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The Role of *O*-Methyltransferase in the Lignification of Douglas-Fir Cultured Tissue

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THE ROLE OF O-METHYLTRANSFERASE IN THE LIGNIFICATION OF DOUGLAS-FIR CULTURED TISSUE

A thesis submitted by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF PHOTOMICROGRAPHS

Man, being the servant and interpreter of Nature, can do and
understand so much, and so much only, as he had observed,
in fact or in thought, of the course of Nature;
beyond this he neither knows anything nor can do anything...
Human knowledge and human power meet in one;
for where the course is not known, the effect cannot be produced.
Nature, to be commanded, must be obeyed.

Bacon

ABSTRACT

O-methyltransferase (OMT) is a key enzyme in the biosynthesis of lignin. This enzyme was isolated and characterized in an effort to understand why Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco] callus tissue does not form appreciable amounts of lignin yet does form large amounts of the related flavonoids and tannins.

It was shown that the OMT in the callus tissue is a cell wall associated, membrane-bound enzyme, in contrast to that of all reported plant species and to Douglas-fir seedlings, which have either a microsomal or soluble OMT. The effect this had on the OMT kinetic constants was studied. It was found that the callus OMT had much higher K_m constants for caffeic acid in both the membrane-bound and free forms compared with seedlings. The callus membrane-bound K_m for caffeic acid is 333 μM. The callus membrane-free K_m for caffeic acid is 250 μM. The seedling K_m for caffeic acid is 90 μM.

The callus OMT activity was shown to be specific for caffeic acid exclusive of the flavonoids. This demonstrates that the OMT studied here is a lignin specific OMT and so the high K_m values are due to an abnormality in the enzyme itself and not just to a low specificity toward lignin precursors as is found in flavonoid specific OMT's.

The levels of caffeic acid present in the callus were measured and found to be low throughout the subculture period. The maximum level of caffeic acid was 66 μM at 12 days after subculture. The average concentration was less than 12 μM.

The consequence of this low steady state caffeic acid concentration and high K_m value for caffeic acid is the formation of little ferulic acid and thus little lignin. The OMT was thus shown to be limiting lignin synthesis due to a functional difference and not to a simple lack of enzyme.

INTRODUCTION

Douglas-fir cultured tissue typically accumulates large quantities of the cinnamic acid-derived flavonoids and tannins. These polyphenolic materials rapidly increase in the tissue to such an extreme that the tissue actually turns brown and has every appearance of being necrotic. Stafford and Lester (1) have reportedly found that up to 40% of the dry weight of Douglas-fir callus consisted of tannins. Work in this thesis found tannin levels up to 60% of the callus dry weight. This phenolic buildup is lessened by the action of growth hormones and conversely, when these hormones are removed in the attempt to induce embryogenesis, the phenolic problem is exacerbated (2).

Although a direct cause and effect relationship between the accumulation of phenolics and the lack of embryogenesis has not been experimentally demonstrated in this tissue, the inactivating effect of tannins on enzymes *in vitro* and the inhibitory effect of phenolic acids on seed germination suggests that this phenolic buildup is detrimental to somatic embryogenesis. Given this behavior, it will be necessary to solve this phenolic problem before somatic embryogenesis can be induced in a controlled manner.

It is known that these phenolics are derived from a biochemical pathway shared with lignin to a large extent. Examination of these cultures shows very little to no lignin formation. Since lignin is the more typical phenolic product in normal plant development, this raises an interesting question and possibility for solving the toxic buildup of phenolics. First of all, why doesn't this tissue form lignin instead of forming the related flavonoids and tannins in very high concentrations? Second, can the lignin pathway be activated, thus redirecting the internally toxic phenolics into the innocuous lignin safely buried in the cell wall?

Figure 1 shows the general pathway for lignin and flavonoid biosynthesis. Stafford and Lester (1) have shown that the tannins of Douglas-fir cultured tissue contain only catechin and epicatechin (Fig. 2). The "B" ring of the flavonoids is derived from the phenylpropanoid pathway. Catechin can be derived either from p-coumaric acid through a para-hydroxylated flavonoid intermediate or from caffeic acid to form a dihydroxy flavonoid, as shown in Fig. 1. It is not clear which pathway is predominant in plants, and it appears to be species dependent. In some plants the pathway from coumaric acid through the monohydroxy flavonoid predominates (3) while in other species the path through caffeic acid predominates (4). It is not known which path predominates in Douglas-fir tissues. However, the large amounts of phenylpropanoid derived phenolics in this tissue indicate that the enzymes producing cinnamic and p-coumaric acid are present in large amounts. The hydroxylating enzyme that forms caffeic acid from coumaric acid may or may not be present in large amounts. It is important to realize that if this hydroxylase is limiting then little lignin would be formed, since little caffeic acid would be formed. On the other hand, if the hydroxylase is present in large amounts then much caffeic acid would be produced and be available for methylation by the next enzyme in the pathway, O-methyltransferase (OMT).

If either this or a subsequent enzyme in the pathway were limiting, the lignin pathway would be blocked, and the phenolic compounds produced would be forced into the embryogenically less desired flavonoid pathway.

In this thesis, a study of the OMT was undertaken in an attempt to determine if it is limiting lignification and, if so, whether the pathway could be made operational by increasing the amount of OMT present.

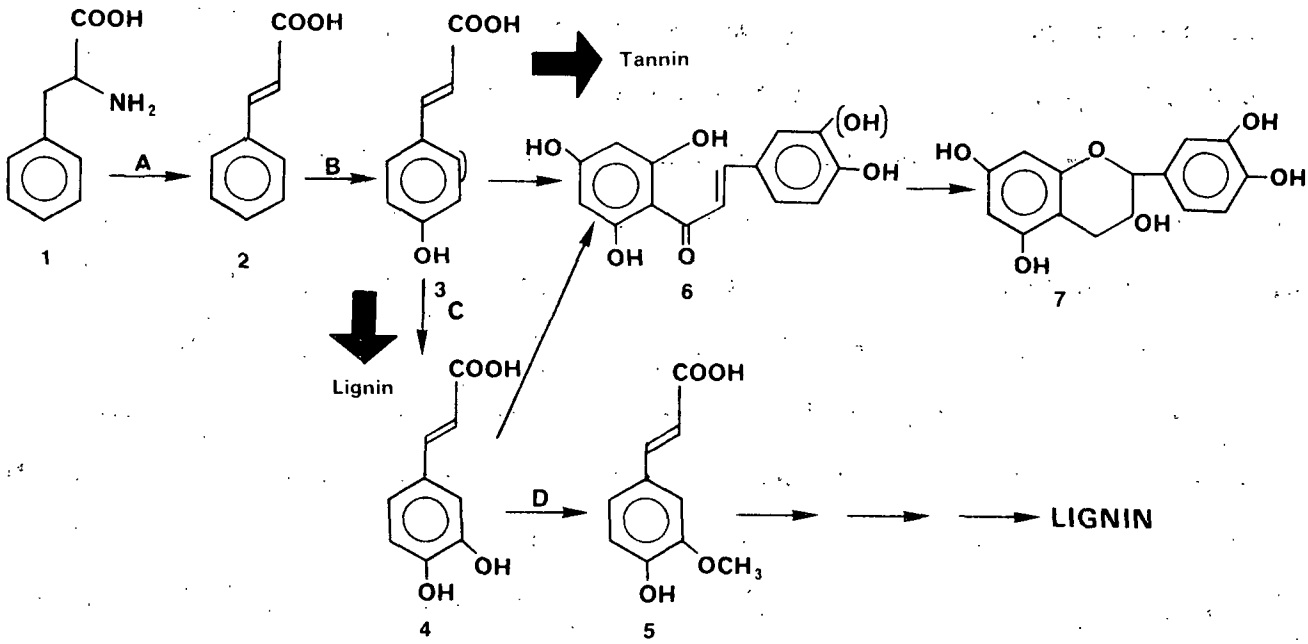


Figure 1. The general phenylpropanoid pathway. 1. Phenylalanine, 2. cinnamic acid, 3. p-coumaric acid, 4. caffeic acid, 5. ferulic acid, 6. a chalcone, 7. catechin. Enzymes: A. phenylalanine ammonia lyase, B. 4-cinnamic acid hydroxylase, C. p-coumaric acid 3-hydroxylase, D. O-methyltransferase.

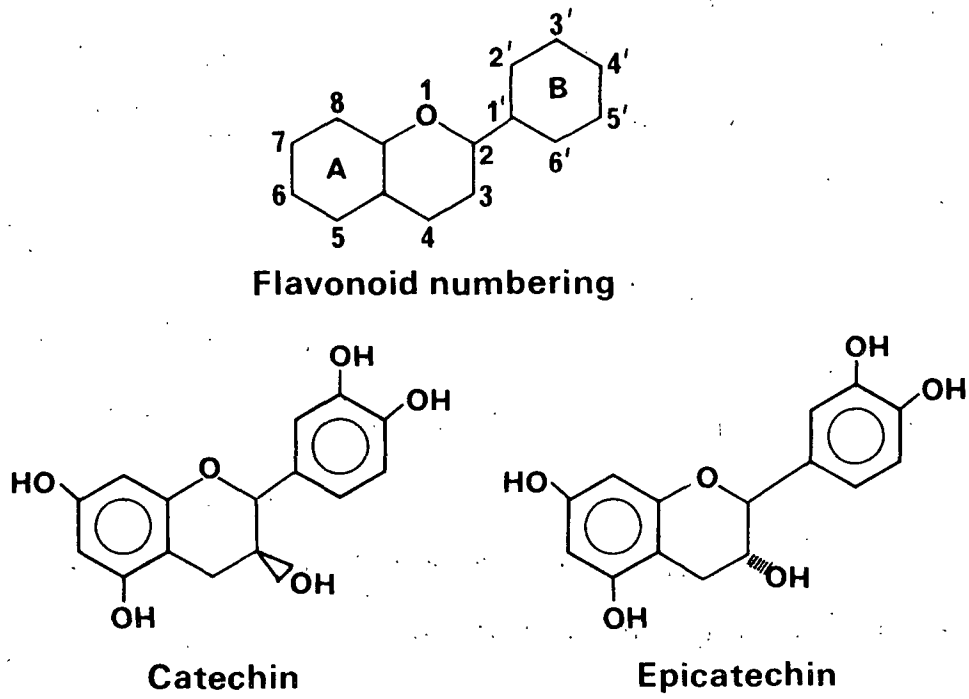


Fig. 2. Flavonoid ring numbering system, catechin, and epicatechin.

LITERATURE REVIEW

The general phenylpropanoid pathway is shown in Fig. 1 for the two main phenolic products in higher plants. However, there are other minor phenolic pathways that lead to such products as phenolic esters, dimers, coumarins, and chalcones. The phenylpropanoid pathway is stimulated by wounding of the tissue and by infections (5-8). In each case, it appears to be an immunological response designed to halt the spread of infection by surrounding the affected area with the microbially toxic phenolics, lignin and flavonoids. It has been suggested that the formation of callus is accompanied by a wound response of the tissue, and the accumulation of phenolics in this tissue is simply that of a wound response. The situation is not so obvious or simple. A wound response is accompanied by lignification, and so the lack of lignification in this tissue indicates that the tissue is not undergoing a simple wound response. The explanation for why this tissue does not lignify still must be addressed.

In healthy plants, the function of lignin is to physically support terrestrial life and to help create an efficient water transport system. The main function of flavonoids is much less clear. Flavonoids are the main coloring material in flower petals and it is thought that they primarily serve to attract insects to the colored flower parts, thus promoting pollination. The purpose of flavonoids in other plant parts is unknown at present, but they may serve to protect the plant from infection by microbes.

LIGNIFICATION

The lignin biosynthetic pathway has been determined primarily by use of labeled precursors in feeding experiments, and the subsequent isolation and characterization of the enzymes involved. The pathway for gymnosperms is shown in Fig. 3. The

compounds shown have all been found to be efficient precursors of lignin, and there would be little argument as to the sequence of compounds in the pathway. The first four enzymes have been fairly well characterized in a number of species (9-13). The other enzymes have been isolated in a few species, and so the main questions remaining regarding lignification are how the precursors are transported to the cell wall, which enzymes are the regulatory enzymes, what is the degree of polymerization of lignin *in vitro*, and what are its monomer building units in the various parts of the cell wall.

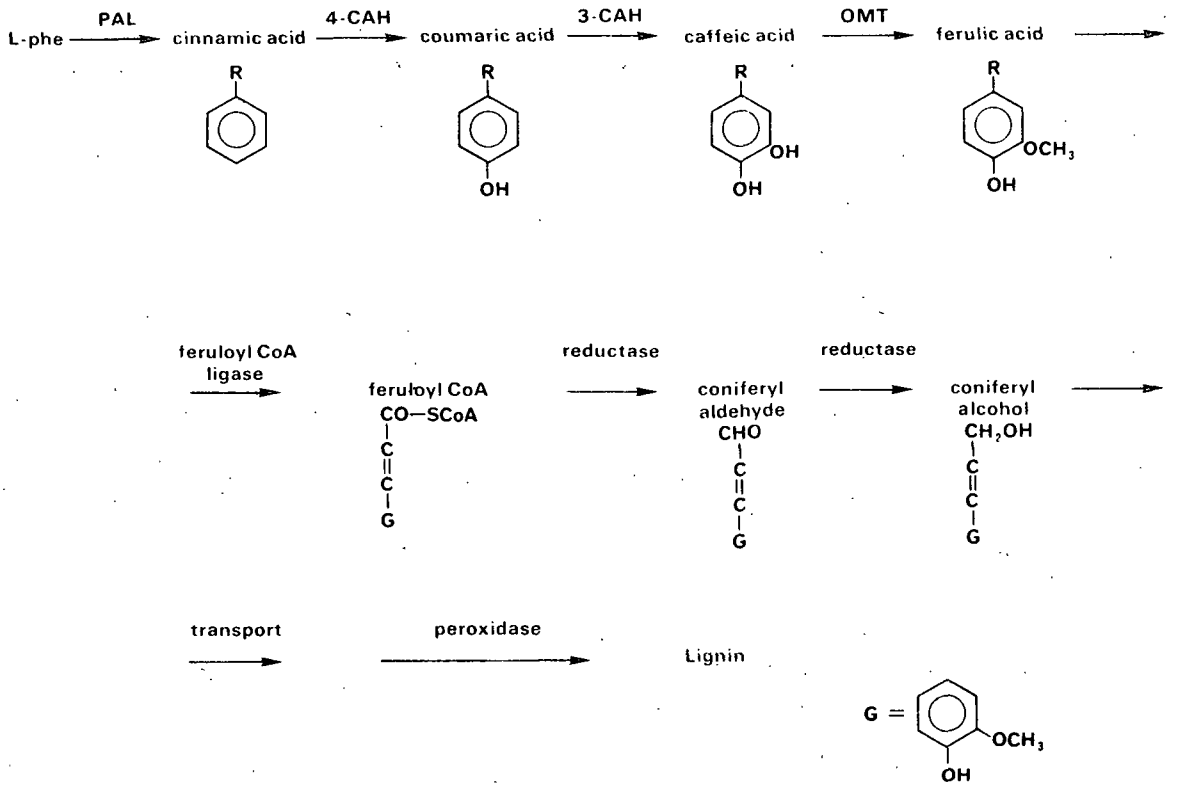


Figure 3. The lignin pathway for guaiacyl-type lignin.

The timing of lignification varies with the plant to some extent, of course, but for woody species in general lignification is a sudden event, taking place within

7-14 days, 70-80 days after germination of seeds or cell division in the mature plant. More precisely, lignification begins when the tracheids begin to differentiate or specialize. On a smaller scale, this means concurrent with the deposition of the secondary wall (14). Fukuda and Komamine (15) found that lignification in Zinnia elegans begins 5 hours after the secondary wall thickening begins.

White light stimulates the phenylpropanoid pathway. In soybean and parsley cultures, Grisebach and Hahlbrock (16) have found that the first three enzymes of the pathway in Fig. 3, PAL, 4-CAH, and 3-CAH, are synchronously stimulated by light in the blue (400-500 nm) and near red (650-705 nm) wavelength region of white light. This stimulation is reversed for these three enzymes by far red light (705-730 nm) by some unknown mechanism. This behavior has also been observed in a number of other species (17-22). In parsley, dilution of the culture with fresh medium has a similar stimulatory effect (23).

A regulatory role has been postulated for three enzymes in lignin biosynthesis. They are the first enzyme in the pathway, phenylalanine ammonia lyase (PAL); O-methyltransferase, which Higuchi has shown to determine the type of lignin formed, i.e., whether a guaiacyl or syringyl type (24); and the feruloyl CoA reductase (25), which controls the redox state of the lignin.

Only the first and second enzymes mentioned will be discussed in any detail. The reductase will not be reviewed, because it is biosynthetically preceded by the O-methyltransferase in the lignin pathway. The first enzyme in the phenylpropanoid pathway, PAL, has been the most studied of all of the pathway enzymes, both in cultured and in natural tissues (26-35). It has been found to be stimulated by white light, and most efficiently by the blue and near red wavelengths. In nearly all cases, this stimulation was accomplished through the synthesis of new PAL. In

one case, a storage form of the enzyme was found (36). In all cases studied, this increase in PAL was closely followed by a large increase in the amount of phenolics present in the tissue (37-41), and a direct relationship to lignification has also been shown (42). Since the latter enzymes of this pathway are not stimulated by light, this means that the stimulation of PAL somehow induced the increased activity of the enzymes further down the biosynthetic pathway, an indication of the regulatory control this enzyme possesses.

Hormones play a role in the stimulation of this pathway. It has been observed that gibberellins and auxins stimulate the synthesis of PAL and phenolics (43). Kinetin also stimulates PAL and lignification in pine cultured tissue (44), but 2,4-D, a synthetic auxin, has been found to inhibit the production of PAL and phenolics in several plant species (45-47).

FLAVONOIDS

The flavonoid pathway is shown in more detail in Fig. 4. It was noted on Page 3 that it is presently unknown at what stage plants hydroxylate the flavonoid "B" ring in general. In fact, recent literature suggests that the "B" ring is hydroxylated both at the C_9 and the C_{15} stage and that which path predominates depends on the species.

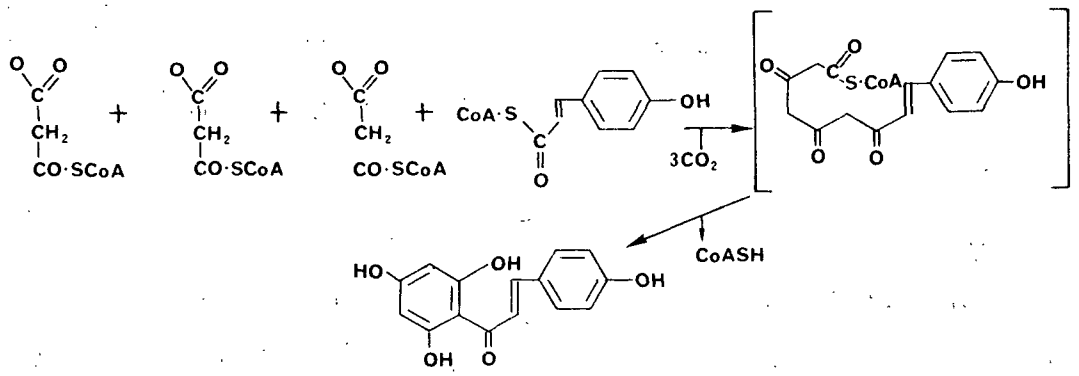


Figure 4. The flavonoid pathway.

Feeding experiments with labelled coumaric acid have shown that the p-coumaric acid is efficiently incorporated into the flavonoids with dihydroxy configurations in the "B" ring, but it was not shown whether this is by hydroxylation of coumaric acid to form caffeic acid and then incorporation into the flavonoids, or by incorporation into the flavonoids followed by hydroxylation. Zaprometov and Buklaeva (48) found a para-hydroxylated chalcone transformed into quercetin (Fig. 5). Vaughan, Butt, Grisebach, and Schill (49) have shown that enzyme preparations from spinach beet (Beta vulgaris L.) will catalyze the 3' hydroxylation of some 4' hydroxylated flavonoids. This would seem to imply hydroxylation after the flavonoid is formed, but Vaughan also found that the above enzyme preparation more efficiently converted p-coumaric acid to caffeic acid, which implies a C_9 primary substrate for the enzyme.

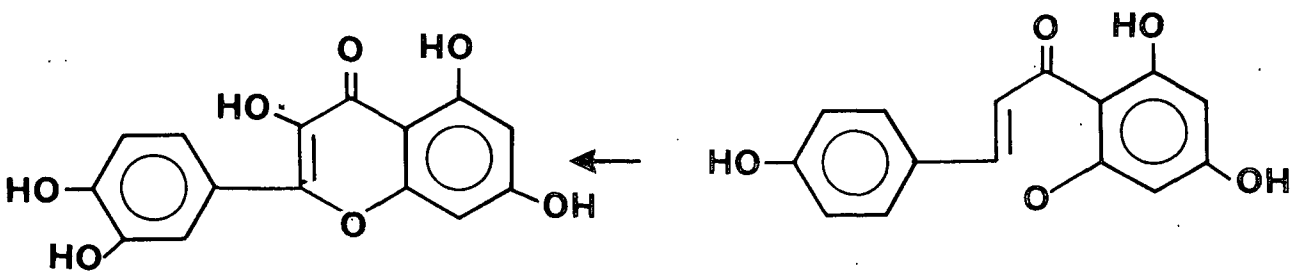


Figure 5. The para-hydroxylated chalcone shown by Zaprometov and Bukleva to be incorporated into quercetin (48).

On the other hand there is a great deal of evidence showing the direct incorporation of substituted cinnamic acids into flavonoids. Meier and Zenk (50) found that 3,4,5-trihydroxycinnamic acid was a better precursor of delphinidin, the 3',4',5'-trihydroxy flavonoid, than either p-coumaric or caffeic acid implying hydroxylation at the C_9 stage is more important. Steiner (51) had a similar finding with sinapic acid in petunia. Further support has been found by many others (52). The conclusion is that the hydroxylation can take place before or after the flavonoid ring system is formed. Which pathway predominates depends on the species being investigated.

O-methylation of flavonoids seems to take place predominantly at the flavonoid stage, however. In one case, ferulic acid was reported to be incorporated into the flavonoids at very low levels, less than 3% (53). Tommimura and Terashima (54) found that C^{14} ferulic acid fed to pine and locust shoots was not transformed into the flavonoids. Other studies show that extensive demethylation does take place before the transformation of the compound into flavonoids (55). Grisebach found p-methoxy cinnamic acid readily incorporated into a flavonoid in black locust, but only after at least 75% demethylation (56). So it seems evident that methylation of caffeic acid precludes incorporation into the flavonoids, at least without demethylation.

TANNINS

Only recently have tannins been studied biochemically to any great extent. Among the difficulties involved in studying them is the trouble in isolating a pure fraction due to differences in their degree of polymerization, and in their chemical reactivity, which is a function of their degree of oxidation. It is generally thought that they are linked as shown in Fig. 6 (57-59), but Roux and coworkers have suggested that the trimer structure of Fig. 7 is more correct (60). Much of the reactivity of tannin is due to its ability to form numerous hydrogen and covalent bonds. In enzyme systems this causes a great deal of trouble by inactivating the enzymes through these bonds.

Tannins are normally stored in the plant cells' vacuoles, where they do no damage to the cell. The tannins are released and oxidized when the cell is lysed or damaged (61-63). The function of tannins in plants seems to be primarily as a feeding deterrent to animals, and as such they are found in large quantities in immature and unripe fruits. It has been shown that the astringent properties of tannins decrease as fruit ripens, and this has been attributed to the increased polymerization of the tannin molecule (64). There is a narrow molecular weight range of 500-3000 in which

the tannins are the most astringent (65). There are some indications that the tannins are also catabolized, i.e., the tannins are not necessarily an end product of the flavonoid pathway as lignin is of the phenylpropanoid pathway, because the tannins may be broken down. This, however, has not been shown conclusively to occur.

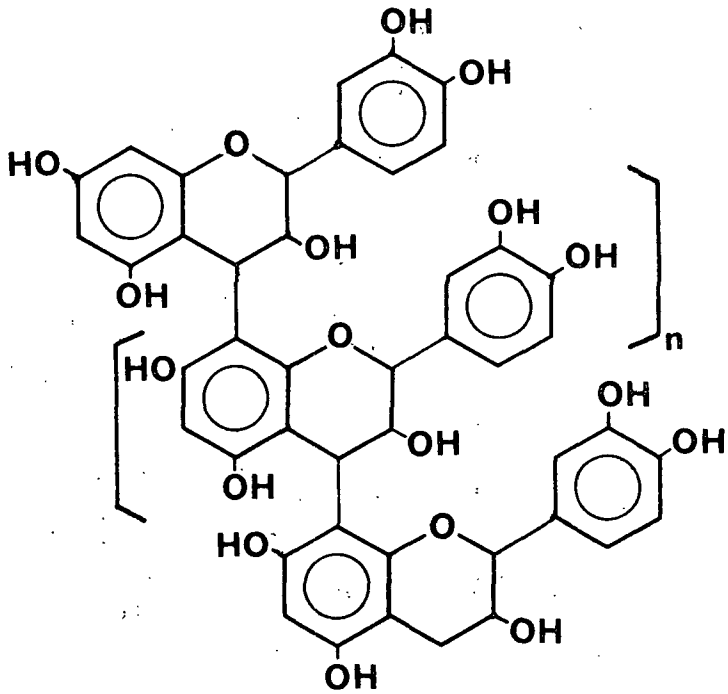


Figure 6. The generally accepted structure of procyanidins.

Stafford has done the greatest amount of work on Douglas-fir cultured tissue tannins (1,66-70). She found that whereas in strawberry, the tannin is comprised solely of catechin, and in avocado of epicatechin only, in Douglas-fir it is a mixture of the two flavonoids in a 3:1 catechin:epicatechin ratio (shown in Figure 2). Douglas-fir cultured tissues contain up to 40% of their dry weight as tannins (1). After homogenization, Stafford found tannins in all cell fractions, which indicates the high degree of contamination and reaction of the tannins with all parts of the

cell. When the cell wall is removed by making protoplasts, she found that no tannin contamination is found in the cell wall. This indicates that tannins are not normally associated with the cell wall and that any tannin found there after tissue disruption is due to contamination by tannin from the vacuole. In contrast to dicotyledonous species, no further tannin accumulation in Douglas-fir was found after the cultures stopped growing (66). This means that the Douglas-fir cells must grow in the presence of this tannin. High tannin levels are not a function of isolated tannin filled cells in a general population of low-tannin cells. This simply means that the measured amount of tannin is more likely to be a true indication of the average amount of tannin present in each of these cells.

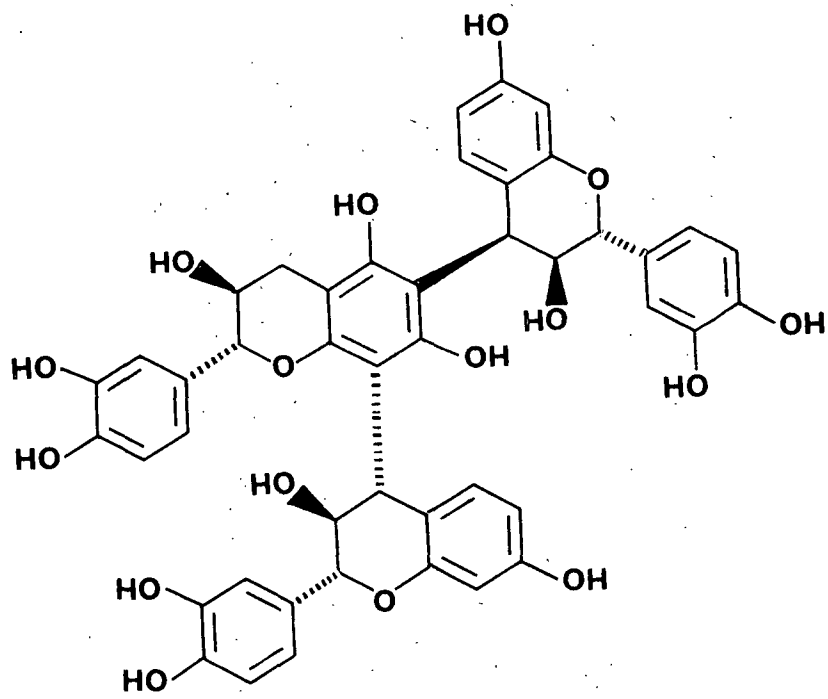


Figure 7. The structure of procyanidin proposed by Roux and coworkers (60).

O-METHYLTRANSFERASE

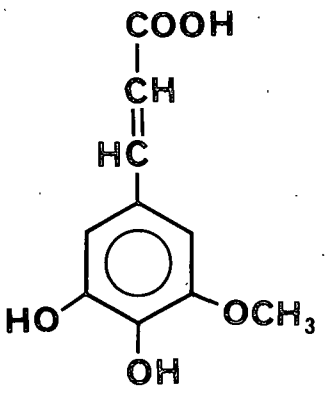
Catechol-O-methyltransferase was first isolated in 1958 from rat livers (71). In 1963 it was first isolated from plants, from an apple tree cambial region and

from the woody shrub Pittosporum crassifolia. Since that time, this enzyme has been studied in many plant species. The OMT mediates the transfer of a methyl group from the activated form of methionine, S-adenosylmethionine (SAM), to an oxygen of a catechol moiety. Strictly speaking, this is the only type of reaction this enzyme is capable of performing, but in practice it has been found that this enzyme can methylate monophenolic oxygens as well, but this depends very much on the source of the enzyme. This enzyme would be better characterized as an O-methyltransferase, rather than a catechol O-methyltransferase. In a number of cases, two and even three separable forms of this enzyme have been isolated (72-76). Since no general methylating enzyme has been found in any plant species (77), this has led to the working hypothesis that there are at least two different types of O-methyltransferases: a lignin-specific OMT and a flavonoid-specific OMT. In all cases where a sufficiently pure OMT has been isolated, this has been found to be the case. These types are not completely exclusive, however. The lignin-specific OMT will methylate flavonoids and vice versa, but only to a small extent, usually less than 5%.

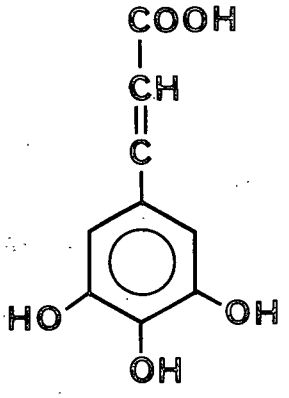
The flavonoid OMT has the broader substrate specificity of the two OMTs. Flavonoid OMTs can methylate in a variety of positions around the C_{15} molecule. Methylation has been shown to take place on the 3' OH (78), the 4' OH position para to the side chain (79), as well as on the 5 and 7 positions of the "A" ring (75). Methylation of chalcones has also been demonstrated (80). Although there is still some controversy about the point of flavonoid methylation in the pathway, whether it is at the C_{15} flavonoid stage or at an earlier point, the general results suggest that the methylation does take place after the C_{15} ring system has been formed. In the few cases studying this aspect it was found that incorporation of ferulic acid into flavonoids took place only after demethoxylation (54-55). In one case where transformation of ferulic acid into a flavonoid was reported, the label was located in

the side chain, not in the methoxyl group, so it is still possible that demethoxylation took place before biogenesis into the flavonoids.

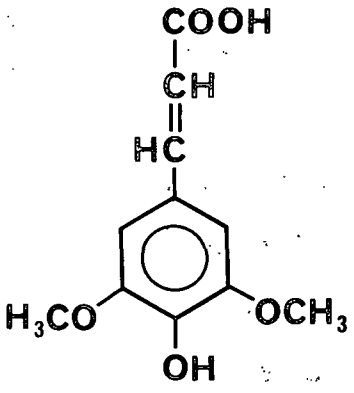
Lignin-specific O-methyltransferase has a much narrower substrate specificity range. It has been established that lignin O-methyltransferases catalyze the methylation of caffeic acid to ferulic acid, 5-hydroxyferulic acid (5-HFA) to sinapic acid, and 3,4,5-trihydroxycinnamic acid to both 5-hydroxyferulic acid and sinapic acid (Fig. 8) (81).



5 - Hydroxyferulic Acid



3,4,5 - Trihydroxycinnamic Acid



Sinapic Acid

Figure 8. The structures of 5-hydroxyferulic acid, 3,4,5-trihydroxycinnamic acid, and sinapic acid.

Higuchi (82-86) has done a great deal of work on the specificity of lignin O-methyltransferases. In 1972 (87) he found that bamboo OMT would methylate 5-hydroxyferulic acid more efficiently than caffeic acid. When he looked at Japanese black pine OMT he found that, conversely, the 5-hydroxyferulic acid was hardly methylated. This was the case for nearly all angiosperms and gymnosperms tested, i.e., angiosperms could methylate caffeic acid and 5-HFA and thus form both guaiacyl and syringyl lignins, but gymnosperms could methylate essentially only caffeic acid and thus could form only guaiacyl lignin. Table I shows some of these results. Higuchi (84) pointed out that this would account for the ratios of syringyl to guaiacyl type lignin found in angiosperms and the relative lack of syringyl lignin in gymnosperms. He thus postulated a regulatory role for this enzyme with respect to the type of lignin formed (88). The OMT for gymnosperms is thus seen to be very substrate-specific. It is essentially capable of methylating only caffeic acid to form ferulic acid. This is not the case for angiosperms where the OMT active site must be different.

TABLE I

OMT SPECIFICITY: SINAPIC ACID/FERULIC ACID SYNTHESIS

Species	SA/FA
Pinus strobus	0.4
P. taeda	0.3
P. densiflora	0.1
P. thunbergii	0.1
Gingko biloba	0.1
Populus nigra	3.0
Magnolia grandiflora	3.0
Salix caprea (callus)	1.7
Pisum sativum	2.7
Phyllostachys pubescens (bamboo)	1.0

The locale of this enzyme has been nearly uniformly stated to be in the soluble enzyme fraction of the cell. The dissenting cases have found the OMT in the microsomal fraction (89,90). It requires a force of at least 100,000 x g for several hours to separate the microsomal fraction from the truly soluble enzyme fraction. Inspection of the actual techniques used in the above experiments shows that a force of 6-30,000 x g for 10-30 minutes was used, so the two fractions were not actually separated. Consequently, it should be pointed out that the OMT in all cases has been found to be either a microsomal or a soluble enzyme (72-76, 91-99) and, as Stafford points out (69), is probably microsomal in all cases. The import of this is that, as a microsomal enzyme the OMT would be membrane-bound, whereas it would not be in the soluble case. This is of some importance because the Douglas-fir callus OMT is later shown to be a cell wall associated, membrane-bound enzyme. This was found to have a large effect on the enzyme kinetics, and so the question arises as to whether this difference is due to the OMT being membrane-bound or cell wall associated. This is discussed in more detail in the Results and Discussion Section.

The timing of OMT increases in the tissue relative to lignification in wheat is shown in Fig. 9 (42). This is the only case where these two related events have been quantitatively studied but, unfortunately, the technique used to quantify the lignin was not revealed. The figure does show, however, that an increase in OMT levels precedes the actual lignification. Another factor to notice is the fluctuation in OMT levels over time. There is no explanation offered for this, but it is reasonable to assume that it is related to growth of the tissue, cell division and precursor levels as well as levels of preceding enzymes such as PAL and CAH, which are known to be light-stimulated. In a number of other systems (100-103) it has been noted that an increase in PAL precedes the observed OMT increase, which is then followed by lignification, but these lignin levels were only qualitatively

followed. Other factors that stimulate OMT increases or lignification are hormones (104). Giberrellins (105) and auxins (106) and kinetins (107) have all been found to increase either OMT synthesis, lignification, or both. Conversely, 2,4-D, a synthetic auxin, has been shown to decrease the amount of OMT and lignin formed by a presently unknown mechanism (108,109). In parsley suspension cultures, as mentioned, dilution into fresh medium causes an increase in OMT levels (23), as does dilution into water (110).

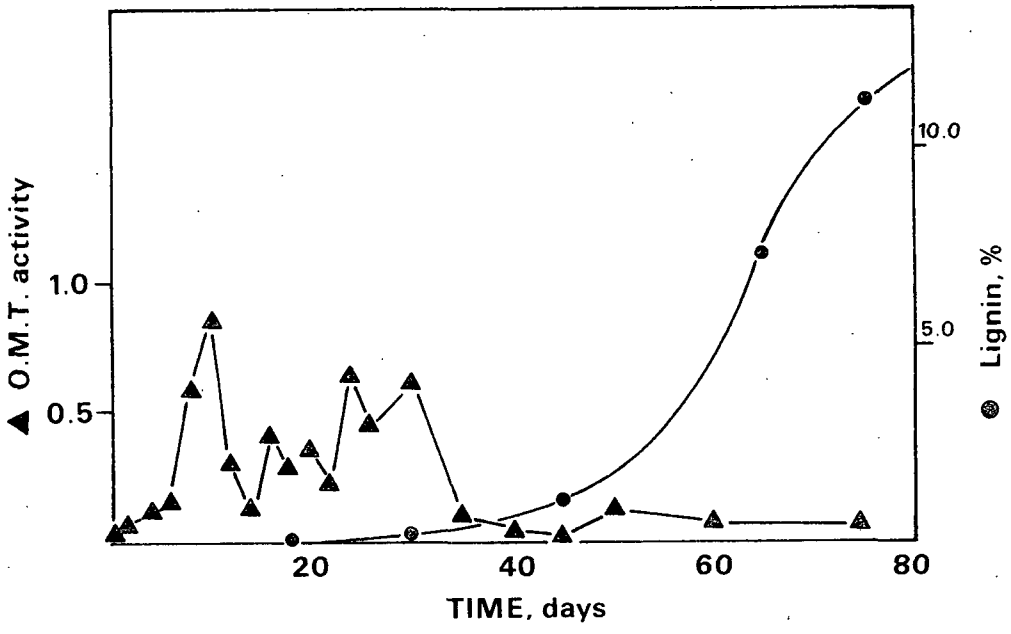


Figure 9. Wheat OMT activity and lignification vs. time (42).

