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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

Wolfgang H. Baur

ENTITLED Regulation of trans-Cinnamic Acid Hydroxylase and

Cytochrome $B_5$ In Pisum Sativum

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

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# REGULATION OF TRANS-CINNAMIC ACID HYDROXYLASE AND CYTOCHROME $B_5$ IN *PISUM SATIVUM*

BY

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THESIS

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TABLE OF CONTENTS



Abbreviations used: trans-cinnamic acid (t-CA), trans-cinnamic acid hydroxylase (t-CAH), p-coumaric acid (p-CA), kilodaltons (kD), N, N'-bisacrylcystamine (BAC)

# INTRODUCTION

Cytochrome P-450 monoxygenases are vital components in the metabolic pathways of prokaryotes and lower eukaryotes as well as all higher organisms. They are protoporphyrin heme-dependent mixed function proteins which use pyridine nucleotide reducing equivalents to reduce and cleave dioxygen to produce functionalized organic substrates and water in a wide variety of reactions including intermediary and peripheral metabolism as well as the detoxification of xenobiotics. They are also responsible for converting some harmless substances into active carcinogens. In mammalian cells (and presumably others), the reactions are of two types: the mitochondrial type enzymes carry out highly stereo- and regio-specific hydroxylations vital for steriod biosynthesis, and the microsomal type enzymes which display much broader substrate specificities that are necessary for detoxification and general metabolism. Their role in hormone biosynthesis in both plants and animals gives them a prominent place in regulation of growth and development. By any standard, the cytochrome P-450 proteins are a critical group of enzymes involved in processes central to eukaryotic organisms.

In plants, however, the cytochrome P-450s are not nearly as well characterized as the Pseudomonas putida camphor-5-exo-hydroxylase ($P\text{-}450_{cam}$) or the LM2 phenobarbitol-induced hepatic cytochrome P-450. The most prominent P-450 monoxygenase

activity detectable in plants appears to be that of trans-cinnamic acid hydroxylase. This enzyme catalyzes the conversion of trans-cinnamic acid to p-coumaric acid. Like all of the P-450 activities characterized in plants so far, it requires $O_2$ and NADPH and is localized in the endoplasmic reticulum. The reaction of t-CA to form p-CA forms a vital link in the production of flavonoids and lignins in the pathway for phenylpropanoid biosynthesis (Figure 1). The t-CAH enzyme comprises approximately forty percent of the P-450 found in artichoke tuber tissue (1) but a somewhat smaller fraction of the total P-450 in wounded pea tissue (2).

Because of the varying end products of the different branches of the phenylpropanoid pathways, a variety of modulators such as red light, fungal elicitors, and mechanical wounding regulate the flow of the substrates through the pathway. The enzyme may also be induced by agents such as 2,4-D, manganese, phenobarbitol, various herbicides (3, 4, 5). Enzyme induction also occurs in response to wounding (4), ageing (6) and light (7).

Although the agents and degree of induction of the plant P-450 enzymes are well known, the mechanisms for regulating expression of these enzymes have not been elucidated. Induction of the t-CAH enzyme potentially occurs by synthesis of the heme protein itself or by synthesis of its electron transfer partners, NADPH-cytochrone P-450 reductase, cytochrome $b_5$, cytochrome $b_5$ reductase. In fact, the number of distinct plant cytochrome P-

450 isozymes or the number of genes corresponding to any of these isozymes are unknown. Trans-cinnamic acid hydroxylase may well be encoded by a multigene subset of the P-450 family, opening up the possibility that some t-CAH genes in the genome may be induced by different environmental signals. Because regulation of P-450 monoxygenase activity may occur via regulation of its electron transfer partners, studies evaluating P-450 monoxygenases must examine the levels of these important electron transfer components. It is even unclear how many forms of cytochrome $b_5$, cytochrome $b_5$ reductase, or P-450 reductase occur in plant microsomes. To unravel these metabolic and regulatory questions our first step has been aimed at the characterization and cloning of the gene encoding cytochrome $b_5$ in Pisum sativum.

## METHODS

CsCl Plasmid DNA Purification. One liter YT broth (5 g yeast extract, 8 g bactotryptone, 5 g NaCl per liter) containing 200 µg/ml ampicillin was inoculated with 0.5 ml stationary cells stored in 50 % glycerol. These cells were grown for 20-24 hours at 37 °C with continuous shaking.

The cells were harvested at 7,000 rpm in GSA rotor (5000 x g) and resuspended in 6 ml Tris·sucrose buffer (50 mM Tris·HCl (pH 8.0), 25% sucrose). The cell suspension was transferred to high speed ultracentrifuge tubes and 3.75 ml 0.20 M EDTA (pH 7.5) and

0.8 ml 20 mg/ml fresh lysozyme were added before the solution was incubated on ice for 30 minutes. 12.5 ml of Triton lysis buffer (1% Triton, 50 mM Tris·HCl (pH 8.0), 60 mM EDTA) were added, and the solution was incubated on ice for 30 minutes. It was centrifuged for 45 minutes at 35,000 rpm in a T865 rotor (124,500 x g) at 5 °C; the supernatant was collected and the volume adjusted to 25 ml with Tris·sucrose buffer. The supernatant was divided into two 30 ml Corex tubes and 12.25 g CsCl and 0.5 ml 10 mg/ml ethidium bromide were added to each tube. They were incubated at room temperature for 30 minutes and centrifuged for 10 minutes at 10,000 rpm in an HB-4 rotor (10,500 x g). The liquid was poured into high speed polycarbonate tubes and centrifuged for 20-24 hrs at 50,000 rpm in a T865 rotor (180,700 x g) at 15 °C. If rebanding the DNA was desired, the plasmid band was pipetted into small high speed polycarbonate tube and recentrifuged for 20-24 hours at 50,000 rpm in a T865.1 rotor (180,700 x g) at 15 °C without adjustment of the CsCl concentration. The plasmid band was removed from the gradient, put into a 30 ml Corex tube, and extracted three times with an equal volume of isopropanol:water (9:1). The phases were separated by centrifuging for 1-2 minutes in a clinical centrifuge at full speed or for 1-2 minutes in a HB-4 rotor at 10,000 rpm (10500 x g). After the third extraction, the interphase and the top layer containing isopropanol were discarded. If the alcohol layer was still pink at the end of

three extractions, the isopropanol extractions were repeated until it became clear.

The aqueous phase was transferred to another sterile Corex tube and diluted it with 2 volumes with TE buffer (10 mM Tris·HCl (pH 8.0), 1 mM EDTA). 1/10 volume 2 M NaCl and 2 volumes of 95 % ethanol were added to the aqueous solution, which was then stored at -20 °C overnight to precipitate the DNA. The DNA was collected by centrifuging for 15 minutes at 10,000 rpm in an HB-4 rotor (10,500 x g) at 4 °C.

