediately centrifuged at 100,000 x g for 60 min. The supernatant fraction was used for enzyme assays. All steps were carried out at 0°.

To ascertain freezing lability, samples were frozen in a dry ice-acetone bath and stored at deep freeze temperatures.

(b) Ammonium Sulphate Precipitation

An $(NH_4)_2SO_4$ precipitation technique was used to partially purify the supernatant fraction. Saturated $(NH_4)_2SO_4$ solution at 0°, pH adjusted to neutrality, was added slowly to the stirring supernatant fraction at ice-water temperatures. Each fraction was allowed to stir for 30 min after completion of the addition of $(NH_4)_2SO_4$ solution and then centrifuged at 6000 x g for 10 min at 0°. The $(NH_4)_2SO_4$-precipitated material was dissolved in a small amount of 0.1 M potassium phosphate buffer, pH 7.4 and either assayed or desalted.

(c) Desalting and Freeze Drying of Preparations

The $(NH_4)_2SO_4$ pellet was dissolved in 1.5 ml potassium phosphate buffer, pH 7.4, and applied to the top of a Sephadex G-25 (2.7 cm ID x 32 cm) column previously equilibrated with buffer. Approximately 8 ml fractions were collected with a 100 ml/h flow rate of potassium phosphate buffer, pH 7.4. Most of the protein was eluted in one fraction and freeze-dried and stored at deep freeze temperatures

until required. Protein was determined according to the method of Lowry et al. (1951), Appendix, Figure A-4.

2. Studies with the Partially Purified System

(a) Enzyme assay with Uracil -6-^{3}H

(i) Incubation mixture

The enzyme prepared as described in Section F.1. above was assayed according to the following method. The standard assay at pH 7.4 contained uracil -6-^{3}H (8.9 mCi/µmole), 0.18 µmoles unlabeled uracil, 1.0 µmole NADPH, 0.2 moles potassium phosphate and approximately 20 mg protein in a final volume of 2 ml. Incubation was at 37° in a shaker bath, and the reaction was stopped by the addition of 0.2 ml of 40% ice cold trichloroacetic acid. Protein was removed by centrifugation at 1,000 x g for 10 min. The supernatant was used for spotting TLC plates.

(ii) TLC system

100 µl of the deproteinized reaction mixture was spotted as a band on a 0.5 mm cellulose-coated 2 inch glass TLC plate. Uracil and dihydrouracil were separated with a t-butanol-methylethyl ketone-water-ammonia (40:30:20:10 v/v/v/v) solvent system (Fink et al., 1956). β-alanine and β-ureidopropionic acid were inseparable in this system. Uracil, β-ureidopropionic acid and β-alanine were separated with a n-butanol-ethanol-water (4:1:5 v/v/v, top layer) solvent system Fritzson and Spaeren (1962). Dihydrouracil trailed uracil slightly.

This did not create any problem since dihydrouracil was not detected in the reaction mixtures. Uracil and β-alanine were characterized by use of authentic labeled compounds. The identity of β-ureidopropionic acid -^{3}H was determined by co-chromatography with unlabeled β-ureidopropionic acid. Dihydrouracil was located by the method of Fink et al. (1956). Uracil was located by its fluorescence by use of a 253.7 mμ ultraviolet lamp. Unlabeled β-alanine was located with a conventional ninhydrin spray.

(iii) Counting Technique

After the TLC plate had dried in a fume hood, 0.5 cm bands were scraped from the plate starting 0.5 cm below the origin and continuing up to the solvent front. The powder was collected by placing a filterpaper disc (cut with an office paper-punch) over the end of 3 mm ID rubber tubing connected to the vacuum line. The collected sample plus disc was released into a scintillation vial containing 0.5 g Cab-O-Sil by pinching the rubber tubing. Scintillation fluor was added, the contents were mixed and counting was determined as described in Section C.5.

(b) General Studies

Experiments were designed to study the effects of NADPH, NADH, Mg^{++}, Mn^{++}, known cofactors of the animal system on the activity of the enzyme preparation. The effects of glutathione and sodium salicylate were also studied.

(c) Assays with Alcohol Dehydrogenase

In experiments where alcohol dehydrogenase was added to the incubation mixture, 1 mg alcohol dehydrogenase and 0.36 µmole acetaldehyde were added to the reaction mixture. The mixture was incubated at 37° for 4 min before the label was added. The reaction was stopped by the addition of 0.2 ml of 40% trichloroacetic acid and protein was removed by centrifugation.

(d) Spectrophotometric Assay for Dihydrouracil Dehydrogenase (EC 1.3.1.2)

The enzyme dihydrouracil dehydrogenase requires reduced pyridine nucleotide as a cofactor. The reaction was studied by a measurement of the absorbance at 340 mµ, the absorption maximum for reduced NADPH. The reaction blanks contained enzyme, NADPH and buffer in a total of 1.0 ml. Reaction samples contained added uracil. Incubation temperature was 37°. A zero time scan was determined on a Perkin Elmer 202 recording spectrophotometer from 300 mµ to 400 mµ . Readings were taken again at 15 min and 30 min incubation times.

3. By Excised Leaves

(a) Label Feeding

Leaves were selected from plants grown under growth conditions #2. Uracil-6-^{3}H (8.9 mCi/µmole) was fed in a fume hood as described in Section C. Small amounts of water were added when label uptake was complete. Leaves were fed for 15, 40, and 120 min at one leaf age and for 15, 60 and 120 min at various leaf ages.

(b) Extraction of Metabolites

Leaves stored in dry ice for at least 2 h were powdered in a mortar and pestle with dry ice. The powder was allowed to warm to room temperature. The grinding and subsequent extractions were carried out in a fume hood. The extractions were made (35 ml/g fresh weight), first with boiling 80% ethanol for 3 min and then twice with boiling water for 5 min and 3 min respectively. After each extraction the mixture was centrifuged at 3000 x g for 5 min at 4°. The combined extract was flash evaporated to dryness. Three ml of water was added and the residue in the flask was loosened with a spatula. The contents of the flask were transferred to a 10 ml centrifuge tube and centrifuged at 26,400 x g for 10 min at 0°. The clear supernatant was used for spotting TLC plates.

(c) Extraction of RNA

The residue from the 80% ethanol extraction for metabolites was used. Ten ml of 1 M NaCl was added per gram of original tissue. The suspension was heated rapidly to 90°, stirred constantly and held at 90° for 7 min. The suspension was cooled rapidly in a dry ice-acetone bath to 0°, and centrifuged at 3000 x g for 15 min at 0°. Potassium hydroxide solution was added to the supernatant layer to give a final concentration of 1.0 M. The solution was left overnight at room temperature. The solution was cooled in ice, 0.25 ml of 0.05 $MgCl_2$ was added per 12 ml solution and precipitation was carried out with 2.0 ml of 12 N perchloric acid. The suspension was centrifuged at 3000 x g for 15 min at 0°. The supernatant contained the hydrolyzed nucleotides. Label

content was estimated by the liquid scintillation system.

(d) TLC and Label Estimation of Metabolite Extracts

Solvent systems and counting procedure were as described in Section F.2. above.

G. Feeding of Orotic Acid to Excised Bean Leaves

1. A Study of the Conversion to β-alanine and Uracil

(a) Label Feeding

Leaves were selected at various ages from plants grown under growth conditions #2. Orotic acid -6-^{14}C (22.6 μCi/μmole) was fed to detached leaves with their petioles placed in Beem capsules. Four leaves at each age were placed in a 2500 ml culture flask and attached to the ethylene collection system as described in Section D.1. Ten g drierite was added to the culture flask to hasten label uptake. Flow rates were the same as for ethylene collections. A silica gel collection tube was connected to adsorb ethylene produced during the feeding experiment (generally 4 h).

For the lithasorb U-tube was substituted a bubble tube containing 2.0 ml of 3.0% potassium hydroxide to trap any CO_2 produced. Counting of an aliquot of the potassium hydroxide gave a measurement of the $^{14}CO_2$ produced from the metabolism of orotic acid -6-^{14}C.

After the feeding experiment was completed, the leaves were frozen in dry ice.

(b) Extraction of Metabolites

The frozen leaves were extracted as outlined in Section F.3. above.

(c) Radioisotope Detection on TLC Plate

100 µl of the above extract was spotted as a band on a 0.5 mm cellulose-coated 2 inch glass TLC plate. The solvent system used was isopropanol-pyridine-water-acetic acid (8:8:4:1 v/v/v/v) (Gordon et al., 1962).

The developed TLC plate was scanned using a Nuclear Chicago Actigraph III, model 1002 equipped with a Thin-Layer-Plate Conveyer system, Model 1006. The apparatus was standardized with known amounts of β-alanine -2-^{14}C applied in a band on a 0.5 mm cellulose plate.

Optimum operating parameters for maximum sensitivity were: time constant 5 sec, collimator slit-width 3 mm, scan speed 30 cm/h, operating voltage 1050 volts and a quench gas (98.7% helium, 1.3% butane) pressure of 7 psi. Counting efficiency obtained was 7.1% with ^{14}C. The Actigraph scans were only used to detect radioactive areas. Accurate counts were determined by scraping the selected areas from the plates and counting them in the liquid scintillation system(Section C.3).

2. Conversion to RNA

The results remaining after the metabolite extraction was extracted as described in Section F.3.

3. Conversion to Ethylene

Throughout all orotic acid-6-^{14}C feeding experiments, ethylene collections were made as described in Section D.1. above. Each sample was analyzed for ethylene content (Section D.2.) as well as ethylene-^{14}C content as described in (Section J.1.) following.

H. Estimation of Endogenous β-alanine Levels

1. Tissue Preparation

Leaves were selected from beans grown under growth conditions #2 (Section B.1.). Approximately 75 g fresh weight of leaves (minus petioles) at various ages were frozen in dry ice and freeze-dried. Dry weight measurements were made.

2. Extraction Procedure

The lyophilied leaf tissue was extracted three times with boiling 80% ethanol for 5 min each (150 ml/g dry wt). After each extraction the suspension was centrifuged at 2000 x g for 10 min at 5°. The combined extract was evaporated to dryness in a rotary flash evaporator. Three ml of water was added to the evaporator flask and the residue was loosened with a spatula. The resulting suspension was centrifuged at 26,400 x g for 10 min at 0°. The supernatant contained the extracted amino acids.

3. Ion-exchange Purification of the Extract

Ion-exchange columns were prepared with Amberlite IR-120 and

washed with 1N HCl. Prior to adsorption of the amino acids, the column (0.8 cm ID x 5.0 cm) was washed to neutrality with water. The supernatant from Section H.2. was placed on the top of the ion-exchange column. Three ml of water was added to wash the column. Amino acids were eluted with 1 N ammonia. The fraction containing the amino acids was collected and flash evaporated to dryness. The residue was dissolved in 0.75 ml of water and stored at refrigerator temperatures.

4. Chromatography

A band of 200 µl of the extracted amino acids (Section H.3.) was applied to a Whatman #1 (46 cm x 57 cm) sheet of chromatography paper. Ten µg of β-alanine was spotted near each edge of the sheet. The amino acids were separated by descending chromatography at 24° with a phenol-water (4:1 v/v) solvent system (Porter et al., 1957), pH 5.5 to 5.7 adjusted with ammonia. The tank had been previously equilibrated with the phenol-water solvent. Time for the solvent to move 48 cm was about 18 h. The chromatogram was allowed to dry a minimum of 24 h in a fume hood.

Strips were cut from both edges including both the β-alanine spot and about 0.2 cm of the separated amino acids. The strips were sprayed with 1% ninhydrin in 95% ethanol containing 1% 8-hydroxyquinoline. Colour development was facilitated by heating the strips to 60° for 30 min. A band with an R_f corresponding to that of β-alanine was cut from the developed chromatogram. A wick, cut from Whatman #3 paper, was attached to the cut strip. Elution of the amino acids was carried out by descending chromatography with water. 400 µg of stock

β-alanine was eluted from an identical strip in an initial 10 ml fraction. The unknown strips were eluted with 20 ml of water. The eluted amino acids were flash evaporated to dryness.

5. Amino Acid Analysis

The dried amino acid eluate was taken up in an appropriate volume of diluting buffer for amino acid analysis in a Spinco Model MS Amino Acid Analyzer. Elution was carried out with a 0.2 N sodium citrate buffer, pH 3.25 followed by a 0.2 N sodium citrate buffer, pH 4.25. β-alanine concentration was determined by conventional peak height-width measurements. Results were expressed as μg β-alanine/g dry wt.

I. Uridine, Uracil, and Dihydrouracil Feedings of Bean Leaves

1. Feeding Procedures

Bean leaves were selected at appropriate ages and placed with their petioles in a 22.3 mM solution of the respective compounds. All feedings were carried out in a fume hood at about 24° in the dark. At the end of a 16-1/2 h feeding period, the petioles were removed and the leaves were frozen in dry ice and freeze-dried.

2. Analysis Procedure

Prior to amino acid analysis the lyophilyzed leaves were extracted, purified and chromatographed as outlined in Sections H.2. to H.4. inclusive. Results were expressed as μg β-alanine produced/ g dry wt per 16-1/2 h.

J. Conversion of β-alanine to Ethylene

1. Estimation of C_2H_4-^{14}C

Ethylene was collected as outlined in Section D above. A flow splitter was utilized to direct 80% of the sample to a Nuclear Chicago gas radiochromatograph (GRC) and the remaining 20% to the gas chromatograph flame. The GRC (85 ml chamber flushed with high purity methane at 30 cc/min) when connected to a 10 mv potentiometric recorder provided a record of radioactivity in the column effluent. The instrument was standardized by injection of known amounts of ethylene -^{14}C (U) into the GC and plotting peak height vs. dpm of ethylene (Appendix, Figure A-5). A comparison of GC and GRC responses during a sample analysis is shown in the Appendix, Figure A-6.

2. Preliminary Experiments

Leaves were selected from plants of about the 16 to 19 day age grown under growth conditions #1. β-alanine -2-^{14}C (0.417 μCi/μmole) was fed and ethylene was collected (Section D.1.). β-alanine-2-^{14}C (3.43 μCi/μmole) was also fed to leaves of a similar age and ethylene was collected. Ethylene -^{14}C determinations were carried out as described in Section J.1. above.

3. The Addition of Unlabeled β-alanine

β-alanine-2-^{14}C (3.43 μCi/μmole) was fed to leaves at various ages selected from plants grown under growth conditions #2. Three different experiments were set up at each age having 0, 1.2, and 3.0 mg

of unlabeled β-alanine respectively added to the feeding solution.

Ethylene collections and ethylene-^{14}C determinations were made as outlined in Section J.1. above.

4. β-alanine Recoveries

(a) Extraction

Leaves were frozen in dry ice after the feeding experiment and extracted as described in Section F.3. with slight modifications. The last two boiling water extractions were substituted for boiling 80% ethanol extractions. The remainder of the isolation procedure was identical.

(b) Label Detection

100 µl of the extract was applied to a 0.5 mm cellulose coated 2 inch wide glass TLC plate. An ethanol-ammonia (120:30 v/v) solvent system as well as a n-butanol-acetic acid-water (4:1:5 v/v/v, top layer) solvent system (Fritzson and Spaeren, 1962) was used for chromatography. Developed TLC plates were scanned on the Actigraph as outlined in Section G.1.

(c) Sugar and Amino Acid Detection Methods

Amino acids on each plate were determined by spraying the plate with 1% ninhydrin in 95% ethanol and heating to 60° for 30 min. Sugars were detected according to the method of Trevelyan et al. (1950) using a silver nitrate-acetone solution followed by a sodium hydroxide-ethanol spray.

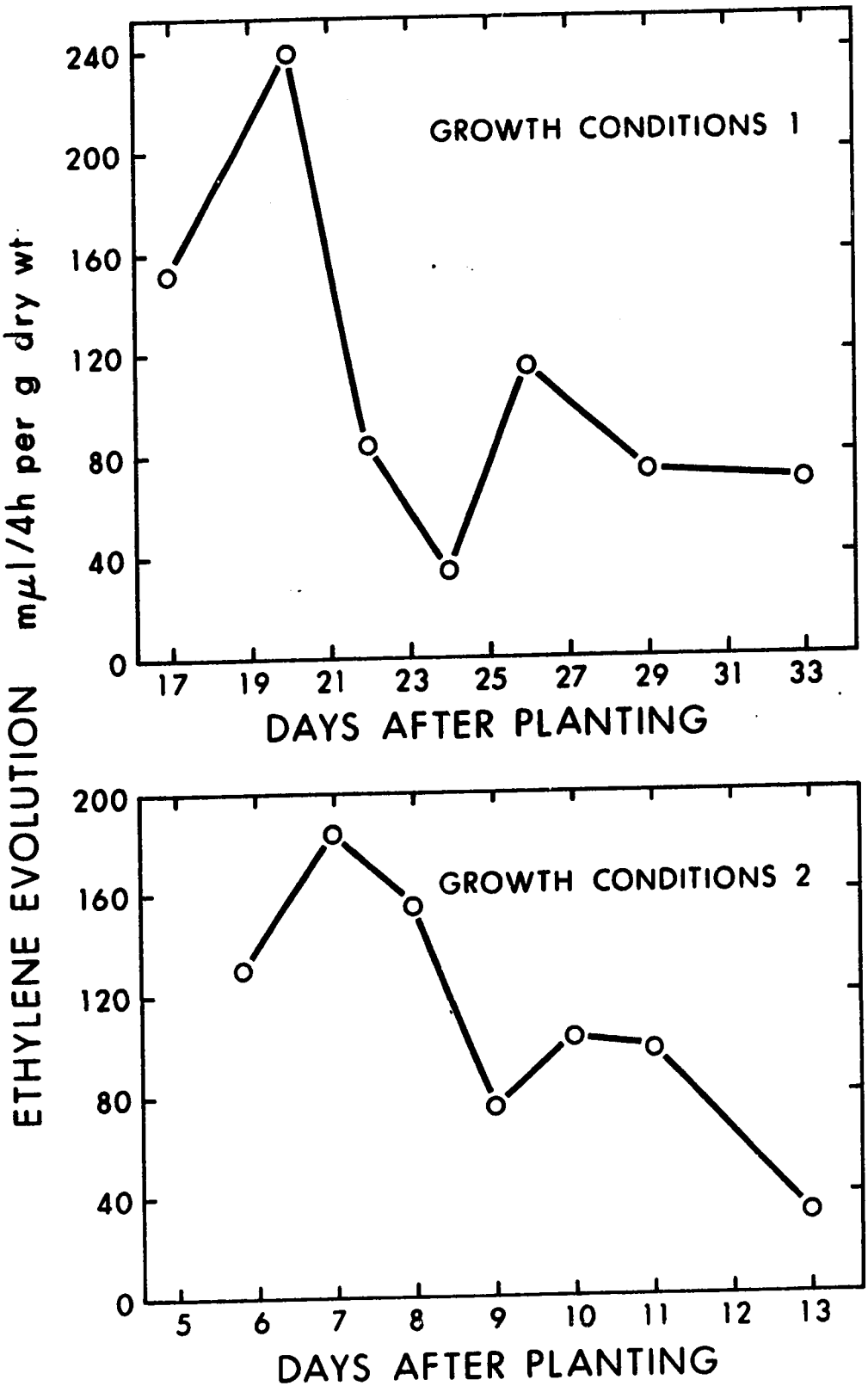
CHAPTER III

ETHYLENE PRODUCTION BY DETACHED BEAN LEAVES

A. Ethylene Production Variation with Leaf Age

Ethylene production from detached bean leaves was estimated with leaves grown under two different sets of growth conditions. The reasons for the two different sets of conditions were listed in Section B, Chapter II. Ethylene collections and estimations followed methods outlined in Chapter II. Appropriate blanks were determined for the ethylene collection system and results given had the ethylene levels of the blank subtracted. The ethylene production pattern from leaves grown under both conditions gave essentially a similar pattern (Figure 2). Figure 2 (top), the ethylene production from primary leaves grown under growth conditions #1, showed a major ethylene peak at 20 days after planting while those grown under growth conditions #2 (Figure 2, bottom) showed a similar peak at 7 days after planting. Both growth conditions produced a second, minor ethylene peak before the ethylene production decreased. Thus the only difference between the two sets of conditions in relation to ethylene production, leaf size, and leaf colour was the time required to reach a comparable state of development. Growth conditions #2 gave a more reproducible result. Under these conditions a variation in leaf age at peak ethylene production of 1 or 2 days from results shown in Figure 2 (bottom) was observed. This was attributed to alterations in the growth cabinet temperature settings (Section B, Chapter II). The overall shape of the ethylene production profile with leaf age was the same. The results shown in Figure 2

Figure 2. Ethylene production from detached bean leaves at various leaf ages. Three leaves were used at each age from plants grown under growth conditions #1 and #2. Growth conditions #1 was a 65° F, 16 h day of about 1200 ft-c with no humidity control. Growth conditions #2 was a 77°F, 14 h day of about 1800 ft-c with a 50% relative humidity. All ethylene collections were made between 8 AM and 12 AM. Results shown are typical of those obtained from 4 series with each set of growth conditions.


