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# MIXED FUNCTION OXIDASES FROM GERMINATING CASTOR BEAN ENDOSPERM

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**Key Word Index**—*Ricinus communis*; Euphorbiaceae; castor bean; endoplasmic reticulum; mixed function oxidase; cinnamic acid 4-hydroxylase; *N*-demethylase.

**Abstract**—Homogenates from germinating castor bean endosperm were fractionated by sucrose density gradient centrifugation and examined for mixed function oxidase activity. Activity of cinnamic acid 4-hydroxylase and *p*-chloro-*N*-methylaniline *N*-demethylase was highest in the endoplasmic reticulum fraction. Activity of both enzymes is dependent on NADPH and on molecular oxygen; both activities are inhibited by carbon monoxide. When challenged with a number of potential inhibitors the enzymes responded in ways fairly typical of mixed function oxidases from other plants and animals. The *N*-demethylase appears to be specific for *N*-methylarylamines. In the absence of NADPH, cumene hydroperoxide is able to support *N*-demethylation. The mechanistic significance of this activity is discussed.

## INTRODUCTION

The technique of sucrose density gradient centrifugation has been used in this laboratory to isolate subcellular fractions from the endosperm of germinating castor beans. Based on a number of criteria a fraction with equilibrium density 1·12 g/ml was identified as membranes of the endoplasmic reticulum [1]. Among these criteria were the presence of cytochromes $P_{450}$ and $b_5$, NADPH-cytochrome *c* reductase (E.C. 1.6.2.3) and antimycin A insensitive NADH-cytochrome *c* reductase (E.C. 1.6.2.1). In liver these cytochromes and reductases are involved in the hydroxylation of endogenous substrates and a variety of foreign compounds [2]. The hydroxylation reactions may be written:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + NADP^+ + H_2O \quad (1)$$

where RH is the substrate. Enzymes catalysing these reactions are called hydroxylases or mixed function oxidases (E.C. 1.99.1).

Mixed function oxidases have been demonstrated in plants. In depth studies include those on cinnamic acid 4-hydroxylase [3,4], hydroxylation reactions of kaurene metabolism [5], geraniol and nerol hydroxylase [6,7], and phenylmethylurea *N*-demethylase [8]; *N*-demethylation may be viewed as a hydroxylation reaction within the context of Eq. 1 [9]:

$$RNHCH_3 + O_2 + NADPH + H^+ \rightarrow RNH_2 + CH_2O + NADP^+ + H_2O \quad (2)$$

The availability of a purified endoplasmic reticulum fraction from castor bean containing components of a hydroxylating system prompted the investigation of a range of compounds as possible substrates of mixed function oxidases. The present paper examines two mixed function oxidases, cinnamic acid 4-hydroxylase (CA4Hase) and *p*-chloro-*N*-methylaniline *N*-demethylase (PCMA *N*-demethylase).

## RESULTS

Preliminary experiments showed that CA4Hase and PCMA *N*-demethylase were present in microsomal suspensions but no aldrin epoxidase or aniline 4-hydroxylase activity was observed. No CA4Hase or demethylase activity was found in the microsomal supernatant.

### *Characteristics of CA4Hase activity*

The characteristics were examined using microsomal suspensions. Activity was dependent on NADPH but NADH gave some activity (Table 1); ascorbate (0·2 mM) was ineffective as a reductant. When microsomal suspensions were preincubated beneath the gas phases $O_2$, air, $N_2$ and CO for 15 min immediately prior to assay in test tubes open to the air, CA4Hase activities obtained were in the ratio 100:100:96:63, respectively. Greater differences were obtained if the gases were applied during assay in serum sealed tubes (Table 2). These results are consistent with an oxygen requirement and the involvement of cytochrome $P_{450}$ in CA4Hase activity [3].

Table 1. Effects of substituents for NADPH on CA4Hase activity

| Nucleotide | Nucleotide-dependent CA4Hase activity (pmol per min/mg of protein) |
|---|---|
| NADPH | 16·2 |
| NADH | 2·0 |
| $NADP^+$ | 2·1 |
| $NAD^+$ | 1·4 |

Nucleotide concentration was 200 μM and incubation time was 30 min.

Table 2. Effects of some gases on CA4Hase activity when applied during incubation

| Gas phase above incubation mixtures | NADPH-dependent CA4Hase activity (pmol per min/mg of protein) |
|---|---|
| $O_2$ | 20·3 |
| Air | 19·3 |
| $N_2$ | 5·0 |
| CO | 2·3 |

The concentration of NADPH was 200 $\mu$M. Gas phases above incubation mixtures contained the various gases for 26 min of the 30 min incubation period; incubation mixtures were open to the air for the remaining 4 min. Gas phases were flushed with the various gases for the first 7 min of the 26 min period.

