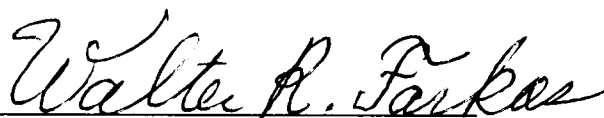
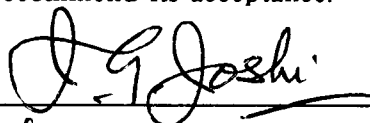
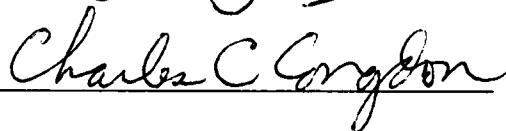


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LYSINOALANINE AS A LYSINE ANTAGONIST: INCORPORATION INTO
PROTEIN AS A POSSIBLE MECHANISM OF TOXICITY

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Ben J. Lifsey, Jr.

June 1987

DEDICATION

To my wife, Daron, for her love, patience, and understanding during the long hours and to my parents, Ben and Barbara, for instilling in me the desire to learn and the perception to gain from my mistakes.

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ABSTRACT

Lysinoalanine [N^{ϵ} -(DL-2-amino-2-carboxyethyl)-L-lysine; LAL] is a nephrotoxic lysine analog formed in proteins as a result of alkali and/or heat treatment. Lysinoalanine induces alterations in the renal epithelial cells of the *pars recta* region of the proximal tubules (Woodard and Alvarez, 1967; Woodard and Short, 1973). This lesion was first observed by Woodard and Alvarez (1967) and designated as nephrocytomegalia by Woodard and Short (1973).

Lysinoalanine was shown to be a competitive inhibitor of lysyl-tRNA-synthetase (EC 6.1.1.6) from prokaryotic and eukaryotic cells *in vitro* when present at micromolar concentrations. The apparent K_i for lysinoalanine for *E. coli* lysyl-tRNA-synthetase was 9.4×10^{-5} M and for rabbit liver lysyl-tRNA-synthetase was 1.5×10^{-4} M. The apparent K_m for the esterification of *E. coli* and rabbit liver tRNAs was 2.0×10^{-5} M and 5.0×10^{-5} M, respectively.

Lysinoalanine inhibited the incorporation of ^{14}C -lysine into protein by a cell-free eukaryotic protein synthesizing system. Inhibition was 69.7% and 18.4% at lysinoalanine concentrations of 1.0 mM and 0.1 mM, respectively.

^{14}C -Lysinoalanine was incorporated into protein by a cell-free eukaryotic protein synthesizing system when present at micromolar concentrations as evidenced by the formation of hot TCA precipitable radioactivity and fluorography of a dried polyacrylamide gel. The proteins labeled with lysinoalanine coelectrophoresed with those labeled with lysine. These results indicate that lysinoalanine is an inhibitor of both prokaryotic and eukaryotic lysyl-tRNA-synthetase at concentrations which might occur *in vivo*. These observations and a possible mechanism of lysinoalanine-induced nephrocytomegalia are presented.

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CHAPTER I

INTRODUCTION

Occurrence of Lysinoalanine in Food Products

Lysinoalanine [N^{ϵ} -(DL-2-amino-2-carboxyethyl)-L-lysine; LAL] is an unusual lysine analog formed as a result of alkali and/or heat treatment of protein. It was first found in alkali treated ribonuclease (Bohak, 1964; Patchornik and Sokolovsky, 1964). Lysinoalanine is toxic and induces alterations in the renal epithelial cells of the *pars recta* region of the proximal tubule (Woodard and Alvarez, 1967; Woodard and Short, 1973).

Alkali treatment is a common practice in the commercial food processing industry to enhance the palatability of the product. A multitude of physical and chemical changes can occur, resulting in altered appearance, texture, taste and digestibility of alkali-treated foods (Sternberg and Kim, 1976). In addition, alkali treatment is used in the purification (Horan, 1974; Saunders *et al.*, 1975; Wu *et al.*, 1977) and preparation (Rosenfield and Hartman, 1974) of spun proteins from alkali-extracted soya isolates used in the production of meat analogs low in cholesterol (Hamdy, 1974; Sternberg and Kim, 1976) and other food products such as "tofu," a popular form of soy protein (Standal, 1961, 1963). The destruction of aflatoxin in peanuts (Sreenivasamurthy, *et al.*, 1967; Goldblatt, 1969), enzymes (Andres, 1979), and digestive enzyme inhibitors (Badenhop and Hackler, 1970) as well as the peeling of fruits and vegetables (Gee *et al.*, 1974; Shultz *et al.*, 1977) employ alkali treatment. Other uses include the modification of properties such as foaming and emulsifying characteristics of proteins intended as food ingredients, enhancing their solubility and stability in solution (Tannenbaum *et al.*, 1970; Circle and Smith, 1972; Betschart, 1974).

DeGroot and Slump (1969) found that exposure of several high protein products to aqueous alkali (pH 10, 40°C, 4 hours) resulted in lysinoalanine formation. Increased severity of the alkali treatment (pH 12.2, 40-80°C, 4-8 hours) caused an increased amount of lysinoalanine formation.

Lysinoalanine is also formed under non-alkaline conditions (pH 2-6.5, 100-120°C, 1-16 hours) (Sternberg *et al.*, 1975; Sternberg and Kim, 1976). Consequently, lysinoalanine is present in a variety of commercial food preparations and ingredients as well as foods prepared in the home under a variety of cooking conditions. Among foods not exposed to alkaline conditions, lysinoalanine content ranged from zero in frankfurters, chicken thighs and fresh egg white before cooking, to as much as 50,000 ppm in a commercial whipping agent. Significant levels of lysinoalanine occur in condensed milk, acid casein, cooked chicken thigh and sirloin steak pan scrapings (Sternberg *et al.*, 1975; Sternberg and Kim, 1976). Table I indicates the presence of lysinoalanine in a variety of food preparations and ingredients.

Lysinoalanine is present in large quantities (1730 ppm) in formula diets designed for administration to patients in a reduced metabolic state (e.g. critical care medicine, gastroenterology and oncology) (Pfaff, 1984) and to infants where food products containing significant levels of lysinoalanine may constitute 100% of the diet (Sternberg and Kim, 1976; Gould and MacGregor, 1976).

Natural Occurrence of Lysinoalanine

Lysinoalanine was first believed to be an artifact of food preparation and had not been identified in native proteins of plant or animal origin. Gross *et al.* (1977) found lysinoalanine to be a naturally occurring amino acid in cinnamycin and duramycin, peptides of microbial origin isolated from *Streptomyces cinnamoneus* and *Streptomyces cinnamoneus forma azacoluta*, respectively. The discovery of lysinoalanine in these peptides was preceded by studies concerning the structural

elucidation of nisin and subtilin from *Streptococcus lactis* and *Bacillus subtilis*, respectively (Gross *et al.*, 1971). These peptides contain numerous residues of the α,β -unsaturated amino acids dehydroalanine and dehydrobutyrine. Dehydroalanine is believed to be a precursor of lysinoalanine (Gross, 1977) and arises from a hydroxide ion catalyzed β -elimination reaction of amino acids with functional groups at the β -carbon atom (Friedman *et al.*, 1984). This group of susceptible amino acids includes serine, cystine and cysteine. Threonine is also susceptible to β -elimination and after reacting with lysine forms β -methyl-lysinoalanine (Gross, 1977). Lysinoalanine has also been detected as a component of native collagen from human dentin (Fujimoto *et al.*, 1981), as well as bovine dentin phosphoprotein (Kuboki *et al.*, 1984).

Mechanism of Lysinoalanine Formation

Lysinoalanine formation is believed to occur via a two step reaction (Gross, 1977; Whitaker and Feeney, 1977) involving lysine and the amino acids mentioned above (Bohak, 1964; Patchornik and Sokolovsky, 1964; Feeney, 1977; Gross, 1977; Friedman, 1978a; Isohata, *et al.*, 1983; Friedman *et al.*, 1984).

Lysine and cystine are the amino acids most easily damaged by processing and/or storage of food (Tucker *et al.*, 1983). Hydroxide ion catalyzed β -elimination of serine, cystine and cysteine residues within the protein give rise to a dehydroalanine intermediate. Dehydroalanine contains a conjugated reactive double bond capable of reacting with the epsilon amino group (ϵ -NH₂) of lysine, forming a lysinoalanine crosslink within the protein (Fig. 1)* (Friedman *et al.*, 1984). Lysinoalanine differs from the common amino acids. It contains two carboxyl groups, two primary amino groups and one secondary amino group (Sternberg and Kim, 1976).

*All Tables and Figures can be found in the Appendix

Factors Affecting Lysinoalanine Formation

The β -elimination reaction is a second order reaction which depends on the hydroxide ion concentration and the concentration of the labile amino acids. In addition, the relative susceptibility of these labile amino acid residues to base-catalyzed elimination and their proximity to ϵ -NH₂ groups of lysine residues are factors (Whitaker and Feeney, 1977).

The reaction between the ϵ -NH₂ group of lysine with the carbon-carbon double bond is also a second order reaction (Friedman and Wall, 1964; Snow *et al.*, 1976), suggesting that the rate of lysinoalanine formation increases with increased protein concentration. Friedman (1978a) reported that the rate of lysinoalanine formation did not increase with increased protein concentration. He proposed that the major factor controlling lysinoalanine formation once the rate determining dehydroalanine intermediate is formed is the location of available lysine residues. Only dehydroalanine and lysine residues in close proximity on the same or adjacent protein chains are able to combine and form crosslinks. Savoie and Parent (1983) suggested that protein conformation and steric factors may play a role in lysinoalanine crosslink formation. Therefore, the degree to which crosslinking occurs appears to be limited by the amino acid sequence of the protein(s) as well as conformational and steric factors.

Determination of the rate of lysinoalanine formation is complicated by the variable effect of pH on the decomposition of alkali-labile amino acids. Cysteine residues decompose at a lower pH than serine and threonine residues (Friedman, 1978a). Therefore, the rate of lysinoalanine formation in different proteins as a function of pH depends on the relative levels of these amino acids.

Lysinoalanine is relatively stable under alkaline conditions. Exposure of free lysinoalanine to 1 N NaOH (pH 11.5 at 100°C) resulted in 87.5% recovery after 24

4.2 N NaOH (Hugli and Moore, 1972; Friedman *et al.*, 1984b) resulted in 82.4% recovery after 24 hours.

Lysinoalanine in the free and protein-bound state is stable to acid hydrolysis (6 N HCl, 100°C for free lysinoalanine, 110°C for protein-bound lysinoalanine) for up to 72 hours (Friedman, 1978a, 1978b, 1979; Friedman *et al.*, 1984). The protein-bound lysinoalanine used in these studies was a component of lactalbumin, soy protein, or wheat gluten.

Lysinoalanine formation in alkali-treated soy protein (0.1 N NaOH, pH 12.5, 3 hours) increased with increased temperature (Friedman *et al.*, 1984). Lysinoalanine formation began at 25°C and increased from 0.49 g/16 g N to 3.07 g/16 g N at 85°C. A concomitant decrease in serine, cystine, threonine and lysine was observed.

The duration of treatment also had an effect on lysinoalanine formation. Significant amounts of lysinoalanine formed in soy protein after 10 minutes in 0.1 N NaOH at 75°C. Lysinoalanine content increased from 0.75 g/16 g N to 3.45 g/16 g N after 8 hours. Again, there was a decrease in cystine, serine, threonine and lysine. After only 10 minutes cystine was completely destroyed (Friedman *et al.*, 1984).

Factors Which Inhibit Lysinoalanine Formation

Considerable interest has been generated concerning the nutritional quality and safety of alkali treated proteins containing lysinoalanine. Strategies to prevent or minimize lysinoalanine formation while maintaining the desirable properties of the protein have been investigated. One of these methods employs the modification of lysine residues by acylation prior to alkali treatment (Friedman, 1978a). Another involves the addition of various compounds which compete with protein components that are involved in the formation of lysinoalanine crosslinks (Finley *et al.*, 1977, 1978; Wartheson and Wood-Rethwill, 1981; Katsuta and Hayakawa, 1984).

Cysteine inhibits lysinoalanine formation in soy protein and casein (Finley *et al.*, 1977,1978). The inhibitory action of added cysteine was later confirmed in casein (Wartheson and Wood-Rethwill, 1981) and in soy protein (Katsuta and Hayakawa, 1984). Katsuta and Hayakawa (1984) found that 2-mercaptoethanol inhibited lysinoalanine formation as well, but not as effectively as cysteine.

Cysteine had a variable influence on different protein systems. The inhibitory effect was less in wheat gluten (10% reduction in lysinoalanine formation) than in casein (38% reduction in lysinoalanine formation) (Wartheson and Wood-Rethwill, 1981). They suggested that the decreased effectiveness of cysteine in wheat gluten was due to the relatively high levels of cysteine within the protein. Finley *et al.* (1978) suggested that added cysteine may not only compete with cysteine residues within the protein, but also reduce the disulfide bonds of cystine crosslinks, thereby slowing the conversion of cysteine to dehydroalanine and reducing the rate of lysinoalanine formation.

There was no significant effect of cysteine on lysinoalanine formation in zein from normal corn. This was attributed to the low level of lysine in zein from normal corn which would limit the formation of lysinoalanine. Consequently, the inhibitory effect of cysteine on dehydroalanine formation was not apparent. In high lysine corn, cysteine caused an increase in lysinoalanine formation (Wartheson and Wood-Rethwill, 1981).

The addition of free lysine during alkaline treatment of casein, gluten and zein was investigated (Wartheson and Wood-Rethwill, 1981). Free lysine contributed significantly to alkali-induced lysinoalanine formation in gluten and zein, 114% and 56.8% respectively. Both proteins are relatively low in lysine. The addition of free lysine to casein, a relatively lysine-rich protein, decreased lysinoalanine formation by 44% during alkali treatment. This same phenomenon was observed in zein from

normal corn when free lysine was added. Wartheson and Wood-Rethwill proposed a mechanism to explain these observations whereby the α -NH₂ group of added free lysine competed with the ϵ -NH₂ group of protein-bound lysine for the reaction with dehydroalanine, reducing the amount of lysinoalanine formed. Reduced glutathione and propionylmercaptoglycine were also effective in reducing lysinoalanine formation in soy isolate (8% and 4% of control, respectively), while oxidized glutathione, a potential source of dehydroalanine, increased the level of lysinoalanine formation nearly 2.5 fold (Finley *et al.*, 1978).

These studies indicate the effectiveness of lysine, reduced glutathione, 2-mercaptoethanol and mercaptoamino acids during alkali treatment on reducing lysinoalanine formation in processed foods. However, it is apparent that added cysteine and lysine do not inhibit lysinoalanine formation under all processing conditions. They may actually serve as promoters of lysinoalanine formation in some situations, depending on the intrinsic nature of the proteins being treated.

Protein acylation (acetylation, succinylation, etc.) of the ϵ -NH₂ groups of lysine residues prevented lysinoalanine formation and lysine destruction in wheat gluten and promine-D (soy protein) when exposed to heat and alkali (Friedman, 1978a). This procedure failed to protect cysteine, serine or threonine residues from destruction.

Metabolic Transit, Biological and Toxicological Consequences of Lysinoalanine in Laboratory Animals

The effects of dietary lysinoalanine were studied in laboratory animals (Woodard and Alvarez, 1967; Woodard and Short, 1973, 1977; Reyniers *et al.*, 1974; Woodard, 1975; Woodard *et al.*, 1975; deGroot *et al.*, 1976; Finot *et al.*, 1977; Struthers *et al.*, 1977, 1978; Leegwater, 1978; Karayiannis *et al.*, 1979; Sternberg and Kim, 1979; Robbins *et al.*, 1980; Abe *et al.*, 1981, 1984). The metabolic fate of

lysinoalanine was investigated by feeding alkali treated protein of known lysinoalanine content to rats and by using free ^{14}C -lysinoalanine, radiolabeled in the lysine moiety, administered to rats, other rodents and Japanese quail (Finot *et al.*, 1977; Struthers *et al.*, 1977).

Considerable lysinoalanine (30-50% of that ingested) was found in the feces of Sprague-Dawley rats fed alkali treated protein (Finot *et al.*, 1977). All lysinoalanine in the feces was protein-bound. Urinary lysinoalanine (10-25% of that ingested) was present in both the free state and protein-bound. Total recovery of lysinoalanine was less than 100%, therefore it must be metabolized and/or retained by the organism or transformed by the intestinal flora. Similar findings were reported by deGroot *et al.* (1976).

Whole body autoradiography of Sprague-Dawley rats, Swiss mice, Syrian hamsters and Japanese quail given ^{14}C -lysinoalanine *per os* indicated that radioactivity was highly concentrated in the kidneys of rodents, but not in quail. ^{14}C was localized in the inner cortex of the kidney in rodents and was highest in the rat. Radioactivity was also incorporated to a lesser degree in the salivary and lachrymal glands, bones and intestinal walls. Radioactivity represented both lysinoalanine and its catabolites (Finot *et al.*, 1977; Struthers *et al.*, 1980). Acetylated derivatives of lysinoalanine were excreted more readily than lysinoalanine by all species studied. The rat eliminated lysinoalanine and its catabolites less effectively than other species. Hepatocytes are not involved in the metabolism of lysinoalanine (Finot *et al.*, 1977).

