To the Graduate Council:

I am submitting herewith a thesis written by Samuel John Ebong entitled " A Study of the Distribution of Plasma Amine Oxidase in some mammals." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with major in Life Sciences.

Walter R. Farkas, Major Professor

We have read this thesis and
recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor and
Dean of The Graduate School.

A STUDY OF THE DISTRIBUTION OF PLASMA AMINE OXIDASE IN SOME MAMMALS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Samuel J. Ebong

December 1991

To Effiong John Ebong and Arit Ebong

Ete ye Eka mmi

(My Parents)

ACKNOWLEDGEMENTS

I would first of all like to offer my most sincere gratitude to my major professor, Dr. Walter R. Farkas. He not only had the belief in me, but also offered critical insights into this study. My gratitude also goes to him for his kindness and for making my stay a pleasant one. I also would like to thank the other members of my committee, Dr. Donita Frazier and Dr. Bill Congdon, for their patience and understanding in guiding me through this study.

At this stage I would like to extend my sincere thanks to my laboratory partners, Mr. Bill Conover and Mr. Tom Stanawitz for their help in getting me started and for their support in urging me. I especially thank Mr. Conover for his help in tracking down the plasma used in this study.

Most importantly, I would like to thank my best friend, Ms. Ana Maria Majano for her unconditional support, for her non-technical help with writing, and for her being there when I really needed a companion.

Last but not the least, I would like to thank my parents for instilling in me the discipline to 'hang in there' by believing in the Lord Jesus Christ and His guidance to succeed in life.

ABSTRACT

Plasma amine oxidase (PAO; EC 1.4.3.6) is an important enzyme in the metabolism of naturally occurring amines. The enzyme is known to contain copper and is generally believed to be present in the plasma of all higher mammals. Earlier evidence pointed to pyrroquinoline quinone (PQQ) as the cofactor required for its function. However, the latest findings indicate that 6 - hydroxydopa may be the true cofactor. PAO will oxidize only primary, medium to long chain amines, and is sensitive to cyanide. The other amine oxidases (mainly cellular) are known to oxidize both primary and secondary amines and are not sensitive to cyanide. We initiated studies to determine the effect of nutritional deficiencies on the plasma enzyme in mice and were surprised to find that control mice fed a normal diet lacked functional PAO. Since the mouse is one of the most frequently used animal models and many compounds of pharmacological and toxicological interest are amines, we initiated a study to find out if sera from eleven different mammals could oxidize a group of amines of biomedical interests. The study used three main assays: benzylamine assay, paper chromatography assay, i.e., determination of the disappearance of reactants, and the assay for the release of ammonia. Our results indicate that the enzyme is not present in all higher mammals. Furthermore, substrate specificity varies from species to species. It is generally believed that polyamines are the natural substrates for this enzyme. However, neither putrescine, spermidine or spermine, the three major polyamines, were oxidized by mouse serum. Furthermore, the absence of PAO activity in mouse serum is not due to the presence of

an inhibitor since mouse serum did not inhibit the oxidation of amines by rabbit, cow, or human.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Plasma amine oxidase (PAO; 1.4.3.6) is one of a group of enzymes important in the biological oxidation of naturally occurring amines. These enzymes are widely distributed in plants, e.g., pea seedling (1), fungi, e.g., Aspergillus niger, animal tissues, e.g., Pig Brain (2), and microorganisms, e.g., Micrococcus rubens (3,4,5).

In general, monoamine oxidases catalyze the oxidative deamination of a primary amine:

$$R\text{-}CH_2\text{-}NH_2 + O_2 + H_2O \xrightarrow[\text{oxidase}]{\text{amine}} R\text{-}CHO + H_2O_2 + NH_3$$

R = aromatic or aliphatic residue.

Plasma amine oxidase is known to take part in the breakdown of dopamine, and also in the inactivation of other exogenous and endogenously formed amines in the bloodstream. It has been suggested that PAO (with predominantly lysyl oxidase), take part in the maturation of connective tissue proteins (6). This suggestion was based on a decrease in PAO activity concurrent with the decreasing rate of connective tissue synthesis during the process of aging (6,7).

There has been confusion as to the nomenclature of plasma amine oxidase. For instance, Tabor et al. (66) reported on the enzyme in the blood plasma of the horse and

other mammals, and called the enzyme benzylamine oxidase since it readily oxidized benzylamine in these mammals whereas they labelled those in sheep and cattle plasma as spermine oxidase because of its high activity on spermine and spermidine. Blaschko et al. (9) suggested that amine oxidases be classified according to their susceptibility to inhibitors rather than their substrate specificity. In this instance, plasma amine oxidase, benzylamine oxidase, spermine oxidase, and pig kidney histaminase, are chemically related since they are inhibited by carbonyl reagents. The mouse liver histaminase and the intracellular amine oxidases present in tissues constitute the second group. Mondovi et al. (10) classified amine oxidases based on their prosthetic groups; i.e., FAD-dependent enzymes include the mitochondrial monoamine oxidases and cytosolic enzymes whereas copper-dependent enzymes include serum amine oxidase and diamine oxidases.

According to the 1984 International Enzyme Nomenclature (11), amine oxidases belong to Class 1 (oxidoreductase) and subclass # 4, which acts on the CH-NH_2, and subgroup # 3; i.e., oxygen is the acceptor. The subgroup has its own set of members used to distinguish between the different forms of the enzyme. Thus, EC 1.4.3.4 stands for the flavin-containing amine oxidase. This oxidase is also referred to as: monoamine oxidase, tyramine oxidase, tyraminase, and adrenaline oxidase, depending on substrate preference. These are the classic intracellular monoamine oxidase enzymes mainly bound to mitochondria. EC 1.4.3.6 is used to classify those enzymes assumed to contain pyridoxal phosphate as a cofactor. Other names for this soluble enzyme include: diamine oxidase, histaminase, serum amine oxidase, and plasma amine oxidase. EC 1.4.3.9 is

another flavin-containing enzyme called tyramine oxidase that act primarily on tyramine.

Plasma amine oxidase is distinguishable from other amine oxidases by its substrate specificity and also by its susceptibility to certain inhibitors. PAO catalyzes the oxidative deamination of mainly primary amines. Because plasma amine oxidase is a copper-containing enzyme, it is highly susceptible to metal chelating agents, for example, sodium cyanide, cuprizone, and sodium diethyldithiocarbamate (DDC) (16,79). Except for DDC, these chelators are all chemicals known to complex with copper. DDC actually removes the copper ion from the enzyme, rendering it inactive (16).

Intracellular amine oxidases are known to catalyze the oxidative deamination of primary, secondary, and tertiary amines. Of these intracellular enzymes, the mitochondrial amine oxidase (MAO) has been one of the most extensively studied. This particular enzyme has been found in every mammal studied (8, p.31). MAO exists in two major forms, type A and type B (12,13), each having different substrate specificities and inhibitor sensitivities. MAO-A is irreversibly inhibited by clorgiline, a non-hydrazine drug, and preferentially deaminates norepinephrine. MAO-B is inhibited by L-deprenyl and preferentially deaminates benzylamine (14).

Presently, the site of synthesis of PAO has not been determined. A hypothesis is that PAO originates from fibroblasts (15). This is based on the fact that corticosteroids decrease PAO activity. Corticosteroids are also known to affect the metabolism of connective tissue by disrupting collagen cross-linking, thereby destabilizing the structure and also affecting PAO activity (15).

Rationale

Initial experiments in the laboratory with germ-free mice showed that, contrary to the widely held assumption that PAO was ubiquitous, the enzyme was not present in all mammals (39). Plasma amine oxidase was not detected in conventional as well as in germ-free mice (Table 19). This is of toxicological significance since many pharmacologically important drugs are amines and the mouse is a commonly used animal model for studying toxicology of these drugs.

Factors that were considered in doing this study included the availability of substrates and species, biomedical and pharmacological significance of substrates as they relate to the different species, especially humans. Also, conflicting reports regarding the distribution of plasma amine oxidase in different species was considered.

Availability. Working at the Veterinary Hospital has been invaluable in obtaining plasma from the selected animals. Generally, the study of a particular substrate and species was dependent on how they could be obtained. In some instances, e.g., the hamster, only one of the three assays could be carried out because of lack of plasma source. Some substrates, especially if radiolabelled, are expensive. In other cases, e.g., amphetamine, benzylamine, these substrates were not readily available in radioactive forms. In these cases, paper chromatography assay was not performed.

Biomedical/pharmacological significance. Some pharmacologically important drugs are amine derivatives and are commonly adminitered intravenously. As already stated, a

general assumption is that PAO is present in all higher mammals. Hence, it is assumed that some, if not all, of the amine administered will be biotransformed. On the other hand, if the animal does not have the enzyme, some of the administered drug will not be immediately biotransformed as expected. This may lead to an increase in drug concentration in the animal if an alternative method of drug clearance is not active. For example, the cat has a deficiency in glucuronic acid conjugation, the pig is deficient in sulfate conjugation, while the dog is deficient in acetylation. If these animals were used for drug testing, the results may be misleading. Furthermore, in cases where results are extrapolated to humans, faulty assumptions may lead to incorrect conclusions.

Conflicting Reports. There are conflicting reports on the distribution of the enzyme in some animal sera. For instance, Blaschko (39) stated categorically that PAO was not present in human serum while McEwen found PAO activity and later purified the enzyme from human serum (42). Crude serum contained low enzyme activity. After purification and concentration the enzyme activity was readily demonstrable.

Purpose

This study was undertaken to investigate:

a) the distribution of plasma amine oxidase in eleven mammalian species, viz : Human, Cow, Cat, Rat, Mouse, Horse, Guinea Pig, Pig, Hamster, Rabbit, and Dog.

b) the differences in substrate specificities in the different species using the following substrates : Amphetamine, Benzylamine, Dopamine, Epinephrine, Histamine, L-Dopa,

Norepinephrine, Putrescine, Spermidine, Spermine, Tryptamine, Tyramine, and Tyrosine. Reports have indicated that for plasma amine oxidase, simple aliphatic amines are among the actively oxidized substrates (43,28). Long chain aliphatic diamines (if oxidized), are better oxidized than short chain diamines (44). Secondary and tertiary amines are not oxidized (39,43).

Plasma levels of catecholamines and their metabolites are frequently used for clinical diagnosis. For example, increased plasma levels of norepinephrine and epinephrine are noticed in cardiovascular diseases such as myocardial infarction, (7) congestive heart failure (7,45), and in pheochromocytoma (46). The catecholamines are dopamine, epinephrine, and norepinephrine. Dopa is a precursor of these catecholamines. These catecholamines are neurotransmitters produced and secreted by the central and autonomic nervous systems. They also function as hormones and are in this instance, secreted mainly by the adrenal medulla. These substrates are also important for pharmacotherapy. For example, L-Dopa is commonly used to treat Parkinson's disease (47), and dopamine to increase cardiac output and blood pressure. It is also thought that dopamine is the inhibitory neurotransmitter effective in treating Parkinson's disease. Epinephrine is useful for treating bronchial asthma and promotes glycogen conversion in the muscle and liver. When administered intravenously, epinephrine increases the blood pressure. By measuring the urine or plasma concentrations of catecholamines and/or their metabolites, an insight may be gained into the possible disease state of the patient. Tyrosine is a natural aromatic amino acid that is a precursor for catecholamine synthesis (Figure 1).

The polyamines used in the study include the long chain aliphatic amines spermine and spermidine, and the short chain diamine, putrescine. They are ubiquitous and considered as natural substrates (39,48,49). They are reported to be oxidized by the plasma of ruminants, e.g. camel, sheep, goat, and cow (8). They are intimately involved in the regulation of normal and neoplastic growth and differentiation. Polyamines are organic cations with ability to bind to nearly all cellular constituents e.g. nucleic acids and acidic proteins (49).

Histamine is present in most mammalian tissues, e.g., lung, liver, and muscle, but the mast cells represent the major site of histamine formation. Histamine has diverse physiological activity, for example, bronchoconstriction, vasodilation, and increased capillary permeability.

Amphetamine (1-phenyl-2-aminopropane) is a sympathomimetic amine with biological effects on the central nervous system. Amphetamine has been abused primarily because of its stimulant and anoretic effects. It is also reported to inhibit PAO activity (8, p55, 68).

Tyramine and tryptamine are sympathomimetic drugs. Tyramine is commonly found in food and food products and is not normally present in mammals in significant amounts. When introduced into the bloodstream, tyramine raises the blood pressure. Tryptamine is a naturally occurring trace amine found in the brain of all mammals. It is pharmacologically active and will readily cross the blood-brain barrier. Tryptamine is known to raise blood pressure and influence behavior. Tryptamine has also been implicated in some neurological and psychiatric disorders (50).

c) since the actual natural substrate for PAO is unknown, the study could be a guide in selecting a particular substrate for a given mammal. The best substrate for PAO is reported to be the primary aromatic amine, benzylamine (44,8). However, benzylamine is not a natural substrate i.e., it is not indigenous to higher mammals. This raises the possibility that some of the animal plasmas may not oxidize benzylamine even though the enzyme may be present in the animal's plasma. As such, the other substrates known to be indigenous in mammals were also tested.

Several assays can be used to measure the activity of plasma amine oxidase: the measurement of hydrogen peroxide formation (51,52,53), oxygen consumption (54), or chromatography (paper or liquid), which is based on the disappearance of substrates. High-Performance Liquid Chromatography (HPLC) has been adapted to test for catecholamines using different detection methods - UV, fluorescence, and electrochemical detection (52,55,56). Gas-Liquid Chromatography (GC) is another chromatographic method available to quantify amines (57). Radioenzymatic methods include the use of phenylethanolamine-N-methyltransferase (PNMT) and catechol-O-methyltransferase (COMT) (58,59). These enzymes catalyze the labelling of cathecholamines by transferring radioactive methyl group to the appropriate catecholamine. Radioisotopic assays have been developed to determine monoamine oxidase in the human plasma (60). Other methods include the measurement of aldehyde product (42), and the measurement of ammonia (61).

Conditions for estimating amine oxidase activity include sensitivity of the assay, ability to use diverse biogenic amines as substrates, and low cost of running the assay.

For this study, three assays, (The Benzylamine Assay, Assay for ammonia release, and Paper chromatography, i.e., determination of the disappearance of reactants), were used to investigate the presence of PAO in eleven mammals. These assays meet the above-listed conditions. However, no one method is adequate to cover all the different substrates chosen. These assays were chosen because they have been used previously and have proven to be effective, reliable, and not time consuming (61,62).

Prosthetic groups

There has been much debate as to the nature of the non-protein part of PAO (16,17,18,19). Plasma amine oxidase is not a single enzyme. Heterogenous isoenzymes have been described using chromatography assays (DEAE cellulose and hydroxyapatite) (20). The isoenzymes differ mainly with regard to their carbohydrate contents (21).

1. **Inorganic**

Of the extracellular amine oxidases (e.g. lysyl oxidase, diamine oxidase, histaminase, PAO, and platelet amine oxidase), PAO is one of the better characterized enzymes. Plasma amine oxidase will not function optimally without oxygen (22,23,24). It is known to contain copper (in Cu^{2+} state) necessary for catalytic activity (16,25). During catalysis, there is no valency change of copper because oxidative deamination proceeds by PAO accepting two electrons from amines and transferring them to molecular oxygen (26). The function of copper is not fully understood but it is thought to be essential in facilitating the transfer (77,8, p.83). No other metals (e.g., Iron, Zinc,

Molybdenum, or Manganese) have been detected in significant amounts (27). The enzyme-bound copper can be removed under non-reducing conditions by dialysis against diethyldithiocarbamate (DDC) or by treating with acidic buffers (16). Enzyme activity is lost when copper is removed. However, activity can be restored by adding appropriate amounts of copper back to the system. Other metals, or hydrogen acceptors (in the case of oxygen), are ineffective in reactivating the enzyme (16,26,8, p.27).

The enzyme has a molecular weight between 170,000 and 195,000 and contains one to two molecules of copper per enzyme molecule (28). PAO also has a cofactor which was later identified conclusively as 6-hydroxydopa quinone (TOPA) (29).