CA4Hase activity was maximal at pH 7·5 in Tricine–KOH buffer and was proportional to protein concentration up to at least 2·7 mg/ml of incubation mixture; activity was abolished when a microsomal suspension was preheated to 80° for 3 min. With untreated microsomal suspension the rate of hydroxylation was constant for 60 min then declined.

The apparent $K_m$ for cinnamic acid was 1·22 $\mu$M while the response to NADPH concentration was sigmoidal; the inflexion point of this curve was at approximately 26 $\mu$M-NADPH.

*Effects of some reagents on CA4Hase activity*

Inclusion of 2 mM-2-mercaptoethanol in extraction medium and in resuspension medium had no effect on CA4Hase activity; the resultant concentration in assay was 0·8 mM. This insensitivity to 2-mercaptoethanol is in contrast to results obtained with CA4Hase from pea seedlings [3]. However, 2-mercaptoethanol is apparently not necessary for activity of CA4Hases from *Sorghum* seedlings and sweet potato tuber [4,10].

When challenged with a number of potential inhibitors CA4Hase responded in ways fairly typical of mixed function oxidases from other plants and animals [4,8,11]. Thus CA4Hase activity was inhibited by thiol reagents and particularly by the electron acceptors menadione, 1,4-naphthoquinone and benzoquinone (Table 3). Possible sites of action of the inhibitors or reasons for lack of inhibition (Table 3) in mixed function oxidase systems have been previously discussed [4,8,11].

Table 3. Effects of some reagents on CA4Hase activity

| Reagent | Concentration ($\mu$M) | Normalised NADPH-dependent CA4Hase activity |
|---|---|---|
| No addition | | 100 |
| Menadione | 100 | 1·5 |
| 1,4-Naphthoquinone | 100 | 2·3 |
| 8-Hydroxyquinoline | 100 | 10 |
| Antimycin A | 5 | 97 |
| *p*-Chloromercuribenzoate | 100 | 11 |
| *N*-Ethylmaleimide | 1000 | 57 |
| $CuCl_2$ | 100 | 41 |
| $NaN_3$ | 1000 | 76 |
| Quinacrine | 100 | 111 |
| Riboflavin | 100 | 38 |
| Benzoquinone | 100 | 5·1 |

The concentration of NADPH was 200 $\mu$M and incubation time was 30 min. The first four reagents were added in ethanolic solution to incubation mixtures; the final concentration of ethanol was 170 mM. The specific activities obtained with no addition and with the control containing 170 mM-ethanol were 25·0 and 27·9 pmol per min/mg of protein, respectively.

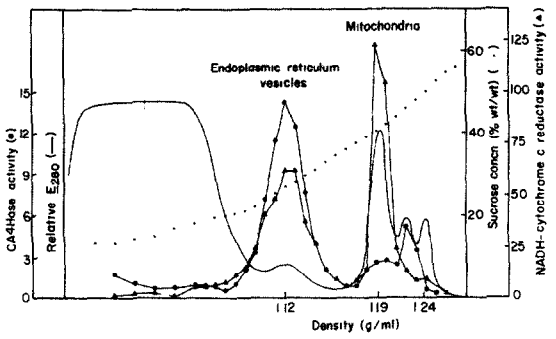


Fig. 1. Localisation of CA4Hase activity after sucrose density gradient centrifugation. CA4Hase activity is in pmole per min/ml of gradient and NADH–cytochrome *c* reductase activity is in nmole per min/ml of gradient.

Piperonyl butoxide, an inhibitor of insect mixed function oxidases [12], and SKF-525A, an inhibitor of liver mixed function oxidases, had no significant effect on CA4Hase activity when tested at 200 $\mu$M.

*Identification of the reaction product*

Identification of the product as 4-hydroxycinnamate was complicated by its behavior on chromatography; it resolved into two or sometimes three spots with $R_f$ values between 0·26 and 0·58. If each of these spots was eluted and rechromatogrammed further spots were resolved, also within the same $R_f$ range. This result suggested that the product consisted of a number of freely interconvertible isomers.

GC–RC analysis of the products eluted from chromatograms revealed that the spots consisted of *cis*- and *trans*-4-hydroxycinnamate [13] in varying amounts. However, GC–RC analysis of an incubation mixture immediately after assay showed that the reaction product was exclusively *trans* isomer. Further, no other radioactive product was found by this method which is able to resolve silylated 2-, 3- and 4-hydroxycinnamates.