The biological consequences of lysinoalanine in the diet are significant both toxicologically and nutritionally. Nutritionally, the presence of lysinoalanine crosslinks reduces the Net Protein Utilization (NPU) and digestibility of the protein (deGroot and Slump, 1969). In addition, lysinoalanine is formed at the expense of

cystine, serine, lysine and possibly other amino acids (deGroot *et al.*, 1976). Heat and alkali treatment of proteins also results in other degradation products, racemization, amino acid-sugar complex formation (Maillard reaction) (Tucker *et al.*, 1983) and crosslinking of amino acids other than lysine, serine and cystine (Walsh *et al.*, 1979; Masters and Friedman, 1980; Thompson and Erdman, 1981).

The inclusion of alkali-treated soy protein in diets intended for animal consumption has been shown to lead to a decrease in the availability of certain trace minerals such as zinc, manganese, copper, and iron (Odell and Savage, 1960). Iron deficiency anemia was induced in monkeys by feeding a soybean protein diet (Fitch *et al.*, 1964). Lysinoalanine is a strong chelator of metals, particularly copper and zinc. Inhibition of the metalloenzymes yeast alcohol dehydrogenase and carboxypeptidases A and B by lysinoalanine at millimolar concentrations was reported by Hayashi (1982). In addition, lysinoalanine was found to be a more effective inhibitor of the zinc-containing enzyme carboxypeptidase A than ethylenediaminetetraacetic acid (EDTA). Inactivation of the enzyme was also observed by lysinoalanine-containing food proteins such as casein, high-lysine zein, soy protein isolate and other proteins after alkali treatment (Friedman *et al.*, 1985). However, Friedman and coworkers also observed inhibition of carboxypeptidase A by alkali treated zein from normal corn containing no lysine and, consequently, no lysinoalanine. Therefore, the mechanism of carboxypeptidase A inhibition by alkali treated protein is unclear at this time and may not involve lysinoalanine.

Microorganisms such as *Bacillus subtilis*, *Aspergillus niger* and *Tetrahymena pyriformis*, a microorganism suggested for use as an indicator of protein nutritional value (Hackler, 1977), and L-lysine dependent mutant strains of *Escherichia coli* and *Erwinia chrysanthemi* (Sternberg and Kim, 1979; Karayiannis *et al.*, 1979) are capable of metabolizing lysinoalanine. Mice exhibited a poor growth response when

fed a wheat gluten diet containing lysinoalanine (8350 ppm) compared to a wheat gluten diet control group (Sternberg and Kim, 1979). This was evidenced by a decreased Protein Efficiency Ratio (PER), indicating that mice are unable to significantly utilize lysinoalanine. Mice have the same essential amino acid requirements as rats and humans (Bauer and Berg, 1943).

Basic amino acids are believed to be reabsorbed by the cells of the *pars recta* region of the proximal tubule (Meister, 1973). The mechanism of basic amino acid (L-lysine) uptake has been described (Lerner, 1978; Lee and Pritchard, 1983; Sepulveda and Pearson, 1985). It appears that lysinoalanine is absorbed by the cells of the *pars recta* region of the proximal tubule as well, where it exerts its toxic effects. Alkali-treated soy protein, when fed to rats, induced changes in the epithelial cells of the *pars recta* region of the proximal renal tubules located in the outer medullary stripe of the kidney (Woodard and Alvarez, 1967; Woodard and Short, 1973). The lesions are characterized by enlargement of the cytoplasm and nucleus, increase in nucleoprotein and disturbances in deoxyribonucleic acid (DNA) synthesis and mitosis (Newberne and Young, 1966; Woodard and Alvarez, 1967; Woodard, 1969; Woodard and Short, 1973, 1977; Reyniers *et al.*, 1974; Woodard *et al.*, 1975). Enlarged nuclei frequently had increased DNA content (Woodard and Alvarez, 1967; Woodard and Short, 1973). Nuclei with DNA values intermediate between diploid and tetraploid were frequently observed as well as an occasional octaploid cell (Woodard and Alvarez, 1967).

This lesion, designated nephrocytomegalia by Woodard and Short (1973), is attributed to lysinoalanine and limited to the outer medullary stripe of the kidney, almost exclusively to the *pars recta* of the proximal tubules (Woodard and Short, 1977). Nephrocytomegalia was also induced by feeding (Woodard and Short, 1973; Woodard *et al.*, 1975; deGroot *et al.*, 1976) and intraperitoneal injection (Woodard *et*

al., 1975) of free synthetic lysinoalanine to rats. Free synthetic L-lysine as a control did not induce nephrocytomegalia when administered in an identical manner (Woodard and Short, 1977).

Microscopic examination of a wide range of organs and tissues (heart, lung, liver, kidney, spleen, brain, gonads, thymus, salivary, prostate, epididymis, thyroid and adrenal glands, uterus, urinary bladder, aorta, gastro-intestinal tract, pancreas and axillary and mesenteric lymph nodes) obtained from rats fed a diet containing a relatively low level of synthetic lysinoalanine (100 ppm) for 13 weeks showed marked damage only in the kidney. The lesions were located in the outer medullary stripe and consisted of necrosis, regeneration and cytomegalia in the proximal tubules. Identical lesions were observed in the kidneys of rats fed higher levels of synthetic lysinoalanine (1000-10,000 ppm) for shorter periods of time (deGroot *et al.*, 1976).

Morphological changes associated with lysinoalanine toxicity include occasional absence of brush border cells, multiple nucleoli, karyomegaly with increased basophilia and frequent cytomegaly (Woodard and Short, 1977). Megalocytes as large in diameter as adjacent tubules were reported (Woodard and Alvarez, 1967). Eosinophilic nuclear inclusions were apparent in cells with normal and enlarged nuclei. These inclusions were not due to cytomegalic inclusion disease or viral infection (Woodard and Short, 1967).

Cortical changes were observed at dietary concentrations of 1000-3000 ppm lysinoalanine. Dilated tubules, groups of tubules with regenerative epithelium, and the presence of periglomerular sclerosis were reported. Hypercellular glomeruli and thickening of Bowman's capsule with hyperplasia of visceral and parietal layers were also observed (Woodard and Short, 1977).

The occurrence of nephrocytomegalia was independent of the route of administration of lysinoalanine (Woodard and Short, 1977; Reyniers, 1979), indicating that lysinoalanine is readily absorbed. Free lysinoalanine is more toxic than protein-bound (deGroot *et al.*, 1976; Woodard and Short, 1977). Reyniers (1979) observed nephrocytomegalia in both germfree and conventional rats when fed either alkali treated soy protein isolate or synthetic free lysinoalanine, indicating that microbial processing was not prerequisite for nephrotoxicity. Incidence and severity of nephrocytomegalia was lower in germfree animals in this study, suggesting that the gut microflora in conventional animals may increase the availability of protein bound lysinoalanine and that microbes may be involved in the production of toxic lysinoalanine metabolites. Lysinoalanine-induced nephrocytomegalia is time and dose dependent (Reyniers, 1979).

Lysinoalanine-induced nephrocytomegalia was first reported to be species specific. DeGroot *et al.* (1967) observed renal lesions in rats fed diets containing lysinoalanine, but no lesions were observed in mice, hamsters, rabbits, quail, dogs or monkeys. However, lysinoalanine induced nephrocytomegalic changes were observed by others in mice (Feron *et al.*, 1978; Sternberg and Kim, 1979; Reyniers, 1979) and in gerbils (Reyniers, 1979). Among different strains of rats, Karayannis *et al.* (1979) reported no qualitative or quantitative differences with respect to nephrocytomegalia in Wistar and Sprague-Dawley rats fed identical diets containing alkali treated soy protein isolate (2630 ppm lysinoalanine).

The clinical significance of lysinoalanine-induced nephrocytomegalia is obscure. The carcinogenicity and teratogenicity of lysinoalanine were tested in rats (Struthers *et al.*, 1978; Feron *et al.*, 1977). The results of both these investigations were negative. There was no effect on conception rate, number of young per litter, fetus weight, or mortality. Pup birth weight was reduced when dams were fed a diet

containing 3000 ppm lysinoalanine or 20-30% alkali-treated soy protein (2000-3000 ppm lysinoalanine). Pups from dams fed lysinoalanine developed nephrocytomegalia by 30 days of age (Struthers *et al.*, 1978). Lysinoalanine was found not to be mutagenic in the Ames test, even in the presence of an induced rat-kidney S-9 preparation (Pintauro *et al.*, 1985). Elevated levels of plasma glutamic-pyruvic transaminase (GPT) and blood urea nitrogen (BUN) were reported with a decrease in total plasma protein in rats fed a 20% alkali-treated soy protein diet (containing 1370-2630 ppm lysinoalanine) after 12 weeks of feeding (Karayiannis *et al.*, 1979). Woodard *et al.* (1975) reported no differences in the excretion of *p*-amino-hippuric acid between treated rats with nephrocytomegalia and normal animals. There was no indication in the literature that an LD₅₀ for lysinoalanine had been determined in any species.

The effects of nephrocytomegalia and the chronic ingestion of lysinoalanine have been studied by only one author (Feron *et al.*, 1978). In rats fed 0 to 200 ppm free synthetic lysinoalanine for one year, nephrocytomegalia developed after 4 weeks. Morphological abnormalities of the affected renal epithelial cells were consistent with those observed at high doses of lysinoalanine in short term studies (Woodard, 1969, 1975). No other adverse effects were observed as a result of chronic ingestion of lysinoalanine.

The nuclear cytochemical alterations associated with nephrocytomegalia were investigated. In addition to karyomegaly, nephromegalocyte nuclear cytochemical characteristics included increased availability of DNA phosphates for cation binding in relation to total DNA, reduced thermal stability of chromatin and an altered ratio of the dye-binding capacity of histone arginine and lysine residues (Reyniers *et al.*, 1974).

Acridine orange binding is exclusive to DNA and is an indicator of the degree of chromatin condensation (Killander and Rigler, 1969). Acridine orange binding in lysinoalanine induced megalocytes indicated a greater availability of DNA phosphates in relation to the amount of DNA, as well as an increased DNA content when compared to normal cells (Woodard, 1967; Reyniers, *et al.*, 1974).

Increased acridine orange binding of chromatin is associated with cellular activation followed by ribonucleic acid (RNA) synthesis in human leukocytes when stimulated with phytohemagglutinin (PHA) (Killander and Rigler, 1965, 1969; Darzynkiewicz *et al.*, 1969). In contrast, mature human spermatozoa, cells in a "deactivated" state, have less acridine orange binding capacity (Killander and Rigler, 1965).

A reduction in chromatin thermal stability has also been associated with cellular activation. Reduced chromatin thermal stability was observed in transplanted murine neoplasias (Alvarez, 1973), hepatocytes activated by partial hepatectomy (Alvarez, 1974) and adrenocorticotrophic hormone stimulated-rat adrenal gland cells (Alvarez and Lavender, 1974). Physicochemical alteration of the chromatin with subsequent RNA synthesis and an increase in cell size has been correlated with an increase in total non-histone nuclear protein (Reyniers *et al.*, 1974).

Histone lysine residues are essential to the mechanism of DNA folding in the nucleosomal core (Jordano *et al.*, 1984). An altered ratio of dye binding by histone lysine and arginine residues may be due to blocking of lysine and arginine residues (Zetterberg and Auer, 1968; Berlowitz and Pallota, 1972). Increased acetylation of the ϵ -NH₂ group of lysine reduces the effectiveness of blocking *in vitro* RNA transcription (Darzynkiewicz *et al.*, 1969; Berlowitz and Pallota, 1972). Post-translational modifications of histones induce changes in the functional state of the nucleus and are major mechanisms for altering the conformation of the eukaryotic

chromosome to allow DNA replication, transcription and mitosis to occur (Ruiz-Carrillo *et al.*, 1976). Condensation of chromatin prior to mitosis is associated with methylation of histone lysine (Olson and Busch, 1974). Methionine, choline and vitamin B₁₂, important dietary sources of methyl groups (Metzler, 1977) have a modulating effect on the development of nephrocytomegalia in rats when fed alkali treated soy protein supplemented with these compounds (Newberne and Young, 1966; Newberne *et al.*, 1968).

Lysinoalanine contains two asymmetric centers and, therefore, four possible stereoisomers (Struthers *et al.*, 1979). The stereoisomers have been designated LD, DL, LL, and DD with the first letter referring to the lysine, and the second to the alanine moiety (Tas and Kleipool, 1976). LD-Lysinoalanine is the most toxic *in vivo* with nephrocytomegalia occurring at only 30 ppm dietary LD-lysinoalanine. The DL form is next in potency, followed by the LL form and the DD form (Feron *et al.*, 1978). By using optically specific amino acid oxidases, Bohak (1964) determined that lysinoalanine formed in alkali treated ribonuclease had the configuration N^ε-(DL-2-amino-2-carboxyethyl)-L-lysine. A racemic mixture with respect to the alanine moiety would be expected since the proposed mechanism of lysinoalanine formation involves the α , β carbon double bond of dehydroalanine and lysine is present in the native protein in the L-form (Gould and MacGregor, 1976). Gould and MacGregor also noted that certain proteins may preferentially give rise to one or the other optical configurations depending on their conformational properties.

Another nephrotoxic amino acid, 2,3-diaminopropionic acid, a potential catabolite of lysinoalanine, induced renal lesions morphologically identical to lysinoalanine-induced nephrocytomegalia. The hierarchy of stereoisomer toxicity with respect to the alanine moiety is identical to lysinoalanine. That is, D-2,3-

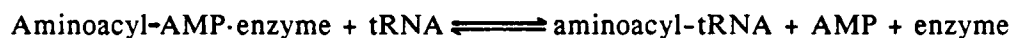
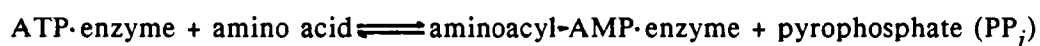
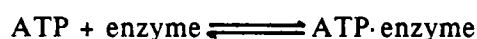
diaminopropionic acid is most toxic and L-2,3-diaminopropionic acid is least toxic (Reyniers, 1979).

The localization of ^{14}C -lysinoalanine (labeled uniformly in the lysine moiety) in renal subcellular fractions obtained from rats fed radiolabeled lysinoalanine was studied (Struthers *et al.*, 1980). A range from 15 to 87% of the radioactivity present in the total subcellular fraction was associated with trichloroacetic acid (TCA) precipitable material. However, upon chromatographic analysis, the ^{14}C -labeled material appeared to be mainly lysine, or possibly a metabolite that cochromatographed with lysine. Lysinoalanine accounted for 0-10% of the total TCA precipitable radioactivity. From these studies, Struthers and coworkers concluded that the rat has some mechanism for converting lysinoalanine to lysine and/or other metabolites reported previously by Finot *et al.* (1977).

Inhibition of Aminoacyl Transfer RNA Synthetase by Amino Acid Analogs

A variety of unusual amino acids occur in nature, often localized in plants or microorganisms (Fowden, 1974; Bell, 1976). These amino acid analogs produce a wide range of toxic effects when ingested by mammals (Knowles and Ballard, 1978). Amino acids perform numerous roles as components of proteins as well as playing essential roles in other biosynthetic pathways (Shive and Skinner, 1963).

Aminoacyl-transfer RNA synthetases, key enzymes in protein biosynthesis, interact with ATP, a specific amino acid and transfer RNA (tRNA) in the following manner (adapted from Allende and Allende, 1971):



In the first two steps, the enzyme aminoacyl-tRNA-synthetase catalyzes a reaction between the carboxyl group of the amino acid and ATP, with the release of

PP_i. The aminoacyl adenylate is firmly attached to the enzyme. Subsequently, the amino acid is esterified to the 2'- or 3'-hydroxyl of the ribose of the terminal adenosine of its cognate tRNA (Novelli, 1967). Following the aminoacylation of tRNA, amino acids are incorporated into protein at the peptidyl transferase center of the ribosome. Peptide chain elongation is accomplished by displacement of the tRNA molecule by the growing peptide chain with the formation of a peptide bond between the α -amino group of the newly attached amino acid and the α -carboxyl group of the previously incorporated amino acid (Novelli, 1967; Metzler, 1977; Stryer, 1981).

The effects of amino acid analogs at all steps of protein biosynthesis have been investigated in a variety of *in vitro* and *in vivo* systems. Many amino acid analogs inhibit the aminoacylation of tRNA by the corresponding aminoacyl-tRNA-synthetases. This inhibition may be manifest by preventing the formation of an aminoacyl adenylate or by preventing the esterification of the amino acid to tRNA. Inhibition at this stage has been investigated by utilizing an assay whereby the ATP-PP_i exchange reaction catalyzed by the specific aminoacyl-tRNA-synthetase is inhibited. This reaction is reversible as demonstrated by the incorporation of radiolabeled pyrophosphate (³²PP_i) into ATP in the presence of the cognate amino acid (Freist and Cramer, 1980).

