METABOLISM AND TOXICITY OF D- AND L-α,γ-DIAMINOBUTYRIC ACIDS

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Lathyrism

The disease lathyrism has been known for a long time. Its occurence has been recorded in a number of countries in Europe, Africa and Asia. It is a neurological disorder resulting in spastic paralysis of the legs. Periodic surveys since 1833 have revealed that this disorder is due to the consumption of L. sativus seeds (1). It was found that the toxic components were in the alcohol soluble fraction of L. sativus seeds. Subsequent analysis of alcohol extracts of the L. sativus seed meal revealed the presence of two new ninhydrin-positive compounds. One was characterized as L-homoarginine (2), the other compound as β-N-oxalyl-L-α,β-diaminopropionic acid (3). However, L. sativus seeds were often contaminated with vicia seeds and it was proposed that the latter might be the actual toxic component (1). Ressler (4) subsequently isolated β-cyanoalanine from vicia seeds, which was found to exert neurotoxic effects in rats.

In 1962, Bell (5, 6) presented preliminary results of a systematic investigation of the acidified 50% ethanol-soluble, ninhydrin-positive materials in 49 species of lathyrus peas. He identified α,γ-diaminobutyric acid (DBA) as one of the unknowns and showed that the three lathyrus species associated with human lathyrism contained homoarginine but no DBA. A compound designated as lathyrine has been characterized

as β-(2-amino-pyridine-4-yl)-alanine, which is a "cyclic-type" derivative of homoarginine (7). β-Aminopropionitrile has been identified as a toxic principle of lathyrus related peas responsible for osteolathyrism and aminoacetonitrile and β-mercaptoethylamine produce a similar toxicity (8, 9).

The L-isomer of DBA appears to be neurotic in rats and mice although it may not be the neurotoxic agent of human lathyrism (10-12). It is a component of bacterial cell walls (13), of certain Lathyrus and related seeds (6, 10, 14-16), and of the polymyxin antibiotics elaborated by some of the Bacillus strains (17). It has been found in bovine brain (18); this is the only species of mammals so far reported to contain this compound.

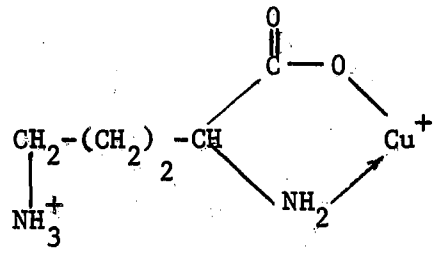
At present, a total of five polymyxins are known (19). Circulin (20), isolated from Bacillus circulans, and polypeptin (21, 22), isolated from Bacillus kremieniewski, also appear to belong to the polymyxin category. The polymyxins differ somewhat in pharmacological properties but are similar in having a narrow antibacterial spectrum that is limited to certain gram negative bacteria, including Hemophilis influenza, Brucella abortus, Salmonella enteritidis, Escherichia coli, and Proteus vulgaris (23).

The polymyxins are related chemically. They are strongly basic, cyclic polypeptides, and yield DBA, threonine, and isopelargonic acid, a branched chain, saturated, optically active fatty acid (6-methyl-octan-1-oic) upon acidic hydrolysis. Most of the DBA residues in polymyxin have free γ-amino groups; most are of the L-configuration (24, 25).

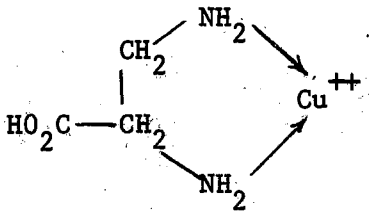
As mentioned previously, the free amino acid L-DBA is neurotoxic in rats and mice. A dose of 680 mg per kilo body weight injected intraperitoneally caused hyperirritability, tremors, convulsions and death (14, 26). The symptoms of toxicity were different and occurred much more

slowly subsequent to L-DBA treatment than did those resulting from lethal doses of other amino acids or ammonia (26).

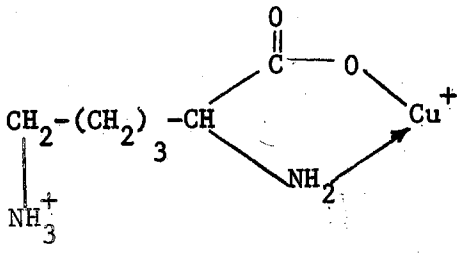
A rather striking observation was made by Christensen and his associates on the concentration of the homologous α,ω-diamino acids within living cells (27). Whereas β-aminoalanine and DBA were very readily concentrated by free-living cells, ornithine and lysine were concentrated to a much smaller extent. Indeed, the concentration of DBA occurs at so rapid a rate as to be frequently fatal to the cells. It is possible that the abrupt change in transfer facility in passing from the lower members of this series to the higher can be explained on steric grounds; the two lower members are capable of forming stable five or six membered rings involving the two amino groups and a metal ion (27, 28). Albert (29) has shown that the copper salts of these two diamino acids involve mainly both amino groups and, to a smaller extent, the α-amino with the carboxyl group, whereas the copper salts of ornithine and lysine have the more usual structure:



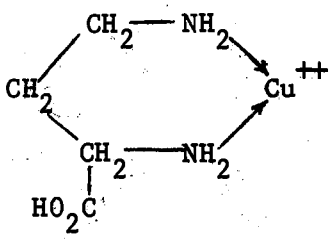
Ornithine



β-Aminoalanine



Lysine



DBA

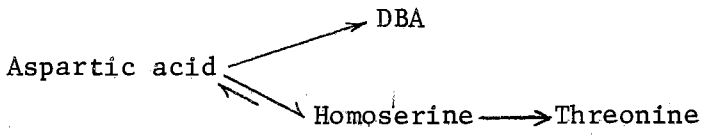
The finding that addition of pyridoxal accelerated the accumulation of amino acids by ascites cells suggested that the transfer reaction involves not only chelation with metals but also the formation, at the same time, of a stable Schiff base with pyridoxal (27). It is possible that the Schiff base of DBA is chelated to both nitrogen atoms (28):

The metal-pyridoxal-amino acid complexes had been prepared by Christensen and Collins as crystals (30). The molar ratio of metal : pyridoxal : DBA depends on the metal and pH, as 1:1:1, 1:2:1 or 1:2:2. These chemical and steric characteristics are very important. They may account, in part, for the physiological differences between DBA and its longer carbon chain analogs, ornithine and lysine. The findings of Christensen and Riggs (27) on the accumulation of amino acids, including the homologous α,ω-diamino acids, within free-living cells agree with this proposal.

Biosynthesis of L-DBA

In the studies of toxic components of Lathyrus peas, Ressler's group encountered β-cyanoalanine as an intermediate in the conversion of asparagine to DBA (10). Instead of finding β-cyanoalanine as a naturally occurring neurolathrogen in L. latifolius, they isolated and identified L-DBA as a component toxic to rats, which might be considered as a

reduction product of β-cyanoalanine (14). During the assimilation of $H^{14}CN$ by the seedlings of Cicia sativa, Tschiersch (31) found that β-cyanoalanine and asparagine were the first compounds to be labelled. L-DBA and an unidentified compound were also highly labelled. This result agrees with the findings of Ressler et al. In 1966, Ressler and Nigram (32) were able to demonstrate that DBA could be synthesized from both homoserine and aspartic acid in the seedlings of L. sylvestris W, and suggested that the pathways for the formation of homoserine and DBA from aspartic acid are related:



However, a new enzyme, β-cyanoalanine synthase, has been isolated and purified from acetone powders of etiolated seedlings of blue lupine by Hendrickson (33), and Blumenthal et al. (34) reported that this enzyme catalyzed the reaction

$$\text{Cysteine} + HCN \longrightarrow \beta\text{-cyanoalanine} + H_2S \ .$$

Probably β-cyanoalanine is hydrolyzed to asparagine but the reverse reaction does not occur (34). During the assimilation of $H^{14}CN$ by the seedlings of blue lupine, sorghum, and common vetch, Blumenthal et al. (34) demonstrated that L-cysteine-3-^{14}C was the source of 3 carbon atoms of asparagine while $H^{14}CN$ was converted into the amide carbon of asparagine without separation of the C and N atoms of the cyanide group. L-cysteine-3-^{14}C is a more efficient precursor of the carbon atoms of asparagine in the three species studied than is L-serine-1-^{14}C (34). Fowden and Bell (35) observed that β-cyanolanine could be converted to γ-glutmyl-β-cyanoalanine in V. sativa by the action of a transpeptidase.

Blumenthal et al. (34) reported that asparagine did not appear to be converted to β-cyanoalanine, and that a cyanogenic glucoside would constitute an alternate source of HCN and perhaps the only source of carbon for the nitrile group of β-cyanoalanine. Tschiersch (36) has demonstrated that phenylalanine-2-^{14}C fed to V. angustifola seedlings formed β-cyanoalanine labelled predominantly in the nitrile carbon. Tyrosine, valine, and isoleucine as well as phenylalanine are known precursors of cyanogenic glucosides (37).

Chemical Synthesis of DBA

DBA has been synthesized chemically. The earliest preparation was made, through the malonic condensation reaction with bromoethylphthalimide, by Fischer (38) in 1901. In a series of studies on the optical configuration of the amino acids, Karrer, Escher, and Widmer (39) prepared L-DBA by the Hofmann degradation of acetyl-L-glutamine in a yield of 18%. DBA obtained by both methods was isolated as the crystalline oxalate. In 1939, Adamson (40), making use of the Schmidt reaction, treated L-glutamic acid with sodium azide in H_2SO_4 solution and isolated DBA as the crystalline dipicrate (mp 178-180°) in a yield of 42%. By using ion-exchange techniques (41), the free amino acid hydrochloride salt can be obtained.

The methods described above, and many others, for the preparations of DBA are of interest but of little practical value in preparing large amounts of the compound. One of the best available procedures for this purpose is that of Carter, Van Abeele, and Rothrock (42), which also has the virtue of being a general method for the synthesis of α,γ-diamino acids. Reaction of diazomethane with ethyl acrylate leads to pyrazoline which, on catalytic hydrogenation in the presence of Raney Nickel at high pressure and subsequent acid hydrolysis, gives DBA in about 80%

yield.

Metabolism of DBA

The mammalian metabolism of D- and L-DBA was first investigated by Mushahwar and Koeppe (41). They observed that a considerable amount of radioactivity from each isomer of DBA-2-^{14}C was excreted in the urine by rats, the L-isomer was more readily converted to respiratory CO_2, and a major urinary catabolite of each isomer was β-alanine-1-^{14}C (41). Although the finding of β-alanine-1-^{14}C suggests removal of the α-amino group and subsequent oxidative decarboxylation, the labeling patterns obtained in tissue glutamate (41) suggest that some gamma deamination and oxidation occurs with the formation of oxalacetate or a related compound. Thus, the metabolism of DBA apparently proceeds via transamination and/or oxidation of the α-amino group with subsequent decarboxylation. This metabolic route is common to most amino acids. The metabolism and metabolites, especially the catabolic rate, may be of value in elucidating the toxic mechanisms of this amino acid.

Transamination

Nonenzymic transamination was first demonstrated by Herbst and Engel (43) in 1934. Pyruvic acid was boiled with O-aminophenyl acetate in aqueous solution. The mechanism proposed involved tautomerization of the Schiff's base. Such Schiff's bases have been crystallized by Witkop and Beiler (44). Nonenzymic transamination was found to take place between a wide variety of amino and keto acids when these are heated together on paper (45). Similar reactions occur in aqueous solution at room temperature, near neutrality, when the keto acid is replaced by

glyoxylate (46-48).

Although enzymic transamination was reported originally as a general reaction, subsequent studies (49-52) indicated that enzymic transamination may be limited to reactions between alanine, glutamate, aspartate and their α-keto analogs. About 1950, when reactants of higher purity and improved analytical procedures permitted more accurate determinations of reaction products, practically all of the naturally occurring α-amino acids were shown to undergo transamination reactions (53). Transamination plays a general role in amino acid metabolism. Although the number of transaminases in any given tissue is unknown, the scope of enzymic transamination is indicated by the fact that almost every amino acid that occurs naturally can be shown to undergo transamination with one or another keto acid in crude extracts (53).

Based on the above knowledge of transamination, it seems likely that both nonenzymic and enzymic transamination reactions should be considered for the case of DBA. The dialyzed crude extracts, should be of interest. Pyruvate, oxalacetate and α-ketoglutarate might serve as amino group acceptors.

A variety of analytical methods suitable for estimating transaminase activity have appeared in recent literature. Among these, the spectrophotometric method is the most convenient (54-58). However, it is limited to a transamination system in which one of the components, either added as substrate or formed as a reaction product, has a measurable characteristic absorption at a suitable wave length. This method is suitable for the transamination system of oxalacetic acid and either DBA or glutamic acid, the rate of disappearance or formation of oxalacetate being measured at 280 mμ.

Oxidative Deamination

The flavoprotein enzymes that catalyze the oxidation of amino acids to the corresponding α-keto acids and ammonia have been known for many years. In 1935, Bernheim et al. (58) reported that resting cells of Bacillus proteus catalyzed the oxidation of certain amino acids. In the same year Krebs published his classical paper on the oxidation of the L- and D-isomers of amino acids by homogenates of mammalian liver and kidney (59). The over-all reaction catalyzed by the flavoprotein amino acid oxidases may be represented as shown in Eq. 1. Reactions 2-4 represent the classical interpretation of the reactions catalyzed by amino

$$RCH(NH_3^+)COO^- + O_2 + H_2O \longrightarrow RCOCOO^- + NH_4^+ + H_2O_2 \quad (1)$$

acid oxidases; reaction 1 is the sum of these three reactions (59-61):

$$RCH(NH_3^+)COO^- + E(FAD)_2 \rightleftharpoons RC(=NH)COO^- + E(FADH)_2 + H^+ \quad (2)$$

$$RC(=NH)COO^- + H_2O + H^+ \rightleftharpoons RCOCOO^- + NH_4^+ \quad (3)$$

$$E(FADH)_2 + O_2 \longrightarrow E(FAD)_2 + H_2O_2 \quad (4)$$

The hydrogen peroxide formed may react nonenzymically with the α-keto acid product (Eq. 5). When catalase is present, reaction 5 does not

$$RCOCOO^- + H_2O_2 \longrightarrow RCOO^- + CO_2 + H_2O \quad (5)$$

take place, and only one atom of oxygen is taken up per mole of amino acid oxidized:

$$RCH(NH_3^+)COO^- + \tfrac{1}{2}O_2 \longrightarrow RCOCOO^- + NH_4^+ \quad (6)$$

In the absence of catalase, the over-all reaction is as depicted in Eq. 7 (59):

$$RCH(NH_3^+)COO^- + O_2 \longrightarrow RCOO^- + NH_4^+ + CO_2 \quad (7)$$

Optical specificity is a striking feature of the L-amino acid oxidase of snake venom and D-amino acid oxidase of mammalian kidney. Both act on a wide variety of amino acid substrates, and although both contain FAD as the prosthetic group, they exhibit absolute specificity for one optical isomer of the substrate in the oxidative deamination of amino acids, as well as in the synthesis of amino acids from α-keto acids and ammonia. The strict antipodal specificity may be ascribed to specific structural features of the enzyme, probably including the nature of the binding of the flavin to the protein (59).

Problems relating to the metabolic and physiological significance of the amino acid oxidases have been considered (60, 62, 63). In the case of D- or L-DBA, the amino acid oxidases of rat kidney may play an important role. Although the activities are low when compared with the L-amino acid oxidase of snake venom, Koeppe and Mushahwar (41) were able to demonstrate β-alanine as a major urinary catabolite of each isomer.

Ammonia Detoxification and Free Amino Acids in Brain

The relatively high content of free amino acids in brain tissue have made them the focal point of many biological studies (64-68). Most of these are non-essential amino acids; glutamic acid, glutamine, γ-aminobutyric acid (GABA), aspartic acid and N-acetyl aspartic acid making up 75 per cent of the total. Of these, N-acetylaspartic acid and GABA appear to be peculiar to nervous tissue.

In a study using several hypoglycemic agents, insulin was the only one which caused major changes in the levels of the amino acids of rat brain (69). Decreases in glutamine, GABA, glutamic acid and alanine, a large increase in aspartic acid content, and no change in the level of

N-acetylaspartic acid in the brain of insulin-treated rats were observed (69-73). The effect of insulin appeared to be directly related to blood sugar levels. When the level of insulin was not sufficient to reduce the blood sugar content to 25 mg per cent, or when glucose was administrated (to insulin treated animals), no effect on the brain amino acid levels was observed (69, 70). O'Neal and Koeppe (74) showed that hypoglycemia produced by ligation of the hepatic artery and portal vein, produced similar results.

In an ammonia toxicity study with rats, a 300 per cent increase in brain glutamine concentration was observed 15 minutes after a $LD_{99.9}$ dose of ammonium acetate had been injected intraperitoneally (75). Berl et al. (76) observed that glutamine was the only cerebral component showing a considerable increase in concentration as a result of ammonia infusion. This increase occurred without a corresponding drop in glutamic acid concentration. From studies of the incorporation of radioactivity from intravenously injected glutamic acid-U-^{14}C and glutamine-U-^{14}C into the amino acids of rat and mouse brain, Lajtha et al. (77) demonstrated that there was an exchange between blood and brain amino acids rather than a net uptake or uptake followed by utilization. In 1953, Flock et al. (78) observed a large increase in the concentration of glutamine, without a corresponding drop in glutamic acid content, in the cerebrum of hepatectomized dogs. Since this increase in glutamine was not proportional to the increase in plasma glutamine, it was thought to result from the brain detoxification of ammonia by de novo synthesis of glutamine.

The detoxification of ammonia via the hepatic Krebs-Henseleit urea cycle has been demonstrated by Greenstein et al. (79, 80), who reported

that L-ornithine, L-citrulline and L-arginine protect against the ammonia released from the injected amino acids (81). The studies of Herrmann et al. (82) showed that L-DBA could competitively inhibit the purified ornithine carbamyl transferase obtained from Neurospora crassa. If these were true in rats intoxicated with L-DBA, L-arginine or L-ornithine might enhance detoxification.

A Proposed Mechanism of Convulsion

An impairment in the biosynthesis of GABA is apparent in a number of convulsive states (83); the administration of large doses of GABA has a definite anticonvulsive action (83). The need for such large doses is attributed to the low permeability of the blood-brain barrier to a polar molecule like GABA. The 2-pyrrolidone has a stronger anticonvulsive action than GABA (84), apparently because it is a less polar molecule that penetrates the brain more easily where it is hydrolyzed to yield GABA (85).

The main catabolic pathway of GABA is its transamination with α-ketoglutaric acid (85). It has been found that the inhibition of this reaction by hydroxylamine or aminooxyacetic acid produces an increase in GABA levels (86, 87) and counteracts the convulsive action of several agents, although the maximal anticonvulsive action does not coincide with the highest cerebral GABA (83). In 1967, Tapia et al. (88) observed an inhibition of glutamic decarboxylase activity, in both brain and liver, by L-glutamic acid-γ-hydrazide or aminooxyacetate at the onset of convulsions, in spite of the simultaneous increase in GABA levels. The results indicate that the inhibition of glutamate decarboxylase activity is an important factor in the production of certain convulsions.

Simultaneous inhibition of GABA-α-ketoglutarate transaminase activity accounts for the increases in GABA concentration. Tapia et al. (88) concluded that the convulsant effect of some substances could be due, in part, to the inhibition of glutamate decarboxylase activity independent of the total brain GABA concentration.

Objectives of This Study

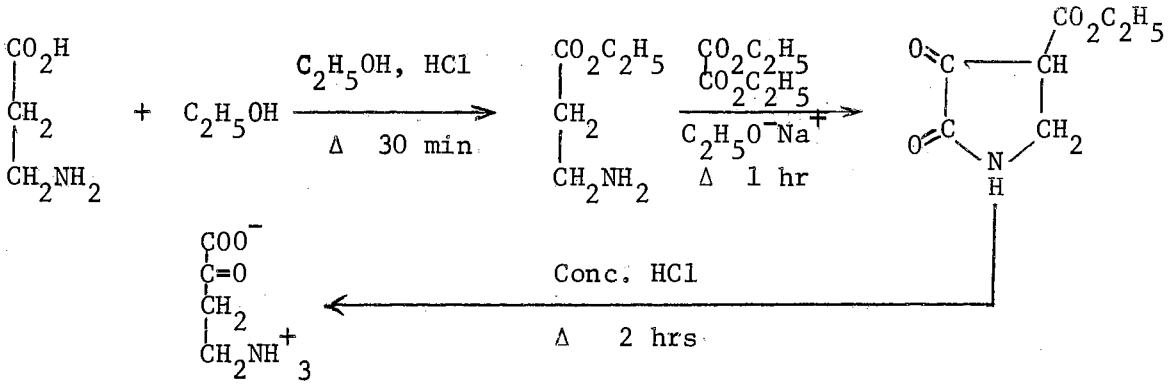
Except for distribution and toxicity studies in mice (12, 89) and uptake studies with ascites tumor cells (27, 89, 90), the only investigations concerning the mammalian metabolism of D- and L-DBA are those conducted by Mushahwar and Koeppe (41). More details remain to be elucidated. The objectives of this study were to attempt to explain the neurotoxicity of this component of certain Lathyrus peas and to elucidate its metabolism by amino acid oxidases and/or transaminases. Work has involved the synthesis of possible metabolic intermediates, e.g., keto amino acids, the determination of changes in certain brain amino acids in rats neurotoxic with DBA and the identification of products of the enzymic reactions of DBA.

