PHENOLIC METABOLISM IN PLANTS:
INTRACELLULAR LOCALIZATION OF SOME ENZYMES

by

EDITH ELLEN CAMM

B.Sc. (Hon.), Queen's University, 1966
M.Sc., University of British Columbia, 1968

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Department of Botany

The University of British Columbia
Vancouver 8, Canada

Date 10.10.72

ABSTRACT

Two aspects of intracellular organization of phenolic metabolism in plants were investigated. The first involved the localization of some reactions in the conversion of tyrosine to plastoquinone, which is accumulated in the chloroplast. More evidence is presented for the formation of p-hydroxyphenylpyruvic acid and homogentisic acid from tyrosine by higher plants, although neither reaction seems to occur in the chloroplast.

The second part of this work involved the intracellular localization of several enzymes involved in the phenolic metabolism of potato. Separation of cell fractions by differential centrifugation and sucrose density gradient separation showed that shikimate dehydrogenase and prephenate dehydrogenase are wholly soluble enzymes. In the cases of phenylalanine ammonia lyase and an O-methyl transferase acting on caffeic acid, 90% of the enzyme was soluble. The remaining 10% of the enzymes that was associated with particulate fractions did not appear to be located in the same

fractions. Most of the cinnamic acid hydroxylase in aged potato was found in the microsomal fraction, but some of this enzyme appeared to be associated with other particles.

All the enzymes studied increased two-fold or more in activity after the potato tuber was sliced and aged in the light. Cinnamic acid hydroxylase increased in activity and also changed from a mainly soluble enzyme in dormant potato to the microsomal enzyme characteristic of actively metabolizing tissue.

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INTRODUCTION

The phenolic metabolism of the plant is especially sensitive to environmental stimuli [Yoshida 1969]. In bright light, for example, the levels of flavonoids are much higher than in dim, and injury or infection can result in the formation of necrotic areas high in phenolic compounds. This study is an investigation of some reactions and enzymes of phenolic metabolism, their location within the cell, and how they are affected by environmental changes.

One approach has been the investigation of changes of levels of enzymes involved in the formation of phenolic compounds. Concomitant increases in levels of phenylalanine ammonia-lyase (PAL) and phenylpropanoids have been demonstrated in many plants and plant tissues. In gherkin seedlings, changes in PAL levels which occur after treatment with blue light, long term red light, wounding or temperature changes are all reflected in the levels of accumulated hydroxycinnamic acids [Engelsma 1967,1967a,1968,

1969]. Fresh potato tissue, devoid of PAL or chlorogenic acid, develops both on incubation in light [Zucker 1965]. Similar increases in PAL and chlorogenic acid have been described in ethylene-treated sweet potato tissue [Imaseki et al. 1968].

Flavonoids, too, are formed in increased amounts after exposure of the plant to light [Scherf and Zenk 1967]. In buckwheat, PAL production and anthocyanin production are affected the same way by red and far-red light [Scherf and Zenk 1967]. Apiin and graveobioside B formation, after illumination of parsley cell cultures, coincides with a peak in PAL activity [Hahlbrock et al. 1971].

Cinnamate hydroxylase, another enzyme of phenylpropanoid metabolism, also shows an increase on exposure of parsley cell cultures to light [Hahlbrock et al. 1971], and after light and wounding in buckwheat seedlings [Amhrein and Zenk 1970].

In parsley, PAL, cinnamate hydroxylase and p-coumarate:CoA ligase increase in parallel after the cell culture has been exposed to light [Hahlbrock et al. 1971]. These enzymes catalyse the first three steps in the conversion of phenylalanine to the flavonoid graveobioside B. The levels of other enzymes concerned mainly with the carbohydrate portion of the molecule increase in parallel also, but with

different kinetics from the first three. Enzymes of basic cell metabolism such as acetate:CoA ligase are unaffected during a similar light stimulation of cell cultures of soybean [Hahlbrock et al. 1971a].

Other enzymes concerned with phenolic metabolism are also sensitive to stimulation. The three phenolases in potato tubers increase 3-fold in 24 hour ageing of tissue slices [Ruis 1972]. In parsley cell cultures, the enzyme methylating the m-hydroxyl of o-dihydric phenols (an O-methyl transferase) is also stimulated by light and ageing [Hahlbrock et al. 1971, Ebel et al. 1972]. The kinetics of stimulation of this enzyme are not the same as those exhibited by PAL.

The approach used in these studies has been the traditional one of the biochemist-investigation of solubilized enzymes from a cell brei. But within the plant cell, enzymes are frequently found associated with specific organelles. In some cases, an intracellular transport system appears to operate between different organelles [Kisaki and Tolbert 1969]. Studies of the control of plant phenolic metabolism must take into account this intracellular organization.

In this study, chloroplasts from leaf tissue, and various particulate fractions from dormant and aged potato

have been examined. The particulate fractions included the microbody-rich, mitochondrion-rich, and microsomal fractions.

The microbody is found in many types of plant tissue [Frederick and Newcomb 1969]. It usually has a buoyant density of 1.25 to 1.26 and contains all the glycolic acid oxidase, uric acid oxidase and catalase of the cell [Tolbert et al. 1968, Vigil 1969]. Microbodies from tissue oxidizing fatty acids contain all the β -oxidation enzymes and enzymes of the glyoxylate pathway. This type of microbody has been termed the glyoxysome [Beevers 1969]. Microbodies from photosynthetic tissue are called peroxisomes, and are involved in the glycolate metabolism of photosynthesis and photorespiration [Tolbert and Yamazaki 1969]. Tissues such as cucumber cotyledons first metabolize fatty acids then become photosynthetic [Trelease et al. 1971]. It appears that peroxisomal enzymes merely replace glyoxysomal enzymes in the same population of microbodies.

Potato tuber tissue (used in this study) is neither photosynthetic nor does it normally metablize fatty acids. Microbodies from this tissue have neither glyoxylate nor glycolate cycle enzymes [Ruis 1971]. However, these particles are of the expected buoyant density and contain catalase and uric acid oxidase.

Microsomal fractions were also examined in this study. The definition of this fraction is a functional one, for it includes those particles which do not sediment after centrifugation at 10,000 times gravity, but form a pellet after 1 hour at 108,000x*g*. This fraction includes ribosomes and fragments of the endoplasmic reticulum, although other particles are almost certainly present.

While most of the work on localization of enzymes has been concerned with oxidative and photosynthetic enzymes, there has been some interest in the localization of enzymes concerned with phenolic biosynthesis (summarized in Figure 1).

Chloroplasts from *Saxifraga* are reported to convert *p*-coumaric acid to caffeic acid [Sato 1966], and secondly, *cis*-caffeic acid to esculetin [Sato 1967]. These chloroplasts and those of several other arbutin-containing plants contain *o*-diphenol oxidase-like enzymes which are quite specific for arbutin, converting it to 3,4-dihydroxyphenyl-β-D-glucoside, and then to the *o*-quinone [Hattori and Sato 1963]. However, these chloroplast preparations might also have contained peroxisomes. Spinach beet chloroplasts contain a phenolase which hydroxylates *p*-coumaric acid. Spinach beet stems, which are non-photosynthetic, lack this enzyme [Parish 1972].

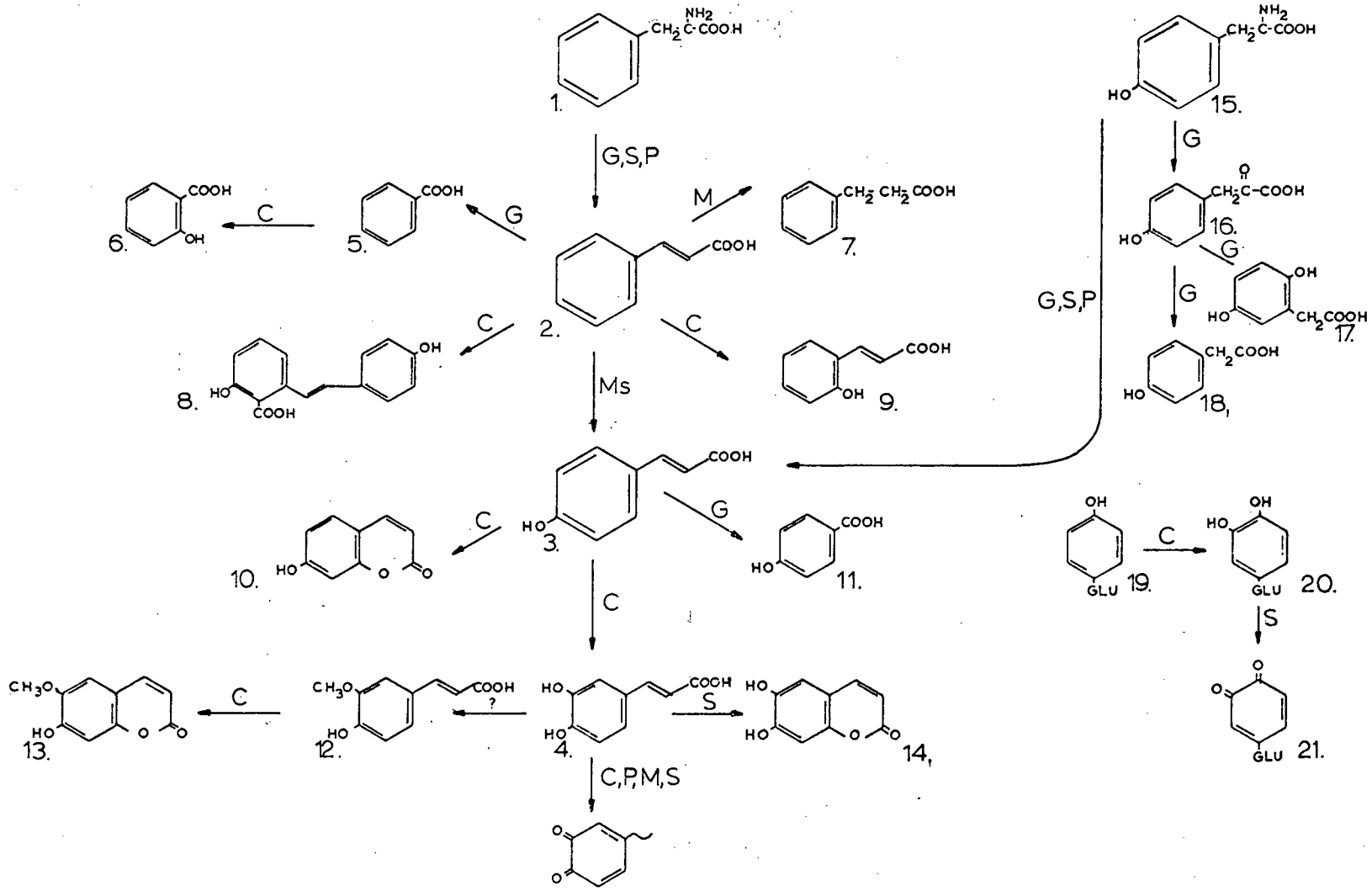
Figure 1

(facing)

Main localized reactions of phenolic metabolism of plant cells.

1, phenylalanine; 2, cinnamic acid; 3, p-coumaric acid; 4, caffeic acid; 5, benzoic acid; 6, salicylic acid; 7, dihydrocinnamic acid; 8, 3,4'-dihydrostilbene-2-carboxylic acid; 9, o-coumaric acid; 10, umbelliferone; 11, p-hydroxybenzoic acid; 12, ferulic acid; 13, scopoletin; 14, esculetin; 15, tyrosine; 16, p-hydroxyphenypyruvic acid; 17, homogentisic acid; 18, p-hydroxyphenylacetic acid; 19, arbutin; 20, 3-hydroxyarbutin.

Major sites of enzyme: S, soluble; G, glyoxysomal; P, peroxisomal; C, chloroplastidic; M, mitochondrial; Ms, microsomal.


1.
G,S,P
M
7.
G
5.
C
6.
2.
C
C
8.
9.
Ms
15.
G
16.
G
G
17.
18,
G,S,P
3.
C
G
10.
11.
C
19.
C
20.
S
21.
GLU
C
S
12.
13.
4.
14,
C,P,M,S

Other reactions which occur in the chloroplast include the o-hydroxylation of cinnamic acid, p-coumaric acid, ferulic acid and benzoic acid [Kindl 1971]. In several cases, the corresponding coumarins are formed. In Hydrangea macrophylla, the intact chloroplasts are able to catalyze the condensation of p-coumaric acid with three acetate units to form the stilbene, 3,4'-dihydroxystilbene-2-carboxylic acid, although the amounts formed are exceedingly low [Kindl 1971a].

Chloroplasts are also able to catalyze the formation of phenylacetaldehyde and phenylacetic acid from phenylalanine [Kindl 1972]. Those from pea and barley can make indoleacetic acid and indolylacrylic acid from L-tryptophan [Kindl 1972].

Mitochondria do not seem to be the site of many enzymes concerned with phenolic metabolism, although cinnamic acid is reported to be reduced to dihydrocinnamic acid here [Kindl 1972]. In spinach beet, DOPA and catechol oxidases are found in the mitochondria [Parish 1972].

The glyoxysomes are the site of considerable phenolic metabolism, Ricinus communis glyoxysomes are capable of converting tyrosine into p-hydroxyphenylacetic acid and homogentisic acid [Kindl and Ruis 1971]. The glyoxysomes are also a site of chain shortening of the

cinnamic acid derivatives to benzoic acid derivatives [Kindl and Ruis 1971].

Preparations of glyoxysomes from castor bean endosperm have the highest specific activities of PAL, tyrosine ammonia-lyase (TAL) and histidine ammonia-lyase, although all the particles separated on a sucrose density gradient showed activity. The authors report this finding in unwashed organelle fractions as a localization of the ammonia lyases in the glyoxysomes, but this statement should be treated with caution. Specific activity depends on the amount of protein present, and a hypothetical organelle containing 90% of the cell complement of enzyme x, plus quantities of storage protein, say, would have a very low specific activity.

The following data are from Ruis and Kindl 1971. The percentage total activity has been calculated from their data (Table 1).

These workers did not investigate the microsomal fraction but even so, 95% of the total activity is found in the soluble fraction. Thus, while the glyoxysomes do appear to contain ammonia-lyases, these enzymes are either very readily solubilized or are mainly soluble <u>in vivo</u>.

Similar work has been carried out on the peroxisomes of sunflower and spinach [Ruis and Kindl 1971]. High

Table I

Distribution of PAL in cell fractions of

Ricinus communis endosperm

fraction	activity (mU)	specific activity (mU/mg protein)	% activity
108,000xg supernatant	20.0	0.014	90
mitochondria	0.5	0.042	2.2
proplastids	0.7	0.2	3.2
glyoxysomes	1.0	0.33	4.5

specific activities of PAL, TAL, and the enzymes forming α,β-unsaturated carboxylic acids from aspartic acid, histidine and tryptophan were present in the peroxisomes. While again most of the activity was in the soluble phase, purified peroxisomal fractions had highest specific activity.

The microbodies of potato, unlike those of castor bean endosperm and spinach, have no PAL activity [Ruis 1971]. These microbodies do, however, contain o-diphenol oxidase activity.

PAL activity is also found in the microsomal fraction from *Sorghum* [Stafford 1969]. Amhrein and Zenk

[1971], working with etiolated buckwheat, found that some PAL activity was located in the microsomes of this plant, too, and that it could not be removed by washing. They found that this particle was not a ribosome, nor was it identical to the particles with which cinnamic acid 4-hydroxylase is associated.

In the work of Russell and Conn [1966] and Russell [1971] the cinnamate hydroxylase of pea was located in the microsomes. In young Sorghum all the cinnamate hydroxylase activity is associated with particles which sediment between 500 and 100,000x*g*, which includes the microsomal fraction [Stafford 1969]. In buckwheat also, the enzyme appears on a microsomal particle [Amrhein and Zenk 1971].

Much information exists on the location within the cell of phenolic compounds and the enzymes involved in their synthesis. Less information exists on the increase of these enzymes after a change in the environment. The present study work combines studies of enzyme localization with those of changes in levels of enzymes.