2. Organic

The presence of an organic cofactor for optimum activity was inferred from PAO sensitivity to carbonyl reagents such as semicarbazide and hydroxylamine. Reports have claimed either flavin adenine dinucleotide (FAD) (17), or pyridoxal phosphate (18), or pyrroloquoinoline quinone (PQQ) (19,30,31,32,33), as possible cofactors. Flavin was ruled out because treatments with strong acids, heat denaturation (18), and also treatment with protease did not liberate any flavins (27). Pyridoxal phosphate was thought to be a cofactor based on the fact that many substances known to inhibit pyridoxal enzyme, for example phenylhydrazine and hydroxylamine, also inhibited PAO, and the spectrum of PAO is similar to that of pyridoxal phosphate (18). Pyridoxal derivatives can also be used to reactivate the enzyme. Up to this point, evidence for a pyridoxal derivative as the organic cofactor have been indirect. Nevertheless, these evidences were stronger than

those advanced for other cofactors, e.g., FAD. Due to indirect and inconclusive evidences, later experiments were geared towards discounting pyridoxal and its derivatives as the cofactor. Watanabe et al. (78), from chromatographic spray test, did not find either phosphate or pyridoxal phosphate, prompting the suggestion that another cofactor might be present in the bovine plasma. Knowles et al. (33), ruled pyridoxal phosphate out from resonance Raman results. This leaves only PQQ, which has been reported to be present only in prokaryotes (31), and its apparent detection in eukaryotic systems was expected to indicate a possible role for PQQ. Like pyridoxal phosphate, no direct evidence supports the presence of PQQ in PAO. Mass spectroscopy and NMR data support 6-hydroxydopa (TOPA) as a cofactor (25,29,34). The presence of TOPA was also supported by Kumazawa et al. using gas chromatography and mass spectrometry (35).

Inhibitors

A major assumption in treating patients with depression is that the sickness results from depletion of some monoamines in the brain. By restoring the levels of the monoamines, the depression can be lifted (36). A way of doing this is by decreasing the activity of amine oxidases.

In higher mammals, MAO inhibitors increase the amount of monoamines (exogenous and endogenous) in the body. Although many of these MAO inhibitors have been produced to function against intracellular amine oxidases, certain MAO inhibitors also inhibit PAO. For example, Parnate sulfate, well established as a MAO inhibitor in

the brain and liver, also inhibits PAO (37). Many of these drugs bind copper, FAD, or the carbonyl end group.

CHAPTER 2

MATERIALS AND METHODS

Animal Species

Conventionally, in performing drug toxicity tests, two species - a rodent and a non-rodent - are commonly used (41). Selection of animal species for this study was based on availability and biomedical and/or pharmacological importance. Also, conflicting reports regarding the distribution of plasma amine oxidase in higher mammals was considered.

The species tested were: cat, cow, dog, guinea pig, hamster, horse, human, mouse, pig, rabbit, and rat.

Serum collection

Plasma was collected from the species to be studied. In order to obtain enough plasma for assays, blood was pooled from more than one animal in smaller species like the mouse. PAO was not purified and concentrated before use, therefore, our results will closely approximate the actual physiological activity of circulating PAO.

Blood was collected into chilled heparinized tubes and then centrifuged for 10 minutes at 2500 rpm. Plasma was aspirated, pooled, and stored at -40^0 C.

Substrates

The following amines were included in the study: amphetamine, benzylamine, spermidine, spermine, tyramine, tryptamine, tyrosine, dopamine, epinephrine, histamine, L-dopa, norepinephrine, and putrescine.

Sources of chemicals

Benzylamine (Phenylmethylamine), Dopamine (3-hydroxytyramine hydrochloride), L-Amphetamine (also, D-Amphetamine sulfate), Putrescine (1,4-Diaminobutane; tetramethylenediamine), Spermine tetrahydrochloride, and Spermidine trihydrochloride (N-[3-aminopropyl]-1,4-butanediamine) were obtained from Sigma Chemical Company, St. Louis, Missouri. L-Dopa (L-beta-3,4-dihydroxyphenylalanine), L-Tyrosine hydrochloride, Tyramine hydrochloride, Tryptamine (3-indole ethylamine HCL), Histamine dihydrochloride, Norepinephrine, and Epinephrine (L-(-)-epinephrine) were obtained from ICN, Costa Mesa, California.

All chemicals were dissolved in 0.2 M, (pH 7.2) phosphate buffer and stored not longer than seven days in a -10^{0}C refrigerator.

The tritiated substrates were: DL [7-^{3}H] norepinephrine (specific activity (SA) = 10 Ci/mmole), levo[N-methyl-^{3}H]-3,4 epinephrine (SA = 85 Ci/mmole); both obtained from American Radiolabelled Chemicals, St. Louis, Missouri. [2,5,6-^{3}H] dopamine, L [3,4-dihydroxyphenyl(1-^{14}C)] alanine (L-Dopa) (SA = 10.3 mCi/mmole), and 5-hydroxy(G-^{3}H) tryptamine creatinine sulphate (SA = 9.3 Ci/mmole) were obtained from Amersham International, Arlington Heights, Illinois. [Ring-^{3}H] tyramine hydrochloride

(SA = 20.1 Ci/mmole), and [^{3}H (G)] histamine (SA = 10 Ci/mmole) were obtained from New England Nuclear Products, Boston, Massachusetts. [3,5-^{3}H] tyrosine (SA = 42 Ci/mmole) was obtained from ICN, Irvine, California.

All chemicals used for this study were analytical reagent grade.

Assays

1. **Benzylamine Assay**

This spectrophotometric assay measures PAO activity by measuring the conversion of benzylamine to benzaldehyde (42):

$$\underset{\text{Benzylamine}}{C_6H_5\text{-}CH_2\text{-}NH_2} + H_2O + O_2 \xrightarrow[\text{oxidase}]{\text{amine}} \underset{\text{Benzaldehyde}}{C_6H_5\text{-}CHO} + NH_3 + H_2O_2$$

The conditions followed that of McEwen's (42,63) with minor modifications (Table 1). Briefly, 0.60 ml of the test plasma was incubated with 0.15 ml of 8 mM buffered Benzylamine* and 0.75 ml of Phosphate Buffer** in 13mm x 100 mm open glass test

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*Obtained by adding 0.05 ml of Benzylamine (Sigma, reagent grade) to 56.25 ml of 0.2M (pH 7.2) phosphate buffer.

**Phosphate buffer was prepared by adding 68.5 ml of 0.2M Na_2HPO_4 with 31.5 ml of 0.2M KH_2PO_4 to yield 0.2M Phosphate buffer, pH 7.2. pH can be adjusted by using the appropriate buffer constituent.

tubes for three hours at 37^0 C with gentle shaking to permit aeration. At the end of incubation, the reaction was stopped by denaturing the proteins with 0.15 ml of 60% perchloric acid (PCA). The major product, benzaldehyde, was extracted into 1.5 ml of cyclohexane and then centrifuged at 2500 rpm to separate the phases. Absorbance of the cyclohexane layer was then read on a Shimadzu (UV 160) spectrophotometer at 242 nm against cyclohexane. Each sample was run in duplicate. One tube was used for zero time control and PCA was added immediately after adding the test serum. The second tube contained the test serum along with the amine. PCA was added after the three hour incubation.

2. Ammonia Assay*

The assay is based on the formation of ammonia during PAO catalyzed oxidative deamination of the appropriate amine:

$$R\text{-}CH_2NH_2 + H_2O + O_2 \xrightarrow[\text{oxidase}]{\text{amine}} R\text{-}CHO + NH_3 + H_2O_2$$

*The kit from Sigma for the enzymatic determination of ammonia was used for the study (Tables 2 and 3) (65).

Quantification of ammonia follows the equation based on the original assay by Tabor et al. (64).

$$\text{2 - Oxoglutarate} + NH_3 + \text{NADH} \xrightarrow{\text{GLDH}} \text{Glutamate} + \text{NAD}$$

GLDH: L-Glutamate dehydrogenase.

Glutamate dehydrogenase catalyzes the transfer of ammonia to 2-oxoglutarate and the measurement of ammonia is proportional to the decrease in absorbance (340nm) of NADH because of the oxidation of NADH to NAD. 2-oxoglutarate and NADH are in excess, so that the limiting reactant is ammonia. Stoichiometrically, the amount of ammonia released is proportional to the amount of substrate oxidized (65), Also, the rate of ammonia released is proportional to PAO activity. Since the amount of substrate is constant, the amount of ammonia released (in microgram per millimeter) per unit time is an indication of PAO activity.

The protocol was the same as for the benzylamine assay, except that at the end of the three hour incubation, PCA was not added since it led to inconsistent readings (Table 3). Instead, the tubes were chilled in an ice bucket and the ammonia assay performed. The substrate concentration was adjusted to 10^{-2} M, giving a final concentration of 10^{-3} M.

Guinea pig plasma was not tested by this assay because of insufficient source of the animal plasma.

In order to reduce false readings from exogenous ammonia, ammonia-free water was used. This was prepared by passing double-distilled H_2O through a Dowex-50 H^+ column. The ammonia-free water was prepared immediately before use.

Time Course. This followed the procedure stated in Tables 2 and 3. Samples were read at 30 minute intervals up to three hours.

Enzyme Concentration. Again the reaction followed that in Table 6 with the following modifications: different amounts (0.3 ml, 0.6 ml, and 0.9 ml) of plasma were added to tubes and the appropriate adjustments made by the amounts of phosphate buffer added to give a final volume in each reaction mix (1.5 ml).

Substrate Concentration. In this instance, different amounts of substrates were added to the tubes and appropriate adjustments made by the amounts of phosphate buffer added to give a final volume of 1.5 ml (Table 5).

Ammonia Kit Test. This was done to verify the consistency and reliability of the ammonia assay kit. The same volume (3.22 ml) suggested in the kit was used with modifications as noted in Table 4. It is expected that increasing concentrations of ammonium sulphate standard should lead to the appropriate increase in optical density at 340 nm irrespective of which sera was being tested.

Calculations. To find how much ammonia is liberated in test samples (65):

$$\text{Samples(ug/ml)}: (\Delta A_{test} - \Delta A_{blank}) \times 44$$

Amount of ammonia in Control solution(ug/ml):

$$(\Delta A_{control} - \Delta A_{blank}) \times 44$$

where the factor 44 $= \dfrac{3.22 \times 17}{6.22 \times 0.2}$

3.22 = total volume (ml) of liquid in cuvette

17 = weight (ug) of 1 umol of Ammonia

6.22 = millimolar extinction coefficient of NADH at 340 nm

0.2 = volume (ml) of sample

ΔA = first reading - second reading

3. Paper Chromatography Assay

The experiment followed that of Benzylamine assay with some modifications (Table 7). The substrate is radioactive and the product was not extracted into cyclohexane. Also, the volumes used were reduced to take into account radioactivity. After incubation, 10uL of sample was spotted on 2 cm x 57 cm strip of Whatman chromatography paper (no.3). Using a solvent of 1-butanol:Acetic Acid (glacial):water (14:4:50), descending chromatography was carried out in a closed tank until the solvent front descended to about 75% of the strip length. The strip was allowed to dry and

subsequently it was cut into 1 cm x 3 cm pieces. These pieces were then placed in scintillation vials containing 10 ml of scintillation cocktail of Toluene and PPO-POPOP*. The radioactivity (counts/minutes) was measured using a Packard Tri-Carb 1500 Liquid Scintillation Analyzer with a 90% counting efficiency. Oxidized amines product (aldehydes) do not migrate; therefore, amine degradation is quantified by measuring radioactivity.

Polyamines, benzylamine, and amphetamine were not used in this assay because of high costs and limited availability. Also, due to insufficient amount of plasma, hamster plasma was not tested. This assay was useful in analyzing biogenic amines since no one solvent is available to study all the substrates selected. The assay does not require the use of high amounts of plasma.

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*40 ml of 5.0 gm. 2,5-diphenyloxazol (PPO) and 0.1 gm. 1,3-bis-2-(5-phenyloxazolyl)-benzene (POPOP) in 1 liter of Toluene was added to 3.75 liters of Toluene.

CHAPTER 3

RESULTS*

Three assays were used to study the distribution and substrate specificity of PAO. Assay for the formation of ammonia was used as the main assay to which others could be compared. The benzylamine assay was used to screen the different plasma for activity.

Benzylamine Assay

Table 18 shows the relative rates of oxidation of benzylamine by the sera of different mammals. For comparison, the highest optical density change, in this case, the Rabbit's, is taken as 100%. Results show that neither the guinea pig, hamster, mouse, nor the rat, have any catalytic activity in their plasma for the oxidation of benzylamine. The human sera oxidized benzylamine at 17% of the rate of rabbit serum. Moreover, it can be seen that there was no inhibitory activity in the non-active plasma. For example, the mouse serum did not inhibit the oxidation of amines by rabbit or cow plasma (Table 19). From Table 20, it can be seen that neither the Human, Hamster, nor the Rat serum had any inhibitory activity. Mixing sera of different species did not reveal any activation or inhibition.

* All results and tables are located in the Appendix.

Ammonia Assay

The formation of ammonia was determined using a kit (Sigma) for the determination of serum ammonia. The assay is based on the glutamic dehydrogenase reaction in which α - ketoglutarate is converted to glutamate while NADH is oxidized to NAD. The reaction is followed at 340 nm. We determined ΔA_{340} at zero (0) time and after three (3) hours of incubation. The ΔA_{340} at 0 time was subtracted from the ΔA_{340} after 3 hours*. A serum that gave a ($\Delta A_{3hours} - \Delta A_{0time}$) less than 0.1 was considered to be so inactive that PAO was essentially absent.

The data in Table 23 gives the ammonia** present in the assay mix in ug/ml. The amount of ammonia released was compared to the standard ammonia sample (5 ug/ml) provided in the kit.

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* Sample calculations:

	Time 0 hour(Blank)	Time 3 hours (Test)
Initial O.D:	1.24	2.45
Final O.D :	0.24	0.05
Difference :	1.00	2.40

$\Delta A_{Test} - \Delta A_{Blank} = 2.4 - 1.0 = 1.4$

This test plasma is considered to have PAO activity against the particular amine (1.4 > 0.1) and is accordingly designated "Y".

** Converted data (ug/ml): ($\Delta A_{Test} - \Delta A_{Blank}$) x 44

1. **Test for Kit reliability**

Figure 13 shows a linear relationship between the amount of ammonium ions added and the NADH oxidized.

2. **Time Course**

This study was undertaken to find out if enzyme activity was time dependent. From Figure 14, the amount of NADH oxidized increased with time up to 180 minutes of incubation at 37^0 C. There is a risk of bacterial contamination if samples are incubated for longer time periods.

3. **Enzyme Concentration**

The NADH oxidized depended on the amount of plasma (enzyme) added to the reaction (Figure 12). Increasing concentrations of plasma resulted in equivalent increase in the amount of NADH oxidized. Results show that the reaction is higher not because of plasma ammonia, but because of PAO activity. For instance, the horse serum did not oxidize histamine but it catalyzed the oxidation of benzylamine. The rate of enzyme reaction is proportional to the enzyme concentration. (Figure 12).

4. **Effect of Cyanide**

The effects of cyanide (sodium cyanide) on plasma amine oxidase activity is shown in Table 24. As can be seen, 0.5mM of cyanide inhibited PAO.

5. Multiple substrates

To check for possible inhibitory effects of multiple substrate on enzyme activity, different mixes of substrates were tested on animal plasma known to be either positive or negative. Table 21 indicates that there is a mild inhibitory effect from the different substrates. Amphetamine has been reported to be a mild inhibitor of PAO (68). This points to the physiological condition in which multiple substrates do exist.

