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TITLE OF THESIS...ETHYENE AND PHENOL METABOLISM IN STORED CARROTS

UNIVERSITY...University of Alberta

DEGREE FOR WHICH THESIS WAS PRESENTED...Ph.D.

YEAR THIS DEGREE GRANTED...1972

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THE UNIVERSITY OF ALBERTA

ETHYLENE AND PHENOL METABOLISM IN STORED CARROTS

by

SUBODH KUMAR SARKAR

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PLANT SCIENCE

EDMONTON, ALBERTA

FALL, 1972

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Ethylene and Phenol Metabolism in Stored Carrots" submitted by Subodh Kumar Sarkar in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

This project was undertaken to study the effects of ethylene on phenol metabolism, particularly to ascertain the role of ethylene in the formation of isocoumarin (the so-called bitter principle) in carrots (*Daucus carota*, L.) after harvest.

In the present research, the effects of exogenously applied ethylene on the harvested carrots were studied.

Ethylene, when applied at moderate level (100 ppm), caused an increase in total phenol content of the roots. It caused an increased accumulation of the phenols normally present in the tissue, especially of isochlorogenic acid. Moreover, relatively longer exposure to a moderate level (100 ppm) and short exposure to high levels (2000 and 50,000 ppm) of ethylene induced the formation of new compounds, *viz*, isocoumarin, eugenin (a member of the chromone series), and two others, yet unidentified. (Intact roots produced about 3.5 nl ethylene per Kg fresh wt per hr. Ethylene production by carrot slices (1 mm thick) was about 1.76 nl per g fresh wt per hr immediately after cutting and about 4.2 nl per g fresh wt per hr after 22 hrs of ageing).

L-phenylalanine ammonia-lyase (PAL) (EC 4.3.1.5) activity in carrots increased on treatment with ethylene (100 ppm), reaching the peak after about 12 hrs of exposure, and then declining.

Studies with 1-^{14}C-acetate, 2-^{14}C-malonate and 3-^{14}C-acetoacetate on the pathways of formation of isocoumarin and eugenin indicated that their probable derivation was *via* the acetate pathway.

Studies with specifically labeled glucose confirmed literature reports that both the Embden-Meyerhof-Parnas and the pentose phosphate pathways operate in carrots. Added ethylene (100 ppm or 2000 ppm) was found to increase the rate of O_2 uptake and CO_2 evolution by carrot slices. It (2000 ppm for 1-8 hrs) preferentially stimulated the EMP pathway. These actions of ethylene on carrots were comparable to those of 2,4-dinitrophenol on carrots. Like ethylene, DNP induced isocoumarin synthesis in carrots. Methylene blue, an electron acceptor, often used for stimulating glucose catabolism *via* PPP, also induced isocoumarin synthesis in carrots. Both the pathways of glycolysis contributed to the synthesis of isocoumarin in carrots. This was concluded on the basis of ^{14}C incorporation into isocoumarin from specifically labeled glucose.

The effect of cycloheximide, an inhibitor of protein synthesis, suggested that the *de novo* synthesis of enzyme protein(s) might be required for isocoumarin synthesis in carrots upon treatment with ethylene.

ACKNOWLEDGEMENTS

I wish to thank Dr. C. T. Phan and Dr. Mary Spencer for their guidance and encouragement throughout all phases of the research and manuscript preparation.

I would like to express my sincere appreciation to Dr. P. R. Gorham for his valuable comments during the manuscript preparation.

I would also like to express my appreciation for the valuable suggestions I have received from the fellow graduate students and postdoctoral fellows, especially Dr. C. H. Drennan, in the South Lab.

A special note of thanks and gratitude is being extended to Dr. Theofanis Solomos (now at UCLA) for his invaluable suggestions and inspirations.

I am indebted to various researchers in Canada, United States and United Kingdom for their generous gifts of various chemical compounds.

I am thankful to Mrs. Lorraine Bendle for her fast and efficient typing of the thesis.

The financial support from the National Research Council of Canada awarded indirectly through research grants to Dr. C. T. Phan and Dr. Mary Spencer, a Dissertation Fellowship, and a special assistance for two months from the University of Alberta are gratefully acknowledged.

I am indebted to my wife for her understanding and encouragement throughout the entire program.

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LIST OF ABBREVIATIONS

A.C.S.	American Chemical Society
ATP	adenosine triphosphate
BSA	N,O-bis(trimethylsilyl)acetamide
Bz	benzene
CAH	cinnamic acid 4-hydroxylase
CoA	coenzyme-A
DHMC	5,7-dihydroxy-2-methylchromone
DNP	2,4-dinitrophenol
dpm	disintegrations per minute
DTT	dithiothreitol
EDTA	ethylenediaminetetracetic acid
EMP	Embden-Meyerhof-Parnas
G-	generally labeled radioisotope
GC	gas chromatograph
HMDS	hexamethyldisilazane
mp	melting point
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
nmr	nuclear magnetic resonance
PAL	L-phenylalanine ammonia-lyase
ppm	parts per million
PPP	pentose phosphate pathway
TCA	tricarboxylic acid
TMCS	trimethyl chloroasilane
U-	uniformly labeled radioisotope
UV	ultraviolet

INTRODUCTION

Recent studies (31,32) have indicated that carrots accumulate increasing amount of phenols during storage. Sometimes, during storage, carrots develop a bitter taste as well. Sondheimer (132) isolated and characterized a phenolic compound, commonly known as isocoumarin, from bitter-tasting carrots and named it a bitter principle in carrots.

No suggestion has been put forward to explain the increase in phenol content in carrots during storage. However, ever since the isolation of isocoumarin several investigators [Bessey *et al.* (8), Carlton *et al.* (25)], have sought the cause of bitterness in carrots. They have suggested that ethylene may induce the synthesis of isocoumarin in carrots. However, their method of isocoumarin assay (*viz*, measuring the change in UV absorbance or fluorescence of the hydrocarbon extract of carrots) after treatment with ethylene is questionable because of the unspecificity of UV absorption and fluroescence. The above investigators did not isolate and characterize isocoumarin.

Condon *et al.* (33,34) in 1963 reported that isocoumarin synthesis in carrots could also be induced by certain fungi, *viz, Ceratocystis fimbriata, Ceratocystis ulmi, Helminthosporium carbonum,* or *Fusarium oxysporum f. lycopersici.* They also commented that ethylene apparently was not a primary causative agent because only *Ceratocystis fimbriata* was able to produce ethylene.

Condon *et al.* (33,34) assumed that changes in the balance between the EMP and the PPP might have occurred in carrot slices which produced

isocoumarin on fungal infection. They also suggested that isocoumarin is produced by the disruption of the normal metabolic processes of carrots.

In this dissertation the author has studied the effects of ethylene on the quantitative as well as qualitative aspects of phenol content in carrots in view of the possible accumulation of ethylene in cold storage rooms.

Attempts have been made to clarify the role of ethylene in the formation of isocoumarin in carrots in the following manner:
(i) to study the effects of ethylene on the quantitative and qualitative aspects of phenolic content of carrots, (ii) to study the effects of ethylene on the respiratory activity of carrots, (iii) to study the effect of ethylene on the glycolytic pathways in carrots and its relation to isocoumarin synthesis.

LITERATURE REVIEW

A. Effects of Ethylene on biological systems

Ethylene, the simplest of the olefin hydrocarbons, is a gas at physiological temperatures, and has a wide range of effects on biological processes. Most of the research on the physiology of ethylene has been done with plants. There are indications that the gas is produced by and has an effect on animal tissues (28). It has also been used as an anaesthetic for mammals, although its use has decreased because of the explosive nature of certain ethylene-oxygen mixtures. the hormonal character of ethylene action had been proposed as early as 1935, but after a prolonged controversy it had only recently been named a plant hormone (111). A number of important reviews dealing with various aspects of ethylene as plant growth regulator have been written recently (1,10,21,57,93,109,134).

a. Physiological effects

A few among the numerous effects of ethylene, which have been discussed in the above reviews, are: acceleration of fruit ripening, acceleration of chlorophyll degradation, enhancement of sprouting of various corms, bulbs, roots, and hardwood cuttings, as well as germination of some species of seeds. The gas causes carnation and other flowers to droop their head as if they were becoming "sleepy".

An early assay for ethylene was based on the "triple response" of pea seedlings. The gas causes a 3-fold effect - leaf epinasty, stem swelling and inhibition of extensive growth. The gas also plays a role in abscission of leaves, fruits, petals and flowers.

Ethylene has been implicated with the development of russet spotting (a physiological disorder of unknown cause) in head lettuce (85,120). Ethylene also appears to have a catalytic effect in the formation of a bitter principle in stored carrot roots (25).

b. Biochemical effects

The gross physiological effects of ethylene discussed in the earlier section have been explained on the basis of experiments not dealing with direct biochemical action of ethylene, but indirectly with biochemical changes which occur in tissues that have been treated with ethylene. Recent reviews of Abeles (1), Spencer (134), Pratt and Goeschl (111), and Phan (109), have dealt with the subject in considerable detail. The biochemical aspects studied were (i) oxidative phosphorylation, (ii) effect(s) on the permeability of the membranes of the cell, both the cytoplasmic membrane enclosing the cell and the membranes surrounding organelles, (iii) effect on enzymes and metabolic pathways, and (iv) effect on nucleic acid metabolism.

c. Ethylene and phenol metabolism

Reports on the effect of ethylene on phenol metabolism in plants are not numerous, but there are now enough of these to be grouped as a separate aspect of ethylene action on plants. For example, Hansen (56) reported that treatment of immature pears with ethylene affected the metabolism of shikimic acid. Stahmann *et al.* (73) studied the effect of ethylene on sweet potato slices. They reported an increase in chlorogenic acid content; less than 10 ppm were required for maximal effect.

Another interesting aspect of the biosynthesis of aromatic compounds is the action of the enzyme L-phenylalanine ammonialyase (PAL). This enzyme (EC 4.3.1.5) catalyses the elimination of ammonium ions from L-phenylalanine to give trans-cinnamate. In higher plants the cinnamic acid thus formed is further metabolized to yield a great variety of phenylpropanoid compounds: lignin, flavonoids such as anthocyanins, conjugates of caffeic acid and p-coumaric acid with quinic acid, etc. (63). This enzyme has been partially purified from several sources and its distribution in the plant kingdom (153) studied. It is reasonable to assume that the enzyme acts at a switching point in metabolism and diverts phenylalanine from the general pool of amino acids used in protein synthesis to the biosynthesis of phenylpropanoid compounds.

There is evidence that ethylene induces an increase in PAL activity, either by *de novo* synthesis or by removal of some inhibitor, or both. The first report of ethylene action on PAL activity originated from Imaseki *et al.* (73). They found an increase in PAL activity of sweet potatoes on ethylene treatment. Riov *et al.* (118) reported that ethylene stimulated PAL activity in the flavedo of intact mature grapefruits; such activity rapidly decreased when the fruit was removed from the ethylene containing atmosphere. Furthermore, from their experiments with inhibitors of protein synthesis they concluded that *de novo* protein synthesis seemed necessary for enhanced PAL activity.

Recently Hyodo and Yang (68) reported a similar stimulation of PAL activity in pea seedlings and excised epicotyl tissue upon ethylene treatment. They, too, concluded from inhibitor studies that the stimu-

lation in PAL activity was due to *de novo* synthesis of protein.

Another enzyme in the biosynthetic pathway of aromatic compounds is cinnamic acid 4-hydroxylase (CAH). This enzyme catalyzes the conversion of cinnamic acid to p-coumaric acid (119). Hyodo and Yang (69) reported a marked stimulation of CAH, in excised epicotyl tissue, on treatment with ethylene. Their results with inhibitors suggested that the increase in CAH activity also resulted from *de novo* synthesis of enzyme protein.

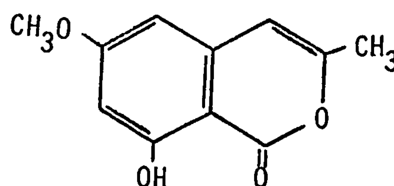
d. Ethylene and isocoumarin synthesis

There are diverse reports in the literature about the synthesis of isocoumarin in carrots. Bessey *et al.* (8) in 1956, Carlton *et al.* (25) in 1961, and more recently Chalutz *et al.* (27) in 1969, have suggested that ethylene might be a factor in inducing the synthesis of this compound in carrots. However, their conclusion was based solely on the observation of changes in ultraviolet absorption of a crude carrot extract. Condon *et al.* (33,34) found that carrots could synthesize isocoumarin on inoculation with a variety of microortanisms, *viz*, *Ceratocystis fimbriata*, *C. Ulmi*, *Helminthosporium carbonum*, and *Fusarium oxysporum f. lycopersici*. However they commented on the basis of their epinastic test of tomato seedlings in presence of different fungi that ethylene apparently was not a causative agent.

Following these findings a new aspect of the problem was opened up by Aue *et al.* (5) by their reported isolation of isocoumarin from a submerged culture of the fungus *Sporormia bipartis* cain. Their report was quickly followed by that of McGahren and Mitscher (95) who isolated

isocoumarin from the cultures of *Sporormia affinis* Sacc., Bomm and Rouss. They suggested that the occurrence of this compound in fungal infected carrots might be due to the fungus itself, thus questioning the findings of Condon *et al.* (33,34) that carrots produced isocoumarin on inoculation with certain fungi.

Curtis in 1968 (42) isolated a closely related compound, 8-hydroxy-6-methoxy-3-methylisocoumarin from the cultures of *Ceratocystis fimbriata* Ell. and Halst, the same fungus which induces the production of isocoumarin in carrots (33,34). This finding was later confirmed by Stoessl (138).



8-hydroxy-6-methoxy-3-methyl isocoumarin

e. Possible mechanisms of ethylene action

On the basis of comparisons of the biological activity of ethylene and other unsaturated compounds as determined by the pea straight growth test, Burg and Burg (22) proposed that "Biological activity requires an unsaturated bond adjacent to a terminal carbon atom, is inversely related to molecular size and is decreased by substituents which lower the electron density in the unsaturated position". Details of their hypothesis have been covered in recent reviews (1,10,21,57,93, 109,134).

Van Overbeek (147) has pointed out that certain responses to both

plant and animal hormones occur much too rapidly to be mediated through an action at the gene level. Venis (148) thought that rapid manifestations of hormone action could result from the direct allosteric modification of extranuclear enzymic or structural proteins. Spencer (134) has expressed a similar view with respect to ethylene and has suggested further that ethylene could accomplish this either by direct interaction with these entities (enzymes or structural proteins) or through the mediation of ethylene hydrates.

In considering other possible mechanisms of action of ethylene, Spencer (134) proposed that ethylene might face attack by an electrophilic reagent, namely, some bound metal of an oxidoreductase system. This ethylene-metal (bound) complex could undergo a reversible oxidation and reduction more rapidly than the bound metal alone. If this redox system was linked to oxygen, a higher oxygen uptake in presence of ethylene would result.

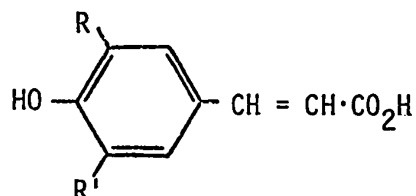
Binding of ethylene with free metal ions was suggested by Spencer (134) as another possible action of the hormone. If these ions were activators or inhibitors for certain enzymes, then change in metabolic activity would follow. She also suggested that ethylene could react through free radical mechanisms in biological systems.

B. Survey of some phenolic compounds of interest in the present study

a. Cinnamic acids and related compounds

One or more of the four acids shown on the following page occur, in combined form, in practically every higher plant. Bate-Smith (6) reports that the frequencies of occurrence in leaves of angiosperms of

p-coumaric, caffeic, ferulic and sinapic acids are 49, 63, 48 and 32% respectively. By contrast, o-hydroxy-cinnamic acids have been



p-Coumaric (R=R'=H) Ferulic (R=OMe, R'=H)

Caffeic (R=OH, R'=H) Sinapic (R=R'=OMe)

reported as occurring rarely. This is at least partly because they are readily cyclized to coumarins during isolation and tend to be recorded as such. O-coumaric acid is the only o-hydroxy-cinnamic acid that has been isolated so far; it has been found to occur with melilotic acid (the dihydro derivative of o-coumaric acid) and coumarin in three out of some eighty leguminous plants that were examined (54). The methylated derivative of sinapic acid, *i.e.*, 3,4,5-trimethoxy-cinnamic acid has been detected in *Polygala senega* and *Rauwolfia* (37,78). Another cinnamic acid, 3,4-methylene-dioxycinnamic acid, has been identified in *Piper tuberculatum* (128). Related methylenedioxy derivatives, *e.g.* piperonylic acid and piperonaldehyde, have been isolated from species of *Piper*.

Caffeic acid, one of the most widespread of all hydroxycinnamic acids, frequently occurs as esters rather than as the free acid (59). The most common caffeic acid ester is chlorogenic acid, the 3-caffeoyl

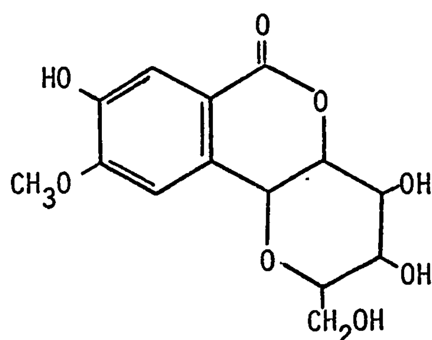
ester of quinic acid. Other isomers of chlorogenic acid are also commonly found in plant extracts. These are neochlorogenic acid and pseudochlorogenic acid. Scarpati and Esposito have shown that neochlorogenic acid is 5-caffeoylquinic acid (125). Isochlorogenic acid, previously described as an isomer of chlorogenic acid, has been identified lately as a mixture of three dicaffeoyl quinic acids (39). A 1,4-dicaffeoyl quinic acid (cynarin) has been found in artichoke, *Cynara scolymus* (107). Quinic acid esters of p-coumaric and ferulic acids have also been reported. For example, 3-p-coumaroyl quinic acid has been found in many plants (60,151) and 3-feruloylquinic acid has been isolated from coffee (38,83). The shikimic acid esters of p-coumaric, ferulic, and caffeic acids have been isolated from the cambium of *Tsuga canadensis* and from the fruit of date, *Phoenix dactylifera* (59). Mono- and dicaffeoyl esters of tartaric acid (the latter being called chicoric acid) have been isolated from the leaves of *Cichorium intybus* (124) and a caffeoyl malic acid, phaseolaric acid, has been found by Scarpati *et al.* in *Phaseolus vulgaris* seeds [In Harborne and Simmonds, (59)]. Rosmarinic acid, a combination of caffeic acid with 3,4-dihydroxy-phenyllactic acid, has been found in leaves of *Rosamarinus officinalis* [In Harborne and Simmonds (59)]. Sugar esters of caffeic acid are also widespread in plants (59).

Caffeic acid has also been found to occur in plants as glycosides. Caffeic acid 3-o-β-D-glucoside has been isolated from potato berries by Corner and Harborne (36). Klostermann and Muggli (79) obtained 4-o-β-glucoside from the hydrolysis of a complex substance in the seeds of *Linum usitatissimum*; it is also formed, along with the 3-o-glucoside when caffeic acid is fed to tomato plants (60).

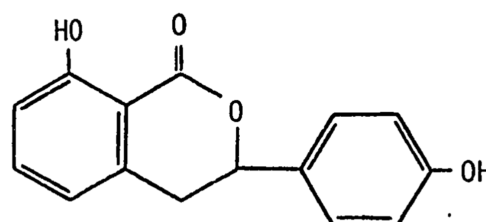
b. Isocoumarins and Chromones

(i) Isocoumarins

In contrast to hydroxycinnamic acids the occurrence of isocoumarins has not been that widespread in the plant kingdom. For example, Bergenin was found in the roots of *Astilbe, Bergenia, Peltoboykinia* and *Rodgersia* species. Recently it has been isolated from the bark of *Sacoglottis gabonensis* by Ogan (106). Hydrangenol, a 3-phenyl substituted isocoumarin, is found specifically in *Hydrangea macrophylla* and is absent from other *Hydrangea* species examined (11).

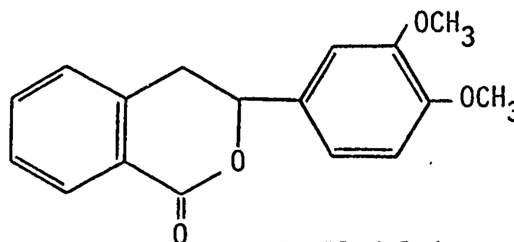


Bergenin



Hydrangenol

A dihydroisocoumarin, phyllodulcin, is the sweet principle of *Hydrangea macrophylla* (4); in contrast another dihydroisocoumarin is responsible for the bitter taste occasionally found in carrots (130). The latter compound has recently been identified as a metabolite of



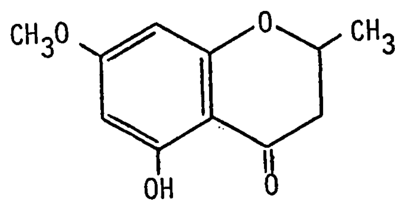
Phyllodulcin

fungi, *Sporormia bipartis* Cain (5) and *Sporormia affinis* Sacc, Bomm and Rouss (95) and most recently it has been isolated from the roots and bark of *Kigelia pinnata* DC (53). The occurrence of kigelin and demethyl kigelin (two new derivatives of dihydroisocoumarin) have been reported in *Kigelia pinnata* DC (53). Isocoumarin derivatives have also been found in the myrobalan, *Terminalia chebula* and *Aspergillus* moulds (59).

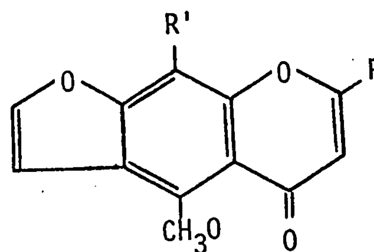
(ii) Chromones

Chromones are structurally related to the coumarins but occur with much less frequency (59). Only a few simple chromones are known and these occur in five genera: *Backhousia* and *Eugenia* (both Myrtaceae), *Eranthis* (Ranunculaceae) and *Peucedanum* and *Ammi* (both Umbelliferae) (59).

Eugenin (I) is a simple chromone present in *Eugenia aromatica*. Khellin (II, R = Me, R' = OMe) and Visnagin (II, R = Me, R' = H) are two of three furanochromones found in the fruit of *Ammi visnaga* (59). The third, khellol (II, R = CH_2OH, R' = H) has also been found in leaves of *Eranthis hiemalis* (46); all three compounds are of pharmaceutical importance.



I
Eugenin



II
Furanochromones

c. Role of phenolics in plants

The fact that plants are rich storehouses of exotic phenolic compounds has led to speculation concerning the metabolic significance of these substances. There is still considerable uncertainty concerning their physiological and biochemical behaviour. That phenolics have a role in plant pathology seems to be well established (41,82,144).

Phenolics occur in great abundance throughout the plant kingdom. Not only are they quantitatively important, but they also demonstrate a tremendous diversity of structure. Some phenolics are distributed ubiquitously, while others are confined to a small number of species. Large concentration differences in phenolics can be found within a particular species, depending on its stage of development, but differences between different species are equally great. For example, chlorogenic acid accounts for 3 per cent of the weight of unroasted coffee beans, but for only 0.0066 per cent of the dry weight of cherries (131). This great diversity in distribution, concentration, and variety of structural types would suggest multifunctional roles for phenolics in plant metabolism (58).

a. Metabolic activities

When administered to angiosperm leaf disks, U-^{14}C-tyrosine yields labelled sugars, organic acids, and glutamic and aspartic acids (71,122). Towers (145) has examined the role of phenols as substrates in the respiration of higher plants. The oxidation of ^{14}C-labelled catechins by tea plant cutting was studied by Zaprometov (155), who could account for 73 to 82 per cent of the absorbed activity as $^{14}CO_2$ after 30 hrs.

A proposal that o-diphenols, together with various phenoloxidases, function as terminal oxidase systems (58) has received considerable attention.

Gortner and Kent (52) showed that many phenols have an effect on the activity of indoleacetic acid oxidase from pineapple *in vitro*. It has also been suggested that dihydric phenols are involved in the direct synthesis of IAA from tryptophan (58). Low concentrations of catechol inhibit the tryptophan oxidizing peroxidase system (94,117). Whether or not phenols have a regulatory effect on IAA levels through an influence on endogenous IAA oxidase activity in intact plants is, however, not yet clear.

Recent reviews have been published on metabolism of phenolic compounds in plants (135) and on the role of phenolic compounds on the regulation of metabolic pathways (58).

b. Phenolic compounds and discoloration of plant products

Many fruits undergo rapid changes in color following mechanical or physiological injury during harvesting and storage (74). Onslow (105) systematically investigated the oxidizing enzymes present in higher plants and segregated them into two groups: those that contained oxygenase and catechol compounds; and those in which oxygenase and catechol compounds were absent. The first group of plants discolors rapidly on injury and includes, according to Onslow, the following fruits: apple, apricot, banana, cherry, fig, grape, peach, pear and strawberry. The second group of plants which do not discolor on injury, includes citrus fruits, red currant, melon, pineapple, and tomato.

Oxidative browning has been reported in apples (149), apricots (110), peaches (55, 110), potatoes (40,62,92,98,99), lettuce (126,127), and carrots (31,32).

Investigations with potatoes, apples and peaches have shown that maturity and both storage temperature and duration affect phenolic content and susceptibility to discoloration. For carrots, total phenols increased during storage for 3 months and were highest at a storage temperature of 50°F (31). Susceptibility to browning increased during the first month of storage (31). The phenolic content was highest in tissues nearest to the root surface, and phenol content at various depths paralleled the observed browning potential (32). However, when the browning intensity of the total root was compared with the total and oxidizable phenol content, no direct relationship was found (31).

Chlorogenic acid has been reported as a browning substrate in sweet potatoes (82) and apples (44). Chubey and Nylund (32) suggest chlorogenic acid to be the major browning substrate in carrots.

c. Phenolic compounds and tastes of plant products

Attempts to relate the tastes of substances to their chemical constitutions are probably as old as chemistry itself. The subject is complex because the palate is extraordinarily sensitive to the minor variations of the structure. The generalizations that inorganic salts are salty, acids are sour, alkaloids bitter and sugars sweet are not always reliable, and they help little in predicting the taste of numerous compounds that do not fall into one of these classes. For

example, phenolic glycosides are known to cause bitterness in citrus fruits (66,67). Data pertaining to the relative bitterness of various phenolic glycosides and their aglycones are presented in Table 1. Quinine dihydrochloride is included for comparison. It will be noted that none of the flavonone aglycones that have been tasted is bitter; only on the addition of a sugar does bitterness appear.

Sondheimer in 1956 isolated and characterized isocoumarin in the hydrocarbon extract of bitter-tasting canned carrots. He concluded tentatively from his findings "that the bitter off-flavor of carrots is caused by the presence of several compounds and that isocoumarin is one of them." Sondheimer also found that when the recrystallized isocoumarin was placed directly on the tongue it did not taste bitter but a 0.015% aqueous solution was found to be bitter by a taste panel (132). Whether bitterness in carrots is due to accumulation or synthesis of some other compound(s) is(are) a matter for further investigation. No mention was made in the report of Sondheimer (130) whether isocoumarin accumulated in carrots as such or in some bound form.

D. Biosynthesis of phenolic compounds

There are two main pathways of formation of aromatic rings in plants, one being the shikimic acid pathway and the other the acetate pathway.

Table 1

Taste and approximate relative bitterness of phenolic rhamnoglycosides and aglycones [After Horowitz (67)]

Compound	Taste	Molar Concn of iso-bitter soln	Relative bitterness
Quinine dihydrochloride	Bitter	1×10^{-5}	100
Hesperitin	Sl. sweet	—	—
Eriodictyol	No taste	—	—
Naringenin	No taste	—	—
Pinocembrin	No taste	—	—
Phloracetophenone	Bitter	—	—
Hesperidin	No taste	—	—
Eriocitrin	No taste	—	—
Naringenin 7-β-rutinoside	No taste	—	—
Isoakuranetin - 7-β-rutinoside	No taste	—	—
Phloracetophenone 4'-neohesperiodoside	Bitter	1×10^{-3}	1
Phloracetophenone 2'-methyl ether 4'-neo hesperiodoside	Bitter	—	—
Phloracetophenone 2',6'-dimethyl ether 4'-neo hesperiodoside	Bitter	—	—
Pinocembrin 7-β-neohesperiodoside	Bitter	5×10^{-4}	2
Neohesperidin	Bitter	5×10^{-4}	2
Neohesperidin oxime	Bitter	—	—
Naringin	Bitter	5×10^{-5}	20
Poncirin	Bitter	5×10^{-5}	20
Phloracetophenone 4'-β-D glucoside	Bitter	—	—
Sakuranetin 5-β-D-glucoside (Sakuranin)	Bitter	—	—
Sakuranetin	No taste	—	—

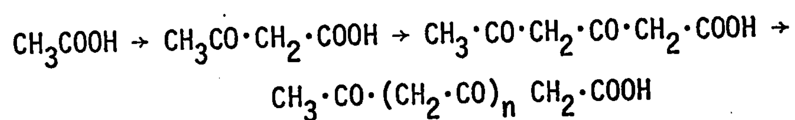
a. The shikimic acid pathway of aromatic compound biosynthesis

The key to this pathway (Fig 1) was the discovery (43,44) that a requirement of mutant strain of *E. coli* for five aromatic compounds (phenylalanine, tyrosine, tryptophan, p-aminobenzoic acid and p-hydroxybenzoic acid) could be satisfied completely by a single compound, shikimic acid. Shikimic acid is produced from carbohydrates or their derivatives (51). Details of the elucidation of this pathway are covered in a number of reviews (51, 102, 103).

It is likely that phenylalanine and tyrosine are synthesized in plants by the same route as in bacteria; all the tracer and enzyme studies in higher plants lend support to the scheme outlined in Fig 1. Neish (101,102,103) and Conn (25) in recent reviews have discussed the research done in this field in considerable detail.

b. The acetate pathway of aromatic compound biosynthesis

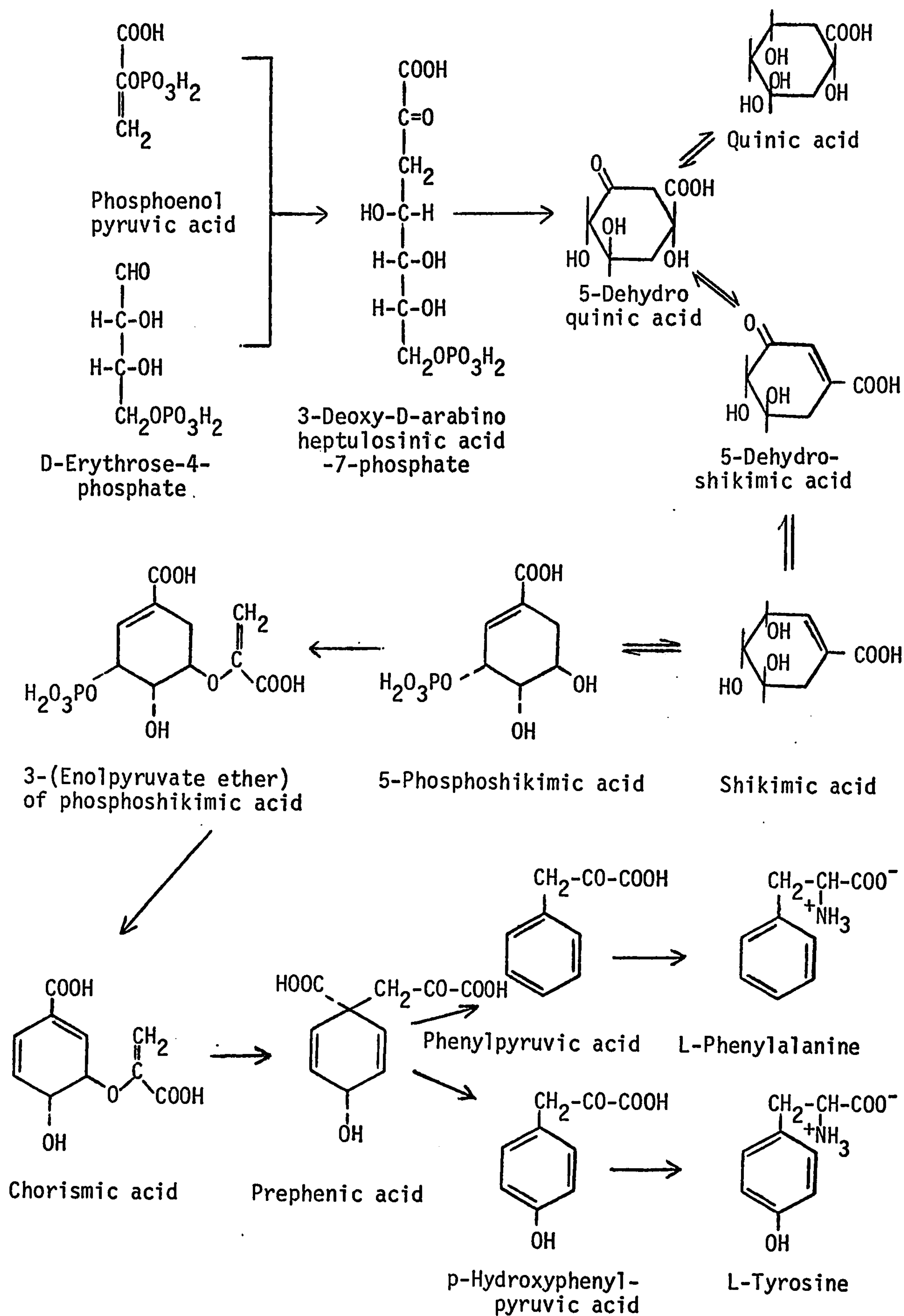
The history of the development of the acetate pathway of aromatic biosynthesis has been discussed by Neish in his review (102). In essence, the acetate hypothesis is as follows:

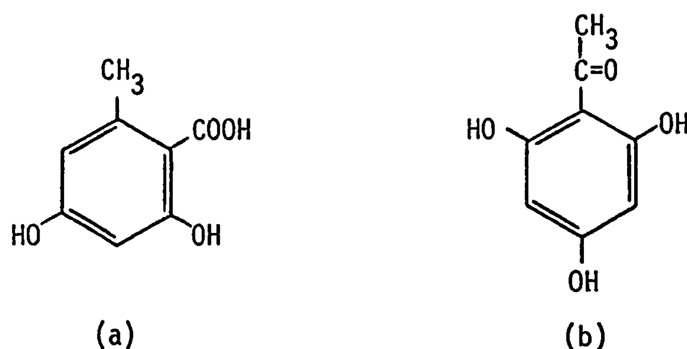


$$CH_3COOH \rightarrow CH_3CO{\cdot}CH_2{\cdot}COOH \rightarrow CH_3{\cdot}CO{\cdot}CH_2{\cdot}CO{\cdot}CH_2{\cdot}COOH \rightarrow CH_3{\cdot}CO{\cdot}(CH_2{\cdot}CO)_n\ CH_2{\cdot}COOH$$

Cyclization of an intermediate of this kind could take place either by an aldol or Claisen type condensation resulting in an acid (a) with an orcinol pattern of hydroxylation or, a ketone (b) with a phloroglucinol ring as shown on the following page. The hydroxyl oxygen is assumed to have arisen from the carboxyl group of the acetic acid and is therefore

Fig 1. Biosynthesis of the phenylpropanoid amino acids, L-phenylalanine and L-tyrosine, from carbohydrates.


COOH
$COPO_3H_2$
CH_2
Phosphoenol pyruvic acid
CHO
H-C-OH
H-C-OH
$CH_2OPO_3H_2$
D-Erythrose-4-phosphate
COOH
C=O
CH_2
HO-C-H
H-C-OH
H-C-OH
$CH_2OPO_3H_2$
3-Deoxy-D-arabino heptulosinic acid -7-phosphate
COOH
OH
HO
OH
5-Dehydro quinic acid
COOH
OH
OH
HO
OH
Quinic acid
COOH
OH
HO
5-Dehydro-shikimic acid
COOH
OH
OH
HO
Shikimic acid
COOH
H_2O_3PO
OH
OH
5-Phosphoshikimic acid
COOH
CH_2
C
COOH
H_2O_3PO
OH
3-(Enolpyruvate ether) of phosphoshikimic acid
COOH
CH_2
C
COOH
OH
Chorismic acid
HOOC
CH_2-CO-COOH
OH
Prephenic acid
CH_2-CO-COOH
Phenylpyruvic acid
CH_2-CH-COO^-
$^+NH_3$
L-Phenylalanine
CH_2-CO-COOH
OH
p-Hydroxyphenyl-pyruvic acid
CH_2-CH-COO^-
$^+NH_3$
OH
L-Tyrosine



on every other carbon atom. In order to explain missing hydroxyls, Birch *et al.* (12,13) postulated reduction of some oxygenated carbons prior to cyclization, and, for hydroxyls in the wrong place, hydroxylation of the benzene ring after cyclization. Other reactions, such as C- and O-methylation and isoprenylation, may also occur after the cyclization (102).

A simple case theoretically is the production of orsellinic acid, since this does not require the reduction of the polyketone intermediate postulated in the case of 6-methylsalicyclic acid. Mosbach (100) has studied biosynthesis of orsellinic acid from acetate labelled with both ^{14}C and ^{18}O in the carbonyl group. He found the distribution of ^{14}C in the orsellinic acid skeleton to fit the head-to-tail acetate theory. He also found that ^{18}O was also incorporated into both the hydroxyl and carboxyl groups.

The biosynthesis of 6-methylsalicyclic acid is a simple example of modification of the skeleton, i.e. reduction at one position. A somewhat more complicated case is the biosynthesis of 3-hydroxyphthalic acid in which, in addition to reduction at one position, a methyl group has been oxidized to a carboxyl group (48). Other phenolic compounds, which are formed by head-to-tail condensation of acetate units, are

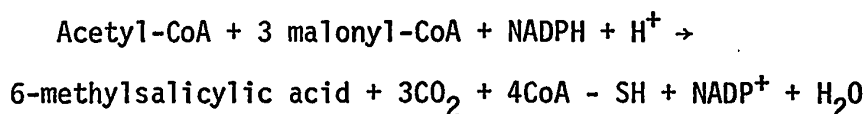
anthraquinones (14, 49, 50), benzoquinones (114,116) and tetracycline antibiotics (116).