A variety of polyfluorinated analogs of tryptophan are competitive inhibitors of the ATP-PP_i exchange reaction (Nevinsky, *et al.* 1974). L-Amphetamine, L-phenylalaninol, 2-phenylethylamine *in vitro* (Santi and Danenberg, 1971) and analogs of N-benzyl-2-phenylethylamine *in vivo* (Santi *et al.*, 1979) were found to inhibit the phenylalanyl-tRNA-synthetase catalyzed ATP-PP_i exchange reaction. Minosine and fluorophenylalanine block phenylalanyl-tRNA-synthetase in the same manner (Smith and Fowden, 1968), as does histidinol in human cells with respect to histidine

(Hansen *et al.*, 1972). Inhibition of the ATP-PP_i exchange reaction also occurs in the inhibition of arginyl-tRNA-synthetase by canavanine (Hirschfield and Bloemers, 1969; Rosenthal *et al.*, 1976). In addition, the D forms of α -aminobutyric acid, valine and norvaline are competitive inhibitors of valyl-tRNA-synthetase (Lotfield, 1973).

Some amino acid analogs may become esterified to tRNA (Vasquez, 1974). At this stage there is no maintenance of translational fidelity so that once an amino acid or an analog has been activated and esterified to tRNA, incorporation into protein is inevitable (Novelli, 1967; Knowles and Ballard, 1978).

A classic case of an amino acid analog becoming incorporated into protein is that of the methionine analog, ethionine. Ethionine has been shown to be incorporated into rat protein *in vivo* (Stekol and Weiss, 1950; Levine and Tarver, 1951; Natori *et al.*, 1961; Natori, 1963). In addition, norvaline, α -aminobutyric acid, S-methylcysteine and homocysteine serve as methionine analogs and are incorporated into protein as well (Old and Jones, 1976). Incorporation of alloisoleucine, canavanine, N-ethylglycine and O-methylthreonine into protein has also been observed (Smulson and Rabinowitz, 1968; Lotfield, 1973).

Several amino acid analogs, when incorporated into protein, result in abnormal procollagen synthesis. Hydroxynorvaline, a threonine analog disrupts the assembly of triple stranded procollagen, resulting in poor recognition by the secretory mechanism (Christner *et al.*, 1975). Similarly, in embryonic chicken tibiae, *trans*-4,5-dehydrolysine, a lysine analog, generated abnormal collagens which were not extruded into the extracellular matrix at the normal rate and were therefore subject to degradation (Christner and Rosenbloom, 1971). Another lysine analog, 4-selenolysine (deMarco *et al.*, 1976) and two proline derivatives, β -thiaproline and γ -thiaproline (Bussiello *et al.*, 1979) were also reportedly incorporated into protein.

The latter two impaired chain elongation by preventing the formation of peptide bonds. Aberrant proteins resulting from the incorporation of amino acid analogs have shortened half-lives. This is true for hemoglobin when valine or lysine analogs are incorporated (Rabinovitz and Fisher, 1961) as well as for canavanine in other proteins (Schachtele *et al.*, 1968; Prouty, 1976).

Involvement of Lysine Residues in the Function of Various Proteins

As mentioned previously, the physiochemical alterations of nucleoprotein (histones) associated with lysinoalanine-induced nephrocytomegalia may in part be due, directly or indirectly, to the role of lysine in nucleoproteins (Woodard, 1967; Zetterberg and Auer, 1968; Darzynkiewicz *et al.*, 1969; Pallota, 1972; Olson and Busch, 1974; Reyniers, *et al.*, 1974; Ruiz-Carrillo *et al.*, 1976; Jordano *et al.*, 1984). Participation of lysine residues in the structural and/or catalytic roles of various proteins in addition to histones has been investigated. In prokaryotes, the ϵ -NH₂ group of lysine residues is important in the recognition and formation of a specific complex between 30-S ribosomal subunits and messenger RNA (mRNA) (Chang and Craven, 1978). Lysine residues are also associated with the binding of cytochrome *c* oxidase and cytochrome *b*₅ with cytochrome *c* (Ahmed *et al.*, 1978; Rieder and Basshard, 1978).

Enzymes involved in the transfer of phosphate groups are known as phosphotransferases or protein kinases. The majority of transphosphorylations involve ATP (Dixon and Webb, 1979). Lysine appears to play an essential role in this class of enzymes. An inspection of amino acid sequences of all protein kinases sequenced to date revealed absolute conservation of lysine at the ATP binding site of these enzymes (Hunter and Cooper, 1985). Lysine residues are essential for protein-tyrosine kinase (Weinmaster *et al.*, 1986) and histone kinase activity (Kochetkov *et al.*, 1977).

Phosphoribosylpyrophosphate synthetase (EC 2.7.6.1) is a key enzyme in the biosynthesis of purines, pyrimidines, pyridine nucleotides, histidine and tryptophan. Inactivation of this enzyme isolated from *Salmonella typhimurium* was achieved by reacting a specific lysine residue at or near the active center of the enzyme with pyridoxal phosphate (Roberts and Switzer, 1978).

Lysine residues play a significant role in the structure of collagen. Procollagen contains hydroxylysine which allows for glycosylation of the protein prior to secretion. Following secretion into the extracellular space and peptidase activity, lysyl amino groups may be oxidized to aldehyde groups, leading to the crosslinking of collagen monomers. Aldol condensation and the elimination of water from these aldehydes results in further crosslinking between four different side chains, giving rise to a histidinohydroxymerodesmosine linkage. Two other lysine derivatives, desmosine and isodesmosine, are found in elastin (Metzler, 1977).

Of particular interest is the amino acid hypusine [N^{ϵ} -(4-amino-2-hydroxybutyl)-lysine], found in the eukaryotic translational initiation factor eIF-4D (Cooper *et al.*, 1983; Park *et al.*, 1984a). Hypusine is formed from lysine by the addition of the butylamine moiety of the polyamine spermidine followed by hydroxylation (Park *et al.*, 1984b; Paz *et al.*, 1984). The role of this modification in protein synthesis and the function of hypusine is not known (Pegg, 1986). Torrelío *et al.* (1984) reported a correlation between the rate of cell growth (protein, DNA synthesis) and the rate of hypusine formation. It should be noted that lysinoalanine after loss of one of its carboxyl groups would be an analog of hypusine.

Characterization of Prokaryotic and Eukaryotic Lysyl-tRNA-Synthetases

Characterization of the kinetics and enzymatic mechanism of lysyl-tRNA-synthetase (EC 6.1.1.6) has been done in both eukaryotic and prokaryotic cells. The reaction mechanism for the enzyme purified from *E coli* B is well established (Hele

and Barber, 1972). The order of addition of substrates is lysine, ATP (in the presence of Mg^{2+}) and tRNA. Hele and Barber (1972) estimated the molecular weight to be 150,000 with two unequal subunits. However, other reports suggest that the enzyme may be polymeric in nature with a minimum molecular weight of 78,000 (Marshall and Zamecnik, 1969). Rymo *et al.* (1972) reported lysyl-tRNA-synthetase from *E. coli* to consist of two subunits of equal molecular weight (62,000) with two lysine and two ATP binding sites.

The Michaelis constant (K_m) for *E. coli* B lysyl-tRNA-synthetase for lysine was determined to be 4.0×10^{-6} M at 25°C over the range 2.5×10^{-6} - 20×10^{-6} M by Hele and Barber (1972) and 2.4×10^{-6} at 25°C over the range of 1.5×10^{-6} - 25×10^{-6} M by Marshall and Zamecnik (1969). Rymo *et al.* (1972) reported a binding constant (K) to be 6.4×10^{-3} M and 3.6×10^{-3} M for lysine by two different enzyme preparations. Waldenstrom (1968) determined the K_m to be 1.8×10^{-5} M lysine at 37°C over an undisclosed range. Similarly, Hirshfield and Yeh (1976) reported a K_m for lysine of 2.5×10^{-5} M and 1.3×10^{-5} M for two different preparations of lysyl-tRNA synthetase from wild-type *E. coli* K-12.

In contrast to the bacterial enzymatic mechanism, the addition of substrates to rat liver lysyl-tRNA-synthetase is tRNA, lysine and then ATP (in the presence of Mg^{2+}). In rat liver, lysyl-tRNA-synthetase is part of a lysyl, arginyl tRNA-synthetase complex (Hilderman *et al.*, 1983). Dang *et al.* (1982) reported that rat liver arginyl-tRNA-synthetase-lysyl-tRNA-synthetase exists as a tetrameric complex with a molecular weight of 285,000 consisting of subunits of 73,000 and 65,000. They found that the reciprocal plot of substrate concentration versus reaction velocity (Lineweaver-Burk plot) was not linear for lysine or arginine. For this reason, they reported the affinity constant as $S_{1/2}$ instead of K_m . The $S_{1/2}$ value for lysine was 1.13×10^{-5} M which is in perfect agreement with the K_m for lysine

reported by Hilderman *et al.* (1983) of 1.13×10^{-5} M at 37°C. There were no studies of rabbit liver lysyl-tRNA-synthetase found in the literature.

CHAPTER II

EXPERIMENTAL

Materials

Tris(hydroxymethyl)aminomethane (Tris), glutathione (GSH), *E. coli* B cells (ATCC 11303), *E. coli* tRNA (Type XXI, Strain W), polyethylene glycol (PEG 6000), ethylenediamine-tetraacetic acid (EDTA), bentonite, lysinoalanine, ammonium persulfate, Coomassie Brilliant Blue R-250, and N-N-N'-N'-tetramethylethylenediamine (TEMED) were purchased from Sigma Chemical Company, St. Louis, MO. Protein standards used in electrophoresis were also from Sigma. Purity of lysinoalanine was determined to be >99.8% on a Beckman Model 120C amino acid analyzer (Fig. 2). 2-Mercaptoethanol and XAR x-ray film was from Eastman Kodak Company, Rochester, NY. All L-amino acids were purchased from Sigma, except for aspartic acid (Nutritional Biochemicals Corp., Cleveland, OH) and tyrosine (Fisher Scientific Company, Fair Lawn, NJ). Rabbit reticulocyte lysate (nuclease treated) and Brome Mosaic Virus RNA (BMV RNA) were purchased from Promega Biotech, Madison, WI. Trichloroacetic acid (TCA) and acetic acid were from Fisher Scientific. Phenol was obtained from J.T. Baker Chemical Company, Phillipsburg NJ. Glycerin and sodium salicylate were from Mallinkrodt Inc., Paris, KY. Bovine serum albumin (BSA) was from Nutritional Biochemicals Corporation. Bovine γ -globulin and Bradford Reagent for protein assays were obtained from Bio-Rad Laboratories, Richmond, CA. Sodium dodecylsulfate (SDS), acrylamide, and N-N'-methylene-bis-acrylamide (bis-acrylamide) were also from Bio-Rad. Bacto Nutrient Broth was from Difco Laboratories, Detroit, MI. Diethyleneaminoethyl (DEAE) cellulose was obtained from Whatman Ltd., Maidstone, England. ^{14}C -Lysine (270-317 mci/mmole, 0.1 mci/ml 0.01N HCl) was purchased from ICN Radiochemicals,

Irvine, CA. ^{14}C -Lysinoalanine [N^{ϵ} -(DL-2-amino-2-carboxyethyl)-L-lysine; 108 mci/mmol, 0.14-0.16 mci/ml] was a generous gift from Jon P. Reyniers, Texas A & I University, Kingsville, TX. ^{14}C -Lysinoalanine was synthesized by the method of Finley and Snow (1977) and purified using an amino acid analyzer (Beckman Model 120C) and thin-layer chromatography (TLC). Figure 3 indicates the minor contamination of ^{14}C -lysinoalanine prior to purification. Purity of ^{14}C -lysinoalanine after cleanup was confirmed by TLC (Fig. 4). Toluene and Scintiverse II were purchased from Fisher Scientific. Omnifluor was from New England Nuclear Research Products, Boston, MA. All inorganic chemicals used were reagent grade or better and water was double glass distilled.

Methods

Preparation of *E. coli* Aminoacyl-tRNA-Synthetase. A modification of the methods described by Baldwin and Berg (1966) and Muench and Berg (1966) for the preparation of aminoacyl tRNA-synthetase was used. All steps were at 4°C unless otherwise noted. Approximately 5 g lyophilized *E. coli* B cells were used to inoculate 2 l Bacto Nutrient Broth (8 g/l) after autoclaving at 15 psi/ 121°C for 15 minutes. Cells were incubated at 37°C for 15 hours with constant air sparge (20 cc/minute). Growth was monitored using a Klett-Summerson photoelectric colorimeter. A bacterial pellet (7.4 g wet weight) was obtained by centrifugation ($1500 \times g$, 15 minutes) during logarithmic phase, suspended in 90 ml 2 mM K_2HPO_4 with the addition of 60 g acid washed glass beads (75-150 μ) and disrupted in a Waring blender 10 minutes. The extract was centrifuged at $5000 \times g$ for 5 minutes to remove glass beads. The beads were rinsed several times with 2 mM K_2HPO_4 . Rinses were added to the extract. This material was centrifuged at $15,000 \times g$ for 25 minutes to remove debris. The volume of the supernatant was adjusted to 300 ml

with 2 mM K_2HPO_4 and 4.8 g solid K_2HPO_4 (1.6 g/100 ml) were added. After incubation at 37°C for 2 hours, the precipitate was removed by centrifugation at 15000 x g for 15 minutes. The supernatant was dialyzed with two changes against 15 volumes 20 mM sodium phosphate pH 7.5, 20 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 10% glycerin (v/v). The dialyzed extract was then subjected to ion-exchange chromatography (DEAE-cellulose, 2.5 x 24.0 cm) after column equilibration with the dialysis buffer. The column was washed with 370 ml of dialysis buffer and then with 1 l of 0.25 M sodium phosphate pH 6.5, 20 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 10% glycerin (v/v). The column effluent was monitored at 280 nm and 5 ml fractions were collected. The fractions containing the first absorbance peak were pooled and concentrated by dialysis against 40 volumes of 1 mM potassium phosphate pH 7.5, 20 mM 2-mercaptoethanol, 10% glycerin (v/v), 15% PEG 6000 (w/v) and then against 300 volumes 1 mM potassium phosphate pH 7.5, 20 mM 2-mercaptoethanol, 10% glycerin (v/v). This preparation served as a source of *E. coli* lysyl tRNA-synthetase and was stored in 1 ml aliquots at -80°C.

Preparation of Rabbit Liver Aminoacyl-tRNA-Synthetase. Livers were obtained from two euthanized New Zealand White rabbits. Livers were weighed (total weight = 64.0 g) and immediately placed in approximately ten volumes of ice cold 0.85% saline. Rabbit liver aminoacyl-tRNA-synthetase was obtained according to the method of Katze and Farkas (1979) with some modification. All steps were carried out at 4°C unless otherwise noted. Livers were homogenized at high speed for 10 minutes in a Waring blender containing 180 ml 1 mM Tris-HCl pH 7.6, 1 mM $MgCl_2$, 10 mM 2-mercaptoethanol, cooling in an ice bath every 2 minutes. The homogenate was centrifuged at 31,000 x g for 30 minutes and the remaining debris was removed by filtration through glass wool. The supernatant was then centrifuged at 105,000 x g for 2 hours. The 105,000 x g supernatant was then subjected to ion-

exchange chromatography (DEAE-cellulose, 2.5 x 15 cm) after column equilibration with Equilibration Buffer (5 mM Tris-HCl pH 7.6, 5 mM MgCl_2 , 5 mM 2-mercaptoethanol). The column was washed with Equilibration Buffer until the effluent absorbance at 280 nm was <0.01 . Protein was eluted with 1 l 10 mM Tris-HCl pH 7.6, 200 mM KCl, 1 mM MgCl_2 , 10 mM 2-mercaptoethanol, 10% glycerin (v/v). The column effluent was monitored at 280 nm and 6 ml fractions were collected. Aliquots of 0.005 ml were taken from every other fraction within the first absorbance peak and assayed for aminoacyl tRNA-synthetase activity. The fractions containing activity were pooled and stored in aliquots of 2 ml at -80°C .

Preparation of Rabbit Liver tRNA. Preparation of tRNA was by the method of Dubrul and Farkas (1976). One New Zealand White rabbit was starved 24 hours prior to sacrifice to reduce hepatic glycogen content. The liver was obtained, weighed (16.3 g), frozen at -80°C , thawed to the consistency of "hard butter," diced into small pieces (approximately 1 cm^3) and homogenized in a Waring blender containing 67.2 ml Buffer A (50 mM Tris-HCl pH 7.6, 50 mM NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA), 67.2 ml phenol and 0.65 ml bentonite (43 mg/ml) for 30 seconds. The homogenate was then placed in an ice bath for 30 minutes and stirred with a magnetic stirrer. The aqueous phase was obtained by centrifugation at $300 \times g$ for 20 minutes. The organic phase was re-extracted with an equal volume of Buffer A. Aqueous phases were pooled. Three volumes of cold 95% ethanol and 1/10 final volume of cold 4.0 M NaCl was added to the aqueous phase and placed at -20°C for 24 hours. Precipitated RNA was obtained by centrifugation at $10,000 \times g$ for 20 minutes at 0°C and dried *in vacuo* for 3 hours at 23°C . A DEAE-cellulose column (1.5 x 4.0 cm) was equilibrated with Buffer B (Buffer A plus 0.25 M NaCl) at 23°C . The RNA was dissolved in 2.0 ml Buffer B and applied to the DEAE column followed by washing with Buffer B until effluent absorbance at 260 nm was

less than 0.01. Transfer RNA was eluted with 20.0 ml Buffer C (Buffer A plus 1.0 M NaCl). One ml fractions were collected and the absorbance of each was determined at 260 nm. Fractions containing the absorbance peak were pooled. Three volumes of cold 95% ethanol were added to this material and kept at -40°C for 3 hours. Precipitated tRNA was obtained by centrifugation and dried *in vacuo* as before. Rabbit liver tRNA was dissolved in 0.5 ml water and the number of absorbance units was determined (A_{260} units/ml). The tRNA concentration was adjusted to approximately 60 A_{260} units/ml and was stored in 1.0 ml aliquots at -80°C .