The DNA was resuspended in 1 ml sterile water and 100 µl 1 M Tris·HCl (pH 8.0) and extracted with 1 ml phenol:chloroform (1:1). The aqueous top layer was removed to a new tube where the DNA was precipitated by adding 1/10 volume 2 M NaCl and 2 volumes 95 % ethanol. This solution was held on wet ice for 30 minutes or overnight at -20 °C. The DNA was pelleted by centrifuging at 10,000 rpm in an HB-4 rotor (10,500 x g) for 10 minutes.

The DNA was reprecipitated by resuspending the pellet in 450 µl sterile water, adding 50 µl 2 M NaCl and 1 ml ethanol and precipitating on wet ice for 30 minutes or at -20 °C for 1-2 hours. It was centrifuged for 10 minutes and reprecipitated two more times. It was dried in a vacuum dessicator, dissolved in 400-1000 µl sterile water or TE buffer, and stored at -20 °C.

Restriction Cuts. Our reactions were set up in Eppendorf tubes as described in the New England Biolabs Catalogue using 25 µg of

miniprep plasmid DNA per 50 µl reaction. The reactions were incubated at 37 °C for two hours after treatment with 1 µg RNAase per 50 µl of reaction for 10 minutes to remove RNA from the low molecular weight range. 1/5 volume 10 M urea loading dye was added and 20-25 µl were loaded on a 1.4 % agarose gel with 5 µl lambda Hind III standard and run for 4 hours at 50 mA when the bromphenol blue dye front reached the bottom. The gel was stained with ethidium bromide and photographed.

Hybridization. Nitrocellulose filters and test strips were placed inside a seal-a-meal bag; sealed; and the appropriate amount of hybridization solution (5 x SSC [0.15 M NaCl, 0.015 M sodium citrate], 1 x Denhardt's solution, 0.2 % lauryl sarcosine) and formamide added. The filters were prehybridized for 1 hour at 25 °C. Throughout our studies, we used the following three sets of hybridization conditions:

| | | |
|---|---|---|
| low stringency | 30 % formamide | 25 °C |
| medium stringency | 30 % formamide | 35 °C |
| high stringency | 40 % formamide | 42 °C |

For our hybridizations we added hybridization solution (5X SSC, 1X Denhardt's solution, 0.2 % lauryl sarcosine) and 1 to 2 x $10^5$ cpm denatured probe per filter. The bag was sealed and hybridization allowed to occur at the appropriate temperature for 20-24 hours. The filter was washed with four changes of 2 x SSC, 0.5 % sarkosyl at 30 °C to 40 °C, depending on the original hybridization temperature. The filters were dried and taped into the cassette, then marked with $^{32}P$ ink to help align

the filters and film after development. The filters and paper were covered with plastic wrap and exposed for 2-7 days at -70 °C.

Nick Translation. These reactions were set up in an Eppendorf tube according to the method of Schuler and Zielinski (8). 2 μg BAC-acrylamide purified DNA were incubated for 1 hour at 14 °C. The reaction was stopped with 20 μl nick translation stop buffer(500 mM TRIS (pH 7.8), 50 mM $MgCl_2$, 1 mg/ml BSA, 10 μM ß-mercaptoethanol). Unincorporated nucleotides were separated out by passing the nick translation reaction over a 5 ml column of Sephadex G-100 and eluting with TE buffer. Nick translated DNA was in the blue dextran peak. 1 μl of each fraction was counted to determine the level of incorporation obtained.

Probe Construction. $A_{260}$ values were measured and used to calculate concentrations of DNA obtained by CsCl preparation of the E. Coli strain containing the soluble cytochrome $b_5$ gene. 50 μg purified plasmid DNA were cut with Pst I in the following reaction:

20 μl 10x Pst I Buffer
60 μl plasmid DNA
110 μl sterile water
10 μl 10 units/μl Pst I (BRL)

This reaction was incubated for six hours in a 37 °C water bath. The buffer was adjusted with 20 μl 1 M TRIS (pH 7.4) to suit the next restriction enzyme, Eco RI. 10 μl 10 units/μl Eco RI

restriction enzyme were added, and the reaction was incubated for six hours at 37 °C.

The extent of the restriction digestion was checked by running the DNA and a lambda Hind III standard on a 1.6% agarose minigel. When the restriction digestion was complete, the entire reaction was stopped by adding one-fifth volume (46 µl) of 10 M urea loading dye.

To purify the desired 340 bp fragment on a 5% BAC-acrylamide gel, the following gel was poured:

12.5 ml 20 % acrylamide:0.5 % BAC-acrylamide stock
5.0 ml sterile 1X TBE buffer
32.5 ml sterile water
320 µl TEMED
800 µl 10% ammonium persulfate

The preparative restriction digestion was electrophoresed at 40 V for 4 hours, stained with 1 % methylene blue, and the desired restriction fragments were cut out. To extract the DNA, 2.5 ml TE buffer and 25 µl ß-mercaptoethanol were added.

A 100 ml DE-52 column was poured in a Pasteur pipette, washed with TE buffer, and the sample was bound to the column by passing the solution over the column three times. The column was washed with two volumes TE buffer to remove monomeric acrylamide and other impurities.

The DNA was eluted from the column using eight 200 µl 2 M NaCl washes per fraction. Four 400 µl fractions were collected in Eppendorf tubes, and 800 µl ethanol were added per fraction. The DNA was precipitated on dry ice for 30 minutes or at -20 °C freezer overnight and spun in Eppendorf centrifuge for 10

minutes. Two more reprecipitations using the same conditions were done to ensure purity.

3 $\mu$l of each fraction were electrophoresed on a 1.6% agarose minigel to evaluate the amount of DNA in each fraction. The $A_{260}$ was measured and used to calculate the DNA concentration.

Western Blot Procedure. The SDS acrylamide gels were run according to the method described in Laemmli (9). The Western blot analysis (10) required equilibrating the gels for 20 minutes in 8-10 volumes of transfer buffer (20% methanol (v/v), 25 mM Tris base, 192 mM glycine). The transfer sandwich was assembled at 4 °C in a glass dish. The 3 mm paper and nitrocellulose were prewet to reduce bubbles. Bubbles created were smoothed out, and the sandwich was removed from the dish and placed in an electrophoresis chamber pre-filled with 4 °C transfer buffer. The 15 % acrylamide gels were transferred for a minimum of 8 hours at 100 V or at 50 V overnight at 4 °C.

