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PHENYLALANINE AMMONIA-LYASE ACTIVITY IN CITRUS FRUIT TISSUE CULTURED *IN VITRO*

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Abstract—The presence of L-phenylalanine ammonia-lyase in citrus fruit tissue cultured *in vitro* is reported. Changes in the level of activity of this enzyme with time were determined in callus derived from the grapefruit (*Citrus paradisi*, Macf. and other citrus species). The influence of light, the substrate and end-products of the enzyme reaction, growth regulators, and cycloheximide on the activity of the enzyme were determined.

INTRODUCTION

PHENOLIC compounds form a large class of secondary metabolites, which play an important role in plant growth and development.[1] They are found in all parts of the plant.[2] In most plants, the principal link between primary metabolism and this class of secondary constituents is the enzyme L-phenylalanine ammonia-lyase (PAL).[3] This enzyme, which is widely distributed in plants,[4,5] catalyses the conversion of L-phenylalanine to *trans*-cinnamic acid. Its activity has been directly correlated with chlorogenic acid biosynthesis in potato tuber discs,[6] and with leucoanthocyanin and other flavonoids in strawberry leaf discs.[7] Recently, Maier and Hasegawa[8] have shown that there is a direct relationship between PAL activity and the rate of accumulation of naringenin glycosides in developing grapefruit. As a result, we have studied the activity of this enzyme in callus derived from the albedo of the grapefruit and other citrus species.

RESULTS

Callus grown in the dark remained colorless, whereas that illuminated started to turn green after 4–5 days in culture. Callus growth, as measured by increase in fresh weight, was greater in dark-grown tissue. Changes in fresh weight of grapefruit callus (*Citrus paradisi*, Macf.) up to 12 days in culture (during which time all measurements of PAL were usually made) are shown in Fig. 1. As can be seen, up to 2 days in culture, there was no difference in weight between light- and dark-grown callus. However, after 4 days in culture

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[1] A. C. LEOPOLD, in *Plant Growth and Development*, p. 144, McGraw-Hill, New York (1964).
[2] J. HARBORNE, in *Biochemistry of Phenolic Compounds* (edited by J. HARBORNE), p. 129, Academic Press, New York.
[3] A. NEISH, in *Plant Biochemistry* (edited by J. BONNER and J. E. VARNER), p. 581, Academic Press, New York (1965).
[4] M. R. YOUNG, G. H. N. TOWERS and A. C. NEISH, *Can. J. Botany* **44**, 341 (1966).
[5] J. KOUKOL and E. E. CONN, *J. Biol. Chem.* **236**, 2692 (1961).
[6] M. ZUCKER, *Plant Physiol.* **40**, 779 (1965).
[7] L. L. CREASY, *Phytochem.* **7**, 441 (1968).
[8] V. P. MAIER and S. HASEGAWA, *Phytochem.* **9**, 139 (1970).

the fresh weight of the dark-grown callus increased at a faster rate. The acetone insoluble solids also increase at a faster rate in dark-grown callus after 7 days in culture (Fig. 1).

Six to eight week old grapefruit callus maintained a low level of PAL activity, on an average of about 1·2 unit/g fr. wt. for dark-grown tissue. On subculturing, there was a large increase in PAL activity with a maximum after 48 hr in culture (Fig. 2). The increase in enzyme activity observed was independent of light. This was followed by a marked loss in activity. However, light-grown tissue always retained a higher level of the enzyme than the corresponding dark-grown tissue. After 12 days in culture, PAL activity was only slightly greater than in the corresponding 6–8 week old callus. To determine whether this behavior of the enzyme was unique to the callus or not, fresh albedo explant tissue from Marsh white grapefruit was cultured *in vitro*, and its PAL activity measured. This albedo tissue also showed the same changes in enzyme activity with time. The albedo, taking from 3 month old grapefruit, had an initial PAL activity of 4·6 unit/g fr. wt. as compared to the average of 1·2 unit found in callus. The activity after 48 hr was doubled, which was of the same order of magnitude observed in callus at that time. Here again, growing the albedo in light led to a higher retention in activity after 4 days in culture.