Protein Concentration Assay. The protein concentration of aminoacyl tRNA-synthetase preparations was determined by the method of Bradford (1976) using the Bio-Rad Protein Assay. Bovine γ -globulin was used to derive a standard curve.

Aminoacylation Assay. The aminoacylation assay was a modification of that described by Farkas *et al.* (1972). The reaction mixture contained 25 mmoles Tris-HCl pH 7.4, 2.5 mmoles MgCl_2 , 2.5 mmoles KCl, 0.25 mmoles ATP, 1.0 mmole GSH, 5 μg BSA, 2.2 A_{260} units tRNA (*E. coli* W Type XXI or rabbit liver), 0.068-0.076 μCi (2.5×10^{-7} moles) or 0.11-0.12 μCi (4.0×10^{-7} moles) ^{14}C -lysine for rabbit liver and *E. coli* assays, respectively, and 100 μg protein containing rabbit liver-lysyl tRNA-synthetase or 1 μg protein containing *E. coli* lysyl-tRNA-synthetase. The final volume was 0.250 ml unless noted otherwise. The reaction mixture was incubated in a water bath at 37°C with constant agitation (60 oscillations/minute) for 5 minutes for rabbit liver or 10 minutes for *E. coli*. Following incubation, tubes were placed in ice cold water and tRNA was precipitated by the addition of 3.0 ml cold 3 M HCL. After 5 minutes the precipitated tRNA was collected by vacuum filtration on Whatman glass microfiber

filter discs (2.5 cm). Tubes were rinsed three times with cold 3 M HCl. This material was collected on the same disc by vacuum filtration as well. Filter discs were washed with 5-10 ml cold 3 M HCl, cold 95% ethanol and dried using an infrared lamp for 15 minutes. Dried filter discs were placed in 10 ml toluene containing 4.0 g/l omniflour and the radioactivity of each sample was determined by liquid scintillation counting using a Packard Tri-Carb 4530 instrument. Counting efficiency for ^{14}C was generally between 90 and 93%. All radioactivity was recorded in dpm and in mmoles substrate/unit time. All assays included appropriate controls which contained all the above components with the exception of the enzyme. All assays were performed in duplicate.

Time Course of ^{14}C -Lysyl-tRNA Formation. Assays were performed as previously described with slight modifications. Excess ^{14}C -lysine was present (6.4×10^{-6} moles, 1.8-1.9 μCi) and the final volume was 0.5 ml for the *E. coli* enzyme. All other components of the reaction were present at the concentrations previously described. Incubation times for *E. coli* lysyl-tRNA-synthetase were 0, 5, 10, 20 and 30 minutes. Incubation times for rabbit liver lysyl-tRNA-synthetase were 0, 2.5, 5, 10, 20, 30, and 40 minutes.

Dependence of ^{14}C -Lysyl-tRNA-Synthetase Activity on Protein Concentration. Assays were performed as previously described with slight modifications. The reaction mixture for the *E. coli* assay contained 0-3.6 μg protein (5 data points) in a final volume of 0.5 ml (the amount of other components listed under Aminoacylation Assay was adjusted accordingly) and excess ^{14}C -lysine (6.4×10^{-6} moles, 1.8-1.9 μCi) was present. For rabbit liver, enzyme was added at 0-700 μg protein (9 data points).

Determination of the Michaelis Constant (K_m) of Lysyl-tRNA-Synthetases for Lysine. Assays were performed as previously described with the exception of substrate (^{14}C -lysine) concentration. A range of $8.0 \times 10^{-7} \text{ M}$ to $3.2 \times 10^{-6} \text{ M}$ (4 data points) was used for *E. coli* and from 5.0×10^{-7} to 2.5×10^{-5} (8 data points) for rabbit liver. Graphical representation and the determination of the apparent K_m for lysine was according to Lineweaver and Burk (1934) as described by Dixon and Webb (1979).

Inhibition of the Aminoacylation of tRNA by Lysinoalanine. The assay for the inhibition of *E. coli* lysyl-tRNA-synthetase was performed as previously described with some modification. Lysinoalanine was held constant at three different levels over a range of lysine concentrations. Lysine concentrations were $2.0 \times 10^{-6} \text{ M}$, $1.25 \times 10^{-6} \text{ M}$, $6.0 \times 10^{-7} \text{ M}$ and $3.0 \times 10^{-7} \text{ M}$. Lysinoalanine was present at 0 M (control), $5.0 \times 10^{-4} \text{ M}$, $1.0 \times 10^{-5} \text{ M}$ and $5.0 \times 10^{-5} \text{ M}$.

Similarly, the assay for the inhibition of rabbit liver lysyl-tRNA-synthetase was performed as previously described with some modification. Lysine concentrations were $3.0 \times 10^{-6} \text{ M}$, $1.1 \times 10^{-6} \text{ M}$, $8.4 \times 10^{-7} \text{ M}$ and $5.6 \times 10^{-7} \text{ M}$. Lysinoalanine was present at 0 M (control), $1.0 \times 10^{-3} \text{ M}$, and $1.0 \times 10^{-4} \text{ M}$. Graphical representation of the data and determination of the apparent K_m for lysine and the apparent K_i for lysinoalanine was according to Lineweaver and Burk (1934) as described by Dixon and Webb (1979).

Time Course of ^{14}C -Lysinoalanyl-tRNA Formation. Assays were performed as previously described for *E. coli* lysyl-tRNA-synthetase except for the substrate used and the length of the 37°C incubation. ^{14}C -Lysinoalanine was used with lysinoalanine present at a concentration of $3.0 \times 10^{-5} \text{ M}$ (0.8 μCi). The reaction was incubated at 37°C for 0, 10, 20, 30, 40, 50 and 60 minutes. A time course of rabbit

liver lysyl-tRNA-synthetase activity using ^{14}C -lysinoalanine as a substrate was not performed due to limited material.

Determination of the Michaelis Constant (K_m) for Lysinoalanine. Assays were performed as previously described with the exception of substrate concentration. A range of 5.0×10^{-6} M to 1.0×10^{-4} M lysinoalanine (7 data points) was used for *E. coli* and a range of 2.0×10^{-5} M to 2.0×10^{-4} M (4 data points) was used for rabbit liver. Graphical representation of the data and the determination of the apparent K_m was according to Lineweaver and Burk (1934) as described by Dixon and Webb (1979).

Inhibition of Cell-Free Protein Synthesis by Lysinoalanine. A cell-free rabbit reticulocyte lysate protein synthesizing system was used. Rabbit reticulocyte lysate was treated with micrococcal nuclease to destroy endogenous messenger RNA (mRNA) and supplemented with creatinine phosphate, creatine phosphokinase, calf liver tRNA and hemin. Brome Mosaic Virus mRNA (BMV RNA, 0.5 mg/ml) was used. The reaction was carried out in a water bath at 30°C with constant agitation (40 oscillations/minutes) in a volume of 0.1 ml containing 0.070 ml reticulocyte lysate, 2.0 μg BMV RNA, 0.25 μmoles ^{14}C -lysine (0.068 μci), 2.0 μmoles of a mixture of 19 L-amino acids pH 7.4(minus L-lysine), and either 0, 0.1 or 0.01 mmoles lysinoalanine. In addition, a control without mRNA or LAL, but containing all of the above components, was run. Aliquots of 0.033 ml were removed at 0, 10, and 20 minutes and immediately placed in 2.0 ml cold (0°C) 10% TCA to precipitate protein. The precipitated material was then incubated at 90°C for 20 minutes to deacylate any remaining aminoacyl-tRNA. The samples were cooled to 4°C and collected on microfiber filter discs by vacuum filtration. Tubes were rinsed three times with 3.0 ml cold 8% TCA and once with cold 95% ethanol. This material

was collected on filter discs as well and rinsed thoroughly with cold 8% TCA and cold 95% ethanol. Filter discs were dried and radioactivity was determined as previously described. All reactions were done in duplicate. The formation of TCA-insoluble radioactivity indicated the incorporation of ^{14}C -lysine into protein.

Incorporation of ^{14}C -Lysinoalanine into Protein by an *in Vitro* Rabbit Reticulocyte Lysate Translation System. The assay was carried out as above except that ^{14}C -lysinoalanine was used in place of ^{14}C -lysine. The lysinoalanine concentration was 0.1 mM (1.1 μCi) in a final volume of 0.1 ml.

Analysis of *in Vitro* Translation Products by Gel Electrophoresis and Fluorography. The *in vitro* rabbit reticulocyte lysate/BMV RNA protein synthesizing system was used as previously described with some modification. The reaction mixture was incubated for 30 minutes at 30°C. A zero message (no BMV RNA) control was run for both ^{14}C -lysine and ^{14}C -lysinoalanine. After the incubation, a 0.010 ml aliquot was removed from each sample and precipitated in hot TCA as previously described to determine the approximate TCA-insoluble radioactivity. One milliliter cold acetone was added to the remaining material (0.090 ml/sample) to precipitate protein. The precipitated protein was collected by centrifugation at 15,000 x g for 10 minutes. Acetone was removed by aspiration and vacuum desiccation. The protein from each sample was solubilized in 0.050 ml of 5 mM Tris pH 6.8 containing 15% glycerol (v/v), 5% SDS (w/v), and 0.02% 2-mercaptoethanol (v/v) and then subjected to electrophoresis. A volume containing approximately 5,000 cpm for (+) mRNA samples was loaded. An equal volume of (-) mRNA samples was loaded. Polyacrylamide gel electrophoresis was performed by a modification of the method of Laemmli (1970). The running gel consisted of 12.5% acrylamide (w/v), 0.34% bis-acrylamide (w/v), 0.38 M Tris-HCl pH 8.8, 0.1% SDS

(w/v) and was polymerized by the addition of ammonium persulfate to 0.06% (w/v) and TEMED to 0.08% (w/v). The stacking gel was 7.4% acrylamide, 0.2% bis-acrylamide, 0.1 M Tris-HCl pH 6.8, 0.1 % SDS and was polymerized by the addition of ammonium persulfate to 0.09% and TEMED to 0.05%. The electrode buffer (pH 8.3) contained 0.025 M Tris-HCl, 0.192 M glycine, 0.1% SDS. The running gel measured approximately 70 x 80 x 1.5 mm thick and was run at 18 mAmps for 90 minutes. The gel was removed and fixed in approximately 100 ml of 40% ethanol, 7% acetic acid for 15 hours (solution changed once) and then stained with Coomassie Brilliant Blue R-250 (0.125% by weight in 40% ethanol, 7% acetic acid) for 1 hour on a rocker platform. The gel was destained for 5-6 hours in 10% acetic acid, 7% ethanol (with several changes) at 37°C. The gel was prepared for fluorography by the method of Roberts *et al.* (1984) by washing with tap water for 30 minutes and then impregnating it with sodium salicylate (1 M solution) for 30 minutes. The gel was then dried under heat and vacuum for 2 hours with white blotter paper as a backing. Radioactive proteins were visualized by exposing the gel to Kodak XAR x-ray film for 7 days at -80°C. The protein standards used were transferrin ($M_r=78,000$), ovalbumin ($M_r=43,000$), chymotrypsinogen ($M_r=25,000$), and ribonuclease A ($M_r=13,899$).

CHAPTER III

RESULTS AND DISCUSSION

In order to characterize the kinetics of an enzymatic reaction and determine the possible effects of a substrate analog on the reaction, several characteristics of the reaction must be established. The formation of product must be shown to be dependent on the length of incubation. In addition, the rate of the reaction must be dependent on the amount of protein present in the reaction. Finally, for aminoacyl-tRNA-synthetases, the optimum substrate (amino acid, ATP, and tRNA) and cofactor (Mg^{2+}) concentrations must be established. The procedure described by Farkas *et al.* (1972) with some slight modifications provided acceptable assay conditions.

Characterization of *E. coli* Lysyl tRNA-Synthetase Activity

The reaction velocity (formation of ^{14}C -lysyl-tRNA) was measured by the formation of acid precipitable (3 M HCl at 0°C) radioactivity and was dependent on the length of incubation at 37°C. The formation of ^{14}C -lysyl-tRNA was also dependent on the amount of protein added to the reaction.

Initially, the activity of *E. coli* lysyl-tRNA-synthetase (2.4 mg protein/ml) was inhibited by some factor in the enzyme preparation (possibly Mg^{2+}). Magnesium ion concentrations have been shown to have a profound effect on synthetase activity (Merault *et al.*, 1978). Dialysis at 4°C against approximately 300 volumes 1 mM potassium phosphate pH 7.5, 20 mM 2-mercaptoethanol to remove $MgCl_2$ and PEG increased enzyme activity approximately ten-fold. A preliminary time course using this material revealed that the enzyme concentration was too high, resulting in very rapid formation of ^{14}C -lysyl-tRNA (>75,000 cpm in 5 minutes). The time course was repeated after diluting the enzyme preparation with 1 mM potassium phosphate

pH 7.5, 20 mM 2-mercaptoethanol. A protein concentration of 60 $\mu\text{g/ml}$ (4 $\mu\text{g/ml}$ in the reaction) gave the best results. The reaction velocity proceeded in a linear fashion with respect to time for up to 15 minutes under these conditions (Fig. 5). All assays with *E. coli* lysyl-tRNA-synthetase and ^{14}C -lysine were incubated for 10 minutes at 37°C.

A linear relationship was observed between the amount of enzyme present and the reaction velocity (Fig. 6). One microgram of protein (4 $\mu\text{g/ml}$ in the reaction) resulted in the most desirable reaction velocity and was used in all *E. coli* enzyme assays. Subsequent assays revealed that storage of the enzyme at -80°C at a protein concentration of 60 $\mu\text{g/ml}$ resulted in a dramatic loss of activity. For this reason, the remainder of the enzyme preparation was stored at -80°C at the original concentration of 2.4 mg/ml. An appropriate amount for individual assays was diluted to 60 $\mu\text{g/ml}$ with 1 mM potassium phosphate pH 7.5, 20 mM 2-mercaptoethanol immediately prior to use.

The apparent K_m of *E. coli* lysyl-tRNA-synthetase for lysine was determined to be 1.9×10^{-6} M. This is in close agreement with the apparent K_m determined by Hele and Barber (1972) of 2.4×10^{-6} M at 25°C and approximately ten-fold lower than that determined by Hirshfield and Yeh (1976) at 37°C ($1.3\text{--}2.5 \times 10^{-5}$ M). The Lineweaver-Burk plot is shown in Figure 7.

Characterization of Rabbit Liver Lysyl-tRNA-Synthetase Activity

The velocity of the reaction catalyzed by rabbit liver lysyl-tRNA-synthetase (14.0 mg/ml) was dependent on the length of incubation at 37°C and on the amount of protein present. The amount of acid-insoluble radioactivity increased rapidly with incubation time (Fig. 8). This is most likely due to the high protein concentration of the rabbit liver lysyl-tRNA-synthetase preparation. Five minutes was chosen as the incubation time and was used in all rabbit liver enzyme assays.

The relationship between the amount of protein present and the reaction velocity is shown in Figure 9.

The apparent K_m of rabbit liver lysyl-tRNA-synthetase for lysine was determined to be 7.9×10^{-7} M. This is approximately fourteen-fold lower than that reported for rat liver lysyl-tRNA-synthetase by Dang *et al.* (1982) and Hilderman *et al.* (1983) (1.13×10^{-5} M at 37°C). The relationship between $1/V$ (mmoles lysyl-tRNA/5 min.⁻¹) and $1/S$ (M lysine⁻¹) was linear ($r=0.991$), whereas Dang and coworkers (1982) reported a non-linear relationship for lysine with the rat liver enzyme. The Lineweaver-Burk plot is shown in Figure 10.

Inhibition of Lysyl-tRNA-Synthetase by Lysinoalanine

The inhibition of lysyl-tRNA-synthetase by lysinoalanine was competitive with respect to lysine for the enzyme from *E. coli* and rabbit liver. The correlation coefficient (r) was at least 0.962 for $1/V$ vs $1/S$ at all lysinoalanine concentrations for both *E. coli* and rabbit liver lysyl-tRNA-synthetases. Figure 11 indicates the competitive nature of lysinoalanine inhibition of *E. coli* lysyl-tRNA-synthetase with an apparent K_i (inhibition constant) of 9.4×10^{-5} M. Competitive inhibition of rabbit liver lysyl-tRNA-synthetase with respect to lysine is plotted in Figure 12. The apparent K_i for lysinoalanine is 1.5×10^{-4} M.