CHAPTER II

ORGANIC SYNTHESIS

Experimental

Synthesis of α-Keto-γ-Aminobutyric Acid (KABA)



Preparation of β-Alanine Ethyl Ester. β-Alanine (50 g) was suspended in absolute ethanol (500 ml, U.S. Industrial Chemicals Co.), and dry HCl (Matheson Co.) was passed into the mixture until a clear solution was obtained. The solution was heated for 30 minutes, concentrated to dryness in vacuo (water pump, using a 30° water bath), and any excess HCl removed by reconcentration in vacuo after the addition of absolute ethanol. The syrup obtained was dissolved in 200 ml of water and chilled in an ice-bath. Ice-cold 28% (100 ml, w/v) NaOH and then 170 g of K_2CO_3 were added over 40 minutes to the solution with stirring to salt out the amine, which was extracted several times with equal volumes of dry ether. Dry HCl was passed into the ether solution until

an aliquot dissolved in 1 ml of water showed pH 3.5 (pH paper). The precipitate was filtered off, washed well with dry ether, and dried over P_2O_5 *in vacuo* overnight to give 74.4 g (87%).

Preparation of 4-Carboethoxy-2,3-Dioxopyrrolidine. The method of Southwick *et al.* (91) with modification was used for preparation. To 1.1 equivalents of sodium ethoxide (23.4 g of sodium metal) in 500 ml of absolute ethanol was added, over 15 minutes, with stirring, 425 ml of an absolute ethanol solution containing 72.3 ml (one equivalent) of diethyl oxalate and 74.4 g of β-alanine ethyl ester. The solution was stirred and heated under reflux for one hr. The yellow reaction mixture was cooled rapidly to room temperature in a cold water bath and poured, with stirring, into 2 liters of water at room temperature. The mixture was acidified by addition of 100 ml of conc. HCl, cooled to 0° and stirred overnight at 4°. The precipitate which formed was filtered off, washed with 95% ethanol, and dried for 2 hrs by drawing air through the filter; then dried over P_2O_5 *in vacuo*. The yield was 42.5 g (50%).

After recrystalization from hot 95% ethanol, the 4-carboethoxy-2,3-dioxopyrrolidine gave white crystals and melted at 183-187° with decomposition (185-186°, decomp., by Southwick *et al.*, 91). The compound is soluble in hot water, hot 95% ethanol and acidic or alkaline solution, and insoluble in cold water and cold 95% ethanol. The compound gives a red color when treated with 5% ferric chloride in 0.1 N HCl solution, a yellow precipitate with 2,4-dinitrophenylhydrazine, and is ninhydrin negative.

Preparation of KABA. Crude 4-carboethoxy-2,3-dioxopyrrolidine (39.7 g) were added to 500 ml of conc. HCl and the solution was flushed with nitrogen immediately to prevent oxidation and then heated at 95° for 12 hrs under a blanket of nitrogen. The hydrolysate was cooled to room temperature in an ice-bath, concentrated to dryness in vacuo, and any residual hydrochloric acid was removed by a second concentration in vacuo at room temperature after the addition of a small amount of ethanol. A syrup was obtained.

Preparation of 2,4-Dinitrophenylhydrazone Derivative of KABA. The KABA sample was converted (92) to the 2,4-dinitrophenylhydrazone derivative for purification. Assuming the yield of previous step to be 70%, an equivalent amount of 2,4-dinitrophenylhydrazine was dissolved in sufficient 2 N HCl to make a 1% solution. The syrup of KABA was dissolved in a small volume of water and added to the 2,4-dinitrophenylhydrazine solution. The mixture was stirred for 7 hrs, filtered and the precipitate was washed with 2 N HCl and then with water. The filtrate and washings were concentrated to a small volume to give an additional 10% of the derivative. The 2,4-dinitrophenylhydrazone of KABA (36.2 g, 52%) was recrystalized from 0.06 N HCl in methanol.

Liberation of KABA. A 1 g sample of KABA 2,4-dinitrophenylhydrazone was dissolved in 300 ml of 2 N HCl at 90° and 0.44 ml of anisaldehyde was added. The mixture was stirred at 90-95° for 5 min, cooled to 15° in ice-bath, and filtered. The filtrate was heated to 80° with 1 g of decolorizing charcoal (Darco G-60) as rapidly as possible with

stirring on the hot plate, cooled in an ice-bath and filtered and the filtrate was then lyophilized to give light yellow crystals. These were washed with absolute ethanol, dried and found to melt at 130-140° with decomposition. The yield was 302 mg (66%). An additional 69 mg (15%) was obtained from the washings.

The crystalline synthetic KABA is very hygroscopic, very soluble in water, and only slightly soluble in ether and absolute ethanol. It gives a yellow color with ninhydrin, an amber color with 5% $FeCl_3$ in 0.1 N HCl solution, and yellow precipitate with 2,4-dinitrophenylhydrazine in 2 N HCl solution (mp 215-221°, decomp.). This keto acid and its 2,4-dinitrophenylhydrazone melt, with decomposition, over a wide temperature range.

Synthesis of D-α,γ-Diaminobutyric Acid

D-α,γ-Diaminobutyric acid (D-DBA) was synthesized by the Schmidt degradation of D-glutamic acid according to the method of Adamson (40) as modified by Koeppe, Hill and Mushahwar (41, 93), without further addition of fuming H_2SO_4 and NaN_3. An amount of 200 mg of D-glutamic acid was used in each reaction. After the reaction, the mixture was transferred to a beaker cooled in ice-bath and titrated to pH 6.5-7.0 (pH meter) with hot saturated barium hydroxide solution. The precipitated barium sulfate was removed by centrifugation. The volume of supernatant solution was reduced in vacuo to about 50 ml and passed through a Dowex-1 x8 (acetate form, 100-200 mesh; 2.4 x 30 cm) column and through a Dowex-1 x8 column (OH^- form, 100-200 mesh; 2.4 x 35 cm). The columns were washed with 100 ml of water. It was assumed the unreacted D-glu-

tamic acid and any excess SO_4^{--} present in the solution were removed by the acetate column. The OH^- column was washed with an additional 200 ml of water to remove any trace of Na^+ and Ba^{++} ions. D-DBA was eluted from the OH^- column with 1 N HCl. The fractions containing D-DBA were combined, the volume was reduced to approximately 50 ml in the rotatory evaporator and the concentrate was titrated to pH 3.5 to 4.0 with pyridine. Ten volumes of absolute ethanol were added to precipitate D-DBA as the monohydrochloride salt. The precipitate was filtered, recrystallized from water-ethanol, washed with absolute ethanol, acetone and dry ether, and dried over P_2O_5 *in vacuo*. The yield was 77%. It was homogeneous on paper chromatography in the solvent systems described by Mushahwar and Koeppe (41). It melted at 234-236° (decomp.) and showed $[\alpha]_D^{23^\circ}$ -23.3° (c 1, 5 N HCl) as determined with the polarimeter. Wilkinson (96) records mp 223-224° (decomp.) for D-form monohydrochloride and $[\alpha]_D^{21^\circ}$ +23.8° for L-form monohydrochloride (c 1.2, 6 N HCl). Fu *et al.* (97) reported $[\alpha]_D^{25^\circ}$ -24.5° for D-form monohydrochloride (c 2%, 5 N HCl). It gives a brown color with ninhydrin, and is soluble in water and insoluble in ethanol and ether.

Preparations of N-Trifluoroacetyl n-Butyl Esters and N-Acetyl Ethyl Esters of Amino Acids

The amino acids or the mixture obtained from nonenzymic transamination were converted to N-trifluoroacetyl n-butyl esters or N-acetyl ethyl esters, which were separated by gas-liquid or thin-layer chroma-

tography and analyzed by mass spectrometry.

Preparation of N-trifluoroacetyl n-butyl Esters. The sample obtained from nonenzymic transamination was evaporated to dryness *in vacuo* after reduction with sodium borohydride. Derivatives of this dried sample or of crystalline amino acids were prepared according to Gehrke and Stalling (94). There are 3 steps. About 60 mg of amino acid was dissolved in 10 ml of 1.25 N HCl (dry) in anhydrous methanol. The flask was stoppered and the solution was stirred for 30 minutes at room temperature. The excess methanol and HCl were removed in the rotary evaporator on a 40° water bath. The methyl ester hydrochlorides were transesterified with n-butanol. Ten ml of 1.25 N HCl (dry) in n-butanol were added. The flask was fitted with a $CaSO_4$ drying tube and placed in an oil bath maintained at 100° by a Multiple Magnetic Hot Plate Stirrer. The solution was stirred for 2½ hrs. The excess n-butanol and HCl were removed in the rotary evaporator on a 40° water bath. The acylation of the n-butyl ester hydrochloride was carried out at room temperature. The dried n-butyl ester was treated with 4 ml of methylene chloride and 1 ml of trifluoroacetic anhydride, and stirred for 2 hrs. The solution was filtered through glass wool, and dried by a stream of nitrogen gas. The oil residue was dissolved in 0.3-0.4 ml of dry ethyl ether. This solution was ready for gas-liquid or thin-layer chromatography and mass spectrometry.

Preparation of N-Acetyl Ethyl Esters. The derivatives are less volatile than those described above, but are useful. The dried sample

of the reduced nonenzymic transamination mixture or amino acids was suspended in 20 ml of absolute ethanol (for about 60 mg of amino acid) and dry HCl gas was passed through to dissolve all the residue. The solution was refluxed for 30 minutes at 90° and the excess ethanol and HCl were removed by concentrating the solution to dryness in vacuo, using a 30° water bath. The gummy residue of ethyl ester hydrochloride was treated with 2 ml of acetyl chloride and stirred for 1 hr. This reaction was poor. Even when triethylamine was added, most of the gummy residue did not dissolve. The solution was concentrated by a stream of nitrogen gas and on the steam plate. The oily residue was dissolved in dry ethyl ether prior to analysis.

Results and Discussions

4-Carboethoxy-2,3-Dioxopyrrolidine

Figure 1 shows alternate reaction schemes for the formation of 4-carboethoxy-2,3-dioxopyrrolidine. It seems likely that a Claisen condensation followed by lactam formation (steps 1 and 2 rather than 3 and 4) may represent the principal reaction path.

The compound is hydrolyzed to KABA when heated under reflux with conc. HCl. Hydrolysis to β-alanine occurs in alkaline solution.

KABA

The synthesis of KABA is shown in Figure 2.

The IR spectrum of our synthetic KABA is the same as that of KABA synthesized by Macholan et al. (Figure 3) using a different method (92, 95).

KABA can be oxidatively decarboxylated to β-alanine by H_2O_2 at any

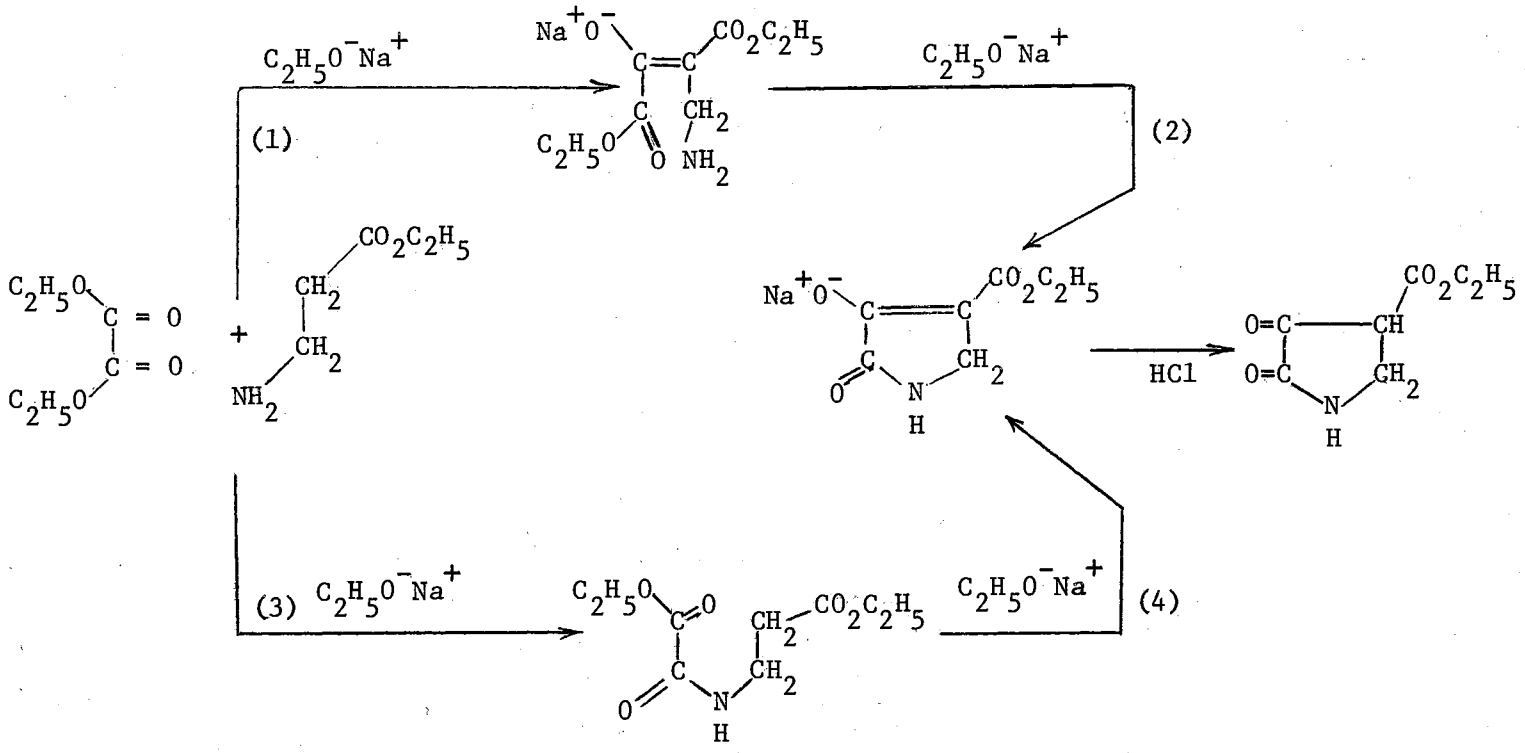


Figure 1. The Synthesis of 4-Carboethoxy-2,3-Dioxopyrrolidine

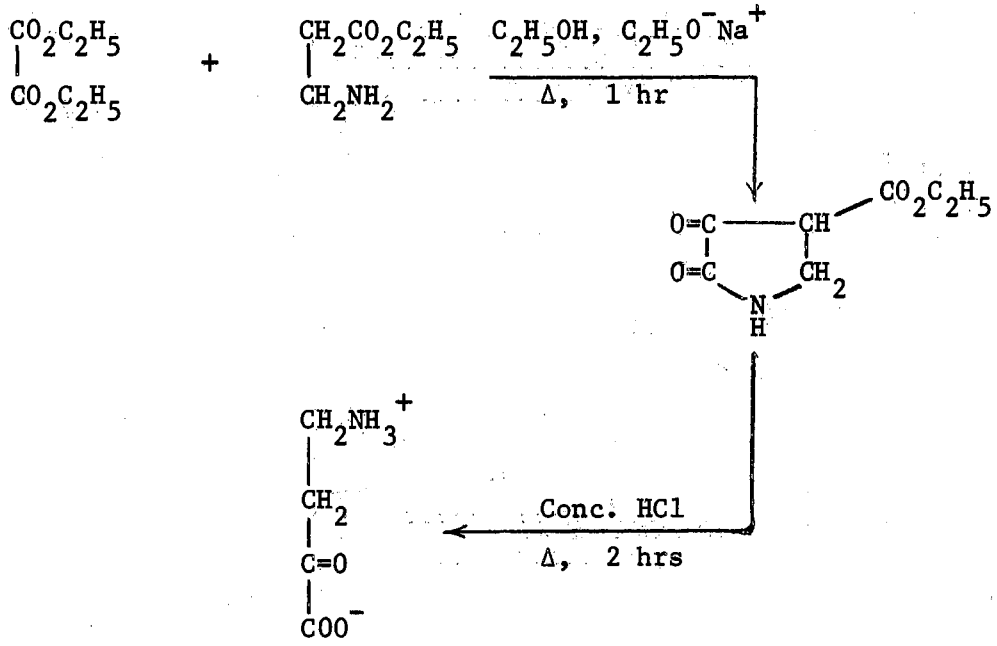


Figure 2. The Synthesis of α-Keto-γ-Aminobutyric Acid

pH, and reduced to α-hydroxy-γ-aminobutyric acid (HABA) (purple color with ninhyrin) with sodium borohydride. The reactions are shown in Figure 17. Both α-keto and α-hydroxy-γ-aminobutyric acids can be identified by paper chromatography in several solvent systems (Table XII).

Concerning the Structure of KABA

The synthetic KABA contains no chloride ion after recrystallization from ethanol-H_2O. When its 2,4-dinitrophenylhydrazone derivative was recrystalized from water or washed well with cold 95% ethanol, or cold water, the IR spectra were modified (Figure 4).

KABA reacts rapidly with 2,4-dinitrophenylhydrazine in 2 N HCl solution, suggesting that in 2 N HCl solution substantial amounts of the keto form exist. The NMR spectra of KABA dissolved in D_2O for 5 minutes and for 24 hours (refrigerated) were identical (Figure 5), except for

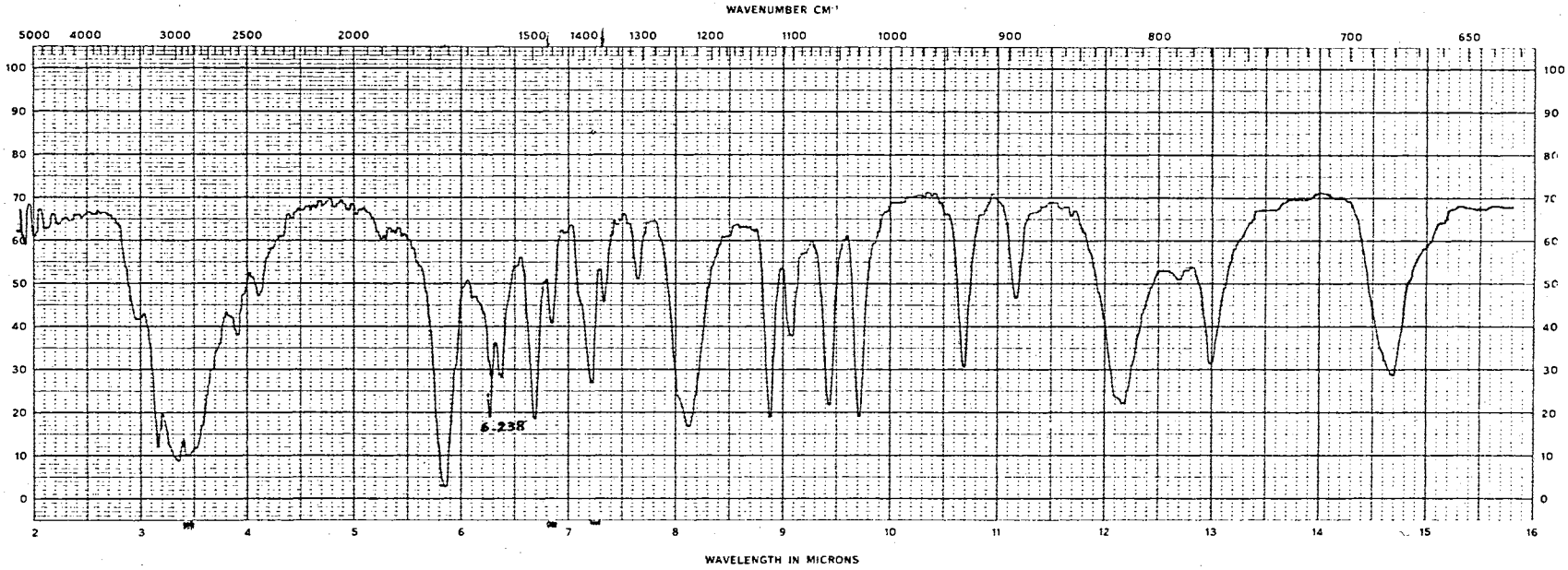


Figure 3. The Infrared Spectrum of α-Keto-γ-Aminobutyric Acid.

