IN VIVO AND IN VITRO DEGRADATION AND METABOLISM OF P-BIS(2-CHLORETHYL)AMINO-L-PHENYLALANINE (MELPHALAN)

by

Thomas Leonard Evans

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STATEMENT BY AUTHOR

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APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

I. Glenn Sipes — July 15, 1979

I. GLENN SIPES, DATE

Associate Professor of Toxicology, Anesthesiology, and Pharmacology

To my wife for her love and understanding.

To my father and mother for their support and encouragement.

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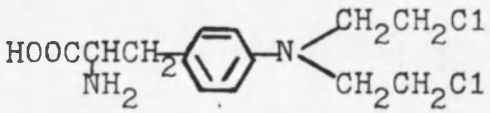
ABSTRACT

L-phenylalanine mustard (L-PAM) an alkylating agent used in cancer chemotherapy, undergoes rapid hydrolysis forming the mono- and dihydroxy degradation products. The hydrolysis rate was dependent on pH, temperature and protein concentrations. L-PAM was stabilized by bovine serum albumin, bovine, canine and human bile fluid and sodium taurocholate solutions. L-PAM was also stable in the presence of chloride ions. The drug was a non-competitive inhibitor of the microsomal enzyme ethyl-morphine N-demethylase. Inhibition of this enzyme was increased by increasing the preincubation time of the microsomes with L-PAM prior to substrate addition. L-PAM disappearance was rapid and first order when incubated in microsomal enzymes in Tris-KCl buffer. Whole liver perfusion studies indicated the rate of L-PAM disappearance was no greater than the L-PAM hydrolysis rate in water. In in vitro plasma recovery studies up to 85% of the ^{14}C L-PAM drug equivalents/ml could be recovered as the mono- or dihydroxy hydrolysis products, protein-bound drug or parent compound. Similarly 85-100% of the ^{14}C-drug equivalents/ml could be accounted for in vivo recovery studies from plasma and urine after oral or intravenous dosing of humans or dogs. It is concluded that L-PAM does not undergo significant in vivo metabolism.

INTRODUCTION

Although attempts are often made to discover new types of anticancer agents by random screening (Rothenberg and Terselic 1970), new drugs are frequently introduced as a result of chance observations or unexpected findings made in fields unrelated to cancer chemotherapy. Once a class of chemicals is known to have anticancer activity, more rational attempts to find new agents can be undertaken by the synthesis of analogues of the active compound in the hope of forming derivatives with reduced toxicity, fewer and milder side effects and perhaps increased anti-tumor potency (Connors 1970).

The classical alkylating agent nitrogen mustard, hardly influences the course of plasma cell myeloma (multiple myeloma). However, conjugation of the mustard nucleus with phenylalanine has generated the active anticancer drug: 1-Phenylalanine Mustard (Melphalan, Alkeran, L-PAM) (Snapper and Kahn 1971). Chemically L-PAM is p-bis(2-chloroethyl) amino-L-phenylalanine, also known as Compound CB 3025 and L-Sarcolysin, and was synthesized in 1953 by Bergel and Stock (Bergel and Stock 1954; Stock 1958). L-PAM has the following structural formula:



and is practically insoluble in water (Blacow 1967; Burroughs Wellcome Co. 1968; Wilson, Grisvold and Doerge 1977) and slightly soluble in alcohol (Burroughs Wellcome Co. 1968; Wilson et al. 1977). As a bifunctional agent (Blacow 1967; Goodman and Gilman 1975; Wintrobe et al. 1975; Anon 1977), L-PAM interferes with DNA replication (Connors 1970; Carter and Slavik 1974) and transcription of DNA and ultimately results in the disruption of nucleic acid function (Gerebtzoff, Lambert and Miescher 1972; Carter and Slavik 1974). L-Pam also exhibits immunosuppressive activity (Chalmers, Burgoyne and Murray 1972; Gerebtzoff et al. 1972; Karchmer 1974) and is the most important drug for the treatment of multiple myeloma and the standard agent for therapy of advanced ovarian carcinoma (Alexanian et al. 1968; Livingston and Carter 1970). It has also proven efficacy in the adjuvant therapy of breast cancer at high-risk of relapse following radical masectomy (Fisher et al. 1975).

CHEMISTRY AND MECHANISM OF ALKYLATION OF L-PAM

The term 'alkylating agent' in its widest sense denotes those compounds capable of replacing a hydrogen atom in another moleucle by an alkyl radical. This involves electrophilic attack by the alkylating agent so that the definition must be extended to include those reactions involving addition of the radical to a molecule containing an atom in a lower valency state (Ross 1962; Warwick 1963). The carbon atom of the radical through which attachment is made is a fully saturated one. Thus the alkylating group may be as simple as a methyl or ethyl group.

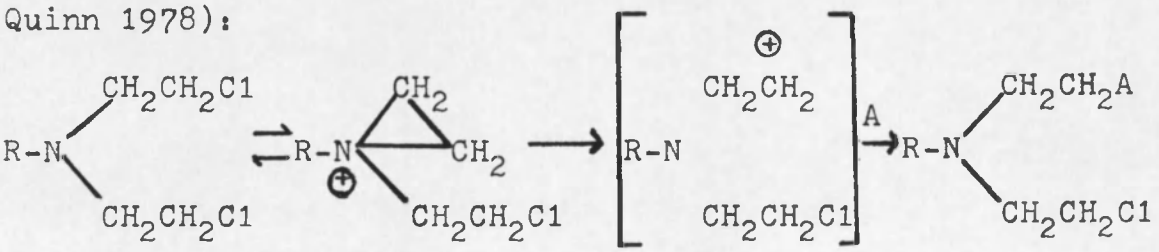
In the process:

$$:X^- + R:Y \longrightarrow R:X + :Y^-$$

RY is the alkylating agent. The driving force for the reaction is the attack of solvent molecules (in aqueous solutions) on the alkylating agent leading to a charge separation. The extent of the displacement reaction will depend on the energy characteristics of the entities involved. The energy required for the heterolytic-bond fission is high, but it is largely compensated for by the energy of solvation of the formed ions. Once formed, the carbonium ion reacts rapidly with any electron-rich center in the system (Ross 1962). In this reaction only one

molecule, the alkylating agent, is undergoing covalency change in the rate determining step, the mechanism is therefore unimolecular and the reaction is S_N1 (Warwick 1963; Hendrickson, Cram and Hammond 1970; Ross 1974). Compounds reacting by the S_N1 mechanism do so at a rate which is largely independent of the concentration of centers capable of alkylation. A feature of the S_N1 reaction is the rate reducing effect of the presence of a 'common ion'. Thus the rate determining ionization stage is reversible and will be retarded if the concentration of an anion (Y^-) in an organic medium is increased.

Ideas on the mechanisms of alkylation by aromatic nitrogen mustards have varied over the years. The most recent evidence favors an aziridinium intermediate (Williamson and Witten 1967; Ludlum 1977; Panthananickal, Hansch and Quinn 1978):



Highly Reactive Intermediate

where, for L-PAM, R=$HOOCCHCH_2$ (C_6H_4) and A=any acceptor molecule. (NH_2)

Internal cyclization of the bis (β -chloroethyl) moiety is necessary for the biological activity of L-PAM

(Livingston and Carter 1970). This intermediate is highly reactive and strained ring scission yields the electrophilic carbonium ion that reacts with water (hydrolysis) or with nucleophilic groups like amino, carboxyl, phosphate, or sulfhydryl groups of proteins and nucleic acids (Connors 1970; Pratt 1976). Biological alkylation mainly occurs at nucleophilic centers such as oxygen, nitrogen, and sulphur atoms (Livingston and Carter 1970) and the bond forming ability towards a saturated carbon atom increases in the order O<N<S (Ross 1962).

The intracellular reaction of importance for L-PAM activity is the formation of a covalent bond between the drug and the 7-nitrogen group of guanine (Harrap 1976). After forming the bond with one molecule, this bifunctional alkylating agent can then undergo a similar cyclization of the second side chain and form a covalent bond with a second nucleophilic group (Ochoa and Hirschberg 1967).

The cytotoxicity of the drug at therapeutic levels is mainly due to inhibition of DNA synthesis (Wheeler 1962; Wheeler and Alexander 1969). The covalent cross-links may be presumed to prevent the two strands of the DNA double helix from separating and hence replicating (Roberts, Brent and Crathorn 1971). Additionally, a DNA strand may be cross-linked to proteins or a protein-protein linkage can occur. However, it has been demonstrated that nitrogen mustard in low doses inhibits the synthesis of DNA by mammalian cells

in culture more rapidly and to a greater extent than it inhibits RNA or protein synthesis (Brewer, Comstock and Aronow 1961).

Interstrand cross-linking is certainly not the only reaction of biological importance. Other possible mechanisms of action have been proposed and studied. Generally these involve the actions of alkylating agents on various enzymes and enzyme systems. This is important because some neoplastic diseases are characterized by an unusual proliferation of cells. This process of growth and division involves ceaseless chemical activity in the form of chemical reactions between enzymes and substrates (Albert 1973).

Recently Tisdale and Phillips (1975) have proposed a possible mechanism of action of bifunctional alkylating agents by inhibition of cyclic 3', 5'-nucleotide phosphodiesterase. Cylic nucleotide phosphodiesterase converts cyclic AMP to 5'-AMP. Cyclic AMP may be an intracellular mediator by which eukaryotic cells control their rate of growth. Other studies have shown that alkylating agents resemble X-irradiation as inhibitors of the development of microsomal enzymes (Tardiff and Dubois 1969). In addition these drugs caused a decrease in the activity of microsomal enzymes in the livers of adult animals <u>in vivo</u>. Studies have demonstrated that certain, but not all, sulphydryl-containing enzymes are inhibited by alkylating agents (Milner 1960).

Other possible mechanisms have been reported. Baker (1959) has proposed that L-PAM acts as an analog of phenylalanine and becomes attached to enzymes at sites occupied by phenylalanine, and the chloroethyl moiety becomes irreversibly attached to the corresponding substrates. Trams, Nadkarni and Smith (1961) have suggested that a large number of biochemical pathways may be susceptible to the action of alkylating agents. Milner et al. (1965) suggests expanding Trams' proposal to include the possibility that at least part of the antitumor effect of L-PAM could be attributed to an effect upon the biochemical processes of tissues other than those of the tumor.

CHEMOTHERAPEUTIC APPLICATIONS

L-PAM has been the most consistently used anti-neoplastic drug for the treatment of multiple myeloma, (Alexanian et al. 1968; Bergsagel 1972; Alexanian 1976; Robins and Chopra 1976) and appears to have at least as good a response rate and impact on survival duration as any other anticancer drug (Alexanian et al. 1968; Anon 1977). It is used alone (Tattersall et al. 1978) and as a component of various chemotherapeutic regimens (Blacow 1967; Beeson and McDermott 1975; Goodman and Gilman 1975; Shirkey 1975; Wintrobe et al. 1975; Modell 1976; Anon 1977).

L-PAM has been demonstrated to be effective in the treatment of women with primary breast cancer, particularly those who are premenopausal (Fisher et al. 1975; Anon 1977; Black 1977; Fisher et al. 1977). It has been evaluated as a single agent in adjuvant chemotherapy (Fisher et al. 1975; Bell, Sullivan, Fone and Hurley 1976) and in combination with other agents both clinically (McElwain 1976; Desai et al. 1977; Fisher et al. 1977; Hansen et al. 1977; Lokich et al. 1977) and in experimental mammary cancers implanted in mice. (Medina and Shepherd 1977). Additionally, L-PAM is a standard anticancer drug in the treatment of ovarian carcinoma (Smith, Rutledge and Taylor 1972; Clarkson 1974;

Black 1977; Frati et al. 1977). Recently it has been tested in advanced prostatic cancer (Houghton, Robinson and Smith 1977) and in the treatment of advanced gastrointestinal cancer (Bullen et al. 1976). Sutow et al. (1975) have reported a four-drug adjuvant chemotherapy regimen, including L-PAM, for post operative treatment of osteogenic sarcoma.

TOXICOLOGIC PROPERTIES

The cytotoxic effects of agents that are able to alkylate biological material under physiological conditions led to the use of such agents in the treatment of neoplastic diseases. It was later established that other classes of compounds which had alkylating ability exerted a cytotoxic action on a variety of rapidly dividing cell types (Ross 1962). This action is not, however, specific for neoplastic tissue, for all rapidly proliferating cells are affected: normal bone marrow, intestinal mucosa, hair forming cells, and testicular and ovarian tissues are also damaged (Ross 1974; McElwain 1976).

L-PAM produces a dose-related depression of bone marrow resulting in anemia, neutropenia, and thrombocytopenia (Anon 1977). Fisher et al. (1977), reported up to 85% of patients experienced Grade I (2.5-3.9 x 10^3 WBC) or Grade II (2.5 x 10^3 WBC) toxicity when receiving L-PAM therapy for five consecutive days at 0.15 mg/kg per day every six weeks. Seventy-five percent of patients receiving L-PAM at 6 mg/M^2/day orally for five consecutive days every six weeks also experienced Grade I or Grade II toxicity. If the leukocyte count falls below 3,000/mm^3 or the platelet count falls below 100,000/mm^3 the drug should be discontinued

until levels increase (Alexanian et al. 1968; Anon 1977). The drug should be used with caution in the presence of leukopenia or thrombocytopenia, and the dosage reduced if these reactions appear (Anon 1977; Fisher et al. 1977). Dose schedules are limited by the effects of L-PAM on marrow production of these hematopietic cells (Santos 1967; Bullen et al. 1976; Fisher et al. 1977). Additionally, L-PAM must be given cautiously to patients receiving radiation or other chemotherapeutic agents that depress bone marrow function.

Nausea and vomiting may occur after large doses (Wiltshaw and Galton 1958; Bullen et al. 1976; Fisher et al. 1977; Hansen et al. 1977; Lokich et al. 1977). L-PAM does not produce alopecia (Frick et al. 1968; Cadman 1977).

Recently L-PAM has been linked to serious hematologic syndromes. Burton et al. (1976), have implied that chemotherapeutics, including L-PAM, may be related to acute leukemia in patients with melanoma on long term chemotherapy. He also cites that "There are now 56 well-documented cases of acute leukemia occurring in myeloma. All were myeloblastic or myelomonocytic and 50 of the 56 patients had received prolonged therapy with alkylating agents -- either Melphalan (L-PAM) or Cyclophosphamide." Other authors have also implicated alkylating agents, and particularly L-PAM, in the de novo development of leukemia especially in patients with myeloma (Holland 1970; Penn 1974; Kyle, Pierre and Bayrd 1975).