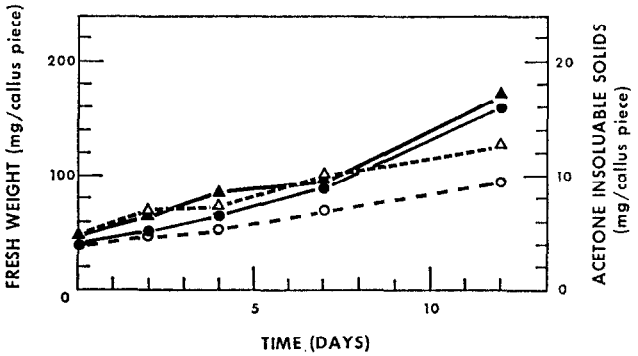

FIG. 1. GRAPEFRUIT CALLUS GROWTH.
(—●—) Fresh weight, dark grown; (—○—) fresh weight, light grown; (—▲—) acetone insoluble solids, dark grown; (—△—) acetone insoluble solids, light grown.

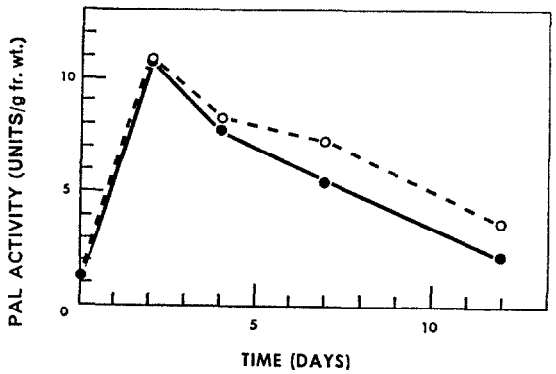

FIG. 2. PAL ACTIVITY IN GRAPEFRUIT CALLUS.
(—○—) Activity, light grown; (—●—) activity, dark grown.

TABLE 1. PAL ACTIVITY IN CALLUS OF DIFFERENT CITRUS SPECIES

Citrus species	PAL activity (units/g fr. wt.)			
	2 days		4 days	
	Dark	Light	Dark	Light
Grapefruit	11·6	11·9	7·7	8·3
Navel orange	7·6	11·2	5·9	8·9
Lemon	7·1	8·8	4·0	4·2
Citrangequat	9·5	17·1	8·1	8·3
Mandarin hybrid	7·0	5·9	7·6	6·5

The activity of PAL in other citrus species was also examined. These were lemon (*Citrus limon*, Burm.), navel orange (*Citrus sinensis*, Osbeck), a Clementine × Wilking mandarin hybrid (*Citrus reticulata*, Blanco), and Thomasville citrangequat [Fortunella sp. × (*Citrus sinesis* × *Poncirus trifoliata*)]. Activities observed after 2 and 4 days in culture can be seen in Table 1. The activity of PAL in these species was similar to that in grapefruit. However, in navel orange and Thomasville citrangequat callus, light increased the maximum PAL measurable after 48 hr, as compared to the dark-grown tissue. No tyrosine ammonia-lyase activity was observed in any of these species.

The effect of L-phenylalanine on the changes in PAL activity with time was determined (Table 2). The addition of the amino acid at 10^{-2} M to the nutrient agar was toxic to the callus, whereas at 10^{-4} M and 10^{-6} M there was a slight stimulating effect on PAL activity. On the other hand, soaking the callus for 2 hr in 10^{-4} M phenylalanine had a slight depressing effect on the activity. Soaking the callus for 2 hr in 10^{-4} M *trans*-cinnamic acid or 10^{-4} M *p*-coumaric acid before planting had a similar depressing effect on the changes in PAL activity.

The effect of growth regulators on PAL activity in light-grown grapefruit callus was also determined. The callus was soaked for 2 hr before planting in the following compounds: abscisic acid (10^{-4} M), indole acetic acid (10^{-4} M), kinetin (10^{-4} M) and gibberellic acid (5×10^{-5}M). They caused a slight reduction in the activity of PAL observed after 48 hr, but led to a higher retention of enzyme activity at 7 days in culture (Table 3).