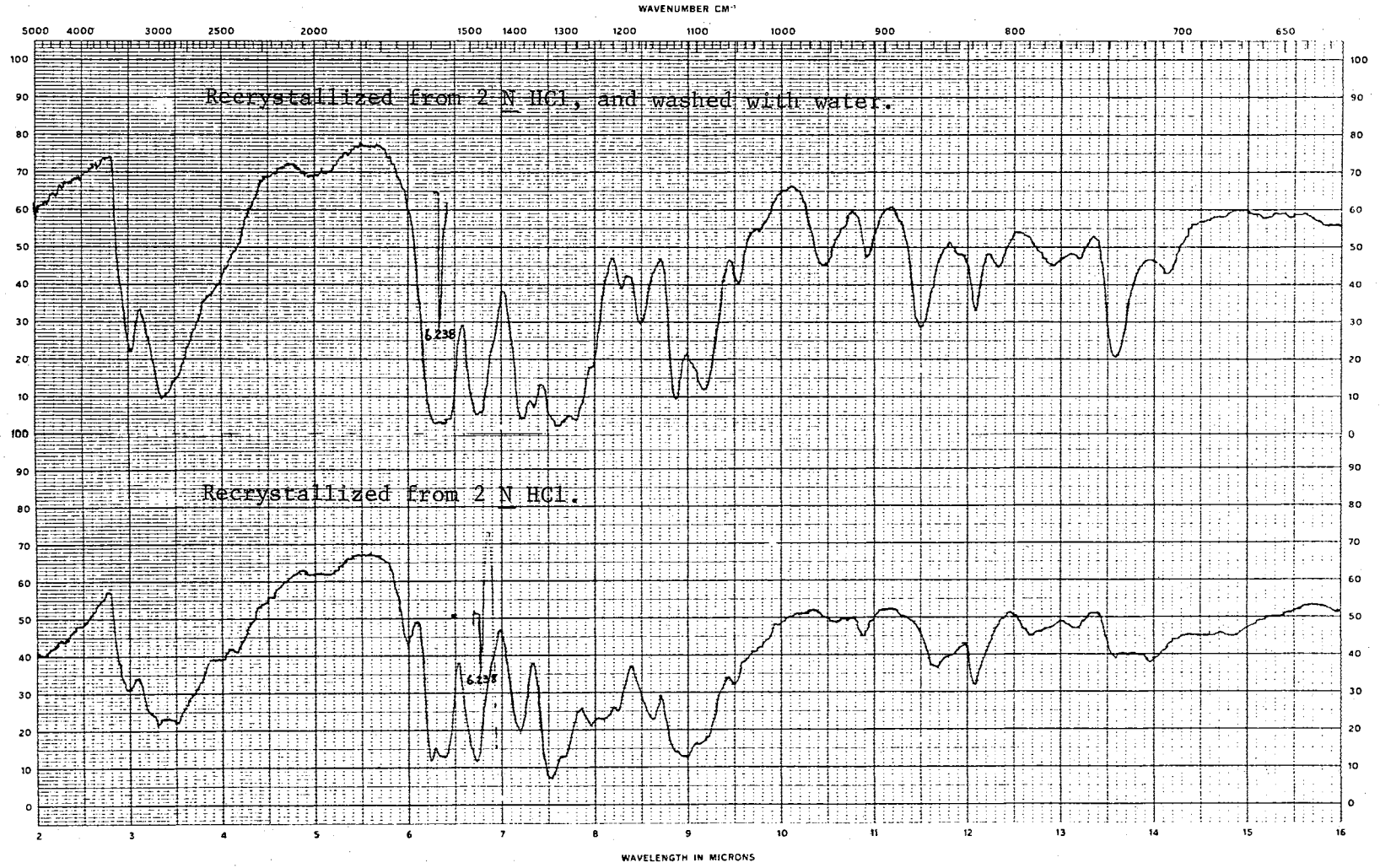


Figure 4. The Infrared Spectra of α-Keto-γ-Aminobutyric Acid 2,4-Dinitrophenylhydrazone.

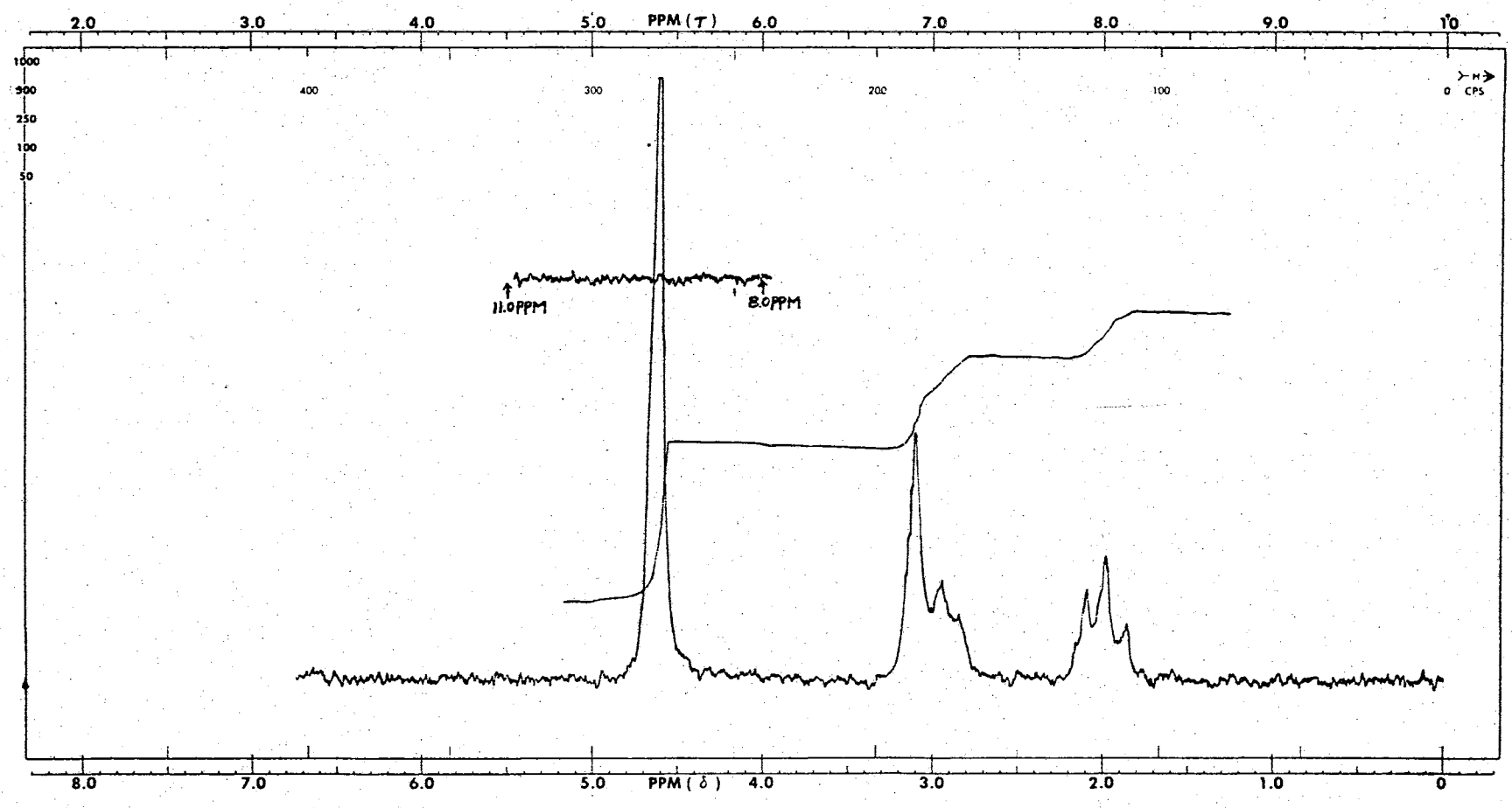
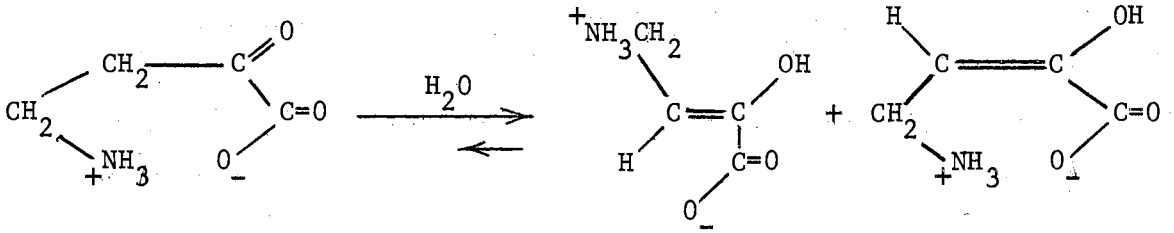


Figure 5. The NMR Spectrum of α-Keto-γ-Aminobutyric Acid in D_2O at 60 Mcps.

the proton ratios given by NMR integration, these ratios were 3.85 : 2 : 1.15 at 5 minutes; 4.12 : 2 : 0.88 at 24 hrs for the three groups of peaks representing a total of 7 hydrogens. No enol hydrogen was observed. Apparently, the enol hydrogen has been exchanged with deuterium.

The fact that KABA does not show a triplet at 3.0 PPM and that integration of the NMR spectrum of KABA shows a proton ratio of 2:1 at 3.0 and 2.0 PPM, respectively, suggests the presence of one vinyl and two methylene hydrogens besides HDO, and that KABA readily enolyzes in aqueous solution (Figure 5). Based on the lack of chloride ion and the preference of the enol form in aqueous solution, the structure of the KABA crystal is hypothesized to be a 6-membered cyclic internal salt. Probably, the compound is a cis or trans enol form in aqueous solution. The vinyl hydrogen may be locked so tightly that it cannot move freely. The structure of KABA would be represented as:



(trans) (cis)

N-Acetyl Ethyl Esters and N-Trifluoroacetyl n-Butyl Esters of Amino Acids

These derivatives were yellow or brown colored oils, soluble in chloroform and ether, insoluble in alcohol. The derivatives could be

separated by thin-layer chromatography and gas-liquid chromatography (GLC) on SE-30 or OV-1 column. The results of the separation by GLC are presented in Chapter V, Figure 24.

CHAPTER III

NEUROTOXICITY

Experimental

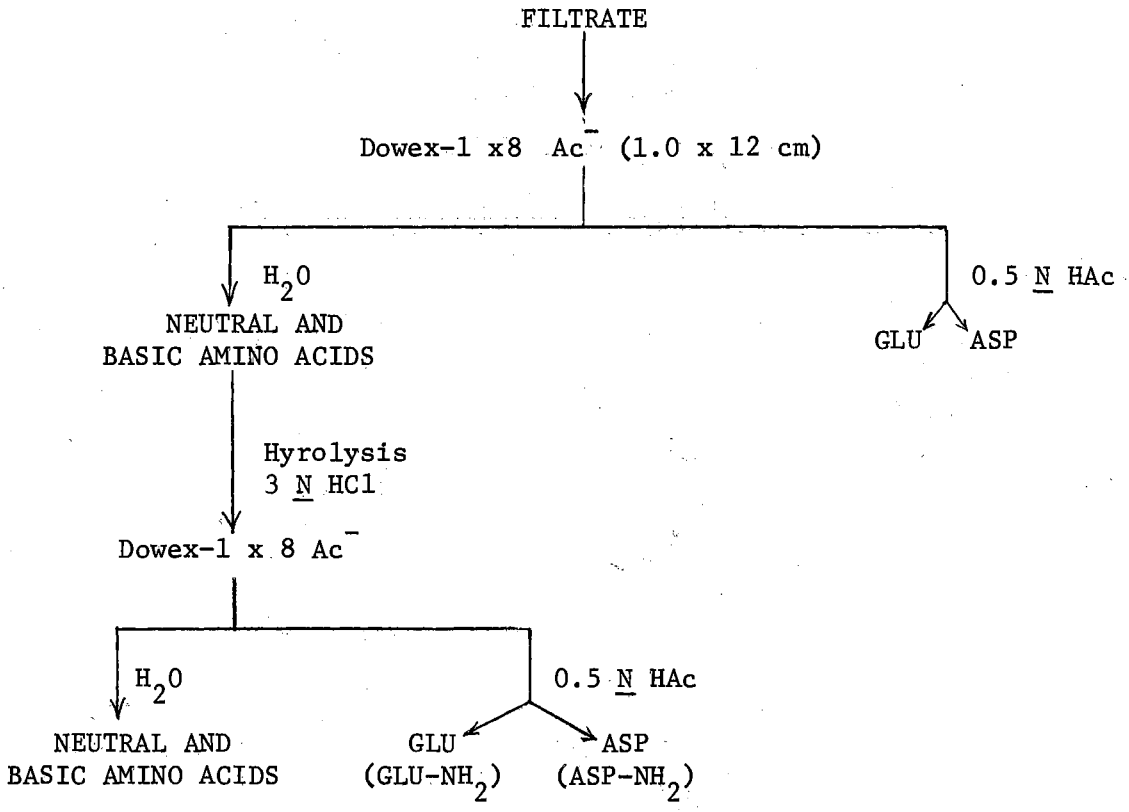
Animals

Male albino rats, obtained from the Holtzman Rat Co., Wisconsin, were given a commercial (Teklad Inc., Iowa) 25% protein rat diet and water ad libitum before the experiments. Leghorn cockerels, obtained from the Stillwater Hatchery were raised on a commercial chick starter ration. Rats weighing 165-320 g and chicks weighing 190-250 g after a 24 hr period of starvation (with water ad libitum) were used for all the intraperitoneal treatment experiments. A group of rats were given 200 µg of thyroxine (titrated to pH 9.0 with 0.1 N KOH) daily for 8 days before beginning the experiments. After the intraperitoneal administration of the test compounds dissolved in 2 ml of water, or an equal volume of water for the control, the animals were allowed to drink water ad libitum for the duration of the experiment and given food 8 hrs after administration. However, in three experiments urine was collected over experimental periods during which no water and food were provided for the rats. For the intracerebral treatment experiments fed rats were used, the test compounds were dissolved in 0.2-0.24 ml of water or saline (0.9% NaCl) and titrated to pH 6.5-7.0. At the termination of the experiment, the animals were decapitated, the desired tissues removed

and prepared for analysis.

Isolation and Assay of Amino Acids

Tissues to be analyzed for amino acids were blotted with filter paper and frozen in liquid nitrogen immediately after dissection. Free glutamate, aspartate and glutamate from glutamine were isolated from the frozen tissue by the method described by Koeppe et al. (74, 98), as shown in the following scheme:



The brain was homogenized with 3 ml (per g tissue) of cold 0.6 N perchloric acid in a Potter-Elvenhjem homogenizer, centrifuged at 10,000 xg for 10 min and re-extracted with an amount of cold 0.33 N perchloric acid equal to that of the first supernatant solution volume. The com-

bined supernatant solutions were neutralized to pH 7.0 with 2 N KOH and left in the refrigerator overnight to complete the precipitation of the $KClO_4$. The $KClO_4$ precipitate was removed by centrifugation at 10,000 xg for 10 minutes or filtration. The liver and kidney tissue were treated the same way except 5 ml instead of 3 ml of perchloric acid was used per g tissue.

The separation of glutamic and aspartic acids was accomplished on a Dowex-1 x8 (Acetate form, 100-200 mesh) column. The neutralized extract was passed over a 1.0 x 12 cm column of Dowex-1 x8 acetate resin at a flow rate of approximately 10 ml/hr. The column was washed with 50 ml of distilled water, and then the free glutamic and aspartic acids were eluted from the column in that order using 0.5 N acetic acid as described by Hirs, Moore, and Stein (99). Collection of 2 ml fractions proved to be very satisfactory for the resolution of glutamic and aspartic acids. The amino acid containing fractions from these columns were identified by spotting aliquots on pH 7.4 phosphate buffered filter paper and developing with ninhydrin. The fractions containing glutamic and aspartic acids were pooled and diluted to 50 ml and 25 ml, respectively, with distilled water.

The water effluent was made 3 N by the addition of conc. HCl, and autoclaved (15 lb/sq in) for 12 hrs. The hydrochloric acid was removed by rotatory evaporation on a 50° water bath *in vacuo* (water pump). The hydrolysates were taken up in 5 to 10 ml of water, neutralized with 0.5 N potassium hydroxide, and passed over a Dowex-1 x8 acetate column as previously described. The glutamic acid and aspartic acid moieties of glutamine and asparagine were eluted from the column as previously described.

The amount of glutamate and aspartate eluted from the column was determined by the ninhydrin method of Rosen (100) as modified by Grant (101) using glutamic and aspartic acids as standards.

The free basic amino acids of brain, liver and kidney were isolated from perchlorate filtrates of these tissues on a column (1.0 x 12 cm) of Amberlite CG-50 (Na^+ form; 100-200 mesh). Five volumes of 1.0 M acetate buffer, pH 4.7, per volume of resin were allowed to flow through the column at a rate of 0.5 ml/min; the column was next rinsed free of excess buffer with water. A portion of the neutral perchlorate filtrate was diluted with water to 25 ml and passed over the buffered Amberlite CG-50 column at approximately 0.5 ml/min. The column was washed with water until (ninhydrin negative) the acidic and neutral amino acids had passed through the column. The basic amino acids were then eluted with 1.0 N HCl. To remove ammonia from the eluates, the pH was adjusted to 11.5 with 2 N KOH and the solution was placed overnight in an evacuated desiccator containing 200 ml of conc. H_2SO_4, as described by Moore and Stein (102). The solution was neutralized with ammonia-free 1 N HCl and the basic amino acids were quantitatively determined using L-DBA hydrochloride as the standard (100, 101). A portion of the isolated basic amino acid solution was desalted by passage over a Dowex-1 x8 (OH^- form; 100-200 mesh) column (1.0 x 12 cm) at a rate of about 20 ml/hr. The column was washed slowly with 100 ml of water and eluted with 0.5 N acetic acid. The acetic acid was removed by evaporation to dryness. The free basic amino acids were dissolved in water and assayed by ascending chromatography on Whatman No. 1 filter paper with isobutyl alcohol methyl ethyl ketone : water : aq. NH_3 (sp. gr. 0.88) *4:3:2:1, by vol.) solvent (103).

Assay of Total Brain Amino Acids by Amino Acid Analyzer

The samples to be analyzed by amino acid analyzer, Beckman Model 120C, were prepared according to the instruction manual with modification.

Brains obtained from treated rats were blotted with filter paper and frozen in liquid nitrogen immediately after dissection. Each brain, 0.8-1.2 g, was homogenized with 15 ml per g tissue of cold 1% picric acid in a Potter-Elvenhjem homogenizer for 20 seconds. The picric acid precipitate was removed promptly by centrifugation at 12,000 xg for 45 minutes. The supernatant solution was treated with approximately 20 g moist Dowex-2 x8, stirred for 30 minutes to remove the excess picric acid, and filtered. The resin was washed with five 4-ml portions of 0.02 N HCl. The filtrate and washings were combined and evaporated on a 28°-30° water bath *in vacuo* (water pump) to a volume of about 1 ml. The contents were transferred with water into a 10 ml-beaker, the total volume being maintained at less than 5 ml. The pH of the solution was adjusted to 6.5 with 1 N NaOH, then 0.15 ml of freshly prepared 0.5 M Na_2SO_3 solution was added. The pH of the mixture was adjusted within the range of 7.2 to 7.5, and the solution was exposed to air for 4 hrs to convert both reduced and oxidized glutathiones to glutathione-S-sulfonate. Both forms of glutathione interfere in this type of chromatography since they emerge (at 32°) as a very broad peak underlying several amino acids from the aspartic acid position to the proline position. Glutathione-S-sulfonate emerges at the column volume, thus eliminating interference with other amino acids or related compounds.