ETHYLENE EVOLUTION mμl/4h per g dry wt
GROWTH CONDITIONS 1
240
200
160
120
80
40
0
17
19
21
23
25
27
29
31
33
DAYS AFTER PLANTING
GROWTH CONDITIONS 2
200
160
120
80
40
0
5
6
7
8
9
10
11
12
13
DAYS AFTER PLANTING

represent typical data obtained on at least 4 trials under each set of conditions.

B. Ethylene Production from Detached Leaves

There are few reports available on the effects of detaching leaves from the plant on the subsequent production of ethylene from these leaves. Pratt (1954) estimated that detached leaves from milk thistle produced 10.8 ml of ethylene from 13.5 kg fresh weight during 4 days. Assuming about a 10 per cent dry weight and uniform production over the period, this would be equivalent to about 330 mμl ethylene/g dry weight per 4 h. This is comparable to results obtained by the author from bean leaves during the initial 4 h after detachment. Pratt's estimation was carried out on a static system over 4 days. He did not notice any visible bacterial contamination but bacterial production of ethylene cannot be discounted. Hall et al. (1957) were unable to detect ethylene production from intact cotton plants during the vegetative stage but estimated a production of 1.96 ml/kg fresh weight per 100 h during the reproductive stage. This was approximately 780 mμl/g dry weight per 4 h if the assumptions of a 10 per cent dry weight and a uniform production are made. This level is nearly double that observed for the author's detached bean leaf system. Detached mature cotton leaves produced about 52 mμl/g dry weight per 4 h with the above assumptions. This is less than one quarter of the amount produced at peak ethylene production by detached bean leaves and is comparable to the amount of ethylene produced from mature bean leaves (Figure 2).

Table 1 shows the results of ethylene production at various

Table 1. Ethylene production by detached bean leaves.

Collection period	Total ethylene
hours (after detachment)	mμl
0-4	99.0
4-8	47.5
8-20	40.5
20-32	3.0
32-44	6.5
44-56	-
56-68	-

Four leaves at day 18 (growth conditions #1) were incubated with β-alanine-2-^{14}C containing about 65 μmoles of β-alanine. Total ethylene was estimated by gas chromatography as outlined in Chapter II. (-) indicated an ethylene level < the limits of detection.

collection periods after detachment. Four young bean leaves produced a total of 99 mμl during the initial 4 h collection period. About half of this amount was collected in the subsequent 4 h period. During the next 12 h only 40 mμl was collected. In the 12 h period after 44 h of detachment no detectable ethylene was produced, although the leaves appeared to be visibly healthy. These results were from a series fed with β-alanine-2-^{14}C. Several other collections from unfed leaves at 0 to 4, 4 to 8, and 8 to 12 h collection periods gave similar results to those in Table 1. These results show that when the leaves were detached from the plant, the ethylene producing ability decreased with time. This loss could be attributed to a loss of enzyme activities associated with its production, an exhaustion of substrates or possibly a reduction in the energy supply. (ATP was shown to be an essential cofactor for the bean subcellular system for ethylene production (Thompson and Spencer, 1966).) Thus the results of Pratt (1954) were probably considerably higher initially than calculated on a uniform production basis because most of the ethylene evolution probably occurred in the initial 20 h after detachment.

C. The Effect of β-alanine on Ethylene Production

Previous theses have proposed β-alanine as a precursor for ethylene (Thompson, 1966; Stinson, 1968). Stinson and Spencer (1969) reported the isolation and partial purification from bean cotyledons of a β-alanine aminotransferase that utilized either oxaloacetate or pyruvate as amino acceptor. This enzyme is involved in the first step of the β-alanine to ethylene pathway proposed by Thompson and Spencer (1966). Stinson (1968) showed that up to and including 90 mM β-alanine

could stimulate ethylene production from a solubilized particulate fraction from bean cotyledons. He was unable to show any inhibitory effects of β-alanine, even at a concentration of 90 mM, suggesting that the K_m of the β-alanine aminotransferase was very high, thus requiring a relatively high concentration for the reaction to proceed.

Figure 3 shows the results of ethylene production from leaves fed exogenously applied β-alanine during feedings of β-alanine-2-^{14}C. The ethylene production peak at day 9 compared to day 7 (Figure 2) was a result of a low growth cabinet temperature rather than an influence of the β-alanine contained in the label feeding solutions. Results on total ethylene production were of importance here. High amounts of exogenously applied β-alanine had an inhibitory effect on this. When only 26 x 10^6 dpm β-alanine-2-^{14}C (specific activity 3.43 μCi/μmole) was fed, the sample actually contained 0.31 mg β-alanine. Similarly when 1.2 and 3.0 mg of β-alanine were added, there was an additional 0.31 mg supplied as part of the labeled material. The 1.2 mg level appeared not to have much effect over the ages studied while 3.0 mg was markedly inhibitory toward ethylene production during the peak of ethylene production. Results in Chapter VIII showed endogenous β-alanine to vary considerably at various leaf ages. The changing endogenous β-alanine levels may be one reason why at certain ages exogenous β-alanine was required to enable a conversion of β-alanine-2-^{14}C to ethylene-^{14}C (Chapter IX).

D. Ethylene Production over a 24 Hour Period from Bean Leaves

Figure 4 shows the ethylene production over a 24 h period for

Figure 3. The effects of exogenously applied β-alanine on ethylene production from detached bean leaves. Ethylene was collected and analyzed from bean leaves at the selected ages as outlined in Chapter II. 26.4×10^6 dpm β-alanine-2-^{14}C (3.43 μCi/μmole) was fed to 4 leaves (growth conditions #2, except at 74°) along with the exogenous β-alanine in a final volume of 700 μl. Ten g drierite was included in the collection flask to hasten label uptake. Total β-alanine fed was about 0.31, 1.51, and 3.31 mg in experiment A, B, and C respectively.

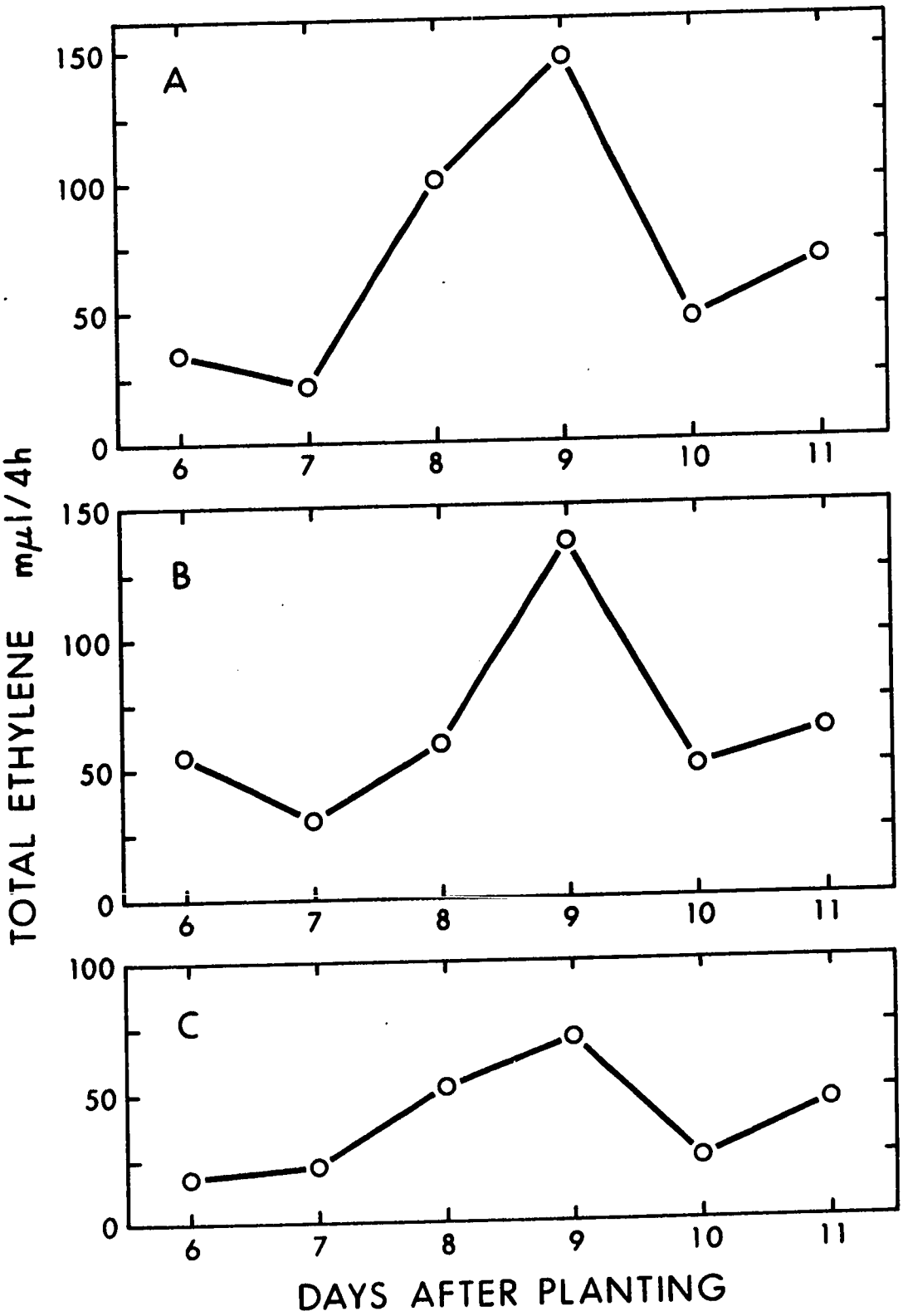
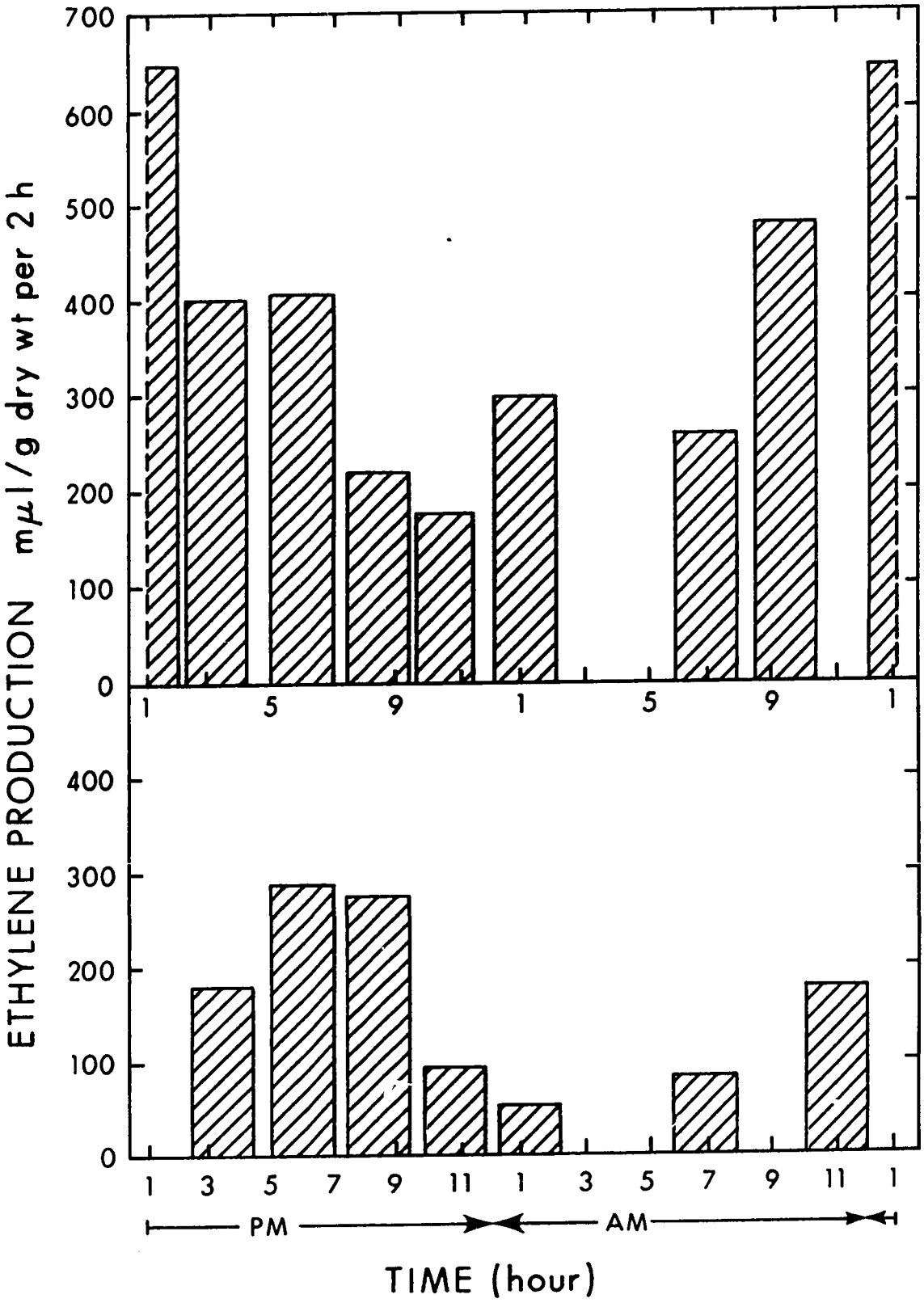

A
B
C
150
100
50
0
6
7
8
9
10
11
TOTAL ETHYLENE mμl/4h
DAYS AFTER PLANTING

Figure 4. Ethylene production during a 24 hour period. Two hour ethylene collections were carried out on 12 day (top) and 14 day (bottom) leaves from plants growing under growth conditions #2. The lights came on at 6 AM and went off at 8 PM. Only the plants under experimentation were in the growth cabinet.


ETHYLENE PRODUCTION mμl/g dry wt per 2 h
700
600
500
400
300
200
100
0
1
5
9
1
5
9
1
400
300
200
100
0
1
3
5
7
9
11
1
3
5
7
9
11
1
PM
AM
TIME (hour)

two separate sets of leaves. Figure 4 (top) and (bottom) shows the ethylene production from leaves selected from bean plants at 12 and 14 days old respectively. For experiments reported in Figure 4 only plants undergoing experimentation were in the growth chamber.

Results included in Figure 4 show marked responses of ethylene evolution to light and darkness. Results indicate that ethylene production during the dark period was generally lower than that for the light period. This agrees with results of Rogers (1969), who found increased ethylene production from orange leaves in the light. One possible explanation for the lower ethylene production in the dark than in the light in Figure 4 could be the higher CO_2 levels in the dark, as pointed out in Chapter IV. The higher CO_2 levels brought about by dark respiration could be antagonistic toward ethylene production or toward the "autocatalytic" effect of ethylene on its own production as pointed out by Spencer (1969). Another explanation for low ethylene production in the dark would be if ATP levels were limiting; this would probably limit ethylene production.

CHAPTER IV

CARBON DIOXIDE PRODUCTION AND ITS POSSIBLE INTERACTION WITH ETHYLENE PRODUCTION

A. Carbon Dioxide Production from Excised Bean Leaves

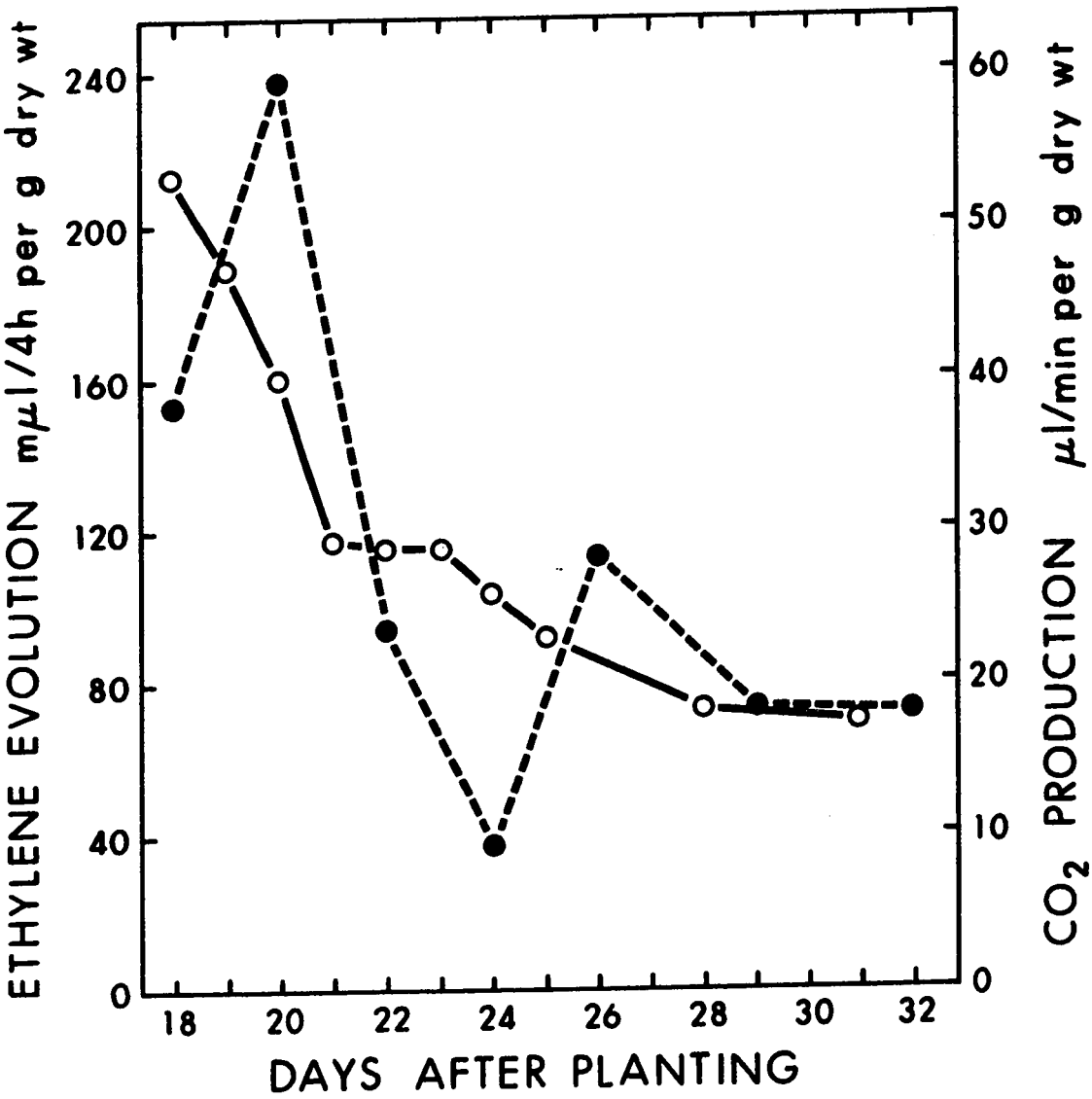
Leaves were selected from bean plants grown under growth conditions #2 and CO_2 estimations were made as outlined in Section E, Chapter II. As seen from Figure 5, the CO_2 production rate decreased from about 55 µl CO_2/min per g dry weight on day 18 to about 18 µl CO_2/min per g dry weight on day 28. The CO_2 production rate from days 28 to 32 appeared to be low and constant. No sharp changes in the CO_2 production rate were noted throughout the ageing period. No climacteric-like respiration rise, such as is evident for many ripening fruits, was noted for leaves during the ages studied. Both ethylene and CO_2 collections were carried out at the same time of day at each day studied. Duplicate leaf samples for ethylene estimation did not give the same absolute result but did give the same relative result. Duplicate leaf samples for carbon dioxide estimations never varied by more than 7 per cent.

The ethylene evolution profile is included for comparative purposes. The initial CO_2 decrease roughly paralleled the ethylene decrease for the first ethylene peak. Thus the CO_2 production rate appeared to continue to decrease with increasing leaf age.

B. Carbon Dioxide Production from Labeled Compounds

Both orotic acid-6-^{14}C and β-alanine-2-^{14}C were used in

Figure 5. Ethylene and carbon dioxide production by bean leaves. Bean plants were grown under growth conditions #1. Three leaves were selected at the respective ages and estimations were carried out as described in Chapter II. Both the ethylene (●——●) and carbon dioxide (0——0) collections were commenced about the same time each day. The results represent typical data obtained from 5 ethylene and 2 carbon dioxide collection experiments over the ageing period.