The phenolic compounds described above are mostly synthesized by fungi utilizing acetate as the sole building unit (102). In contrast, with the exception of chromones (61), higher plants do not normally synthesize phenols that are derived exclusively from the acetate. However, phenolic compounds resulting from co-operation of the two pathways (shikimic acid and acetate) are widespread in the plant kingdom. Examples are flavonoids, isoflavones and isocoumarins (102).

c. <u>Mechanism for biosynthesis of aromatic rings from acetate</u>

The mechanisms for aromatic ring formation via head-to-tail condensation of acetate units is most probably very similar to that of fatty acid synthesis. These pathways seem to be related, since, in each system, a head-to-tail condensation of activated acetate units is involved. Recent work on biosynthesis of fatty acids has been reviewed by Stumpf (139) and Lynen (87). The mechanism of fatty acid synthesis in animals, plants and microorganisms is now well established. In this synthesis, acetyl-CoA gives rise to the CH_3CH_2- group of long chain fatty acids. The other carbons come from the methylene and ester carbons of the malonyl-CoA. The carboxyl group of malonyl-CoA is lost as carbon dioxide so the malonate carbons incorporated are those of the acetate unit in acetyl-CoA (102). If synthesis of aromatic compounds from acetate by head-to-tail condensation is analogous to fatty acid synthesis, then the $CH_3C(H)$ group should come directly from

acetyl-CoA and the rest of the acetate derived carbons should be incorporated *via* malonyl-CoA. Birch *et al.* (15) incubated 1-^{14}C-acetate and unlabelled diethyl malonate with mycelia of *Penicillium urticae* and found the 6-methyl-salicylate formed had a higher isotopic content in carbon 6 (from acetyl-CoA carboxyl) than in the carboxyl group (from malonyl-CoA carboxyl). Bu'Lock and Smalley (20) fed diethyl 2-^{14}C malonate and found it to be readily incorporated into 6-methyl salicylate, but there was no activity in the methyl group which is assumed to come directly from acetyl-CoA. Various other experiments have been reported which amply corroborate the close relationship between fatty acid biosynthesis and the formation of aromatic compounds by acetate-malonate condensation. Lynen and Tada (88), using a cell-free extract of *Penicillium patulum*, were able to demonstrate that 6-methylsalicylic acid was synthesized from acetyl-CoA and malonyl-CoA by the overall reaction:



$$\text{Acetyl-CoA} + 3\ \text{malonyl-CoA} + \text{NADPH} + H^+ \rightarrow$$
$$\text{6-methylsalicylic acid} + 3CO_2 + 4\text{CoA - SH} + \text{NADP}^+ + H_2O$$

It seemed to Lynen (87) that the enzyme system catalyzing the formation of aromatic structures resembled the enzyme complex encountered in the studies of fatty acid synthesis. He concluded that the specific arrangement of a number of common enzyme activities within the complex determines the direction of synthesis, that is, whether fatty acids, aromatic compounds, or macrolide structures are formed from malonyl-CoA and NADPH in the presence of a suitable "primer", such as acetyl-CoA and the same component enzymes.

MATERIALS AND METHODS

A. Materials

Carrots, var. Imperator 11, were obtained from the Horticultural Research Station in Brooks, Alberta. They were stored at 3±1° with no light and no control of humidity, and used whenever required.

Routine chemicals of reagent grade (meeting A.C.S. specifications) were bought from Canadian Laboratory Supplies, Fisher Scientific Company, and Aldrich Chemicals. Other chemicals *viz*, N,O-bis(trimethylsilyl)actamide (BSA), trimethyl chlorosilane (TMCS), and hexamethyldisilazane (HMDS), and silylation grade pyridine were obtained from Pierce Chemical Company. Glucose-1-^{14}C, specific activity 48.6 mCi/mmole, and glucose-6-^{14}C, specific activity 42.0 mCi/mmole were bought from Schwarz/Mann and glucose-3,4-^{14}C, specific activity 10.7 mCi/mmole was bought from New England Nuclear. Dithiothreitol (DTT; Cleland's reagent) was bought from Sigma Chemical Company. Chlorogenic acid was bought from Aldrich Chemicals and isochlorogenic acid was obtained from K & K Rare and Fine Chemicals. Hydroxycinnamic acids, *viz* p-coumaric, ferulic and caffeic acids were bought from Aldrich Chemicals. Silicic acid (100 mesh) was bought from Mallincrodt Chemical Works. Gas-chrom Q (80-100 and 100-120 mesh) and silcone DC 560 were bought from Applied Science Laboratories, Inc. Cellulose MN 300HR was supplied by Macherey, Nagel & Company.

Polyclar AT powder was obtained as a gift from Chemical Development of Canada Ltd.; Porpak Q (80-100 mesh) was a gift from Dr. S. K. Chakraborty, Research Council, Edmonton, Alberta. A standard sample of isocoumarin was kindly provided by Dr. W. J. McGahren (Lederle

Laboratories, Pearl River, New York). Standard eugenin samples were kindly given as gifts by Drs. W. Steck (Prairie Regional Laboratory, Saskatoon, Saskatchewan), H. A. Linke (Allied Chemical Corporation, Morristown, New Jersey), and R. D. H. Murray (Chemistry Department, University of Glasgow, U.K.). Standard samples of 8-hydroxy-6-methoxy-3-methylisocoumarin were kindly provided by Drs. R. F. Curtis (Head, Chemistry Division, Food Research Institute, Colney Lane, Norwich, England), and A. Stoessl (Research Institute, Research Branch, Canada Department of Agriculture, London, Ontario). G-^{3}H-5,7-dihydroxy-2-methylchromone and 5,7-dihydroxy-2-methylchromone were obtained as gifts from Dr. W. Steck (Prairie Regional Laboratory, Saskatoon, Saskatchewan).

B. Methods

The centrifugations were performed on an International Refrigerated Centrifuge (Model B-20). All the gas chromatographic determinations were made using a Varian Aerograph model 1740 gas chromatograph equipped with a flame ionization detector. The ultraviolet absorbances were measured on a Beckman DU-2 spectrophotometer whereas the complete spectra of compounds were recorded on a Perkin-Elmer 202 recording spectrophotometer.

The nmr spectra were run on a Varian A-60 as well as an HA-100 instrument with tetramethyl silane as internal reference. Mass spectra were run on an AE 1 MS 9 high resolution, direct inlet mass spectrometer.

Radioactivity measurements were made using a Nuclear Chicago Unilux II liquid scintillation counter.

1. Estimation of total phenol content

To determine the total phenol content of carrots at one month intervals during storage 10 different healthy carrots were used for each sample. Surface tissue (1 mm) was peeled from these carrots and then the peels from all these carrots were randomized. Two to five grams of the surface tissue were extracted with 70% methanol by refluxing for one hour. At the end of the refluxing period the tissue was transferred to a Virtis homogenizing flask and homogenized at medium speed for 3 min. The homogenate was then filtered through Hyflo Super Cel (a general purpose filtering aid supplied by Fisher Scientific Co.). The filtrate and the washings were combined and then concentrated under reduced pressure at a temperature of 35°. Upon removal of most of the methanol the extract always turned greenish. It is known (119) that green substances are formed by oxidation of caffeic acid esters in the presence of ammonia or amino acids. To remove any amino acids, the extract, after evaporating most of the methanol, was passed through a column of analytical grade (Fisher certified) Rexyn 101 (H^+ form 16-50 mesh) cation exchange resin and eluted with water. The completion of elution was checked for by ultraviolet absorption as well as by color reaction of phenols with Folin-Ciocalteau reagent. (The recovery experiments with known amounts of chlorogenic acid indicated no loss of chlorogenic acid on passage through the column). The effluent did not become green on concentration. This would suggest that the green color could have been a result of interaction between the caffeic acid esters and the amino acids as noted above (119). The effluent was then concentrated, made up to a definite volume, generally 100 ml, and 1 ml

aliquot was used to determine the phenolic content. The method used was that of Swain and Hilis (140) except that Folin-Ciocalteau reagent was used in place of Folin-Denis reagent. All extractions were performed in duplicate and all readings were taken in duplicate, and occasionally in triplicate. Appropriate blanks with water and other reagents were always used. A standard curve (Fig A-1) of mg chlorogenic acid *vs.* absorbance (at 725 nm) was prepared and all the results were expressed as mg of chlorogenic acid per 100 g fresh weight or as mg of chlorogenic acid per g dry weight of tissue.

2. Separation and identification of ether-insoluble phenols

Ten to fifteen different carrots of varying sizes were cut into thin transverse slices (1 mm) and then randomized. Five grams of slices were weighed and extracted with 70% methanol in water by refluxing for one hour. The slices were then homogenized as described in Section 1. The filtrate was freed of methanol and passed through a column of Rexyn 101 (H^+ form). The effluent was concentrated and then extracted with purified ether (3 x 30 ml) in a separatory funnel. The ether and aqueous layers were separated. The ether extract was kept aside (Extract 1). The aqueous layer was freed of ether and concentrated to a viscous residue on a rotary evaporator. The viscous residue was subjected to acid and alkaline hydrolysis as described by Ibrahim and Towers (70). The hydrolysates were extracted with ether as described by the above authors. This extract was (Extract 2) . Extracts 1 and 2 were mixed together and concentrated under reduced pressure. The concentrated ether solution was divided into two portions: (1) transferred to a 5 ml vial and evaporated to dryness under a jet of nitrogen and

then closed with a serum stopper; (2) transferred to a small Erlenmeyer flask and saved for paper chromatography.

3. Gas chromatography of ether extract after hydrolysis

Gas chromatography was performed on the crude extract (portion 1 above) by forming the trimethyl silyl derivatives of the hydroxy acids according to the method of Davis *et al.* (45). The standard acids were recrystallized and dried overnight in a vacuum desiccator before preparing their silyl derivatives. The silylation was accomplished with N,O-bis(trimethylsilyl)acetamide (BSA). A 5 ft (150 cm) x ¼ inch (6.25 mm) column, packed with 10% silicone DC 560 coated on gas-chrom Q (80-100 mesh) was used for the separation. The temperature settings were: 210° for the column, 230° for the injection port, and 250° for the detector. The inlet pressure of the carrier gas (N_2) was 17 psi and the chart speed was 0.2 inch per min. Two microliter of silyl derivative solution was injected into the column. Individual standards as well as mixture of the three acids were prepared for identification of the peaks.

4. Thin layer chromatography of the ether extract

Thin layer chromatography (0.25 mm thick coating) was used for separation purposes. Silica gel G and a (1:1) mixture of cellulose MN 300 HR and silica gel G were used as the adsorbents. For silica gel G plates the solvent system toluene: ethyl formate: formic acid (5:4:1) (135) was used, whereas for the plates of the mixture of cellulose and silica gel G, the solvent system was the organic phase of benzene: acetic acid: water (2:2:1) (72). Appropriate standards

were run at the same time.

5. Identification and estimation of chlorogenic and isochlorogenic acids

In the extract (portion 2, Section 2), chlorogenic acid was separated by paper chromatography on Whatman 3MM paper and was identified by co-chromatography with the standard chlorogenic acid, and also by comparison of the ultraviolet spectrum.

For estimation, the effluent from the Rexyn 101 column (H^+ form) was evaporated to dryness under reduced pressure at a temperature not exceeding 35°. The residue was leached several times with methanol and the methanol solution was made to either 2 or 5 ml. A 50 or 100 μl aliquot was applied to the paper as a streak. The chromatogram was developed with the organic phase of n-butanol: acetic acid: water (4:1:5). Equilibration of the paper did not improve the separation and so it was not practised in subsequent runs. The solvent was allowed to descend about 40 cm from the base line. The paper was then dried and the band ascribed to chlorogenic acid was cut out and eluted with methanol. The eluant was centrifuged to remove floating fibres, made to either 2 or 5 ml with methanol and the absorbance at 330 nm determined. The results are expressed as absorbance per 5 g fresh weight of tissue in order to compare these with the values obtained for isochlorogenic acid, whose identity is unsettled. (Isochlorogenic acid has been reported in the literature (39) as being a mixture of three dicaffeoyl quinic acids. The acid that was bought from K & K Rare and Fine Chemicals was given a chemical formula (by the supplier) corresponding to 5-caffeoyl quinic acid, which is commonly known as neo-chlorogenic acid. The author has made some attempts to identify it.

were run at the same time.

5. <u>Identification and estimation of chlorogenic and isochlorogenic acids</u>

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The spectral and mass spectrometric data suggest that the above acid may be primarily 4,5-dicaffeoyl quinic acid).

A band having the same Rf value and a UV spectrum similar to that of isochlorogenic acid was found to be quite prominent, particularly in carrots treated with ethylene. This band was cut out and eluted with methanol. The absorbance was measured at 330 nm after making the eluant to either 2 or 5 ml with spectrograde methanol. The results were expressed as absorbance per 5 g fresh weight of tissue.

6. Extraction of the enzyme: L-phenylalanine ammonia-lyase (PAL)

The activity of PAL could not be detected in the extract of whole carrots. However, by using only the 1 mm outer layer (portion richest in phenolic compounds) an active preparation was always obtained.

The carrots were surface sterilized by dipping into a 1% (w/v available chlorine) NaOCl solution for 5 min. The roots were then rinsed under running tap water for another 5 min. Extraction and assay of PAL were carried out according to the method of Zucker (156) with a slight modification. Ten grams of the 1 mm thick outer layer of carrots was cut into small pieces and then homogenized, along with 1 g of purified polyclar AT, in 25 ml of 0.05 M boric acid-borate buffer (Na^+), pH 8.7, 1 mM in DTT and 1 mM in EDTA, using a high speed Virtis homogenizer. After homogenization for 1 min the homogenate was strained through 4 layers of cheese cloth. The filtrate was centrifuged at 18,000 g for 10 min and the resulting supernatant was further purified by passing it through a column (2.5 x 45 cm) packed with Sephadex G-25 (Medium). The protein fraction was eluted with 0.05 M borate buffer, pH 8.7 and used directly for enzyme assay.

All the above operations were carried out at 0-4°.

An acetone powder of the 1 mm thick carrot peel tissue was prepared by the method described by Rahe *et al.* (112).

7. Enzyme assay for PAL

The assay mixture contained 2 ml of 0.05 M borate buffer, pH 8.7, which was 1 mM in DTT and 1 mM in EDTA, 1 ml of 0.05 M L-phenylalanine solution in the above buffer, and 2 ml of enzyme extract in a total volume of 5 ml. The control had 1 ml of extra buffer instead of the L-phenylalanine solution. Incubations were carried out for 4 hours at 33°. All the experiments were done in duplicate and many in triplicate. Enzyme activity was determined spectrophotometrically by following the increase in absorbance at 290 nm (81). The absorbance was converted to mg of cinnamic acid (Fig A-6) and the activity was expressed in nmoles of cinnamic acid produced per mg protein per hour.
Protein in the extract was determined by the method described by Lowry *et al.* (84) [See Fig A-7].

8. Identification of the product of the enzymatic reaction

Following the incubation period 2 ml of 6N HCl were added to each incubation mixture. The solutions were then centrifuged and the supernatant was extracted with ether. The ether extract was dried over anhydrous Na_2SO_4 and evaporated to dryness. As no cinnamic acid could be detected after thin layer or paper chromatography of the residues, trimethylsilyl ethers prepared from the components of the residues were separated and estimated by gas chromatography as indicated below. Standard cinnamic acid was also silylated for direct comparison.

Silylation was performed as follows: 0.4 ml of BSA, 0.1 ml (trimethyl chlorosilane) TMCS, 0.2 ml (hexamethyl disilazane) HMDS, and 0.3 ml of dry pyridine were added to the residues from the test and control runs. A solid precipitate which was obtained in the flasks was avoided while withdrawing the solution by means of a syringe. A 6 ft (180 cm) x $\frac{1}{8}$ inch (3.1 mm) stainless steel column, packed with 10% silicone DC 560 on gas chrom Q (100-120 mesh) was used for the separation purposes. The temperature settings were: 160° for the column, 227° for the injection port, and 260° for the detector. The inlet pressure of the carrier gas (N_2) was 16.5 psi.

9. Isolation of large amounts of isocoumarin and eugenin for subsequent characterization

About 600 g of carrot slices, 4 mm in thickness, were placed on a perforated porcelain support in a large desiccator. Water was added at the bottom of the desiccator to keep the slices moist. Slices were separated from each other by means of glass wool. A constant stream of 100 ppm ethylene was passed through the desiccator for 7 days at a temperature of 23±2°. After 7 days the slices were added to boiling methanol, refluxed for two hours, then homogenized and filtered. The residue was washed several times with 70% methanol until the washings turned colorless. The filtrate was evaporated almost to dryness under reduced pressure, then treated with 80% methanol. Most of the carotene crystallized out on standing in the refrigerator overnight. The mother liquor after filtration was evaporated to dryness. The resultant residue was chromatographed on a column containing 10 g of silicic acid

(100 mesh) and eluted with benzene (Bz) and benzene-methanol mixtures (Bz: MeOH) as indicated below:

Fraction No.	Elution solvent	Volume eluted	Remarks
1	Bz	80 ml	No phenolic compounds
2	Bz 95%, MeOH 5%	80 ml	Single phenolic compound
3	Bz 95%, MeOH 5%	80 ml	Mixture of phenolic compounds
4	Bz 50%, MeOH 50%	80 ml	Mixture of phenolic compounds

Fraction No. 2 was evaporated and the residue crystallized from ether-pentane. 500 g of carrot slices yielded about 100 mg of crystalline material.

Fractions 3 and 4 above were combined and rechromatographed on a column packed with 15 g of silicic acid (100 mesh). The elution pattern is given below:

Fraction No.	Eluting solvent	Effluent volume	Remarks
1	Benzene (Bz^1)	150 ml	$Iso^+ + U^*$
2	Benzene (Bz^1)	125 ml	less iso^+ more U^*
3	Benzene (Bz^1)	50 ml	only U^*
4	Benzene (Bz^1)	50 ml	only U^*
5	Benzene (Bz^1)	75 ml	$Mixture^{**}$
6	Benzene (Bz^1)	50 ml	$Mixture^{**}$

Fraction No.	Eluting solvent	Effluent volume	Remarks
7	[1]Bz: MeOH (99:1)	100 ml	Mixture**
8	[1]Bz: MeOH (50:50)	150 ml	Mixture**

Bz[1] = Benzene

Mixture** = Iso+ + U* + others

Iso+ = isocoumarin

U* = Unknown

Fractions 5-8 were combined and rechromatographed on a separate column of silicic acid. Eventually enough of the unknown compound was isolated in the pure form to allow crystallization from ether-pentane. 500 g of carrots yielded about 35 mg of the crystalline material.

10. Isolation and quantitative estimation of isocoumarin in carrot slices treated with ethylene

Eight to ten grams of carrot slices (4 mm thick) were added in each flask and were selected from the randomized pile of slices from 10 to 15 carrots for treatment with ethylene free air, or 100 ppm ethylene at room temperature. Moisture was maintained by putting water at the bottom of the Erlenmeyer flask. The water was then covered with some glass beads. At the end of each required period of exposure the slices from each flask were dropped in boiling 70% methanol and refluxed for one hour. The slices were then homogenized and filtered. The residue was washed several times with 70% methanol until the washings were colorless. This was found to be a good indicator of the completeness of extraction, as evidenced by thin layer chromatography

followed by fluorescence detection. The filtrate was freed of methanol on a rotary evaporator keeping the temperature of the bath at about 30°. The residue was thoroughly extracted with ether (4 x 15 ml) in a separatory funnel. The completeness of the extraction was checked as described above.

The ether solution was dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was dissolved in chloroform and made to 2 ml in a volumetric flask. Fifty microliter were applied to a 0.25 mm thick silica gel G plate. The chromatogram was developed with toluene: ethyl formate: formic acid (5:4:1). The band due to isocoumarin was scraped off and extracted with reagent grade ether (3 x 10 ml). This gave about 99% recovery. The extract was evaporated to dryness and freed completely of ether. The residue was made to 2 ml with spectrograde methanol and the absorbance was measured at 267 nm (see Fig A-2) against a similar extract of silica gel coating developed in a similar manner. The absorbance value was then converted to mg of isocoumarin from the standard curve (Fig A-8). All experiments were done in duplicate.

11. <u>Measurement of O_2 uptake and CO_2 evolution by carrot slices</u>

Whole carrots were washed in cold water and then patted dry with a paper towel. The outer 1 mm thick layer was removed from the roots and cut into small pieces about 5 mm long. These tissues were termed "skin tissues". Cylinders of root tissue without the outer layer were removed with a cork borer (6 mm diam.) and sliced into disks about 1 mm thick. These were termed "inner tissues". The disks were washed in three changes of distilled water and used for respiration measurement.

In most of the experiments, 10-15 disks were placed in each flask.

The gas exchange was measured manometrically at 25° by the direct Warburg method (146). No buffer was employed. A 20% KOH solution (0.3 ml) was used in the centre well for CO_2 absorption. Readings were made for 30-40 min in either 100 ppm or 2000 ppm ethylene (in air) or air (ethylene free). After that the Warburg flasks were disconnected and placed in large bottles and a constant stream of 100 or 2000 ppm ethylene or air was passed through the bottles to expose the tissues continuously with ethylene or air. Moisture was maintained by keeping a layer of water on the bottom of the large bottles. After the required period of exposure the centre well KOH solution was replaced and measurements were made as described before. The same procedure was followed for subsequent runs. All measurements were made in duplicate.

12. <u>Measurement of CO_2 production by carrot slices after feeding specifically labeled glucose</u>

All the glucose samples were converted to the same specific activity. All compounds were infiltrated in aqueous solution under a vacuum of 12 mm of mercury from a water aspirator. Three grams of 1 mm slices of carrots were used for this purpose. After infiltration the slices were washed with water and the washings were added to the remainder of the ^{14}C-glucose solution. The radioactivity was counted by the method of Baxter *et al.* (7). Sixty-five to seventy per cent efficiency was obtained with aqueous samples.

13. <u>Absorption of CO_2 and determination of its radioactivity</u>

$^{14}CO_2$ was absorbed in a mixture of ethanolamine and methanol

(20:80) and counted after adding toluene based scintillation solvent according to the method described by Boggiolini *et al.* (18). A counting efficiency in the range of 55-59% was obtained.

Counts per min was always converted to dpm with the help of a quench curve prepared by counting ^{14}C-quenched standards (Fig A-13).

14. Collection of ethylene

The method described by Knight (80) was used as such except that copper U-tubes were used in place of glass U-tubes. A continuous stream of air was freed of ethylene by passing through mercuric perchlorate absorbed on silica gel (28-200 mesh) in a U-tube that was placed in an ice bath (this ethylene-free air hereafter will be referred to as air). The air flowed into the flasks containing 5 or 10 g of carrot slices, or into the jar containing whole carrots through a U-tube (id 1.5 cm) containing drierite (8 mesh) to remove moisture. The gas stream was then passed through 1 g silica gel (28-200 mesh) in a copper U-tube (id 4 mm) kept in dry ice-acetone bath. The gas mixture evolved from whole carrots was swept out of the flask containing whole tissue, at a flow rate of 100 ml per min, and at 25 ml per min from flasks containing carrot slices by means of high pressure purified air.

Immediately prior to collecting ethylene, the copper U-tube containing 1 g silica gel was heated in a boiling water bath for about 30 min. During this heating period, a stream of purified nitrogen was passed continuously through the tube to remove all the gases that were evolved from silica gel. The tube was sealed with a short piece of

rubber tubing and transferred to the collection system.

15. Estimation of ethylene

Ethylene was estimated by gas chromatography using a 5 ft (1.50 cm) x $\frac{1}{8}$ inch (3.1 mm) stainless steel column, packed with activated alumina, 60-80 mesh. The gas from the U-tube was directly delivered into the column by means of an auxiliary carrier gas (He) flow mechanism. One end of the copper U-tube was connected to the auxiliary carrier gas flow system (back flush, 4 point valve) keeping the U-tube in dry ice-acetone bath. The valve was opened for 30 sec and the U-tube was flushed with helium. After 30 sec the valve was closed and the other end of the copper U-tube was connected to helium flow system. With the valve closed the U-tube was heated in a water bath at 49° for 3 min to release the gases from silica gel. The valve was then opened and the contents of the U-tube were flushed onto the GC column. One min was found to be sufficient to scavenge all the ethylene absorbed on to silica gel. The temperature settings were as follows : column - 50°, injection port - 125°, and detector - 200°. Carrier gas flow rate was 24 ml per min.

The peak heights in the chromatogram were plotted against known amounts of standard ethylene in nl (Fig A-9 and A-10). From these calibration curves the amount of ethylene in an unknown sample was determined from the peak height of that sample. The peak height instead of area was used because of the relative sharpness of ethylene peak in the gas chromatogram. The instrumental error was kept at a minimum by maintaining the same conditions in all the runs.

16. <u>Estimation of acetaldehyde and ethanol</u>

About 5 g of 1 mm thick carrot slices were either exposed to air or to 100 ppm, or 0.2% (2000 ppm) ethylene for a known length of time. At the end of the exposure period the slices were transferred to two different flasks connected to two different cold traps leading to the vacuum pump. The flasks containing the slices were placed in a water bath at 50°. The cold traps were cooled by liquid N_2 and the distillation under a pressure of 0.1 mm of mercury was done for 45 minutes.

The air or ethylene stream coming out of the flasks containing carrot slices were passed through empty U-tubes (internal diameter 1.5 cm) cooled in a dry ice-acetone bath (108). This would condense any acetaldehyde or ethanol coming from the tissue. The distillate and the condensate were mixed together for each of the two samples - control and test, and then made to 5 ml in a volumetric flask. The estimation of acetaldehyde and ethanol in these samples was accomplished by injecting 4 μl of the solution into a porapak Q (80-100 mesh) column and separated by gas chromatography. A 6 ft (180 cm) x $\frac{1}{8}$ inch (3.1 mm) stainless column was used for the separation purpose. Carrier gas (He) was flown at the rate of 28 ml per min. The temperature settings were as follows: column - 100°, injection port - 148°, and the detector was at 193°.

Two standard curves, one for acetaldehyde (Fig A-11) and the other for ethanol (Fig A-12) were prepared under similar conditions of the gas chromatograph as described above, based on areas under the peaks as the instrument response. Areas were measured with a planimeter. By measuring

area of an unknown peak the amount of either acetaldehyde or ethanol was calculated from the standard curves.

17. Feeding of radioactive tracers

Labelled compounds were administered in aqueous solutions either by an infiltration technique or by shaking the slices continuously with the solutions of the labelled compounds. The infiltration was done by agitating the slices in the solutions of labelled compounds for 1 and ½ min under a vacuum of 12 mm of Hg (with the help of a water aspirator).

A continuous stream of 0.2% ethylene was passed through the flasks in all the cases.

The per cent conversion was calculated on the basis of the amount of labelled compound taken up, not on the amount of labelled compound metabolized.

18. Determination of specific activities of isocoumarin and eugenin

Isocoumarin and eugenin were extracted from carrot slices as described before (B.10). They were separated as bands by thin layer chromatography. The bands were scraped and extracted with appropriate solvents (ether in most cases but acetone or methanol were used for 5,7-dihydroxy-2-methyl-chromone). After evaporating the eluting solvent the absorbance was measured in methanol at 267 nm in the case of isocoumarin (Fig A-2) and 248 nm in the case of eugenin (Fig A-3) and 5,7-dihydroxy-2-methyl chromone (29). After measuring the absorbance the solutions were quantitatively transferred back to the flask. The solution was evaporated and the residue was

quantitatively transferred to a scintillation vial by repeated washing of the residue with the scintillation solvent. The activity in the residue was then counted. For isocoumarin and eugenin, toluene based scintillation solvent was used and for 5,7-dihydroxy-2-methylchromone toluene and methanol based scintillation cocktail was used according to the method described by Baxter *et al.* (7).

Counts per min was always converted to dmp with the help of a quench curve prepared by counting ^{14}C-quenched standards (see Fig A-13) or ^{3}H-quenched standards (see Fig A-14).

19. Treatment of carrot slices with inhibitor solutions

Inhibitors, *viz*, DNP, methylene blue, arsenite and cycloheximide, were either dissolved in glass-distilled water or in buffer solutions. All these inhibitor solutions were infiltrated either into 1 mm or 4 mm thick carrot slices. For infiltration purposes, a vacuum (of 14 mm of Hg) was used for 1 min for 1 mm thick and 1½ min for 4 mm thick slices. The slices were then removed from the vacuum flask and patted dry on paper towel.

RESULTS AND DISCUSSION

Section I

Effect of storage on the phenol content of carrots

The carrots that were stored for 9 months were tested periodically at one month's interval for total phenol content. The total phenolic extract was then divided into two portions (1) ether-soluble and (2) ether-insoluble. The qualitative composition of these two fractions were determined by paper, thin layer or gas chromatography.

A. Centripetal distribution of total phenols in carrots

In order to find out whether some parts of the root were richer in phenol content than others, the root was cut into three portions: (1) "peel" refers to the skin, about 1 mm thick, (2) "phloem" represents the tissue portion between the outer wall of central core or the pith and the outer portion of the root (without the 1 mm thick skin), (3) "xylem" represents the central core or the pith.

Three different tissue portions were separated from 10 different carrots and were piled up separately. Each pile was extracted as described under Materials and Methods (B.2). Extraction was done in triplicate for each tissue portion. Total phenol in each extract was determined in triplicate. The average for triplicate values from each extract had a standard deviation of ±0.03, whereas the average of three different extracts had a standard deviation of ±0.11. The result of a typical experiment was recorded in Table 2. The data showed that about 85% of total phenol was present in the "peel", 10% in the "phloem", and 5% in the "xylem". Chubey and Nylund (31) found that the total phenol and

Table 2

Centripetal distribution of total phenol content in carrots

Tissue portion	Total phenol: mg of chlorogenic acid per g dry wt.	Distribution % (of total)
Peel	19.67	85%
Phloem	1.98	10%
Xylem	1.05	5%

"Peel" refers to about 1 mm thick outer layer, "phloem" to the tissue portion between the xylem and the 1 mm thick outer layer), and the "xylem" to the central core or the pith of the root.

oxidizable phenol contents decreased substantially from the surface to the second layer; thereafter they varied only slightly. (In their study the surface referred to 1.5 mm thick outermost layer, and the second layer represented the region 1.5-3.0 mm below the root surface). Thus, most of the phenols were located in the 1 mm thick skin.

B. Variation of total phenol content of carrots during storage at 3±1°

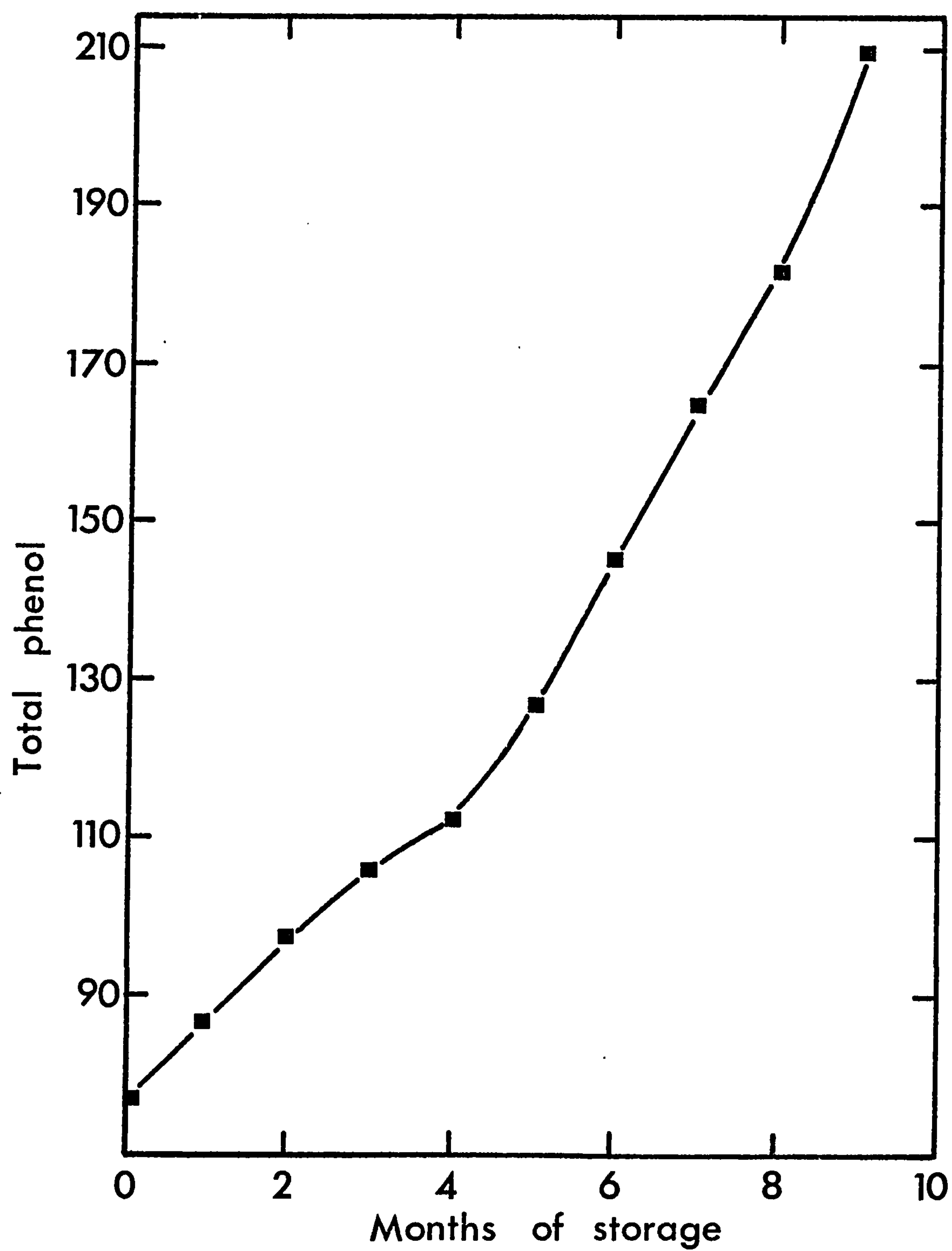
The carrots were stored in plastic bags (thickness 1 mil) for the entire period of 9 months. About 1 mm thick skin was removed from 10 intact roots, cut into 1 cm small pieces and then mixed together. About 5 g was accurately weighed and used for total phenol determination. The extraction was done in triplicate and the determination of total phenol in each extract was made in triplicate. The standard deviation for the average of triplicate determination was ±0.04, and that for the average of triplicate extraction was ±0.09. Fig 2 shows that the total phenol content of carrots increases steadily on storage. Chubey and Nylund (32) reported similar observation in carrots. They found that phenol content of carrots increased during storage and was highest at a storage temperature of 50°F. They stored the carrots for 3 months. Mondy *et al.* (98) reported similar relationship in potatoes. However, Craft *et al.* (40) reported that storage up to five months did not affect the phenol content in potatoes.

C. Qualitative composition of phenolic compounds in the aqueous carrot extract

(i) Paper chromatography of ether-insoluble phenols

The whole aqueous extract was partitioned with ether, the ether-

Fig 2. Variation of total phenol content of carrots during storage at 3±1°. Total phenol is expressed as mg chlorogenic acid per 100 g fresh weight.


210
190
170
150
130
110
90
Total phenol
0
2
4
6
8
10
Months of storage

soluble fraction was kept aside. The ether-insoluble fraction was concentrated and then streaked on a 3 MM paper. On development, the chromatogram showed several bands distinguishable as due to phenols by the usual color reactions, and by color and fluorescence under ultraviolet light. Rf values are presented in Table 3.

On repeating the qualitative analysis of phenols in carrots during the entire storage period of nine months no detectable change in the qualitative composition was observed. Throughout the entire storage period the same qualitative composition (Table 3) was maintained by the carrot roots.

(ii) Isolation and characterization of ether-insoluble phenols

The bands at Rfs 0.82, 0.76, and 0.64 (Table 3) were identified as due to caffeic, isochlorogenic, and chlorogenic acids, respectively. This was accomplished by co-chromatography and by comparing the ultraviolet spectra with those of authentic standards. Whether caffeic acid is an artifact of isolation is not clear at the moment. The chemical composition of isochlorogenic acid is unsettled. This has been described under Materials and Methods (B.5).

Little is known about the identities of the other four bands (Table 3), but they may be conjugates of caffeic, p-coumaric or ferulic acids, as all the three acids have been identified after acidic and alkaline hydrolysis, by thin layer as well as by gas chromatography. Fig 3 is a diagram showing the locations of three phenolic acids on the thin layer plates. The mixture of cellulose and silica gel G gave a better separation than silica gel G alone. The color reactions of these phenolic acids together with their ultraviolet fluorescence are

Table 3

R_f values of ether-insoluble

phenols separated by paper chromatography

R_f	Identity
0.82	Caffeic acid
0.76	Isochlorogenic acid
0.64	Chlorogenic acid
0.57	Not identified
0.52	Not identified
0.39	Not identified
0.31	Not identified

Solvent system was the organic phase of the mixture: n-butanol: acetic acid: water (4:1:5).