The pH of the sample was brought to 2.0-2.2 with 1 N HCl, and the volume raised to 25 ml with 0.20 N sodium citrate buffer, pH 2.2. One

ml and two ml aliquots of sample were analyzed on long and short columns, respectively, with an automatic recording amino acid analyzer, Beckman Model 120C, as described by Spackman et al. (104). The neutral and acidic components were eluted and separated from each other on the long column (52.3 x 0.9 cm) with 0.20 N sodium citrate buffer, pH 3.25, which was changed to pH 4.25 after 155 minutes. The temperature was set at 32° and raised to 55° 100 minutes later. The basic amino acids remained on the long column; these were assayed by a separate run, using the short column. The neutral and acidic components emerged from the short column (19.3 x 0.9 cm) at column volume, while the basics were eluted and separated from each other with 0.38 N sodium citrate buffer, pH 4.26. The temperature initially 32°, was raised to 55° after 185 minutes. The time required for the complete analysis is 375 minutes and 325 minutes for the short and long columns, respectively. Beckman Custom Spherical Resins were used: short column, PA-35 resin, 13 μ size with 7.5% cross-linking; long column, PA-28 resin, 16 μ size with 7.5% cross-linking. The columns were equilibrated with buffer. For the quantitative determination of amino acids the amino acid analyzer was standarized with a mixture containing amino acids at known concentrations.

Determination of Tissue Ammonia and Urea and Blood Glucose

Protein free filtrates for ammonia analysis were prepared by precipitation with 20% (blood) or 10% (brain) (w/v) trichloroacetic acid according to the methods of Nathan et al. (105) and Tews et al. (106). For determination of ammonia the microdiffusion method described by Seligson et al. (107) was combined with the ninhydrin colorimetry of Rosen (100) and Grant (101). Protein-free filtrates of blood, brain,

and liver for urea determination were prepared by precipitation with $ZnSO_4$ and $Ba(OH)_2$ as described by Somogyi (108). Samples of urine were preserved with toluene. The method of Archibald (109) was adopted for the urea quantitative assay method, in which urea reacted with α-isonitrosopropiophenone and measured at 540 mμ. The procedure presented by Nelson and Somogyi (110, 111) was used to determine the concentration of blood glucose.

Tissue K^+ and Na^+ Analysis

Liver, brain and kidney tissues obtained from L-DBA or water treated rats were blotted, weighed and ashed at 550° in a muffle furnace. The residue was dissolved in 3.6 N HCl and analyzed for K^+ and Na^+ with an atomic absorption spectrophotometer (112) by Mr. Thomas E. Nelson, Animal Science Department.

Histopathological Examination of Tissue

The brain, liver and kidney obtained from L-DBA, or water treated rats were blotted, cut into 5-10 mm. pieces and fixed in 10% (w/v) formalin. Sections 4-6 μ thick were prepared and stained with haematoxylin-eosin for examination. The examination was made by Dr. James B. Corcoran, of Veterinary Pathology Department.

Incubation and Analysis of Liver Slices

Liver slices (0.5 mm thick) were prepared with a microtone (113). Slices of approximately 0.5 g wet weight were incubated for 30 minutes at 37° in 25 ml Erlenmeyer flasks containing 4 ml of Krebs-Ringer bicarbonate buffer, pH 7.4 (114). In some of the experiments liver slices

were incubated with L-DBA, L-ornithine, or L-homoarginine during the 30 minutes before the addition of the ammonium chloride. Then 1 ml of 25 mM NH_4Cl was added and the incubation continued for 1 hr (80). During the entire experiment, the solutions were gassed with O_2 + CO_2 (95:5). The addition of 0.3 ml of 3% (w/v) sodium lactate, pH 7.3, to the incubation medium (115), which enhanced the utilization of ammonia and production of urea by the slices, was used in two of the later experiments. The incubations were terminated by addition of 1 ml of 0.6 N perchloric acid. Control flasks were prepared similarly except that NH_4Cl was added after $HClO_4$. The mixtures were centrifuged at 15,000 xg for 15 minutes; the clear supernatants were analyzed for ammonia and urea. After diffusing for 3 hrs (107), the ammonia was quantitatively determined by Nesslerization (116). The iodinated Nessler's solution was prepared by dissolving 30 g of KI, 41 g of HgI_2 and 2 g of I_2 in water and diluting to 200 ml with water. Urea was determined by the method of Archibald (109).

Ornithine Transcarbamylase (EC 2.1.3.3) Assay

Rats fasted for 24 hrs were killed by decapitation, the liver was removed promptly and chilled in ice. A 1 g sample of liver was passed through a cold Harvard tissue press and homogenized with 19 ml of ice-cold water (117). The assay system of Brown and Cohen (118) was used to determine the enzymic activity in the water homogenate by measuring the rate of citrulline formation. The incubation mixture consisted of L-ornithine, 20 μmoles; homogenate, 0.02 ml; glycylglycine buffer, pH 8.3, 90 μmoles; carbamyl phosphate, 20 μmoles; and L- or D-DBA, 0-40

μmoles. The total volume was made up to 2 ml by the addition of water. After preincubation for 10 minutes, the reaction was started by adding carbamyl phosphate, and terminated by the addition of 0.6 N perchloric acid. The controls were similar except that carbamyl phosphate was added after perchloric acid. The precipitate was removed by centrifugation, and the clear supernatant was analyzed for citrulline by the method of Archibald (119) as modified by Ratner (120).

Materials

L-α,γ-diaminobutyric acid monohydrochloride (A grade), L-homoarginine hydrochloride (A grade), L-ornithine hydrochloride (A grade), L-glutamine (A grade), glycylglycine (A grade) and dilithium carbamyl phosphate (B grade) were obtained from California Corp. for Biochemical Research, Los Angles, Calif. D-α,γ-diaminobutyric acid monohydrochloride was prepared by the Schmidt degradation of D-glutamic acid as described in Chapter II.

Results

Effect of L-α,γ-diaminobutyric Acid (L-DBA) on Blood Sugar

At 16 or 26 hrs after the intraperitoneal administration of 4.4 mmoles of L-DBA/kg body weight (14) to fasted rats, the blood glucose levels of the treated rats were comparable to those of the controls.

Effect of Amino Acids and Ammonia on Free Amino Acids of Brain and Ammonia of Brain and Blood

A short time (15-30 minutes) after the intraperitoneal administration of $LD_{99.9}$ (28) doses of L-leucine and L-lysine hydrochloride/kg

body weight to rats or 22.5 mmoles of ammonium acetate/kg body weight to chicks, the animals developed the typical symptoms of toxicity described by Greenstein et al. (28) : dyspnoea, extreme prostration and in some cases coma. The administration of smaller doses of ammonium acetate (7.0 or 12.0 mmoles/kg body weight) to chicks resulted in no such symptoms of toxicity. Giving homoarginine at a dose of 5 mmoles/kg body weight to rats caused no noticeable symptoms of toxicity, but doses of 10 mmoles/kg body weight resulted in hypersensitivity and death after 15-20 hrs.

None of the treatments mentioned above changed the concentrations of glutamate or aspartate in rat or chick brain. However, brain glutamine concentrations of these animals were higher than those of controls (Table I), the increase being comparable with the increase in free glutamine of rat brain following treatment with a $LD_{99.9}$ dose of ammonium acetate (74). The blood ammonia concentration of rats increased after treatment with the above mentioned amino acids, in agreement with the findings of Greenstein et al. (28).

Intraperitoneal administration of 4.4 mmoles of L-DBA/kg body weight resulted in hyperirritability, tremors and convulsions in 12-17 hrs, followed by death in 3-8 days. The effect of this treatment on major free amino acids, brain ammonia and blood ammonia is shown in Tables I and II. At 30 hrs after treatment with L-DBA the brain glutamine concentration was significantly higher than normal. Shorter periods of treatment with L-DBA also resulted in higher concentrations of brain glutamine, but the increases were smaller than those of 30 hr rats. At 30 hrs after treatment with L-DBA, the ammonia concentration in brain was slightly but not significantly, higher than that in control

TABLE I

THE EFFECT OF AMINO ACIDS AND AMMONIA ON THE CONCENTRATION OF FREE GLUTAMIC ACID, ASPARTIC ACID AND GLUTAMINE OF BRAIN AND AMMONIA OF BRAIN AND BLOOD

Animal	Compound	Dose[a]	Duration of Treatment	Brain				Blood Ammonia
				Glutamic Acid	Aspartic Acid	Glutamine	Ammonia	
		mmoles/kg body wt	hr	μmoles/g Tissue[b]				μmoles/ml[b]
Rat	Water	-	30	8.5±0.24 (3)	1.9±0.18 (3)	4.1±0.09 (3)	1.3±0.01 (10)	1.1±0.18 (12)
Rat	L-DBA	4.4	30	9.4±0.29 (4)	2.0±0.09 (4)	9.4±0.45 (4)	1.7±0.16 (4)	1.8±0.38 (5)
Rat	L-DBA	4.4	25	10.0 (1)	1.5 (1)	7.2 (1)		
Rat	L-DBA	4.4	20	9.6 (2)	2.3 (2)	6.6 (2)		
Rat[c] (Thyroxine-) treated	L-DBA	4.4	30	9.9±0.78 (3)	1.9±0.11 (3)	5.3±0.43 (3)		0.9±0.22 (3)
Rat	Water	-	1	8.4±0.30 (3)	1.8±0.20 (3)	3.6±0.40 (3)		1.4±0.28 (3)
Rat	L-Homoarginine ·HCl	20	1/4	9.6 (2)	2.2 (2)	5.5 (2)		10.0 (2)
Rat	L-Lysine·HCl	36	1/2	9.2±0.20 (3)	2.1±0.10 (3)	4.9±0.63 (3)		5.4±0.88 (3)
Rat	L-Leucine	64	1/3	9.3 (2)	2.2 (2)	7.8 (2)		6.9 (2)
Chick	Water	-	1/4	8.5 (1)	2.4 (1)	5.3 (1)		
Chick	Ammonium Acetate	22.5	1/4	7.3 (2)	1.5 (2)	6.3 (2)		
Chick	Water	-	26	12.5 (2)	2.5 (2)	6.1 (2)		
Chick	L-DBA	12.9	26	9.1 (2)	2.1 (2)	8.2 (2)		

[a]The compounds were dissolved in 2.0 ml water and intraperitoneal injection, and equal volume of water was used for control rat injection.

[b]Data are mean values followed by their standard errors with number of determinations shown in parentheses.

[c]Rats were given 200 μg thyroxine daily for 8 days before given L-DBA.

TABLE II

THE EFFECT OF INTRAPERITONEAL TREATMENT WITH L- AND D-DBA ON THE CONCENTRATION OF FREE AMINO ACIDS OF RAT BRAIN

Treatment	Dose[a]	Duration of Treatment	Toxicity	Glu	Asp	Glu NH_2 Asp NH_2	Gly	Ala	Thr	Ser	P Ser	GABA	Orn DBA	Ammonia	Lys	Arg	Urea
	μmoles/kg body wt	hr		μmoles/g tissue													
Control	–	30	No	11.9	2.9	3.1	0.8	0.6	0.5	1.1	1.0	2.0	0.1	2.0	0.3	0.1	1.6
Control	–	30	No	11.3	2.7	4.1	0.8	0.7	0.5	1.3	1.1	2.1	0.0	2.1	0.3	0.1	1.1
L-DBA	4.4	30	Yes	12.2	2.3	7.0	1.4	1.3	0.9	1.7	0.9	4.1	2.9	2.1	0.7	0.2	3.6
L-DBA	4.4	30	Yes	12.2	2.2	10.4	1.1	0.9	0.8	1.6	0.7	2.8	3.4	3.0	0.7	0.2	3.2
L-DBA	4.4	30	Yes	9.5	2.0	9.3	1.0	1.0	0.7	1.5	0.7	3.3	4.0	2.2	0.5	0.2	2.8
L-DBA	6.6	30	Yes	11.6	1.9	7.7	1.2	1.5	1.2	1.7	0.6	3.5	3.3	3.3	0.7	0.2	3.5
L-DBA	6.6	30	Yes	9.7	2.1	13.3	1.1	1.8	1.3	2.2	0.4	3.0	4.2	1.8	0.6	0.1	2.6
L-DBA	6.6	30	Yes	9.6	2.1	10.8	1.3	1.4	1.1	2.1	0.6	4.1	5.3	2.1	0.6	0.1	2.9
L-DBA	6.6	60[b]	Yes	6.2	1.4	7.9	0.7	0.9	0.6	2.1	0.4	2.9	4.6	1.5	0.5	0.1	1.8
L-DBA	6.6	101[b]	Yes	9.5	2.2	7.4	0.8	0.8	0.6	1.4	0.6	2.1	1.5	2.0	0.3	0.3	2.7
L-DBA	6.6	130[b]	Yes	6.8	2.8	12.8	0.8	0.5	0.7	1.2	0.5	2.0	1.1	2.1	0.4	0.2	5.6
L-DBA	4.4	30	No	13.5	2.6	3.1	0.7	0.6	0.5	1.2	1.1	3.5	1.1	2.1	0.4	0.2	2.4
L-DBA	6.6	30	No	11.6	2.7	4.6	0.8	0.6	0.5	1.2	0.7	2.2	0.2	2.0	0.2	0.2	2.5
D-DBA	4.4	30	No	11.1	2.8	4.2	0.8	0.7	0.5	1.2	1.2	2.4	0.1	1.9	0.2	0.1	3.4
D-DBA	6.6	30	No	11.8	2.5	3.7	0.7	0.6	0.5	1.2	0.8	2.1	0.1	2.1	0.2	0.1	2.6
D-DBA	13.2	30	No	13.5	3.0	4.0	0.8	0.6	0.5	1.2	0.6	2.4	0.2	1.8	0.2	0.2	2.2

[a]The compounds were dissolved in 2.0 ml water and administrated by intraperitoneal injection, and equal volume of water was used for control rat injection.

[b]The animals were killed near death.

rats. This same treatment resulted in a blood ammonia concentration higher than that in controls, but much lower than those observed a few minutes after treatment with L-homoarginine hydrochloride, L-lysine hydrochloride or L-leucine (Table I). Blood ammonia concentrations measured at 2, 6, 12 and 20 hrs after L-DBA treatment were not higher than the average value 30 hrs after treatment.

When the intraperitoneal dose of L-DBA was increased to 6.6 mmoles/kg body weight, the rats showed typical toxic symptoms in 10-12 hrs and died in 3-5 days. Intraperitoneal administration of 4.4-13.2 mmoles of D-DBA/kg body weight resulted in no toxic symptoms or decrease in growth (about 20-30 g per week).

The effect on the concentrations of free amino acids (which were analyzed in the amino acid analyzer) of rat brain are shown in Table II. At 30 hrs after treatment of rats with 4.4 mmoles/kg body weight of L-DBA, the concentrations of glycine, alanine, threonine, γ-aminobutyric acid (GABA) and lysine in brain were higher than in control rats along with glutamine and ammonia increases comparable to those shown in Table I. The concentration of DBA in brain was 3-4 μmoles/g. Although the sum of serine and phosphoserine was comparable to that of controls, the concentration of serine was increased while that of phosphoserine decreased. Increasing the dosage of L-DBA to 6.6 mmoles/kg body weight resulted in the same changes of amino acid concentrations in brain (after 30 hrs), except for higher concentrations of DBA and GABA (Table II). At death the same treatment gave concentrations of glycine, alanine, threonine, GABA, serine and ammonia comparable to that of controls, while the glutamine concentration remained high and phosphoserine was slightly lower than that of controls (Table II).

L-DBA at a dose of 4.4 mmoles/kg body weight or higher was not always fatal. The fatality was about 80% at a dose of 4.4 mmoles/kg body weight and about 95% at a dose of 6.6 mmoles/kg body weight. Some treated rats survived without toxic symptoms; few survived after showing toxic symptoms. Rats receiving D-DBA at a dose up to 13.2 mmoles/kg body weight showed neither toxic symptoms nor decrease in growth rate (about 20-30 g per week). The concentrations of the free amino acids in brains of surviving rats treated with either D- or L-DBA, were comparable to those of controls. Less than 0.2 μmoles/g brain tissue of D-DBA, and less than 1.2 μmoles/g brain tissue of L-DBA were observed in surviving animals (Table II).

The administration of 3.22 or 6.45 mmoles of L-DBA/kg body weight to chicks resulted in no symptoms of toxicity and no decrease in growth rate. The injection of 12.9 mmoles of L-DBA/kg body weight to chicks did not cause the hyperirritability, tremors and convulsions observed in rats, but it appeared to inhibit growth and resulted in increased levels of brain glutamine (Table I).

Effect of L-DBA on the Free Basic Amino Acids, Na^+ and K^+ of Liver and Brain

After the intraperitoneal administration of L-DBA to rats, the free basic amino acid concentration of liver was higher than in normal rats (Table III). At 1 hr after treatment the free basic amino acid level of liver was five times higher than that of controls. The difference between DBA-treated and control rats decreased with increasing time of treatment. However, 30 hrs after L-DBA treatment, the liver still contained a small amount of DBA. Although all the differences between the levels of free basic amino acids in the control and L-DBA-treated rat

TABLE III

THE EFFECT OF L-DBA ON THE CONCENTRATION OF FREE BASIC AMINO ACIDS OF RAT LIVER AND BRAIN

Treatment[a]	Duration of Treatment	Basic Amino Acids[b]	
		Liver	Brain
	hr	μmoles/g Tissue	
Control	30	6.6± 0.35 (2)	3.5±0.63 (4)
L-DBA	1	34.0±4.15 (3)	4.1±1.25 (3)
L-DBA	2	23.6±0.40 (2)	
L-DBA	15	15.2 (1)	
L-DBA	30	9.4±0.55 (2)	5.7±0.43 (3)

[a]Intraperitoneal injection of 4.4 mmoles/kg body weight of L-DBA or equal volumes of water to rats.

[b]Data are reported as mean values followed by their standard errors with the number of determinations shown in parenthesis.

livers can not definitely be attributed to the accumulation of DBA, paper chromatography of the desalted basic amino acids from rat livers demonstrated the presence of larger amounts of DBA than lysine or arginine. The R_f values of L-DBA, lysine and arginine obtained by paper chromatography (see Experimental) were 0.29, 0.23 and 0.18, respectively. This accumulation of DBA by liver agrees very well with results obtained by other workers (41, 89).

The concentration of free basic amino acids in brain 1 hr after L-DBA treatment was only slightly different from that of the controls. However, 30 hrs after L-DBA treatment the average concentration of free basic amino acids in brain was increased from 4.1 to 5.7 μmoles/g (Table III) and paper chromatography revealed the presence of DBA. Thus, it appears that a slight amount of an intraperitoneal dose of L-DBA does enter the brain and the accumulation increased with time. Slight penetration of L-DBA into rodent brain has been previously reported by Kessel (12).

The changes in concentrations of free basic amino acids in brain was readily observed when the automatic recording amino acid analyzer was applied to assay the tissue extracts. Although DBA could not be separated from ornithine by the short column of the analyzer, the concentration of ornithine in control rats was very low (0.02-0.08 μmoles/g) and was assumed not to change with DBA treatment. The concentrations of brain basic amino acids, including GABA and ammonia, measured at 0, 1, 3, 9 and 15 hrs after the onset of toxic symptoms of rats treated intraperitoneally with 4.4 mmoles/kg body weight of L-DBA, are shown in Table IV. The lowest concentration of L-DBA observed at the onset of toxic symptoms was 2.25 μmoles/g brain tissue, reaching a maximum at 9

TABLE IV

CHANGES IN CONCENTRATIONS OF BASIC AMINO ACIDS OF L-DBA-TREATED RAT BRAIN AFTER THE ONSET OF SYMPTOMS

Treatment[a]	Duration[b] after Symptom	Basic Amino Acids					
		GABA	Orn DBA	Am	Lys	Hist	Arg
	hr	µmoles/g Tissue					
Control[c]	-	2.0	0.1	2.0	0.3	0.1	0.1
Control[c]	-	2.1	0.0	2.1	0.3	0.0	0.1
L-DBA	0	3.7	2.6	2.2	0.3	0.1	0.1
L-DBA	0	3.3	3.1	2.6	0.4	0.1	0.1
L-DBA	0	3.4	2.3	2.7	0.2	0.1	0.1
L-DBA	1	3.4	3.1	2.9	0.3	0.1	0.1
L-DBA	3	5.0	3.1	3.0	0.5	0.2	0.1
L-DBA	3	4.8	3.4	1.8	0.5	0.1	0.1
L-DBA	9	5.6	4.7	7.0	0.7	0.2	0.2
L-DBA	9	4.9	3.2	2.5	0.9	0.2	0.2
L-DBA	9	5.3	3.5	3.0	0.7	0.2	0.2
L-DBA[d]	15	4.1	2.9	2.1	0.7	0.2	0.2
L-DBA[d]	15	2.8	3.4	3.0	0.7	0.1	0.2
L-DBA[d]	15	3.3	4.0	2.2	0.5	0.1	0.2

[a] As mentioned in Table III.