ETHYLENE EVOLUTION mμl/4h per g dry wt
240
200
160
120
80
40
0
60
50
40
30
20
10
0
CO2 PRODUCTION μl/min per g dry wt
18
20
22
24
26
28
30
32
DAYS AFTER PLANTING

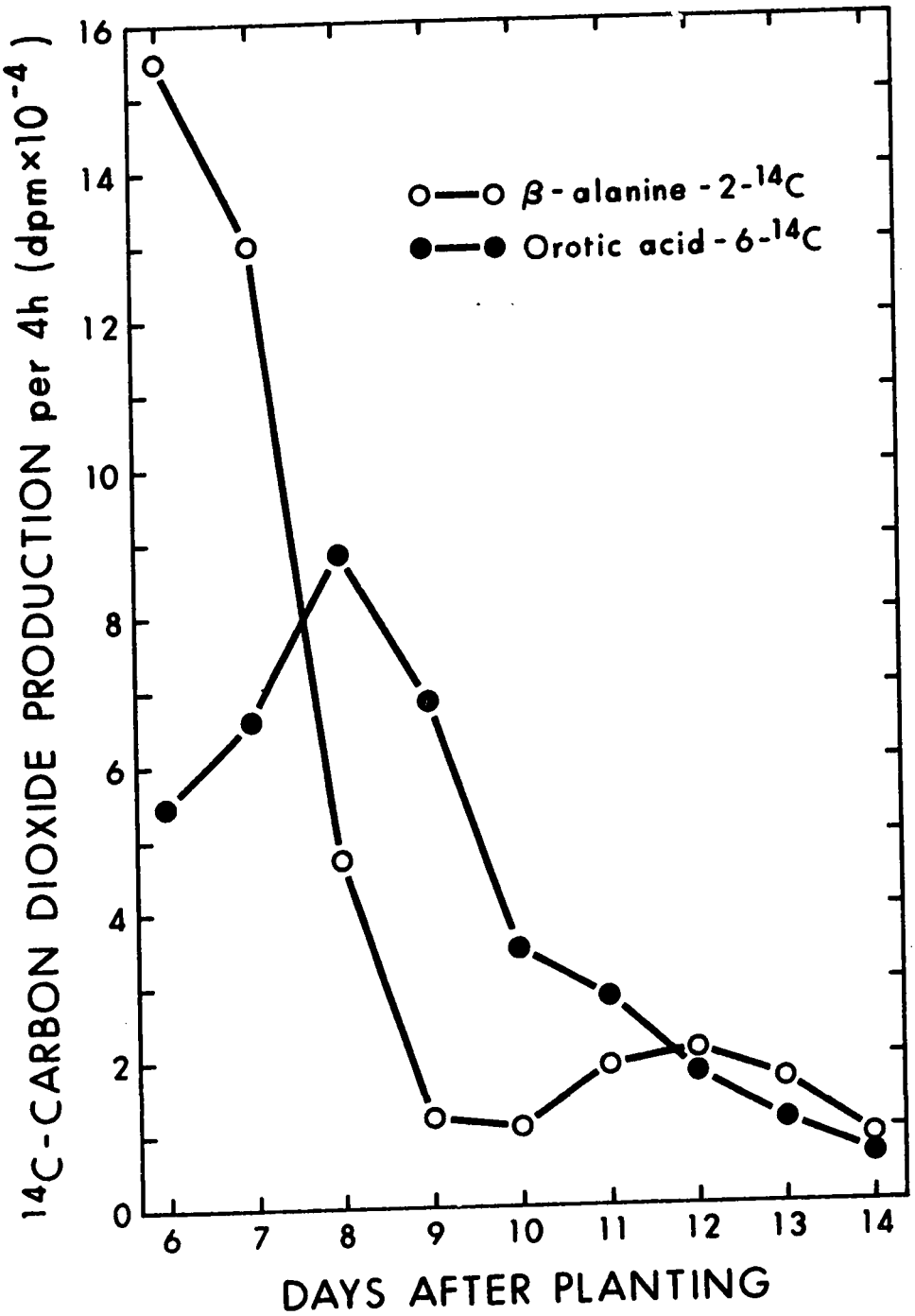
studies of precursors of ethylene. During the ethylene collections, $^{14}CO_2$ was trapped in potassium hydroxide and the radioactivity determined as described in Section C, Chapter II. This measurement indicated the rates of conversion of the administered compounds to CO_2 at the different ages (Figure 6). A decrease in the amount of $^{14}CO_2$ produced could be attributed to a decreased respiration rate as indicated by the total CO_2 produced, a decrease in the amount of the compounds being metabolized to CO_2, their metabolism to other compounds that produce CO_2 only slowly, or a change in the permeability of subcellular and/or cellular membranes not permitting the penetration of the labeled compounds at the older ages. From Figure 5 it appeared that the respiration rate was decreasing at the older ages. With β-alanine-2-^{14}C a slight increase was noted around day 12 before a further decrease became apparent. This second $^{14}CO_2$ peak coincided with the second ethylene peak (Chapter III).

C. Carbon Dioxide Levels During the Growth of the Bean Plants

Throughout the ethylene collection experiments variable results were obtained, especially on the ethylene-^{14}C production from leaves fed with β-alanine-2-^{14}C. The incubation conditions were rigidly controlled as were leaf handling techniques. Results varied among leaves from different replicates as well as from duplicate runs at different times of the day.

One possible explanation for the variable results was that the CO_2 levels in the growth cabinet interfered with the ethylene production mechanism (Section E, Chapter I). Thus, an investigation was undertaken to estimate the CO_2 levels in the growth cabinet.

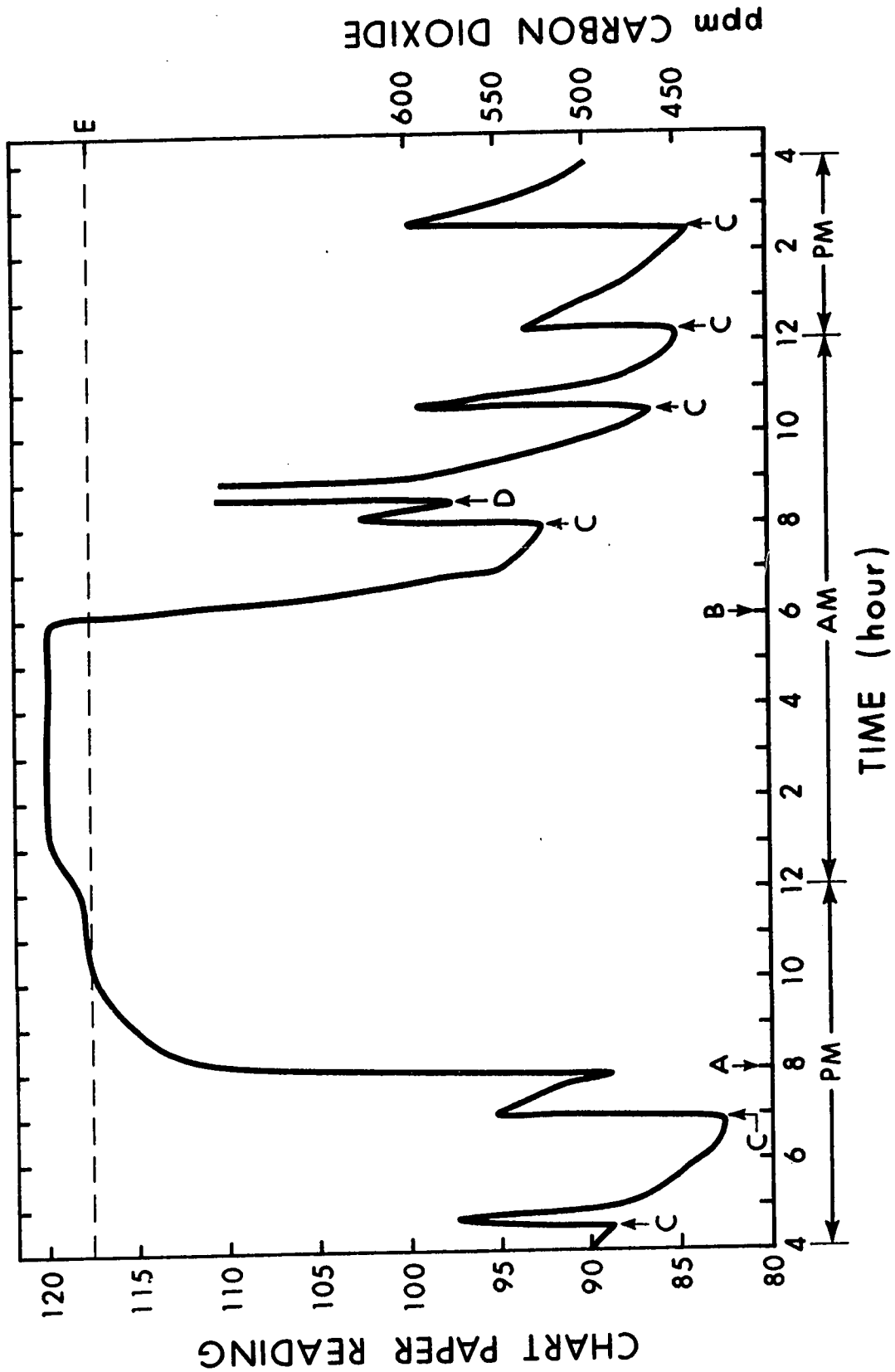
Figure 6. $^{14}CO_2$ production from detached bean leaves incubated with β-alanine-2-^{14}C and orotic acid-6-^{14}C. Bean plants were grown under growth conditions #2. $^{14}CO_2$ collections and determinations were outlined in Chapter II. For the β-alanine-2-^{14}C feedings, 8 leaves were incubated with 27.5×10^6 dpm (3.43 μCi/μmole) at each leaf age. For the orotic acid-6-^{14}C feedings, 8 leaves at day 6 and 7 and 4 leaves thereafter were incubated with 10.6×10^6 dpm (22.6 μCi/μmole). Incubation time was 4 h for both β-alanine-2-^{14}C (O———O) and orotic acid-6-^{14}C (●———●).


16
14
12
10
8
6
4
2
0
6
7
8
9
10
11
12
13
14
O—O β - alanine - 2-14C
●—● Orotic acid - 6-14C
14C-CARBON DIOXIDE PRODUCTION per 4h (dpm×10-4)
DAYS AFTER PLANTING

The infrared analyzer was set up to monitor CO_2 levels in the cabinet over 24 h periods. (The beans were grown in a growth cabinet (Growth Conditions #2) that allowed very little exchange with the external atmosphere). Under the light regime used, lights came on at 6:00 AM and went off at 8:00 PM. The CO_2 levels varied considerably from a low of about 450 ppm at mid-day to a high of about 750 ppm prior to the lights coming on in the morning (Figure 7). This determination was made with 10 flats of 14 day old and 5 flats of 21 day old beans in the cabinet. The figure given for the higher level is only approximate as the data supplied with the instrument did not allow for accurate measurements above 600 ppm CO_2. If we assume that the standard CO_2 curve for the instrument could be extended above 600 ppm, night time levels would reach 800 or 900 ppm CO_2. Normal air contains about 300 ppm CO_2.

Another fact that is worthy of note was that the CO_2 concentration outside the growth cabinet was consistently above the upper limit of the standard curve supplied with the instrument and close to the level reached during the dark period (Figure 7). The physical location of the cabinet aids in the explanation for this. It was located in the basement of the greenhouse about 20 steps below ground level. Since greenhouse atmosphere are normally above atmosphere levels with respect to CO_2 (Heinicke and Hoffman, 1933), the atmosphere surrounding the cabinet in question would certainly be much higher since CO_2 has a density greater than air. Thus when the greenhouse staff watered the beans in the mornings (about 3 h after the lights came on) the CO_2 level increased and commenced to decrease only about

Figure 7. Carbon dioxide levels during a 24 hour interval in the growth cabinet. The growth cabinet contained 10 flats of 14 day old and 5 flats of 21 day old bean plants (growth conditions #2). The infrared analyzer monotored CO_2 levels at flow rates of 100 cc/min against compressed breathing air (0 ppm CO_2) as a reference gas (Section E, Chapter II). The following letters included in the Figure indicate: A, lights off at 8 PM; B, lights on 6 AM; C, growth cabinet door opened for a 30 to 60 sec time interval; D, growth cabinet door opened for about 180 sec for watering plants; and E, CO_2 level in greenhouse air surrounding the growth cabinet.


ppm CARBON DIOXIDE
600
550
500
450
E
C
C
C
D
C
B
A
C
C
120
115
110
105
100
95
90
85
80
CHART PAPER READING
4
6
8
10
12
2
4
PM
AM
PM
TIME (hour)

one-half hour after the doors were closed. It took about one hour to reduce CO_2 levels nearly to the concentration prior to a 3 minute opening of the doors. This time was observed with 15 flats of 7 to 21 day old plants (a large total photosynthetic capacity). This effect was repeated throughout the day whenever leaf samples were removed. If only 5 flats (less total photosynthetic capacity) were present, levels seldom decreased to much less than 590 ppm at mid-day even if the doors were only opened once for watering.

D. Possible CO_2 Interaction with Ethylene Production

To the present no information is available on CO_2 levels that might be antagonistic to ethylene production in the leaf system. In controlled atmosphere storage of fruit CO_2 levels are increased to about 5 per cent.

One can only speculate on the effect that the observed CO_2 levels had on bean leaf metabolism and specifically on the ethylene producing system. It would be possible to explain conflicting results if CO_2 concentrations about double atmospheric levels (about 600 ppm) were able to control the ethylene producing system. Thus when the lights went off, dark respiration increased CO_2 levels above the postulated 600 ppm critical level. The opposite trend was noted when the lights came on - CO_2 levels decreased because CO_2 requirements for photosynthesis exceeded the CO_2 production from respiration. During this period when the CO_2 level was below 600 ppm, the ethylene system was not affected.

Ethylene production from bean leaves was sampled over a 24 h period. Results although varying (Chapter III) did show a higher ethylene production during the light period than during the dark period with 5 flats of 12 and 14 day old bean leaves in a chamber by themselves (Growth Conditions #2). The changes were not attributable to the lights as such. During the 2 h collection period, a light intensity of about 1500 ft-c shining on leaves harvested during the night failed to increase ethylene levels appreciably. If 600 ppm were a critical level for ethylene production, respiration rates at night soon increased CO_2 levels above 600 ppm, and ethylene production decreased.

Although conclusive evidence was not obtained that CO_2 did have an antagonistic effect on the ethylene producing system, the high CO_2 levels must be considered when interpreting results, especially when only a few flats of beans were present in the growth cabinet.

CHAPTER V

RNA LEVELS IN AGEING BEAN LEAVES

Total RNA has been shown to progressively decrease during senescence of leaves (Srivastava and Ware, 1965; Osborne, 1962; Shaw et al., 1965; and Fletcher and Osborne, 1965, 1966). The majority of these reports dealt with the effect of growth regulators on the levels of total RNA. However, little work has been described with regard to the qualitative differences in RNA of senescing leaves. There has been some documentation of the qualitative changes occurring in senescing storage tissue (peanut cotyledons, Cherry, 1964; pea cotyledons, Barker and Hollinshead, 1967; and avocado fruit, Richmond and Biale, 1967). Knight (1966) showed a marked decrease in the ribosomal components of RNA in senescing excised radish leaves.

The present investiagtion was initiated to study the qualitative changes in the RNA components of bean leaves over an extended growth period. In addition, a study of $^{32}P_i$ incorporation into RNA was undertaken in order to ascertain relative synthesis of RNA at various ages of bean leaves.

A. Total RNA Changes at Various Leaf Ages

Total RNA was estimated by absorbancy at 260 mμ measurements after phenol extraction from the leaves and prior to MAK column fractionation. Table 2 shows the results for the total RNA extracted at the various ages of leaves studied. Total RNA decreased with increasing leaf age. The amount on day 28 was about 30 per cent of the day 13 level.

Table 2. Total RNA from bean leaves of various ages.

Leaf age days	Total RNA	
	Absorbance	260mμ/10g fresh wt
13		336
16		329
19		206
22		190
25		116
28		108

RNA was extracted from 10 g (fresh wt) of bean leaves grown under growth conditions #1 (Chapter II). An aliquot of the dialyzed RNA was taken for absorbance measurements and corrected for sample size to 260 mμ give total RNA. Results are representative of 4 separate series over the identical ageing period.

At day 28 the leaves showed some visible symptoms of senescence (a slight yellowing of the leaves).

B. MAK Column Fractionation of RNA

MAK column elution profiles of RNA from bean leaves of three different ages are shown in Figure 8. Only slight differences were noted in the sizes of the soluble RNA, the DNA-RNA and the light-r-RNA areas of the elution profile at the three ages. However, the heavy-r-RNA peak decreased with increasing leaf age. It should be noted that 75 absorbancy $_{260\ m\mu}$ units of RNA were applied to each column. This amount was found to give excellent MAK column separations (Knight, 1966). Since the total amount of RNA extracted from increasing ages of bean leaves decreased (Table 2), only a fraction of the day 13 sample and nearly all of the day 28 sample was applied to the MAK columns for the respective separations. Table 3 shows the quantitative estimation of the RNA components corrected to the total RNA extracted from the leaves. Over the ageing period, a slight decrease is noted in the DNA-RNA component. A greater decrease over the ageing period is evident for soluble RNA and light-r-RNA. A marked decrease is apparent for the heavy-r-RNA fraction.

The decrease observed in total RNA could be a reflection of a decreased synthesis of RNA, an increased degradation rate of RNA, or a combination of both with the rate of degradation exceeding the rate of synthesis. The marked decline of the heavy-r-RNA component with increasing age would perhaps indicate an enhanced degradation of this component as leaf age increased. The nature of this degradation has not

Figure 8. MAK column fractionation of RNA isolated from leaves of three different ages. RNA was isolated from 10 g fresh wt of bean leaves grown under growth conditions #1 as described in Section C, Chapter II. Approximately 75 $\text{absorbancy}_{260\ m\mu}$ units of RNA was subjected to MAK column fractionation at each age. Nucleic acids were eluted with a linear 0.3 M to 1.4 M NaCl (in 0.05 M phosphate buffer at pH 6.7) gradient and 5 ml fractions were collected. Absorbance at 260 mμ measurements were determined on each fraction to construct the elution profile. Days 13, 22, and 28 are representative MAK column fractionation of the series comprising the date for Table 3.

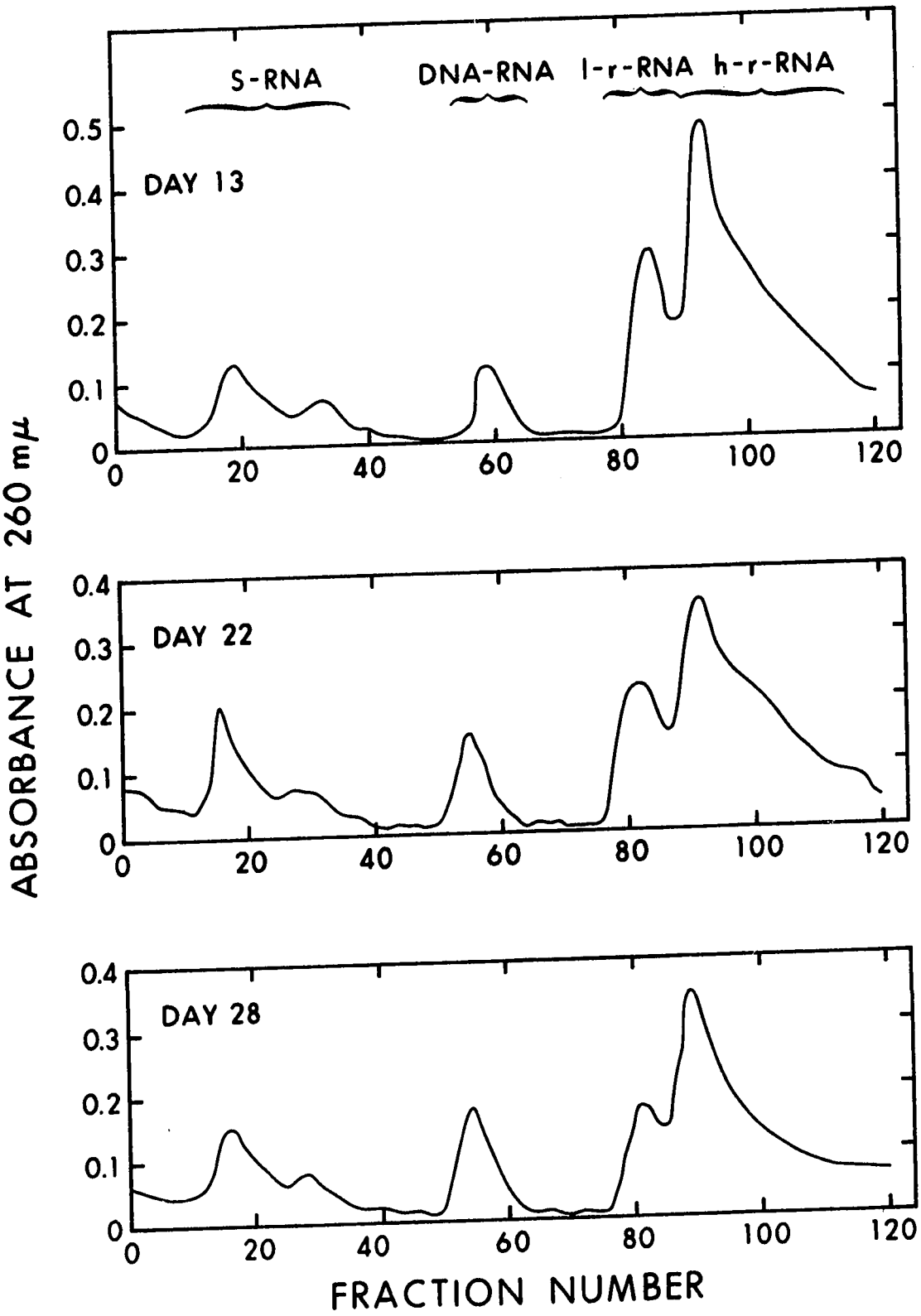

S-RNA
DNA-RNA
l-r-RNA
h-r-RNA
DAY 13
DAY 22
DAY 28
ABSORBANCE AT 260 mμ
FRACTION NUMBER
0
0.1
0.2
0.3
0.4
0.5
20
40
60
80
100
120

Table 3. Estimation of RNA component levels at various bean leaf ages.