Two aspects of phenolic metabolism were investigated. The first part of this study involved the chloroplast and a group of phenolic compounds found there, the plastoquinones (Figure 2). Light causes the level of these compounds to rise in etiolated barley [Lichtenthaler 1969]. It has been shown that

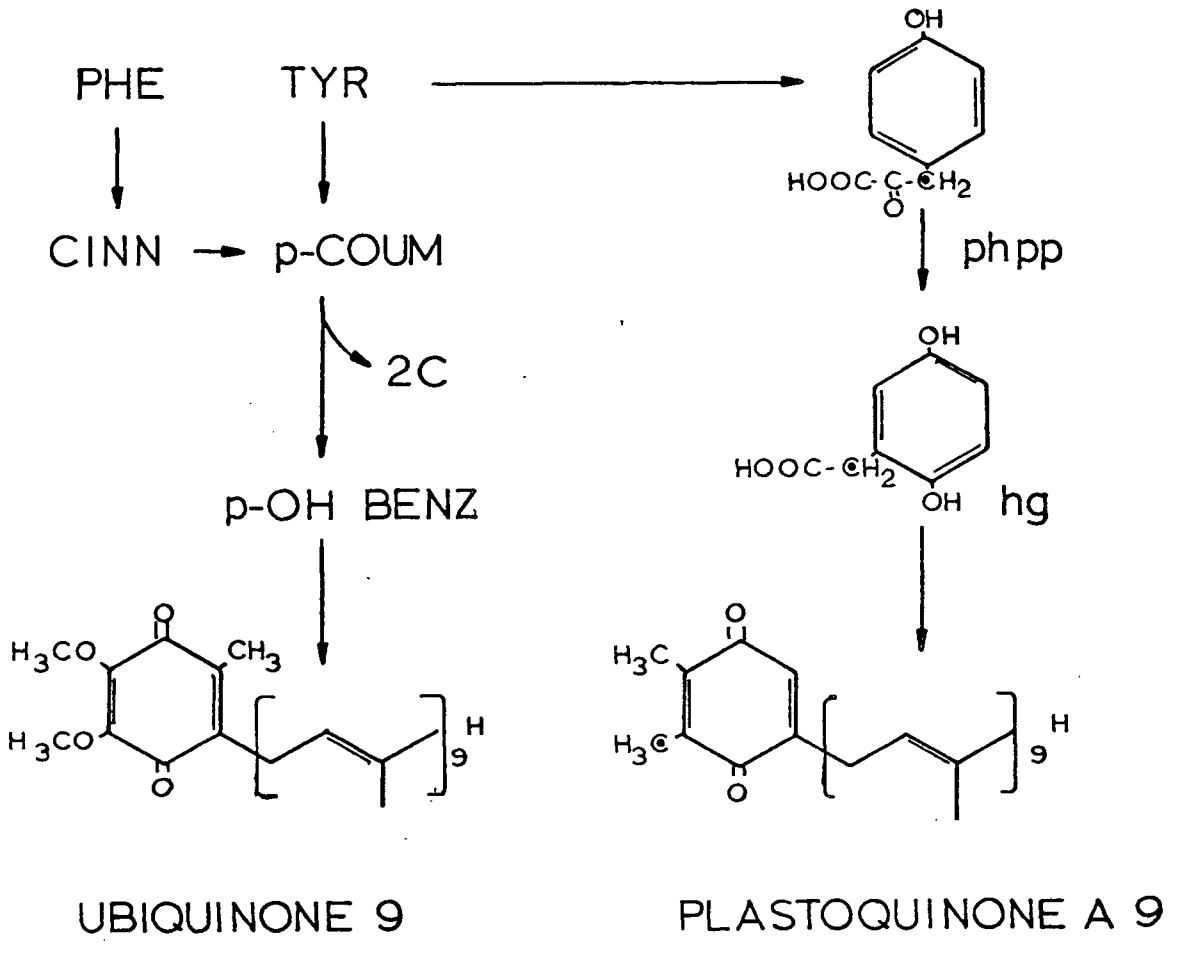


Figure 2. Formation of ubiquinone and plastoquinone from phenylalanine and tyrosine [after Whistance and Threlfall 1967].

phe: phenylalanine, tyr: tyrosine, cinn: cinnamic acid, p-coum: p-coumaric acid, p-OH benz: p-hydroxybenzoic acid, phpp: p-hydroxyphenylpyruvic acid, hg: homogentisic acid.

D- and L-tyrosine, and in some plants, phenylalanine, are very good precursors of these compounds, and that the methyl group meta to the polyprenyl chain is derived from the β-carbon of tyrosine [Whistance and Threlfall 1968]. The most logical and direct route for the conversion of tyrosine to plastoquinone is via p-hydroxyphenylpyruvic acid (PHPP) and homogentisic acid (HG) [Whistance and Threlfall 1967]. Although the enzymes for these conversions are known in bacteria they are not all known in plants. Further credence is given this suggestion, however, by the findings that radioactive PHPP [Threlfall and Whistance 1968] and HG [Whistance and Threlfall 1970] are well incorporated into plastoquinone.

Similarly, the ubiquinones accumulate in the mitochondria [Griffiths et al. 1967]. The ubiquinones have been shown to be formed from administered phenylalanine or tyrosine via p-coumaric acid and p-hydroxybenzoic acid [Whistance and Threlfall 1968]. Some of the enzymes capable of carrying out these reactions have been located within the cell (Figure 1).

In order to settle the problem of the role of each organelle in a specific area of metabolism, the enzymes involved must be located with respect to these organelles. If, for example, all the enzymes synthesizing the ring of

plastoquinone were found to be extrachloroplastidic, then the presence of plastoquinones in the chloroplast after illumination would be a result of transport and not of *in situ* synthesis.

The second part of the work involves the increase in phenolic acids in potato after ageing in the light [Zucker 1963]. The intracellular location of enzymes involved in this increase was investigated in dormant potato tissue and in disks aged in the light. It was felt that the combination of the approaches of localization and changes in enzyme levels would yield a more integrated picture of phenolic metabolism in the tissues investigated.

MATERIALS AND METHODS

Chemicals

Most biochemical reagents and materials for the preparation of media and cell fractions were obtained from Calbiochem, La Jolla, Calif. These items include Miracloth, tricine buffer, sorbitol, dithiothreitol, ATP, all pyridine nucleotides and amino acids, glucose-6-phosphate dehydrogenase, 3-phosphoglyceraldehyde and 3-phosphoglyceric acid kinase.

p-Hydroxyphenylpyruvic acid was obtained from Calbiochem because their product (although labelled B grade) was purer than the best grades from other companies.

L-Amino acid oxidase (Naja naja), shikimic acid, malic acid, caffeic acid, ferulic acid, p-hydroxybenzaldehyde and m-hydroxybenzaldehyde were obtained from Koch-Light, Colnbrock, Buck., England.

Ficoll, Dextran T 40, and Sephadex G-200 were obtained from Pharmacia (Canada) Ltd., Montreal.

Vanillin was obtained from Aldrich Chemicals, Milwaukee, Wisc. Benzaldehyde was from BDH Chemicals, Toronto.

Radioactive tyrosine, phenylalanine and cinnamic acid were obtained from New England Nuclear, Boston, Mass., as were all reagents used in preparation of samples for liquid scintillation counting.

S-Adenosylmethionine-methyl-^{14}C was obtained from International Chemical and Nuclear Corporation, Irvine, Calif.

Silica gel G and GHR were obtained from E. Merck, Darmstadt, Germany. MN cellulose was purchased from Machery, Nagel and Co., Düren, Germany.

All other chemicals and solvents were of reagent grade, but were from various sources.

Biological Material

Spinach

Seeds were planted in flats of soil maintained in a growth chamber at 20°C on 12 hour days. Plants were harvested at 4-6 weeks. Other spinach was purchased locally.

Barley

Seeds were surface sterilized in 2% sodium hypochlorite, rinsed and sown in sterile vermiculite in flats. For sterile experiments using etiolated barley, the seeds were rinsed and sown using sterile technique in moist vermiculite in a large steel beaker covered by foil. An air vent was covered with a cotton plug. No watering was required if the seedlings were used before 9 days old.

Tomato

Tomato fruits were surface sterilized in ethanol. Using sterile technique, seeds were removed and 6 were placed on the surface of nutrient agar* in a 250 ml flask. The seedlings were maintained at 23°C in 12 hour days.

The seedlings were used in the flask, or were harvested thus: 30 ml of sterile water was added to the flask, and the seedlings were cut off under the water with curved scissors (5" arteriotomy scissors).

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*Nutrient agar: per l, 1.32 gm $Ca(NO_3)_2$; 0.25 gm of KH_2PO_4; 0.25 gm $MgSO_4 \cdot 7H_2O$; 5 gm agar and tap water.

Tissue culture

Ruta graveolens tissue was maintained from stocks obtained from Dr. O. Gamborg, Prairie Regional Laboratory National Research Council, Saskatoon. Tissue was maintained in shake culture on B_5 or B_5C_2 medium at 23°C in 16 hour days. Material was subcultured every three weeks.

Tissue culture medium (B_5C_2)

	1 ℓ	
$NaH_2PO_4 \cdot H_2O$	150 mg	
KNO_3	3.5 gm	
$(NH_4)_2SO_4$	134 mg	
$MgSO_4 \cdot 7H_2O$	250 mg	
$CaCl_2 \cdot 2H_2O$	150 mg	
Fe (sequestrene)*	28 mg	
KI	0.75 mg	
Sucrose	20 gm	
N-Z amine**	2 gm	(omit for B_5 medium)
2,4-D	1 mg	
vitamin solution	10 ml	
micronutrient sol.	1 ml	
pH	5.5	

*Geigy Industrial Chemicals, Ardsley, New York.

**Sheffield Chemicals, Norwich, New York.

Vitamin solution (in 100 ml)

nicotinic acid	10 mg
thiamine	100 mg
pyridoxine	10 mg
myo-inositol	1 gm

Micronutrient solution (in 100 ml)

$MnSO_4 \cdot H_2O$	1 gm
$H_3 BO_3$	300 mg
$Zn SO_4 \cdot 7H_2O$	300 mg
$Na_3MoO_4 \cdot 2H_2O$	25 mg
$CuSO_4$	25 mg
$CoCl_2 \cdot 6H_2O$	25 mg

Potato

Large firm tubers of potato variety 'Netted Gem' were washed and cylinders were removed with a sharp cork borer. Disks approximately 2 mm thick were cut from the cylinders and were washed in cold running water for 15 minutes. The discs were either used fresh, or aged 18 hours under fluorescent lights at 23°C in Petri dishes

(5 gm/dish with 5 ml water). After 18 hours, the disks were washed and chilled before grinding.

Fractionation Procedures

Preparation of chloroplasts

Chilled washed tissue was cut with scissors and then homogenized in a blendor for three 2-second bursts in twice the volume of sorbitol medium [after Cockburn *et al.* 1968; sorbitol 0.33 M, $MgCl_2$ 5 mM, sodium isoascorbate 2 mM, pH 6.7 sodium pyrophosphate buffer 10 mM] or modified Honda medium [Ranaletti *et al.* 1969; Ficoll 2.5%, Dextran T-40 5%, sucrose 0.25 M, mercaptoethanol 8 mM, tricine buffer 0.1 M, $MgCl_2$ 1 mM]. The homogenate was filtered through Miracloth, then centrifuged at 270x*g* for 5 minutes. The supernatant was centrifuged at 6000x*g* for 5 minutes. This was termed the 'chloroplast-rich' preparation. A 'mitochondrion-rich' preparation was made by centrifuging the supernatant at 20,000x*g* for 20 minutes.

Non-aqueous cell fractionation

Plant tissue was frozen in liquid nitrogen, placed over P_2O_5 (anhydrous) or H_2SO_4 in the cold and evacuated

with a pump for two hours. The material was stored in a desiccator in a freezer. Tissue culture lumps have little cuticle and can be freeze-dried in an ordinary lyophilizer.

About 0.2 gm of freeze-dried tissue was thoroughly ground in a VirTis Omnimixer in an ice-cold mixture of chloroform-hexane with specific gravity (ρ) 1.40. This was strained through cheesecloth (8 layers) and 5 ml of the mixture was added to a 15 ml glass centrifuge tube. On top were layered 2 ml each of mixtures of chloroform and hexane with ρ of 1.35, 1.30 and 1.20. The density gradients were centifuged at 20,000x*g* for 20 minutes in a Sorvall RC-2B centrifuge using the HB swinging bucket rotor. Bands were collected from the top of the gradient with a pipette.

Preparations of potato fractions by differential centrifugation

Plant material was ground or chopped in an equal volume of grinding medium [after Ruis 1971; sucrose 0.4 M, tricine buffer 0.165 M pH 7.5, KCl .01 M, EDTA 1 mM, $MgCl_2 \cdot 6H_2O$ .01 M, dithiothreitol .01 M], then filtered through Miracloth to remove the cell clumps. The filtrate was centrifuged in a refrigerated centrifuge 5 minutes at 270x*g* to remove whole cells. The supernatant was centifuged for 10 minutes at 10,000x*g*. This pellet was termed the

particulate fraction and was suspended in fresh grinding medium. The supernatant was centrifuged in a Spinco Model L2-65B preparative ultracentrifuge from 1 to 4 hours in a 50Ti rotor at 40,000 rpm. The supernatant from this treatment was termed the soluble fraction, or 100,000x*g* supernatant and the pellet, the microsomal fraction or 100,000x*g* supernatant.

Preparation of cell fractions by sucrose density gradient separation

The 10,000x*g* pellet from the above procedure was resuspended in fresh medium and was layered gently on a previously prepared gradient.

The discontinuous gradients were formed by layering with a pipette. A typical gradient consisted of 5 ml of 70% w/v sucrose, 10 ml of 60% w/v sucrose, 10 ml of 51% w/v sucrose and 8 ml of 44% w/v sucrose in a 1x3½" cellulose nitrate tube. All solutions used for gradients contained sucrose, tricine buffer .05 M, pH 7.5, and 1 mM EDTA. On top of this was layered about 6 ml of resuspended pellet.

The tubes were centrifuged from 4 to 18 hours in a SW27 rotor in a Spinco Model L2-65B preparative ultracentrifuge.

Fractions were collected from the gradient with a capillary tube (gently inserted to the bottom of the centrifuge tube) attached to a LKB Varioperpex pump. The pump was adjusted to a flow rate of 1 ml/min, and 2 min fractions were collected with an ISCO Model 272 fraction collector.

Refractive indices were measured with a Bausch and Lomb Abbé refractometer.

Extraction and Chromatography Procedures

Extraction and chromatography of phenolic acids

Plant material was extracted with hot 80% ethanol. The extract was dried then taken up in a small volume of hot water. The hot water/extract was filtered through a pad of Celite (Johns-Manville Company) to remove lipids. The lipid-free extract was acidified to pH 2 and extracted continuously 4 to 6 hours in a liquid-liquid extractor. If the lipid content of the ethanolic extract were low, then the ethanol was removed *in vacuo* and the aqueous extract was extracted with ether directly. The ether extract was dried over anhydrous Na_2SO_4 taken to dryness *in vacuo* and made to a small volume for chromatography.

Several solvent systems were employed for chromatography of compounds on MN cellulose thin layer plates.

System A) ben zene:acetic acid:water (10:7:3, upper phase)

System B) butanol:pyridine:water (7:1.5:1.5)

System C) anisole:acetic acid:water (70:29:1) (excellent for resolution of HG and tyrosine)

System D) 2% formic acid (generally used for the second dimension)

System E) butanol:acetic acid:water (40:10:1)

System F) toluene:ethyl formate:formic acid (5:4:1)

Phenolic compounds were visualized under UV, and by spraying with diazotized p-nitroaniline reagent. (Spray with a fresh mixture of 5 ml of 0.3% p-nitroaniline, 1 ml of 5% $NaNO_2$ and 15 ml of 20% Na acetate. Overspray while still damp with 5% NaOH.)

Formation, extraction and chromatography of 2,4-dinitrophenylhydrazones (DNPH) [after Katsuki et al. 1968]

To 4 or 5 ml of material (deproteinized if necessary with 1% $HClO_4$) was added 1 ml of DNPH reagent (1 mg

2,4-dinitrophenylhydrazine in 1 ml 2 N HCl). After 30 minutes at 30°C, the reaction mixture was extracted with ethyl acetate, and the ethyl acetate extract was evaporated to dryness. This was shaken with 10 ml benzene and 20 ml of 0.1 N sodium bicarbonate. The bicarbonate layer contained the keto acid DNPH, and the benzene layer, the other DNPH and excess reagent.

The following solvent systems were used: butanol: 3% ammonia (4:1), and water-saturated isoamyl alcohol: butanol (3:1), both on Silica Gel G. The unknowns were compared with standard DNPH prepared from authentic compounds in the above manner.

Extraction and chromatography of lipids (including plastoquinones) [after Griffiths et al. 1967]

In dim light freeze-dried tissue was extracted with several volumes of ethanol:ethyl ether (1:2) and thrice with 1 ml of acetone until colourless. The acetone and ethanol were removed by washing with water. The water extract was back extracted with ether. The ethereal extracts were dried over anhydrous Na_2SO_4 and taken to dryness

in vacuo. The extracts were made to volume in petroleum ether (30°-60° b.p.) and stored at -20°C.

Chromatography was on Silica Gel GHR (Merck) in chloroform:heptane (4:1). Standard Plastoquinone A(45) was obtained from Roche, Basel, Switzerland.

The Preparation of p-Hydroxyphenylpyruvate From L-Tyrosine-^{14}C Using L-Amino Acid Oxidase

Five μCi of L-tyrosine-^{14}C (specific activity 5 μCi/umole), 0.1 mmoles of KCl, 1 mg catalase powder, 0.1 mmoles of tris buffer pH 8.0, and 4 units of L-amino acid oxidase were mixed in a flask. The mixture was incubated at 37°C.

Radiotracer Methods

Liquid scintillation counting

Liquid samples were dissolved in, and solid samples suspended in, toluene-based scintillation counting fluids (4 gm PPO, 100 mg POPOP per 1 toluene). To the powdered samples were added 2 cc of Cabosil to aid suspension. Samples were counted in a Nuclear Chicago model 720 liquid scintillation counter.

Examination of chromatograms

Tnin layer and paper chromatograms were placed with X-ray film (Kodak Blue Brand) and developed after two to three weeks with Kodak X-ray developing solutions.

One-dimensional chromatograms were scanned on a Nuclear Chicago Actigraph II.

Protein Determination

The method of Lowry et al. [1951] was used for the experiments with chloroplasts. For protein measurements of sucrose density gradient fractions, the E_{280}/E_{260} method was used [Chaykin 1966]. Optical density at 540 nm was used as an estimate of turbidity.

The method of Bramhall et al. [1969] which was not suitable for use with the heavy sucrose from the gradient fractions was used at all other times. Samples of 10 µl, containing less than 25 µgm of protein were spotted on 2x2 cm squares marked on Whatman #50 paper. The squares were dried, cut apart, and the protein was fixed in cold 7.5% trichloroacetic acid. This was heated to 80°C for one-half hour to hydrolyze nucleic acids. The papers were washed in ethanol:ethyl ether (1:1), dried, and stained with a solution of xylene brilliant cyanin G (Edward Gurr Ltd.,

London) (10 mg/ml in 7% acetic acid) for 15 minutes at 50°C. It was important that the papers were occasionally swirled, but not allowed above the surface of the stain solution. The stain could be re-used four or five times. Excess stain was removed in 7% acetic acid at 50°C. The squares were blotted and placed in separate test tubes, and de-stained with 5 ml each of methanol:water:3% ammonia (66:34:1). The absorbance of the solutions was read at 610 nm.