Paper Chromatography Assay

In the experiments summarized in Figures 4-11, we incubated a radioactive amine with plasma in phosphate buffer (0.2 M, pH 7.2). An aliquot was taken at zero (0) time and after three (3) hours of incubation, spotted on chromatography paper, and the chromatograms were developed with n-butanol:acetic acid:water, 14:4:50 (upper phase). The paper was dried, cut into strips, and the radioactivity determined with a scintillation counter. We measured the change in mobility of a substrate (usually tritium-labelled) after it was oxidized by the enzyme. The spot to which the substrate moved should have lower counts if it has been oxidized. No change in the number of counts indicates that either enzyme activity is absent, or that the substrate is a poor one. This is seen in a plot of a chromatogram of the control and that of the test plasma and substrate. The figures show the count per minute (cpm) as a function of distance migrated. Any plasma that showed a decrease of 50% of the zero time value was termed positive. Based on this criterion, Epinephrine, Norepinephrine, Tryptamine, L-Dopa, and Tyrosine were not oxidized by the plasma of any the mammals tested (Table 22). In

general, the paper chromatography results agree with both the benzylamine assay and the assay for ammonia release.

CHAPTER 4

DISCUSSION

This study was undertaken to investigate the distribution of PAO in different species of higher mammals and also to study the oxidation of different substrates by different sera. The study used only the "crude" enzyme, that is, enzyme that was not purified from plasma. Results reflect more closely the physiological conditions than would be expected using a purified enzyme. Purification may alter the enzyme structure, thereby changing its activity (73). Blaschko reported that the ability to oxidize 5-hydroxytyramine (serotonin) by pig plasma amine oxidase was lost upon purification (75).

In the case of the polyamines, results from this study and also from Tabor (70) indicated that spermidine and spermine are excellent substrates only for sera from the ruminant (cow). This activity has been called polyamine oxidase. Moreover, bovine plasma oxidized benzylamine, dopamine, histamine, and tyramine. According to Tabor et al. (64), spermine is oxidized at both terminal amino ends to form a dialdehyde, and spermidine is converted to the monoaldehyde. Blaschko (39) suggested that polyamine oxidation in ruminants is a result of the presence of microorganisms found in the rumen. These microbes produce the oxidase that can catalyze the oxidation of polyamines. Results from this study support the suggestion. For instance, newborn calf plasma showed no activity against spermine but was active against benzylamine (Table 23). Five

week old calf plasma showed slight activity against spermine while adult bovine showed considerable activity against both spermine and benzylamine. At this stage the rumen is well developed and has established the microflora known to produce polyamine oxidase. The rumen of the newborn has not developed a gut flora. Siler (69) believes that there is no function for the high polyamine oxidase activity by ruminants on polyamines. Of interest would be a study in which bovine plasma from germ-free animals are tested for the presence of activity against spermine both in the calf and adult. If no activity is observed at both stages, one can definitely conclude that the microbes produce the oxidase.

Of the phenylalkylamines (epinephrine, norepinephrine, tyramine, and dopamine), only tyramine and dopamine were oxidized by some of the plasma (cow and rabbit). McEwen et al. (43,61,69), used enzyme that had been purified 5000 times and assayed for enzyme activity using a higher substrate concentration (5mM). No activity was seen on epinephrine or norepinephrine in this study or from other species studied by other workers. Histamine oxidation was observed in cow, pig, and rabbit. Tabor et al. (70) did not find any activity in cow serum. This activity has been called Histaminase. McEwen observed slight activity using purified enzyme from the human serum against histamine. However, histamine cannot be accepted as the natural substrate since some species either attack it very slowly, or do not attack it at all. Studies show that histamine is a better substrate for the intracellular amine oxidases (2,3,6).

The benzylamine assay is fast and inexpensive. This assay measures the oxidation of benzylamine to benzaldehyde and can be followed spectrophotometrically

at 242 nm (66). Benzylamine is not a natural substrate, therefore, it is possible that some sera may not oxidize benzylamine even though they have plasma amine oxidase. In this study, benzylamine oxidation was observed in cow, dog, horse, pig, and rabbit sera using 1mM final substrate concentrations. This agrees with other authors. Tyramine oxidation was observed in the cow and rabbit. Tabor (70) did not observe activity against tyramine using purified bovine plasma while Blaschko (72), using 10^{-2} M substrate concentration, observed slight activity in dog and pig sera. McEwen (7,43,61) observed high activity against tyramine in human and rabbit sera. Buffoni and Della Corte (68), using a 10mM concentration (pH 7.4), found the pig enzyme to be active with benzylamine, dopamine, histamine, and tyramine.

Paper chromatography is used extensively, especially in pharmaceutical areas, to quantitatively estimate drug concentration and also to identify drugs. Descending paper chromatography is fast and was adapted for this study. The results found with the paper chromatography assay was helpful in identifying plasma of interest. The assay is more sensitive than the benzylamine assay and is particularly useful in studying the different plasma activity against biogenic amines. Radioactive materials pose a health risk and disposal problems. Furthermore, there exists an availability problem: they are either not readily available, e.g., ^{3}H Amphetamine, or they are extremely expensive, e.g., ^{3}H Benzylamine. Radioenzymatic assays are widely used and are suitable for routine analysis, but they are quite expensive. Gas - Liquid chromatography is very sensitive and may be the best assay method. However, like the radioactive assay, it is expensive.

In none of these studies was plasma amine oxidase activity found in either the

mouse, guinea pig, or the rat. This study has shown that these animals do not have significant enzyme activity against any of the substrates studied. An implication is that since it has generally been assumed that PAO is present in all higher mammals, and these mammals (mouse, rat, guinea pig) are common models for studying important biogenic and other pharmaceutically important amines, present forms of kinetic studies may not be valid.

The most obvious conclusion from this study is that there is no particular natural substrate for all the animals tested. The substrate specificity of plasma amine oxidase depends mainly on source of the enzyme, and each source has a different rate of oxidation for various amines. When substrate oxidation was analyzed for a given species, a particular substrate typically stood out, albeit with low response in most cases. Whether this is the natural substrate for the enzyme for this species cannot be claimed since all possible substrates were not tested. Furthermore, since benzylamine, not endogenous to higher mammals, is readily (and in some cases, rapidly) oxidized by most plasma, it is possible that a similar compound must exist in higher mammals that may be the natural substrate.

Because there is no unequivocal reference to the natural substrate, discussion of the physiological significance and the significance of plasma amine oxidase distribution becomes limited. Hence, some of the functions attributed to this enzyme are based partly on what their intracellular counterparts are known to do, i.e., biological inactivation of naturally occurring amines, implying that PAO and the classical amine oxidases may be isoenzymes. This definitely fits in with the definition of isoenzyme by the international

enzyme nomenclature (11). Both forms of enzyme follow the same general equation during the oxidation of amines (see page 1), so they must be closely related despite their multiple forms, inhibitor and substrate specificities, and prosthetic group differences.

The different degrees of PAO activities could be due to the presence of inhibitors in the serum. For instance, Buffoni et al. (80) showed that the human plasma amine oxidase is low because there is an inhibitor present in the plasma. Also, our results from Table 21 have shown that the effect of a mild inhibition due to multiple substrates could lead to a reduced enzyme activity seen in some of the results. With this in mind, it may be that with pure enzyme, observations for all species could be the same. Blaschko (39) suggested that the different reactivities of amine oxidases from different origins must be due to enzyme protein variation, leading to different structural conformations and affecting the substrate and metal specificities. Such protein variations could result from mutations which, through time, became incorporated into the animal system.

The biological role of plasma amine oxidase is not limited to the inactivation of endogenous amines. In a situation where there is exposure to toxic amines, it would be expected that some of the amine would reach the bloodstream through the gastrointestinal tract. These amines are inactivated by PAO. Also, the products of amine oxidation have been shown to have biological activities, e.g., polyamine oxidation products in ruminants (74). Polyamine oxidation products usually undergo secondary reactions. One product is acrolein, known to be toxic to parasites e.g., Trypanosoma equiperdum, as well as to animal and plant viruses. Acrolein is also toxic to tumor cells (74).

The presence of plasma amine oxidase along with the other amine oxidases can thus be viewed as a positive evolutionary selection because amines, both endogenous and exogenous, can be oxidized and eliminated from the system.

This study has shown that not all mammals have the PAO enzyme. In this respect caution should be taken when performing toxicological tests on pharmaceutically significant drug which are amines (especially primary amines).

SUMMARY

Plasma amine oxidase is thought to be ubiquitous. It has been isolated from various sources. The enzyme functions to actively inactivate endogenous and exogenous amines.

This study was undertaken to assess the distribution of plasma amine oxidase in diverse mammalian species. Results with three different assays (benzylamine assay, paper chromatography assay, and assay for the release of ammonia) show that the enzyme is not present in all mammals. In some plasma, e.g., human, the activity recorded was present but low. High activities were observed in the cow, pig, and rabbit. However, activities depended highly on the substrate being tested. The commonly used animal models (murine, guinea pig, hamster) did not exhibit any activity toward the amines tested. Future studies on the metabolism of primary amines should consider that the rodent may not be an appropriate model. The rabbit, another commonly used animal model, is much more active than human in the oxidation of amines.

It has been suggested that polyamine may be the natural substrates for the enzyme. This study has shown that this is not the case since only the ruminant (and only the adult ones) showed activity against polyamines.

The study was unable to identify a natural substrate for this enzyme, but it is assumed that the substrate would be similar to benzylamine (for example, tyramine, dopamine, or β-phenylethylamine).

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APPENDIX

Abbreviations

Amp = Amphetamine

Ben = Benzylamine

Dop = Dopamine

Epi = Epinephrine

His = Histamine

L-do = L-Dopa

Nor(e) = Norepinephrine

Put = Putrescine

Spd = Spermidine

Spe = Spermine

Try = Tryptamine

Tya = Tyramine

Tyo = Tyrosine

Ref = Reference

Y or ++ = Yes (plasma amine oxidase activity present)

N or -- = No (No enzyme activity)

NA = Not Tested

Table 1. Benzylamine Assay reaction chart.

	1	2
Phosphate buffer (0.2 M, pH 7.2)	0.75 ml	0.75 ml
*Benzylamine	0.15 ml	0.15 ml
Plasma	0.60 ml	0.60 ml
60% PCA (0.15 ml)	0 hour	3 hours
Cyclohexane	1.50 ml	1.50 ml

For tube # 1, benzylamine was added after 3 hours of incubation at 37 degrees in a water bath with a shaker. Cyclohexane was added after incubation and the reaction tube vortexed briefly to obtain an emulsion. After 15 minutes, the tube was again briefly vortexed and centrifuged at 2500 rpm for 5 minutes to separate the phases.

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* Diluted in phosphate buffer (0.2M, pH 7.2) to give a final concentration of 8 mM.

Table 2. Reaction chart for obtaining samples for ammonia assay (Test samples)

	1	2
Phosphate buffer (0.2 M, pH 7.2)	0.75 ml	0.75 ml
*Substrate	0.15 ml	0.15 ml
Plasma	0.60 ml	0.60 ml

After 3 hours of incubation, 0.15 ml of substrate was added to tube # 1. The tubes were then chilled in an ice bucket and the ammonia assay carried out as in Table 3.

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* Diluted in phosphate buffer (0.2 M, pH 7.2) to give a final concentration of 10^{-2}M.

Table 3. Ammonia assay reaction chart.

	Blank	Control	Test
*Ammonia Assay Solution	3.0 ml	3.0 ml	3.0 ml
Water (Ammonia-free)	0.2 ml	------	------
**Ammonia Control Solution	------	0.2 ml	------
Test Samples	------	------	0.2 ml
L-Glutamate Dehydrogenase	0.02 ml	0.02 ml	0.02 ml

Each cuvette was gently mixed by inversion. After
5 minutes, the cuvette was read on a spectrophotometer
(Shimadzu UV-160) at 340 nm versus ammonia-free water
This is the first reading.
The enzyme L-glutamate dehydrogenase was then added.
For the second reading, each cuvette was again mixed
by inversion and read after 5 minutes.

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* Reconstituted with ammonia-free water to give 2 mmol
of 2-oxoglutarate, 0.12 mmol of NADH, and 3000 U of
Bovine Lactate Dehydrogenase.
** 5 ug/ml of Ammonia (Ammonium sulfate) as the positive control.

Table 4. Reaction chart to test for reproducibility
and reliability of Sigma Ammonia detection kit.

Tube #	1	2	3	4
Ammonia Assay Solution	3.0 ml	3.0 ml	3.0 ml	3.0 ml
Test Sample	0.05 ml	0.05 ml	0.05 ml	0.05 ml
Water (ammonia-free)	0.15 ml	0.10 ml	0.05 ml	------
Ammonium Sulfate	------	0.05 ml	0.10 ml	0.15 ml
L-Glutamate Dehydrogenase	0.02 ml	0.02 ml	0.02 ml	0.02 ml

Sample was gently mixed and read at 340 nm on
a spectrophotometer (Shimadzu UV-160). This is the first reading.
L-glutamate dehydrogenase was then added.
For the second reading, sample was again mixed by inversion and
read after 5 minutes.

Table 5. Reaction chart for checking the effect of substrate concentrations on plasma amine oxidase activity.

	1,2	3,4	5,6	7,8
Phosphate buffer (0.2 M, pH 7.2)	0.85 ml	0.75 ml	0.60 ml	0.30 ml
Plasma	0.15 ml	0.15 ml	0.15 ml	0.15 ml
*Substrate	0.05 ml	0.15 ml	0.30 ml	0.60 ml

Substrates were added to the odd-numbered tubes after 3 hours of incubation in a water bath shaker set at 37 degrees. After incubation, the tubes were chilled and ammonia assay carried out as in Table 3.

Table 6. Reaction chart to check for the effect of increasing levels of plasma (enzyme).

	1,2	3,4	5,6
Phosphate buffer (0.2 M, pH 7.2)	1.05 ml	0.75 ml	0.45 ml
*Substrate	0.15 ml	0.15 ml	0.15 ml
Plasma Enzyme	0.30 ml	0.60 ml	0.90 ml

The odd-numbered tubes were used as control and serum was added after the 3 hour incubation. The tubes were then chilled and ammonia assay carried out as in Table 3.

---

* Diluted in phosphate buffer (0.2 M, pH 7.2) to give a final concentration of 10^{-2}M.

Table 7. Reaction chart for Paper Chromatography Assay.

	1	2
Phosphate buffer (0.2 M, pH 7.2)	0.12 ml	0.12 ml
Plasma	0.02 ml	0.02 ml
Substrate (Tritium-labelled)	0.10 ml	0.10 ml
60% PCA (0.20 ml)	0 hour	3 hours

Tubes were incubated for 3 hours at 37 degrees in a water bath shaker. Amine was then added to tube # 1 and PCA added immediately to both tubes to stop the reaction.

Table 8. Spectrophotometric results from ammonia assay (at 340 nm) for the cat

Compound	Control	Test	*Change	Response
Amp	0.26	0.26	0.00	N
Ben	0.03	0.34	0.32	Y
Dop	0.24	0.28	0.04	N
Epi	0.28	0.23	0.00	N
His	0.34	0.36	0.02	N
L-do	0.09	0.08	0.00	N
Nore	0.21	0.21	0.00	N
Put	0.07	0.07	0.00	N
Spd	0.03	0.03	0.00	N
Spe	0.03	0.03	0.00	N
Try	0.07	0.08	0.01	N
Tya	0.07	0.09	0.02	N
Tyo	0.08	0.07	0.00	N

* Change = Test O.D - Control O.D

Table 9. Spectrophotometric results from ammonia assay (at 340 nm) for the cow.

Compound	Control	Test	* Change	Response
Amp	0.06	0.06	0.00	N
Ben	0.15	0.64	0.49	Y
Dop	0.27	0.68	0.41	Y
Epi	0.23	0.26	0.03	N
His	0.35	0.50	0.15	Y
L-do	0.07	0.06	0.00	N
Nore	0.20	0.21	0.01	N
Put	0.03	0.08	0.05	N
Spd	0.42	0.88	0.46	Y
Spe	0.12	0.75	0.63	Y
Try	0.06	0.09	0.03	N
Tya	0.06	0.34	0.28	Y
Tyo	0.07	0.07	0.00	N

Table 10. Spectrophotometric results from ammonia assay (at 340 nm) for the Dog.