Leaf age days	RNA components from MAK column			
Area:	s-RNA $(inch)^2$	DNA-RNA $(inch)^2$	l-r-RNA $(inch)^2$	h-r-RNA $(inch)^2$
13	21.5	8.9	22.4	64.0
16	16.1	5.5	11.3	49.7
19	16.6	6.1	14.6	46.3
22	13.4	6.7	12.4	38.0
25	7.0	3.6	4.5	18.0
28	7.0	4.0	3.6	16.7

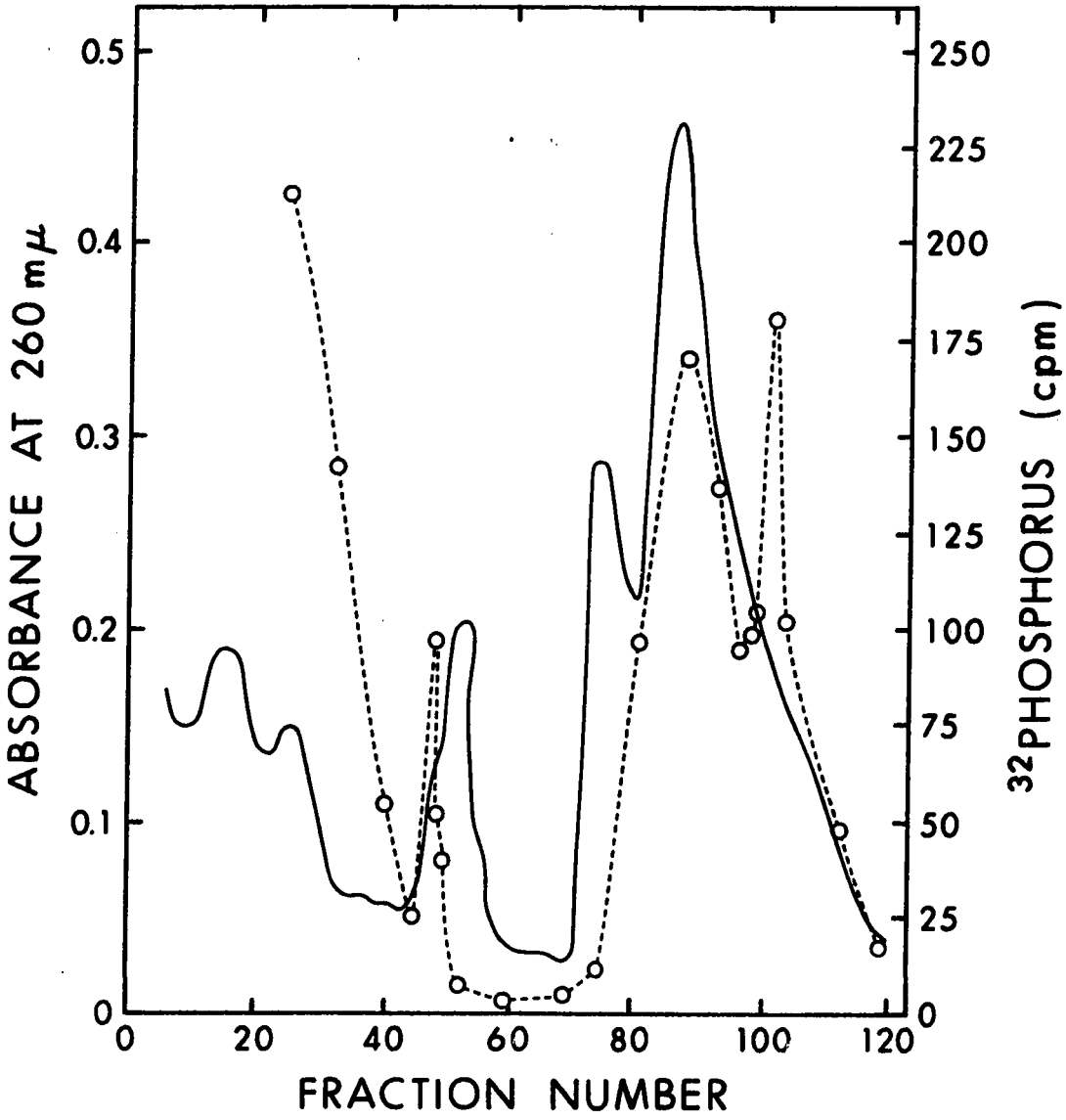
RNA was extracted from 10 g (fresh weight) of bean leaves grown under growth conditions #1 (Chapter II). Planimetry measurements (square inches) were determined on the MAK column elution profiles. The measurements were corrected for total RNA extracted to yield the above data. Results are representative of 3 separate estimations over the ageing period.

been documented for plant leaf tissue. Beevers and Guernsey (1966) estimated nucleotide and RNA levels in the axis and cotyledons of germinating peas. They found no nucleotide accumulation in cotyledons but noted an accumulation in the axis. This axial accumulation was accompanied by a decrease in total RNA in the cotyledons. This would be indicative of a further RNA degradation than that of the transportable RNA proposed by Oota (1964). There exists the possibility of a complete degradation to nucleotide, nucleoside, or base level with a movement from older to younger leaves for re-synthesis to new RNA.

C. The Incorporation of $^{32}P_i$ into RNA

$^{32}P_i$ incorporation studies were carried out to ascertain the ability of leaves to synthesize RNA at various leaf ages. The 13, 17, and 20 day old leaves (Growth conditions #1) showed incorporation into RNA. The radioactivity was detected to the leading edge of the DNA-RNA peak and to the right of the heavy-r-RNA peak (Figure 9). The highest radioactivity occurred in the youngest leaves studied. A 22 day sample indicated slight incorporation of radioactivity in the two peaks while a 26 day sample gave no detectable incorporation. The existence of a radioactive peak in the leading edge of the DNA-RNA was consistent with the findings of other workers (Hulme and Abeles, 1967; Cherry and Lessman, 1967). Lonberg-Holm (1967) stated that this peak could be a result of bacterial contamination. If this were true, the present author should have shown more, or at least some incorporation into the older bean leaves that are probably more susceptible to bacterial

Figure 9. MAK column fractionation of RNA isolated from leaves incubated with $^{32}P_i$. Eight leaves at day 20 (growth conditions #1) were incubated with 6 x 10^6 dpm $^{32}P_i$ for 4 h. The RNA was extracted and the total amount was fractionated by MAK column chromatography as outlined in Section C, Chapter II. Five ml fractions were collected from a 0.3 M to 1.4 M NaCl (in phosphate buffer, pH 6.7) elution gradient. Absorbance at 260 mμ measurements (———) were taken on each fraction to construct the elution profile. 0.5 ml aliquots were taken from selected fractions for the ^{32}P estimations (0-----0).


ABSORBANCE AT 260 mμ
0.5
0.4
0.3
0.2
0.1
0
250
225
200
175
150
125
100
75
50
25
0
32PHOSPHORUS (cpm)
0
20
40
60
80
100
120
FRACTION NUMBER

growth. Lonberg-Holm worked with tissue slices; the present author used whole leaves. The radioactivity present in the ribosomal region of the MAK column was probably newly synthesized ribosomal RNA and/or messenger RNA. Thus the observed labeling pattern was indicative of a decreasing ability of progressively ageing leaves to synthesize RNA.

CHAPTER VI

THE URACIL TO β-ALANINE PATHWAY

Enzymes of the uracil catabolic pathway have been isolated and characterized from rat liver by Fritzson (1959), Grisolia and Wallach (1955), and Caravaca and Grisolia (1958). The pathway has been shown to be active in plant tissue (pine, Barnes and Naylor, 1962; and rape seedlings, Tsai and Axelrod, 1961) by the detection of intermediate metabolites. Only dihydrouracil hydrase has been isolated and characterized from pea plants (Mazus and Buchowicz, 1968). This enzyme cleaves the dihydrouracil ring during the formation of β-ureidopropionic acid. Because dihydrouracil dehydrogenases is a rate limiting enzyme in the animal uracil catabolic system, the detection of its activity in the bean leaf system was of prime importance. It was not the object of the present thesis to characterize individual enzymes but rather to study the overall pathway in bean leaves and compare cofactors and activators with those known for the animal system. Supernatant, ammonium sulfate-precipitated, and freeze-dried protein preparations were prepared from bean leaves as described in Section F, Chapter II. These preparations were used in the study of the uracil to β-alanine pathway. Detached bean leaves were used to study the catabolism of uracil of β-alanine and the incorporation of uracil into RNA.

A. In Vivo Metabolism of Uracil by Bean Leaves

Detached bean leaves (growth conditions #1) were incubated with uracil-6-^{3}H as outlined in Chapter II. As indicated in Table 4, Experiment A and B, significant conversions to β-alanine occurred. The

Table 4. Metabolism of uracil-6-^{3}H by detached bean leaves.

Experiment	Leaf age	% conversions to:			Label fed	Uracil-6-^{3}H recovered
		β-alanine-^{3}H	H_2O-^{3}H	RNA-^{3}H		
	days				dpm x 10^{-6}	%
A	17	12.5	1.8	-	6.4	5.8
	20	16.7	1.4	-	3.4	4.5
	23	14.1	1.6	-	6.4	3.6
B	12	16.2	2.0	4.4	9.0	4.5
	14	22.5	1.5	3.0	10.4	4.1
	16	18.9	1.3	3.2	12.4	-
	30	4.5	-	1.7	6.6	2.8

Four leaves from plants grown under growth conditions #1 were incubated with uracil-6-^{3}H (8.9 mCi/μmole) for 2 h, extracted with boiling 80% ethanol, and label was estimated as described in Chapter II. (-) indicates no measurement was made.

conversion to β-alanine varied considerably but decreased at the older ages, especially the 30 day sample. The amount of unmetabolized uracil-6-^{3}H decreased with increasing leaf age. RNA estimations indicated a decreased incorporation of uracil into RNA at increasing leaf ages (Table 4, Experiment B). The estimation of H_2O-^{3}H gave an indication of the amount of β-alanine subsequently metabolized. This was similar to the $^{14}CO_2$ estimation from β-alanine-2-^{14}C feedings. The results on H_2O-^{3}H would be minimum levels since some H_2O-^{3}H could conceivably be lost by transpiration during the incubation period or during the flash evaporation of the extracted metabolites. Stock uracil-6-^{3}H contained a minimum of 1.1% H_2O-^{3}H.

In a separate experiment uracil-6-^{3}H was incubated with 18 day old leaves (growth conditions #1) for 20, 40 and 120 minute intervals. Results (Table 5) show conversions to β-alanine-^{3}H increase, as expected, for longer incubations. Unmetabolized uracil decreased to non-detectable levels after the 120 min period. RNA levels increase slightly from the 40 to 120 min incubation. The amount of H_2O-^{3}H increased slightly, indicative of some β-alanine-^{3}H being metabolized during the longer incubation period.

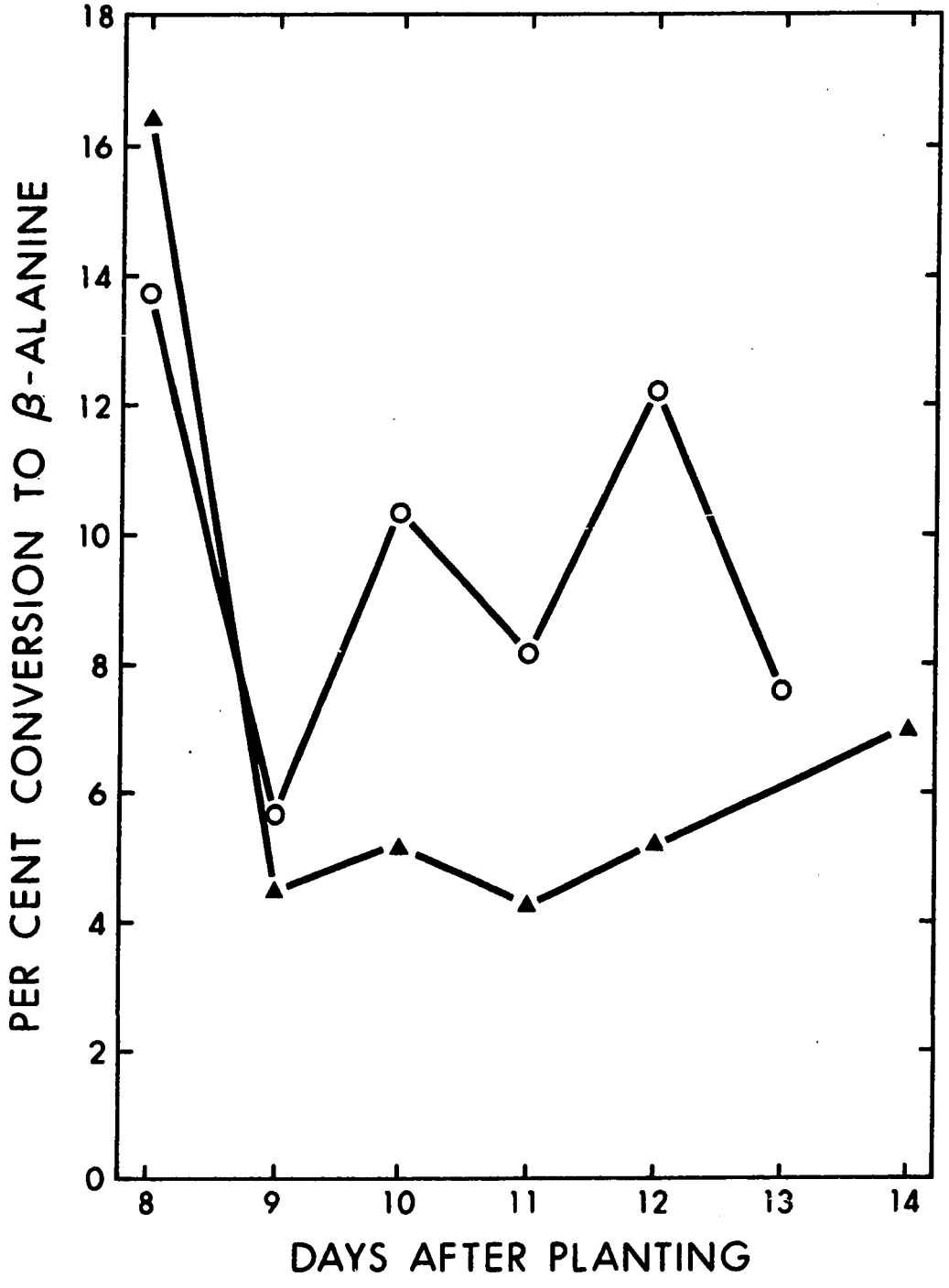
Figure 10 shows the results of 15 and 60 min incubations of detached bean leaves with uracil-6-^{3}H. The results cannot be compared on an age relationship basis with results in Tables 7 and 4 because of alterations in growth conditions (foot-note to Figure 10). However, it was noted that the youngest leaves produced the highest level of β-alanine-^{3}H and conversions then generally decreased with increasing

Table 5. Uracil-6-^{3}H metabolism by detached bean leaves during different incubation periods.

Incubation time	Percent conversion to:			Label fed	Uracil-6-^{3}H recovered
min	β-alanine-^{3}H	H_2O-^{3}H	RNA-^{3}H	dpm x 10^{-6}	%
20	12.5	0.96	-	6.6	12.7
40	17.9	1.10	10.6	12.1	4.9
120	19.6	1.26	12.7	12.4	0.0

Four leaves selected from 18 day old plants grown under growth conditions #1 were incubated with uracil-6-^{3}H (8.9 mCi/ μmole) for the times indicated. Label estimations were as described in Chapter II. (-) indicates no determination was made.

Figure 10. Production of β-alanine from uracil-6-^{3}H during a 15 and 60 min incubation with bean leaves of different leaf ages. Bean plants were grown under growth conditions #1 except new lights were installed, resulting in higher intensities and a more rapid bean growth rate. Four bean leaves were incubated with 5.5 x 10^{6} dpm and 11.0 x 10^{6} dpm of uracil-6-^{3}H (8.9 mCi/μmole) for 15 (▲——▲) and 60 (0——0) min respectively. Leaf extraction and label estimations followed procedures described in Chapter II.


PER CENT CONVERSION TO β-ALANINE
0
2
4
6
8
10
12
14
16
18
8
9
10
11
12
13
14
DAYS AFTER PLANTING

leaf age. The 1 h incubation period did not show such a clear pattern, probably a result of some leaf wilting toward the end of the incubation period.

B. Dihydrouracil Dehydrogenase Activity as Measured by a Spectrophotometric Assay

The activity of this enzyme was measured by utilizing the 340 mμ absorption properties of reduced pyridine nucleotides, known cofactors of the enzyme isolated from animal or bacterial sources. Uracil itself did not contribute significantly to the absorption at this wavelength.

Table 6 shows the results of a typical determination on each of the various enzyme preparations. The presence of the enzyme was confirmed by a stimulation of NADPH oxidation upon uracil addition to the incubation medium (Chapter II). Considerable oxidation in the absence of uracil was indicative of the presence of other components also oxidizing NADPH. The spectrophotometric assay was used to indicate the presence of dihydrouracil dehydrogenase and not to estimate relative activities among different preparations.

C. Stability and Cofactor Requirements of Dihydrouracil Dehydrogenase

The supernatant enzyme preparation lost all dihydrouracil dehydrogenase activity while stored at 0 to 4° for 60 min. The freeze-dried preparation showed about 20% of its activity after 18 days storage at deep freeze temperatures. NADH was unable to substitute for NADPH as a 'Hydrogen-donor' in the dehydrogenase reaction. Thus the bean leaf system was similar in its pyridine nucleotide requirement to

Table 6. Spectrophotometric assay for dihydrouracil dehydrogenase isolated from bean leaves.

Preparation	Leaf age	Protein in reaction mixture	Enzyme activity	
	days	mg	Δ Absorbancy$_{340\ m\mu}$/15 min	
Supernatant layer	16	0.35	+ uracil	0.150
			- uracil	0.071
30-45% Ammonium sulfate fraction	20	0.25	+ uracil	0.250
			- uracil	0.150
Freeze-dried, desalted, 30-45% ammonium sulfate fraction	20	0.30	+ uracil	0.33
			- uracil	0.11

The enzyme preparations were described in Section F, Chapter II. Reaction mixtures contained 0.20 μmoles NADPH, enzyme, and 47.5 μmoles potassium phosphate buffer, pH 7.4, in a final volume of 1.0 ml. The reaction was initiated by the addition of 0.18 μmoles uracil and incubation was at 37°. Spectrophotometric readings were taken immediately after uracil addition and after 15 min.

the animal system.

The dihydrouracil hydrase enzyme in the animal system was known to have the absolute requirement for Mg^{++} or Mn^{++} ions as activators. In the bean leaf system no requirement was shown which agreed with results of Mazus and Buchowicz (1968) on the hydrase isolated from wheat.