[b] The range of onset of symptoms is 12-18 hrs.

[c] Including GABA and ammonia.

[d] The data are adopted from 30 hr treatment with L-DBA in Table II.

hrs after the onset of toxic symptoms. These results are in accord with the data in Table III. The increase in concentrations of GABA and lysine in rat brain was parallel to the increase of DBA. At 9 hrs after the onset of toxic symptoms, GABA was increased to a maximum of 2 to 3 times that of the controls, whereas the sum of lysine, arginine and histidine was 3 times that of the controls (Table IV).

The concentrations of Na^+ or K^+ in brain or liver measured at 1, 15 or 30 hrs after intraperitoneal treatment with L-DBA were not consistently different enough from those found in the controls to indicate a definite effect due to L-DBA treatment. However, 1 and 15 hrs treatment with L-DBA lowered the concentration of K^+ in liver slightly.

Effect of Intracerebral Treatment of L-Glutamine on the Concentrations of Free Glutamate, Aspartate and Glutamine of Rat Brain

The intracerebral injection of 11.5 mg (78.8 μmoles) of L-glutamine in 0.2 ml saline to rats had no effect on either the concentrations of free glutamic acid and aspartic acid (Figure 6), which were comparable to those of controls, or on growth rate. The penetration of glutamine into brain tissue occurred within one minute, and reached a maximum after 30 minutes. After 100 minutes, brain glutamine concentration was decreased to the level found in controls without changes in concentrations of glutamic and aspartic acids. The highest concentration of glutamine observed was 9.12 and 9.24 μmoles/g at 30 and 50 minutes, respectively, which represented about 20% of the injected dose. There was no difference between saline and water used as solvents for the glutamine (Figure 6).

The position of this intracerebral treatment is between cerebrum and cerebellum. This treatment with glutamine in saline or water, and

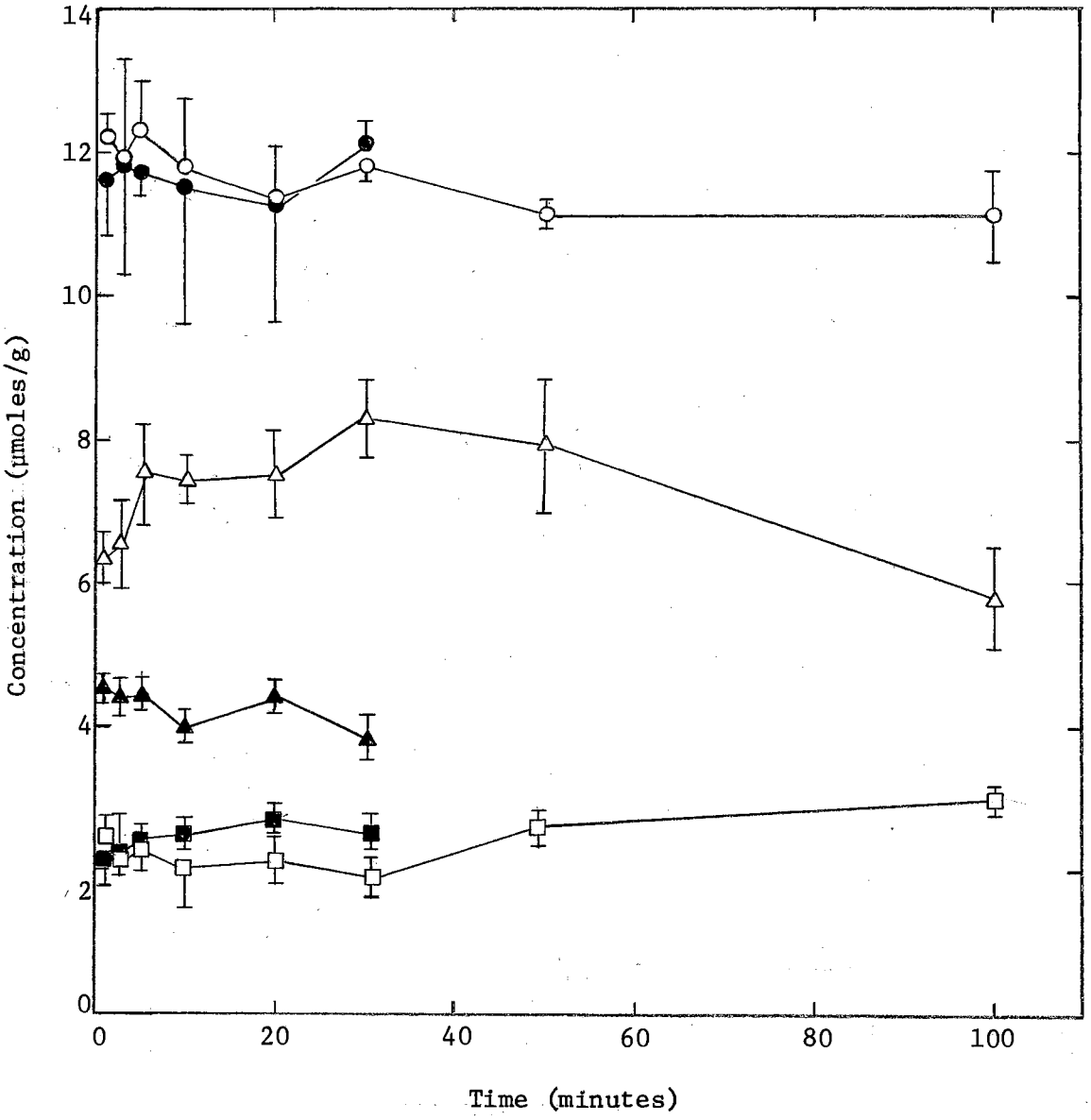


Figure 6. Effect of Intracerebral Treatment of L-Glutamine on the Concentrations of L-Glutamate, L-Aspartate and Glutamine of Rat Brain.

The values reported are averages of 5 treated rats and of 3 control rats.

—○— Glutamate, —●— Control.

—□— Aspartate, —■— Control.

—△— Glutamine, —▲— Control.

saline to rats resulted in uncontrollable spasms and jumping immediately after injection, lasting approximately 10 minutes. The same effects were observed in intracerebral treatment of rats with 2 mg of ammonium acetate, recovery requiring about 30 minutes. The injection of water had no observable effect on the rats.

Effect of Intracerebral Treatment with L-DBA and D-DBA in Rats on the Concentrations of Brain Free Amino Acids

The rats were extremely prostrate immediately after the intracerebral treatment with L-DBA or D-DBA, but recovered after 30-40 minutes in the case of L-DBA. At 1-1½ hrs after intracerebral treatment with 7 mg of L-DBA, the rats showed the toxic symptoms previously observed 30 hrs after intraperitoneal treatment of rats with 4.4 mmoles/kg body weight of L-DBA. Administration intracerebrally of 6 mg of D-DBA resulted in the same toxic symptoms in 15-20 minutes. Death followed in 1-2 days.

Tables V and VI show the effects of intracerebral treatment of rats with L- and D-DBA, respectively, on the brain free amino acids. As high as 3.34 μmoles/g of D-DBA was observed to penetrate the brain within 5 minutes, whereas L-DBA took about 10 minutes to reach a concentration greater than 2 μmoles/g.

There were no clear relationships between the toxic symptoms and the changes in concentrations of brain free amino acids, except that glutamine concentration was lower than that of controls when the penetration of DBA began, and phosphoserine concentration was decreased 50% when rats were near death. The occurrence of toxic symptoms did not coincide with the penetration of DBA into brain over 2 μmoles/g as was observed in intraperitoneal treatment. There was a latent period of 10-20 minutes for D-DBA treatment and of 60-80 minutes for L-DBA treat-

TABLE V

THE EFFECT OF INTRACEREBRAL TREATMENT WITH L-DBA ON CONCENTRATIONS OF FREE AMINO ACID OF RAT BRAIN

Treatment[a]	Duration of Treatment	Free Amino Acids													
		Glu	Asp	Glu NH_2 Asp NH_2	Gly	Ala	Thr	Ser	P Ser	GABA	Orn DBA	Am	Arg	Lys	Urea
	hr	μmoles/g Tissue													
Control	1/6	9.7	2.3	4.5	0.9	0.7	0.5	1.5	0.6	1.7	0.0	3.0	0.1	0.3	2.7
Control	1/3	10.9	2.1	4.6	0.7	0.6	0.5	0.9	0.6	1.9	0.0	1.7	0.1	0.2	2.5
L-DBA	1/12	11.3	2.5	2.9	0.8	0.7	0.5	1.0	0.7	1.8	0.3	2.7	0.1	0.2	1.9
L-DBA	1/6	10.1	2.2	4.2	0.7	0.8	0.5	1.1	0.6	1.8	2.3	0.9	0.1	0.2	0.8
L-DBA	1/2	9.0	2.7	4.4	0.7	0.6	0.5	0.9	0.5	1.9	2.8	0.9	0.1	0.2	1.1
L-DBA	1/2	9.8	2.4	4.6	0.8	0.7	0.4	1.2	0.6	1.9	2.5	1.2	0.1	0.2	1.5
L-DBA	1 1/2	10.6	1.8	3.4	0.8	0.7	0.6	1.1	0.8	3.4	3.5	3.4	0.1	0.4	1.8
L-DBA	1 1/2	8.4	2.5	4.9	0.6	0.4	0.5	1.1	0.7	2.6	2.8	1.0	0.1	0.3	1.9
L-DBA	5	9.9	1.8	4.5	0.8	0.5	0.5	1.0	0.6	3.3	1.1	1.0	0.0	0.1	1.4
L-DBA	5	11.6	2.5	3.5	0.9	0.8	0.6	1.1	0.7	2.7	1.2	1.9	0.1	0.3	1.9
L-DBA	5	7.3	1.8	4.4	0.8	0.6	0.6	1.2	0.5	4.2	3.1	1.1	0.2	0.5	1.5
L-DBA	17	8.2	1.9	4.8	0.9	0.5	0.7	1.1	0.3	2.8	2.6	1.2	0.1	0.7	0.8
L-DBA	17	7.7	1.9	4.3	0.9	0.5	0.9	1.0	0.3	3.8	2.2	1.2	0.1	0.7	1.8
L-DBA	27	9.9	1.8	6.1	1.1	0.9	0.6	1.3	0.3	3.9	2.3	1.5	0.1	0.6	1.9
L-DBA	27	9.5	2.4	8.4	1.1	0.5	0.8	1.4	0.3	3.0	3.1	1.5	0.2	0.6	2.9
L-DBA	20 (days)	11.9	2.3	5.6	1.1	0.6	0.6	1.2	0.9	1.8	0.0	1.5	0.1	0.2	1.8

[a]A dose of 7 mg of L-DBA dissolved in 0.2 ml water, pH 7.0, or equal volumes of water was given to rat between cerebrum and cerebellum.

TABLE VI

THE EFFECT OF INTRACEREBRAL TREATMENT WITH D-DBA ON CONCENTRATIONS OF FREE AMINO ACID OF RAT BRAIN

Treatment[a]	Duration of Treatment	Free Amino Acids													
		Glu	Asp	Glu NH_2 Asp NH_2	Gly	Ala	Thr	Ser	P Ser	GABA	Orn DBA	Am	Arg	Lys	Urea
	hr	μmoles/g Tissue													
Control	1/6	9.7	2.3	4.5	0.9	0.7	0.5	1.5	0.6	1.7	0.0	3.0	0.1	0.3	2.7
Control	1/3	10.9	2.1	4.6	0.7	0.6	0.5	1.0	0.6	1.9	0.0	1.7	0.1	0.2	2.5
D-DBA	1/12	10.4	2.2	2.8	0.7	0.7	0.4	0.9	0.7	2.0	3.3	3.0	0.1	0.1	1.8
D-DBA	1/12	9.7	2.6	2.9	0.7	0.6	0.5	0.8	0.7	2.0	3.0	3.1	0.1	0.2	1.7
D-DBA	1/6	10.0	2.6	3.5	0.7	0.8	0.5	1.0	0.8	2.0	2.0	7.7	0.1	0.2	2.0
D-DBA	1/6	9.7	2.4	3.8	0.7	1.3	0.5	1.0	0.6	2.4	2.1	2.7	0.1	0.3	1.4
D-DBA	1/3	8.3	2.5	4.7	0.7	1.2	0.5	1.0	0.7	2.5	2.2	3.1	0.1	0.2	2.3
D-DBA	1/3	8.6	1.7	3.7	0.6	1.0	0.4	1.0	0.5	1.4	1.3	3.8	0.1	0.2	1.0
D-DBA	1/2	7.6	1.5	3.0	0.5	0.7	0.4	0.8	0.6	1.8	1.5	1.7	0.1	0.1	2.0
D-DBA	1/2	8.4	1.7	4.0	0.6	0.8	0.4	1.0	0.5	1.8	2.2	1.6	0.1	0.2	1.6
D-DBA	1 1/2	9.0	1.3	9.4	0.9	1.1	0.5	1.2	0.7	2.8	2.4	1.6	0.1	0.3	1.6
D-DBA	1 1/2	9.3	5.5	4.0	0.9	1.0	0.6	1.4	0.4	2.7	3.6	2.5	0.1	0.5	1.5
D-DBA	5	11.7	2.3	4.0	0.9	0.8	0.6	1.2	0.6	2.1	1.3	1.9	0.1	0.3	2.8
D-DBA	5	11.0	2.7	6.2	0.8	0.6	0.8	1.2	0.6	4.1	3.2	1.1	0.3	0.9	1.8
D-DBA	17	7.8	1.8	5.0	0.6	0.7	0.5	1.0	0.4	1.1	0.9	1.2	0.1	0.2	1.2
D-DBA	17	8.2	2.0	6.1	0.8	0.6	0.5	1.0	0.2	1.6	1.4	1.2	0.1	0.2	2.8
D-DBA	27	9.6	2.1	5.1	0.9	0.8	0.6	1.2	0.3	1.5	0.7	1.2	0.1	0.2	1.3
D-DBA	28	10.3	2.3	5.2	0.7	0.6	0.5	1.0	0.2	1.5	0.6	1.4	0.1	0.2	2.1
D-DBA	20 (days)	7.7	1.9	4.6	0.6	0.3	0.3	0.8	0.3	1.7	0.1	1.1	0.1	0.2	1.8

[a]A dose of 6 mg of D-DBA dissolved in 0.2 ml water, pH 7.0 or equal volume of water was given to rat between cerebrum and cerebellum.

ment for development of typical toxicity symptoms.

Throughout the intracerebral studies, the concentrations of brain free glycine, alanine, threonine, serine and glutamic acid were comparable to those of controls. Aspartate concentration was very irregular, and, in most cases, slightly lower than in controls. Near death, in both treatments, brain glutamine reached higher concentration than in controls (Tables V and VI). However, the increment is lower than that of intraperitoneal treatment (30 hrs) with higher doses of L-DBA (Table I). An increase in GABA was observed after the intracerebral treatment with L-DBA, but not with D-DBA (Tables V and VI).

Effect of L-Thyroxine Treatment on DBA Toxicity in Rats

Thyroxine treatment protected rats against the toxicity of L-DBA when treated intraperitoneally with a dose of less than 7.7 mmoles/kg body weight of L-DBA. The same thyroxine-treatment had no protective effect when rats were given L- or D-DBA intracerebrally. The thyroxine-protected rats (L-DBA given intraperitoneally) had normal concentrations of blood ammonia and brain glutamine (Table I).

Detoxification by Ornithine and Aspartic Acid

The intraperitoneal administration of 10-20 mmoles/kg body weight of ornithine alone, or with 4 mmoles of aspartic acid, resulted in a decrease in the fatality from 80% (L-DBA alone) to 50% (with ornithine alone, or with aspartate before treatment with L-DBA, based on 53 rats examined). However, in some cases, the toxicity was enhanced (rats died after 40 hrs instead of 3-8 days).

Effect of L-DBA on Urea Synthesis of Liver Slices

The intraperitoneal injection of 4.4 mmoles of L-DBA/kg body weight to rats 30 hrs prior to the preparation of liver slices resulted in significant inhibition of ammonia utilization and urea production (Table VII). In another series of experiments the addition of either 14.02 or 6.46 mM L-DBA to the incubation media significantly inhibited the utilization of ammonia and urea production by normal rat liver slices (Table VII), while the presence of 4.22 mM L-homoarginine hydrochloride in the incubation media had no effect.

Effect of L-DBA on Tissue Urea and Urine Excretion

Analysis of the urea synthesized by the liver slices revealed that liver tissue of L-DBA-treated rats contained considerably more urea than that of controls. Urea determination of tissue extracts obtained from L-DBA-treated and control rats showed that the above observation was correct and that blood and brain tissue of L-DBA-treated rats also had higher levels of urea than corresponding tissues of control rats (Table VIII). After treatment with 4.4 mmoles of L-DBA/kg body weight, rats excreted up to 5 times as much urine than control rats over a 30 hr period during which no water was provided for either group. Thus, diuresis probably caused the elevation in tissue urea concentrations.

Histopathologic Examination of Liver, Kidney and Brain 20 Hrs After L-DBA Treatment

This examination revealed changes in the brain tissue of one of the three L-DBA-treated animals examined. This tissue showed cellular loss in all parts of the hippocampus with some demyelinization. These lesions

TABLE VII

INHIBITION OF AMMONIA UTILIZATION AND UREA FORMATION BY RAT LIVER SLICES WITH L-DBA

Type of Expt.	Treatment	No. of Rats Used	Amount of L-DBA	Ammonia[a] Used	Urea[a] Produced
			mmoles/kg body wt.	µmoles/g wet wt./hr.	
in vivo	Control	9	-	17.2 (20)	5.5 (6)
in vivo	L-DBA	9	4.4	11.2 (20)	0.8 (6)
in vitro	Control	4	(mM)	19.4 (8)	6.0 (6)
in vitro	L-DBA	4	14.0	8.4 (8)	1.7 (6)
in vitro	Control	2	-	24.4 (4)	5.0 (4)
in vitro	L-DBA	2	6.5	15.2 (4)	1.7 (4)

[a]The data are reported as mean values with the number of determinations shown in parentheses. The P values of variance were less than 0.10.

appeared similar to those produced by some neurotoxins or by relatively acute anoxia. No lesions were observed in any of the other tissues examined. Therefore, the neurotoxic effect of L-DBA might be the result of gross structural damage to brain, and not to liver and kidney.

Noncompetitive Inhibition of Ornithine Transcarbamylase by L-DBA

The effect of L-aspartate, L- and D-DBA, thyroxine and ATP *in vitro* on the activity of ornithine transcarbamylase of rat liver is shown in Figure 7 and Table IX. The activity of ornithine transcarbamylase was inhibited by L-DBA, whereas L-aspartate, ATP, thyroxine and D-DBA had no effect or caused a very slight activation. The activity in a reaction mixture containing 10 mM of ornithine was inhibited approximately 40% by 20 mM of L-DBA. The inhibition could be reduced to 22% and 14% in the presence of 20 mM of D-DBA and 1.5 mM of ATP, respectively. L-aspartate and thyroxine had no effect on this inhibition. When L-DBA in the reaction mixture was increased to 40 mM, neither D-DBA, L-aspartate, ATP nor thyroxine had any effect (Table IX). 10 or 15 mM of L-homoarginine in a reaction mixture containing 7.5 mM of ornithine gave no appreciable inhibition.

Figure 8 shows Lineweaver-Burk plots of the activity of ornithine transcarbamylase as a function of ornithine concentration in the presence of three concentrations of L-DBA. Increased L-DBA concentration resulted in an increased slope, with a common point of intersection on the abscissa. From the three points of intersections on the ordinate, the values of K_I obtained were 22.9, 23.2 and 23.2 mM, respectively, with an average K_I of 23.1 mM at pH 8.3, 37°. The K_m and V_{max} are 3.3 mM and 0.95 μmoles/min., respectively, for ornithine at pH 8.3, 37°. A K_m

TABLE VIII

THE EFFECT OF L-DBA ON THE CONCENTRATION OF UREA OF LIVER, BRAIN AND BLOOD

Treatment[a]	Urea Concentration[b]		
	Liver	Brain	Blood
Control	7.2 (1)	4.4±1.15 (2)	6.2±2.25 (2)
L-DBA	10.8 (1)	10.8±0.36 (2)	12.6±1.4 (2)

[a]As mentioned in Table III.

[b]As mentioned in Table III.