Preparation of Potato PAL

Three hundred gm of potato slices incubated 20 hours in the normal manner were ground in a chilled blendor with 200 ml of 0.1 M tris buffer pH 8.6. The slurry was filtered through Miracloth and the filtrate centrifuged 20 minutes at 10,000x*g*. The 30-60% ammonium sulfate fraction was prepared, and redissolved in 20 ml of .05 M tris buffer pH 8.6. Fifteen ml of this were put on a Sephadex G-200 column (27 x 2.6 cm). PAL activity was measured spectrophotometrically (see Assays) and the most active fractions pooled. Protein was precipitated in 70% ammonium sulfate and was taken up in 14 ml of potato grinding medium [Ruis 1971] and stored at -20°C overnight.

Enzyme Assays

All the following assays were carried out at 30°C. The spectrophotmetric assays were carried out on a Unicam SP500 with SP45 attachment and constant temperature cell holder.

Catalase [E.C. 1.11.1.6; after Aber 1970]

In a 1 ml cuvette were mixed 0.9 ml 0.05 M phosphate buffer pH 7.0 and 0.1 ml enzyme. The reaction was started by the addition of 0.02 ml of 1.8% hydrogen peroxide (6% dilution of 30% reagent grade). The reaction was followed at 240 nm. The molar extinction co-efficient of hydrogen peroxide is 43,600.

Fumarase [L-malate hydrolyase, E.C. 4.2.1.2; from the method of Racker 1950]

In a 1 ml cuvette were mixed 0.9 ml of .05 M sodium malate in 0.1 M tricine buffer pH 7.5, and 0.1 ml enzyme. The appearance of fumaric acid was followed at 240 nm. The molar extinction co-efficient of fumaric acid was calculated as 2360 from the data of Racker.

PAL [phenylalanine ammonia-lyase, E.C. 4.2.1.5; from the method of Zucker 1965]

In 3 ml cuvette were mixed 1.5 ml of 0.05 M tris buffer pH 8.6 at 30°C, and 0.5 ml enzyme. The reaction was started by the addition of 1.0 ml of 2.5 mM L-phenylalanine in the buffer. The appearance of cinnamic acid was followed at 290 nm. The molar extinction co-efficient of cinnamic acid at 290 nm is 10,600.

When the enzyme activity was very low, the reaction time was extended to overnight.

O-Methyl transferase acting on caffeic acid [E.C. 2.1.1.; from the method of Basmadjian and Paul 1971]

Caffeic acid (72 µmoles/ml in .05 M tricine buffer pH 7.5), 0.5 µCi S-adenosylmethionine methyl-^{14}C (specific activity 52 µCi/umole) in buffer, and 0.1 ml enzyme were mixed in a test tube. After one hour incubation, the reaction was stopped with 0.2 ml of 0.1 1 N HCl, and 1 ml of water was added. The mixture was extracted three times with 2 ml ethyl acetate, and the combined extracts were washed with .05 N HCl and dried over anhydrous Na_2SO_4. The extract was transferred to a scintillation vial, dried, then taken

up in 0.1 ml of ethanol. The normal volume of toluene scintillation fluid was added for counting.

The identity of the reaction product was established by co-chromatography of the ethyl acetate extract of the reaction product with authentic ferulic acid.

Cinnamic acid 4-hydroxylase [E.C. 1.99.1; after Russell and Conn 1971]

The $NADPH_2$-generating system consisted of 5 µmoles glucose-6-phosphate, 2 µmoles NADP, and 0.4 units of glucose-6-phosphate dehydrogenase in a total of three ml of 0.1 M phosphate buffer pH 7.5. This was incubated at 30°C for five minutes.

The hydroxylating system consisted of 1 ml enzyme, 0.3 mg cinnamic acid, 0.1 µCi cinnamic acid ^{14}C (1.4 µCi/µmole, 1.0 ml of buffer, and 0.24 mg mercaptoethanol. The addition of the $NADPH_2$ generating system started the reaction. Incubation time was one hour. It was not necessary to shake the reaction mixture during incubation if the reaction vessels were uncovered. This seemed to provide enough oxygen to allow the reaction to proceed. The reaction was stopped with 0.1 ml of 6 N HCl.

The reaction mixture was extracted three times with 4 ml of ethyl acetate, and the extracts pooled. The

extract was then dried and the whole extract spotted with authentic p-coumaric acid on Whatman #1 paper and chromatographed in one dimension in solvent system A. The spot corresponding to p-coumaric acid was located under UV light after fuming with ammonia vapour, cut out and counted directly in a scintillation vial.

p-Coumaric acid hydroxylase

Several procedures were investigated.

a. The system as described for cinnamic acid 4-hydroxylase was employed with the substitution of p-coumaric acid for cinnamic acid.

b. A modification of method of Sato [1966] was used. The reaction mixture consisted of 1 ml of enzyme, 2 ml 0.1 M phosphate buffer pH 6.7, 0.1 ml of 0.02 M p-coumaric acid and .06 µCi of p-coumaric acid-^{14}C (prepared by B. Ellis of this laboratory).

In both cases a. and b., after the extraction of the reaction mixtures the material was repeatedly chromatographed in 1 dimension in solvent A to separate the caffeic acid from the origin.

c. The method of Nambudiri [1971] was followed. The tissue was ground in the buffered sucrose medium prepared without mercaptoethanol. The enzyme was incubated with 2 µmoles of p-coumaric acid and 2 µmoles of phosphate buffer pH 7.5. The p-coumaric acid formed was measured by the method of Arnow [1937].

d. A modification of the method of Stafford and Dresler [1972] was used. The tissue was ground in buffered sucrose with mercaptoethanol, but was passed through a Sephadex G-25 column in an attempt to remove the mercaptoethanol. The incubation mixture consisted of 2 µmoles of p-coumaric acid-^{14}C, 0.2 ml enzyme, .4 µmoles of ascorbic acid, and 150 µmoles of phosphate buffer pH 6.0. After ½ hour at 30°C the reaction was stopped with 0.1 ml 2N HCl, and the mixture was extracted with ethyl ether. The extract was chromatographed with authentic caffeic acid in solvents A and E on cellulose TLC plates. Caffeic acid spots were scraped and counted in scintillation vials.

Shikimic acid:NADP oxidoreductase [E.C. 1.1.1.25; after Balinsky and Davies 1961]

Nine µmoles of shikimic acid, 1 µmole of NADP, and 160 µmoles of glycine:NaOH buffer, pH 9.0 were mixed in a 1 ml cuvette. Up to 0.4 ml of enzyme was added. The reaction was followed at 340 nm.

Prephenic acid:NADP oxidoreductase [E.C. 1.1.1; after the method of Gamborg 1966]

Preparation of prephenic acid from the barium salt — [After the method of Gamborg and Simpson 1964]. Fifty mg of prephenic acid barium salt (obtained from Dr. O. Gamborg) were dissolved in 10 ml of .05 M tris. A slight excess (1.48 mg) of potassium sulfate was added. The mixture was stirred for 15 minutes, then filtered through glass fiber paper.

Estimation of prephenic acid concentration — To 50 µl of the above solution were added 50 µl of water and 0.1 ml 1N HCl to convert the solution to PHPP. After 15 minutes incubation at 30°C, 2.3 ml of 0.5 N NaOH were added. The molar extinction coefficient at 320 nm of the resulting p-hydroxybenzoic acid is 16,000, and the optical density of the solution at hand was 0.8. This indicates a prephenic acid concentration in the original solution of 2.5 µmoles/ml.

The pH of the solution of prephenic acid was lowered to 8.0 for use in the enzyme assay. In a 1 ml cuvette were placed 50 µmoles of pyrophosphate buffer pH 7.5, 0.5 µmoles of NADP, and 1 µmole of prephenic acid. Up to 0.4 ml of enzyme were used. The reaction was followed at 340 nm.

Alcohol dehydrogenases [after Davies et al. 1972]

Alkyl alcohol dehydrogenase — Five μmoles of acetaldehyde, .25 μmoles of $NADH_2$, 50 μmoles of phosphate buffer pH 7.5, and potato enzyme to a total of 1 ml were mixed in a 1 ml cuvette. The reaction was followed at 340 nm. $NADPH_2$ was also tried in place of $NADH_2$.

Aromatic alcohol dehydrogenase — The above proportions were used, with vanillin, benzaldehyde, m-hydroxybenzaldehyde, and p-hydroxybenzaldehyde as the substrates. Both $NADPH_2$ and $NADH_2$ were used.

D-Glyceraldehyde-3-phosphate:NADP oxidoreductase (phosphorylating) (E.C. 1.2.1.9)

The reaction mixture consisted of 3-phosphoglyceraldehyde, 2 μmoles, 100 μmoles of phosphate buffer, pH 7.0, 10 μmoles of $MgCl_2$, 5 μmoles of reduced glutathione, 5 μmoles of ATP, 0.2 units of 3-phosphoglyceric acid kinase. This mixture was incubated one minute at 30°C. Enzyme and 0.2 μmoles $NADPH_2$ were added to a total volume of 1 ml. The reaction was followed at 340 nm.

PART I

METABOLISM OF TYROSINE BY ISOLATED CHLOROPLASTS

RESULTS

In most of the following experiments, labelled substrate of low specific acitivity was used. With high specific activity substrate, even a small amount of bacterial contamination can result in conversions which are misleading [Gnanam *et al*. 1969]. The present study was an investigation of the possible metabolism of tyrosine to *p*-hydroxyphenylpyruvic acid (PHPP) and homogentisic acid (HG). Since both these compounds are produced by the bacterial breakdown of tyrosine, it is especially important to use low specific activity substrates.

Experiments with Spinach Chloroplasts

(i) In order to form HG from supplied tyrosine *via* the expected pathway to plastoquinone [Whistance and Threlfall, 1967, 1968, 1970], the chloroplasts would first have to convert the tyrosine to PHPP and then to HG. The

following experiments were an attempt to find the second step of this pathway; the conversion of PHPP to HG.

The amino acid oxidase system (see Methods) was used to generate PHPP from tyrosine-UL-^{14}C (specific activity 10 μCi/umole). After five minutes pre-incubation of the oxidase system, spinach chloroplasts from 20 gm of spinach (isolated in Honda medium) were added. After 30 minutes incubation, the material was extracted and chromatographed as for plastoquinone determination. Chromatography in heptane:chloroform and in systems A and D (2 dimensions) showed no radioactive compounds other than the PHPP generated by the oxidase. Either there had been no further metabolism, the turnover of the HG was very fast, or the HG had been degraded in the extraction procedure.

(ii) To prevent chemical breakdown during isolation and chromatography, the following experiment was devised. Incubation mixtures were set up as before, with tyrosine-UL-^{14}C. After five minutes pre-incubation, fractions from a non-aqueous preparation of spinach were added. At the end of 30 minutes incubation, 9.5 mg of HG were added to each fraction. After extraction and chromatography, it appeared that from the entire extract, equivalent to 300 mg of freeze-dried spinach, only 920 cpm were recovered in HG.

Thus, there appears to be only a low level of metabolism of tyrosine into phenolic compounds. The spinach plants used above were young (not over one month old), and were quite green. Leaf lipids are formed rapidly on exposure of the leaf to light [Lichtenthaler 1969], and greening tissue might be the best source of enzymes metabolizing tyrosine and PHPP.

Experiments with Greening Barley

(i) The following experiment was designed to verify that plastoquinones were being formed from tyrosine.

Shoots (100) of etiolated 9 day old barley grown under sterile conditions were placed in 10 ml of water with 7.5 µCi DL-tyrosine-UL-^{14}C (specific activity 50 µCi/µmole). After the plants had been allowed to green for 29 hours, they were extracted in dim light with ether and acetone. While chromatography of the phenolic acid fraction showed no label in HG, chromatography of the lipids on Silica Gel GHR showed many radioactive fluorescent bands including one corresponding to authentic plastoquinone A 45 (Figure 3). The UV spectra in methanol of compounds 2, 3 and 4, but not 5, showed shifts with $NaBH_4$ characteristic of quinones.

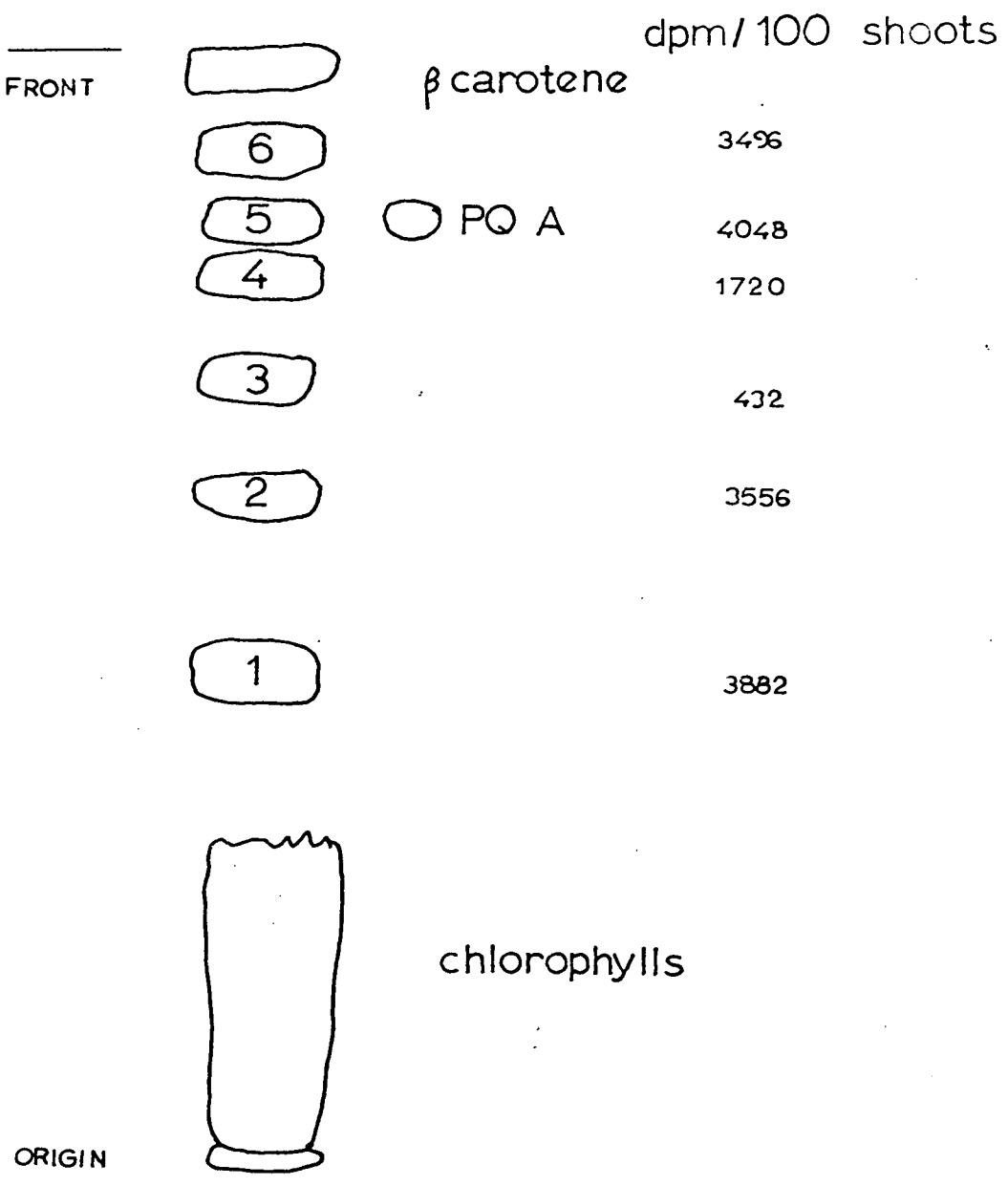

Figure 3. Chromatogram of lipid extract from barley shoots administered tyrosine-UL-^{14}C.

coating: silica gel GHR
solvent: chloroform:heptane 4:1
PQ A : authentic plastoquinone A 45

This experiment suggests that turnover of HG into plastoquinones is very rapid, since there is no accumulation of labelled material, although the end product is labelled.

(ii) Since it is known that PHPP is an unstable compound, an attempt was made to prevent breakdown by the formation of the 2,4-dinitrophenylhydrazine derivative (DNPH).

Chloroplasts were prepared in Honda medium from 9.5 gm of young barley. The chloroplasts were resuspended in 4 ml of buffer and the supernatant was saved. A brei was prepared from a second batch of barley (10.5 gm). Tyrosine-UL-^{14}C, 1 µCi/umole, 1 µCi/reaction) was added.

The reaction was stopped after one hour by the addition of 2,4-DNP reagent. Extraction, separation and chromatography were carried out as in Methods.

The low counts of PHPP (Table 2) indicate that this compound does not accumulate in the plant, if indeed it is formed at all.

(iii) In an attempt to separate chloroplasts with active enzymes from the rest of the cell, a non-aqueous fractionation was carried out with 7-day old barley. The

Table 2

Radioactivity in 2,4-dinitrophenyhydrazine derivatives of compounds from tyrosine-^{14}C fed to all fractions of young barley

Barley Fraction	Volume Reaction Mixture (ml)	CPM in Keto Acid DNPH	CPM in PHPP DNPH	CPM in Other DNPH
chloroplast	4	2248	181	1407
supernatant	16	—	22	1410
brei	20	1700	66	486

chloroplast-rich fraction was of specific gravity 1.30-1.35 as judged by the chlorophyll-protein ratio and the presence of 3-phosphoglyceraldehyde dehydrogenase (NADP). To the fractions were added 1.0 ml 0.1 M tris buffer pH 7.4, and 1 uCi tyrosine-3-^{14}C (8.8 µCi/µmole). After 30 minutes at 37°, the reaction was stopped by freezing. Chromatography of the ether extract showed one radioactive compound which agreed in R_f value in two solvent system and in colour reaction (diazotized p-nitroaniline) with authentic p-hydroxy-benzaldehyde.

