A study of the evolution of volatiles from etiolated Pisum sativum.

Gregory Raymond Jolicoeur

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LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L'AVONS REÇUE
A STUDY OF THE EVOLUTION OF VOLATILES FROM ETIOLATED *Pisum sativum*.

by

Gregory Raymond Jolicoeur

A Thesis submitted to the Faculty of Graduate Studies through the Department of Biology in partial fulfillment of the requirements for the degree of Master of Science at the University of Windsor.

Windsor, Ontario, Canada.
1982
DEDICATION

This thesis is dedicated to my wife
Lynda,
and our two children,
Adrian and Jason.
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ABSTRACT

The low molecular weight volatiles produced by segments of etiolated Pisum sativum epicotyls were studied using gas chromatography, and identified as ethylene, methanol, acetaldehyde and ethanol.

The effects of temperature and tissue crushing on the evolution of the volatiles were examined. When compared with the vapour pressures calculated for the volatiles at the experimental temperatures, and based on the actual changes measured in the segments, the results show that the present 15 min sampling period causes little perturbation to the segments over the sampling period. Therefore, evolution of volatiles due to wounding will not affect the evolution of volatiles produced by the experimental treatments. Crushing of segments was found to decrease the rate of evolution of ethylene and ethanol but did not have any effect on the evolution of methanol and acetaldehyde when compared to evolution from uncrushed segments.

The effect of another type of tissue trauma, segment cutting, on the rate of evolution of volatiles was studied by comparing evolution of volatiles from equal weights of segments cut to various lengths. Ethylene and ethanol evolution rates did not change measurably with different sample lengths. However, methanol and acetaldehyde evolution rates increased when the segments were cut into shorter lengths.
The effects of a synthetic and a naturally occurring auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) and Indole-3-acetic acid (IAA) respectively, on *Pisum sativum* epicotyls were studied by spraying the auxins onto 14 day old etiolated pea seedlings grown under aseptic conditions. When the effect of 2,4-D was examined over a 38 hr period, the evolution of ethylene and acetaldehyde was stimulated after 2 hr. The evolution of methanol and ethanol was also stimulated shortly after this time. IAA was found to have similar effects except in the case of methanol the rate of evolution did not change when compared to controls. The effect that 2,4-D exerted on the plants was maintained over a much longer period of time when compared to IAA. While the response initiated by IAA was found to be short-lived, it was shown to be capable of reinitiation in the present system by a renewed application of IAA.

The effect of 10 mmolar cycloheximide applied as a spray prior to a spray containing 5.7 mmolar IAA on the evolution of volatiles was monitored 5.5 hr after exposure to IAA. Ethylene evolution was strongly inhibited; in contrast, the evolution of the other volatiles was stimulated—sharply in the case of methanol and modestly in the cases of acetaldehyde and ethanol. While cycloheximide strongly inhibited the ethylene response to the IAA application, it did not eliminate it.

The evolution of methanol as a volatile in plants has
not been reported in the literature up to this time. In order to test a hypothesis that methanol which arose as a result of responses involving protein methylation and produced by *Pisum* might be from the 'methanol-forming enzyme', protein methylase II (protein-carboxyl O-methyl transferase); tritiated methyl-labelled methionine was incorporated into segments of *Pisum sativum*. *Pisum* was shown to contain an enzyme of the protein methylase II type, which may possibly account for the evolution of methanol.
INTRODUCTION

Ethylene, an intensively studied component of illuminating gas since the turn of the century has wide ranging effects in plants. Its evolution in plants due to various factors, i.e., wounding or hormone application (1-11,13,17, 21,23-26,28,29,31,33,34,37,39,41-43,45,47-57,60-64,68,70,71, 74,76-78,80,81,83,85-87,91,92), and responses to inhibitors (35,46,52,62), have received renewed attention in the last two decades as a result of the commercial availability of the gas chromatograph (Lieberman,57). The evolution of ethylene has been reported to be associated with an intact cell wall-membrane complex (4) and to be greatly decreased in disrupted cell systems (4,45,64). This may be due to the presence of free radical scavengers (e.g. benzoate and propylgallate) which inhibit (in the case of benzoate) by scavenging hydroxy radicals (10). CO₂ exposure or anaerobiosis also stops ethylene production (47,76). Since excised plant tissue is used in this and other studies, the effect of wounding on the volatiles is important in selecting methodology, and in interpretation of results. Wound ethylene (ethylene evolved by cutting, excising or by exposure to toxic chemicals) was found to be delayed for 2 hr when the temperature was below 10 C or above 36 C in etiolated peas (62,76). The magnitude of the response was reported to vary from experiment to experiment, however the time sequence of the response was constant. This variability may be due, in part, to different maturation
times of successive plantings, and variability in the gas chromatograph (see Appendix 3). Also, rates of evolution are related to the region of the plant that is used for the assay (5,77). Variability also arises when controls are kept in close proximity to the treated plants. Ethylene produced by the treated plants can cause a response in the controls that is almost indistinguishable from that in the treated plants (8). Excessive levels of ethylene may also result when sections or parts of plants are sealed in flasks for long periods of time. In 1976, Jackson and Campbell (47) demonstrated that measurements of ethylene production should be made not later than 20-30 mins after excision otherwise discrepancies which arise from the wound response can be particularly misleading when the results obtained using segments are related to the intact plant. Short incubation periods also ensure that the O₂ concentration within the flasks does not fall to levels which may inhibit the biosynthesis of ethylene. The morphological and physiological aspects of ethylene and auxin responses are diverse. Ethylene causes retardation of extension growth as a result of deposition of longitudinal microfibrils in the cell walls. Cross-linking bonds in the polysaccharide matrix prevent their separation. Swelling of the epicotyl caused by lateral expansion of cells exposed to IAA which involves breakage or inhibited formation of these cross-linking bonds (78). However, swelling of the epicotyl as a result of exposure to ethylene is the result of
wall thickening (25). The ethylene produced as a result of hormone application blocks DNA synthesis and subsequently, mitosis (6). Enlargement in the epicotyl then is due to the swelling of individual cells and not cell division. Apelbaum and Burg (6), and others (85) reported an increased incorporation of glucose into the cell wall as a response to ethylene.

In contrast to ethylene, other small molecular weight carbon compounds, such as methanol, acetaldehyde and ethanol found in this study have received little attention. Studies of acetaldehyde and ethanol (16,19,36,44,49,74,76,79) have been mainly concerned with metabolism of endogenous substrate rather than their evolution as volatile products.

It is now generally accepted that ethylene is produced from methionine via s-adenosylmethionine (SAM) and l-aminocyclopropane-1-carboxylic acid (ACC); (Lieberman,57). The regulation of its production is not completely understood. Acetaldehyde and ethanol are components of the glycolytic cycle and can be monitored during the course of an experiment to shed some light on the degree of turnover of this portion of glycolysis. Ethanol is formed as an end product of glycolysis (anaerobic metabolism) in plants (18,19,58,67,73,74,104) with acetaldehyde as its immediate precursor.