The kinetics of this enzyme have not been widely reported, but information is available for some plant species. Table II shows the results for the Michaelis-Menten constant K_m, and for V_{max} (see glossary). The K_m values do show some interesting trends. The K_m values for caffeic acid with lignin-specific OMTs are generally in the range of 50-100 μM. For the flavonoid-specific OMTs the K_m range for caffeic acid is much higher, and for flavonoids themselves it is correspondingly

lower. In several cases, more than one OMT is found. The parsley suspension data appear rather high for some reason. The aspen K_m values are also higher than seems normal. This could be due to the isolated OMT being a flavonoid-specific rather than a lignin-specific OMT. The first tobacco reference shows the OMT I as the lignin-specific OMT, and OMTs II & III as flavonoid-specific OMTs. In the other tobacco case only two forms of OMT were found.

TABLE II

REPORTED OMT KINETIC CONSTANTS

	K_m, μM			V_{max},	K_i[c],	
	Caffeic	SAM	Quercetin	pkat	μM	Reference
Tobacco[a] OMT I	100	4.5		5200	5	
OMT II	250	3		425	5	
OMT III	200	3		640	5	(76)
Tobacco[b]	100	4		171		(75)
Aspen	380					(97)
Medicago sativa	58	4.1			0.44	(91)
Pinus thunbergii	51	40.6				(82)
Parsley suspension culture	1600	150				(93)
Bamboo	50					(87)
Chrysosplenium americanum		70	1.4			(111)
" " OMT I		70	1.4			
OMT II		90	0.5			
OMT III		50	2.5			(99)

[a]Nicotiana tobacum, cv. Samsun NN.
[b]Nicotiana tobacum, cv. Wisc. No. 38.
[c]Inhibition due to SAH.

These observations summarize what is known about plant OMTs. They help explain why and how flavonoid and lignin OMTs are different. They also indicate why angiosperm lignin has both guaiacyl and syringyl units, while gymnosperm lignin consists of primarily guaiacyl units. These results also suggest ways to stimulate the synthesis of OMT, but they do not show how or why this occurs.

If the lack of lignification in the Douglas-fir cultured tissues is to be better understood, the OMT in this tissue will have to be more closely investigated. This thesis is an attempt to provide that information.

THESIS OBJECTIVES

Douglas-fir needle callus typically accumulates polyphenolic flavonoids and tannins rather than lignin to the apparent detriment of embryogenesis. O-methyltransferase was hypothesized to be the key enzyme limiting lignification in this tissue by limiting the formation of ferulic acid. This bottleneck in the pathway would then force the p-coumaric acid and caffeic acid formed into the flavonoid and tannin pathway.

The objective of this thesis was to determine if the O-methyltransferase is indeed limiting lignification and, if so, to determine if the limitation can be overcome by inducing more OMT to be produced by this callus. To determine this required that the OMT be isolated and characterized. Thus, the objectives were

1. to determine if any OMT is present in this callus
2. to optimize the extraction and assay of the OMT from this callus
3. to determine the enzyme activity as a function of time from subculture
4. to determine the specificity of the OMT, whether it is a lignin-specific or flavonoid-specific enzyme
5. to localize the enzyme within the cells
6. to determine the kinetic characteristics of the OMT
7. to use a ^{14}C-labelled precursor to measure the extent of conversion into the flavonoid pathway *vs.* the lignin pathway
8. to quantify the amounts of the cinnamic acids, catechin, and tannin in this callus as a function of time from subculture.

The results of research based upon these objectives were used to determine whether or not the OMT is limiting lignification and if it is, what could be done to alleviate the phenolic problem.

METHODS AND MATERIALS

PLANT MATERIAL

The Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco] needle callus originated from Douglas-fir shoots supplied by Weyerhaeuser Co. The shoots were obtained organogenically from Douglas-fir needle callus. The needles from these shoots were surface sterilized and placed on a modified Murashige-Skoog medium (Appendix I) containing 5 mg/L NOAA and 0.1 mg/L BAP.

Once initiated, the callus was maintained in a Percival model E54 incubator with a 16 hour day at 2000 lux and 22°C, and 8 hours darkness at 18°C. The cultures were subcultured onto fresh medium every 4-6 weeks. The callus was initiated in July, 1978 and was labeled clone L-11. A new line of needle callus labeled clone DFN was started in May, 1980 to serve as a backup line.

Douglas-fir seedlings were grown under greenhouse conditions in a mixture of peat and vermiculite. The seeds were sown after either cold treatment for three weeks or peroxide-mediated stratification for 30 minutes. The seedlings used to determine the OMT kinetics were 30 days old, i.e., they were harvested 30 days after shoots appeared above the soil. The seedlings used to determine the OMT locale were four months old. It normally took two to three weeks after sowing for germination to occur and for shoots to appear.

ENZYME PREPARATION

The callus tissue was removed from the agar nutrient media and used without any further treatment. The seedlings were pulled from the soil and thoroughly washed in tap water followed by a distilled water rinse. The needles were then cut from the stem, and the stem and roots were then used as the enzyme source.

The callus and seedling tissue was homogenized at 4°C in 2 times (W/V) 0.1M potassium phosphate buffer (KPi) at a pH of 7.6, containing 10% PolyClar AT (based on the plant tissue wet weight), and 5 mM each of EDTA, reduced glutathione, and ascorbic acid, except where noted otherwise. PolyClar AT is an insoluble polyvinylpyrrolidone which serves to bind and sequester plant phenolics when the tissue is homogenized. The phenolic material must be removed to protect the enzymes from the inactivating tendency of these compounds, and PolyClar AT (known as PVP) has been shown to effectively protect plant enzymes from phenolic inactivation. In nearly all cases a Brinkman Polytron homogenizer model PF-34 with an ST-24 generator was used to disrupt the tissue and liberate the enzymes in a large test tube or beaker cooled in an ice bath. This allowed rapid homogenization of the tissue with a minimum of heat generated. In other cases, either a Ten Broeck hand-held glass tissue homogenizer, or a cooled mortar and pestle was used when gentle disruption of the tissue was desired, in order to preserve the cell organelles.

The whole cell homogenate generated was filtered through 4 layers of cheese cloth to remove whole cells and undisrupted tissue. It was then used without further treatment for the OMT assay, except when the purified enzyme system was investigated.

ENZYME ASSAY

The O-methyltransferase was assayed radiochemically by following the transfer of a ^{14}C-methyl group from S-adenosylmethionine to caffeic acid to form ferulic acid (72). The ferulic acid was then isolated and counted in the Beckman scintillation counter. In general, 1 mL of enzyme solution in 0.1M KPi buffer at pH 7.6 was assayed with 100 µL caffeic acid in buffer and 20 µL of [methyl-C^{14}] S-adenosylmethionine. This corresponded to concentrations of 1000 µM caffeic acid and 200 µM

SAM. There were slight variations in the volumes of the substrates needed to attain the desired concentrations. This is especially true of the kinetic experiments, where the concentrations were varied over wide ranges. These variations were accounted for in the data concerning the purified OMT, but in the homogenate case, the dilution of the enzyme preparation with substrate approximately equaled the dry matter in the homogenate and so no concentration corrections were needed. The assay ran for one hour in a shaking waterbath heated to 30°C unless otherwise noted.

The homogenates contained PVP, EDTA, glutathione, and ascorbic acid in the assays, because these were needed to protect the enzyme during disruption of the tissue. They were not removed for the assay itself. For the purified OMT, these additives were removed by centrifugation and dialysis. Their addition to the enzyme solution had no effect on the enzyme activity, so they were not added back for the purified OMT assays.

The assay was stopped by first adding 1-2 μmole of ferulic acid in 100 μL of 50% ethanol to act as a carrier and then by immediately adding 200 μL 5N HCl. This amount of ferulic acid was 100-1000 times the amount of ^{14}C ferulic acid formed, so little of the labeled product was lost in the workup procedure. This amount also gave a visible and discrete spot on the TLC plate, which made isolation that much easier. The ferulic acid was extracted from the acidified assay mixture by shaking with 3 x 5 mL diethyl ether. It was found that 95% of the ferulic acid was extracted in the first ether extract. The ether extract was then dried under nitrogen, and the ferulic acid was spotted onto a silica gel G TLC plate with methanol. The TLC plate was developed in benzene:acetic acid:water (10:10:2, organic phase), which was found to give good separation of the cinnamic acids. After drying the plate, the ferulic acid spot was scraped from the plate into a scintillation vial.

Several scintillation cocktails were used throughout this thesis. Phasar, purchased from Amersham Corp. was initially used. However, most of the counting done in this work was with Scintisol, purchased from Isolab Corp. The quench curves for these cocktails are contained in the appendix. The amount of radioactivity was determined in the Beckman scintillation counter, model DPM-100, after the addition of 10 mL of the scintillation cocktail.

ENZYME PURIFICATION

The crude homogenate described above was stirred with the detergent, digitonin, at 0.5-1% based on the tissue wet weight, for 3-5 hours in the cold (4°C). The digitonin serves to liberate enzymes from membranes by interacting with and disrupting the lipid bilayer of the membrane. Solubilization by digitonin is generally taken as evidence for a membrane-bound enzyme (112). The resultant slurry was centrifuged at 20,000 x g for 20-30 minutes and the pellet was discarded. The supernatant was placed in dialysis tubing and dialyzed against 10 volumes of 0.1M KPi buffer for 1-2 hours to remove the digitonin. The buffer was replaced once, and the dialysis was continued for several more hours. The bag contents were then removed, and the enzyme preparation was fractionated with ammonium sulfate. The enzyme preparation was fractionated as follows: 0-25%; 25-35%; 35-50%; 50-80%; and 80-100% saturation. The 25-35% fraction had the highest OMT activity, and so was used in further experiments.

The 25-35% fraction was resuspended in buffer and placed in dialysis tubing, where it was dialyzed against 10 volumes of buffer with a change of buffer after several hours. This fraction was divided into 5 mL samples and stored in the freezer at -20°C until needed. Assays in this thesis showed that freezing had no detrimental effect on the enzyme activity at this level of purity.

O-METHYLTRANSFERASE LOCALE

It was necessary to disrupt the tissue gently to preserve the integrity of the cell organelles. To do this a cooled mortar and pestle were used, with gentle hand-grinding of the tissue. A one-to-one ratio of buffer to tissue wet weight was used to maintain a high OMT concentration in the final extract. The grinding medium contained 30 mM sucrose to prevent the organelles from bursting, and 5 mM each of EDTA, reduced glutathione, and ascorbic acid to insure that the OMT activity was preserved in 0.1M, pH 7.6 KPi buffer. The slurry was strained through 4 layers of cheesecloth after grinding, and the filtrate was centrifuged for 15 minutes at 20,000 x g. The supernatant was removed and used for the microsomal and soluble enzyme fraction. The pellet was resuspended in buffer and centrifuged at 1000 x g, which left only the mitochondria in suspension. Resuspension of the pellet and centrifugation at 200 x g for 15 minutes left the chloroplast fraction in suspension, and the cell wall fraction as the pellet. Resuspension of the pellet gave the last of the four fractions used. All fractions were diluted with 5 mL of buffer except the microsomal-soluble fraction which was diluted to 5 mL. Figure 10 schematically shows the plan used. Microscopic examination at 66X of the different fractions showed no significant cross contamination, i.e., the mitochondrial and microsomal-soluble fractions contained no visible cell wall debris. After ultrasonic disruption of the organelles present, each fraction was assayed using the standard assay procedure, except that 40 μM SAM was used rather than 200 μM. The caffeic acid concentration was 1000 μM.

RADIOTRACER

2-C^{14} cinnamic acid was fed to callus cells in the radiotracer experiments. Previous experiments in this lab (113) showed detrimental effects on growth when C^{14} compounds were fed at levels of 4 μCi/mL packed cell volume, or about 4 μCi/gm wet weight of tissue. The concentration of cinnamic acid in this experiment was kept at

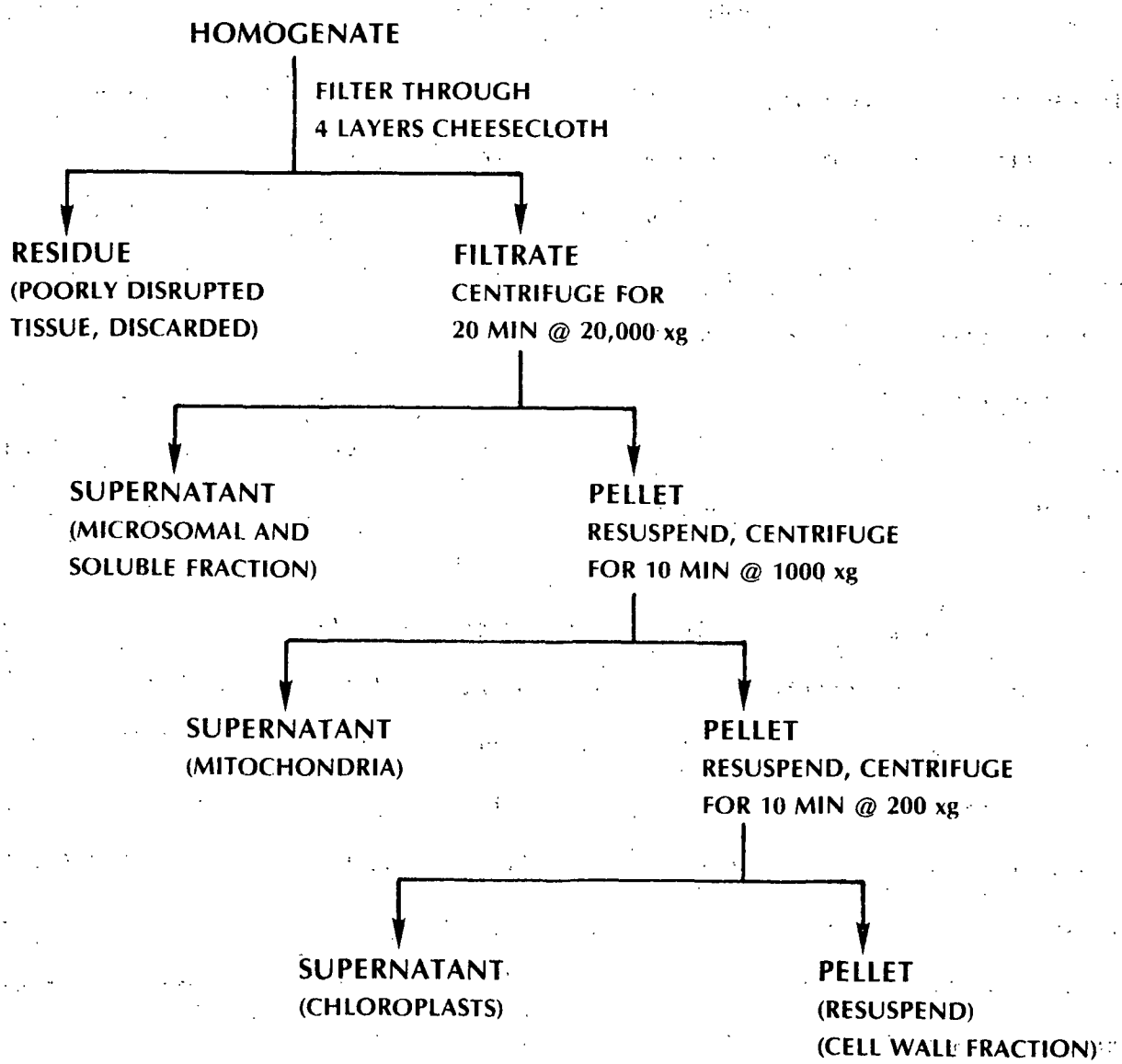


Figure 10. Centrifugation flow sheet for organelle isolation.

1 μCi/mL buffer for this reason. To a glass vial containing 1 mL of 0.1M KPi buffer at pH 7.6 was added 10 μL C^{14} cinnamic acid (purchased from Amersham Corp., cat. No. CFA 346, sp. act.). Ten-day-old callus, 100-200 mg, was added, and the tissue was incubated for 2 or 12 hours at room temperature in the light. Ten day old callus was used because it had the highest measured level of OMT activity over subculture time. The tissue was removed and washed with buffer. The cells were then homogenized in several mLs of water in a glass Ten Broeck homogenizer with 0.1 mg each of cinnamic,

p-coumaric, caffeic, and ferulic acids and 0.1 mg of catechin added as carriers. The resultant homogenate was acidified with 5N HCl, and the free cinnamic acids were extracted with diethyl ether, 3 x 5 mL. The ether was dried under a stream of nitrogen gas and spotted onto a silica gel G TLC plate with methanol. The plate was developed as before in benzene:acetic acid:water (10:10:2, organic phase). The free acids and catechin were located visually under UV light and by a Berthold model LB 2760 TLC Scanner, scraped off into vials and counted in the scintillation counter.

The acidified, ether-extracted homogenate was adjusted to 5% HCl, and was boiled for 1 hour to hydrolyze the glycosidically-bound storage forms of the cinnamic acids. The products of this reaction were similarly extracted and isolated. The acids were then removed and counted as before.

The resultant homogenate was neutralized with NaOH and adjusted to 5% NaOH. This was then boiled for 1 hour also, to liberate any alkaline hydrolyzable storage forms. The homogenate was extracted with ether and treated as above after reacidification.

The data from these three extracts were then used to determine the level of these compounds for the lignin and flavonoid pathways.

ISOLATION OF ENDOGENOUS CINNAMIC ACIDS, CATECHIN, AND TANNIN

Callus tissue, 1-2 g, was sampled every other day for 30 days and frozen at -20°C. At the end of this time three samples of 0.14-0.5 g from each time point were taken and homogenized in water. This slurry was then acidified and extracted with 3 x 5 mL diethyl ether. The ether extracts were dried under nitrogen, and the residue was spotted on silica gel G TLC plates along with known standards in parallel and developed as before. The cinnamic acids and catechin spots were scraped

off, and extracted with methanol. The silica gel was removed by filtering through glass wool disks. Each sample was extracted three times with 1-2 mL methanol.

The filtrate was taken and the absorbance of each sample was read in the Perkin Elmer UV spectrophotometer at the appropriate wavelengths for the particular compound extracted. Given the absorbance reading, the extinction coefficient (Appendix V), and the mass of tissue extracted, the concentration of the particular cinnamic acid in the original tissue was determined. This yielded the levels of the free compounds in the tissue.

The ether-extracted homogenate was acidified to 5% HCl with 10N HCl and boiled for 1 hour in dim light. This would free the acid labile forms of the cinnamic acids or flavonoids. The compounds were extracted, isolated, and quantified as above. The remaining residue was neutralized and adjusted to 5% NaOH with 10N NaOH and also boiled for 1 hour in dim light. After acidification, the acids and flavonoids were extracted and treated as before. The proanthocyanidin was determined according to the procedure of Stafford and Cheng (66). This meant boiling a known weight of tissue for 30 minutes in dim light in 95/5 (V/V) n-butanol concentrated HCl. The proanthocyanidin is thus extracted and hydrolyzed to form cyanidin, which is a red-colored compound absorbing light at 550 nm. The absorbance of each sample at this wavelength was taken, and comparison with a standard curve yielded the amount of tannin present in the original tissue.

The proanthocyanidin for the standard curve was also isolated according to the method of Stafford and Cheng (66). Douglas-fir callus tissue was extracted with 70% methanol, and the methanol was removed with a rotary evaporator under reduced pressure. The remaining water phase was frozen and lyophilized. The residue was extracted with absolute methanol which was then diluted 1:1 with ethyl acetate.

Upon cooling to 0-4°C, the procyanidin precipitated and was collected by filtration on glass wool and used for making the standard curve.

All of these data were standardized to 1 g fresh weight of tissue for plotting on the graphs.

RESULTS AND DISCUSSION

CALLUS GROWTH CHARACTERISTICS

The Douglas-fir needle callus exhibited growth patterns typical of this species. Figure 11 shows the change in callus fresh weight over time for triplicate samples. The curve is sigmoid, which is normal for cultured tissues (114). This experiment was purposely extended to 43 days. However, the normal subculture period was 30 days long. Under this schedule, the tissue was just reaching the stationary phase when it was subcultured onto fresh growth medium. It was noticed that handling the tissue by picking it up with sterile forceps and replacing it seemed to increase the browning of the tissue. Checking this phenomenon led to the results in Fig. 12, the growth curve for the handled tissue. It can be seen that the handled tissue reached accelerated growth at the end of the experiment, long after the unhandled control reached such growth. Tissue this old normally has reached a stationary phase, having gone through the entire growth cycle, as Fig. 11 shows. The handled tissue has only grown through one-half of the normal growth cycle. Figure 13 shows some data from measurements of the amounts of tannin present in these tissues. It is easily seen that the handled tissue has roughly three times the amount of tannin in the cells as the unhandled material. Since the only difference between these samples was that the handled tissue was picked up from its dish, weighed individually in a sterile plate, and replaced, it is apparent that such mechanical manipulation of the tissue has an adverse effect on the health of the tissue. Similar behavior was noted qualitatively by R. Smeltzer in his thesis on loblolly pine (115). To say that this is a wound response of the tissue or a gaseous or humidity effect would be premature given this particular set of data, but this aspect of the cultured tissue deserves further study because of its implications about the effects of agitation on the suspension cultures.

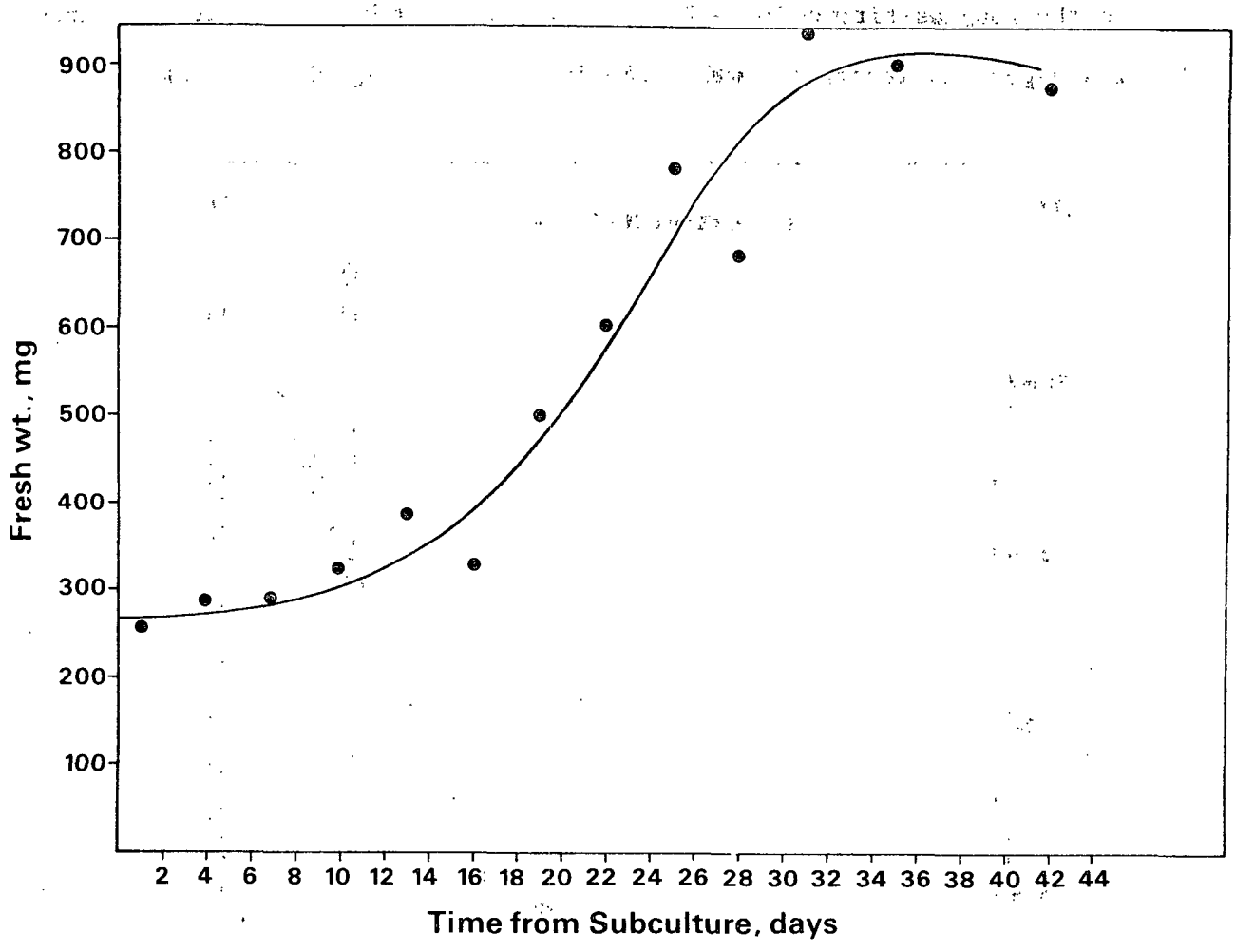


Figure 11. Callus growth curve, callus fresh weight vs. time from subculture.

Two other morphological features of the tissue were followed over time: they were the secondary wall deposition and lignification. The first was followed by utilizing the fact that ordered lamellar structures, such as cellulose fibrils in cell walls, will birefringe in polarized light (116). Using an Olympus Model Vanox PM-10-A microscope wih polarizing filters, the deposition of the secondary cell wall was followed. Photographs 1 and 2 show the birefringence of Douglas-fir at 7 and 30 days after subculture. There was little secondary wall material deposited in that time. Photographs 3 and 4 show loblolly pine needle callus at days 7 and 30. In

contrast to the Douglas-fir needle callus, it is evident that much secondary wall material was deposited over the growth cycle in the loblolly pine callus.

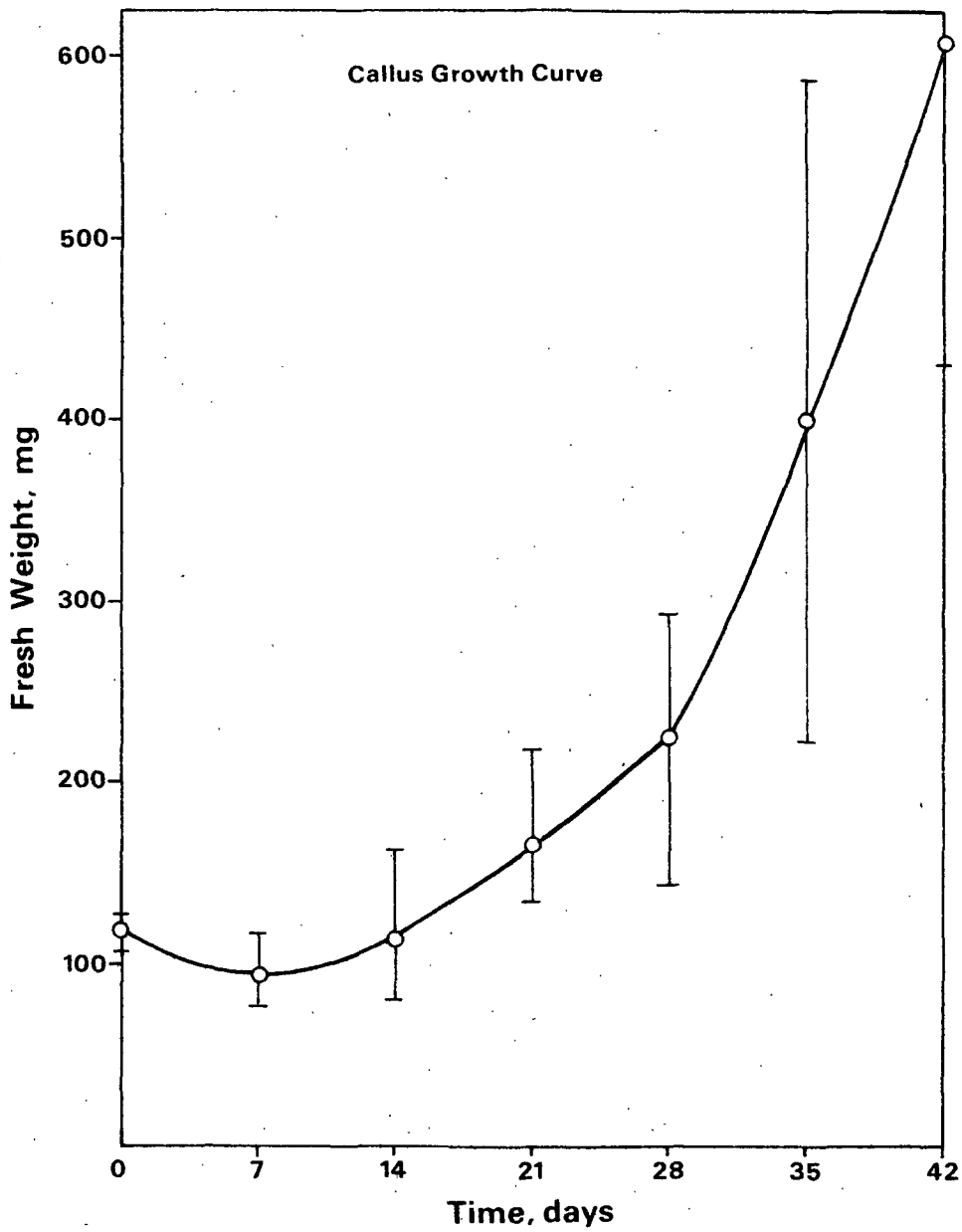


Figure 12. Handled callus fresh weight vs. time from subculture.

Photomicrographs 5 through 8 show the amount of lignin present in these tissues. The phloroglucinol-HCl stain gives a red product upon reaction with coniferyl

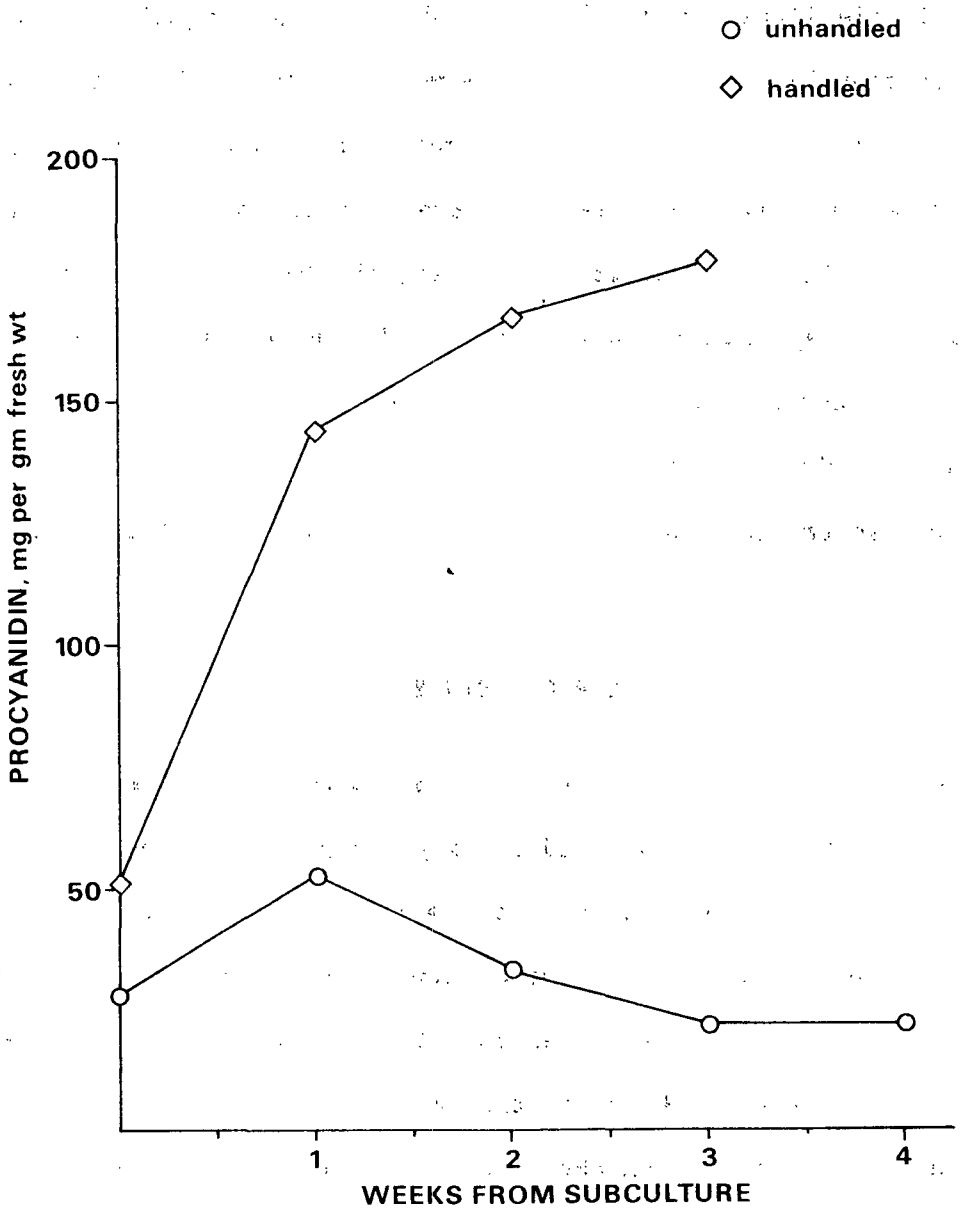


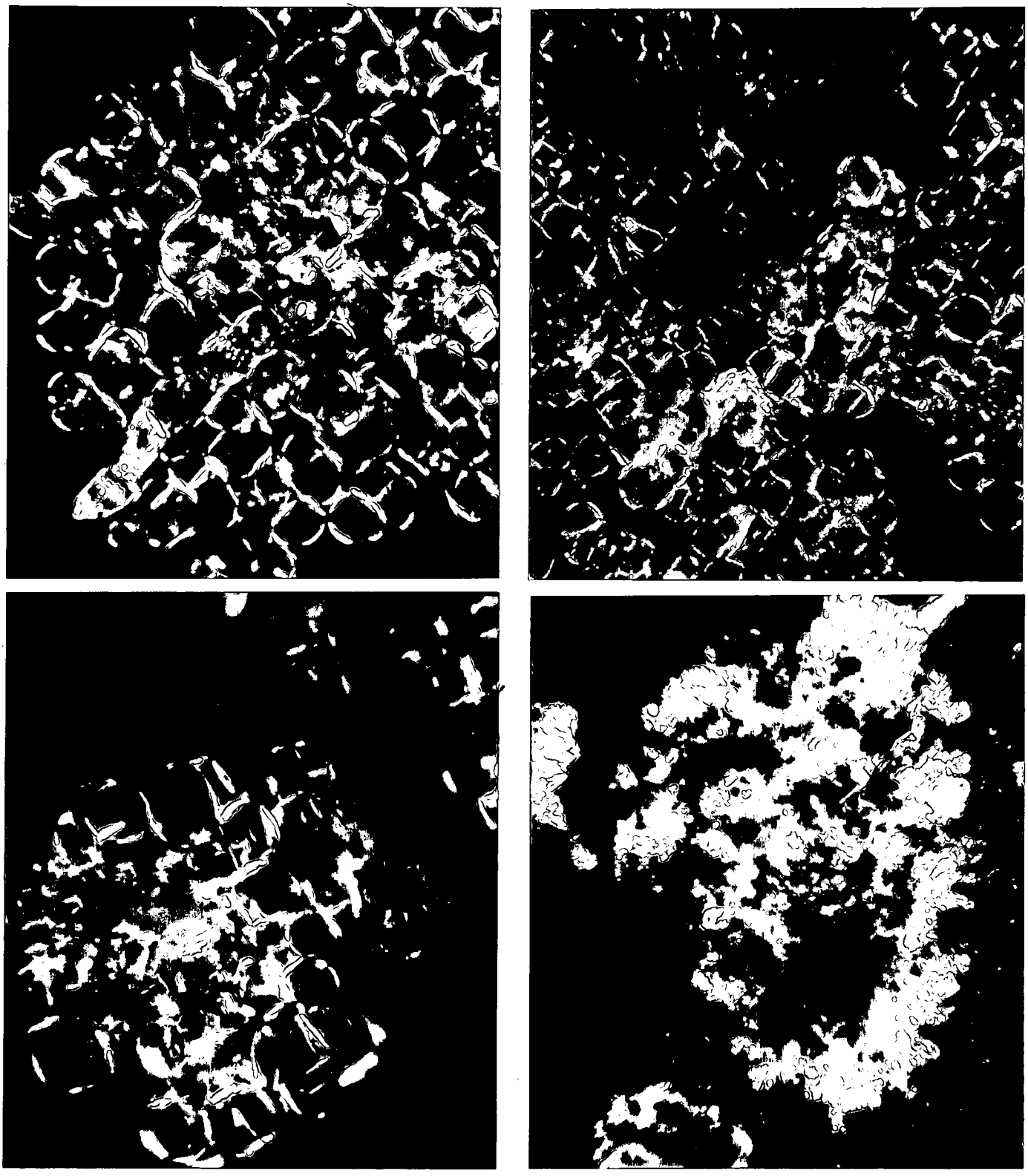
Figure 13. Comparison of tannin vs. time in handled and unhandled callus tissue.

aldehyde groups in the lignin molecule (117). In the redox environment of these cells coniferyl alcohol can react as well. Once again, it is clear that the Douglas-fir tissue forms little lignin, while the loblolly pine callus forms a great deal. Photomicrographs 5 and 6 show that the Douglas-fir callus starts with little lignin, and little or no lignin is formed over the growth cycle. Loblolly pine, on the other

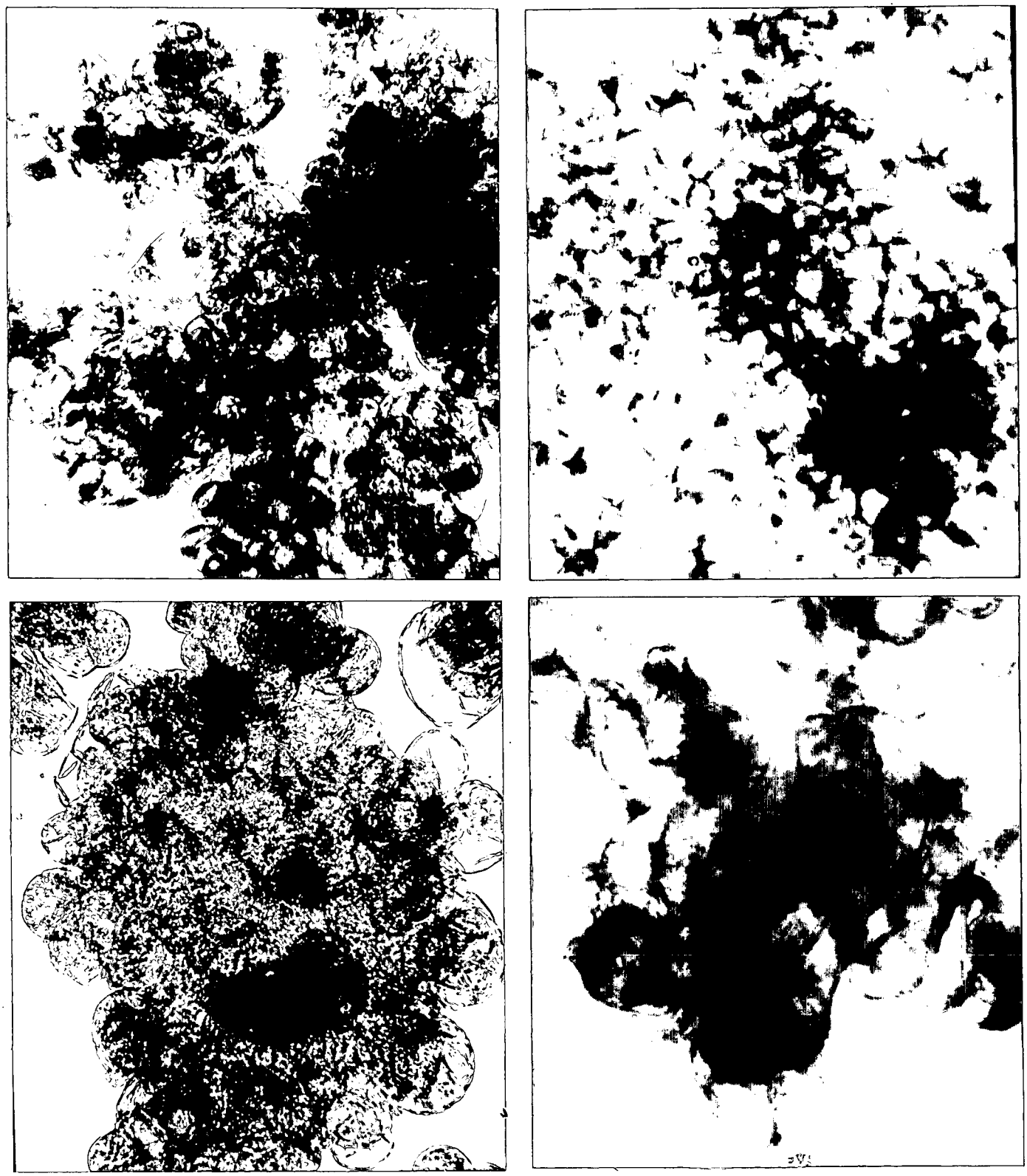
hand, starts with little lignin at day 7 but it shows a substantial increase in the amount of lignin present by day 30. It would seem, therefore, that significant secondary wall deposition is required before lignification begins. Consequently, the Douglas-fir callus does not increase its lignin content over time because no further secondary wall thickening occurs. However, the Douglas-fir callus does show a small degree of secondary wall thickening at day 7, and whether or not the amount of lignin actually present is low is open to question. Given the small amount of secondary wall material in the Douglas-fir callus, it may be possible to increase the amount of lignin in the cell wall and thereby help alleviate the internal phenolic buildup.