*Localisation of CA4Hase activity*

Activity was principally associated with the endoplasmic reticulum fraction [1] (Fig. 1). With gradients constructed so as to resolve the denser fractions more clearly, it was found that the activity at higher densities was associated with the mitochondrial and glyoxysomal fractions as judged by coincidence with the marker enzymes fumarase and catalase, respectively. However, the bulk of the activity was always associated with the endoplasmic reticulum fraction, marked by NADH–cytochrome *c* reductase activity at density 1·12 g/ml (Fig. 1).

*Some characteristics of PCMA N-demethylase activity*

Formaldehyde formation from PCMA was identified by the absorption spectrum of the yellow pigment formed upon addition of Nash reagent and by the method of Chrastil and Wilson [14]. When *p*-chloroaniline was used as substrate the activity was 9% of that obtained with PCMA which suggested that the origin of the formaldehyde was the *N*-methyl group of PCMA. In early experiments the demethylase was assayed by primary amine formation [15]; the results obtained were similar to those in Table 4 and to those obtained in

Table 4. Some cofactor requirements of PCMA *N*-demethylase activity

| Change in complete incubation mixture | $10^3$ $E_{412}$ |
|---|---|
| No change | 512 |
| PCMA deleted | 59 |
| NADPH deleted | 18 |
| $NADP^+$ in place of NADPH | 40 |
| NADH in place of NADPH | 267 |
| $NAD^+$ in place of NADPH | 65 |
| Microsomes heat treated | 67 |
| Microsomes deleted | 51 |

The complete reaction mixture was as described in the experimental section except that the concentration of PCMA in the complete incubation mixture was 2·5 mM. Protein concentration was 4·35 mg/ml. Under these conditions the PCMA-dependent activity was 1·05 nmol per min. Heat treatment of microsomes was at 80° for 2 min.

enzyme localisation experiments (see later). PCMA-dependent activity with NADH was about half of that with NADPH (Table 4).

Demethylase assays were done in Tricine buffer at pH 7·5 although this was not the optimum pH (8) in Tricine. Activity in KPi buffer between pH 6·2 and 7·7 was about 14% of the activity in Tricine buffer between pH 7·5 and 8·6. This result is in contrast to results obtained with an *N*-demethylase from cotton hypocotyl [8] where KPi was an effective buffer and Tricine was inhibitory.

Demethylase activity was stable if microsomal suspensions were quick frozen and stored at −80° while storage at 2° for 2 days resulted in a 73% loss in activity.

*Some kinetic properties and substrate specificity of PCMA N-demethylase activity*

PCMA-dependent activity was constant for 90 min in incubation mixtures containing 4·6 mg of protein per ml. Activity increased with protein concentration up to approximately 3·6 mg per ml of incubation mixture; higher concentrations were slightly inhibitory. The cause of this inhibition is not known. The apparent $K_m$ for NADPH was 217 μM with little or so sigmoidal component, and the apparent $K_m$ for PCMA was 63 μM (Fig. 2).

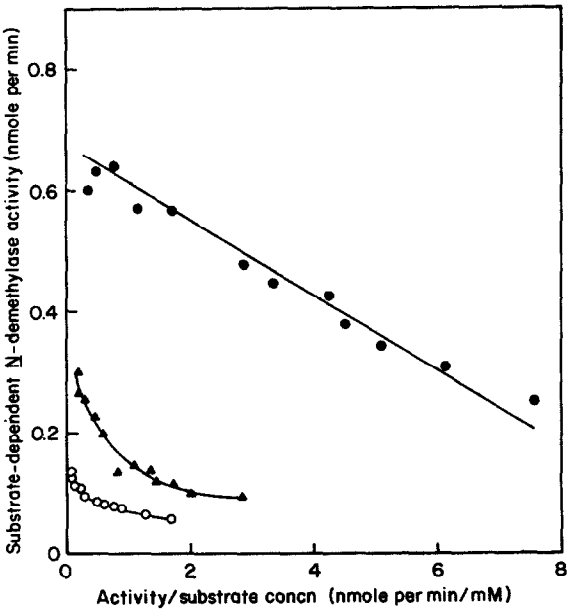


Fig. 2. Eadie–Hofstee plot of the effects of PCMA (●), *N*-methylaniline (▲) and *N*,*N*-dimethylaniline (○) on substrate-dependent *N*-demethylase activity. Protein concentration was 1·86 mg/ml of incubation mixture.