Historically, IAA and auxin have been almost synonymous. With the development of many synthetic auxins and gibberellins, the definition of auxin had to be generalized to include compounds that were capable of inducing elongation of cells
in growing shoots, and specifically, etiolated oat coleoptiles which elongate exclusively with exposure to auxin.

Auxins can be subdivided into different groups including natural auxins which are indole auxins. Other classes are phenoxyacetic acids (2,4-D), benzoic acids, some dithiocarbamates and napthalene carboxylic acids. All of the indole auxins can be transformed in the plant to IAA. The side chains of all active auxins can be degraded to acetic acid. The side chains of almost all auxins thus terminate in a carboxylic acid group. As a clue to the possible coupling to cellular processes, the most active auxins can form structures in which a positive charge is 5.5 Angstroms away from a negatively charged carboxylate oxygen. The molecular site or sites of auxin action presumably have an electrical and spatial coordination with the structures of active auxins (72).

While 2,4-D is a synthetic analog of IAA, its effects are not identical to those of IAA. Part of the reason for this is the very slow breakdown of 2,4-D in endogenous plant systems. Response to 2,4-D typically include increased ethylene evolution (7,51,57,64,85,87). From the data obtained by following the breakdown of labelled 2,4-D in the plants, Morgan and Hall (64) proposed that 2,4-D was not the source of ethylene evolution by itself. Therefore, it can be surmised that 2,4-D affects cellular processes that will ultimately produce ethylene. If this is true, then the production of additional cellular metabolites may be affected. For this
reason, the evolution of methanol, acetaldehyde and ethanol after treating *Pisum sativum* seedlings with 2,4-D was monitored in the present study. When IAA was applied, ethylene evolution was found to increase after 2 hrs and to taper off to control values by 15-20 hrs. This occurred in pea root tips (81) and in banana tissue (87) and has been reported by others also (see above). Franklin and Morgan (37) proposed that the initial response of ethylene due to auxin occurs even when protein synthesis was blocked. These results were similar to those of Steen and Chadwick (81) using cycloheximide.

Cycloheximide is a well-known inhibitor of protein synthesis (81), blocking peptide bond formation by binding to the 80s ribosome of eukaryotes (Lehninger (101), Price (72), Fuchs and Lieberman (39)). This inhibitor was used to determine the possible cycloheximide sensitivity of volatile evolution stimulated by IAA.

Etiolated pea seedlings were used in the present study since metabolism is carried out by the use of stored food reserves (mainly starch). This relieves some of the complications brought about by the initiation of chlorophyll synthesis and other light induced reactions. It is interesting to note also, that growth of green pea stem tissue is insensitive to ethylene, while etiolated tissue is highly sensitive (Evans 29), and is therefore a good system for study in this regard.

Very recently protein methylation has been proposed as
a possible mechanism for hormone action (69). Protein methylation is a post-translational modification of protein amino acid residues. Examples of covalent modifications include: methylation, acetylation, phosphorylation, hydroxylation, ADP-ribosylation, nucleotidylation, carboxylation and glucosylation (69). While the regulatory significance of phosphorylation in carbohydrate metabolism is well understood, the biological significance of the other side chain modifications is not clearly understood. Protein methylation is thus only one of a host of post translational modifications. Although the biochemical significance of most of the side chain modification reactions has yet to be fully understood, it is apparent that they constitute potentially one of the most efficient biochemical control mechanisms. Only a small fraction of ATP is spent in transforming a protein into a functionally different form, thereby saving the organism from synthesizing the protein de novo, thus avoiding the expenditure of energy as well as occupying the biochemical machinery with de novo synthesis (69).

If plants contain an enzyme of the protein methylase II type, the enzyme, also known as the 'methanol-forming enzyme' may possibly account for the production of this alcohol, detected in the present study. Protein methylase II (PM II, or S-adenosyl-methionine: protein-carboxyl O-methyl transferase) methylates (esterifies) the free carboxyl groups of protein with S-adenosyl-L-methionine as methyl donor (Fig. 1a; 69). The enzyme has been found to be widely distributed in mammalian
FIGURE 1a.
organs (100). Subcellular localization within several tissues indicates that it is located in the cytosol (96). A striking feature of the reaction is the formation of an unstable protein-methyl ester that yields methanol non-enzymatically in weak aqueous alkaline solutions. Since methanol is known to be affected by the application of hormones (3, and the present study), it was desirable to design an experiment to determine: 1) whether pea plants might contain carboxy-methylated proteins, a potential source of methanol and; 2) whether auxin might influence the carboxyl methylation of plant protein.
MATERIALS AND METHODS

A model 402 Hewlett Packard high efficiency gas chromatograph with a 2 metre column packed with Poropak Q was used. Nitrogen, air and hydrogen flow rates were adjusted to 60, 300 and 40 ml/min respectively. The column oven was operated isothermally at 125 C. The Pisum sativum seeds (cv.361) were generously supplied by Green Giant of Canada. After soaking in distilled water overnight, seeds were surface sterilized with 0.5% sodium hypochlorite for 10 mins. The seeds were rinsed with approximately 2 1 of sterile distilled water and sown aseptically on a 41 X 66 cm aluminum pan containing 3 Kimtowels (Merchants Paper Co.) and 275 mls of water previously autoclaved for 15 mins at 121 C. The seeds were grown in the dark for 14 days at 24 C.

For the chromatograph assay the first 10 mm. of each epicotyl including the apical hook was discarded and the second 10 mm. segment retained. One gram fresh weight of epicotyl segments was added to a 5 ml vial which was capped immediately with a serum stopper. The initial handling of the plant tissue varied, as described below. In the first experimental procedure, three separate incubations were carried out at 0, 25 and 40 C. The vials for the 0 C determination were kept in an ice bath for 13 min and then warmed for 2 min in a 40 C water bath in order to bring the contents to a measured 25 C. The other two incubations were made for 15 min in water baths kept at the appropriate temperature. This
adjustment was performed so that any difference in volatile evolution could be distinguished from a temperature dependent change in vapour pressure. In order to determine the effect disruption of the tissue organization might have on the evolution of volatiles, tissue samples from each temperature treatment were either crushed or left intact before sampling. The tissue was crushed by inserting a thin stainless steel spatula blade through the serum stopper and crushing the tissue against the glass for approximately 15 sec, to obtain a smooth paste. Two ml air samples were withdrawn from the vials while depressing the stoppers to make up the volume being sampled. These samples were then injected into the gas chromatograph. Triplicate samples were run at each temperature over two experiments.

In order to evaluate cut-induced trauma, the first 10mm of each epicotyl including the apical hook were discarded as before and segments measuring either 9, 4.5 or 3mm were cut to obtain samples each of 1 gm fresh weight. Samples were enclosed in 5 ml vials for 15 min and sampled as described previously. After evaluating treatments as described above, the following protocol for tissue handling was selected: 1 g fresh weight of epicotyl segments, each approximately 10 mm long, was incubated for 15 min at 25 C in a stoppered vial prior to sampling of the volatiles produced.