TABLE 2. EFFECT OF DIFFERENT COMPOUNDS ON PAL ACTIVITY IN GRAPEFRUIT CALLUS

Compound	PAL activity as % of control		
	2 days	4 days	7 days
L-Phenylalanine 10^{-2} M*	17·2	18·7	23·1
L-Phenylalanine 10^{-4} M*	110·0	111·4	129·0
L-Phenylalanine 10^{-6} M*	106·0	114·8	102·0
L-Phenylalanine 10^{-4} M†	88·5	86·5	110·0
trans-Cinnamic acid 10^{-4} M†	88·4	73·5	93·0
p-Coumaric acid 10^{-4} M†	87·0	77·5	102·0

* Incorporated into nutrient medium.
† Incubation in test solution for 2 hr before planting.

TABLE 3. EFFECT OF GROWTH REGULATORS ON PAL ACTIVITY IN GRAPEFRUIT CALLUS

Growth regulator	PAL activity as % of control		
	2 days	4 days	7 days
Indole acetic acid*	67·8	121·0	132·0
Kinetin*	72·6	94·7	143·2
Gibberellic acid†	88·7	114·0	160·0
Abscisic acid*	80·6	86·0	137·8

* 10^{-4} M.
† 5×10^{-5} M.

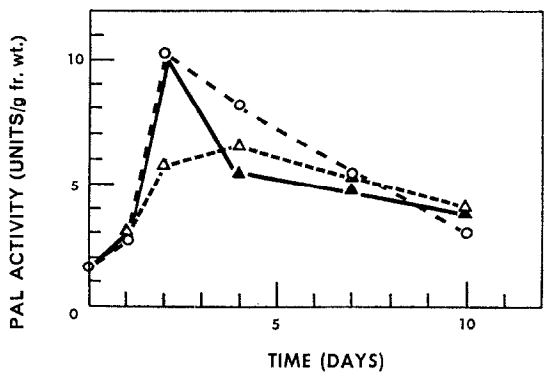

FIG. 3. EFFECT OF CYCLOHEXIMIDE ON PAL ACTIVITY IN GRAPEFRUIT CALLUS.
(—○—) Control; (—△—) 10^{-4} M cycloheximide at 0 hr; (—▲—) 10^{-4} M cycloheximide at 48 hr.

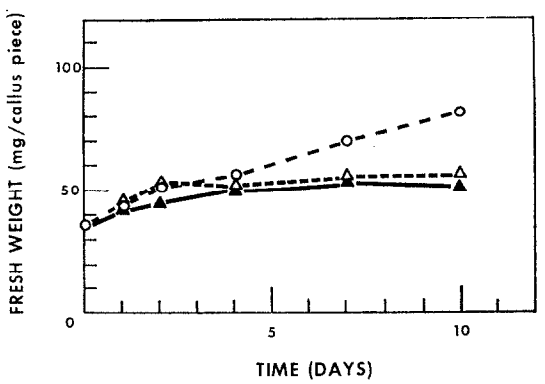

FIG. 4. EFFECT OF CYCLOHEXIMIDE ON GRAPEFRUIT CALLUS GROWTH.
(—○—) Control; (—▲—) 10^{-4} M cycloheximide at 0 hr; (—△—) 10^{-4} M at 48 hr.

The effect of cycloheximide on the changes in level of PAL activity in grapefruit callus was also examined. When cycloheximide at a concentration of 5×10^{-5} M was in the nutrient medium there was no effect on the rate of increase or the activity of PAL measurable after 48 hr. However, there was less loss of activity, during the subsequent growth of the callus, up to 11 days in culture. This light-grown callus, with the inhibitor, failed to turn green and its growth rate was much slower. On the other hand, when the tissue was soaked in the inhibitor for 2 hr, in a concentration of 10^{-4} M, a different picture emerged (Fig. 3). Soaking the callus at 0 hr had no effect on the initial increase in PAL activity

TABLE 4. CONVERSION OF L-PHENYLALANINE TO *trans*-CINNAMIC ACID IN GRAPEFRUIT CALLUS

Incubation Time (hr)	Phenylalanine (cm^2/g fresh tissue)*	Cinnamic acid (cm^2/g fresh tissue)
6	28·6	3·3
24	12·6	15·2
48	5·0	26·8

* See Experimental.

during the first 24 hr, but reduced the rate of increase during the next 24 hr, so that the PAL activity measured was about half that of the control. This activity slowly decreased after 4 days in culture. When the callus was incubated in the inhibitor for 2 hr, after 48 hr in culture, the treated callus lost activity faster than the control. However, after 7 days in culture, there was no difference in the level of PAL activity between the treatments. However, treatment in cycloheximide at 0 or 48 hr, had a marked inhibiting effect on the growth of the callus (Fig. 4).