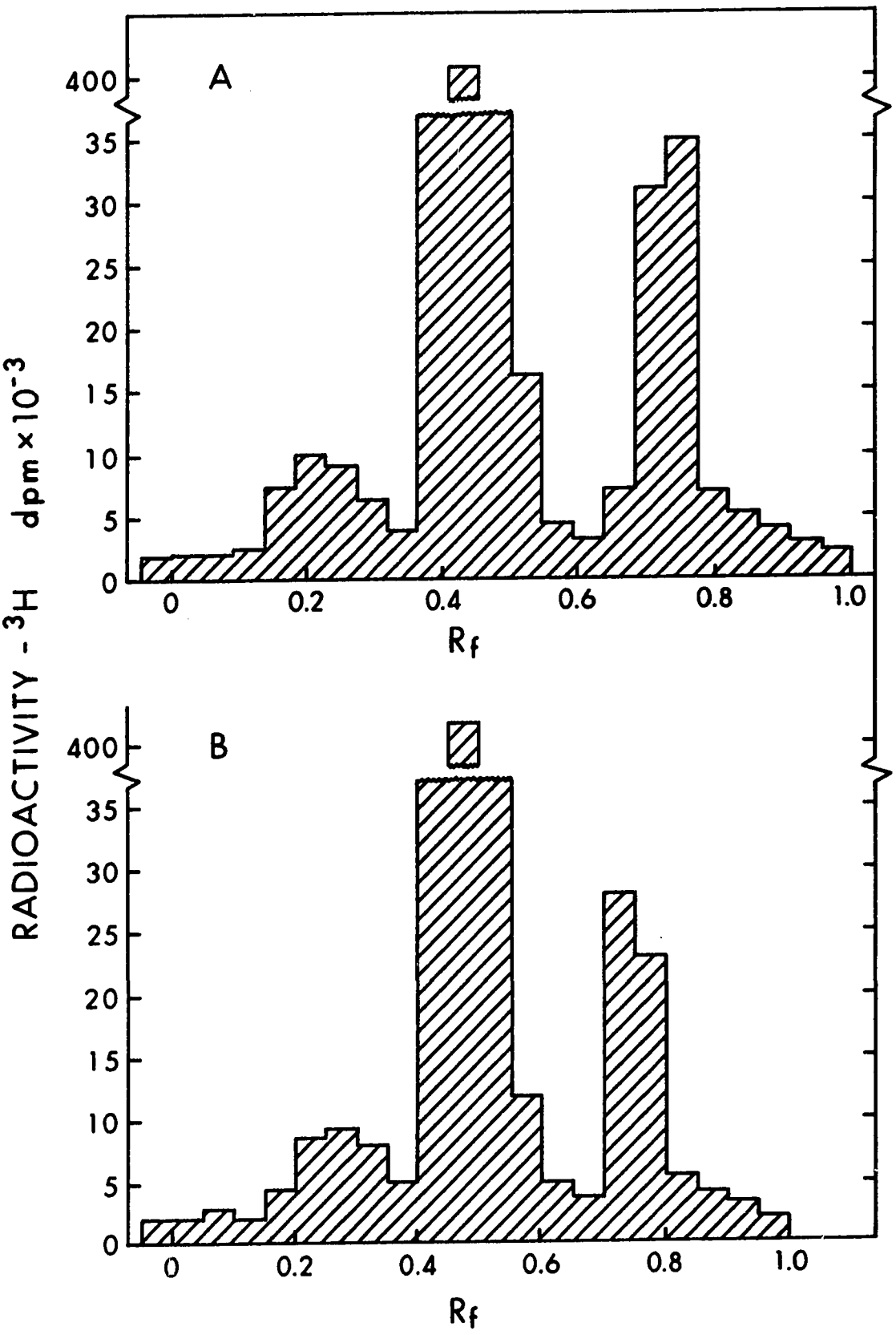
D. Variation in Dihydrouracil Dehydrogenase Activity with Leaf Age

By the spectrophotometric assay dihydrouracil dehydrogenase activity was detectable in leaves 16 to 25 days old (growth conditions #1). No detectable dihydrouracil dehydrogenase activity was noted in either younger leaves or those of day 14. Similarly leaves of about 30 days old had no enzyme activity. The assay was only used to detect activity and comparisons of enzyme activity were not made because of the lability of the preparation.

E. Studies with the Freeze-Dried Preparations

Leaves about 20 days old were used for freeze-dried preparations. (Supernatant preparations from 16 to 25 day old leaves showed the presence of uracil catabolic enzymes). A freeze-dried, desalted 30 to 45% ammonium sulphate protein fraction was most active toward the conversion of uracil-6-^{3}H to β-ureidopropionic acid-^{3}H, 45 to 60% and 60 to 80% fractions, similarly prepared, contained considerably less activity. Low amounts of β-alanine-^{3}H were detected. Figure 11A shows the TLC separation of an incubation mixture containing the above active preparation. A stimulation by NADPH of β-ureidopropionic acid formation

Figure 11. Thin layer chromatograms showing the metabolism of uracil-6-^{3}H by a freeze-dried protein preparation. The freeze-dried preparation was described in Section F.1., Chapter II. Reaction mixtures contained 0.18 μmoles uracil, 1.85×10^{6} dpm uracil-6-^{3}H (8.9 mCi/μmole), 100 μmoles potassium phosphate buffer, pH 7.4, and 20 mg freeze-dried enzyme preparation in a final volume of 2.0 ml. (A) contained 1.0 μmole NADPH and (B) was the control (0 NADPH). Incubation time was 15 min at 37°. The reaction was stopped with 0.2 ml 40% cold trichloroacetic acid. Protein was removed by centrifugation and 100 μl of the supernatant was spotted on 0.5 mm cellulose TLC plates, chromatographed in n-butanol-ethanol-water (4:1:5 v/v/v, top layer). Samples were scraped and counted as outlined in Chapter II. The radioactivity shown has been corrected for sample application size. R_fs : β-alanine, 0.21; dihydrouracil, 0.44; uracil, 0.48; and β-ureidopropionic acid, 0.67.


A
B
400
35
30
25
20
15
10
5
0
0
0.2
0.4
0.6
0.8
1.0
R_f
RADIOACTIVITY - ^3H dpm x 10^{-3}

over the control was noted (A vs. B). However, a considerable conversion to β-ureidopropionic acid, in the absence of NADPH, was also noted (Figure 11B). Results similar to those obtained in Figure 11 were obtained from reactions with uracil-6-^{3}H and supernatant preparations.

Since NADPH is a cofactor for the first step in the reaction, it was possible that the enzyme preparations contained bound NADPH. Alcohol dehydrogenase and its substrate acetaldehyde were selected as a possible mechanism for utilizing NADPH in the control mixtures. The rapidity of the alcohol dehydrogenase reaction is shown in Figure 12. Alcohol dehydrogenase and acetaldehyde, when pre-incubated with the freeze-dried preparation and incubation medium prior to the addition of uracil-6-^{3}H, was able to negate the background conversion of β-ureidopropionic acid (Figure 13A).

Quantitative results of radioactivity determinations on the TLC separations shown in Figure 11 are tabulated in Table 7. The n-butanol-ethanol water solvent system (4:1:5 v/v/v) was not able to separate uracil and dihydrouracil. However t-butanol-methylethyl ketone-water-ammonia (4:3:2:1 v/v/v/v) was capable of separating uracil and dihydrouracil and showed no detectable dihydrouracil in the incubation mixture (Figure 13B). It should be noted that the quantities of β-alanine-^{3}H in Figure 11A and Figure 13B are different. The uracil-6-^{3}H contained about 0.9% impurity which chromatographed with a similar R_f to β-alanine in the n-butanol-ethanol-water system. In the t-butanol-methylethyl ketone-water-ammonia system this impurity spread over the chromatogram.

Figure 12. Alcohol dehydrogenase oxidation of NADPH with acetaldehyde as substrate. The reaction mixture contained 1 mg alcohol dehydrogenase, 0.30 µmoles NADPH, 0.36 µmoles acetaldehyde, and 47.5 µmoles potassium phosphate buffer in a final volume of 1.0 ml. Incubation was at 37°; absorbance measurements were made at 340 mµ as described in Chapter II.

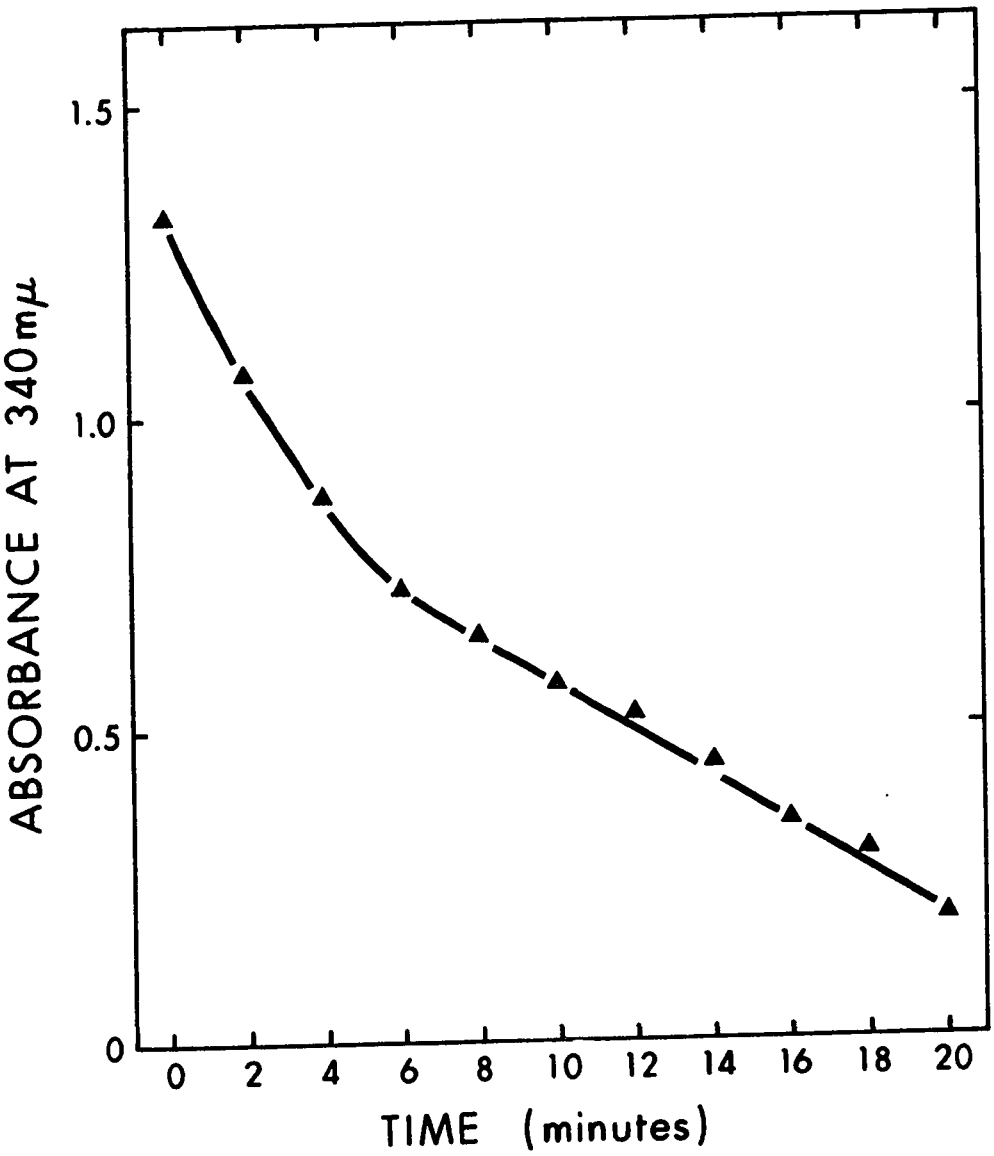

ABSORBANCE AT 340mμ
1.5
1.0
0.5
0
0
2
4
6
8
10
12
14
16
18
20
TIME (minutes)

Figure 13. Thin layer chromatograms showing the metabolism of uracil-6-^{3}H by a freeze-dried preparation with an alcohol dehydrogenase pre-incubation, with no additions, and with sodium salicylate added. The freeze-dried preparation was described in Section F.1., Chapter II. Reaction mixtures contained 0.18 µmoles uracil, 1.85×10^{6} dpm uracil-6-^{3}H (8.9 mCi/ µmole), 1.0 µmole NADPH, 100 µmoles potassium phosphate buffer, pH 7.4, and 20 mg freeze-dried enzyme preparation in a final volume of 2.0 ml. In addition (A) contained 1 mg alcohol dehydrogenase and 1.5 µmoles acetaldehyde and was pre-incubated for 4 min prior to the addition of uracil-6-^{3}H. (C) contained 10 µmoles sodium salicylate. Incubation time was 15 min at 37°. The deproteinized supernatant (100 µl) was applied on 0.5 mm cellulose TLC plates. (A) and (C) were separated with n-butanol-ethanol-water (4:1:5, v/v/v, top layer) and (B) was separated with t-butanol-methyethyl ketone-water-ammonia (4:3:2:1 v/v/v/v). R_f values in the latter solvent system were: β-alanine 0.32; β-ureidopropionic acid, 0.34; uracil, 0.38; and dihydrouracil, 0.67. The radioactive measurements have been corrected for sample application size.

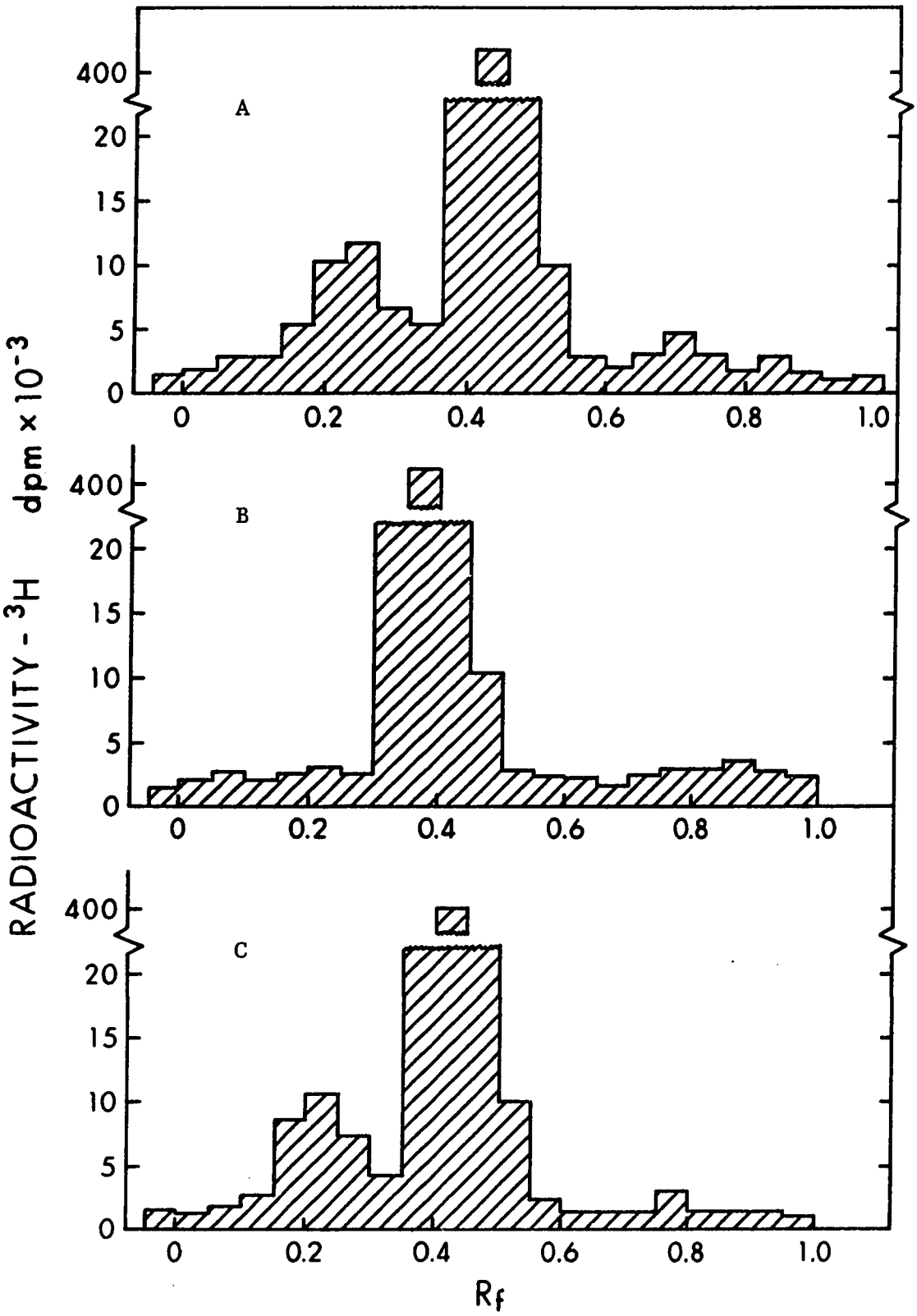

RADIOACTIVITY - ^3H dpm x 10^{-3}
A
B
C
400
20
15
10
5
0
0
0.2
0.4
0.6
0.8
1.0
R_f

Table 7. Quantitative determination of β-alanine and β-ureidopropionic acid on TLC chromatograms.

Figure number	Total label determined			
	β-alanine-^{3}H		β-ureidopropionic acid-^{3}H	
	dpm	%	dpm	%
11 A (+ NADPH)	24,130	1.3	70,300	3.8
11 B (- NADPH)	20,629	1.1	46,048	2.5

The TLC chromatograms were scraped and individual samples were counted as described in Chapter II. Data contained in this table has been corrected for background absorption as determined by counting samples at either end of the TLC chromatogram.

The effects of salicylate (an activator of dihydrouracil dehydrogenase in the animal system (Reichard and Skold, 1963) and reduced glutathione were studied on the enzyme system. Both glutathione and salicylate eliminated any conversion to β-ureidopropionic acid (Figure 13C). Boiling of the enzyme preparation prior to incubation gave a similar chromatogram. The peak on the TLC chromatogram at the R_f of β-alanine was the impurity mentioned above. No detectable dihydrouracil was present in the reaction mixture. If the first step in the conversion of uracil to β-alanine were rate limiting in the plant system, as it is in the animal system, dihyrouracil when formed would be rapidly converted to β-ureidopropionic acid. This appeared to be true. As will be shown later, β-alanine was produced from uracil-6-^{3}H in intact leaves. The absence of a rapid conversion of β-ureidopropionic acid to β-alanine could be attributed to a labile enzyme, a low activity of the enzyme in vitro or the nonexistence of the enzyme in the 30 to 45% ammonium suphate protein fraction.

The above experiments indicated the presence of an active system for the catabolism of uracil to β-alanine in bean leaves. The incorporation of uracil into RNA decreased with increasing leaf ages. No definite pattern of labeled β-alanine levels and leaf age was realized.

CHAPTER VII

FEEDING OF OROTIC ACID TO BEAN LEAVES

A. The Incorporation of Orotic Acid into Excised Bean Leaves

The ethylene evolution pattern has been observed to show a two peaked profile; one peak for young leaves, the second peak for older leaves (Chapter III). RNA analysis have indicated extensive decreases in the ribosomal RNA complement at older leaf ages (Chapter V). The possibility existed that some of the RNA degradation products could serve as ethylene precursors. Feeding experiments with orotic acid-6-^{14}C were attempted for several reasons. The first, to check the possibility of C_2H_4-^{14}C being evolved when orotic acid-6-^{14}C was fed. Secondly, to estimate the net RNA synthesis of leaves at various ages by studying the amount of orotic acid incorporated into RNA. Thirdly, to estimate the *in vivo* conversion of orotic acid-6-^{14}C to uracil and β-alanine, probable precursors of ethylene. Orotic acid-6-^{14}C was chosen to study the conversion to ethylene because uracil-6-^{14}C was not readily available from commercial sources. Uracil-2-^{14}C was no value because in the normal degradation pathway C-2 is lost as $^{14}CO_2$ when β-alanine is formed. With the use of a C-6 label both β-alanine and ethylene, if formed would be ^{14}C labeled. If uracil-6-^{3}H were supplied, any C_2H_4-^{3}H formed would not be as easily detectable as C_2H_4-^{14}C on the gas radiochromatograph.

Leaves were excised from plants grown under growth condtions #2 (Chapter II). Collection flasks contained 10 g drierite to hasten label uptake. Feeding time with the label was 4 h unless otherwise

specified. At the end of this time leaves were frozen and extracted as outlined in Chapter II. After the metabolites were extracted the remaining residue was extracted for RNA with the boiling NaCl-KOH procedure (Chapter II).

B. The Production of Ethylene-^{14}C from Orotic Acid -6-^{14}C

No detectable C_2H_4-^{14}C was obtained from any orotic acid-6-^{14}C feeding. This is understandable if one considers conversions obtained to date. Maximum orotic acid-6-^{14}C to β-alanine-^{14}C has been about 10% (Table 8). Maximum conversion of β-alanine-2-^{14}C to C_2H_4-^{14}C calculated from results in Table 12, Chapter IX has been about 0.0034%. On the basis of these conversion figures, a feeding of 22.2 x 10^6 dpm orotic acid-6-^{14}C would be expected to produce 75 dpm of ethylene. This would not be detectable by GRC. In a separate experiment, 55.0 x 10^6 dpm of orotic acid-6-^{14}C (22.6 μCi/μmole) was fed to 4 leaves at day 10 (growth conditions #2) for 4 h. No C_2H_4-^{14}C was detected.

C. The Production of $^{14}CO_2$ from Orotic Acid-6-^{14}C

In the first feeding experiment (Table 8, Experiment A) unlabeled β-alanine was added to trap most of the β-alanine-^{14}C formed. The results in Table 8, Experiment A indicate about equal amounts of $^{14}CO_2$ formed for the 3 ages studied.

In the second experiment (Table 8, Experiment B) where no unlabeled β-alanine was added, considerably more $^{14}CO_2$ was produced. Leaves at the day 6 produced 9,300 dpm while those at the day 11 produced

Table 8. The conversion of orotic acid-6-^{14}C to various metabolites at different leaf ages.