In mammalian systems a wide range of substrates are hydroxylated by liver microsomes. NADPH and $O_2$-dependent *N*-, *S*- and *O*-dealkylations may be viewed as examples of hydroxylation reactions [9]. Equation 2 illustrates *N*-demethylation. Measurement of formaldehyde arising from demethylation provided a convenient method of screening compounds to determine the range of substrate specificity. PCMA *N*-demethylase appears to be specific for *N*-methylarylamines (Fig. 2) since no formaldehyde was detected on testing the following compounds, each of which has at least one *N*, *S*, or *O*-methyl group: hexobarbital, antipyrine, ethylmorphine, 3,4,5-trimethoxybenzoate, 3,4,5-trimethoxycinnamate, ephedrine, 5-methoxytryptamine, 5-methoxy-*N*,*N*-dimethyltryptamine, 3-methoxyphenylacetate, caffeine, aminopyrine, methamphetamine, benzphetamine, 4-methylthiobenzoate, and ricinine, a compound native to castor bean tissues. Also, no demethylation occurred with ricinine when NADH was used in place of NADPH. In all the above tests PCMA-dependent activity was observed.

*Effects of some reagents on PCMA N-demethylase activity*

As shown in Table 5, *N*-demethylase activity responded to potential inhibitors in ways typical of mixed function oxidases (Table 3) [4,8,11]. Piperonyl butoxide and SKF-525A (both 167 μM) had no significant effect on activity. Addition of oxidised cytochrome *c* (18 nmol) caused an approximate 80% loss in activity, considerably more than would be expected on purely stoichiometric grounds since the incubation mixture contained 600 nmol of NADPH and only 62 nmol of formaldehyde was formed in the absence of cytochrome *c*. The significance of this result is unknown. Geraniol hydroxylase from *Vinca rosea* [7] was markedly sensitive to cytochrome *c*.

Phospholipase C (E.C. 3.1.4.3) (13 μg, 17 nkat) inhibited demethylase activity by 60%, while phospholipase A (E.C. 3.1.1.4) (145 μg, 17 nkat) inhibited by 24%. Large amounts of superoxide dismutase (E.C. 1.15.1.1) were required to elicit a response. Inhibition by 51 μg

Table 5. Effects of some reagents on PCMA *N*-demethylase activity

| Reagent | Concentration (μM) | Normalised PCMA-dependent activity |
|---|---|---|
| No addition | | 100 |
| Menadione | 100 | 7 |
| 1,4-Naphthoquinone | 100 | 24 |
| 8-Hydroxyquinoline | 100 | 78 |
| Antimycin A | 5 | 103 |
| *p*-Chloromercuribenzoate | 100 | 34 |
| Quinacrine | 100 | 97 |
| Benzoquinone | 100 | 72 |
| KCN | 100 | 90 |

The first four reagents were added to incubation mixtures in ethanolic solution; the final concentration of ethanol was 140 mM. The activities obtained with no addition and with the control containing 140 mM-ethanol were 1·01 and 0·86 nmole per min, respectively. Protein concentration was 2·03 mg per ml of incubation mixture.

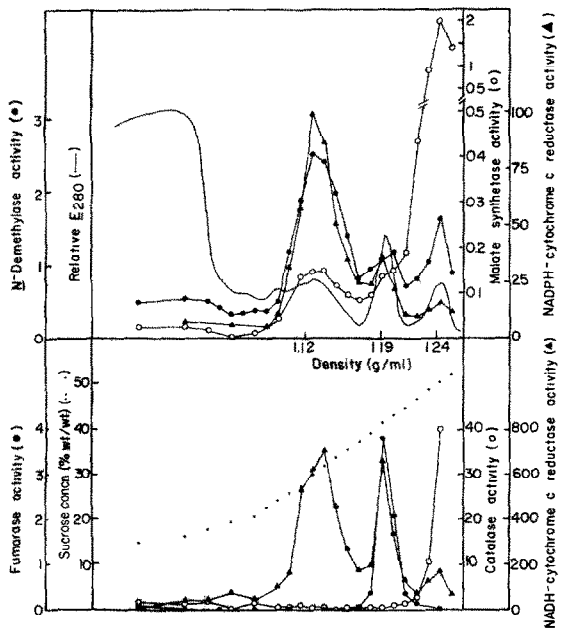


Fig. 3. Localisation of PCMA *N*-demethylase activity after sucrose density gradient centrifugation. All enzyme activities are expressed per ml of gradient; demethylase and cytochrome *c* reductase activities are in nmol per min, malate synthetase activity is in $\mu$mol of CoA per min, catalase activity is in mmol of $O_2$ per min, and fumarase activity is in $\mu$mol of fumarate per min.