Fig 3. Thin-layer chromatography of cinnamic acids.

a. Adsorbent: Silica gel G

Solvent: Toluene: ethyl formate: formic acid (5:4:1 v/w)

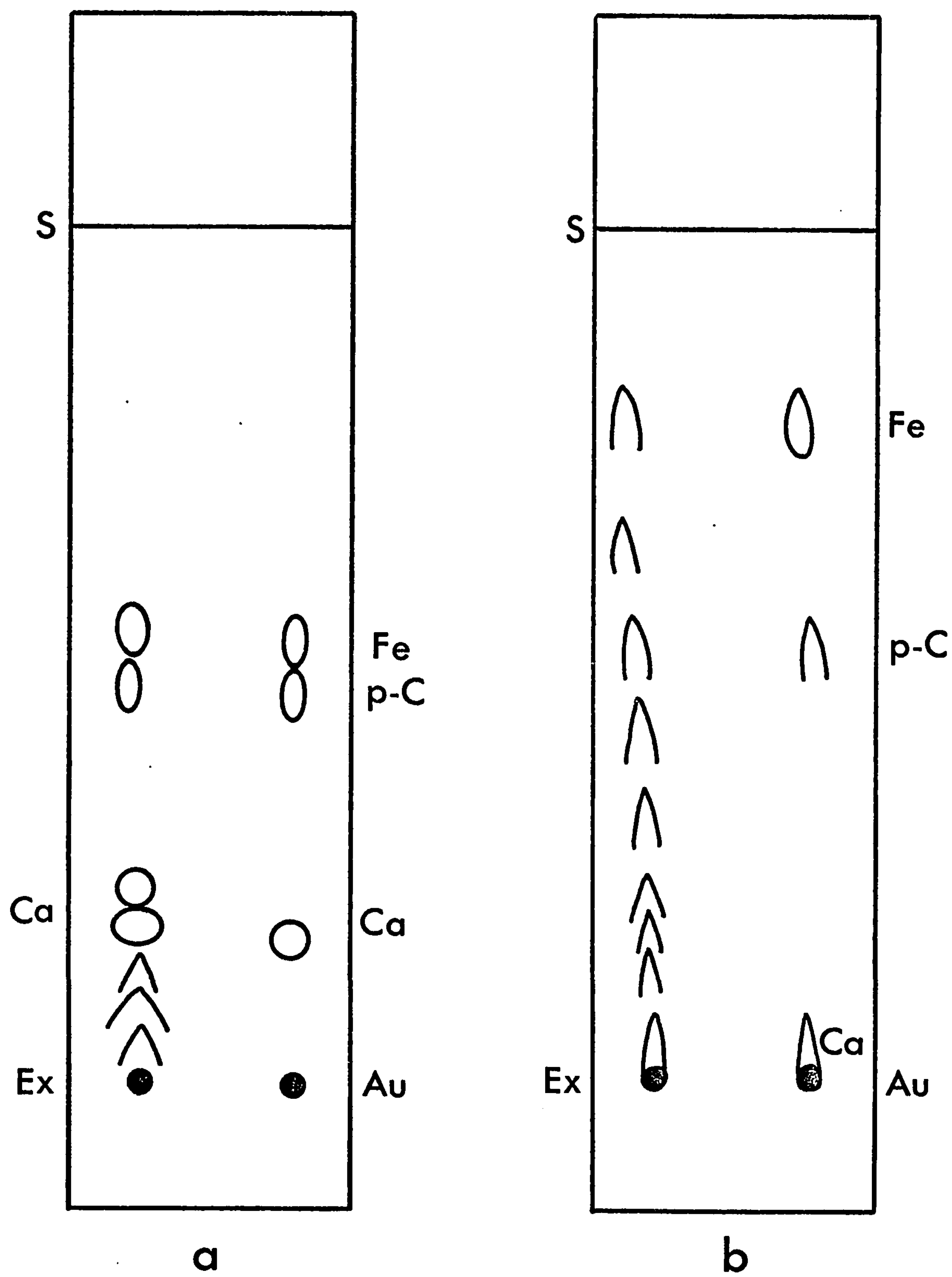
b. Adsorbent: Cellulose MN300HR: silica gel G (1:1)

Solvent: Organic phase of benzene: acetic acid: water (2:2:1 v/w)

S = solvent front; Fe = ferulic acid;

p-C = p-coumaric acid; Ca = caffeic acid;

Ex = extract; Au = authentic standard mixture


S
Fe
p-C
Ca
Ca
Ex
Au
a
S
Fe
p-C
Ca
Ex
Au
b

listed in Table 4. Fig 4 shows the gas chromatogram obtained with the extract after carrying out silylation with BSA. The identity of the three acids in the extract was established by comparing the retention time (Table 5) as well as by co-chromatography with the standards (Fig 4). When the chromatogram was run under programmed temperature conditions there was another prominent peak as well as a number of other smaller ones. The identities of these were not determined.

(iii) Thin layer chromatography of ether-soluble phenols

The ether-soluble fraction of the aqueous carrot extract, which was kept aside, was concentrated and streaked on 0.25 mm thick silica gel G coating. Rf values were recorded in Table 6. No attempts were made to identify any of the phenolic compounds present in the ether-soluble fraction of carrot extracts during storage. This was because the abundances of ether-soluble phenols were negligible compared to those of ether-insoluble ones. No isocoumarin was detected during the storage period. Probably the level of isocoumarin was too low to be detected by the methods used in this study. The method used in this investigation enables one to detect as low as 0.1 μg of isocoumarin by UV fluorescence and determine about 15 μg by UV absorption.

Conclusions on Section I

In summary, the carrot roots contain several phenolic compounds of common occurrence in other plants. Most of the phenols are located in the 1 mm thick skin of the root. During storage the phenols increased quantitatively without any apparent change in the qualitative composition. The above results also suggest that under normal storage conditions no detectable amount of isocoumarin is accumulated.

Table 4

Color reactions and ultraviolet fluorescence of hydroxy-cinnamic acids

Acid	UV fluorescence	Color reactions		
		Diazotized p-nitro aniline/NaOH	Diazotized Sulfanilic acid	1% $FeCl_3$ (alcoholic)
p-Coumaric	Dark blue	Blue	Light brown	None
Ferulic	Sky blue	Bluish green	Purple	None
Caffeic	Bluish white	Light brown Fading to white	Buff	Dark green

Fig 4. Gas chromatograms of cinnamic acids.

a: standard mixture

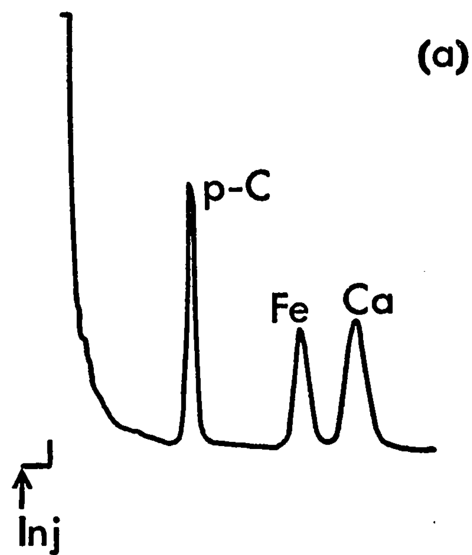
b: extract

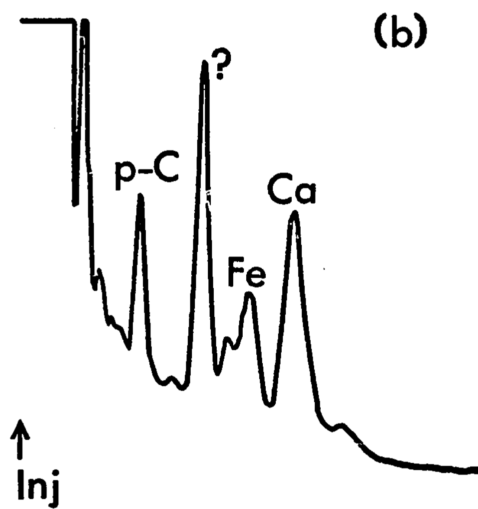
c: extract + internal standards

p-C = p-coumaric acid; Fe = ferulic acid

Ca = caffeic acid; ? = unidentified

Inj. = injection point


(a)
p-C
Fe
Ca
Inj


(b)
?
p-C
Ca
Fe
Inj

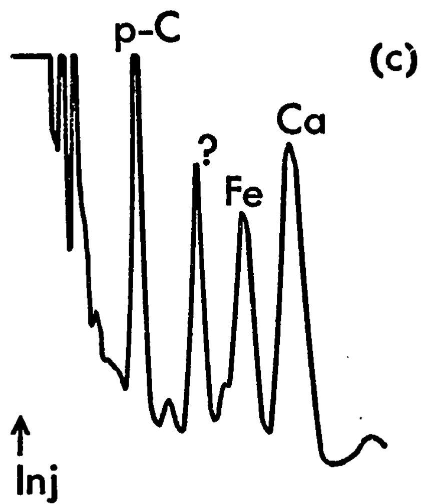

p-C
(c)
Ca
?
Fe
Inj

Table 5

Retention times of the cinnamic acids

(as calculated from the chromatograms of Figure 4)

Compound	Retention time	Identified as
Pure Standards:		
p-Coumaric acid	14 min. 12 sec.	
Ferulic acid	25 min. 24 sec.	
Caffeic acid	30 min. 30 sec.	
Extract:		
First peak	14 min. 30 sec.	p-Coumaric acid
Second peak	20 min. 54 sec.	?
Third Peak	25 min. 48 sec.	Ferulic acid
Fourth peak	30 min. 15 sec.	Caffeic acid
Co-chromatography:		
First peak	14 min. 45 sec.	p-Coumaric acid
Second peak	20 min. 57 sec.	?
Third peak	25 min. 42 sec.	Ferulic acid
Fourth peak	30 min. 30 sec.	Caffeic acid

? indicates unknown

Table 6

R_f values of ether-soluble compounds*

separated by thin layer chromatography

R_f	Color in UV	Color with Folin-ciocalteau reagent	Color with $FeCl_3$
0.95	BWF**	None	None
0.83	Reddish purple	None	None
0.52	Dull blue	None	None
0.46	Blue	Bluish green	Green
0.30	Blue	Blue	Green

*None of these compounds have been identified.

**BWF - bluish white fluorescence.

The solvent system was toluene: ethyl formate: formic acid (5:4:1).

Section II

Effects of ethylene on the quantitative and qualitative composition of phenols in carrots

A. On the centripetal distribution of total phenol content in carrots

It was seen earlier (Results and Discussion, Section IA) that about 85% of the total phenol of carrots was present in the 1 mm thick skin, 10% in the "phloem" and 5% in the "xylem". The effect of ethylene on the above distribution of total phenols in carrot roots was recorded in Table 7. Each datum is average of triplicate determinations. The standard deviation of the average for both air-treated and ethylene-treated samples was ±0.03.

The results show that the same distribution pattern exists for the phenolic content of "peel", "phloem" and "xylem", upon air or ethylene treatment. This observation was repeated with different batches of carrots.

Whether phenols are synthesized at one location and then transported to different parts of the tissue, or whether they are synthesized at different locations, the effect of ethylene is felt throughout the cross-section of the tissue to the same extent, and the phenolic content in "peel", "phloem", and "xylem" doubles the value obtained on air treatment.

B. On the total phenol content of carrots

The change in phenol content with days of exposure to air or 100 ppm ethylene was shown in Fig 5. The carrots were exposed to constant stream of air and 100 ppm ethylene for the times indicated at room temperature (25±0.5°). Total phenols were determined in

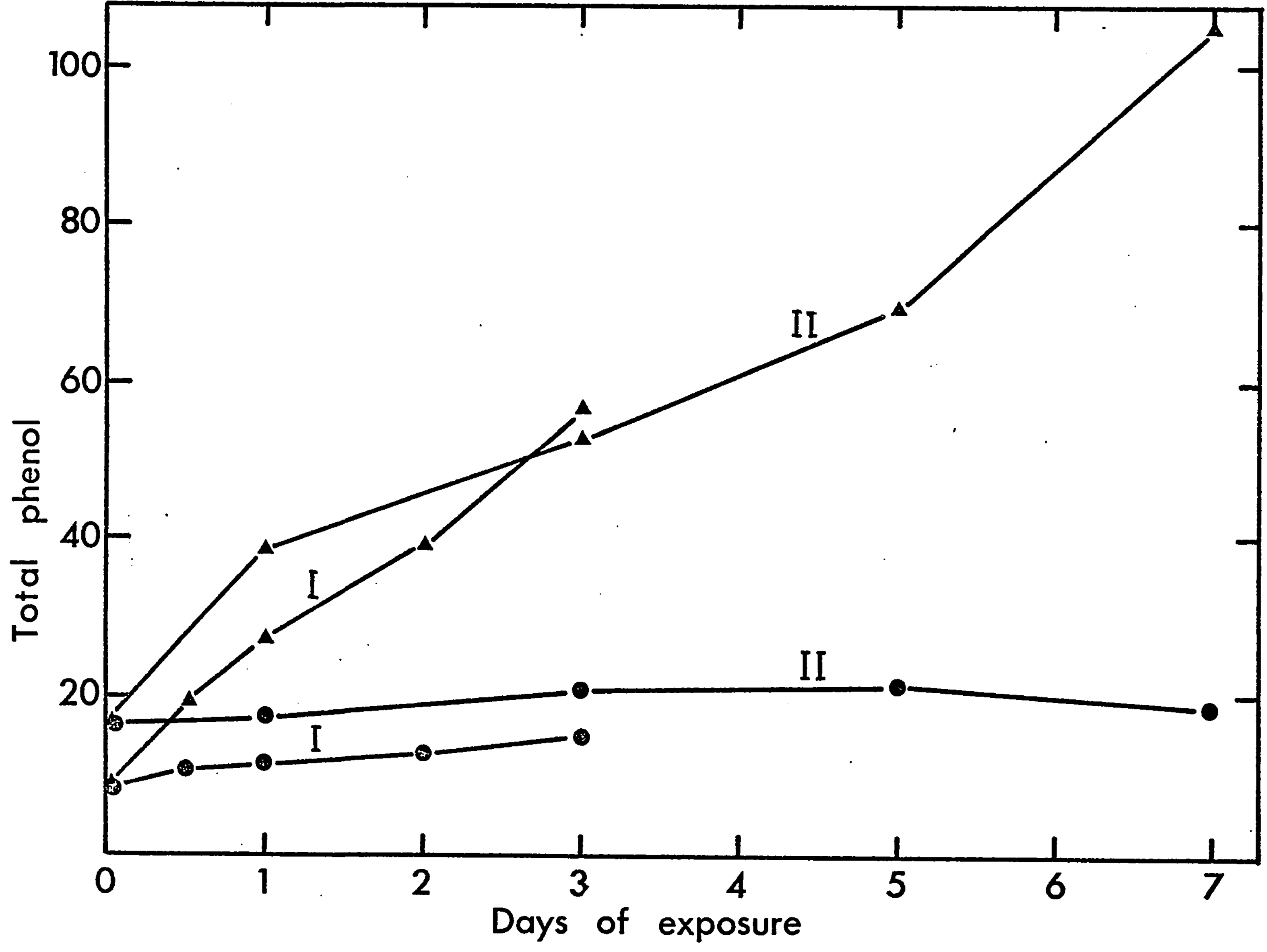
Table 7

Centripetal distribution of total phenol content in carrots, exposed to air and 100 ppm ethylene for 2 days

Tissue portion	Total phenol content: mg chlorogenic acid per g dry wt.	
	Exposed to air	Exposed to ethylene
"Peel"	21.87	43.21
"Phloem"	2.26	4.46
"Xylem"	1.35	2.88

"Peel", "phloem" and "xylem" have the same meaning as elsewhere.

Fig 5. Effect of continued exposure to 100 ppm ethylene on the total phenol content of carrots. Total phenol was expressed as mg chlorogenic acid per g dry wt. (▲——▲) represents total phenol content in ethylene-treated carrots, and (●——●) represents total phenol content in air-treated carrots. I and II represent two different experiments.



100
80
60
40
20
Total phenol
II
I
II
I
0
1
2
3
4
5
6
7
Days of exposure

the combined "peel" tissue from 10 carrots treated either with air or 100 ppm ethylene. The data are the average of triplicate determinations. The standard deviation of the averages for both air-treated and ethylene-treated samples was ±0.03. The results show that the amount of phenol increased 5-fold on 3-day exposure and about 7-fold on 7-day exposure to 100 ppm ethylene. Could ethylene affect phenol synthesis in carrots during storage?

C. <u>On the qualitative composition of phenols</u>

a. <u>On ether-insoluble phenols</u>

(i) <u>Separation of ether-insoluble phenols by paper chromatography</u>

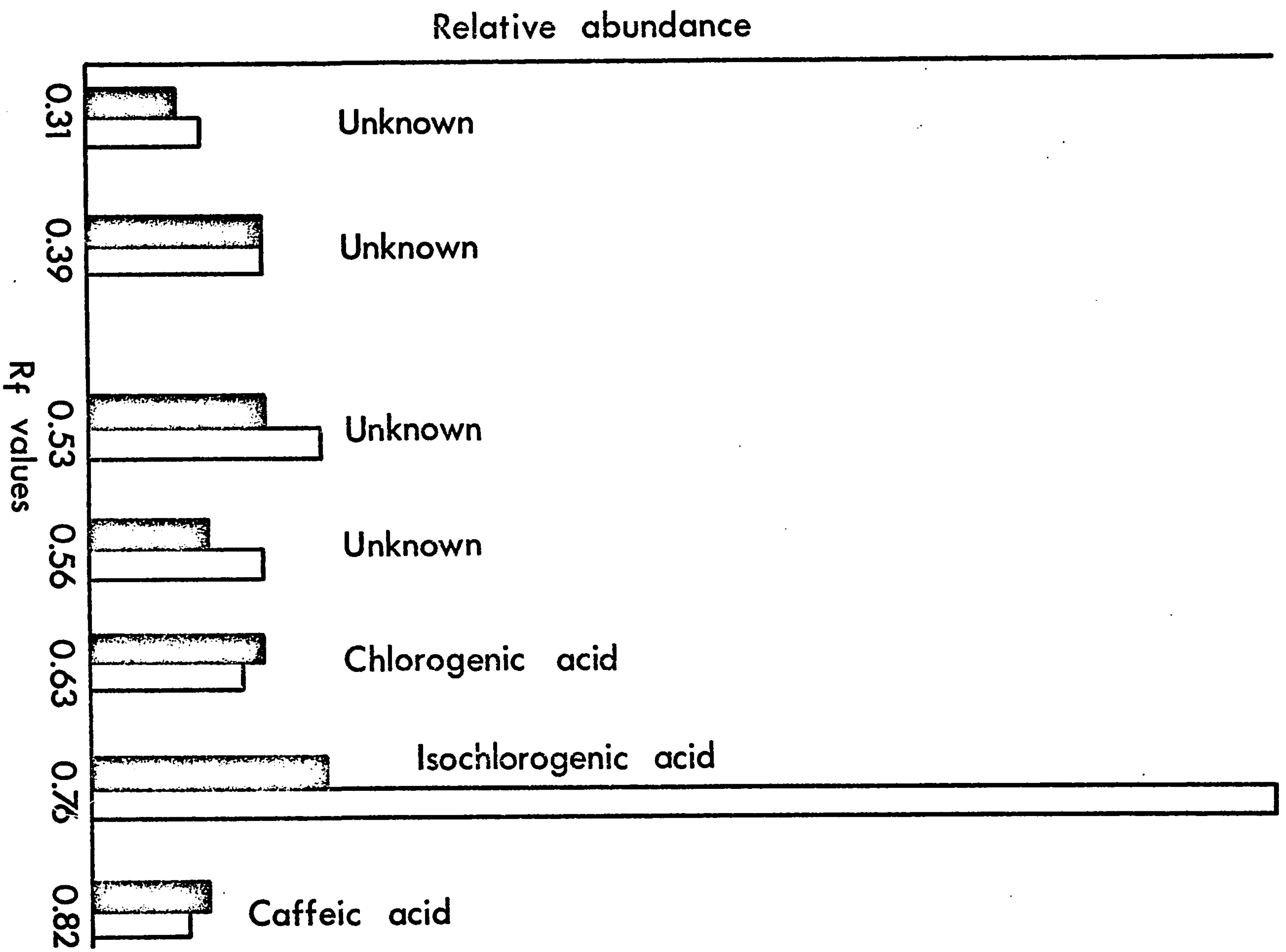
It was found earlier (Results and Discussion Section IC) that there were at least 7 phenols in the ether-insoluble portion of the carrot extract. Three of them were identified as caffeic, isochlorogenic, and chlorogenic acids. The effect of ethylene on the above qualitative composition of ether-insoluble phenols was studied. Carrots (numbering 10) were exposed to 100 ppm ethylene or air for 4 days at room temperature. Phenols were then extracted as described before (Materials and Methods, B.2). Rf values and relative abundances were recorded in Fig 6. Relative abundances were estimated roughly by the intensity of fluorescence and color under UV light. The results revealed that on exposure to air neither the qualitative composition nor the relative amounts of the individual component changed significantly. On exposure to 100 ppm ethylene one component demonstrated marked prominence over others. This compound was identified as isochlorogenic acid (as supplied by K & K Rare

and Fine Chemicals). Isochlorogenic acid increased almost 5-fold on exposure to 100 ppm ethylene (Fig 6). This observation was repeated several times.

(ii) Effect of exposure time on ethylene-induced increased isochlorogenic acid formation in carrots

The results of Fig 6 indicated an increased isochlorogenic acid formation in carrots upon ethylene treatment. The time course of increased isochlorogenic acid formation was recorded in Figs 7 and 8. The graph was plotted with 330 nm (in MeOH) per 5 g fresh weight of carrots against days of exposure to either 100 ppm ethylene or air. Because isochlorogenic acid could not be obtained in pure form the absorbance was not converted to mg of isochlorogenic acid. The absorbance of chlorogenic acid could have been converted to the corresponding figure in mg but it was recorded as absorbance in Figs 7 and 8 for the sake of comparison with that of isochlorogenic acid. The molar absorbance at 330 nm for chlorogenic acid was found to be 21,000. Corse *et al.* (39) reported a value of about 20,000 for the molar absorbance at 330 nm for isochlorogenic acid. Therefore, a comparison of absorbance of chlorogenic and isochlorogenic acid seemed appropriate. On the paper chromatogram as low as 0.5 μg of chlorogenic and about 1.0 μg of isochlorogenic acid could be detected by UV fluorescence. However, for quantitative estimation purposes about 25 μg of chlorogenic and about 50 μg of isochlorogenic acid was found appropriate to record a reliable reading on the spectrophotometer. This difference was due perhaps to lower sensitivity of absorption

Fig 6. R_f values and relative abundances of ether-insoluble phenols separated by paper chromatography. The organic phase of n-butanol: acetic acid: water (4:1:5) was used as the developing solvent. Solid bar represents phenols in air-treated carrots, and blank bar represents phenols in 100 ppm ethylene-treated carrots. Relative abundances were estimated visually on the basis of area of fluorescence of different compounds under UV light.


Relative abundance
Unknown
Unknown
Unknown
Unknown
Chlorogenic acid
Isochlorogenic acid
Caffeic acid
0.31
0.39
0.53
0.56
0.63
0.76
0.82
Rf values

compared to fluorescence (72). From Figs 7 and 8 two important observations could be made: (1) both ethylene free air and 100 ppm ethylene brought a slight decrease in chlorogenic acid content. (2) in case of isochlorogenic acid, a slight increase in air and a marked increase in 100 ppm ethylene was obtained. The rate of isochlorogenic acid formation in carrots treated with ethylene can not be matched by the rate of decrease in chlorogenic acid. From zero to a half day of exposure to 100 ppm ethylene (Figs 7 and 8) the isochlorogenic acid formation increased at a steady rate, between a half and one day it was almost constant, and between one and two days and two and three days of exposure the isochlorogenic acid formation increased, but at different rates.

(iii) Centripetal distribution of chlorogenic and isochlorogenic acid

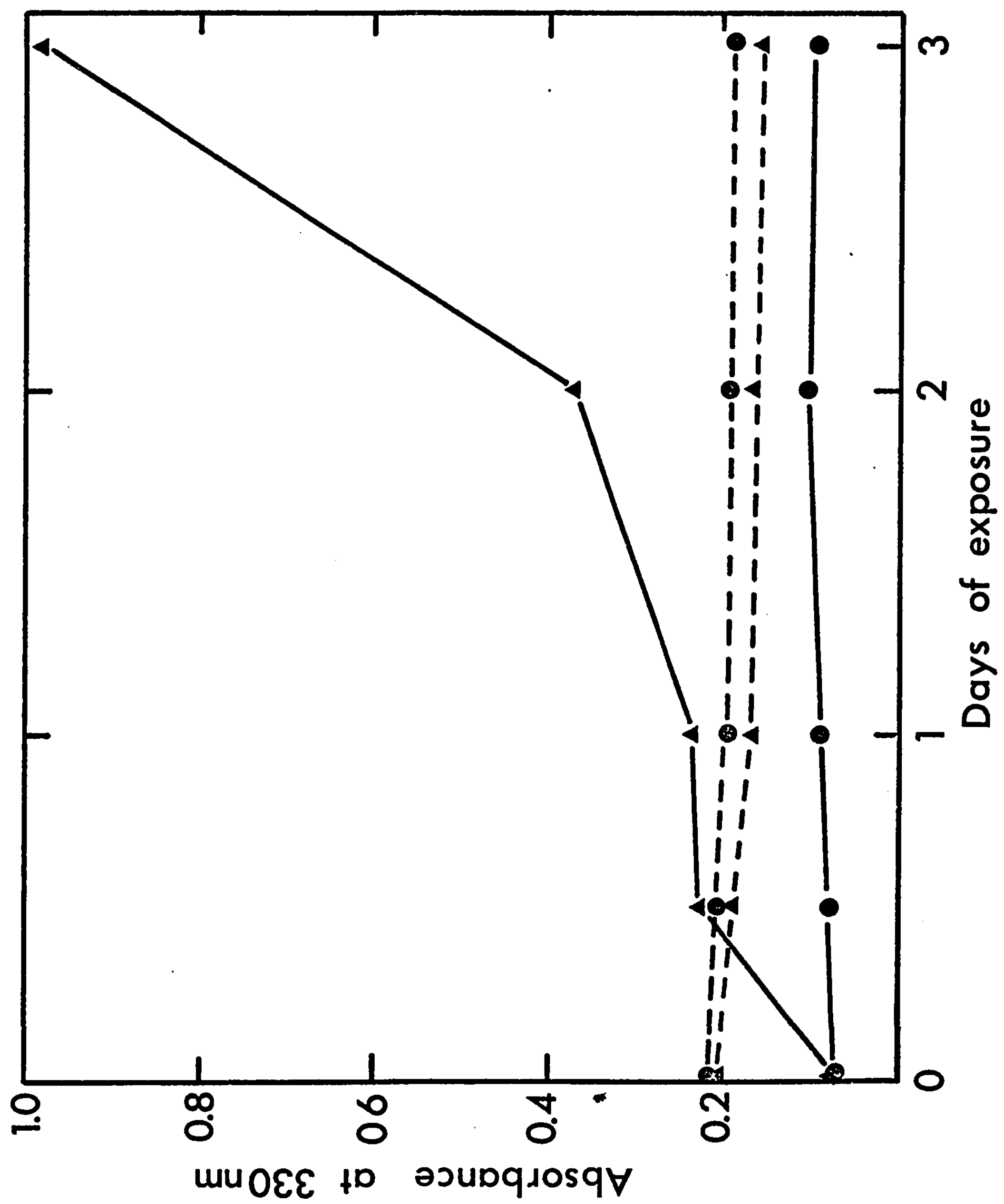
In order to find out whether ethylene-induced increased isochlorogenic acid occurred in "peel", "phloem" or "xylem", the amounts of chlorogenic and isochlorogenic acids in these tissue portions were determined after treatment with air and 100 ppm ethylene (of carrot roots) for 2 days. The results were recorded in Table 8. Most of the chlorogenic and isochlorogenic acids were present in the "peel". Trace amounts (about 0.5-1.0 μg of chlorogenic acid and 1.0-2.0 μg of isochlorogenic acid) were present in the "phloem" and "xylem".

b. On ether-soluble phenols

(i) Separation of ether-soluble phenols by thin layer chromatography

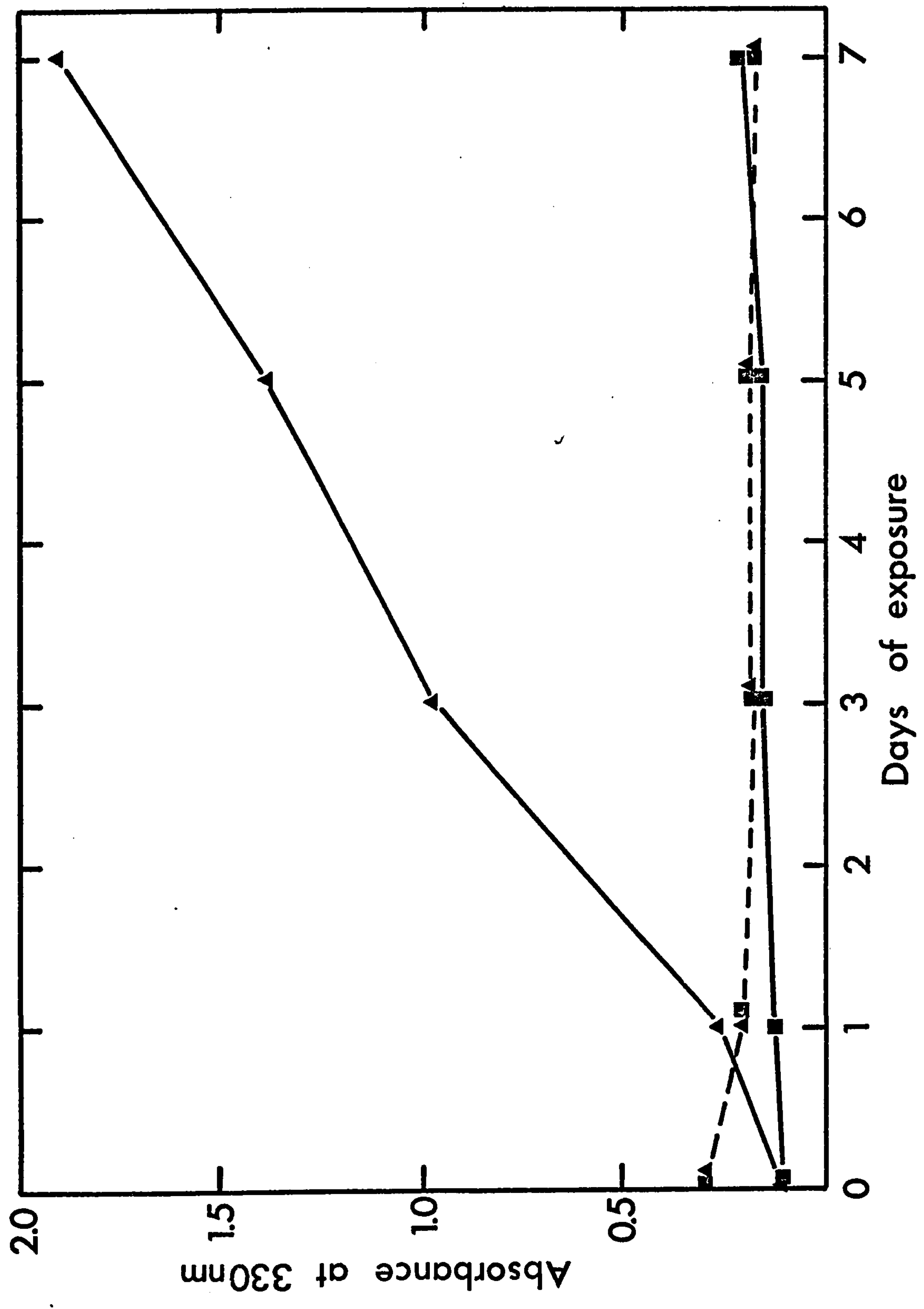
Ether-soluble phenols which were separated by thin layer chromatography during storage were relatively less abundant than the ether-

Fig 7. Time course of isochlorogenic acid accumulation in carrots upon 3 day exposure to ethylene (100 ppm). Amounts of chlorogenic and isochlorogenic acids were expressed as absorbance at 330 nm per 5 g of carrots. (▲——▲) represents isochlorogenic acid in ethylene-treated, and (●——●) represents isochlorogenic acid in air-treated carrots. (▲- - -▲) represents chlorogenic acid in ethylene-treated, and (●- - -●) represents chlorogenic acid in air-treated carrots.



Absorbance at 330nm
1.0
0.8
0.6
0.4
0.2
0
1
2
3
Days of exposure

Fig 8. Time course of isochlorogenic acid accumulation in carrots upon 7 day exposure to ethylene (100 ppm). Amounts of chlorogenic and isochlorogenic acid were expressed as absorbance at 330 nm per 5 g of carrots. (▲——▲) represents isochlorogenic acid in ethylene-treated, and (■——■) represents isochlorogenic acid in air-treated carrots. (▲----▲) represents chlorogenic acid in ethylene-treated, and (■----■) represents chlorogenic acid in air-treated carrots.



Absorbance at 330nm
2.0
1.5
1.0
0.5
0
1
2
3
4
5
6
7
Days of exposure

Table 8

Effect of air and 100 ppm ethylene on the centripetal distribution of chlorogenic and isochlorogenic acids

Tissue portions	Chlorogenic acid		Isochlorogenic acid	
	Air treated	100 ppm ethylene treated	Air treated	100 ppm ethylene treated
"Peel"	0.190	0.235	0.230	0.810
"Phloem"	*Trace	*Trace	*Trace	*Trace
"Xylem"	*Trace	*Trace	*Trace	*Trace

Chlorogenic and isochlorogenic acids are expressed as absorbance at 330 nm/5g fresh weight.

*Trace = Not determinable by its UV absorption.

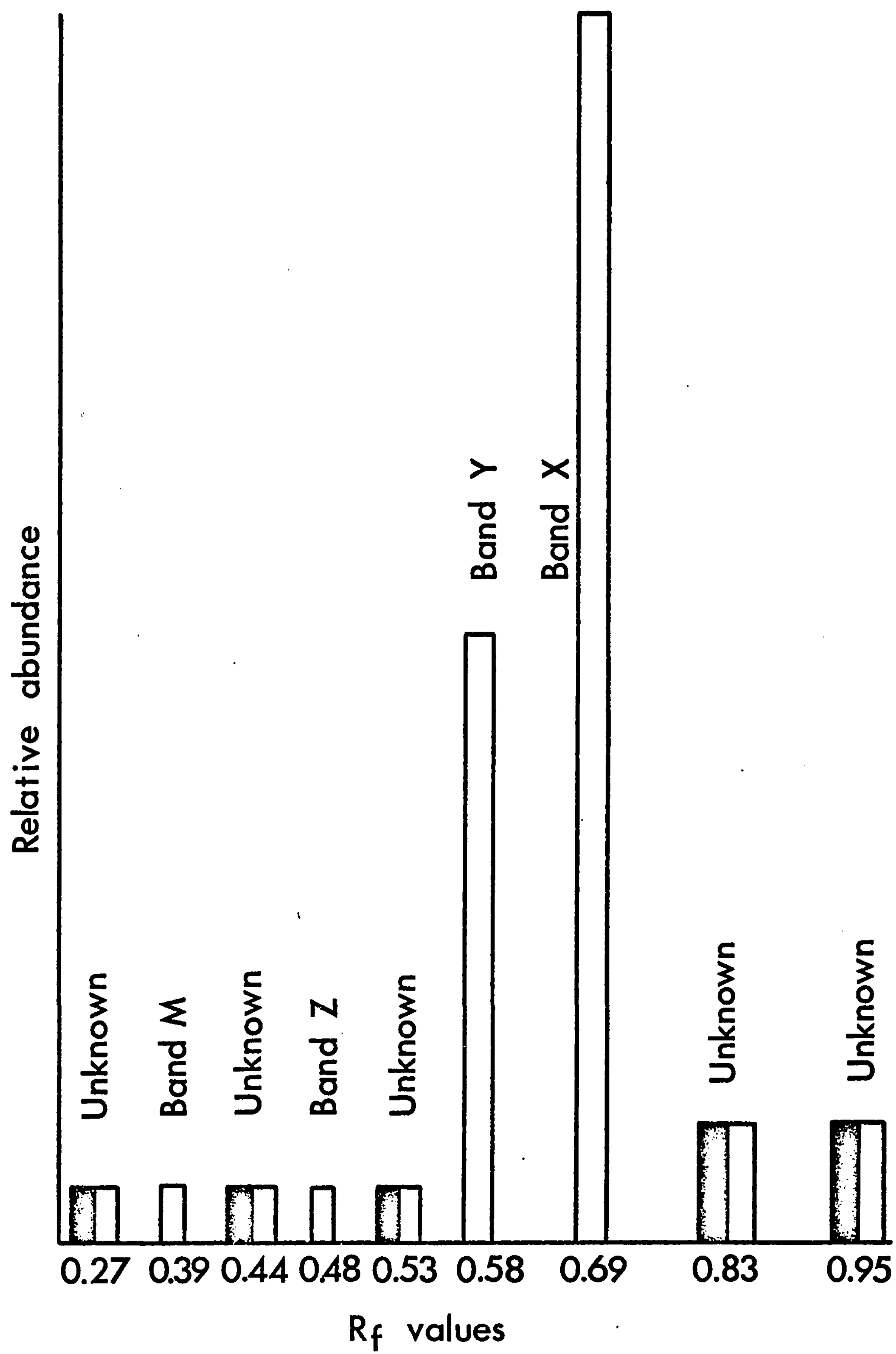
insoluble ones. But ethylene produced a pronounced effect on the qualitative composition of these phenols. In these experiments carrots were treated with 100 ppm ethylene or air for 4 days at room temperature (25±0.5°). As shown in Figure 9, four new phenolic compounds were detectable on the chromatogram by their color or fluorescence under UV light. Two of these four compounds were accumulated in considerable amounts. As the sensitivity of the detecting technique is important in view of the above findings the sensitivity was determined for the two relatively abundant compounds. Band X was detectable as a bluish-white fluorescent band under UV light. Band Y did not show any fluorescence but it became light purple on standing and did not change the color under UV light. For band X, 0.1 μg was detectable by its fluorescence whereas for band Y 1-2 μg was found necessary for its detection. Other two bands, *viz*, Z and M exhibited bluish-white fluorescence under UV light. The sensitivity for their detection was not determined, but it was felt that they too would have a lower limit of 0.1 μg for their detection.

