Phytochemistry, 1971, Vol. 10, pp. 1989 to 1997. Pergamon Press. Printed in England.

THE EFFECT OF ETHYLENE ON THE RESPIRATION AND ON THE ACTIVITY OF PHENYLALANINE AMMONIA LYASE IN SWEDE AND PARSNIP ROOT TISSUE

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(*Received* 22 *October* 1970)

Abstract—It has been shown that the treatment of intact storage roots of swede and parsnip with low levels of ethylene leads to an increase in respiration and a large rise in the activity of phenylalanine ammonia lyase (PAL). It is shown that in disks of swede aged in the presence of ethylene there is also an increase in the extractable activity of PAL. The increase in PAL activity is very sensitive to the level of ethylene; 1 ppm of ethylene being sufficient to give a half maximal response. IAA at 10^{-4} M stimulates an increase in endogenous ethylene production of the disks and an increase in PAL activity, but this increase in PAL activity is small in relation to that given by saturating levels of ethylene. It is likely that the ethylene induced increase in PAL activity is due to *de novo* protein synthesis since it is inhibited by the presence of a range of inhibitors of RNA and protein synthesis. The characteristics of the response are discussed in relation to the other known effects of ethylene.

INTRODUCTION

RECENTLY, there have been many reports in the literature of responses of plant tissue to applied ethylene[1] which have led to the view that ethylene acts as a regulatory hormone[2] in a variety of physiological changes occurring at many stages in the ontogeny of the plant. Many of these changes effected by applied ethylene involve complex alterations in the pattern of development of the tissue or organ in question. There is, at the present time, very little known about the mechanism by which ethylene acts in these responses. It has been suggested[3] that the primary locus of ethylene action involves the binding of the gas to a metallo-protein. However, neither the nature of the specific binding protein nor the nature of the metabolic changes which result from the formation of the ethylene-protein complex is known.

In the present work we have set out to study some aspects of the mode of action of ethylene in initiating physiological changes in plant tissues. For this work we have sought a plant material which only produces very low levels of ethylene and yet which will give responses to the gas without major changes in the pattern of growth and differentiation of the tissues. Mature plant storage organs seem to fulfil some of these specifications and we have shown that mature swede and parsnip roots will respond to ethylene by giving not only an increased respiration, as has also been shown for the potato,[4] but also a 25–50-fold increase in the activity of the enzyme, phenylalanine ammonia lyase, PAL.

[1] L. W. MAPSON and A. C. HULME, *Advance. Phytochem.* **2**, 343 (1970).
[2] S. P. BURG and E. A. BURG, *Science* **148**, 1190 (1965).
[3] S. P. BURG and E. A. BURG, *Plant Physiol.* Lancaster **42**, 144 (1967).
[4] M. S. REID and H. K. PRATT, *Nature* **226**, 976 (1970).

RESULTS

Figure 1 shows the response of intact swede roots to applied ethylene (43 ppm) in terms of increased PAL activity and respiration as compared with roots maintained in air. The activity of PAL extracted from untreated swedes is very low (<0·2 EU/10 g tissue) and remains so throughout the 9-day period at 15°. The activity of the enzyme increases in ethylene treated swedes after a lag period of about 1 day, and reaches a peak (*ca.* 7·0 EU/10 g tissue) after a treatment of 4 days. On transfer of treated swedes from ethylene to air, the extractable activity of PAL falls rapidly and the control roots, on later exposure to ethylene, show a rising activity after a lag phase. The respiration of swedes rises rapidly on exposure to ethylene; there is a 250 per cent increase in the rate of CO_2 production which is completed within one day. The respiratory response is completed while the response in terms