TABLE IX

THE EFFECT OF L-ASPARTATE, D-DBA, THYROXINE AND ATP ON THE ACTIVITY OF ORNITHINE TRANSCARBAMYLASE INHIBITED BY L-DBA

Compounds Added					Rate[a]	% Rate
L-DBA	D-DBA	L-Asp	ATP	Thyroxine		
μmoles				μg	μmoles/min	
-	-	-	-	-	0.49	100
40	-	-	-	-	0.29	59
40	20	-	-	-	0.31	63
40	40	-	-	-	0.38	77
40	-	20	-	-	0.30	61
40	-	40	-	-	0.32	65
40	-	-	3	-	0.42	86
40	-	-	6	-	0.33	67
40	-	-	-	1.5	0.30	61
40	-	-	-	3	0.32	65
80	-	-	-	-	0.26	53
80	40	-	-	-	0.23	47
80	-	40	-	-	0.26	53
80	-	-	6	-	0.23	47
80	-	-	-	3	0.22	45

[a]Rate of citrulline formation. The incubation media consisted of ornithine, 20 μmoles. Total volume is 2 ml.

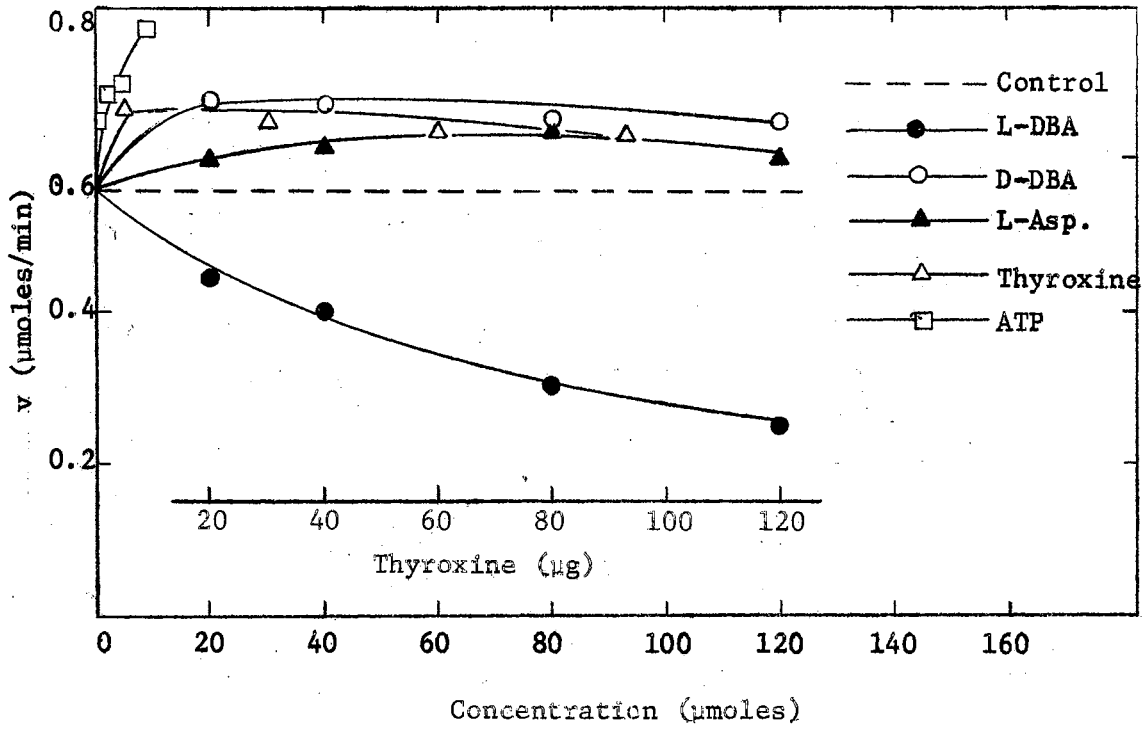


Figure 7. Effect of L-Aspartate, D-DBA, L-DBA, Thyroxine and ATP on the Activity of Ornithine Transcarbamylase. Incubation media consisted of 20 μmoles of L-Ornithine in 2 ml.

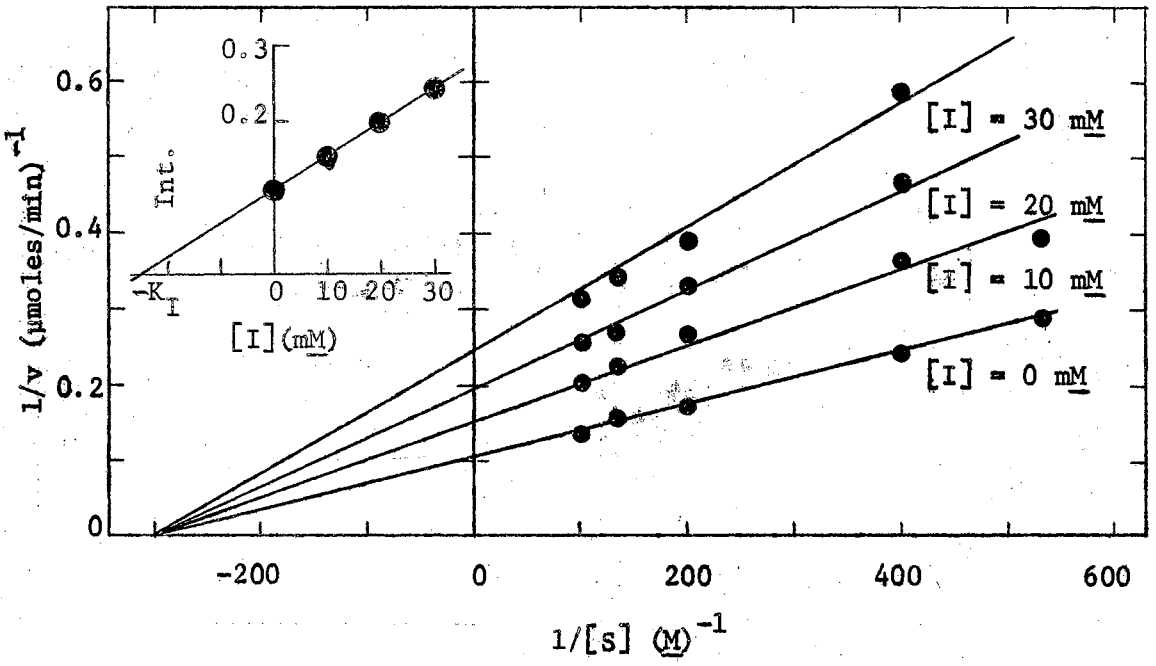


Figure 8. Double Reciprocal Plots of the Inhibition of Ornithine Transcarbamylase from Rat Liver by L-DBA.

value of 1.4 mM of rat liver ornithine transcarbamylase for ornithine at pH 7.3-7.4 had been reported by Reichard (121). These Lineweaver-Burk plots suggest that L-DBA acts as a classical noncompetitive inhibitor for ornithine, although L-DBA acts as a competitive inhibitor for ornithine in the studies of Herrmann et al. (82) with purified ornithine transcarbamylase obtained from Neurospora Crassa.

Discussion

The physiological effects of L- and D-DBA were compared with those of other amino acids and, in the case of L-DBA, found to differ in several ways. The dose of L-DBA necessary to produce toxic effects was considerably lower than that of the other amino acids examined, and of the toxic levels of ammonia and other amino acids reported by Greenstein et al. (28). The symptoms of toxicity after treatment with L-DBA were different and occurred much more slowly than those resulting from lethal doses of ammonia. Lethal doses of other amino acids and ammonia resulted in dyspnoea, extreme prostration and in some cases coma, as described by Greenstein et al. (28), and in a high blood ammonia level shortly after administration (Table I), whereas L-DBA resulted in hyperirritability, tremors and convulsions starting 12-15 hrs after treatment and in slight increases in the brain and blood ammonia levels (Table I). These observations suggest that the mechanism of toxicity for L-DBA differs from that for certain other amino acids and ammonia, and that the catabolic rate might be the key point accounting for this difference.

Although the other amino acids or ammonia resulted in an acute ammonia toxicity, the slight increase in ammonia concentration in blood of L-DBA-treated rats suggested either no ammonia toxicity (from L-DBA)

or a chronic toxicity. The acute toxicity of the other amino acids is believed to result from a rapid release of ammonia from the large dose of the amino acid, thus exceeding the capacity of liver urea synthesis to detoxify ammonia. This results in an accumulation of large excesses ammonia in blood and other tissues (26, 28).

Liver is known as the major site of urea synthesis. L-DBA has been demonstrated to inhibit the ammonia utilization and urea production both *in vivo* and *in vitro* and to inhibit the ornithine transcarbamylase, which may account for the slight increase in blood ammonia concentration and in the marked increase in brain glutamine concentration. The observations that D-DBA has no effect on ornithine transcarbamylase *in vitro* and D-DBA-treated (intraperitoneally) rats have normal brain glutamine and no toxic symptoms suggest that the inhibition of ornithine transcarbamylase may account for the slight increase in blood ammonia concentration, thus resulting in the marked increase in brain glutamine concentration. Moreover, the observation that a uricotelic animal, the chick, was not susceptable to acute ammonia toxicity up to a dose of 12.0 mmoles/kg body weight of ammonium acetate, but L-DBA caused the inhibition of growth in the chick suggested that chronic ammonia toxicity may not be the cause of death of L-DBA-treated rats.

The marked increase in brain glutamine concentration in rats given L-DBA intraperitoneally indicates that the brains of these animals are detoxifying appreciable amounts of ammonia (26). The high concentration of glutamine in rat brain probably does not cause toxic symptoms, because the intracerebral treatment with glutamine resulted in an accumulation of glutamine in rat brain (Figure 5) without toxic effects.

Although it was observed that D-DBA had no effect on the ornithine

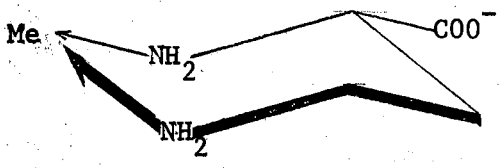
transcarbamylase of rat liver while L-DBA acted as a noncompetitive inhibitor, the intracerebral treatments with small doses (6-7 mg) of L- and D-DBA resulted in rapid (see Results) development of the same toxic symptoms observed after intraperitoneal treatment. However, no toxicity was observed after intraperitoneal treatment with D-DBA at a dose up to 3 times that of L-isomer. These observations suggest that the primary mechanism of toxicity of L- and D-DBA may not be inhibition of the rate of liver urea synthesis by these compounds but rather their presence in brain (Table II).

Rather large doses of homoarginine were required for toxicity. The toxic symptoms and effect on the concentrations of blood ammonia and brain glutamine were similar to those resulting from lethal doses of other amino acids or ammonia. Therefore, even though significant quantities of homoarginine have been found in seeds of L. sativas, L. cicera or L. clymenum, the species associated with neurolathyrism in man, horses and cattle (5), the findings in this study indicate that it is not a primary lathyrogenic factor in these plants, but has toxicity characteristics similar to other amino acids (26).

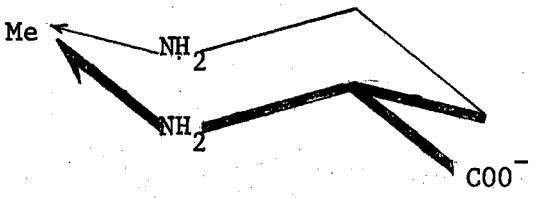
The report (122) that thyroxine treatment decreases the penetration of DBA into rat brain and protects against the neurotoxicity that it causes suggests that the entry of DBA into brain is necessary for some of its neurotoxicity. In this study we observed that thyroxine treatment protected against L-DBA toxicity only when L-DBA was given intraperitoneally in a dose less than 7.7 mmoles/kg body weight. Thyroxine failed to prevent L-DBA (given intracerebrally) toxicity, even when the dose of L-DBA was as low as 6 mg. Moreover, after intraperitoneal treatment with 4.4 mmoles/kg body weight of L-DBA the thyroxine-protected

rats had normal concentrations of brain glutamine and blood ammonia, and thyroxine had no effect on the activity of ornithine transcarblamylase from rat liver *in vito* or *in vivo*, and on the activities, L- or D-DBA as a substrate of L-amino acid oxidase from *Crotalus adamanteus* venom and D-amino acid oxidase from hog kidney *in vitro*. These observations suggest that the prevention, by thyroxine, of the toxicity of L-DBA may not be related to the deamination or transaminations processes, although Vivanco *et al*. (122) have suggested that the protective effect of thyroxine on the toxicity may be through an influence on the deamination or transamination processes which render these substances non-toxic.

Liver was known (12, 41, 89) and shown in this study (Table III) to accumulate DBA. The rapid transfer of this amino acid was explained on steric grounds. The two amino groups of DBA can chelate with metal (27-29) to form a stable six membered ring and form, at the same time, a stable Schiff base with pyridoxal (27). This complex can be arranged in a stable chair conformation and the carboxylic group in both the D- and L-isomer is on an equatorial bond so that DBA is greatly stablized.



L-DBA



D-DBA

The nontoxic effect of D-DBA (given intraperitoneally) may be due to the rapid excretion by kidney, resulting in a limited chance for absorption by brain. Although L-DBA inhibits ornithine transcarbamylase noncompetitively for ornithine, while D-DBA has no effect, it is not known to

date whether this is a factor in the nontoxic effect of D-DBA (given intraperitoneally).

The studies of the concentrations of free amino acids of brain indicate that the toxic symptoms of intraperitoneal DBA-treated rats are related to the penetration of DBA into brain and the modification of some brain free amino acids (Tables II and IV - VI). The increase in glutamine accounts for the detoxification of ammonia by brain; however, whether changes in concentration of some other brain free amino acids is related to toxicity is not known.

The intracerebral treatment with L- and D-DBA also showed that the toxicity of DBA is not an ion-exchange problem, because the symptoms right after treatment with DBA and saline were different (see Results). The observations that there was a similar latent period for the appearence of the symptoms of toxicity after an accumulation of 2.0 μmoles of DBA in brain, which was a minimal amount observed to be required for the toxic symptoms, and that DBA reacted with oxalacetate nonenzymically at an appreciable rate (see Results in Chapter V) suggest that the carbonyl trapping ability of DBA may be responsible for a part of the neurotoxicity. Thiosemicarbazide, a carbonyl trapping agent, has been reported as a convulsant (88).

Also observed in this study the fact that nonenzymic and/or enzymic reactions might exist in the rat brain during storage in the deep freezer (-20^{o} to -30^{o}). The changes in concentration of free amino acids of rat brain during storage are shown in Tables X and XI. The decrease in glutamine plus glutamate is approximately equal to the increase in GABA. Loss of glutamine accounts for much of the ammonia increment. The increase in aspartate is presumed, but not proven, to be due to

TABLE X

CHANGES IN CONCENTRATION (μmoles/g tissue) OF FREE AMINO ACIDS OF RAT BRAIN DURING STORAGE IN THE DEEP FREEZE

Amino Acid or Compound	0 Week	16 Weeks
	μmoles/g tissue[c]	
Glutamic Acid	10.4±0.5 (9.7~10.9)	8.7±1.4 (7.1~10.6)
Aspartic Acid	2.3±0.1 (2.1~2.4)	5.3±0.7 (4.4~5.9)
Glutamine[a]	4.1±0.7 (3.0~4.5)	1.8±1.1 (0.3~2.8)
Alanine	0.6±0.1 (0.6~0.7)	1.9±0.4 (1.5~2.4)
γ-Aminobutyric Acid	1.9±0.2 (1.7~2.3)	5.9±0.54(5.5~6.7)
Ethanolamine	0.3±0.2 (0.1~0.4)	1.9±0.1 (1.8~2.1)
Ethanolamine Esters[b]	1.0±0.1 (0.9~1.2)	0.6±0.0 (0.5~0.6)
Ammonia	2.3±0.5 (1.7~3.0)	7.8±1.8 (5.5~9.4)

[a]Include Asparagine

[b]Phosphoethanolamine + Glycerophosphoethanolamine

[c]Data are mean values followed by their standard errors with the range shown in parentheses.

TABLE XI

CHANGES IN FREE AMINO ACIDS OF RAT BRAIN DURING STORAGE IN THE DEEP FREEZE, EXPRESSED AS PER CENT OF 0 TIME CONTROLS

Amino Acid or Compound	4 Weeks	8 Weeks	12 Weeks	16 Weeks
Glutamic Acid	91	86	80	83
Aspartic Acid	121	152	190	231
Glutamine[a]	68	46	42	45
Alanine	235	255	215	323
α-Aminobutyric Acid	230	232	255	305
Ethanolamine	359	437	431	583
Ethanolamine Esters[b]	76	59	58	57
Ammonia	239	268	242	342

[a]Includes Asparagine

[b]Glycerophosphoethanolamine + Phosphoethanolamine.

hydrolysis of acetylaspartate while the ethanolamine increment is only in part accounted for by a decrease in ethanolamine esters. These results indicate that during the storage period enzymic or nonenzymic amidase, decarboxylase, transaminase and esterase activity occurred.

CHAPTER IV

OXIDATIVE DEAMINATION

Experimental

Preparation of L-Amino Acid Oxidase from Rat Kidney

The method of Blanchard et al. (123) was modified to prepare the L-amino acid oxidase from rat kidney. Because of inadequate amounts of tissue, the enzyme was isolated and purified only through the first sodium sulfate precipitate step and dialyzed.

Step 1

The tissue was passed through a Harvard press and homogenized in a Waring blender for 20 seconds, three times, with 10 volumes of acetone cooled to -10° to -15° with dry ice. The temperature was maintained in the range (-10° to -15°). After homogenization, the suspension was rapidly filtered under suction. The procedure was repeated twice using the residue of the filtration for rehomogenization. After the final filtration, the filter cake was pulverized to facilitate drying in the air. The dry, light brown colored powder was stored in a freezer when not in use.

Step 2

The acetone powder was extracted with 20 volumes of water, and the

suspension was stirred for 30 minutes. The insoluble particles were removed by centrifugation at 600 xg for 10 minutes. Anhydrous sodium sulfate was added (15 g per 100 ml of supernatant) to the supernatant in 5 portions over 20 minutes with constant stirring. Acetic acid, 0.5 N, was then added to bring the pH to 5.1. The adjustment of pH should be carried out electrometrically with a glass electrode. The protein precipitate was collected by centrifugation at 10,000 xg for 10 minutes and resuspended in water to a concentration of 1.5 ml/g acetone powder. All the procedures in this step were performed at room temperature.

Step 3

The above enzyme solution was dialyzed overnight at 0^{o} against 0.025 M phosphate buffer, pH 7.3 (5 ml enzyme per liter buffer), with several changes of buffer. The dialyzed enzyme solution was then used for assays.

Assay of L-amino Acid Oxidase from Snake Venom and Rat Kidney

The rate of oxidation was measured with a Yellow Springs Model 53 polarographic oxygen electrode, which is a Clark type, membrane-coated electrode, by following the change in % of saturation. Calibration of an oxygen electrode apparatus can be accomplished in a number of ways (124). For example, since the solubility of oxygen in water is known, an approximate calibration can be achieved by setting the meter at 100% with air saturated H_2O (23.87 µl/ml at 37^{o}) and assuming a linear meter response in all other situations (i.e., buffer solutions).

The incubation media consisted of substrate (amino acids), 16.6 mM; Tris-HCl buffer (for the pH range of 7.0 to 9.0), or glycine-NaOH buffer

(for the pH range of 9.0-10.0), 0.2 mM; catalase, 2-4 µg; and crude enzyme, 0.2 ml (1.3 g rat kidney), or pure enzyme (snake venom), 0.03-0.05 mg (as recommended by Worthington Co. (125)) in a total volume of 3.0 ml. In the case of snake venom L-amino acid oxidase, 40 mM of KCl were added. The mixture was preincubated in a 37° water bath for 10 minutes, and the reaction was started by adding the enzyme. The oxygen consumption at 37° was measured with the Yellow Springs Model 53 oxygen electrode. Readings were taken at 3 minute intervals until the saturation was decreased to nearly 10% or, for the slow reactions, during the period (30-40 minutes) when the oxygen utilization is constant (Figure 9). The rate of oxidation was measured by the slope of the initial reaction (Figure 9), which was multipled by 2 because catalase regenerated 0.5 moles of oxygen from one mole of H_2O_2 formed, and was expressed as µmoles of amino acid oxidized or µl O_2 consumed per hr per mg of protein. When crude enzyme was used, results were expressed per ml enzyme preparation. The substrate concentration used for assay was in the first order region of the velocity vs. substrate curves (e.g. 0.4 K_m). However, the range of enzyme concentration used is linear with rate constant k' (Figures 10 and 11).