Azar (1973), also reports secondary neoplasa following L-PAM treatments. He suggests that the new neoplastic population is possibly from the oncongenic effects or the immunosuppressive effects of the alkylating agent. Cytotoxic agents are clearly carcinogenic in animals, as studies have demonstrated (Harris 1975; Medina and Shepherd 1977). There is concern that they may be carcinogenic in humans, particularly when administered on a long-term basis in doses that are effective for cancer-cell kill (Costanza 1975).

Bell et al. (1976) have postulated two roles for L-PAM in secondary tumorgenesis similar to Azar (1973). One is the reduced immunological resistance to a hypothetical virus or the failure of the immunological surveillance mechanism to eliminate the malignant clone. In effect, L-PAM may have contributed to the immunological paralysis in the patient. Alternatively, L-PAM may have acted as a carcinogenic agent. Evidence of this role is aided by work showing that L-PAM is mutagenic in the Ames Test with and without the S-9 fraction for bioactivation (Minnich et al. 1976). Chemicals which alkylate and/or bind to DNA, such as L-PAM, exert a variety of effects on cells. In normal cells, non-lethal damage may be repaired. However, if repair is inaccurate, a mutant and/or a transformed cell may arise and develop into a cancer if host defense systems are inadequate (Harris 1975).

It can be argued that the development of second neoplasms in patients receiving cancer chemotherapy is to be expected, as individuals with one malignancy are liable to develop other neoplasms (Gunz and Angus 1965; Regelson et al. 1965; Penn 1976). Therefore, whether leukemia is part of the natural history of myeloma or is directly due to prolonged chemotherapy is controversial. However, occurrence with an agent (L-PAM) commonly used in the treatment of myeloma, adds weight to the concept that this complication is related to the chemotherapy rather than the nature of the primary neoplasm (Burton et al 1976).

DEVELOPMENT OF ANALYTICAL METHODS

L-PAM is believed to be well absorbed following oral administration (Blacow 1967; Burroughs Wellcome Co. 1968; Goodman and Gilman 1975) and reportedly remains detectable in the blood for six hours (Burroughs Wellcome Co. 1968; Beeson 1975; Goodman and Gilman 1975). Its' distribution metabolism and excretion in humans or animals have not yet been determined (Blacow 1967; Chalmers et al. 1972).

One of the major reasons information on L-PAM pharmacokinetics has been lacking is due to its rapid hydrolytic degradation common to alkylating agents (Freedman and Boger 1961; Linford 1961; Chirigos and Mead 1964) as shown in Figure 1. L-PAM is completely hydrolyzed in water at 37°C after eight hours (Chang et al. 1978b). However the most important reason pharmacokinetic data is lacking is the absence of a rapid and sensitive analytical procedure.

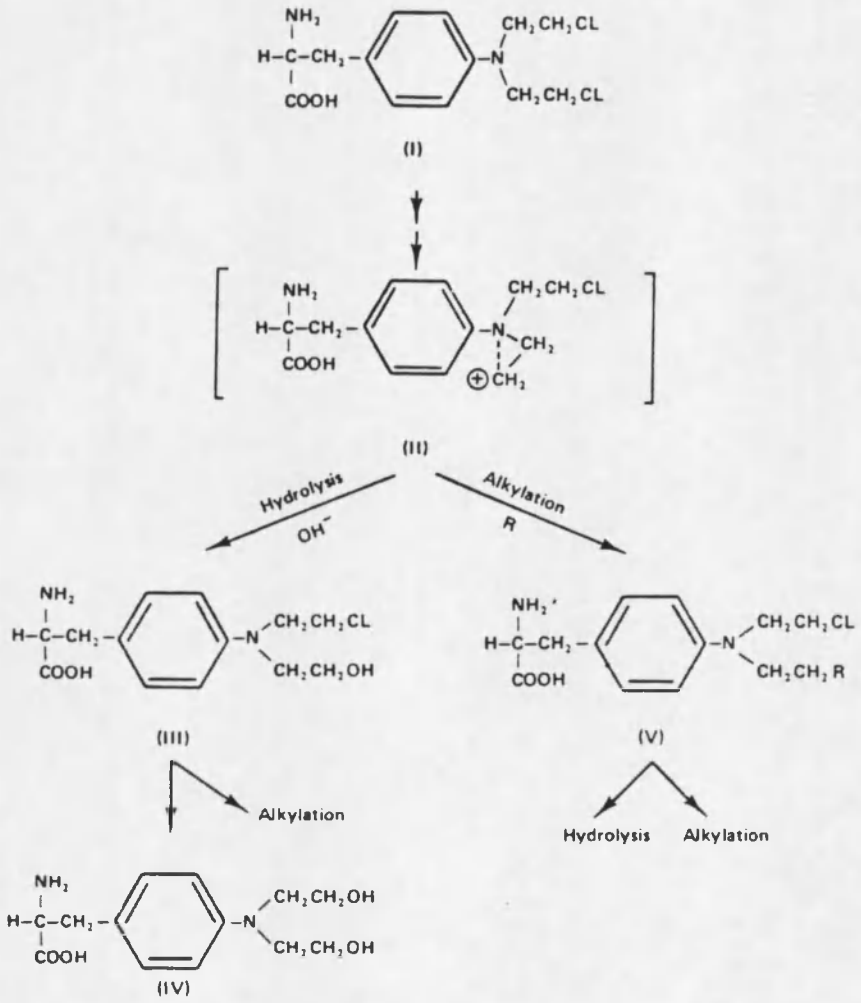


Figure 1. Scheme of L-PAM hydrolysis and alkylation taken from the Journal of Pharmaceutical Science, Chang et al 1978a by permission.

Early analytical methods were directed towards colorimetric procedures to estimate low concentrations of alkylating agents in nonaqueous solvents (Epstein, Rosenthal and Ess 1955). One procedure involved the reaction of the alkylating agent with p-(4-nitrobenzyl) pyridine (NBP) followed by alkalinization. No attempt was made to develop a procedure for quantitative estimation of the reacting compounds or to determine the limits of sensitivity. In 1960 Klatt, Griffin and Stehlin refined this colorimetric procedure for L-PAM. It was adapted from Epsteins procedure utilizing NBP and optimal conditions for determining L-PAM were established. However the assay was not sensitive enough and too complex to study the pharmacokinetic properties of L-PAM. At about the same time, Friedman and Boger (1961) also developed a modified NBP procedure to determine nitrogen mustards. This method was sensitive to less than 1 μg/ml. However, like the modified NBP procedure of Klatt et al. it was too complex for routine analysis or pharmacokinetic studies (Petering and Van Giesson 1963).

Chirigos and Mead (1964) have described a sensitive spectrophotofluorometric method for the quantitative estimation of L-PAM. The assay was sensitive to 20 ng/ml, but like the NBP methods, it was not practical because of high background interference and complexity. Additionally, the

NBP and fluorimetric methods were not specific since they measured the total concentration of alkylating agents including one of the hydrolysis products (Chang et al. 1978b).

Goras, Knight and Irvanmoto (1970) have described a GLC method to determine L-PAM. Chrysene was used as an internal standard and L-PAM was silyated with bis (trimethylsilyl) acetamide and chromatographed as the trimethylsilyl derivative. However, the method has not been applied to biological samples.

Today research as been directed towards assays utilizing high pressure liquid chromatography (HPLC) sensitive for L-PAM. Furner et al. (1976) have developed an assay for L-PAM in mouse and dog tissue. They have separated the parent compound, L-PAM, from its hydrolytic products, the mono- and dihydroxy degradation products. The method employed a solvent system composed of 2-methoxyethanol/0.1% acetic acid, a solvent programming system with a step gradient and micro-Bondapak C_{18} column. Recently, Chang et al. (1978a), have also developed a sensitive and rapid HPLC assay for L-PAM and its metabolites. The method utilized an isocratic solvent system and L-PAM was quantitated using an internal standard (Dansylproline). They have also used mass spectrometry and a ^{14}C-radiolabelled parent compound (in the chlorethyl moiety) to supplement the HPLC method for the measurement of L-PAM and its

metabolites in biological samples. The assay is rapid (15 minutes/sample) and sensitive to 10ng/ml.

STATEMENT OF PROBLEM

The action of most drugs is the result of a series of interactive processes relating to the physical and chemical properties of the drug, the manner and site of administration, the nature of the drugs absorption, distribution and metabolism and the interaction of the drug with cellular processes. A knowledge of the pharmacokinetics of useful anticancer drugs may eventually prove helpful to increase antitumor efficacy and decrease toxicity to normal tissue. Therefore, the objective of this research project was to investigate some of the physical and chemical properties of L-PAM, its interaction with a cellular process and the possible metabolism of L-PAM by using *in vitro* and *in vivo* techniques.

As described earlier, L-PAM disposition was relatively unknown due to the lack of a sensitive assay. By using the procedure of Chang et al. (1978a), it was possible to quantitate L-PAM and its mono- and dihydroxy hydrolysis products (structures III and IV, Figure 1) in *in vitro* incubations and in body fluids and excreta. Primary emphasis was placed on *in vitro* studies in order to distinguish between enzymatic biotransformation and non-enzymtic degradation of L-PAM.

Several *in vitro* techniques were used to study L-PAM degradation. Initially L-PAM was incubated in water to characterize the rate of hydrolysis. It was then incubated in various aqueous media such as: bovine serum albumin; sodium taurocholate; Tris-KCl buffer; Tris buffer alone; mono-phosphate buffer; bilirubin and increasing concentrations of KCl.

L-PAM was also incubated in selected biological fluids including human, bovine or canine bile because of earlier findings by Furner et al. (1976). Additionally, L-PAM and ^{14}C L-PAM were incubated in human plasma *in vitro* to determine the extent of non-enzymatic degradation. These plasma studies were later compared to plasma levels obtained after oral or intravenous dosing in humans and dogs with L-PAM.

The *in vitro* metabolism studies were performed using rat hepatic microsomal enzymes. In order to verify the enzyme activity of the microsomes an ethylmorphine N-demethylase reaction was run. Active enzymes were compared to heat denatured enzymes (which would represent degradation through non-enzymatic paths). Additionally, an isolated perfused whole rat liver was used to determine the effect of an intact organ on L-PAM metabolism and/or degradation.

To study the *in vivo* disappearance of L-PAM, plasma or urine from humans and dogs were analyzed. Subjects received ^{14}C-radiolabelled drug either orally or

intravenously. ^{14}C-bound drug equivalents were determined in both fluids by HPLC separation and liquid scintillation.

MATERIALS

L-Phenylalanine Mustard was obtained from the Division of Cancer Treatment, NCI, Bethesda, Maryland. ^{14}C L-Phenylalanine Mustard (^{14}C labelled in the chloroethyl moiety) was obtained through the National Cancer Institute from the Stanford Research Institute, Menlo Park, California.

Bovine bile was purchased from the Busby Meat Company, Tucson, Arizona. Canine bile was obtained from the Departments of Veterinary Science and Microbiology, University of Arizona, Tucson, Arizona. Human bile was obtained from Dr. I. Glenn Sipes, Department of Toxicology, University of Arizona and had been previously stored at $-20^{\circ}C$ for four years. Human or canine plasma and urine were obtained from Dr. David S. Alberts, Department of Internal Medicine, University of Arizona.

Bovine serum albumin (BSA), bilirubin, potassium chloride (KCl), sodium taurocholate (Na.T.), Trizma® buffer, and potassium monophosphate (KH_2PO_4) were purchased from the Sigma Chemical Corporation, St. Louis, Missouri. Dansylproline, the internal standard (I.S.), was purchased from the Pierce Chemical Company, Rockford, Illinois. Medium 199 and Fetal Calf Serum were purchased from the Grand Island Biological Company (GIBCO), Grand

Island, New York. Ethylmorphine HCl was purchased from the Mallinckrodt Chemical Works, St. Louis, Missouri. All solvents were purchased from Burdick and Jackson, Muskegon, Michigan. NADP (oxidized, monosodium salt), glucose-6-phosphate (G-6-P) glucose-6-phosphate dehydrogenase (G-6-PD, 2000 I.U./ml), were purchased from Calbiochem. An N_2 gas purifier containing Drierite and a molecular sieve 5A was purchased from the Alltech Associates Inc., Arlington Heights Illinois.

Animals

Male, Sprague-Dawley rats (Hilltop), 200-250 gms and virus free at the time of the experiments, were housed in wire-bottomed cages. Animals were fed a standard diet and received water ad libitum. Rats were kept in a 22°C room with a 12 hour light; 12 hour dark cycle. Food was withheld from the animals for 24 hours prior to use. The animal facilities complied with Public Law 89-544, The Laboratory Animal Welfare Act of August 24, 1966.

METHODS

Assay of L-Phenylalanine Mustard

L-PAM was analyzed according to the method reported by Chang et al. (1978a) utilizing high pressure liquid chromatography (HPLC). Two Waters 6000 pumps (Waters Associates Company, Milford, Massachusetts), a 600 solvent programmer (Waters Associates Company), and a dual 440 detector (Waters Associates Company) were used. A μc-18 column (4mm x 25 cm, Waters Associates Company) was used for all analysis. An isocratic solvent system of distilled deionized water and methanol (1:1) with 1% acetic acid was delivered at a flow rate of 2 ml/minute. L-PAM and its mono- and dihydroxy hydrolysis products were detected by UV absorbance at 254 nm. Total analysis time per sample was approximately 15 minutes.

Sample Preparation Procedure

Five micrograms of dansylproline (the Internal Standard-I.S.) were added to a one ml aliquote of human or canine plasma in 13mm x 100mm glass test tubes (Scientific Products, McGaw Park, Illinois) in ice. Two ml of methanol (4°C) was added to the sample and mixed vigorously for 20 seconds on a Vortex mixer followed by immersion into an acetone/dry ice bath at -60°C. The samples were then

centrifuged in the cold at 3,000 rpm for 3 minutes (Sorval RC-3, HG-41 rotor, Sorval Inc., Newton Conn). Five to 100 µl of the clear methanolic solution were injected directly into the µC-18 column.

For the analysis of human or canine urine, one ml was added to a clean glass test tube in ice. The pH was adjusted to between 5-7 with 1 N NaOH. Ten µg I.S. was added and five to 100 µl of the resulting solution were injected directly into the µC-18 column.