Compound	Control	Test	* Change	Response
Amp	0.06	0.07	0.01	N
Ben	0.07	0.17	0.10	Y
Dop	0.24	0.29	0.05	N
Epi	0.23	0.23	0.00	N
His	0.36	0.36	0.00	N
L-do	0.08	0.08	0.00	N
Nore	0.21	0.21	0.00	N
Put	0.05	0.06	0.01	N
Spd	0.03	0.03	0.00	N
Spe	0.03	0.03	0.00	N
Try	0.06	0.09	0.03	N
Tya	0.07	0.12	0.05	N
Tyo	0.09	0.08	0.00	N

* Change = Test O.D. - Control O.D.

Table 11. Spectrophotometric results from ammonia assay (at 340 nm) for the Hamster.

Compound	Control	Test	* Change	Response
Amp	0.08	0.09	0.01	N
Ben	0.10	0.13	0.03	N
Dop	0.14	0.16	0.02	N
Epi	0.08	0.09	0.01	N
His	0.17	0.18	0.01	N
L-do	0.12	0.13	0.01	N
Nore	0.12	0.12	0.00	N
Put	0.06	0.06	0.00	N
Spd	0.10	0.09	0.00	N
Spe	0.13	0.13	0.00	N
Try	0.08	0.08	0.00	N
Tya	0.07	0.12	0.05	N
Tyo	0.09	0.08	0.00	N

Table 12. Spectrophotometric results from ammonia assay (at 340 nm) for the Horse.

Compound	Control	Test	* Change	Response
Amp	0.23	0.23	0.00	N
Ben	0.09	0.28	0.19	Y
Dop	0.24	0.31	0.07	N
Epi	0.24	0.25	0.01	N
His	0.35	0.38	0.03	N
L-do	0.06	0.06	0.00	N
Nore	0.24	0.24	0.00	N
Put	0.04	0.04	0.00	N
Spd	0.03	0.03	0.00	N
Spe	0.05	0.05	0.00	N
Try	0.24	0.28	0.04	N
Tya	0.05	0.15	0.10	Y
Tyo	0.04	0.04	0.00	N

* Change = Test O.D. - Control O.D.

Table 13. Spectrophotometric results from ammonia assay (at 340 nm) for Human.

Compound	Control	Test	* Change	Response
Amp	0.21	0.21	0.00	N
Ben	0.07	0.07	0.00	N
Dop	0.05	0.06	0.01	N
Epi	0.05	0.06	0.01	N
His	0.18	0.17	0.00	N
L-do	0.07	0.07	0.00	N
Nore	0.21	0.21	0.00	N
Put	0.05	0.06	0.01	N
Spd	0.05	0.05	0.00	N
Spe	0.06	0.06	0.00	N
Try	0.07	0.06	0.00	N
Tya	0.06	0.08	0.02	N
Tyo	0.07	0.07	0.00	N

Table 14. Spectrophotometric results from ammonia assay (at 340 nm) for the Mouse.

Compound	Control	Test	* Change	Response
Amp	0.07	0.07	0.00	N
Ben	0.06	0.07	0.01	N
Dop	0.25	0.25	0.00	N
Epi	0.04	0.05	0.01	N
His	0.20	0.21	0.01	N
L-do	0.10	0.10	0.00	N
Nore	0.08	0.07	0.00	N
Put	0.02	0.02	0.00	N
Spd	0.11	0.13	0.02	N
Spe	0.07	0.07	0.00	N
Try	0.07	0.06	0.00	N
Tya	0.10	0.09	0.00	N
Tyo	0.10	0.10	0.00	N

* Change = Test O.D. - Control O.D.

Table 15. Spectrophotometric results from ammomnia assay (at 340 nm) for the Pig.

Compound	Control	Test	* Change	Response
Amp	0.22	0.22	0.00	N
Ben	0.07	0.24	0.17	Y
Dop	0.08	0.14	0.06	N
Epi	0.08	0.08	0.00	N
His	0.37	0.53	0.16	Y
L-do	0.07	0.07	0.00	N
Nore	0.07	0.07	0.00	N
Put	0.06	0.07	0.01	N
Spd	0.06	0.06	0.00	N
Spe	0.04	0.05	0.01	N
Try	0.06	0.06	0.00	N
Tya	0.06	0.06	0.00	N
Tyo	0.07	0.06	0.00	N

Table 16. Spectrophotometric results from ammonia assay (at 340 nm) for Rabbit.

Compound	Control	Test	* Change	Response
Amp	0.20	0.20	0.00	N
Ben	0.07	0.21	0.14	Y
Dop	0.23	0.40	0.17	Y
Epi	0.21	0.28	0.07	N
His	0.37	0.51	0.14	Y
L-do	0.10	0.09	0.00	N
Nore	0.06	0.06	0.00	N
Put	0.06	0.06	0.00	N
Spd	0.05	0.05	0.00	N
Spe	0.12	0.09	0.00	N
Try	0.06	0.13	0.07	N
Tya	0.01	0.21	0.20	Y
Tyo	0.05	0.06	0.01	N

* Change = Test O.D. - Control O.D.

Table 17. Spectrophotometric results from ammonia assay (at 340 nm) for the Rat.

Compound	Control	Test	* Change	Response
Amp	0.07	0.07	0.00	N
Ben	0.07	0.07	0.00	N
Dop	0.23	0.14	0.00	N
Epi	0.09	0.10	0.00	N
His	0.15	0.15	0.00	N
L-do	0.07	0.10	0.03	N
Nore	0.06	0.06	0.00	N
Put	0.05	0.06	0.01	N
Spd	0.04	0.04	0.00	N
Spe	0.12	0.12	0.00	N
Try	0.06	0.05	0.00	N
Tya	0.06	0.07	0.01	N
Tyo	0.08	0.09	0.01	N

* Change = Test O.D. - Control O.D.

Table 18. Results from Benzylamine Assay in diverse species.

Animal	A242	*Relative oxidation of Benzylamine (%)
Cat	1.40	59.00
Cow	2.24	93.00
Dog	2.40	96.00
Guinea Pig	0.00	0.00
Hamster	0.05	2.00
Horse	2.19	88.00
Human	0.43	17.00
Mouse	0.04	1.00
Pig	2.33	94.00
Rabbit	2.50	100.00
Rat	0.00	0.00

* Highest change in O.D. at 242 nm (2.50) is taken as 100%

Table 19. Mouse Plasma does not oxidize Benzylamine nor does it inhibit the oxidation of Benzylamine by Rabbit plasma.

PLASMA	A242
MOUSE	0.042
RABBIT	1.180
RABBIT + MOUSE	1.200
BLANK	0.020

Table 20. Plasma with no amine oxidase activity does not inhibit the oxidation of Benzylamine by other plasma

Plasma Mix	A242
RABBIT + HUMAN	2.39
MOUSE + RAT	0.01
DOG + COW	2.30
COW + HAMSTER	2.31

Table 21. Possible effects of mixing substrates on plasma amine oxidase activity.

Plasma	Substrate mix	Ammonia release ug/ml*	Individual ammonia release ug/ml**
Cow	Ben + His	7.32	25, 6
	Spd + Amp	4.82	15, 0
Hamster	Spd + Try	4.58	15, 1.5
	Spd + Ben	0	0, 1.5
	Ben + Amp	4.61	10, 0
	Ben + Try	4.50	10, 2
Horse	Ben + Tyo	4.78	10, 0
	Dop + Tyo	1.02	3, 0
Pig	Ben + His	4.61	9, 7
Rabbit	Dop + L-do	3.87	8, 0
Rat	Dop + L-do	0	0, 0
Pig + Rat	Ben + Amp	3.06	-----
Standard	Ammonium sulfate	5.0	5.0

* Represents reaction mixture, not plasma concentration.
** Ammonia released for each substrate from each plasma.

Table 22. Results from Paper Chromatography Assay.

Species / Substrate	Cat	Cow	Dog	Guinea Pig	Horse	Human	Mouse	Pig	Rabbit	Rat
Dop	*10	76	3	3	18	7	2	7	83	4
Epi	4	6	2	5	0.8	2	6	7	8	2
His	0.4	82	6	7	10	6	8	81	71	8
L-do	8	4	2	5	8	5	2	4.6	5	8
Nore	3	5	10	3	9	9	5	4	10	10
Try	0.8	4	8	0.3	3	3	0.3	1.4	1	2
Tya	0.7	73	19	3	79	8	2	8	82	2
Tyo	4	0	4	7	10	3	7	9	7	8

* % oxidation : [Peak(control) - Peak(test)] / Peak(control) x 100. See Text.

Table 23. Results from Ammonia Release Assay (ug/ml).

Species / Substrate	Cat	Cow	Dog	Hamster	Horse	Human	Mouse	Pig	Rabbit	Rat
Amp	0.08	0	0.48	0.22	0.08	0	0.17	0.22	0	0
Ben	3.8	24.86	4.53	1.14	9.55	0.04	0.4	8.65	6.51	0
		*(8.10)								
Dop	2.55	19.27	1.89	0	2.82	0.44	0	2.68	7.17	0
Epi	0	0.79	0	0	0.29	0.66	0.97	0	0.84	0
His	0.84	5.78	0.38	0.53	1.14	0	0.4	6.82	6.16	0
L-do	0	0	0	0.26	0.04	0	0.18	0.04	0	0
Nore	0	0	0	0.13	0	0	0	0.08	0.31	0.22
Put	0	1.12	0.31	0	0	0	0.04	0.22	0	0
Spd	0	14.77	0	0	0	0	0.74	0	0.22	0
Spe	0.31	27.9	0.13	0.31	0.09	0.09	0.26	0.44	0	1.62
		*(0.00)								
Try	0.57	1.27	1.14	0.09	1.58	0	0.09	0	2.77	0
Tya	0.88	12.58	1.9	0.75	3.9	0.8	0	0.09	6.12	0.48
Tyo	0	0.26	0	0	0	0	0.09	0	0.04	0

*Calf plasma, less than 24 hours old.
Standard = 5 ug/ml

Table 24. The effect of cyanide (sodium) on plasma amine oxidase activity. Cyanide inhibits enzyme activity.

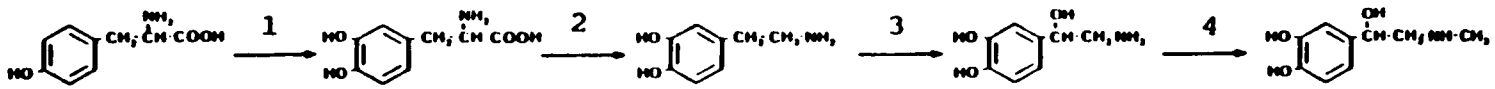
Plasma	Substrate	A340	
		with cyanide	without cyanide
Cow	Ben	0.00	2.24
	Dop	0.00	2.04
Horse	Ben	0.19	2.19

Table 25. Comparison of the distribution of Plasma Amine Oxidase from various authors.

Species	Substrate													
	Amp	Ben	Dop	Epi	His	L-do	Nor	Put	Spd	Spe	Try	Tya	Tyo	Ref.
Cat	--	--	--	--	--	--	--	--	--	--	--	--	--	*
Cow	--	++ @,++ %,+	++	-- %,--	++ %,--	--	-- %,--	--	++ @,+ %,++	+++ %,+++	-- %,--	++ %,--	-- --	* 70 70
Dog	--	++ %,++	--	--	-- %,--	--	--	-- %,+	-- %,--	-- %,--	-- %,+	-- %,+	--	* 72
Guinea Pig	--	--	--	--	--	--	--	--	--	--	--	--	--	*
Hamster	--	--	--	--	--	--	--	--	--	--	--	--	--	*
Horse	--	++ %,++	--	--	-- %,--	--	--	-- %,--	-- %,--	-- %,--	-- %,+/-	-- %,+/-	--	* 72
Human	--	--	-- @,++ @,++	--	-- @,+ @,+	--	-- @,- @,--	-- @,-	-- @,-- @,-	--	-- @,++ @,++	-- @,++ @,+++	--	* 61 7
Mouse	--	--	--	--	--	--	--	--	--	--	--	--	--	*
Pig	--	++ %,+ %,++	-- %,++	--	++ %,+ %,++	--	-- %,--	-- %,-- %,--	-- %,-- %,--	-- %,-- %,--	-- %,+ %,--	-- %,+ %,+	--	* 72 68
Rabbit	--	++	++ @,++	--	++ @,+/-	--	-- @,--	-- @,--	-- @,--	-- @,--	-- %,+ @,+	++ @,++	--	* 43 70
Rat	--	--	--	--	--	--	--	--	--	--	--	--	--	*

* This thesis

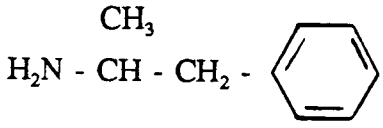
@ = ammonia assay; % = oxygen consumption; ++ = Enzyme present; -- = Enzyme absent



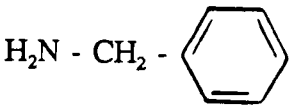
Tyrosine Dopa Dopamine Norepinephrine Epinephrine

1 = Tyrosine hydroxylase
2 = Dopa decarboxylase
3 = Dopamine β-hydroxylase
4 = Phenylethanolamine N-methyltransferase

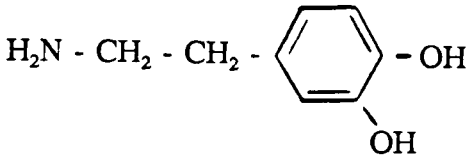
Figure 1. Catecholamine biosynthetic pathway



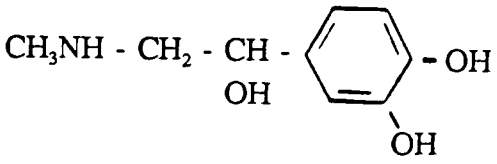
Amphetamine



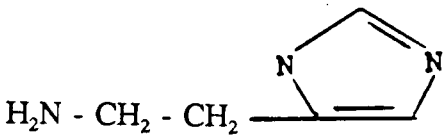
Benzylamine



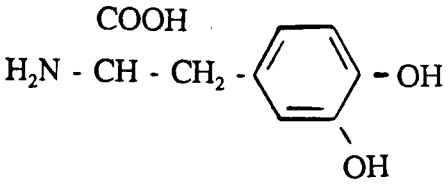
Dopamine



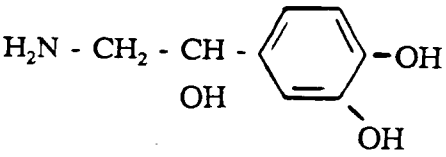
Epinephrine



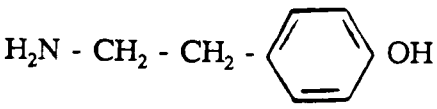
Histamine



L-Dopa

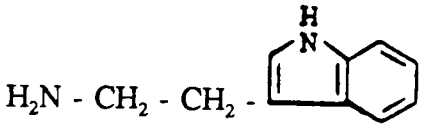


Norepinephrine

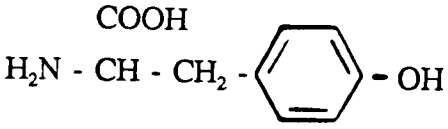


Tyramine

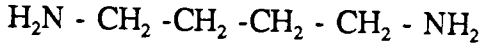
Figure 2. Substrate structures.



Tryptamine

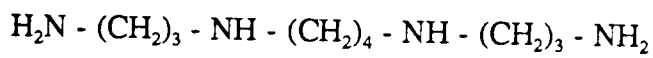


Tyrosine



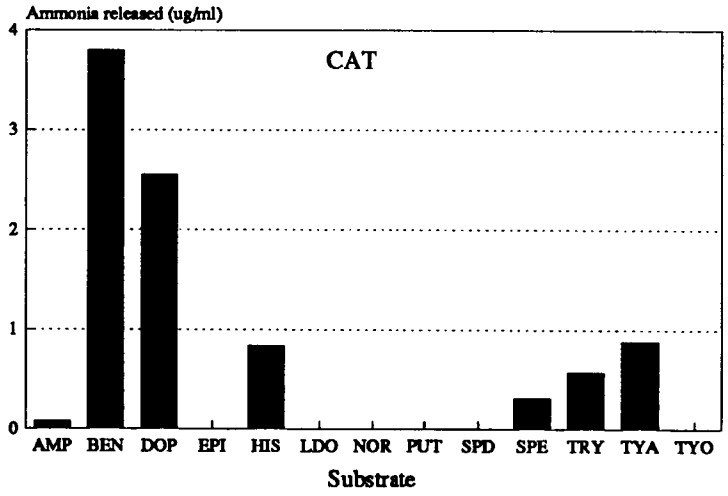
Putrescine

Spermidine



Spermine

Figure 2. Substrate structures. Continued.