RADIOTRACER STUDY

The radiotracer study was an attempt to follow quantitatively the substrate, cinnamic acid, through the phenylpropanoid pathway. Table III shows the amounts of each cinnamic acid derived product found in the Douglas-fir tissue. Single samples were used for this experiment. Two incubation times were used, and it can be seen that little difference was found for the amount of cinnamic acid taken up by these cells. This was probably due to the concentration of the cinnamic acid in the buffer in which the tissue was placed. From the table it is immediately apparent that little of the cinnamic acid was utilized by the phenylpropanoid enzymes. Only about 5% of the cinnamic acid taken up was hydroxylated to form p-coumaric acid. Only 10% of this 5% was further hydroxylated to form caffeic acid, while the amounts of ferulic acid and catechin were about the same. A larger amount of activity was found at the TLC plate origin. This was assumed to be the proanthocyanidin which is derived from catechin. This was previously found to be slightly soluble in ether and was also found to remain at the origin in the solvent system used to develop the TLC plate. The butanol-HCl test showed this spot contained procyanidin. Additionally, the



Micrographs 1-4. Douglas-fir and loblolly pine cultured tissue cells birefringence. Upper left. Douglas-fir cells, day 7. Upper right. Douglas-fir cells, day 30. Lower left. Loblolly pine cells, day 7. Lower right. Loblolly pine cells, day 30. Magnification: Upper 99X. Lower left 99X. Lower right 39.6X.



Micrographs 5-8. Phloroglucinol-HCl lignin stain for Douglas-fir and loblolly pine cultured tissue. Upper left. Douglas-fir cells, day 7. Upper right. Douglas-fir cells, day 30. Lower left. Loblolly pine cells, day 7. Lower right. Loblolly pine cells, day 30. Magnification: Upper 99X. Lower left 99X. Lower right 297X.

ether-extracted aqueous homogenate, from which the free cinnamic acids were derived, was boiled in 5% acid to hydrolyze any glycosides present, and this had the added effect of testing for the presence of procyanidins in a manner similar to the method of Stafford and Cheng (66). This homogenate showed a great deal of proanthocyanidin present, so it is likely that the amount of procyanidin is actually much higher than is indicated in the table.

TABLE III

RADIOTRACER STUDY

Compound	^{14}C, CPM per Sample	
	Incubation Time	
	2 hour	12 hour
Cinnamic acid	444,633	493,211
p-Coumaric acid	18,725	23,688
Caffeic acid	1,911	2,327
Ferulic acid	2,921	1,932
Catechin	2,357	1,260
Origin-proanthocyanidin	6,319	3,781
Cell wall debris	391	1,541
Incubation buffer-wash	1,196,988	1,104,570
Total ^{14}C-cinnamic acid	1,940,000	1,940,000
Recovery, %	84%	86%

From these results in Table III, it appears that slightly more of the added cinnamic acid is shunted to the tannin pathway, rather than to the lignin pathway, but in neither case does the radioactivity appear to be very high. It should be pointed out, however, that the tissue used was 10 days old, which corresponds to a maximum level of OMT in the callus, but an unknown level of the flavonoid enzymes.

In addition, after this maximum spike in the OMT activity, the level of OMT in this tissue drastically decreases. So, this amount of ferulic acid formed shows an optimum condition, which may not be the case for the flavonoids. It is obvious that the levels of total flavonoids formed would be much higher than that of lignin if the flavonoid enzymes remained at a relatively higher level than the OMT over the entire subculture period. The small amount of caffeic acid seemingly formed could be due to just a small, steady-state concentration in the tissue. This would seem to be the case, given the larger total amounts of proanthocyanidin and ferulic acid. The relatively small amount of ferulic acid, compared to the combined activity of the catechin and proanthocyanidin, does imply that there is a problem with the OMT in this tissue. The rest of this thesis is an attempt to explain exactly what that problem is.

O-METHYLTRANSFERASE EXTRACTION

In soy cultures, Stafford found the OMT to be part of an enzyme cluster in the microsomal fraction, which could be sedimented by centrifugation at 100,000 x g (69). All other reports concerning plant O-methyltransferase reported the enzyme to be in the soluble cell fraction. As mentioned earlier, these reports left open the possibility that the enzyme was actually a microsomal enzyme, but they did uniformly find the enzyme in the supernatant after centrifugation at 6-30,000 x g. Because of these reports, preliminary assays in this thesis focused on the soluble cell fraction of Douglas-fir callus, i.e., on the supernatant after centrifugation at 10,000 x g. These assays were uniformly negative for OMT activity.

When whole cell homogenates were used, the OMT was found in abundance. Consequently, cell homogenates were used to optimize the extraction of the enzyme. Acetone powders of the enzyme, made at -20°C, destroyed its activity. The use of acetone

powders is a well-known technique for isolating enzymes which has several advantages for working with plant tissues. The organic solvent nature of the acetone precipitates the enzymes while solubilizing the enzyme-inactivating phenolics. Centrifugation of the homogenate then sediments the enzymes and other proteins while removing the phenolics in the supernatant. Acetone also solubilizes and removes the lipid membranes from membrane-bound enzymes. A number of reports concerning OMT (8,42,93) have used acetone powders for isolating the enzyme, which raises the possibility that other membrane-bound forms of OMT were solubilized by the technique used. A drawback with acetone powders is the severely inactivating nature of organic solvents at temperatures above -15°C. For this reason, the temperature of the acetone used in the isolation of OMT from Douglas-fir was carefully maintained at -20°C. Unfortunately, inactivation of the enzyme still occurred. This behavior is found for some enzymes, for reasons not understood at the present time. Since acetone powders destroyed OMT activity, it was necessary to work through a direct aqueous extraction. This meant finding a way of protecting the enzyme from the high levels of inactivating phenolics present, which would be expected to bind to the protein upon oxidation, following tissue disruption. Insoluble polyvinylpyrrolidone (PVP) has been found by many to be helpful in protecting plant enzymes during extraction. It works by hydrogen bonding reduced phenolics, and has been estimated to be 92% effective in plant homogenates (118). That is, 92% of the phenolics present will be safely bound to the PVP. To maintain a reduced state of the phenolics, previous research has found mercaptoethanol, ascorbic acid, and other reducing agents effective. Table IV summarizes the results found with Douglas-fir OMT in 4 different experiments.

TABLE IV

OMT EXTRACTION OPTIMIZATION

Assay Solution	Ferulic Acid, nmole/g wet wt./hr
Part 1	
Buffer only	3.4
Buffer + XAD	4.0
Buffer + PVP	9.0
Buffer + mercaptoethanol	1.6
Buffer + mercaptoethanol + PVP	4.9
Part 2	
Buffer + PVP	6.7
Buffer + PVP + cysteine (5mM)	20.0
Buffer + PVP + EDTA	49.0
Part 3	
Buffer + PVP	0.28
Buffer + PVP + BSA	0.15
Buffer + PVP + oxidized glutathione	0.24
Buffer + PVP + metabisulfite	0.29
Buffer + PVP + ascorbic acid	0.36
Part 4	
Buffer + PVP	2.8
Buffer + PVP + EDTA	5.6
Buffer + PVP + EDTA + BSA	4.4
Buffer + PVP + EDTA + cysteine	3.2
Buffer + PVP + EDTA + glutathione, reduced	6.1
Buffer + PVP + EDTA + metabisulfite	3.7
Buffer + PVP + EDTA + ascorbic acid	8.3

The absolute differences between the sections of this table are unimportant. Calli at different ages were used for the individual sections, so the amount of OMT activity present varies. However, within a single section the comparisons are valid. The assays with PVP serve as controls to enable comparison between sections.

From Table IV it is apparent that PVP is an important protector of enzyme activity. It is also apparent that EDTA, reduced glutathione, and ascorbic acid are important in extracting a maximal amount of OMT from these tissues. The deleterious

effect of mercaptoethanol is at variance with other reports concerning OMT in other species (76-78,81,91,93). Since the Douglas-fir callus OMT is not a soluble enzyme, as the OMT is reported to be in other species, it could be conjectured that this factor causes the different response to mercaptoethanol. It is possible that the enzyme requires both oxidized and reduced portions to remain active and that the glutathione is more efficient at maintaining this redox state than is mercaptoethanol. Glutathione is a zwitterion while mercaptoethanol is not ionized and so there may be a large difference in their mobilities.

It was possible to survey changes in the OMT levels in whole cell homogenates over time, with this optimized extraction of OMT. Figures 14 and 15 show this change on a wet- and dry-tissue basis. Five replicates were used for each data point. The graphs show a peak of activity at day 10, and then some fluctuation in activity that may represent subsequent catabolism and synthesis of the enzyme. Following the results of this experiment, all further experiments were done with tissue 10-14 days old.

It can be pointed out here that it is felt that these fluctuations are indicative of the metabolism of the system as a whole and not just the normal variability found in living systems. This type of variation was found repeatedly in this tissue with many different compounds, and it has been found in loblolly pine callus as well, specifically in tannin and polyamine contents (113). It may be a function of partial synchronization of cell division, or some other periodic factor not now evident (119).

SPECIFICITY

Table V shows the results of a series of experiments to determine the specificity of the purified Douglas-fir callus OMT. Duplicate samples were run. Since

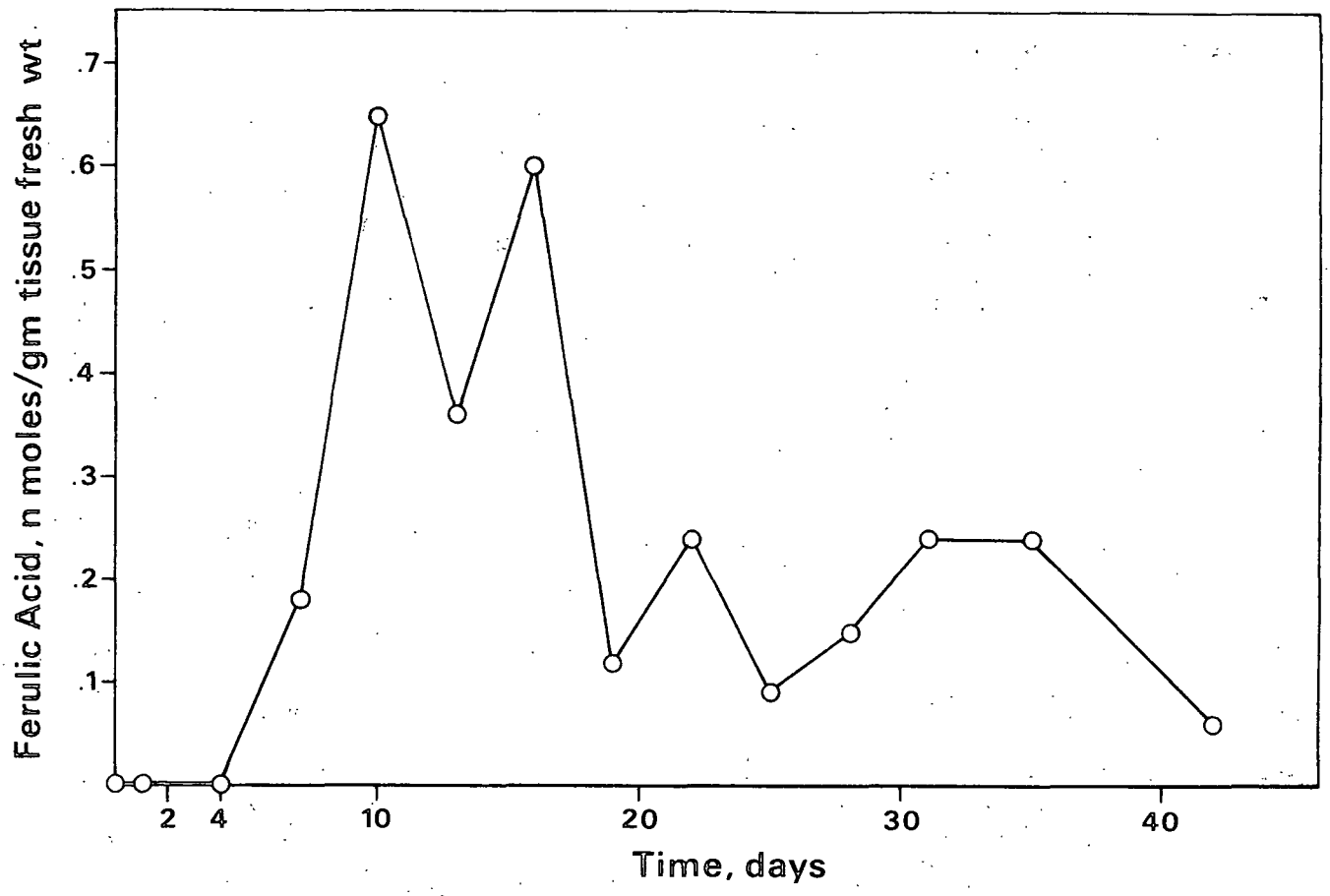


Figure 14. Callus OMT activity/fresh weight vs. time from subculture.

there are two forms of the enzyme possible, a lignin-specific and a flavonoid-specific form, it is necessary to determine which form is being investigated here. Each substrate was assayed individually under the same conditions. Each substrate was assayed at 1000 μM concentration with 40 μM S-adenosylmethionine for 1 hour at 30°C in a constant temperature water bath. Caffeic acid, and caffeic acid with magnesium were run to serve as a control and to confirm the beneficial nature of magnesium on OMT activity as found by other researchers. The four flavonoids most characteristic of Douglas-fir were also assayed. Catechin and epicatechin are the two flavonoids that make up procyanidin in Douglas-fir callus cultures (1).

Taxifolin and quercetin are two of the more abundant flavonoids in the Douglas-fir tree (120,121). Aesculetin is a coumarin formed by the cyclization of caffeic acid, and as such has the potential to be methylated. Coumaric acid was included to determine if this OMT could methylate in the para position, and catechol was used to see if the enzyme only required a small aromatic diol for activity.

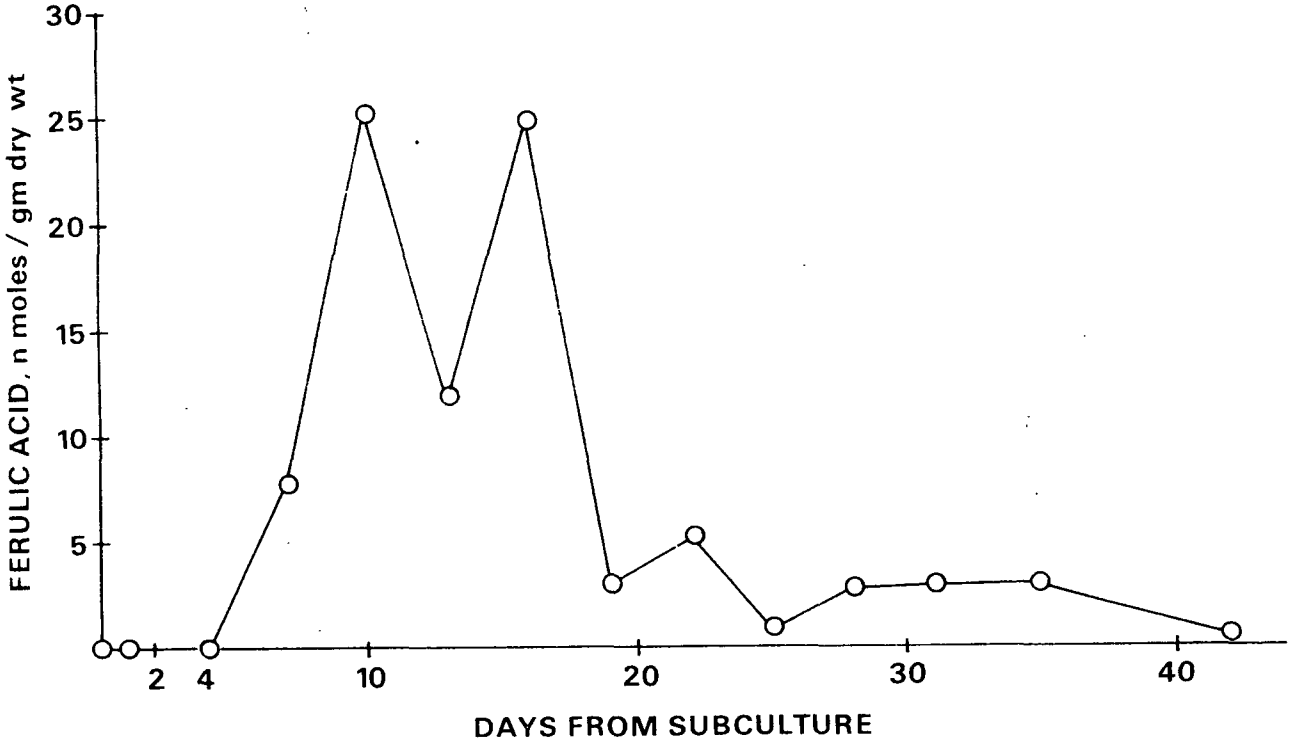


Figure 15. OMT activity/dry weight vs. time from subculture.

When caffeic acid is used as the control and set at 100% relative activity, it is easily seen that this OMT is a lignin-specific enzyme. No flavonoids were methylated significantly, below 5% in every case, with quercetin having the highest level of methylation. The other substrates are likewise relatively unmethylated. The p-coumaric acid was completely unmethylated, which indicates the inability of this enzyme to methylate para-hydroxyl groups. These results confirm the beneficial nature of magnesium, while also showing that it is apparently not a required cofactor.

This is not conclusively proven, because of the possibility of the enzyme having a magnesium or other metal atom already sequestered before isolation, so it can only be said that this enzyme does not require an exogenously supplied cofactor.

TABLE V

CALLUS OMT SUBSTRATE SPECIFICITY

Compound	Cpm	Activity, %
Caffeic acid	1190	100
Caffeic acid + 5 mM $MgCl_2$	1600	135
Taxifolin	13	1.1
Quercetin	56	4.9
Catechin	12	1.0
Epicatechin	0	0
Aesculetin	23	2.0
p-Coumaric acid	0	0
Catechol	13	1.1

In conclusion, it can be said that this OMT is a lignin-specific OMT with essentially no activity toward flavonoids. This means that the enzyme has no other substrates competing with the caffeic acid in this tissue, so the lack of ferulic acid formed and the lack of lignification must be due to some other property of the enzyme as it exists in the tissue. There are three possibilities: one is that the OMT is inhibited *in vivo* by some factor that is removed upon purification. Add-back experiments with dialyzates and other removed fractions showed no inhibition of the enzyme, indicating that there is no endogenous inhibitor. The second possibility is that there is a problem with the functioning of the enzyme itself. Characterization

of the enzyme itself is required to determine if this is indeed the case. A third possibility is a physical separation of the enzyme from the substrate *in vivo* due to compartmentation.

O-METHYLTRANSFERASE LOCALE

It has been noted earlier that the Douglas-fir callus OMT is not cytoplasmic in origin, in contrast to all plant species reported to date. This observation was based on the fact that the Douglas-fir callus OMT activity, lacking in the supernatant upon centrifugation at high g forces, was found in appreciable quantities in the crude whole-cell homogenate. Further, this activity was solubilized by the detergent, digitonin, which liberates membrane-bound enzymes (112). The possibility that the OMT was simply precipitated by the tannins present in the tissue was rejected, because of the inability of detergents to break the tannin-protein bonds that would have been formed. This leaves only the possibility that the enzyme is membrane-bound, which as noted earlier may be the natural state for all plant OMTs. But the difference with this tissue is that the OMT is not found in the supernatant after centrifugation at low g forces, as is the case for all of the other reported OMTs. This indicates a possibly important difference of Douglas-fir callus OMT, and thus requires a more accurate determination of its location.

The cell fractions were isolated by gentle grinding of the tissue with a mortar and pestle in a buffered medium containing 30 mM sucrose as an osmoticum to prevent the cell organelles from lysing. The whole cell homogenate was then fractionated into cell fractions by centrifugation at varying speeds. These fractions were the cell wall fraction, the chloroplast fraction, the mitochondrial fraction, and the microsomal and soluble fraction, at low to higher g forces, respectively. Triplicate samples were run. The chloroplast fraction was used because of previous reports

that up to 29% of the total phenylalanine ammonia lyase was found there in other plant species (122). Other phenolic enzymes have also been found associated with the chloroplast fraction as well (123,124).

Table VI shows that the cell wall fraction contains the greatest total activity of OMT in the callus tissue. The activity in the other fractions is much lower and could be due to contamination from the cell wall fraction even though microscopic examination of the various fractions showed little cross-contamination at 66X magnification. This data does indicate an important difference in the Douglas-fir callus OMT locale, when compared with the reported locale of the OMT in all other plant species investigated. This piece of information, when combined with the fact that digitonin solubilizes the OMT, shows that the OMT in this tissue is membrane-bound in the cell wall region and is not just precipitated by the tannin in the cell, nor nonspecifically bound by the precipitating cell wall. This is an important difference, which may account for the seemingly abnormal behavior of these tissues with respect to lignin and flavonoid biosynthesis. It is thus important to determine whether this is a species difference found in Douglas-fir seedlings as well, or if it is a consequence of putting the tissue into culture.

TABLE VI

CALLUS OMT LOCALE

Fraction	Ferulic Acid, DPM, 1 mL sample	Protein, mg/mL	Specific Activity, DPM/mg protein
Cell wall	11,143	0.375	29,715
Chloroplast	2,937	0.340	8,638
Mitochondria	3,575	0.475	7,526
Microsomal	1,641	0.520	3,156
Cytoplasmic	2,664	0.160	16,650

The amount of total ferulic acid formed is more important here than is the specific activity, which is simply an indication of the purity of the enzyme. It is interesting to note that there is relatively little protein that is truly soluble and that of the soluble enzymes present, a relatively large amount is OMT. It is unclear if this high soluble OMT is present in vitro or if it is merely due to liberation of the membrane-bound OMT upon homogenization. It would be necessary to make a protoplast isolation to check this possibility.

Decays per minute (DPM) were used here because the samples were counted in Phasar scintillation cocktail using the ^{14}C window only. The counting efficiency was 20-30%. The counts per minute (CPM) were then corrected to 100% efficiency or DPM.

SEEDLING O-METHYLTRANSFERASE LOCALE

Table VII shows the locale of the OMT in Douglas-fir seedlings. The seedlings' bark, and perhaps its cambial layer, were stripped off the stem and handled separately from the xylem region of the stem. The tissue was again gently ground in a mortar with a pestle, in a buffered medium containing 30 mM sucrose as an osmoticum. The cell fractions were separated as before, and to each pelletized fraction was added 5 mL of buffer to keep the relative dilutions of each fraction the same. These fractions were then assayed as usual.

Counts per minute (CPM) were used here because the samples were counted in Scintisol scintillation cocktail in the full $^{14}C/^{3}H$ window. This yielded a 90% counting efficiency which was not then converted to 100% efficiency or DPM.

The results show that by far the largest amount of OMT activity is in the xylem, as might be expected, but the enzyme in this region is a microsomal-soluble

enzyme in contrast to the locale of this enzyme in the callus. Over 50% of the xylem OMT is found in the microsomal-soluble fraction, with about equally small amounts found in the other three fractions. This could be due to nonspecific binding of the enzyme to the other fractions during centrifugation.

TABLE VII

SEEDLING OMT LOCALE

Fraction	Ferulic Acid, CPM/mL Region		Xylem	
	Cambial-Bark	Xylem	Protein, mg/mL	Sp. Act., cpm/mg
Homogenate	87	2,694	1.32	2,041
Cell Wall	283	894	1.50	596
Chloroplast	221	686	0.38	1,805
Mitochondrial	98	787	0.30	2,623
Soluble-microsomal	57	2,231	0.44	5,070

The cambial and bark region contains almost no OMT. However, what OMT is present seems to be contained in the cell wall and chloroplast fractions. This raises some interesting questions in itself about the origin of the callus, but the results are too incomplete to draw any conclusions at this time.

These results do indicate, however, that the membrane-bound OMT is not due to any species difference. Douglas-fir seedlings, like other plant tissue reported to date, have a microsomal or soluble OMT. The membrane-bound OMT of the callus may be an effect of putting the tissue into culture. So, it is shown here that there is a significant difference between the Douglas-fir callus OMT and that of other species. What effect this has on the actual kinetic characteristics of the enzyme will be discussed in the next section.

O-METHYLTRANSFERASE KINETICS

Work by Sharma and Brown (124), using an affinity column linking ferulic acid to the fixed column matrix, has indicated that the O-methyltransferase operates by an ordered Bi-Bi mechanism. This simply means that two substrates are required and two products are formed and the order of substrate addition is fixed. The general reaction scheme for this reaction is:

In this case E is the enzyme, SAM is S-adenosylmethionine, CA is caffeic acid, the P and Q are the reaction products, S-adenosylhomocysteine and ferulic acid. It was observed that the enzyme must first bind SAM before it can bind a caffeic acid molecule, probably due to an allosteric interaction where the SAM binding changes the active site in such a way that only then can caffeic acid be bound. The order of release has not been demonstrated.

Derivation of the kinetic equation for this type of reaction is very complex, involving a number of assumptions about the enzyme-substrate interactions. Experimentally, the situation is greatly simplified by supplying one substrate in such an excess of saturating levels that only 5% or less of the substrate is utilized in the incubation period (125). This keeps the enzyme kinetics at the zero order state with respect to this substrate, while the second substrate is varied over a low to high concentration range. The kinetic equation then reduces to a simple unireactant case of Michaelis-Menten kinetics where the reaction velocity, v, equals:

$$v = V_{max} \frac{[S]}{K_m + [S]} \qquad (1)$$

where V_{max} is the maximum reaction velocity, [S] is the variable substrate concentration, and K_m is the composite equilibrium constant for the enzyme. When K_m = [S], v = 1/2 V_{max}. Rearranging Eq. (1) gives:

$$\frac{1}{v} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}}$$

Which is in the form of an equation for a straight line:

$$y = mx + b$$

By plotting the reciprocal of the reaction velocity versus the reciprocal of the substrate concentration, one can determine the V_{max} and K_m values for the enzyme, which then characterize the enzyme. This double reciprocal plot is known as a Lineweaver-Burke plot. The V_{max} and K_m values characterize the enzyme by indicating how much product can be synthesized by the enzyme under optimum conditions, and by indicating the level of substrate concentrations required for this. The K_m value lacks a precise physical definition, but it does offer an indication of how sensitive an enzyme is to the presence of the substrate (125). Segel (126) feels that the K_m value establishes an approximate value for the intracellular level of substrate concentration. He says that the substrate concentration is unlikely to be significantly less than or greater than this K_m value, because in the first case v would be very sensitive to changes in [S] and most of the catalytic potential of the enzyme would be wasted, and in the second case it would make no physiological sense having [S] >> K_m since v cannot exceed V_{max}. But it must be remembered that in the cell there is a dynamic flux and ebb of both the substrate and the enzyme concentrations. Whereas the kinetic constants measure the characteristics of the enzyme itself, the requirement for the enzyme product may be quite small, and so the enzyme

and substrate concentrations may increase dramatically for only a short period of time during the cells' life cycle. This, of course would not be reflected in the actual kinetic constants of the enzyme, and so the K_m value may not accurately represent the substrate concentration *in vivo*. This may be more the case in plant secondary products metabolism, where the products are made when the tissue differentiates. However, this may provide a good indication of the substrate concentration at the point of highest enzyme activity during the plant cells' life cycle.

Figure 16 shows a typical plot of v *vs.* [S] for any enzyme. At the K_m value, v equals $\frac{1}{2}$ V_{max}. It is a general rule of thumb that a substrate concentration of 10 K_m is necessary to insure saturation of the enzyme with that substrate. The plot shows that this will give more than 90% of the V_{max} value for the enzyme.

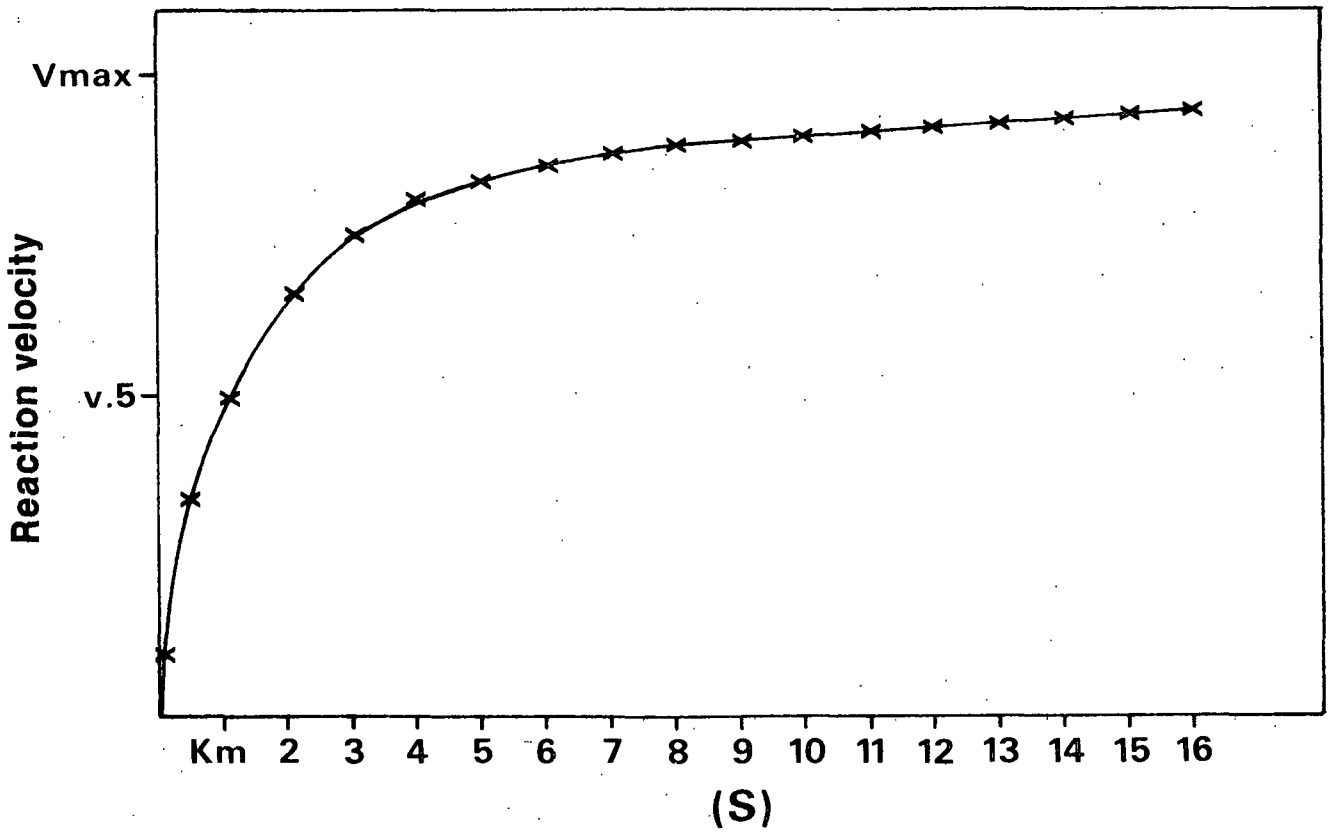


Figure 16. Calculated enzyme velocity *vs.* substrate concentration using Michaelis-Menten equation.

Figures 17 through 20 show the results of kinetic experiments using whole cell homogenates of ten day old callus. The line drawn in Fig. 17 and 18 is the least squares fit of the data with the line fixed at zero. Each data point represents three sample replicates. Whole cell homogenates were used to provide a comparison between the membrane-bound form of the OMT in the homogenate and the membrane-free purified form of the enzyme. This is necessary, despite some of the difficulties and assumptions made with homogenates, because of the possibility that the free and bound forms would show differences in their kinetic constants. It could be that the membrane somehow activates the enzyme and causes the V_{max} to increase and the K_m to decrease, or it could turn out that the soluble form has greater accessibility to substrate and so it has the higher V_{max} and the lower K_m. It could also happen that the membrane makes virtually no difference to the values of the kinetic constants. We must look to animal OMT to find a case where the membrane-bound and free forms of the OMT were compared. Tong and D'Iorio (127) found that rat liver, membrane-bound OMT had a K_m value three to eight times lower than the K_m for the membrane-freed enzyme. However, others working with human brain OMT have reported the opposite to be the case (128), i.e., they found the membrane-bound OMT to have a higher K_m value than the solubilized form. So it is obvious that this phenomenon must be studied on a case-by-case basis. Given the difficulty of knowing the exact, effective substrate concentration in a whole cell homogenate, and given the presence of other enzymes that may also utilize and/or bind the substrate, the data still provides a good indication of the OMT kinetics.

Figure 17 shows the increase in product, ferulic acid, over time at four different caffeic acid concentrations. They are 50, 100, 500, and 1000 μM. The SAM concentration was kept at 500 μM for this set of data. Triplicates of each sample concentration were run.

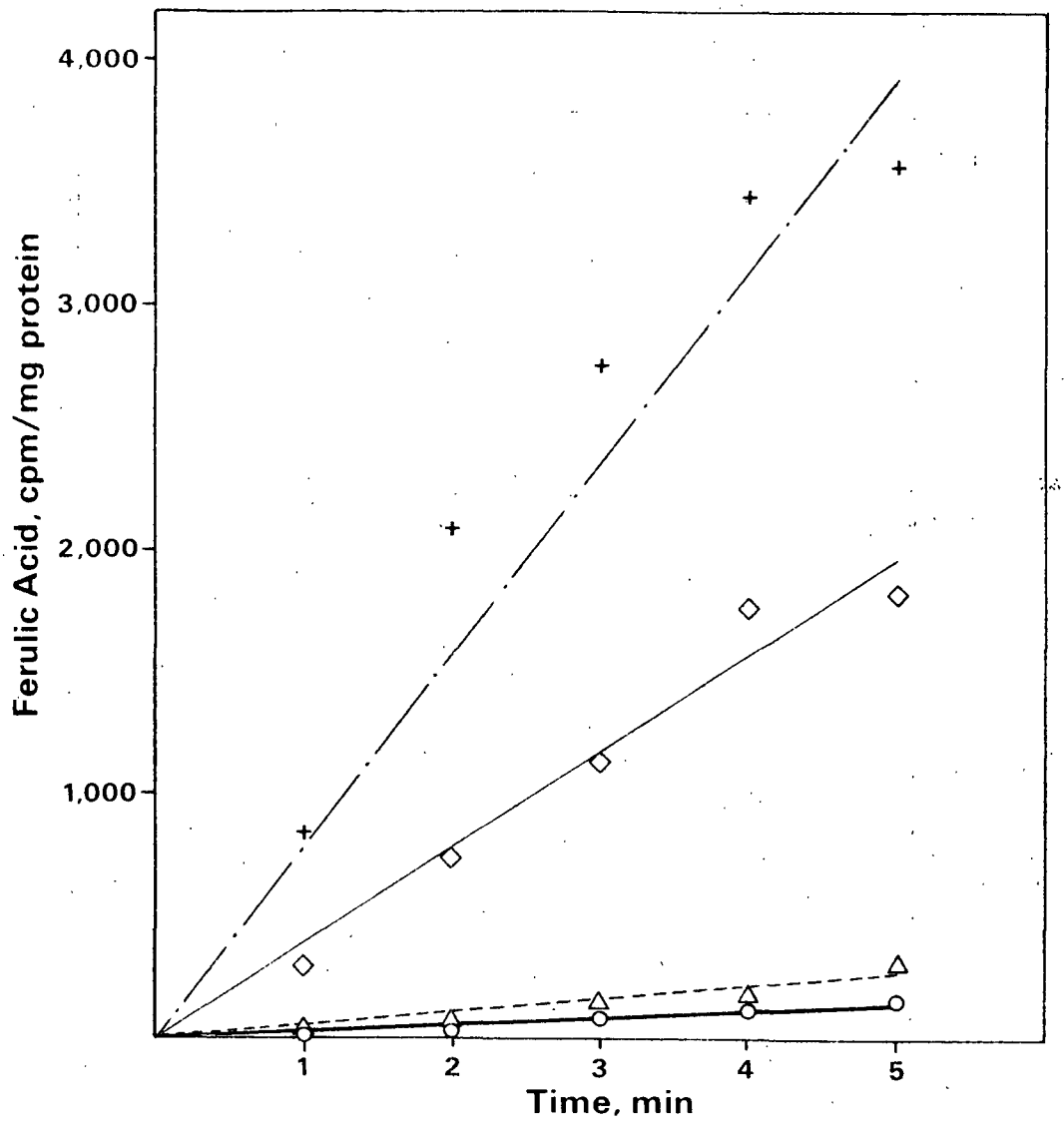


Figure 17. Callus homogenate kinetic data, ferulic acid vs. time. SAM fixed at 500 μM. Caffeic acid concentration: 0-0 50 μM; Δ-Δ 100 μM; ◇-◇ 500 μM; +- · -+ 1000 μM. (The lines are least squares fits fixed at zero.)