The calculation of L-PAM concentrations (according to Chang et al. 1978a) were as follows:

$$\mu g \text{ L-PAM} = \frac{\text{response of L-PAM sample (cm)}}{\text{response of internal standard sample (cm)}} \times \frac{\mu g \text{ of internal standard added}}{\text{relative weight response}}$$

where:

$$\text{relative weight response} = \frac{\text{response of L-PAM (cm)}/\mu g \text{ L-PAM}}{\text{response of internal standard (cm)}/\mu g \text{ I.S.}}$$

The disappearance rate constant (k) of unchanged drug (L-PAM) in incubation media *in vivo* or *in vitro* was calculated according to $k=2.3 \log C_o / C_t / T$; where C_o and C_t are the concentrations of L-PAM at time zero and time T, respectively. Because there are two basic phenomena of chemical reactions occurring with L-PAM (hydrolysis and alkylation), the term "disappearance constant" rather than "hydrolysis constant" is used. This is because at any given moment, the determination of unchanged drug does not represent

the hydrolysis of L-PAM nor its alkylation, but the recovery of all unreacted drug. Additionally, because of poor L-PAM solubility, low concentrations between 50 and 100 µg/ml were used. Published data (Chang et al. 1978ab) have shown good recovery of parent drug and very good detection by HPLC of low concentrations of L-PAM (10ng/ml).

DEGRADATION STUDIES

Degradation Studies in Aqueous Media

To perform degradation studies 100 μg of L-PAM and 200 μg of dansylproline (I.S.) dissolved in methanol, were added to incubation tubes immersed in ice and evaporated by purified N_2 gas. One ml of incubation media (1.15% KCl; 5.0% KCl; 0.05 M Tris Buffer, pH 7.4; 0.05 M Tris 1.15% KCl Buffer, pH 7.4; 0.05 M KH_2PO_4 Buffer, pH 7.4; or distilled water) at 37°C was added to the dried incubation tubes. The tubes were mixed for 10 seconds on a Vortex mixer and placed in a water bath at 37°C. At selected time points, 0.1 ml aliquots were taken for analysis and added to 0.2 ml cold (4°C) methanol, vortexed 20 seconds and placed in an acetone/dry ice bath at -60°C. Samples were then stored at -20°C until assayed as previously described.

Degradation Studies in Biological Fluids

For studies in biological fluids, 100 μg of L-PAM and 200 μg of I.S. were added to incubation tubes as outlined above. One ml of incubation fluid (Human, Bovine, or Canine bile; 75 mg Bovine Serum Albumin (BSA)/ml, 125 mg BSA/ml, or 250 mg BSA/ml; 2.49 mg bilirubin/ml of water; or distilled water) at 37°C was added to the incubation tubes. Tubes

were vortexed 10 seconds and placed in a water bath at 37°C. At selected times, 0.1 ml aliquots were taken for analysis as described above.

Sodium taurocholate (Na.T.) dissolved in methanol was added to L-PAM (plus 100 μg I.S.) in the following Na.T.: L-PAM ratios: 1000:1; 500:1; 10:1; and 1:1. The resulting solutions were added to incubation tubes immersed in ice and evaporated with purified N_2 gas. The dried Na.T.: L-PAM was reconstituted by adding distilled water at 37°C and sonicated for 10 seconds.

At designated intervals 0.1 ml aliquots were taken for analysis as previously described.

TLC ASSAY OF SODIUM TAUROCHOLATE IN BILE FLUIDS

In order to determine taurocholate levels in the experimental animals, dog and bovine bile were directly spotted on TLC plates (Silica Gel 60, EM Laboratories Inc., Elmsford, New York) with 5 mg/ml, 10 mg/ml, and 15 mg/ml sodium taurocholate as references. The plates were developed in a solvent system of chloroform, methanol, acetic acid and water (65 : 20 : 10 : 5) (Eneroth, 1976) and visualized by iodine vapor.

MICROSOMAL INCUBATION STUDIES

Isolation of Microsomes

The microsomal preparation was performed as described by Mazel (1971). The animals were sacrificed by decapitation and the livers perfused with cold 0.9% saline in situ. The livers were removed and placed in breakers of cold buffer (0.05 M Tris-1.15% KCl, pH 7.4) that were submerged in ice. Six to seven grams of liver were placed into homoganization tubes (Potter Elvehjam, 30 ml, Thomas Company, Philadelphia, Pennsylvania) that were submerged in ice and contained three volumes of cold buffer. The liver sections were homogenized with seven strokes of a teflon pestle attached to an electric hand drill. The resulting homogenate was poured into polypropylene centrifuge tubes (Nalgene, 28.7mm x 103mm, 50 ml, Scientific Products, McGaw Park, Illinois) and spun at 9,000 x G (Sorval RC2-B, SS-34 Rotor, Sorval Inc., Newton, Connecticut) for 30 minutes at 4°C.

Floating lipids were aspirated from the top of the 9,000 x G supernatant. The lipid free supernatant was then poured into ultracentrifuge tubes (Beckman Ultracentrifuge Bottles with Cap 1" x 3.5", Beckman Instruments, Inc., Palo Alto, California). The tubes were filled to the top with cold buffer, capped, and spun in a Beckman Ultracentrifuge (L5-50, SW 30 Rotor) at 105,000 x G for 75 minutes.

The microsomal pellet was resuspended in cold buffer by transferring it with a glass rod to a glass homogenizing tube and homogenization was performed as above. A biuret protein assay (Gornall, Bardwill and David 1949) was performed so that the concentration of protein could be adjusted with cold beffer to 6 mg/ml.

Microsomal N-demethylase Activity

To determine the effect of L-PAM on the activity of microsomal drug metabolizing enzyme, the activity of ethylmorphine N-demethylase was determined (Mazel 1971). Incubations were performed with and without the addition of L-PAM at various time sequences according to the following protocol. Incubation mixtures were prepared that contained: 1 ml microsomal suspension (6 mg/ml); 1 ml Substrate (0.05 mM, 1.0 mM, 2.5 mM, 5.0 mM, 10 mM, 20 mM, 30 mM, or 40 mM ethylmorphine in Tris Buffer) or 1 ml Tris Buffer (as a tissue blank); 1 ml Complete Media (see below) and 5 µl L-PAM solution (5 µg/ul) or 5 µl methanol (L-PAM solvent). The final protein concentration was 2 mg/ml.

Complete Media (NADPH-Generating System)

NADP (TPN)	8 mg
Glucose-6-Phosphate	30 mg
Glucose-6-Phosphate Dehydrogenase	20 units
Tris-KCl Buffer with 7.65 gm/L $MgCl_2$	10 ml

The incubation process was begun by mixing the microsomal suspensions in 20 ml glass vials (Scientific Products, McGaw Park, Illinois) with 1 ml of complete media at 37°C. L-PAM was then added to the appropriate vials and methanol to the controls. The reaction was started by adding the ethylmorphine solution after zero time and one, three and five minute pre-incubations with L-PAM.

After a 10 minute incubation at 37°C the reactions were stopped by the addition of 1 ml $ZnSO_4$ (15%) into each vial. In order to adjust to the optimal pH of 5.5 to 6.5 for the Nash reaction, 1 ml saturated $Ba(OH)_2$ was added to each vial. Particulate matter was removed by centrifugation of the incubation solution for 20 minutes at 1,000 x G in a Sorval GLC-2 Table Centrifuge (HL-4 Rotor).

Two and a half ml of the clear supernatant were placed into test tubes followed by the addition of 1 ml Nash Reagent (30.0 gm Ammonium Acetate; 0.4 ml Acetyl Acetone; Distilled Water to 100.0 ml). After mixing, the test tubes were placed into a 60°C water bath for 30 minutes, cooled and read at 412 nm on a Beckman Model 24 Spectrophotometer.

Microsomal Degradation of L-PAM

To determine if L-PAM was biotransformed by the liver, it was incubated in microsomal drug metabolizing

enzymes. Incubation mixtures were prepared that contained; one ml microsomal suspension (6 mg protein/ml); 0.016 nM L-PAM (10 μg); and one ml complete media. The final protein concentration was 3 mg/ml.

The incubation process was initiated by adding th L-PAM to 20 ml glass vials that contained the microsomal suspension and complete media. At designated time points 0.1 ml aliquots were removed and added to tubes containing 0.2 ml ice cold methanol plus 5 μg Internal Standard to be analyzed as described earlier. Heat denatured (60^{o}C for one hour) microsomes were substituted in certain incubations to assess the degree of non-enzymatic degradation of L-PAM.

For the incubations in the phosphate buffer, the same procedure was followed. However, the microsomes and complete media were suspended in 0.05 M KH_2PO_4 buffer (ph 7.4) with the required $MgCl_2$ co-factor added.

ISOLATED PERFUSED WHOLE RAT LIVER STUDIES

Isolation of whole rat livers were performed according to the method of de Galdeano, Bressler and Brendel (1973). Rats were anesthetized with ether throughout the procedure. The perfusion apparatus was filled prior to liver isolation with 200 ml of a Modified Medium 199 Perfusion Buffer (GIBCO) with and without 5% Fetal Calf Serum and equilibrated with 0_2 at 37°C.

The abdominal cavity was opened and a polyethylene catheter (PE-10) inserted in the bile duct. The portal vein was isolated and a cannula of the perfusion apparatus (grooved 15 gauge SS needles) inserted and tied at the general locus of the lienal branch. The vena cava was opened at the level of the right kidney, and the perfusion was started while the animal was still alive. At this time 700 ug L-PAM in 20% DMSO:H_2O was added to the perfusion apparatus and allowed to equilibrate. The perfusing liver was surgically removed from the animal, trimmed of any extraneous tissue, rinsed out, and placed into the perfusion vessel. Approximately 10 ml of perfusate was then collected into a clean test tube, capped and inserted into the perfusion vessel as a positive degradation control. The cannulated bile was collected outside of the apparatus or

allowed to flow freely into the perfusion media. One ml samples were taken from the control tube and from the perfusion media bathing the liver at designated time points and analyzed by HPLC as previously described.

IN VITRO DEGRADATION OF L-PAM IN HUMAN PLASMA

^{14}C-L-PAM was incubated in human plasma to determine the extent of protein binding, the amount of monohydroxy and dihydroxy degradation products formed and the percent of recoverable ^{14}C-drug equivalents. Incubation mixtures were prepared that contained: 10 ml human plasma; one mg L-phenylalanine mustard; and 0.01 mCi ^{14}C-L-PAM (23.6 mCi/mMole).

One ml of L-PAM solution (1 mg/ml in methanol) was added to a 20 ml glass vial in ice and evaporated with purified N_2 gas. Then, 0.01 mCi ^{14}C-L-PAM was added. The incubation was begun by adding 10 ml of human plasma at 37°C into the vial and vortexing for 20 seconds. At designated time points 50 µl samples were removed and added to 10 ml Aquasol (New England Nuclear, Boston, Massachusetts). Radioactivity was determined by counting the samples for 10 minutes on a Beckman LS-230 Liquid Scintillation System with a pre-set error setting of 0.5%. For determination of L-PAM and the mono- and dihydroxy products 0.5 ml of plasma was taken and added to one ml cold methanol in glass test tubes maintained in ice. Five µg I.S. were added to each tube and the tubes vortexed 20 seconds and spun at 3,000 rpm for 3 minutes in the cold (0-5°C) as described earlier.

Samples were stored at -20°C until analysis by HPLC and liquid scintillation.

The HPLC procedure of Chang et al. (1978a) was modified for separating and collecting the parent compound L-PAM and the two degradation products the mono- and dihydroxy hydrolysis products. Instrument conditions were changed to produce a flow rate of one ml/minute of 2% glacial acetic acid in distilled water and methanol (55:45). Samples (100µl) of the clear methanolic solution obtained after centrifugation were injected directly into the µC-18 column and the eluate collected in 20 ml glass scintillation vials. Aquasol (10 ml) was added to each vial and the samples counted for total extractable ^{14}C drug equivalents which included the mono- and dihydroxy products and L-PAM. The amount of ^{14}C drug equivalent bound to proteins was calculated by subtracting the total methanol extractable ^{14}C drug equivalents from the total plasma ^{14}C drug equivalents.

IN VIVO RECOVERY OF L-PAM AND ITS DEGRADATION PRODUCTS

Quantitation of the mono- and dihydroxy products of L-PAM was obtained by using the ^{14}C radiolabelled L-PAM (^{14}C in the chloroethyl moiety) as previously described for in vitro plasma studies. Human plasma and urine (50 ml urine plus 5 ml concentrated HCl) stored at -80°C were used. Partially hydrolyzed L-PAM from a stock solution was injected into the HPLC to obtain relative retention times for the mono- and dihydroxy products and L-PAM in plasma or urine.

One ml aliquots of urine were adjusted to pH 5-7 with 1N NaOH. From this solution 100 µl were counted for total ^{14}C drug equivalents and 100 µl were injected directly into the HPLC. The eluate was collected in 20 ml glass scintillation vials and counted with Aquasol as described earlier. The percent of ^{14}C drug equivalents recovered as the mono- and dihydroxy products and L-PAM in urine were calculated as follows: the sum total of the two degradation products and L-PAM were divided by the total ^{14}C drug equivalents in urine and multiplied by 100. Additionally the 24 hour excretion of drug equivalents were calculated by multiplying the ^{14}C equivalents of L-PAM and the mono- and dihydroxy products by the urine volume collected at each time point.

RESULTS

The percent recovery of L-PAM as the parent compound from various incubation media at 37°C and the recovery rate constants are listed in Table 1. L-PAM was more stable in a 5% KCl media at 37°C when compared to 1.15% KCl or water (0.13 hr^{-1} vs. 0.26 hr^{-1} vs. 0.85 hr^{-1}, respectively). The stability of L-PAM in KCl was dependent on the concentration of KCl since the recovery from 5% KCl is greater than at 1.15% KCl. There was no difference in disappearance rate constants when 0.05 M Tris-1.15% KCl buffer was compared with 1.15% KCl alone. Any stabilization of L-PAM in Tris-KCl buffer is due solely to the presence of KCl. Tris buffer alone shows a disappearance rate constant twice as high as 1.15% KCl (0.53 hr^{-1} vs. 0.26 hr^{-1}, respectively) where greater disappearance rate constants indicate decreasing L-PAM stability. However, Tris buffer does show some increased stabilization when compared to distilled water or 0.05 M monobasic phosphate buffer (0.53 hr^{-1} vs. 0.85 hr^{-1} vs. 0.99 hr^{-1}, respectively). The monobasic phosphate buffer was less stabilizing for L-PAM at 37°C than water (0.99 hr^{-1} vs. 0.85 hr^{-1}, respectively). Both distilled water and 0.05 M KH_2PO_4 buffer show rapid degradation with less than 10% of the parent compound being detected after

Table 1. Disappearance rate constants (k) and percent L-PAM recovered at various time points in non-biological fluids at 37°C and pH 7.4.

	k^1	% L-PAM Recovered at: 1Hr	2Hr	3Hr
5.0% KCl	0.13	87.5	76.5	66.5
0.05M Tris-1.15% KCl	0.25	78.0	60.6	47.2
1.15% KCl	0.26	77.2	59.5	46.0
0.05M Tris-1.15% KCl plus CM[2]	0.26	77.0	59.3	45.8
0.05M Tris	0.53	58.5	34.6	20.4
Distilled H_2O	0.85	42.0	17.7	7.6
0.05M KH_2PO_4	0.99	35.5	13.5	5.0

1. k is a first order kinetics constant equal to 2.3 log $C_o/C_t/T$ as described earlier.