The four peaks obtained by gas chromatography were identified and calibrated by comparison with standards. The vap-
Our pressure for the four volatiles at the various temperatures used in the first experiments, was calculated using the equation (102):

\[ \log_{10} P = (-0.2185 \frac{A}{K}) + B \]

where \( P \) = pressure in Torr (mm. Hg.)
\( K \) = temperature in degrees Kelvin
\( A \) = molar heat of vapourization in calories/g.mole.
\( B \) = constant

A curve was obtained by comparing the vapour pressures at 25 and 40 C. to the vapour pressure at 0 C. and calculating the fold difference between the values in order to determine the effect of temperature, per se, on vapour pressure. This could then be compared to the difference in the rates of evolution of ethylene, ethanol, methanol and acetaldehyde at 0, 25 and 40 C. in order to distinguish between purely physiological increases in vapour pressure and temperature dependent physiological responses.

2,4-D was mixed at a concentration of 0.0045M or 0.1% with 0.01% detergent (Alconox) in water at pH 6.0. This was sprayed onto the seedlings until dripping. The controls received a similar spray with 2,4-D omitted. The chromatographic assay procedure and growth conditions were the same as described previously.

The inhibitor, cycloheximide, was made to a concentration of 10 mmolar with 0.01% detergent in water at pH 6.0. The controls received an aqueous spray that included 0.01% det-
ergent at pH 6.0. The solutions were sprayed onto the plants until dripping. The cycloheximide was applied 30 mins prior to the IAA spray with the hormone application being considered as zero time.

In the experiment on protein methylation, etiolated Pisum sativum seedlings handled as described above were decapitated by removing the epicotyl hook including the top 10 mm with sharp scissors 6 hrs prior to the experiment. This removal of the main site of IAA synthesis was aimed at minimizing endogenous IAA in the subapical sections used in the experiments. At zero time, 10 mm terminal epicotyl sections, totaling 1 gm in fresh weight were harvested and placed in 10 ml vials with 2 mls of 0.2M tris buffer at pH 7.2. Treated segments were bathed in the buffer which included 1mM IAA. Both treated and controls received 20 microliters of L-(methyl-3H) methionine. The labelled methionine was purchased from Amersham Corp., (Oakville, Ont.) and was supplied with a specific activity of 250 microCuries (9.25 MBq) in an aqueous solution containing 0.2% 2-mercaptopethanol. The addition of the labelled methionine marked the beginning of a 3 hr incubation time on a metabolic shaker set to 175 oscillations per minute. After 2 hrs, the reaction was terminated by washing with 2 rinses of tris buffer. Liquid nitrogen was then used to freeze the tissue which was ground with a chilled mortar and pestle. 1 ml of tris buffer was added after grinding and the suspension centrifuged for 15 min in a bench-top centrifuge. The super-
nate was acidified with 20% TCA to a final concentration of 10%, to precipitate the protein. One ml of ethanol was overlayed and the samples were centrifuged again for 15 mins. The precipitate was washed 3 times with 5 ml of TCA (10%), once with chloroform:ether:ethanol (1:2:2 v/v) and once with ethanol (99,103).

At this point half of the treated and untreated samples were prepared for counting and the other half were suspended in 0.2M phosphate buffer (pH 7.2) and placed in a boiling water bath for 5 mins. This decomposed the labile protein methyl ester and allowed methanol to boil off. The protein was reprecipitated with 5 mls of TCA, washed once with TCA and once with ethanol. These precipitates were prepared for counting by redissolving in 0.5 mls of 0.1N NaOH: 0.1 ml was used in 5 mls of liquid scintillation cocktail (toluene, ethylene glycol, PPO; 2,5-diphenyloxazole and POPP; 1,4-Bis (2-(5-phenyloxazole)) benzene) in a Beckman liquid scintillation counter (105).
RESULTS

By comparing Fig.1b with Fig.2a and 2b, it can be noted that the evolution of the volatiles as a function of temperature did not simply follow the expected increase of vapour pressure with temperature. This comparison indicates that the observed volatile evolution differs markedly from the values expected on the basis of exclusively physical effects on vapour pressure. Figs.2a and 2b show the differences in rates of volatile evolution between crushed and uncrushed tissue with increasing temperature. Rates of evolution of ethylene and ethanol with temperature are sensitive to tissue disruption while methanol and acetaldehyde rates of evolution appear to be insensitive. It was found (Fig.2b) that rates of evolution of methanol and acetaldehyde at 25°C decreased below the 0°C rate before increasing again at a higher temperature. From Figures 3a and 3b, ethylene and ethanol are seen not to increase significantly with decreasing sample length. In other words, their evolution rates were unaffected by cut-surface trauma. In contrast, methanol increased from 18 to 30 ppM over 15 mins/gm tissue and acetaldehyde levels also increased from 0.1 to 10 ppM when wounding was increased by cutting segments from 9 mm (2 cut ends per segment) to 3 mm (6 cut ends per original 9 mm segment) lengths.

With 2,4-D, the evolution of ethylene (Fig.4) was observed to increase at about 2 hours. The ultimate level attained was approximately 60X greater than control values at 30 hours after
which a steady decline occurred. In Fig. 5 it can be seen that methanol evolution began to increase in treated plants by 4.5 hrs reaching a peak 1.5X greater than control values at 19 hrs after which a steady decline was observed until 38 hrs by which time, the levels had fallen to control values. Increases in acetaldehyde as seen in Fig. 6 occurred after 2.5 hrs with levels peaking at 3.9X control values at 30 hrs after which a steady decline is noted. Ethanol evolution as documented in Fig. 7 has 2 peaks of evolution at 6.5 and 29 hrs. From 6.5-29 hrs a decline with a minimum at 19 hrs can be observed. This coincides with the time of maximum swelling of the epicotyl in response to hormone treatment (25). Ethanol levels just begin to increase after 2 hrs. After 29 hrs, there is a decline until 38 hrs when the rate of evolution is similar to the controls.

From Fig. 8 it can be observed that ethylene evolution increases dramatically 1 hour after IAA spray application. A peak is attained at 8 hrs that is 35 times greater than control levels. After this, there is a gradual decrease until 30 hrs to near control values. There is no change in methanol evolution (Fig. 9) until 25 hrs, when evolution in treated plants decreases to 1.6 fold below control levels. Fig. 10 shows that acetaldehyde evolution is also affected; however induction was delayed until after 4 hrs and maximum increases were not observed until 18 hrs had passed. The levels remained 2.5 times greater than controls up to and including 38 hrs.
Ethanol evolution (Fig.11) increases beginning at 4 hrs to a maximum at 8 hrs with levels that are approximately 2 times greater than controls. Then follows a slow decrease to control values by 38 hrs. Respraying of IAA at 48 hrs, (see arrows in Figs.8-11), induced increases in ethylene, acetaldehyde and ethanol by 3.5 hrs. No change in levels of methanol were detected within this time period. From Fig.12, it can be seen that, compared to controls, ethylene evolution increased from less than 0.2 pmoles at 1 hr to 9 pmoles by 5.5 hrs in the IAA treated seedlings. The timing of the response was similar when cycloheximide was sprayed previous to IAA except that the rate of evolution at 5.5 hrs had decreased by 83% when compared to the plants treated with IAA alone. As displayed in Fig.13, the rate of methanol evolution did not change markedly when IAA was applied, but when IAA treatment was preceded by the cycloheximide spray, methanol evolution increased from 41 to 65 micromoles at 5.5 hrs. In Fig.14, acetaldehyde evolution is shown to increase from 9 to 41 micromoles when IAA was applied. Cycloheximide appears to elicit a slight initial inhibition, followed by a stimulus to 52 micromoles at 5.5 hrs. Ethanol evolution (Fig. 15) increased in IAA treated seedlings from 6 to 14 micromoles at 5.5 hrs. When preceded by the cycloheximide spray, ethanol evolution increased to 4.3 micromoles.