The nitrocellulose was removed and the face which abutted the gel was marked to keep that side up. 200 ml of 1X TBST (10 mM Tris-HCl (pH 8.0), 15 mM NaCl, 0.05% Tween 20) containing 3 % gelatin dissolved by heating were prepared. The filter was covered with the cooled TBST and gelatin solution for 30 minutes and rinsed with several changes of TBST.

The filter was incubated with the primary antibody for 30 minutes at room temperature in 100 ml of 1X TBST containing 0.5 % gelatin. The antibody dilution must be determined empirically

for each new batch of serum. In most cases we used a 1:2000 dilution of the polyclonal anti-cytochrome $b_5$ antiserum. The filter was washed once for 15 minutes in 250 ml 1X TBST, then twice for 10 minutes in 250 ml of 1X TBST without gelatin.

The Western was crossreacted with goat anti-rabbit alkaline phosphatase conjugate (Promega Biotech) for thirty minutes at 1:10,000 dilution in 1 X TBST containing 0.5 % gelatin. The filter was washed three times for 20 minutes in 250 ml of 1 X TBST without gelatin.

The solution was removed from the dish and 10 ml of alkaline phosphatase buffer (100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$), 35 $\mu$l BCIP reagent (1.5% BCIP powder in dimethylformamide) and 70 $\mu$l NBT reagent (3% NBT powder in 70% dimethylformamide) were added for a one hundred fifty square centimeter filter. The color development was stopped by adding 10 mM EDTA. The filter was blotted dry on a paper towel and stored in the dark, as exposure to light increases the background color.

## RESULTS AND DISCUSSION

We considered various approaches to understanding the regulation of the trans-cinnamic acid hydroxylase (t-CAH) gene. Since t-CAH requires cytochrome $b_5$ for electron transfer when carrying out its monoxygenation reactions, our attention has focused on understanding the regulation of cytochrome $b_5$ at the

protein level. In addition, we have focused on determining the number of cytochrome $b_5$ genes present in the pea genome.

A rat liver cytochrome $b_5$ gene (11) was used as a probe to detect plant cytochrome $b_5$ genes in the pea genome. This synthetic gene, constructed using E. coli codon frequencies, codes for the mammalian cytochrome $b_5$ present in rat hepatic tissue. This gene has been used as a probe because cytochrome $b_5$ is very well conserved in eukaryotes and homologous to the desired pea cytochrome $b_5$ (12; Table 1.). This paper investigates the level of cytochrome $b_5$ homology in mammalian and plant systems.

The cytochrome $b_5$ in rat hepatic microsomal membranes has a soluble core domain of 13.5 kD and a complete size of 18.1 kD (12) Calf liver cytochrome $b_5$ has 135 residues and a molecular weight of 16 kD (14). Western analysis using antibody directed against the mammalian cytochrome $b_5$ has indicated that the pea cytochrome $b_5$ has a molecular weight of about 16.4 kD (13). Indeed, pea cytochrome $b_5$ and pea cytochrome $b_5$ reductase can transfer electrons to the t-CAH cytochrome P-450 (13), so the difference between plant and mammal can be seen to be minimal at the functional level (13).

To ascertain the level of protein homology, several of the cytochrome $b_5$ amino acid sequences available in the literature were compared. The sequences used in the comparison include rat, pig, ox, human, and chicken. This comparison shows that the level of protein homology between any two of the sequences

used ranges from 98.4 % to 82.9%. When all five sequences are compared, 51.3 % of the positions are completely conserved at the amino acid level (see Table 1 and Figure 6).

To determine whether the synthetic cytochrome $b_5$ gene could be used to detect plant cytochrome $b_5$ genes, we have calculated the expected degree of nucleotide homology. In this analysis, codons used in the rat hepatic cytochrome $b_5$ were compared with the average codon frequencies in the genes for a Glycine max storage protein (15), a Medicago sativa glutamine synthase (16), the Arabidopsis thaliana alcohol dehydrogenase and ribulose bis-phosphate carboxylase activase (17, 18), and Phaseolus vulgaris storage protein (14; Table 2). When these average plant codon frequencies are compared with the codons used in the mammalian cytochrome $b_5$ sequence (11) used as a probe, homology is found to exist with the preferred, frequently used plant codons at 220 of 291 nucleotides, or 75.6 %. For comparison, in the worst case scenario where degeneracy in the code allows for maximum mismatch, we find that 178 of 291 or 61.2 % nucleotides can be expected to match.

Using this estimate of 38.8 % mismatch the minimum Tm was calculated assuming the presence of identical but degenerate codons. The base $T_m$ of 84.9 °C in 1 X SSC is decreased by 1.4 °C (500/340 bp) to compensate for the short length of the probe. The mismatch adjustment of 38.8 % X 0.7 °C gives a final Tm of 59.1 °C. Assuming that maximum hybridization occurs 15 °C below

the $T_m$, the optimal hybridization should occur at 44.1 °C. Our experimental hybridization temperature was 40 °C.

The results of the genomic Southern analysis (Figure 2) show distinct hybridization with two of the three genomic DNAs. Several bands in both the barley and pea libraries show that these libraries contain the cytochrome $b_5$ gene in single copy abundance. The bands' shifts in position reflect the differing successful cuts by the Eco RI, Bam HI and Hind III restriction enzymes. Since none of the bands is darker than the others we can assume that the copies of the gene are scattered throughout the chromosomes in different locations. The tomato genome must also contain a gene similar to that in barley and pea, yet the probe has not hybridized to such a site. It is unlikely that this particular genome is missing a cytochrome $b_5$ gene, which suggests that under the hybridization conditions used the tomato gene is not homologous enough to crosshybridize.

Because the size of the pea cytochrome $b_5$ protein is nearly identical to mammalian cytochrome $b_5$ we suspect that this protein is highly conserved. Such a conserved pea cytochrome $b_5$ was expected to exhibit crossreactivity with antibodies prepared against purified mammalian cytochrome $b_5$. Under relatively stringent crossreaction conditions (1:2000 dilution), plant cytochrome $b_5$ crossreacts well with the mammalian cytochrome $b_5$ antibody (Figure 3). This indicates that substantial structural similarity as well as sequence similarity exists between the mammalian and plant enzymes.