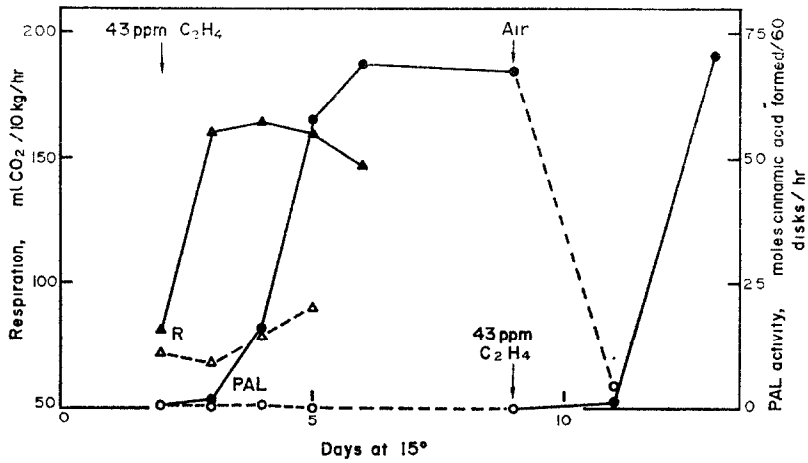

FIG. 1. THE EFFECT OF ETHYLENE (43 ppm) ON THE RESPIRATION AND PHENYLALANINE AMMONIA LYASE ACTIVITY OF WHOLE SWEDE ROOTS

Swedes were stored at 15° in air (open symbol) or air containing 43 ppm ethylene (closed symbol). The respiration of the swedes (△, ▲) was measured daily and at intervals swedes were extracted and their PAL activity (○, ●) measured. After 8 days, the ethylene treated swedes were returned to air and the control roots were given ethylene and the change in PAL activity again followed The times at which the ethylene was applied are shown with arrows.

of PAL activity is still within the lag phase. Mature parsnip roots respond to ethylene treatment (Fig. 2) in a manner similar to the swede roots, although the increase in PAL activity is smaller and the respiratory response tends to be somewhat slower to develop.

When disks (10 × 2 mm) of swede root tissue are incubated ('aged') at 25° in a suitable medium through which a stream of 7 ppm of ethylene was passed, there is a marked rise in the activity of PAL in extracts prepared from the disks (see Fig. 3). The curve of the increase in activity shows an initial lag phase followed by a rapid increase in activity leading to a steady value after 16 hr ageing. In similar disks aged in the absence of applied ethylene, there is a rise in activity, but this is much smaller than in the ethylene treated disks and, in the experiment shown in Fig. 3, the level of activity is only one-third of that developed in the ethylene treated tissue. The ratio of developed activity in ethylene treated and control disks varied from experiment to experiment within the range of 3–10:1. We have shown that intact swede roots produce small amounts of ethylene (0·03 mμl/g/hr at 25°).

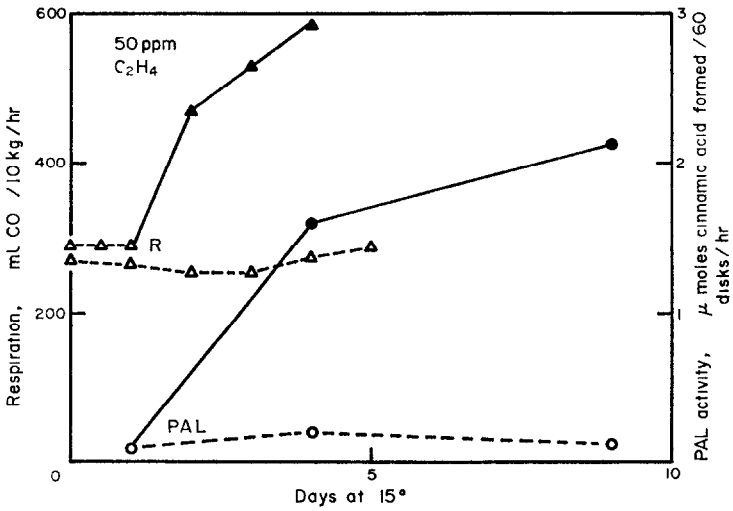

FIG. 2. THE EFFECT OF ETHYLENE (50 ppm) ON THE RESPIRATION AND PHENYLALANINE AMMONIA LYASE ACTIVITY OF WHOLE PARSNIP ROOTS.

Samples of whole parsnip roots were stored in air for 2 days at 15° prior to treatment of one sample with ethylene at a concentration of 50 ppm. The respiration of the two samples (▲—▲ ethylene; △---△ air) was followed daily and at intervals parsnips were extracted and their PAL activities measured (●—● ethylene; ○---○ air).