Determination of the K_m for Lysinoalanine

A time course of *E. coli* lysyl tRNA-synthetase activity with ¹⁴C-lysinoalanine as the substrate indicated that reaction velocity (formation of ¹⁴C-lysinoalanyl-tRNA) with respect to incubation time was essentially linear from zero to 20 minutes (Fig. 13). Due to limited material, a time course of rabbit liver lysyl-tRNA-synthetase activity using ¹⁴C-lysinoalanine as a substrate was not performed.

The apparent K_m for lysinoalanine was calculated to be 2.0×10^{-5} M for *E. coli* lysyl-tRNA-synthetase (Fig. 14) and 5.0×10^{-5} M for rabbit liver lysyl-tRNA-synthetase (Fig. 15). A summary of the kinetic data for lysyl-tRNA-synthetase from both *E. coli* and rabbit liver is shown in Table 2.

The effect of other amino acids on the esterification of ^{14}C -lysinoalanine to tRNA by rabbit liver lysyl-tRNA-synthetase is shown in Table 3. ^{14}C -Lysinoalanine and other amino acids shown in the table were present at equimolar concentrations (5×10^{-5} M) in a final volume of 0.125 ml. The addition of all other components listed under Aminoacylation Assay (p. 27) to the reaction was adjusted to compensate for the final volume used. The nature of the inhibition (competitive or noncompetitive) by L-tryptophan and the L-amino acid mix was not determined. The L-amino acid mix contained all the naturally occurring amino acids except L-lysine.

Inhibition of *in Vitro* Translation by Lysinoalanine.

Inhibition of *in vitro* incorporation of ^{14}C -lysine into hot TCA precipitable radioactivity (protein) with a rabbit reticulocyte lysate/BMV RNA translation system was observed when lysinoalanine was present at 0.1 and 1.0 mM. After 20 minutes incubation at 30°C, inhibition was 18.4% at 0.1 mM lysinoalanine and 69.7% at 1.0 mM lysinoalanine (Figure 16).

Incorporation of ^{14}C -Lysinoalanine into Protein by a Cell-Free Protein Synthesis System.

The recovery of hot TCA insoluble radioactivity indicated that ^{14}C -lysinoalanine was incorporated into protein. The data in Figure 17 is expressed as $\Delta^{14}\text{C}$ -DPM/unit time because of high zero time counts and high zero mRNA counts. There are several possibilities to explain this. First, lysinoalanine may be

nonspecifically (noncovalently) bound to proteins present in the lysate. Second, a small TCA precipitable radioactive peptide formed during the synthesis of ^{14}C -lysinoalanine which cochromatographs with lysinoalanine may be present. However, a more likely and less elaborate explanation is that cellulose debris from the TLC purification of ^{14}C -lysinoalanine may be introduced into the reaction mixture along with the radiolabeled lysinoalanine. ^{14}C -Lysinoalanine may be adsorbed to these cellulose fibers causing the radioactivity to be retained by the filter disc during vacuum filtration. This possibility is supported by the fact that the exclusion of the enzyme, BSA, and tRNA from the reaction mixture failed to reduce the high background radioactivity. A small amount of TLC cellulose fiber was added to one reaction mixture inadvertently. The amount of radioactivity retained by the filter disc during vacuum filtration was much greater than that with a "standard" reaction mixture (75,000 vs. 1000 cpm). Although the ^{14}C -lysinoalanine purified by TLC was centrifuged to remove cellulose debris (p. 24), it is possible that minute fibers were still present and were added to the reaction mixture, resulting in high background radioactivity.

High zero mRNA counts may be due to incomplete nuclease destruction of endogenous mRNA in the rabbit reticulocyte lysate. Based on the certificate of analysis supplied with each shipment of lysate the success of this treatment varies among commercial preparations of lysate. The cause of these high background counts was not determined since it was clear that ^{14}C -lysinoalanine was being esterified to tRNA (Fig. 14 and 15) and incorporated into protein (Fig. 17).

Fluorography of the dried polyacrylamide gel indicated that ^{14}C -lysinoalanine was incorporated into BMV mRNA coded proteins which comigrate with proteins containing ^{14}C -lysine (Figure 18).

CHAPTER IV

GENERAL DISCUSSION AND CONCLUSIONS

The work presented in this thesis indicates that lysinoalanine is incorporated into protein *in vitro*. The incorporation of other unusual amino acid analogs sometimes results in the formation of proteins which have compromised function. The possibility that the same is true for lysinoalanine exists and may be manifest directly or indirectly by the abnormal cellular morphology exhibited by renal epithelial cells when animals are exposed to lysinoalanine. Indeed, the mechanism of toxicity for this unusual amino acid may very well be more complex than just amino acid antagonism. Work done by Hayashi (1982) and Friedman *et al.* (1985) indicate that lysinoalanine is an effective chelator of metal ions which may inhibit the catalytic activity of enzymes involved in normal cellular metabolism or induce those involved in "cellular activation." Reyniers *et al.* (1974) suggested that lysinoalanine megalocytes may represent "activated" cells. In addition, a number of metabolic derivatives are possible and may be toxic themselves. Reyniers (1979) reported that 2,3-diaminopropionic acid, a potential lysinoalanine catabolite, was toxic *in vivo*, resulting in abnormal cellular morphology identical to lysinoalanine-induced nephrocytomegalia.

As mentioned previously, the structure of lysinoalanine is very similar to that of polyamines. The toxicity of lysinoalanine, especially following decarboxylation, as a polyamine antagonist cannot be discounted. Polyamines play numerous roles in cellular metabolism at the level of nucleic acid (DNA and RNA) synthesis and protein synthesis (Tabor and Tabor, 1976). The synthesis of hypusine, a lysine/spermidine derivative found in eukaryotic translation initiation factor eIF-4D (Cooper *et al.*, 1983; Park *et al.*, 1984a) has been suggested as a modulator of cell

growth (Torrelío *et al.*, 1984). Decarboxylation of lysinoalanine would result in a structural analog of hypusine and, if incorporated into initiation factor eIF-4D, could conceivably have an effect on the interaction of this lysine/spermidine derivative with other cellular components in the regulation of protein synthesis.

Histones are characteristically basic proteins, high in lysine and arginine (Johns, 1971). Reyniers *et al.* (1974) reported an altered ratio of the dye binding capacity of histone arginine and lysine residues in nephromegalocytes. Although the precise role(s) of histones on cellular metabolism is not known, they appear to have an effect on a number of cellular activities (Allfrey, 1971), as well as serving as structural components of chromatin dictating the degree of DNA folding (Metzler, 1977). The altered acridine orange binding by DNA phosphates, as well as the altered thermal stability observed by Reyniers *et al.* (1974), suggests that the chromatin structure (interaction between DNA and protein) may be altered in nephromegalocytes. Suppression of RNA synthesis and RNA-dependent cell functions occurs with chromatin (DNA associated with histones) as opposed to pure DNA in the presence of RNA polymerase. This has been demonstrated with polymerases of plant, animal, and bacterial origin (Allfrey, 1971). In addition, histones are frequent sites of post-translational modification. The mammalian H1 histone becomes phosphorylated by a special protein kinase during the initiation step of mitosis (Bradbury *et al.*, 1974). Other histones undergo modifications such as acetylation and phosphorylation (reversible) and methylation (irreversible) (Ruiz-Carrillo *et al.*, 1975). Many of these modifications occur at the ϵ -NH₂ group of lysine (Darzynkiewicz *et al.*, 1969; Berlowitz and Pallota, 1972; Olson and Busch, 1974; Ruiz-Carrillo *et al.*, 1975; Ruiz-Carrillo, 1976). The function of these modifications is unknown. The presence of lysine residues in histones and in the mammalian initiation factor are particularly attractive possibilities for lysinoalanine

toxicity considering the nuclear cytochemical evidence concerning quantitative and qualitative differences between normal cells and nephromegalocytes (Reyniers *et al.*, 1974).

In conclusion, the kinetic data presented in this thesis provide evidence that lysinoalanine is incorporated into protein in place of lysine. The fluorography of *in vitro* translation products containing radiolabeled lysinoalanine further demonstrates this claim. The incorporation of lysinoalanine into protein *in vitro* is presented as a possible mechanism of the nephrotoxicity observed *in vivo*.

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APPENDIX

Table I. Occurrence of Lysinoalanine in Various Foods.
(Adapted from Sternberg *et al.*, 1975)

Name	LAL ppm/protein
<u>Commercial Food Preparation</u>	
Corn chips	390
Pretzels	500
Hominy	560
Tortillas	200
Taco shells	170
Milk, infant formula (5 commercial samples)	150-640
Milk, evaporated (2 commercial samples)	590-860
Milk, skim, evaporated	520
Milk, condensed (2 commercial samples)	360-540
Simulated cheese	1,070
<u>Food Ingredients</u>	
Egg white solids, dried (4 samples)	160-1,820
Calcium caseinate (2 samples)	370-1,000
Sodium caseinate (5 samples)	430-6,900
Acid caseine (3 samples)	70-190
Hydrolyzed vegetable protein (18 samples)	40-500
Whipping agent (2 different types)	6,500-50,000
Soya protein isolate (45 samples)	0-370
Yeast extract	120

Table I (continued)

Name	LAL ppm/protein
<u>Home-cooked Foods</u>	
Frankfurter (as purchased)	0
Frankfurter (boiled)	50
Frankfurter (fried)	50
Frankfurter (oven-baked)	170
Frankfurter (charcoal-broiled)	150
Chicken thigh (as purchased)	0
Chicken thigh (charcoal-broiled)	150
Chicken thigh (retorted)	100
Chicken thigh (microwave)	200
Pan scrapings (fried sirloin steak)	130
Egg white (fresh)	0
Egg white (boiled 3 minutes)	140
Egg white (boiled 10 minutes)	270
Egg white (boiled 30 minutes)	370
Egg white (fried 10 minutes at 150°C)	350
Egg white (fried 30 minutes at 150°C)	1,100

Table 2. Summary of Kinetic Data

Enzyme Source	Substrate	App. K_m	App. K_i
<i>E. coli</i>	Lysine	1.9×10^{-6} M	-----
	Lysinoalanine	2.0×10^{-5} M	9.4×10^{-5} M
Rabbit Liver	Lysine	7.9×10^{-7} M	-----
	Lysinoalanine	5.0×10^{-5} M	1.5×10^{-4} M

Table 3. Effect of Natural Amino Acids on the Formation of ^{14}C -Lysinoalanyl-tRNA

Amino Acid	^{14}C -Lysinoalanyl-tRNA Formed (% of control)
None	100
L-Lysine	46.1
L-Alanine	100
L-Tryptophan	52.1
L-Amino Acid Mix (minus L-Lysine)	50.4

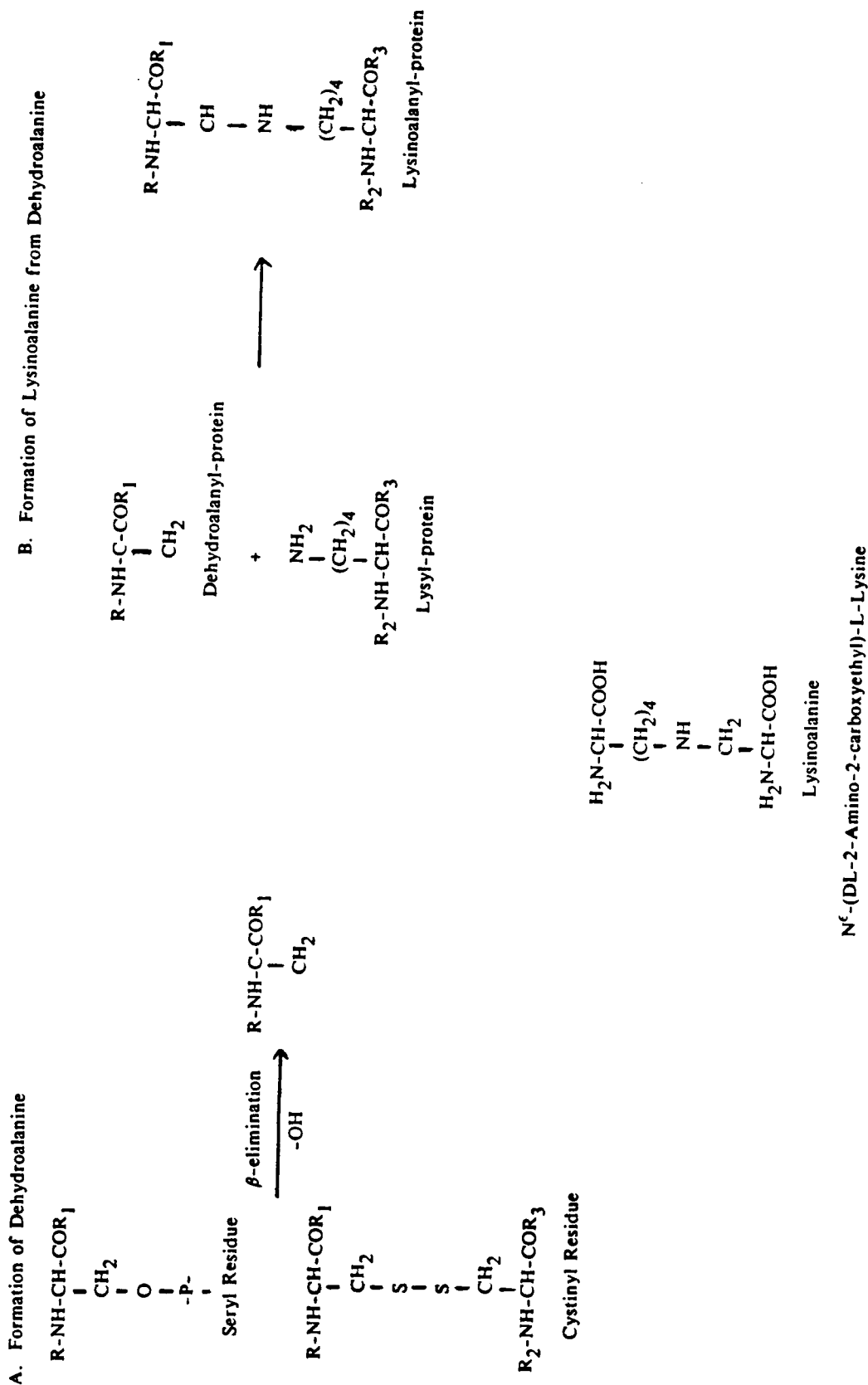


Figure 1. Mechanism of Lysinoalanine Formation (adapted from Sternberg and Kim, 1976).

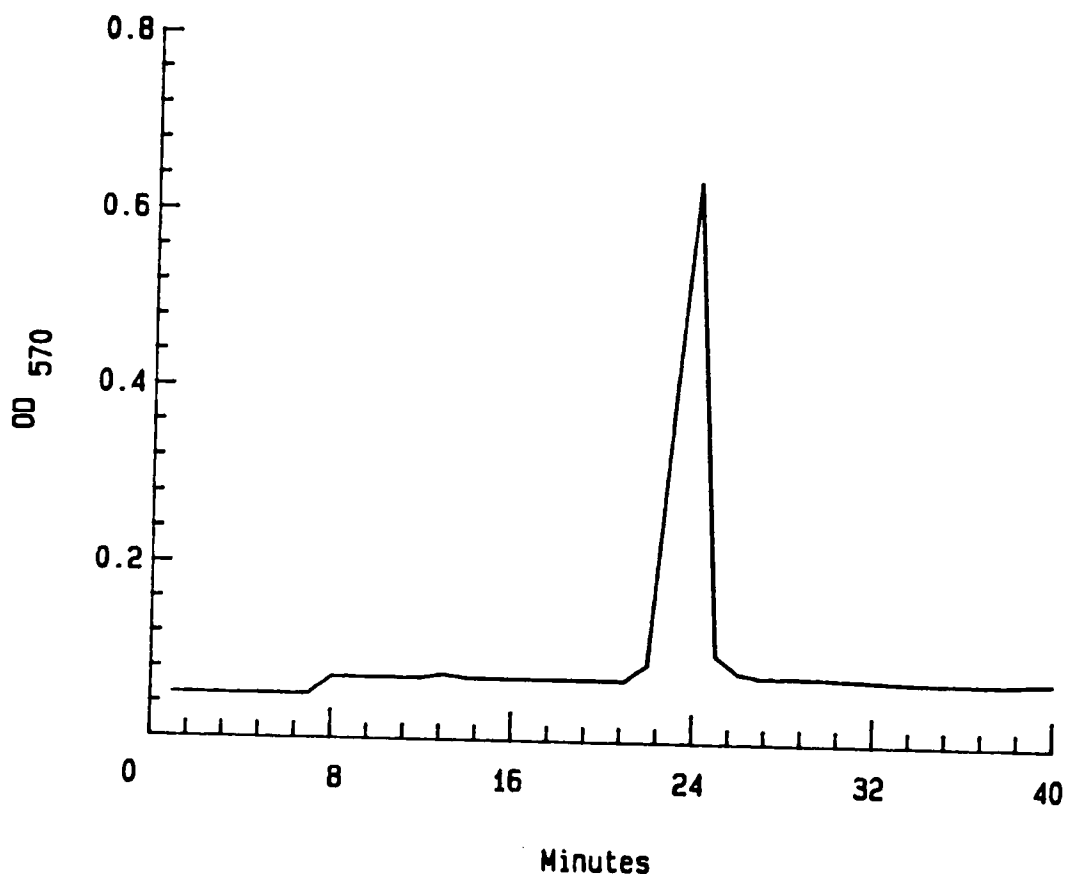


Figure 2. Amino Acid Analysis of Lysinoalanine. Lysinoalanine was obtained from Sigma Chemical Co. Analysis was carried out on a Beckman Model 120C Amino Acid Analyzer (courtesy of Raymond A. Popp, Biology Division, Oak Ridge National Laboratory). Chromatography was performed on a short, basic (1 x 8 cm, Dowex 50 x 12 resin) column at 58°C. Lysinoalanine was dissolved in 0.35 M sodium citrate buffer pH 2.2, and 0.25 μ moles (0.3 ml) was adsorbed to the resin. Material was eluted from the column at a flow rate of 1.2 ml/minute with 0.35 M sodium citrate buffer pH 5.3. The column effluent was reacted with ninhydrin and monitored for absorbance at 570 nm. Lysinoalanine was eluted at 24 minutes.