Up to this point, tyrosine of low specific activity had been used in an effort to minimize the effect of bacterial contamination. However, this meant that the specific activity of the product was also low, and if there were only a few molecules of the product present, detection and analysis became difficult. The use of sterile material and substrate was thought to overcome this problem and permit the use of tyrosine of higher specific activity.

Experiments with Tomato Seedlings

(i) The inhibitor α, α'dipyridyl prevents the enzymic oxidation of HG in mammalian systems [La Du and Zannoni 1955], and was used in an attempt to prevent further metabolism of any HG that might be formed.

Ten ml of a sterile solution of .02 M α, α'dipyridyl in .06 M phosphate buffer pH 6 were poured into two flasks of six 4-week old plants. Control plants received buffer alone.

After 12 hours (by which time the inhibitor had turned the agar in those flasks pink) 1.25 μCi of sterile tyrosine-UL-^{14}C (50 μCi/μmole) were added. After an additional 60 hours, when the experimental plants were becoming necrotic, the plant material was removed, rinsed well and extracted with boiling 80% ethanol.

Five main compounds were labelled, and their characteristics are shown in Table 3.

Table 3

Radioactive compounds after administering tyrosine-^{14}C to tomato seedlings

Spot	R_f System		Colour, pNA*	Spectrum	Relative Label	
	B	D			inhibitor	control
1	.78-.84	.57-.61		(p-hydroxy-benzoate)	++++	+++
2	.78-.84	.77-.80		peak 382	++++	+
3	.67-.73	.87		(HG)	++++	+
4	.39-.40	.83	green	332,sh300 (caffeate)	+	+++
5	.22-.30	.90-.93	reddish	378, sh386	++++	++++

*pNA - diazotized p-nitroaniline reagent.

Compound 3 co-chromatographs with HG. This experiment indicates that the metabolism of tomato is affected by the inhibitor, and that a buildup of HG seems to occur.

Because the presence of the inhibitor caused the plants to become necrotic, it was impossible to prepare chloroplasts from them, to find where the radioactive compounds were localized. Therefore, the use of the inhibitor was discontinued.

(iii) In the above experiment, the entrance of substrate depended upon transport across the membranes of the root hairs and stem. In an attempt to get the radioactive material to enter the plants as quickly as possible, sterile tyrosine was fed through cut stems. Sterile techniques were used throughout.

Ten stems of tomato seedlings were placed in each of three vials in a large sealed chamber. The sterile feeding solution (3 ml) was 5 μCi each of L-tyrosine-UL-^{14}C (50 μCi/μmole) in 0.1 M phosphate buffer pH 6.3. At 1, 2, and 4 hours, one vial was removed and the plants used for chloroplast and mitochondrion preparations. Chloroplast-rich, mitochondrion-rich and supernatant fractions were extracted and chromatographed.

Tyrosine was found to be taken up in both chloroplastidic and non-chloroplastidic protein (Figure 4), and into the ether-extractable material (Figure 5).

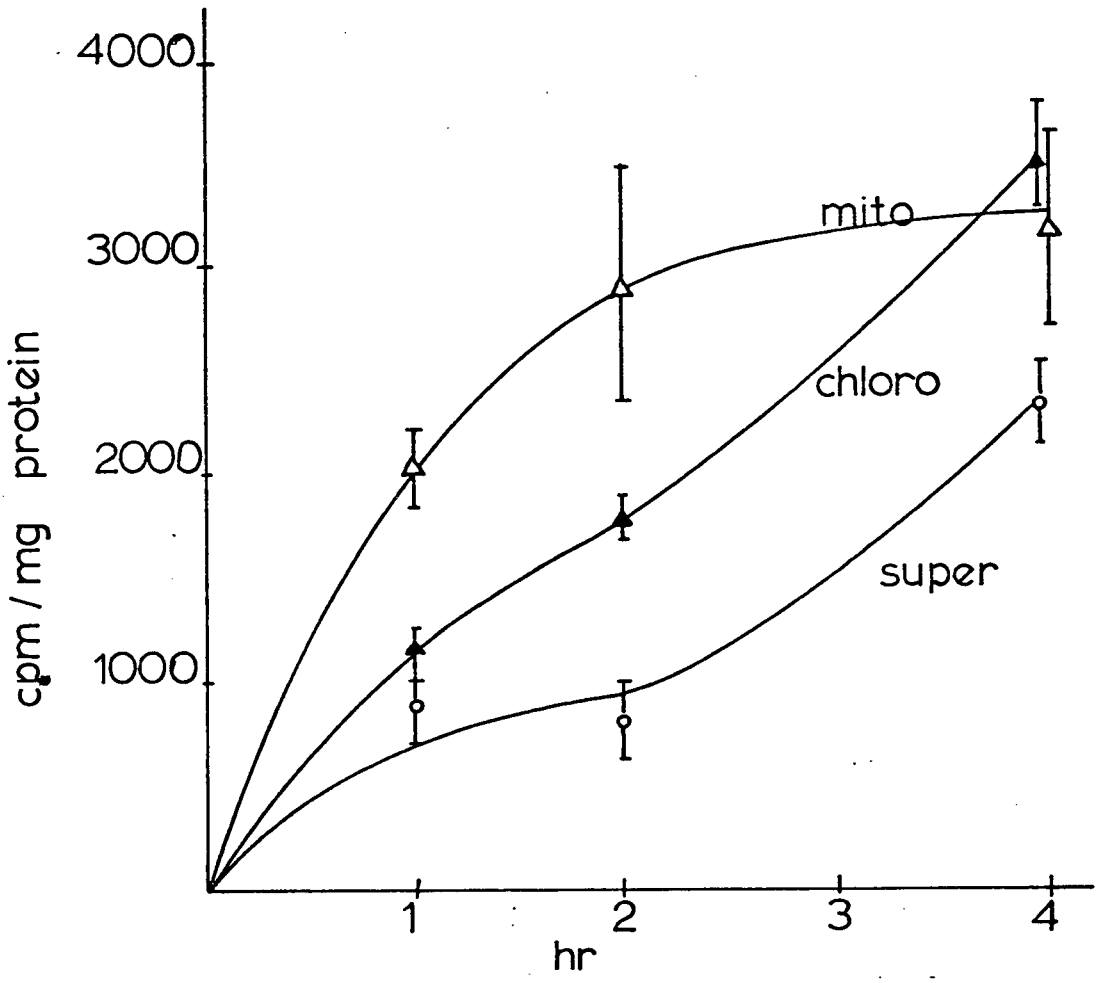

Figure 4. Increase with time of radioactivity in the protein of subcellular fractions of tomato plants after the administration of tyrosine-UL-^{14}C.

chloro: chloroplast-rich fraction ▲——▲

mito : mitochondria-rich fraction △——△

super : supernatant fraction o——o

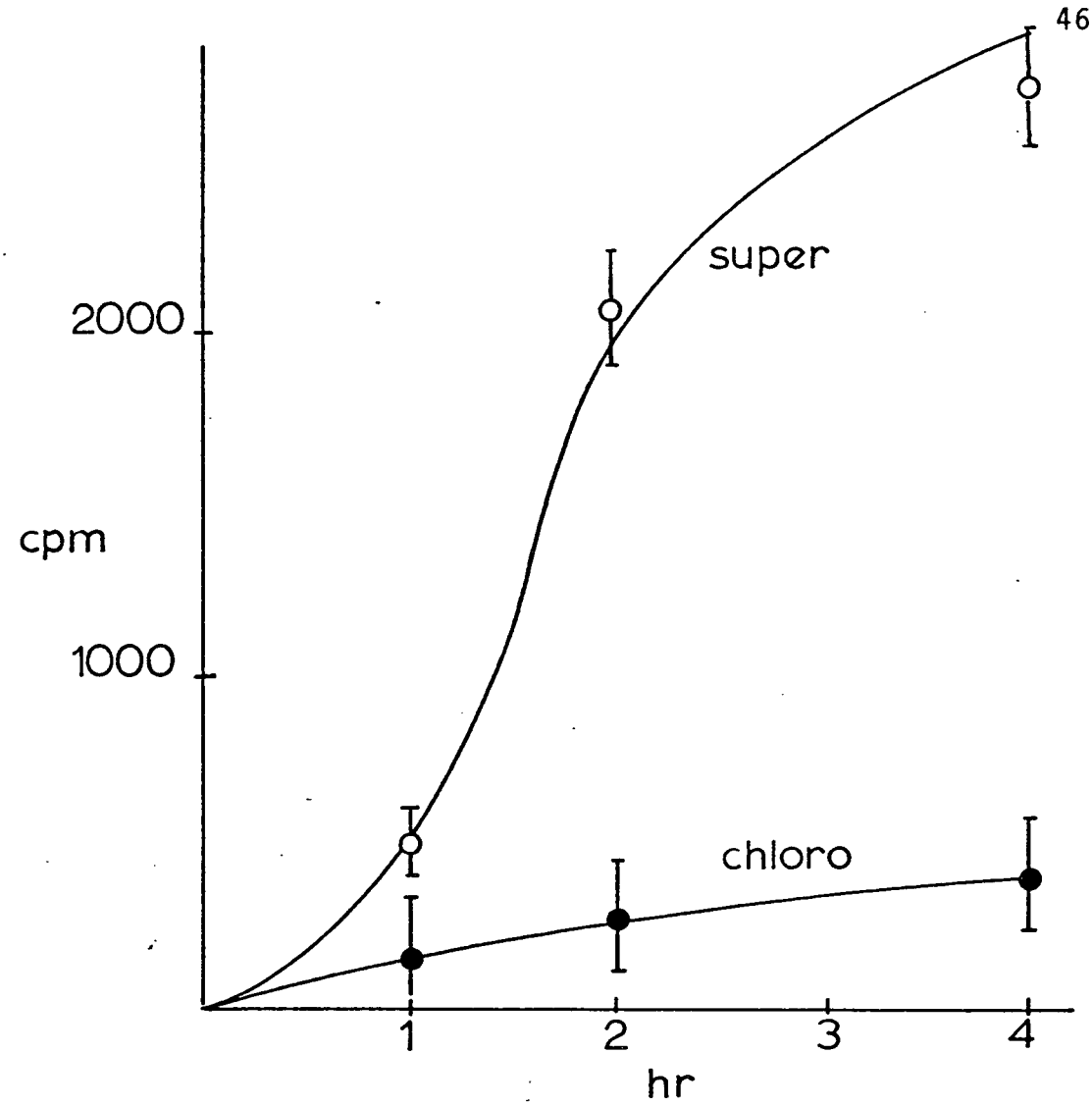

Figure 5. Increase with time of radioactivity in the ether extract of subcellular fraction of tomato plants after the administration of tyrosine-UL-^{14}C.

chloro ●——● : chloroplast-rich fraction

super o——o : cell supernatant

However, chromatography, followed by liquid scintillation counting of the appropriate spots on the chromatograms of the ether extracts showed no radioactivity in HG or any of the other expected phenolic acids in either supernatant of chloroplast fractions.

Experiments with *Ruta* Tissue Culture

The dual requirements of large amounts of material for chloroplasts extraction, and of sterility for long term feeding suggested the use of tissue culture. The strain of Ruta graveolens used does not form etiolated tissue that greens readily in the light.

(i) Using sterile techniques throughout, tissue about one month old was chopped and rinsed in 1% sucrose. Aliquots of about 0.5 gm were resuspended in 8 flasks; 4 in normal B5C2 medium and 4 in B5 medium without added amino acids. Into each flask were introduced 2 μCi tyrosine-UL-^{14}C of specific activity 350 μCi/μmole. The flasks were incubated at 20°C with shaking, in room light up to seven days. Pairs of flasks were removed at various times and chloroplasts were prepared from the cells. Figure 6 shows the uptake with time of tyrosine into protein. The tissues actively

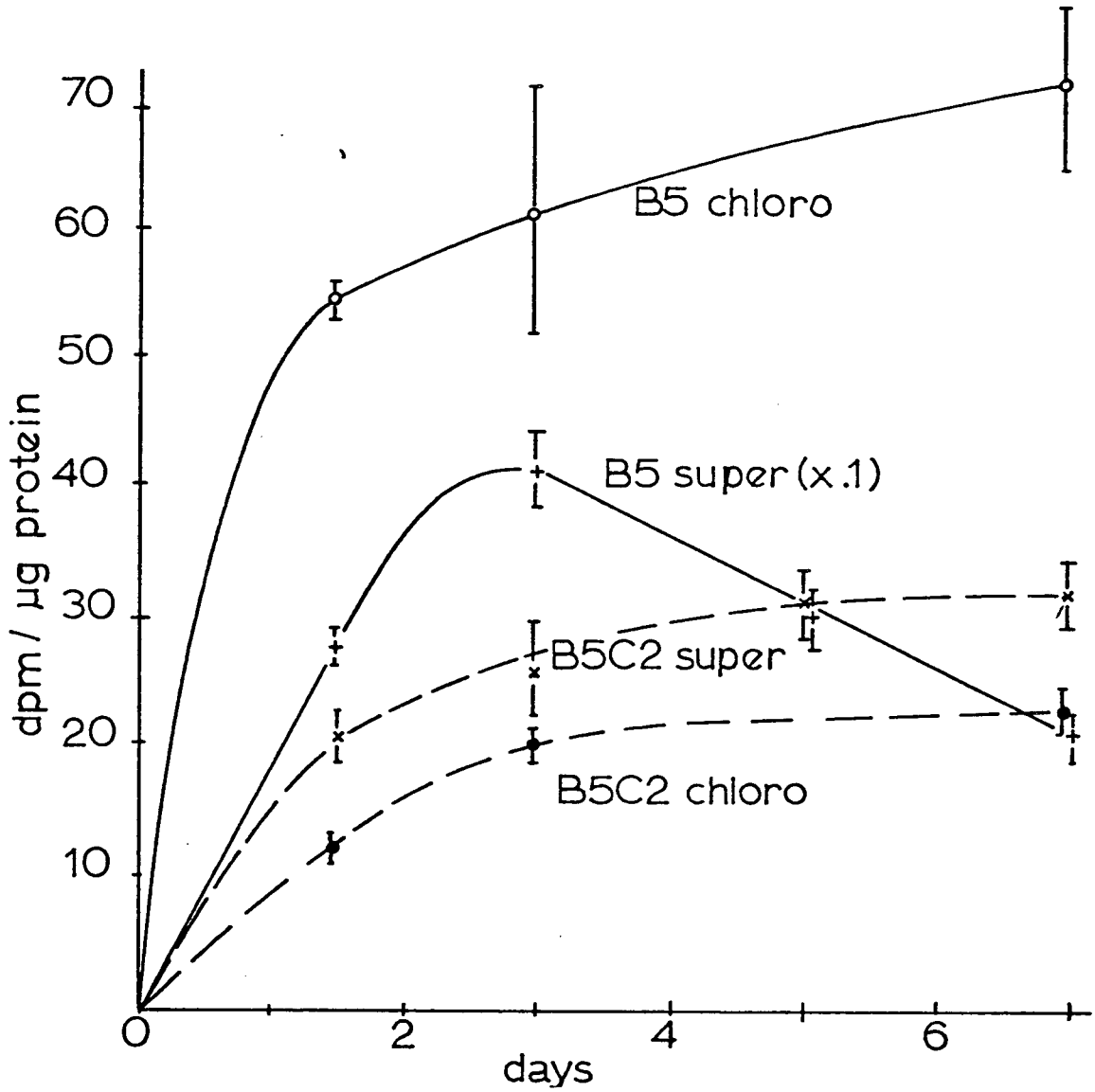

Figure 6. Increase with time of radioactivity from tyrosine-UL-^{14}C in the protein of subcellular fractions of Ruta cultures, growing on B_5 or B_5C_2 medium.

Ruta grown on B_5C_2 : chloroplast protein ●---●
: cell supernatant protein x---x
grown on B_5 : chloroplast protein o——o
: cell supernatant protein* +——+

*values shown are 1/10 actual values

metabolized the supplied material. Figure 7 shows that radioactivity recovered in the spots corresponding to HG increased with time, in the ether extract of the chloroplast-poor supernatant.

The Ruta tissue grown on the medium without added amino acids produced more HG than did the Ruta grown on B5C2 medium.

Chromatography of the chloroplast fractions followed by liquid scintillation counting showed no radioactivity at all in the spots corresponding to HG.

(ii) Compounds other than HG are formed by the Ruta cultures, however. A feeding similar to the last was set up, but in B5C2 medium for better growth. Up to two days, HG production appears the same in cells grown in both media (Figure 7).

Cells were removed at times to two days and extracted with boiling 80% ethanol. Radioactivity in at least two compounds increased with time. In solvent C these compounds have R_f values of 0.24 (yellow fluorescent under UV) and 0.52 (blue fluorescent under UV). The yellow spot corresponds to HG in R_f and fluorescence.