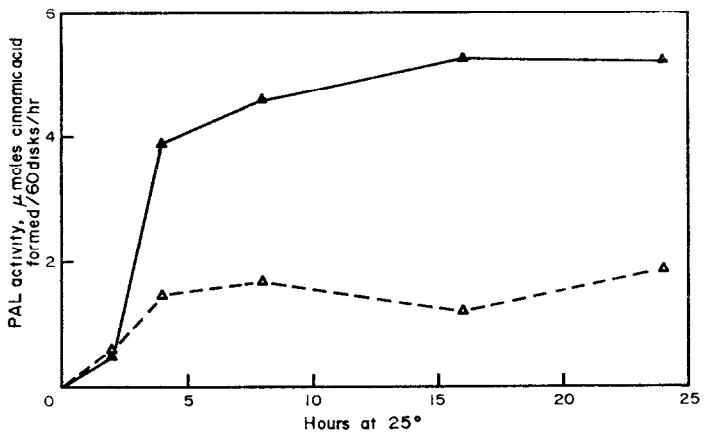

FIG. 3. THE TIME CURVES OF CHANGES IN PAL ACTIVITY EXTRACTED FROM DISKS (10 × 2 mm) OF SWEDE ROOTS AGED FOR PERIODS OF UP TO 24 hr AT 25° (SEE EXPERIMENTAL SECTION) IN AIR (△---△) OR IN AIR CONTAINING 7 ppm OF ETHYLENE (▲—▲).

Disks cut from such swedes produce larger amounts of ethylene (of the order of 0·3 mμl/g/hr) and this could lead to intracellular ethylene concentrations capable of stimulating some increase in PAL activity. The effect of varying concentrations of ethylene, applied during ageing, on the levels of activity of PAL in disks aged for 24 and 48 hr is shown in Fig. 4. In these results no account has been taken of endogenous ethylene production, but the results show that an applied concentration of about 1 ppm is sufficient to give a half maximal increase in activity and concentrations above 4 ppm are saturating.

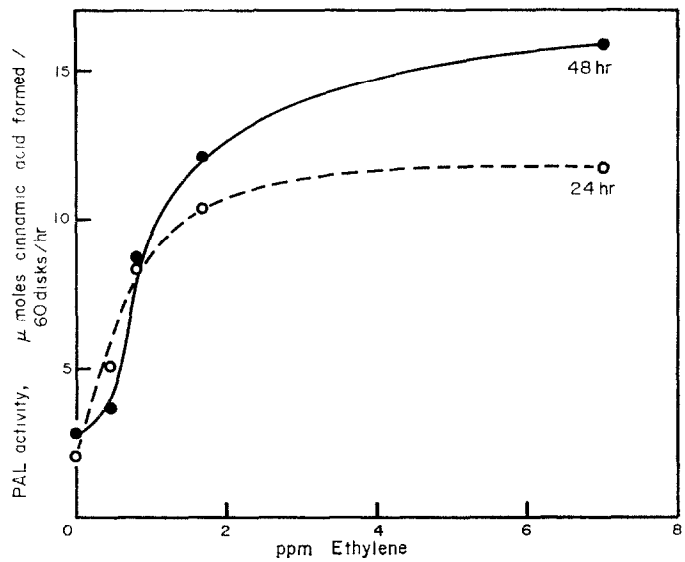

FIG. 4. THE EFFECT OF VARIOUS CONCENTRATIONS OF ETHYLENE ON THE LEVEL OF PHENYLALANINE AMMONIA LYASE DEVELOPED IN DISKS OF SWEED ROOTS AGED FOR 24 AND 48 hr.

The effect of other hydrocarbons in inducing an increase in PAL activity in disks of swede roots aged for 24 hr is shown in Table 1. Neither ethane nor acetylene give increases in PAL activity over and above that given by the air control disks. Ethylene in this experiment gives a 4·5-fold stimulation, while propylene treatment leads to a somewhat smaller (2·6-fold) stimulation. This reduced response is given at a propylene concentration more than twice that of ethylene.