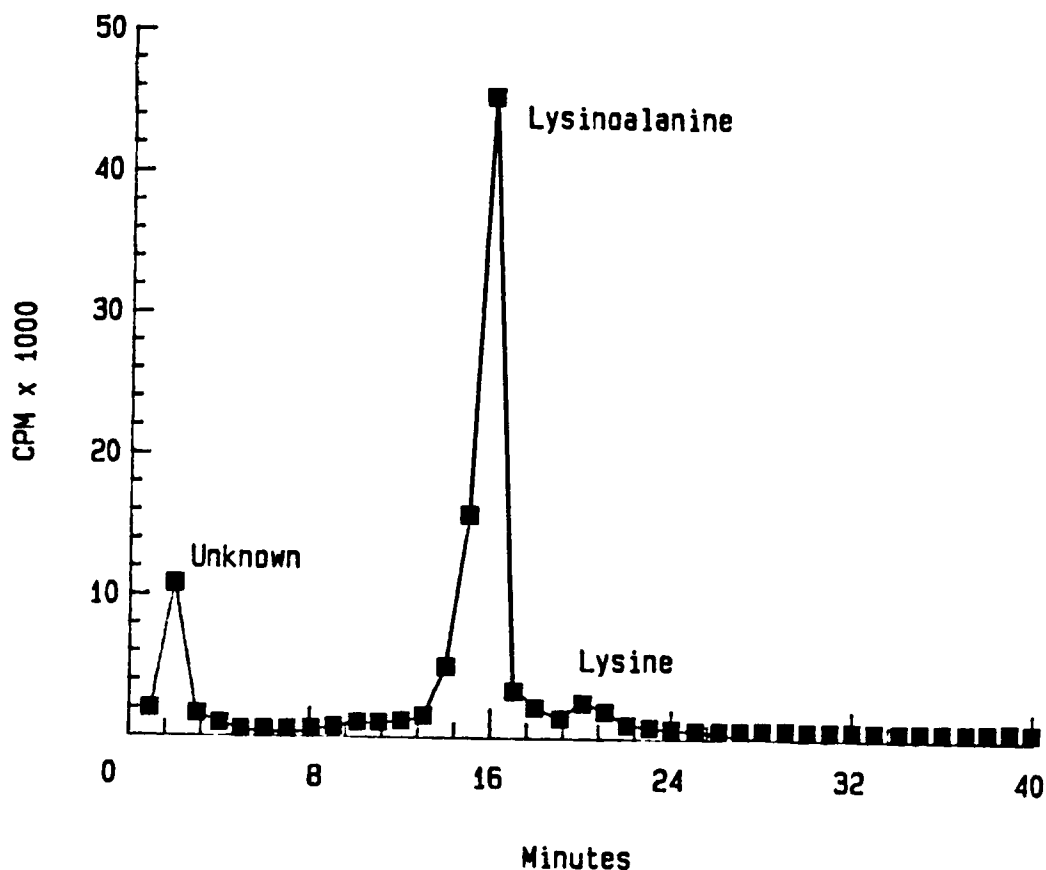


Figure 3. Amino Acid Analysis of ^{14}C -Lysinoalanine Prior to Purification. Analysis was carried out essentially as in Figure 2. Two separate runs were performed. In the first, $0.046 \mu\text{moles } ^{14}\text{C}$ -lysinoalanine were chromatographed, reacted with ninhydrin, and the absorbance at 570 nm was monitored. In the second run, approximately 100,000 cpm ^{14}C -lysinoalanine were chromatographed. The column effluent was collected in 1 minute fractions directly from the bottom of the column and placed in 10 ml Scintiverse II. Radioactivity was determined as described in the text. Profiles of absorbance at 570 nm and radioactivity were adjusted to compensate for the delay time (8 minutes) required for the ninhydrin reaction. All ninhydrin positive material (profile not shown), with the exception of the ammonia peak, was coincident with radioactivity. All remaining ^{14}C -lysinoalanine on hand was chromatographed in this manner without ninhydrin detection. The radioactivity of fractions collected was determined by removing 0.0025 ml aliquots and counting in 5 ml Scintiverse II as described in the text. ^{14}C -Lysinoalanine was concentrated by lyophilization. Further purification and removal of salt was accomplished by TLC. TLC methodology was developed and performed by Jon P. Reyniers, Texas A & I University.

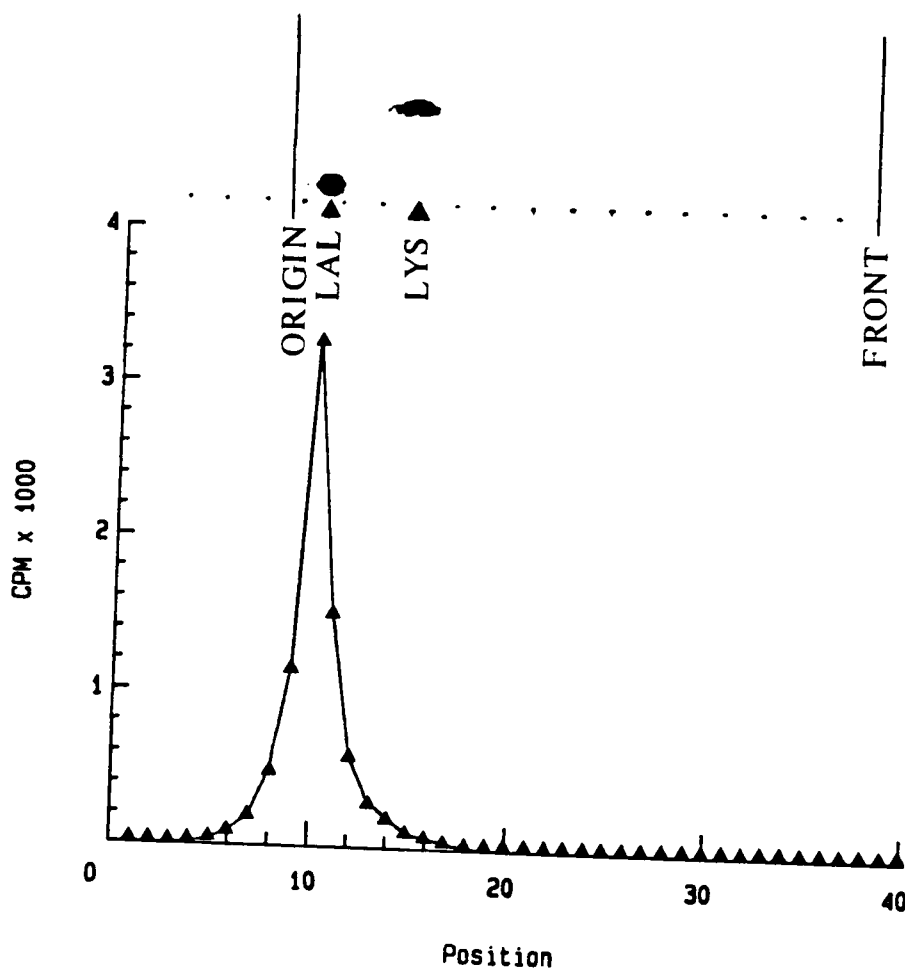


Figure 4. TLC Analysis of Purified ^{14}C -Lysinoalanine. Chromatography was performed by Jon P. Reyniers, Texas A & I University using Eastman cellulose TLC sheets without fluorescent indicator, developed in a solvent containing HPLC-grade n-butanol:formic acid:water (23:4:5, v/v) over a distance of 12.5 cm. Detection was by reaction with ninhydrin and radioactivity. All radioactivity was in the lysinoalanine spot (J. P. Reyniers, personal communication).

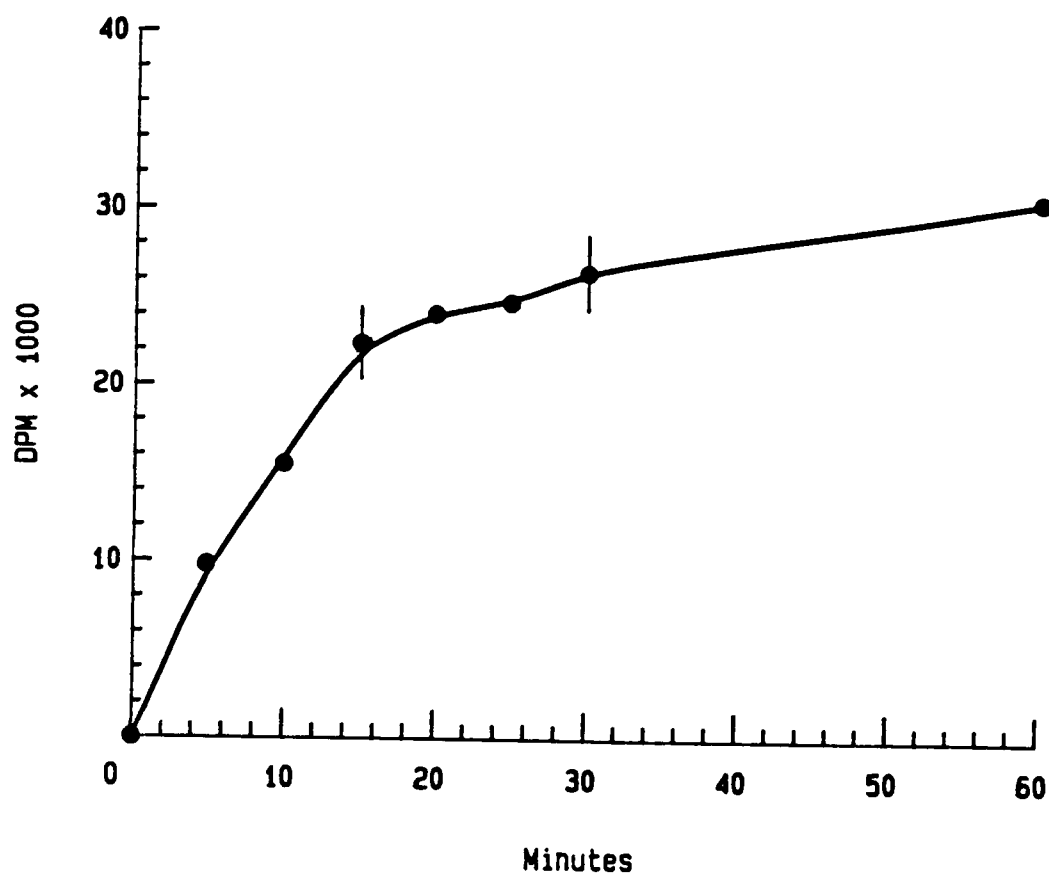


Figure 5. Time Course of *E. coli* Lysyl-tRNA-Synthetase Activity with Lysine. Assay conditions were as described in the text. An incubation time of 10 minutes provided an adequate level of enzyme activity.

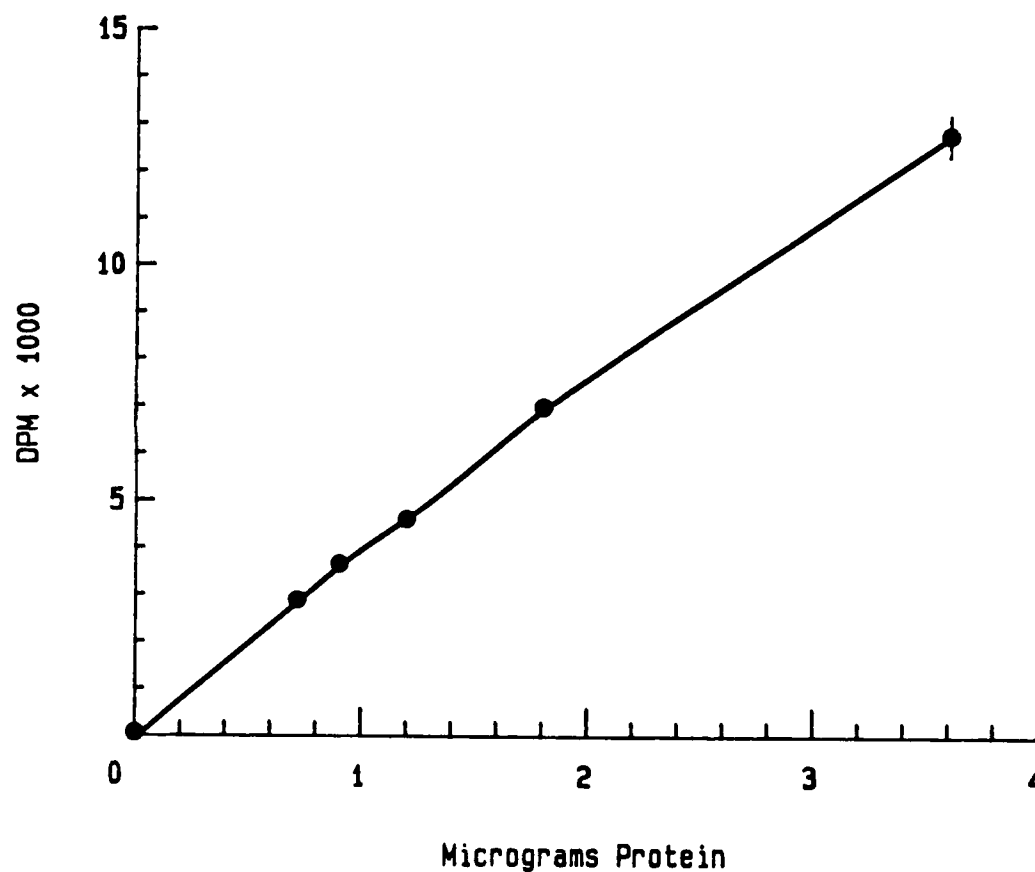


Figure 6. Dependence of *E. coli* Lysyl-tRNA-Synthetase Activity on Protein Concentration. The assay was performed as described in the text. A protein concentration of 4 $\mu\text{g}/\text{ml}$ in the reaction mixture was used in all subsequent assays. The reaction volume was 0.5 ml.

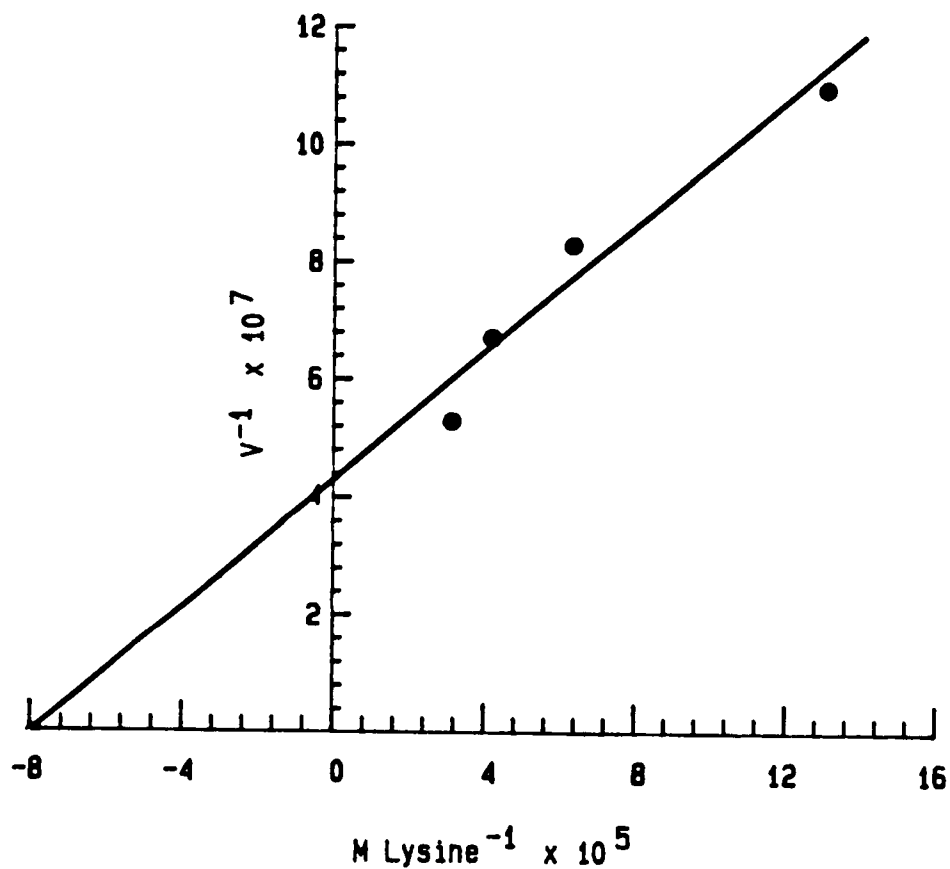


Figure 7. Lineweaver-Burk Plot of *E. coli* Lysyl-tRNA-Synthetase with lysine. Velocity (V) represents mmoles lysyl-tRNA/10 minutes. The apparent K_m was $1.9 \times 10^{-6} M$.