In order to determine if the levels of cytochrome $b_5$ protein are induced in response to stimuli which induce microsomal P-450s, I have analyzed the levels of cytochrome $b_5$ after wounding. In this analysis, microsomal proteins were prepared from seedling tissues at 0 to 10 hours after wounding. In this time course, t-CAH activity is typically induced at 6 hours. The Western blots of microsomal proteins probed with antiserum directed against purified rat hepatic cytochrome $b_5$ demonstrated the pea cytochrome $b_5$ has an apparent molecular weight of 16,500 D (Figure 3). The standard has an apparent molecular weight of 16,000 D. It might be expected that a microsomal preparation such as the one used here would give a value closer to the 18.1 kD of the mammalian protein, but the pea protein more closely approximates the 16 kD soluble mammalian core protein. Given the easily cleaved nature of this hydrophobic tail region this result is not unusual. In fact, a faint band of approximately 17.9 kD apparent molecular weight can be seen on the Western blot. In general, however, it must be noted that SDS-PAGE gel determinations of membrane proteins' molecular weights is more error-prone due to their inherently greater hydrophobicity and the resulting potential for partial folding affecting how these proteins run, and thus the molecular weights obtained must be considered apparent molecular weights. In addition, some membrane proteins bind non-standard amounts of SDS.

The purified rat hepatic cytochrome $b_5$ standard was prepared from *E. coli* as described in von Bodman *et al*. (11). In this

cytochrome $b_5$ preparation an extra band is seen in the Coomassie blue stained gels (Fig. 4) and in Western blots (Fig. 3). This additional protein is not due to the presence of the heme prosthetic group because its molecular weight is only 600 D. It is possible that this protein represents a cytochrome $b_5$ dimer formed during the purification. It is more likely that this extra band is a copurified contaminant. Since the standard was purified by the same method used to purify protein for the antibody preparation, some non-specific cross-reactivity may be expected.

The Western blot suggests an increase in amount of pea cytochrome $b_5$ (Fig. 3) at 6 hours after wounding. This suggests that cytochrome $b_5$ may be coregulated with t-CAH. This suggests that cytochrome b5 is not at saturating levels in the pea microsomes. Coregulation may be suggested since the time course of induction of cytochrome $b_5$ seems similar to that of t-CAH and phenylalanine ammonia lyase in its rise over the 8-10 hour range (Figure 5). Note that the antibody cross-reacts with several higher molecular weight proteins or complexes as well. This may be indicative of either the formation of cytochrome $b_5$ aggregates or of non-specific antibody crossreactivity.

In this paper we have demonstrated that cytochrome $b_5$ is a viable probe with which to study the regulation of plant cytochrome $b_5$ and attempt to clone this enzyme in peas. The genomic DNA Southern analysis is hopeful, in that our probe hybridizes to discrete bands in the proper size range. In

addition, we have shown by our Western blot analysis that the regulation of cytochrome $b_5$ parallels the regulation of t-CAH. The next logical step will be to purify and sequence the t-CAH enzyme itself and use the genetic code to construct a matching oligodeoxyribonucleotide probe to search for this clone and its regulatory mechanisms.

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FIGURES

Figure 1. Phenylpropanoid Pathway

Figure 2. Genomic DNA Southern analysis of tomato, pea, and barley libraries with mammalian cytochrome $b_5$ probe. Each library was cut with Eco RI, Bam HI, and Hind III (l to r). The pAB96 control on the far right was cut with Pst I to release pBR322. The libraries were hybridized with 2 x $10^6$ cpm of wild type cytochrome $b_5$ probe at 40° in 5x SSC, 1 x Denhardt's solution and then washed at 30° and exposed for 2 weeks at -70°.

Figure 3. Western blot analysis of cytochrome $b_5$ levels after wounding. Microsomal proteins were collected at 0, 2, 3, 6, 8, and 10 hours after wounding and prepared for analysis as described in Stewart and Schuler (6). 75 $\nu$g of each sample were electrophoresed on a 15 % SDS-polyacrylamide gel, the proteins transferred to nitrocellulose and cross-reacted with antibody directed against the the mammalian cytochrome $b_5$ at a 1:2000 dilution for 30 minutes. Lane 1, 10 $\nu$g cytochrome b5 standard, lanes 4-8, 0, 2, 3, 6, 8 hours after wounding, lane 9, 10 hours after induction, lane 10, 1 $\nu$g molecular weight standards and lane 11, 25 $\nu$g cytochrome $b_5$ standard.

Figure 4. Coomassie blue-stained 15 % acrylamide gel of a transferred time-course induction Western blot using cytochrome $b_5$. This gel was blotted for 16 hours at 80 V. Lane 1, low molecular weight standards, Lane 2, mammalian cytochrome $b_5$ standard, Lane 3 is 10 hours after wounding, lanes 8-12, 8, 6, 3, 2, and 0 hours after wounding.

Figure 5. Time course induction of t-CAH and PAL activities by wounding. Pea epicotyl tissue was sectioned, incubated in 5 mM sodium phosphate buffer and duplicate samples were assayed at the designated times. t-CAH activity was determined by the HPLC analytical method described in Stewart and Schuler (Submitted); PAL activity was determined by the method of

Schopfer and Mohr (1972). Figure taken from Stewart and Schuler, manuscript in preparation.

Figure 6. Amino acid comparison of cytochrome $b_5$ from rat microsomes and mitochondria, and porcine, human, ox, and chicken microsomes. The sequences are taken from (11) and (12).

TABLES

Table 1. Cytochrome $b_5$ Homology

| | Amino Acid Homology | |
|---|---|---|
| Rat Microsomal vs. | | |
| Rat Mitochondrial | 121/123 | 98.4 % |
| Human Microsomal | 110/122 | 90.2 % |
| Pig Microsomal | 115/126 | 91.3 % |
| Ox Microsomal | 115/127 | 90.6 % |
| Chicken Microsomal | 97/117 | 82.9 % |
| All Conserved Positions | 58/113 | 51.3 % |
| | **Nucleotide Homology** | |
| Plant Codons | 220/291 | 75.6 % |
| Worst Case | 178/291 | 61.2 % |

Table 2. Averaged Plant Codon Usage

| Amino Acid | Codon | Usage | Amino Acid | Codon | Usage |
|---|---|---|---|---|---|
| Phe | UUU | 32 | Ser | UCU | 22 |
| Phe | UUC | 68 | Ser | UCC | 17 |
| Leu | UUA | 4 | Ser | UCA | 15 |
| Leu | UUG | 26 | Ser | UCG | 4 |
| Leu | CUU | 34 | Pro | CCU | 30 |
| Leu | CUC | 18 | Pro | CCC | 17 |
| Leu | CUA | 6 | Pro | CCA | 45 |
| Leu | CUG | 9 | Pro | CCG | 8 |
| Ile | AUU | 42 | Thr | ACU | 29 |
| Ile | AUC | 42 | Thr | ACC | 43 |
| Ile | AUA | 16 | Thr | ACA | 16 |
| Met | AUG | 100 | Thr | ACG | 8 |
| Val | GUU | 44 | Ala | GCU | 44 |
| Val | GUC | 16 | Ala | GCC | 25 |
| Val | GUA | 5 | Ala | GCA | 28 |
| Val | GUG | 32 | Ala | GCG | 3 |
| Tyr | UAU | 36 | Cys | UGU | 57 |
| Tyr | UAC | 64 | Cys | UGC | 43 |
| Stop | UAA | | Stop | UGA | |
| Stop | UAG | | Trp | UGG | 100 |
| His | CAU | 42 | Arg | CGU | 20 |
| His | CAC | 58 | Arg | CGC | 8 |
| Gln | CAA | 60 | Arg | CGA | 8 |
| Gln | CAG | 40 | Arg | CGG | 3 |
| Asn | AAU | 27 | Ser | AGU | 16 |
| Asn | AAC | 73 | Ser | AGC | 20 |
| Lys | AAA | 47 | Arg | AGA | 28 |
| Lys | AAG | 53 | Arg | AGG | 33 |
| Asp | GAU | 49 | Gly | GGU | 41 |
| Asp | GAC | 51 | Gly | GGC | 10 |
| Glu | GAA | 49 | Gly | GGA | 12 |
| Glu | GAG | 51 | Gly | GGG | 12 |