(2·5 $\mu$kat) and 102 $\mu$g was 16 and 22%, respectively; bovine serum albumin (200 $\mu$g) did not inhibit activity.

Inclusion of $MgCl_2$, $FeCl_2$ and $FeCl_3$, each 500 $\mu$M in incubation mixtures decreased demethylase activity by 5, 63 and 33%, respectively. The decrease in activity with iron contrasts with the iron requirement of the $O_2$ and NADPH-requiring but CO-insensitive $GA_1$ hydroxylating system of *Phaseolus* seeds [16].

*Localisation of* N-*demethylase activity*

Activity was principally associated with the endoplasmic reticulum fraction [1] (Fig. 3). Similar results were obtained when slightly younger or older tissue was used. However, in all instances, substantial activity was associated with mitochondrial and glyoxysomal fractions.

The distribution of malate synthetase (E.C. 4.1.3.2) (Fig. 3) is consistent with an ontogenetic relationship between endoplasmic reticulum and glyoxysomal membranes [17]. Similarly consistent is the distribution of NAD(P)H-cytochrome *c* reductases and *N*-demethylase. With fractions selected from sucrose gradients it was found that glyoxysomal demethylase activity has the same response to various gases as the endoplasmic reticulum demethylase activity; whether assayed by primary amine formation [15] or formaldehyde formation, activities were in the order $O_2$ > air > $N_2$ > CO. This result is consistent with an oxygen requirement and $P_{450}$ involvement. Accordingly, glyoxysome membranes were examined by differential spectroscopy for the presence of cytochromes [1]; these experiments were done in conjunction with Dr. R. P. Donaldson. Glyoxysome membranes were prepared from sucrose gradient purified glyoxysomes by osmotic shock [18]. Cytochrome $b_5$ was demonstrated but not cytochrome $P_{450}$; a CO-dependent absorption peak could not be demonstrated at 450 nm with dithionite-reduced membranes. The failure to detect $P_{450}$ may be due to the presence of contaminating pigments.

Table 6. Effects of preheating microsomes on various enzyme activities

| Formaldehyde formation supported by | PCMA-dependent activity* (nmole of formaldehyde in 80 min) | |
|---|---|---|
| | With untreated microsomes | With preheated microsomes, 80° for 3·5 min |
| NADPH (1 mM) and $O_2$ | 75·0 | 0 |
| CHP (1 mM) | 95·4 | 58·4 |
| $H_2O_2$ (1 mM) | 0 | 6·5 |
| No addition | 0 | 0 |

NADPH-cytochrome *c* reductase activities were 13·3 and 0 nmol of cytochrome reduced per min/mg of protein for untreated and heat treated microsomes, respectively. The corresponding catalase activities were 0·64 and 0 mmol of $O_2$ per min/mg of protein. Protein concentration in *N*-demethylase incubations was 1·95 mg/ml. *Where activity is shown as zero, the reading obtained without PCMA was slightly higher than that obtained with PCMA.

*PCMA* N-*demethylase activity with peroxides*

In the presence of liver microsomes or purified cytochrome $P_{450}$, peroxides are able to support aromatic hydroxylation or *N*-demethylation of a variety of compounds, in a reaction that does not require reduced pyridine nucleotide or $O_2$ [19–21]. The activity with microsomes was due to cytochrome $P_{450}$.

Cumene hydroperoxide (CHP) supported castor bean microsomal PCMA *N*-demethylation but with reduced activity after microsomes were preheated to 80° for 3·5 min (Table 6). Activity with NADPH was abolished by this pretreatment while activity with $H_2O_2$ occurred only after pretreatment. All activity was abolished by preheating microsomes to 100° for 5·5 min, and no activity was observed if microsomal resuspension medium was used in place of microsomes. Catalase (E.C. 1.11.1.6), a contaminating enzyme, and NADPH-cytochrome *c* reductase were rendered completely inactive by preheating to 80° for 3·5 min (Table 6). Measurement of peroxide concentrations in incubation mixtures containing 6 mM-CHP demonstrated that CHP was not a substrate of castor bean catalase.

The substrate specificity of the CHP activity was tested with PCMA, ephedrine, antipyrine, ethylmorphine and methamphetamine, each 0·83 mM. With both untreated and heat treated (80° for 3·5 min) microsomes, formaldehyde was formed with PCMA only. The same result was obtained with untreated microsomes in the presence of NADPH.