The results of ^{14}C phenylalanine studies are shown in Table 4. Two major radioactive peaks were observed on TLC strips after development. These peaks were identified as phenylalanine (R_f 0·59) and cinnamic acid (R_f 0·80) by comparison with authentic samples. It was found also that the rate of cinnamic acid production was directly proportional to time of incubation.

DISCUSSION

Irrespective of whether fresh weight or acetone insoluble solids is used as a measure of growth, the callus started to show an increase in weight within 24 hr (Figs. 1 and 4). That this initial increase in weight is not due to the uptake of water is indicated by the increase in acetone insoluble solids during this period. The weight increase began before cell division begins in the callus. New cells are formed by 7 days in culture with lemon callus,[9] but their growth probably starts as early as 4–5 days. It should also be noted that the growth rate of the dark-grown callus is higher than for the corresponding light-grown tissue. This light inhibition of the callus growth is thus in keeping with observations made on the intact plant.[10,11]

[9] D. H. P. TUCKER, Ph.D. Dissertation, University of California, Riverside (1966).
[10] M. W. PARKER, S. B. HENDRICKS, H. A. BORTHWICK and F. W. WENT, *Am. J. Botany* **36**, 194 (1949).
[11] J. A. LOCKHARD, *Am. J. Botany* **48**, 387 (1961).

Callus tissue, whether grown in the dark or the light, always maintained some PAL activity. The presence of PAL in callus was confirmed by ^{14}C tracer studies. As shown in Table 4, labelled phenylalanine was converted to cinnamic acid in callus tissues and the rate of this conversion was linear with time. This represents the first report concerning the presence of PAL in callus. The level of activity, although low, was higher for light-grown tissue, and was several times higher than that observed in flavedo discs from mature grapefruit.[12] On subculturing, there is a large increase, greater than 9 fold, in the activity of the enzyme. Whereas the increase in the first 24 hr was minimal (Fig. 3), the bulk of the increase in PAL took place during the next 24 hr. Thus, the period of greatest increase in PAL activity occurs before cell division begins. The rate of increase of the enzyme is much slower than that generally observed.[6,12–16] If the increased activity were purely a response to the wounding, then the rate of increase in PAL should compare with that observed in the excision of the storage tissue of such plants as potato,[6] sweet potato[13] or Jerusalem artichoke,[14] and such a prolonged lag period should not be observed. The lag period was even longer in albedo, taken from mature grapefruit and its significance is unclear. PAL activity measured after 48 hr was about the same for callus and fresh albedo, despite the fact that the latter initially contained nearly four times the PAL activity usually observed in callus.

The behavior of PAL was similar in the different citrus species examined. Although the maximum activity attained varied with the species (Table 1), it was nevertheless of the same order of magnitude. The only difference appeared to be a light-dependent increase of PAL in Thomasville citrangequat and navel orange. However, the amount of PAL observable after 48 hr in the dark was about the same for all the species, i.e. about 8 unit/g fr. wt. If light plays a part, its major role during the period of PAL increase seems to be to increase the maximum obtained. This result differs from that of most workers, who have found a light dependence for PAL induction,[6,14,17,18] but agrees with the findings of Riov *et al.*[12] on grapefruit flavedo discs and Walton[16] on excised bean axes. However, Atridge and Smith[19] found that a single photoactivation of phytochrome with red light was enough to cause a large increase in PAL in Alaska peas. Under our conditions, since planting took place under dim fluorescent light, we are unable to assign a positive or negative role for phytochrome in this process.