The results of the experiments on protein methylation are summarized in Table 1.
In these experiments, the extent of protein carboxymethylation is indicated by the percentage of radioactivity lost during heat treatment under alkaline conditions (69). Experiments 1 and 2 (Table 1) indicate auxin stimulation of protein carboxy-methylation of 19.6 and 24.0% respectively. The results from the unboiled samples also show that increased incorporation of labelled methionine into protein occurred in the presence of IAA.
Figure 1b. Calculated values of Vapour Pressure for Ethylene (squares), Methanol (circles), Acetaldehyde (triangles), and Ethanol (hexagons). Values for the y-axis were derived from the fold difference in the value of vapour pressure between 0 C. and the indicated temperature. A dashed line indicates that there is no further increase in vapour pressure of the volatile from the last indicated temperature, since the boiling point of the volatile has been attained at 1 atm.
Figure 2a. Fold differences in emissions of ethylene (squares) and ethanol (hexagons) between 0 C. and indicated temperatures for crushed (closed symbols) vs. uncrushed (open symbols) tissue. Standard deviations not shown are within the point markings.

Figure 2b. Fold differences in emissions of methanol (circles) and acetaldehyde (triangles) between 0 C. and the indicated temperatures for crushed (closed symbols) vs. uncrushed (open symbols) tissue.
Figure 3a. Evolution of methanol (circles) and acetaldehyde (triangles) with decreasing sample length. Standard deviations not shown are within the point markings.

Figure 3b. Evolution of ethylene (squares) and ethanol (hexagons) with decreasing sample length. Standard deviations not shown are within the point markings. (Arrows show to which axis the curves correspond).
Figure 4. The evolution of ethylene (squares) from 2,4-D treated Pisum epicotyl segments. (closed symbols) vs. controls (open symbols). Vertical bars denote standard deviations when larger than point markings.
Figure 5. The evolution of methanol (circles) from 2,4-D treated plants (closed symbols) vs. controls (open symbols). Vertical bars denote standard deviations when larger than the point markings.
Figure 6. The evolution of acetaldehyde (triangles) from 2,4-D treated (closed symbols) vs. controls (open symbols). Vertical bars denote standard deviations when larger than point markings.
Figure 7. The evolution of ethanol from 2,4-D treated plants (closed hexagons) vs. controls (open hexagons). Vertical bars denote standard deviations when larger than the point markings.
Figure 8. The effect of IAA, applied at zero time, on the evolution of ethylene (closed squares) as compared to controls (open squares). The arrow indicates a second IAA treatment. Vertical bars denote standard deviations when larger than the point markings.
Figure 9. The effect of IAA, applied at zero time, on the evolution of methanol (closed circles) as compared to controls (open circles). The arrow indicates a second IAA treatment. Standard deviations are represented by vertical bars when larger than the point markings.
Figure 10. The effect of IAA, applied at zero time, on the evolution of acetaldehyde (closed triangles) as compared to controls (open triangles). The arrow indicates a second IAA treatment. Standard deviations are denoted by vertical bars when the deviation is greater than the point marking.
Figure 11. The effect of IAA, applied at zero time, on the evolution of ethanol (closed hexagons) as compared to controls (open hexagons). The arrow indicates a second IAA treatment. Standard deviations are represented by vertical bars when larger than the point markings.
Figure 12. The effect of cycloheximide applied 30 mins prior to zero time, on the evolution of auxin-induced ethylene (IAA applied at zero time); untreated controls (open squares), IAA treated only (half-closed squares), and IAA preceded by cycloheximide (closed squares). Standard deviations are represented by vertical bars when larger than the point markings.
Figure 13. The effect of cycloheximide, applied 30 mins prior to zero time, on the evolution of auxin-induced methanol (IAA applied at zero time); untreated controls (open circles), IAA treated only (half-closed circles), and IAA preceded by cycloheximide (closed circles). Standard deviations are represented by vertical bars when larger than the point markings.
Figure 14. The effect of cycloheximide, applied 30 mins prior to zero time, on the evolution of auxin-induced acetaldehyde (IAA applied at zero time); untreated controls (open triangles), IAA treated only (half-closed triangles), and IAA preceded by cycloheximide (closed triangles). Standard deviations are represented by vertical bars when larger than the point markings.
Figure 15. The effect of cycloheximide, applied 30 mins prior to zero time, on the evolution of auxin-induced ethanol; (IAA applied at zero time); untreated controls (open hexagons), IAA treated only (half-closed hexagons), and IAA preceded by cycloheximide (closed hexagons). Standard deviations are represented by vertical bars when larger than the point markings.
### TABLE 1

<table>
<thead>
<tr>
<th>Exp.</th>
<th>TREATMENT</th>
<th>NOT BOILED</th>
<th>BOILED</th>
<th>% OF RADIOACTIVITY LOST IN BOILING</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NO IAA</td>
<td>21611</td>
<td>14020</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>IAA</td>
<td>24474</td>
<td>11109</td>
<td>54.6</td>
</tr>
<tr>
<td>2</td>
<td>NO IAA</td>
<td>35474</td>
<td>25843</td>
<td>27.1</td>
</tr>
<tr>
<td></td>
<td>IAA</td>
<td>48393</td>
<td>23652</td>
<td>51.1</td>
</tr>
</tbody>
</table>

The effect of IAA on protein carboxymethylation which is indicated by the percentage of radioactivity lost on boiling, and on total incorporation of $^3$H-methyl into protein (not boiled).
DISCUSSION

The variations in rates of evolution of volatiles with temperature (Figs. 2a and 2b), are not consistent with a simple temperature/vapour pressure relationship (Fig. 1b). In Fig. 2b, methanol and acetaldehyde rates of evolution can be seen to dip below the 0 C rate at 25 C. Since enzymatic processes and metabolism are brought almost to a halt in the 0 C treatment, the decline as temperature rises to 25 C. may be attributable to 1) active metabolism of methanol and acetaldehyde or 2) inhibited synthesis of these two volatiles, methanol and acetaldehyde at 25 C. Crushing had little effect on the evolution of methanol and acetaldehyde. If non-enzymatic breakdown was occurring at all temperatures, it is difficult to understand why the rate of evolution would drop to a minimum level below the 0 C. rate. The insensitivity of the methanol and acetaldehyde generating systems to mechanical disruption by crushing is consistent with the possibility that they may be located in the cytosol.