The effects of various gases on the NADPH and CHP activities was examined using untreated and heat treated microsomes (Fig. 4). The activity with NADPH showed the expected $O_2$ stimulation and CO inhibition. The CHP activity was similarly affected by these gases whether it was expressed as PCMA-dependent (Fig. 4), or CHP-dependent.

## DISCUSSION

The demonstration of CA4Hase activity in germinating castor bean endosperm confirms the original finding

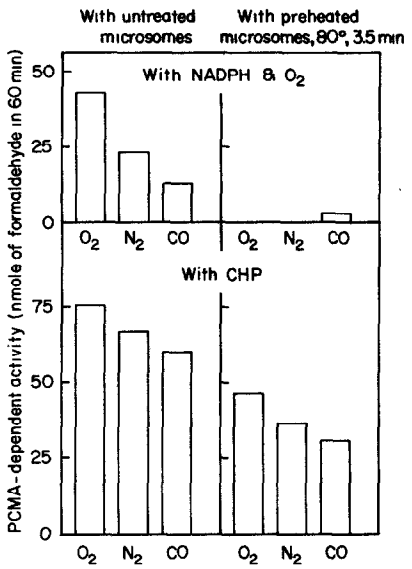


Fig. 4. Effects of some gases on PCMA *N*-demethylase activity supported by NADPH and $O_2$, and CHP. The volume of incubation mixtures was 0·4 ml and the concentration of CHP was 1 mM. Protein concentration was 2·25 mg/ml. Gas phases above incubation mixtures contained the various gases for 57 min of the 60 min incubation period; incubation mixtures were open to the air for the remaining 3 min. Gas phases were flushed with the various gases for the first 5 min of the 57 min period. NADPH–cytochrome *c* reductase activities were 11·7 and 0 nmol of cytochrome reduced per min/mg of protein for untreated and heat treated microsomes, respectively.

of Kindl and Ruis [22]. Localisation of most of the activity in the endoplasmic reticulum fraction, density around 1·12 g/ml (Fig. 1), is consistent with the localisation of CA4Hase from other plant sources in the microsomal fraction obtained by differential centrifugation [3,4,23]. Further, using sucrose density centrifugation, Potts *et al.* [4] demonstrated that CA4Hase from etiolated *Sorghum* seedlings equilibrated with a fraction of density around 1·13 mg/ml; by analogy to the results obtained by Lord *et al.* [1] this fraction, called light membrane fraction, is probably endoplasmic reticulum vesicles. The subcellular localisation of CA4Hase from sweet potato tuber was similarly examined by density gradient centrifugation [10]; the bulk of the activity equilibrated around density 1·13 g/ml.

PCMA *N*-demethylase activity was principally located in the endoplasmic reticulum fraction (Fig. 3). The phenylmethylurea *N*-demethylase activity of cotton hypocotyl was similarly located in the endoplasmic reticulum fraction as determined by differential and density gradient centrifugations [8].

Many authors have demonstrated $P_{450}$-type spectra with extracts from diverse plant sources [1,4,7,24–26]. The substrates for these cytochromes are in many instances not known. However, some instances where the true substrate is known suggest that $P_{450}$-dependent mixed function oxidase activities are widespread if not universal in higher plants. Thus the $P_{450}$-dependent reactions of kaurene metabolism in *Echinocystis* endosperm are involved in gibberellin synthesis *in vivo* [5]; gibberellins are widespread in higher plants. Similarly, CA4Hase is an enzyme central to phenylpropanoid metabolism in plants which leads to compounds such as lignin [27]. However, other modes of hydroxylation in plants have been described which are not CO sensitive and therefore are not dependent on cytochromes of the $P_{450}$-type. Examples of hydroxylation systems where $P_{450}$ is probably not involved include the soluble $GA_1$ hydroxylating system of *Phaseolus* seeds [16], the microsomal system for hydroxylation of oleyl-CoA to ricinoleate in developing castor bean [28] and the soluble 4-hydroxycinnamate 3-hydroxylase activity of beet leaves [29].

Experiments with potential substrates suggest that the specificity of PCMA *N*-demethylase is restricted, although the true substrate is not known. Restricted specificity has been observed with the phenylmethyurea *N*-demethylase of cotton hypocotyl [8] and with *Sorghum* CA4Hase which showed no activity with phenylacetate, benzoate, salicylate or phenylalanine [4]. Senescent leaves of castor plant *N*-demethylate exogenous ricinine [30]. It is clear that PCMA *N*-demethylase is not the enzyme responsible for this activity. The above results indicate that plant mixed function oxidases have a limited range of substrates and may show some tissue specificity. To account for the wide range of substrates attacked by the mixed function oxidase system in liver it appears that a series of cytochromes $P_{450}$ (the substrate binding molecules) exists [31,32].