TABLE 1. THE EFFECT OF VARIOUS HYDROCARBONS ON THE PHENYLALANINE AMMONIA LYASE ACTIVITY OF DISKS AGED FOR 24 hr

Material	Hydrocarbon added to air stream bubbled through medium in which disks were aged	Phenylalanine ammonia lyase activity μmoles cinnamic formed 1 hr/10 g fr. wt.
Freshly prepared disks	—	~0
Disks aged for 24 hr	Nil	0·78
	Ethylene 1·8 ppm	3·50
	Propylene 4·2 ppm	2·05
	Acetylene 3·9 ppm	0 95
	Ethane 2·6 ppm	0 98

Table 2 shows the effect of the addition of indolylacetic acid at various concentrations to the medium in which the disks were aged on the development of PAL activity in swede disks aged in air. Concentrations of IAA between 5×10^{-4} and 10^{-4} M gave stimulations in activity over the air controls. Concentrations below 10^{-4} M were ineffective, while those above 5×10^{-4} M tended to be inhibitory. In all cases, however, the stimulations given by IAA were small compared with those given by saturating concentrations of applied ethylene.

TABLE 2. THE EFFECT OF INDOLYLACETIC ACID ON THE DEVELOPMENT OF PHENYLALANINE AMMONIA LYASE ACTIVITY IN SWEDE ROOT DISKS AGED IN AIR

Material	Additions to medium in which disks were aged	Phenylalanine ammonia lyase activity μmoles cinnamic formed 1 hr/10 g fr. wt. Experiment No.	
		1	2
Freshly prepared disks	—	~0	~0
Disks aged for 24 hr	No addition	0·93	0·27
	IAA 10^{-3} M	—	0·15
	5×10^{-4} M	—	1·74
	10^{-4} M	2·30	—
	10^{-5} M	1·30	—
	10^{-6} M	1·30	—
	Ethylene 8 ppm	6·0	8·1

In similar experiments, it was shown that gibberellic acid and kinetin applied at concentrations of 10^{-4} M were ineffective in promoting an increase in PAL activity above that of the air controls.

Various inhibitors of RNA and protein synthesis were tested in the experiments recorded in Fig. 5 for their effectiveness in inhibiting the increase in PAL activity in swede root disks

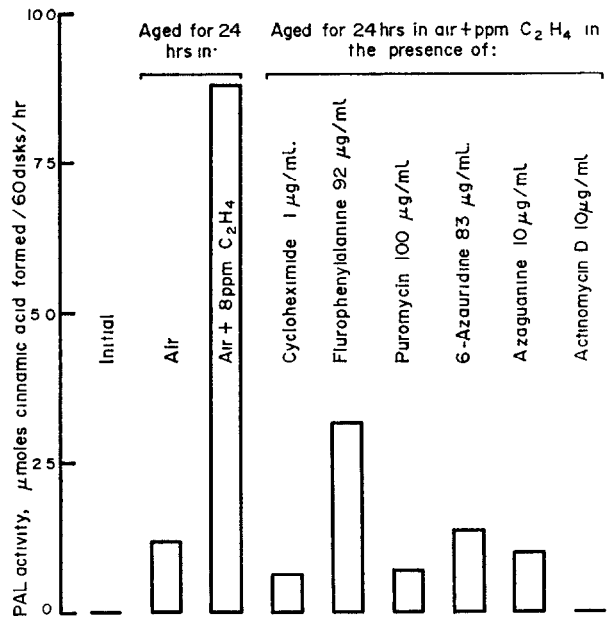

FIG. 5. THE EFFECT OF VARIOUS INHIBITORS OF RNA AND PROTEIN SYNTHESIS ON THE DEVELOPMENT OF PHENYLALANINE AMMONIA LYASE ACTIVITY IN DISKS OF SWEDE ROOTS AGED FOR 24 hr IN THE PRESENCE OF 8 ppm OF ETHYLENE.

These results are represented as histograms and show the levels of activity in initial disks, in disks aged in air, in 8 ppm of ethylene and in the presence of ethylene (8 ppm) together with the inhibitors at the concentrations shown.

aged in the presence of saturating levels of ethylene. The inhibitors of protein synthesis, cycloheximide (1 μg/ml) and puromycin (100 μg/ml), almost completely prevent development of the enzymic activity, while the amino acid analogue, fluorophenylalanine (90 μg/ml) reduces the increase in activity to about a third of that in its absence. The inhibitor of DNA dependant RNA synthesis, actinomycin D[5] (10 μg/ml), completely prevents the development of PAL activity, while the other RNA synthesis inhibitors, azauridine (80 μg/ml) and azaguanine (10 μg/ml), reduce the increase in activity to that of the air control. With all these inhibitors it was shown that they do not directly inhibit the enzyme during assay, but only the development of the activity during the 24 hr ageing period.