The results of the above experiments are well-founded by their established repeatibility. The above thin layer profile (Fig 9) has been confirmed with other solvent system, *viz*, benzene: methanol (95:5), and on polyamide II coating with 95% ethanol as developing solvent.

(ii) Characterization of ether-soluble phenols

The compounds corresponding to Rf values 0.69 (band X) and 0.58 (band Y) were isolated in fairly large amounts by column

Fig 9. R_f values and relative abundances of ether-soluble compounds separated by thin layer chromatography. Silica gel G coating (0.25 mm thick) and the solvent system, toluene: ethyl formate: formic acid (5:4:1) was used. Solid bars represent phenols in air-treated, and blank bars represent phenols in ethylene-treated carrots.



Relative abundance
Unknown
Band M
Unknown
Band Z
Unknown
Band Y
Band X
Unknown
Unknown
0.27
0.39
0.44
0.48
0.53
0.58
0.69
0.83
0.95
Rf values

chromatography and were crystallized (Materials and Methods, B.9). The steps involved in their characterization are given below:

1. Characterization of band X compound

The band X compound yielded crystals, mp 75-75.5° (unc); $[\alpha]^{25}D$--52.0±3.5[c 1.0, MeOH] lit. (95) mp 75.5-76°; $[\alpha]^{25}D$-51.0±3.0 (c 1.0, MeOH)]; λ_{max} (MeOH) 302 nm (ε, 4900), 267 (ε,12,400), and 217 (ε, 19,600); ν_{max} ($CHCl_3$) 1668, 1630, 1586, 1505, 1375, 1310, 1250, 1160, 1120, 1075, 1038, 968 cm^{-1}; nmr ($CDCl_3$) at δ1.52 (3 protons-doublet, J = 6 cps assigned to $-CH_3$), δ2.87 (2 protons-doublet, J = 8 cps, assigned to $-CH_2-$), δ3.82 (sharp singlet, 3 protons, assigned to $-OCH_3$), δ4.67 (multiplet, assigned to -CH-), δ6.25 and 6.36 (1 proton each, split singlets, J = 2 cps. assigned to m-coupled aromatic protons), and δ11.20 (1 proton singlet, exchangeable with D_2O, assigned to hydrogen bonded phenolic -OH). Microanalytical data are presented below:

Calculated for $C_{11}H_{12}O_4$: C, 63.45; H, 5.81; mol wt, 208.
Found: C, 63.35; H, 5.71; mol wt, 208 (M^+ peak in mass spectrum).

The most abundant peak at *m/e* 164 (400%) arose because of loss of acetaldehyde. A metastable peak at *m/e* 129.3 confirmed this loss from the molecular ion. The loss of $CH_3CO\cdot$ accounted for the peak at *m/e* 165 (10%), and the other peaks were the same as reported (95).

All these results were further corroborated by comparison with an authentic sample of 6-methoxy-mellein, (or isocoumarin as it is commonly called).

Isocoumarin was found to occur free in carrots. No bound form was detected in the carefully prepared extracts.

2. Characterization of band Y compound

The band y compound yielded crystals that melted at 119-119.5°(unc); ν_{max} (MeOH) 227 (ε, 21,400), 248 (ε, 25,500), 255 (ε, 25,500), 289 (ε, 9,750), ν_{max} ($CHCl_3$) 1660, 1630, 1595, 1508, 1445, 1420, 1388, 1350, 1335, 1270, 1215, 1195, 1160, 1120, 1080, 1040, 990, 930, cm^{-1}, nmr ($CDCl_3$) at δ2.31 (3 protons, sharp singlet, assigned to $-CH_3$), δ3.88 (3 protons, sharp singlet, assigned to $-OCH_3$). δ6.1 (singlet, 1 proton, assigned to olefinic proton), δ6.44 (2 protons, singlet, assigned to aromatic protons), δ12.76 (1 proton, exchangeable with D_2O, assigned to intramolecularly hydrogen-bonded proton). Microanalytical data are presented below:

Calculated for $C_{11}H_{10}O_4$: C, 64.07; H, 4.89; mol wt, 206
Found: C, 64.01; H, 4.95; mol wt, 206 (M^+ peak in mass spectrum).

The most abundant peak in the mass spectrum was at *m/e* 206 (100%), the molecular ion. Second most abundant peak at *m/e* 177 (78%) arose due to the loss of CHO·. A metastable peak at *m/e* 152.3 confirmed this loss from the molecular ion. The loss of another CHO· resulted in a peak at *m/e* 149 (18%). A possible mode of fragmentation is given on the following page.

The spectrochemical and analytical data led to the possibility of the structures I and II (the following page).

The mp and other physico-chemical properties of 8-hydroxy-5 or 6-methoxy-3-methylisocoumarin (I) were not comparable with those found for band Y compound. So, the possibility that the compound in question could be a derivative of I was ruled out. On comparing

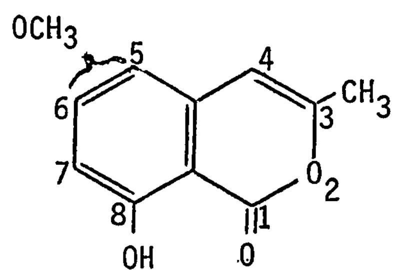
A possible mode of fragmentation in the mass spectrometer of band Y compound:

m/e 206

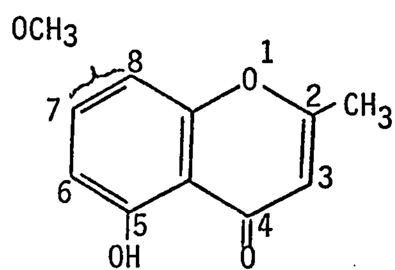
(M-29) *m/e* 177

(M-28)

(M-57) *m/e* 149



I



II

the mp and other physico-chemical properties of derivatives of II with those of band Y compound, it was found that 5-hydroxy-7-methoxy-2-methylchromone (II) was the compound representing band Y. Comparison with an authentic synthetic material has amply confirmed the identity since then.

Furthermore, it was confirmed by preparing the acetyl derivative, mp 152.5-153.5° (unc) (96); infrared spectrum ($CHCl_3$), no hydrogen-bonded hydroxyl absorbtion, ν_{max} 1770, 1662, 1580, 1500, 1435, 1390, 1368, 1345, 1312, 1172, 1148, 1098, 1055, 982 cm^{-1}; nmr ($CDCl_3$) at δ2.30 (3 protons, sharp singlet, assigned to $-CH_3$), δ2.40 (3 protons, sharp singlet, assigned to $-COCH_3$), δ3.88 (3 protons, sharp singlet, assigned to $-OCH_3$), δ6.06 (1 proton, singlet, assigned to olefinic proton), δ6.68 and δ6.85 (2 protons, pair of split singlets, J = 2 cps, assigned to m-coupled aromatic protons). Micro-analytical data are presented below:

Calculated: C, 62.90 H, 4.87; mol wt, 248
Found: C, 62.88; H, 4.91; mol wt, 248 (M^+ peak in mass spectrum).

The most abundant peak was again at 206 (46 times the M^+ peak). This was obtained by the loss of CH=C=O from M^+. This was further confirmed by the presence of a metastable peak at 171.1. Next most abundant peak at *m/e* 177 (16 times the M^+ peak) was obtained by the loss of CHO· from peak at 177. This was further confirmed by the presence of a metastable peak at *m/e* 152.1. Another quite important peak at *m/e* 149 (38 times the M peak) was obtained by the loss of CO from the peak at *m/e* 177. This was confirmed by the presence of a

metastable peak at *m/e* 125.4.

Thus, two of the four compounds, *viz*, band X and Y, were crystallized and characterized. Band X represents the so-called isocoumarin or 6-methoxy mellein, as it is sometimes called and band Y represents a compound that has not been found in carrots previously, but is found in other plants. This is a simple chromone, 5-hydroxy-7-methoxy-2-methyl-chromone, commonly known as eugenin. The other two compounds, representing bands Z and M, have not been identified but their UV spectra and their color reaction to phenol detecting reagents (Table 9) are very similar to those of isocoumarin. This will suggest the presence of a similar chromophore in all these compounds.

Why these phenols are synthesized in carrots on ethylene treatment is a matter of profound interest to the plant physiologists as well as to the plant biochemists. It adds a new dimension to the study of ethylene on plant processes. Particularly, the synthesis of isocoumarin in carrots as well as in fungi poses a problem to the comparative biochemists.

D. Rate of isocoumarin formation in carrots on ethylene treatment

After characterization of isocoumarin further amounts of it were isolated to serve as standards for thin layer chromatography and for making calibration curve. It was mentioned earlier [Results and Discussion, Section II C.b(i)] that as little as 0.1 μg was detectable as a spot on thin layer plates. For quantitative estimation purposes 15 μg or more was needed to produce a reliable reading on the spectrophotometer. To compare the effects of 100 ppm ethylene with that of

Table 9

UV* maxima and color reactions with $FeCl_3$ of bands Z and M on a thin layer** chromatogram

Band	R_f	λ_{max}, MeOH	Color with $FeCl_3$
Z	0.39	217, 262 and 300 nm	Purple
M	0.48	217, 263 and 300 nm	Purple

*The spectra are reproduced in the appendix (Figs A-5 and A-6).

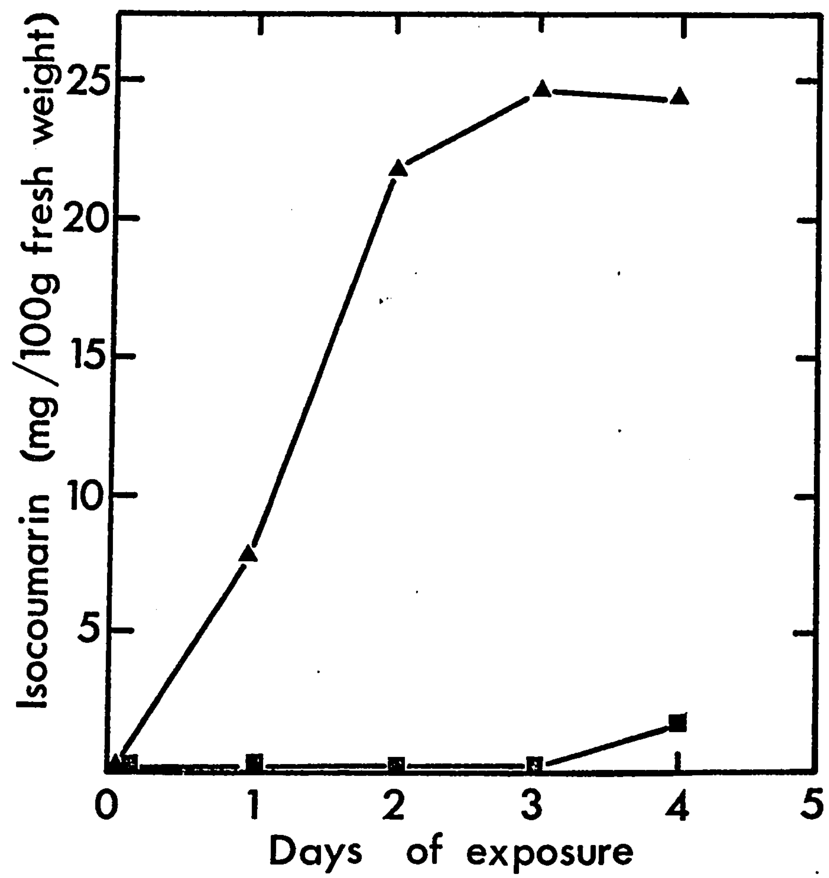
**Silica gel G coating (0.25 mm thick). Solvent system used was toluene: ethylformate: formic acid (5:4:1).

air, the carrot slices were exposed continuously to 100 ppm ethylene and air at room temperature (25±0.5°). The flow rates for each of these gases were the same (40 ml/min). The method of isolation and quantitative estimation has been described before (Materials and Methods, B.10). This differed from that of others, Carlton *et al.* (25) and Chalutz *et al.* (27), who estimated isocoumarin quantitatively by reading the increase in absorbance at 267 nm of the crude hexane extract of carrots. Results of these workers will be referred to as "isocoumarin".

The reproducibility of the method was found to be within the limit of experimental error, *viz*, 98-99% of isocoumarin added before the isolation was recovered at the completion of the isolation procedure. This was repeated several times. The quantitative estimation after separation on thin layer plate was repeated without any appreciable variation, e.g., 99% of pure isocoumarin was recovered after its separation on thin layer and subsequent elution. On carrying out quadruplicate quantitative estimation of isocoumarin from a single extract the average was found to have a standard deviation of ±0.05.

The results to be presented below were carried out to evaluate the effects of ethylene on the accumulation of isocoumarin in carrots. Each experiment was repeated several times in order to be sure about its reproducibility. Figure 10 showed the rate of isocoumarin formation in carrot slices on exposure to 100 ppm ethylene. Up to day 2 the accumulation of isocoumarin continued at a steady rate and then from day 2 to day 3 it increased at a slower rate, and after day 3 it stayed nearly unchanged. The slices exposed to air did not show any

Fig 10. Rate of ethylene-induced isocoumarin synthesis in carrot slices at 25±0.5°. Carrot slices (4 mm thick) were exposed continuously to 100 ppm ethylene and air for number of days as indicated. (▲——▲) represents isocoumarin in ethylene-treated and (■——■) represents isocoumarin in air-treated carrots.


Isocoumarin (mg/100g fresh weight)
25
20
15
10
5
0
1
2
3
4
5
Days of exposure

isocoumarin until day 3, at day 4 a small amount of isocoumarin was detectable. When intact carrots were exposed to 100 ppm ethylene similar observations were made. The results were recorded in Figure 11. The carrots treated with 100 ppm ethylene showed increase in isocoumarin content up to day 2 and then between day 2 and 3 it remained almost unchanged. The control samples did not show the presence of any isocoumarin.

A time course study for the accumulation of isocoumarin in carrots on 100 ppm ethylene treatment was done to see the initial rate of isocoumarin formation. As shown in Figure 12 there was no accumulation of detectable amount of isocoumarin up to 16 hrs of exposure. After 16 hrs isocoumarin content increased until it reached a plateau after 48 hrs. This was contrary to what Chalutz *et al.* had reported (27). They reported a continued increase in "isocoumarin" content of carrots up to day 7 upon exposure to low level of ethylene concentration. It was felt that the discrepancy might have been due to two factors; (1) the possible presence of other 267 nm absorbing material in the extract, (2) the concentration of ethylene they used was lower than that used in the present study.

The effects of higher concentrations of ethylene on isocoumarin formation in carrots were also studied. The results were recorded in Figure 13. Carrot slices on treatment with 0.2% (2,000 ppm) and 5% (50,000 ppm) ethylene (in air) accumulated detectable amounts of isocoumarin as early as 4 hr after exposure. The author has carried out some experiments (not reported here) which indicated that as high as 25% ethylene (in air) did not eliminate the lag period of about 3 hrs

Fig 11. Rate of ethylene-induced isocoumarin synthesis in intact carrots. Intact carrots were exposed continuously to 100 ppm ethylene and air for number of days as indicated. (△——△) represents isocoumarin in ethylene-treated, and (○——○) represents isocoumarin in air-treated carrots.

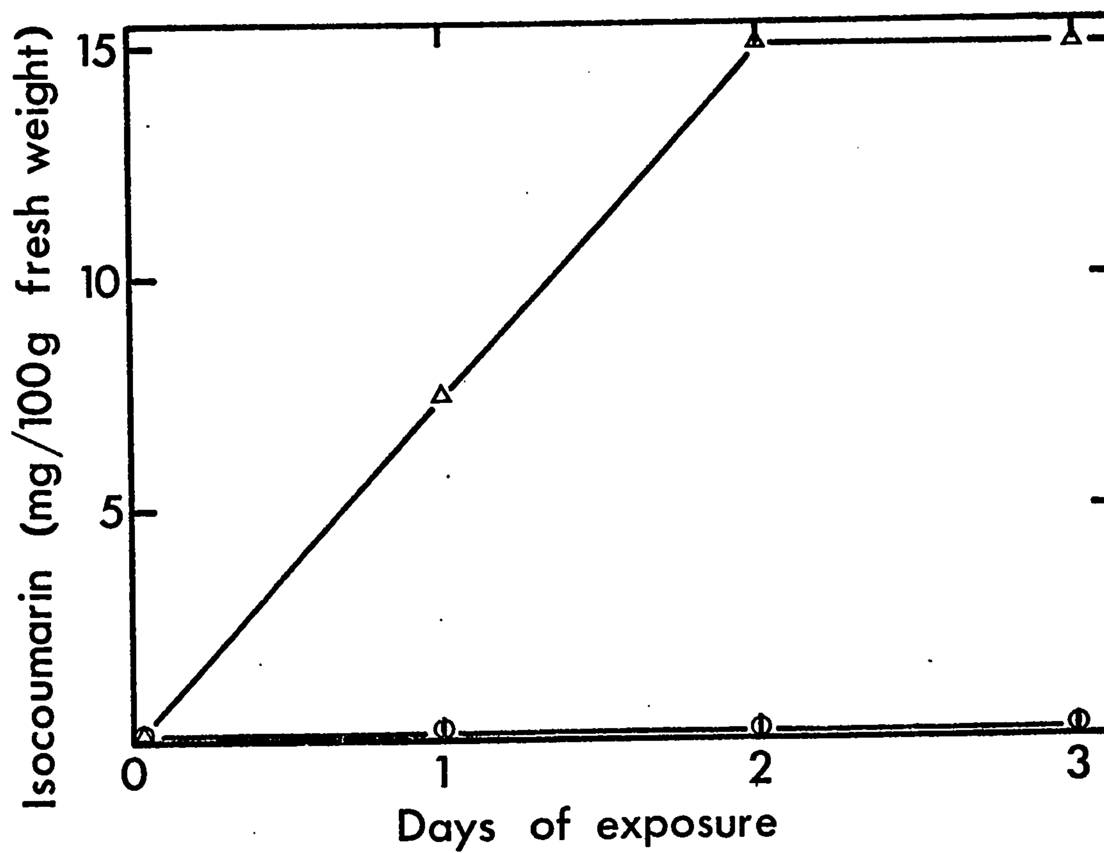

Isocoumarin (mg/100g fresh weight)
15
10
5
0
1
2
3
Days of exposure

Fig 12. Time course of isocoumarin formation in carrots on ethylene treatment. Carrot slices (4 mm thick) were exposed to 100 ppm ethylene. Curves (▲——▲) and (●——●) refer to 2 different batches of carrots.

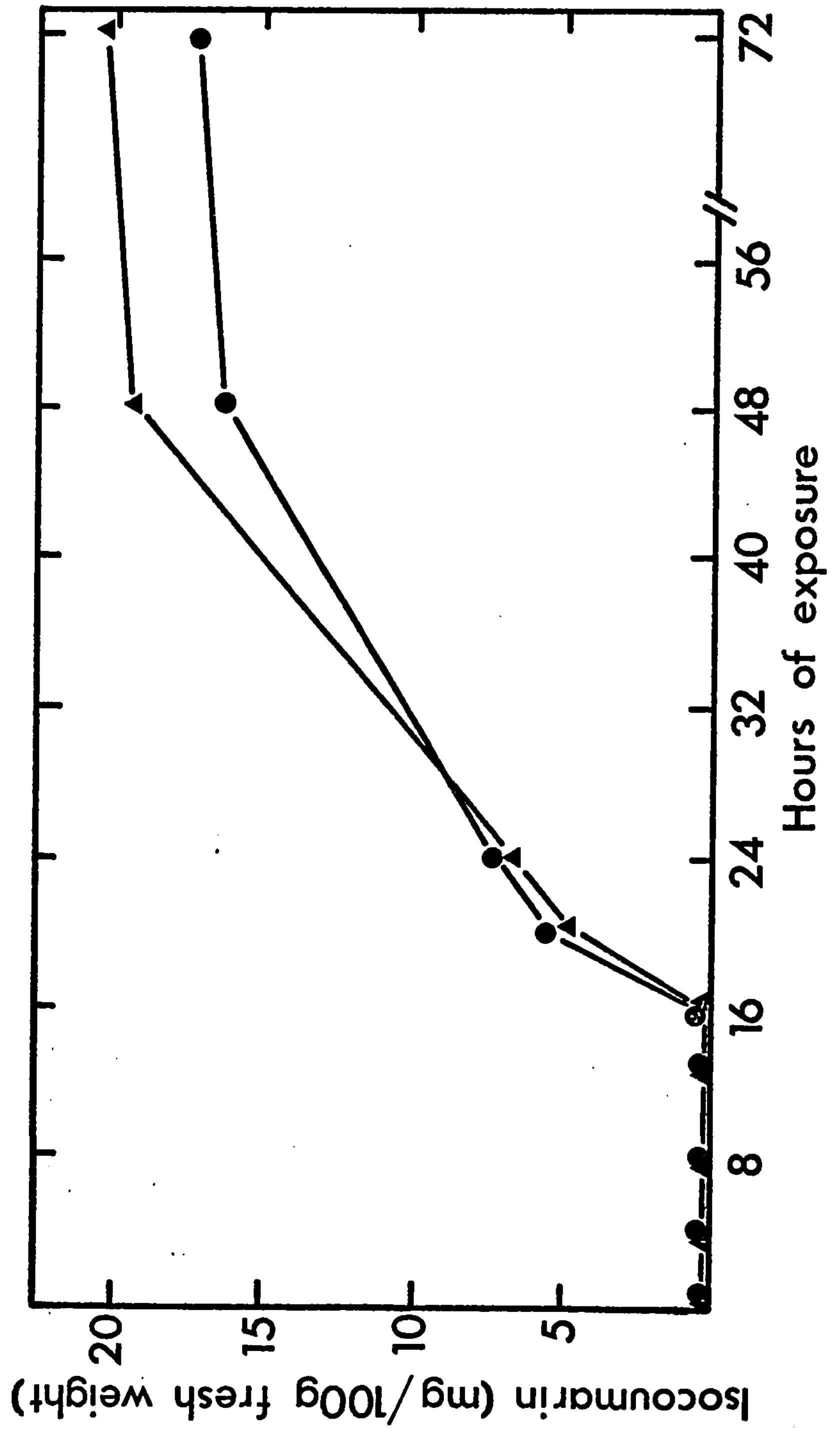
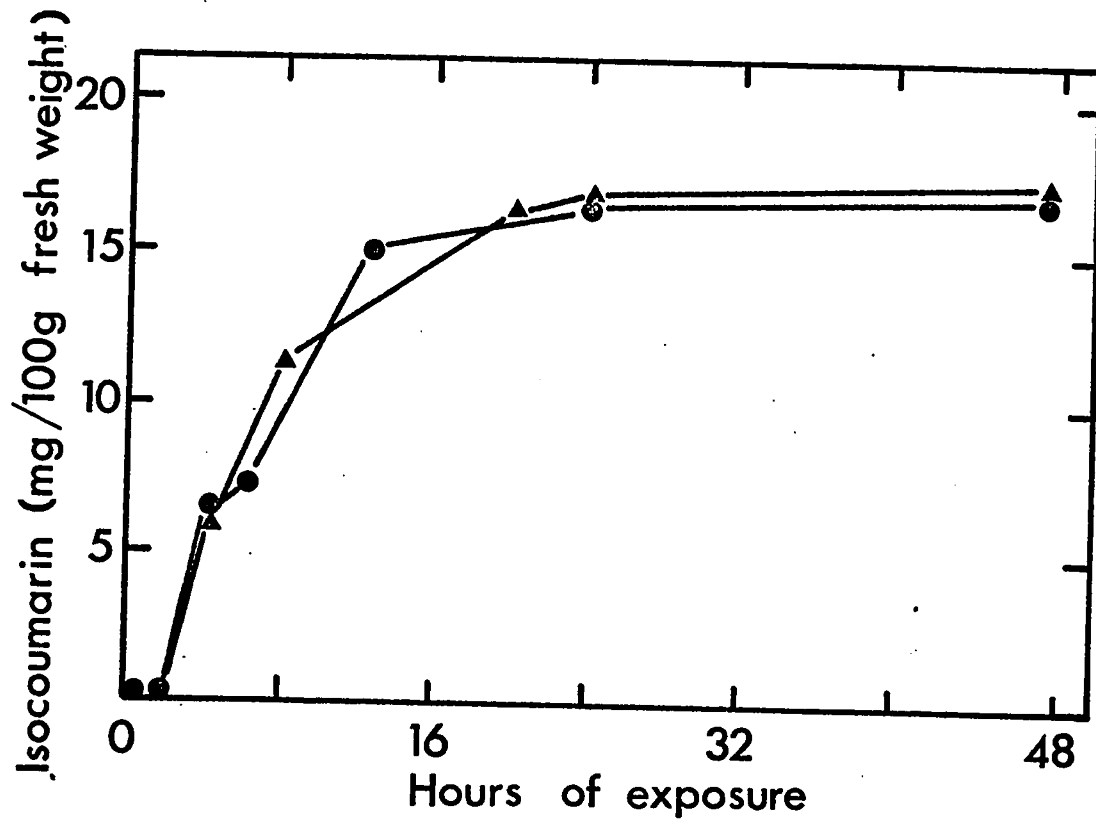

Isocoumarin (mg/100g fresh weight)
20
15
10
5
8
16
24
32
40
48
56
72
Hours of exposure

Fig 13. Rate of ethylene-induced isocoumarin synthesis in carrot slices treated with 0.2% and 5% ethylene (in air). Carrot slices (4 mm thick) were exposed continuously to above concentrations of ethylene. (●——●) represents isocoumarin formation in 0.2% ethylene-treated, and (▲——▲) represents isocoumarin in 5% ethylene-treated carrots.


Isocoumarin (mg/100g fresh weight)
20
15
10
5
0
16
32
48
Hours of exposure

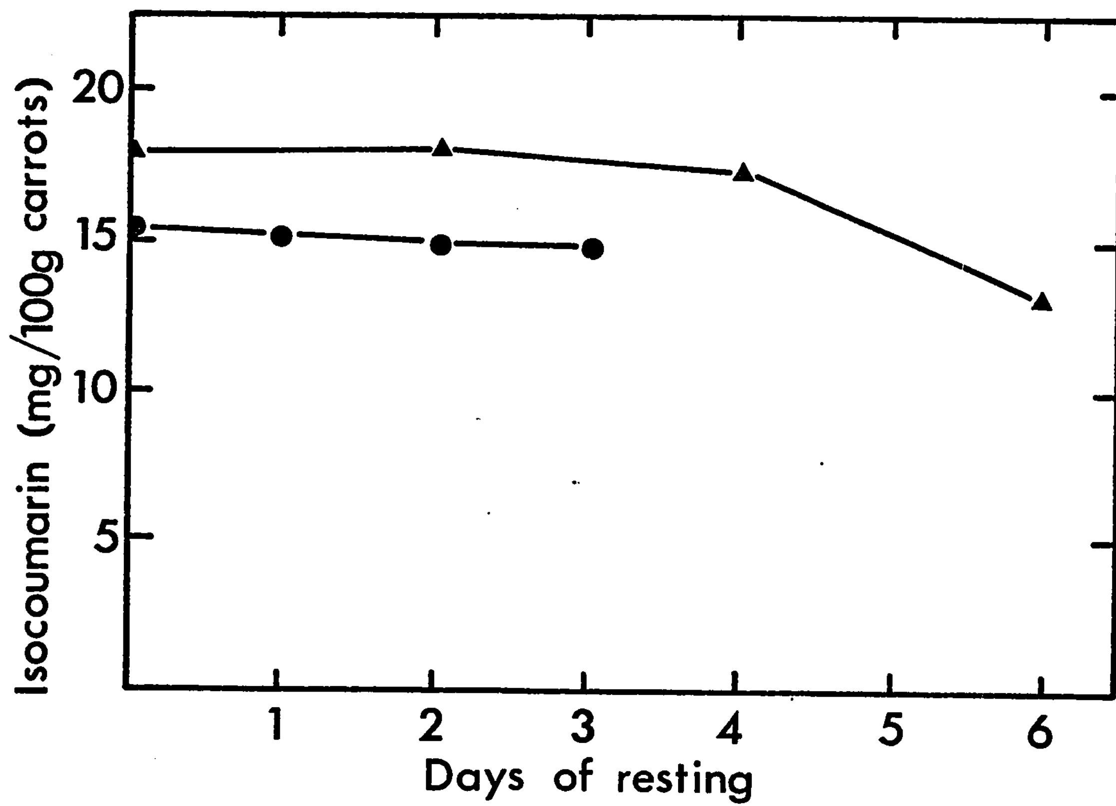
before the presence of isocoumarin could be detected.

The above results also led to an interesting observation; that both 0.2% and 5% ethylene increased the initial rate of isocoumarin synthesis, but these (concentrations of ethylene) had little effect on the final amount of isocoumarin accumulated. The level (of isocoumarin accumulation) that was reached after 2 days with 100 ppm ethylene was achieved in one day with 0.2% or 5% ethylene.

E. Effect of re-aeration on the isocoumarin content of carrots after exposure to ethylene

In order to find out whether isocoumarin once synthesized might be catabolized again, or in other words if the accumulation of isocoumarin was reversible, the effect of re-aeration of the slices (that had already accumulated certain amounts of isocoumarin) was studied. The carrot slices (4 mm thick) were exposed to 100 ppm ethylene for 2 days at room temperature (25±0.5°). After determining the amount of isocoumarin at 0 day of resting the slices were exposed to a continuous stream of air for 4 days in one experiment and for 6 days in the other. The results as recorded in Figure 14 showed little change in isocoumarin content up to day 4 of re-aeration. After day 4 there was a decline in isocoumarin content. The decline might have been due to metabolic activity or some non-enzymatic oxidation typical of phenolic compounds in presence of air. Chalutz *et al*. (27) reported that on withdrawing ethylene from the tissue the production of isocoumarin ceased and its concentration in the tissue declined immediately after withdrawal from ethylene. Incompatibility of their

Fig 14. Effect of reaeration on the isocoumarin content of carrot slices after prior exposure to 100 ppm ethylene. The carrot slices (4 mm thick) were exposed to 100 ppm ethylene for 2 days and then allowed to rest in presence of a continuous stream of air (25 ml/min) for number of days as indicated. (●——●) represents 3 day resting and (▲——▲) represents 6 day resting.



Isocoumarin (mg/100g carrots)
20
15
10
5
1
2
3
4
5
6
Days of resting

results with those of the author might be due to the differences in methods of isolation and determination of isocoumarin as discussed before (Results and Discussion, Section II, D).

F. Centripetal distribution of ethylene-induced synthesis of isocoumarin in carrots

On studying centripetal distribution of isochlorogenic acid [Section II.b(i)] it was found to be confined mostly to the "peel". In order to find out whether the synthesis of isocoumarin in carrots was confined to a certain part or parts the relative amounts of isocoumarin were determined in "peel", "phloem", and "xylem". The data were presented in Table 10. From the data which were typical of such determinations, it was evident that the accumulation of isocoumarin occurred almost to the same extent in the three above mentioned regions of the root.

G. Production of ethylene by carrots

The onset of increased synthesis of isochlorogenic acid and probable *de novo* synthesis of at least four phenolic compounds in carrots on treatment with ethylene have been observed. To find out how much ethylene was given out by carrots itself, the rate of ethylene production by slices was studied at different times after slicing. The experiment was done at room temperature (25±0.5°). The measurement of ethylene was done in triplicate.

The figure on ethylene production (Table 11) by carrots indicated two things; (1) the slices produced ethylene at a much higher rate (about 600 times) than the whole carrots at room temperature,

Table 10

Centripetal distribution of

ethylene-induced isocoumarin synthesis in carrots

Tissue portion	Isocoumarin*	
	Air treated	Ethylene treated
"Peel"	None	1.09
"Phloem"	None	1.07
"Xylem"	None	0.93

The carrots were exposed to air or ethylene (100 ppm) for 2 days.

"Peel", "Phloem" and "Xylem" have the same meaning as elsewhere.

*Isocoumarin was expressed as mg/g dry wt.

Table 11

Production of ethylene* by carrot slices (1 mm thick)

Collection time	Ethylene**	
	nl/g fresh weight/hr	ppm
0 - 1 hr and 15 min	1.76	0.0011
1 hr and 15 min - 4 hr and 30 min	2.36	0.0015
22 hr - 23 hr	4.2	0.0028

*Ethylene production by intact carrots: The ethylene production by the intact roots, after keeping them for 8 hrs at room temperature, averaged 3.5 nl/Kg fresh weight/hr for the first 2 hrs.

**The amounts of ethylene were obtained after subtraction of ethylene from the blank (air).

(2) as expected, on aging, the rate of ethylene production increased; after 22 hours it became about 3 fold. However, the increased rate of ethylene production by slices did not seem to be sufficient enough to cause the induction of isocoumarin synthesis, because the slices treated with air did not produce any of the four compounds mentioned earlier (Results and Discussion, Section II C.b(i)).

Conclusions on Section II

All the above findings taken together lead to the conclusion that ethylene is responsible for the increased synthesis of phenols in carrots. Certain phenols, *e.g.*, isochlorogenic acid accumulates in increasing amount in carrots upon treatment with ethylene. The increase in isochlorogenic acid is mostly confined to the "peel", the 1 mm thick skin of the root.

More important are the findings that ethylene induces (depending on the limit of detection) the synthesis of at least four compounds, two of them in considerable amounts. The two major components have been characterized as isocoumarin and eugenin. Isocoumarin, which was held responsible for bitter taste in canned carrots, could not be detected in normal carrots. Ethylene was suggested (25,27) to be a causative agent for inducing isocoumarin synthesis in carrots, but isocoumarin was not isolated and characterized after treatment with ethylene. This is the first report of isolation and characterization of isocoumarin in carrots exposed to ethylene. Besides, a chromone, eugenin (5-hydroxy-7-methoxy-2-methylchromone) has been isolated and characterized in carrots after treatment with ethylene. Other

Two compounds which are synthesized in carrots on ethylene treatment have not been characterized, but preliminary investigations lead to the suggestion that these compounds are structurally closely related to isocoumarin.

That ethylene-induced isocoumarin synthesis occurs in the tissues of the carrot root itself (and might not be a result of microbial contamination) was substantiated by the fact that isocoumarin was synthesized by carrot slices as well as whole roots under aseptic conditions.

The rate of isocoumarin formation in carrots seems to be dependent on the concentration of ethylene applied, but the final amount accumulated is independent of ethylene concentration used. Further proof of this conclusion (that the isocoumarin formation in carrots is dependent on ethylene concentration) is obtained from the data of ethylene production by carrot slices. Though the rate of ethylene production increases on ageing, yet the concentration in terms of ppm may be much lower than what triggers the isocoumarin formation in carrots.

The present finding does not indicate that the accumulation of isocoumarin might be reversible as reported (27). The accumulation of isocoumarin in the three regions of the tissue ("peel", "phloem" and "xylem") occurs almost to the same extent. This is an indication that the site of formation or transformation of isocoumarin is different from that of isochlorogenic acid.

Section III

Effects of ethylene on the biosynthetic pathways of phenols

A. On the shikimic acid pathway (Studies on L-phenylalanine ammonia-lyase, PAL)

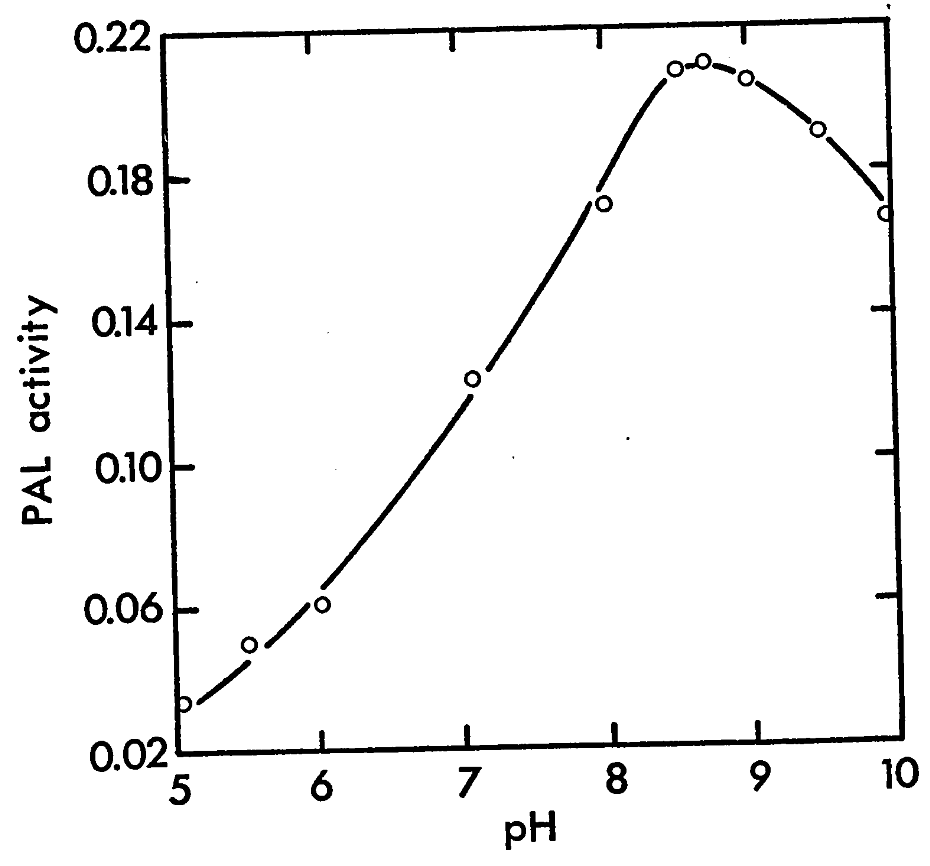
The activity of PAL could not be detected in the extract of whole carrots but PAL activity was always detected in the extract of 1 mm thick skin of the roots (Materials and Methods B.6). This might suggest two possiblities: (1) if most of the phenols in carrots were synthesized in the 1 mm thick skin the enzyme PAL might be confined to the skin and therefore extraction of the enzyme from whole carrots might lead to a mass dilution of the enzyme with other protein; (2) some inhibitor of PAL might be present in the inner portion of the root. Search for such an inhibitor was not made.