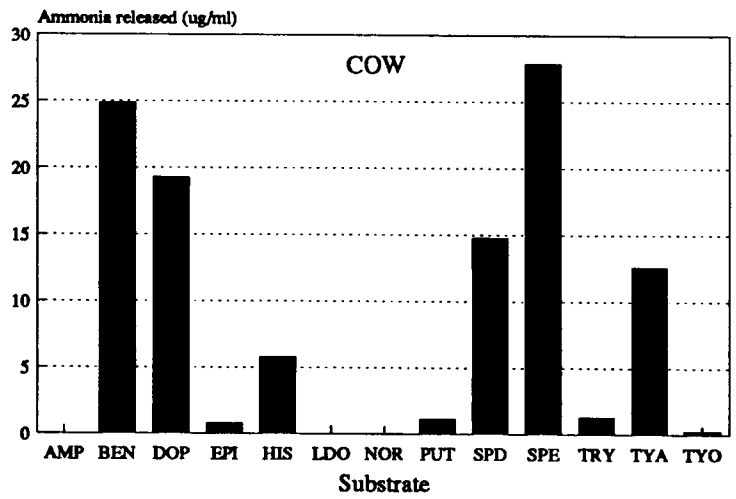
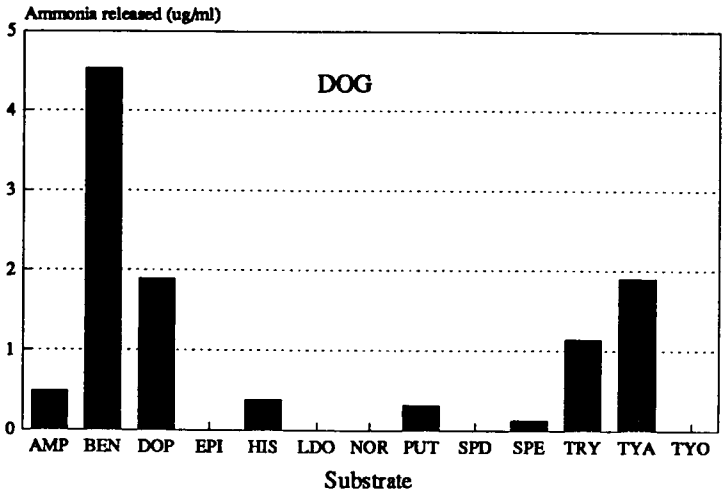
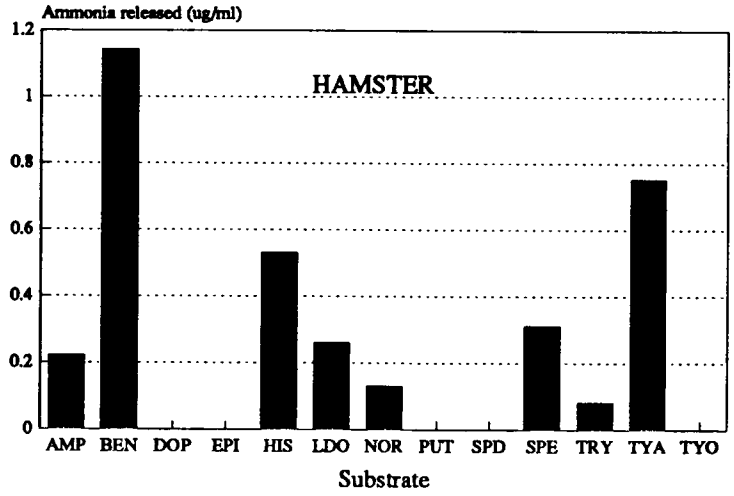
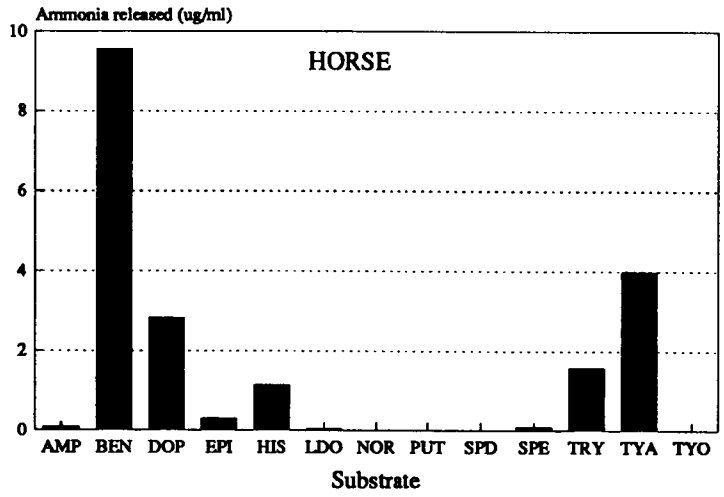


Figure 3. Ammonia Release Assay (340 nm) for Diverse Plasma.







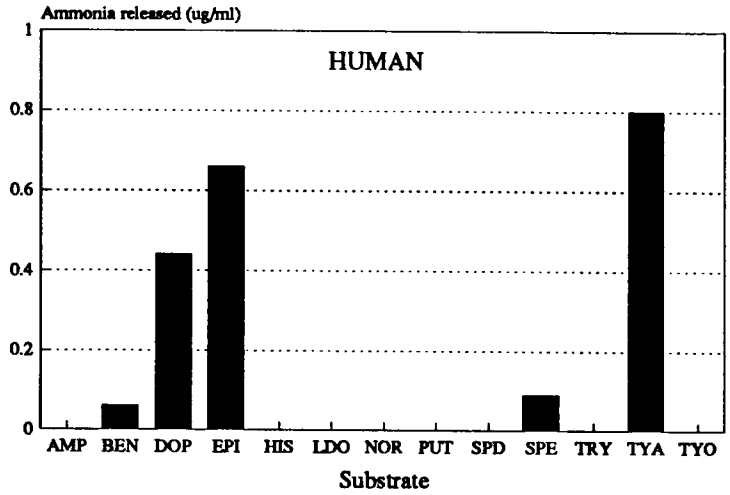
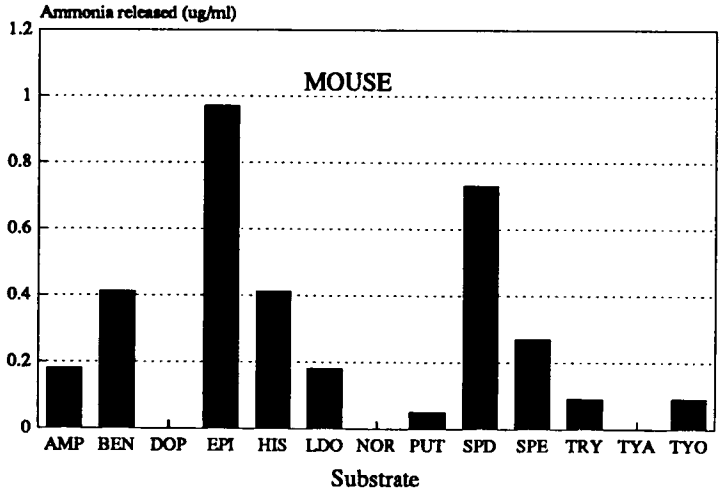
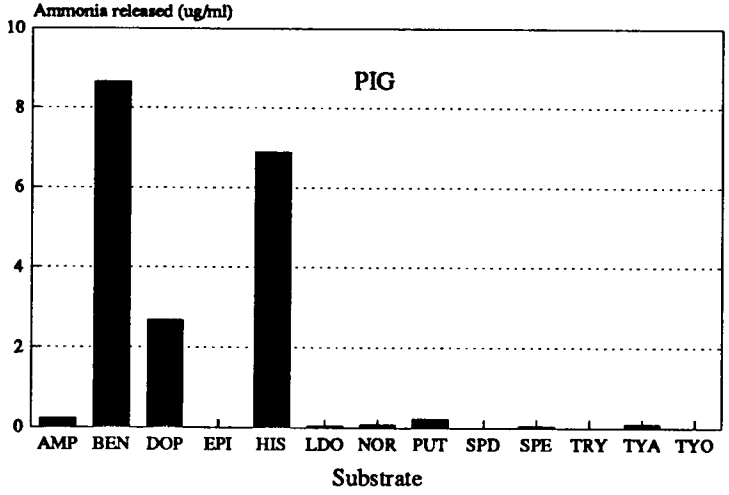


Figure 3. Continued.





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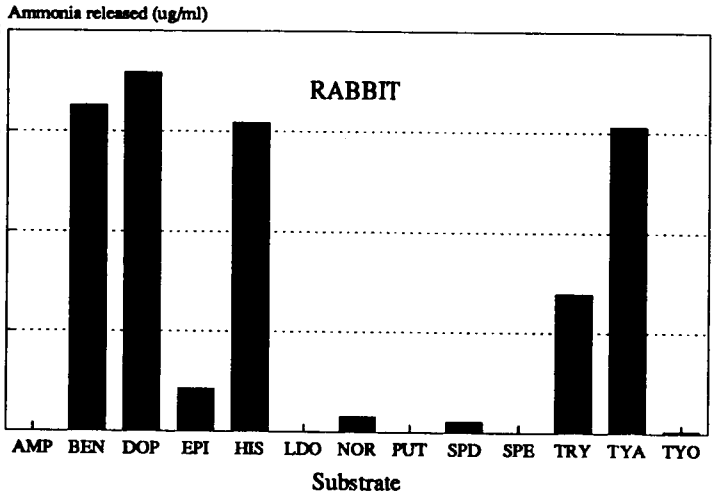
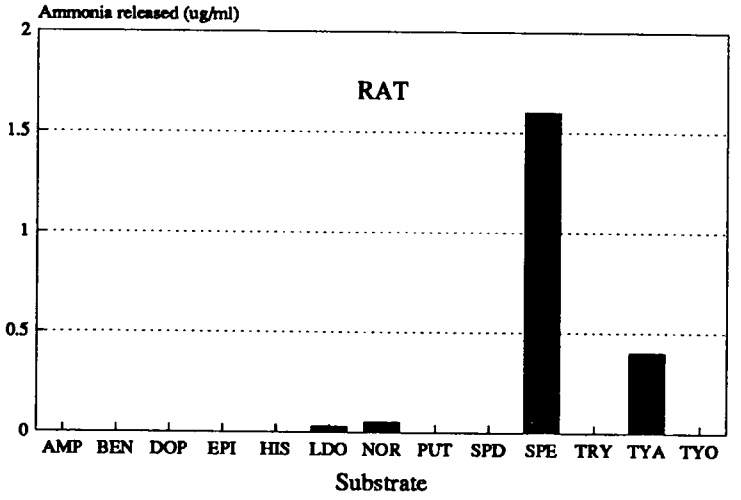




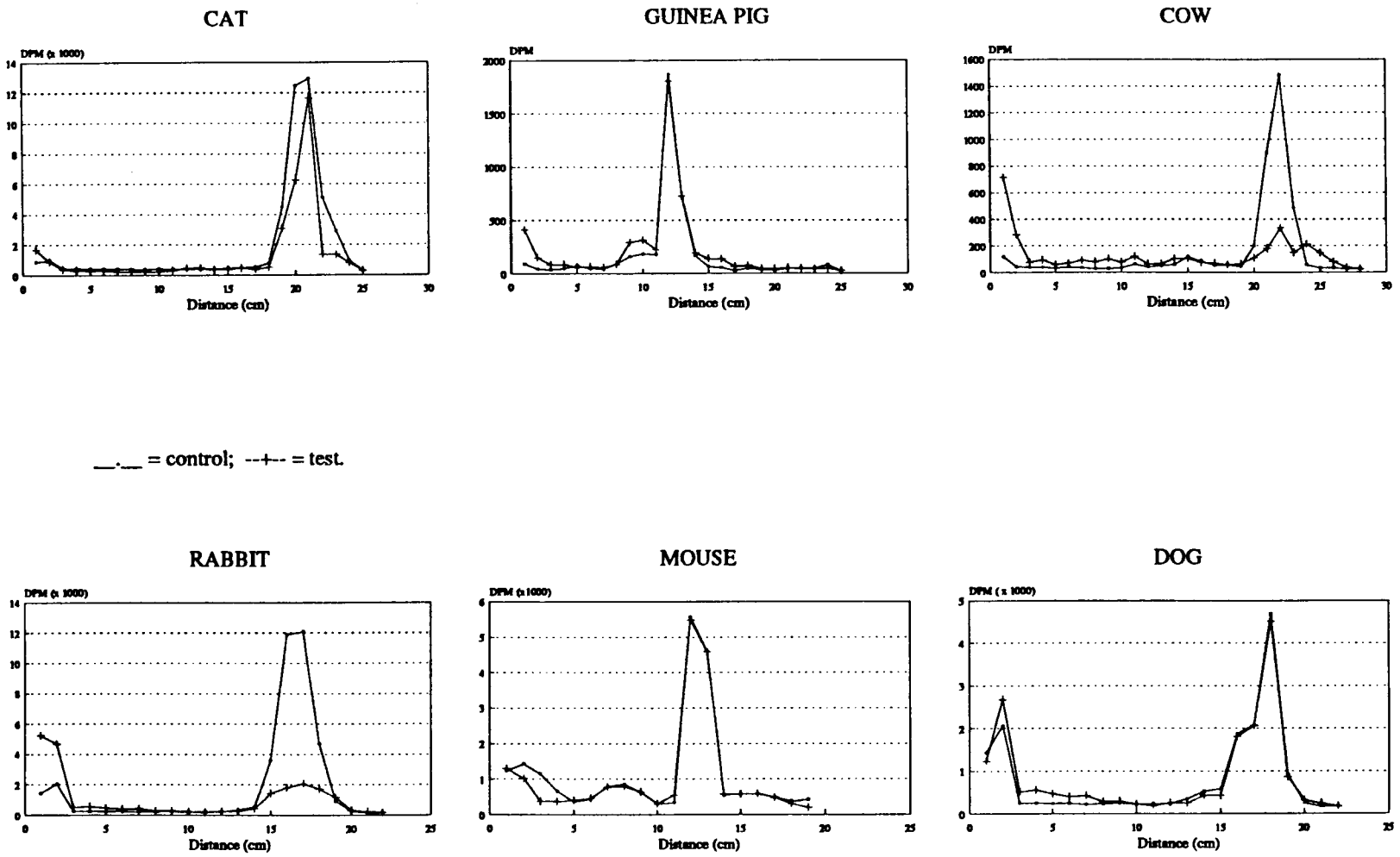
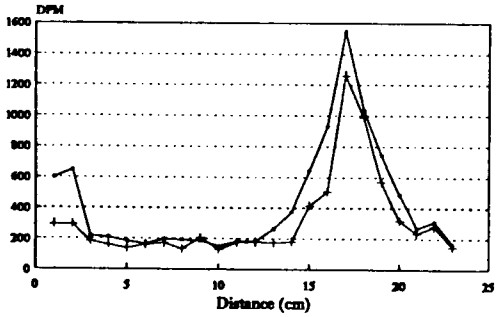


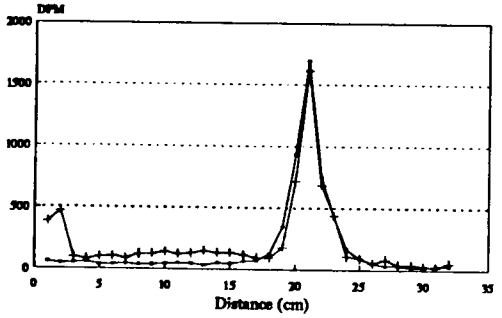
Figure 4. Paper Chromatography Assay results on Dopamine

HORSE

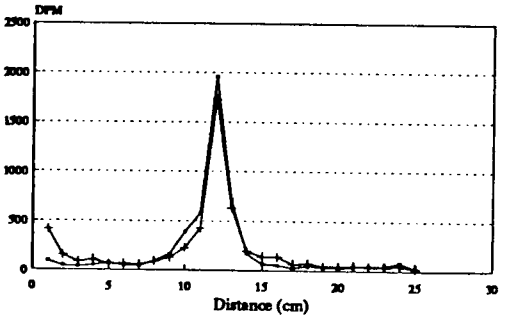


--.-- = control; --+-- = test.