Figure 18 shows the product vs. time plot for five S-adenosylmethionine concentrations at a constant caffeic acid concentration of 1000 μM. The SAM concentrations used were 10, 50, 100, 500, and 1000 μM. In this figure it can be seen that the two highest concentrations caused a decrease in the reaction velocity. This is indicative

of substrate inhibition of the enzyme, and becomes clearer in the Lineweaver-Burke plots.

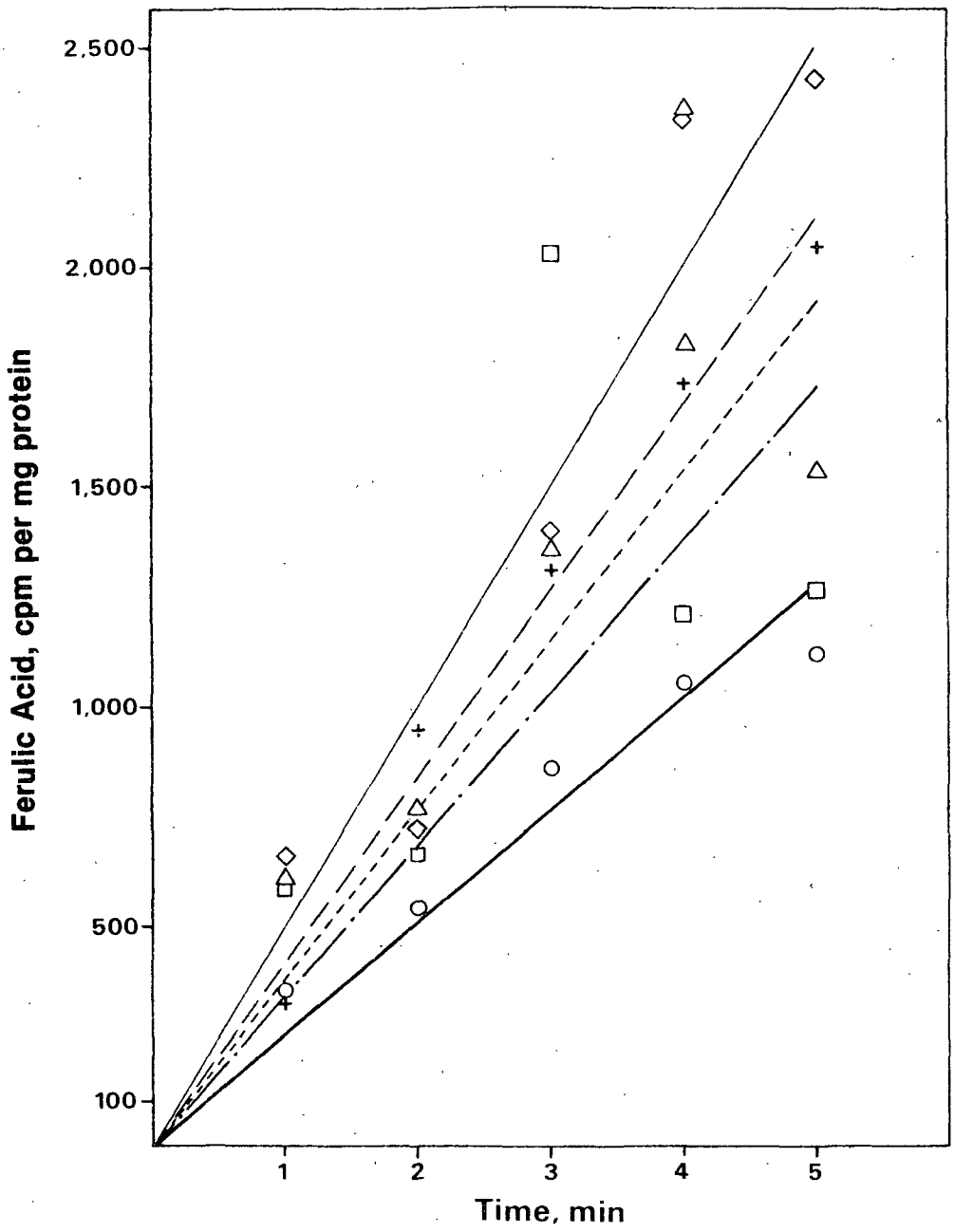


Figure 18. Callus homogenate kinetic data, ferulic acid vs. time. Caffeic acid fixed at 1000 μM. SAM concentration: O-O 10 μM; Δ- • -Δ 50 μM; ◇ - ◇ 100 μM; □ ----- □ 50 μM; + ---+ 1000 μM. (The lines drawn are least squares fits fixed at zero.)

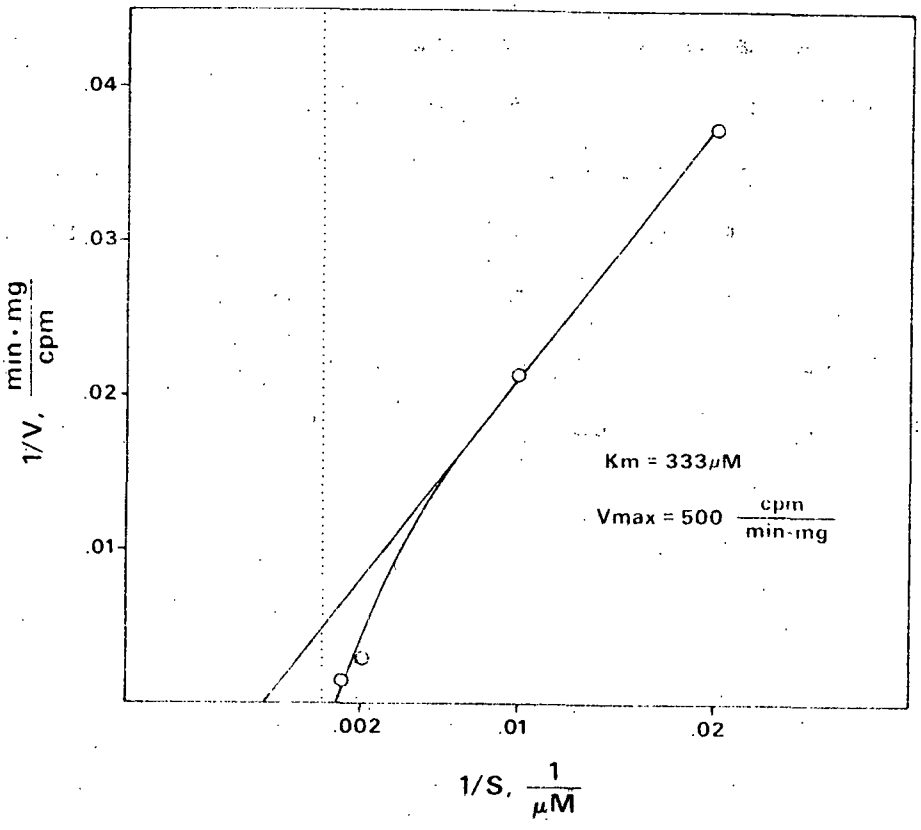


Figure 19. Lineweaver-Burke plot: SAM fixed at 500 μM.

Figure 19 shows the Lineweaver-Burke plot for the homogenate using caffeic acid as the substrate. The plot indicates that there is enzyme activation by this substrate, as indicated by the downward curve of the plot as the Y axis is approached, i.e., as the substrate concentration is increased above 100 μM. This yields the nonideal plot as shown. A line tangential to the curve gives a minimum K_m and V_{max} values for the cell wall, membrane-bound OMT, using caffeic acid as the substrate. The V_{max} value was 500 cpm per minute per mg protein. This corresponds to a rate of 0.5 nanomoles of ferulic acid/min/mg protein under the reaction conditions used, where 1000 cpm equals 1nmole. The K_m value is 333 μM, which is several times higher

than the generally reported K_m value range of 50-100 μM (see Table II). The implication of this high K_m value is that the callus OMT in this cultured tissue is less efficient at utilizing the caffeic acid than that of other species. It is unlikely that the caffeic acid concentration in the tissue would be this high, and so little or no ferulic acid would be formed, meaning little or no lignification. It is necessary once again to determine if this difference is a characteristic of the species in general, or of the cultured tissue only. The results for this are reported below, after the results for the callus OMT with SAM as substrate are examined.

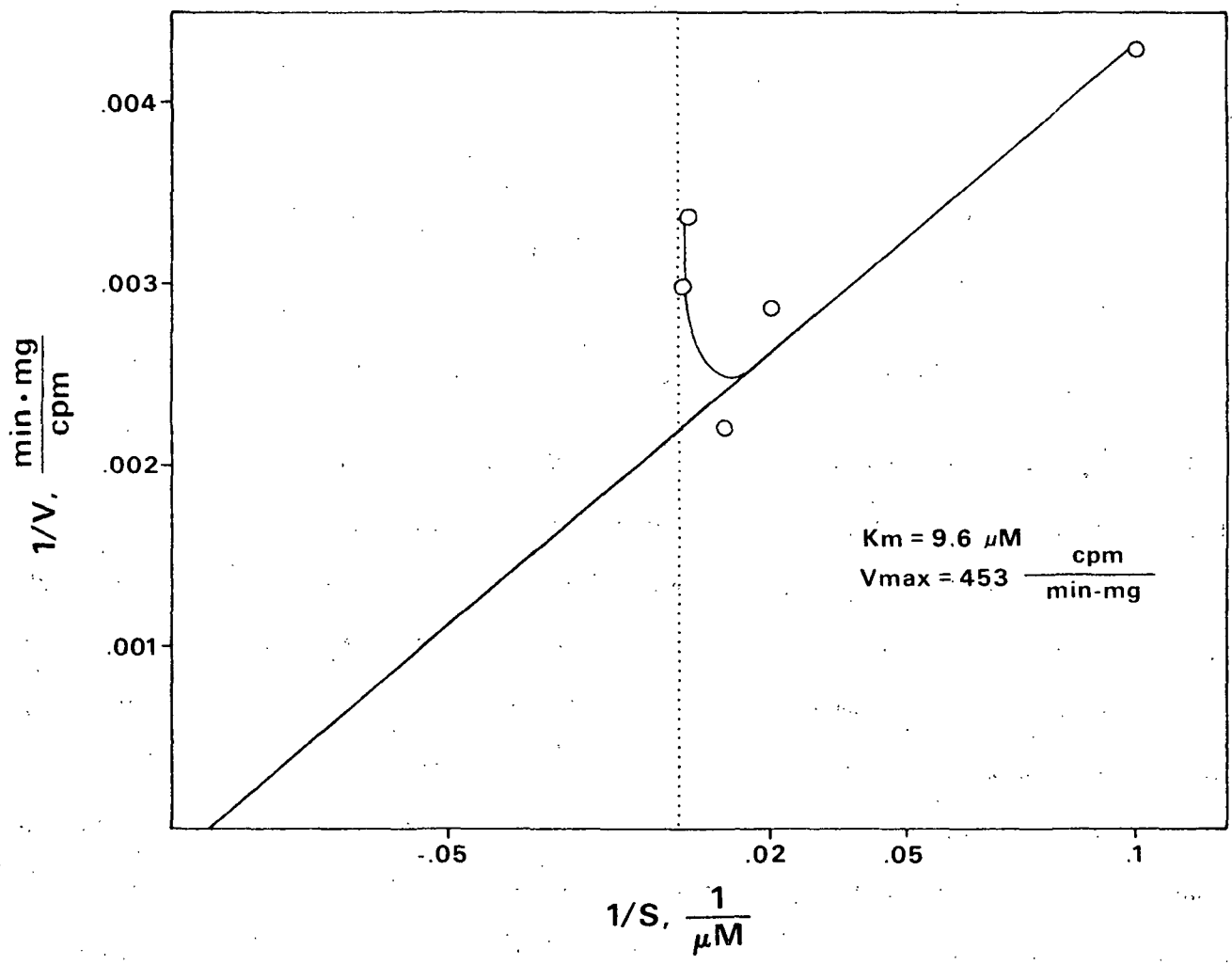


Figure 20. Lineweaver-Burke plot: SAM. Caffeic acid fixed at 1000 μM.

Figure 20 shows the Lineweaver-Burke plot for SAM as the variable substrate at a constant caffeic acid concentration of 1000 μM. The plot is nonideal also, but in this case it shows classic substrate inhibition at the higher substrate concentrations. As the substrate concentration rises beyond a specific concentration, the reaction velocity falls instead of increasing or leveling off. The inhibition is greatest at 500 μM, with 1000 μM showing some recovery from the inhibition. The kinetic constants can still be estimated from the remaining three points, although with less accuracy. The V_{max} and K_m values determined from this plot are 453 cpm/min/mg protein, or 0.453 nmole ferulic acid/min/mg protein, and 9.6 μM. This K_m value is in good agreement with the literature values of 4-9 μM for other plant OMTs, especially when considering the high probability of other SAM-utilizing enzymes in the homogenate, which would tend to increase the apparent K_m value. This K_m then, can be considered as an upper limit on the actual K_m value of SAM with Douglas-fir callus homogenate OMT.

The fact that the V_{max} values are very close together also shows that these results are internally consistent. Since the V_{max} value is the highest velocity attainable by this enzyme under optimum, saturating substrate conditions, the V_{max} values for each substrate should be the same. This is approximately the case here.

However, while the caffeic concentration was varied, the SAM concentration was fixed at 500 μM, which Fig. 21 shows to be inhibitory to the OMT activity. It is not possible to determine the exact extent of the inhibition from this data, but it appears to be about 30-35% inhibitory. The effect this has on the K_m value is unknown. Depending on the type of inhibition, the K_m value measured here may be too high, too low, or equal to the real K_m value. It can only be said at this point that there is apparent substrate inhibition of the Douglas-fir callus O-methyltransferase

by S-adenosylmethionine, and that the K_m value for caffeic acid is higher than that generally found for other plant species. Table VIII shows the kinetic constants determined for the Douglas-fir callus homogenate.

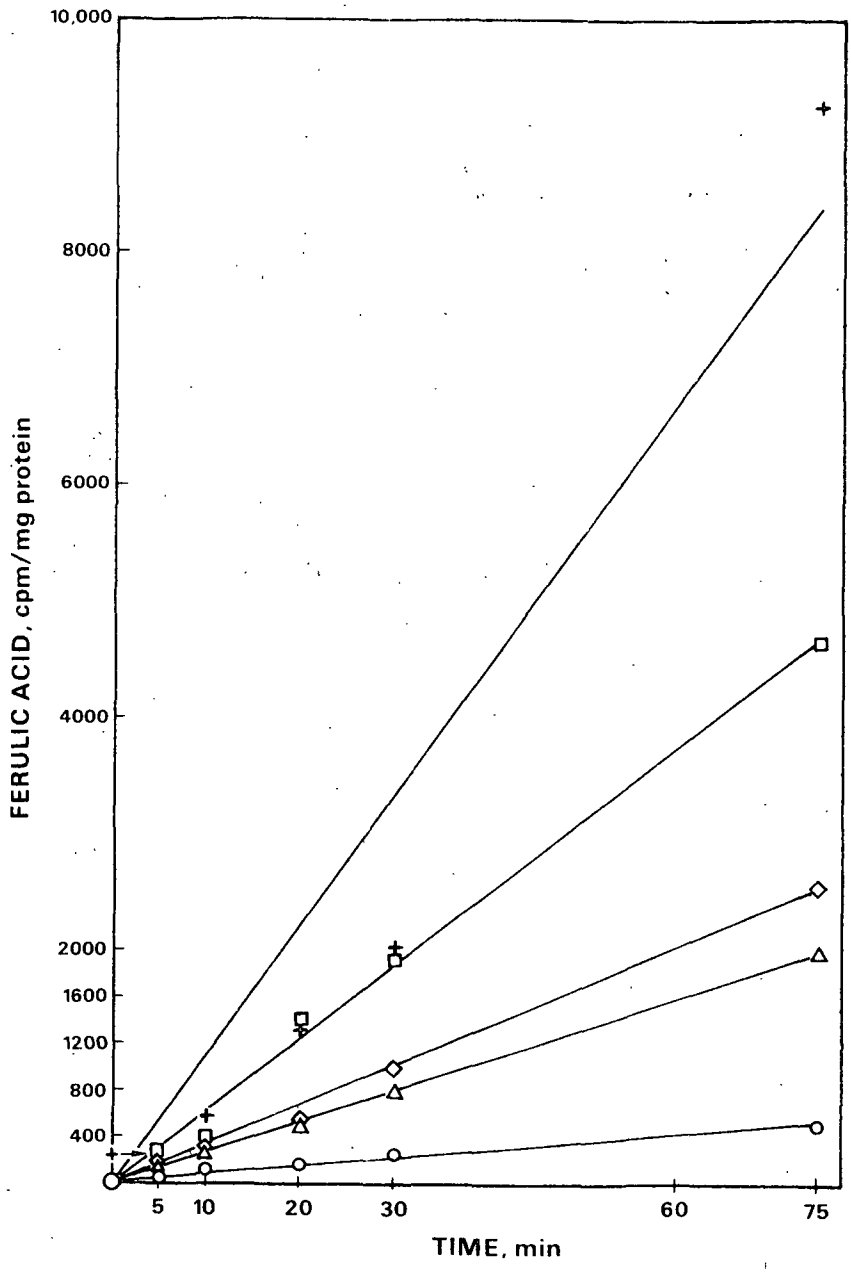


Figure 21. Seedling homogenate kinetic data, ferulic acid vs. time. SAM fixed at 500 μM. Caffeic acid concentration: 0-0 10 μM; Δ-Δ 50 μM; ◇ - ◇ 100 μM; □ - □ 500 μM; + - + 1000 μM. (The lines are least squares fits fixed at zero.)

TABLE VIII

HOMOGENATE KINETIC CONSTANTS

Substrate	V_{max} Ferulic Acid, nmole/min-mg	K_m μMolar
Caffeic acid	0.500	333
S-adenosylmethionine	0.453	9.6

[a]FA = ferulic acid.

SEEDLING O-METHYLTRANSFERASE KINETICS

Before determining the type of inhibition actually shown for the callus OMT, the kinetic constants for Douglas-fir seedling's OMT were determined. As mentioned, this was necessary in order to determine whether or not the high K_m value for caffeic acid was due to a species difference or to placing the tissue into culture. The seedlings were harvested 30 days after shoots appeared. A seedling stem and roots homogenate was used under the same kinetic conditions as the callus, even though there were some inhibition problems with the high SAM concentration used in the callus experiment. This will allow direct comparison of the callus and seedling results. Figure 21 shows the product versus time plot for the data at five different caffeic acid concentrations. They were: 10, 50, 100, 500, and 1000 μM. Only the caffeic acid was studied here, since it was the aberrant substrate. Figure 22 shows the Lineweaver-Burke plot for this data. The V_{max} determined from this plot is 66.9 cpm/min/mg protein, or 0.067 nmoles ferulic acid/min/mg protein. The K_m value is 90.7 μM. This K_m value is within the range of values typically reported for OMT in other species, and is considerably less than that found in the callus homogenate under the same reaction conditions. So it is possible that the high caffeic acid K_m value of the callus is a function of placing the tissue in culture and is not a property of the species itself.

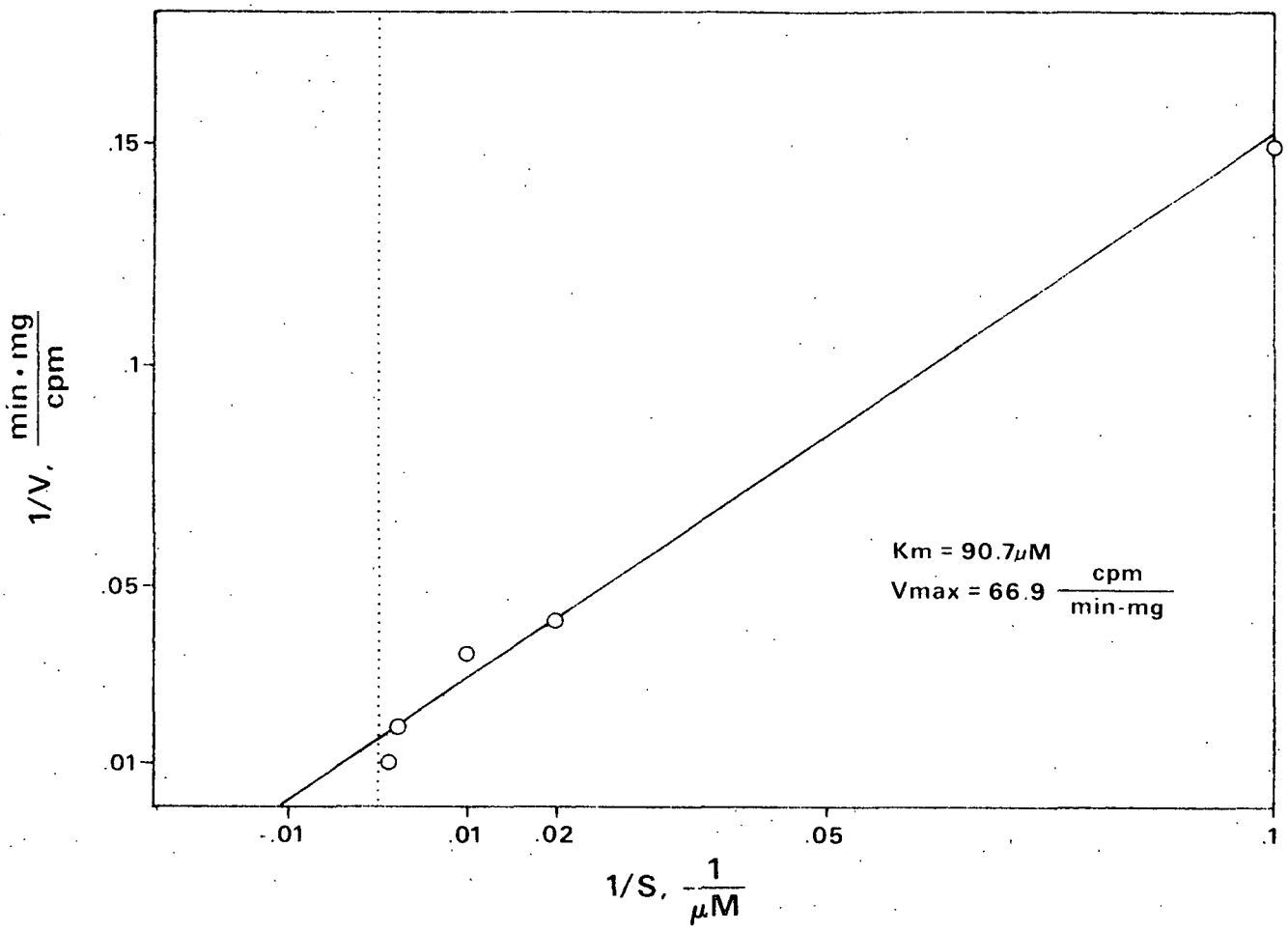


Figure 22. Lineweaver-Burke plot: caffeic acid. SAM fixed at 500 μM.

The V_{max} value of the seedlings is also less than that for the callus, but this is not of much concern. The seedling homogenate was not characterized as to its point of highest OMT activity over time, as was the callus tissue. In fact, the seedlings were harvested soon after shoots appeared and before the woody pith could be formed, in an effort to ease the isolation of the enzyme from the tissue. It is quite likely, then, that the level of OMT activity in the seedlings was not anywhere near its highest level. Although the kinetic constants are supposed to be characteristics of the enzyme alone, this is true only of the purified enzymes. This is one

of those complications in using homogenates mentioned earlier in this thesis. Simply put, there is no way to tell if the ratio of OMT to the other enzymes present in the tissue is the same for the callus and the seedling homogenates tested. Whereas, when the callus was tested the OMT level per gram of tissue was at its highest in the growth cycle, it is probable that this was not the case with the seedlings. The actual amount of OMT present was probably small, since the seedlings do not lignify to a great extent when they are this young. This means that when the results are divided by the amount of protein present to normalize the data to one mg protein, the seedling data is divided by a larger number. This would make the V_{max} value appear smaller on a per mg basis than it actually is at the time of highest OMT concentration which would precede lignification. So the V_{max} comparison between the seedlings and the callus OMT may be skewed toward the callus. The K_m term is not affected, however, because it is not dependent on protein content.

ENZYME PURIFICATION

It is desirable to compare the homogenate kinetic results with those of the purified enzyme because of the questions raised by using the whole cell homogenates. While the purified O-methyltransferase kinetic constants may differ from those of the cell wall, membrane-bound OMT, due to the removal of the membrane the purified system offers more control over the reaction conditions. This will result in a more accurate determination of the kinetic constants for this enzyme form, and will also give a range indicative of these constants.

The purification scheme for isolating the OMT from the callus consisted of treatment of the homogenate with digitonin, the detergent, to disrupt the membrane which binds the enzyme. This was followed by a narrow band salt fractionation which separated the OMT from most of the other enzymes present in the tissue. Further

attempts at purification failed. Reasons for this and other results concerning the extent of purification are discussed below.

Previous locale work showed that the OMT activity of the callus was located in the cell wall fraction, apparently membrane-bound there. Purification required that the enzyme be solubilized, and this was done by stirring the homogenate with 0.5-1% digitonin for several hours at 0-4°C with a magnetic stir bar. The other naturally soluble enzymes of the callus were not removed prior to the digitonin treatment, because they protected the OMT activity during solubilization.

After the digitonin treatment, centrifugation at 16,000 x g for 15 minutes left the now soluble OMT in the supernatant. The enzyme was precipitated in the 25-35% ammonium sulfate fraction after dialysis against two changes of 10 volumes of buffer to remove the digitonin. This 25-35% salt fraction was collected by centrifuging the solution at 16,000 x g for 15 minutes. The OMT was found in the pelletized protein fraction. The pellet was resuspended in buffer and dialyzed against two changes of 20 volumes of buffer to remove the ammonium sulfate salt. Table IX shows the resulting enzyme purification obtained.

Figure 23 shows the results of electrofocusing this OMT preparation, in comparison with a commercial grade of the enzyme catalase. A pure enzyme with no isozymes would show only one band. Many enzymes, however, do have isomeric forms and so would show several bands. It is seen from the figure that there are many bands in the OMT preparation, which are most probably due to contamination. However, the level of contamination in the OMT fraction appears to be substantially less than that found in the catalase. This shows that, although the OMT preparation is not a pure sample of the enzyme, it is still a relatively clean preparation. Table X

shows some results for the purity of OMT preparations as found in other species as reported. These are reported as nanokatals (nkat) rather than picokatals (pkat).

TABLE IX

DOUGLAS-FIR CALLUS OMT PURIFICATION

Fraction	Activity, cpm/mg	Protein, mg/mL	Specific Activity nmol/mg/hr	Specific Activity pkat/mg
Homogenate	9,350	0.51	18.34	5.1
Digitonin freed	5,167	0.51	10.12	2.8
0-25% amm. sulfate	2,106	0.27	7.8	2.2
25-35%	19,480	0.38	51.26	14.
35-55%	14,842	1.36	10.91	3.
55-80%	7,446	1.52	4.90	1.4
80-100%	1,326	1.14	1.16	0.3

Further attempts at purification failed for one of three reasons: the enzyme was merely diluted, not separated from the other enzymes present; the enzyme was inactivated; or the enzyme failed to elute from the column used in column chromatography.

Sephadex G-25 and G-100 gel filtration columns yielded no further purification. The enzyme was diluted with these columns, but its specific activity, which is a measure of its purity, was unchanged. Similar results were obtained with hydroxyapatite columns and with γ alumina.

Ion exchange and affinity chromatography methods bound the enzyme to the column matrix from which it could not be removed either by pH changes or by high salt solutions (0.2-1M). The OMT was bound to the DEAE cellulose ion exchange column and to two different affinity columns, a ferulic acid-Sepharose 4B column and

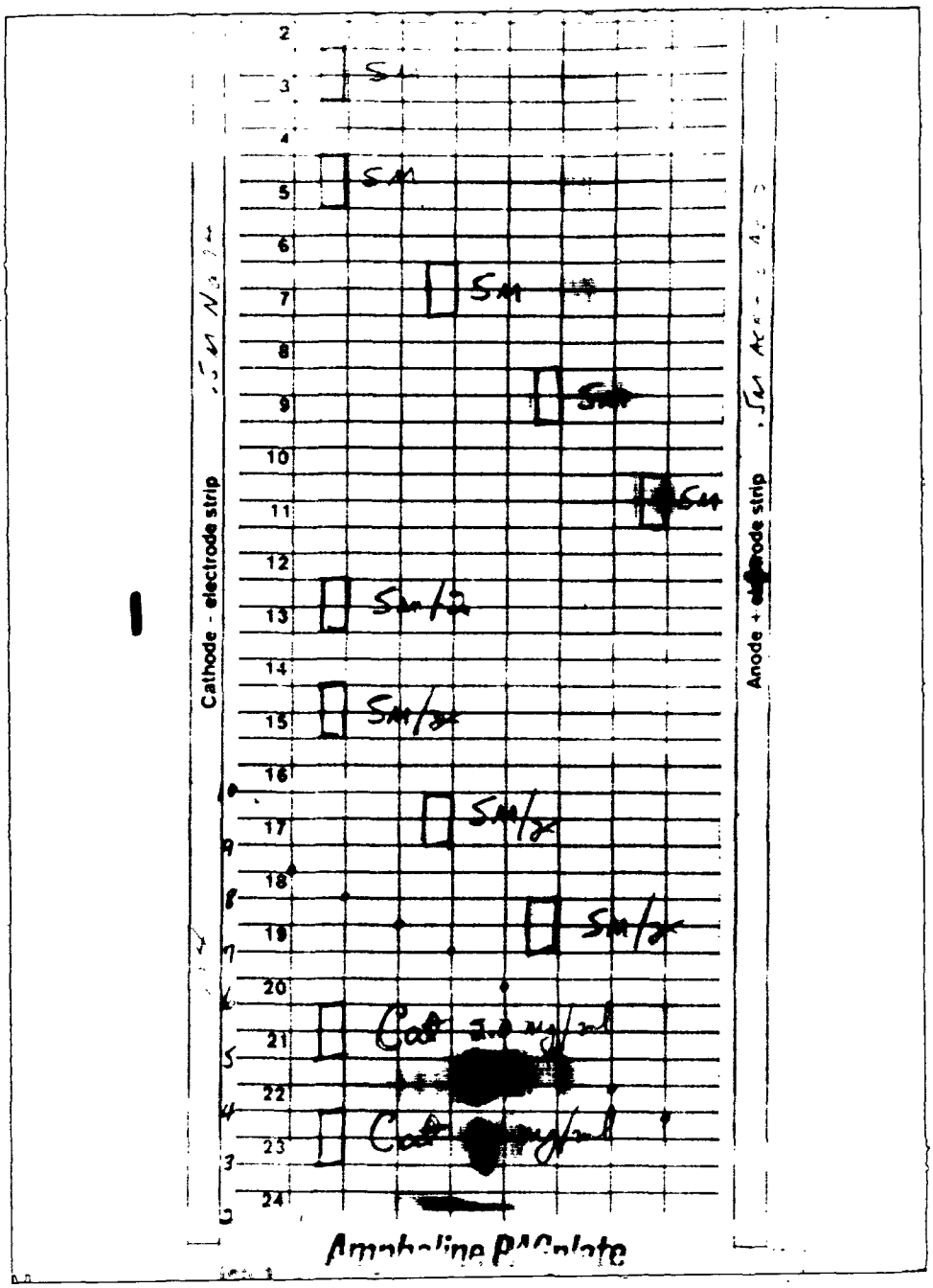


Figure 23. Electrofocusing gel of callus OMT.

a S-adenosylhomocysteine-Sepharose 4B column. Assays of the top portions of the cellulose and ferulic acid-Sepharose 4B columns found the enzyme present and still active. The S-adenosylhomocysteine column matrix was not assayed, since the lack of OMT activity in the column eluate indicated that no OMT purification would be found.

TABLE X

OMT REPORTED PURITY

Source	Purity, nkat/mg protein	Reference
Tobacco[a]	0.125	(72)
Tobacco[b]	4.8	(71)
Aspen	183	(93)
Alfalfa	0.0284	(87)
Pinus thunbergii	265	(78)
Parsley suspension	0.45	(89)

[a]Nicotiana tabacum cv samsun NN.
[b]Nicotiana tabacum L cv Wisc. No. 38.

The binding nature of the enzyme may be a function of the phenolics present in this tissue. Table XI shows the amount of procyanidin present in each cell fraction upon callus homogenization. It shows that there is considerable tannin present in each fraction, and this tannin would be expected to bind to the protein in solution. It is probable that these phenolics bind to the OMT without inactivating it, however. The OMT is a phenolic-utilizing enzyme, and as such may be less susceptible to inactivation by phenolics. The tannin present could then bind to the column matrix and thus immobilize the enzyme. This would also help to explain the results of the ultracentrifugation study of the OMT. Briefly, the enzyme was very mobile, which suggests a low molecular weight; yet it dispersed very slowly, which indicates a

very high molecular weight. This behavior could be explained if the OMT was a relatively small enzyme, many of which would be bound in a loose configuration by the tannin. The resulting association would present a small effective diameter to the flow, thus giving quicker diffusion through the buffer while preventing the common dispersion of the molecules.

TABLE XI

DOUGLAS-FIR CALLUS TANNIN DISTRIBUTION

Locale	mg Proanthocyanidin/mL
Homogenate	1.22
Cell wall	1.69
Chloroplast	1.49
Mitochondrial	1.78
Microsomal and soluble	1.54

This behavior of the enzyme, its tendency to bind to column materials, effectively prevents the use of the more typical enzyme purification techniques and leaves only the brute force physical methods. As seen in Table XII, these methods yield an inactivated enzyme, not a purified OMT. The conclusion is that it is impractical to purify the OMT beyond that level given by the ammonium sulfate fractionation. Consequently, this enzyme preparation was used for the purified OMT kinetic studies and the specificity work.

PURIFIED ENZYME KINETICS

The kinetic data for the OMT, purified in the manner described, was derived using the following fixed substrate concentrations: 1000 μM caffeic acid, and 200 μM S-adenosylmethionine. In general, one must use 10 times the K_m value to ensure saturating conditions for the enzyme throughout the reaction time. This condition

was met for SAM where the upper limit on the homogenate K_m value is 9.6 μM. At the 200 μM level used the SAM is certainly saturating. It should also be noted here that, although the SAM concentration of 500 μM was inhibitory to the homogenate OMT, the 100 μM level was not. Given similar conditions for the purified system, the 200 μM level used should not have had any inhibitory effects. For caffeic acid, the solubility limit is exceeded at 10 times the K_m value. Since it is possible that the K_m for the purified OMT may be less than the homogenate value, and since the conversion rate is less than 1% over the assay period at a caffeic acid concentration of 1000 μM, it is assumed that this concentration is saturating in practice. Another consideration in determining which caffeic acid concentration to use is the possible inhibitory effect of high concentrations of this aromatic diol. Taking both of these factors into account, the 1000 μM concentration used is a good working compromise.

TABLE XII

PURIFICATION EFFORTS

Method	Ferulic Acid, Cpm, 1/2 mL sample	Protein, mg/mL	Sp. Act., nmol FA/mg protein
Control	9,350	0.69	27.1
Hydroxyapatite	5,904	0.50	23.8
C γ alumina	8,258	0.54	30.6
Heat (70°C-30 min)	0	0.61	0
TCA[a]	0	0.75	0
Foaming	8,740	0.70	24.9
pH (to 5)	129	0.56	0.46

[a]Trichloroacetic acid.

Figures 24 and 25 show the amount of product formed over time at the various substrate concentrations. Figure 26 shows the Lineweaver-Burke plot for caffeic acid as the variable substrate. The K_m value measured here is 114 μM with a V_{max} value of 2.44 nmole ferulic acid produced/min/mg protein. The K_m value here has decreased to the region more commonly reported for OMT in other species.