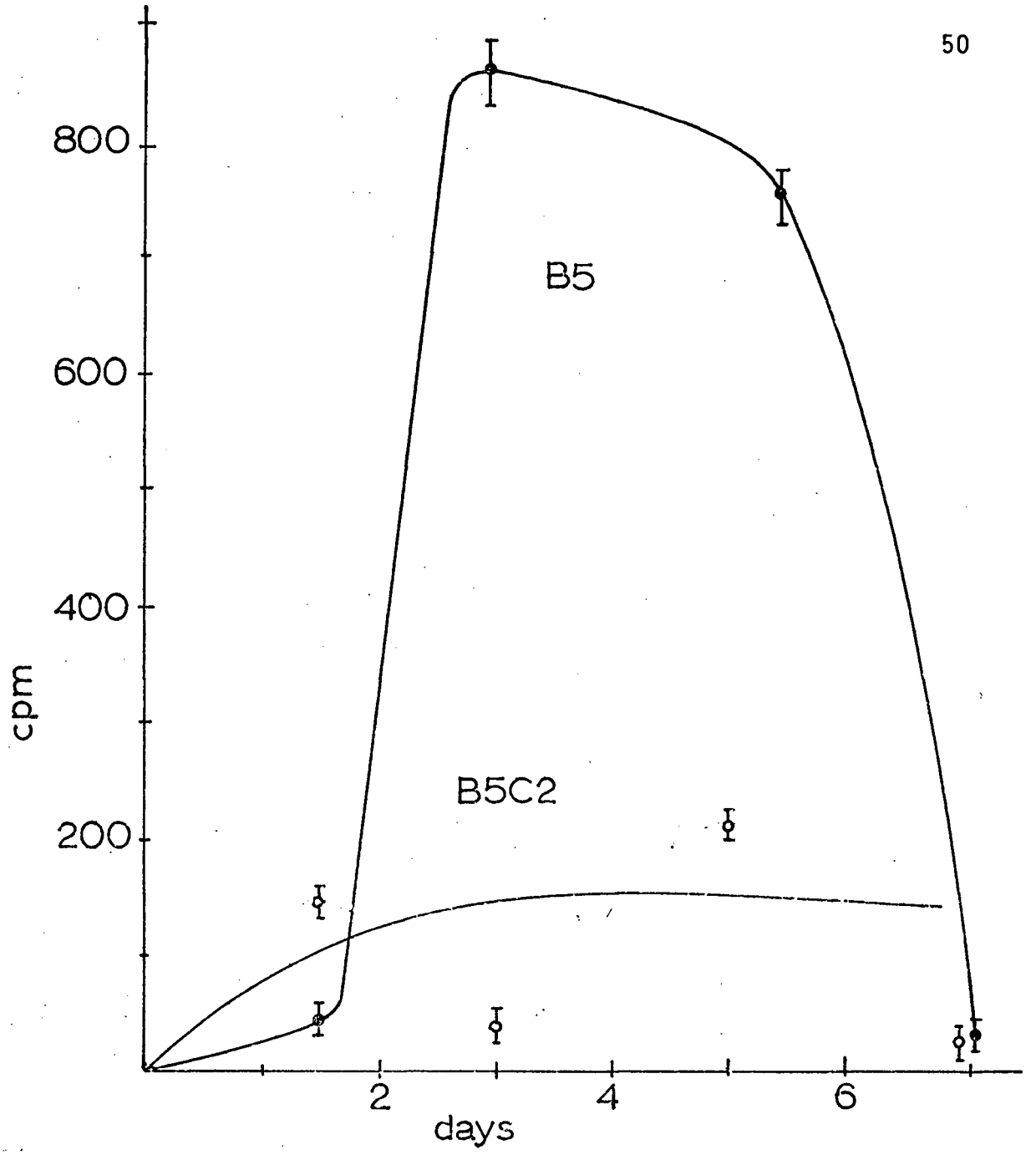

Figure 7. Change with time of radioactivity in homogentisic acid from Ruta cultures administered tyrosine-UL ^{14}C (see text for details).

grown on B_5C_2 medium o——o

grown on B_5 medium ●——●

(iii) The two experiments above consisted of a radioactive feedings, followed by extraction of chloroplasts, in an attempts to localize the product of a reaction. The following is an attempt to find the site of the reaction itself.

A non-aqueous cell fractionation was made of 4-week old Ruta tissue. The fractions were supplied with 0.5 µCi tyrosin-UL-^{14}C of 1 µCi/umole in 0.5 ml of 0.1 M tris buffer, pH 7.5. After one hour at 30° the reaction was stopped with o.1 ml of 1 M HCl, and the reaction mixture was extracted with ether. Chromatography followed by liquid scintillation counting showed that HG was scarcely radioactive but a blue fluorescent spot of R_f 0.15 in system C showed some radioactivity. Most conversion had taken place in the fraction of ρ 1.20, although the chloroplast fraction is ρ 1.30-1.35.

DISCUSSION

There are several general conclusions to be drawn from this series of experiments. Firstly it appears that HG is formed from tyrosine by sterile plant material. This confirms the work of Ellis [1970]. The formation of PHPP in this material is not definitely known, although it is almost certainly the intermediate in the formation of HG from tyrosine. Secondly, HG is not stored in the chloroplast, as shown by the experiments in which chloroplasts were prepared from plants fed radioactive substrate. Thirdly, either these conversions do not take place in the chloroplasts, or the chloroplastidic enzymes have such a high turnover number that the intermediates are never detected.

The simplest explanation would be that the ring of the plastoquinone nucleus is formed outside the chloroplast and then transported into it. Some support for this idea comes from the discovery of L-amino acid oxidase in glyoxysomes [Beevers 1970]. This enzyme would form PHPP from

tyrosine. Further, in Ricinus glyoxysomes, tyrosine is converted to HG, although this was not a sterile preparation [Kindl and Ruis 1971].

The stage at which the p-dihydric ring is oxidized to the quinone is unknown, as is the site of this oxidation. While phenolases with o-diphenol oxidase activity are present in chloroplasts, mitochondria [Parish 1972], microsomes, microbodies and soluble fractions [Ruis 1972], the location of p-diphenol oxidase is unknown.

It appears most probable that the attachment of the plastoquinone isoprenoid tail occurs in the chloroplast, since lipid metabolism is very active in these organelles [Rogers et al. 1968].

With information like this, a picture of intracellular transport can gradually be built up. It is still not certain that this is the way that plastoquinones are formed; it is merely a plausible route.

PART II

INTRACELLULAR LOCALIZATION OF ENZYMES OF PHENYLPROPANOID METABOLISM IN POTATO TUBER

RESULTS

General Observations

Nature of the bands observed after density gradient centrifugation

Potato — In most preparations, about four main bands were noted. These correspond in Figures 8 to 12, to peaks of protein content, or of absorbance at 540 nm. Band 1 always contained catalase (an enzyme which is characteristic of the microbody) in high specific activity. The buoyant density of this band was 1.25 to 1.26. Band 3 contained fumarase, a mitochondrial enzyme, and had a buoyant density of 1.18 to 1.19. Bands 1 and 3 corresponded to the microbody and mitochondrion bands identified in potato by Huang and Beevers [1971].

A band of density 1.22 was not described by these workers, although a low peak of enzyme activity appeared here in their graphs of gradient profiles. This band appeared in the gradient profiles of other workers as well [Ruis 1971].

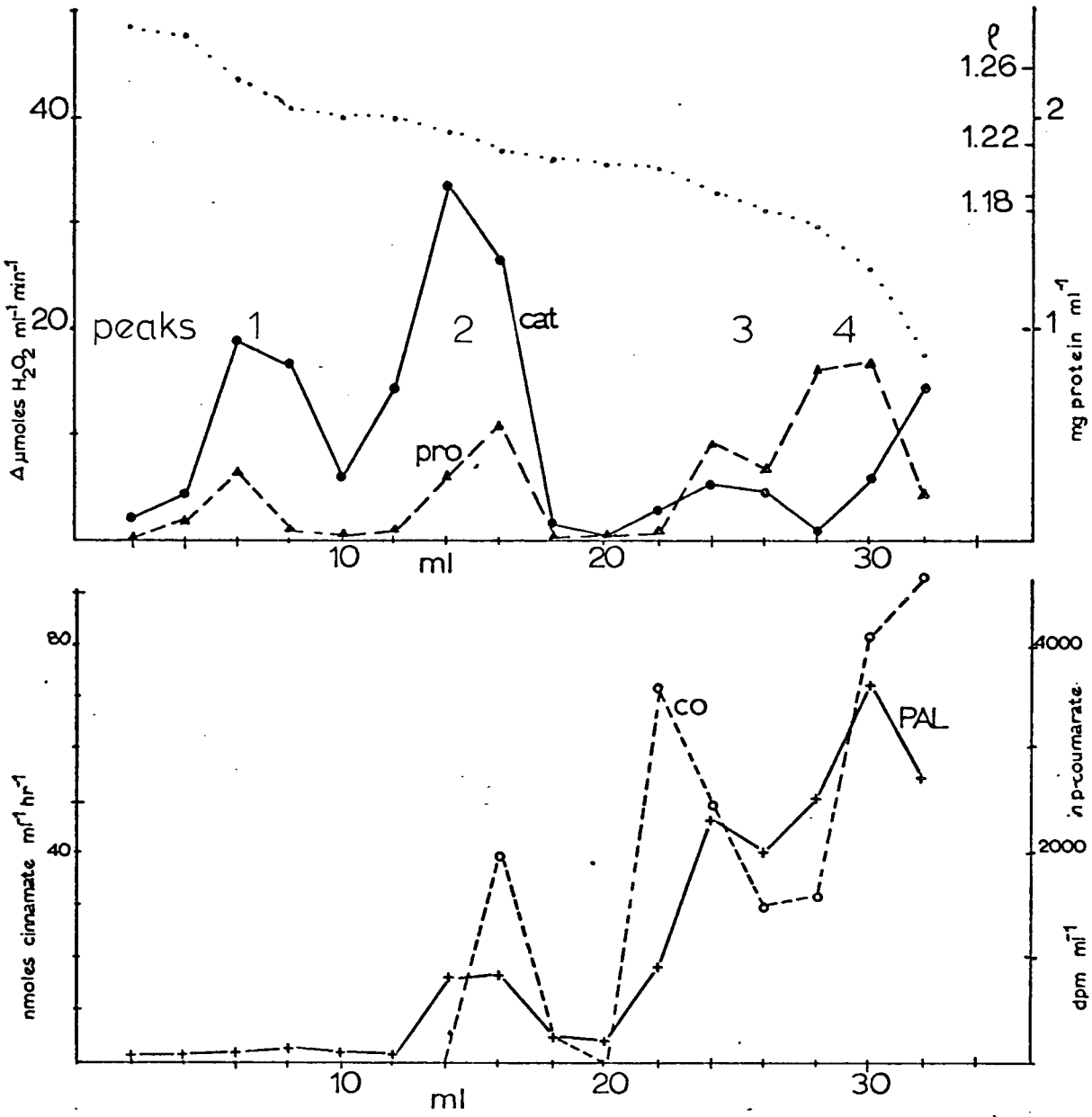

Figure 8. Gradient profile of enzymes from the 10,000xg pellet from 100 gm of potato disks aged in the light.

protein: pro ▲---▲, catalase: cat ●——●, PAL: +——+, cinnamic hydroxylase: co o---o, specific gravity: ρ ●···●

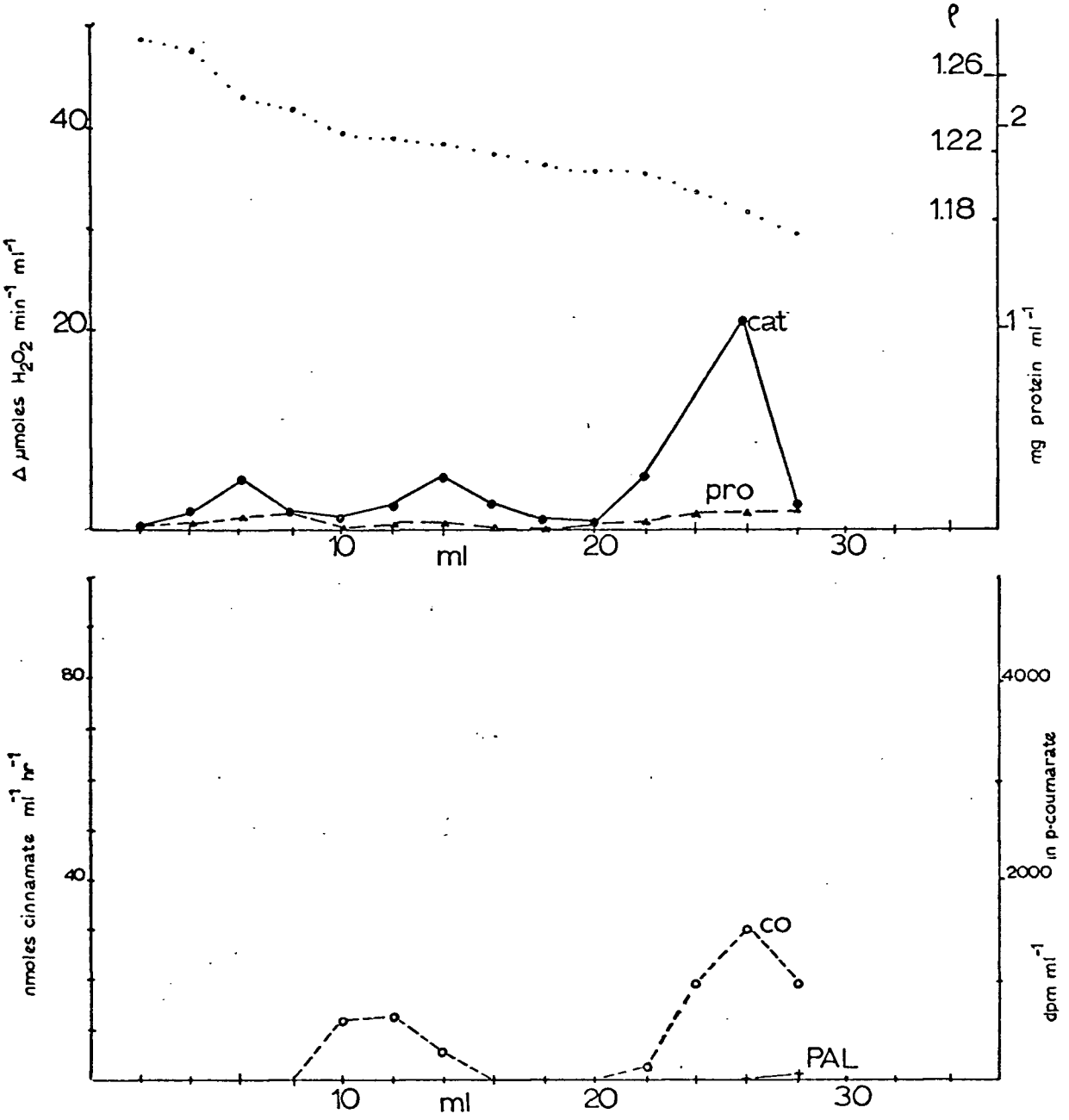

Figure 9. Gradient profile of enzymes from the 10,000xg pellet from 100 gm of dormant potato.

Protein: pro ▲---▲, catalase: cat ●——●, PAL: +——+, cinnamic hydroxylase: co o---o, specific gravity: ρ ●···●

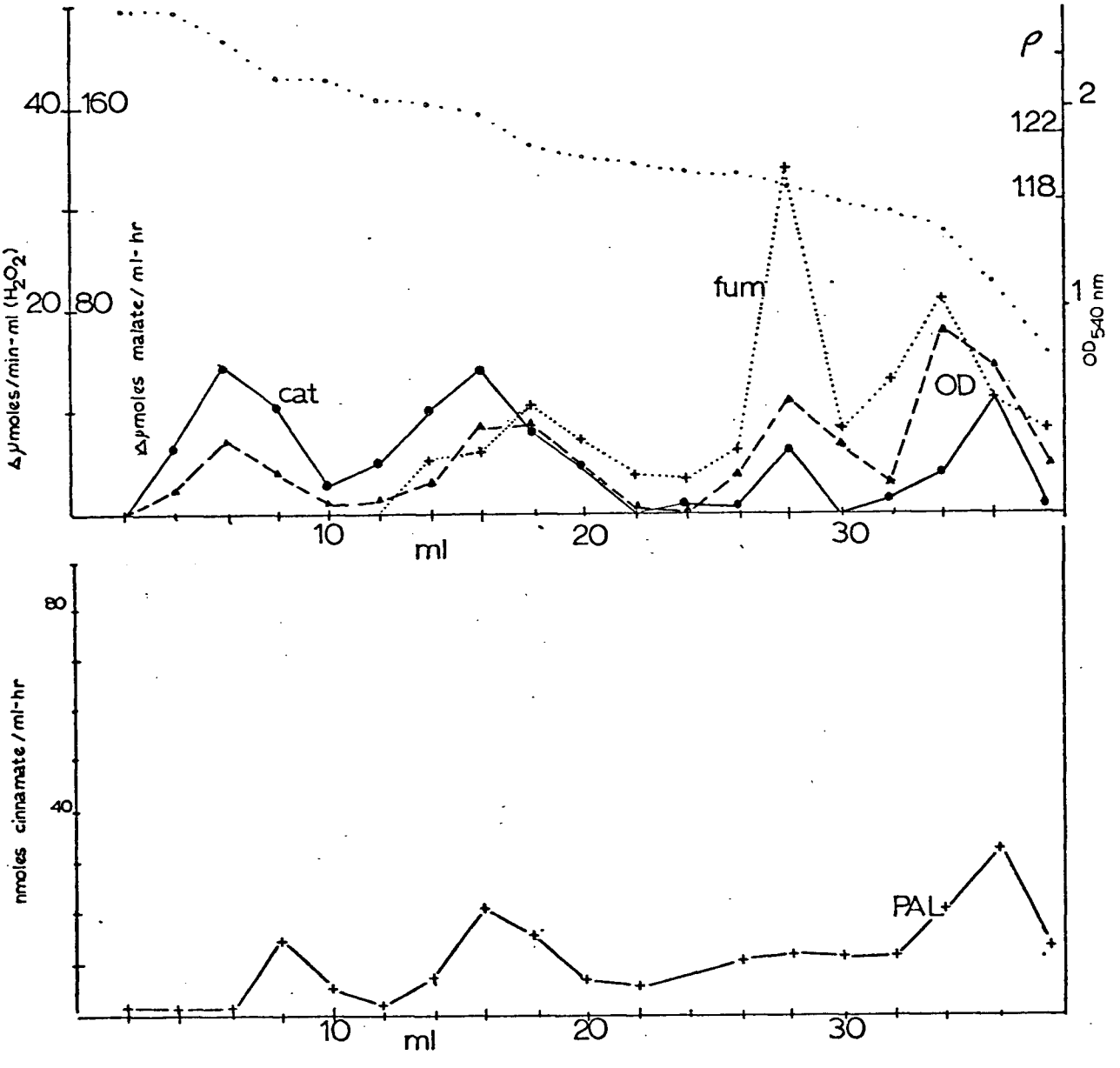

Figure 10. Gradient profile of enzymes from the 10,000xg pellet from 70 gm of potato disks aged in the light.