The time courses of changes in respiration (measured monometrically as O_2 uptake) of swede root disks aged for periods up to 24 hr at 25° in the presence and absence of 7 ppm ethylene are shown in Fig. 6. The untreated disks show the characteristic time course of

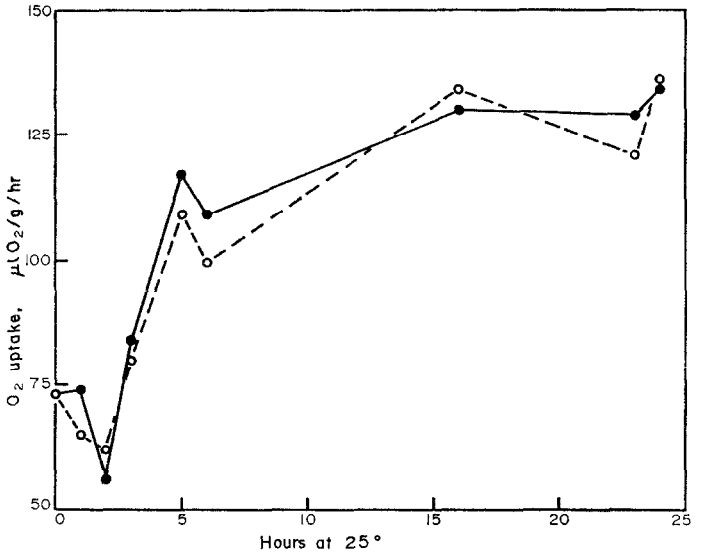

FIG. 6. THE TIME CURVE OF CHANGES IN RESPIRATORY ACTIVITY OF DISKS OF SWEDE ROOTS AGED IN THE PRESENCE (●—●) OR ABSENCE (○—○) OF 7 ppm ETHYLENE.
The respiration (O_2 uptake) of samples of disks taken at intervals during ageing was measured manometrically (see Experimental section).

development of respiratory activity of disks of plant storage organs during ageing.[6] This shows an initial lag phase lasting about 2 hr followed by a rapid phase of developing respiratory capacity leading to a 2-fold increase in rate after 16 hr of ageing. The application of 7 ppm of ethylene to such disks does not significantly alter either the time course of development or the final rate of respiration achieved.

DISCUSSION

Mature swede roots have been shown to respond to ethylene giving an increase in respiration and in the activity of phenylalanine ammonia lyase (PAL). Disks of untreated swedes on ageing in an aerated medium will give a rise in respiration and a small increase

[5] J M. KIRK, *Biochim. Biophys. Acta* **42**, 167 (1960).
[6] G. G. LATIES, *Plant Physiol. Lancaster* **39**, 654 (1964)

in PAL activity. The application of ethylene to such ageing disks leads to a marked stimulation of the increase in PAL activity (3–10-fold) but has no effect on the development of increased respiratory activity. The increase in PAL activity in the untreated control disks is probably, therefore, induced by the small endogenous ethylene production caused by the damage involved in cutting the disks. The effect of ethylene in causing an increase in PAL activity during ageing has many characteristics in common with other plant responses to ethylene. For instance, the concentrations of ethylene required for stimulation are very small; 1 ppm for half maximal response and 4 ppm for saturation. The effect is promoted by other hydrocarbons, e.g. propylene, but the concentration required for an equivalent response is higher than with ethylene.[3]

Indolylacetic acid can partially replace the action of ethylene in the system. Relatively high concentrations of IAA (10^{-4} M) are required to give the response, and we have shown that such levels of IAA will cause a 4-fold stimulation in ethylene production by the disks, (*ca.* 1·3 mμl/g/hr) compared with control disks (0·3 mμl/g/hr). Therefore, it is likely that the IAA effect on PAL activity is at least partly due to the IAA stimulation of ethylene production. Other growth substances, kinetin and gibberellic acid, are ineffective at the concentration tested (10^{-4} M) in replacing ethylene. In contrast to some other plant tissues,[7] light does not appear to be an important factor in the control of PAL activity in the swede, since disks of swede roots will respond to ethylene equally well whether they are aged at high light intensities (1000 lux) or in darkness.