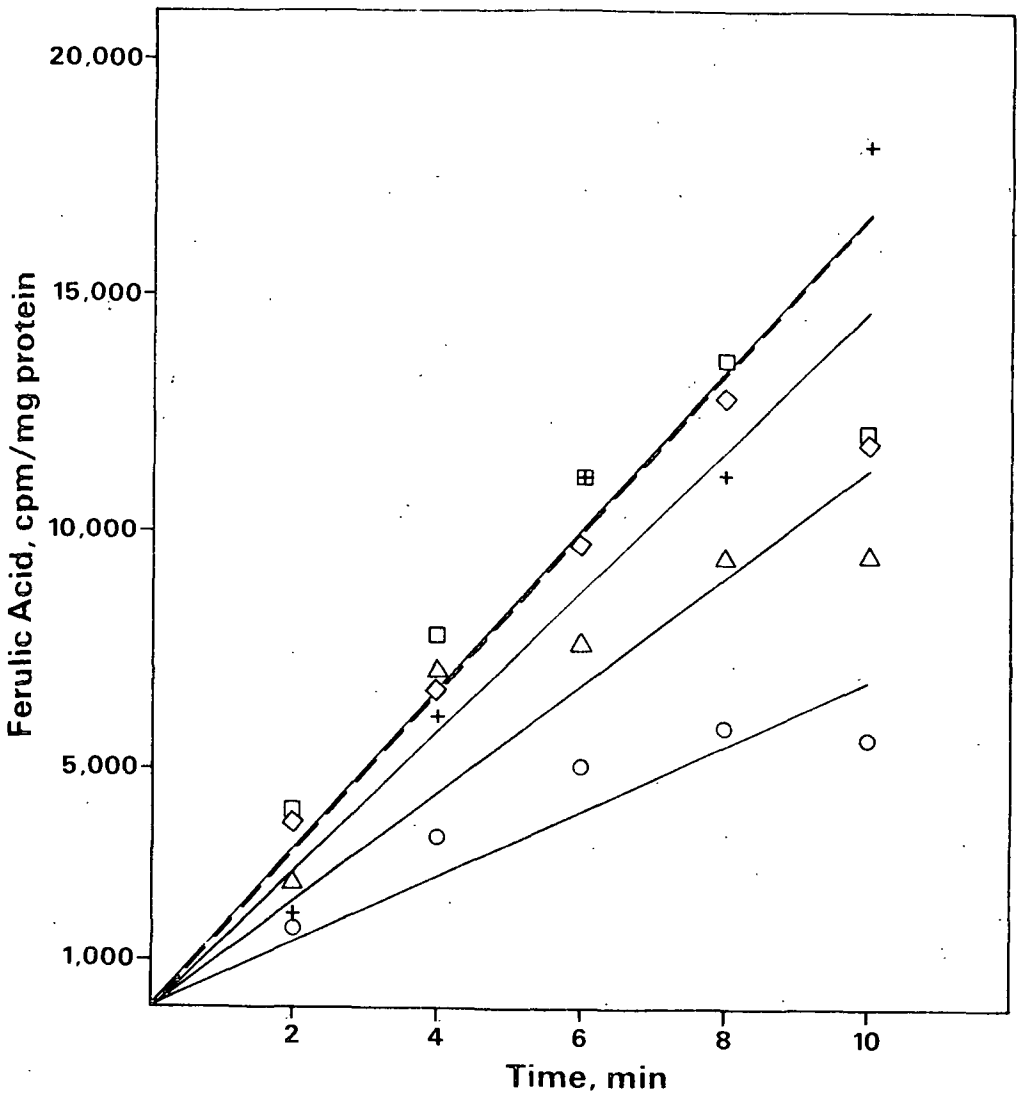


Figure 24. Purified callus OMT kinetic data: ferulic acid vs. time. SAM fixed at 200 μM. Caffeic acid concentration: 0-0 50 μM; Δ-Δ 100 μM; ◇ - ◇ 300 μM; □ - □ 500 μM; + --- + 1000 μM. (The lines drawn are least squares fits fixed at zero.)

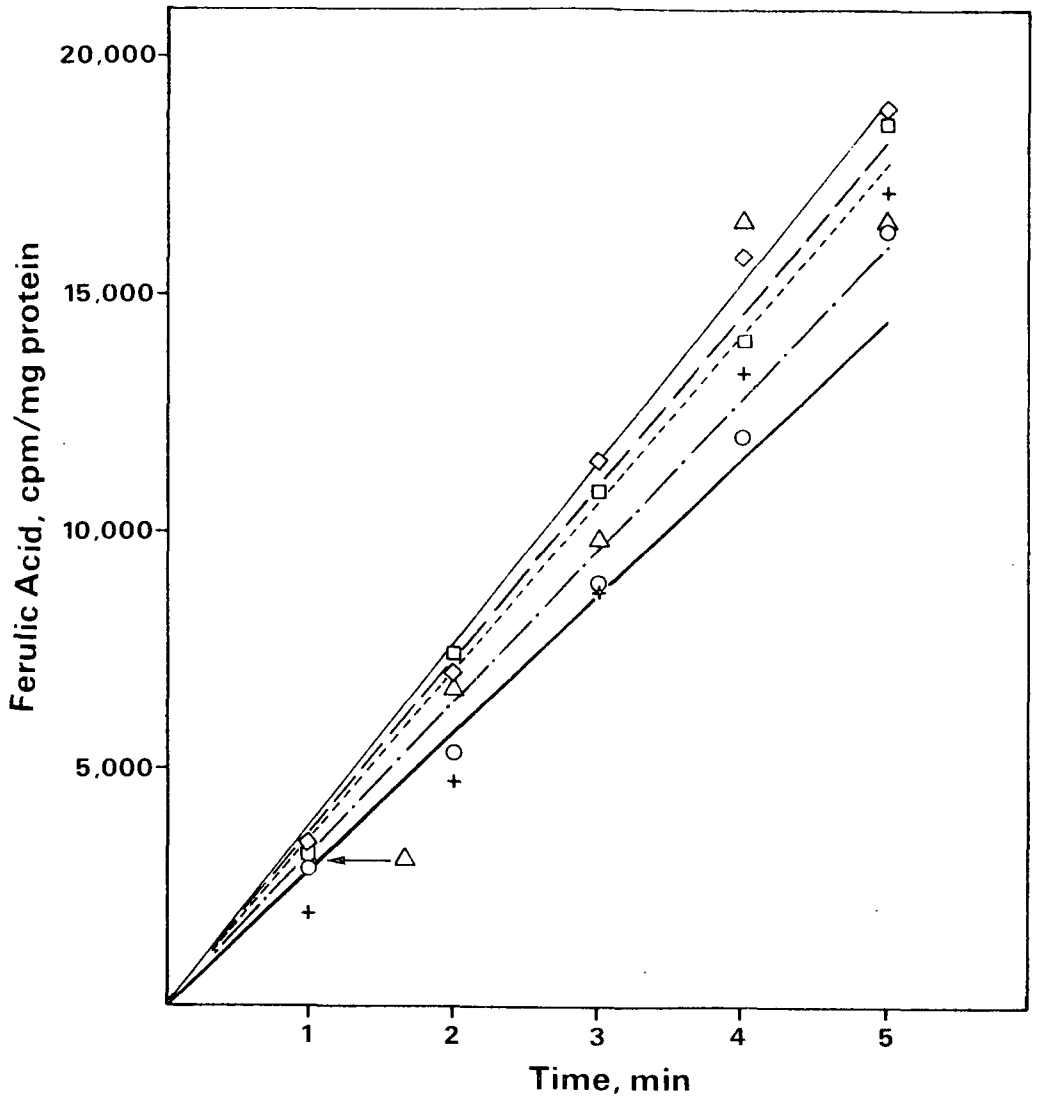


Figure 25. Purified callus OMT kinetic data: ferulic acid vs. time. Caffeic acid fixed at 1000 μM. SAM concentration: 0-0 20 μM; Δ-----Δ 40 μM; ◇ - ◇ 60 μM; □---□ 80 μM; + -·- + 100 μM. (The lines drawn are least squares fits fixed at zero.)

Figure 27 shows the kinetic plot for S-adenosylmethionine with the purified enzyme. The apparent V_{max} is 2.13 nmoles ferulic acid/min/mg protein, which again agrees well with the homogenate value found for caffeic acid, thus indicating internal consistency. The K_m value is 7.7 μM, which is a little lower than that found for

the homogenate. This is expected, because the homogenate would have other methylating enzymes utilizing SAM. Consequently, the K_m would be lower with less competition for the substrate.

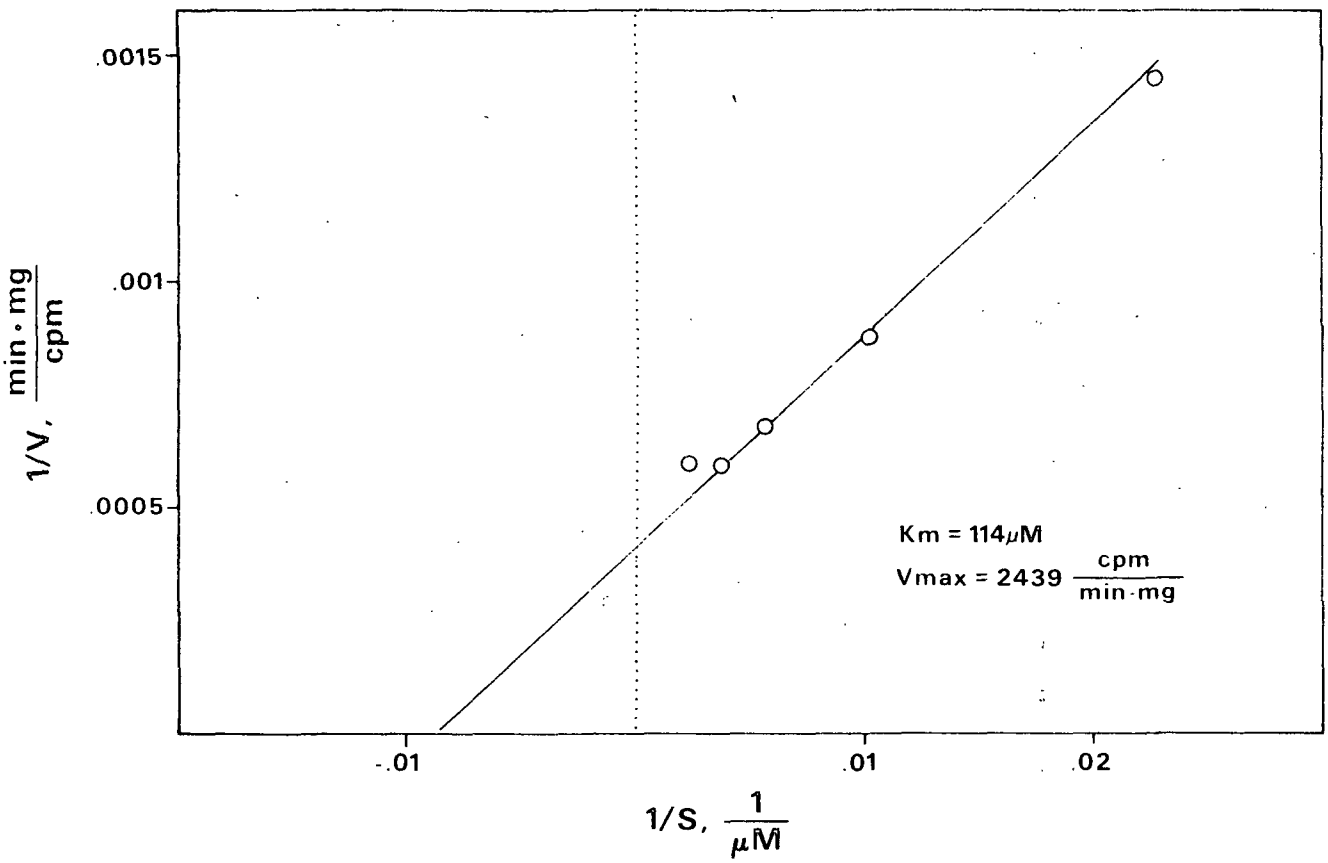


Figure 26. Lineweaver-Burke plot: SAM fixed at 200 μM.

Figure 27 shows, once again, classic substrate inhibition at the higher SAM concentrations. This time, it is seen that 80 and 100 μM SAM levels are inhibitory to the enzyme activity. So, the kinetic constants for SAM are again determined with fewer points than desired, which will have some small effect on their accuracy. But more importantly, these results raise questions about the accuracy of the kinetic constants of caffeic acid, which were determined with the now inhibitory level of

200 μM SAM. As mentioned before, the kinetic values may increase, decrease, or stay the same depending on the type of inhibition present. The next section of this thesis considers this aspect of the Douglas-fir callus O-methyltransferase.

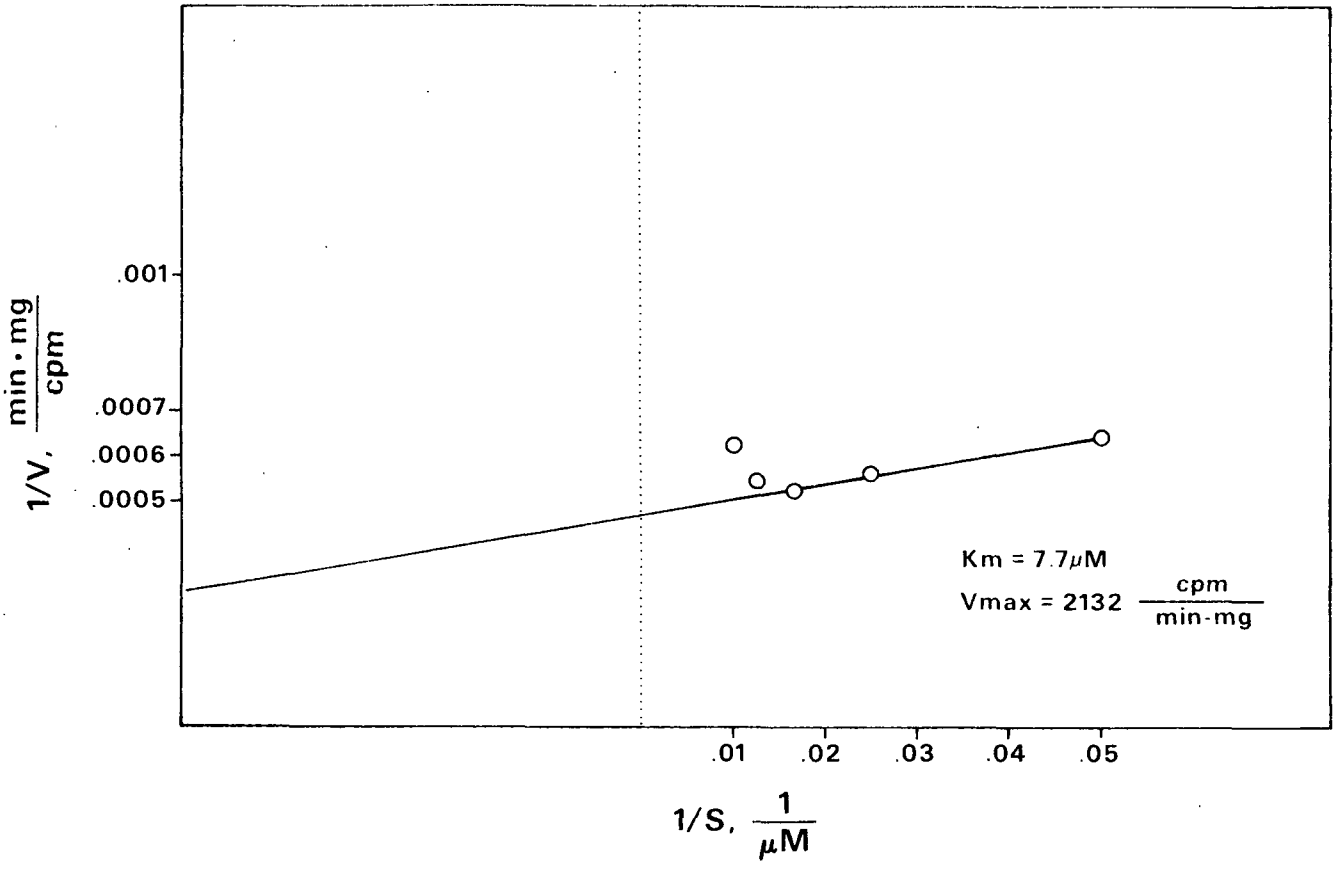


Figure 27. Lineweaver-Burke plot: Caffeic acid fixed at 1000 μM.

SUBSTRATE INHIBITION

SUBSTRATE CONTAMINANT INHIBITION

There is a possibility that the OMT inhibition shown is really due to a contaminant in the substrate. The SAM used in this thesis was specially purified for enzyme assay work, as made commercially by Amersham Corporation. The SAM, however,

had a purity limit of 98% due to the unstable nature of the compound. SAM, under normal circumstances, can break down to methanol and S-adenosylhomocysteine (SAH), adenosine and methionine, or adenine-methionine and ribose. This breakdown occurs at the rate of 3% per day at room temperature, while at -20°C, the rate is less than 5% per year. For this reason, the SAM was normally stored at -20°C. There still remains the original 2% impurity in the SAM as it is received from the manufacturer. If all of the impurity were SAH, then in a 200 μM concentration of SAM, a 4 μM concentration of SAH would be present.

In other plant species, it was found that SAH is a powerful inhibitor of OMT activity. The K_i values denote the inhibitor concentration at which 50% of the enzyme activity is inhibited. The K_i values found for SAH and OMT were from 0.4-4 μM (75,76,91). It is obvious, then, that if the K_i value for SAH with Douglas-fir callus OMT is at the lower end of this range, the contaminating SAH in the commercial SAM would severely inhibit the enzyme activity. So, before looking at the substrate inhibition itself, it was decided to determine the K_i value of the most probable inhibitor present: SAH.

There are basically three types of inhibition possible: competitive, noncompetitive, and uncompetitive. Each has a different effect on the graphical behavior of the data, and on the experimentally determined kinetic constants. Competitive inhibition occurs when the inhibitor binds only to the free enzyme, to the exclusion of the proper substrate. Conversely, when the substrate is in place on the enzyme, there is no place for the inhibitor to bind. Most often, this means that the inhibitor and substrate are structurally and chemically very similar, and so each binds to the active site of the free enzyme. If the substrate binds to the enzyme, the proper reaction takes place, of course. If the inhibitor binds to the active site,

no enzyme reaction can take place until the inhibitor is removed, generally by diffusion. This has the effect of reducing the effective concentration of the enzyme in solution, and so reduces the overall reaction and is seen as inhibition. It is also possible that the inhibitor binds to another part of the free enzyme, and allosterically prevents binding of the substrate by changing the configuration of the active site. In general, competitive inhibition can be overcome by increasing the concentration of the substrate and thereby increasing the probability of an enzyme-substrate interaction. But this cannot be done in the case of a contaminant in the substrate, because there is a constant ratio of contaminant to substrate.

In the case where there is a competitive inhibitor in the substrate, the kinetic analysis yields Eq. (4):

$$v = \left(\frac{V_{max}}{1 + \frac{xK_m}{K_i}}\right)\left(\frac{[S]}{\frac{K_m}{1 + \frac{xK_m}{K_i}} + [S]}\right) \tag{4}$$

where x is the constant concentration of inhibiting contaminant in the substrate. This equation is in the same form as the kinetic equation without the inhibitor. The reciprocal equation [Eq. (5)] shows that the Lineweaver-Burke plot still gives a straight line.

$$\frac{1}{v} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \left(\frac{1 + \frac{xK_m}{K_i}}{V_{max}}\right) \tag{5}$$

So, it is concluded that in the case of a competitive contaminant in the substrate, there would be no aberrant Lineweaver-Burke plot (121). Since the Lineweaver-Burke

plot for the purified OMT definitely did show inhibition with SAM, competitive inhibition due to a contaminant in the substrate can be ruled out.

Equation 6 shows the velocity equation for the noncompetitive inhibition case. Notice the denominator contains a $[S]^2$ term which would make the Lineweaver-Burke plot nonlinear.

$$\frac{1}{v} = V_{max} \frac{[S]}{K_m + [S]\left(1 + \frac{xK_m}{K_i} + \frac{x[S]}{K_i}\right)} \tag{6}$$

In this case, the inhibitor binds randomly to either the free enzyme or to the enzyme-substrate complex. In either case the inhibitor binds at a point other than the active site, and changes the active site's nature so that the enzyme reaction cannot proceed normally.

The inhibitor in the uncompetitive case binds only to the enzyme-substrate complex. The kinetic analysis yields Eq. (7).

$$v = V_{max} \frac{[S]}{K_m + [S]\left(1 + \frac{x[S]}{K_i}\right)} \tag{7}$$

This also yields a nonlinear Lineweaver-Burke plot for the enzyme.

In summary, for the case of a contaminant in the substrate, only the noncompetitive and uncompetitive cases give nonlinear double reciprocal plots while the competitive inhibition case still yields a linear plot. Table XIII shows the effect these types of inhibitions have on the experimentally determined kinetic constants in relation to the real kinetic constants of the enzyme.

TABLE XIII

INHIBITION EFFECT ON KINETIC CONSTANTS

Inhibition	Determined Constants	
	K_m apparent	V_{max} app.
Competitive	> K_m real	= V_{max} real
Noncompetitive	= K_m real	< V_{max} real
Uncompetitive	< K_m real	< V_{max} real

Ruling out the competitive case because of the nonlinear behavior of the plot experimentally obtained, leaves only the non- and uncompetitive cases to be considered. This means that either the experimentally determined K_m equals, or is less than, the real Km value.

S-adenosylhomocysteine was obtained from Sigma Chemical Company and was stored in a desiccator at -20°C. The SAH was added to the experimental assays at three concentrations: 1, 10, and 20 μM, at each of three caffeic acid concentrations of 200, 500, and 1500 μM, with a constant SAM concentration of 40 μM. An assay run without any added SAH served as a fourth data point and as a control. The assays were run over 50 minutes, with samples pulled every 10 minutes over the course of the experiment. Because of a very limited amount of purified OMT available, no duplicates were run nor were zero time samples pulled. This was unfortunate, but necessary in order to have enough of the purified enzyme available for further determinations of the kinetic constants of the purified OMT. A few samples were randomly duplicated to serve at least as a check on the experimental reproducibility. The duplicates did agree well with the original samples.

Figures 28-32 show the results from this inhibition experiment using SAH. Figure 28 shows the results when 200 μM caffeic acid was used, which is near the apparent K_m value and so is not saturating the enzyme. Little inhibition is seen at 1 μM SAH, but significant inhibition is found at 10 and 20 μM. Figure 29, with a higher

caffeic acid concentration, shows more enzyme inhibition at 1 μM and again significant inhibition at 10 μM. At the highest caffeic acid concentration, 1500 μM, which should be saturating the enzyme, the inhibition at 1 and 10 μM SAH seems to be overcome. Only the 20 μM SAH shows significant inhibition.

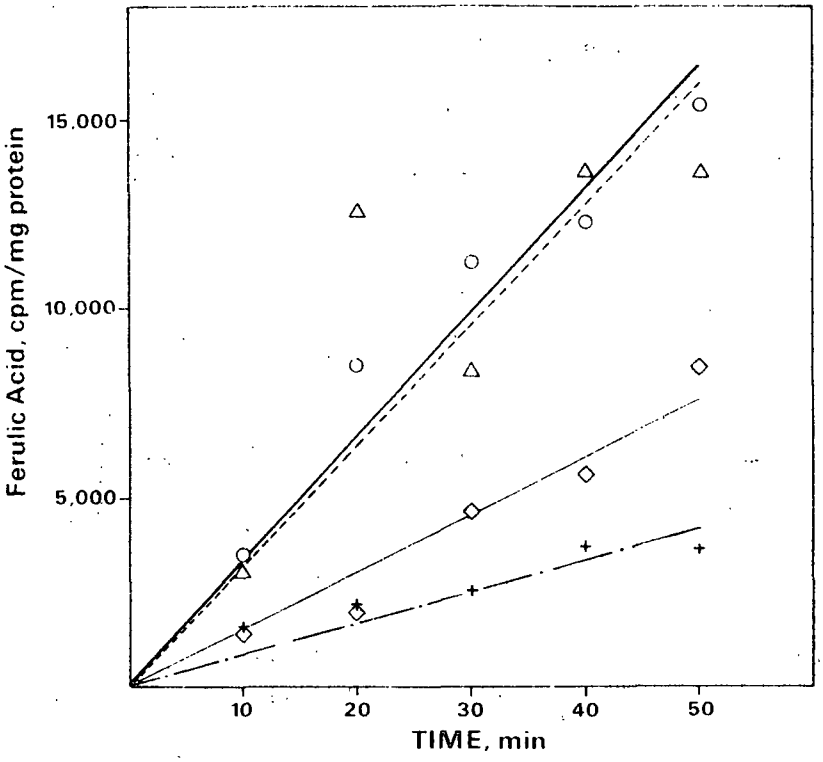


Figure 28. SAH inhibition. Caffeic acid fixed at 200 μM, SAM fixed at 40 μM. SAH concentration: 0–0 0 μM; Δ----Δ 1 μM; ◇–◇ 10 μM; + - • - + 20 μM. (The lines drawn are least squares fits fixed at zero.)

Figure 31 shows the plot of 1/v vs. I for the three caffeic acid concentrations. The two lowest concentrations show seemingly noncompetitive inhibition with a K_i of 6.7 μM. The highest caffeic acid concentration, which overcame the SAH inhibition to a large extent, has a K_i of 13 μM or about double that for the other caffeic acid

levels. This reversal of the inhibition could be due to enzyme activation, which seemed to occur with the callus homogenate at the higher caffeic acid concentrations. A Dixon replot of the "y" intercepts vs. I is shown in Fig. 32 (121,122). This shows a K_i value of 15.1 μM.

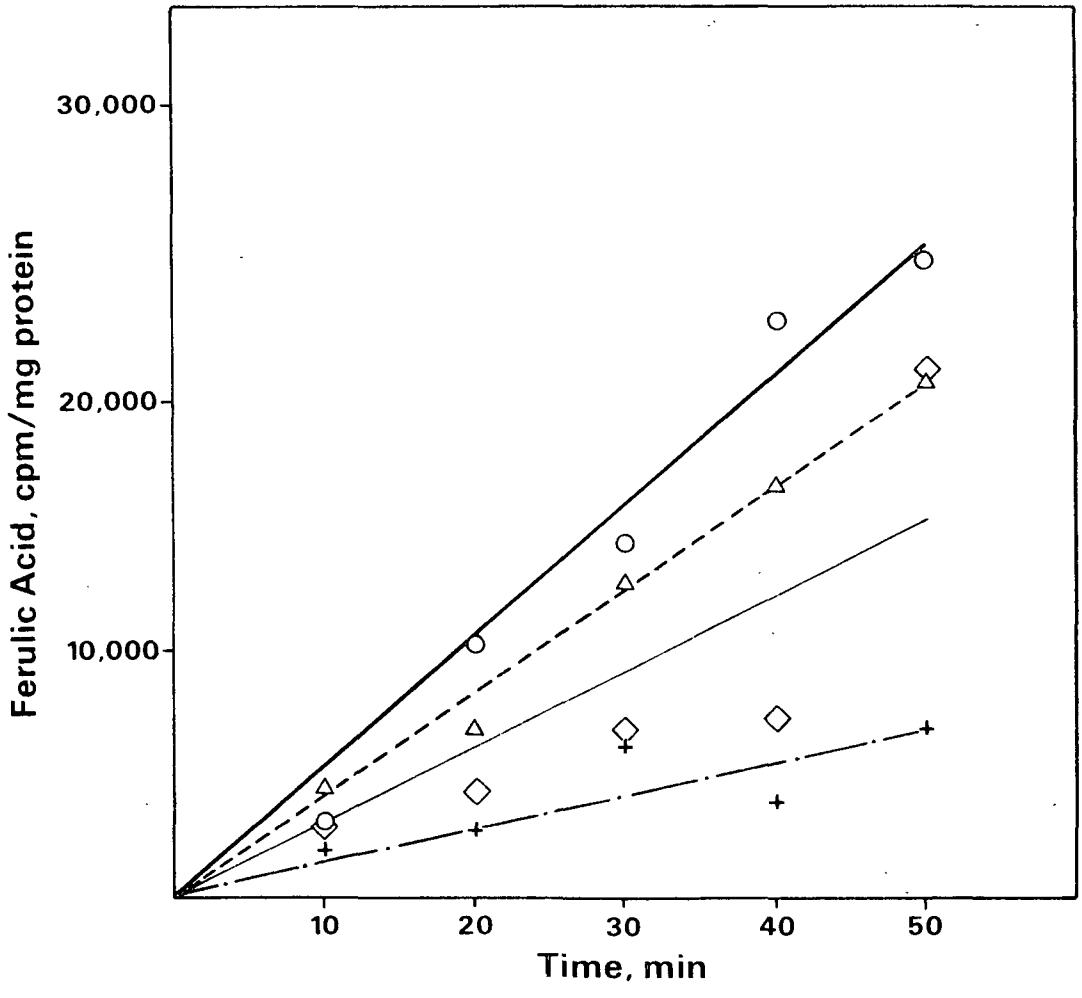


Figure 29. SAH inhibition. Caffeic acid fixed at 500 μM, SAM fixed at 40 μM. SAH concentration: 0-0 0 μM; Δ----Δ 1 μM; ◇-◇ 10 μM; + - • - + 20 μM. (The lines drawn are least squares fits fixed at zero.)

It appears that the inhibition due to SAH is a rather complicated phenomenon, showing noncompetitive inhibition that is overcome by activation of the enzyme due

to high caffeic acid concentrations. The results do indicate that the K_i for SAH in this system is a relatively high value. The K_i is at least 6.5 μM for low caffeic acid concentrations, and is 15 μM for saturating caffeic acid levels. Assuming that the entire 2% contamination in the SAM is SAH, it would take a 325 μM concentration of SAM to reach the lower K_i value and 750 μM SAM to reach the higher K_i value. This would still give only 50% inhibition of the OMT activity.

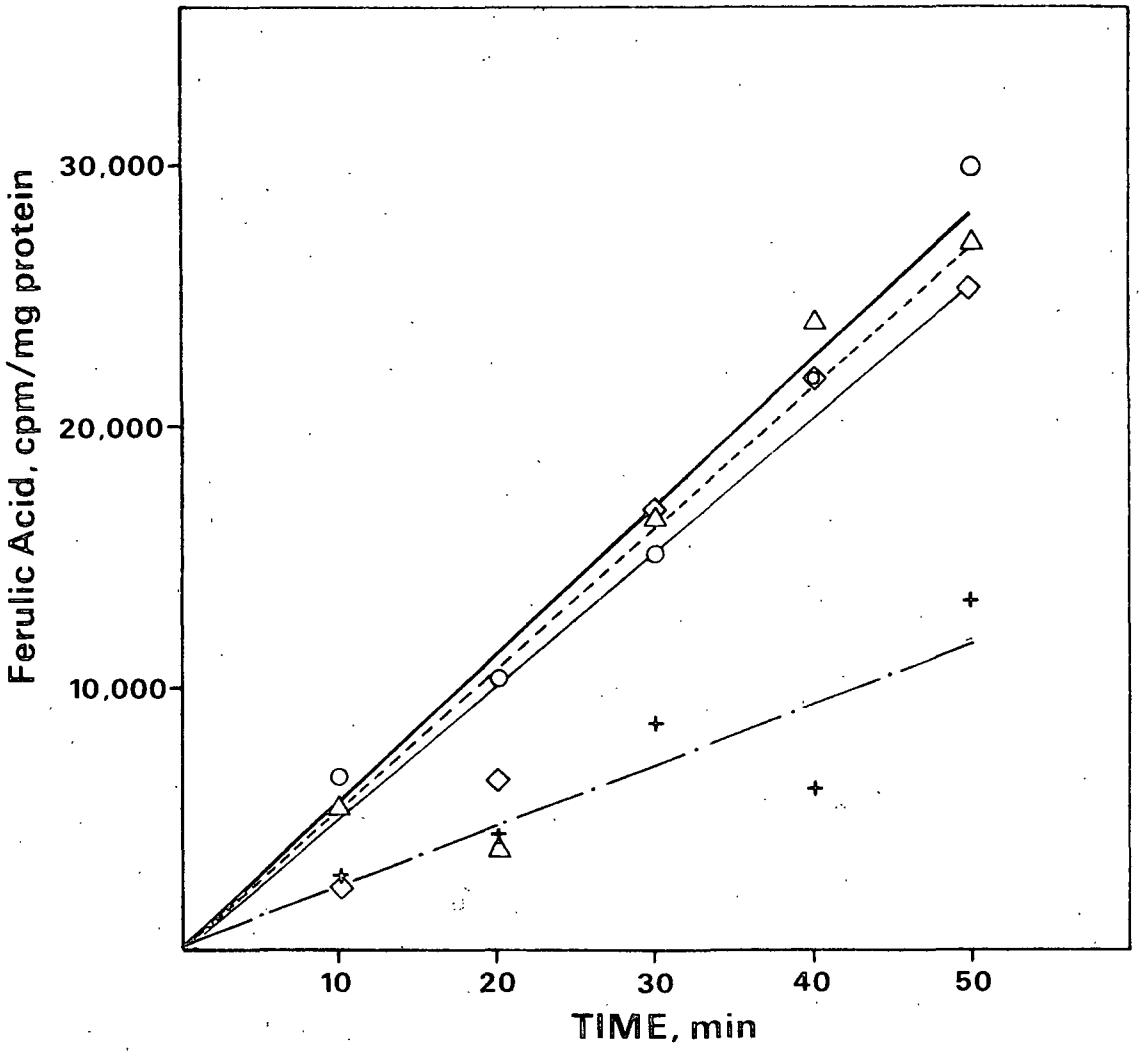


Figure 30. SAH inhibition. Caffeic acid fixed at 1500 μM, SAM fixed at 40 μM. SAH concentration: 0–0 0 μM; Δ----Δ 1 μM; ◇–◇ 10 μM; + – ∘ – + 20 μM. (The lines drawn are least squares fits fixed at zero.)

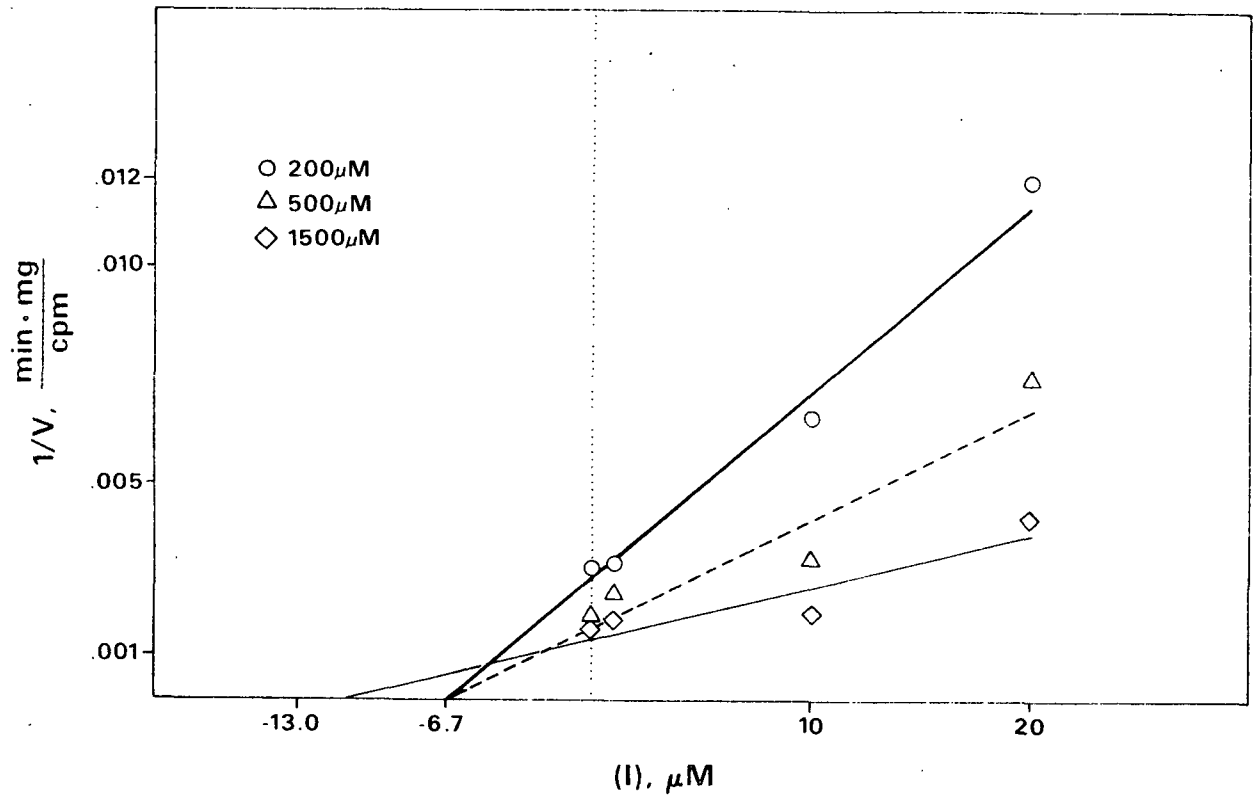


Figure 31. SAH inhibition, K_i determination. SAM fixed at 40 μM. Caffeic acid concentration: 0-0 200 μM; Δ----Δ 500 μM; ◇-◇ 1500 μM.

Significant inhibition of the OMT would not be expected at 80 and 100 μM concentrations if the inhibition was due solely to SAH. Since inhibition is found at these levels, it seems likely that the inhibition is due to actual substrate inhibition, and so the type of inhibition caused by the substrate must be determined.

S-ADENOSYLMETHIONINE INHIBITION

It is possible to have competitive substrate inhibition that yields a non-linear Lineweaver-Burke plot. While the general equation for the reaction velocity is the same as in the competitive contaminant case [Eq. (4),(5)], it is possible that the inhibition is nonlinear with respect to the substrate concentration. This would be especially true at the very high relative substrate concentrations, where

substrate inhibition often occurs. So, all three of the potential inhibition types are possible. This means that once again the kinetic constants may increase, decrease, or stay the same in the inhibited system.