Ethanol evolution seems to be related to that of ethylene in its dependence on an intact tissue. While the evolution of ethanol in the intact plant may simply reflect local anaerobic conditions in the epicotyl, its possible role as a volatile growth regulator cannot be excluded. In 1962, Cossins and Turner (18) showed that the addition of ethanol to pea cotyledon slices elicited an increase in respiration. They also postulated that ethanol concentrations might incr-
ease in germinating tissues as a result of anaerobiosis and that the ethanol might eventually be metabolized by the plant. The possibility of subsequent metabolism of the ethanol might be dependent on transport from an anaerobic production site to a relatively aerobic site where ethanol oxidation can occur.

It was postulated that the cutting of the tissue into segments might constitute a wounding response that may affect interpretation of the data. A wounding response has been variously described (2, 17, 25, 53, 76, 77, 91) as a repeatable physiological response in the plant or plant part. Most commonly, only ethylene levels have been monitored while the specific type of wounding experiment is carried out. It has been previously observed that evolution of ethylene and ethanol increased only slightly with temperature in crushed tissue as opposed to a large increase in uncrushed tissue. Abeles and Yang (2, 91) have described a wounding response in which a sudden increase in ethylene is observed only after 1 hr. Since the present sampling procedure requires a 15 min holding period after cutting, the time does not allow for an accumulation of ethylene that was evolved in response to wounding. An increase was observed for methanol and acetaldehyde which are water soluble and which evolve at greater rates when the sections are cut into smaller lengths or when the temperature is increased above 35 C (Fig. 2b).

When sections were crushed, the evolution of ethylene and ethanol decline. The increases with increasing temper-
nature may be attributable to rising vapour pressure. When sections are cut into smaller lengths, little change in the rate of evolution is noted. It may have been expected that an increase in evolution of ethylene and ethanol due to increasing surface area might occur. This was not observed. Even though sections are made with sharp razor blades, some crushing (shearing) and leakage of the tissue fluid results. In other words, the increase in evolution expected as a result of increasing area is offset by the leakage of fluid or mechanical trauma resulting from cutting to smaller segments. The increases in methanol and acetaldehyde are expected because of the increasing surface area and are observed, because, as previously shown, their evolution is not affected by crushing. The similarities between ethylene and ethanol evolution rates cannot be reconciled on the basis of similar chemical properties. Ethylene is insoluble in water and is a gas at room temperature, while ethanol is water soluble and boils at 78.5 °C. The lack of increase with increasing area of cut surface leads one to speculate that some form of degradation is occurring when these volatiles are exposed to increasing amounts of tissue fluid, either through crushing or the mechanical trauma resulting from cutting into smaller pieces. This could be due to a specific or non-specific enzymatic breakdown or a chemical reaction. That ethanol and ethylene evolution is greatly decreased when the tissue is crushed leads us to conclude either that the respective producing
systems are dependent on the structural integrity of the cell wall or plasma membrane (as has been proposed in 4,57), or that these volatiles are being degraded when exposed to the tissue fluid (10).

From the results, it can be concluded that the experimental assay procedure selected for this work after the preliminary studies, does not allow sufficient time for volatiles produced as a result of wounding to accumulate in the vials. The results of Jackson and Campbell (47) have supported the contention that if segments or tissue slices are kept in a closed container and assayed after 20-30 mins, then the likelihood of the segments responding to the assay procedure itself is almost certain. In our experiments, the assay procedure involved excising epicotyl segments and allowing the volatiles to accumulate for only 15 min. This is well within the time limit recommended by Jackson et al. and from our experiments we have not been able to discern any response resulting from the procedure itself.

Suggestions for research in this area may include the following precautions; avoid crushing or unnecessary handling of segments; keep sample times short (less than 30 mins) to avoid wound-induced accumulation of volatiles caused by the cutting procedure; if buffers are used, levels of water soluble volatiles should be determined in the liquid medium as well as in the head space of the reaction vessels. The aforementioned considerations were applied to the selection of
the experimental protocols used in subsequent work.

2,4-D causes increases in the evolution of ethylene, acetaldehyde, methanol and ethanol. It appears unlikely that the increases in acetaldehyde and ethanol are induced by ethylene because the rates of these volatiles increase concurrently with ethylene. This is consistent with reports (5, 87) that show increases in respiration and glucose utilization which would lead to greater evolution of these 3 metabolic intermediates.

The 2 hr delay in the response to 2,4-D is not a result of uptake limiting since 2,4-D is taken up in an initial diffusion process which is followed by a slow uptake. This occurs within 1 hr due to the metabolic utilization of 2,4-D (Leguay and Guern, 55). The delay is within the amount of time necessary for de novo synthesis of protein and new tRNA's which suggests a coordination of the response, at least in part, at the level of the DNA.

The results of this work have an interesting parallel with the recent work by Davies and Schuster (22) who have demonstrated a large increase in polysome formation in aged pea stems as a result of wounding. This response was detectable in 30 mins, massive by 3 hours, and sustained for over 24 hours. The kinetics of the wounding response thus coincide quite well with those of the emission of ethylene in response to 2,4-D, consistent with the possibility of a common mechanism.
It has previously been demonstrated that IAA causes a rapid increase in ethylene evolution followed by a gradual decline (51). However, by comparison of Fig. 4 with tests on 2,4-D effects, it can be observed that in the same system, 2,4-D, which is fairly stable in plant tissues, maintains a steady increase in the rate of ethylene evolution up to 28 hrs after which a slow decline is observed. The effect on acetaldehyde evolution rates is interesting in that the increase appears after the rise in ethylene and is sustained even after ethylene levels drop. This is consistent with the possibility that ethylene may induce other cellular reactions which affect acetaldehyde production. Auxin typically increases the overall metabolic rate (87) and cell wall synthesis (85) resulting in the swelling response of pea epicotyls and stems to auxin application. Maximal acetaldehyde production in response to auxin, coincides with the time of maximum epicotyl swelling at 18 hrs (25).

Increased glucose utilization as documented by Abdul-Baki and Ray (1) when pea stem segments are exposed to IAA could account for an increase in glycolysis with a concomitant increase in glycolytic intermediates namely, acetaldehyde and ethanol. Ethanol evolution rates begin to increase at the same time as acetaldehyde evolution rates, with ethanol reaching a maximum by 8 hours as compared to 18 hours for acetaldehyde.