2. Complete Media (an NADPH generating system, formula presented in Methods) used in microsomal experiments

L-PAM (100 μg/ml) was incubated at 37°C in various incubation media for three hours. Values were obtained from three separate trials.

three hours incubation at 37°C. Figures 2 and 3 show rapid first order disappearance kinetics for L-PAM in all incubation media. Tris-KCl buffer and Tris-KCl plus complete media were very similar in appearance (Figure 3).

The recovery of L-PAM from incubations containing increasing concentrations of bovine serum albumin (BSA) in water at 37°C is shown in Figure 4. Disappearance rate constants of L-PAM were; 0.17/hr (250 mg BSA/ml); 0.34/hr (125 mg BSA/ml); and 0.41/hr (75 mg BSA/ml). When these values are compared with the recovery of L-PAM in distilled water (0.85/hr), it is obvious that L-PAM stability was dependent on protein concentration as reported by Linford (1961), Sanderson, McKenna and Blakemore (1965), Hopwood and Stock (1971), and Chang et al. (1978b).

The recovery of L-PAM from various biological fluids at 37°C is shown in Figure 5. Incubation of L-PAM in bile from various species retarded the hydrolysis of L-PAM. The disappearance rate constants for L-PAM were 0.090/hr for canine bile from three separate animals (0-3 hrs); 0.183/hr for bovine bile from one animal and 0.441/hr for human bile from one patient. These values are compared with the recovery of L-PAM in 75 mg BSA/ml and distilled water (0.41 hr^{-1} and 0.85 hr^{-1}, respectively). It is interesting to note that L-PAM showed an unusual stability from three to six hours in the bile from the three dogs.

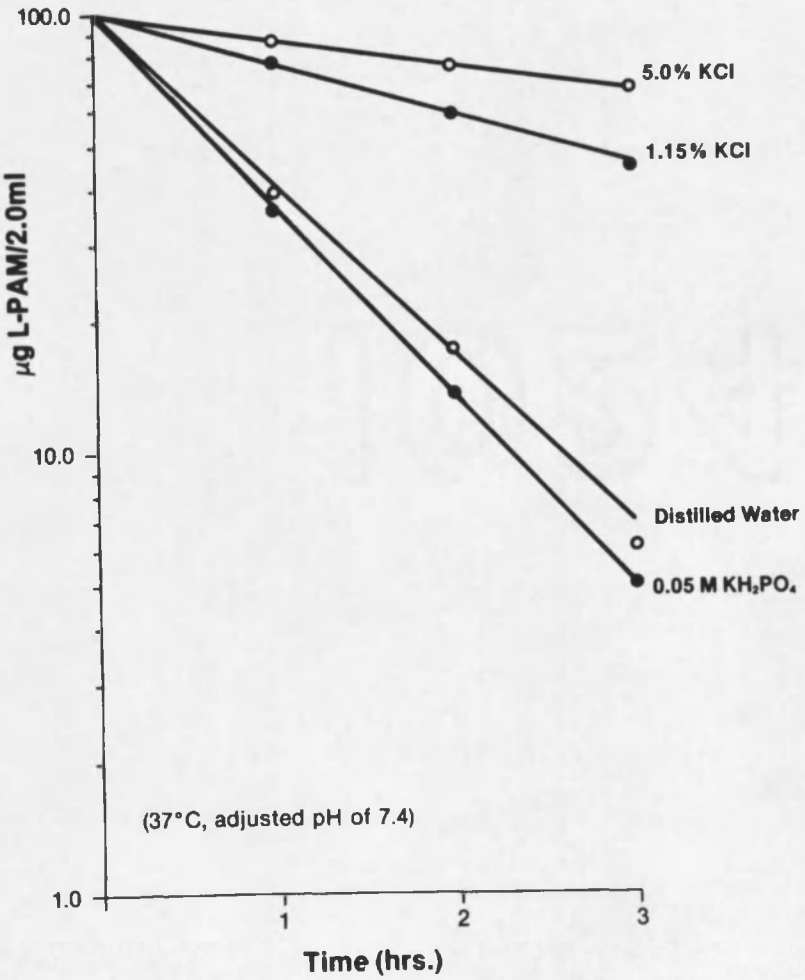


Figure 2. Recovery of L-PAM from various incubation media at 37°C. -- L-Pam (100 µg/ml) was incubated at 37°C in 1.15% KCl (●-●); 5.0% KCl (0-0); 0.05M KH_2PO_4 (▲-▲); and distilled water (△-△) for three hours. Time points represent the average for triplicate samples.

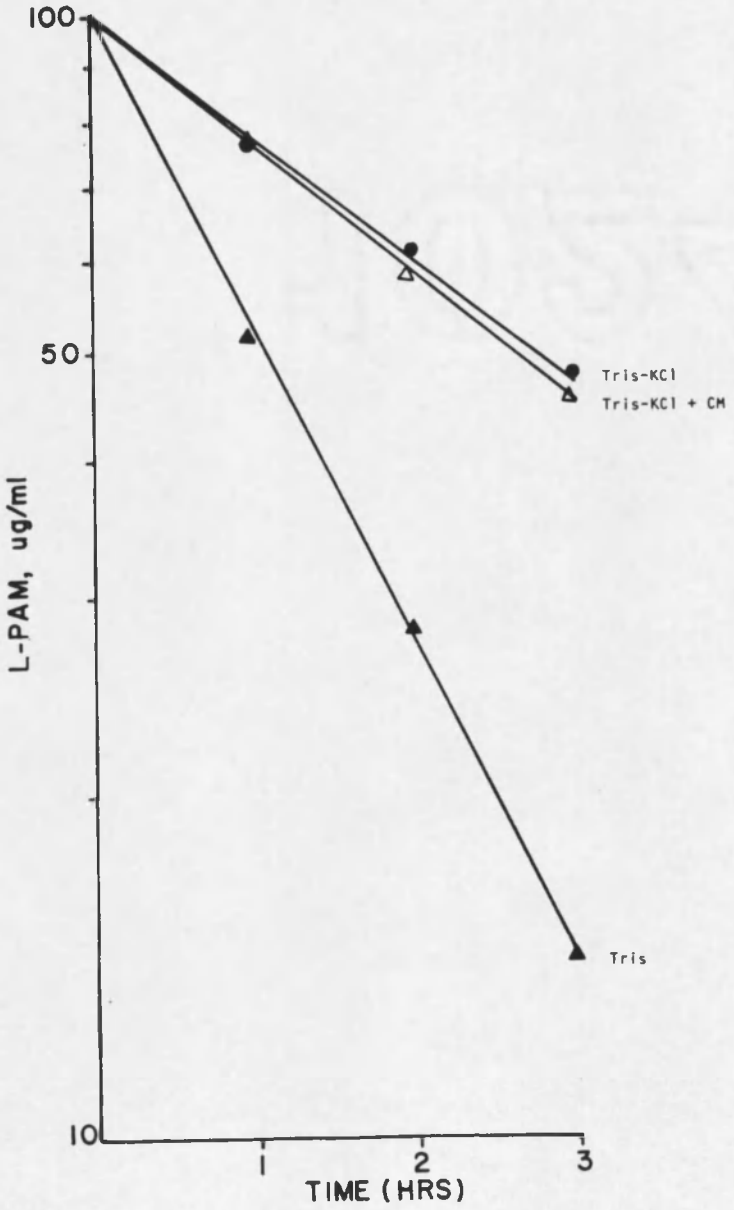


Figure 3. Recovery of L-PAM from various incubation media at 37°C. -- L-PAM (100 µg/ml) was incubated at 37°C in 0.05M Tris-1.15% KCl (●-●); 0.05M Tris-1.15% KCl plus Complete Media (Δ-Δ); and 0.05M Tris buffer (▲-▲) for three hours. Time points represent the average of triplicate samples.

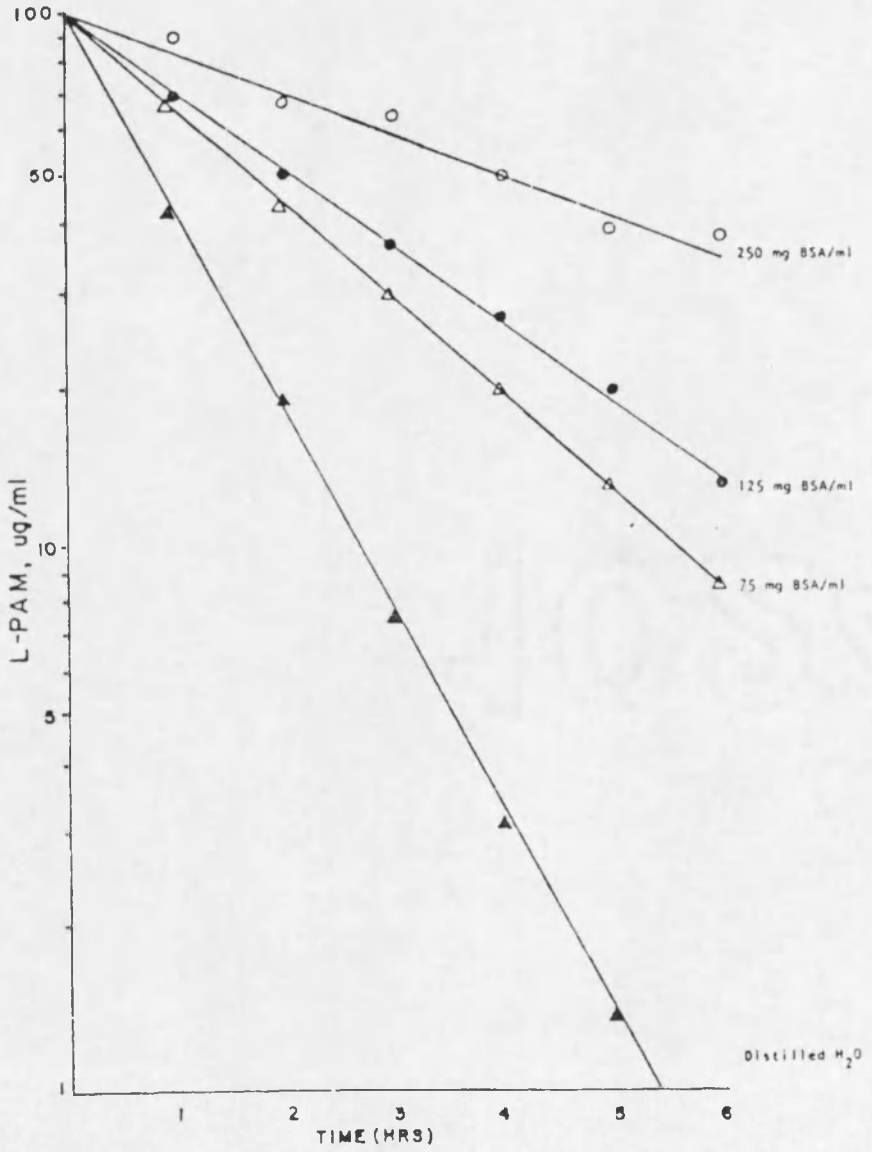


Figure 4. Recovery of L-PAM from incubation media as a function of increasing protein concentration and time at 37°C. -- L-PAM (100 µg/ml) was incubated at 37°C for six hours in increasing concentrations of bovine serum albumin (BSA) and distilled water (▲-▲). Selected protein concentrations were 75 mg-BSA/ml (Δ-Δ); 125 mg BSA/ml (●-●); and 250 mg BSA/ml (O-O). Time points are the average of triplicate samples.

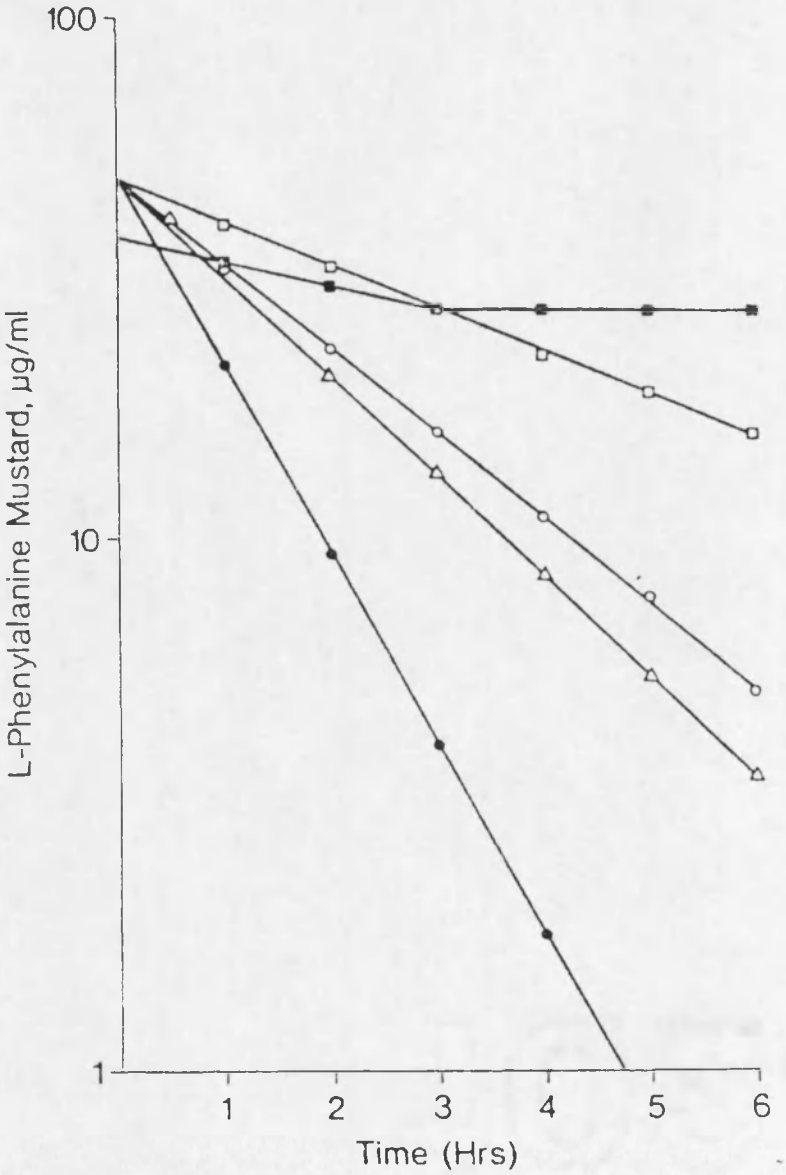


Figure 5. Recovery of L-PAM from bile fluids bovine serum albumin solution and water at 37°C. -- L-PAM (50 µg) was added to one ml of various incubation mediums (canine bile ■-■ ; bovine bile □-□ ; human bile △-△ ; bovine serum albumin, 75 mg/ml 0-0; and distilled water ●-●) at 37°C. Samples were analyzed at designated time points and values are the average of triplicate samples.