The assay procedure (Materials and Methods, B.7) was found to be reliable and reproducible. For a particular extraction 5 replicate determinations gave an average with a standard deviation of ±0.04.

(a) The effect of pH on PAL activity

The acetone powder extract of carrots was extracted with buffer solutions of different pH and the activity of PAL was determined. The results were recorded in Fig 15. The pH optimum was found to be 8.7. Havir and Hanson (64) reported a pH optimum of 8.7 for the same enzyme (from potatoes).

Fig 15. The effect of pH on PAL activity. PAL activity was expressed as absorbance at 290 nm per mg protein per 4 hr. The enzyme was assayed as described under Materials and Methods (B.7).



0.22
0.18
0.14
0.10
0.06
0.02
PAL activity
5
6
7
8
9
10
pH

(b) Effect of ethylene on isolated enzyme: PAL

The effects of ethylene on the isolated enzyme were studied in the following manner: (1) The acetone powder extract of carrot roots was treated with 100 ppm ethylene for 20 min at 50 ml/min. The buffer and the substrate solutions were also treated in a similar manner with air. All the operations were done at 33°. The incubation at 33° was done for 4 hrs. The results were recorded in Table 12. (2) The buffer extract of fresh roots was treated with 100 ppm ethylene and air as described under (1). The results were recorded in Table 13. (3) The buffer extract of fresh carrots was treated continuously with 100 ppm ethylene and air while being incubated. The flow rate for both was adjusted to 10 ml/min. The results were recorded in Table 14.

The data in the tables are the averages of triplicate determinations with standard deviation in the range ±0.03 to ±0.05.

The results presented in Tables 12, 13, and 14 showed no apparent effect of ethylene on PAL activity. It would thus seem that ethylene did not act by activating the enzyme (PAL) as such under these conditions.

(c) The PAL activity extracted from intact roots exposed to ethylene

The roots were exposed to ethylene or air for specific lengths of time. The enzyme was then extracted from these roots. All observations were repeated and all the data were the averages of triplicate determinations with a standard deviation in the range of ±0.03 to ±0.05.

Table 12

Effect of ethylene (brief exposure)
on PAL activity of an acetone powder extract of fresh carrots

Experiment number	PAL activity	
	In air	In 100 ppm ethylene
1	0.107	0.105
2	0.112	0.114
3	0.118	0.116

The enzyme was assayed as described under Materials and Methods (B.7). PAL activity was expressed as absorbance at 290 nm per mg protein per 4 hrs.

Table 13

Effect of brief exposure to ethylene on PAL (extracted from fresh roots) activity

Experiment number	PAL activity	
	In air	In 100 ppm ethylene
1	0.105	0.107
2	0.109	0.110
3	0.117	0.118

The enzyme was assayed as described under Material and Methods (B.7). PAL activity was expressed as absorbance at 290 nm per mg protein per 4 hrs.

Table 14

Effect of continuous exposure to ethylene
on PAL (extracted from fresh roots) activity

Experiment number	PAL activity	
	In air	In 100 ppm ethylene
1	0.121	0.119
2	0.128	0.125

The enzyme was assayed as described under Materials and Methods (B.7). The PAL activity was expressed as absorbance at 290 nm per mg protein per 4 hrs.

To study the effect of ethylene concentration on the stimulation of PAL activity the carrots were treated with different concentrations of ethylene (in air) and air for 12 hrs - this was the time required to induce highest stimulation of PAL activity. The results that were recorded in Fig 16 showed that 100 ppm ethylene, the highest concentration tried, produced the highest stimulation. In subsequent studies of ethylene effect on the stimulation of this enzyme, 100 ppm ethylene was used.

The effect of 100 ppm ethylene on the development of PAL activity was recorded in Fig 17. The maximum stimulation was attained after 12 hrs of exposure to ethylene and then the activity declined. Similar observations were made by Hyodo and Yang (68) on the effect of ethylene on PAL activity in excised pea epicotyl segments. They found that the peak of PAL activity was reached after 30 hours of exposure and then declined. If PAL were the rate limiting enzyme for the biosynthesis of isochlorogenic acid, then it would be difficult to explain such a decline in PAL activity in carrots, particularly with regard to the increased isochlorogenic acid synthesis on exposure to ethylene. Whether some other alternate pathway, *viz* synthesis of cinnamic acid *via* β-phenyl-lactic acid, of aromatic biosynthesis, is operative as well. Attempts to isolate an active extract of β-phenyl-lactic acid-hydrolyase met with failure. This does not rule out, however, the possibility of the existence of such a pathway *in vivo*.

Another rationalization for such a contrast between phenol

Fig 16. Effect of ethylene concentration on PAL activity. The roots were maintained at specified concentrations of ethylene by passing a continuous stream of appropriate concentration of ethylene for 12 hrs at 25°. The enzyme was extracted and assayed as described under Material and Methods (B.7). (▲———▲) represents PAL activity in ethylene-treated, and (●———●) represents PAL activity in air-treated carrots. PAL activity was expressed as absorbance at 290 nm per mg protein per 4 hr.

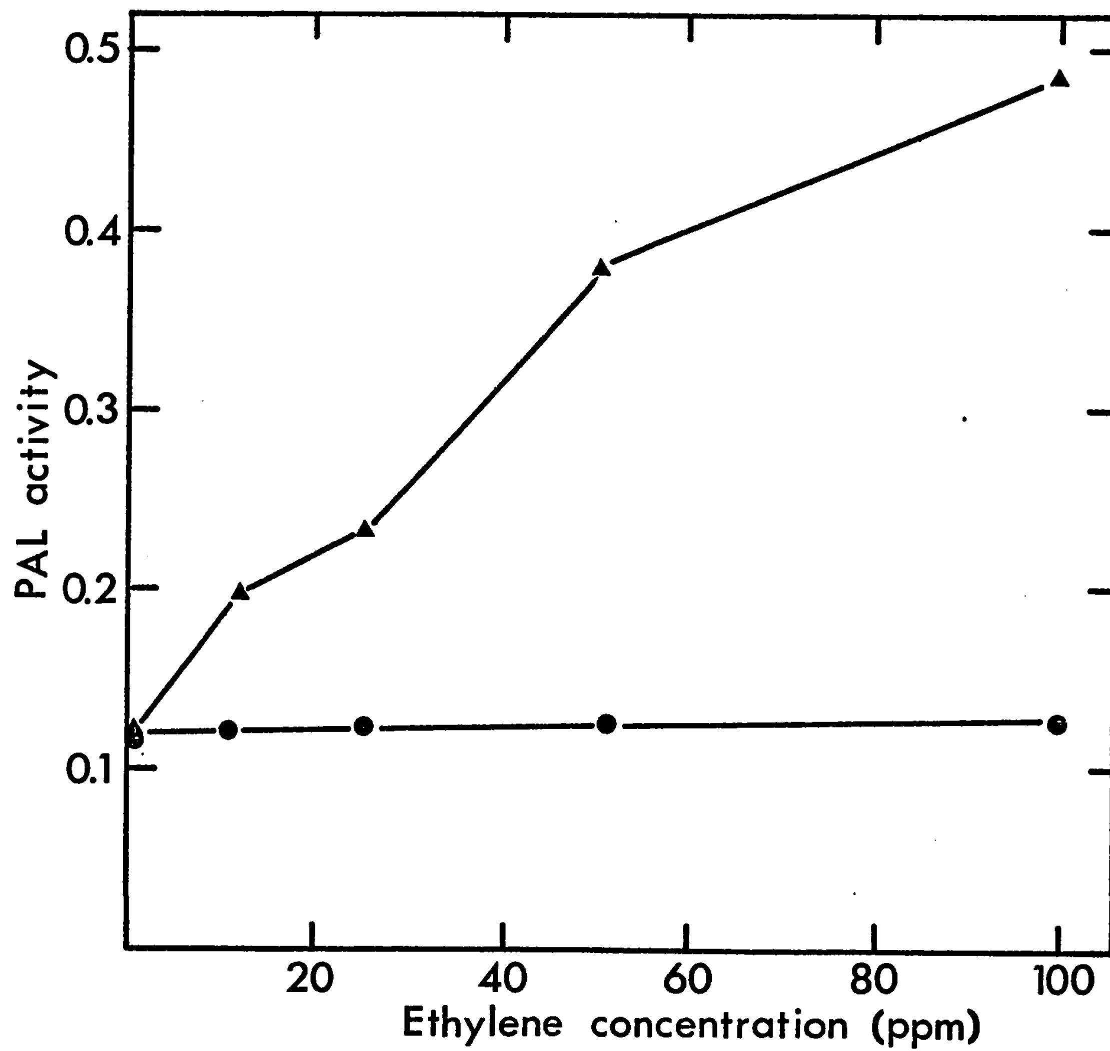
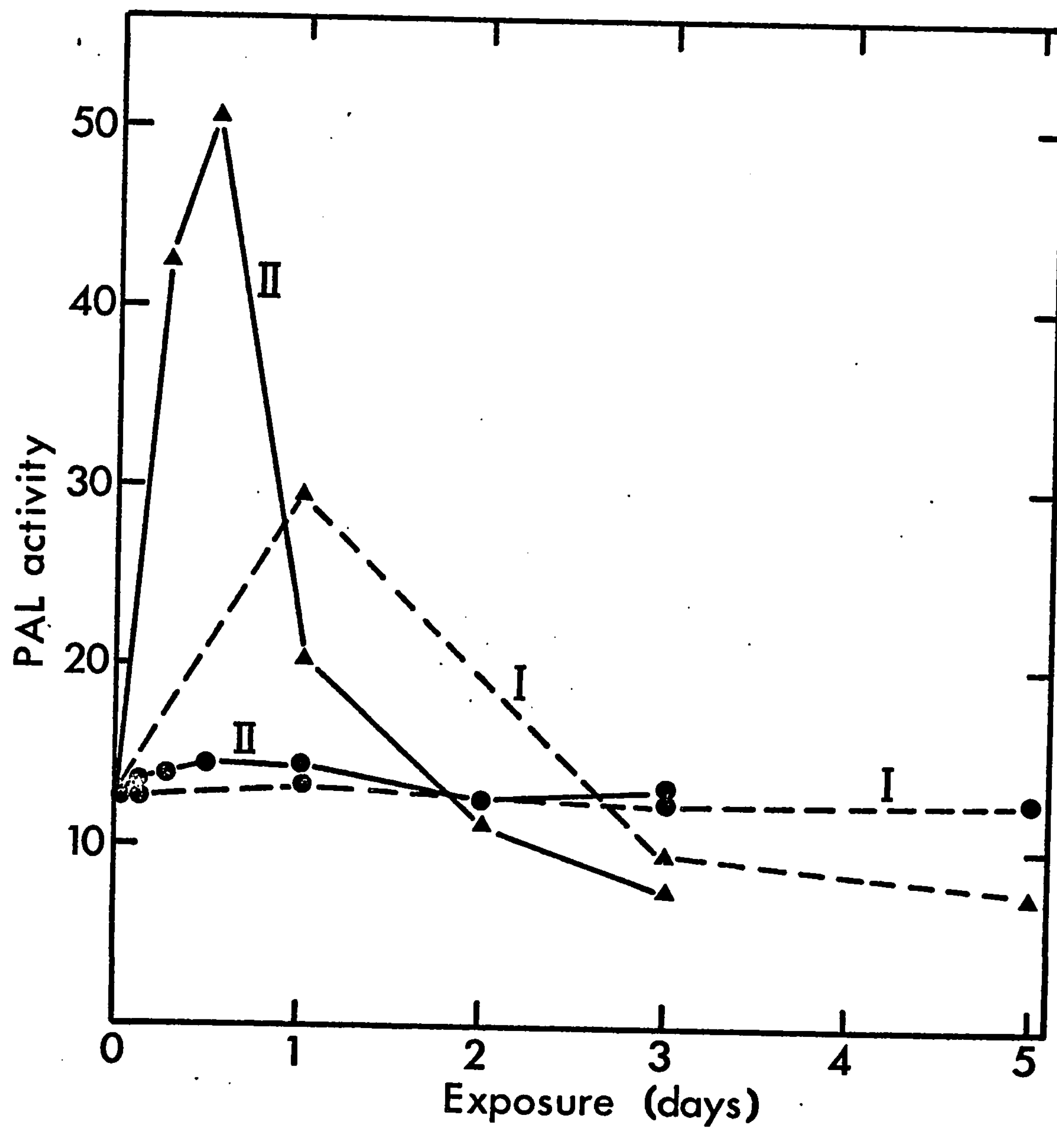

0.5
0.4
0.3
0.2
0.1
PAL activity
20
40
60
80
100
Ethylene concentration (ppm)

Fig 17. Effect of exposure time on the development of PAL activity in carrots. The roots were continuously exposed to either 100 ppm ethylene or air at 25°. The enzyme was assayed as described under Materials and Methods (B.7). The enzyme activity was expressed as nmole cinnamic/mg protein/hr. I and II represent two different experiments. For I, (▲- - -▲) represents PAL activity in ethylene-treated, and (●- - -●) represents PAL activity in air-treated carrots. For II, (▲——▲) represents PAL activity in ethylene treated, and (●——●) represents PAL activity in air-treated carrots.



50
40
30
20
10
PAL activity
0
1
2
3
4
5
Exposure (days)
II
I
II
I

content and PAL activity could be the possible deactivation of enzyme by (a) phenols during isolation, (b) subsequent synthesis of a system capable of degrading or inactivating the phenylalanine ammonia-lyase, as described by Zucker (156).

B. On the acetate pathway

The ether-soluble phenols that were synthesized in carrots on exposure to ethylene are structurally quite different from the hydroxycinnamic acids. Structural analysis of these compounds, particularly of isocoumarin and eugenin, suggests that these compounds are most likely derived *via* the acetate pathway. Condon *et al.* (34) reported that the biogenesis of isocoumarin in carrots seemed to be *via* the acetate-malonate pathway of aromatic biosynthesis.

The biosynthesis of eugenin as such has not been reported so far, but its immediate precursor, 5,7-dihydroxy-2-methylchromone has been shown to be acetate derived in *Ammi visnaga* by Harrison *et al.* (61). These workers also found that 5,7-dihydroxy-2-methylchromone was metabolized to other chromones in *Ammi visnaga*.

In the present work feeding experiments with ^{14}C-substrates were carried out to throw some light on the biosynthesis of isocoumarin. Data are also presented to support the hypothesis that like eugenin, isocoumarin is also acetate derived. Furthermore, evidence is presented to indicate that 5,7-dihydroxy-2-methyl-chromone is a natural precursor of eugenin.

a. Effect of ethylene on the biosynthesis of isocoumarin

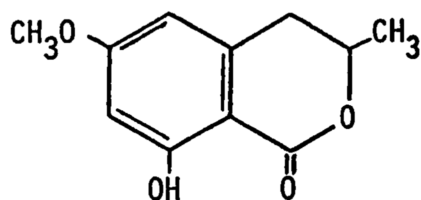
Biosynthesis of isocoumarin was studied by feeding ^{14}C-labeled

acetate, malonate and acetoacetate to 1 mm thick carrot slices treated with 0.2% ethylene (in air). The incubation was done for 2 days. Methods of feeding, extraction and isolation of labeled products were described under Materials and Methods (B.10 and 17). Specific activities were determined after purifying the products by thin layer chromatography. Details were described under Materials and Methods (B.18).

(i) <u>Incorporation of 1-^{14}C-acetate and 2-^{14}C-malonate into isocoumarin</u>

Carrot slices (1 mm thick) were fed 1-^{14}C-acetate and 2-^{14}C-malonate by continuous agitation of the aqueous solution of the respective compounds along with the tissue slices. The results (Table 15) showed that 1-^{14}C-acetate and 2-^{14}C-malonate were both good precursors of isocoumarin. The percent conversion of acetate was 1.57 and that of malonate was 1.33. Condon *et al.* (34) reported 0.26% conversion of acetate and 0.33% of malonate.

Recalling the (mechanism of) acetate pathway one finds that for a single polyketomethylene chain before its cyclization to aromatic compounds, only the two terminal carbon atoms come from the acetate, the rest come from malonyl units, for example, in the case of isocoumarin,



the CH_3-CH unit will only be derived from acetate, the rest should come from malonate. Thus, on using ^{14}C-acetate and ^{14}C-malonate of the same specific activity and same molar concentration (1.82×10^{-4}M) the

Table 15

Incorporation of 1-^{14}C-acetate and 2-^{14}C-malonate into isocoumarin

Substrate	Activity taken up x 10^{-6} dpm	Activity added x 10^{-6} dpm	Activity in isocoumarin x 10^{-5} dpm	% conversion	Specific activity of isocoumarin µCi/mmole
1-^{14}C-acetate, specific activity, 10.4 mCi/mmole	9.39	12.72	1.48	1.57	135
2-^{14}C-malonate, specific activity, 10.4 mCi/mmole	8.85	12.72	1.17	1.33	104

One mm carrot slices were shaken with aqueous solutions of the labeled compounds for 2 days.

Percent conversion was calculated on the basis of activity taken up.

The specific activity of isocoumarin was determined as described under Materials and Methods (B.18).

specific activity of the product should have been higher in the latter case, provided both acetate and malonate were taken up with equal ease and the endogenous malonate pool was comparable to that of acetate. The present finding was the other way around, indicating the possibility of the existence of either a barrier to malonate uptake or a larger endogenous malonate pool compared to that of acetate. Canvin (24), working with fatty acid synthesis in developing castor beans, reported slight absorption of malonate by the tissue. However, the data in Table 15 did not indicate any significant barrier to malonate absorption by carrot slices.

(ii) Effect of addition of unlabeled malonate on the incorporation of 1-^{14}C-acetate into isocoumarin

Acetate-1-^{14}C alone or acetate-1-^{14}C mixed with unlabeled malonate were infiltrated into the slices after 8 hrs of prior exposure to ethylene. The slices were then exposed to ethylene for another 40 hrs. The same experiment was repeated with a slight modification, *viz*, the acetate-1-^{14}C alone, or together with unlabeled malonate were fed by shaking 1 mm thick tissue slices with the aqueous solution of labeled compound for 2 days in the presence of 2000 ppm ethylene.

If acetate is first converted to malonate before being incorporated into an acetogenin, as is generally the case (16), then on feeding ^{14}C-acetate along with unlabeled malonate one should see a dilution of specific activity of isocoumarin compared to that synthesized on feeding ^{14}C-acetate alone, provided of course, that both compounds are taken up by the tissue with similar ease. Results in Table 16, where ^{14}C-acetate had been infiltrated into the tissue along with unlabelled

malonic acid, showed there was a decrease in the specific activity of isocoumarin as the ratio of 1-^{14}C-acetate to unlabeled malonate increased. In view of the acetate-malonate hypothesis for aromatic biosynthesis one would expect much greater decrease in specific activity of isocoumarin in presence of unlabeled malonate (if both acetate and malonate were taken up by the tissue with equal ease). Whether acetate and malonate were taken up by the tissue with equal ease in presence of one another was not determined.

When the carrot slices were incubated with the solution of 1-^{14}C-acetate and unlabeled malonate with constant shaking, 0 to 3 fold addition of malonate did not cause any change in the specific activities of isocoumarin isolated (Table 16). One might only speculate from these results that either malonate was not involved in the synthesis of isocoumarin, or that malonic acid was not taken up to any significant extent, particularly in presence of acetate.

(iii) Incorporation of 3-^{14}C-acetoacetate alone or together with unlabeled acetate into isocoumarin

The extent of incorporation of 3-^{14}C-acetoacetate alone as well as in presence of large excess of unlabeled acetate was studied to find out if acetoacetate was broken down into acetate prior to its utilization.

The results in Table 17 indicated the involvement of polyketomethylene intermediate as proposed by Collie [in Neish (102)] in the biosynthesis of isocoumarin. On feeding 3-^{14}C-acetoacetate alone or together with unlabeled acetate (up to 30-fold) it was found that the addition of unlabeled acetate did not have any effect on the

Table 16

Effect of addition of unlabeled malonate on the incorporation of 1-^{14}C-acetate into isocoumarin

Experiment	Acetate-1-^{14}C specific activity 54.7 mCi/mmole	Unlabeled malonate	Activity added x 10^{-6} dpm	Activity in isocoumarin dpm	Specific activity of isocoumarin µCi/mmole
1	3.5×10^{-5}M	None added	11.22	16,520	8.04
	3.5×10^{-5}M	3.5×10^{-5}M	11.22	10,077	7.30
	3.5×10^{-5}M	7.0×10^{-5}M	11.22	17,421	4.95
	3.5×10^{-5}M	10.5×10^{-5}M	11.22	15,542	3.55
2	3.5×10^{-5}M	None added	11.22	0.82×10^{5}	51.82
	3.5×10^{-5}M	3.5×10^{-5}M	11.22	1.69×10^{5}	75.00
	3.5×10^{-5}M	7.0×10^{-5}M	11.22	1.54×10^{5}	75.00
	3.5×10^{-5}M	10.5×10^{-5}M	11.22	1.57×10^{5}	56.82

In experiment number 1, acetate alone, or acetate mixed with malonate was infiltrated into the slices after 8 hrs of prior exposure to ethylene (2000 ppm). The slices were then exposed for another 40 hrs at 25°.

In experiment number 2, acetate-1-^{14}C alone, or together with unlabeled malonate was fed by shaking 1 mm tissue slices at 25° with the aqueous solutions of labeled compound for 2 days in the presence of ethylene (2000 ppm). The specific activity of isocoumarin was determined as described under Materials and Methods (B.18).

Table 17

Incorporation of 3-^{14}C-acetoacetate alone or together with unlabelled acetate into isocoumarin

3-^{14}C-acetoacetate Specific activity 11.63 mCi/mmole	Unlabeled acetate	Activity added x 10^{-6}dpm	Activity taken up x 10^{-6}dpm	Activity in isocoumarin	% conversion	Specific activity of isocoumarin µCi/mmole
2.1 x 10^{-5}M	None added	3.5	1.00	17,122	1.70	4.55
2.1 x 10^{-5}M	2.1 x 10^{-4}M	3.5	0.96	6,396	6.67	4.23
2.1 x 10^{-5}M	4.2 x 10^{-4}M	3.5	1.03	7,749	0.75	5.32
2.1 x 10^{-5}M	6.3 x 10^{-4}M	3.5	0.99	18,351	1.86	5.73

Acetoacetate-3-^{14}C alone, or together with unlabeled acetate was fed by infiltration into the tissue after 8 hrs of prior exposure to ethylene (2000 ppm). The incubation was done for another 40 hrs at 25° in the presence of same concentration of ethylene. The specific activity of isocoumarin was determined as described under Materials and Methods (B.18).

specific activity of isocoumarin. This suggested that the isocoumarin might have been synthesized *via* the hypothetical polyketomethylene chain and acetoacetate was most likely incorporated as such (without first being broken down into acetate) otherwise a dilution of specific activity would have occurred when a 30-fold excess of unlabeled acetate was used along with 3-^{14}C-acetoacetate.

(iv) Effect of specific activity of 1-^{14}C-acetate used on the specific activity of isocoumarin synthesized

In order to find out whether the specific activity of 1-^{14}C-acetate administered had any effect on the specific activity of ^{14}C-isocoumarin isolated, the incorporation studies with 1-^{14}C-acetate of three different specific activities were made.

The data in Table 18 showed the relationship between specific activity of acetate used and that of isocoumarin synthesized. If isocoumarin was derived from acetate and if the acetate pool in carrots was not large, then on feeding ^{14}C-acetate one would notice only reasonable dilution of specific activity in isocoumarin. Using three different specific activities of ^{14}C-acetate, namely, 54.7 mCi/mmole, 10.4 mCi/mmole and 2 mCi/mmole, ^{14}C-isocoumarin was found to have increasing specific activities, namely, 51.82 μCi/mmole, 135 μCi/mmole and 670 μCi/mmole respectively. This kind of inverse relationship between the precursor and the product is, perhaps, due to a relatively large acetate pool in the carrots treated with ethylene, compared to the amount of ^{14}C-acetate added exogenously. If the endogenous acetate pool in carrot was small compared to exogenous ^{14}C-acetate added, the dilution of specific

Table 18

Effect of specific activity of 1-^{14}C-acetate used on the specific activity of isocoumarin synthesized

Substrate	Specific activity mCi/mmole	Activity added dpm	Activity taken up dpm	Activity in isocoumarin dpm	% conversion	Specific activity of isocoumarin µCi/mmole
1-^{14}C-acetate	2	3.90×10^7	3.84×10^7	4.7×10^5	1.22	670
1-^{14}C-acetate	10.4	9.72×10^6	9.39×10^6	1.48×10^5	1.57	135
1-^{14}C-acetate	54.7	11.22×10^6	11.15×10^6	0.82×10^5	0.73	51.82

The carrot slices (1 mm thick) were agitated continuously for 2 days at 25° with the solutions of labeled compound in the presence of ethylene (2000 ppm).

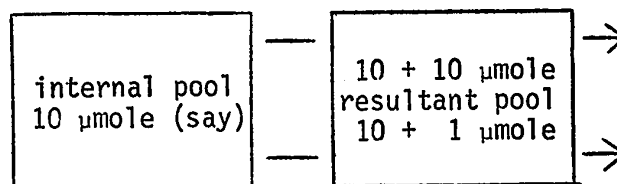
The specific activity of isocoumarin was determined as described under Materials and Methods (B.18).

activity of isocoumarin would not be so much dependent on the specific activity of ^{14}C-acetate administered. Following illustration will assist in the argument:

10 μci activity
to be added

(1) Specific activity = 1 μci/μmole

(2) Specific activity = 10 μci/μmole



(1) Specific activity = 0.5 μci/μmole
dilution = 2 times

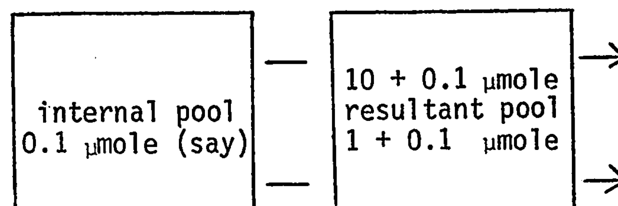
(2) Specific activity = 0.91 μci/μmole
dilution = 10.9 times

In contrast, if the pool size was smaller, then the ^{14}C-acetate would be diluted to a much smaller extent as can be seen below:

10 μci activity
to be added

(1) Specific activity = 1 μci/μmole

(2) Specific activity = 10 μci/μmole

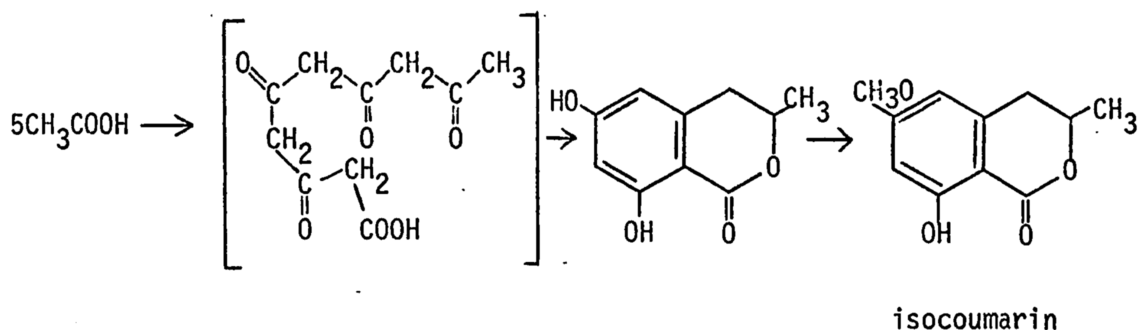


(1) Specific activity = 0.99 μci/μmole
dilution = 1.01 times

(2) Specific activity = 9.09 μci/μmole
dilution = 1.1 times

All the above results taken together would indirectly indicate the origin of isocoumarin *via* the acetate-malonate pathway. This is in agreement with the report of Condon *et al.* (34). They had also tried ^{14}C-phenylalanine as a precursor, but they could not detect any ^{14}C-isocoumarin from such experiments. However, degradative studies will be needed before one could say categorically that all the carbon atoms in isocoumarin are derived from acetate *via* the polyketomethylene chain.

The proposed biosynthetic route may be the following:



isocoumarin

b. Effect of ethylene on the biosynthesis of eugenin

In the study of the biosynthesis of eugenin the slices were exposed to a constant stream of 0.2% (2000 ppm) ethylene for two days prior to either infiltration or incubation with the labeled compounds. The method of feeding labeled compounds has been described under Materials and Methods (B.17). Irrespective of the mode feeding, after administering the labeled compounds the incubation period was another two and a half days at room temperature (25±0.5°) in presence of 2000 ppm ethylene.

Eugenin, which is synthesized in carrots upon ethylene treatment,

has a chemical structure typical of an acetogenin. Chemically, it is 5-hydroxy-7-methoxy-2-methylchromone, one of the simplest of naturally occurring chromones.

If the polyketomethylene chain was involved in its synthesis from acetate, then 5,7-dihydroxy-2-methylchromone could be the most likely intermediate, provided methylation did not occur before the ring closure took place. Birch (16) had suggested that the addition of "extra" groups such as CH_3- (from methionine) could occur at a β-polyketone stage or at a later phenolic stage.

(i) <u>Incorporation of G-^{3}H-5,7-dihydroxy-2-methylchromone (DHMC) into eugenin</u>

G-^{3}H-5,7-dihydroxy-2-methylchromone was infiltrated into 1 mm thick carrot slices after 2 days of prior exposure to ethylene. The same experiment was repeated with a slight modification, *viz*, the slices were agitated continuously with the aqueous solution of G-^{3}H DHMC in presence of 2000 ppm ethylene.

Data in Table 19 showed that on feeding G-^{3}H-5,7-dihydroxy-2-methyl-chromone, specific activity; 104 mCi/mmole, by infiltration into carrots eugenin of very high specific activity was isolated. Dilution was 68 times, probably due to the fact that a little of the labeled material was taken up because of its slight solubility in water. Experiment number 2 (Table 19) demonstrated a little lower dilution of specific activity (28 times) than the experiment number 1 and the specific activity of G-^{3}H-5,7-dihydroxy-2-methylchromone was diluted indicating the presence of an internal pool of 5,7-dihydroxy-2-

Table 19

Incorporation of G-^{3}H DHMC into eugenin

Experiment number	Specific activity of G-^{3}H DHMC mCi/mmole	Activity added x 10^{-7}dpm	Activity taken up dpm	Activity in eugenin	% conversion	Specific activity(μCi/mmole)*	
						eugenin	leftover DHMC
1	104	17.49	13.8×10^{6}	7.74×10^{-6}	56	1530	Not determined
2	104	8.75	4.34×10^{-7}	2.04×10^{-7}	47	3727	11,905

In experiment number 1, the aqueous solution of G-^{3}H-DHMC (5,7-dihydroxy-2-methyl chromone) was infiltrated into the tissue after prior exposure to ethylene (2000 ppm) for 2 days. The tissue was then incubated with the labeled compound for 60 hrs at 25° in the presence of same concentration of ethylene. The specific activity of eugenin and DHMC were determined as described under Materials and Methods (B.18).

*Figures have not been corrected for loss of tritium through substitution.

In experiment number 2, the aqueous solution of G-^{3}H DHMC was shaken with the tissue for 60 hrs at 25° in presence of ethylene (2000 ppm) after prior exposure of the tissue to 0.2% (2000 ppm) ethylene for 2 days.

methylchromone in carrots. These results clearly indicated that 5,7-dihydroxy-2-methylchromone could serve as a precursor of eugenin and was probably a natural precursor of eugenin in carrots.

(ii) Incorporation of 1-^{14}C-acetate into eugenin in absence or presence of unlabeled 5,7-dihydroxy-2-methylchromone (DHMC)

In order to verify whether 5,7-dihydroxy-2-methylchromone was a natural precursor of eugenin, incorporation of 1-^{14}C-acetate in presence of unlabeled 5,7-dihydroxy-2-methylchromone was studied.

The Table 20 showed the results of feeding carrot slices with 1-^{14}C acetate alone and together with unlabeled 5,7-dihydroxy-2-methylchromone. On feeding acetate-1-^{14}C alone, eugenin of fairly high specific activity was obtained suggesting its origin *via* the acetate pathway, which confirmed the reports of Egger (46) and Chen *et al.* (29,30) that chromone nucleus was derived from acetate. When acetate-1-^{14}C was fed along with carrier 5,7-dihydroxy-2-methylchromone, considerable activity was isolated in the carrier after 12 hours of incubation. Also the specific activity of eugenin was diluted many times compared to that obtained by feeding acetate-1-^{14}C alone.

Harrison *et al.* (61), studied the biosynthesis of furanochromones in *Ammi visnaga* with G-^{3}H-5,7-dihydroxy-2-methylchromone (DHMC) as a precursor. They suggested DHMC a natural constitutent of *Ammi visnaga* on the basis of the observed dilution of labeled compound recovered and by the labelling of the trapping compound (unlabeled DHMC) in feeding experiment with 1-^{14}C-acetate.

The above results suggested that eugenin was biosynthesized in

Table 20

Incorporation of 1-^{14}C-acetate in the presence or absence of unlabeled DHMC

Substrate	Metabolic period	Activity added $\times 10^{-7}$ dpm	Activity taken up dpm	% conversion	Activity in eugenin dpm	Specific activity* (μCi/mmole)	
						eugenin	DHMC
1-^{14}C-acetate Specific activity 2 mCi/mmole	60 hrs	3.90	3.74×10^{7}	0.79	2.95×10^{5}	154	-
1-^{14}C-acetate Specific activity 2 mCi/mmole + 5 mg of unlabeled DHMC	12 hrs	3.90	-	-	0.83×10^{5}	18	4.82

The solution of acetate alone or along with unlabeled DHMC was shaken with 1 mm thick tissue slices after 2 days of prior exposure to 2000 ppm ethylene in the presence of same concentration of ethylene.

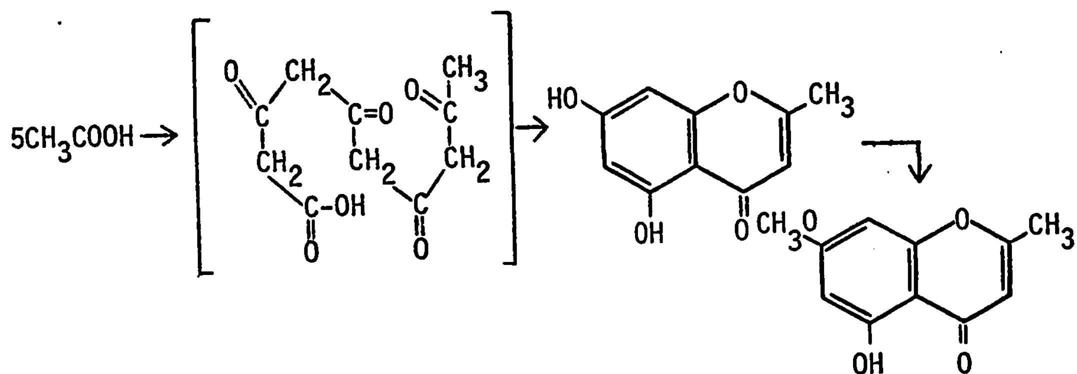
(-) indicates not determined.

*The specific activity of DHMC is after dilution with the carrier.

carrots (that had been treated with ethylene) *via* the acetate pathway, and 5,7-dihydroxy-2-methylchromone was probably a natural precursor of eugenin. This was evident from the two findings, *viz*, (1) on feeding 5,7-dihydroxy-2-methyl-chromone of known specific activity and then reisolating, considerable dilution of its specific activity occurred; (2) on feeding 1-^{14}C-acetate, ^{14}C-5,7-dihydroxy-2-methylchromone was trapped by using unlabeled 5,7-dihydroxy-2-methylchromone.

The above results also indicated that the O-methylation most likely occurred after the aromatization of the benzene ring in eugenin. Rhodes *et al.* (114) found, in the biosynthesis of griseophenones in *Penicillium patulum*, that at least some O-methylations occurred after aromatization. Rickards (116) too found during the biosynthesis of mycophenolic acid in *Penicillium brevi-compactum* that O-methylation occurred after aromatization.

Thus, the biosynthetic route may be as follows:



Conclusions on Section III

In conclusion, ethylene stimulates the enzyme phenylalanine ammonia-lyase for a short initial period, thus explaining the increased synthesis of isochlorogenic acid in the initial period after exposure to ethylene. However, the steady increase in isochlorogenic acid on

ethylene treatment can not be explained on the basis of observed initial enhancement followed by decline in PAL activity. One may only speculate that either some other enzyme(s) is(are) the controlling factor for the increase in isochlorogenic acid, or it may be that *in vivo* the activity of PAL keeps on increasing in contrast to that which is found *in vitro*. There is possibility that during the isolation procedure either the phenols may have deactivated the enzyme (PAL) or the enzyme has been deactivated by some PAL deactivating system (156).

So far as the phenols derived from the acetate pathway are concerned, it is probable that ethylene induces the acetate pathway for the biosynthesis of isocoumarin and eugenin, because these compounds are not detectable in carrots normally.

Thus, ethylene stimulates both pathways of aromatic biosynthesis in carrots.

Section IV

Effects of ethylene on the respiratory activity and glycolytic pathways of carrots

Reported effects of ethylene on various enzyme syntheses have been described earlier (Literature Review, A.b). Those reports together with the author's observation on increased synthesis of phenolic compounds in carrot roots on ethylene treatment indicate the fact that ethylene somehow must stimulate the basic metabolic processes in the cells in order to produce considerable amount of energy as well as many precursors for the syntheses of either different enzyme proteins or compounds like the ones isolated and characterized.