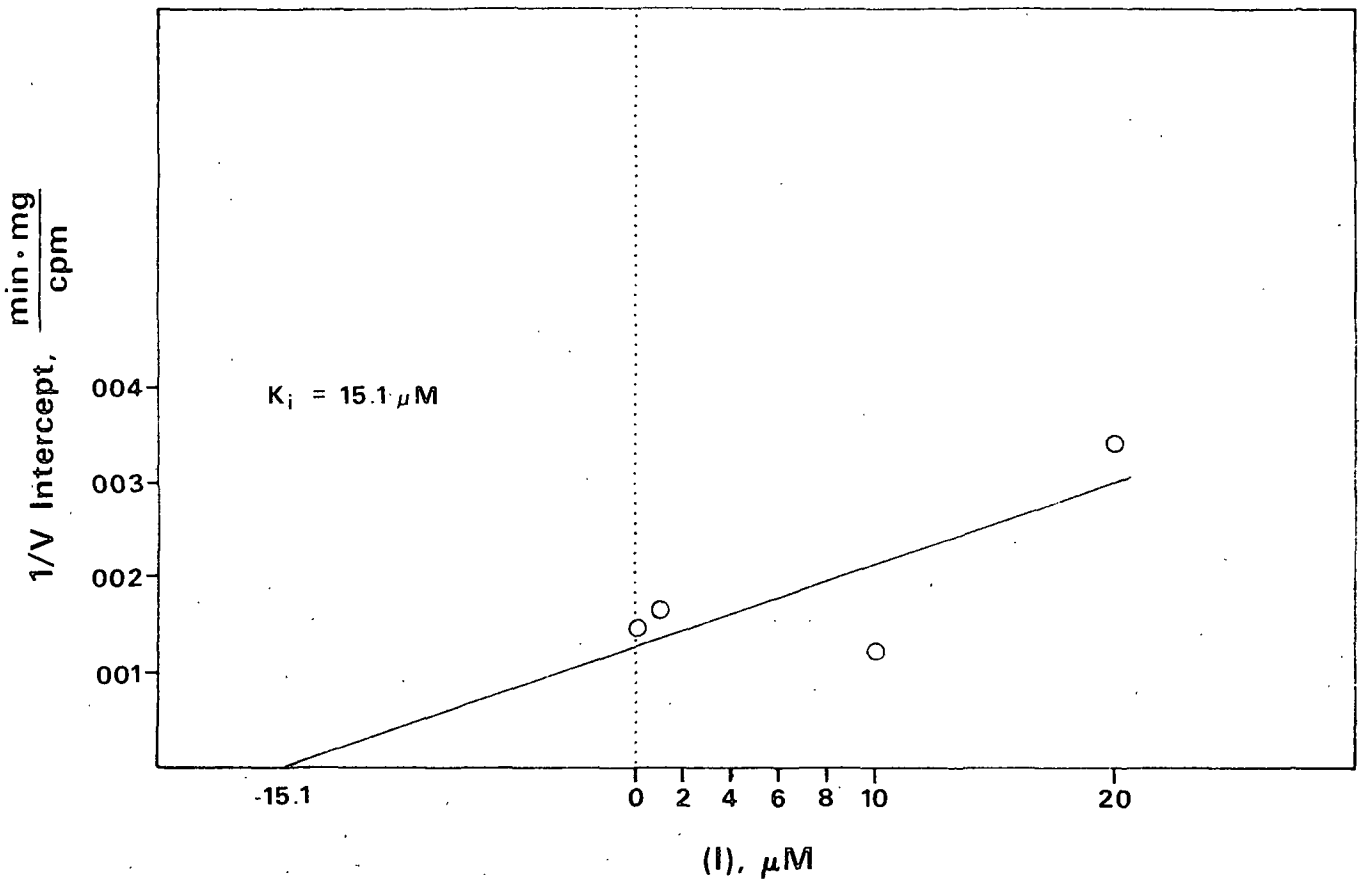


Figure 32. SAH inhibition, K_i determination. Dixon replot.

The technique used to investigate substrate inhibition was to vary the inhibiting substrate concentration at different fixed concentrations of the other substrate. This yields a series of plots as shown in Fig. 33. Each type of inhibition has a different type of series, as shown, and so one can determine the type of inhibition from the shape of the experimentally determined series of Lineweaver-Burke plots.

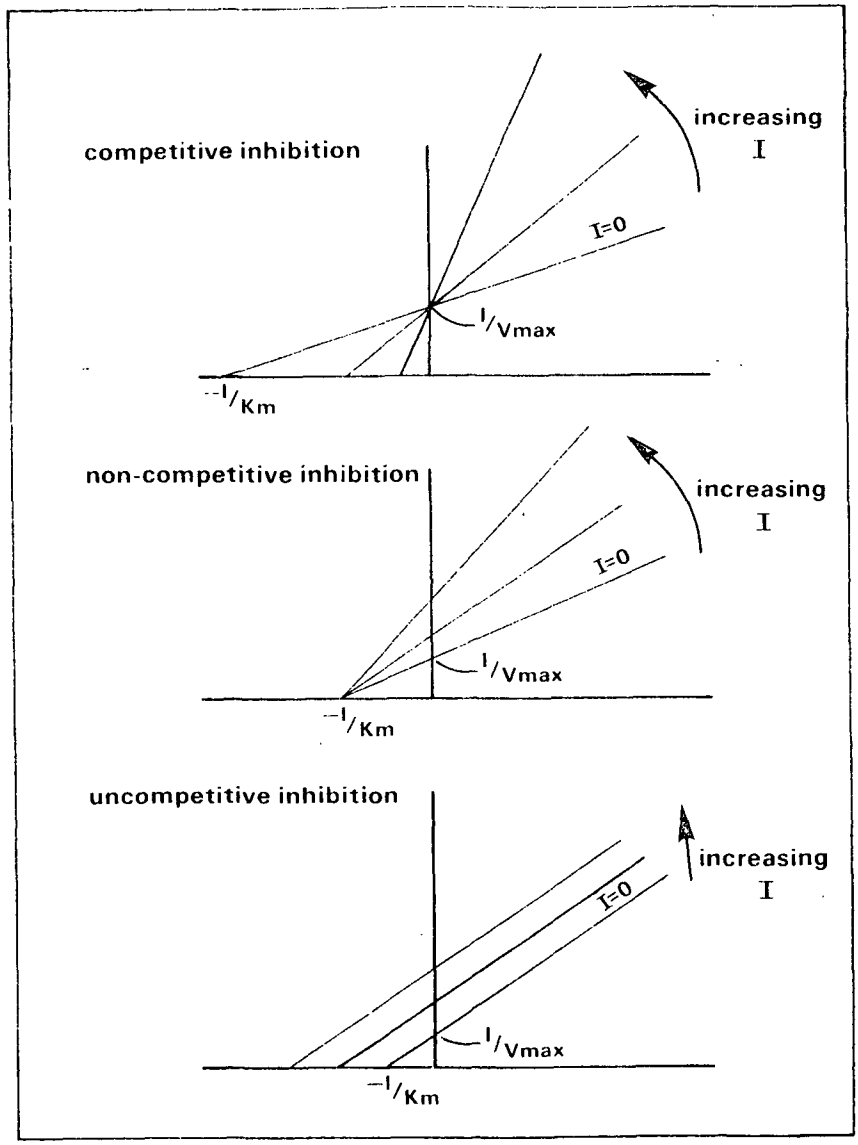


Figure 33. Lineweaver-Burke plots for inhibited enzyme systems. The figures show the effect the three different types of inhibition have on a series of Lineweaver-Burke plots.

The data in Fig. 34 and 35 were generated to find a noninhibitory level of substrate to use for the varying fixed substrate. Figure 34 shows the effect of

caffeic acid on the reaction rate at a constant SAM concentration of 200 μM. The reaction rate increases until 1000 μM is reached, at which point it levels off until 2000 μM is attained. Above 2000 μM the reaction rate decreases significantly. This inhibition is technically substrate inhibition, but it is possibly due simply to the reactive and enzyme-inactivating nature of quinones that would be formed by caffeic acid upon oxidation.

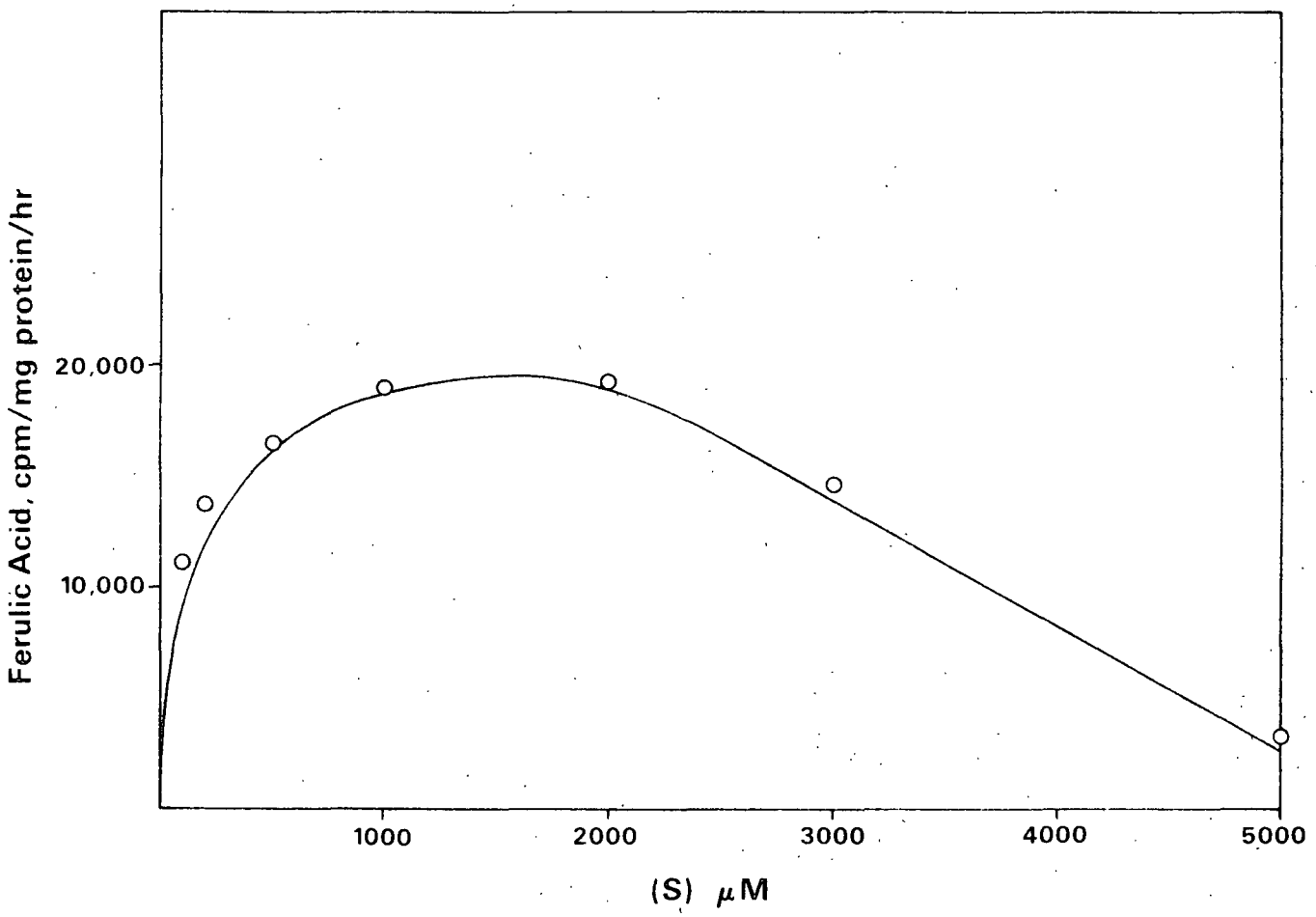


Figure 34. Purified callus OMT, enzyme activity vs. substrate concentration. The figure shows inhibition of the OMT above 2000 μM caffeic acid.

Figure 35 shows the effect of SAM on the OMT activity at a fixed caffeic acid concentration of 2000 μM. These data show a rapid leveling off of the enzyme activity

even at very low substrate concentrations. As Fig. 16 showed, enzyme activity vs. substrate concentration does level near the V_{max} value, so this curve has the correct shape. Simply comparing reaction velocities with the V_{max} value shows that there is some inhibition here. Homogenates had a V_{max} value of 500 cpm/min/mg protein. The apparent V_{max} value in Fig. 35 is 300 cpm/min/mg protein. For the lower substrate concentrations this inhibition is probably not very significant, but for the concentrations above 80 μM it has already been shown that there is significant substrate inhibition.

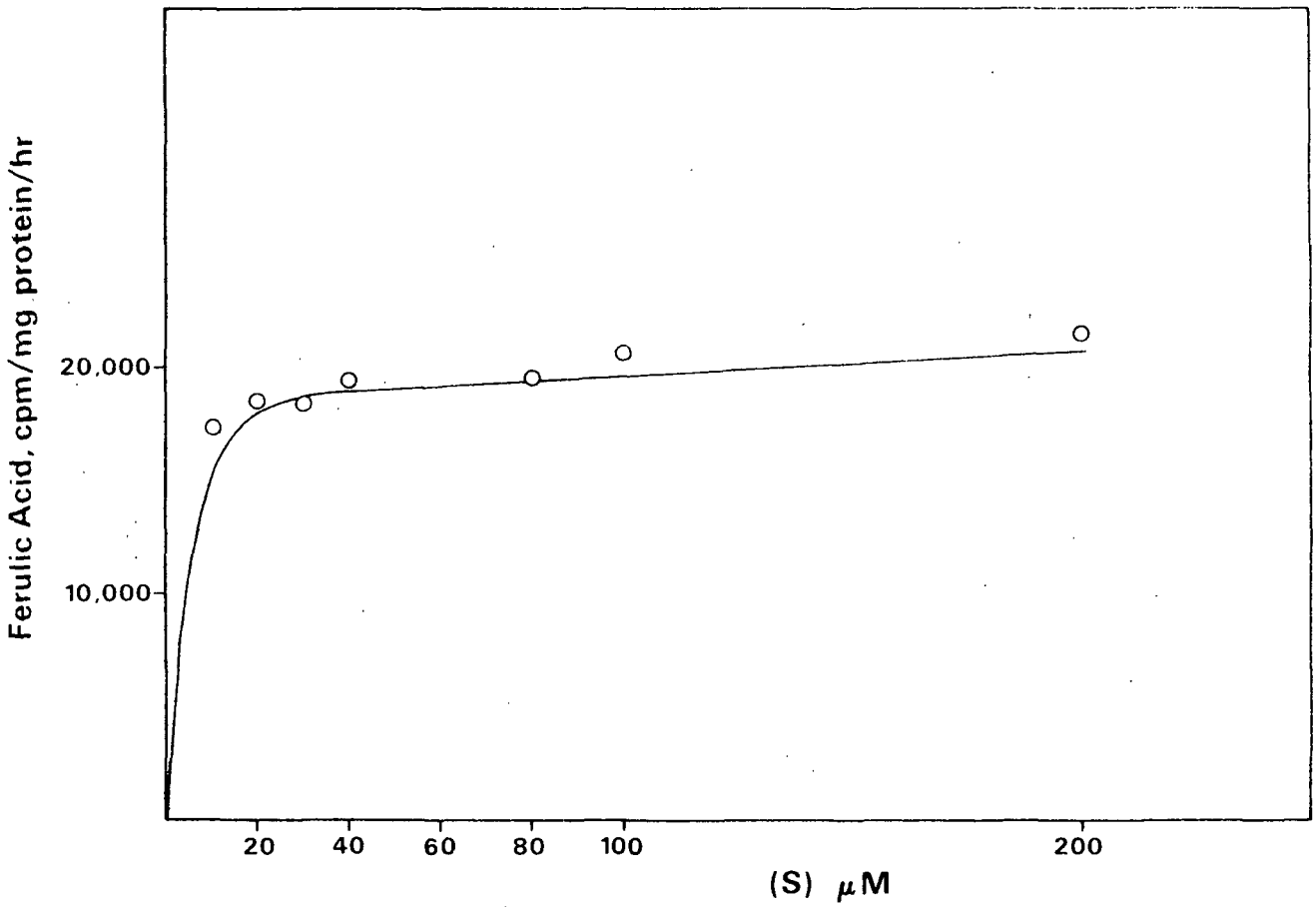


Figure 35. Purified callus OMT, enzyme activity vs. substrate (SAM) concentration.

In light of these results, it was decided to vary the SAM concentration from 10, 20, 30, to 40 μM at each of five caffeic acid concentrations. These concentrations

were: 100, 200, 300, 500, and 1500 μM. Figures 36 through 39 show the results of the product formed vs. time plots for the fixed SAM concentrations. Here, once again, the lack of purified enzyme forced the use of single samples for each data point with random duplication.

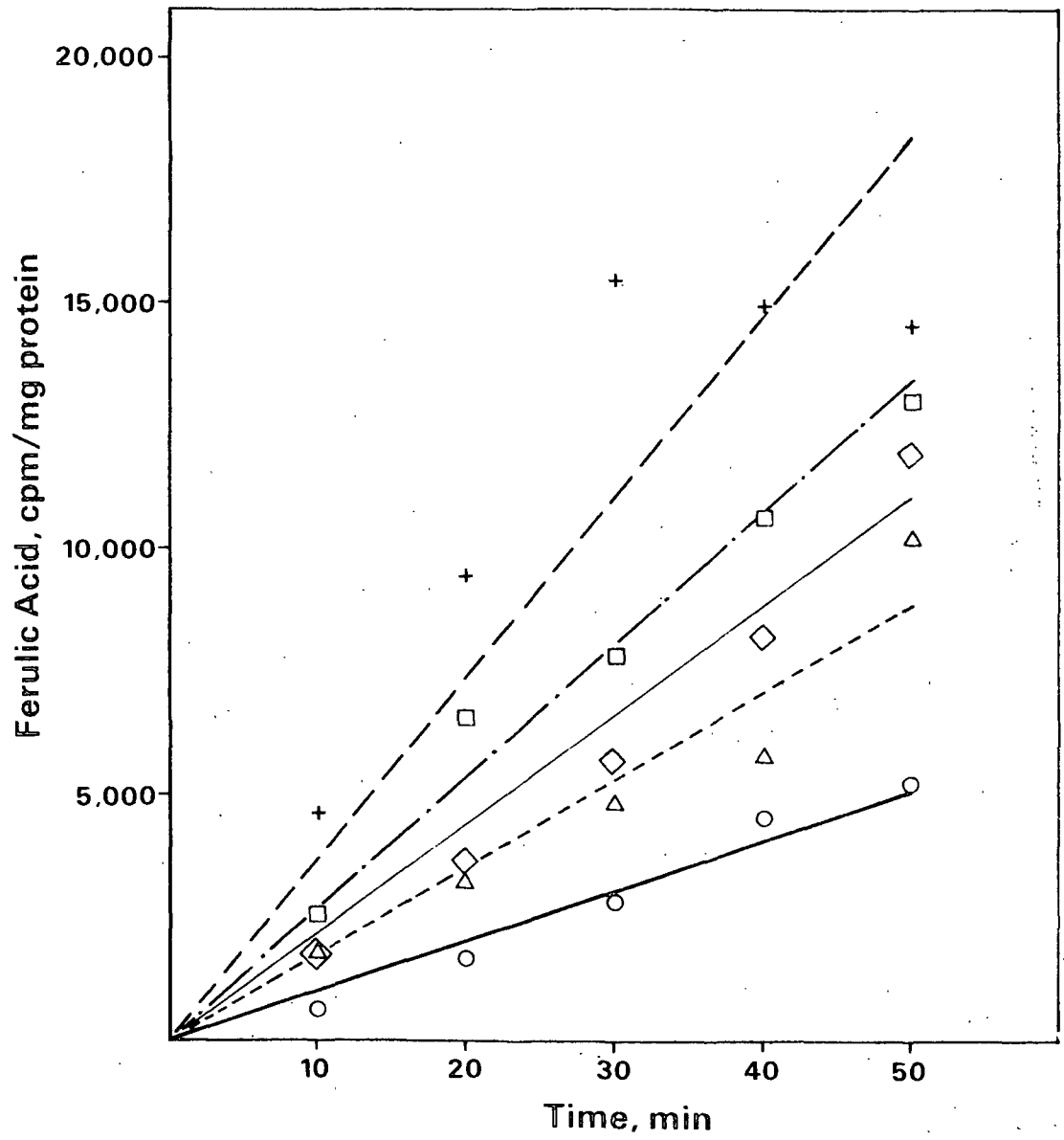


Figure 36. Purified OMT kinetic data, noninhibited system, ferulic acid vs. time. SAM fixed at 10 μM. Caffeic acid concentration: 0–0 100 μM; Δ---Δ 200 μM; ◇-◇ 300 μM; □-·-□ 500 μM; + --- + 1500 μM. (The lines drawn are least squares fits fixed at zero.)

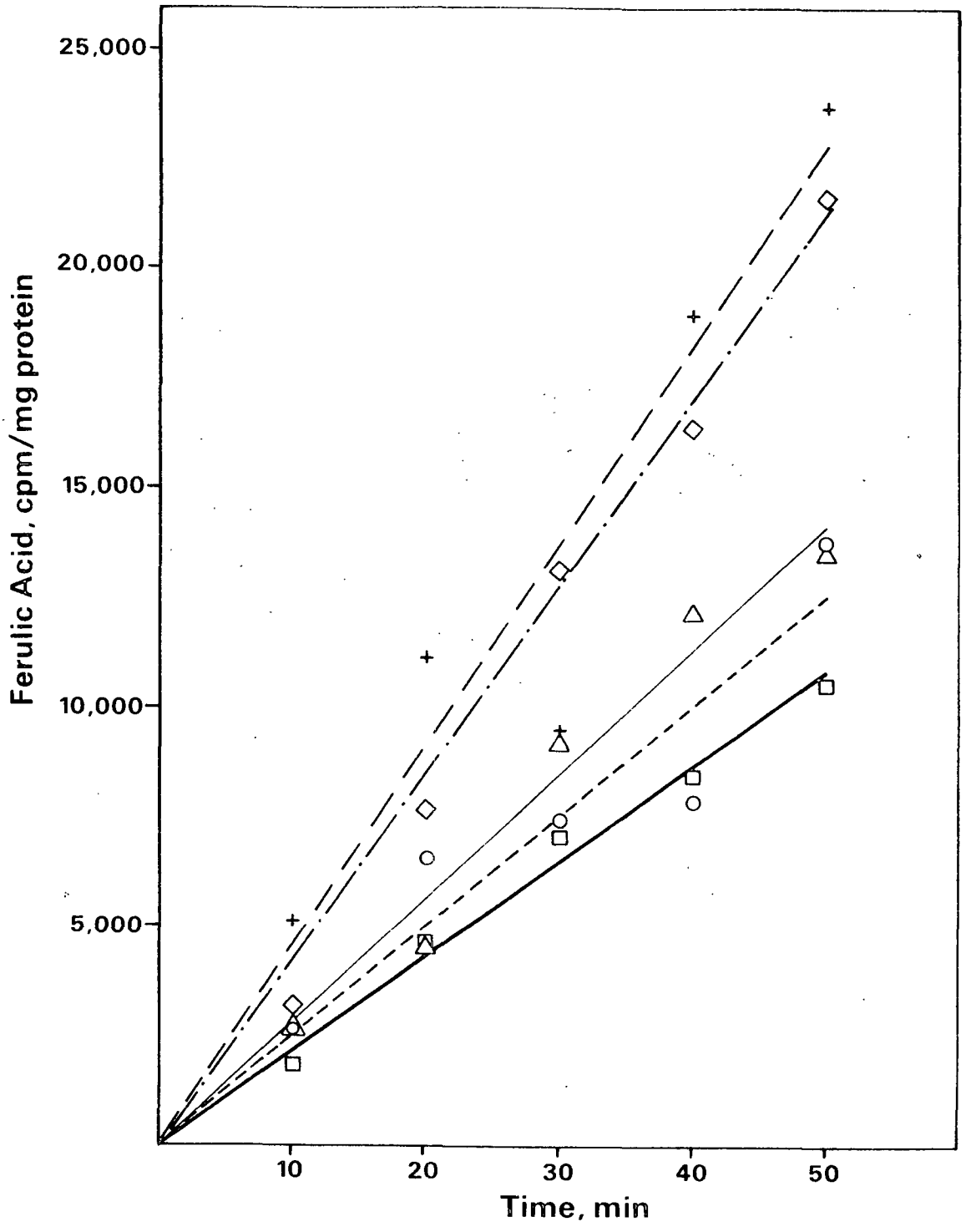


Figure 37. Purified OMT kinetic data, noninhibited system, ferulic acid vs. time. SAM fixed at 20 μM. Caffeic acid concentration: 0-0 100 μM; Δ---Δ 200 μM; ◊ - ◊ 300 μM; □-•- □ 500 μM; + --- + 1500 μM. (The lines drawn are least squares fits fixed at zero.)

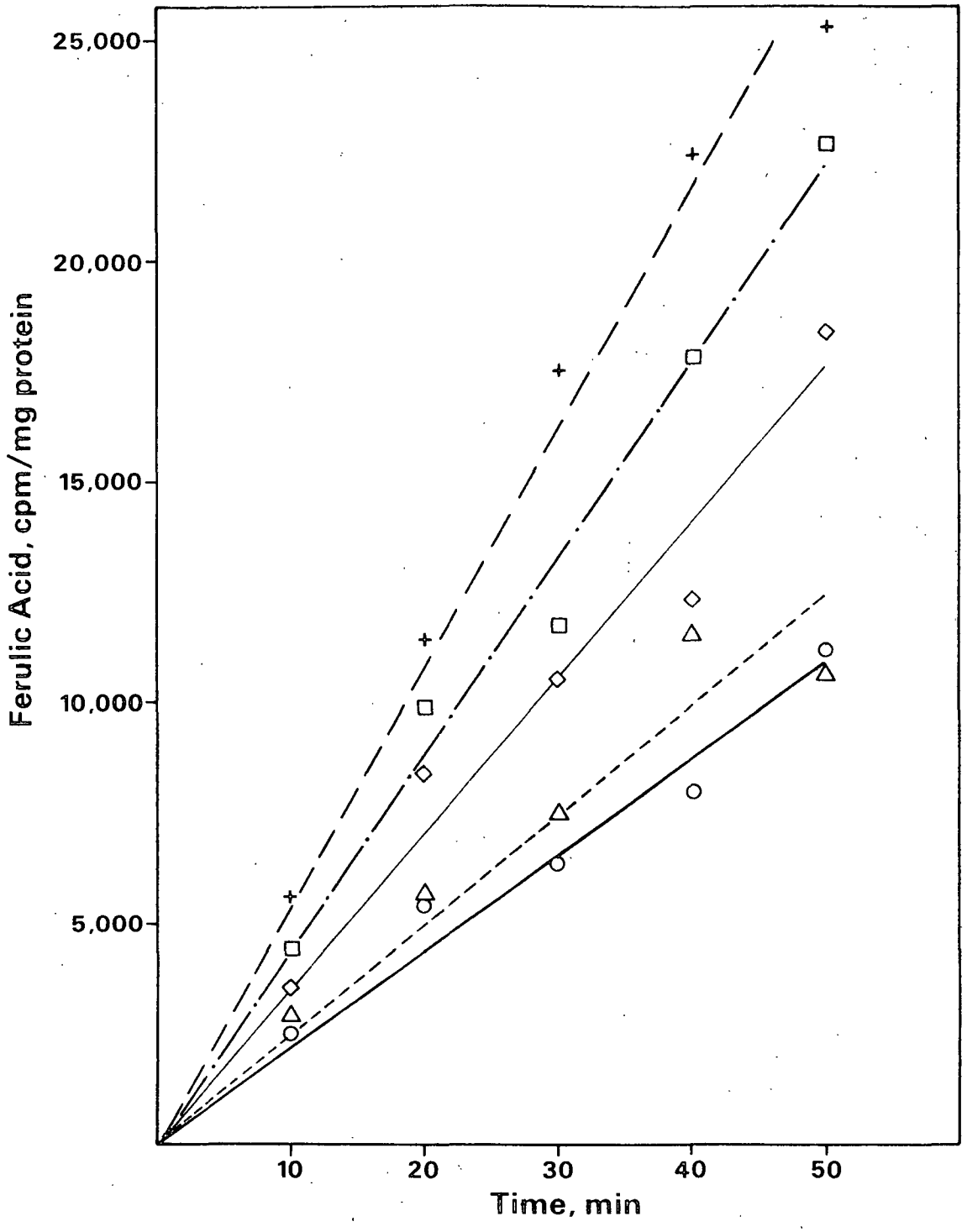


Figure 38. Purified OMT kinetic data, noninhibited system, ferulic acid vs. time. SAM fixed at 30 μM. Caffeic acid concentration: 0-0 100 μM; Δ---Δ 200 μM; ◊ - ◊ 300 μM; □-•- □ 500 μM; + --- + 1500 μM. (The lines drawn are least squares fits fixed at zero.)

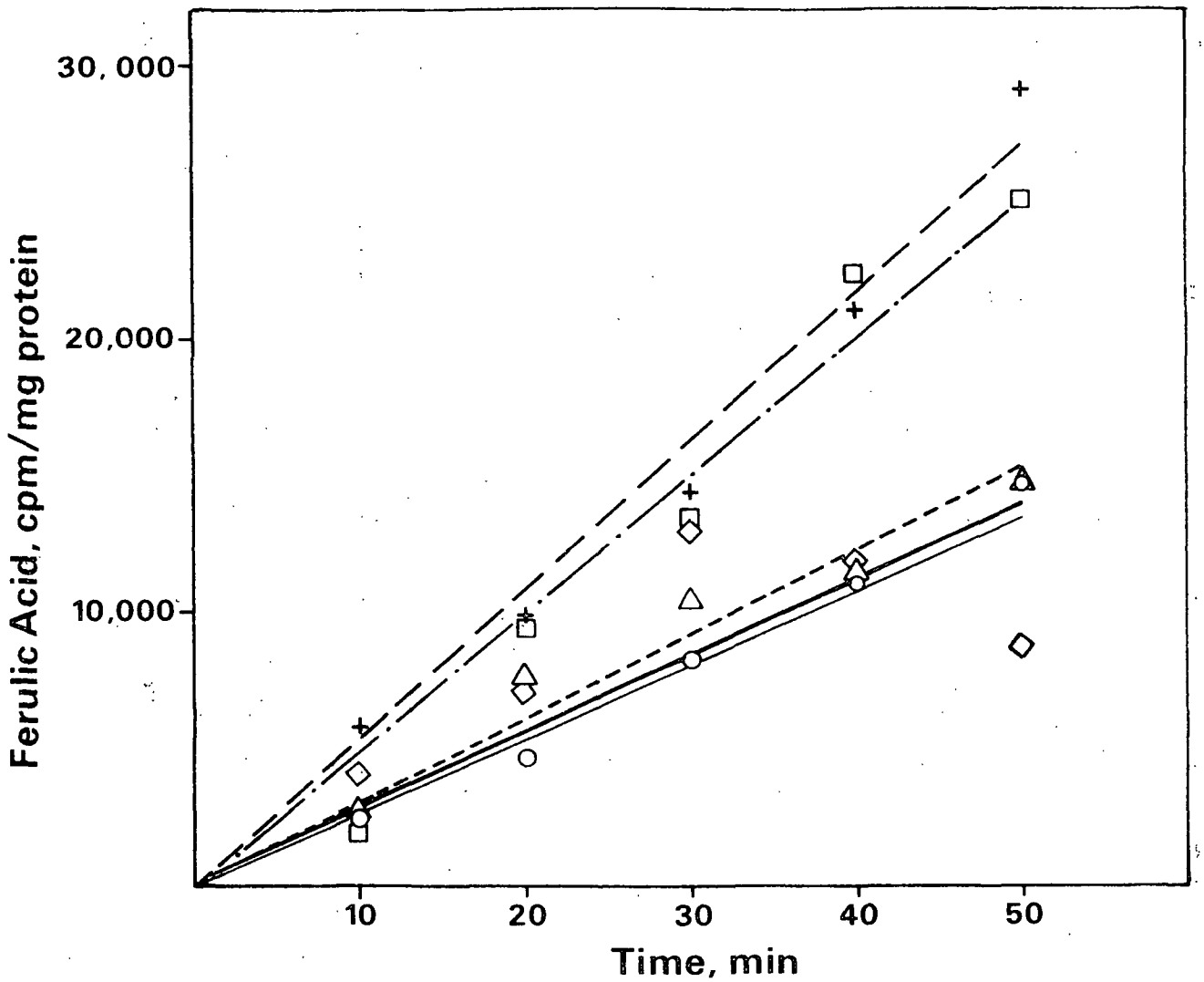


Figure 39. Purified OMT kinetic data, noninhibited system, ferulic acid vs. time. SAM fixed at 40 μM. Caffeic acid concentration: O–O 100 μM; Δ---Δ 200 μM; ◇-◇ 300 μM; □-·-□ 500 μM; + --- + 1500 μM. (The lines drawn are least squares fits fixed at zero.)

Figure 40 is the Lineweaver-Burke plot for these data. Although there appear to be some complicating factors, the overall shape of this plot implies a noncompetitive type of inhibition with the K_m value of 8 μM. In this type of inhibition, theory predicts the inhibited K_m value will be unchanged and the inhibited V_{max} value will be less than the real value. In the previous, inhibited, system the SAM K_m value

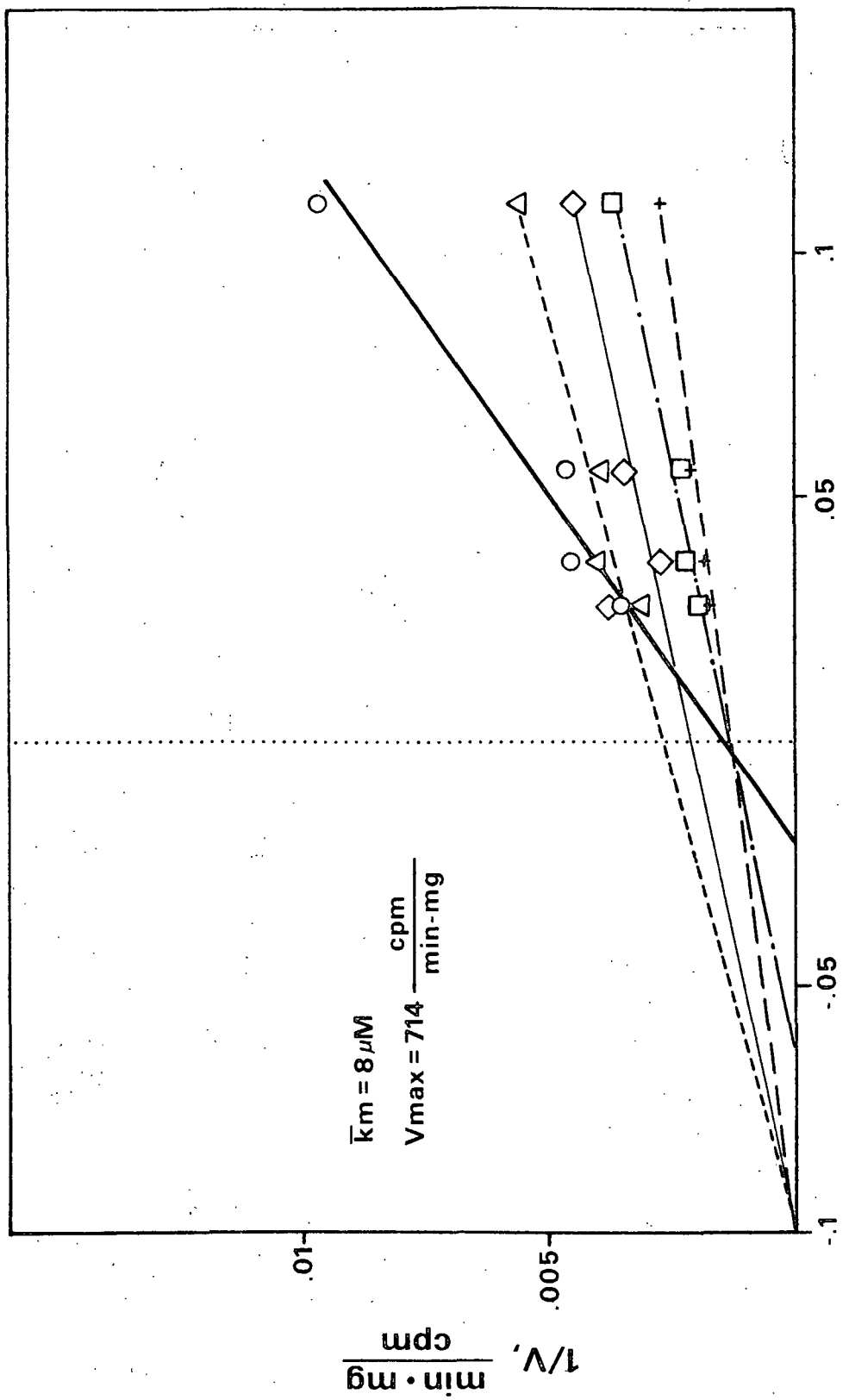


Figure 40. Lineweaver-Burke plot, purified OMT kinetic data, noninhibiting system. Caffeic acid concentration: 0–0 100 μM; Δ---Δ 200 μM; ◇-◇ 300 μM; □-·-□ 500 μM; + --- + 1500 μM.

was determined to be 7.7 μM, which agrees very well with this new, uninhibited value of 8 μM. This tends to support the interpretation of the Lineweaver-Burke plot as indicative of noncompetitive inhibition. The V_{max} value, however, does not behave as well. The inhibited V_{max} value was 2190 cpm/min/mg protein or 2.19 nmole ferulic acid/min/mg protein. The uninhibited V_{max} value is 714 cpm/min/mg protein or 0.714 nmole ferulic acid/min/mg protein. This represents a very significant decrease in the V_{max} value, where the theory predicts an increase. In fact, none of the inhibition types predict this decrease. This behavior is unexplained. One possible explanation would be to attribute the increase in the V_{max} value to a synergistic effect of caffeic acid and SAM on the inhibited OMT activity.

Figures 41-46 show the reaction velocity plots for caffeic acid at the four SAM concentrations. Figure 46 shows the Lineweaver-Burke plot for this data. Again, with the exception of the lowest concentration of SAM, the inhibition appears to be of one type. In this case the inhibition is uncompetitive, with an averaged K_m value of 229 μM. This is double what the inhibited K_m value was for caffeic acid, which is in agreement with what would be expected for uncompetitive inhibition, where the real K_m is larger than the inhibited value. In this case, the V_{max} also decreased the inhibited, relative to the noninhibited, case. However, the V_{max} values for each substrate do agree well.

Table XIV shows the summarized results for the membrane-bound and the liberated, uninhibited Douglas-fir callus OMT.