Optical density at 540 nm: ▲---▲,
catalase: cat ●——●, fumarase: fum +•••+,
PAL: +——+, specific gravity: ρ ●•••●

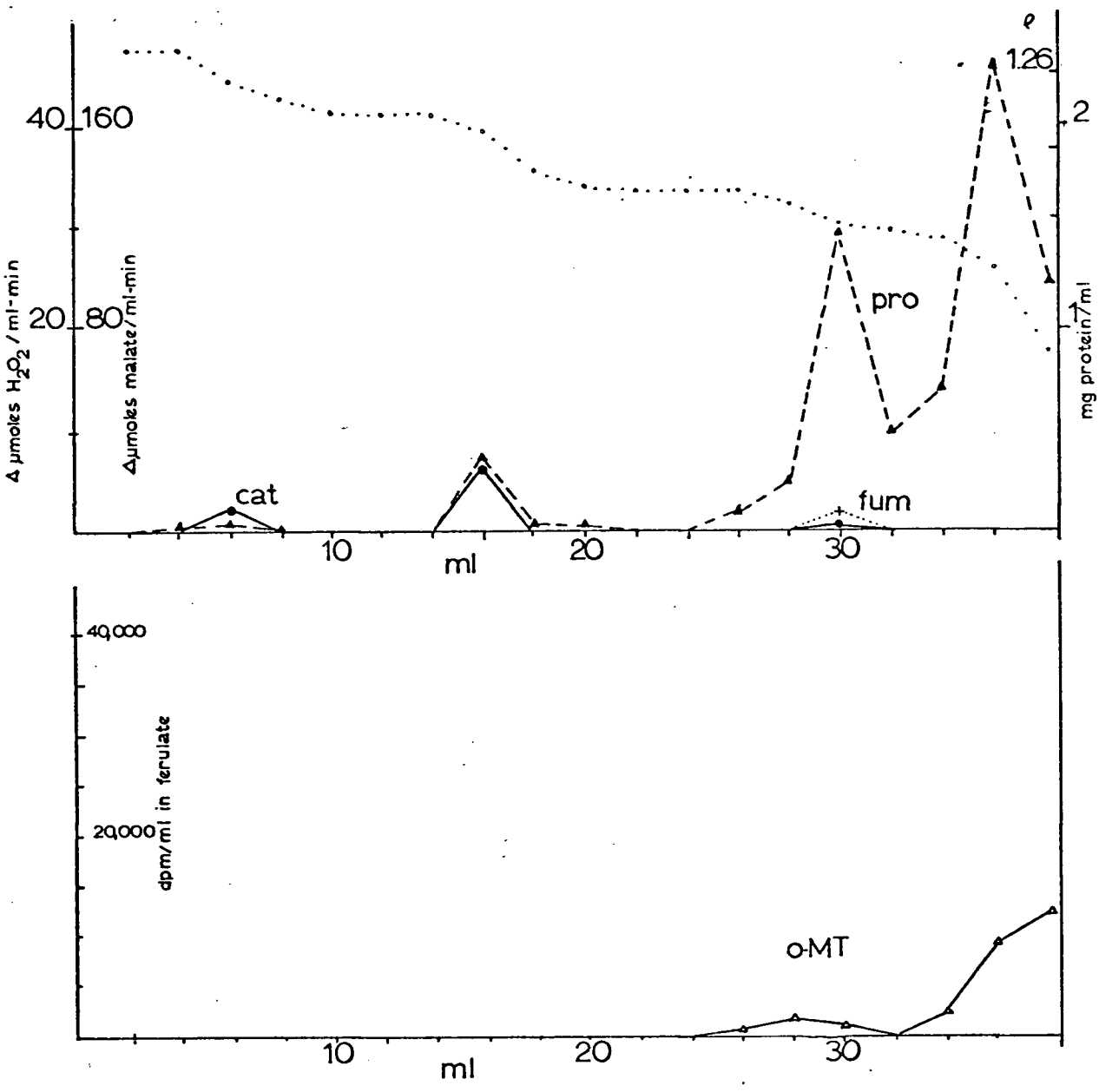

Figure 11. Gradient profile of enzymes from the 10,000x<u>g</u> pellet from 45 gm of dormant potato.

protein: pro ▲---▲, catalase: cat ●——●, fumarase: fum +•••+, O-methyl transferase: O-mt Δ——Δ, specific gravity: ●•••●

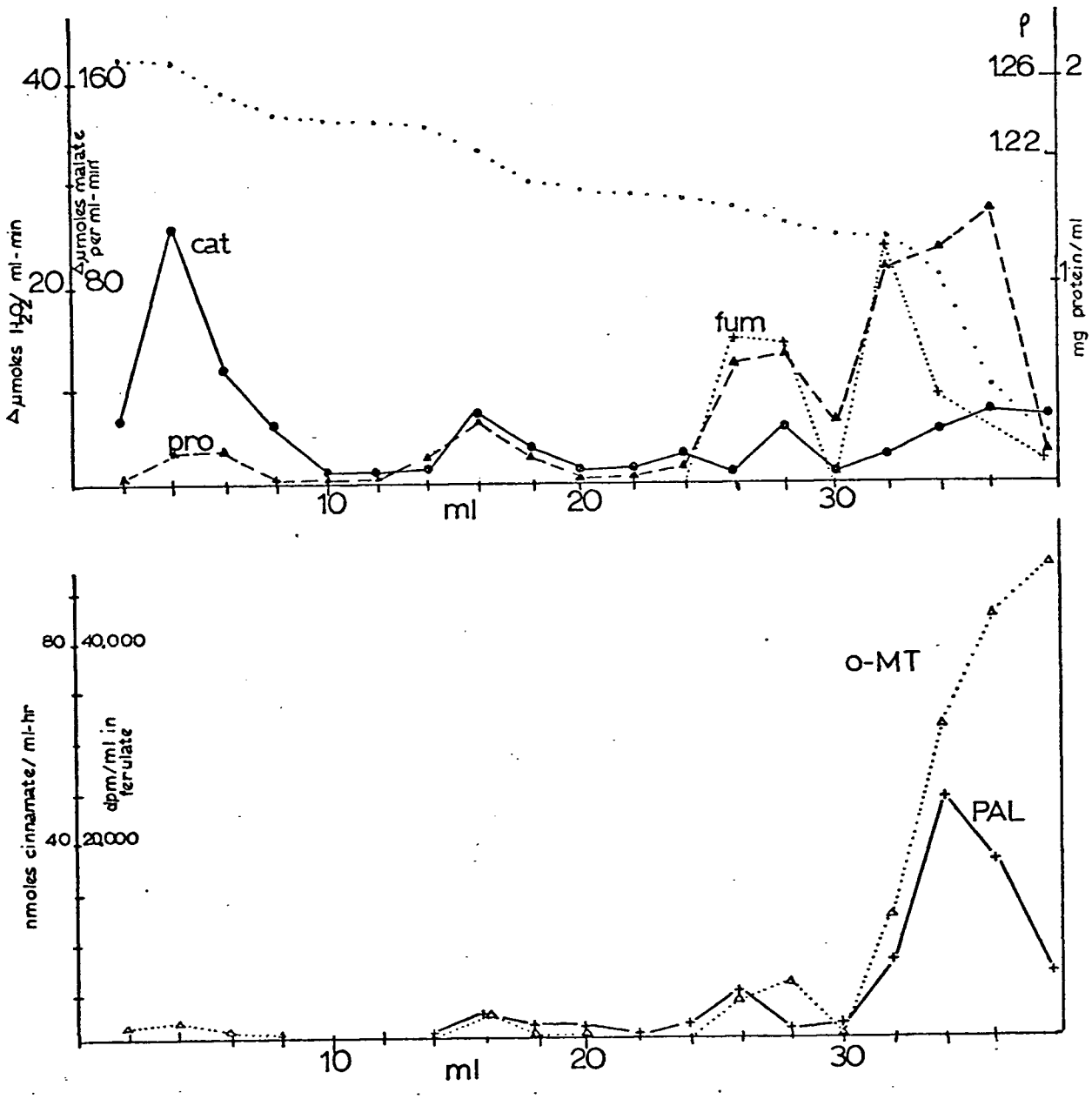

Figure 12. Gradient profile of enzymes from the 10,000x*g* pellet from 80 gm of potato disks aged in streptomycin in the light.

protein: pro ▲---▲, catalase: cat ●——●,
fumarase: fum +•••+, PAL: +——+,
O-methyl transferase: O-mt Δ•••Δ,
specific gravity: ρ ●•••●

In many plants e.g. spinach, the mitochondria are of this buoyant density [Huang and Beevers 1970], but in potato band 3 was always found to contain more fumarase than band 2. Since the size and enzyme activities of this band were extremely variable, it well might be that it represented broken and aggregated microbody and mitochondrion particles. It is known that damaged castor bean glyoxysomes sediment with the mitochondria [Gerhardt and Beevers 1970].

Further evidence for this interpretation comes from Figure 10, which represents an experiment in which only one-half the usual volume of grinding medium was used. During disruption, the plant material browned slightly. This, then, was obviously a damaged preparation and it is only in this preparation that PAL was located in band 1, and fumarase in band 2, as well as in the usual bands.

The fourth band, of buoyant density 1.15 to 1.17, corresponded to the heterogenous band investigated by Ruis [1971]. Particles which sediment from the applied layer, but which do not penetrate the gradient, contained some of the o-diphenolase of the potato tuber. It was later shown that this enzyme is present in the microsomes of potato [Ruis 1972], so perhaps there was some microsomal contamination in band 4. Fumarase, too, was also found in band 4;

perhaps this represented the 'light mitochondria' found in preparations from sliced potato [Verleur et al. 1970].

Spinach — Spinach preparations also displayed 4 main bands (Figure 13).

Band 1 contained only catalase, and is probably peroxisomal in nature. Band 2 contained a very high level of catalase also, but since this band was also rich in fumarase, it probably represented the mitochondrion-rich fraction. This also might be a case of damaged microbodies sedimenting with the mitochondria [Gerhardt and Beevers 1970]. Band 3 contained a low level of fumarase, a high catalase level, and a very high level of protein. Almost all the chlorophyll in the preparation was located in this band; it probably represented the chloroplasts. Band 4 seemed to be very heterogenous, and its nature is unknown.

Effect of antibiotics on the presence of enzymes

Most experiments were performed on potato tissue that had been incubated for 18 hours in distilled water. However, there is a significant bacterial flora inside the

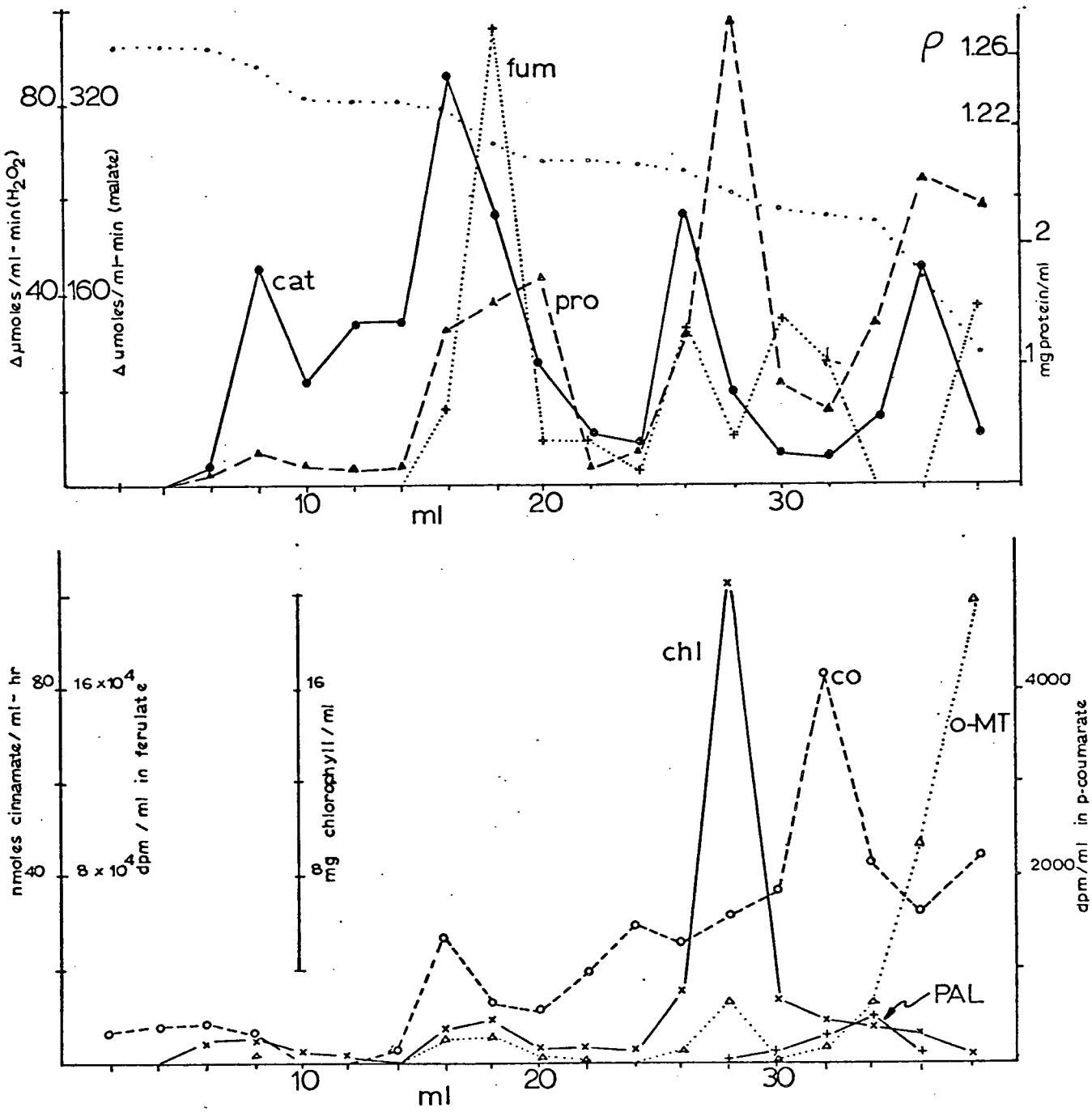

Figure 13. Gradient profile of enzymes from the 10,000x<u>g</u> pellet from 50 gm of spinach.

protein: pro ▲---▲, catalase: cat ●——●, fumarase: fum +•••+, specific gravity: ρ o•••o, cinnamic hydroxylase: co o---o, O-methyl transferase: O-mt Δ•••Δ, chlorophyll: chl x——x, PAL: +——+

potato tuber* [R. Bandoni, pers. comm.] and this, as well as those contaminants introduced during non-sterile handling could result in a massive growth of bacteria during the incubation period. To see if bacteria made any difference to the activity of some of the enzymes in question, potatoes were incubated in the presence of streptomycin sulfate (1 gm/l).

Figure 12 shows the gradient profile of this tissue. There did not seem to be any significant effects of the antibiotic.

Tissue incubated in neomycin sulfate was used for the preparation of May 16 (Table 4), and again, there did not appear to be any significant differences in PAL production or distribution.

It seems that the presence or absence of bacteria did not markedly affect the production and distribution of several of the enzymes examined in this study.

Phenylalanine ammonia-lyase (E.C. 4.3.1.5)

Potato tissue, especially after slicing and ageing, contains many aromatic compounds [Hanson and Zucker 1963].

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*Electron micrographs of the 10,000xg pellet showed microbodies and mitochondria, but no bacterial cells that could be positively identified.

Table 4

Distribution of PAL in subcellular fractions of potato aged in the light

Cell Fraction	Experiment 1		Experiment 2		Experiment 3		
	S.A.*	%	S.A.*	%	S.A.*	%	
10,000x*g* pellet	13.9	2.4	7.14	4.3	5.4	2.6	microbodies 0% band 2 .13 mitochondria .24 band 4 and soluble enzymes 2.02 2.4%
100,000x*g* pellet (microsomes)	8.75	4.8	3.03	1.8	20.4	9.9	
100,000x*g* supernatant	164.0	92.8	156	94.0	180.1	87.6	
	186.6	100.0	166.2	100.1	206.9	100.1	

*Specific Activity in μmoles cinnamic acid formed hr^{-1} gm fresh $weight^{-1}$.

It is therefore a danger that the measurement of PAL by change in optical density at 290 nm might be confounded by the release during incubation of previously bound aromatic compounds. On the other hand, the destruction of particles on long incubation reduces the apparent optical density in those fractions.

To evaluate these factors, the change in optical density measured in aliquots of material incubated overnight in buffer without added phenylalanine was compared to the changes obtained during the usual PAL assay. Figure 14 indicates that the errors introduced by overnight incubation with undialysed particulate fractions were relatively minor.

The 10,000x*g* pellet, 100,000x*g* pellet and the 100,000x*g* supernatant from aged potato were examined for the presence of PAL (Table 4). While 90% of the enzyme appeared to be soluble, there was considerable activity in both the organelle and microsomal fractions (10,000x*g* and 100,000x*g* pellets, respectively). The latter fraction contains about 10% of the total activity of the cell; it is unlikely that 10% of the soluble material would be carried by the unwashed pellet. Thus it appears that some of the enzyme is soluble and some sediments with the organelles.