The protein concentration of crystalline L-amino acid oxidase of snake venom was determined with the Cary 14 spectrophotometer by measuring the absorbance at 275 mµ. A_{275} x 0.56 = mg/ml (125).

Assay of D-Amino Acid Oxidase from Hog Kidney

Conditions for this assay were the same as for the assay of L-amino acid oxidase with a modification in the incubation media. The mixture consists of substrate, 33.3 mM; enzyme, 0.1-0.2 mg; and pyrophosphate

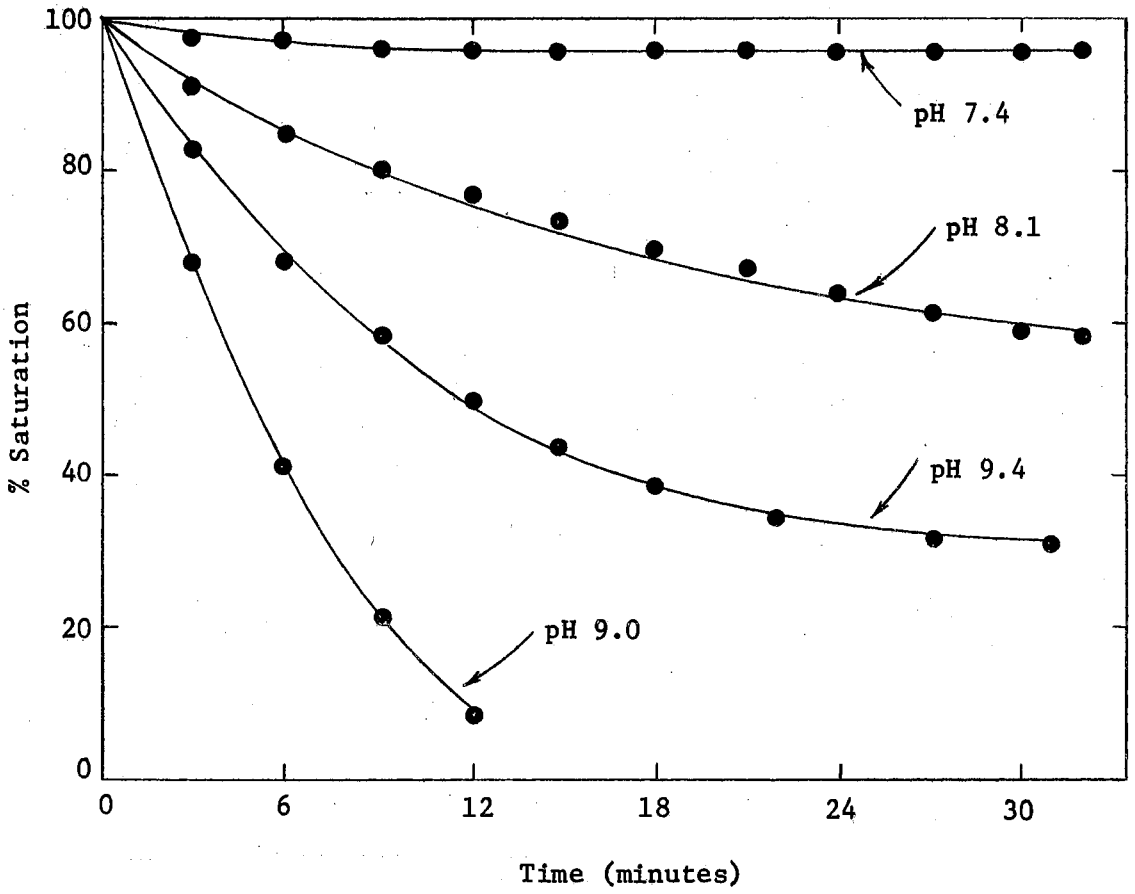


Figure 9. The Oxygen was Measured with the Yellow Springs Model 53 Oxygen Electrode.

Incubation media consisted of L-DBA, 16.6 mM; Tris or glycine buffer, 0.2 mM; KCl, 40 mM; and L-amino acid oxidase from snake venom, 0.005 mg/ml. Assayed at 37°.

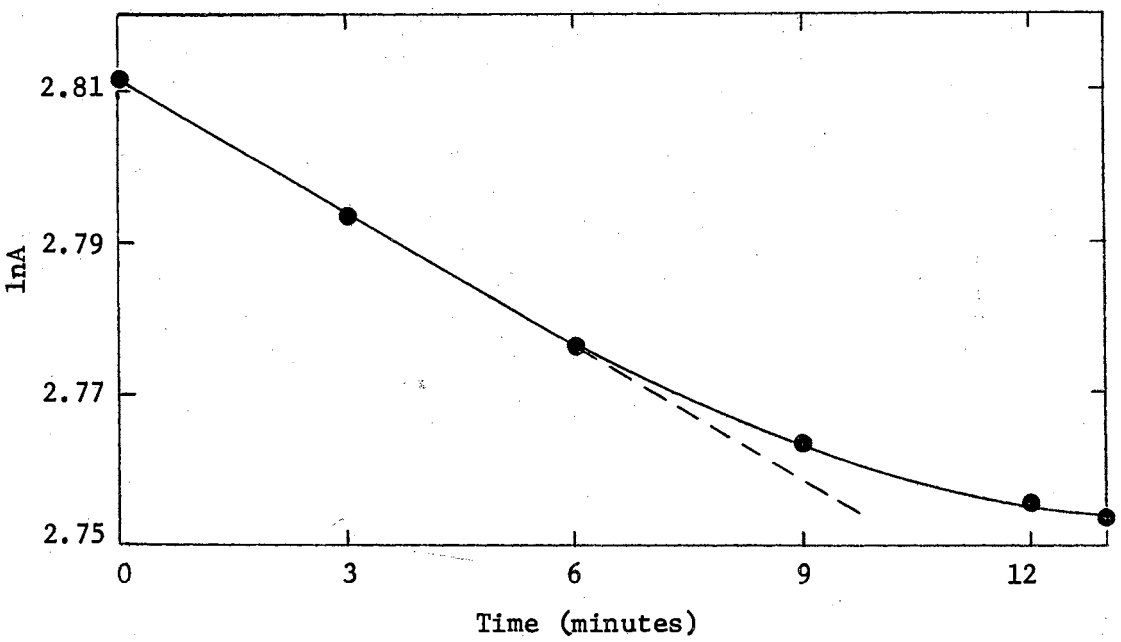


Figure 10. The "lnA vs t" Plot of the Assay of L-DBA with L-Amino Acid Oxidase from Snake Venom.

A is remaining concentration of L-DBA at time t. The rate constant k' is calculated from slope. 0.0147 mg/ml enzyme was used for assay.

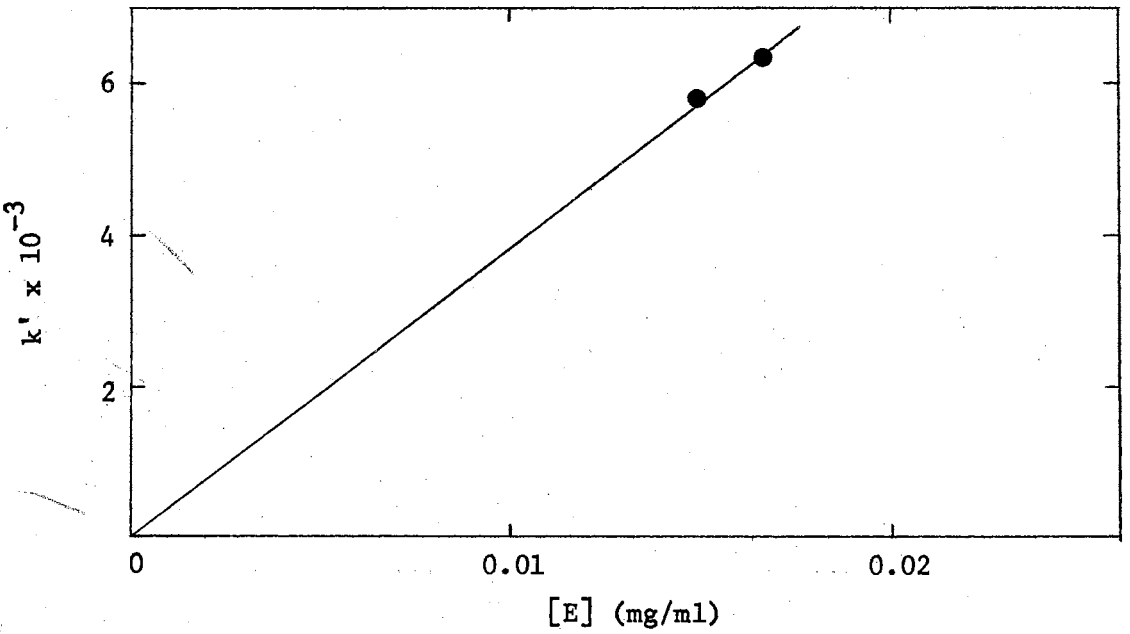


Figure 11. The "k' vs [E]" Plot of the Assay of L-DBA with L-Amino Acid Oxidase from Snake Venom.

buffer (pH from 7.5 to 9.0), 13.3 mM. The concentrations of substrate and enzyme used for assay were chosen in the same manner as those for the assay of L-amino acid oxidase (Figures 12 and 13). The protein concentration was determined spectrophotometrically by measuring the absorbance at 280 mμ. A_{280} x 0.625 = mg/ml (126).

Identification of the Products of the Enzymic Reaction

The mixture, as described previously, was incubated in a 37° water bath for one hr, with or without catalase, gassed with a small stream of oxygen or exposed to air. The reaction was terminated by adding an equal volume of 6 N HCl. The precipitate was removed by centrifugation. The reaction products were then identified by paper chromatography. Due to the difficulty in isolating small amounts of keto acid products, they were prepared as 2,4-dinitrophenylhydrazine derivatives and examined by infrared spectroscopy as described in Chapter II, or treated with H_2O_2 or sodium borohydride prior to identification by paper chromatography.

Results

Effect of pH on the Activity of Respective Amino Acid Oxidases with L- and D-DBA

With crystalline L-amino acid oxidase from Crotalus adamanteus venom and D-amino acid oxidase from hog kidney, the optimum pH obtained for L-DBA and D-DBA are 8.6-9.2 and 9.2-9.4, respectively (Figure 14). In the case of L-amino acid oxidase, the enzyme was inactivated after 10 minutes at a pH above 9.4.

The potency of enzymic activity of partially purified and dialyzed L-amino acid oxidase from rat kidney (12 μl O_2/hr/g wet tissue for

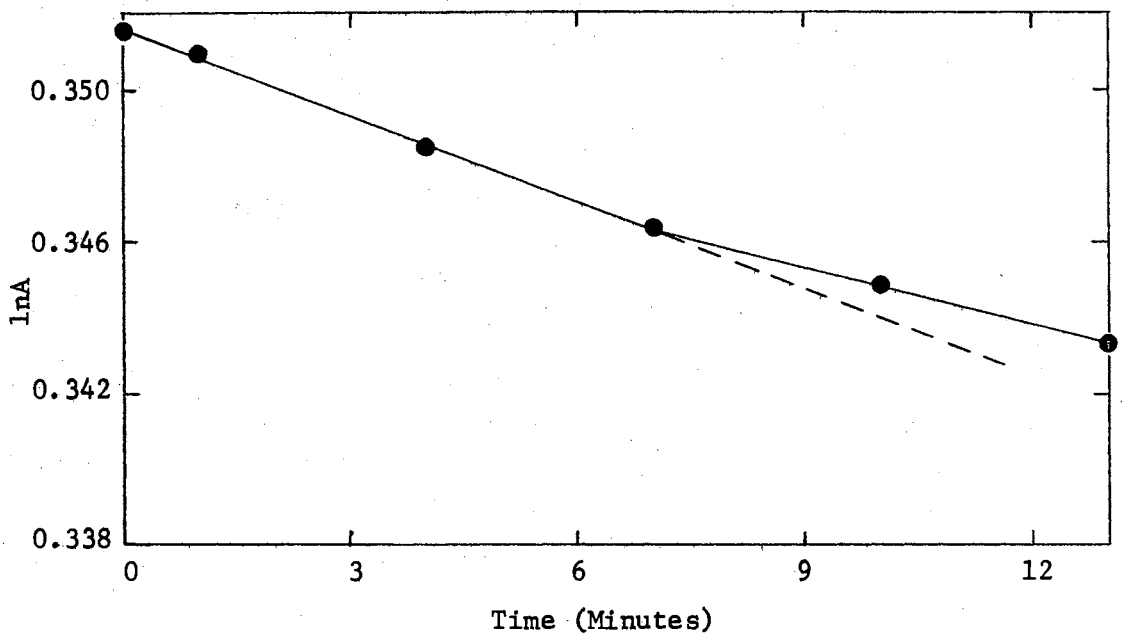


Figure 12. The "lnA vs t" Plot of the Assay of D-DBA with D-Amino Acid Oxidase from Hog Kidney.

A is remaining concentration of L-DBA at time t. The rate constant k' is calculated from slope. 0.0277 mg/ml enzyme was used for assay.

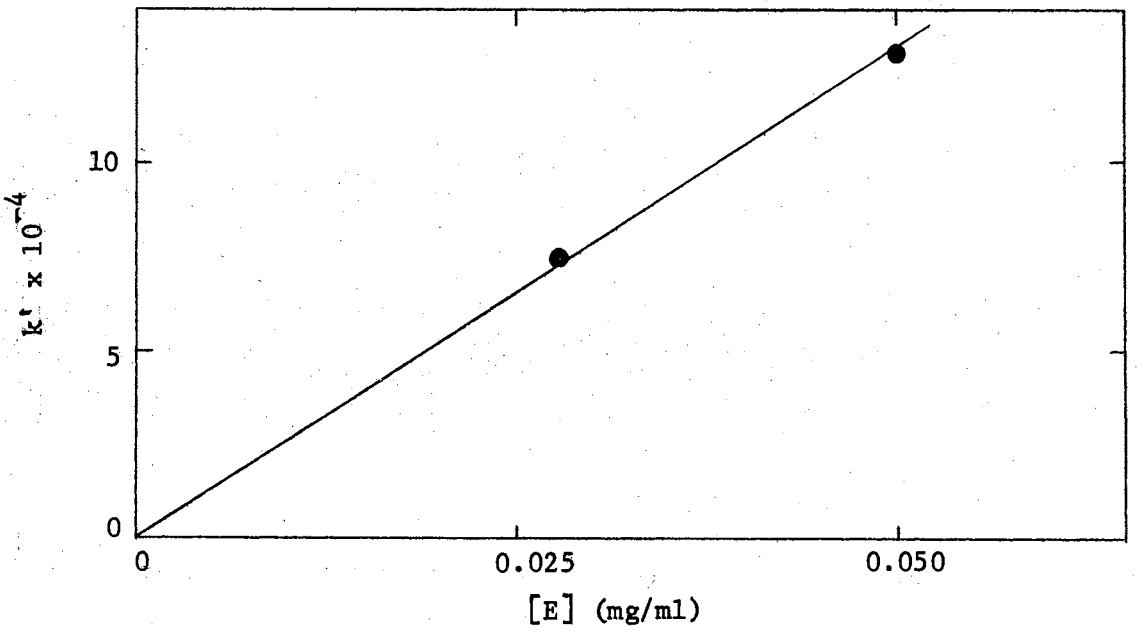


Figure 13. The "k' vs [E]" Plot of the Assay of D-DBA with D-Amino Acid Oxidase from Hog Kidney.

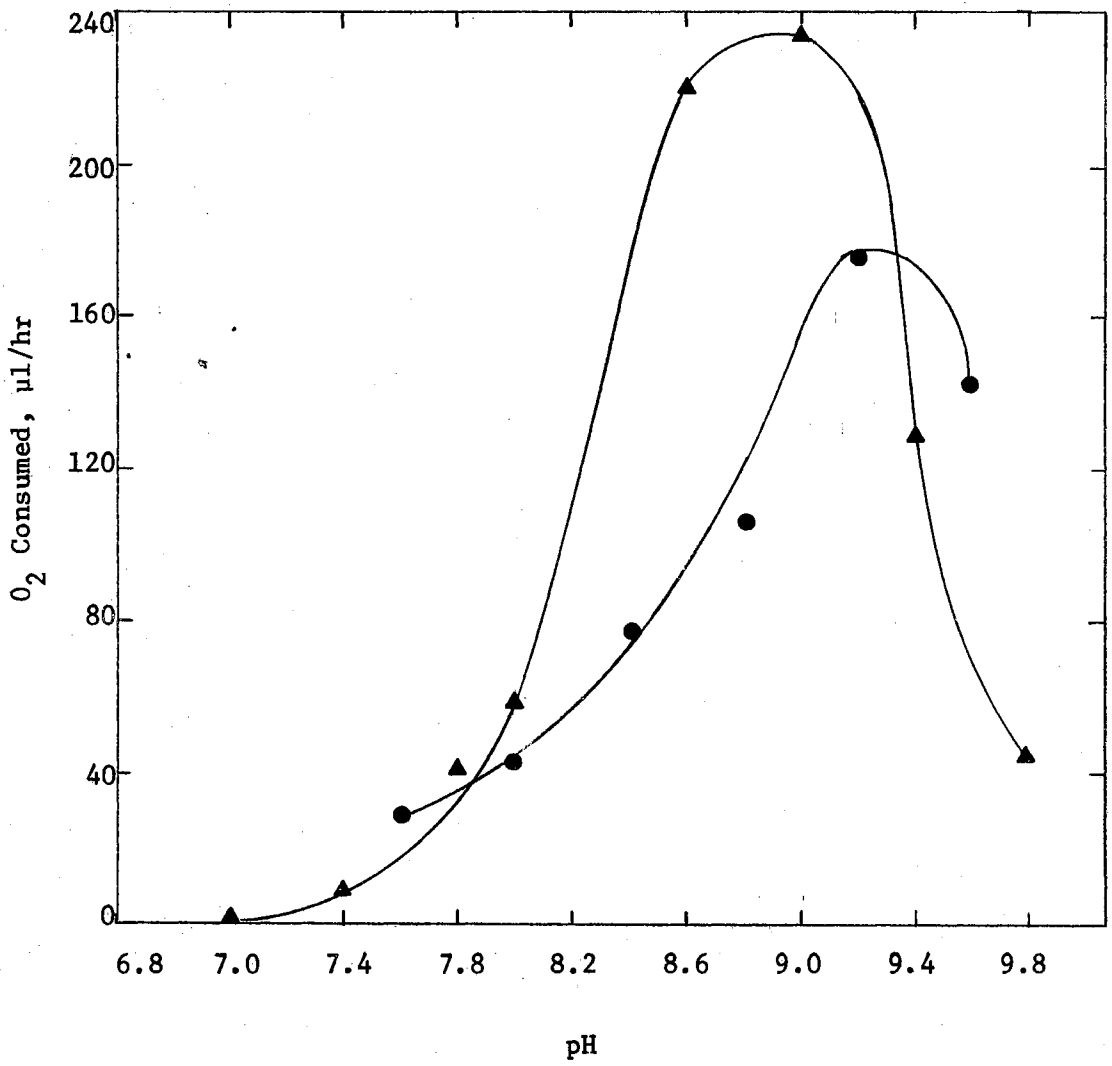


Figure 14. The pH-Curves of L- and D-DBA with Respective Amino Acid Oxidases.

The enzymic activity was determined with a polarographic oxygen electrode, YSI Model 53. ▲ indicates 50 µmoles of L-DBA with 0.015 mg of L-amino and oxidase from Crotalus adamanteus venom, in 0.2 M Tris and glycine buffer, and ● indicates 85.7 µmoles of D-DBA with 0.083 mg of D-amino acid oxidase from hog kidney, in 0.02 M pyrophosphate buffer.

L-leucine) towards L-DBA was too low to measure accurately. The same problem has been observed by Park and Kim (127). Although the optimum pH obtained was 7.3-7.8 for L-DBA with L-amino acid oxidase from rat kidney (about 1.3 g of kidney was used for each assay), the activity observed was only slightly greater than the controls, from which only L-DBA was omitted. The net activity (3 µl O_2/hr at pH 7.4) may be experimental error, since amino acid analysis showed the presence of amino acids in the dialyzed rat kidney enzyme preparation.