Sodium Taurocholate (Na.T.) one of the major components in human bile, was effective in retarding L-PAM hydrolysis (Figure 6). At the selected molar concentration ratios of 1000:1, 500:1, 10:1, and 1:1 (Sodium Taurocholate : L-PAM) the recovery rate constants were; 0.15/hr, 0.28/hr, 0.74/hr, and 0.82/hr, respectively. An expected molar concentration ratio of total bile salts to L-PAM *in vivo* would be far greater than 10,000 to 1 if L-PAM concentration in bile is less than 10μg/ml. Normal L-PAM plasma concentrations in humans after oral dosing are usually less than one microgram per milliliter (Alberts et al. 1978). The concentration of Na.T. in bile as determined by TLC assay, indicated that canine levels were at least twice as much as bovine levels (8-9 mg/ml for canine and 4 mg/ml for bovine). Human levels could not be determined, however, total cholate levels in human gallbladder are reported to range from 7-64 mg/ml (Miettinen 1973 and Latner 1975).

Bilirubin, another constituent of bile, was less effective than sodium taurocholate in stabilizing L-PAM (k=0.52/hr for bilirubin; k=0.15/hr for Na.T. at 1000 molar excess; see Figure 6). However, it was more effective than water as a stabilizing agent (k=0.85/hr). There was considerable difficulty in solubilizing the desired amount of bilirubin in water. The incubation mixture had a large amount of undissolved bilirubin and it is possible that there was very little association of L-PAM to the bilirubin.

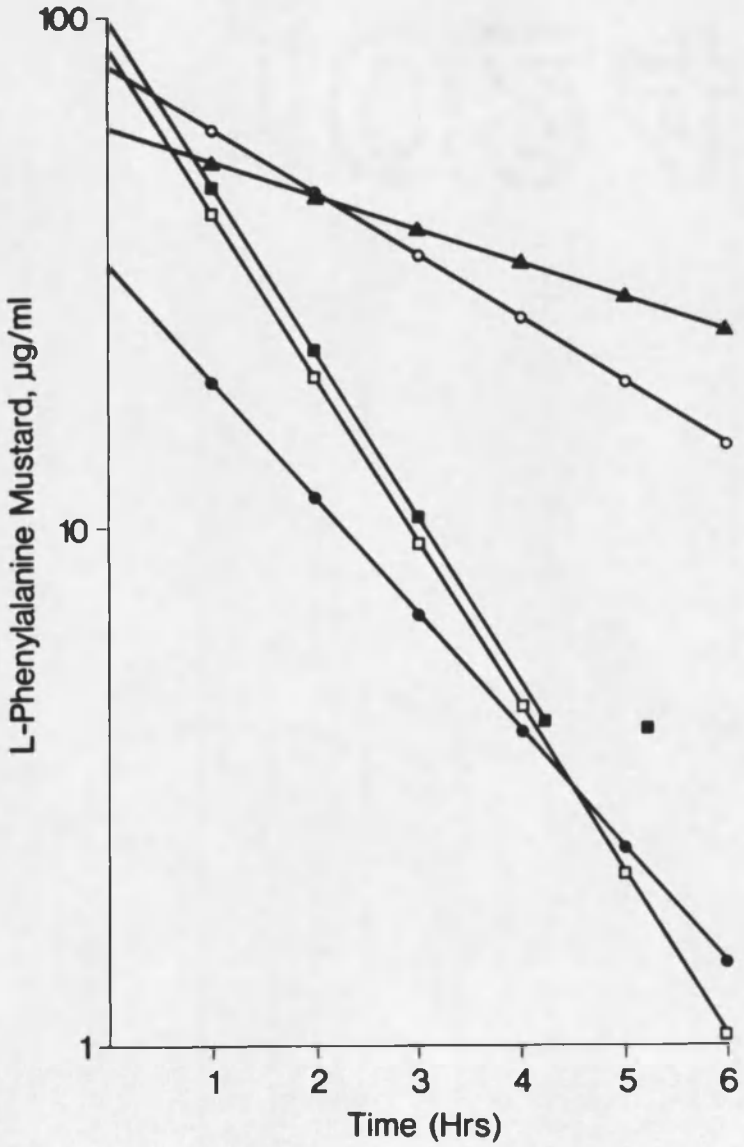


Figure 6. Effect of sodium taurocholate and bilirubin on the recovery of L-PAM at 37°C. -- L-PAM (100 µg) was added to one ml of various molar concentrations of sodium taurocholate (Na.T.) at ratios of: 1000 Na.T.:1L-PAM (▲ - ▲): 500:1 (0-0); 10:1 (■ - ■); and 1:1 (□-□). Fifty µg of L-PAM was added to one ml of bilirubin (2.49 mg/ml H_2O, ● - ●). All mixtures were adjusted to a pH 7.4 and incubated at 37°C and analyzed in triplicate at selected time points.

Effects of L-PAM on Microsomal Ethylmorphine N-demethylase

Figure 7 illustrates the effect of increasing pre-incubation time with L-PAM on the rate of microsomal ethylmorphine N-demethylase. The calculated values for Vmax were: 6.25 nanomoles HCHO/mg protein/minute for controls; 4.83 nanomoles HCHO/mg protein/minute when L-PAM was added immediately prior to substrate; 3.89; 2.15; and 1.89 nano moles HCHO/mg protein/minute for one, three and five minute preincubation of microsomal suspensions with L-PAM, respectively. The time of pre-incubation with a fixed concentration of L-PAM did not change the Km significantly. Km values were: 1.12 mmolar for controls and the three minute pre-incubation; 1.11 mmolar for the one minute and five minute pre-incubation; and 1.04 mmolar for the zero time pre-incubation. These data, showing a change in the Vmax but no change in the Km when increasing the pre-incubation time suggest that L-PAM acts as non-competitive inhibitor of the ethylmorphine N-demethylase system.

Microsomal Degradation of L-PAM

The recovery of L-PAM from active and heat denatured microsomal enzymes is shown in Figure 8. The microsomal suspension media was 0.05 M Tris-1.15% KCl buffer, pH 7.4. The disappearance rate constants were 0.33/hour for heat denatured microsomes and 2.05/hour for active microsomes. Rapid first order kinetics of L-PAM disappearance

Figure 7. Lineweaver-Burke plots comparing the effects of increasing preincubation time of microsomal suspensions with L-PAM on ethylmorphine-N-demethylase activity. -- Twenty µg of L-PAM (4 µg/µl in methanol) were added to microsomal suspensions with complete media and incubated for 0-minutes (▲-▲); 1-minute (0-0); 3-minutes (■-■); and 5-minutes (◇-◇) prior to addition of ethylmorphine. Controls (Δ-Δ) received five ul of methanol at the same time and all incubations were 10 minutes at 37°C. Plotted values are the average of six samples for controls; quadruplicate samples for the 0 and 1 minutes pre-incubations; and one sample each for the 3 and 5 minute pre-incubations.

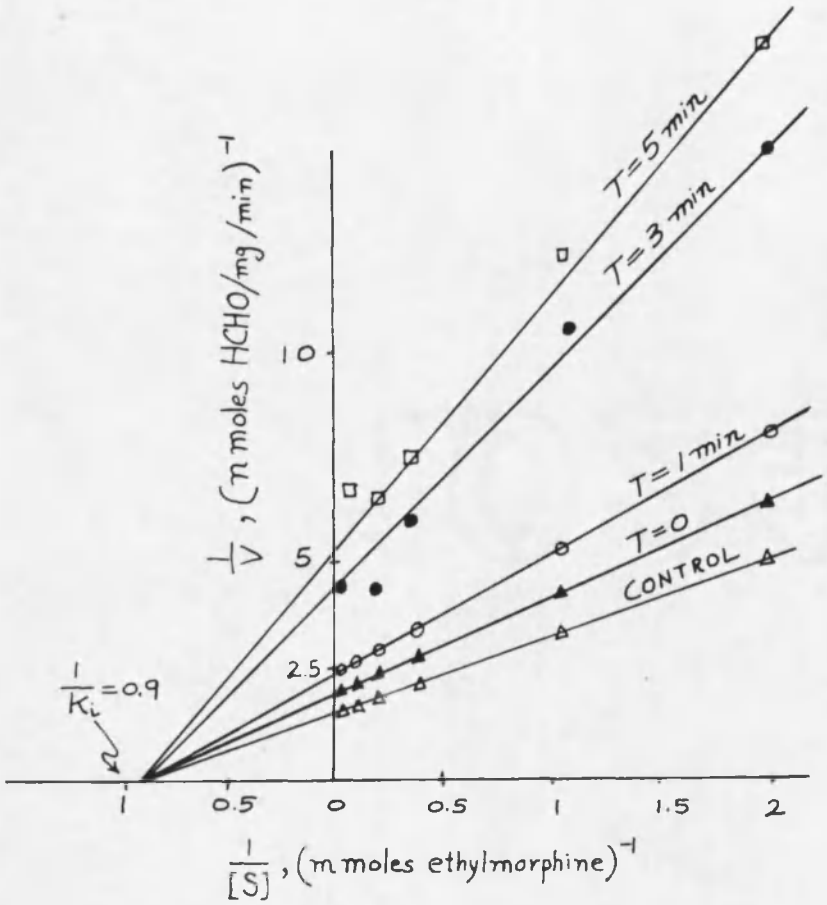


Figure 7. Lineweaver-Burke plots comparing the effects of increasing preincubation time of microsomal suspensions with L-PAM on ethylmorphine-N-demethylase activity.

Figure 8. Recovery of L-PAM from active and heat denatured microsomes suspended in 0.05M Tris-1.15% KCl buffer or 0.05M KH_2PO_4 buffer at 37°C. -- L-PAM (10 µg) was incubated in one ml of a microsomal suspension (6 mg protein/ml) and one ml complete media for 30 minutes at 37°C. Microsomes were suspended in a 0.05M Tris-1.15% KCl buffer or a 0.05M KH_2PO_4 buffer adjusted to a pH 7.4. Disappearance rate constants were: 0.33/hr for denatured enzymes in Tris-KCl (0-0); 0.70/hr for denatured enzymes in KH_2PO_4 (▲-▲); 0.97/hr for active enzymes in KH_2PO_4 (Δ-Δ); and 2.05/hr for active enzymes in Tris-KCl (●-●).

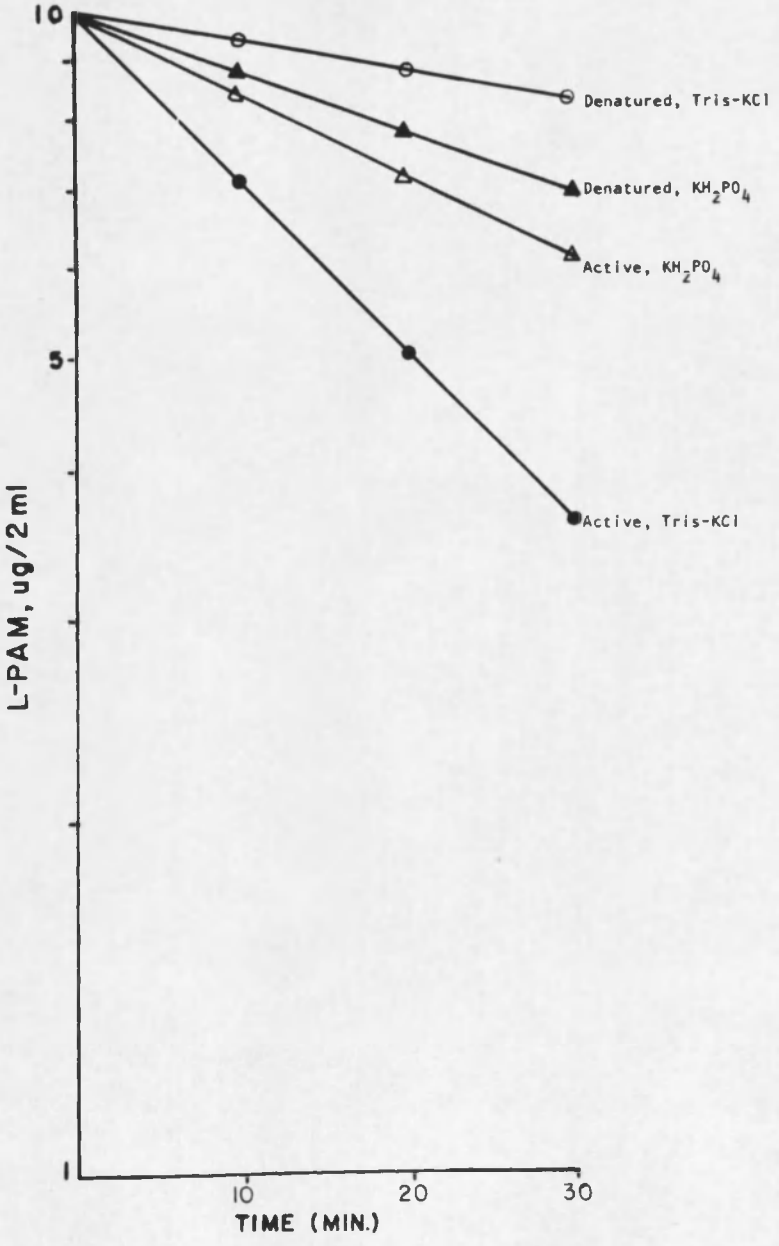


Figure 8. Recovery of L-PAM from active and heat denatured microsomes suspended in 0.05M Tris-1.15% KCl buffer or 0.05M KH_2PO_4 buffer at 37°C.

were shown for the active enzyme system. The heat denatured microsomes showed an increase in L-PAM stability ($0.33\ hr^{-1}$) when compared to distilled water ($0.85\ hr^{-1}$) or the active enzyme system ($2.05\ hr^{-1}$).