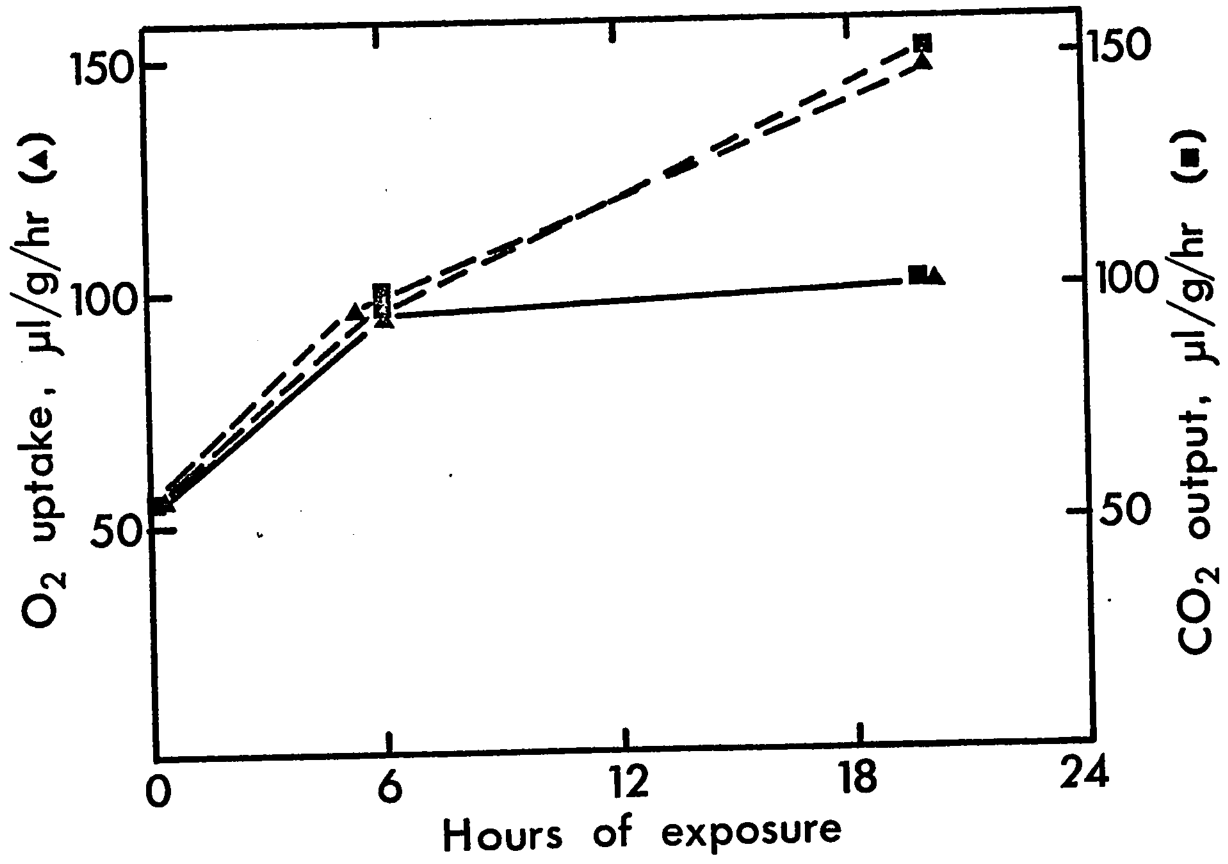
The present study was undertaken to see if ethylene stimulated the processes in carrots which yielded the required energy and the intermediates for the observed synthesis of new compounds. Present study was confined to the respiratory activity of carrots on ethylene treatment and to the operation of two well known pathways of carbohydrate breakdown. In order to see whether ethylene stimulated the respiration rate in carrots and also to compare the effects of some other agents, namely DNP and methylene blue, which affect the respiration both quantitatively and qualitatively in various plant tissues (9) including carrots with that of ethylene, the following approach was taken. What similarities and dissimilarities existed among ethylene action, the action of DNP and the action of methylene blue, on carrots. Moreover, experiments were carried out to determine the relationship between glycolytic pathways and the synthesis of isocoumarin.

A. <u>On the respiratory activity</u>

The effect of ethylene on carbohydrate breakdown was studied by measuring the rate of oxygen uptake and carbon dioxide evolution without exogenous glucose added. The intact root was segregated into two portions, (1) the "skin" (1 mm thick outer layer) and (2) the rest, without the 1 mm thick outer layer, this was called the "inner tissue". The effect of ethylene on the rates of O_2 uptake and CO_2 output by these segments were studied separately. Two different concentrations of ethylene were used. These were 0.01% (100 ppm) and 0.2% (2,000 ppm). The values in graphs are the averages of duplicate, or in most cases triplicate determinations. The rates of O_2 uptake and CO_2 evolution were measured as described under Materials and Methods (B.11). The results were further confirmed by repeating the individual experiment with different batches of carrots.

The results of oxygen uptake and CO_2 evolution by "inner tissue" of carrot roots (recorded in Fig 18)indicated that up to 6 hrs of either air or 100 ppm ethylene treatment the rates of oxygen uptake and CO_2 evolution remained almost the same. The difference occurred after 6 hrs when the rate of respiration of aerated samples reached a plateau, whereas that of ethylene-treated tissue kept on increasing at a somewhat reduced rate. Similar results were obtained with a higher concentration of ethylene, 0.2 per cent (2000 ppm) in air (Fig 19). This was in disagreement with the results of Rhodes and Wooltorton (115), who found ethylene to have no effect on the respiration rates of swede and parsnip disks. However, the concentration of ethylene which they used was lower than that used in this study.

Fig 18: Effect of ethylene (100 ppm) on the rate of O_2 uptake and CO_2 output by "inner tissue". "Inner tissue" refers to the portion of roots without the 1 mm thick skin. (▲——▲) and (■——■) represent oxygen uptake (in air) and CO_2 output (in air) respectively. (▲- - -▲) and (■- - -■) represent O_2 uptake (in ethylene) and CO_2 output (in ethylene) respectively.


150
100
50
O2 uptake, µl/g/hr (▲)
150
100
50
CO2 output, µl/g/hr (■)
0
6
12
18
24
Hours of exposure

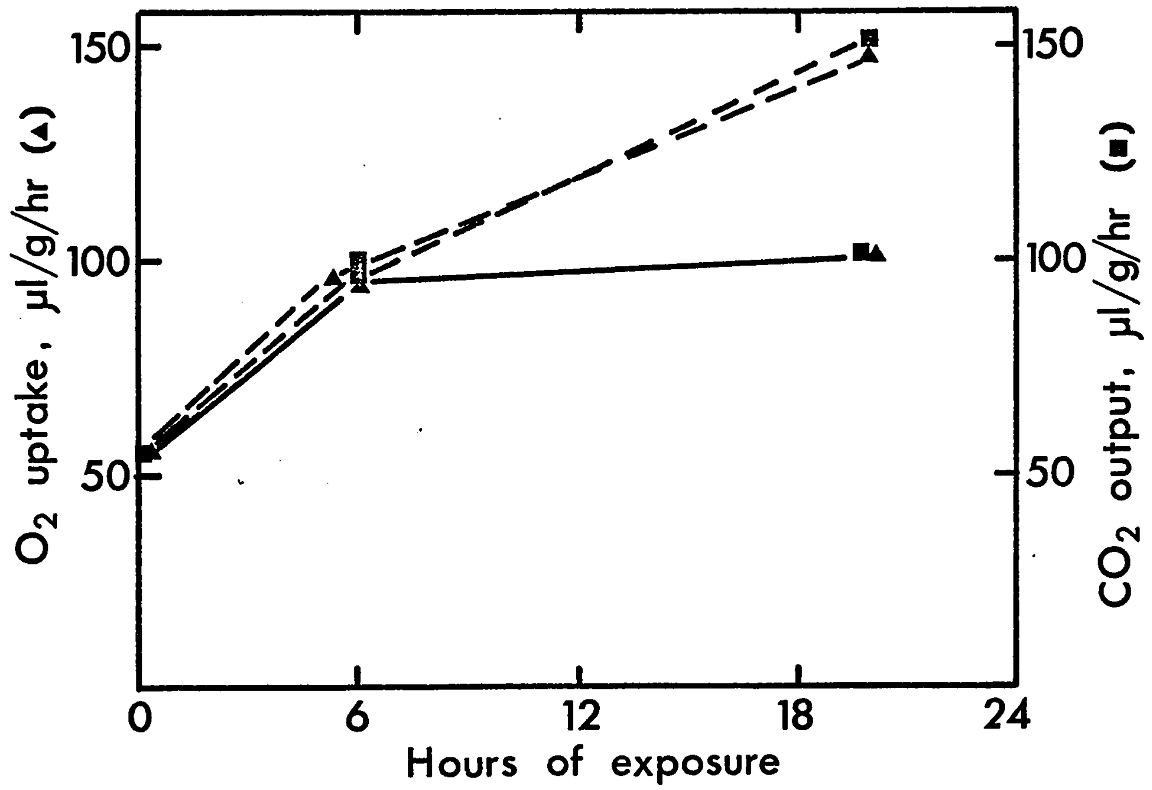
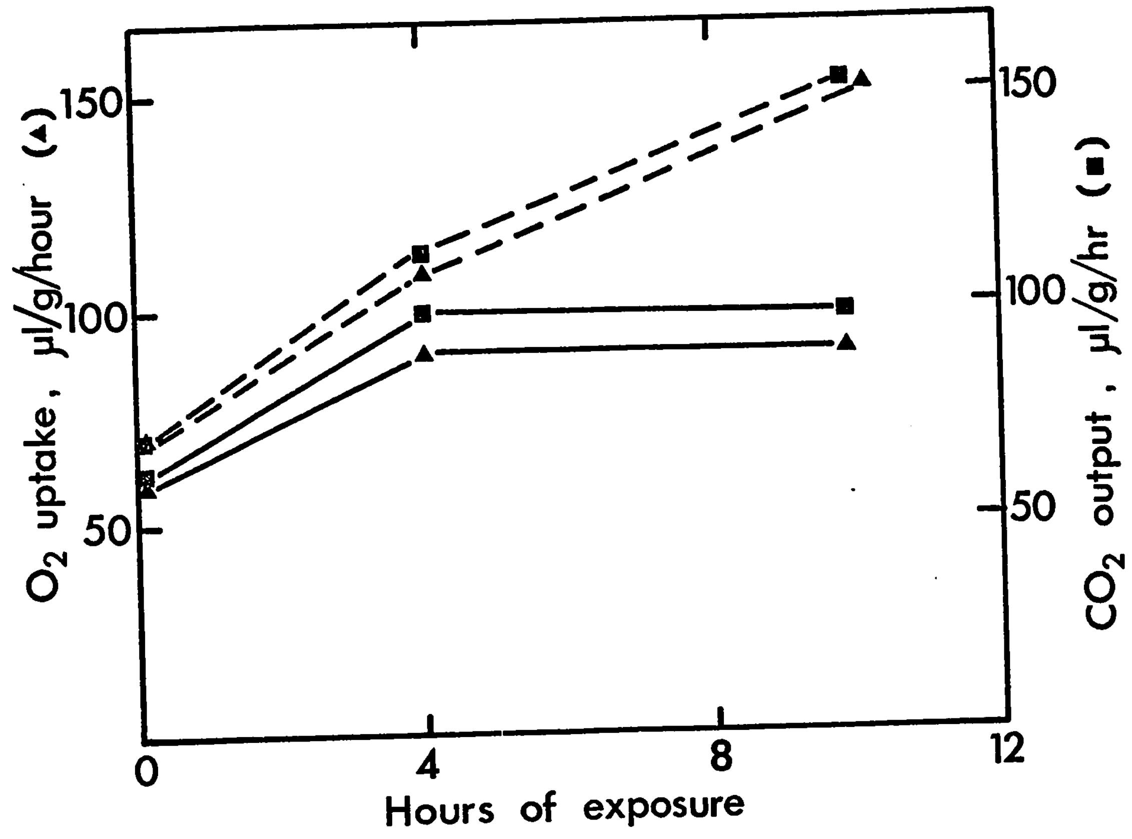

150
100
50
0
6
12
18
24
Hours of exposure
O_2 uptake, µl/g/hr (▲)
CO_2 output, µl/g/hr (■)

Fig 19. Effect of 0.2% (2000 ppm) ethylene on the rate of oxygen uptake and CO_2 evolution by "inner tissue". Inner tissue refers to the portion of the roots without the 1 mm thick skin. (▲——▲) and (■——■) represent O_2 uptake (in air) and CO_2 output (in air) respectively. (▲---▲) and (■---■) represent O_2 uptake (in ethylene) and CO_2 output (in ethylene) respectively.



O_2 uptake, µl/g/hour (▲)
150
100
50
CO_2 output, µl/g/hr (■)
150
100
50
0
4
8
12
Hours of exposure

Fig 20 recorded the results of ethylene treatment on O_2 uptake and CO_2 evolved by the "skin" of carrots. The rate of respiration of this tissue was much higher than that of "inner tissue". The effect of ethylene on this tissue was slightly different from that on the inner tissue. Here, after 6 hours, the rate of respiration in the air-treated sample did not reach a plateau but increased at a much lower rate than that of ethylene-treated sample. Similar results were obtained with a higher concentration of ethylene, 0.2 per cent (2000 ppm) in air (Fig 21). So, it was apparent from these results that ethylene increased the rate of respiration in carrot slices. One point should be made here however, that ethylene did not produce any change in the RQ of carrot slices. This would suggest that other pathways of CO_2 production, *viz*, alcoholic fermentation or activity of malic enzyme were presumably not stimulated.

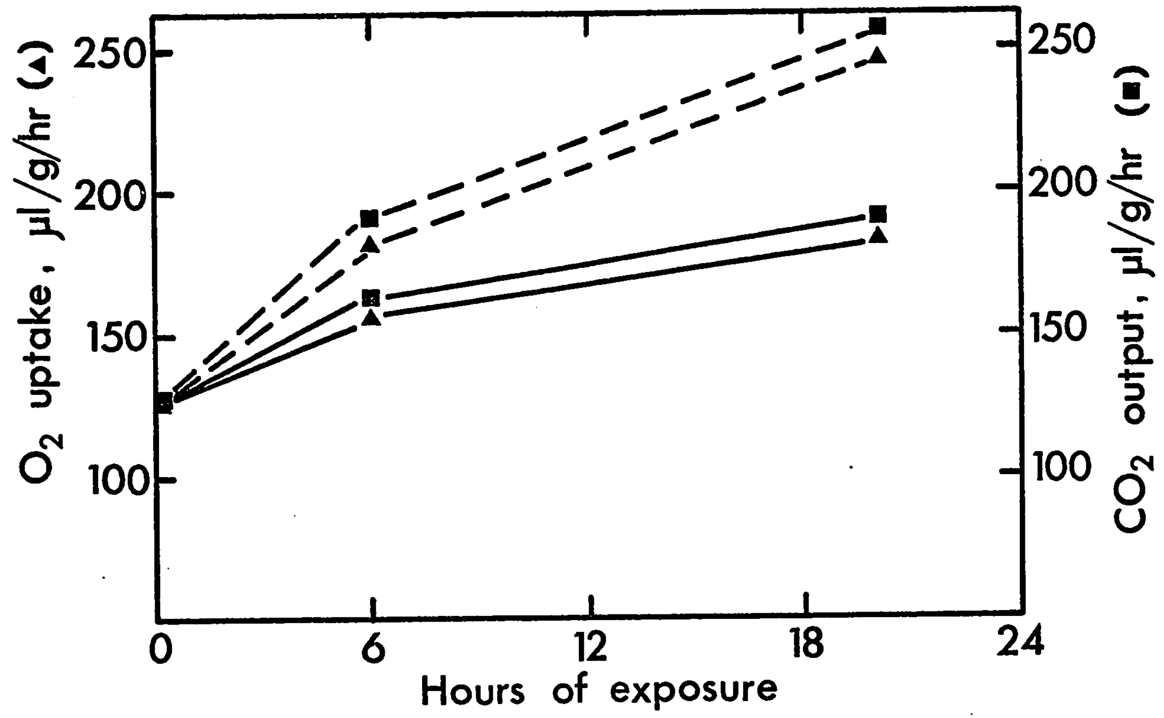
B. <u>On glycolytic pathways</u>

The next step was to see whether ethylene behaved like some other stimulatory agents of respiration, namely DNP and methylene blue, in changing the balance between the Embden-Meyerhof-Parnas and the pentose phosphate pathway.

DNP at certain concentrations stimulates glucose breakdown *via* the EMP pathway (3,23) and methylene blue, by accepting electrons from NADPH, stimulates glucose breakdown *via* the pentose phosphate pathway (65). Whether ethylene could have a selective action on the pathways of glucose utilization by plant tissues had not been studied previously.

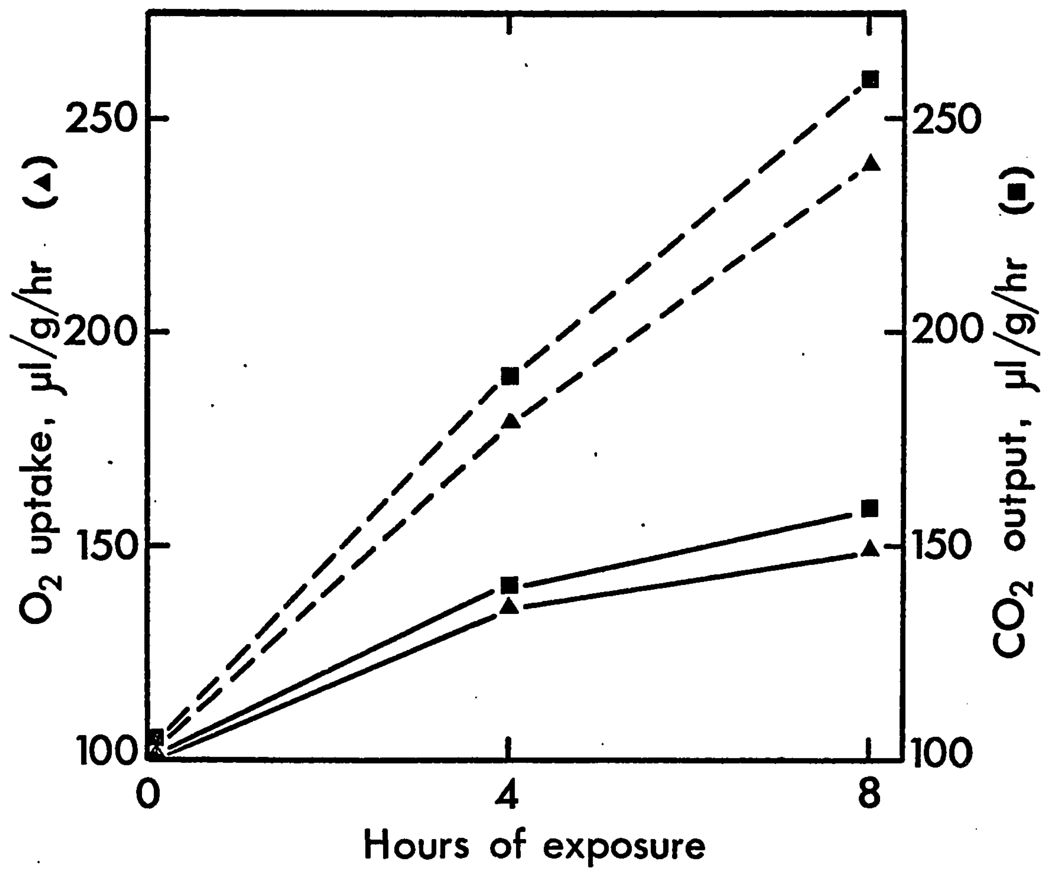
The effects of ethylene on CO_2 production by 1 mm carrot slices after

Fig 20. Effect of 100 ppm ethylene on the rate of O_2 uptake and CO_2 output by 1 mm thick skin tissue at 25°. (▲——▲) and (■——■) represent O_2 uptake (in air) and CO_2 output (in air) respectively. (▲---▲) and (■---■) represent O_2 uptake (in ethylene) and CO_2 output (in ethylene) respectively.



O_2 uptake, µl/g/hr (▲)
250
200
150
100
0
6
12
18
24
Hours of exposure
CO_2 output, µl/g/hr (■)
250
200
150
100

Fig 21. Effect of 0.2% (2000 ppm) ethylene on the rate of oxygen uptake and CO_2 output by 1 mm thick skin tissue at 25°. (▲———▲) and (■———■) represent O_2 uptake (in air) and CO_2 output (in air) respectively. (▲----▲) and (■----■) represent O_2 uptake (in ethylene) and CO_2 output (in ethylene) respectively.



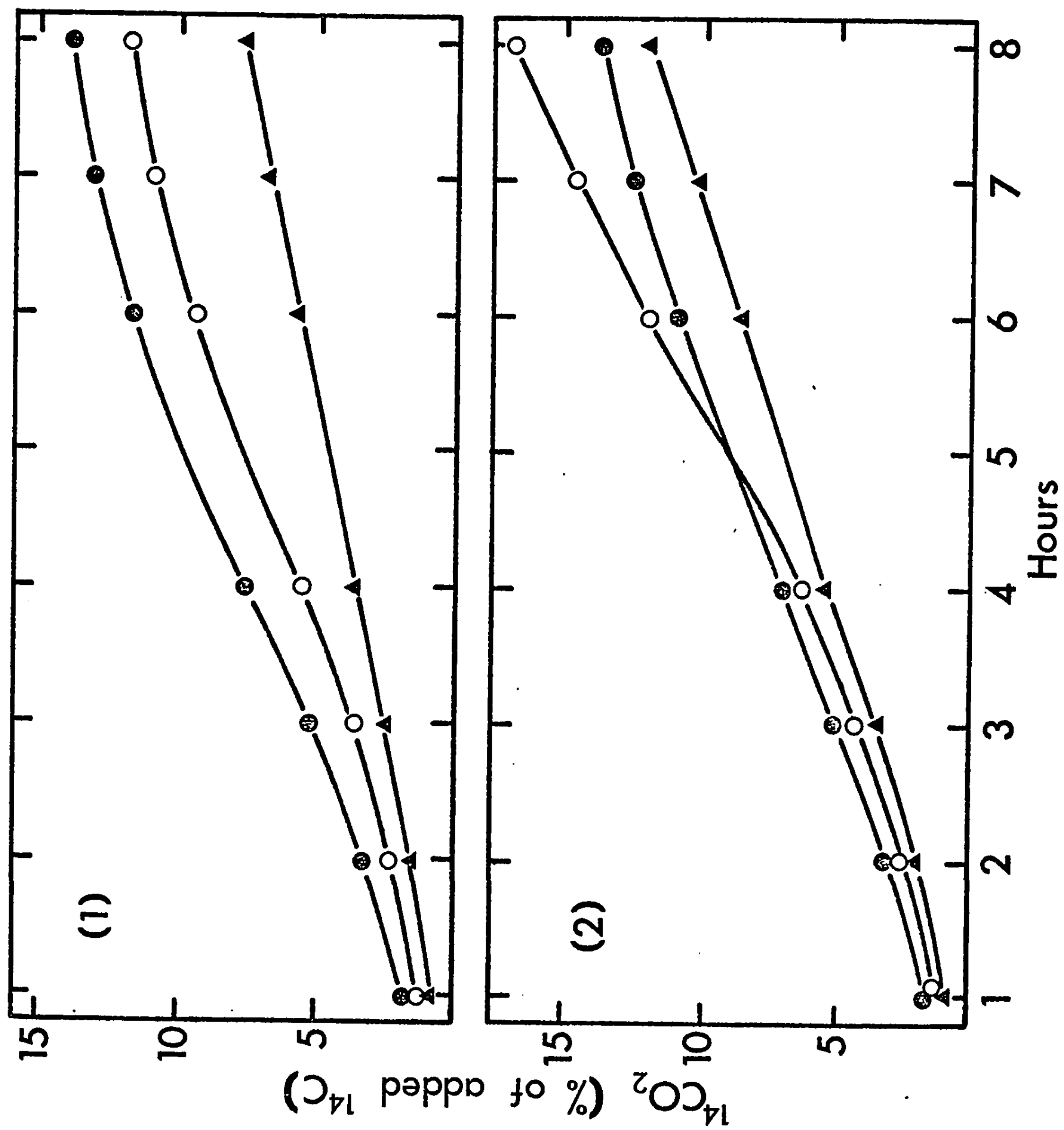
250
200
150
100
250
200
150
100
0
4
8
Hours of exposure
O_2 uptake, µl/g/hr (▲)
CO_2 output, µl/g/hr (■)

feeding 1-^{14}C-glucose, 6-^{14}C-glucose, and glucose-3,4-^{14}C were studied with a single concentration of ethylene (2,000 ppm in air). The values in graphs and tables are the averages of duplicate determinations. Moreover each experiment was repeated with different batches of carrot slices.

It was clear from the Fig 22 that the rate of CO_2 production from glucose-1-^{14}C in air was higher than that from either glucose-6-^{14}C or glucose-3,4-^{14}C. This would indicate glucose utilization *via* both the pathways of carbohydrate breakdown. This was further confirmed by calculating the C-1/C-6 ratios. These ratios were always more than one (Table 21) indicating the operation of both the pathways. However, after 5 hrs of ethylene (2,000 ppm) treatment the rate of CO_2 production from glucose-3,4-^{14}C was higher than that from glucose-1-^{14}C (Fig 22). Also, the rate of CO_2 production from glucose-6-^{14}C (on ethylene treatment)(Fig 22)was 175% of the control (air-treated). The rate of CO_2 production from glucose-1-^{14}C was slightly depressed (compared to that of control) on ethylene treatment. The increased CO_2 production from glucose-6-^{14}C resulted in lowering of C-1/C-6 ratios (Table 21). This observation would suggest a preferential stimulation of the EMP pathway compared to the pentose phosphate pathway. However, no quantitative assessment was possible due to complications arising out of close association of the two pathways (75) and because of long incubation period used in this study.

Two pathways of glucose dissimilation are present in carrots is well-founded (3), and that it is possible to change the balance between them by using inhibitors and other chemical agents has also

Fig 22. Effect of ethylene (2000 ppm) on the $^{14}CO_2$ evolution from carrots fed with specifically labeled glucose. The slices were kept at 25°. Absorption of $^{14}CO_2$ and determination of its radioactivity has been described under Materials and Methods (B.12). (▲——▲), (○——○), and (●——●) represent $^{14}CO_2$ evolved from 6-^{14}C-glucose, 3,4-^{14}C-glucose, and 1-^{14}C-glucose respectively. (1) in presence of air and (2) in presence of ethylene. The slices were incubated for the length of time as indicated.



(1)
(2)
15
10
5
$^{14}CO_2$ (% of added ^{14}C)
1
2
3
4
5
6
7
8
Hours

Table 21

Effect of ethylene on release of *C-1 and **C-6 of glucose as CO_2

Experiment number	Time, hrs	Control			Ethylene (2000 ppm) treated		
		% of C-1 converted to CO_2	% of C-6 converted to CO_2	Ratio C-1/C-6	% of C-1 converted to CO_2	% of C-6 converted to CO_2	Ratio C-1/C-6
1	0-3	5.23	2.47	2.11	4.80	3.41	1.40
	3-6	6.65	3.40	1.95	6.00	4.80	1.25
2	0-2	3.75	1.56	2.40	2.81	1.61	1.61
	2-4	5.46	2.65	2.06	4.97	3.73	1.33
3	0-2½	2.79	1.28	2.18	2.23	1.40	1.59
	2½-5	3.98	2.13	1.87	3.55	2.54	1.39

*C-1 release was determined using 1-^{14}C-glucose.

**C-6 release was determined using 6-^{14}C-glucose.

One mm thick carrot slices (3 g for each sample) were infiltrated with the aqueous solutions of labeled glucose. The method of infiltration has been described under Materials and Methods (B.17). A continuous stream (25 ml/min) of 2000 ppm ethylene or air was passed at 25° and $^{14}CO_2$ was collected as described under Materials and Methods (B.12).

been reported. Ap Rees and Beevers (3) found that DNP changed the balance between the two pathways in carrots by stimulating the EMP pathway whereas methylene blue changed the balance by stimulating the pentose phosphate pathway. The present findings suggest a preferential stimulation of the EMP pathway on ethylene treatment. Tager (141) had suggested a similar effect of ethylene on ripening bananas. He found that in preclimacteric fruit the respiration was mediated through the pentose phosphate pathway and there was a partial shift to the EMP pathway during climacteric period. The concentration of ethylene used in the present study far exceeded the physiological level needed for its hormonal action. Whether ethylene at physiological concentration would have similar effect would be of interest to explore in the future studies with ethylene.

C. Effect of ethylene on the pathways of glucose dissimilation and isocoumarin synthesis

It was shown in the preceeding results (Fig 22) that ethylene might have stimulated the EMP pathway preferentially in carrots. It was also found that both the pathways of glucose dissimilation were present in carrots. It was discussed earlier (Results and Discussion, Section I.B) that isocoumarin was derived most likely from acetate. When both pathways of glucose dissimilation are present in a tissue one of the methods to calculate quantitatively their relative operation is to determine the acetyl derivative ratios [*viz*, fatty acid ratios, as was done by Abraham and Chaikoff (2)] from glucose-1-^{14}C and glucose-6-^{14}C. This method has been described by Katz and Wood

(75,76) and Katz *et al.* (77). In the present study carrot slices were fed glucose-1-^{14}C and glucose-6-^{14}C. After incubating the carrot slices (1 mm thick) for 2 days in presence of ethylene (2000 ppm) isocoumarin was isolated and the radioactivity was determined as described under Materials and Methods (B.18). The quantitative assessment of the two pathways was not done because the incubation time was too long to justify the assumptions (77) needed in such a calculation.

All the values in the table are the averages of duplicate determinations. Moreover each experiment was checked for its reproducibility. The results that were recorded in Table 24 showed more incorporation from glucose-6-^{14}C than glucose-1-^{14}C into isocoumarin. This would suggest the operation of a pathway other than the EMP pathway, such as the pentose phosphate pathway (as suggested earlier with regard to $^{14}CO_2$ production from ^{14}C-glucose). If carrots had utilized glucose by only the EMP pathway the percent conversion of glucose-1-^{14}C and glucose-6-^{14}C into isocoumarin would have been equal.

Further evidence pointing to the operation of a pathway other than the Embden-Meyerhof-Parnas in carrots treated with ethylene is provided in the study with glucose-3,4-^{14}C. When glucose so labeled was incubated with the carrot slices, a significant conversion of the ^{14}C to isocoumarin was observed (Table 22). After a glucose molecule traverses the Embden-Meyerhof-Parnas sequence of enzymatic reactions, its two central carbon atoms 3 and 4 are lost as CO_2, thereby making available 2 acetyl-COA molecules for further metabolism *via* the TCA cycle, fatty acid formation, etc. The present finding of a significant conversion of the two central glucose carbons into isocoumarin in

Table 22

Incorporation of specifically labeled glucose into isocoumarin

Substrate	Expt. No.	Activity added x 10^{-6}dpm	Activity taken up x 10^{-6}dpm	isocoumarin dpm	% conversion	Specific activity μCi/mmole
Glucose-1-^{14}C, Specific activity 10.7 mCi/mmole	1	18.10	15.33	77,284	0.504	7.27
	2	15.32	13.50	75,104	0.556	7.09
Glucose-6-^{14}C, Specific activity 10.7 mCi/mmole	1	18.10	17.29	119,800	0.693	11.69
	2	15.32	13.88	107,496	0.774	11.27
Glucose-3,4-^{14}C, Specific activity 10.7 mCi/mmole	1	18.10	16.85	17,025	0.101	2.93
	2	15.32	12.99	15,055	0.115	2.84

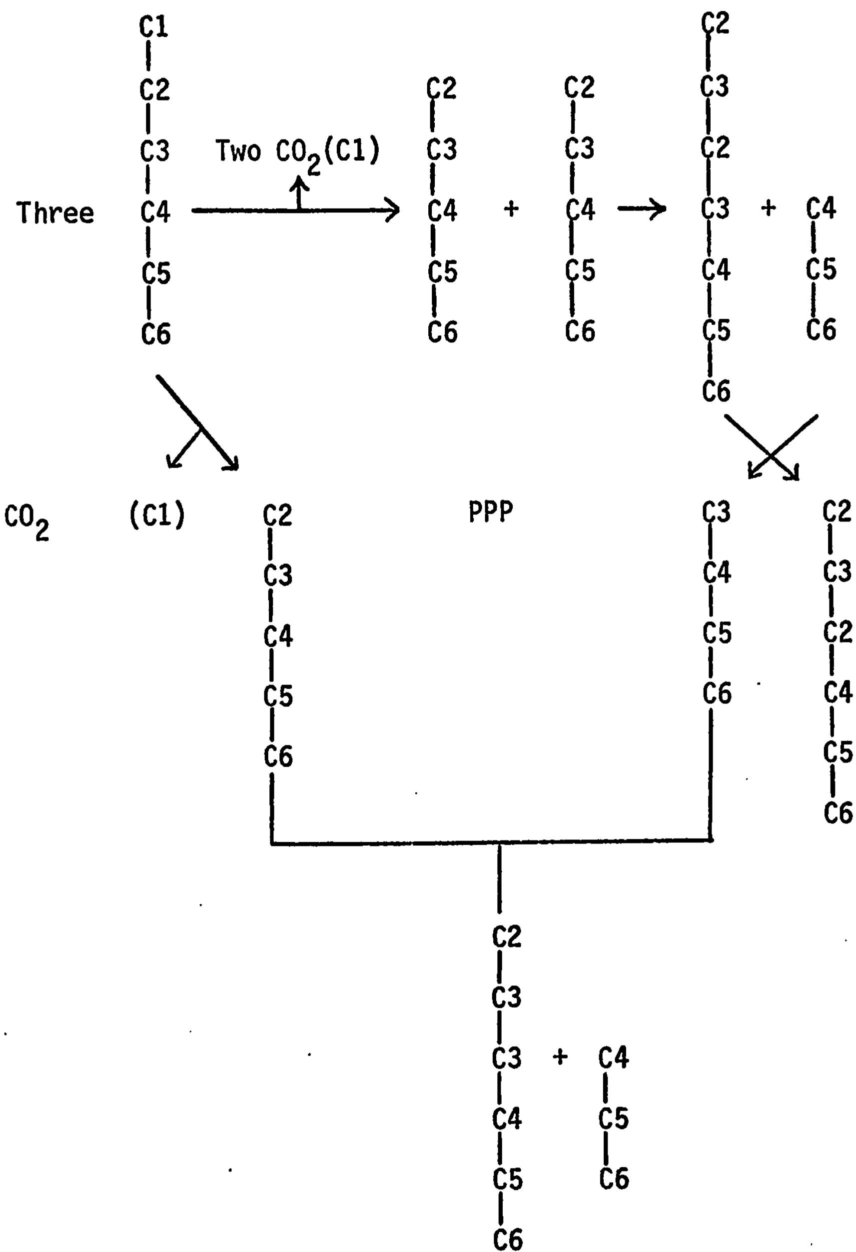
Percent conversion was determined on the basis of labeled glucose taken up by the tissue.

One mm thick carrot slices (3 g for each sample) were agitated with the aqueous solutions of labeled glucose at 25° in the presence of 2000 ppm ethylene for 2 days.

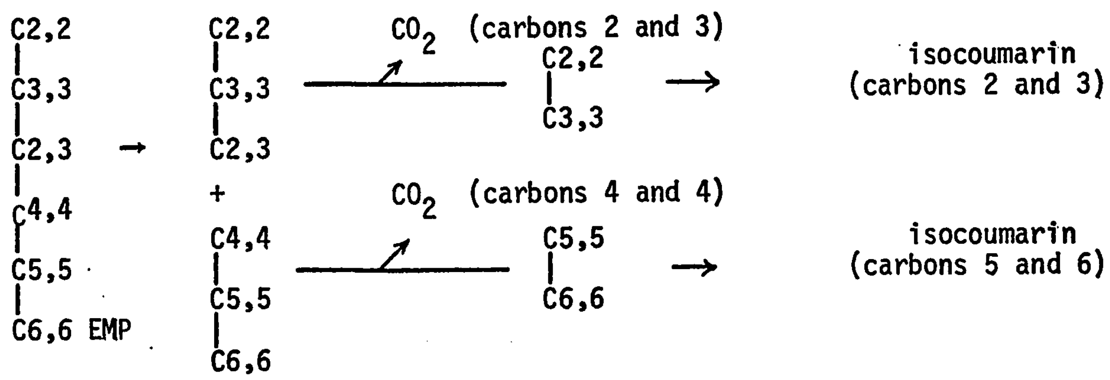
incompatible with the sole operation of the EMP pathway in the carrot tissue treated with ethylene. If the pentose cycle operates, carbon 3 of glucose appears in positions 1 and 2, as well as 3, of the reformed hexose monophosphate. When the latter is cleaved by Embden-Meyerhof-Parnas enzymes, the resulting triose phosphate will contain ^{14}C in carbons 2 and 3 as well as in the carboxyl carbon. Upon decarboxylation, the acetyl-CoA formed will now contain ^{14}C. Carbon 4 of glucose does not migrate as a consequence of the pentose cycle reactions, but appears as the carboxyl carbon of a triose. Thus, carbon 3, not 4, of glucose undergoes conversion first to acetyl-CoA which is converted to isocoumarin in carrots or to other acetogenins in tissues in which there is an extensive operation of the pentose phosphate pathway. The above reactions are elaborated in the following diagram [after Abraham and Chaikoff (2)] (Fig 23).

The relationship between pentose phosphate pathway and activity of the shikimic acid pathway for aromatic compound synthesis is obvious. PPP may be the most important source of intermediate, such as 4-erythrose phosphate, of the shikimic acid pathway. Because the isochlorogenic acid is derived from the shikimic acid pathway the pentose cycle must be operative in carrots treated with ethylene. Moreover, reduced pyridine nucleotide (NADPH) has to be available for the reductive steps in aromatic biosynthesis *via* the acetate-malonate pathway (87). The pentose cycle is one of the major sources of reduced NADPH. The operation of the EMP pathway is also essential in order to produce pyruvate and energy currency needed to maintain the cellular activites. So, the operation of both the EMP and PPP

Fig 23. Incorporation of various glucose carbons into isocoumarin *via* a combination of the pentose phosphate (PPP) and the Embden-Meyerhof-Parnas (EMP) pathways.