The decrease in enzyme activity after 2 days, on the other hand, seems light dependent, since light-grown tissue always contained a higher level of PAL. However, if one measures the enzyme activity in equal amounts of acetone powder from corresponding light, and dark-grown tissue, it is found that the activities are similar. Apparently, since the dark-grown tissue is growing at a greater rate (Fig. 1), the actual amount of PAL present is diluted to a larger extent, and the apparent higher retention of the enzyme activity in light-grown tissue may not be real. The significance of this is unclear. It is possible that the kinetics of both the increase and decrease in PAL activity are independent of light in citrus tissue culture. Zucker[20] has suggested that at least part of the role of light in PAL

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[13] T. MINAMIKAWA and I. URITANI, *J. Biochem.* **57,** 678 (1965).
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[16] D. C. WALTON, *Plant Physiol.* **43,** 1120 (1968).
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[18] G. ENGELSMA, *Planta* **82,** 355 (1968).
[19] T. H. ATTRIDGE and H. SMITH, *Biochem. Biophys. Acta* **148,** 805 (1967).
[20] M. ZUCKER, *Plant Physiol. Suppl.* **43,** S-26 (1968).

maintenance in Zanthium leaf discs lies in the production of photosynthate. Also, Creasy[7,21] has shown that both sucrose and carbon dioxide increase PAL activity in strawberry leaf discs. Under tissue culture experimental conditions, carbohydrates would not be limiting as the medium contains 5 per cent sucrose. Perhaps the absence of a significant light effect on PAL behavior in callus may be explained on this basis.

Creasy[7] has found that phenylalanine, cinnamic acid and *p*-coumaric acid all affected the level of PAL activity in strawberry leaf discs; lower concentrations inhibiting and relatively higher concentrations increasing the content of the enzyme. Zucker[6] has shown that phenylalanine and cinnamate at relatively high concentrations inhibit the induction of PAL in potato tuber explants.

We have found that these compounds produced small positive effects on PAL activity of grapefruit callus when incorporated in the medium at low non-toxic concentrations (Table 2). However, when the callus tissue was soaked directly in solutions of the compounds before planting all three compounds produced small negative effects on PAL activity. Thus, grapefruit callus responds in a manner similar to potato tuber explants. These negative effects began to disappear after 4 days. L-Phenylalanine at 10^{-2} M in the medium proved toxic to the callus. This sensitivity of callus to higher levels of L-phenylalanine is in marked contrast to leaf discs which when floated on 10^{-2} M phenylalanine showed no toxic effect and gave a large positive PAL effect.[7] The results of radio tracer studies (Table 4) show good uptake of phenylalanine by callus.

The growth regulators slightly reduced the maximum amount of PAL measurable after 48 hr (Table 3). However, this reduction was minimal and within the limits of the amount of PAL observable in grapefruit callus at that time. The higher retention of PAL after 7 days in culture may simply be due to the dilution effect, as the control tissue grew at a faster rate. The nutrient medium contains an auxin (2,4 dichlorophenoxyacetic acid), and a cytokinin (kinetin) at optimum amounts for callus growth. It is therefore not surprising that the additional auxin and cytokinin slightly inhibited growth. Gibberellin had no effect on the callus growth and was therefore omitted from the nutrient medium. The effect of abscisic acid on the callus growth was not determined. Walton and Sondheimer[22] found that abscisic acid influenced both the development and retention of PAL activity in excised bean axes. Cheng and Marsh[23] have shown that gibberellic acid promoted PAL activity in dwarf pea. Ethylene has also been shown to increase PAL activity in citrus flavedo.[12] Under our experimental conditions the effects of the growth regulators on PAL activity in grapefruit callus are not large.

Cycloheximide has been shown to influence both the development and deactivation of PAL in gherkin seedlings,[15] bean axes[16,22] and potato tuber discs.[24] Cycloheximide inhibited the development of PAL activity in grapefruit flavedo discs.[12] Under *in vitro* culture conditions with grapefruit callus, when the inhibitor was incorporated in the medium, it failed to affect the rate of increase or the maximum amount of the enzyme measurable after 48 hr. On the other hand, when the tissue is soaked in the inhibitor, the effect is more clear cut (Fig. 3). Incubating it at 0 hr in the cycloheximide did not affect the lag phase, but did substantially effect the log phase of PAL development. The subsequent decrease of activity after 4 days in culture may be due to a dilution effect. When the tissue is treated with

[21] L. L. Creasy, *Phytochem.* **7**, 1743 (1968).
[22] D. C. Walton and E. Sondheimer, *Plant Physiol.* **43**, 467 (1968).
[23] C. K-C. Cheng and H. V. Marsh, Jr., *Plant Physiol.* **43**, 1755 (1968).
[24] M. Zucker, *Plant Physiol.* **43**, 365 (1968).