The recovery of L-PAM from active and heat denatured microsomal enzymes in 0.05 M monophosphate (KH_2PO_4) buffer is also shown in Figure 8. The disappearance rate constants were 0.70/hour for the heat denatured system and 0.97/hour for the active enzyme system. When these values were compared, degradation in the phosphate buffer in the presence of active enzymes (0.97/hour) did not exceed the rate of L-PAM degradation in phosphate buffer alone (0.99/hour, Figure 2). The disappearance rate constant for L-PAM in heat denatured enzymes suspended in a phosphate buffer was twice as much as the heat denatured, Tris-KCl system (0.70/hour vs. 0.33/hour, respectively). Conversely, the disappearance rate of the active enzymes suspended in the Tris-KCl buffer was twice the rate of active enzymes suspended in phosphate buffer (2.05/hour vs. 0.97/hour, respectively). The phosphate and Tris-KCl buffered systems presented conflicting evidence of L-PAM metabolism in the presence of biotransformation enzymes. However, the rapid L-PAM degradation seen in the active Tris-KCl buffered enzyme system, may be an artifact unique to the *in vitro* system and not seen *in vivo* in the presence of endogenous phosphate buffers.

Whole Liver Perfusion Studies

L-PAM has shown variable absorption after oral dosing in humans (Alberts et al. 1978). This may be due to a rapid degradation in the gut, a formulation problem, the inability to absorb the drug through the gut wall or possibly a first pass effect in the liver with subsequent excretion into the bile. In fact Furner et al. (1976) have shown unusual L-PAM stability and deposition in bile fluid in dogs. In order to investigate some of these possibilities, isolated rat livers with cannulated bile ducts were perfused with a modified Medium 199 with and without 5% albumin. Both the buffers contained 3.5 ug L-PAM/ml fluid.

Figure 9 shows the disappearance of L-PAM from the buffers as they perfuse the isolated liver (lines b, d, and e). The controls (lines a and c) represent the loss of drug due to hydrolysis in the buffered media. These data show an increased stability of L-PAM in the presence of albumin (disappearance rate constants: 0.54/hour for buffer controls and 0.30/hour for albumin controls; lines c and a, respectively). This same increased L-PAM stability was seen when comparing the perfusate with albumin to the perfusate without albumin (disappearance rate constants: 0.46/hour vs. 0.67-0.71/hour, lines b vs. e, respectively) in the cannulated bile duct system. However, when the bile was allowed to flow freely into the

Figure 9. Disappearance of L-PAM from the perfused whole rat liver in medium 199 or medium 199 plus 5% albumin at 37°C. -- L-PAM (700 µg in 20% DMSO: H_2O) was added to the perfusion apparatus prior to the addition of the isolated livers. Ten mls of perfusate were taken as controls in stoppered vials. The bile ducts were either cannulated and the bile collected outside the apparatus or not cannulated allowing the bile to flow freely into the perfusion media. Time points are the average of duplicate samples.

a. albumin control (non-circulating buffer plus 5% albumin).
b. albumin (buffer plus 5% albumin circulating through liver).
c. buffer control (non-circulating buffer).
d. albumin plus bile (circulating buffer plus 5% albumin in the presence of bile).
e. buffer/buffer plus bile (circulating buffer and circulating buffer in the presence of bile.

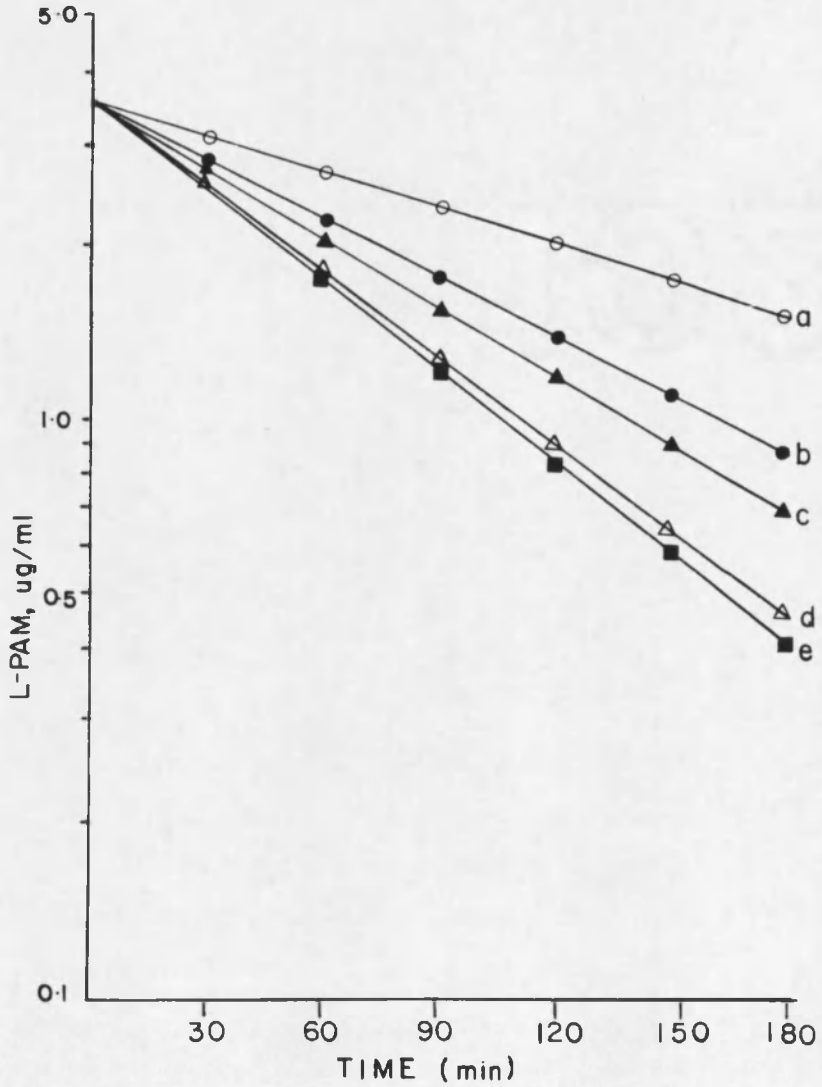


Figure 9. Disappearance of L-PAM from the perfused whole rat liver in medium 199 or medium 199 plus 5% albumin at 37°C.

perfusion media, the disappearance of L-PAM in the presence of albumin was rapid and had a similar disappearance rate constant as buffer without albumin (0.68/hour vs. 0.71/hour, lines d vs. e, respectively). This decreased L-PAM stability in the presence of small amounts of bile suggest a drug displacement phenomena by the bile salts for plasma binding sites on the albumin.

In Vitro Plasma Decay Studies

Figure 10 shows the percent recovery of ^{14}C L-PAM drug equivalents versus time for plasma #1 incubating at 37°C. The disappearance rate constants were: 0.409/hour for plasma #1 and 0.436/hour for plasma #2 (data in Appendix A, Table A1). L-PAM disappearance was rapid and followed first order kinetics. The percent of ^{14}C present as the monohydroxy degradation product (MOH) showed a rapid increase and peaked by two hours before beginning to decline. The loss of monohydroxy product was by hydrolysis to the dihydroxy degradation product (DOH) or alkylation by the remaining chloroethyl moiety to plasma proteins. The percent of the ^{14}C present as the dihydroxy degradation product of L-PAM continuously rose in both plasma samples to about 20% of total ^{14}C in plasma #1 and 46% of total ^{14}C in plasma #2 by 24 hours. The difference in percent ^{14}C DOH was probably due to the protein concentration of the individual plasmas. The total bound drug equivalents

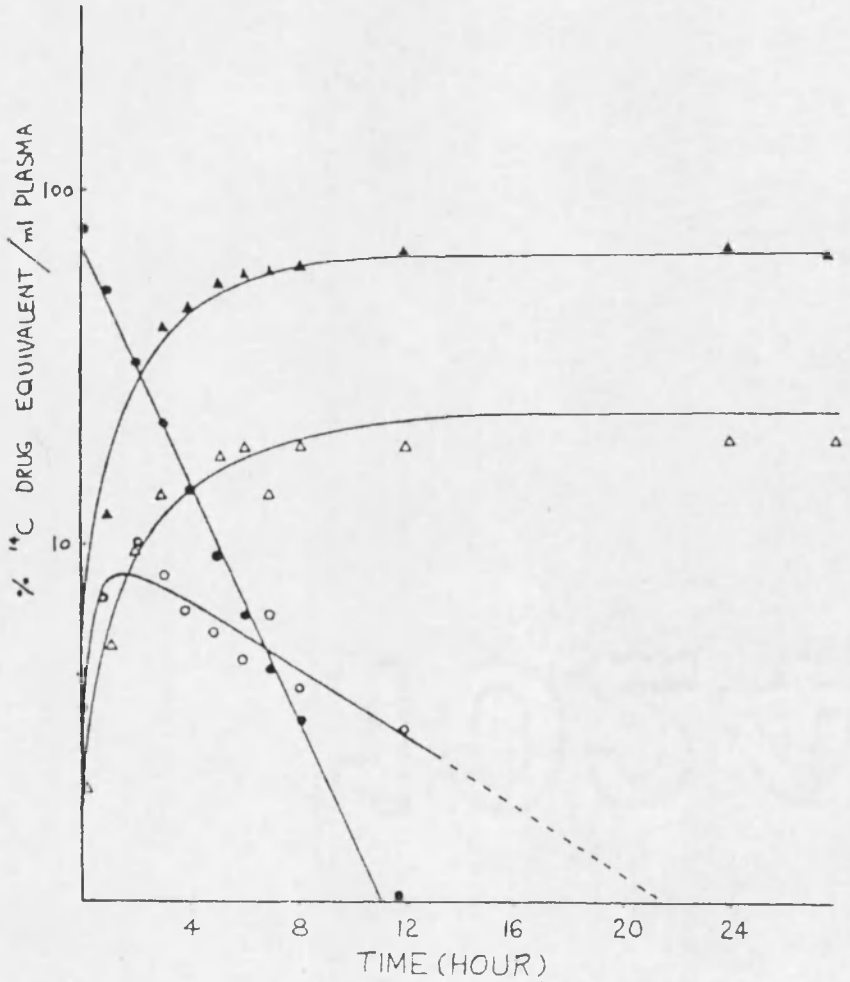


Figure 10. Percent total ^{14}C L-PAM drug equivalents per one ml human plasma incubated at 37°C *in vitro*. -- L-PAM (21 μg/ml, 0.01 mCi) was incubated in human plasma *in vitro* at 37°C. Samples were taken and counted for total ^{14}C drug equivalents. The HPLC eluate was collected and counted to obtain the percent ^{14}C as: Protein bound drug (▲-▲); Dihydroxy product (△-△); Monohydroxy product (○-○); and L-PAM (●-●).

(as determined by subtracting the methanolic extractable ^{14}C from total plasma ^{14}C at each time point -- see methods) steadily rose with increasing incubation time for the first six hours. The percent total bound fluctuates from six hours till the end of the experiment probably because of the breakdown of the plasma proteins to smaller, soluble peptides. At any time point, an average of 85% of the total ^{14}C drug equivalents could be recovered as: the mono- and dihydroxy hydrolysis products; protein bound drug or the unreacted parent drug L-PAM. The remaining 15% of ^{14}C drug equivalents that could not be accounted for were probably bound to small, methanol soluble peptides which adsorbed to the micro-C18 column.

In Vivo L-PAM Disappearance after Oral or Intravenous Dosing

L-PAM disappearance in human plasma in vitro was studied. An average of 85% of the ^{14}C-drug equivalents could be recovered as the mono- and dihydroxy degradation products, protein bound drug or the parent compound L-PAM (Plasma #1, Appendix A, Table A1). This study compares the in vivo L-PAM disappearance after an oral or intravenous dosing in humans or animals with the in vitro disappearance in human plasma.

Figure 11 illustrates the percent L-PAM ^{14}C-drug equivalents per one ml plasma up to 24 hours after oral dosing of L-PAM from patient J.O. (See also Appendix A for

Figure 11. Percent total ^{14}C L-PAM drug equivalents in one ml of human plasma after oral dosing in patient J.O. -- L-PAM, 0.6 mg/kg body weight, in tablet form, was administered orally with ^{14}C L-PAM (100 μCi) that had been mixed with orange juice. Blood samples were obtained at various time points from a heparin lock and collected in tubes containing 100 I.U. heparin. L-PAM was extracted from the plasma and 100 μl of the organic layer injected into the HPLC. The eluate was collected every 20 seconds and counted for ^{14}C drug equivalent.

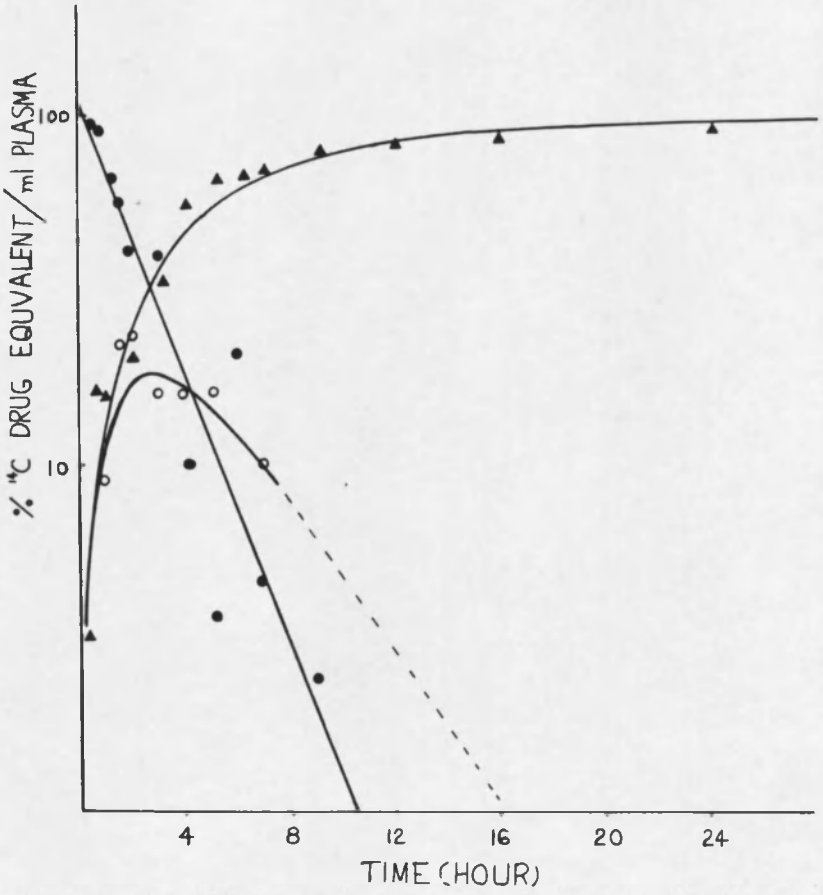


Figure 11. Percent total ^{14}C L-PAM drug equivalents in one ml of human plasma after oral dosing in patient J.O.

other patients studied). Figure 12 shows the percent L-PAM ^{14}C-drug equivalents per one ml plasma after an intravenous dosing in patient M.M.. In both figures, the parent compound L-PAM, is the predominant species found in plasma during the first one to two hours after drug administration. In vitro experiments showed the same pattern for the parent compound. The percent L-PAM in each sample decreases rapidly with time in all experiments whether studied in vitro or in vivo, or after oral or intravenous dosing (Figures 10, 11 and 12; Appendix A, Tables A2 and A3). The percent protein bound drug in a one ml sample was 80-98% by twelve hours in both the oral or intravenous studies and was a much higher percent than found in in vitro incubations. This higher percent may reflect the body's ability to eliminate the free mono- and dihydroxy products. The percent of the dihydroxy product remains constant in vitro overtime, but rapidly falls in vivo probably reflecting its rapid excretion. The percent monohydroxy drug equivalents peaks between one and two hours and this was similar to the in vitro studies.