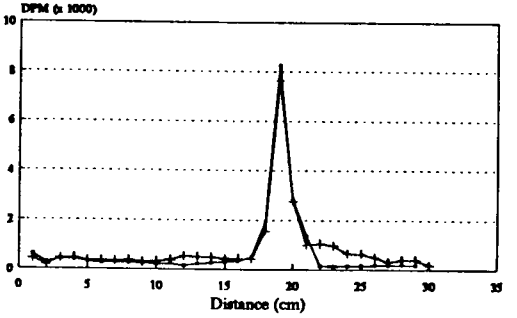
RAT



PIG



HUMAN



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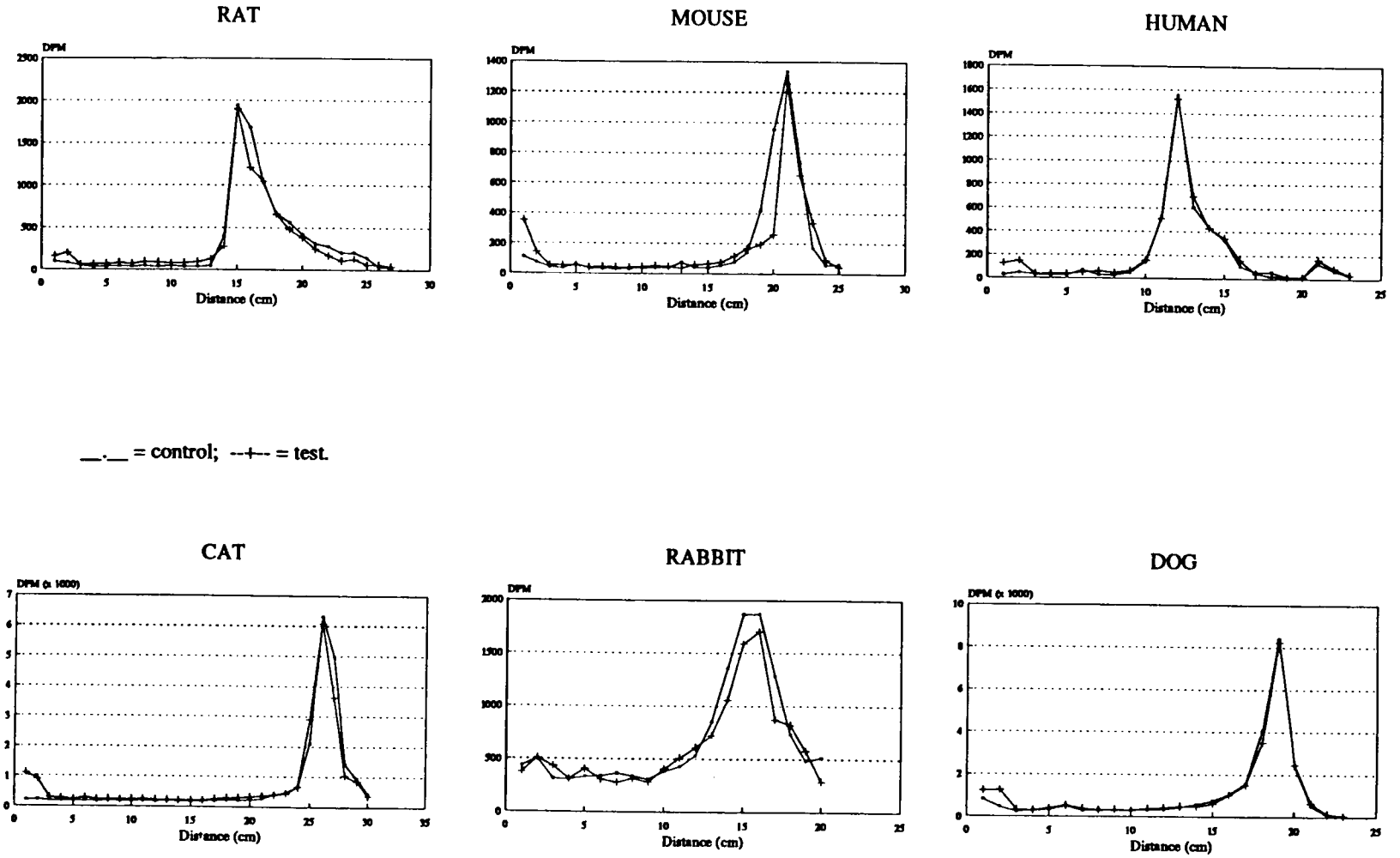
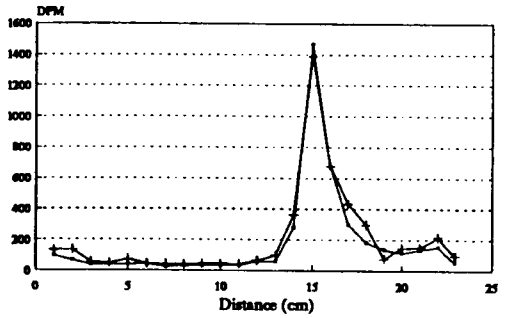


Figure 5. Paper Chromatography Assay results for Epinephrine

GUINEA PIG



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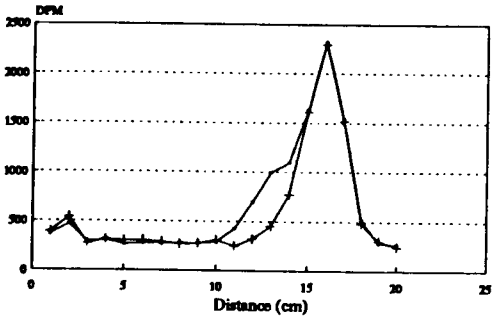
HORSE



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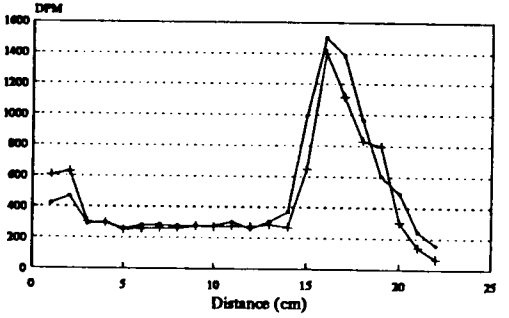
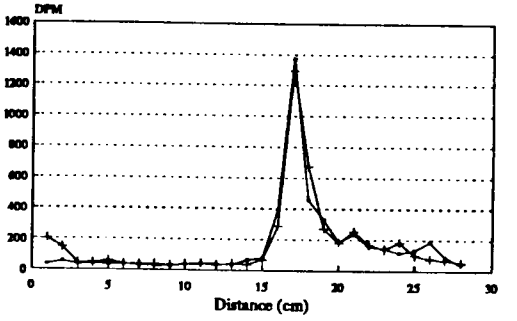


COW



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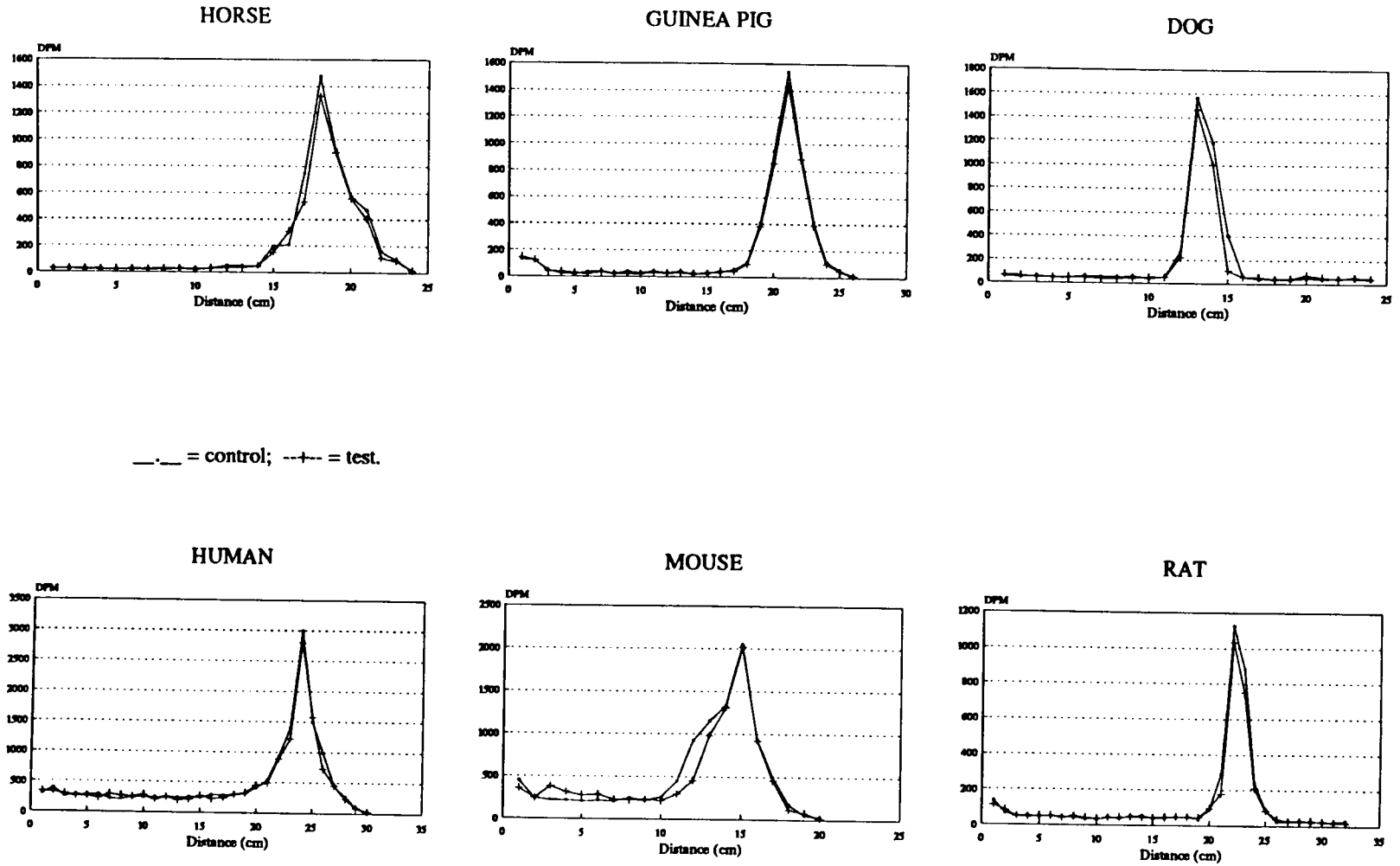
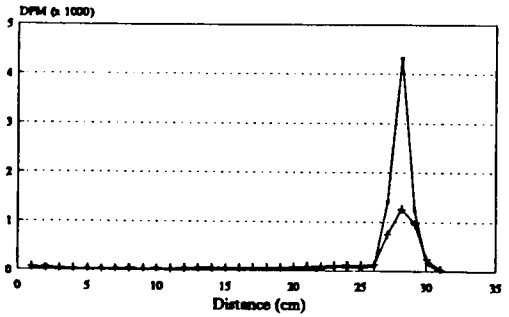


Figure 6. Paper Chromatography Assay results for Histamine.

RABBIT



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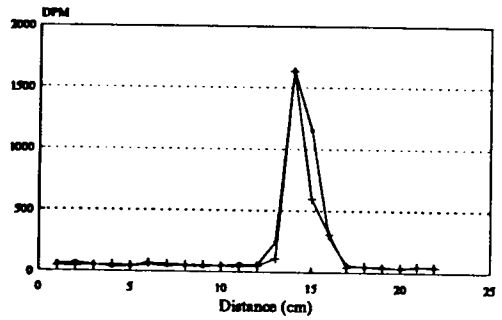
CAT



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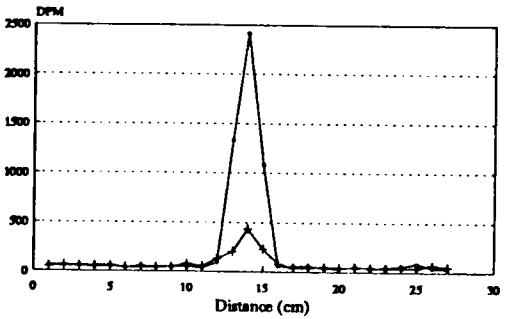

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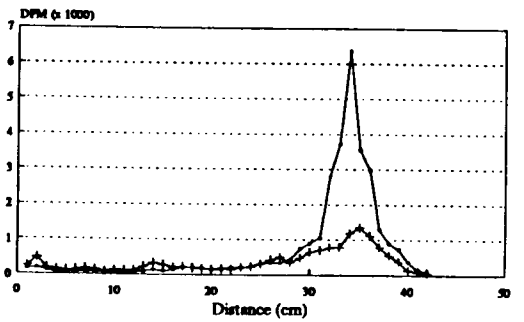

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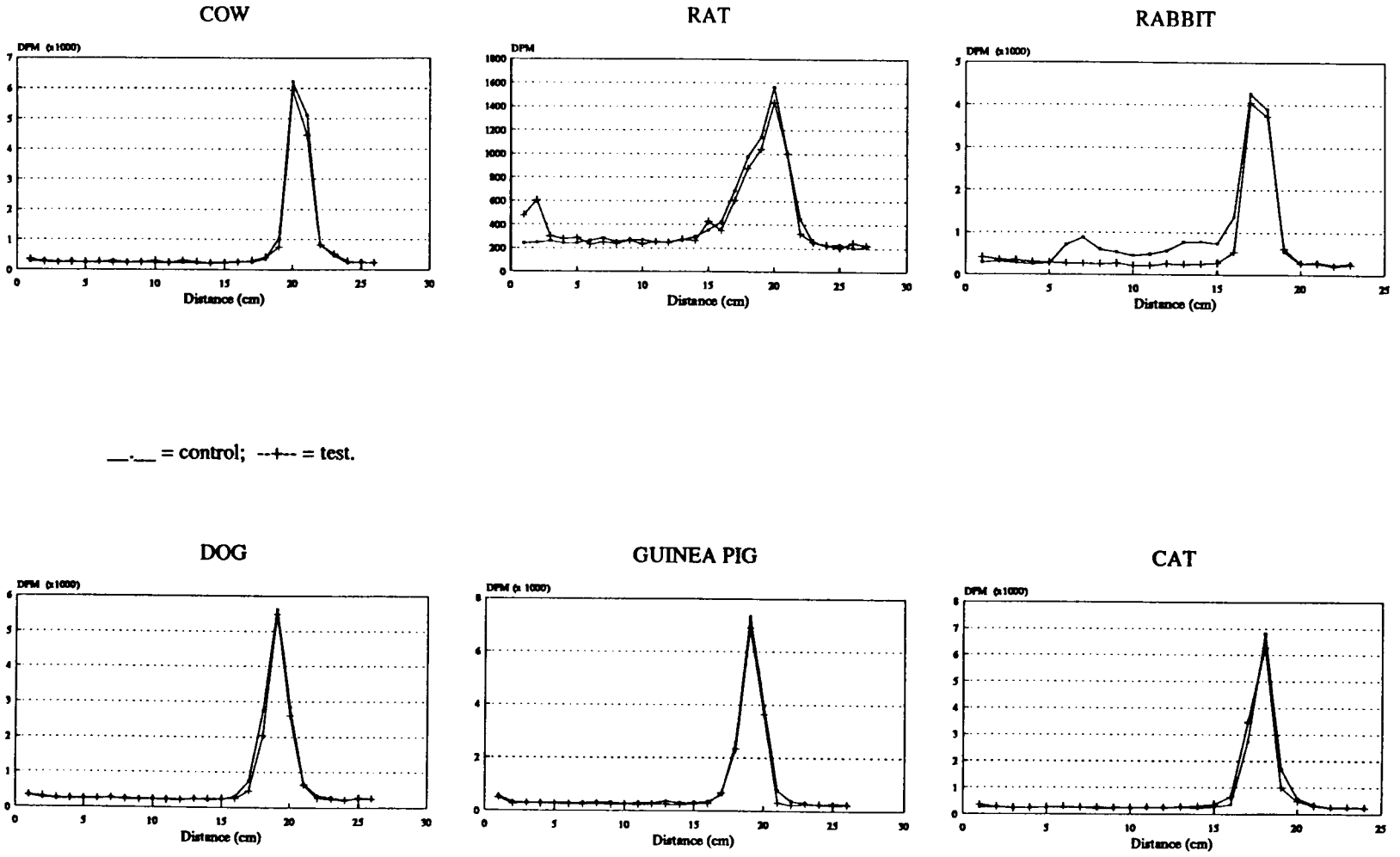
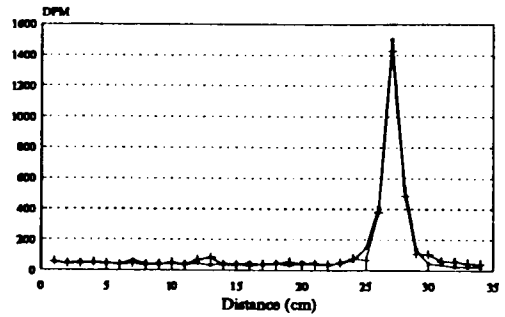


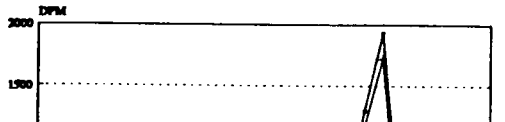
Figure 7. Paper Chromatography Assay results on L-Dopa.