The percent usage average is taken from the values derived from the average usage from the sequences of a Glycine max storage protein, a Medicago sativa glutamine synthase, the Arabidopsis thaliana alcohol dehydrogenase and ribulose bis-phosphate carboxylase activase, and a Phaseolus vulgaris storage protein.

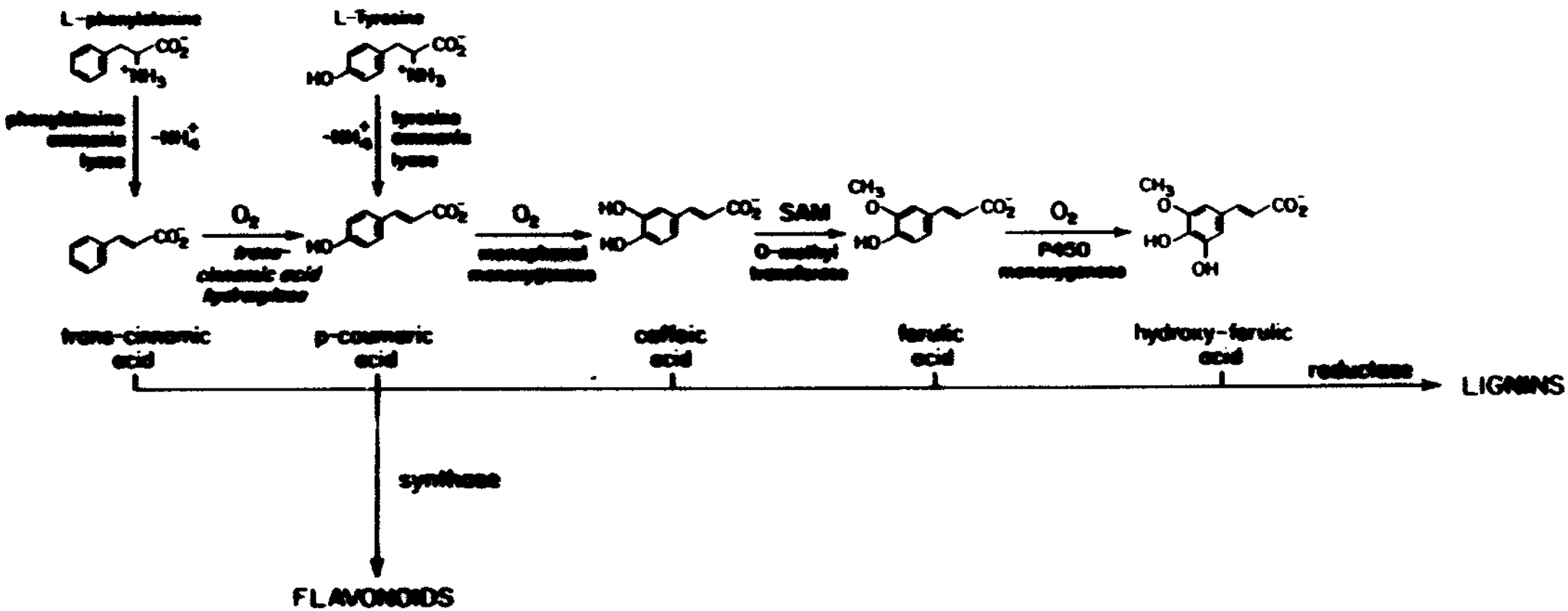



**Figure 1. Phenylpropanoid Pathway**

Figure 2.