When ethylene evolution after IAA application is comp-
ared to evolution as a result of 2,4-D, it can readily be observed that the effect of IAA diminishes relatively rapidly. A second application of IAA at 48 hrs strongly stimulated ethylene evolution. Thus, the plant retains sensitivity to auxin at this time. 2,4-D, presumably because it is more resistant to metabolism, maintains a response over a longer period of time. 2,4-D does not stimulate ethanol in the same fashion as IAA. Two increases, from 0-10 hrs and 20-35 hrs approximately are observed. Work undertaken by Kang, Newcomb and Burg (51) has shown that free IAA levels in subapical sections of Pisum sativum seedlings decrease to control values by 12 hrs. Response to IAA is therefore considered to be a transient response resulting from an exposure to a level of IAA that will induce the observed responses, i.e., swelling and increased volatile evolution.

The effect of cycloheximide on the volatile generating system was interesting. There is some variation in the extent of cycloheximide inhibition of protein synthesis (59). Also, cycloheximide can inhibit many cellular processes (59,72,81). In this system, ethylene evolution as a response to IAA was markedly depressed but not eliminated. This is related to work by Steen et al. (81) that demonstrated inhibition of an ethylene response due to auxin in pea root tips. In their research, they demonstrated that initial production of ethylene as a result of auxin application occurred even when protein synthesis was blocked with cycloheximide. The subsequ-
ent evolution of ethylene was inhibited. A complete response requires an inhibitor-free system for ethylene possibly because new protein must be made to enhance the endogenous ethylene forming rate. The other 3 volatiles, especially methanol, are undiminished and actually increase in their rates of evolution. This is not unexpected if one considers that protein synthesis may not be required for glycolysis to continue provided that the existing enzymes have sufficiently extended half-lives and that pools of precursors and intermediates exist in the cell. Similar stimulation has been noted elsewhere (81) and may be due to a wound response induced by cycloheximide.

In the literature, cycloheximide is reported to inhibit induction of ethylene evolution associated with ripening (38), or wounding (2,13,14,22,45,47,76,91). It was considered that if IAA acted at the level of protein synthesis to elicit changes in volatile evolution, then a response should be modified when an inhibitor of protein synthesis was used. The present results are generally consistent with the results of the above mentioned workers. However, if one compares the experimental systems, it can be seen that when sections or parts of plants were used, inhibition of protein synthesis and ethylene evolution occurred. When the whole plant is treated, cycloheximide and other inhibitors of protein synthesis (eg. thiouracil, azaguanine, tetracyclin) have been reported to increase auxin induced elongation (10,39). From the results
in the literature, it has been inferred that RNA and protein synthesis are required for a sustained and complete response to IAA application but is not necessary for an initial response. The reason that a measurable response occurs in the experiments involving the intact plant as opposed to sections may be due to the availability of some essential factor transported from elsewhere in the plant.

In summary, IAA-induced ethylene evolution is sharply reduced by cycloheximide but not eliminated in the 5.5 hr test period. Auxin-induced ethylene synthesis appears to depend on protein synthesis. This agrees with the literature (76,77, 81). In contrast, volatiles other than ethylene seem to be produced by a constitutive mechanism that may in general be modulated by IAA, but which is resistant to, and may in some cases be stimulated by cycloheximide.

When the volatiles other than ethylene are examined, one must not lose sight of the fact that a response following auxin application may be mediated by an enhanced production of ethylene. Since, wall deposition is occurring (6), swelling of the epicotyl (25) is taking place, glucose uptake (1) and respiration is stimulated (5), it seems probable that the levels of acetaldehyde and ethanol would rise as a result of greater turnover in glycolysis. Methanol so far has been unreported as a plant volatile product. In bacteria, the following method for methanol production has been postulated (72): Hydrogenases serve to release H₂ from excess NADH that may
arise because active growth of cells requires biosynthesis of many components such as amino acids. When glucose is the sole source of carbon, the metabolic reactions involve an excess of oxidation steps over reduction steps. The excess reducing equivalents may be released as \( \text{H}_2 \), or may be used to form highly reduced products like succinate. In the plant, excess reducing power could presumably be used to reduce formate to formaldehyde then to methanol and finally to methane. At high partial pressure (within the cell), hydrogen can only be released from: i) pyruvate (pyruvate-ferrodoxin oxidoreductase + hydrogenase), or ii) formate (formate-hydrogen lyase). At low partial pressures, hydrogen evolution from NADH \(_2\) (NADH \(_2\)-ferrodoxin reductase + hydrogenase) and other H-carriers becomes possible. Below a partial pressure of \( \text{H}_2 \) of 0.001 atm, the free energy change of \( \text{H}_2 \) evolution from NADH \(_2\) is negative, driving the reaction in that direction. Because of the relation of the reducing equivalents to the electron transport chain, there is a possible coupling of the \( \text{H}_2 \) in the production of ATP by electron transport phosphorylation with the formation of methanol and methane. If the excess \( \text{H}_2 \) is being used to drive electron transport phosphorylation to produce ATP, then the evolution of methanol and possibly methane could be expected in the process. Perhaps, the plant is using these compounds as a \( \text{H}_2 \) acceptor before oxygen. More research needs to be conducted in order to ascertain the reason for
the presence of methanol and whether or not it exists in other plant systems.

An alternative mechanism for methanol production in plants could be via the activity of protein methylase II. Pea shoots appear to contain protein methylase II activity which is enhanced by auxin. The labile methyl groups produced may possibly be a source of the methanol which is evolved by the plant material. Protein carboxymethylation would seem in addition to be an attractive candidate for a role in mediating the plant cell response to IAA. Adler and Dahl (93) were the first to report the involvement of protein methylation in bacteria as a response to a chemical stimulus. They discovered that a methionine auxotroph of Esherichia coli was motile, but not chemotactic unless methionine was supplied exogenously. Armstrong (94) observed that an ethionine-resistant mutant that was not chemotactic had a low amount of S-adenosylmethionine synthetase and a reduced S-adenosyl-L-methionine pool, showing a good correlation between the pool size and the degree of chemotaxis. The requirement of S-adenosyl-L-methionine for bacterial chemotaxis was then correlated with the methylation of a specific protein from the plasma membrane (97). A number of E. coli mutants demonstrated abnormalities in the methylation pattern of this specific methyl-accepting chemotaxis protein, ranging from a total absence to a severalfold increase over the methylation level of the wild type. However, a continuous supply of methionine was required for chemotaxis,
indicating a labile nature of the methyl group that was incorporated into the protein. A possible correlation between protein carboxy-methylation and the chemotaxis of leukocytes was studied in a mammalian system (rabbit, 98), in which active neutrophils were incubated with a chemotactic tripeptide, N-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe) and S-adenosyl-L-(methyl-14C) methionine, an increase in carbonyl-methylation of the endogenous substrate protein was observed. Also, chemopeptide antagonists blocked the chemopeptide-induced protein-carboxyl-methylation. Since it had been previously demonstrated by Aswanikumar et al. (95) that specific chemotactic receptors were present on the neutrophils by the fact that fMet-Leu-Phe inhibited the binding of the radiolabelled chemopeptide to the neutrophils, O'Dea et al. (98) concluded that the receptor on the neutrophil membrane was the actual endogenous protein methylated in response to the binding of the peptide. Then, methylation of carboxyl groups of this receptor initiates the signal which directs the cellular motion of the leukocyte.