PIG

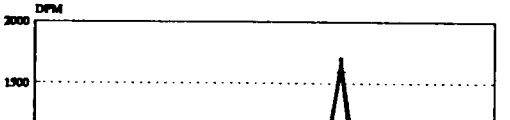


__.__ = control; --+-- = test.

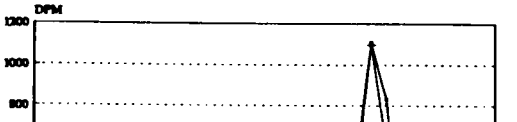
MOUSE



HUMAN



HORSE



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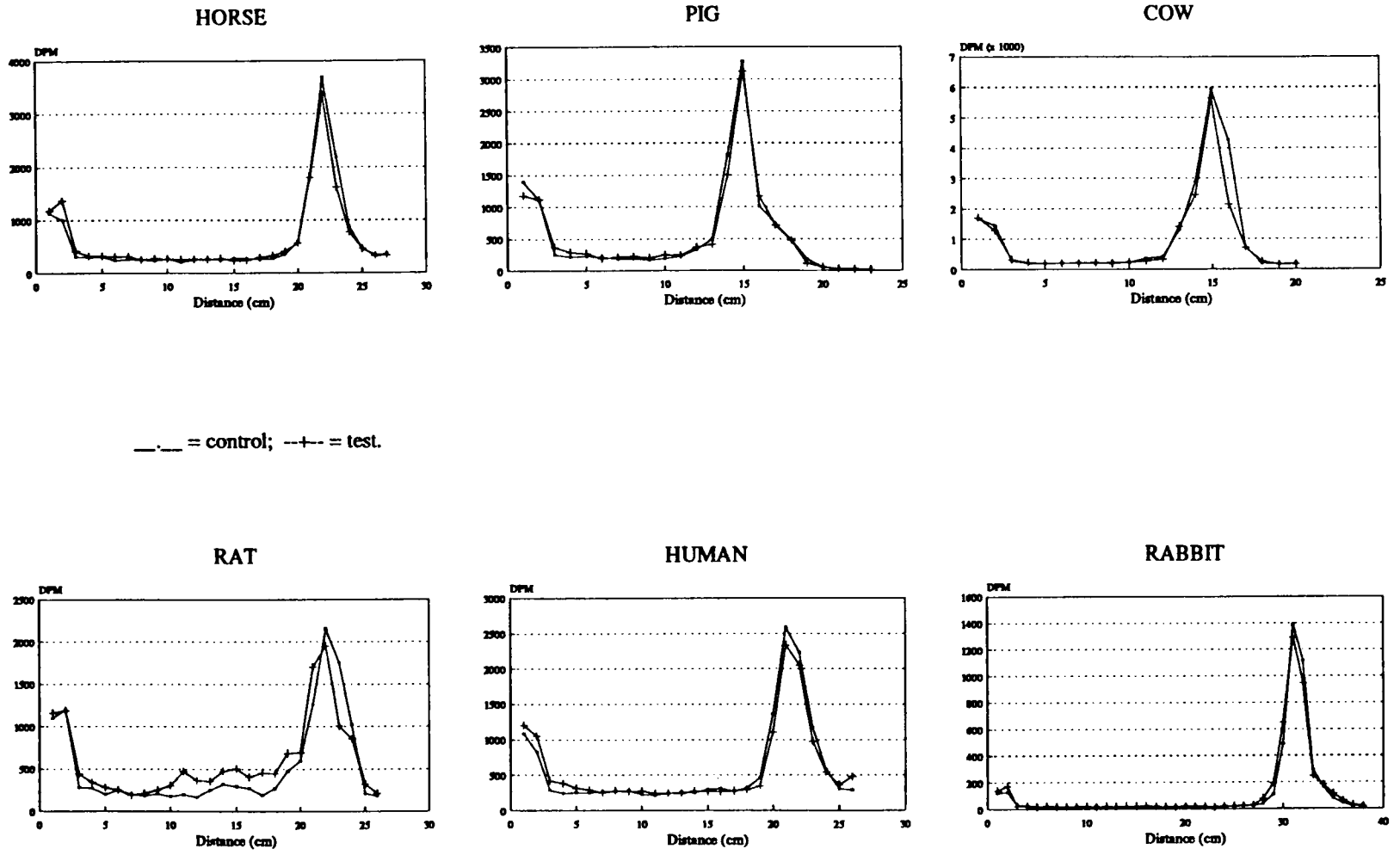
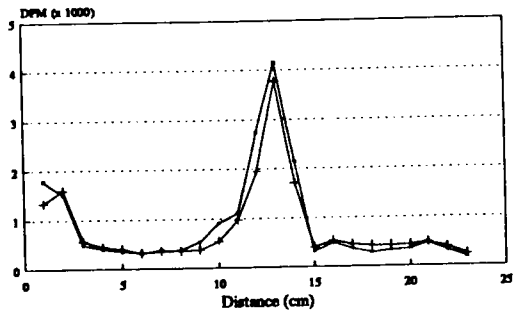


Figure 8. Paper Chromatography results on Norepinephrine.

DOG



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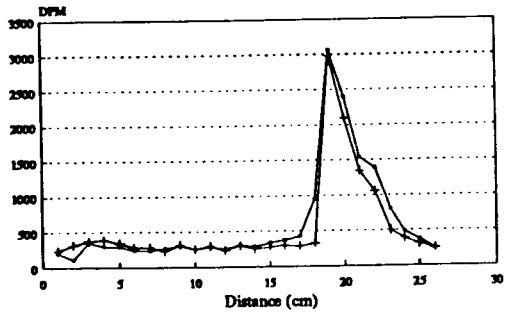
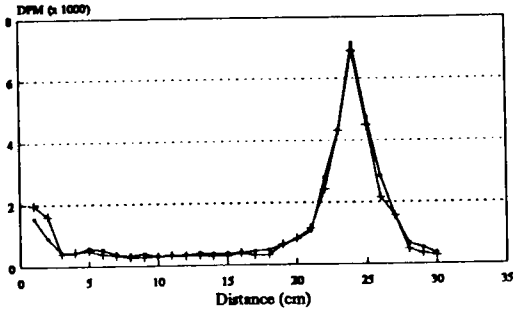
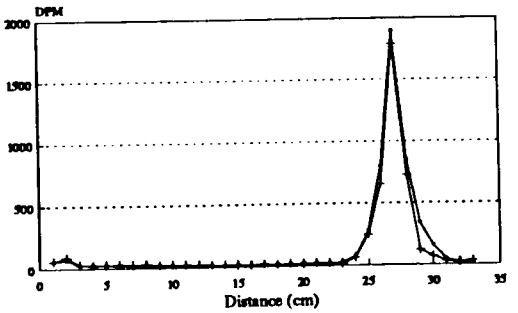
CAT



GUINEA PIG



MOUSE



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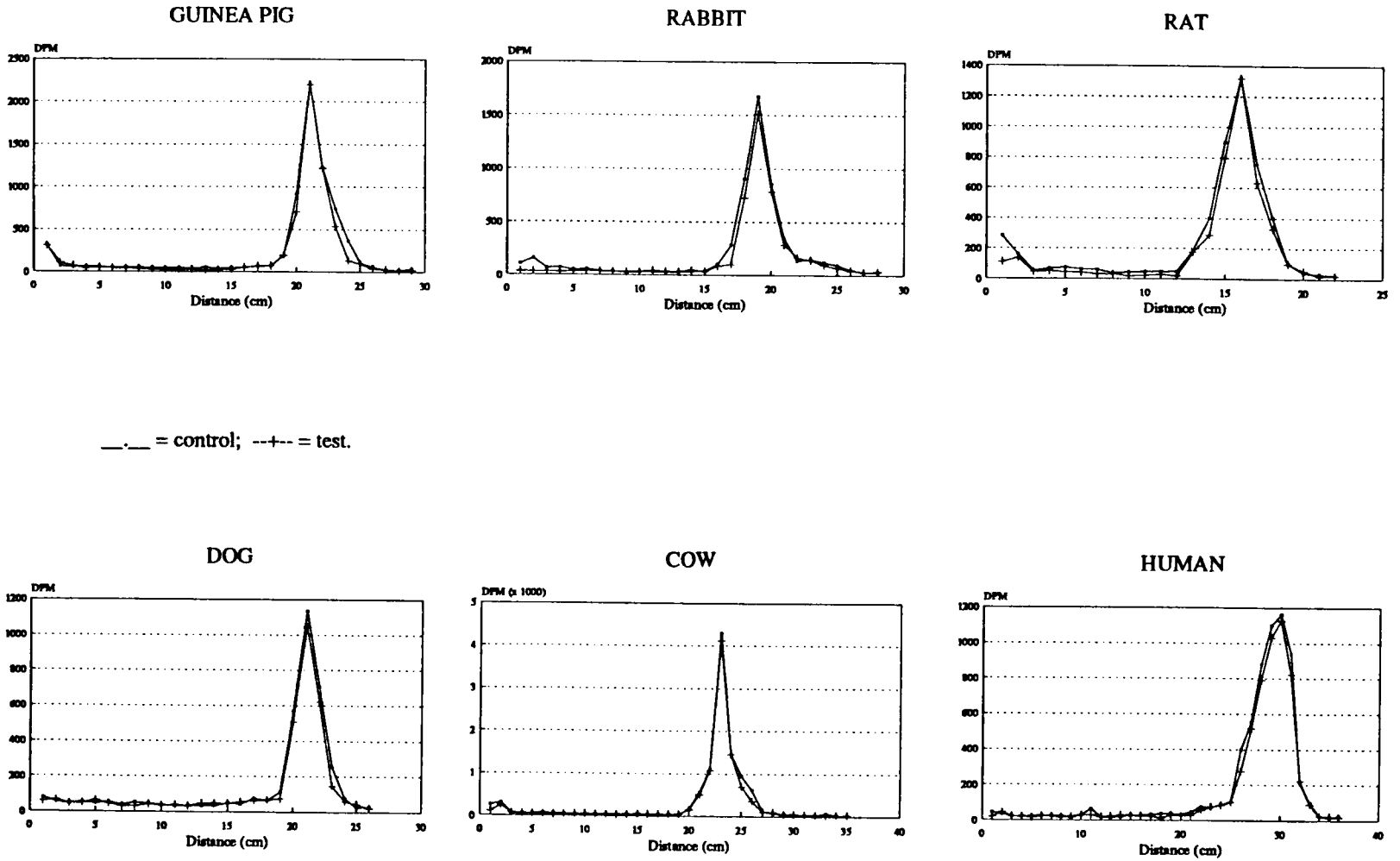
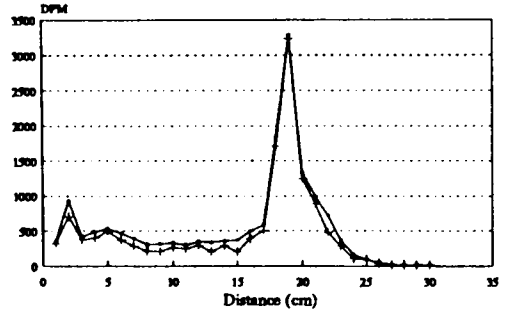


Figure 9. Paper Chromatography Assay results on Tryptamine.

PIG



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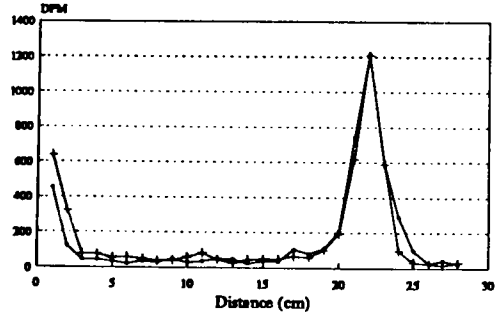
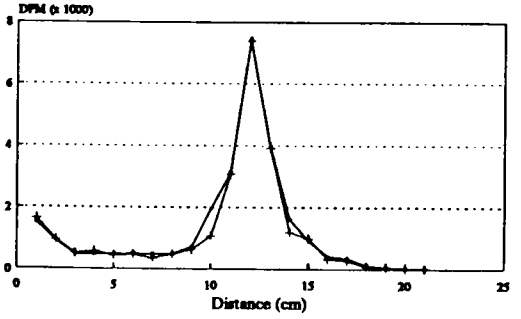
MOUSE



CAT



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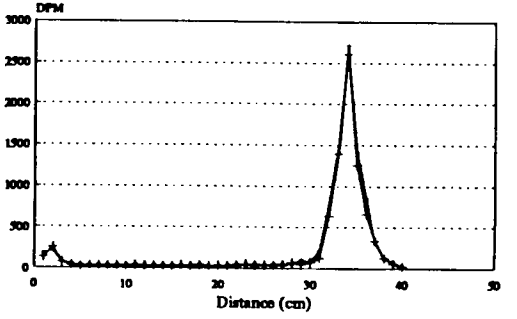