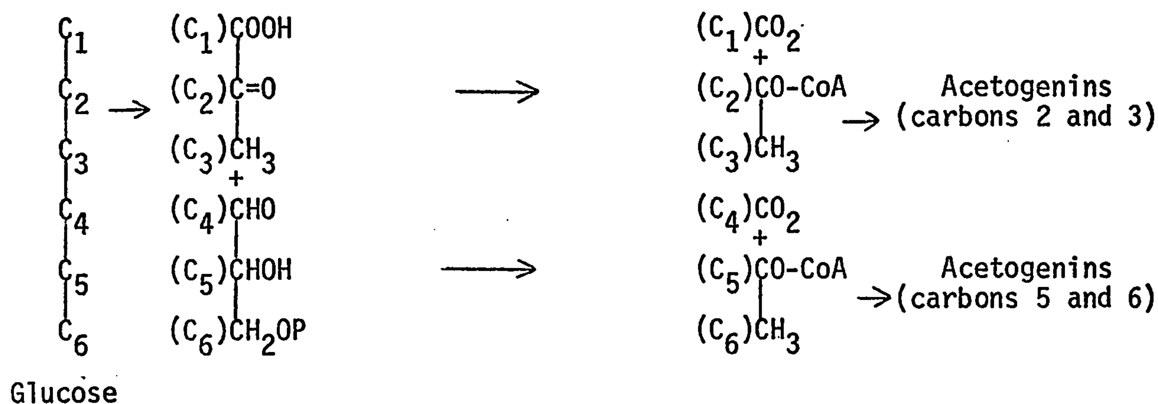
C1
C2
C3
C4
C5
C6
Three
Two CO_2(C1)
C2
C3
C4
C5
C6
+
C2
C3
C4
C5
C6
C2
C3
C2
C3
C4
C5
C6
+
C4
C5
C6
CO_2
(C1)
C2
C3
C4
C5
C6
PPP
C3
C4
C5
C6
C2
C3
C2
C4
C5
C6
C2
C3
C3
C4
C5
C6
+
C4
C5
C6



C2,2
C3,3
C2,3
C4,4
C5,5
C6,6 EMP
C2,2
C3,3
C2,3
+
C4,4
C5,5
C6,6
CO_2 (carbons 2 and 3)
C2,2
C3,3
isocoumarin
(carbons 2 and 3)
CO_2 (carbons 4 and 4)
C5,5
C6,6
isocoumarin
(carbons 5 and 6)

in ethylene-treated carrots is in agreement with the manifestation of ethylene effects on the tissue.

A number of present findings on glucose metabolism of the carrot slices are also compatible with the operation of the Entner-Doudoroff pathway (47). In this scheme of glycolysis, which is based on observations with microorganisms, 6-phospho-gluconate is cleaved, yielding pyruvate and glyceraldehyde 3-phosphate, with the distribution of glucose carbons shown in the diagram below:



The compatible findings are: (a) the recovery of more $^{14}CO_2$ from glucose-1-^{14}C than from glucose-6-^{14}C and glucose-3,4-^{14}C; (b) the conversion of ^{14}C of glucose-3,4-^{14}C to isocoumarin; and (c) the greater conversion of glucose carbon 6 to isocoumarin than of glucose carbon 1. However, there has been no report so far regarding the operation of this pathway in plants. Therefore, PPP will be considered as the only alternate pathway of carbohydrate breakdown in carrots.

Ethylene stimulated the respiration of carrot slices and changed the balance between the two pathways of glucose breakdown by stimu-

lating the EMP pathway preferentially. Ap Rees and Beevers (3) found that DNP at 2.5 x 10^{-5} M concentration stimulated the respiration of carrot slices and also stimulated the EMP pathway preferentially. Thus, the effects of ethylene on carrots have at least two similarities with those of DNP on carrot slices.

D. Effect of ethylene on alcohol and acetaldehyde production by carrots

In the previous section ethylene was shown to stimulate the EMP pathway compared to the PPP. To find out whether the stimulation of the EMP pathway was manifested in increased production of alcohol and acetaldehyde in presence of air (negative Pasteur effect) the estimation of alcohol and acetaldehyde (in carrots) was made after ethylene treatment. Two different concentrations of ethylene were used, *viz*, 100 ppm and 2,000 ppm. The values in the tables are the averages of duplicate determinations. Each experiment was repeated several times with different batches of carrots.

The results recorded in Tables 23 and 24 showed a small but consistent decrease in alcohol and acetaldehyde production by carrots upon ethylene treatment. It was felt that the decrease might have been due to increased synthesis of isocoumarin upon ethylene treatment. Isocoumarin, as suggested earlier, might have been derived from acetate. The acetate is derived most likely from pyruvate, and if the bulk of the pyruvate is directed towards isocoumarin synthesis as well as towards increased TCA cycle activity (as evidenced by increased CO_2 output and O_2 consumption) on ethylene treatment, little

Table 23

Effect of ethylene on alcohol production by carrot slices

Ethylene concn ppm	Duration of treatment hrs	Expt. No.	Alcohol produced, μg/g fresh weight	
			Air treated	Ethylene treated
100	4	1	1.12	0.91
100	8	2	1.71	1.52
100	4	3	2.08	1.12
100	8	4	2.72	1.95
2000	9	1	1.52	0.72
2000	9	2	1.52	0.91

Collection and estimation of ethanol was done as described under Materials and Methods (B.16).

Table 24

Effect of ethylene on acetaldehyde production by carrot slices

Ethylene concn ppm	Duration of treatment hrs	Expt. No.	Acetaldehyde produced; μg/g fresh weight	
			Air treated	Ethylene treated
100	4	1	1.91	1.65
100	8	2	8.38	5.00
100	4	3	3.44	3.09
100	8	4	5.95	5.23
2000	9	1	8.38	2.65
2000	9	2	5.29	3.73

Collection and estimation of acetaldehyde was done as described under Materials and Methods (B.16).

pyruvate would probably be used by the fermentative pathway. As a result of this, a decrease in acetaldehyde and alcohol production by carrot roots on ethylene treatment, would be observed. It was found earlier that the effects of ethylene and DNP on carrot slices had certain similarities. However, DNP at certain concentration was found to stimulate the production of acetaldehyde and alcohol in plants (9), yeast (137), and animals (23) in presence of air. The above observation on the effect of ethylene did not show any stimulation of alcoholic fermentation in carrots.

Conclusions on Section IV

In summary, ethylene has been shown to stimulate both the oxygen uptake and carbon dioxide evolution by carrot slices. However, the RQ always remained close to one. This suggests that other pathways of CO_2 evolution, *viz*, fermentative pathway and the activity of malic enzyme are not stimulated by ethylene in carrots.

Investigations with carrots after feeding glucose-1-^{14}C,glucose-3,4-^{14}C, and glucose-6-^{14}C led to the conclusion that both the EMP and the PPP were operative in carrot slices, and ethylene caused a preferential stimulation of the EMP pathway. Both the pathways contributed to the biosynthesis of isocoumarin. This was evidenced by greater contribution towards ^{14}C-isocoumarin formation by glucose-6-^{14}C than glucose-1-^{14}C. Also, formation of ^{14}C-isocoumarin from glucose-3,4-^{14}C corroborated the above conclusion.

A slight decrease in ethanol and acetaldehyde production by carrots was observed upon ethylene treatment. This suggested that there was no stimulation of fermentative pathway in ethylene-treated carrots.

Section V

Effects of metabolic inhibitors on carrots (in regard to ethylene and isocoumarin production)

Metabolic inhibitors such as DNP, methylene blue, sodium arsenite, and cycloheximide have been used to throw some light on the possible control sites for ethylene-induced isocoumarin synthesis in carrots. Though these inhibitors are not always specific but their action on certain metabolic steps are well-established. For example, DNP has been shown to uncouple phosphorylation from oxidation in the mitochondrial electron transport chain. It induces aerobic fermentation in yeast (137), animals (23) and plants(9,104). It also stimulates the pyruvate kinase in the yeast (129). Methylene blue has been widely used as a stimulant of pentose phosphate pathway in animals (23) and plants (9). It accepts the electrons from the pyridine nucleotides and thus simulates the processes that produce them (the pyridine nucleotides). Arsenite has been held responsible for causing inhibition of transport processes in the cells (150). It is suggested by Maizels (91) that a permeability change may be caused by arsenicals through a depression of oxidative metabolism. It is believed that arsenite inhibits the generation of ATP rather than interferring with its utilization, and this perhaps is accomplished by an inhibition of pyruvate oxidation (150). The cycloheximide is an inhibitor of protein biosynthesis. It interferes in the peptide bond forming steps.

All the inhibitors were infiltrated into the carrot slices as aqueous solutions by the technique described under Materials and Methods (B.19).

A. Effects of DNP and methylene blue on ethylene production by carrot slices

Carrot slices (1 mm thick) were infiltrated with 1×10^{-4} M DNP solution in 0.05 M phosphate buffer (pH 5.0), and 1×10^{-3} M methylene blue solution in 0.05 M phosphate buffer (pH 5.0). Slices serving as control were infiltrated with only phosphate buffer. The results were recorded in Table 25. The values are the averages of triplicate determinations. The values (Table 25) revealed that DNP depressed the ethylene production (almost 8-fold) whereas methylene blue stimulated its production (about 7-fold). Spencer (133) also observed a depression in ethylene production by tomato fruits on DNP treatment. Perhaps DNP made ATP, necessary for ethylene production (143), less available.

B. Effect of DNP on carrots (in regard to isocoumarin formation)

Carrot slices (4 mm thick) were infiltrated with different concentrations of DNP solution in a 0.05 M buffer solution (pH 5.0). After infiltration the slices were aerated for different lengths of time. The results were recorded in Table 26. All the values were the averages of duplicate determinations. Furthermore, each observation was verified by repeating the experiment with different batches of carrots. Appropriate controls were always run. It was found that DNP, at pH 5.0 and 1×10^{-4} M and pH 7.1 and 1×10^{-3} M concentration, induced the formation of considerable amounts of isocoumarin, but no eugenin was detected. The production of isocoumarin by DNP could not be mediated *via* ethylene because DNP depressed the production of

Table 25

Effect of DNP and methylene blue on the rate of ethylene production by carrot slices

Expt. No.	Collection period, hrs	Inhibitor	Ethylene, nl/g/hr fresh weight	
			With inhibitor	Control
1	4	None		0.400
2	4	None		0.925
1	4	DNP	0.050	
2	4	DNP	0.090	
1	4	Methylene blue	2.750	
2	4	Methylene blue	5.240	

DNP and methylene blue solutions were prepared in 0.05 M (pH 5.0) phosphate buffer. Phosphate buffer (0.05 M) was used with the slices serving as control. The inhibitors and buffer solution were infiltrated into the carrot slices as described under Materials and Methods (B.19).

Table 26

Effect of DNP on carrots (in regard to isocoumarin synthesis)

Expt. No.	Duration of aeration after DNP* treatment	Molarity and pH (of DNP soln) respectively	Isocoumarin**, mg/100 g carrots	
			***Control	DNP treated
1	3 days	1×10^{-3}M; 7.1	Non detectable	22.17
	3 days	1×10^{-4}M; 7.1	Non detectable	Non detectable
	3 days	1×10^{-5}M; 7.1	Non detectable	Non detectable
2	1 day	1×10^{-3}M; 7.1	Non detectable	10.76
3	2 days	1×10^{-3}M; 7.1	Non detectable	31.85
4	2 days	1×10^{-3}M; 5.0	Non detectable	Non detectable
	2 days	1×10^{-4}M; 5.0	Non detectable	17.45
	2 days	1×10^{-5}M; 5.0	Non detectable	Non detectable

*DNP solution (in phosphate buffer) was infiltrated as described under Materials and Methods (B.19).

**Isocoumarin was isolated and estimated as described under Materials and Methods (B.10).

***The slices (serving as control) were infiltrated with phosphate buffer solution.

ethylene by carrots. Condon *et al.* (34) reported DNP to have no apparent effect on isocoumarin treatment in carrots. This perhaps was due to the concentration of DNP they used; the concentration they used might have been different from that which could trigger isocoumarin synthesis.

The above finding reveals one more similarity between the action of ethylene and that of DNP on carrots (Section IV.C).

Ethylene, like DNP, stimulates glycolysis, which in turn may produce pyruvate at a much higher rate than can be utilized by the TCA cycle as acetate. The excess acetate then most likely would be channeled to other pathways of acetate utilization. As isocoumarin and eugenin are probably both acetate derived it would not be unreasonable to rationalize the formation of these compounds from the available excess acetate. In his findings about induction of aerobic fermentation in carrots on DNP treatment, Beevers (9) suggested the utilization of excess pyruvate by the fermentative pathway for the production of acetaldehyde and alcohol.

C. Effect of methylene blue on carrots (in regard to isocoumarin synthesis)

Carrot slices (4 mm) were infiltrated with 1×10^{-3} M solution of methylene blue and then aerated for different lengths of time. Controls were infiltrated with water in all experiments. The results were recorded in Table 27. The values are the averages of duplicate determinations. Each observation was checked several times for its repeatibility. Methylene blue induced the production of isocoumarin in carrots after a day of aeration, but no eugenin was detected even

Table 27

Effect of methylene blue on carrots (in regard to isocoumarin synthesis

Expt. No.	Duration of aeration after treatment	Isocoumarin*, mg/100 g fresh wt	
		Control**	Methylene blue *** treated
1	1 day	None	10.08
2	2 days	None	31.35

*Isocoumarin was isolated and estimated as described under Materials and Methods (B.10).

**Slices (serving as control) were infiltrated with water.

***Methylene blue solution (1×10^{-3}M) in water was infiltrated into the slices as described under Materials and Methods (B.19).

after 4 days of aeration. Whether the formation of isocoumarin in carrots on methylene blue treatment was mediated through ethylene or not could not be answered with certainty, because the production of ethylene by carrot slices was stimulated by methylene blue (Table 25). However, the fact that methylene blue did not produce any eugenin was an argument against such a mediation. Ap Rees and Beevers (3) found that methylene blue stimulated glycolysis beside stimulating pentose phosphate pathway, although to a much smaller extent. They also suggested that in carrots both the pathways of carbohydrate breakdown were closely integrated and there was a common pyruvate pool. The author's finding (Table 22) that ^{14}C-isocoumarin was formed from glucose-3,4-^{14}C lent support to the above suggestion. Thus, if there was a common pyruvate pool in carrots the stimulation of PPP by methylene blue might result in increased isocoumarin synthesis in carrots. Condon *et al.* (34) investigated the effect of methylene blue on isocoumarin synthesis in carrots, but reported inhibitory effect on isocoumarin formation. The concentration of methylene blue they used was the same as that used in the present investigation.

D. Effect of arsenite on isocoumarin production in carrots

Carrot slices (4 mm), after infiltration with different concentrations of sodium arsenite solution, were treated with 100 ppm ethylene for 1 or 2 days. Controls (infiltrated with water) were similarly treated with 100 ppm ethylene. The results were recorded in Table 28. The values are the averages of duplicate determinations. Each experiment was repeated with different batches of carrots.

The data (in Table 28) revealed that a 10 millimolar solution of arsenite completely inhibited the production of isocoumarin in carrots on ethylene treatment. A 1 millimolar solution of arsenite partially inhibited its formation and a 0.1 millimolar solution stimulated the production of isocoumarin in carrots on ethylene treatment. This kind of stimulatory (instead of inhibitory) action of arsenite has been reported in the case of certain enzymes. For example, Blackley and McDoughall (17) reported a stimulation of dydrofolate reductase (about 17%) by a 10 millimolar arsenite solution.

E. Effects of cycloheximide on the formation of isocoumarin in carrots

Carrot slices (1 mm thick) were infiltrated with 1×10^{-4} M cycloheximide solution either before or at a later stage after exposure to ethylene. The results were recorded in Table 29. All the values are the averages of duplicate extractions and triplicate determinations. Moreover, each experiment was repeated with different batches of carrots. It was observed that infiltration of cycloheximide before exposure to ethylene completely inhibited isocoumarin formation, whereas infiltration after a brief exposure to ethylene only partially inhibited the isocoumarin formation in carrots. This would suggest a probable involvement of *de novo* enzymes synthesis in carrots upon ethylene treatment. The occurrence of a lag phase (Fig 13) in the production of isocoumarin in carrots on ethylene treatment lends support to such a possibility.

Table 28

Effect of arsenite on the synthesis of isocoumarin in carrots

Expt. No.	100 ppm C_2H_4	Molarity of arsenite solution*	Isocoumarin,***mg/100 g carrots	
			Control**	Arsenite treated
1	1 day	None added	11.65	
	1 day	1×10^{-2}M		None
	1 day	1×10^{-3}M		7.19
	1 day	1×10^{-4}M		18.19
2	2 days	None added	19.85	
	2 days	1×10^{-2}M		None
	2 days	1×10^{-3}M		16.89
	2 days	1×10^{-4}M		25.67

*Arsenite solutions were infiltrated into the slices as described under Materials and Methods (B.19).

**Water was infiltrated into the slices serving as control.

***Isocoumarin was isolated and estimated as described under Materials and Methods (B.10).

Table 29

Effect of 1 x 10^{-4}M cycloheximide solution on the synthesis of isocoumarin in carrots

Expt. No.	Prior ethylene treatment, hrs	Infiltration of cycloheximide followed by ethylene treatment	Isocoumarin, mg/100 g carrots
1	None	Yes	Not detectable
	4	Yes	7.91
	8	No	11.6
2	None	Yes	Not detectable
	4	Yes	6.15
	8	No	9.80

Infiltration was done under a vaccum (equivalent to 14 mm of Hg). The slices were exposed to 0.2% ethylene for a maximum period of 8 hrs.

Conclusions on Section V

Studies on the effects of metabolic inhibitors, *viz*, DNP, methylene blue, arsenite, and cycloheximide revealed the following: (1) DNP, which has been shown to stimulate respiration in a variety of plant tissues including carrots and stimulates the EMP pathway preferentially in carrots, induced the formation of isocoumarin in carrots. It also depressed ethylene production by carrot slices. (2) Methylene blue, which has been used to stimulate pentose phosphate pathway in animals and plants, also induced the formation of isocoumarin in carrots. However, neither DNP nor methylene blue induced the formation of eugenin in carrots. This makes ethylene a unique influencing agent for the synthesis of eugenin in carrots. (3) Arsenite which has been suggested as an inhibitor of pyruvate oxidase, completely inhibited the formation of isocoumarin (at certain concentration) in carrots on treatment with ethylene. This leads to the conclusion that pyruvate may be involved in isocoumarin synthesis. It was shown earlier that isocoumarin was probably derived from acetate. The acetate, in turn, might have been derived from pyruvate by the action of pyruvate oxidase, which could have been inhibited by arsenite.

Inhibition of isocoumarin synthesis in carrots by cycloheximide suggests the possibility of a *de novo* synthesis of enzyme(s). The occurrence of a lag period in the synthesis of isocoumarin in carrots on ethylene treatment corroborates this suggestion.

General Conclusions

An increase in total phenol content of carrots during cold storage was observed. This agreed with the report of Chubey and Nylund (31). The increase was manifested as an additional accumulation of the existing phenols in the roots. No change in the qualitative composition of the phenols was observed under normal storage conditions. In an attempt to find a possible explanation for the increased phenol accumulation in carrots during storage, the effects of ethylene on the qualitative and quantitative aspects of phenol content in carrot roots were studied. It was shown that ethylene induced an increased accumulation of phenols in carrot roots. One of the common phenols, isochlorogenic acid, accumulated in increasing amounts in carrots upon ethylene treatment. Furthermore, the gas induced the formation of at least 4 phenolic compounds (in carrots) that were not present originally. One of these has been characterized as isocoumarin, the so-called bitter principle in carrots (132). Thus, ethylene has been established as a cause for the formation of isocoumarin in carrots. Besides isocoumarin another phenolic compound, commonly called eugenin, is produced in carrots upon treatment with ethylene. The remaining two were not characterized, but there were indications that these were related to isocoumarin.

It has been shown in a number of plants that isochlorogenic acid is biosynthesized *via* the shikimic acid pathway of aromatic compounds. An increased accumulation of isochlorogenic acid in carrots upon treatment with ethylene seemed to indicate an increased activity of

the said pathway. A key enzyme, L-phenylalanine ammonia-lyase (PAL) for the biosynthesis of aromatic compounds has been shown to be stimulated by ethylene in a number of plants. The activity of PAL in carrots and its relation to ethylene action on carrots were studied. Ethylene seemed to stimulate the activity of this enzyme, at least initially. On studying the biosynthesis of isocoumarin and eugenin in carrots after administering 1-^{14}C-acetate, 2-^{14}C-malonate, and 3-^{14}C-acetoacetate it was apparent that isocoumarin and eugenin were probably synthesized *via* the acetate pathway of aromatic biosynthesis.

Attempts were made to find a plausible physiological and biochemical rationale for the effects of ethylene on phenol metabolism in carrots. So far no single mechanism has been found to explain ethylene's numerous effects on plant processes. The present research adopted a different approach for the explanation of its action on phenol metabolism in carrots. It was found that, like DNP, ethylene also stimulated glycolysis *via* the EMP pathway. Both compounds induced the production of isocoumarin in carrots. Unlike DNP (9), ethylene did not induce alcoholic fermentation in carrots. Furthermore, from 1-^{14}C- and 6-^{14}C-glucose catabolism studies, it was found that both pathways of glucose breakdown operate in carrots before and after ethylene treatment, and both the pathways contributed to the synthesis of isocoumarin in carrots in the presence of ethylene. That pentose phosphate pathway contributed to the isocoumarin synthesis in carrots was further evidenced by the formation of isocoumarin in carrots on treatment with methylene blue. Methylene blue is an electron

acceptor and has been frequently used as a stimulant of PPP in various organisms (3,23).

It is being proposed that ethylene enhances the production of pyruvate by stimulating glycolysis. The pyruvate thus formed produces more acetyl CoA than can be used by TCA. The excess acetyl CoA is then diverted to the synthesis of acetogenins, *viz*, isocoumarin and eugenin. Beevers (9) explained the increased production of ethanol and acetaldehyde in carrots on DNP treatment in a similar fashion. An inhibitor of keto acid synthesis (150), arsenite, blocked the synthesis of isocoumarin, thus lending support to the above proposal.

Finally, it was concluded from the study with cycloheximide, an inhibitor of protein synthesis, that the formation of isocoumarin in carrots probably followed the *de novo* synthesis of enzyme(s) system(s).

On the basis of the foregoing findings it seems certain that ethylene causes an increased accumulation of existing phenolic compounds in carrots. Ethylene has also been established as a cause for inducing the synthesis of isocoumarin in carrots. Although the concentrations of ethylene used in these studies were much higher than the physiological levels, yet the above findings might be extrapolated to the effects of lower concentrations of ethylene. It is suggested that low levels of ethylene produced by carrots may induce slow production of isocoumarin in carrots during storage and may render them bitter. What effect(s) eugenin and other phenols (synthesized upon ethylene treatment) may have on the taste and other qualities of carrots will be worth studying. The relationships between isocoumarin and eugenin

syntheses and the metabolism of fatty acids and other acetogenins (*viz*, carotenoids and terpenoids) will be worth exploring in view of their origin probably from a common precursor.

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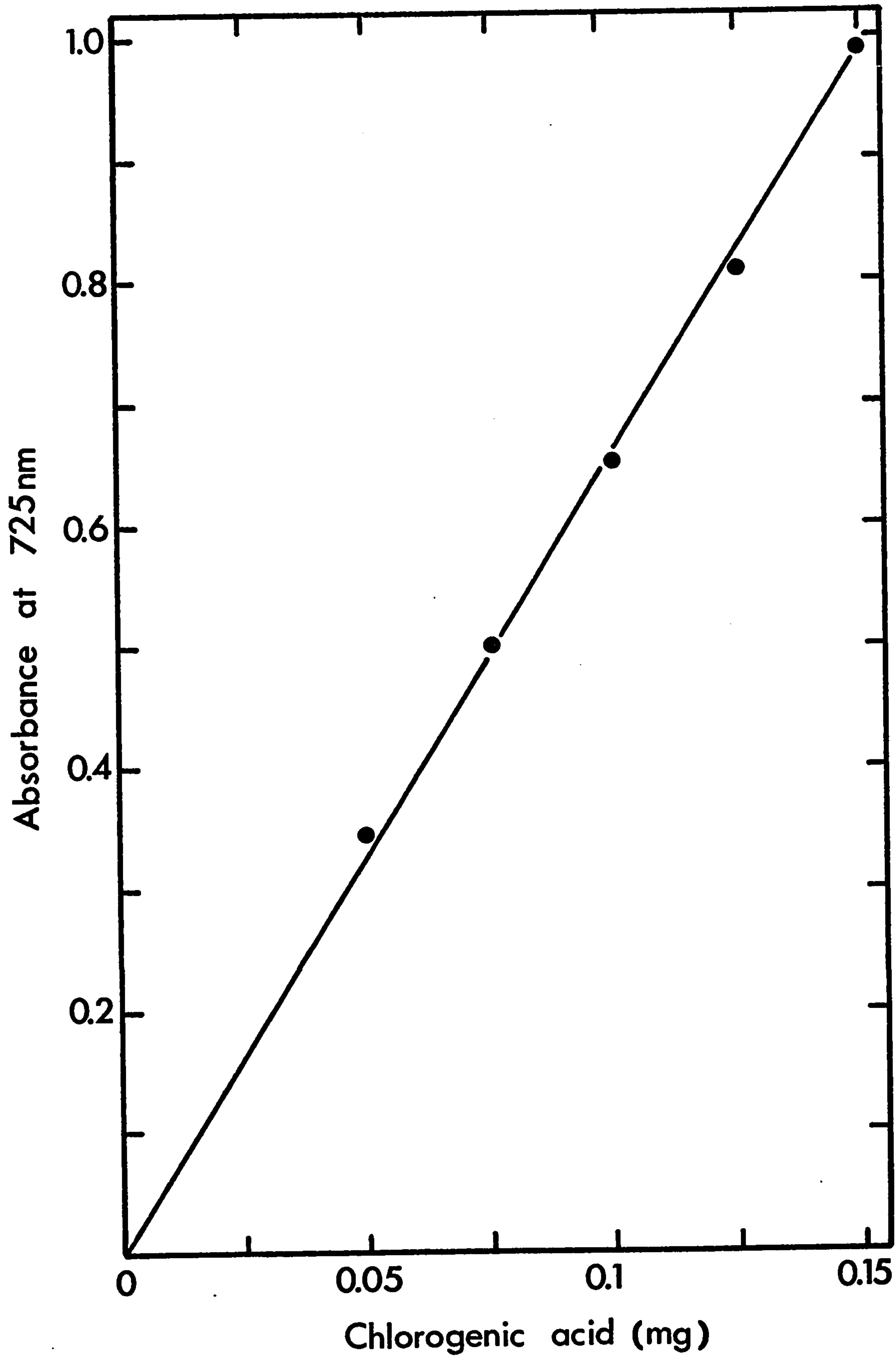
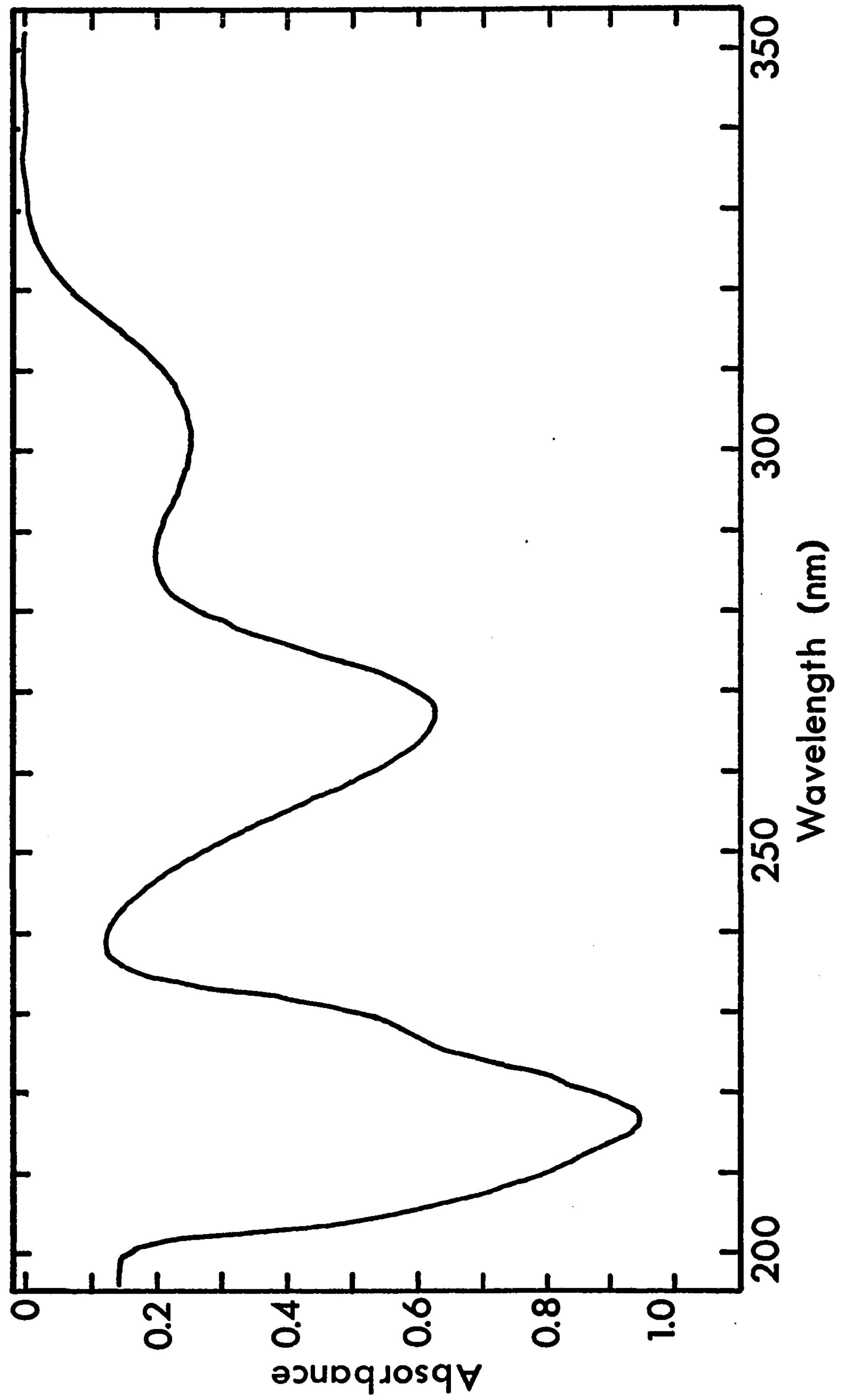
APPENDIX

Fig A-1. Standard curve for total phenol estimation. The method has been described under Materials and Methods (B.1). Total phenol was expressed as mg chlorogenic acid per 1.0 ml of total phenol extract of carrots.



Absorbance at 725nm
1.0
0.8
0.6
0.4
0.2
0
0.05
0.1
0.15
Chlorogenic acid (mg)

Fig A-2. The ultraviolet spectrum of isocoumarin. The spectrum of isocoumarin solution in methanol was recorded in a recording spectrophotometer.



Absorbance
0
0.2
0.4
0.6
0.8
1.0
200
250
300
350
Wavelength (nm)

Fig A-3. The ultraviolet spectrum of eugenin (in methanol solution). The spectrum was recorded in a recording spectrophotometer.

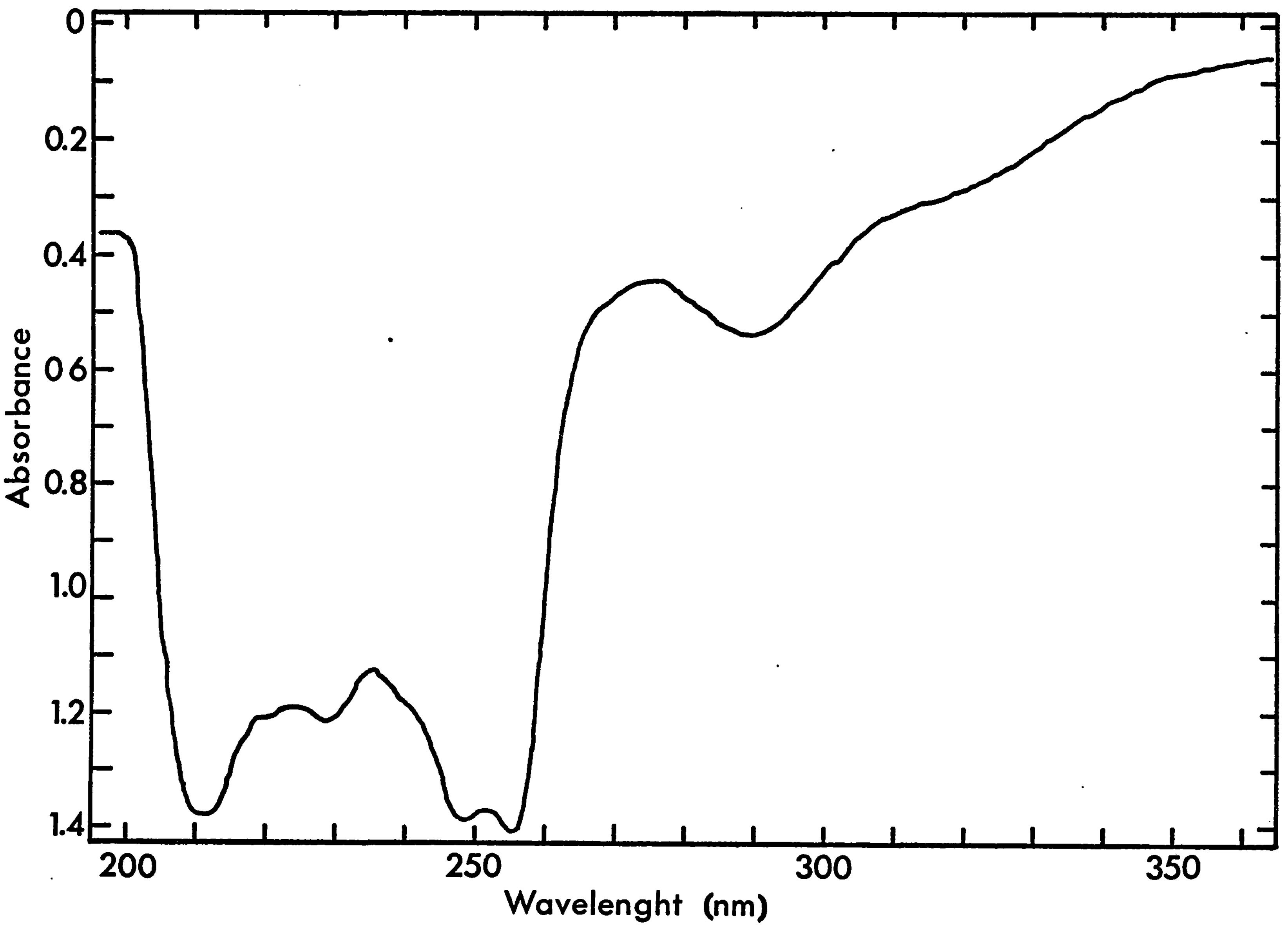

0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Absorbance
200
250
300
350
Wavelenght (nm)

Fig A-4. The ultraviolet spectrum of band Z. The spectrum of methanolic solution was recorded in a recording spectrophotometer.