Resolution and purification of liver mixed function oxidase systems has demonstrated that activity in reconstituted systems is dependent on NADPH-cytochrome $P_{450}$ reductase, phosphatidylcholine and cytochrome $P_{450}$, although cytochrome $b_5$ is required for maximal activity in some systems [2,33]. Exogenously supplied cytochrome *c* or certain other oxidants are able to act as alternate electron acceptors to cytochrome $P_{450}$. Phosphatidylcholine is required for both electron transfer to $P_{450}$ and substrate binding [34].

A considerable amount of evidence suggests that plant mixed function oxidases may be similarly organized. For instance, NADPH–cytochrome *c* reductase activity occurs in cotton hypocotyl microsomes along with phenylmethylurea *N*-demethylase [8] and co-equilibrates in sucrose gradients with PCMA *N*-demethylase (Fig. 3). Büche and Sandermann [23] elegantly showed a phospholipid dependence of parsley CA4Hase, and geraniol hydroxylase [7] and PCMA *N*-demethylase activities were inhibited by phospholipases A and C. Most significant to date however is the resolution and partial purification of geraniol hydroxylase from *Vinca rosea* by Madyastha and Coscia [7] who identified a reductase fraction, a phospholipid fraction, and a cytochrome $P_{450}$ fraction although this fraction also contained cytochrome $b_5$. Maximum activity in a reconstituted system was dependent on the presence of the three fractions.

The hypothesis that plant mixed function oxidases are dependent on reductase, phospholipid and cytochrome $P_{450}$ provides one explanation of the results shown in Table 6 if it is further assumed that the cytochrome $P_{450}$ is more heat stable than the reductase and catalase, a contaminating enzyme. Nordblom *et al.* [21] have shown that the peroxidative activity of purified rabbit liver cytochrome $P_{450}$ is dependent on phospholipid but not on reductase (nor NADPH and $O_2$). Heat treatment at 80° for 3·5 min of castor bean microsomes (Table 6) abolished the NADPH/$O_2$ activity of the demethylase which is consistent with the loss in reductase activity, measured

as NADPH-cytochrome *c* reductase. However, substantial CHP activity was retained which suggests that reductase is not necessary for the peroxidative activity. When catalase activity was abolished by heat treatment $H_2O_2$ was able to react in the same way as CHP.

The experiments with possible alternate substrates indicate that the CHP activity is specific for PCMA in the same way as the NADPH/$O_2$ activity, which suggests that the same molecule, probably cytochrome $P_{450}$, binds the substrate in both instances. The experiment with heated microsomes (80° for 3·5 min) suggests that the specificity for PCMA is independent of the reductase which is inactivated by heat treatment.

Many of the results discussed above collectively demonstrate that plant mixed function oxidases are similar in many respects to those from liver. However, the effects of $O_2$ and CO on the CHP activity with PCMA *N*-demethylase (Fig. 4) are in marked contrast to the results obtained with the peroxidative activity of rabbit liver $P_{450}$ where no stimulation by $O_2$ nor inhibition by CO was observed [21]. No effect by these gases is to be expected since $P_{450}$ is oxidised by peroxides [35], and only the reduced form of $P_{450}$ can bind $O_2$ and CO.

## EXPERIMENTAL

*Preparation of subcellular fractions.* Castor beans (*Ricinus communis* var. Hale) were soaked in running tap water for 12 hr then germinated in darkness in moist vermiculite at 30°. After 4 days the endosperms were removed and homogenized with razor blades to a fine pulp in extracting medium: 13% (wt/wt) sucrose in 150 mM-Tricine–KOH (pH 7·5), 1 mM-EDTA, and in some experiments 10 mM-KCl. The ratio of tissue to grinding medium was around 1·1 g/ml. After filtration through nylon cloth the homogenate was subjected to differential centrifugation for the preparation of microsomes or to sucrose density gradient centrifugation for the preparation of endoplasmic reticulum membranes and other organelles. Linear sucrose gradients were prepared in a manner similar to that described by Lord *et al.* [1] except that in the *N*-demethylase studies Tricine and KCl were omitted from the gradients. The homogenate was usually centrifuged (270 *g*, 10 min) to remove cell debris and 14 ml of the supernatant solution was layered on the gradients contained in 60 ml polyallomer tubes. After centrifugation at 21 000 rpm for 2·5 hr in an SW 25·2 rotor (Beckman) 1·2 ml fractions were collected (ISCO fractionator, model 640).