The percent composition of ^{14}C-drug equivalents in human and canine urine after oral or intravenous doses can be seen in Appendix A, Tables A4 and A5. Except in patient J.O., 80 to 100% of the ^{14}C-drug equivalents could be recovered as the mono- or dihydroxy products and L-PAM within 24 hours. In patient J.O. the average recovery was 73%.

Figure 12. Percent total ^{14}C L-PAM drug equivalents in one ml of human plasma after intravenous dosing in patient M.M. -- L-PAM (0.6 mg/kg body weight) and ^{14}C L-PAM (100 μCi) were combined and injected as a bolus into a running intravenous administration set. Blood samples (10ml) were obtained from a heparin lock and collected in tubes containing 100 I.U. of heparin. L-PAM was extracted from the plasma and 100 μl of the organic layer injected into the HPLC. The eluate was collected every 20 seconds and counted for ^{14}C drug equivalents.

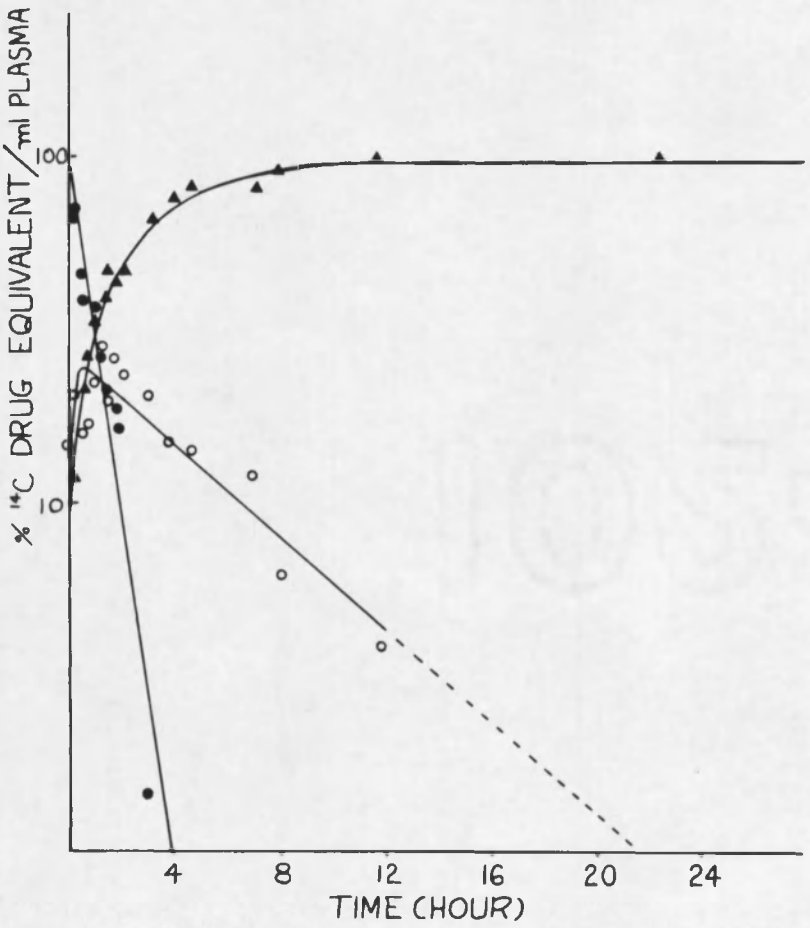


Figure 12. Percent total ^{14}C L-PAM drug equivalents in one ml of human plasma after intravenous dosing in patient M.M.

However, this patient had a considerable amount of solids in the urine and some of the drug equivalents could have been alkylated to them. In all subjects the predominant urinary species excreted within 24 hours was the dihydroxy product. Additionally, urine from one patient (E.S.) was separated by HPLC and the eluate collected in glass scintillation vials and counted for ^{14}C-drug equivalents. Five different samples from times up to 24 hours were examined and at each time point, at least 85-96% of the ^{14}C could be accounted for as the two hydrolysis products or L-PAM.

The very close similarities between the *in vivo* and *in vitro* studies on percent drug equivalents as bound drug, mono-or dihydroxy products and L-PAM suggest that L-PAM disappearance *in vivo* is primarily through hydrolysis and alkylation to proteins and not by enzymatic biotransformation.

DISCUSSION

Degradation Studies

The hydrolysis of L-PAM has been described (Ross 1962; Chirigos and Mead 1964; Chang et al. 1978b) and characterized to occur by first order kinetics (Gardiner 1969; Ross 1974). In this study L-PAM hydrolysis or disappearance exhibited rapid first order kinetics after incubations in various aqueous media at 37°C (Figures 2 and 3). The mechanism is unimolecular and the reaction is S_N1 (Warwick 1963; Hendrickson, Cram and Hammond 1970; Ross 1974). The addition of the Cl^- ion decreased the rate of hydrolysis of L-PAM. The stability of L-PAM seen in Figure 1 is probably not a result of the "common ion" effect as described by Ross (1962). However, recent and unpublished observations have shown that the rate at which the reactive carbonium ion formed does not change in the presence of Cl^- ions. Greater L-PAM stability likely resulted from the stronger electronegativity of the Cl^- ion compared to the OH^- ion and thereby regenerating L-PAM. This is seen in Figure 2 where the KCl concentration has been increased.

A 0.05 M Tris buffer did show some ability to decrease L-PAM disappearance when compared to water. The increased recovery of L-PAM in Tris may be due to the presence

of Cl^- ions as in 1N HCl was used to adjust the pH. The Tris buffer did not appear to play any major role in L-PAM stability when combined with a 1.15% KCl (Figure 2). L-PAM degradation was increased when incubated in a 0.05 M KH_2PO_4 buffer. The recovery of parent compound was less than that of water. The increased degradation may be due to the phosphate and hydroxide ions. This rapid L-PAM degradation in phosphate buffer may be important clinically. At present, the parental dosage form of L-PAM utilizes a dibasic potassium phosphate buffer and it may be worthwhile to change to normal saline and take advantage of the Cl^- ion to increase L-PAM stability.

Furner, Brown and Duncan (1977) reported that at thirty minutes 11% of the total I.V. dose given to dogs was found in the bile fluid of the gallbladder. Upon analysis, it was discovered that 86% of the drug equivalents in bile at thirty minutes was the parent compound as compared with less than 2% present as parent compound in the liver. This unusually high concentration of parent drug in canine bile seemed to contradict the previously reported labile chemical characteristics of L-PAM (Friedman and Boger 1961; Ross 1962; Chirigos and Mead 1964; Furner et al. 1976; Chang et al. 1978b).

It was thought that perhaps bile acted as a reservoir for L-PAM by retarding its degradation. Increased stability of L-PAM in the presence of proteins (BSA) has

been reported by Chang et al. (1978b) and was shown in Figure 4. However, it is unlikely that protein plays a role in the decreased degradation observed in bile. The protein concentration in bile was examined and less than 7 mg/ml protein was found in canine or bovine bile. Normal protein concentration in human bile are low (0.3-3 mg/ml), consisting mostly of serum albumin (Schoenfield 1977). The stability may be in part related to the ion concentration in bile. Literature value for Cl^- ion concentration in human hepatic bile is 88-115mEq/L. (Schoenfield 1977). These values are less than the Cl^- ion concentration in 1.15% KCl solution (154 mEq/L) and considerably less than the 5.0% KCl (671 mEq/L). However, the gallbladder does have the ability to concentrate bile through the diffusion of water out of the bladder. It is reported that humans have the ability to concentrate up to ten fold the bile pigments and acids and that dogs can concentrate bile up to twenty fold (Schoenfield 1977).

Sodium taurocholate, one of the major components of human bile was also effective in retarding L-PAM hydrolysis and degradation. At a high molar excess, the sodium taurocholate slowed L-PAM disappearance the same as bovine bile or the 5.0% KCl solution. The stability of L-PAM in bile is probably due to the formation of miscellular emulsions of taurocholate molecules. These amphipathic molecules have both hydrophobic and hydrophilic

regions and are probably spherical in shape with a central non-polar core and an external, polar region (Kaneko and Cornelius 1971). Fatty acids, monoglycerides, other lipids, and lipophilic molecules (such as L-PAM) are solubilized when they enter the central core of the micelle and are covered by the outside polar coat. Once L-PAM is protected by the micelles, its chloroethyl moiety probably becomes inaccessible to nucleophilic attack by hydroxy radicals. The bile salt micelles and the presence of a high Cl^- ion concentration present a very reasonable explanation for the unusual stability of L-PAM in bile fluid *in vivo* and *in vitro*. Additionally, the high percentage of parent compound seen in canine bile *in vivo* suggest the possibility of an important L-PAM enterohepatic circulation. Finally, since L-PAM undergoes rapid *in vitro* degradation, a liposomal delivery system (Gregoriadis 1976) for intravenous or oral dosing of L-PAM, could prove useful in increasing L-PAM efficacy and absorption from the GI tract. However, if the liposomal emulsions increase the amount o L-PAM absorbed or help obtain higher plasma levels, L-PAM toxicity may also increase requiring a reduction in the dose given.

Microsomal Studies

DNA is considered to be the probable target for the cytotoxic and antitumor actions of bifunctional alkylating agents such as L-PAM (Ewig and Kohn 1977). Since

bifunctionality greatly increases cytotoxicity and anti-tumor activity, it is reasonable to suppose that the essential reaction is a cross-link of DNA (Stacey et al. 1958). As cited earlier, other possible mechanisms of action have been proposed and studied. Of particular interest was the work by Tardiff and Dubois (1969). Their study demonstrated that alkylating agents resemble X-irradiation as inhibitors of the development of microsomal enzymes. In addition, these drugs caused a decrease in the activity of microsomal enzymes in the livers of adult animals. Mechlorethanine (HN2) and Cyclophosphamide (Cytoxan^{R}), the two nitrogen mustards studied, were shown to be ineffective as inhibitors of the N-demethylase, O-demethylase and the EPN detoxification systems *in vitro*.

The addition of L-PAM (also a nitrogen mustard) to the N-demethylase system *in vitro* produced a non-competitive inhibition. This contradicts the results of Tardiff and Dubois (1969) on the effects of nitrogen mustards *in vitro* on microsomal enzymes. However, those investigators used a 0.05 M Na^{+}/K^{+} phosphate buffer while this work was done using a 0.05 M Tris-1.15% KCl buffer. It was shown earlier that L-PAM stability is increased in the presence of the Cl^{-} ion supplied by this 1.15% KCl and decreased in the presence of phosphate ions (Figure 2). Because of the increased stability of L-PAM in the Tris-KCl buffer, more of the parent-lipid soluble drug is available to cross

the membrane barriers of the microsomal enzymes or to alkylate randomly and inhibit the reaction in a non-competitive manner. This inhibition may be unique and specific only for the Tris-KCl buffered system. However if the inhibition of the N-demethylase system is not unique to Tris-KCl buffered systems and occurs *in vivo*, therapy with L-PAM may produce a possible potentiation of the toxicity of drugs normally detoxified by hepatic microsomal enzymes.

Presently, the known degradative paths of L-PAM *in vitro* are hydrolysis and alkylation (Figure 1). Hydrolysis *in vivo* may follow oxidative or reductive biotransformation (Williams 1969). It is not known if L-PAM is actively metabolized *in vivo*. When incubated in microsomal enzymes in a Tris-KCl buffer L-PAM showed rapid disappearance above the rate of water hydrolysis. The ability of the microsomal suspensions to increase L-PAM disappearance was probably related to the unusually high Cl^- ion concentration not found *in vivo*. Because the Cl^- ions helped regenerate the lipophilic parent compound, L-PAM was able to pass through the lipoprotein membranes and gain access to the enzymes active sites. When L-PAM was added to microsomal suspensions in a phosphate buffer, the disappearance rate was the same as the earlier degradation studies using the phosphate buffer alone (Figure 2). The apparent difference in results from the Tris-KCl system

and phosphate buffered system can be explained by the rapid L-PAM degradation in the phosphate buffer thereby decreasing the amount of parent compound available to cross the lipoid membranes.

Although the microsomal systems do not agree with each other the phosphate buffered is more important because it is similar to *in vivo* conditions whereas the Tris-KCl is not an endogenous buffer. If L-PAM is metabolized *in vivo*, the rat liver may not be the organ to study for metabolism. Work by Cohn (1957) using Merphalan, the racemic mixture of L-PAM and labeled with ^{14}C at the β position supports this idea. Cohns' work showed the protein fraction of the rat kidneys had a specific radioactivity at least three times that of any tissue. Additionally cytosolic and microsomal protein of the kidney had the highest radio-activity but no such difference was found in similar cell fractions from the liver. Unpublished data from Alberts et al. has shown an increased L-PAM toxicity in dogs with an impared renal flow. The reason is not known nor has the toxic species (either through metabolism or non-enzymatic degradation) been identified. It would be beneficial therefore to conduct microsomal experiments to compare kidney microsomes with liver microsomes.

Perfused Whole Rat Liver Studies

As shown earlier, the addition of protein (bovine serum albumin) increased the stability of L-PAM in the

degradation studies. A similar protein stabilizing effect has been reported by Chang et al. (1978b). When albumin was added to the buffered media used in the perfusion studies, the stability of L-PAM was increased in the controls and in the perfusate circulating through the liver. However, in the presence of a small amount of bile, L-PAM stability decreased in the buffer containing 5% albumin. This effect of decreased L-PAM stability was opposite from that seen in the degradation studies using bovine, canine or human bile.

There are two possible reasons for the decreased L-PAM stability in the presence of small amounts of bile. One, the bile salts form polymolecular aggregates of micelles once their concentration increases above a certain level known as the critical micellar concentration, or CMC (Sleisenger 1967; Kaneko and Cornelius 1971). In this system, the CMC was never reached and the protective micelles could not be formed. Secondly, the bile salts themselves become bound to serum proteins. This binding would displace L-PAM from drug binding cites and allow it to be more rapidly hydrolyzed. In addition the bile salts may disrupt hydrophobic areas that afford protection to L-PAM. The effect of high plasma bilirubin levels of L-PAM stability in vivo has been reported by Tattersall et al. (1978) and supports the idea of decreased L-PAM stability by possible drug displacement.