The nature of the 2-3% of the total enzyme that is located in the 10,000x*g* pellet was investigated with a

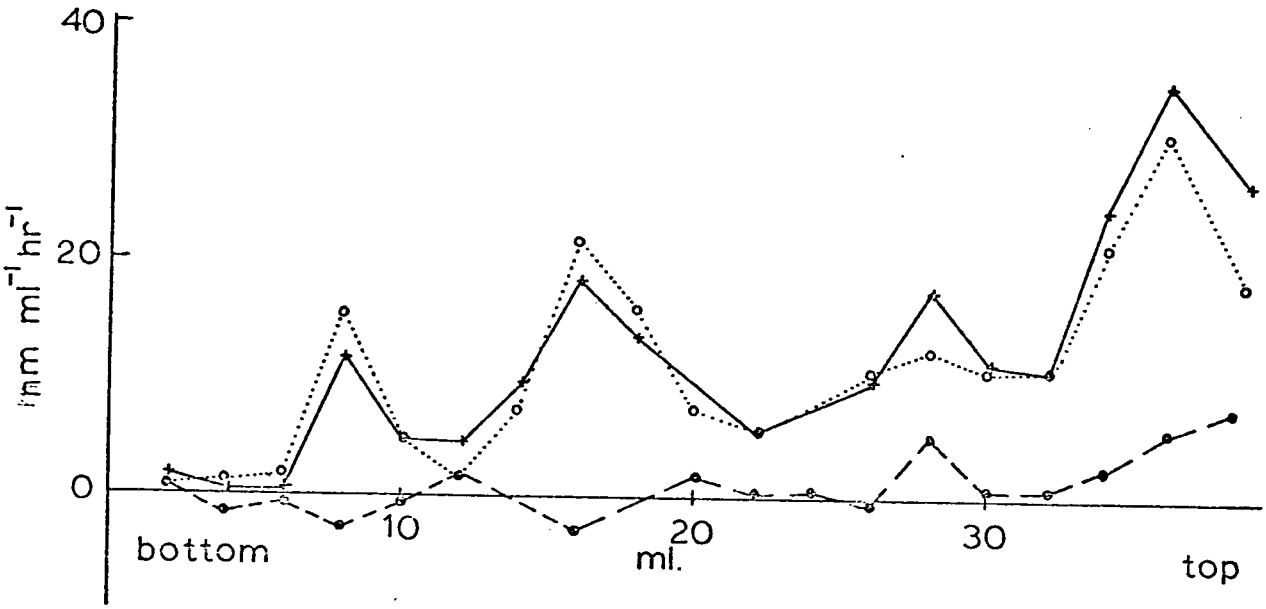

Figure 14. Assessment of errors in the PAL assay due to overnight incubation.

usual assay: x——x

control (without phenylalanine): ●---●

actual change in nmoles/ml/hr: o•••o

discontinuous gradient. Figures 8 and 12 are typical gradient profiles. PAL appeared in the soluble fractions of all preparations. PAL also appeared consistently in the bands 2, 3 and 4.

There is the possibility that the enzyme molecules (or particles) were adsorbed onto, or adhered to the surfaces of the larger organelles. To test this possibility, a partially purified preparation of PAL from 18-hour old potato disks was put through a Sephadex G-200 column and this preparation was incubated with the particulate fraction from fresh potatoes, which had little or no PAL (see Figure 9). If there had been adherence of PAL to the organelles from the fresh potato, then this should have been distinguishable by the distribution of the enzyme on a sucrose density gradient. Figure 15 shows the results of such a gradient separation, and shows that, within experimental error, there was no 'smearing' of the soluble PAL on the gradient.

Cinnamic Acid 4-Hydroxylase (E.C. 1.99.1)

The effects of ageing in the light on the production of this enzyme were examined in potato discs. Table 5 shows that specific activity increased after ageing when expressed either as units per mg protein or per gm fresh weight.

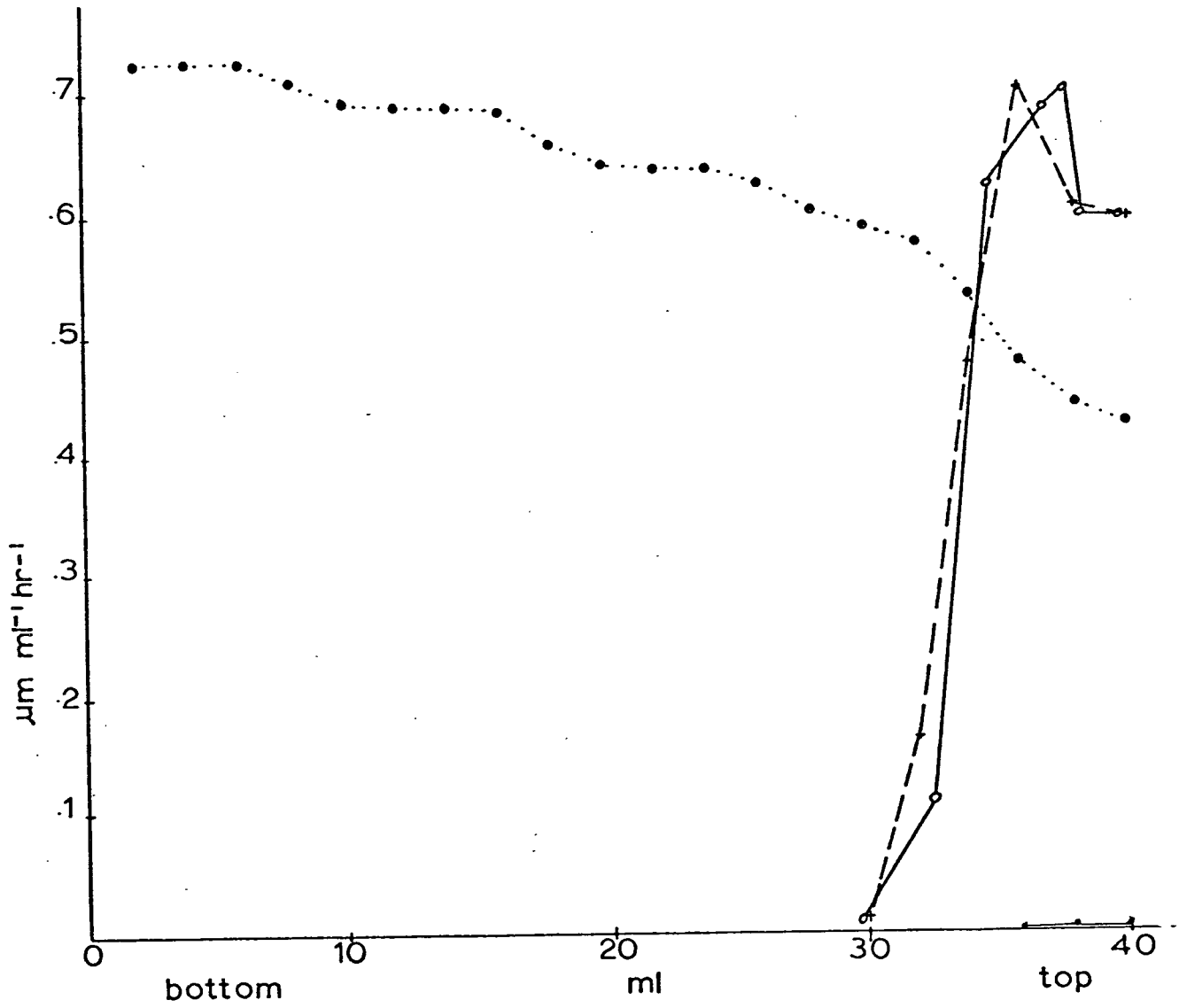

Figure 15. Gradient profile of partially purified PAL mixed with organelles from dormant potato.

PAL alone: o——o

organelles alone: ●——●

PAL plus organelles: +---+

specific gravity: ●···●

Table 5 also shows that most of the enzyme appeared in the 0-30% ammonium sulfate fraction in contrast to PAL, which was found in the 27-40% fraction [Havir and Hanson 1968].

Table 5

The effect of ageing in the light on the activity of cinnamic acid 4-hydroxylase in potato

	AGE	
	Dormant	Aged 18 Hours
Experiment 1*	641±13% dpm/gm fr wt	1440±4% dpm/gm fr wt
Experiment 2**		
0-30% ammonium sulfate	652 cpm/mg protein	20,800 cpm/mg protein
30-70% ammonium sulfate	142 cpm/mg protein	1730 cpm/mg protein

*Experiment 1: material chopped to preserve organelles.

**Experiment 2: material ground for complete extraction.

While there was relatively little activity in the 10,000x<u>g</u> pellet in the fresh material, there was up to 20% of the total activity in this fraction in aged material (Table 6). Much less of the total activity of cinnamate

Table 6

Subcellular distribution of cinnamic acid 4-hydroxylase in dormant potato tuber and tissue aged in the light

Cell Fraction	Dormant Tissue			Aged Tissue		
	activity*†	%	organelles	activity*†	%	organelles
10,000xg pellet	.165	2.4±.2		3.16	20.4±3.1	0%
including:						
microbodies			.06%			0%
band 2			.06%			1.9%
mitochondria			.17%			6.3
band 4			2.1 %			12.2
100,000xg pellet (microsomal)	.900	13.7±4.6		9.76	67.3±4.0	
100,000xg supernatant (soluble)	5.52	84.0±20		1.17	12.2±.72	
	6.59			14.7		

*Activity in nmoles/hr/gm fresh weight.

†Determinations done in duplicate.

hydroxylase was found in the soluble fraction in aged than in fresh potato; it appears that the enzyme becomes particulate as the potato ages.

Of the material which is found in the 10,000x*g* pellet, there appears to be the same distribution of enzyme as was shown by PAL; cinnamate hydroxylase occurred in all organelles except the microbodies.

In spinach (Table 7, Figure 14), some cinnamate hydroxylase did appear to be associated with the peroxisomes.

Table 7

Distribution of cinnamic acid 4-hydroxylase in the 10,000x*g* pellet of spinach

peroxisomes	6.8%
mitochondria	11.7%
chloroplasts	22.7%
band 4	58.8%

O-Methyl Transferase Methylating Caffeic Acid (E.C. 2.1.1)

Ageing in light caused a 2-fold increase in the amount of this enzyme (Table 8), but there did not appear to

Table 8

Subcellular distribution of O-methyl transferase in dormant potato tuber and tissue aged in the light

Cell Fraction	Dormant Tissue		Aged Tissue	
	activity*†	%	activity*†	%
10,000xg pellet	.009	4.7±.98	.015	2.9±.84
100,000xg pellet (microsomal)	.004	2.1±.47	.008	1.4±.21
100,000xg supernatant (soluble)	.185	93.2±16	.453	95.6±15.3
	.198		.476	

*nmoles/hr/gm fresh weight.

†determinations done in duplicate.

be any change in the distribution. In both fresh and aged tissue, about 95% of the total activity was soluble.

In two different experiments (Figures 11 and 12), the 10,000x*g* pellet was examined on a sucrose gradient. In both cases, there appeared enzyme activity with the various bands, but only about 5-10% of the applied activity was recovered in these bands, and the rest of the activity remained in the supernatant above the sucrose gradient.

The gradient distribution in spinach is very similar (Figure 13).

Shikimic Acid:NADP Oxidoreductase (E.C. 1.1.1.25) (SOR), Prephenic Acid:NADP Oxidoreductase (E.C. 1.1.1) (POR)

These two enzymes behaved in a similar manner. Both increased in activity on ageing, but the extent of increase in unknown, for in one experiment (Table 9), neither enzyme could be located in fresh potato, while in another experiment, considerable activity was present before ageing. In any event, there was at least a two-fold increase on ageing.

These enzymes showed very similar distribution patterns; both were exclusively soluble. While SOR is quite stable during the period of centrifugation, 40% of the original activity in POR was lost during fractionation.

Table 9

Subcellular distribution of shikimic acid oxidoreductase (SOR) and prephenic acid oxidoreductase (POR) in dormant potato tuber and tissue aged in the light

Experiment	Enzyme	Tissue	Fraction	Activity µmoles/hr/gm f.w.	% Total
1	SOR	dormant	10,000xg pellet	0	
			microsomal	0	
			soluble	0.524	100%
		aged	10,000xg pellet	0	
			microsomal	0	
			soluble	1.17	
	POR	dormant	10,000xg pellet	0	
			microsomal	0	
			soluble	0.250	
		aged	10,000xg pellet	0	
			microsomal	0	
			soluble	0.626	100%
2	SOR	dormant	10,000xg pellet	0	
			microsomal	0	
			soluble	0	
		aged	10,000xg pellet	0	
			microsomal	0	
			soluble	1.56	100%
	POR	dormant	10,000xg pellet	0	
			microsomal	0	
			soluble	0	
		aged	10,000xg pellet	0	
			microsomal	0	
			soluble	0.786	100%

In spinach, POR was present in the 10,000x*g* supernatant in trace amounts. It was not established whether the enzyme was soluble or particulate. Higher activities of SOR were present, and the activity was all soluble.

p-Coumaric Acid Hydroxylase

No hydroxylation of *p*-coumaric acid to caffeic acid by extracts of aged potato discs could be detected by any of the methods tried (see Methods).

Alcohol Dehydrogenases

Three alcohol dehydrogenases separable on Sephadex G-200, and with different specificities have been found in fresh potato [Davies *et al*. 1972]. One enzyme requires NAD and is active with alkyl alcohols; the second is an isoprenoid alcohol enzyme using NADP or NAD, and the third enzyme oxidizes aromatic alcohols with NADP.

In crude enzyme preparations from 'Netted Gem' slices aged in the light and preparations from which low molecular weight compounds had been removed, the aromatic alcohol dehydrogenase (NADP) as described by Davies *et al*. was not present. Table 10 compares the activities found in

'Netted Gem' with those which were found in the potatoes examined by Davies *et al*.

Table 10

Potato alcohol dehydrogenases as described by Davies *et al*., and reduction of aldehydes by extracts of aged 'Netted Gem' slices

Substrate	Davies *et al*. Relative Activity*		'Netted Gem' ΔOD_{340}/min/ml	
	NADH	NADPH	NADH	NADPH
acetaldehyde	1	0	.066	.026
benzaldehyde	0.012	1	0.14	.008
vanillin	0	8	0	0
p-hydroxybenzaldehyde	0	4.9	0	0
m-hydroxybenzaldehyde	0	1.3	0	0

*Relative to the rate with acetaldehyde for the alkyl ($NADH_2$) enzyme and to the rate with benzaldehyde for the aryl ($NADPH_2$) enzyme.

Biosynthetic Capabilities of Subcellular Fractions Supplied with Radioactive Substrate

Potato discs aged 18 hours in the light in neomycin sulfate were separated into a particulate fraction (10,000x*g* fraction) containing microbodies and mitochondria, a microsomal pellet (100,000x*g* pellet) and a soluble fraction (100,000x*g* supernatant). Tyrosine-2-^{14}C (50 µCi/µmole, 0.5 µCi/feeding) and phenylalanine 1-^{14}C (16.5 µCi/umole, 0.5 µCi/feeding) were fed to aliquots of each fraction. After one hour of incubation, the fractions were extracted and chromatographed in the usual manner. Compounds corresponding to fogged areas on radioautograms were eluted and re-chromatographed with various authentic compounds in solvents A, D and F.

Phenylalanine feeding: In all cases, only cinnamic acid was labelled. This suggests that the enzyme catalyzing chain shortening is very active. No *p*coumaric acid became labelled, but this is to be expected as the cinnamic acid 4-hydroxylase reaction requires the presence of $NADPH_2$.

Tyrosine feeding: In aged potato there was no trace of TAL activity. Compounds which co-chromatographed in three solvent systems with *p*-hydroxyphenylacetic acid (PHPA) and *p*-hydroxyphenylpyruvic acid (PHPP) were formed from the

administered tyrosine. It would appear that the fate of tyrosine in potato is transamination followed by chain shortening.

The microsomal fraction formed very little PHPA but it did form PHPP. The extract of the particulate fraction contained both compounds. It is concluded that the first reaction took place in both fractions, while the second reaction took place mainly in the larger organelles.

DISCUSSION

Distribution of PAL in Subcellular Fractions from Aged Potato Tuber

In aged potato, PAL was found in every subcellular fraction except the microbody fraction (Table 4). Ruis [1971] could find no trace of PAL in these organelles. Since these fractions were all unwashed, it would be a mistake to claim that an enzyme is localized in an organelle because it is found in a fraction rich in that organelle.

Such a distribution could arise in any of several ways. Firstly there could be several isozymes formed, and each might be associated with a separate organelle. Another explanation could be the adsorption of PAL molecules to the surfaces of organelles after cell disruption. Or, bits of cytoplasm containing PAL could be attached to the organelles. Yet another explanation is that aggregates of PAL molecules could sediment with the cell fractions, depending of course, on size and density.

The first explanation is unlikely, since in a detailed investigation only two forms of PAL were found in aged potato [Havir and Hanson 1969]. Absorption of PAL molecules is also unlikely: certainly PAL molecules do not adsorb onto organelles from fresh tissue (Figure 15).

The suggestion that cytoplasmic contamination might account for the distribution is more reasonable. A photography of a microbody-rich fraction from cucumber cotyledons [Trelease et al. 1971] shows portions of cytoplasm included within microbodies. If such inclusions were associated with other organelles, distribution of PAL might be found.