The ethylene induced changes in enzyme activity reported in this paper are very large compared with other ethylene dependent changes that have been reported by other workers. Swede tissue has an advantage over many tissues used in these studies in that its endogenous rate of ethylene production is very low, and the stimulation of ethylene production on cutting the tissue into disks is small compared with many other tissues.[8,9] This has enabled us to demonstrate ethylene dependent stimulation of PAL activity, not only in the intact root, but also with disks of root tissue. This is in contrast to the situation with grapefruit flavedo reported by Riov *et al.*[8] who showed that ethylene treatment of the intact fruit led to an increase in PAL activity in the flavedo tissue, but that when disks of grapefruit flavedo were cut and incubated, massive endogenous ethylene production resulted in a large rise in PAL activity in the absence of applied ethylene. Imaseki *et al.*[9] showed that PAL activity increases by about two-fold in response to ethylene treatment (5 ppm) of disks of sweet potato root. Other workers have reported increases in activities of a few other enzymes in response to ethylene treatments of tissues or disks; these include peroxidase in sweet potato root disks,[9,10] phenolase in potato tuber tissue[11] and cellulose in pea stem tissue.[12]

It has been proposed that ethylene has a general role in the defence mechanisms of plants and that it may 'act as stimulus of localized metabolic changes leading to necrotic and hypersensitive reactions in plants following infection'.[11] Stahman *et al.*[11] treated blocks of sweet potato root tissue with ethylene and found an increased resistance of the tissue to infection by the black rot fungus, *Ceratocystis fimbriata.* In the present case it could well be that ethylene produced by injured swede tissue could lead to the induction

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[8] J. Riov, S. P. Monselise and R. S. Kahan, *Plant Physiol. Lancaster* **44**, 631 (1969).
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of PAL activity and the biosynthesis of phenolic compounds which play a role in forming some chemical or physical barrier to infection. The nature of the enzymic response to ethylene would be expected to vary from tissue to tissue. In tissues rich in phenolics the response may be via the increased rate of oxidation of pre-existing phenolic compounds, e.g. increase in phenolase activity in the potato, but in the swede, which has a relatively low content of phenolic compounds and a low phenolase activity (unpublished results), the response could be in the activation of the pathway of phenolic biosynthesis by the induction of a key enzyme in the pathway of phenolic biosynthesis.[13] We have shown that the activity of other enzymes in the pathway of phenolic biosynthesis, i.e. tyrosine ammonia lyase, shikimate dehydrogenase and the transaminase controlling the formation of phenylalanine from phenylpyruvate are not affected by ethylene treatment. Similarly, neither peroxidase nor phenolase activity increases on ethylene treatment of the whole swede or of aged disks. An example of ethylene treatment affecting the metabolism of phenolic compounds in root tissue is in the carrot, in which it has been shown that a bitter tasting principle develops when carrots are stored in the presence of ethylene at 100 ppm.[14] This compound was shown by Sondheimer[15] to be 3-methyl-6-methoxy-8-hydroxy-3-4-dihydroiso coumarin, and it could be here that the role of ethylene in promoting bitterness in the carrot is mediated via an increase in enzyme activity and increased 150-coumarin biosynthesis.

The observed increase in PAL activity could be the result of a number of causes which include the *de novo* synthesis of the enzymic protein or the activation, either by destruction of an inhibitor or synthesis of an activator, of a pre-existing protein moiety. We have looked for the possible presence of inhibitors in our initial disks by assaying for PAL in extracts of 24 hr aged disks by themselves and with the addition of extracts of initial disks. In these experiments we have found no evidence for the presence of inhibitors in the initial disks and here, therefore, inhibitors do not appear to play a controlling role in the development of PAL activity as they do in other systems.[16] The evidence from the use of antimetabolites of RNA and protein synthesis is highly suggestive that the increase in activity involves *de novo* protein synthesis. In recent years the specificity of many so called specific inhibitors has been called into question, particularly in relation to the effects of some of them on the processes of plant respiration.[17] However, taken together, the data makes the possibility of *de novo* protein synthesis very likely. The present data gives no information on the way in which ethylene induces the RNA and protein synthesis resulting in increase in PAL. At present, work is underway to obtain further evidence on the involvement of RNA and protein synthesis in the ethylene induced increase in PAL activity and the acceleration of phenolic biosynthesis in swede tissue.