	Leaf age	Label fed	$^{14}CO_2$	Orotic acid recovered	Orotic acid conversion to:					
					β-alanine		Uracil		RNA	
	days	dpm x 10^{-6}	dpm	%	dpm x 10^{-6}	%	dpm x 10^{-6}	%	dpm x 10^{-6}	%
	7	12.3	670	4.5	1.25	10.2	0.15	1.2	0.33	2.7
A	8	9.9	625	9.7	1.72	17.4	3.44	34.7	0.12	1.2
	9	22.2	765	9.8	2.23	10.3	4.73	21.3	2.44	1.1
	6	10.3	9,310	12.2	1.03	10.3	0.43	4.2	0.70	6.8
B	8	10.6	20,980	4.1	0.76	7.2	4.15	39.1	0.42	4.1
	9	10.6	17,020	3.8	0.50	4.7	4.21	39.6	0.38	3.7
	11	10.7	22,950	3.2	0.43	4.1	4.28	42.8	0.32	3.1

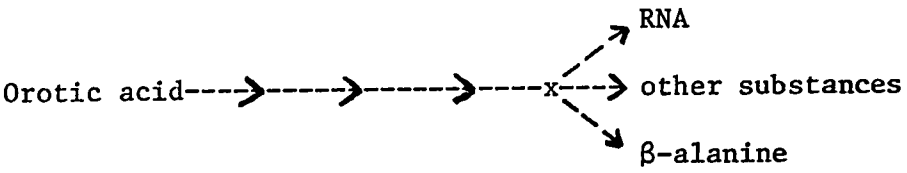
Bean plants were grown under growth conditions #2. Label feeding and extraction techniques were described in Chapter II. Label uptake was complete in all experiments. (A) Four leaves were fed orotic acid-6-^{14}C (22.6 μCi/μmole) for 4-1/2, 4-3/4, and 4-3/4 h at the 7, 8, and 9 day age, respectively. Nine mg unlabeled β-alanine was supplied with the label. (B) Four leaves were fed orotic acid-6-^{14}C (22.6 μCi/μmole) for 4 h at each age. No unlabeled β-alanine was added to the label.

about 23,000 dpm. These data could be interpreted in a number of ways.

(a) Orotic acid-6-^{14}C penetrated the 6 day old leaves more slowly than the 11 day age.

(b) If $^{14}CO_2$ came only from β-alanine-2-^{14}C, the pathway of orotic acid to β-alanine functions slower in the younger leaves.

(c) The concentration of an intermediate metabolite(s) (x) from orotic acid changes with age.



i.e. x could be more readily incorporated into RNA in young tissue than in older tissue and thus would not be available to be degraded to β-alanine and subsequently to CO_2.

(d) If all $^{14}CO_2$ came from β-alanine-^{14}C, the endogenous β-alanine level could be higher in the younger leaves thereby diluting the newly formed β-alanine-^{14}C. This would result in less $^{14}CO_2$ formed from this pool. This would be similar to the above experiment where unlabeled β-alanine was added to trap any β-alanine-^{14}C formed. This, in fact, gave a very low $^{14}CO_2$ production.

(e) A combination of the above.

D. Orotic Acid-6-^{14}C Incorporation into RNA at Various Leaf Ages

Orotic acid is incorporated rapidly into RNA and thus is used extensively to label RNA. The radioactivity from the NaCl-extracted, KOH-digested material was estimated. The assumption was made that all the label came from RNA. Less than 0.1% of extractable metabolites remained after the ethanol extractions. The results in Table 8, Experiment A and B, indicate a conversion of orotic acid-6-^{14}C into RNA. In Table 8, Experiment B, 6.82% of the label fed to the day 6 leaves was present in RNA. This decreased to 3.14% in the day 11 leaves. This coincides with results obtained with MAK column estimation of total RNA. Less RNA was present in the older tissues. One would assume that RNA synthesis would be curtailed if the total RNA was decreasing. Thus the results of Table 8 indicate that the RNA synthetic ability of the leaves decreases with increasing age of the leaves. This argument assumes that the basic assumption that no pool changes in intermediates between orotic acid and RNA occurred.

E. The Conversion of Orotic Acid-6-^{14}C to Uracil

Results in Table 8, Experiment A and B, indicate the youngest age in each case gave very low conversions of labeled orotic acid to uracil. Uracil is not on the direct pathway of orotic acid to RNA and thus would not appear as a metabolite of this pathway. In both experiments significant increases in the labeled uracil formed occurred as the leaf age increased. The larger amounts formed in the older ages could be explained by an increase in the rate of operation of the orotic

acid to uracil pathway, or by trapping of the labeled uracil by an increasing uracil pool size generated by degraded RNA. The latter idea may be the least probable because the same drastic change is not observed in the β-alanine labeling. If it were just a dilution phenomenon, a corresponding decrease in β-alanine-^{14}C formed might be expected. However other factors may limit the conversion of uracil to β-alanine.

F. β-alanine Formation from Orotic Acid

The initial experiment was designed to trap any β-alanine-^{14}C formed in the high β-alanine pool supplied. Results in Table 8, Experiment A, show that β-alanine-^{14}C was formed in varying amounts at the 3 ages studied. The varying results may not be significant because they are expressed as a per cent of label fed and the amount of label fed varied considerably.

In the second experiment (Table 8, Experiment B) where no exogenous β-alanine was applied, significant β-alanine-^{14}C was detected, but the amount formed decreased with the increasing age of the leaves. This decrease could be linked to *in vivo* changes in the uracil levels. If uracil is a metabolite between orotic acid and β-alanine, an increased uracil pool size could dilute any uracil-^{14}C formed thus having the effect of lowering the amount of β-alanine-^{14}C. Uracil pool sizes could be increasing because results above indicated less RNA synthetic ability of older leaves. MAK column results (Chapter V) indicated less total RNA in older leaves. This loss in RNA would lead to a net increase in the endogenous uracil pool.

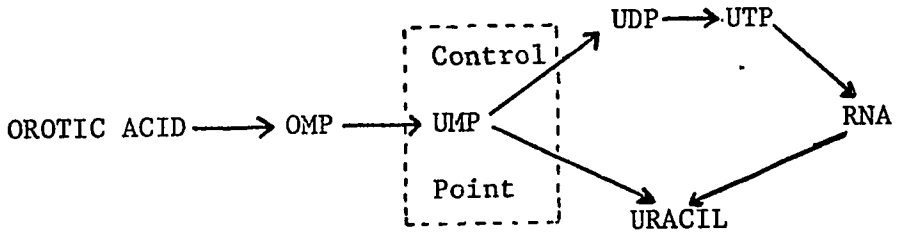
G. Metabolite Formation During Orotic Acid Feedings

Metabolites were extracted (Chapter II) and separated by TLC on 0.5 mm cellulose plates using an isopropanol-pyridine-water-acetic acid (8:8:4:1 v/v/v/v) solvent system. The Actigraph scan (Figure 14) of the extract indicated 7 radioactive peaks. Peaks, 2,4, and 7 represent β-alanine, orotic acid and uracil respectively. These were characterized by adding known labels with the extract. Peak 1 was very prominent at the day 6 stage and nearly non-existent at the 11 day age. It could possibly be an intermediate in RNA synthesis. Peaks 3, 5, and 6 do not change a great deal over the various ages.

H. Controls

Table 8, Experiment A and B, indicated that the age where the greatest decrease in incorporation of orotic acid into RNA occurred was the exact age where the greatest increase in uracil-^{14}C was detected. A control mechanism may exist.

i.e.



Possibly the rate of the RNA synthetic mechanism is inhibited or changed when the RNA begins to be degraded, orotic acid then is metabolized to uracil instead of the UMP to RNA sequence. A large increase in the uracil-^{14}C has been noted at this age. It was also at

Figure 14. Actigraph scan of a thin layer chromatographic separation of an extract from 9 day old bean leaves fed with orotic acid-6-^{14}C. Bean plants were grown under growth conditions #2. Four leaves were fed 22.2 x 10^6 dpm of orotic acid-6-^{14}C (22.6 μCi/μmole) for 4-3/4 h. The label extraction procedure was described in Chapter II. An isopropanol-pyridine-water-acetic (8:8:4:1 v/v/v/v) solvent system was used. Actigraph operating parameters were: time constant 5 sec, collimator slit-width 3 mm, scan speed 30 cm/h, operation voltage 1050 volts and a quench gas (98.7% helium, 1.3% butane) pressure of 7 psi. The full scale chart range is indicated on the Figure.

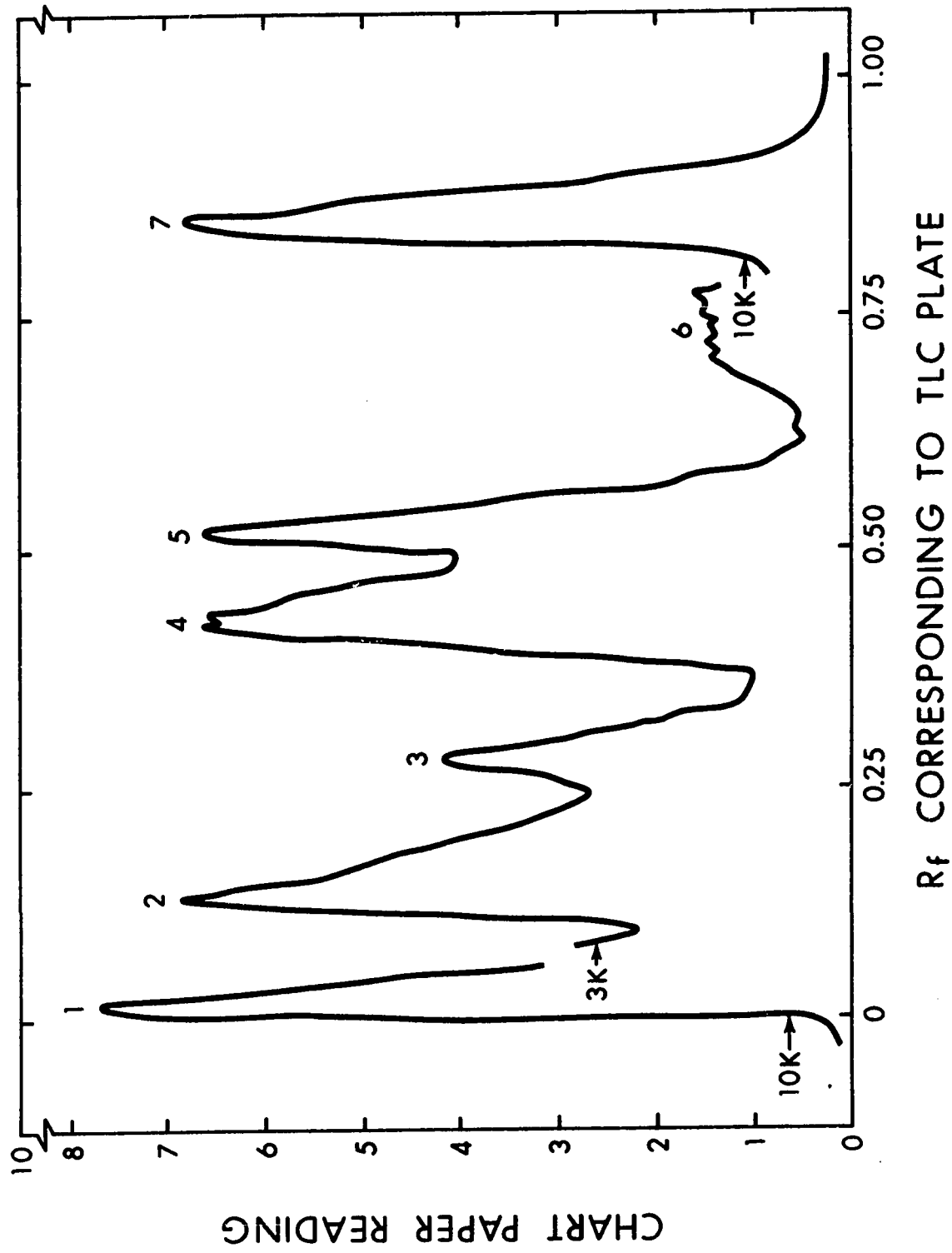


CHART PAPER READING
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8
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2
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10K
3K
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1
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5
6
7
0
0.25
0.50
0.75
1.00
Rf CORRESPONDING TO TLC PLATE

this very age that the major ethylene peak occurred. Could ethylene be:

(i) regulating the shut off of RNA synthesis thereby triggering senescence of the leaf?

(ii) or could a product formed by the metabolism of the increased uracil production (ie. β-alanine and/or ethylene) have a function elsewhere in the plant?

CHAPTER VIII

β-ALANINE, ENDOGENOUS LEVELS AND PRODUCTION

β-alanine has long been known to be a product of uracil metabolism in animal systems (Canellakis, 1956 and Fritzson, 1957). It has also been shown to be a product of uracil feeding in pine callus tissue (Barnes and Naylor, 1962). Thompson (1966) and Stinson (1968) have shown β-alanine to be a precursor of ethylene by using an enzyme system from germinating bean cotyledons. Results in Chapter III showed a two peaked ethylene production pattern during ageing of bean leaves. Either or both of these peaks could be related to changes in β-alanine metabolism. Buchowicz and Reifer (1964) showed an inhibition by β-alanine of uracil incorporation into polynucleotides in winter wheat. A decrease in total RNA, especially r-RNA, as leaves aged was shown in Chapter V, and would probably result in increased amounts of low molecular weight degradation products. An active uracil catabolic system producing β-alanine could be involved in the regulation of nucleic acid synthesis in older leaf ages.

A. Endogenous β-alanine Levels in Bean Leaves at Various Ages

Endogenous β-alanine levels, estimated over various ages as outlined in Chapter II, are shown in Table 9. The level was high initially, decreased to a minimum on day 7 and then increased to a maximum on day 13. Levels decreased after day 13. The β-alanine level on day 5 in bean leaves corresponded closely with endogenous levels in pine callus tissue (Barnes and Naylor, 1962). The peak on day 13

Table 9. Endogenous levels of β-alanine from bean leaves.

Leaf age days	Micrograms of β-alanine/g dry wt of leaf tissue
5	55.6
7	5.4
11	28.4
13	72.5
16	17.1

Bean plants were grown under growth conditions #2 as described in Chapter II. About 75 g (fresh wt) were harvested and analyzed as outlined in Chapter II. The results are based on one determination at each leaf age.

corresponded with the second ethylene evolution peak. It is plausible that the increased endogenous β-alanine levels could give rise to this second peak of ethylene. The initial high endogenous β-alanine level of day 5 does not coincide as well with the major ethylene peak at day 7, although ethylene-^{14}C was produced from β-alanine-2-^{14}C over 5 to 8 day ages (Chapter IX). Stinson (1968) showed that the first enzyme in the β-alanine to ethylene sequence required relatively high levels of β-alanine for the reaction to proceed.

B. β-alanine Production from Uracil Feeding to Bean Leaves

Table 10 shows that the β-alanine production from uracil varied with the age of the leaves. Production by young leaves was high, decreased by day 9, peaked from day 11 to 13 and decreased again on days 16 and 18. The peak around 11 and 13 days corresponded to the second ethylene peak as well as to the endogenous β-alanine peak. This could be indicative of an active uracil to β-alanine pathway at the 11 to 13 day age. Uracil could arise from enhanced RNA degradation at the older ages and thus serve as a source of β-alanine in these leaf ages.

The uracil to β-alanine pathway has been well documented by Canellakis (1956) and Fritzson (1957) in rat liver slices. Their findings indicated that the uracil to dihydrouracil conversion, mediated by dihydrouracil dehydrogenase, was a rate limiting step in the overall pathway. Dihydrouracil dehydrogenase could be inactive or slightly active in young leaves and become active or more active around the 11 to 13 day age. If dihydrouracil dehydrogenase activity were limting in leaves also, then the assumption was made that the remaining enzymes in the sequence have a relatively constant activity over all leaf ages.

Table 10. β-alanine levels in bean leaves fed with uracil.

Leaf age days	Micrograms β-alanine/g dry wt of leaf tissue
5*	537
6	637
9	285
11	345
13	343
16	275
18	215

Bean leaves were grown under growth condition #2. Leaves were fed with a 22.3 mM uracil solution for 16-1/2 h at 24° in the dark. β-alanine determinations were made as described in Section H, Chapter II. *This sample was fed for only 7 h. The results are based on one determination at each leaf age. The results have not been corrected for zero time controls.

C. The Conversion of Uridine to β-alanine at Three Bean Leaf Ages

The estimations (Table 11) indicate an increased ability at day 10 to produce β-alanine from uridine, over that of day 5. The 15 day age was low relative to days 5 and 10. The β-alanine levels shown in Table 11 are actually a combination of the low but varying endogenous β-alanine levels in Table 9 and the results of β-alanine production from uridine and β-alanine utilization by the cell metabolism.

D. β-alanine Formation from Dihydrouracil Feeding

The figures for dihydrouracil feeding in Table 11 do not show the same increase in β-alanine at day 10 as was apparent in the uridine feeding. The bean leaves were able to produce four and one half times as much β-alanine from dihydrouracil at day 15 as from uracil at day 16. The high β-alanine production was probably indicative of an active pathway from dihydrouracil to β-alanine over the ages studied. The marked differences between the results of uracil and dihydrouracil feedings (Table 10 and 11 respectively) show that dihydrouracil dehydrogenase is rate limiting in the conversion of uracil to β-alanine. Feedings with uracil resulted in a β-alanine peak at days 11 and 13 and the uridine feeding showed a β-alanine increase at day 10. Since dihydrouracil feeding showed a high level over the ages studied, it might be concluded that the activity of dihydrouracil dehydrogenase was responsible for the observed β-alanine peak and possibly the endogenous β-alanine peak.

Table 11. β-alanine levels in bean leaves fed with uridine and dihydrouracil at three different ages.

Material fed	Leaf age days	Micrograms β-alanine/g dry wt of leaf tissue
1. Uridine	5	370
	10	424
	15	113
2. Dihydrouracil	5	2155
	10	1900
	15	1243

Bean leaves were grown under growth conditions #2. About 25 g of leaves were fed with 22.3 mM uridine and dihydrouracil solution respectively for 16-1/2 h at 24° in the dark. β-alanine determinations were as detailed in Section H, Chapter II. The results are based on one determination at each leaf age. The results have not been corrected for zero time controls.

CHAPTER IX

CONVERSION OF β-ALANINE TO ETHYLENE

A. The Requirement for Rapid β-alanine-2-^{14}C Uptake

Excised leaves were used for all feeding experiments. A rapid uptake of label was required because total ethylene production decreased after the leaves were removed from the intact plant (Table 12, Experiment A). Twice the amount of ethylene was produced in the initial 4 h period after excision as in the succeeding 4 h interval. Thus a low C_2H_4 -^{14}C production may not be indicative of a lack of a mechanism to convert β-alanine-2-^{14}C to C_2H_4-^{14}C but rather could result from a slow penetration of the label to the site of ethylene synthesis.

B. The Effect of the Specific Activity of β-alanine on Ethylene-^{14}C Production

In initial experiments β-alanine-2-^{14}C (0.417 μCi/ μmole) was used. In one experiment 63.47 x 10^6 dpm was fed into six excised leaves through petioles that had been cut under water. Results in Table 12, Experiment A showed a decrease in total ethylene produced in the successive collections. $^{14}CO_2$ production increased, indicative of β-alanine-2-^{14}C having entered the tissue and having been metabolized. Low initial rates of $^{14}CO_2$ production may indicate slow penetration of the label. Radioactivity was only detected in ethylene in the initial 4 h collection period. This was reasonable because the ability of the leaves to produce ethylene decreases with time. In the 20 to 32 h

Table 12. Ethylene, ethylene-^{14}C and $^{14}CO_2$ production during β-alanine-2-^{14}C feeding of bean leaves

Experiment number	Collection period h	Total ethylene mμl	Ethylene-^{14}C dpm	$^{14}CO_2$ dpm
A	0-4	99.0	352	3,170
	4-8	47.5	-	5,475
	8-20	40.5	-	32,580
	20-32	3.0	-	55,750
	32-44	6.5	-	94,800
	44-56	-	-	312,200
	56-68	-	-	592,000
B	0-4	75.0	-	2,360
	4-9 1/2	25.0	-	21,443
C (i)	0-5 1/2	65.0	1065	10,000
	0-5	47.5	175	12,000
(ii)	5-11	21.0		35,000

All leaf tissue was grown under growth conditions #1. Label feeding and ethylene collection procedures were outlined in Chapter II. Ten g drierite was added to the collection chamber to hasten label uptake. Small feedings of water were added after label uptake was complete. The following amounts of label were fed.

A. 63.47×10^6 dpm β-alanine-2-^{14}C (0.417 μCi/μmole) was fed to 4 leaves at 18 days old.

B. 40.0×10^6 dpm β-alanine (3.43 μCi/μmole) was fed to 4 leaves at 19 days old.

C. (i) 32.7×10^6 dpm β-alanine-2-^{14}C (3.43 μCi/μmole) with 3.0 mg unlabeled β-alanine added was fed to 4 leaves at the 18 days old.

(ii) 30.74×10^6 dpm β-alanine-2-^{14}C (3.43 μCi/μmole) with 3.0 mg unlabeled β-alanine added was fed to 4 leaves at 19 days old.

period the ethylene production is very low; thus any C_2H_4-^{14}C, if the specific activity were similar to the initial 4 h collection, would not be detectable.