In conclusion of the kinetic section of this thesis, the SAM K_m value is within the range for OMT as reported for other species, but the caffeic acid K_m value is significantly higher. This could very well account for the lack of lignification in these tissues, if the substrate concentrations never reach values high enough to be

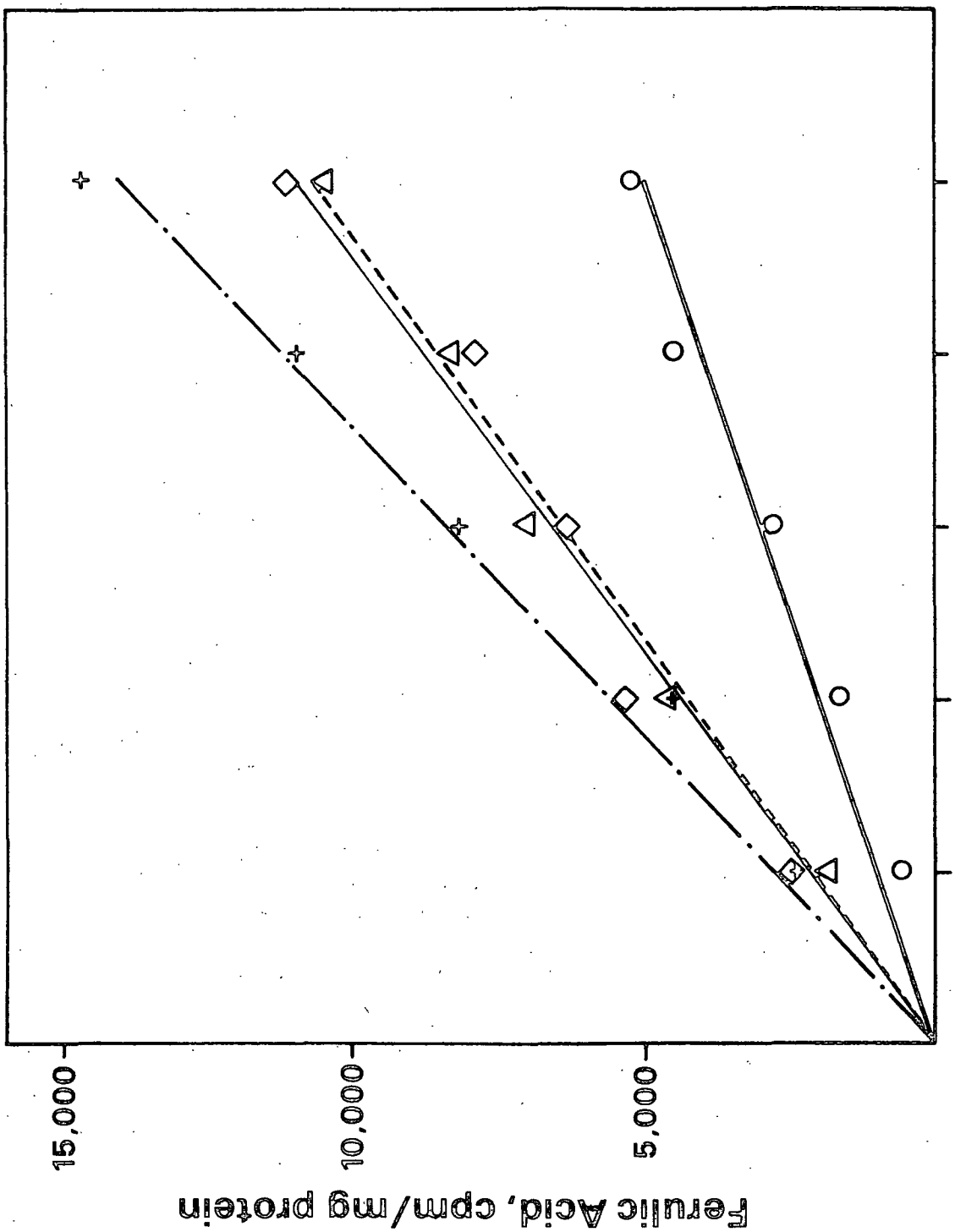


Figure 41. Purified OMT kinetic data, noninhibiting system, ferulic acid vs. time. Caffeic acid fixed at 100 μM. SAM concentration: O-O 10 μM; Δ--Δ 20 μM; ◇-◇ 30 μM; + --- + 40 μM. (The lines drawn are least squares fits fixed at zero.)

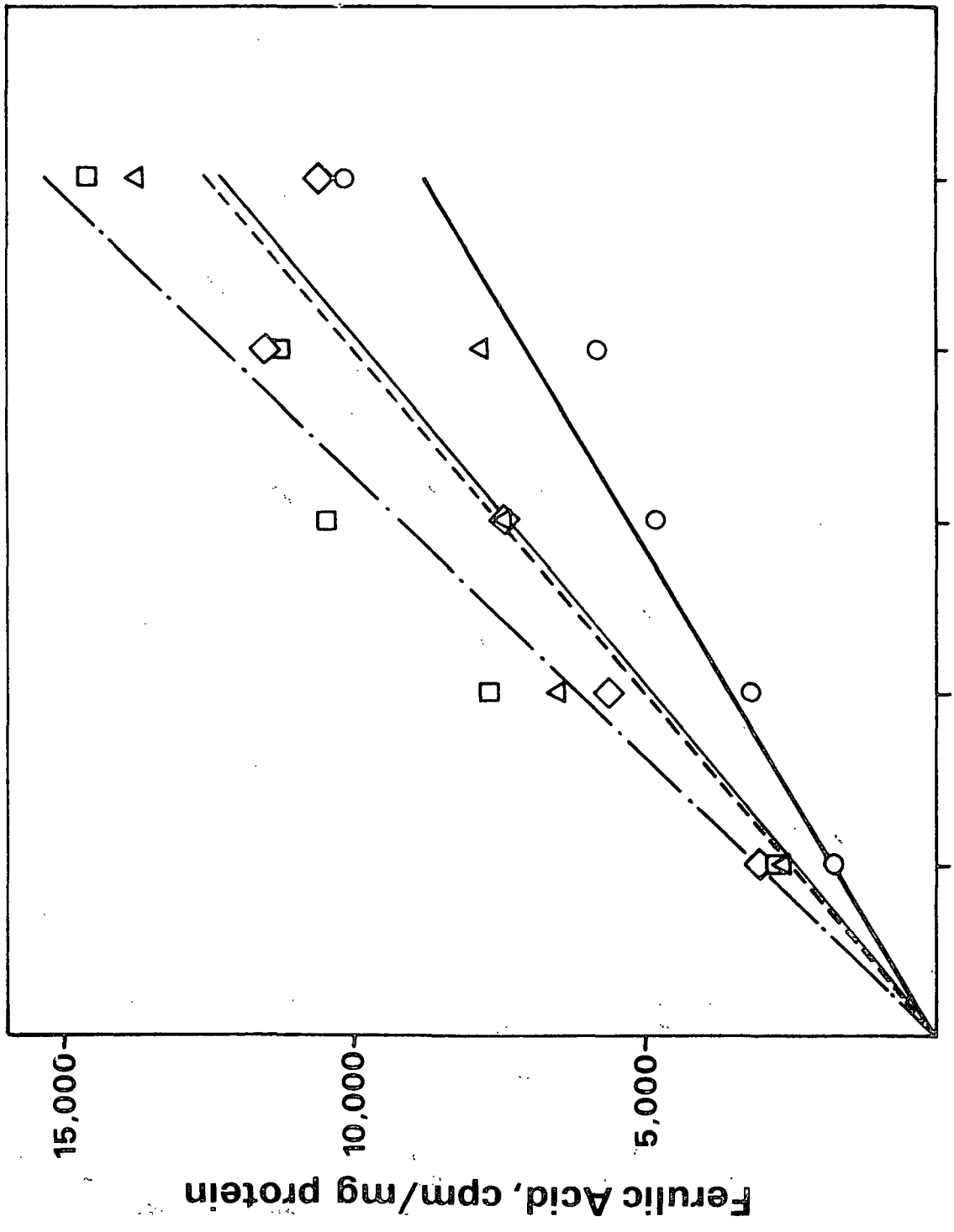


Figure 42. Purified OMT kinetic data, noninhibiting system, ferulic acid vs. time. Caffeic acid fixed at 200 μM. SAM concentration: 0-0 10 μM; Δ---Δ 20 μM; ◇ - ◇ 30 μM; □-•-□ 40 μM. (The lines drawn are least squares fits fixed at zero.)

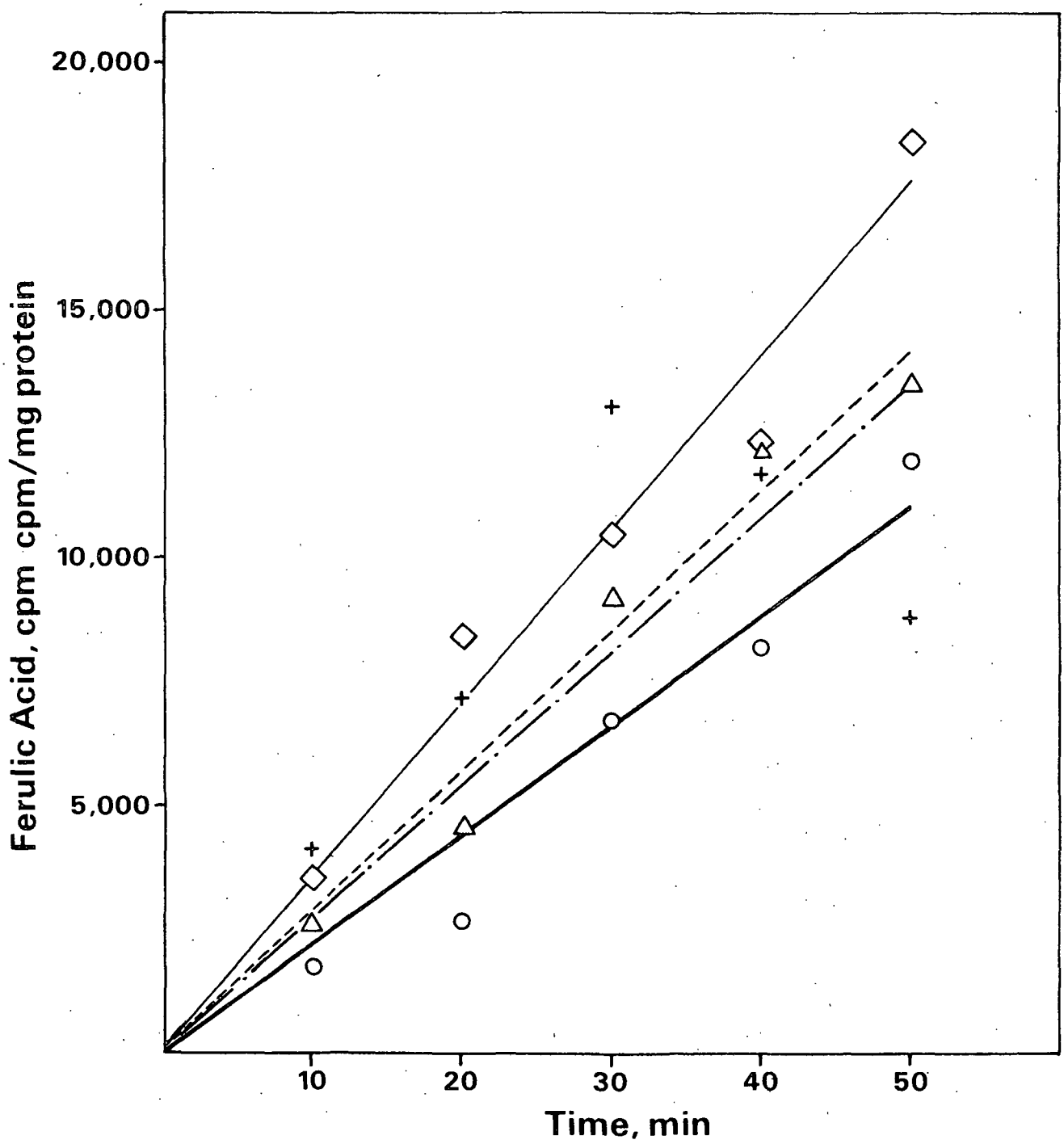


Figure 43. Purified OMT kinetic data, noninhibiting system, ferulic acid vs. time. Caffeic acid fixed at 300 μM. SAM concentration: 0-0 10 μM; Δ---Δ 20 μM; ◇-◇ 30 μM; + -•- + 40 μM. (The lines drawn are least squares fits fixed at zero.)

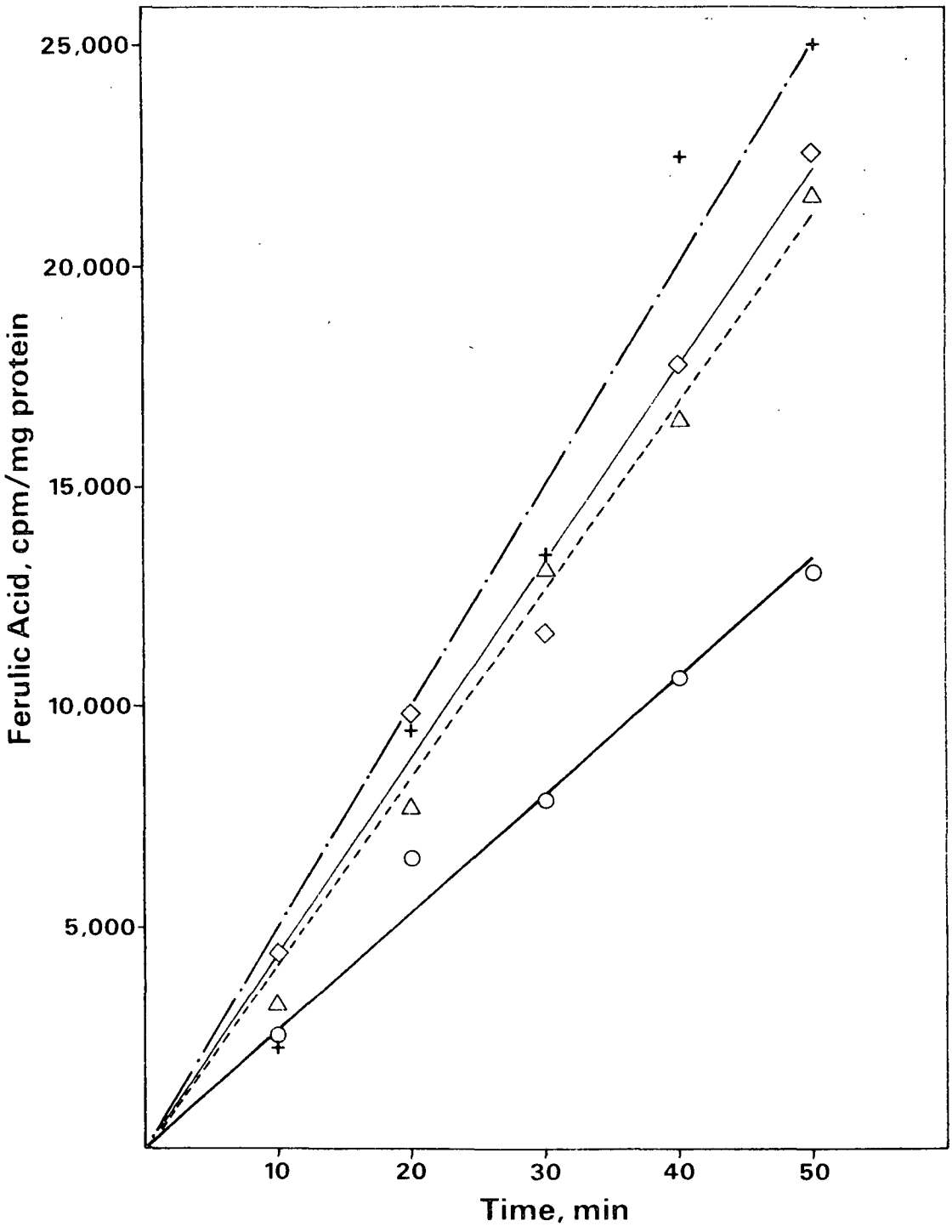


Figure 44. Purified OMT kinetic data, noninhibiting system, ferulic acid vs. time. Caffeic acid fixed at 500 μM. SAM concentration: 0-0 10 μM; Δ---Δ 20 μM; ◇ - ◇ 30 μM; + -•- + 40 μM. (The lines drawn are least squares fits fixed at zero.)

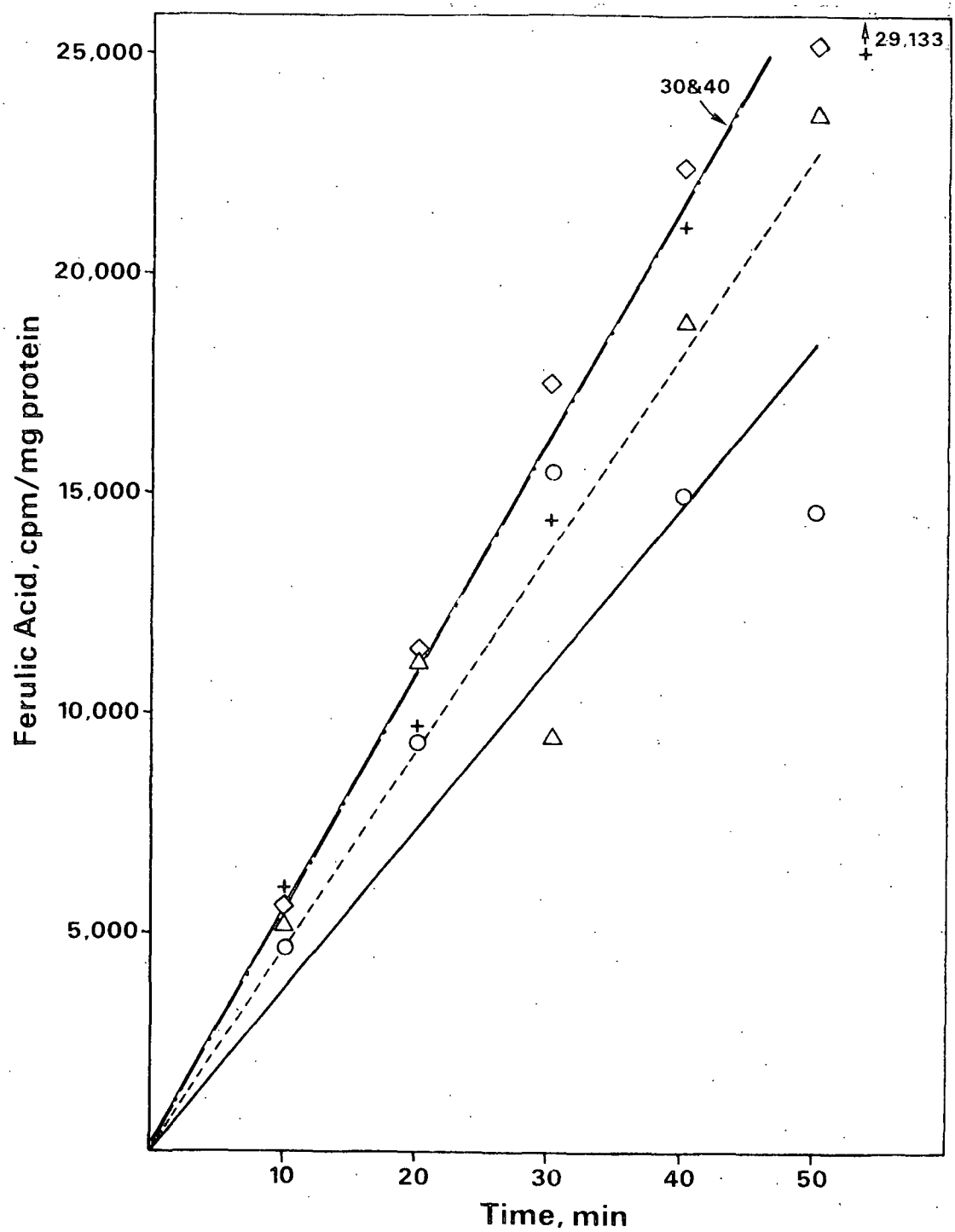


Figure 45. Purified OMT kinetic data, noninhibiting system, ferulic acid vs. time. Caffeic acid fixed at 1500 μM. SAM concentration: 0-0 10 μM; Δ---Δ 20 μM; ◇ - ◇ 30 μM; + -•- + 40 μM. (The lines drawn are least squares fits fixed at zero.)

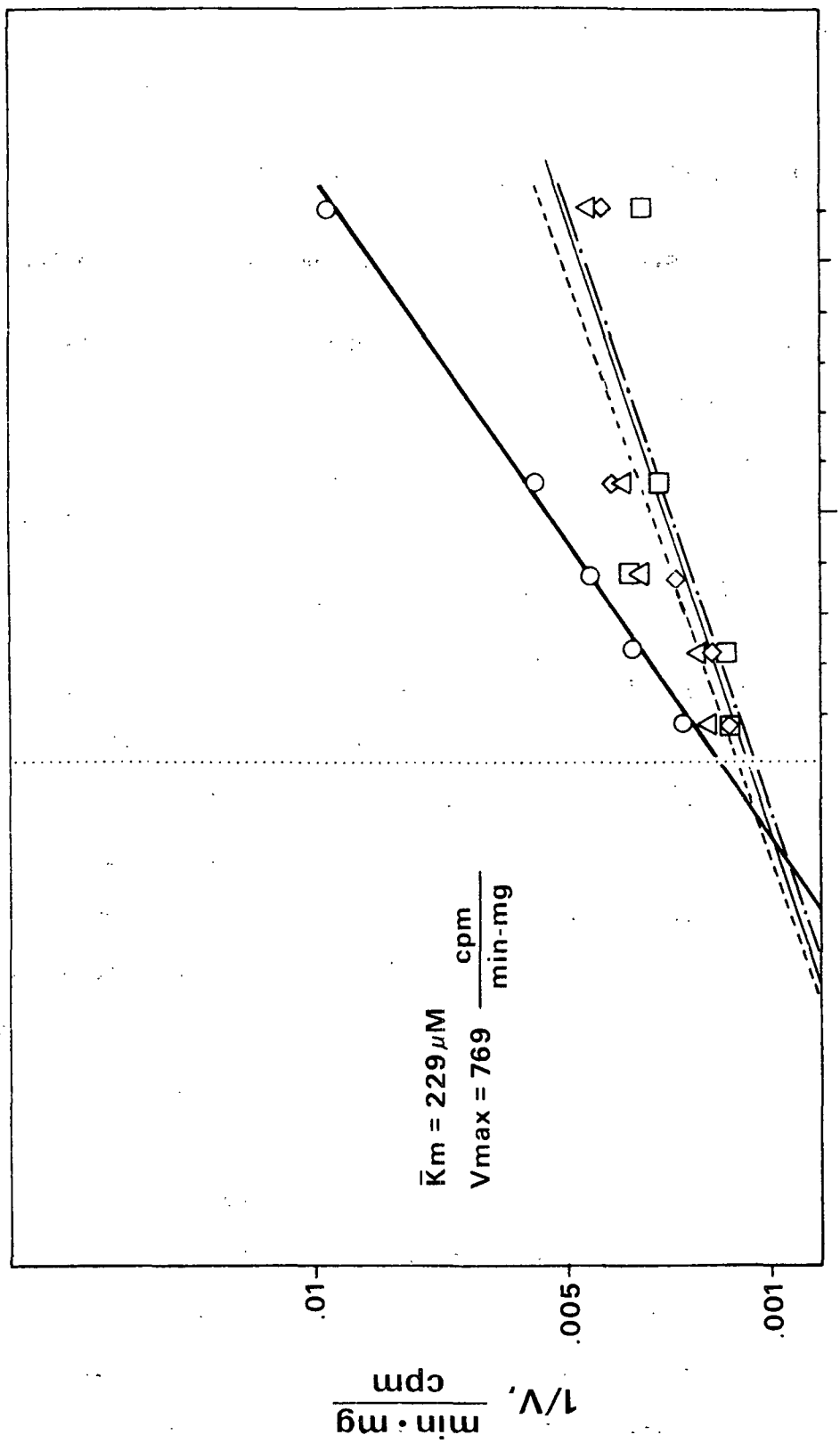


Figure 46. Lineweaver-Burke plot for caffeic acid, purified OMT kinetic data, noninhibited system. SAM concentration: 0-0 10 μM; Δ---Δ 20 μM; ◇ - ◇ 30 μM; □-•-□ 40 μM.

effectively used by this enzyme. Investigation of this aspect of the tissues comprises the remainder of this thesis.

TABLE XIV

DOUGLAS-FIR CALLUS OMT KINETIC CONSTANTS

Source	Substrate	V_{max}, nmol/min/mg protein	K_m, μMolar
Homogenate	Caffeic acid	0.500	333
Homogenate	S-adenosylmethionine	0.453	9.6
Purified	Caffeic acid	0.769	250
Purified	S-adenosylmethionine	0.714	8.0

ENDOGENOUS CINNAMIC ACIDS, CATECHIN, AND TANNIN VS. TIME

Figure 47 shows the average fluctuations in the free cinnamic acids (cinnamic, p-coumaric, caffeic, and ferulic acid) over the subculture period for three replicates taken at each time point. It can be seen that a peak is reached at 6 and 12 days, and that further fluctuations follow one another closely. It is noted that the caffeic acid concentration reaches a maximum concentration of 75 μM at day 12, but otherwise the concentration never surpasses 30 μM. At these levels it is quite evident that very little ferulic acid will be formed. This in effect will force the caffeic acid into the flavonoid pathway, if the enzymes of this pathway are more sensitive to the presence of caffeic acid (i.e., have a lower K_m value). There is no information available about the kinetic constants of these enzymes. But because more flavonoids and tannin than lignin are formed in the Douglas-fir callus tissue, and because there is a small steady-state concentration of caffeic acid, it seems reasonable to conclude that the flavonoid pathway is indeed more sensitive to caffeic acid, if catechin is, indeed, derived primarily from caffeic acid.

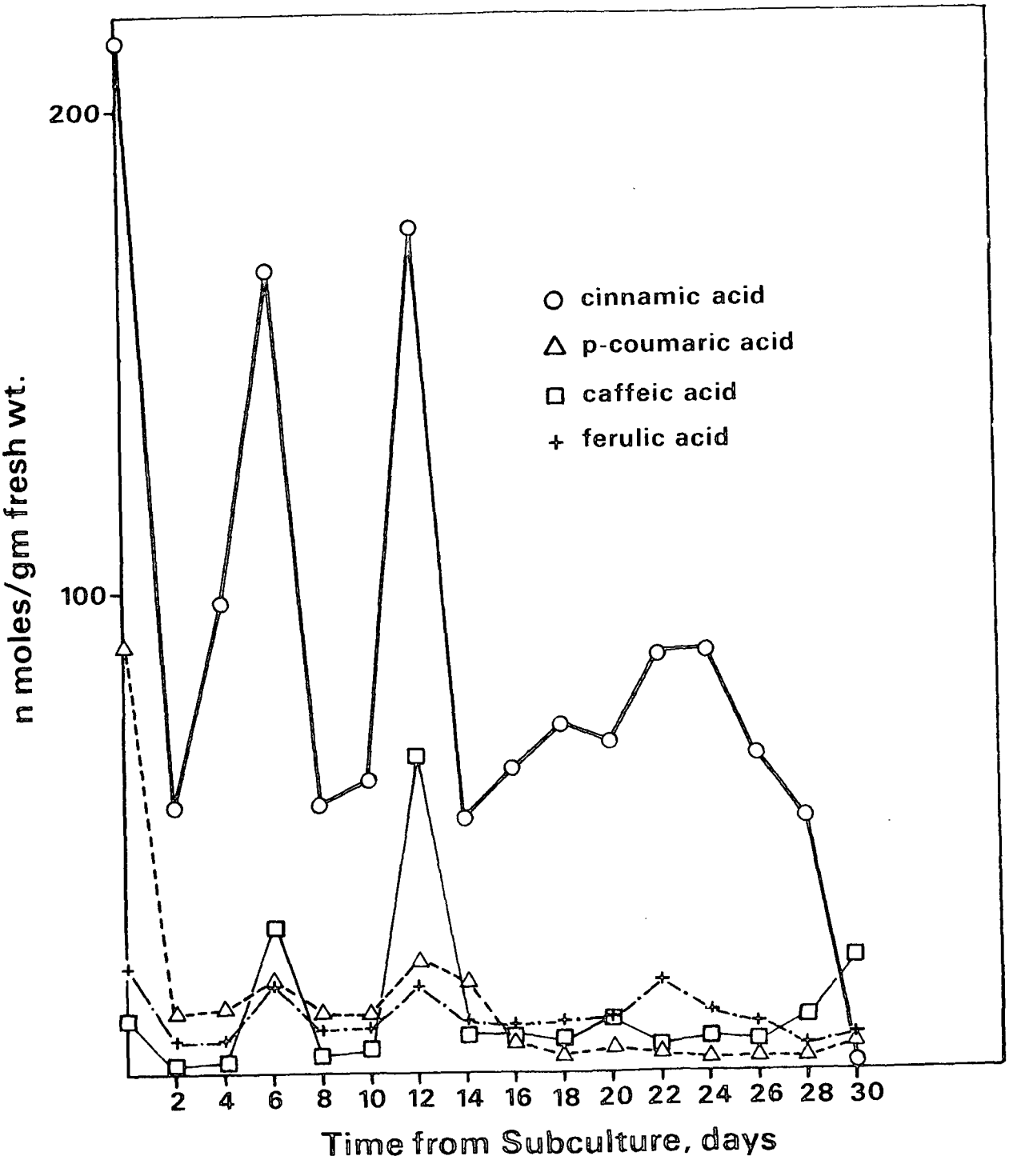


Figure 47. Douglas-fir callus free endogenous cinnamic acids vs. time. O–O cinnamic acid, Δ---Δ p-coumaric acid, □-- □ caffeic acid, + -•- + ferulic acid.

Figure 48 shows the amount of free catechin in these tissues. The cinnamic acid is shown to provide an idea of the scale. It is quite obvious that the amount of catechin is nearly an order of magnitude higher than the amount of cinnamic acid. It also fluctuates, but at points slightly removed from the cinnamic acid, as would be expected in sequential reactions.

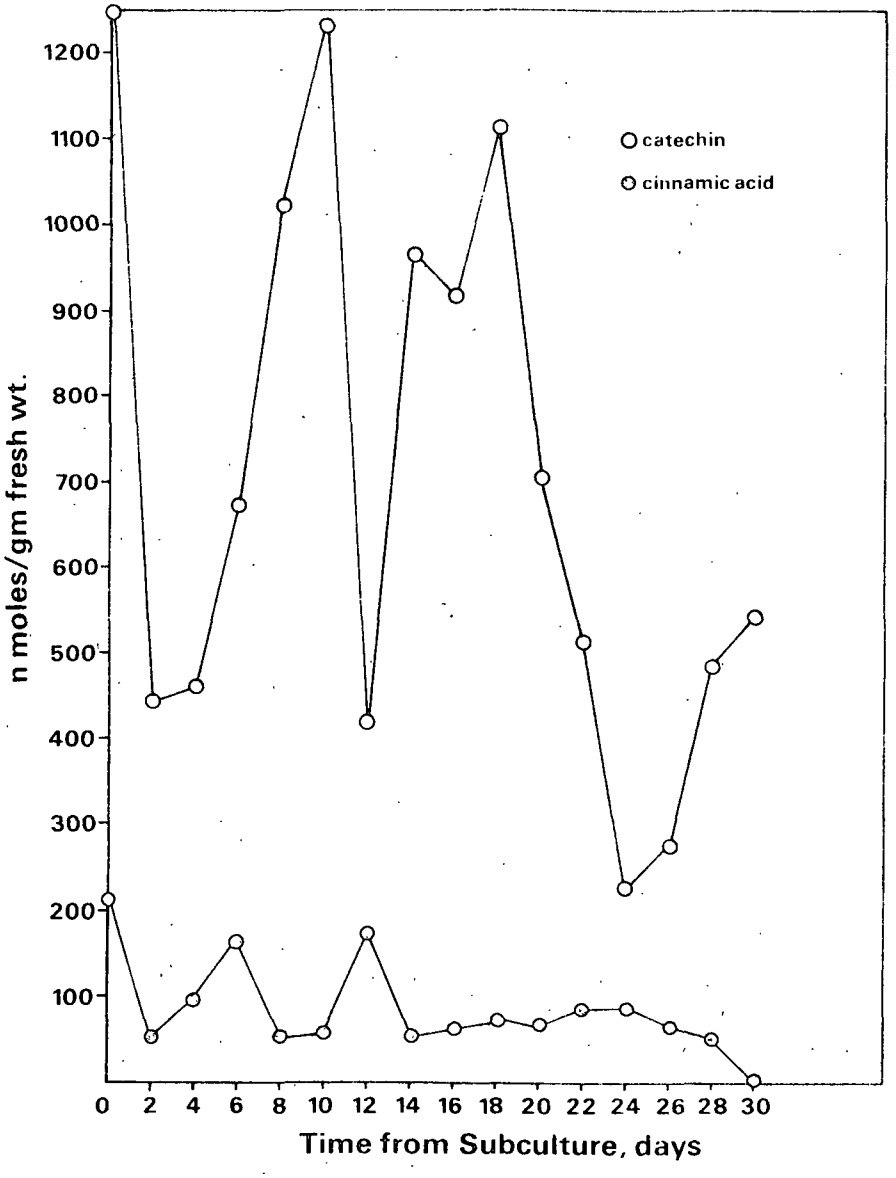


Figure 48. Douglas-fir callus free endogenous catechins vs. time. O-O catechins, O-O free cinnamic acid. The cinnamic acid is included for comparison.

Figure 49 shows the levels of condensed tannin or proanthocyanidin present in these tissues. The catechin levels are shown to provide a sense of scale. The catechin was converted to a weight per gm fresh weight because no molecular weight for the proanthocyanidin is available. It will be noted that up to 5% of the tissue fresh weight consists of tannin. The fluctuation in tannin levels over time is confusing since the tannin is thought to be an end product. It would not be expected to fall over time, as utilized compounds do. This type of fluctuation in proanthocyanidin has also been noted in loblolly pine callus tissue. This could possibly be due to uptake of water as the cells swell and enlarge, which would thereby dilute the tannin on a per gram fresh weight basis. If the cell division were synchronous, this could happen, but if the division were random this fluctuation would not be observed.

Figures 50-52 show the acid and alkaline hydrolyzed cinnamic acids over time. These are the storage forms of the acids, and were measured to determine if large amounts of caffeic acid could be available for the OMT from storage. These results indicate that this is not the case.

In conclusion, the free caffeic acid concentration is low over the subculture period, predominantly under 10 μM concentration. The other cinnamic acids are also low, which is in contrast to the high levels of the catechins. These data suggest that the biosynthesis of catechin is a major pathway for the cinnamic acids produced in this tissue, to the near exclusion of lignification.

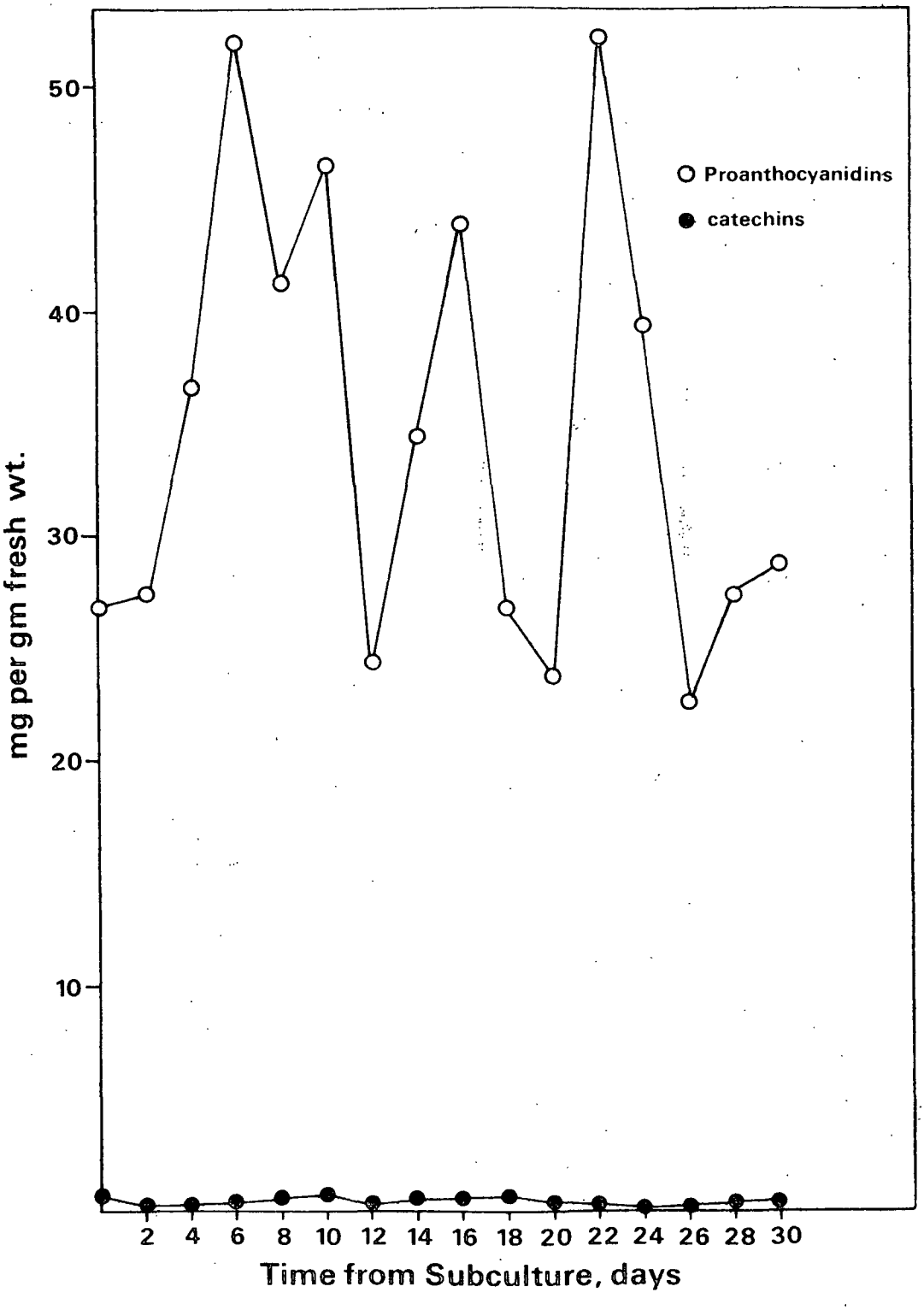


Figure 49. Douglas-fir proanthocyanidin vs. time. O-O proanthocyanidin, O-O free catechins. The free catechins were included for comparison.