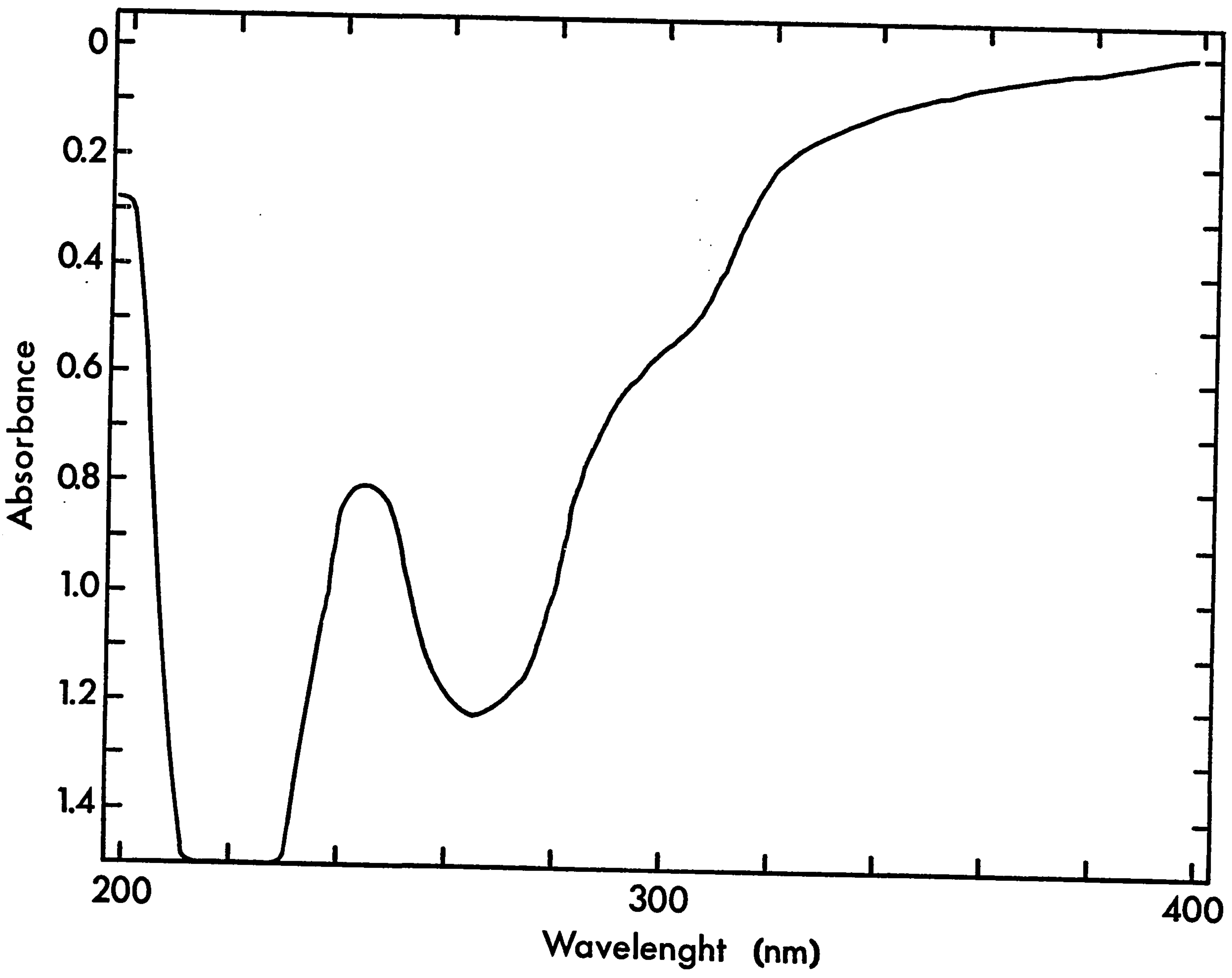
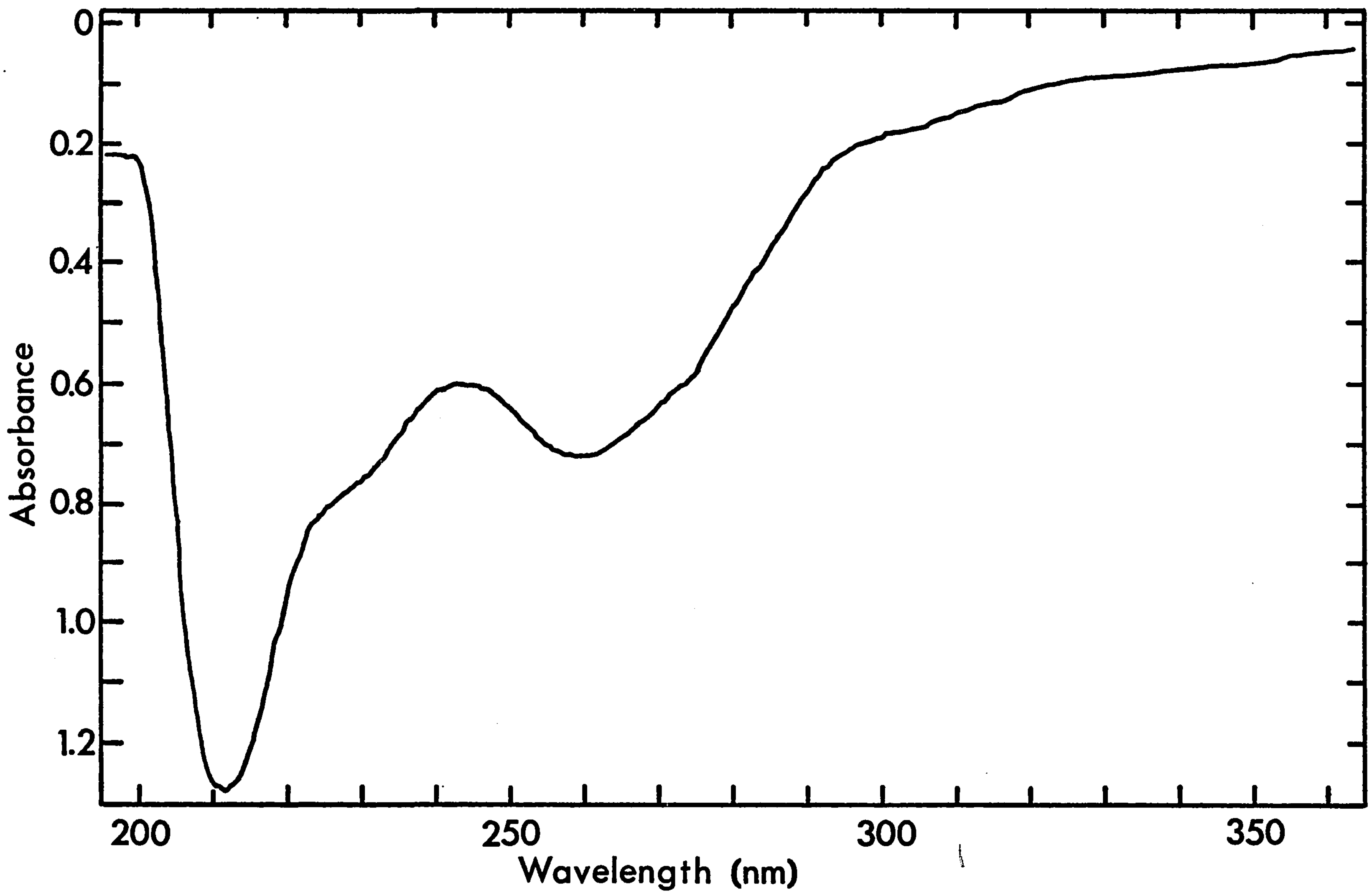

Absorbance
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
200
300
400
Wavelenght (nm)

Fig A-5. The ultraviolet spectrum of band M. The spectrum of methanolic solution was recorded in a recording spectrophotometer.



Absorbance
0
0.2
0.4
0.6
0.8
1.0
1.2
Wavelength (nm)
200
250
300
350

Fig A-6. Standard curve for cinnamic acid. Absorbance at 290 nm was plotted against mg of cinnamic acid in borate buffer, 0.05 M (pH 8.7), 1 mM in EDTA and 1 mM in DTT. The absorbance was converted to nmoles of cinnamic acid for expressing PAL activity.

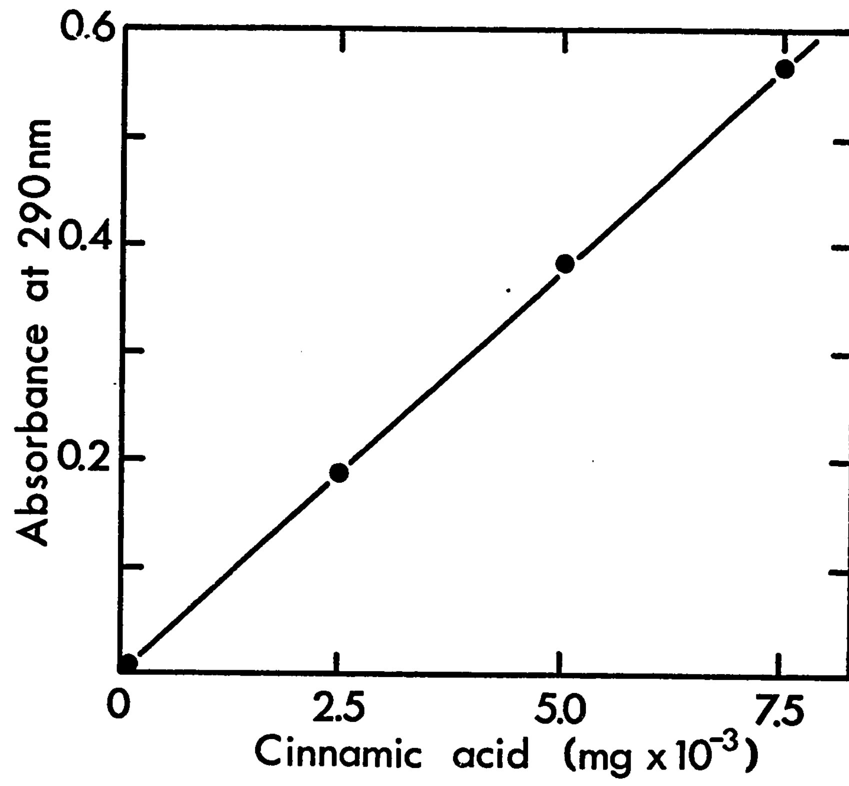

0.6
0.4
0.2
Absorbance at 290nm
0
2.5
5.0
7.5
Cinnamic acid (mg x 10⁻³)

Fig A-7. Standard curve for protein determination (Lowry *et al.* (86)). Protein was expressed as μg in the reaction mixture.

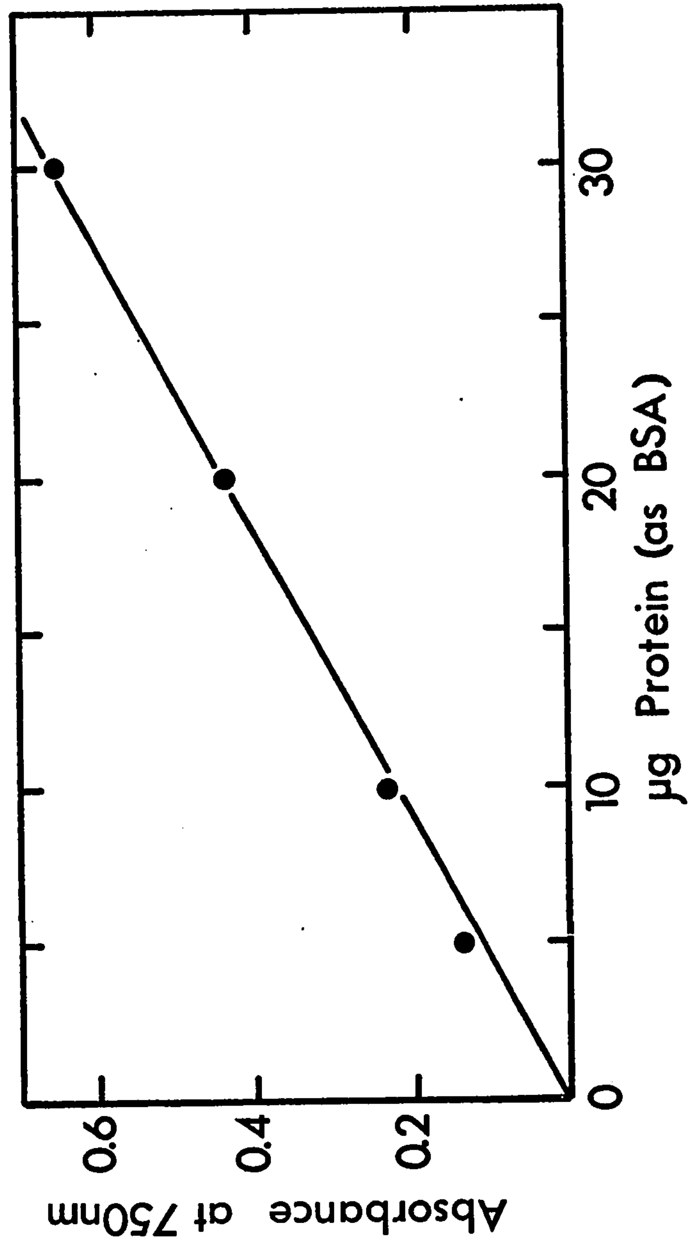

Absorbance at 750nm
0.6
0.4
0.2
0
10
20
30
μg Protein (as BSA)

Fig A-8. Isocoumarin standard curve. After scraping and eluting the band of isocoumarin on thin layer chromatogram the absorbance at 267 nm was measured.

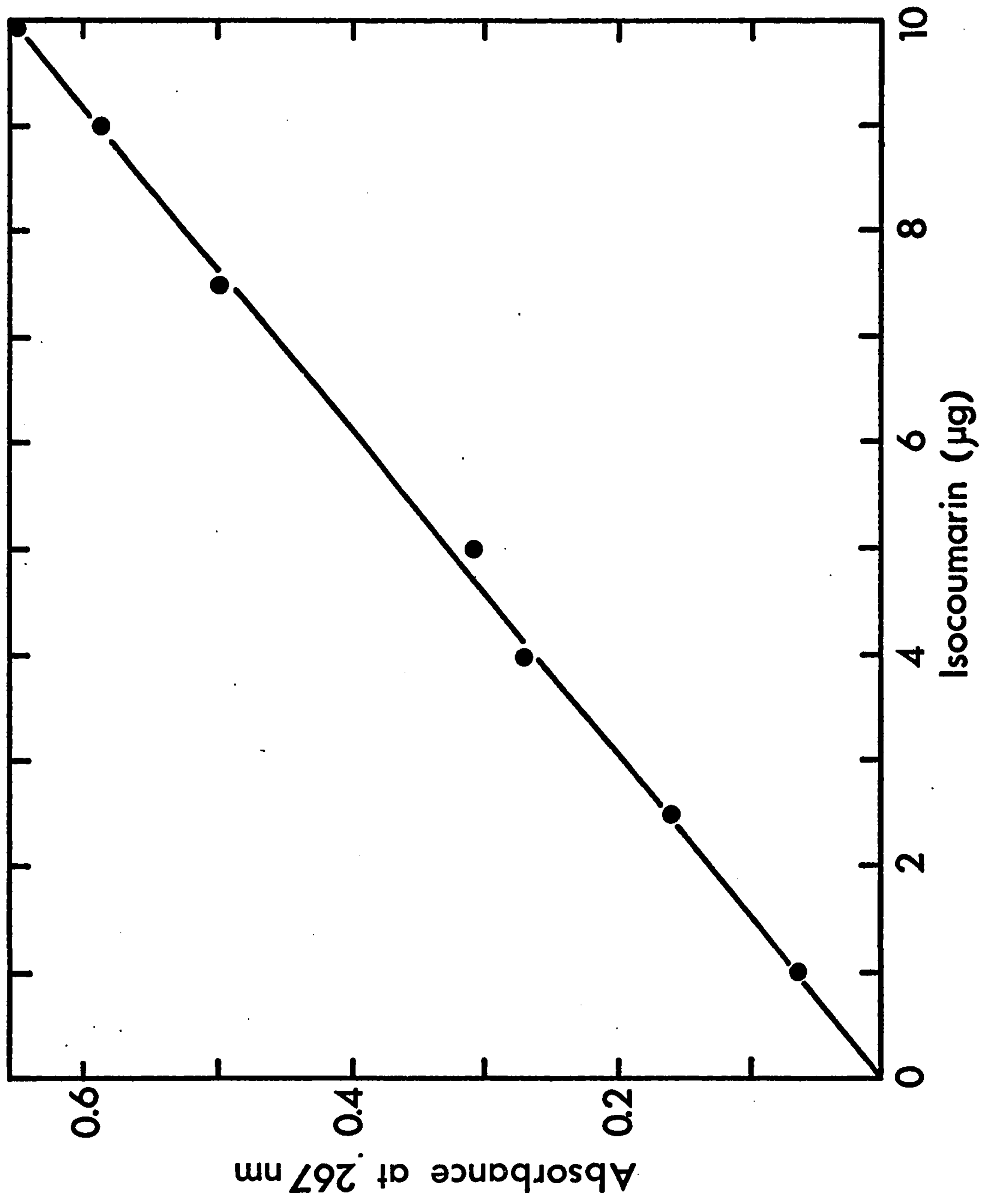
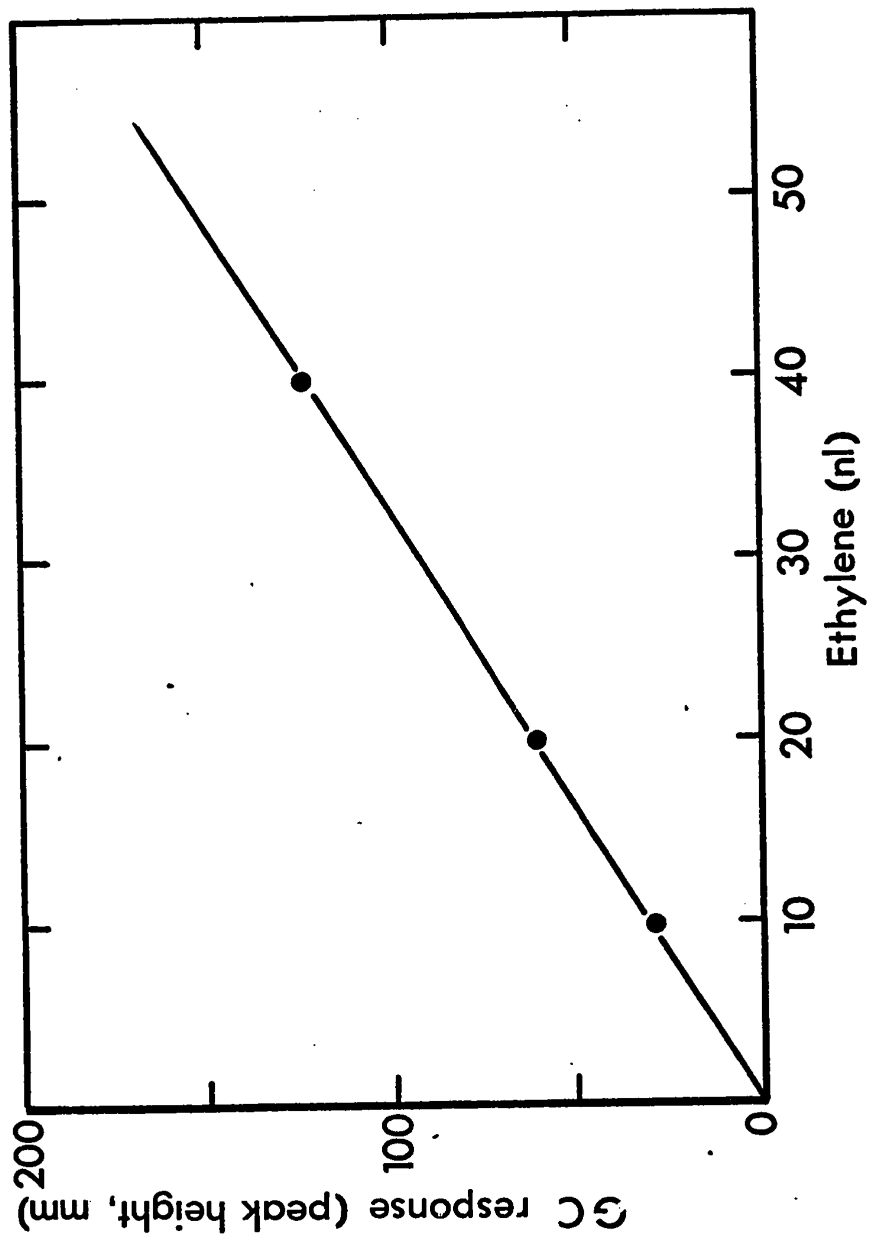

Absorbance at 267 nm
0.6
0.4
0.2
Isocoumarin (μg)
0
2
4
6
8
10

Fig A-9. Ethylene standard curve for lower concentration. The gas was analysed by gas chromatography and was resolved by an activated alumina column. The column temperature was 50°.



GC response (peak height, mm)
0
100
200
Ethylene (nl)
10
20
30
40
50

Fig A-10. Ethylene standard curve for higher concentration. The gas was analysed by gas chromatography and was resolved by an activated alumina column. The column temperature was 50°.

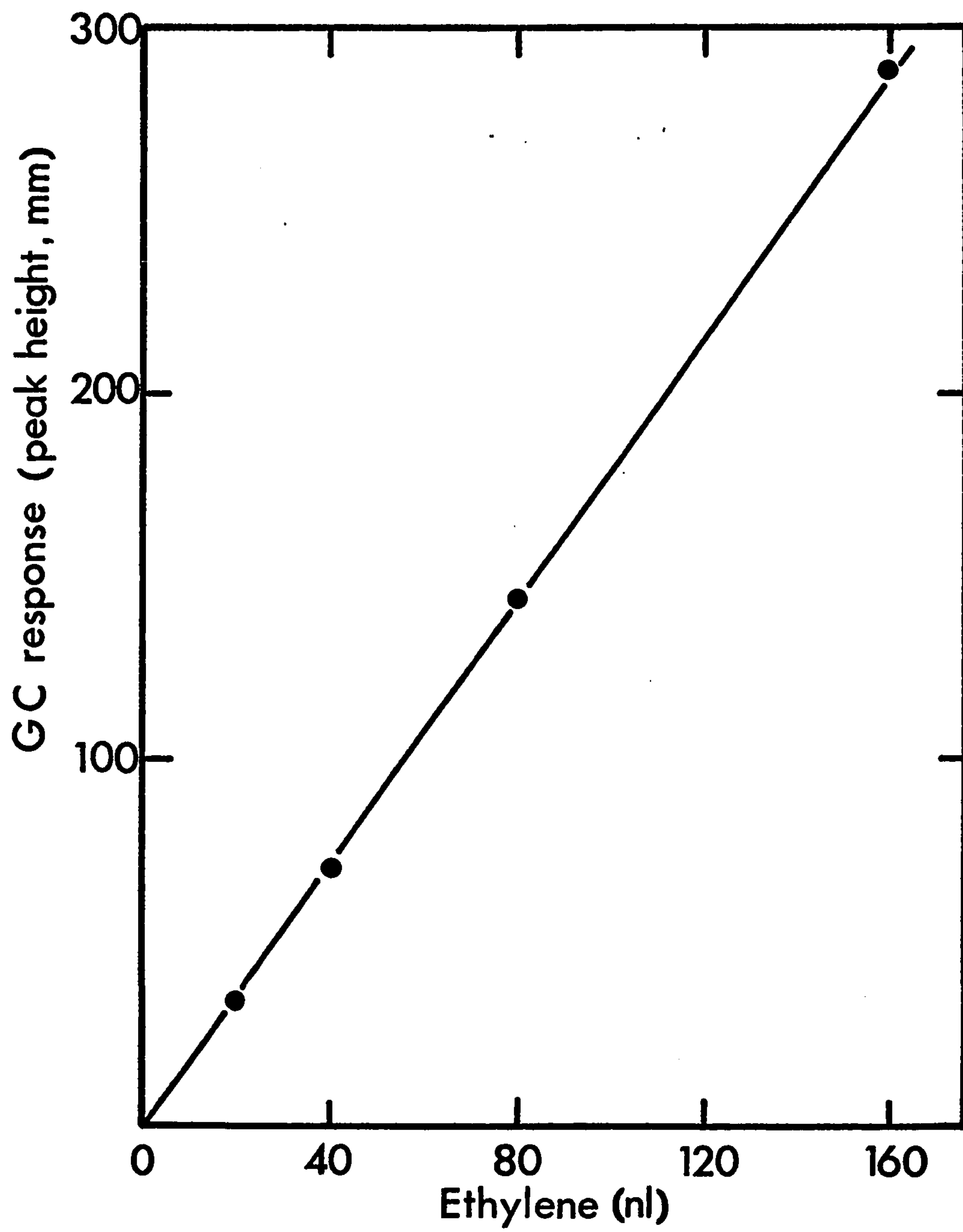

300
200
100
GC response (peak height, mm)
0
40
80
120
160
Ethylene (nl)

Fig A-11. Acetaldehyde standard curve. The volatile was analysed by gas chromatography and was resolved by a Porapak Q column.

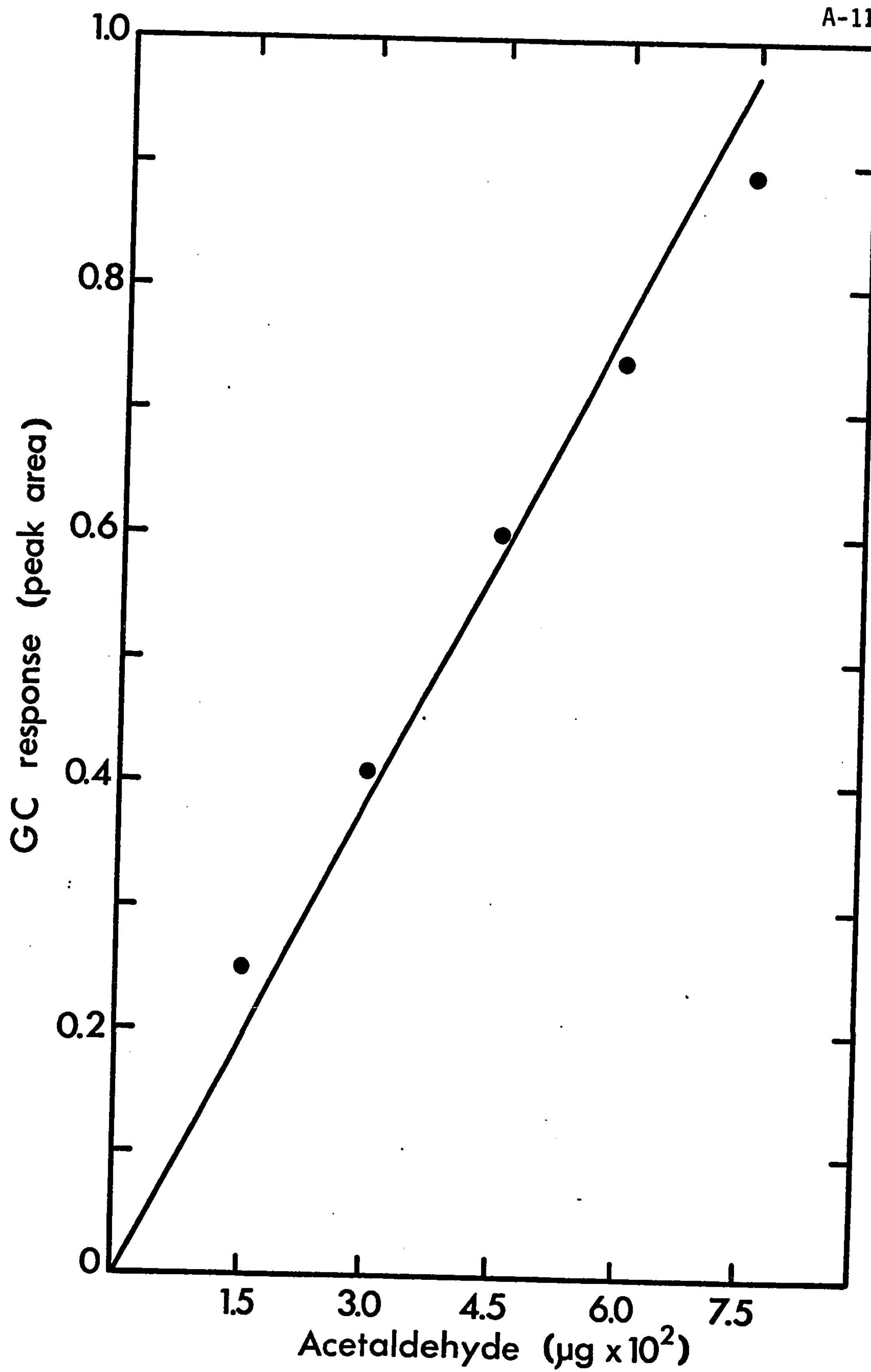

1.0
0.8
0.6
0.4
0.2
0
GC response (peak area)
1.5
3.0
4.5
6.0
7.5
Acetaldehyde (μg x 10²)

Fig A-12. Ethanol standard curve. Ethanol was analysed by gas chromatography and was resolved by a Porapak Q column.

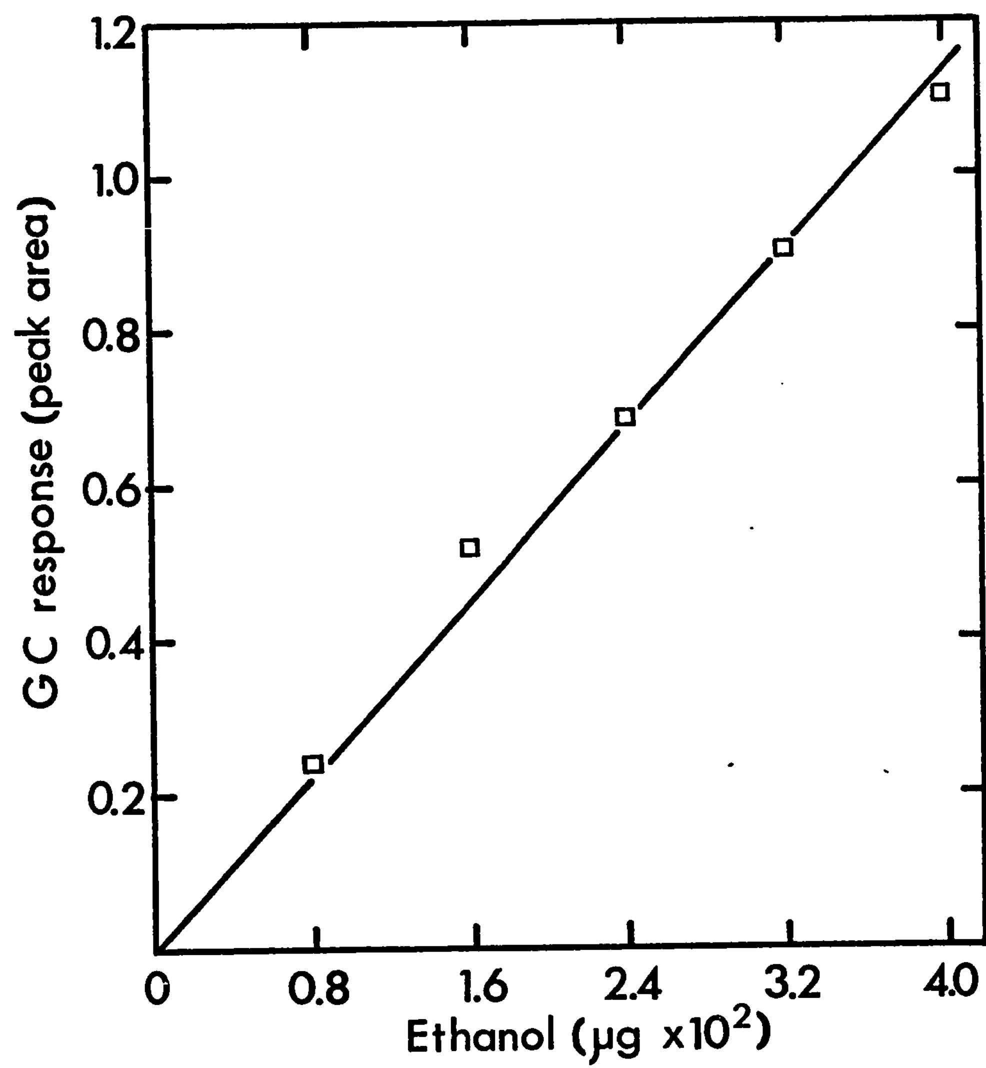
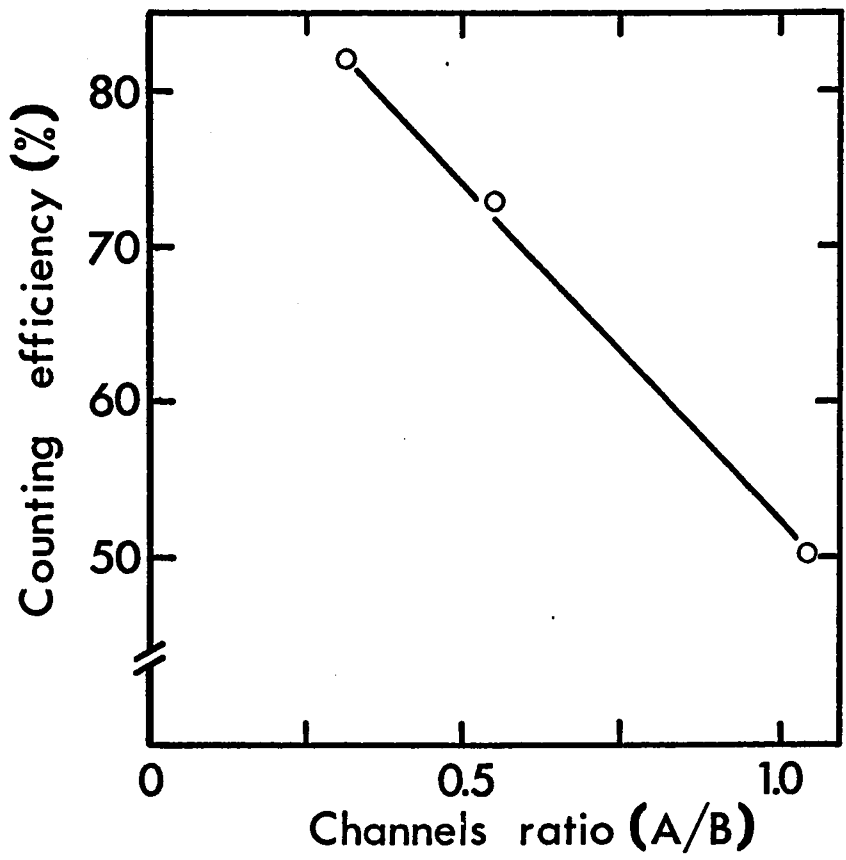

1.2
1.0
0.8
0.6
0.4
0.2
GC response (peak area)
0
0.8
1.6
2.4
3.2
4.0
Ethanol (µg x10²)

Fig A-13. ^{14}C quench curve. From quenched standards in sealed scintillation vials (Amersham-Searle). Channel A represents lower energy channel and channel B represents higher energy channel.


80
70
60
50
Counting efficiency (%)
0
0.5
1.0
Channels ratio (A/B)

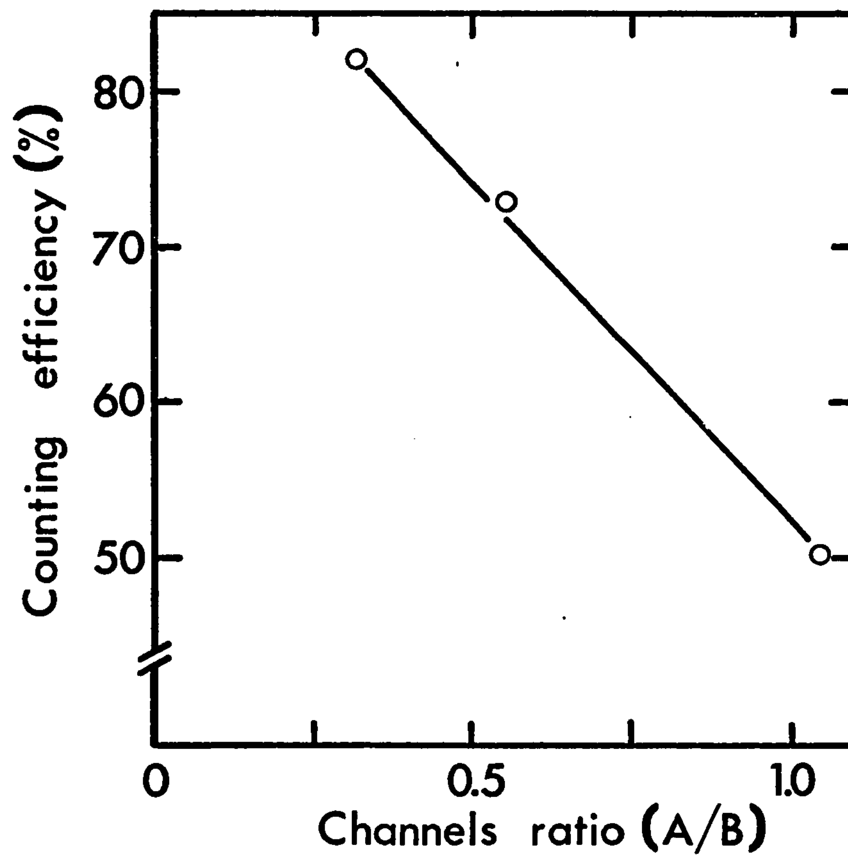
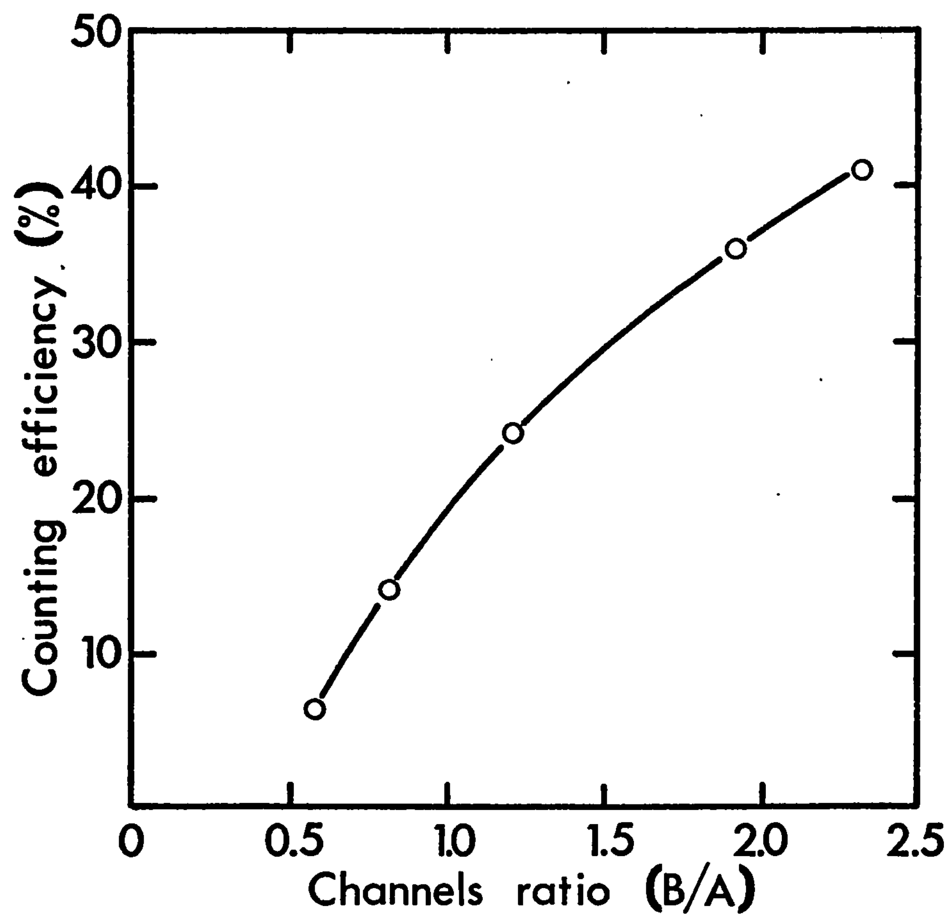

Counting efficiency (%)
80
70
60
50
0
0.5
1.0
Channels ratio (A/B)

Fig A-14. ^{3}H quench curve. From quenched standards in sealed scintillation vials (Nuclear-Chicago). Channel A was set to monitor the lower energy portion while channel B is set to monitor the spectrum at balance point using the ratio A/B.



50
40
30
20
10
Counting efficiency (%)
0
0.5
1.0
1.5
2.0
2.5
Channels ratio (B/A)