EXPERIMENTAL

The swedes (*Brassica napo-brassica*) or parsnips (*Pastinaca sativa*) used in the experiments were purchased locally and stored for short periods at 3°. For measurement of the responses of the whole roots to ethylene, samples of roots were placed in chambers at 15° and a stream of CO_2-free air or CO_2-free air containing a low concentration of ethylene was passed over them at a constant rate. The respiration (CO_2 production) of the roots was measured daily by withdrawing samples of the air which had passed over the

[13] A. C. NEISH, in *Plant Biochemistry* (edited by J. BONNER and J. E. VARNER), p. 581, Academic Press New York (1965).
[14] B. C. CARLTON, C. E. PETERSON and N. E. TOLBERT, *Plant Physiol. Lancaster* **36**, 550 (1961).
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tissue and measuring its CO_2 content by GLC.[18] At intervals, individual roots were withdrawn, extracts prepared and the activity of PAL measured.

Preparation and Ageing of Root Disks

Swedes were taken from storage and 1 cm wide borings made from top to bottom through the tissue with a cork borer. The cylinders of tissue were cut into 2 mm thick transverse slices with a Mickle tissue slicer (Mickle Laboratory Engineering Co., Gomshall, Surrey). The slices were washed thoroughly with a medium containing 0·25 M mannitol, 0·05 M phosphate pH 6·0 and 50 μg/ml chloramphenicol. The disks were aged in this same medium (1 ml medium per disk) in flasks covered in aluminium foil. Air, or the appropriate gas mixture (air plus ethylene, propylene, etc.) was gently bubbled through the medium in which the disks were aged by means of fine tubes set into the side of the flasks. Throughout the ageing the flasks were agitated in a shaking water bath at 25°. At intervals during ageing samples of disks were taken from the medium and assayed for respiratory activity, ethylene production or enzymic activity. The rate of ethylene production by the disks was measured by taking samples of 24 disks (*ca.* 2 g fr. wt.) in 3 ml of medium and enclosing them in sealed flasks for 1 hr. Samples of the head space gas were taken after 1 hr and the ethylene content measured by GLC as previously described.[19] The respiratory activity of the disks was measured on 12 disks (1 g) samples by the normal Warburg manometric methods. The effect of growth substances or inhibitors was studied by adding these compounds to the normal medium at the concentration stated in the text.

The Extraction and Estimation of Phenylalanine Ammonia Lyase

Samples of tissue, either 5 g of tissue or 60 disks ($\simeq$ 5 g) were homogenized in an ultraturax homogenizer for 1 min in 30 ml of extraction medium (0·2 M Tris, 0·001 M EDTA, 0·25 M sucrose and 1% PVP at pH 8·0). The homogenate was centrifuged at 6000 *g* for 10 min. The residue was homogenized in a further 10 ml of the extraction medium and again centrifuged. The combined supernatants were adjusted to pH 8·9 with 2 N KOH and made up to 50 ml with ice-cold H_2O. All were operations carried out at 0–4°.

Two methods for estimation of PAL were used, but mainly the method of Zucker.[20] 1·5 ml of the extract was incubated at 40° for 1 hr with 50 μmoles L-phenylalanine and 300 μmoles Tris buffer pH 8·9 in a final volume of 3 ml. The mixture was then chilled in ice and the absorptivity read at 290 nm against a suitable control. (Cinnamic acid has an E_{290} of 10^4 at pH 8·9.) The endogenous level of phenylalanine in swede extracts is very low and did not give cinnamic acid in our control incubations.[21] The rate of formation of cinnamic acid is proportional to the amount of extract and is linear with time over 2 hr.

The second method of assay of PAL involves acidification of the assay mixture after incubation and extraction of the cinnamic acid with Et_2O. The Et_2O was extracted with 0·5 N NaOH and the absorptivity of the latter at 278 mμ (λ_{max} for cinnamic acid) determined using a suitable control. This method enabled us to establish that cinnamic acid is the product of the reaction. The spectrum was identical with authentic *trans*-cinnamic acid and the product co-chromatographs with, *trans*-cinnamic acid on paper chromatography using *n*-BuOH–HOAc–H_2O (4:1:5).

Acknowledgements—The authors wish to express their thanks to Dr. A. C. Hulme for his help and encouragement and to Mr. P. Harkett and Miss S. Edwards for their excellent technical assistance

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