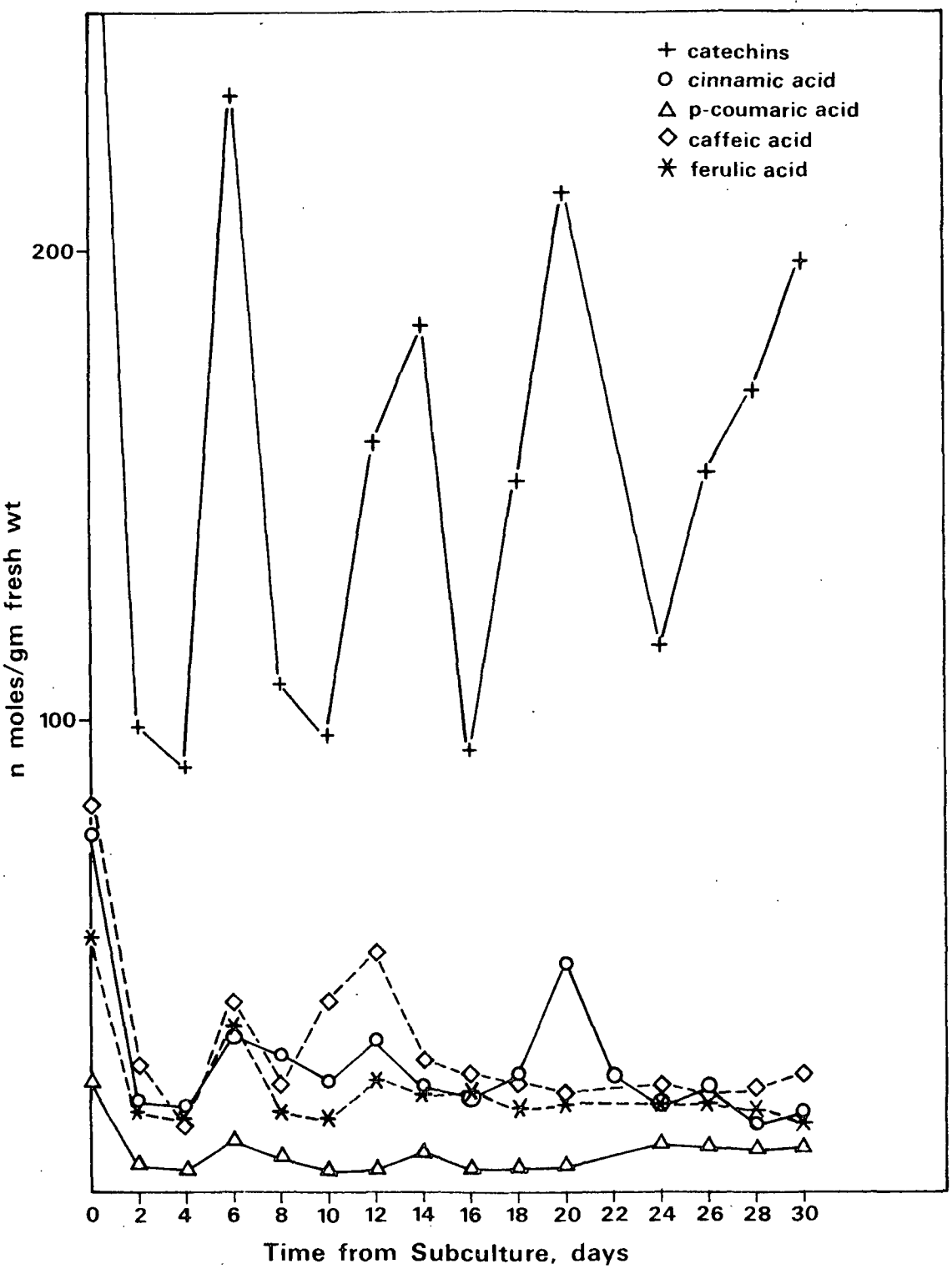


Figure 50. Douglas-fir callus acid hydrolysis liberated cinnamic acids and catechins vs. time.

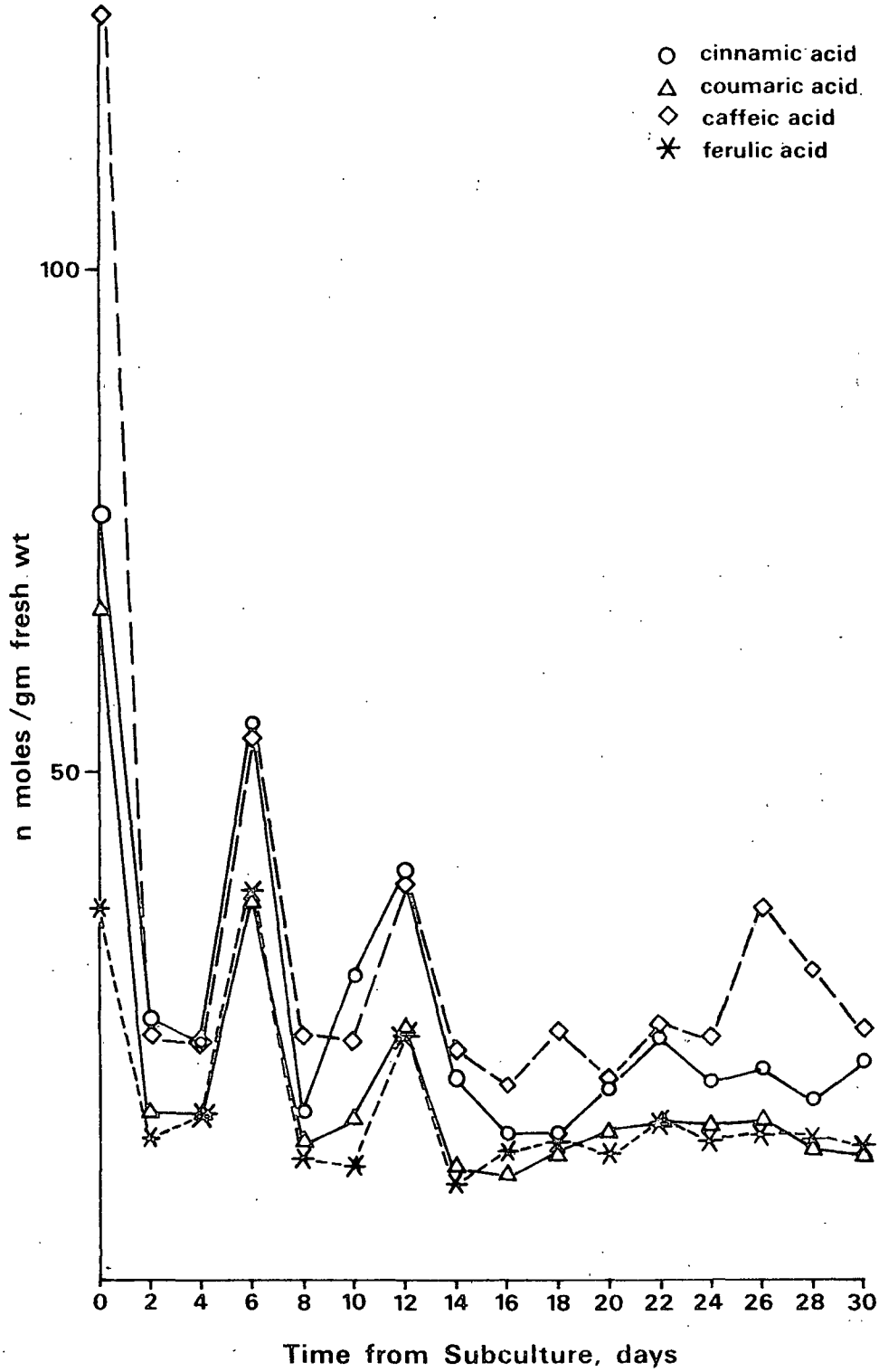


Figure 51. Douglas-fir callus alkaline hydrolysis liberated cinnamic acids and catechins vs. time.

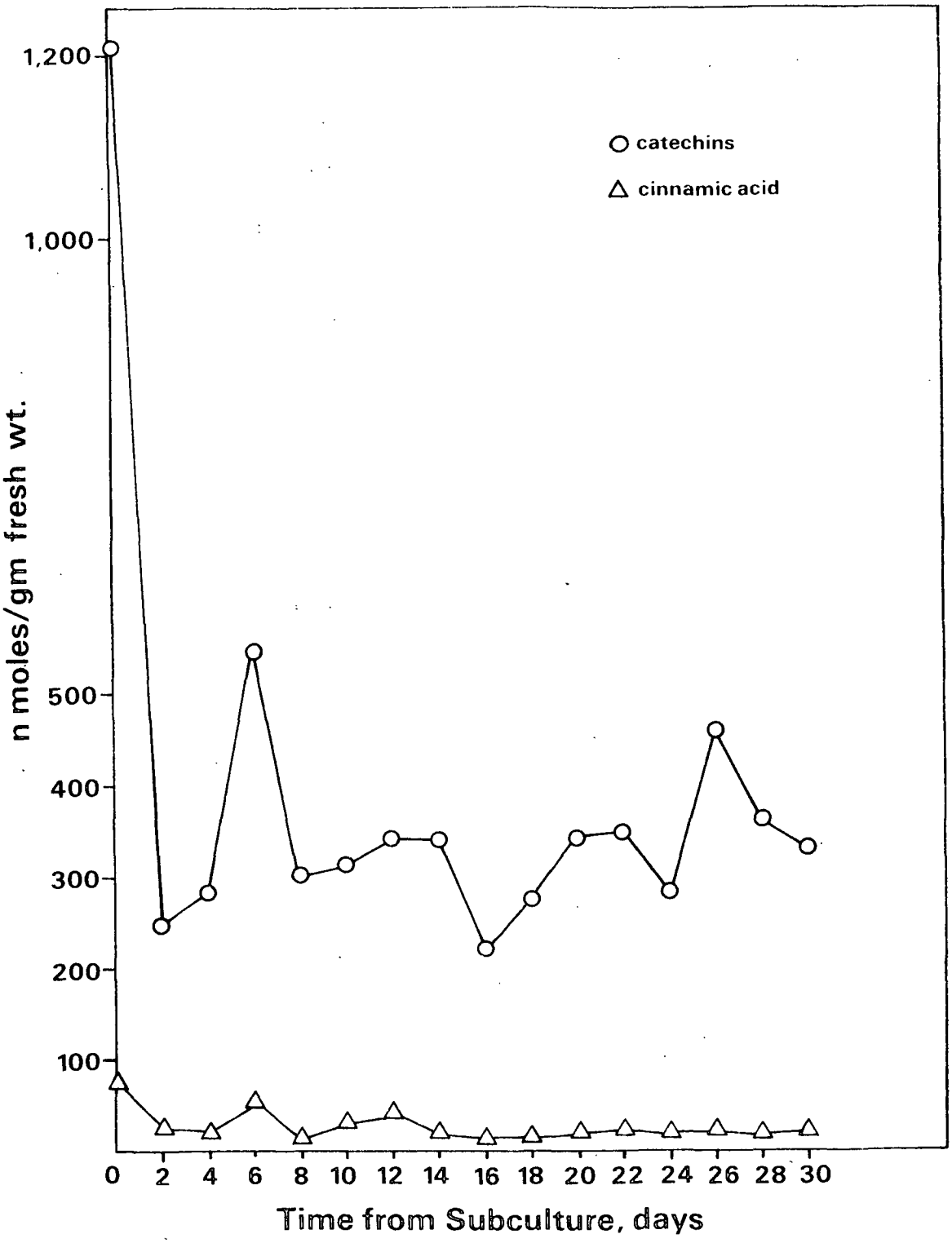


Figure 52. Douglas-fir callus alkaline hydrolysis liberated catechins vs. time.

CONCLUSIONS

The Douglas-fir callus O-methyltransferase is a membrane-bound, cell wall associated enzyme which has an abnormally high K_m value for caffeic acid. This is coupled with a low endogenous caffeic acid concentration, which makes the OMT functionally limiting lignification in this tissue. These factors force the caffeic acid that is formed into the flavonoid pathway, which leads to the observed high buildup of polymeric tannins or proanthocyanidins. Given this sequence of events, it is not feasible to overcome this limiting of lignification by OMT simply by increasing the level of O-methyltransferase in the tissue. Consequently, it is not feasible to shunt the phenolics from the flavonoid pathway to the lignin pathway by this means.

This thesis was begun with the working hypothesis that the O-methyltransferase in Douglas-fir cultured tissue was limiting lignification by not producing enough ferulic acid. The ultimate goal was to increase the levels of OMT present to induce the cinnamic acid precursors to follow the lignin pathway instead of the detrimental flavonoid/tannin pathway. It was found, however, that the OMT is present in these cells in a significant quantity when assayed under optimum conditions. The OMT fluctuates in activity over the subculture period as would be expected. The enzyme required no exogenously supplied cofactors.

The OMT has been shown to be lignin-specific. The data from the specificity experiment demonstrated that the OMT will not significantly methylate the four flavonoids most commonly found in Douglas-fir tissue. In fact, the OMT is very specific toward caffeic acid compared to the compounds tested.

The major difference between this Douglas-fir callus OMT and that from other plants is the fact that this OMT is associated with the cell wall in a membrane-bound state. It was shown that this is a difference peculiar to this cultured

tissue, since the Douglas-fir seedling OMT is a microsomal/soluble enzyme as all reported plant OMTs have been shown to be to date. The difference of the callus OMT locale is not a species difference, then, and so must be due to placing the tissue in culture. Whether this is an actual change in the genetic expression for this enzyme or a selection of the type of cells in the explant is not now known.

The results of the radiotracer study with C^{14} labeled cinnamic acid are not so clear, but it does show that as much catechin is formed as ferulic acid, and more ether-soluble tannin is formed than lignin. These results are at the highest OMT levels in the tissue and at unknown levels of the flavonoid enzymes, and so may be skewed more toward the lignin pathway than is actually the case in this tissue. It is interesting to note that even in the optimum OMT case, the amount of ferulic acid and lignin formed is still less than the amount of flavonoids and tannin formed.

The kinetic determinations are where the limiting nature of the OMT is most clearly shown. In both the homogenate and the purified, uninhibited cases, the K_m value for caffeic acid was found to be very high. The K_m value is 333 $\mu\underline{M}$ in the homogenate, membrane-bound OMT, and 250 $\mu\underline{M}$ in the purified case as compared to 90 $\mu\underline{M}$ for the Douglas-fir seedling. This high K_m value could effectively prevent the formation of ferulic acid by the Douglas-fir cultured tissue if the steady-state concentration of caffeic acid were too low to be effectively used by the OMT.

The kinetic data also indicated substrate inhibition by the SAM and caffeic acid in the purified case and activation by caffeic acid in the homogenate case. The SAM is a noncompetitive inhibitor and the caffeic acid is an uncompetitive inhibitor. S-adenosylhomocysteine is also a noncompetitive inhibitor of the OMT with a K_i of 15.1 $\mu\underline{M}$, which is higher than that generally reported for other plant species.

When the kinetic data are considered together with the actual caffeic acid concentrations in this tissue, it is apparent that the OMT is indeed limiting lignification. The highest caffeic acid concentration reached over the subculture cycle of the callus tissue is still only 1/4 of the K_m value of the enzyme, and more generally the caffeic acid concentration is only 3-5% of the K_m value. This would mean that the enzyme reaction velocity would be less than 5% of the V_{max} value at best, and when the competing flavonoid pathway is considered, the value will be reduced even further.

The measured concentrations of the flavonoid catechin and the tannin show this clearly. The catechin concentration is an order of magnitude greater than that of the caffeic acid. The proanthocyanidin concentration is an order of magnitude greater than the catechin, or 100 times the level of caffeic acid in the tissue. This indicates the magnitude of the phenolic problem and dramatizes the ineffectiveness of the OMT in utilizing the caffeic acid that is present in this tissue.

FUTURE WORK

It does not appear to be possible to redirect the flow of phenolics from the flavonoid-tannin pathway to the lignin pathway by increasing the amount of OMT in the tissue. Because the OMT is limiting lignification, further research of the other enzymes in the lignin pathway beyond OMT would not be fruitful. Likewise, attempts to increase the levels of caffeic acid present in the tissue so that lignification could take place, would be counter-productive to the ultimate goal of limiting flavonoid and tannin formation.

Therefore, the only way to limit the phenolic buildup in these tissues is to block the formation of the phenolics, either at the flavonoid stage or at the initial point of formation of cinnamic acid. While acknowledging that the inorganic compound balance is a critical factor in phenolic metabolism (129), and that there is the possibility of fortuitously finding an optimum medium for the growth and development of somatic embryos without the concurrent buildup of phenolics, it seems clear that, until that time, research should be concentrated on blocking the flavonoid formation either at the beginning of the phenylpropanoid pathway or where the malonyl CoA is added to the C_9 unit. The preferred choice of the two points to block the flavonoid biosynthesis would be at the very beginning of the pathway, at phenylalanine ammonia lyase, because of possible alternate pathways for the formation of the flavonoids that are not now expressed.

Because blue and near red light stimulate the de novo synthesis of phenylalanine ammonia lyase (15,16-21), the effect of light quality on the cultures' growth should be studied. If possible, all light should be eliminated from the growth of these cultures. Natural embryogenesis does not require light, and the artificial reproduction of this process should likewise not require light.

There currently are several compounds reported in the literature that selectively inhibit PAL synthesis. They are O-benzylhydroxylamine (OBHA) (130), hydroxylamine (131), and L-α-aminooxy-β-phenylpropionic acid (132-134). These should be studied as possible blockers of this pathway. OBHA was looked at in the course of this thesis, using loblolly pine suspension cultures, but no PAL inhibition was found. This could, however, bear more research.

The detrimental effects of the phenolic buildup on embryogenesis demand a solution by some means or another. The above suggestions are but an initial attempt to control this presently little understood area. As more is learned about the factors controlling the expression of this pathway, other possible solutions should suggest themselves.

Several other areas of fruitful study come out of this thesis. The first would be a study of why this OMT is membrane bound. The different types of tissue in the original explant could be investigated with regard to the OMT locale. The actual cell source of the callus could be studied along with what nutrient or hormonal changes affect the origin of the callus cells. Allied with this is the genetic component of the OMT, which gene controls the expression of OMT and how is it changed with culturing and over time.

The kinetic results of the inhibited, purified OMT raises some interesting questions about activation and synergism between substrates. The V_{max} increased instead of decreasing as theory predicts. This could use more study, as well.

As is perhaps most typical of scientific endeavors, this thesis has generated more questions than answers, but it has increased our knowledge of plant tissue culture, however slightly. The mass propagation of Douglas-fir trees through tissue culture embryogenesis still proves elusive but by incrementally adding to our

understanding of the differentiation process through such basic biochemical research, our ultimate success is assured. To effect the controlled embryogenesis of conifers, and of all higher plants, it is best that the embryogenic process can be understood.

GLOSSARY AND SPECIAL ABBREVIATIONS

3-CAH. p-Coumaric acid 3-hydroxylase, an enzyme of the general phenylpropanoid pathway yielding caffeic acid from p-coumaric acid

4-CAH. Cinnamic acid 4-hydroxylase, an enzyme of the general phenylpropanoid pathway yielding p-coumaric acid from cinnamic acid

CPM counts per minute, a measure of radioactivity

DPM disintegrations or decays per minute, the calculated degree of radioactivity

2,4-D 2,4-dichlorophenyoxyacetic acid, a synthetic plant auxin, a growth hormone

EDTA ethylene diamine tetraacetate, a chelating agent

kat a katal, a measure of enzyme activity with units of moles product formed/second

K_m The Michaels-Menten constant. This rate constant lacks a precise physical definition because it is a composite of several rate constants, but it has the unique feature in Michaelis-Menten enzyme kinetics that when [S] = K_m, the reaction velocity v = 1/2 V_{max} the maximum reaction velocity

K_i the inhibition rate constant. At this concentration, the inhibitor stops 50% of the reaction

KPi short hand for potassium phosphate buffer

µCi microcurie, a unit of radioactivity roughly equal to 2.2×10^6 cpm

PVP polyvinylpyrrolidone, a phenolic binding polymer

OMT O-methyltransferases, the methylating enzymes in lignin and flavonoid biosyntheses

PAL phenylalanine ammonia lyase, the first enzyme of the general phenylpropanoid pathway yielding cinnamic acid from phenylalanine

SAH S-Adenosylhomocysteine, the reaction product of OMT along with ferulic acid. It is also typically a potent inhibitor of OMT activity

SAM S-Adenosylmethionine. The methylating substrate of OMT, also the precursor of SAH

V_{max} A kinetic constant of enzymes. It is equal to the maximum reaction velocity under optimum conditions, i.e., the maximum reaction rate an enzyme can reach

ACKNOWLEDGMENTS

I would like to acknowledge and express my gratitude to my thesis advisory committee, Drs. J. D. Litvay and W. F. W. Lonsky, and in particular to my thesis advisor, Morris A. Johnson. They were always available with perceptive and cogent advice. It was a privilege to have worked with them.

I am grateful to all of the many others who also contributed in no small measure to the completion of this thesis. To list them here would be an inappropriately lengthy exercise, so I will simply thank them as a group.

I would be remiss, however, if I did not specifically mention my gratitude to John Carlson for his help with electrofocusing and ultracentrifuging my enzyme and for sparing my samples in his periodic purges of the lab. I also thank Shirley Verhagen and Gail Mignon for helping keep my callus cultures growing well.

To The Institute of Paper Chemistry, I extend a special thanks for the educational opportunity provided.

Well Chris, it appears that I am about to become one of those woolly-headed scientific chaps one always reads about. Here's to radical empiricism.

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APPENDIX I

CALLUS GROWTH MEDIUM

Calli were grown on Winton's Medium 11, the composition of which is listed below. This represents the basal medium used to perpetuate Douglas-fir needle callus.

COMPOSITION OF WINTON'S MEDIUM 11

Inorganic Nutrients	ppm	Organic Supplements	ppm
NH_4NO_3	1650	Nicotinic acid	0.5
KNO_3	1900	Pyridoxine • HCl	0.1
$CaCl_2 \cdot 2H_2O$	440	Thiamine • HCl	0.1
$MgSO_4 \cdot 7H_2O$	370	Myo-inositol	100
KH_2PO_4	170	Napthalenoxy acetic acid	5
H_3BO_3	6.2	N^6-Benzylaminopurine	0.1
$MnSO_4 \cdot H_2O$	16.9	Sucrose	30,000
$ZnSO_4 \cdot 7H_2O$	10.6	Agar	4-8000
KI	0.83	pH	5.8 ± 0.2
$NaMoO_4 \cdot 2H_2O$	0.25		
$CuSO_4 \cdot 5H_2O$	0.025		
$CoCl_2 \cdot 6H_2O$	0.025		
Fe EDTA	5.6		

APPENDIX II

SCINTILLATION COCKTAIL QUENCH CURVES

Scintillation cocktail quench curves were made using a known amount of a standard ^{14}C compound, ^{14}C-n-hexadecane. The external standard was run and plotted against the % efficiency of the counting. The sample was quenched sequentially with $FeCl_3$ yielding the following curves for two different cocktails.

These curves were used to determine the efficiency of counting, and where that efficiency was below 80%, the curves were used to calculate the decays or disintegrations per minute (DPM).

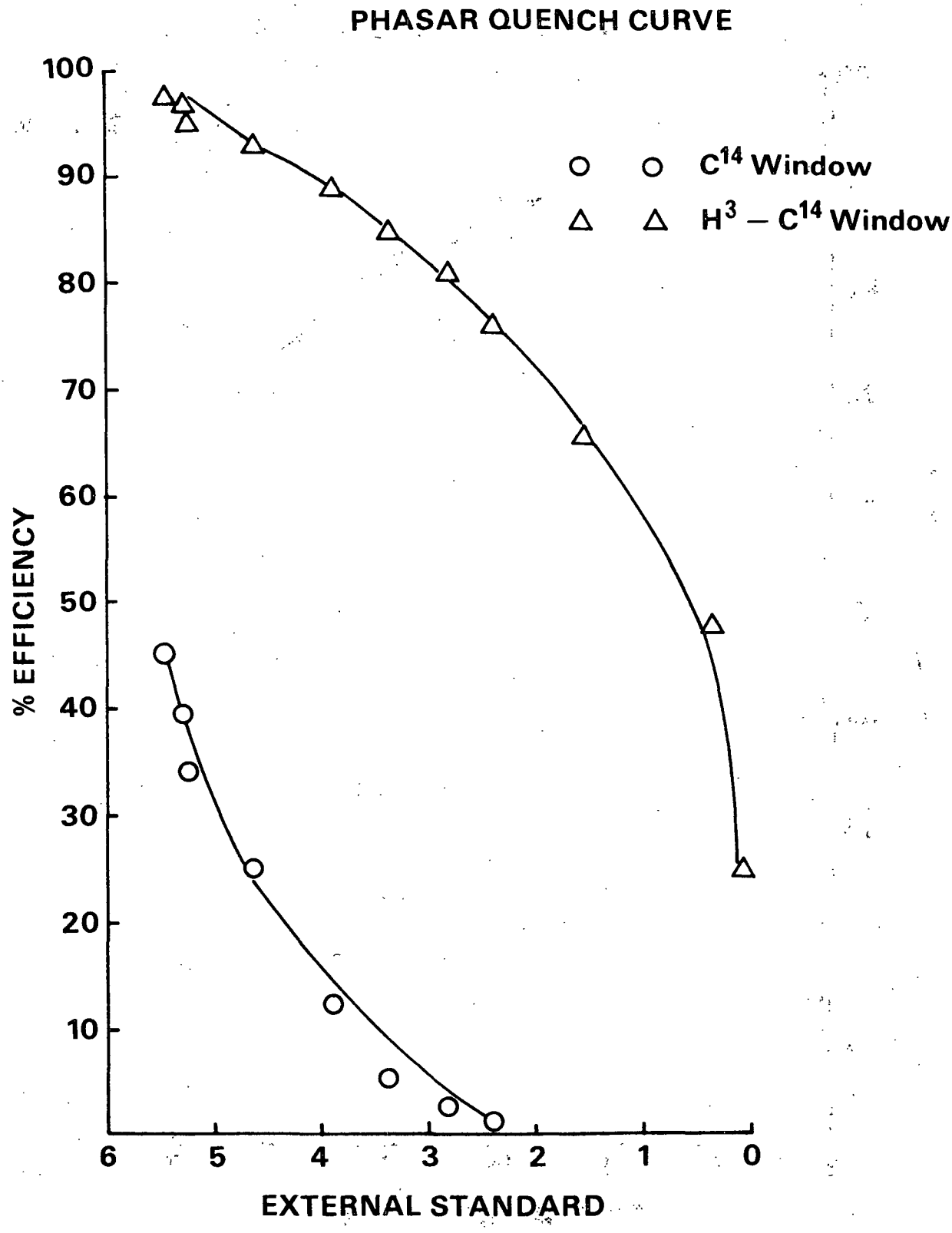

Figure 53. Scintillation cocktail quench curve - phasar cocktail.

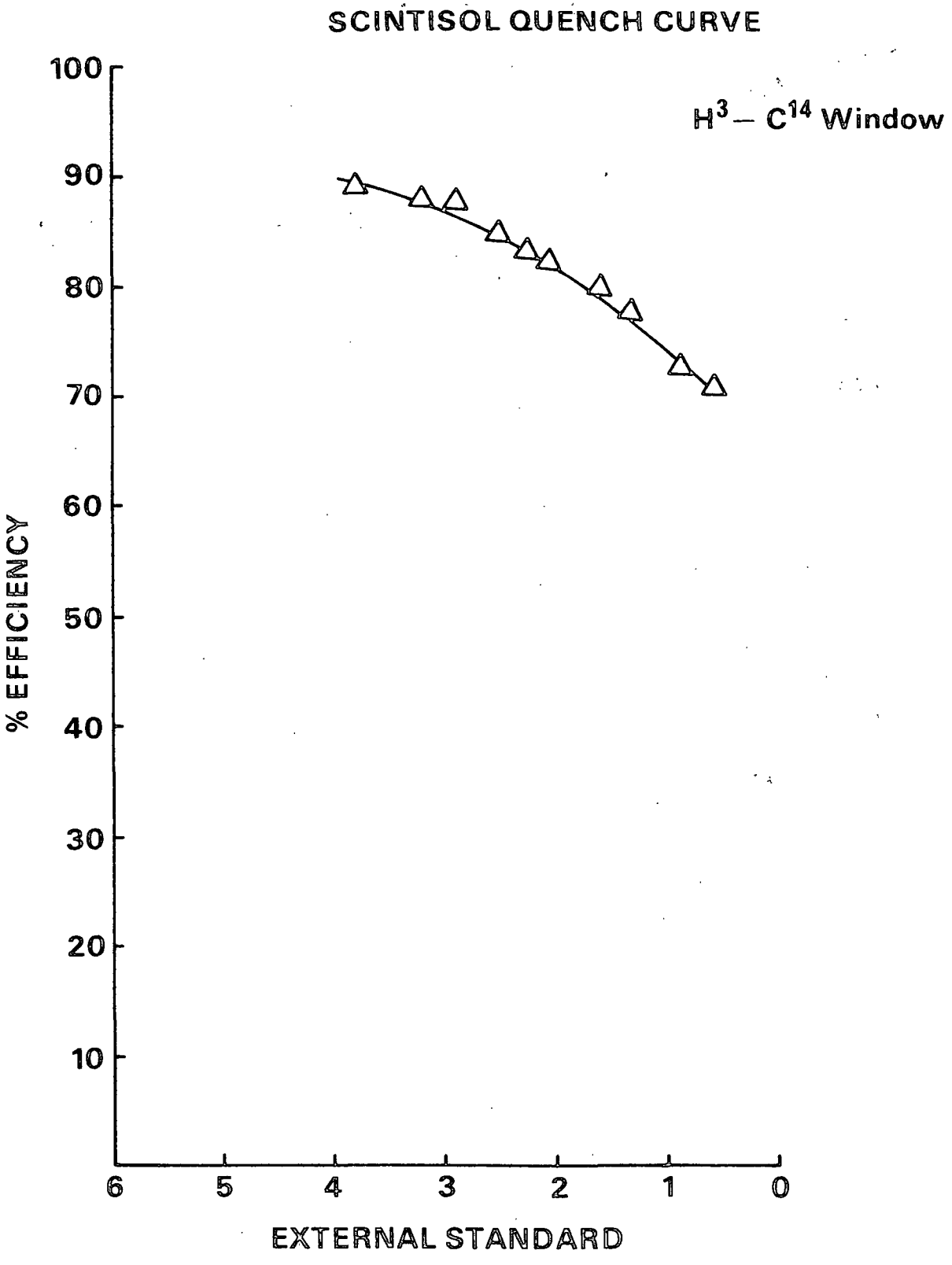

Figure 54. Scintillation cocktail quench curve - scintisol cocktail.

APPENDIX III

PROCYANIDIN STANDARD CURVE

A procyanidin standard curve was made using a procyanidin fraction isolated according to the method of Stafford and Cheng (66). The procyanidin was determined by boiling the sample in 95/5 (v)/v) butanol/conc. HCl for 30 minutes in dim light. The sample was then cooled and the absorbance at 550 nm was read.

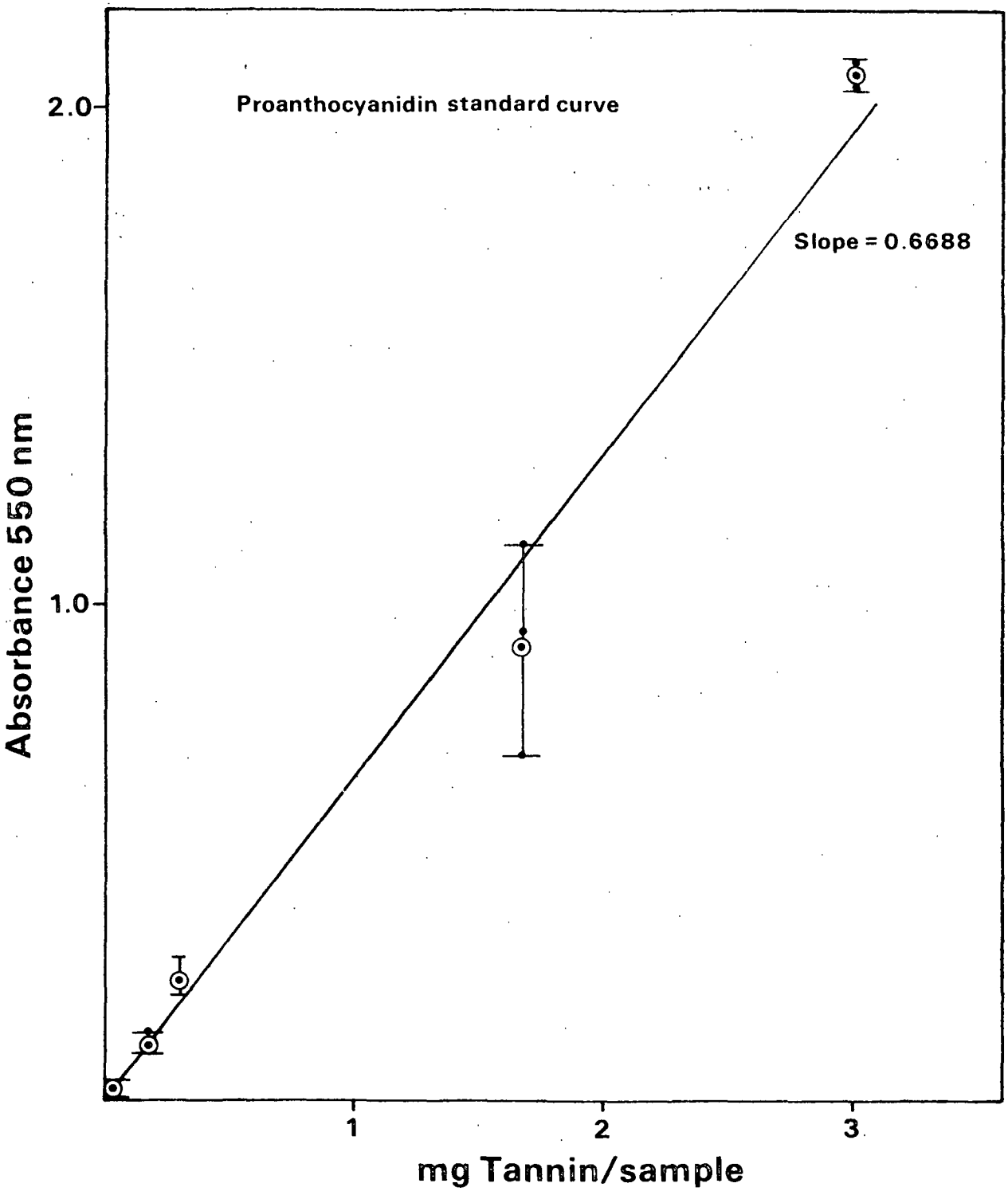

Figure 55. Douglas-fir callus procyanidin standard curve.

APPENDIX IV

PROTEIN DETERMINATIONS

Protein concentrations were determined by the Bradford method. The sample contained 0.3 mL of the protein solution and 2.7 mL of the Bradford reagent. The absorbance at 595 nm was read two minutes after mixing against a reference containing the Bradford reagent only. The protein concentration was determined with a standard calibration curve made with bovine serum albumin.

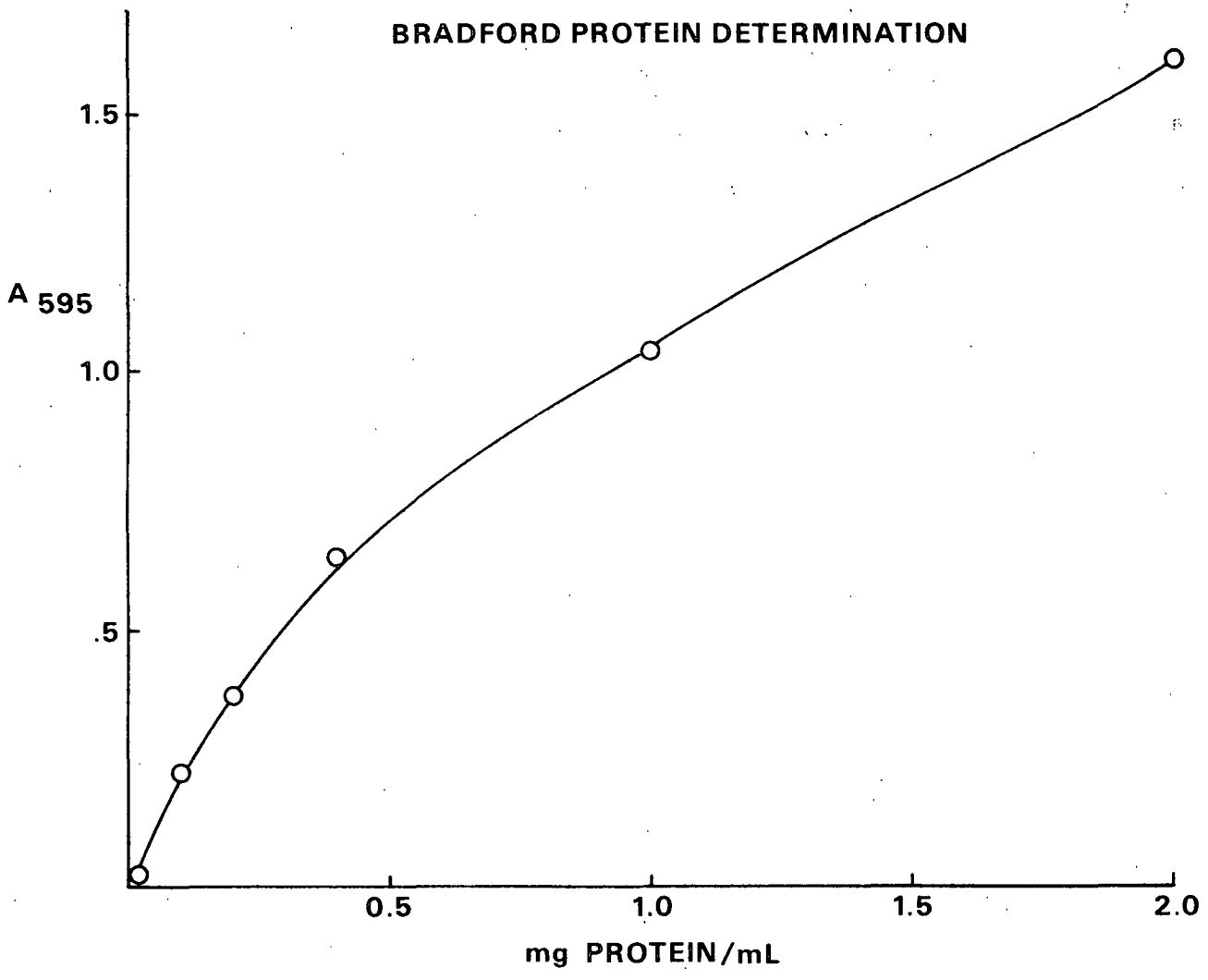

Figure 56. Protein standard curve.