In plants, geo- and photo-tropism, both mediated by auxins and usually operating in conjunction with ethylene, are behavioral counterparts to the tactic movements of bacterial and eukaryotic cells. While the detailed mechanism of plant tropisms remains unknown, biochemical unity would seem to favour involvement of protein methylation. If this speculation finds support, S-adenosylmethionine (SAM) would
find placement in a key regulatory position as a substrate not only for ethylene (57), but also for protein methylation. Guided by the bacterial model, it might be hypothesized that methylation of membrane proteins might be implicated in the modulation of auxin transport which occurs in response to geo- or photo-stimulation of a plant axis.
APPENDIX 1

GROWTH OF ETIOLATED Pisum Sativum SEEDLINGS

In order to minimize extraneous influences on the experimental systems, it is necessary to grow seedlings free of fungal and bacterial contamination. It is also desirable, for the sake of repeatability, to grow the plants in an environment that is as nearly uniform as possible in temperature, density of growth, and supply of water and nutrients. Close packing of seeds encourages contamination, so attention to detail is necessary.

Initially, untreated Pisum Sativum seeds (cv. Little Marvel) were sowed onto 30 X 36 X 8 cm plastic greenhouse flats containing Vermiculite to a depth of approximately 3 cm. About 250 gms of seeds were sowed directly onto the Vermiculite after soaking in 1 litre of distilled water overnight and allowing to grow uncovered in the dark at room temperature with occasional watering. This led to rather extensive fungal growth, and concomitant stunting and abnormal seedling growth.

Next, seeds were surface sterilized before sowing with a 10 min. immersion in a 10% aqueous solution of commercial bleach (Javex) followed by a thorough distilled water rinse then planted in Vermiculite. Fungal contamination decreased only slightly.

Vermiculite was abandoned in favour of a less confining medium, i.e. paper towels. While fungal growth was reduced, the seeds could dry out overnight even when excess water was
used. Covering the tray with aluminum foil in order to reduce evaporative water loss increased fungal proliferation.

Finally, plastic greenhouse trays were replaced in favour of aluminum roasting pans approximately 41 x 66 cm. in size. These could be autoclaved together with the required amount of water and paper towels in order to eliminate contamination contributed by the medium. The pans, covered with aluminum foil were sterilized for 15 mins. at 121 deg. C. Surface disinfested seeds sowed aseptically onto this medium grew with small amounts of bacterial and fungal contamination, introduced by the seeds themselves. The paper did not retain sufficient water to supply the seedlings without daily watering, which increased the possibility of contamination. The paper towels were then replaced with Kim-towels, (Merchants Paper Co.) a disposable, highly absorbent paper towel applied 3/pan. These could each hold approximately 90 mls. of water, allowing seedlings to grow for 10-14 days undisturbed. Contamination was now reduced to 10-15%. The commercial seeds were then replaced by a series of cultivars donated by Green Giant of Canada. These seeds were coated with Captan 75 and Methoxychlor 5, fungal inhibitors. A growth test was conducted along with observations for contamination.
TABLE 2. GROWTH AND CONTAMINATION TEST OF 7 CULTIVARS

<table>
<thead>
<tr>
<th>VARIETY</th>
<th>GROWTH (1-4)</th>
<th>CONTAMINATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>361</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>346</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>510</td>
<td>2</td>
<td>MOLD</td>
</tr>
<tr>
<td>250</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>386</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>531</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>451</td>
<td>1</td>
<td>BACTERIA</td>
</tr>
</tbody>
</table>

(Growth - a comparison of heights at 14 days. Tallest = 4 and smallest = 1).

From the growth test varities #361 and 346 were used for further experimental work. After this test, seeds for all subsequent work were surface sterilized and treated as described previously. This led to completely contaminant-free growth of seedlings to 6-10 cm. height in 10-14 days when sowed at a rate of 250 gms/pan.
APPENDIX 2

PRINCIPLE AND DESCRIPTION OF OPERATION OF THE GAS CHROMATOGRAPH.

The gas chromatograph is an instrument capable of detecting the components of mixtures that have been separated on a column contained within the instrument. For the purposes of these experiments the column used was a stainless steel tube 6 ft. in length with an inner diameter of 4 mm. The column was packed with Porapak Q (Waters Associates, Maple St., Mass.). This packing is a cross-linked polymer produced by copolymerizing styrene and divinyl benzene. Differences in pore size and surface area can be obtained by varying the amount of cross-linking agent, divinyl benzene. This is used directly in the gas chromatographic column without further coating with a liquid phase. Separation of molecules occurs through the difference in hydrogen bonding characteristics of the components. This makes it suitable for use in separating alcohols, water, free acids, gases and organic solvents. However, separation of these compounds are usually achieved at temperatures at least 100 deg. C. higher than those required using conventional liquid phases on diatomaceous earth supports. The range of compounds is usually limited to those with boiling points less than 250 deg. C., with fewer than 10-12 carbons for hydrocarbons, or with molecular weights less than about 200.

The instrument has an oven that maintains the column
temperature very precisely. This temperature usually exceeds the boiling point of the volatiles being separated so that condensation will not occur on the column. The sample is injected into the column through a sealing septum (usually rubber) that allows the pressure to be maintained on the column while the sample is injected. Nitrogen is used as the inert carrier gas because it will not oxidize in the detector. The carrier gas flows into the column at the sample injection point. This point is heated by a flash heater that immediately vaporizes the sample which is then swept onto the column. Here, the components of the volatiles collected from the experimental systems are adsorbed onto the column for varying lengths of time. At the opposite end of the column is a hydrogen flame detector that senses conductivity changes due to oxidation of components in the gas stream exiting from the column. The flame burns with pure hydrogen supported by dry compressed air which is supplied immediately below the flame through a diffuser. A potential difference is maintained between a metal collector surrounding the flame and the hydrogen jet. (See Fig. 16). Oxidation and ionization of molecules causes a change in the conductivity through the flame that is detected and amplified by the gas chromatograph electrometer. The amplifier output is connected to a chart recorder where the signal is measured as a discrete series of peaks above a baseline. This baseline will vary with temperature, septum bleed and column bleed...
FIGURE 16.
but can be compensated for by a balance and zero control that allows the user to establish the baseline as zero on one side of the chart paper. The sensitivity and thus the response of the detector varies with the sample component. It tends to increase linearly with increasing number of carbon atoms and the speed with which the components elute from the column. A high, sharp peak is indicative of a reactive compound, easily ionized and low in molecular weight. Ethylene is a good example. The entire column ethylene load will elute in approximately 1-2 seconds as compared to ethanol which takes approximately 1-2 minutes to elute completely. The times for the volatiles and water are tabulated below:

<table>
<thead>
<tr>
<th>VOLATILE</th>
<th>TIME TO MID-ELUTION AFTER INJECTION</th>
<th>TIME TO ELUTE TOTAL SAMPLE FROM COLUMN</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETHYLENE</td>
<td>42 seconds</td>
<td>9 seconds</td>
</tr>
<tr>
<td>WATER</td>
<td>66 &quot;</td>
<td>12 &quot;</td>
</tr>
<tr>
<td>METHANOL</td>
<td>2.5 mins</td>
<td>35-45 &quot;</td>
</tr>
<tr>
<td>ACETALDEHYDE</td>
<td>3.5 mins</td>
<td>35-45 &quot;</td>
</tr>
<tr>
<td>ETHANOL</td>
<td>5.5 mins</td>
<td>1-2 mins</td>
</tr>
<tr>
<td>ACETONE</td>
<td>9 mins</td>
<td>3 mins</td>
</tr>
</tbody>
</table>

All gases need to be pure, dry and clean in order to detect small sample component concentrations and reduce the noise background. The amplifier can be adjusted for gain in order to increase or decrease the output to the recorder (102).
APPENDIX 3

Conversion

The calculations required to change ppM and ppB to moles follow:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Density g/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>.79 at 20 deg. C.</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>.78</td>
</tr>
<tr>
<td>Ethanol</td>
<td>.79</td>
</tr>
<tr>
<td>Ethylene</td>
<td>.00126 at 0 deg. C.</td>
</tr>
<tr>
<td></td>
<td>and 760 mm Hg.</td>
</tr>
<tr>
<td></td>
<td>= 1.116 mg/ml at 24 deg. C.</td>
</tr>
</tbody>
</table>

Ethylene, a gas requires special consideration. Since at 24 deg. C. density is 1.116 mg/ml, it follows that:

1 ppM = 1.116 ng/ml.

and 1 ppB = 1.116 pg/ml. 1 ppB is therefore equivalent to 32.5 pmoles.

1 ppM of Methanol = 79 micrograms/ml which is equivalent to 25.3 micromoles.

1 ppM of Acetaldehyde = 78 micrograms/ml which is equivalent to 34.3 micromoles.

1 ppM of Ethanol = 79 micrograms/ml which is equivalent to 36.3 micromoles.

Most experiments measured volatiles evolved in closed vials containing 1 gm fresh weight of pea epicotyls over a period of 15 min. It is sometimes desirable to convert the
concentration data (ppM and ppB) to the rate of volatile evolution (micromoles/min/gm). For methanol, 1 ppM is equivalent to 25.28 micromoles/gm/15 min which, in the experimental system equals 1.685 micromoles/gm/min. Therefore, acet-aldehyde at 1 ppM = 34.32 micromoles/g/15 min which is equivalent to 2.288 micromoles/g/min. Ethanol, at 1 ppM = 36.34 micromoles/g/15 min which is equivalent to 2.422 micromoles/g/min. Ethylene, at 1 ppB = 32.5 pmoles/g/15 min which is equivalent to 2.16 picomoles/g/min.
APPENDIX 4

Calibration of Peak Areas of Volatiles in the Gas Chromatograph.

For methanol, acetaldehyde and ethanol, serial dilutions were made in water to known concentrations and then injected into the gas chromatograph. The averages for several injections were used to plot graphs for concentration vs. peak area in mm.

Ethylene however, is a gas at room temperature and is insoluble in water. Dilutions were accomplished using a 500 ml bulb with a vacuum fitting and a system for sample withdrawal. The bulb was first evacuated with a vacuum pump capable of evacuating the bulb to a least 0.01 Torr. The bulb was then sealed and a known amount of ethylene gas (obtained from a pressurized cylinder of pure ethylene) was injected into the bulb. Ethylene free air was then allowed to enter the bulb slowly until atmospheric pressure was reached. A sample was withdrawn with a clean syringe and the dilution process was either repeated or the sample was injected into the gas chromatograph.
Figure 17. Calibration for methanol. The relationship between concentration, peak area and evolution rate.
Figure 18. Calibration for acetaldehyde. The relationship between concentration, peak area and rate of evolution.
Figure 19. Calibration for ethanol. The relationship between concentration, peak area and rate of evolution.
Figure 20. Calibration for ethylene. The relationship between concentration, peak area and rate of evolution.
APPENDIX 5

COLUMN PERFORMANCE INDICATORS

The fundamental goal of the chromatographic analysis is the separation of samples into their components for qualitative and quantitative analysis. The greatest influence on obtaining this goal is the use of an adequately selective column packing. Separations of components to the baseline are subject to two factors that are indicators of column performance. These are the narrowness of peaks and absence of tailing.

The narrowness of peaks is calculated in terms of the number of theoretical plates and is referred to as the efficiency of the column - instrument system.

The higher the number of theoretical plates, the better the separation of closely eluted components. The expression for calculating the number of theoretical plates is:

\[ N = 16 \left( \frac{X}{Y} \right)^2 \]

where the distances X and Y are measured as shown in Figure 21 (102)

Excessive peak tailing interferes with the next component eluted. This increases the problem of quantitation and the resolving of small component volumes. The extent of tailing can be measured quantitatively by taking the ratio of the widths of the front and rear halves of the peak. Therefore, the tailing factor is actually a measure of peak symmetry. A convenient method for estimating the tailing factor is to take the ratio of the total width of the peak at 1/20th the peak
height to twice the width of the front half of the peak at the same height. Tailing = a/2b where the distances a and b are measured as shown in Figure 21

<table>
<thead>
<tr>
<th>VOLATILE</th>
<th>THEORETICAL PLATES</th>
<th>TAILING FACTOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETHYLENE</td>
<td>1820</td>
<td>1</td>
</tr>
<tr>
<td>METHANOL</td>
<td>759</td>
<td>1.17</td>
</tr>
<tr>
<td>ACETALDEHYDE</td>
<td>2567</td>
<td>0.83</td>
</tr>
<tr>
<td>ETHANOL</td>
<td>807</td>
<td>1.25</td>
</tr>
</tbody>
</table>

The values for tailing factors above 1 show slight tailing of the components peaks. A value of 1 shows equal symmetry or no tailing and a value of less than 1 demonstrates that the component elutes suddenly and quickly from the column.
CALCULATING COLUMN EFFICIENCY.

CALCULATING THE TAILING FACTOR.

FIGURE 21
REFERENCES


3. Analabs Chromatography Chemicals and Accessories. Published 1980 by Foxboro Analytical, 80 Republic Dr., North Haven, CT. 06473.


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VITA AUCTORIS

Gregory Raymond Jolicoeur was born in Windsor on January 30, 1954. He attended P.J. Brennan High School in September 1967, and graduated from grade 13 in June 1972. From September 1972 to May 1974, Greg studied at the University of Windsor in the Department of Biology. From May 1974 until September 1976, Greg was employed at Valente Modco in Windsor. During this time, in July 1975, he was married to Lynda Adam. In September 1976, he returned to the University and completed an Honours B.Sc. degree by May 1978. In September 1978, he began study in the M.Sc. program under the supervision of Dr. D. Thomas in the Department of Biology.