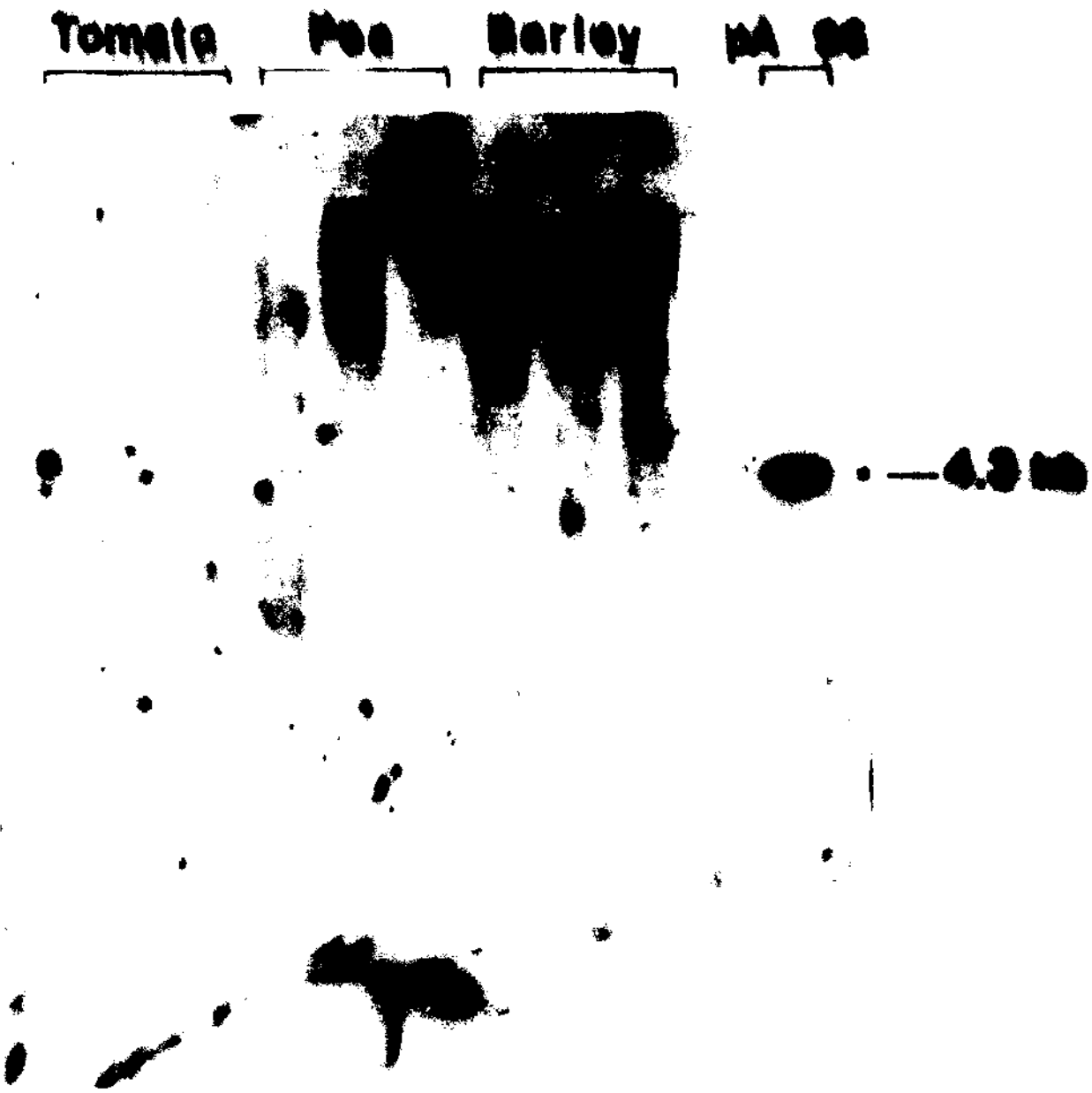


Figure 3.

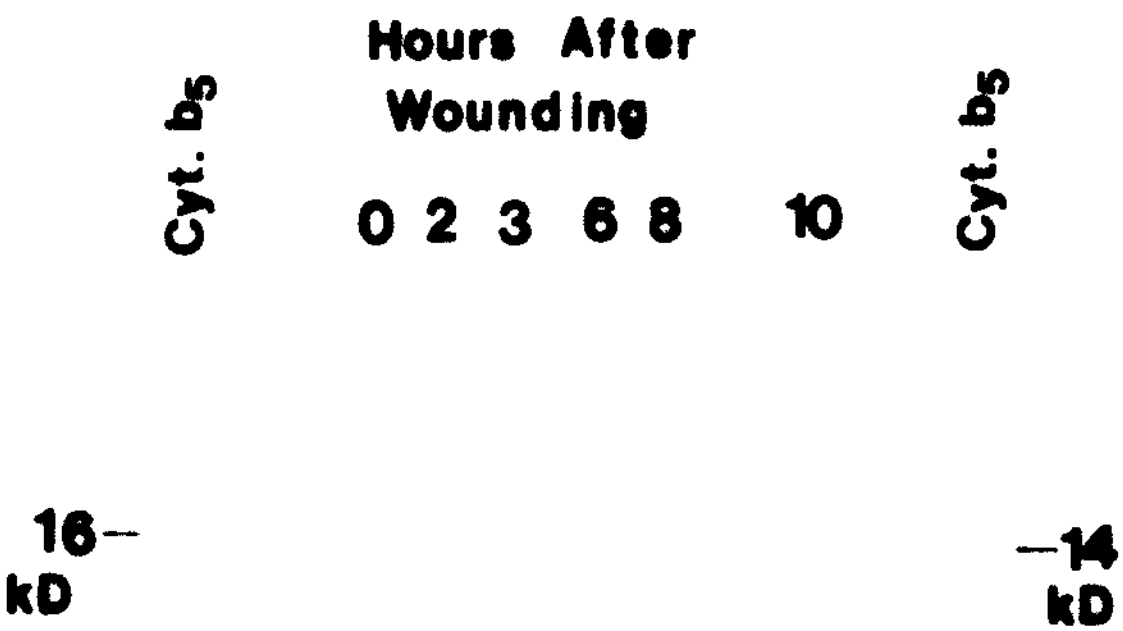


Figure 4.