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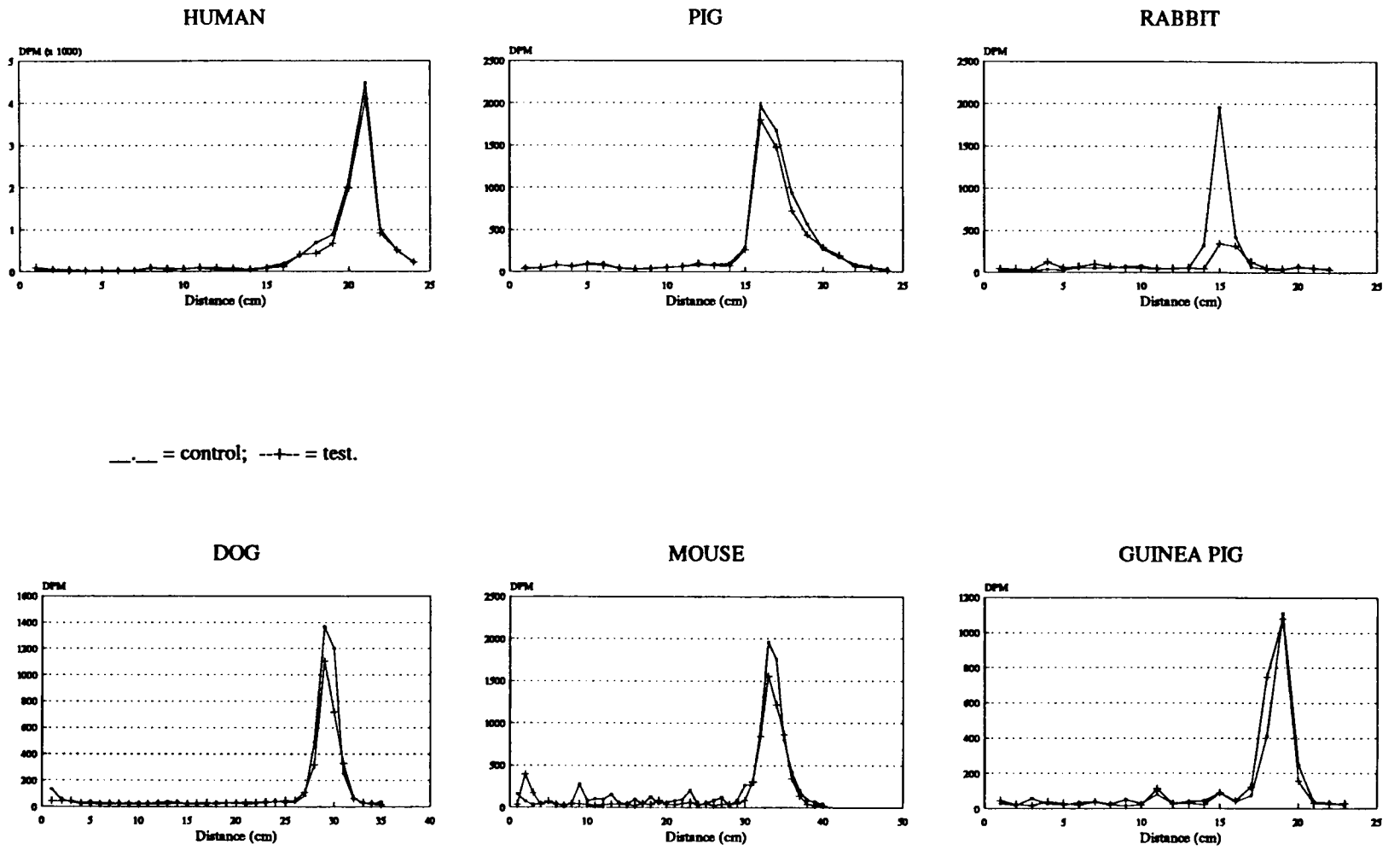
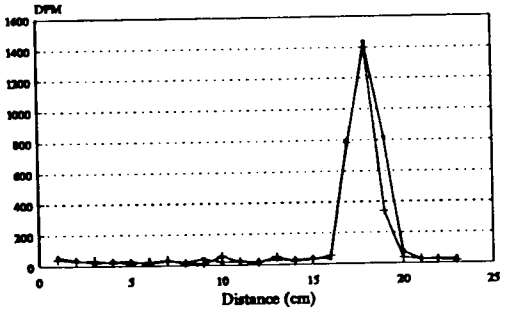


Figure 10. Paper Chromatography Assay results on Tyramine.

RAT



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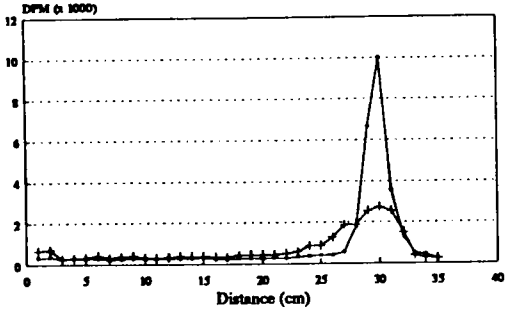
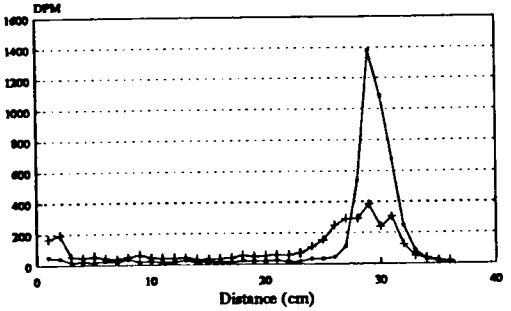
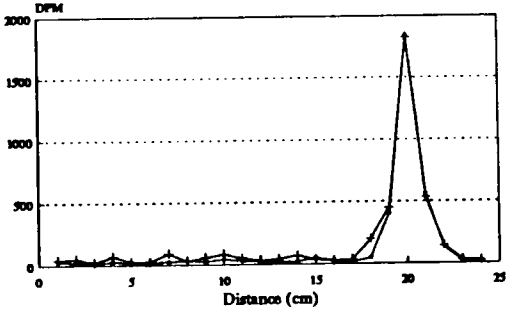
COW



HORSE



CAT



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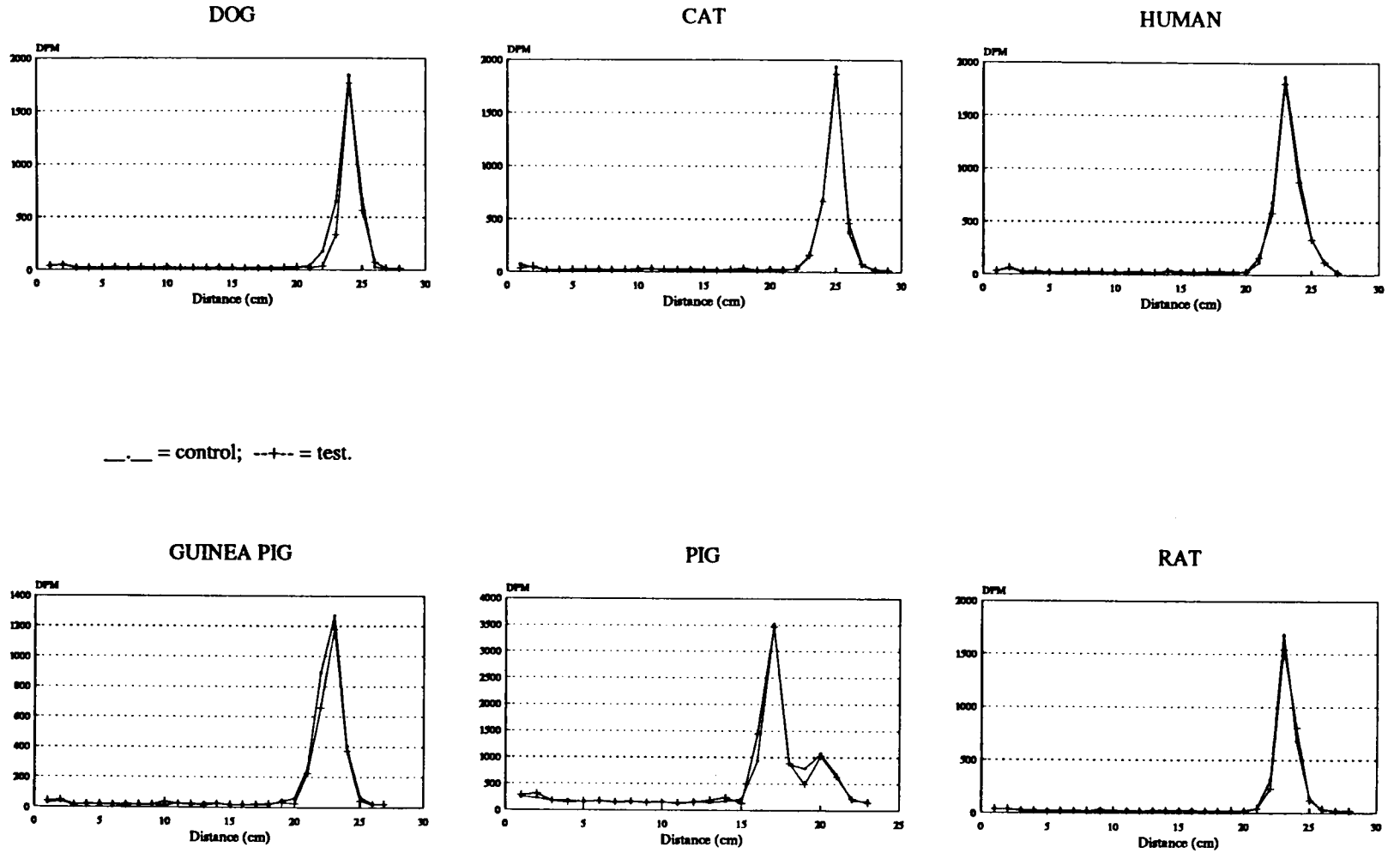
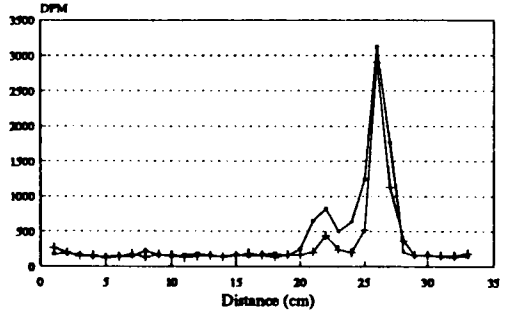


Figure 11. Paper Chromatography Assay results on Tyrosine.

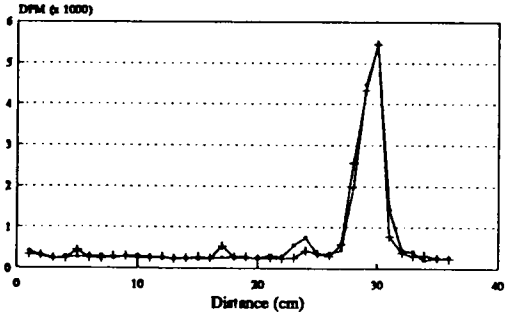
RABBIT



77

—·— = control; --+-- = test.

COW



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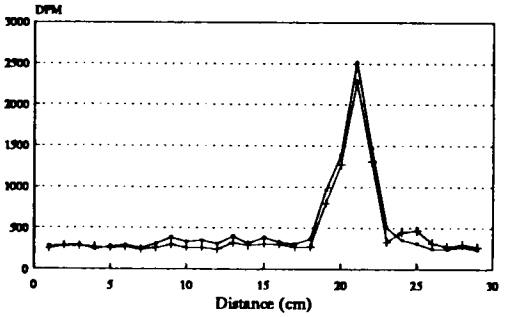

MOUSE

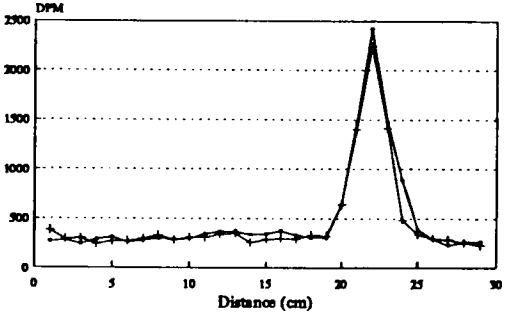

Figure 11. Continued.

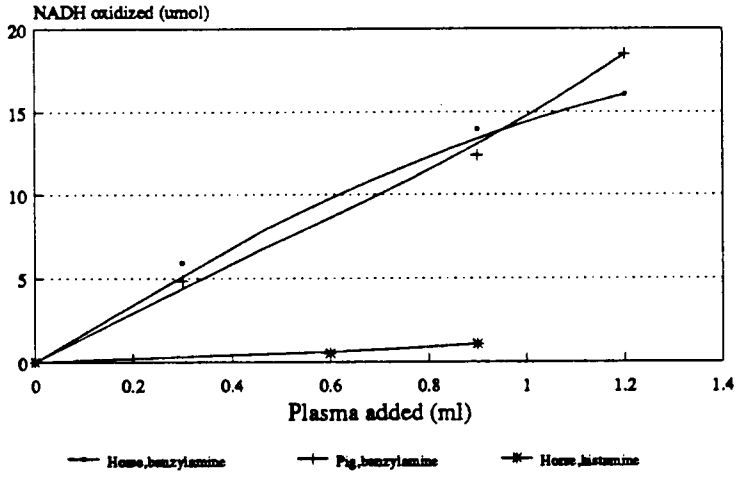

Figure 12. The rate of ammonia produced is proportional to the amount of plasma used.

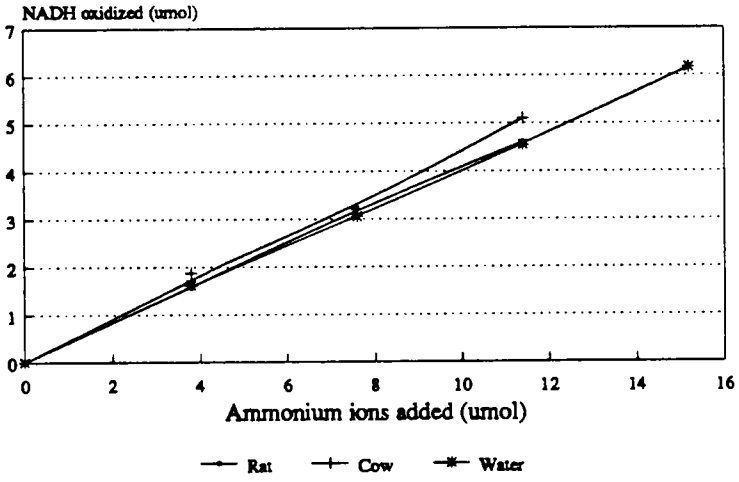

Figure 13. Ammonia Assay in plasma is quantitative.

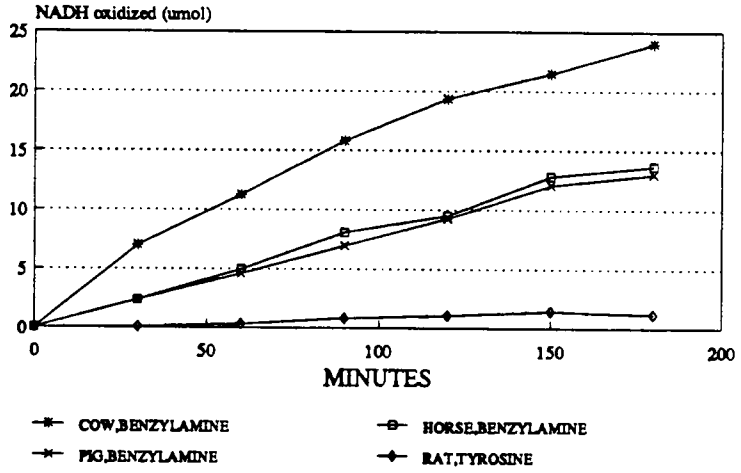

Figure 14. Time Course check on plasma amine oxidase activity.

VITA

Samuel John Ebong was born May 11, 1958 in Abakaliki, Nigeria, the son of Effiong John Ebong and Arit Ebong. He attended Massey Square School for his primary education and secondary school at Birch Freeman Secondary School, Lagos before transferring to Holy Trinity Secondary School , Mbiakong, Cross River State of Nigeria, where he finished in 1977. He later attended the School of Arts and Science, Uyo, where he obtained Certification in advanced level studies in Physics, Chemistry, and Biology at a sitting. In 1979, Samuel came to the United States for his studies. He graduated in 1982 with a degree in Microbiology. After a working hiatus, Samuel accepted a Teaching Assistantship and enrolled as a graduate student in Environmental Toxicology in 1988.

While working, he was awarded a Honorary Citizenship of Knoxville for his community work with children. Other awards were received from annual meetings of the Southeastern Society of Parasitologists (1987), Southeastern Society of Toxicologists (1990), and the American College of Toxicologists (1991).

The author received his Master's degree in Life Sciences in December, 1991.