A different supply of β-alanine-2-^{14}C was then obtained having a specific activity of 3.43 μCi/μmole. In experiments with this material (Table 12, Experiment B) no C_2H_4-^{14}C was detected, although significant $^{14}CO_2$ was produced, indicating the penetration of β-alanine-2-^{14}C into the tissue. However when unlabeled β-alanine was added to bring the specific activity down to that used in the initial experiment (Table 12, Experiment A), C_2H_4-^{14}C was produced (Table 12, Experiment C).

This suggests that a certain β-alanine concentration is required for the β-alanine to ethylene pathway to operate. Stinson (1968) found that 5 mM or higher β-alanine was required for ethylene formation with a solubilized particulate enzyme system from bean cotyledons.

C. <u>The Effects of Leaf Age and Added β-alanine on Ethylene-^{14}C Production</u>

The C_2H_4-^{14}C production was apparent in the younger leaves and decreased or was absent in the older leaves (Table 13). β-alanine-2-^{14}C (3.43 μCi/μmole) was fed in all experiments. Since at day 9 the leaves were still producing ethylene and no radioactivity was detected possibly β-alanine-2-^{14}C was not reaching the site of ethylene formation because of permeability changes. Equally plausible would be a dilution of the applied label by increasing endogenous β-alanine levels. The latter

Table 13. Ethylene -^{14}C produced leaves at various ages with no added β-alanine and with varying amounts of added β-alanine.

Experiment Number	β-alanine added mg	Age of leaves in days 5	6	7	8	9
1	0				-	
	1.2	312	-	-	312	-
	3.0	437	trace	-		-
2	0		625	294	trace	
	1.2		550	125	-	
3	9			800	-	

Approximately 13.2 x 10^6 dpm β-alanine-2-^{14}C (3.43 μCi/μmole) was fed to 4 leaves (growth conditions #2) for each determination. Ethylene collection was continuous during the 4 h collection period. Ten g drierite was added to the culture flask to hasten label uptake. Ethylene collection and analysis followed procedures outlined in Chapter II. (-) indicates < detectable radioactivity.

idea appears attractive when one considers experiment (2) with 1.2 mg unlabeled β-alanaine added. At all ages in experiment (2) less C_2H_4-^{14}C was produced in each collection where unlabeled β-alanine was added than the collections with no unlabeled β-alanine additions. Ethylene-^{14}C production with varying levels of unlabeled β-alanine and studies with β-alanine of different specific activities probably indicate that the endogenous β-alanine concentration is critical to the ethylene production. Only when suitable exogenous β-alanine was added to bring the endogenous concentration within this specific range did one detect β-alanine-2-^{14}C metabolized to C_2H_4-^{14}C.

D. Conversion of β-alanine-2-^{14}C to $^{14}CO_2$

$^{14}CO_2$ was produced whenever β-alanine-2-^{14}C was fed to excised leaves (Table 12). The labeled CO_2 can arise from a branch or branches of the β-alanine to ethylene pathway (Chapter I) as outlined by Stinson (1968). In no experiment did the $^{14}CO_2$ produced exceed 5% of the label fed to the excised leaves. The rate of $^{14}CO_2$ production may indicate the rate of entry of the label into the cell. This is suggested by the fact that $^{14}CO_2$ production was higher in the presence of drierite in the metabolism chamber than in its absence. The effect was especially pronounced in the 0-4 h collection period.

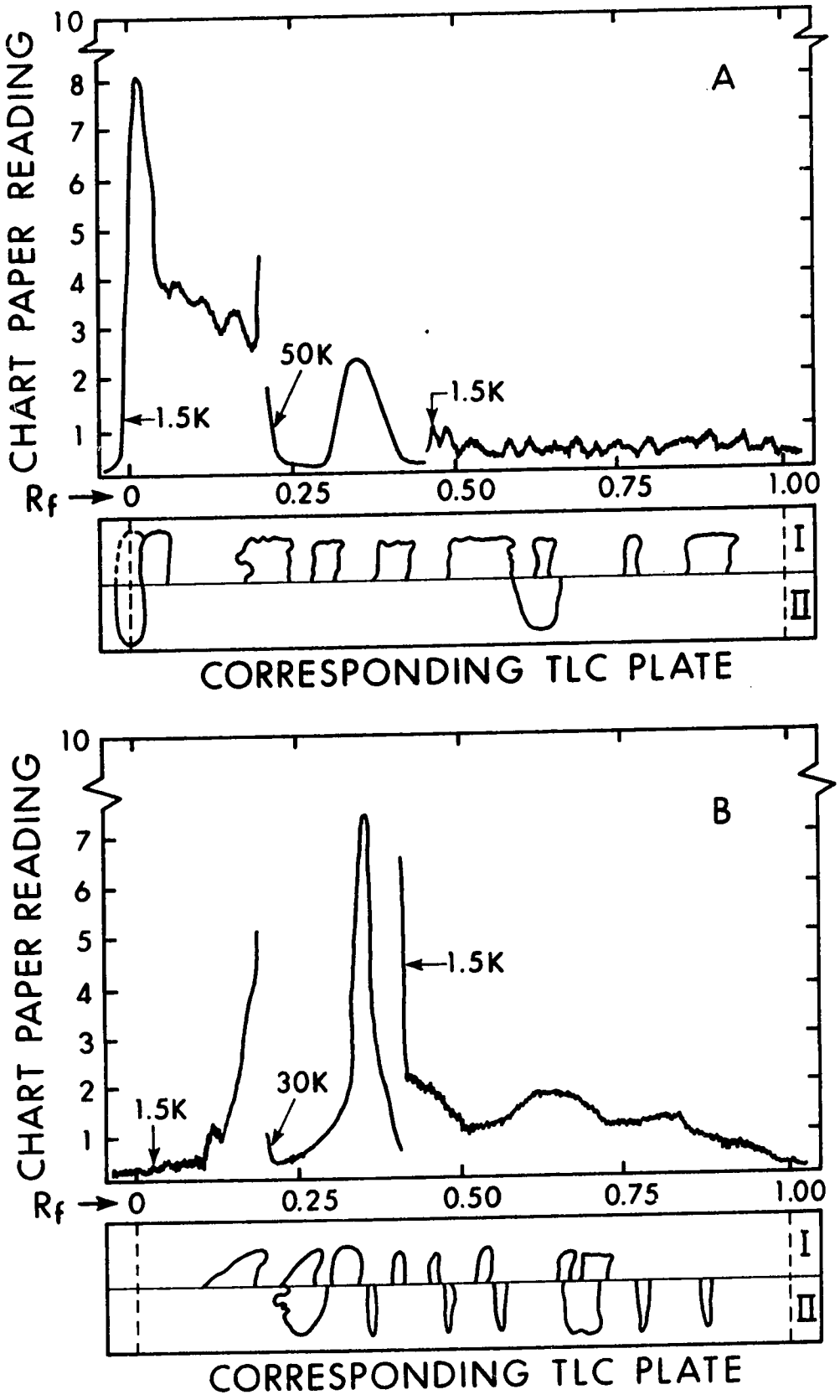
E. Estimation of Unmetabolized β-alanine after Feeding Experiments

After the C_2H_4-^{14}C collections were completed the leaves were homogenized and extracted as outlined under Section J, Chapter II. The extract was separated by ascending TLC on 0.5 mm cellulose plates.

With an ethanol-ammonia (120:30 v/v) solvent system, 2 radioactive peaks were evident on an Actigraph scan of the plate (Figure 15A). Using a n-butanol acetic acid-water (4:1:5 v/v/v, top layer) solvent system only one radioactive peak was present (Figure 15B). The regions of the 2 peaks from Figure 15A and the one from Figure 15B were scraped from the plate and placed in scintillation vials with Cab-O-Sil for counting. Results indicated that counts on plate A equalled counts on plate B. A sugar detecting spray indicated most of the sugars on plate A were near the origin. Sugars on plate B had separated over the length of the plate. It appeared that some of the β-alanine was bound to the sugars and remained so in the basic solvent system. However these bonds were severed in the acidic solvent system and the previously bound counts moved with the major β-alanine peak. Phan, (1960) has shown that β-alanine can bind to sugars, the bonds being labile in acidic conditions.

Results from TLC with an acidic solvent system indicated that up to 95 per cent of the fed label could be recovered as unmetabolized β-alanine-2-^{14}C. Considering the maximum 5 per cent $^{14}CO_2$ produced and small amounts of C_2H_4-^{14}C, most of the β-alanine was thus accounted for.

Figure 15. Actigraph scans of thin layer chromatographic separations of an extract from bean leaves fed with β-alanine-2-^{14}C. Four 20 day old bean leaves (growth conditions #1) were fed with 44.4 x 10^6 dpm β-alanine-2-^{14}C (3.43 μCi/μmole) for 4 h. The leaves were extracted as outlined in Chapter II. Plate A was chromatographed in an ethanol-ammonia (120:30 v/v) solvent and plate B in a n-butanol-acetic acid-water (4:1:5 v/v/v, top layer) solvent system. Each plate was divided and sprayed for (1) amino acids by the conventional ninhydrin spray and (11) sugars by the method of Trevelyan et al. (1950). The actigraph operating parameters were: time constant 5 sec, collimator slit-width 3 mm, scan speed 30 cm/h, operating voltage 1050 volts and a quench gas (98.7% helium,1.3% butane) pressure of 7 psi. The full scale chart range is indicated on the figure.


A
CHART PAPER READING
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1.5K
50K
1.5K
Rf → 0
0.25
0.50
0.75
1.00
I
II
CORRESPONDING TLC PLATE
B
CHART PAPER READING
10
7
6
5
4
3
2
1
1.5K
30K
1.5K
Rf → 0
0.25
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I
II
CORRESPONDING TLC PLATE

CHAPTER X

GENERAL DISCUSSION AND CONCLUSIONS

This work was directed towards investigation of the possible linkage between the RNA decrease in ageing bean leaves and ethylene production. It involved studies of the catabolic pathway for uracil, endogenous β-alanine levels, β-alanine conversions to ethylene, and the metabolism of selected compounds. In the past decade considerable research has been carried out on RNA metabolism in ageing tissue. It is generally agreed that RNA decreases as tissue age increases. The fate of the RNA that disappears has not been elucidated. Barnes and Naylor (1962) provided evidence indicating the presence of a catabolic system for uracil with a production of β-alanine in pine callus tissue. The metabolites detected indicated a similar pathway to that operating in animal tissue. That β-alanine is a precursor of ethylene in bean cotyledon systems is based on conclusions of recent theses from this laboratory.

Detached primary wax bean leaves were used throughout this investigation. The detached tissue would not be expected to be an exact expression of the metabolic state of an intact leaf. However detachment facilitated ethylene and carbon dioxide collections and label feedings.

Two different sets of growth conditions were used in the study. The two conditions had different day lengths, light levels, temperatures, and humidity control. The two conditions used resulted from greenhouse space availability rather than a desire to

study ethylene production under the two sets of conditions. Both sets gave essentially an identical ethylene production pattern, the difference being that the pattern obtained with growth conditions #1 (lower light level, lower temperature than growth conditions #2) was extended over a longer age period. Since actual time after germination thus could not be used, ethylene production was used to identify relative leaf age for comparisons between the two sets of conditions. Under both sets of conditions, the two-peaked ethylene production profile was always obtained; a major peak followed by a minor peak. At the major ethylene production peak, the primary leaves had just reached full expansion and the cotyledons had withered and commenced to fall. This was true for both sets of growth conditions.

A study of the RNA levels in the bean leaves over an extended period indicated marked decreases in the heavy-r-RNA component. Other RNA fractions decreased to a lesser extent. To ascertain whether this decrease was a result of an increased degradation or a decreased synthesis of RNA, incorporation studies were designed. The highest incorporation of either $^{32}P_i$, uracil-6-^{3}H, or orotic acid-6-^{14}C into RNA occurred in the youngest leaves studied in both sets of conditions. MAK column fractionation of RNA from leaves of increasing age showed no new detectable components in the older leaves compared to the younger ones. The degraded RNA must have been broken down to segments not retained by the RNA precipitation and dialysis procedure. RNA synthesis was shown to decrease and the total RNA complement decreased.

Because the possibility existed for the production of low molecular weight compounds from the degradation of RNA, it was of

paramount importance to show the conversion of uracil to β-alanine in bean leaves. Young adult leaves produced significant conversions of uracil-6-^{3}H to β-alanine-^{3}H while older leaves produced less. In studies where unlabeled uracil was fed to leaves grown under growth conditions #2, young leaves showed high levels of β-alanine, then a decrease, and a further increase at days 11 to 13 (the minor ethylene production peak age). Endogenous β-alanine levels also were initially high, decreased and then showed a secondary increase coinciding with the minor ethylene peak.

Fritzson (1959) has shown dihydrouracil dehydrogenase to be a rate limiting enzyme in rat liver tissue. The detection of this enzyme's activity in leaf tissue was important as an indication of the operation of the catabolic pathway from uracil to β-alanine. Dihydrouracil dehydrogenase activity was shown in 20 to 26 day old bean leaves grown under growth conditions #1. The activity was not detected in very young leaves (day 14) or in very old leaves (day 30). The presence of the activity between days 20 to 26 inclusive confirmed the existence of at least the first step of the uracil to β-alanine pathway. Reactions with a partially purified enzyme preparation from bean leaves, and uracil-6-^{3}H showed the conversion of the uracil to β-ureidopropionic acid. The absolute cofactor requirement of NADPH was shown; NADH would not act as a substitute.

For the second enzyme, dihydrouracil hydrase, no cofactor requirement was found in the plant system. The lack of detection of dihydrouracil in either the <u>in vivo</u> feedings or the <u>in vitro</u> incubations of bean leaves was not unexpected. If the enzyme is rate

limiting in the plant system as it is in the animal system, dihydrouracil when formed would be converted to β-ureidopropionic acid and β-alanine, and would not accumulate. In the feedings of unlabeled dihydrouracil to detached leaves, very high levels of β-alanine formed compared to the amounts formed with the uracil feedings at the ages studied. This is suggestive that the operation of the pathway from uracil to β-alanine is present at all ages, but the enzyme catalyzing first step, dihydrouracil dehydrogenase, is more active at certain ages. The presence of β-alanine and β-ureidopropionic acid formed from uracil-6-^{3}H confirmed the presence in bean leaves of a catabolic pathway similar to that proposed by Barnes and Naylor (1962).

β-alanine was shown to be formed from each of orotic acid, uracil, and uridine. That β-alanine acted as a precursor of ethylene in bean leaves was shown by the formation of ethylene-^{14}C from β-alanine-2-^{14}C. Ethylene-^{14}C was only produced from leaf ages comparable to the major ethylene peak. No ethylene-^{14}C was detectable when β-alanine-2-^{14}C was fed at the minor ethylene peak age. The actual concentration was important to ethylene formation because at some ages the addition of unlabeled β-alanine to the β-alanine-2-^{14}C was required to produce ethylene-^{14}C. This probably was the result of varying endogenous β-alanine levels. Stinson (1968) showed that at least 5mM β-alanine was required for ethylene formation from a solubilized subcellular preparation from wax bean cotyledons. Thus the observed variation from 70 to 5 μg β-alanine per g dry wt leaf tissue, at the compartmentalized subcellular or enzyme level, would probably be adequate to alter the pathway operation.

The changing endogenous β-alanine levels could result in the observed two-peaked ethylene production profile. β-alanine has been shown to inhibit the incorporation of uracil into polynucleotides (Buchowicz and Reifer, 1964). One can speculate that the initial high β-alanine levels in the younger leaves may inhibit RNA synthesis by curtailing the incorporation of uracil. This impairment of RNA synthesis may be one signal that triggers the senescence of the leaf. The second ethylene peak could result from the increased β-alanine levels produced from the uracil generated from RNA and converted by the active dihydrouracil dehydrogenase. Another observation suggesting the operation of a complicated control mechanism was noted during studies on the conversion of orotic acid to β-alanine. Very low conversions to uracil were noted in very young leaves (about 1.2%) and this changed to 34% within one or two days. This could reflect a control mechanism switching off the RNA synthetic mechanism and increasing the degradation pathways. Uracil is not on the normal orotic acid to RNA pathway. The observed increases would have to result from a degradation of UMP to uracil, as UMP is a precursor in RNA synthesis.

The ethylene production profile over the ages studied showed a two-peaked production pattern. As well as variations with leaf age, variations in ethylene production during a 24 h period were observed. Ethylene levels during light periods were always higher than in the dark. The change between light and dark was abrupt and not a direct result of light. CO_2 is known to be a probable antagonist to ethylene production. Daylight levels of CO_2 in the growth cabinet reached about 450 ppm CO_2; night levels were much greater than 600 ppm and possibly reached 800 to 900 ppm. Dark respiration quickly

increased CO_2 levels when the lights went off; this was accompanied by a decrease in the ethylene production. If one postulates a critical level somewhere between 450 and 800 to 900 ppm for CO_2 to become an effective antagonist of ethylene production, the observed pattern was as expected.

Results obtained by the author with bean leaves have indicated a two-peaked ethylene production pattern, a conversion of β-alanine to ethylene, a conversion of uracil to β-alanine and, as bean leaf age increases, a decrease in the incorporation of orotic acid and uracil into RNA, a changing endogenous β-alanine level, and a decrease in total RNA.

These observations give a picture of some of the relationships between RNA metabolism and ethylene evolution and suggest some possible controls for the production of the hormone.

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APPENDIX

Figure A-1. ^{14}C quench curve. Scintillation vials contained 247,000 dpm β-alanine-2-^{14}C and various amounts of a leaf extract prepared as outlined in Section F.3., Chapter II. The inclusion of an amount of cellulose equivalent to that scraped for a sample from a TLC chromatogram did not change the counting efficiency.

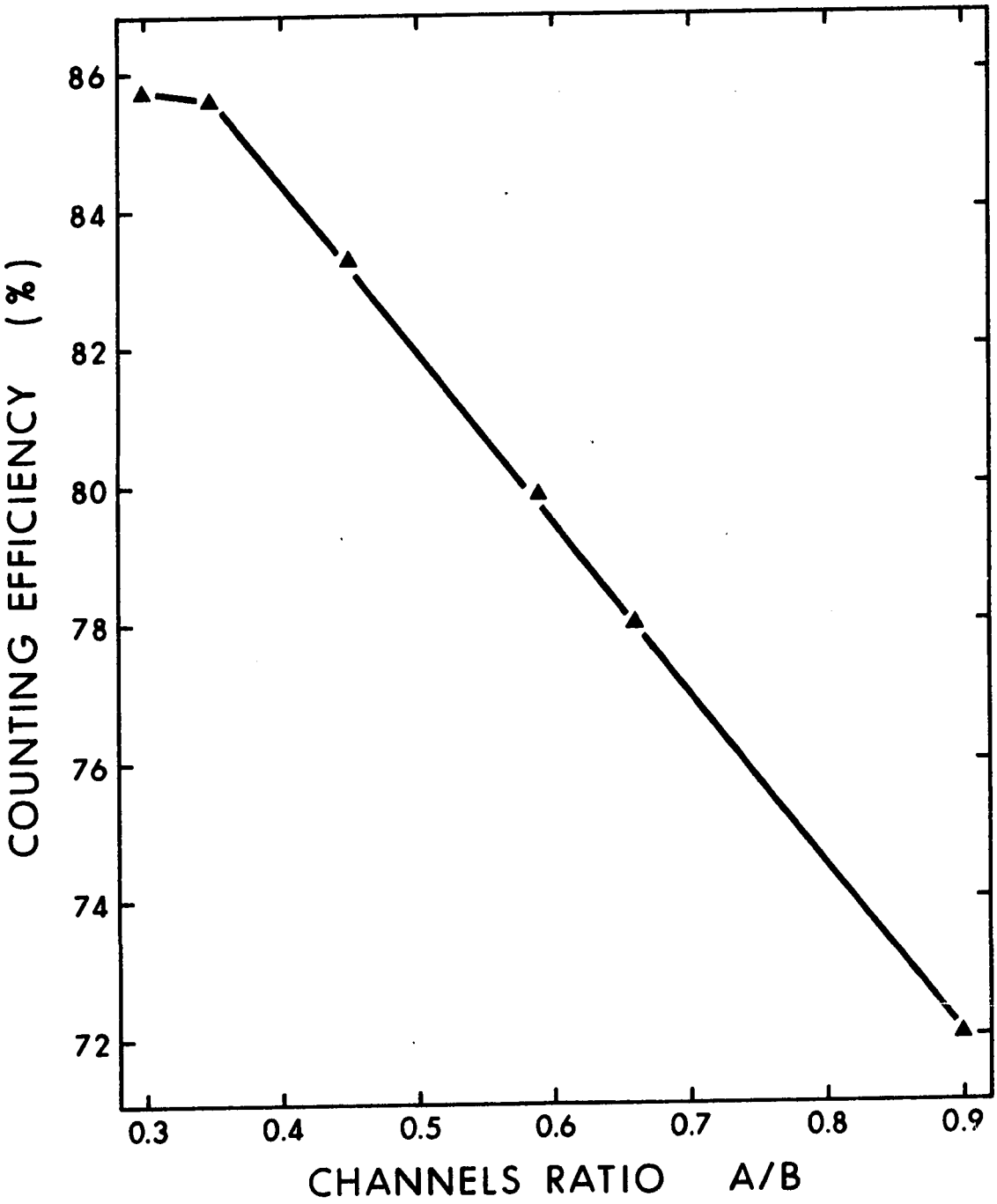

COUNTING EFFICIENCY (%)
86
84
82
80
78
76
74
72
0.3
0.4
0.5
0.6
0.7
0.8
0.9
CHANNELS RATIO A/B

Figure A-2. ^{3}H quench curve. Scintillation vials contained 925,000 dpm uracil-6-^{3}H, a standard amount of cellulose, and various amounts of a deproteinized incubation mixture (similar to that described in Section F.2., Chapter II) as quenching agent.