the inhibitor after 48 hr in culture, the rapid loss of activity could be attributed to it having a greater effect on the synthesis of PAL than on a PAL degrading enzyme,[24] which was apparently being synthesized by that time. This explanation would account for the higher level of PAL observable after 4 days in the control tissue, as compared with the tissue incubated with cycloheximide at 48 hr in culture. Apparently, although there are residual effects of the inhibitor on the callus (e.g. growth and chlorophyll synthesis inhibition), there is none on PAL activity by 7 days in culture. Since the callus always maintains some activity the rate of synthesis of the enzyme appears to remain higher than its rate of degradation.

Although, as pointed out by Filner *et al.*,[25] the use of a general inhibitor of protein synthesis, such as cycloheximide, does not in itself show that an enzyme is being synthesized, nevertheless, it would appear from our results (that the large increase in PAL activity observed (over 9 fold) in citrus callus does represent increased synthesis of this enzyme. A similar conclusion was reached by Riov *et al.*,[12] with regard to PAL development in grapefruit flavedo discs. Similarly, the rapid loss of activity between 2 and 4 days in culture is best explained on the basis of the synthesis of a PAL degrading enzyme system.

The ability of callus to metabolize ^{14}C phenylalanine was explored under shake (liquid) culture conditions. Although phenylalanine was converted to cinnamic acid as mentioned previously, there was no detectable formation of or label in naringenin or its glycosides. Thus, grapefruit callus differs significantly from the intact fruit[8] with regard to the accumulation of flavonoids.

EXPERIMENTAL*

All of the fruit except the lemon used to obtain callus were collected at the USDA Date and Citrus Station, Indio, California. The lemon fruit were obtained from the University of California, Riverside, California. Both Marsh white and Ruby red grapefruit were used in the studies. The method of obtaining callus from the albedo and/or juice vesticles of young grapefruit and other citrus species was the same as reported by Murashige and Tucker[26] for lemon. Their lemon medium was modified by the addition of 1 g/l. of Difco Casamino acids and 5% reconstituted pure frozen orange juice. The callus was subcultured at 5–6 weekly intervals on the above medium, solidified with 0·9% Difco Bacto-agar. Initiation of the callus and subculturing of it was carried out in the dark in a room maintained at 28 ± 1°. Irradiation of the cultures was by means of Grolux lamps ($1{\cdot}7 \times 10^3$ lx). Only callus which had been subcultured at least twice was used in any experiment. The age of such experimental callus was 6–8 weeks in the transfer. Test substances were either incorporated into the medium before autoclaving, or filter sterilized and added to the medium, after it was sterilized, but before it had solidified. Test solutions used for incubating were autoclaved or filter sterilized, depending on their lability, after their pH's were adjusted to 6·0 ± 0·1.

The method for making acetone powder from the callus and extracting the enzyme from it was the same as reported by Maier and Hasegawa.[8] 200 mg of acetone powder was used per enzyme assay. One enzyme unit is defined as the amount of enzyme that catalyses the production of 1 mn mole of cinnamic acid/min under the conditions described by them.

Conversion of radioactive phenylalanine to *trans*-cinnamic acid in grapefruit callus was carried out as follows. Callus incubation took place under light on a gyrotory shaker on the above (standard) medium minus orange juice and agar but including 5×10^{-4} M L-phenylalanine and a suitable dilution of ring-labelled DL-phenylalanine for periods up to 48 hr. After incubation, the tissues (about 1·5 g) were removed, washed with H_2O and ground with H_2O. The filtrate was transferred onto the top of a $1{\cdot}5 \times 5$ cm Dowex-1 column (acetate form). The column was washed with H_2O and eluted with 0·5 N NH_4OH. The effluent was dried and re-dissolved in 0·5 ml H_2O. Samples were separated on a cellulose TL plate developed with t-BuOH–HCOOH–H_2O (70:1:30). A Vanguard Automatic Chromatogram Scanner was used for locating and estimating relative concentrations of the radioactive spots. The relative concentrations of the radioactive spots were determined by measuring area under peaks on the radiochromatograms and the results were expressed as area/unit tissue weight.

* Reference to a company or product name does not imply approval or recommendation by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

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