The untoward effect of bile on plasma protein and L-PAM stability may be important clinically. Recently Furner et al. (1976) have suggested using L-PAM for cancer of the bile ducts by taking advantage of the unusual L-PAM stability in bile fluid. However, these patients would likely have elevated serum bilirubin. Thus L-PAM may degrade more rapidly thereby reducing the amount of parent drug reaching the cancer. It would be important to study the effect of serum bilirubin on L-PAM stability. Additionally, since L-PAM is used with other drugs their effect on L-PAM protein binding and stability should also be investigated.

In Vitro and In Vivo L-PAM Plasma Disappearance

Furner et al. (1976), have reported at least three L-PAM metabolites, in addition to the mono- and dihydroxy hydrolysis products, in mice and dog tissue. Results from the in vitro and in vivo studies did not show L-PAM to be metabolized. Greater than 85% of the total ^{14}C drug equivalents in plasma or urine could be accounted for both in vitro and in vivo. The parent compound L-PAM, the two hydrolysis products (the mono- and dihydroxy degradation products) and protein bound drug accounted for 85% recovery. The remaining 15% which were lost in both in vitro and in vivo studies, were probably small-methanol soluble peptides which adsorbed to the μC18 column and could be removed

from the column by washing with methanol and one to two mls of DMSO reagent. The plasma profile of percent ^{14}C drug equivalents versus time after oral or intravenous dosing or *in vitro* incubations were similar in appearance and suggest that L-PAM is not significantly metabolized. Tattersall et al. (1978) have also indicated that L-PAM may not be metabolized but that it becomes highly protein bound with time. This is in agreement with the *in vitro* and *in vivo* studies reported here.

CONCLUSIONS

L-PAM showed unusual stability in various incubation media. The mechanism of L-PAM stability in the presence of KCl appeared to be different than that reported for its' stability in bovine serum albumin (Chang et al. 1978b). The stability of L-PAM in bile fluid and high molar concentrations of sodium taurocholate was probably related to hydrophobic interactions between the chloroethyl moiety of L-PAM and taurocholate type molecules, possibly by forming micellular structures.

In <u>in vitro</u> studies, L-PAM was shown to be a noncompetetive inhibitor of the microsomal enzyme system ethylmorphine N-demethylase. The percent inhibition was increased by increasing the pre-incubation time of the microsomal suspensions with L-PAM prior to ethylmorphine addition. L-PAM was rapidly degraded by microsomal enzymes suspended in a Tris-KCl buffer. The effect was thought to be an artifact produced by the Cl^- ions maintaining L-PAM in its parent form and thereby lipid soluble for access to membrane bound enzymes. L-PAM was not rapidly degraded in a phosphate buffered enzyme system above the rate of L-PAM degradation in the phosphate buffer alone.

Whole liver perfusion studies showed that L-PAM was more stable in a buffered media containing albumin. L-PAM did not degrade above the rate of water hydrolysis in the presence of the liver. When small amounts of bile were allowed to flow into the perfusion apparatus, a possible drug displacement effect on L-PAM from protein binding sites was seen.

In in vitro plasma recovery studies, 85% of ^{14}C L-PAM drug equivalents per ml could be recovered at any time up to 56 hours as the mono- and dihydroxy products, protein-bound drug or parent compound. Similarly, 85-100% of the ^{14}C-drug equivalents per ml could be accounted for in in vivo recovery studies from plasma or urine after oral or intravenous dosing of humans and dogs. Both in vitro and in vivo plasma studies strongly suggest that L-PAM does not undergo significant metabolism. Instead, L-PAM disappearance in vitro and in vivo occurs mainly by a non-enzymatic hydrolysis reaction and/or alkylation.

APPENDIX A

PERCENT RECOVERIES OF ^{14}C L-PAM DRUG EQUIVALENTS FROM HUMAN INCUBATED IN VITRO AT 37°C OR HUMAN AND CANINE PLASMA AND URINE FROM IN VIVO PHARMACOKINETIC STUDIES

Table A-1. Percent recovery of L-PAM ^{14}C drug equivalents from human plasma incubated at 37°C *in vitro*.

Time[1]	Total Plasma[2]	% Total ^{14}C Drug Equivalents/ml Plasma				
		A Bound[3]	B DOH	C MOH	D L-PAM	A+B+C+D
0	21.40 µg/ml	4.21	1.92	2.52	78.04	86.69
1	19.97 "	12.72	5.56	7.01	52.28	77.42
2	20.29 "	35.58	9.66	10.05	32.68	84.97
3	19.83 "	40.95	13.97	7.92	22.09	84.93
4	20.26 "	47.19	14.41	6.32	14.51	82.41
6	21.41 "	58.41	18.41	4.58	6.50	87.90
8	20.26 "	59.38	18.41	3.90	3.31	85.00
12	20.77 "	67.45	18.34	3.03	0.96	89.79
24	20.77 "	67.60	19.11	0.00	0.00	86.71
32	19.54 "	55.58	28.71	0.00	0.00	84.29
48	20.99 "	60.36	29.20	0.00	0.00	89.57
56	22.38 "	62.42	24.44	0.00	0.00	86.86

Table A-1--continued. Percent recovery of L-PAM ^{14}C drug equivalents

Plasma #2						
Time[1]	Total Plasma[2]	Bound	DOH	MOH	L-PAM	A+B+C+D
0	89.57 µg/ml	4.57	2.93	7.84	65.87	81.20
1	87.64 "	4.05	8.08	16.00	50.80	78.93
2	89.74 "	24.27	11.52	17.25	29.54	82.58
4	87.55 "	31.81	30.11	11.46	12.74	86.12
5	84.91 "	36.32	36.32	15.31	9.49	84.10
24	83.27 "	36.56	46.18	10.30	1.27	94.32

1. Time in hours after drug administration.

2. Total ^{14}C drug equivalents in ug per one ml human plasma as determined by liquid scintillation after HPLC separation.

3. Non-extractable ^{14}C drug equivalents bound to plasma protein.

L-PAM (210 µg, 0.01 mCi for plasma #1; 1 mg, 0.01 mCi for plasma #2 was added to 10 ml human plasma at 37°C and incubated up to 56 hours. Samples were taken for analysis at designated time points and fractionated into the mono- and dihydroxy degradation products and L-PAM by using an HPLC method previously described.

Table A-2. Percent recovery of L-PAM ^{14}C drug equivalents from human and canine plasma after oral dosing.

Time[1]	Total Plasma[2]	% Total ^{14}C Drug Equivalents/ml Plasma			
		A Bound[3]	B MOH + DOH	C L-PAM	A+B+C
Patient J.O.					
0.50	0.020 µg/ml	-	-	90.00	90.00
0.75	0.109 "	16.51	-	92.66	109.17
1.00	0.225 "	15.56	8.89	67.11	91.56
1.50	0.377 "	-	21.75	56.50	78.25
2.00	0.433 "	20.09	23.79	41.80	85.68
3.00	0.396 "	34.34	15.66	39.65	89.65
4.00	0.445 "	55.73	15.73	10.11	81.57
5.00	0.416 "	65.59	16.11	3.61	86.30
12.00	0.220 "	85.00	-	-	85.00
24.00	0.179 "	91.62	-	-	91.62
Dog S					
0.08	0.37 "	2.70	21.62	81.08	105.41
0.27	0.95 "	5.26	29.47	61.05	95.79

Table A-2--continued. Percent recovery of L-PAM ^{14}C drug equivalents

Time[1]	Total Plasma[2]	A Bound[3]	B MOH + DOH	C L-PAM	A+B+C
Dog S					
0.50	1.16 µg/ml	14.66	26.72	49.14	90.52
0.75	1.04 "	21.15	34.62	31.73	87.50
1.00	1.05 "	28.57	38.10	27.62	94.79
1.50	0.94 "	29.79	39.36	9.57	78.72
2.00	0.78 "	46.15	38.46	7.69	92.31
3.00	0.71 "	47.89	64.79	-	112.68
4.00	0.68 "	63.23	44.12	-	107.35
5.00	0.56 "	67.86	28.57	-	96.43
12.00	0.39 "	89.74	5.13	-	94.87
24.00	0.41 "	90.24	4.89	-	95.12

1. Time in hours after drug administration.

2. Total ^{14}C drug equivalents in ug/ml.

3. Non-extractable ^{14}C drug equivalents bound to plasma protein.

L-PAM (0.6 mg/kg body weight for humans, 1 mg/kg body weight for dogs and ^{14}C L-PAM (100 µCi) were administered orally. Blood samples were

Table A-2--continued. Percent recovery of L-PAM ^{14}C drug equivalents

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obtained as described earlier. L-PAM was extracted from one ml plasma with two ml methanol and 100 µl of the organic layer injected into the HPLC. The eluate was collected and counted for ^{14}C drug equivalents.

Table A-3. Percent recovery of L-PAM ^{14}C drug equivalents from human plasma after intravenous dosing.

Time[1]	Total Plasma[2]	% Total ^{14}C Drug Equivalents/ml Plasma			
		A Bound[3]	B MOH + DOH	C L-PAM	A+B+C
Patient M.M.					
0.08	5.174 µg/ml	11.62	14.80	67.39	93.76
0.25	2.503 "	-	20.53	70.52	91.05
0.50	2.302 "	21.85	15.81	46.35	84.01
0.75	1.876 "	27.72	16.90	38.54	83.16
1.00	1.826 "	33.79	22.62	35.93	92.33
1.50	1.777 "	46.76	19.36	21.05	87.17
2.00	1.804 "	46.56	23.45	16.57	86.59
3.00	1.190 "	65.13	20.50	1.43	87.05
4.00	1.267 "	81.61	14.05	-	95.66
8.00	0.863 "	91.54	6.26	-	97.80
12.00	0.917 "	98.80	1.96	-	100.77
22.50	0.563 "	100.00	-	-	100.00

Table A-3--continued. Percent recovery of L-PAM ^{14}C drug equivalents

Time[1]	Total Plasma[2]	A Bound[2]	B MOH + DOH	C L-PAM	A+B+C
Patient J.P.					
0.08	2.429 µg/ml	21.86	11.94	52.70	86.50
0.25	1.885 "	-	14.75	72.25	87.75
0.50	0.910 "	21.98	13.85	52.52	88.35
0.75	0.961 "	44.75	13.73	31.84	90.32
1.00	0.787 "	48.41	16.40	14.49	79.29
1.50	0.802 "	63.34	15.00	12.22	90.65
1.75	0.778 "	66.71	10.03	8.74	85.48
4.08	0.623 "	100.00	-	-	100.00
5.00	0.601 "	100.00	-	--	100.00
6.00	0.553 "	100.00	-	-	100.00
24.00	0.375 "	100.00	-	-	100.00

1. Time in hours after drug administration.

2. Total C-14 drug equivalents in µg/ml.

3. Non-extractable C-14 drug equivalents bound to plasma proteins.

Table A-3--continued. Percent recovery of L-PAM ^{14}C drug equivalents

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L-PAM (0.6 mg/kg body weight) and C-14 L-PAM (100 μCi) were combined and injected as a bolus into the side of a running intravenous administration set. Blood samples (10ml) were obtained at various time points and L-PAM extracted from one ml plasma with methanol.

Table A-4. Percent recovery of L-PAM ^{14}C drug equivalents from human and canine urine after oral dosing.

Time[1]	Total[2]	% Total ^{14}C Drug Equivalents/ml Urine A DOH	B MOH	C L-PAM	A+B+C
Patient J.O.					
3.00	5.069 mg	20.89	21.04	29.91	71.83
4.25	1.388 "	28.47	20.18	28.11	76.76
6.25	1.090 "	36.42	17.73	14.45	68.59
7.00	0.608 "	48.89	16.89	10.67	76.44
24.00	1.972 "	53.26	13.59	5.43	72.28
24 hr Total	10.127 mg	31.58%	19.45%	22.07%	73.18%
Dog S					
Time	Total	DOH + MOH		L-PAM	%A+B
1.00	1.318 mg	37.31		37.40	74.71
2.00	2.087 "	64.52		16.62	81.14
4.00	2.054 "	84.58		4.44	89.02
6.00	0.897 "	94.26		3.91	98.12
24.00	2.017 "	99.60		2.97	102.57
24 hr Total	8.373 mg	76.82%		12.31%	89.13%

1. Time in hours after drug administration.

2. Total C^{14} drug equivalents in milligrams (mg).

L-PAM (0.6 mg/kg body weight for humans, 1 mg/kg body for dogs) plus ^{14}C L-PAM (100 μCi) were administered orally. Urine collections were taken for the first 8 hours after drug administration and then at known intervals for up to 24 hours. Urine samples (100 μl, adjusted to pH 7.4 with 1N NaOH) were injected into the HPLC system. The eluate was collected and counted for ^{14}C drug equivalents.

Table A-5. Percent recovery of L-PAM ^{14}C drug equivalents from human and canine urine after oral dosing.

Time[1]	Total[2]	% Total ^{14}C Drug Equivalents/ml Urine			
		A DOH	B MOH	C L-PAM	A+B+C
Patient M.M.					
4.67	2.244 mg	45.24	22.59	23.61	91.44
9.50	8.045 "	69.23	18.96	6.40	94.59
24.00	0.514 "	76.01	17.34	8.08	101.43
24 hr Total	10.803 mg	64.57%	19.63%	10.05%	94.25%
Patient J.P.					
Time[1]	Total[2]	A DOH	B MOH	C L-PAM	A+B+C
1.25	3.418 mg	18.46	24.66	46.66	89.79
2.00	2.365 "	45.17	27.91	23.51	96.60
4.00	3.711 "	47.86	27.51	19.99	95.35
24.00[3]	2.06 "	78.64	13.59	5.83	98.06
24 hr Total[4]	9.494 mg	36.56%	26.55%	30.45%	93.56%

1. Time in hours after drug administration.

2. Total ^{14}C drug equivalents in milligrams (mg).

3. Urine volume was not recorded and total is in µg/ml. Urine was separated into the MOH, DOH and L-PAM fractions.

4. Values do not include the 24 hour urine data.

L-PAM (0.6 mg/kg body weight) and ^{14}C L-PAM (100 µCi) were combined and injected as a bolus into the side of a running intravenous administration set. Urine collections were taken for the first eight hours after drug administration and then at known intervals for up to 24 hours. A urine sample (100 µl, adjusted to pH 7.4 with 1N NaOH) was injected into the HPLC system and the eluate collected and counted for ^{14}C drug equivalents.

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