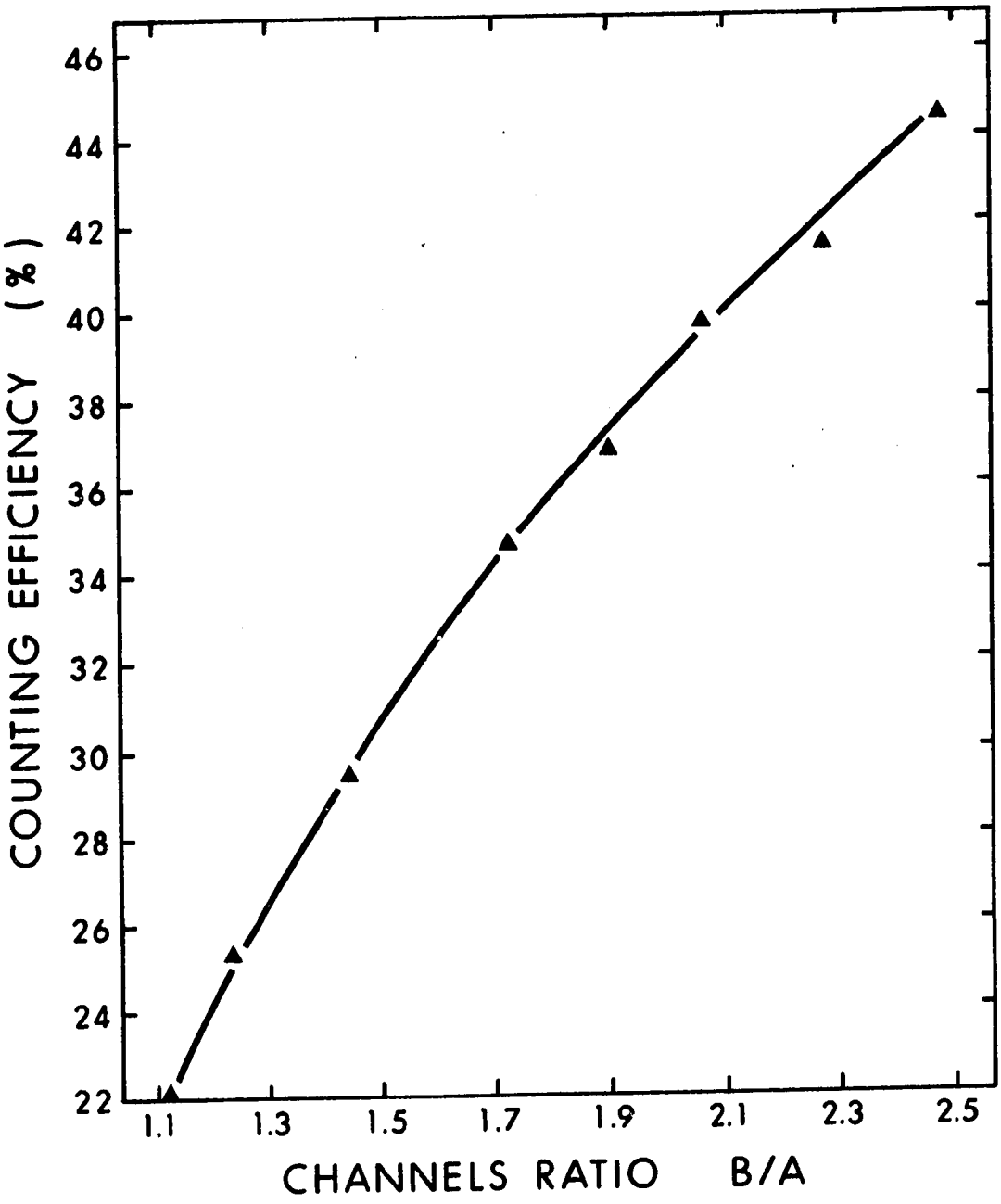

COUNTING EFFICIENCY (%)
46
44
42
40
38
36
34
32
30
28
26
24
22
1.1
1.3
1.5
1.7
1.9
2.1
2.3
2.5
CHANNELS RATIO B/A

Figure A-3. Ethylene standard curve. The gas was analyzed by gas chromatography and was resolved by a column (50 cm x 6 mm ID) of activated alumina with 2-1/2% silicone 550. He, H_2, and air flow rates were 64 cc/min, 37 cc/min, and 200 cc/min, respectively. Column temperature was approximately 20° and the detector temperature was approximately 125°.

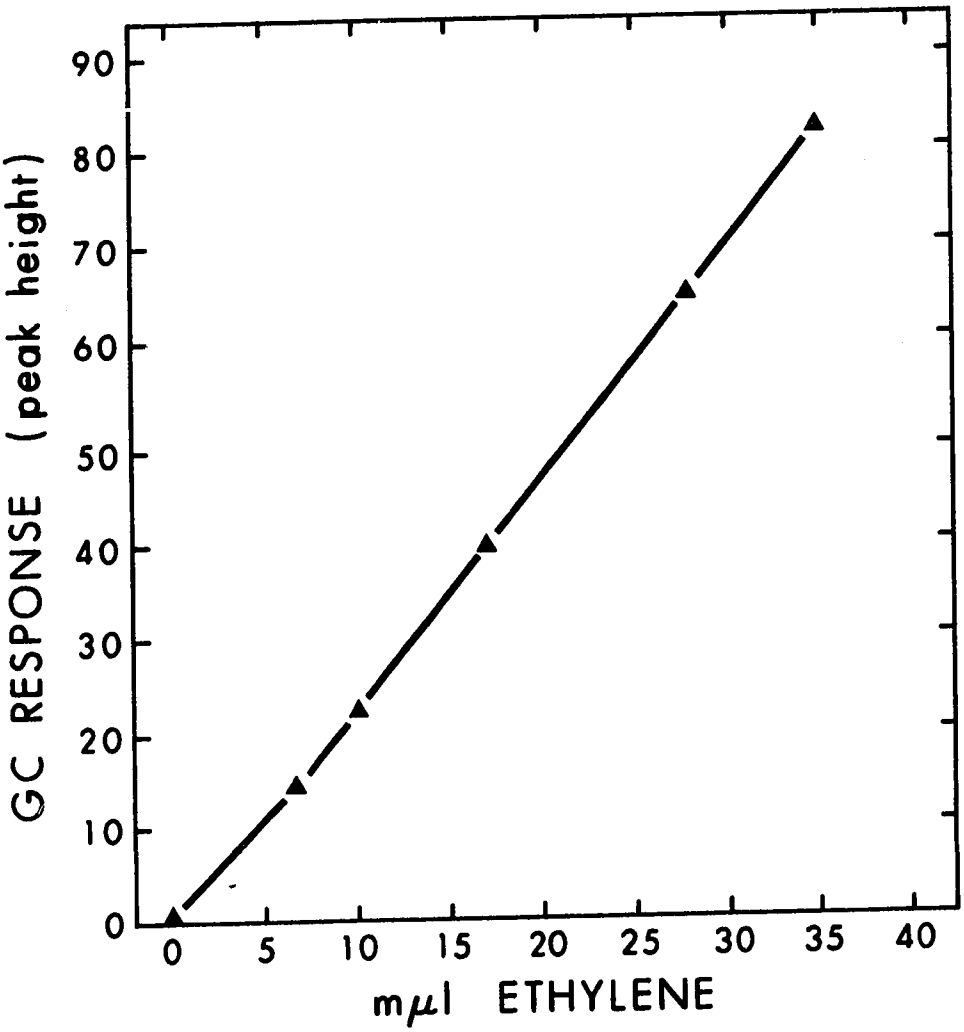

GC RESPONSE (peak height)
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
mμl ETHYLENE

Figure A-4. Protein standard curve. Protein was estimated by the procedure of Lowry et al. (1951). Protein concentration was expressed as µg per 1.0 ml of an appropriately diluted enzyme preparation.

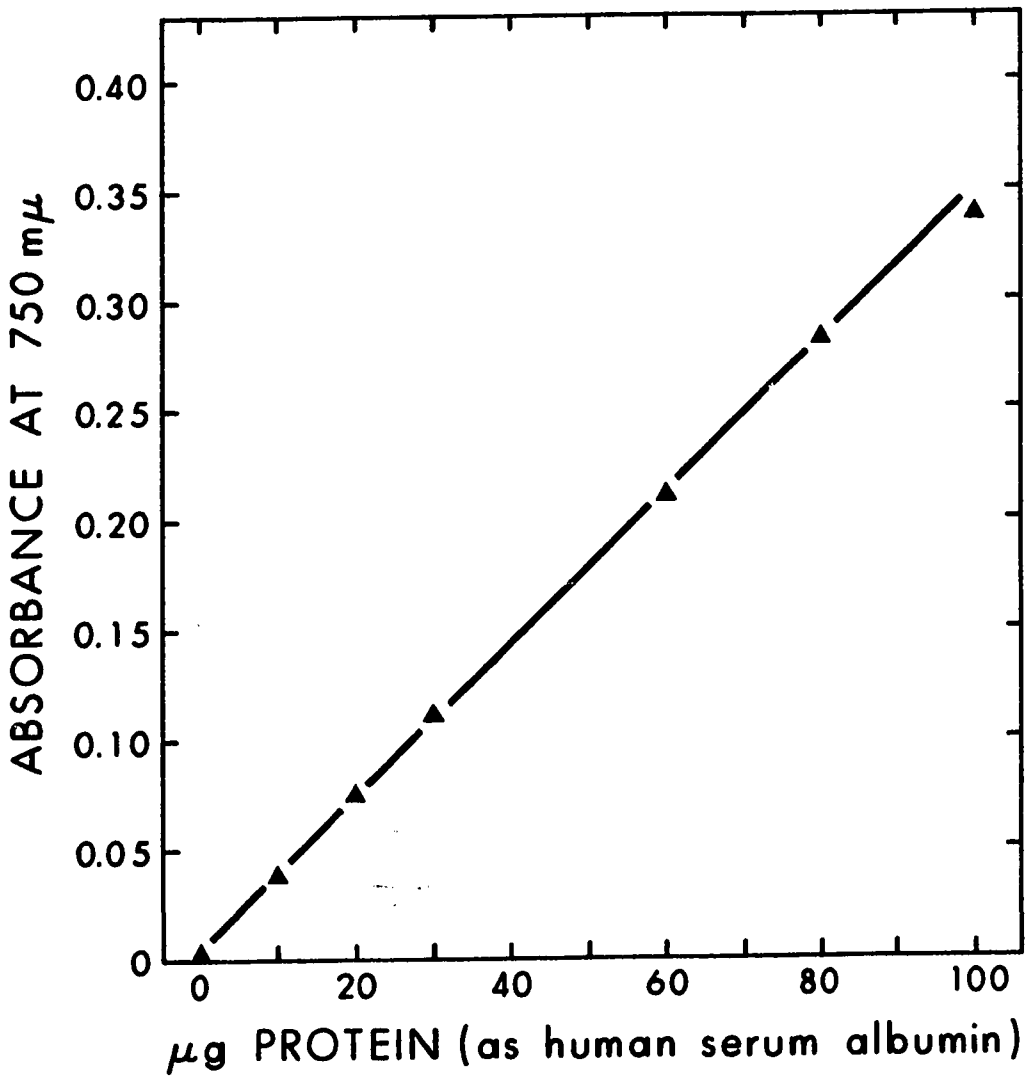

ABSORBANCE AT 750 mμ
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
0
20
40
60
80
100
μg PROTEIN (as human serum albumin)

Figure A-5. Calibration curve of the gas radiochromatograph (GRC). The 85 ml chamber was flushed with high purity methane (30 cc/min). Polarizing voltage was 2700 volts, the time constant was 10 sec, and full scale on the recorder (100) represented 1500 dpm. Various amounts of ethylene-(U)-^{14}C were injected into the gas chromatograph attached to the GRC as described in Section J, Chapter II.

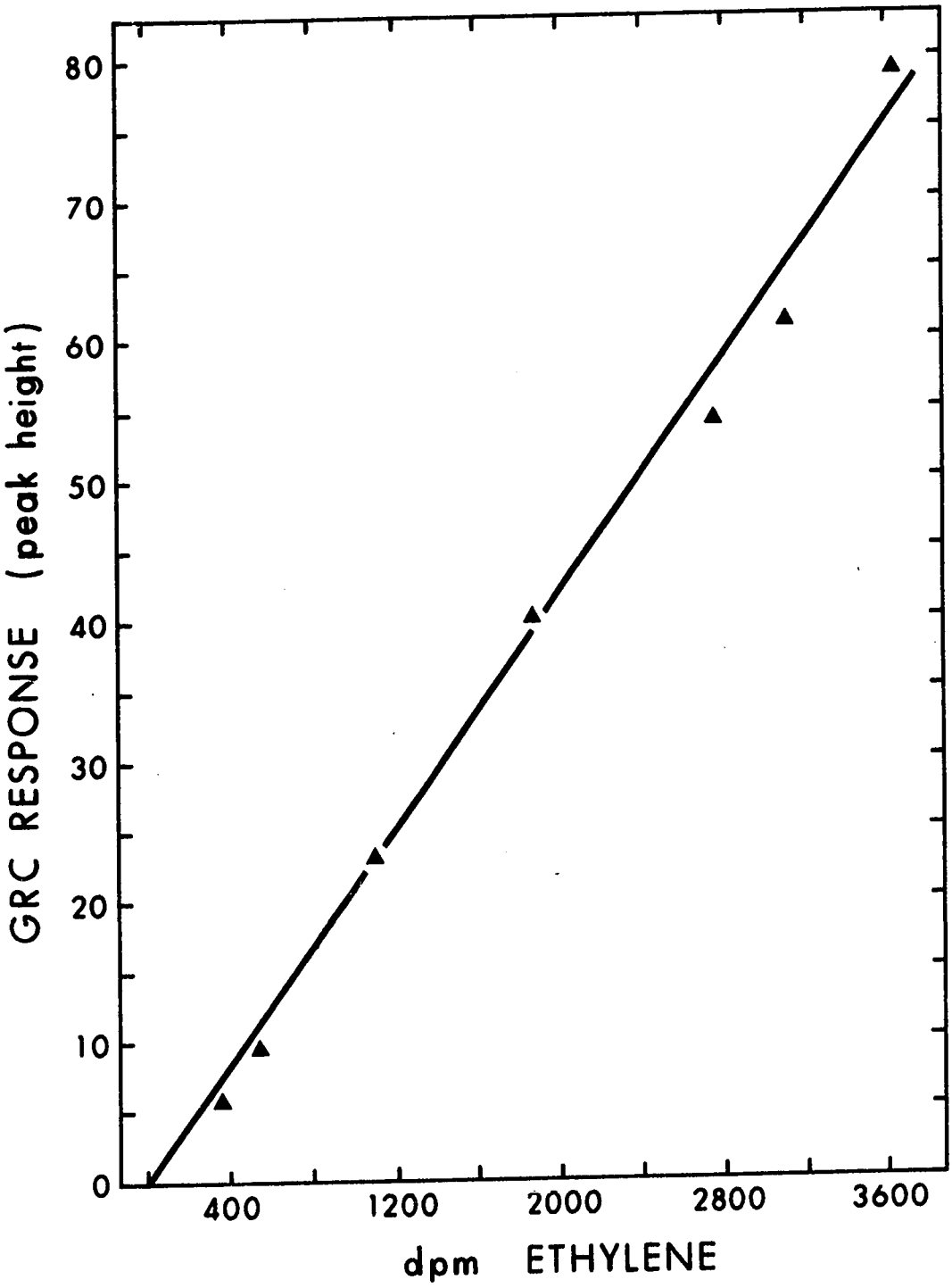
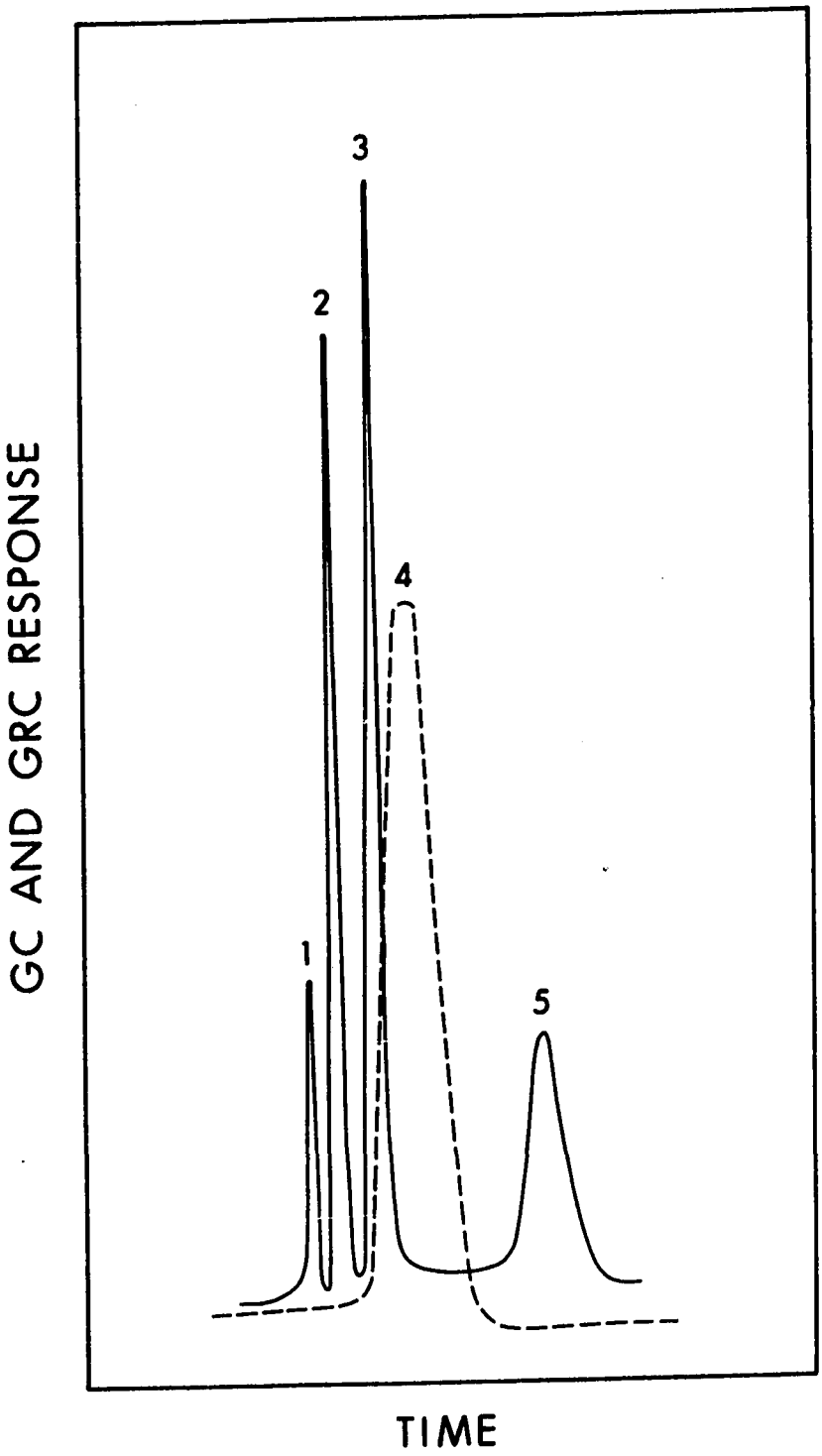

GRC RESPONSE (peak height)
80
70
60
50
40
30
20
10
0
400
1200
2000
2800
3600
dpm ETHYLENE

Figure A-6. Superimposed tracings of the gas chromatograph (GC) and the gas radiochromatograph (GRC) recordings of a sample analyzed for ethylene and ethylene-^{14}C. The solid tracing is the GC response and the dashed line is the GRC response. The baseline of the GRC tracing was purposely lowered. Peaks are as follows: 1, air and unknown; 2, ethane; 3,ethylene; 4,ethylene-^{14}C; and 5, unknown. Total abscissa width represents 19 minutes; time between peaks 1 and 5 reprsents 6.5 minutes.


GC AND GRC RESPONSE
1
2
3
4
5
TIME