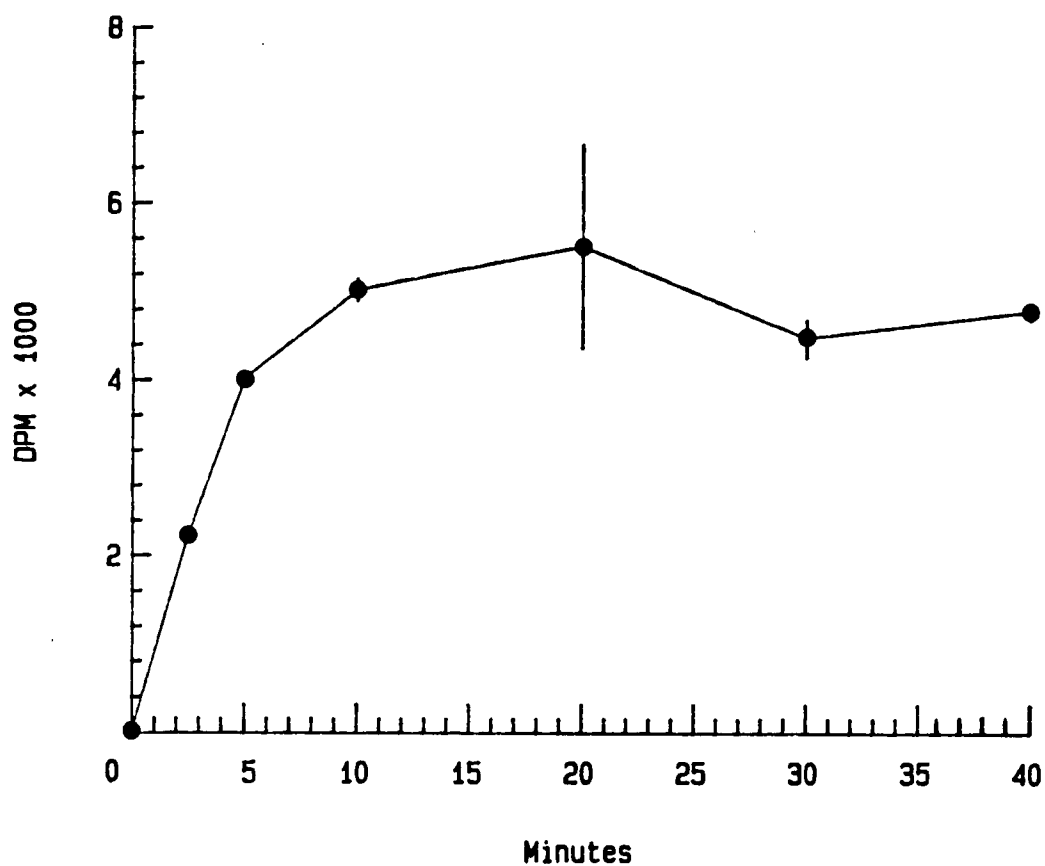


Figure 8. Time Course of Rabbit Liver Lysyl-tRNA-Synthetase Activity with Lysine. Assay conditions were as described in the text. An incubation time of 5 minutes provided an adequate level of enzyme activity.

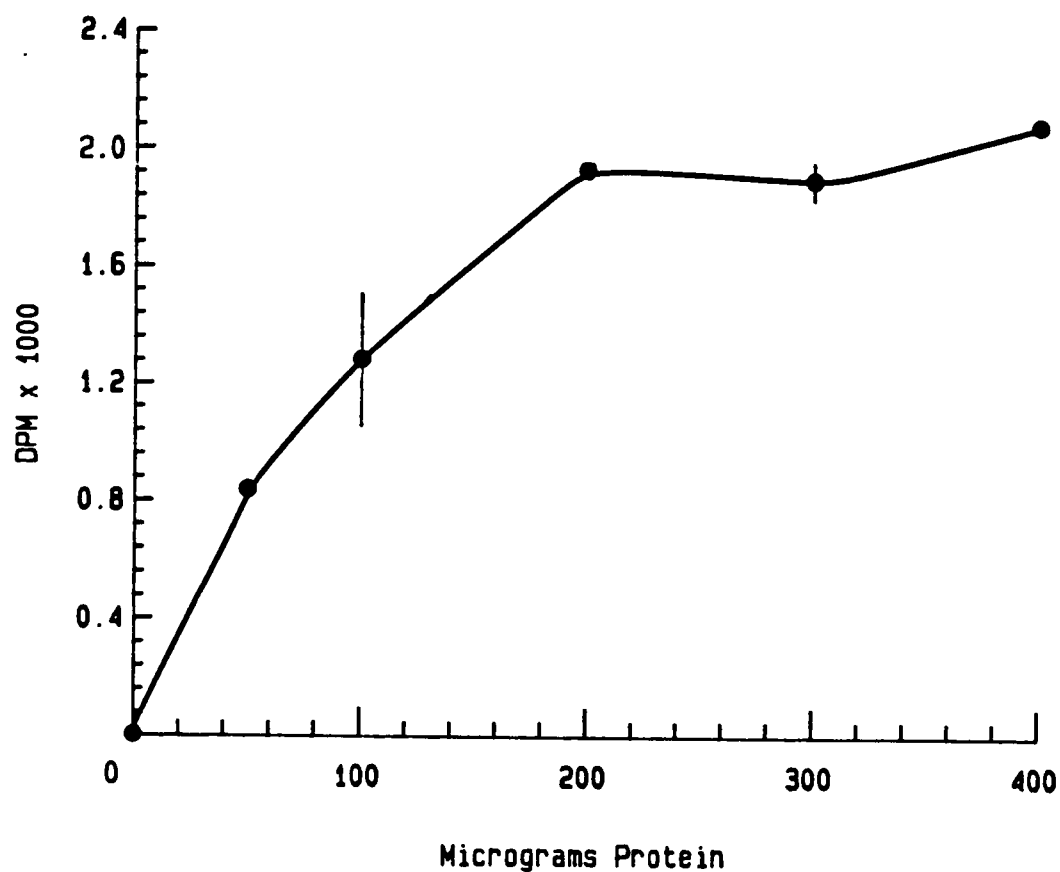


Figure 9. Dependence of Rabbit Liver Lysyl-tRNA-Synthetase Activity on Protein Concentration. The assay was performed as described in the text. 100 Micrograms of protein in a final volume of 0.25 ml was used in all subsequent assays.

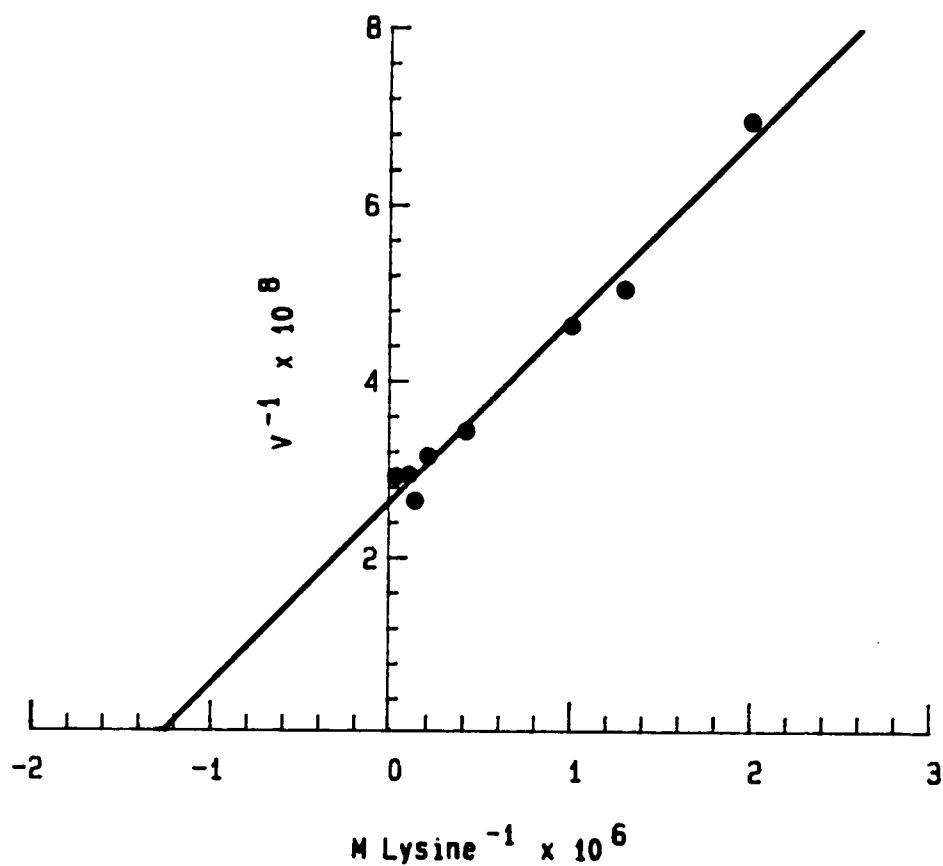


Figure 10. Lineweaver-Burk Plot of Rabbit Liver Lysyl-tRNA-Synthetase Activity with Lysine. Velocity (V) represents mmoles lysyl-tRNA/5 minutes. The apparent K_m was 7.9×10^{-7} M.

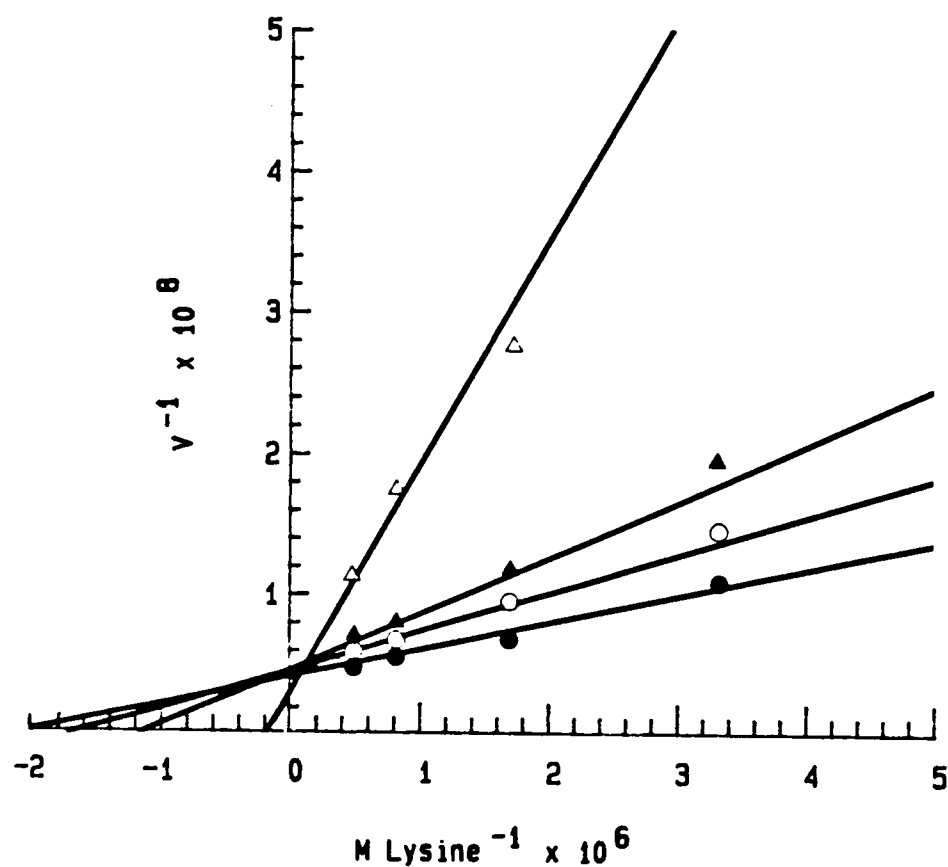


Figure 11. Lineweaver-Burk Plot of the Inhibition of *E. coli* Lysyl-tRNA-Synthetase by Lysinoalanine. Velocity (V) represents mmoles lysyl-tRNA/10 minutes. Lysinoalanine was present at 0 M (●), 0.5 mM (△), 0.01 mM (▲), and 0.005 mM (○). The apparent K_i for lysinoalanine was 9.4×10^{-5} M.

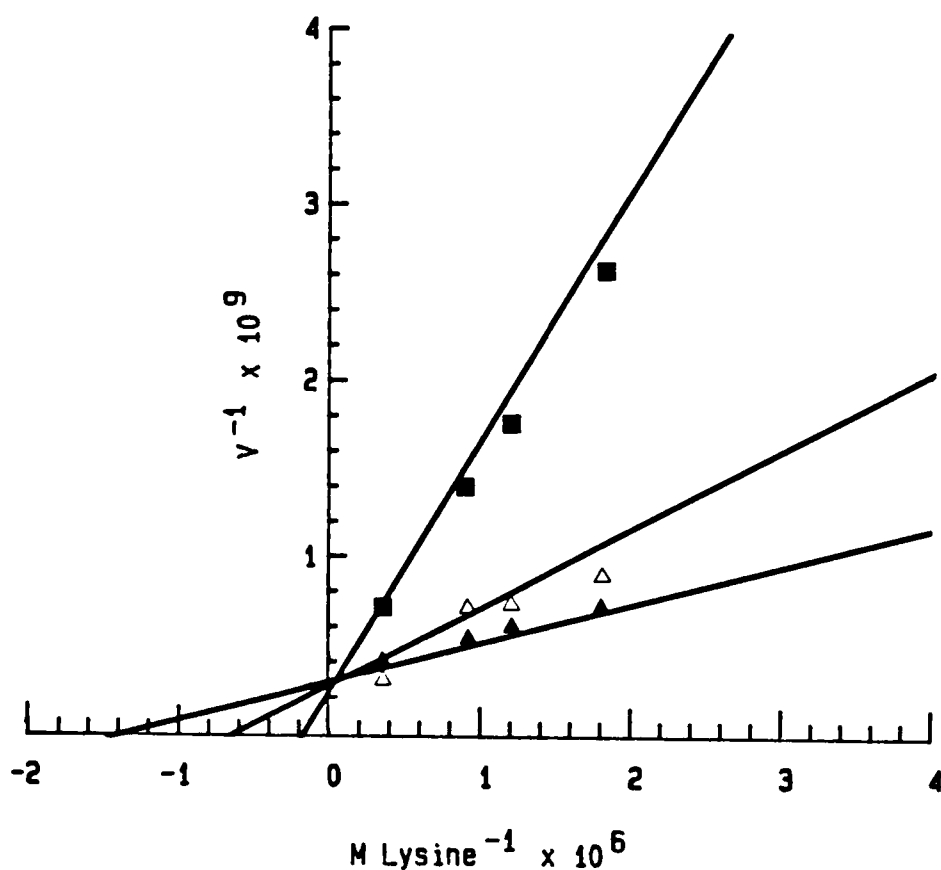


Figure 12. Lineweaver-Burk Plot of the Inhibition of Rabbit Liver Lysyl-tRNA-Synthetase by Lysinoalanine. Velocity (V) represents mmoles lysyl-tRNA/5 minutes. Lysinoalanine was present at 0 M ($\blacktriangle$), 1.0 mM ($\blacksquare$), and 0.1 mM ($\triangle$). The apparent K_i for lysinoalanine was 1.5×10^{-4} M.