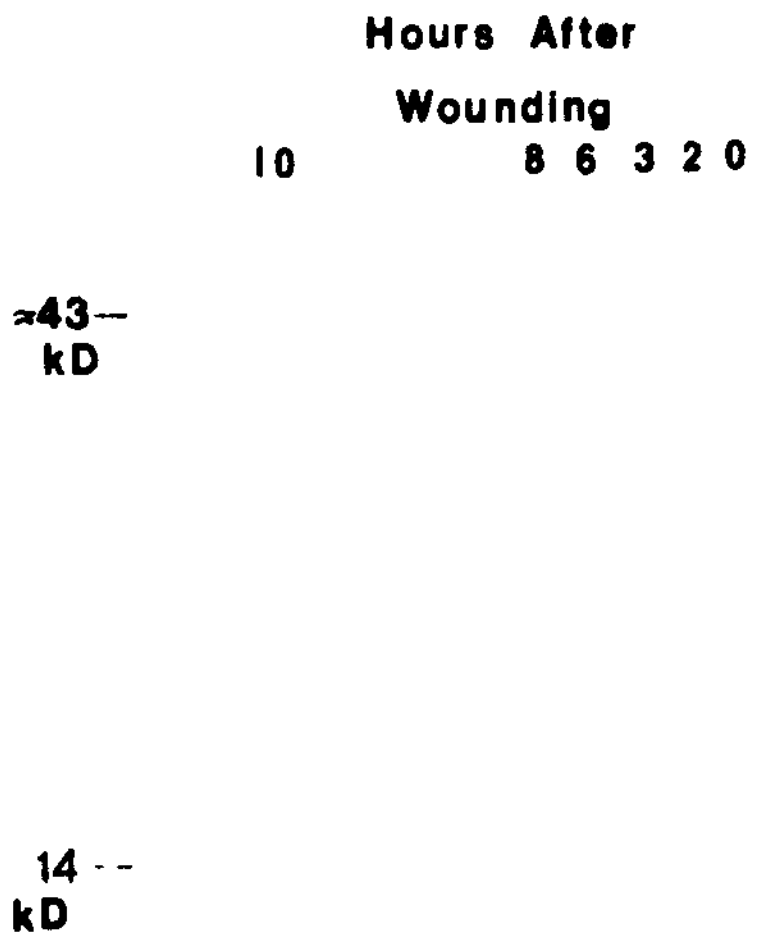


**Figure 5.**

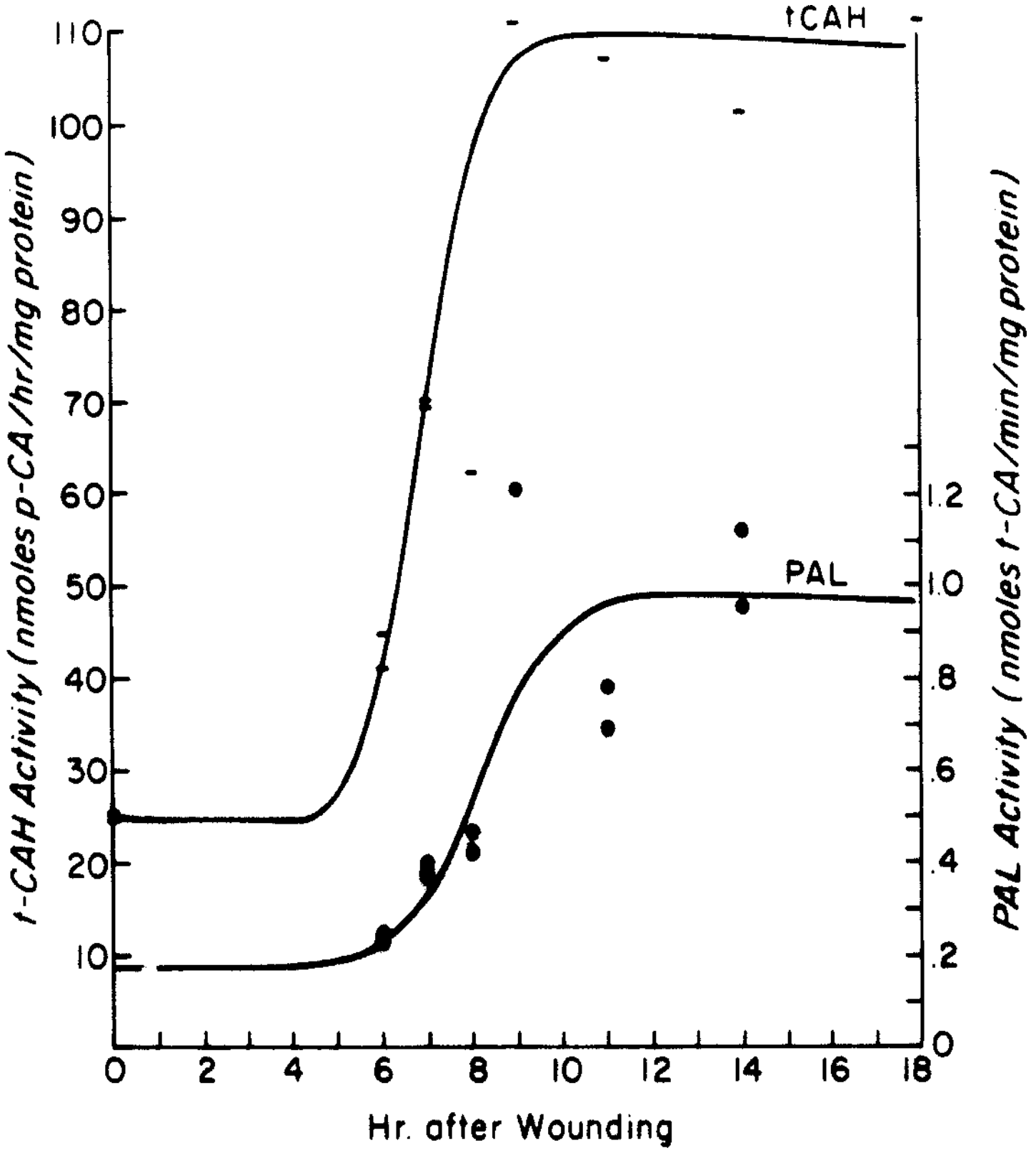
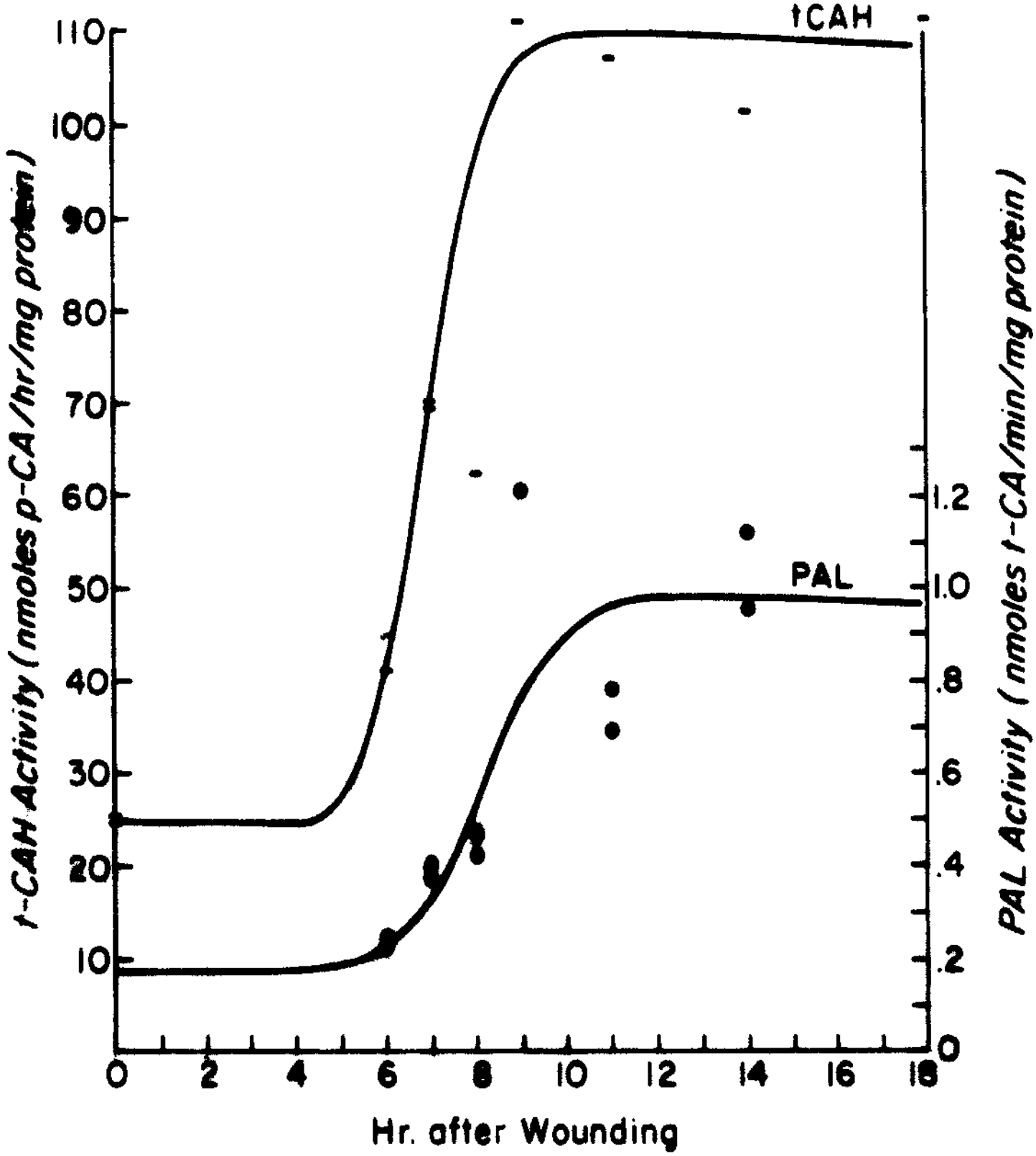