Differential centrifugation was at 270 *g* for 10 min, then 12000 *g* for 15 min; the pellets and fat were discarded. The supernatant was centrifuged for 1 hr at 42000 rpm in a "65" rotor (Beckman), yielding the microsomal pellet which was resuspended in 30 mM-Tricine–KOH, pH 7·5, 1 mM-EDTA (the microsomal suspension). All procedures were done between 0° and 4°.

*Enzyme and other assays.* Incubation mixtures for cinnamic acid 4-hydroxylase [3] contained between 12 and 45 $\mu$M-[3-$^{14}$C] *trans*-cinnamic acid (Schwartz/Mann, Van Nuys, Calif.), $K^+$ salt, 0·5 $\mu$Ci, 200 or 400 $\mu$M $Na_4$ NADPH, 50 mM-Tricine–KOH, pH 7·5, and enzyme in EDTA and Tricine–KOH, pH 7·5, final vol. 0·25 ml in 13 ml tubes. Incubations at 30° were started by addition of enzyme. After 30 or 40 min without shaking they were stopped with 0·25 ml of 0·33 M-HCl. NADPH was omitted from control incubations. Phenylpropenoic acids were extracted with EtOAc (1·5 ml). EtOAc was removed by evaporation with a stream of $N_2$ and the residue was dissolved in 20 $\mu$l of EtOH, and spotted on Gelman (Ann Arbor, Mich.) ITLC Si gel chromatograms; these were developed on $C_6H_6$–HOAc (199:1). Radioactive areas were located by autoradiography, cut out and counted in a dioxane based scintillant; counting efficiency was constant. Activity is expressed as 4-hydroxycinnamate formation.

The reaction product was identified by marker spots on Si gel chromatograms and by GC–RC [36]. For GC–RC, phenylpropenoic acids were silylated with Sil-Prep (Applied Sciences, Penn.). Radioactive material was collected in chilled capillaries periodically inserted in an exit stream splitter then counted as above. The column (10% (wt/wt) SE-52 on Gas Chrom Q, Applied Sciences) used in these experiments was able to resolve *cis* and *trans* isomers of silylated 4-hydroxycinnamic acid.

*N*-Demethylase incubation mixtures contained 1 mM-$Na_4$NADPH, 90 mM-Tricine–KOH, pH 7·5, 0·83 mM-*p*-chloro-*N*-methylaniline–HCl (Calbiochem, La Jolla, Calif.), and enzyme in EDTA and Tricine–KOH, pH 7·5, final vol. 0·6 ml also in 13 ml tubes. The substrate was omitted from control incubations. Incubations at 30° were started by addition of enzyme. After 80 min without shaking they were stopped by chilling and by the addition of 0·15 ml of satd $Ba(OH)_2$ followed by 0·15 ml of 0·25 M-$ZnSO_4$. The ice cold supernatant was incubated (45 min, 38°) with 0·3 ml of Nash reagent but at a concn fourfold that used by him [37]. The colour due to formaldehyde was measured at 412 nm. Activity is expressed as formaldehyde formation.

*N*-Demethylase activity was also measured by primary amine formation [15] and in one instance formaldehyde was measured by the tryptophan method [14].

The effects of various gases on hydroxylase and demethylase activities during assay were examined by changing the gas phase above incubation mixtures. Flush time was short to minimise evaporation.

Fumarase was assayed by fumarate formation [38], catalase by $H_2O_2$ disappearance [39], and malate synthetase by CoASH formation [40]. NADH and NADPH-cytochrome *c* reductases were assayed by monitoring cytochrome *c* reduction at 550 nm, at pH 9·2 and 7·5 [1], respectively. Activities are expressed as nucleotide-dependent cytochrome *c* reduction.

Protein in hydroxylase assays was measured by Ellman's method [42] and in demethylase assays by Lowry's method. Sucrose concentrations were determined refractometrically. Cumene hydroperoxide (Matheson, Coleman and Bell, Los Angeles, Calif.) and $H_2O_2$ concentrations were monitored during incubation by the ferrithiocyanate method. In these experiments KPi buffer 30 mM, pH 7·5 was used in place of Tricine–KOH, pH 7·5. Aniline 4-hydroxylase activity was measured colorimetrically [43] and aldrin epoxidase was assayed in the manner of Krieger and Wilkinson [44]; dieldrin was measured by Dr. J. Hill, Davis, Calif.

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