There are arguments against this suggestion. First, why would cytoplasmic contamination occur in all fractions but the microbodies? Secondly, if PAL were a soluble cytoplasmic enzyme, then the ratio of specific acitivities of PAL and the mainly cytoplasmic enzyme O-methyl transferase should be the same in each fraction. Since it is not (Table 11), the distribution of the enzymes cannot be the same, and the presence of PAL cannot be due to cytoplasmic contamination.

The fourth suggestion deals with the presence of PAL in aggregates. In buckwheat, PAL has been located in particles that sediment at 100,000x*g* but are not ribosomes

Table II

Ratio of PAL and O-methyl transferase activities in subcellular fractions of aged potato

Fraction	PAL nm $ml^{-1}hr^{-1}$	O-methyl transferase dpm $ml^{-1}hr^{-1}$	Ratio
microbodies	0	3815	-
band 2	7.5	4220	564
mitochondria	15.0	16050	1065
band 4 and soluble	115.9	180500	1560

[Amrhein and Zenk 1971]. Such particles could well be trapped in the packed pellet after centrifugation at 10,000x*g*. Schopfer [1971] suggest that such particles might be aggregation products, since mustard PAL isolated in tris buffer of low concentration formed aggregates of molecular weight $\geq 4 \times 10^6$, which might sediment with the ribosomes. But in the present study, the enzyme was never in buffer of concentration less than 0.05 M, and this is a less severe condition than used by Schopfer to obtain aggregates. Further, partially purified PAL prepared in 0.05 M tris buffer is retarded in a Sephadex G-200 column, which implies a molecular weight

$\leq$ 800,000 for a globular protein (Pharmacia technical bulletin 1966).

On the other hand, PAL from potatoes ground in too little buffer did appear at greater buoyant densities than usual. Thus, the PAL molecules from potato may form aggregates under conditions other than those mentioned by Schopfer.

The most reasonable explanation for the sedimentation behaviour of PAL is that the enzyme was mostly soluble but also occurs in a particulate form. This particle was larger than a ribosome, but too small to sediment with the larger organelles. The presence of PAL in these larger organelles could have been due to contamination by, or further aggregation of, these particles. Such particles would be difficult to identify with electron microscopy.

In any event, more work is needed with purified organelle fractions before the endogenous presence of PAL can be unequivocally demonstrated.

PAL from fresh potato (present at very low levels) did not appear with any of the larger organelles, and may be mostly soluble. This finding agrees with earlier work on potato [Ruis 1971].

Distribution of Cinnamic Acid 4-Hydroxylase

Most of the cinnamate hydroxylase in aged potato seems to be associated with particulate fractions (Table 6). Summing the percentages found in the 10,000x*g* and 100,000x*g* pellets, 88% of the total activity was not soluble. This agrees well with Stafford's finding (1969) that all the cinnamate hydroxylase in *Sorghum* was particulate.

As in the case of PAL, no activity was associated with the microbodies, and a large amount of activity was associated with band 4. It is interesting that in spinach, the enzyme appeared in the peroxisomal as well as the other fractions.

Amrhein and Zenk showed by gradient centrifugation of the 100,000x*g* pellet from buckwheat that the microsomal particles containing PAL were quite different from those containing cinnamate hydroxylase. The situation needs to be determined in potato also.

The Effect of Ageing on Enzymes of Phenylpropanoid Metabolism of Potato

The most obvious effect of ageing on the enzymes of Table 12 is a strong increase in activity. The increase of SOR on ageing was noted by Sacher *et al*. [1972]. They found

Table 12

The effect of ageing in the light on some enzymes of phenolic metabolism in disks of potato tuber

Enzyme	Experiment	Increase in Ageing (%)
SOR	expt. 1	(no activity in fresh tissue)
	expt. 3	220
POR	expt. 2	(no activity in fresh tissue)
	expt. 3	250
PAL	expt. 3	(no activity in fresh tissue)
cinnamate hydroxylase	expt. 1	220
	expt. 3	3500
O-methyl transferase	expt. 4	240

a 500-600% increase on ageing and this might be a reflection of differences between potato varieties or conditions of ageing.

Gamborg [1966] was not able to detect POR in tissue culture from potato tuber, although SOR was present. Perhaps

high levels of the first enzyme are involved in responses to wounding.

PAL is the only enzyme of those studied present consistently in only trace amounts in fresh potato. It well might be the enzyme limiting phenolic biosynthesis in dormant tissue.

Cinnamic hydroxylase and O-methyl transferase are known to increase in the light in parsley cell cultures [Halhbrock *et al.* 1971]. Cinnamate hydroxylase behaves like PAL, the levels peaking 18 hours after the start of illumination, while O-methyl transferase levels continue to increase in activity even after 24 hours.

Although no hydroxylation of *p*-coumaric acid to caffeic acid could be detected in extracts from aged disks, this reaction was carried out by extracts from the peel of 'Kennebec' potatoes [Patil and Zucker 1965]. The potato disks used in the present study were free of peel. Perhaps the monophenol oxidase activity is restricted to the outer layers of the tuber.

Chlorogenic acid is the quinic acid ester of caffeic acid, and this compound accumulates even in the stelar region of potato upon ageing of slices [Levy and Zucker 1960]. The major route of formation of this compound is *via* the quinic acid esters of cinnamic acid and *p*-coumaric acid. It

well may be that the hydroxylation occurs only when the p-coumaric acid is present as the quinic acid ester.

Although no monophenol oxidase was detectable in the potato discs, earlier work has shown that the o-diphenol oxidases of potato were present and increased 3-fold on ageing [Ruis 1972; Sacher et al. 1972]. The distribution of the soluble, microsomal and microbody forms of the enzyme did not change on ageing of the tissue [Ruis 1972].

The distribution of cinnamate hydroxylase does undergo a change upon ageing of potato in the light. In dormant tissue, the enzyme is largely soluble; in aged tissue it is largely particulate. The same phenomenon might occur with PAL, for indications are that the enzyme is soluble in dormant tissue and at least partly particulate in aged tissue. However, there is so little enzyme in the fresh tissue that it is difficult to tell.

Such changes of form are not unknown in other tissues. In rhesus monkey liver, a mitochondrial tyrosine transaminase was detectable in early fetal life, while a soluble form reached significant activity just before birth. Activity of both forms increased at birth [Koler et al. 1969]. In wheat leaves, the typical catalase-containing microbodies are not formed until the fourth day after germination [Feierabend and Beevers 1972]. Before this time, the enzyme

is associated with particles of low specific gravity. These are believed to be components of the microbodies which develop later. While in dormant potato catalase is found in a fraction with the specific gravity typical of microbodies, a series of changes analogous to those taking place in germination might take place on ageing of potato. The soluble cinnamate hydroxylase may be characteristic of dormant tissue; the particulate form might be characteristic of the actively metabolizing tissue.

Formation of new cinnamate hydroxylase could take place in either of two ways. The production of both forms could increase, although to a higher extent in the case of the particulate form. This would be similar to the case in monkey tissue. Alternatively, the soluble form might become particulate as changes in the intracellular organization occur, in a manner similar to the behaviour of catalase in wheat leaves. It is known that general membrane structure changes as potato slices age [Castelfranco et al. 1970]. These changes might include aggregation or organization of cinnamate hydroxylase molecules into particles.

Tyrosine Metabolism by Aged Potato Tissue

In *Ricinus*, the proplastids and glyoxysomes deaminate tyrosine to *p*-hydroxyphenylpyruvic acid (PHPP) and thence to *p*-hydroxyphenylacetic acid (PHPA) [Ruis and Kindl 1971]. In potato, the first reaction seems to be mainly microsomal, although some conversion takes place in the 10,000x*g* pellet also. This pellet includes the potato microbody, as well as other organelles, and in this fraction the PHPP loses a carbon atom to become PHPA. Since this reaction is glyoxysomal in *Ricinus*, it would seem likely that in potato it would occur in the microbodies. This is not certain, however, for extrapolations from one plant to another are not always valid. For example, cinnamate hydroxylase and PAL have been shown in the present study to be present in the peroxisomal fraction of spinach but not in the microbody fraction of potato.

General Conclusions

To this time, work has been done on the increase in phenylpropanoid derivatives in potato on ageing, but the present study presents the most coherent picture to date of the intracellular changes involved in this increase. Figures 16 and 17 summarize these changes.

DORMANT POTATO

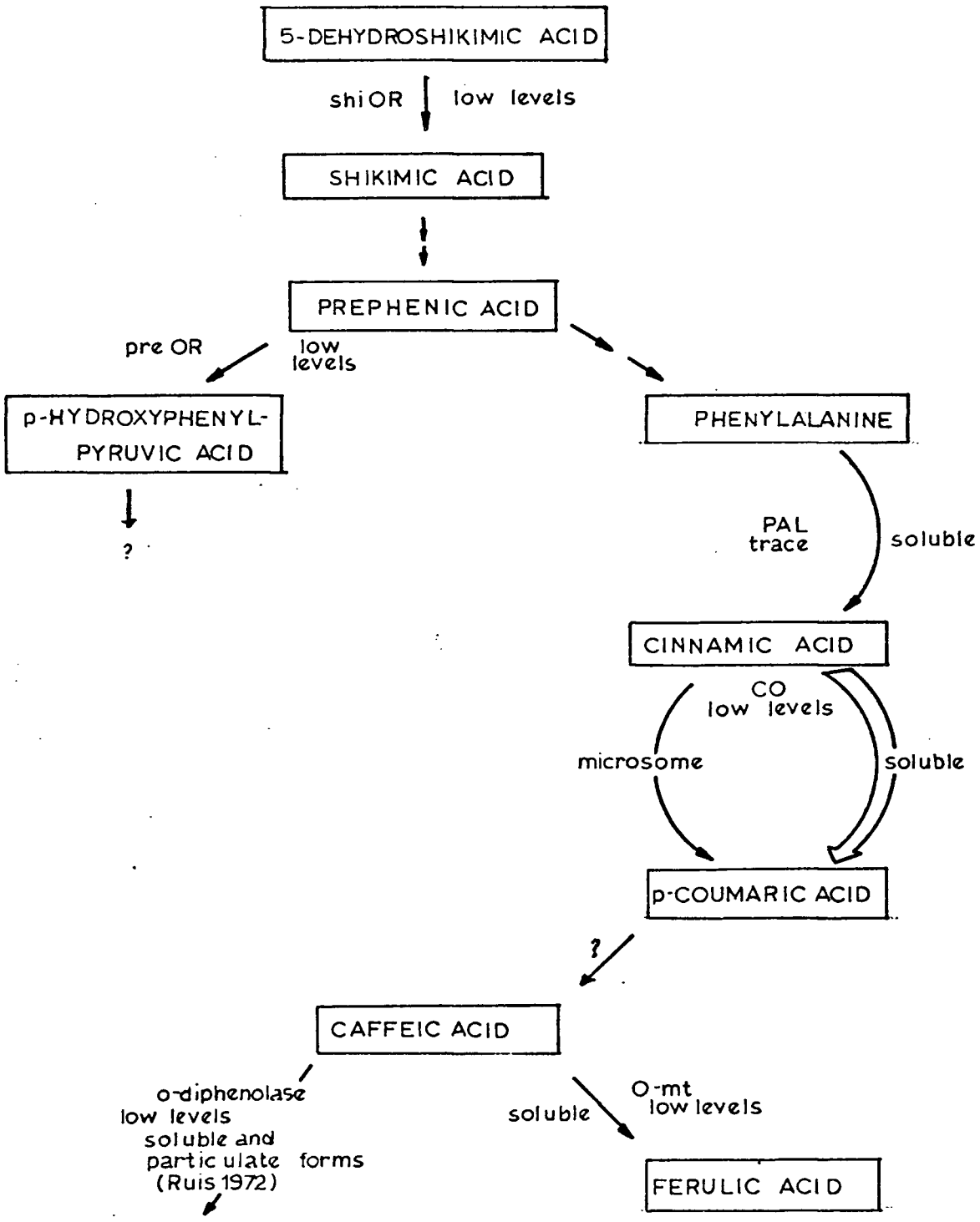

Figure 16. Localization of phenylpropanoid reactions in dormant potato.

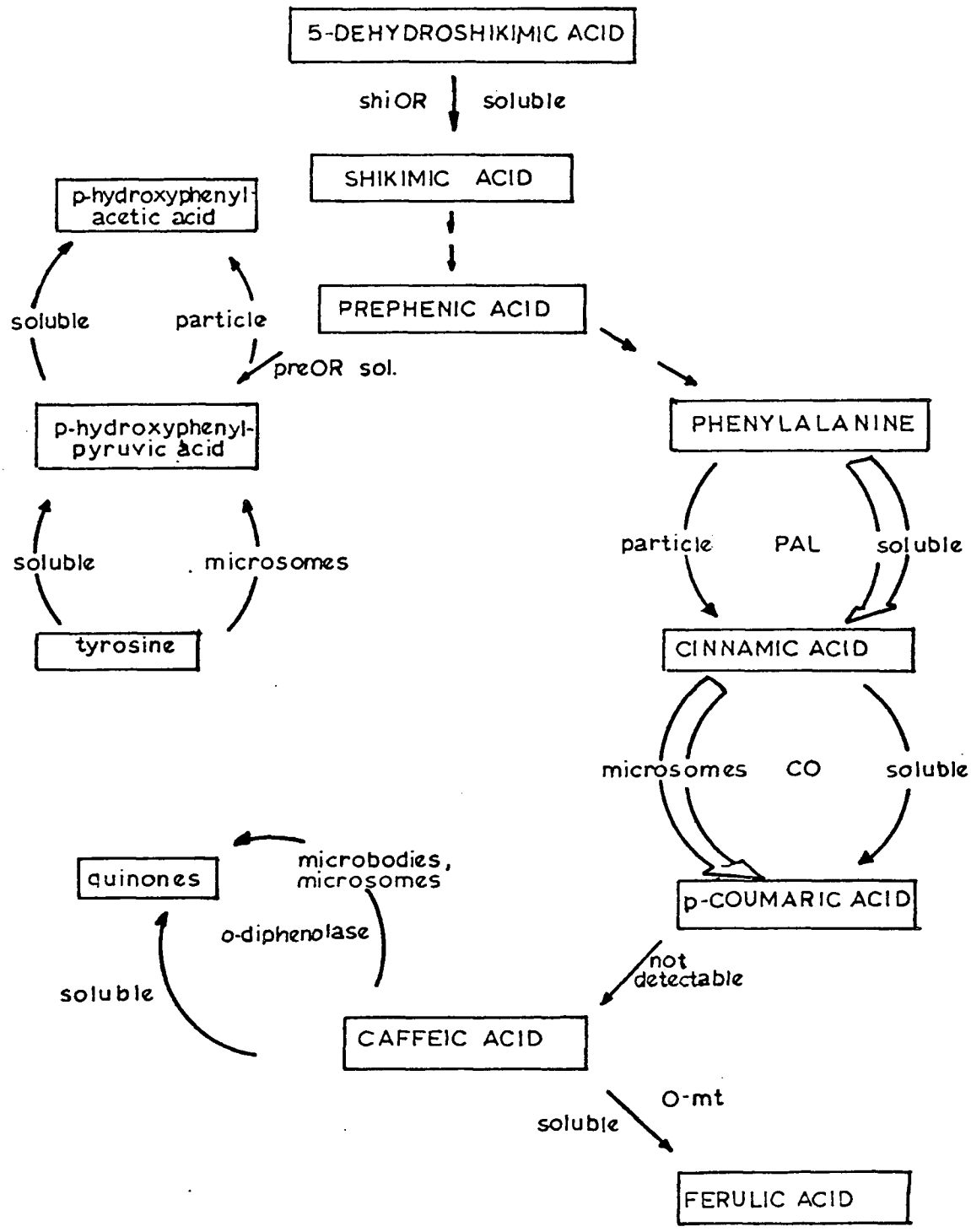

Figure 17. Localization of phenylpropanoid reactions in potato aged in the light.

The increase in SOR noted by others [Sacher et al. 1972] has been verified. This implies that an increase in carbon flow is permitted through the whole shikimic acid pathway. A higher flow of carbon to tyrosine is also permitted through the increase in POR discovered in this study. There have not been any reports of organisms in which these two enzymes are under co-ordinate control, so it seems likely that in potato also this would provide a mechanism for channeling of carbon into phenylalanine or tyrosine.

While phenylalanine is deaminated by PAL to cinnamic acid in potato, tyrosine is not converted to p-coumaric acid by TAL. Instead, tyrosine is converted to p-hydroxyphenylpyruvic acid and p-hydroxyphenylacetic acid. Even before ageing, there is a significant level of cinnamate hydroxylase activity, and after ageing, there is probably a high enough level to supply any p-coumaric acid needed by the plant.

The significance of the particulate and soluble forms of PAL and cinnamate hydroxylase is unknown. There are two isoenzymes of PAL in oak [Boudet et al. 1971], one of which is subject to inhibition by phenylpropanoid derivatives, the other by benzoic acid derivatives. The two tyrosine transaminases, soluble and mitochondrial, in monkey tissue are also subject to different control mechanisms

[Koler *et al*. 1969]. A similar situation might be in operation in the case of potato. In any event, the nature of the different enzymic forms of PAL and cinnamate hydroxylase, whether aggregates or isoenzymes, whether present in organelles or merely contamination, remains to be seen.

The absence of a *p*-coumarate hydroxylase but the presence of an active O-methyl transferase of caffeic acid, is a puzzle. As discussed before, the hydroxylation of *p*-coumaryl-quinate apparently does occur in potato tissue. An increase in the enzyme methylating caffeic acid does not necessarily imply an increase in that substrate. This (or these) enzymes(s) might have very broad specifities, and an ester of caffeic acid might be the substrate for the enzyme *in vivo*.

The potato is an excellent tissue for the investigation of intracellular control of phenylpropanoid metabolism. The relatively dramatic step-up in production of phenylpropanoid derivatives under reproducible conditions means that models of carbon flow and behaviour can be assembled. The present study is a first step in that direction.

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