t-CAH
PAL
t-CAH Activity (nmoles p-CA/hr/mg protein)
PAL Activity (nmoles t-CA/min/mg protein)
Hr. after Wounding
110
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
1.2
1.0
.8
.6
.4
.2
0

Figure 5.


t-CAH
PAL
t-CAH Activity (nmoles p-CA/hr/mg protein)
PAL Activity (nmoles t-CA/min/mg protein)
110
100
90
80
70
60
50
40
30
20
10
1.2
1.0
.8
.6
.4
.2
0
0
2
4
6
8
10
12
14
16
18
Hr. after Wounding

| | 1 | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Rat Microsomal $b_5$ | (MET) | ALA | GLU | GLN | SER | ASP | LYS | ASP | VAL | LYS | TYR | TYR | THR |
| Rat Mitochondrial $b_5$ | (MET) | ALA | GLU | GLN | SER | ASP | LYS | ASP | VAL | LYS | TYR | TYR | THR |
| Human $b_5$ | (MET) | ASX | GLX | GLU | GLU | ALA | SER | ASP | GLU | ALA | VAL | LYS | TYR | TYR | THR |
| Pig $b_5$ | (MET) | GLX | GLX | ASP | ALA | SER | . | LYS | ALA | VAL | LYS | TYR | TYR | THR |
| Ox $b_5$ | (MET) | SER | . | LYS | ALA | VAL | LYS | TYR | TYR | THR |
| Chicken $b_5$ | (MET) | GLY | ARG | TYR | TYR | ARG |

| | 14 | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LEU | GLU | GLU | ILE | GLN | LYS | HIS | LYS | ASP | SER | LYS | SER | THR | TRP | VAL |
| | LEU | GLU | GLU | ILE | GLU | LYS | HIS | LYS | ASP | SER | LYS | SER | THR | TRP | VAL |
| | LEU | GLU | GLU | ILE | GLU | LYS | HIS | ASN | HIS | SER | LYS | SER | THR | TRP | LEU |
| | LEU | GLU | GLU | ILE | GLU | LYS | HIS | ASN | ASN | SER | LYS | SER | THR | TRP | LEU |
| | LEU | GLU | GLU | ILE | GLU | LYS | HIS | ASN | ASN | SER | LYS | SER | THR | TRP | LEU |
| | LEU | GLU | GLU | VAL | GLN | LYS | HIS | ASN | ASN | SER | GLX | SER | THR | TRP | ILE |

| | 29 | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ILE | LEU | HIS | HIS | LYS | VAL | TYR | ASP | LEU | THR | LYS | PHE | LEU | GLU | GLU |
| | ILE | LEU | HIS | HIS | LYS | VAL | TYR | ASP | LEU | THR | LYS | PHE | LEU | GLU | GLU |
| | ILE | LEU | HIS | HIS | LYS | VAL | TYR | ASP | LEU | THR | LYS | PHE | LEU | GLU | GLU |
| | ILE | LEU | HIS | HIS | LYS | VAL | TYR | ASP | LEU | THR | LYS | PHE | LEU | GLU | GLU |
| | ILE | LEU | HIS | TYR | LYS | VAL | TYR | ASP | LEU | THR | LYS | PHE | LEU | GLU | GLU |
| | ILE | VAL | HIS | HIS | ARG | ILE | TYR | ASP | ILE | THR | LYS | PHE | LEU | ASP | GLU |

**Figure 6.**

44 HIS PRO GLY GLY GLU GLU VAL LEU ARG GLU GLN ALA GLY GLY ASP

HIS PRO GLY GLY GLU GLU VAL LEU ARG GLU GLN ALA GLY GLY ASP

HIS PRO GLY GLY GLU GLU VAL LEU ARG GLU GLN ALA GLY GLY ASP

HIS PRO GLY GLY GLU GLU VAL LEU ARG GLU GLN ALA GLY GLY ASP

HIS PRO GLY GLY GLU GLU VAL LEU ARG GLU GLN ALA GLY GLY ASP

HIS PRO GLY GLY GLU GLU VAL LEU ARG GLU GLN ALA GLY GLY ASP

59 ALA THR GLU ASN PHE GLU ASP VAL GLY HIS SER THR ASP ALA ARG

ALA THR GLU ASN PHE GLU ASP VAL GLY HIS SER THR ASP ALA ARG

ALA THR GLU ASP PHE GLU ASP VAL GLY HIS SER THR ASP ALA ARG

ALA THR GLX ASP PHE GLU ASP VAL GLY HIS SER THR ASP ALA ARG

ALA THR GLU ASP PHE GLU ASP VAL GLY HIS SER THR ASP ALA ARG

ALA THR GLU ASP PHE GLU ASP VAL GLY HIS SER THR ASP ALA ARG

74 GLU LEU SER LYS THR TYR ILE ILE GLY GLU LEU HIS PRO ASP ASP

GLU LEU SER LYS THR TYR ILE ILE GLY GLU LEU HIS PRO ASN ASP

GLU MET SER LYS THR PHE ILE ILE GLY GLU LEU HIS PRO ASP ASP

GLU LEU SER LYS THR PHE ILE ILE GLY GLU LEU HIS PRO ASP ASP

GLU LEU SER LYS THR PHE ILE ILE GLY GLU LEU HIS PRO ASP ASP

ALA LEU SER GLU THR PHE ILE ILE GLY GLU LEU HIS PRO ASP ASP

89 ARG SER LYS ILE ALA LYS PRO SER GLU THR LEU

ARG SER LYS ILE

ARG PRO LYS

ARG XXX XXX ILE ALA LYS PRO SER GLU THR LEU ILE THR THR VAL

ARG SER LYS ILE THR LYS PRO SER GLU SER ILE ILE THR THR ILE

ARG PRO LYS LEU ARG

104 GLU SER ASN SER SER TRP TRP

ASP SER ASN PRO SER TRP TRP