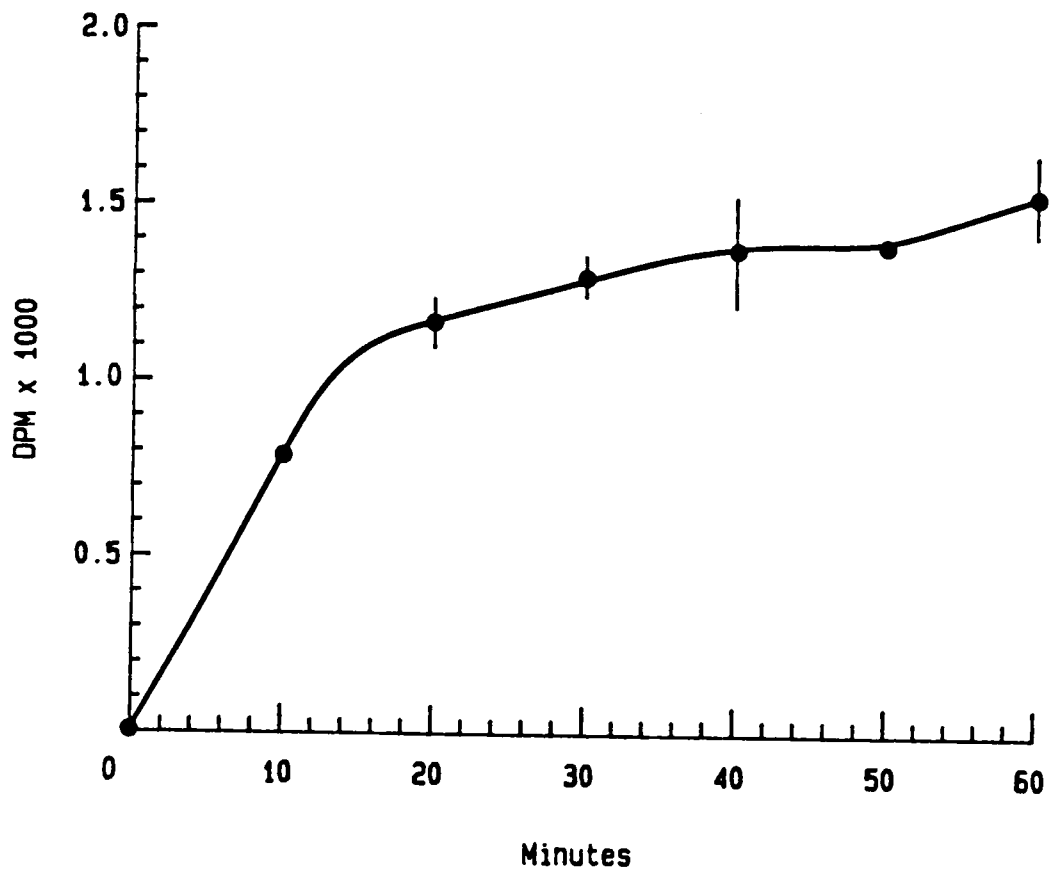


Figure 13. Time Course of *E. coli* Lysyl-tRNA-Synthetase Activity with Lysinoalanine. Assay conditions were as described in the text. An incubation time of 10 minutes was used in all assays.

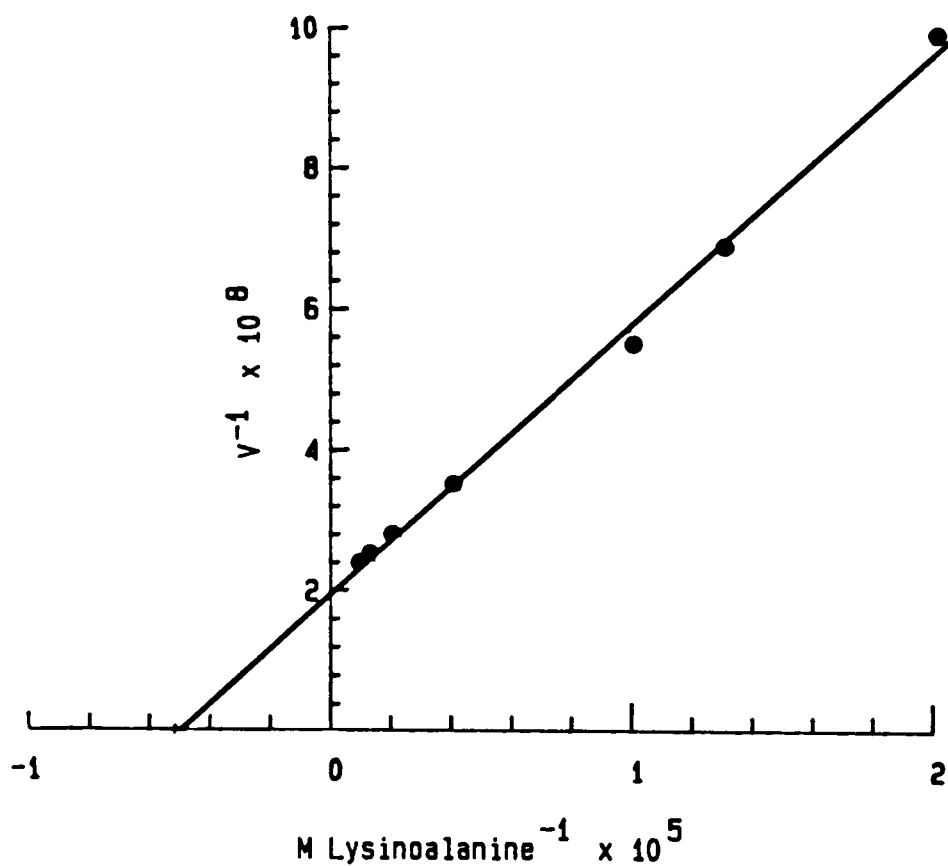


Figure 14. Lineweaver-Burk Plot of *E. coli* Lysyl-tRNA-Synthetase with Lysinoalanine. Velocity (V) represents mmoles lysinoalanyl-tRNA/10 minutes. The apparent K_m was 2.0×10^{-5} M.

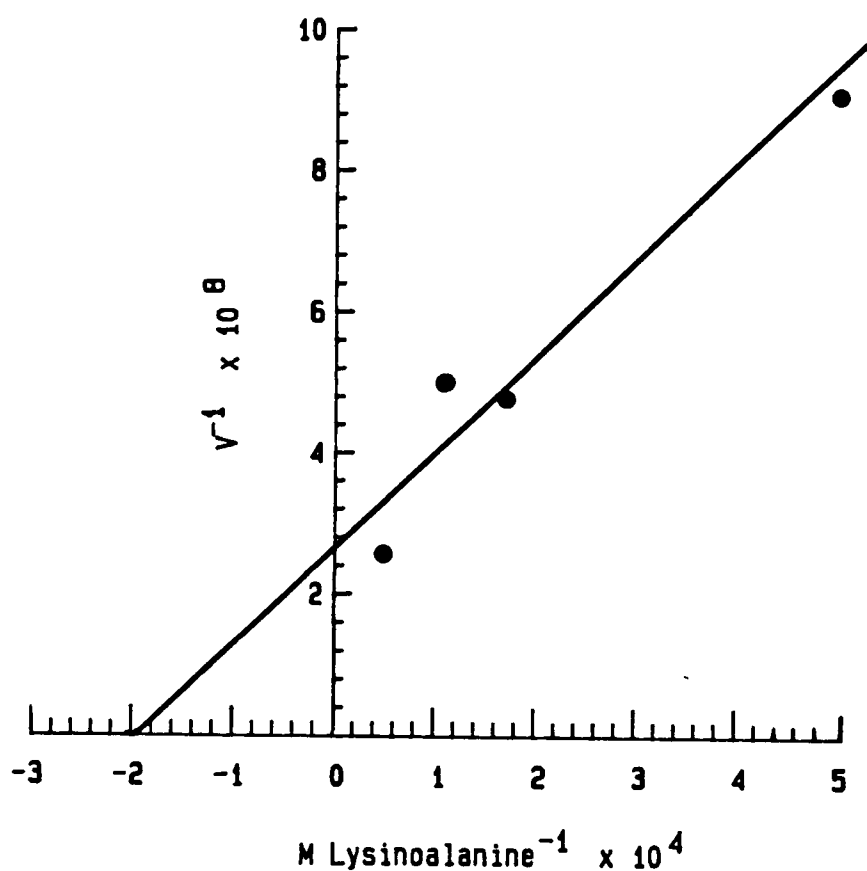


Figure 15. Lineweaver-Burk Plot of Rabbit Liver Lysyl-tRNA-Synthetase with Lysinoalanine. Velocity (V) represents mmoles lysinoalanyl-tRNA/5 minutes. The apparent K_m was 5.0×10^{-5} M.

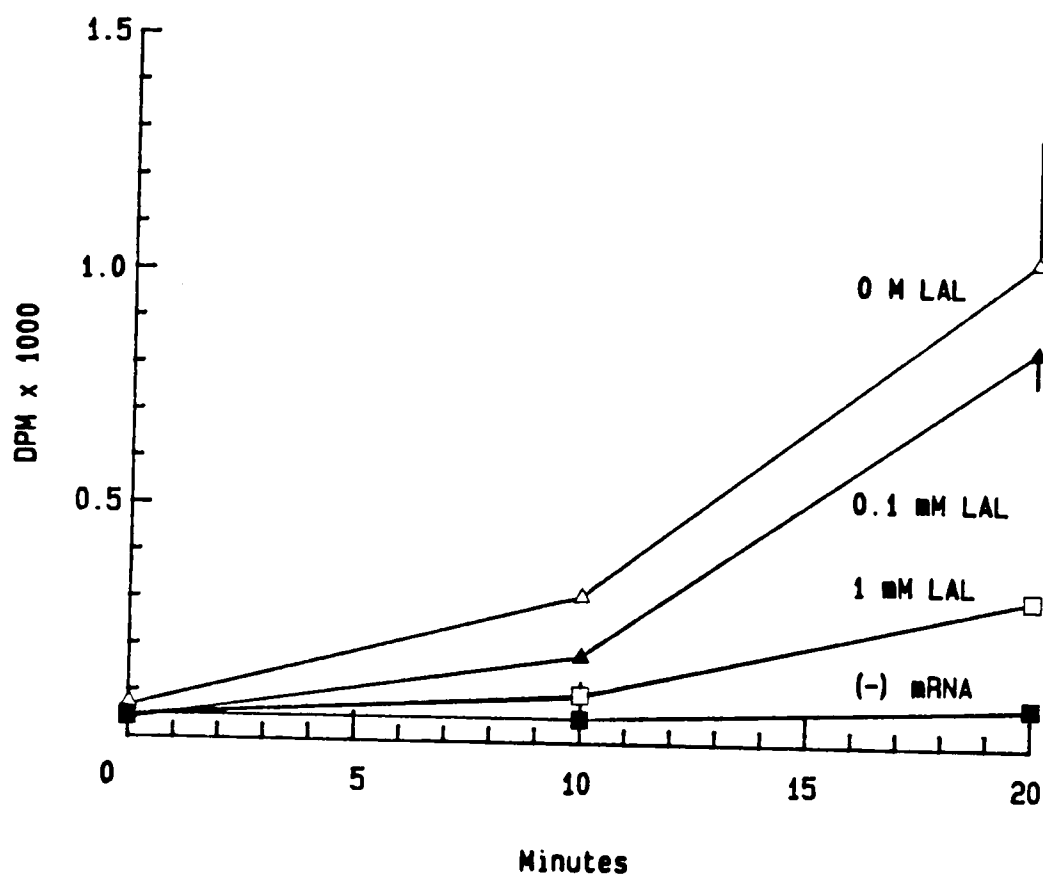


Figure 16. Inhibition of *in Vitro* Protein Synthesis by Lysinoalanine. Assay was performed as described in the text. At 20 minutes, inhibition was 18.4% and 69.7% at 0.1 mM and 1.0 mM lysinoalanine, respectively.

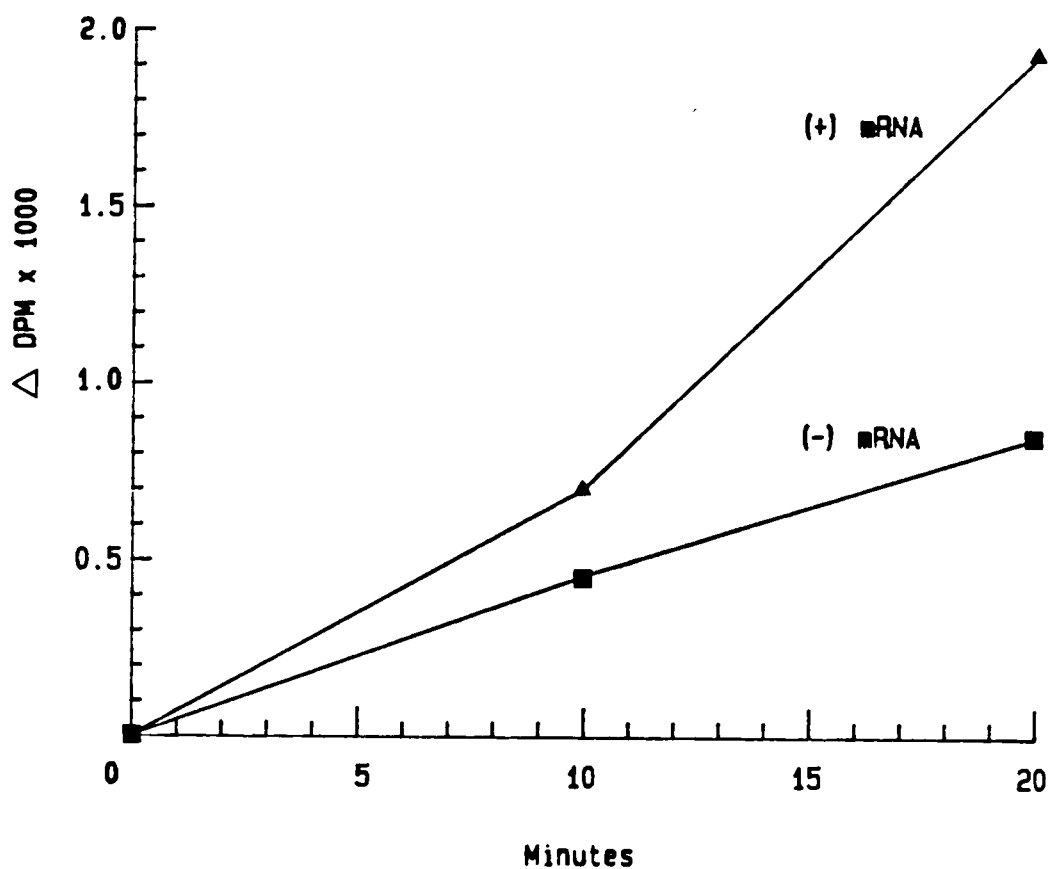


Figure 17. *In Vitro* Incorporation of ^{14}C -Lysinoalanine into Protein. Assay was performed as described in the text. Data is presented as ΔDPM because of high (-) mRNA counts and high (+) mRNA zero time counts.

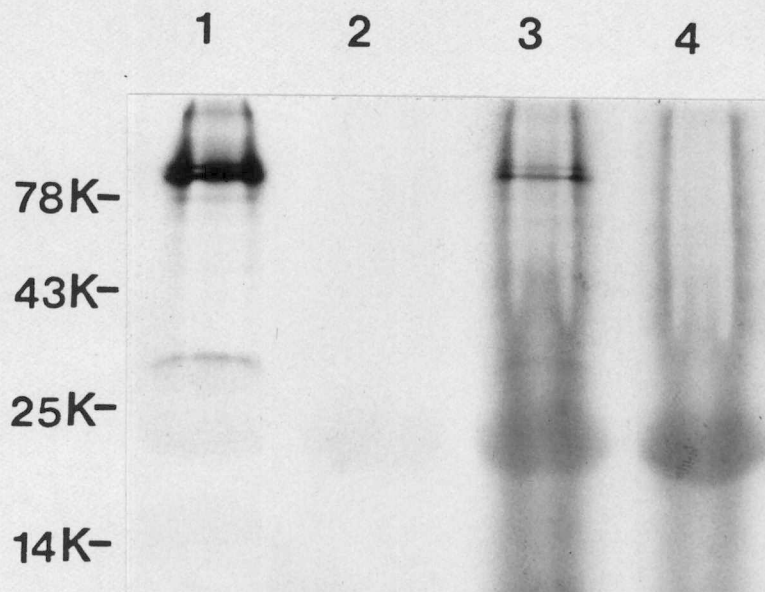


Figure 18. Fluorography of *in Vitro* Translation Products Containing ^{14}C -Lysine and ^{14}C -Lysinoalanine. *In Vitro* translation was performed as described in the text. Protein containing ^{14}C -lysine (lane 1) and ^{14}C -lysinoalanine (lane 3) were electrophoresed and the gel was fluorographed as described in the text. In each case, (-) mRNA controls (lane 2 for ^{14}C -lysine and lane 4 for ^{14}C -lysinoalanine) were run. Approximately 5000 cpm (hot TCA precipitable cpm) per lane for (+) mRNA samples and an equal volume of the corresponding (-) mRNA samples were loaded onto the gel. Lanes 1 and 3 indicate that protein synthesis did occur in response to added mRNA. Proteins containing ^{14}C -lysinoalanine (lane 3) comigrated with proteins containing ^{14}C -lysine (lane 1).

VITA

Ben J. Lifsey, Jr. was born in Jackson, Tennessee on June 1, 1960. He attended elementary schools in that city and was graduated from Old Hickory Academy in June 1978. That summer he entered The University of Tennessee, and in June 1982 he received a Bachelor of Science degree in Agriculture. In the summer of 1984 he became employed as a graduate research assistant at The University of Tennessee College of Veterinary Medicine, Department of Environmental Practice and began study toward a Master's degree in Life Sciences. In July 1985 he accepted a research assistantship for the continuation of his study. In June 1986, while completing his degree, he became employed by The University of Tennessee, Department of Animal Science as a research assistant. He received a Master of Science degree with a major in Life Sciences with Environmental Toxicology as a major area of interest in June 1987.

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