Kinetics of L- and D-Amino Acid Oxidases with L- and D-DBA

With crystalline enzyme, the double reciprocal plots (the Lineweaver-Burk plots) of the activities of amino acid oxidases as a function of DBA concentrations are shown in Figures 15 and 16. The Michealis constant of D-amino acid oxidase from hog kidney for D-DBA is 35.7 mM; the V_{max} is 2.75 x 10^2 µmoles/hr/mg protein at pH 9.2, 37° (Figure 15). The L-amino acid oxidase from Crotalus adamanteus venom has a same K_m (40 mM at pH 9.0, 37° for L-DBA) and a ten times higher V_{max} (2.26 x 10^3 µmoles/hr/mg protein, Figure 16). Thus, per mg of the enzymes studied, the L-isomer of DBA can be oxidized faster than D-isomer.

The 0.15 mg of D-amino acid oxidase used for each enzymic assay are equivalent to approximately 4.5 g of hog kidney (Massey et al. 126).

Effect of Thyroxine on the Oxidative Deamination of L- and D-DBA

The presence of 0.01-2.0 µg of L-thyroxine at pH 9.0 in the incubation media had no effect on the enzymic activities of both D-amino acid oxidase from hog kidney and L-amino acid oxidase from snake venom.

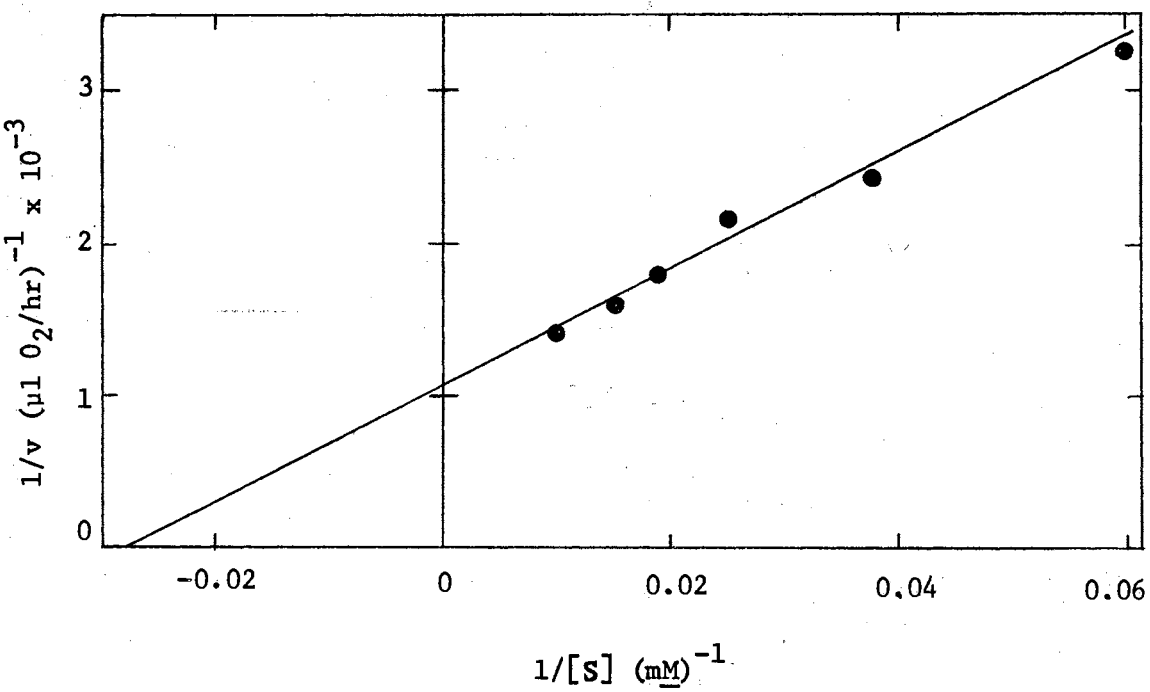


Figure 15. Double Reciprocal Plot of the Activity of D-Amino Acid Oxidase from Hog Kidney as a Function of D-DBA Concentration.

Rate was measured at pH 9.2, 37°, and 0.15 mg of enzyme.

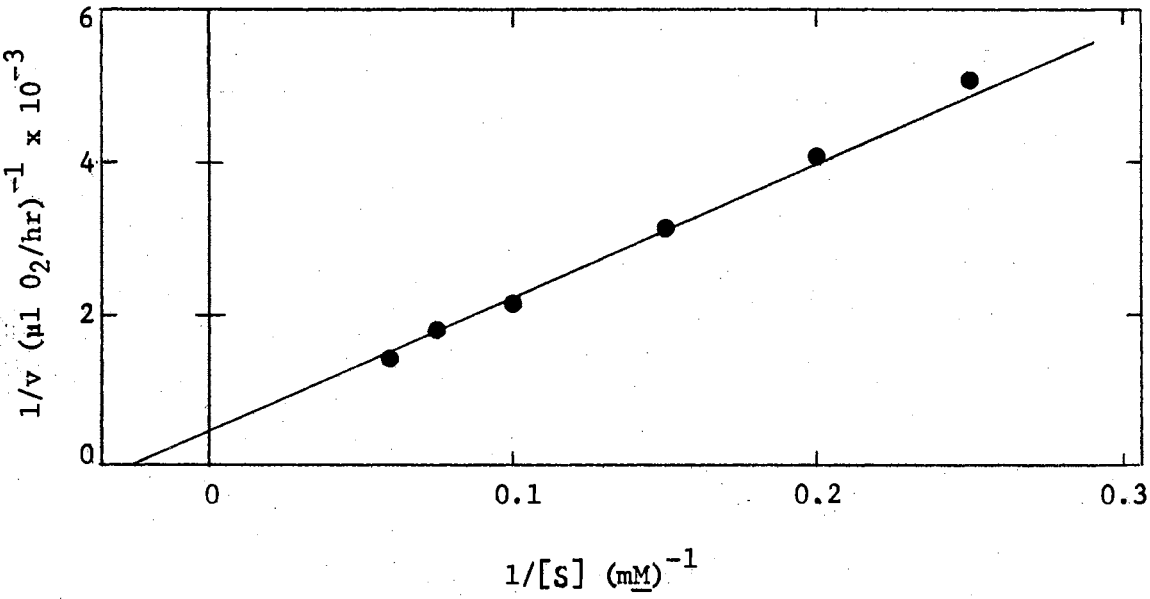


Figure 16. Double Reciprocal Plot of the Activity of L-Amino Acid Oxidase from Crotalus adamanteus Venom as a Function of L-DBA Concentration.

Rate was measured at pH 9.0, 37°, and 0.0442 mg enzyme.

The intraperitoneal administration of 200 μg L-thyroxine (pH 9.0) to rats daily for eight days before the preparation of L-amino acid oxidase of kidney, resulted in no observable effect on the oxidase activity toward L- and D-DBA.

The Oxidative Deamination Products of L- and D-DBA

Instead of the γ-amino group of both isomers of DBA, the α-amino group was oxidized to give the α-keto amino acid in reactions catalysed by either D- or L-amino acid oxidase. In the presence of catalase (2-3 μg), α-keto-γ-aminobutyric acid (KABA) was formed; β-alanine was formed instead of KABA when catalase was absent. This keto amino acid can be isolated as its 2,4-dinitrophenylhydrazone derivative. The infrared spectra of both the keto acid and its derivative are the same as those of synthetic compounds (Figures 3 and 4). KABA and β-alanine were identified by paper chromatography in several solvent systems (Table XII). KABA can be reduced by sodium borohydride to give α-hydroxy-γ-aminobutyric acid (HABA), which was also identified by paper chromatography (Table XII). The identification of KABA, HABA and β-alanine was enhanced by the availability of synthetic or commercial (β-alanine) authentic compounds. KABA gives a yellow color with ninhydrin, HABA, a purple color and β-alanine, a blue color.

The enzymic reactions of L-DBA with L-amino acid oxidase from snake venom, including the chemical reactions, are shown in Figure 17. The reactions of D-DBA with D-amino acid oxidase follow the same pattern.

Paper chromatography failed to identify the products from the reaction of L-DBA with rat kidney L-amino acid oxidase and the amino acid analyzer revealed that the hydrogen peroxide treated reaction mixture

TABLE XII

IDENTIFICATION OF KABA[a], HABA[b] AND β-ALANINE BY PAPER CHROMATOGRAPHY

Solvent	R_f					
	KABA	Yellow Spot	β-ala	Blue Spot	HABA	Purple Spot
EtOH:H_2O (76:24	.23	.23	.37	.36	.25	.25
tBuOH:HOAc:H_2O (4:1:2)	.18	.18	.39	.38		
MeOH:H_2O:Pyridine (20:5:1)	.36	.36	.36	.36	.35	.35
Pyridine:H_2O (65:35)	.30	.30	.28	.28	.20	.19

[a]KABA = α-Keto-γ-Aminobutyric Acid.

[b]HABA = α-Hydroxy-γ-Aminobutyric Acid.

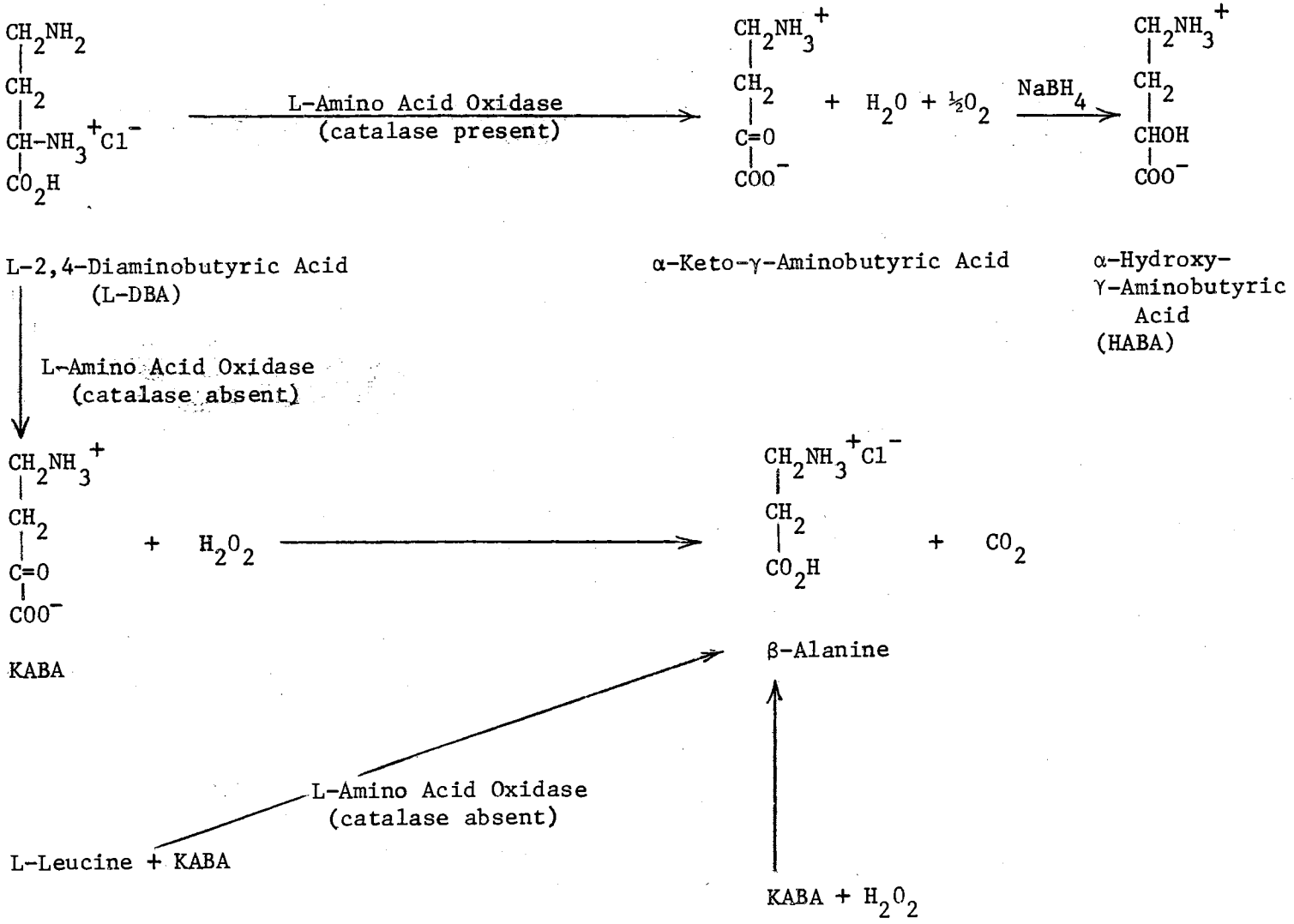


Figure 17. The Formation, Reduction and Decarboxylation of α-Keto-γ-Aminobutyric Acid (KABA)

did not contain detectable amounts (0.002 μmoles/ml) of β-alanine, glycine or aspartic acid. However, it cannot be concluded that kidney is not able to oxidize L-DBA. It may be that the enzymic activity is very low and the amount of enzyme used was not sufficient to give a detectable reaction.

Discussion

The L- and D-isomers of DBA can be rapidly oxidized by the respective crystalline L- and D-amino acid oxidases from snake venom and hog kidney. They are better substrates than L-leucine for L-amino acid oxidase and D-alanine for D-amino acid oxidase, respectively (Table XIII). However, L-amino acid oxidase from rat kidney showed a great preference for L-leucine compared to L-DBA. Although the L-amino acid oxidase prepared from rat kidney had activity of 12 μl O_2/hr/g wet tissue for L-leucine (9 μl O_2/hr/g wet tissue by Blanchard et al, 123), L-DBA was oxidized at a rate of 3 μl O_2/hr/g wet tissue, which may be experimental error. No oxidation product of L-DBA with rat kidney L-amino acid oxidase was detected either by paper chromatography or with the amino acid analyzer, while KABA was identified as the oxidation product of both isomers of DBA with the crystalline enzymes.

L-amino acid oxidase, where it occurs in a mammalian tissue, is very weakly active (128); rat kidney and liver being the only satisfactory sources of this enzyme (59). On the other hand, it is present to a surprisingly high level in the venoms of a number of species of snakes (129). Therefore, the failure to observe L-DBA oxidation with rat kidney oxidase was not surprising. Blanchard et al. (123) reported that there was only 1 unit (μmoles/min) per 100 g tissue in the crude extract

TABLE XIII

COMPARISON OF ENZYMIC OXIDATION RATES BETWEEN L- AND D-ISOMERS WITH RESPECTIVE AMINO ACID OXIDASES

Substrate	Substrate Concentration	pH	Enzyme Source	Velocity	References
	mM			μmoles/hr/mg protein	
L-leucine[a]	4.2	7.2	Snake venom[b]	36	Greenstein et al. (128)
L-DBA	4	9.0	Snake venom	196	Our Results
L-leucine	16.6	7.8	Snake venom	84	Park and Kim (127)
L-DBA	16.6	9.0	Snake venom	698	Our Results
D-alanine[a]	4.2	8.2	Hog kidney	1.7	Greenstein et al. (128)
D-DBA	16.6	9.2	Hog kidney	13.7	Our Results

[a]The rate is an approximate value which is calculated (divided by 6.25) from μmoles/hr/mg N

[b]Crotalus adamanteus

and 0.01 units per 100 g tissue in three ammonium sulfate fractionations, and the yield in the preparation was only 1.4%. The oxidation of L-DBA is difficult to examine unless an appreciable amount of purified enzyme is available.

Although the content of D-amino acid oxidase in rat kidney and liver was not known, hog kidney has 4.8 units per g tissue in the homogenate, and from 30 g of kidney Massey et al. (126) obtained 1 mg of crystalline enzyme with a specific activity of 12.5 units/mg protein. The study with this enzyme (s.a. 2.15, from Worthington Co.) and L-amino acid oxidase from Crotalus adamanteus venom (s.a. 2.3, from Worthington Co.) herein reported, showed that V_{max} for L-DBA was 10 times higher than that for D-DBA, with approximately the same K_m values. The observation that KABA, the oxidation product of both L- and D-DBA, was decarboxylated to give β-alanine when catalase was absent or inadequate is in agreement with the findings of Koeppe and Mushahwar (41).

CHAPTER V

TRANSAMINATION

Experimental

Animals

As described in Chapter III, the fed rats were used for experiments.

Tissue Preparations

The method of Rowsell (130) was used for the preparation of tissue homogenates. After decapitation, rat liver and kidney were removed and chilled in ice. The tissues were passed through a Harvard press and homogenized in an ice-cold Potter-Elvenhjem homogenizer with cold 0.1 M phosphate buffer, pH 7.4, and filtered through surgical cotton gauze. Homogenates were diluted with buffer to eight times the volume of the tissue and centrifuged at 10,000 xg for 10 minutes. The 10,000 xg pellet was resuspended in buffer such that 5 ml of the solution was equivalent to 1 g of tissue. This was called the mitochondrial fraction. Nine volumes of the mitochondrial fraction and a portion of the original homogenate were treated with one volume of 2% Triton X-100. All fractions, (homogenate, mitochondria and 10,000 xg supernatant), with or without Triton X-100,were dialyzed against buffer at room temperature for 20 hrs, with several changes of buffer. The dialyzed crude transaminase preparations were used for assay.

Spectrophotometric Assay

This method was used only with a transamination system in which one of the substrates was oxalacetate (131). The decrease in concentration of oxalacetate was measured with a Cary 14 spectrophotometer by following the change in absorbance at 280 mμ at 25°. In principle the method requires that the other components do not have an interfering absorption at the wavelength used for a given component. Oxalacetate has a very high millimolar extinction coefficient at 280 mμ, 0.53 in 0.05 M phosphate buffer, pH 7.4 (131) and 0.47 in 0.1 M phosphate buffer, pH 7.4. All other possible components have comparatively low extinction coefficients, among them, KABA is the highest at 0.040.

The enzymic assay mixture consisted of NaH_2PO_4-K_2HPO_4 buffer, 87.5 mM (0.1 M, pH 7.4); amino acid, 20 mM; oxalacetate, 2 mM; pyridoxal-5-phosphate, 10 μg; the enzyme preparation, 25λ; and water to make a total volume of 1 ml. A reference cuvette consisted of the same reagents excluding oxalacetate. The nonenzymic assay mixture was varied by the desired experimental conditions, in which the buffer was changed or omitted, and in some cases metal ions were added. This rate was measured by following the changes in the absorbance at 260 mμ. Oxalacetate has a shoulder near 260 mμ in the spectrum. The assay is much more sensitive at 260 mμ than at 280 mμ. However, the millimolar extinction coefficient (0.962 in aqueous solution, pH 7.0) at 260 mμ must be carefully checked, occasionally, during a series of assays.

Qualitative Filter Paper Chromatography

The final products of transamination are of interest even without

quantitative considerations. The method of Rowsell (130) was applied with modification. To a 15 ml Corex centrifuge tube were added 40 µmoles of sodium pyruvate, α-ketoglutarate, or oxalacetate, 40 µmoles of amino acid, and 20 µg of pyridoxal-5-phosphate (total volume, 1 ml). After the preincubation in a 37° water bath for 5 minutes, one milliter of dialyzed crude enzyme was added and the tube was immediately gassed with N_2 and sealed with a rubber stopper. The contents of the tubes were mixed and incubated at 37°. At timed intervals, 0.1 ml of the contents were removed and added immediately to 0.1 ml of 2 N HCl to denature the protein, which was removed by centrifugation. The reaction tube was regassed with N_2 and sealed for further incubation. A 10 to 25 µl aliquot of each sample was spotted on phosphate buffered filter paper (132) and developed in several solvent systems by the ascending technique. Authentic amino acids were included on each chromatogram. After development, the papers were dried in a hood at room temperature. Then they were sprayed with 0.2% ninhydrin in 95% ethanol, dried and heated at 110° for 30 seconds.

The nonenzymic reactions were performed in similar fashion, the cofactor, enzyme and buffer being omitted. At the termination of incubation, the mixture was brought to pH 2.2 with 6 N HCl, refrigerated, and assayed by paper chromatography and with an amino acid analyzer.

Sodium Borohydride Reduction and Amino Acid Derivatives

The reaction mixtures mentioned above were also treated with excess sodium borohydride at pH 7.0 while stirring at room temperature for 4 hrs. A portion of the reduced products were acidified to pH 2.2 and analyzed with an amino acid analyzer. Their N-acetyl ethyl esters or

N-trifluoroacetyl n-butyl esters, whose preparations are described in Chapter II, were analyzed with GLC on OV-1 or SE-30 column, using a temperature program of 2^{o}/min from 100^{o} to 250^{o}. The modified Barber Coleman Model 5000 with a hydrogen flame detector was used. Some of the GLC peaks were assayed in the mass spectrometer.

Results

Nonenzymic Transamination Between L-DBA and Oxalacetate

There should exist other nonenzymic transaminations with L-DBA besides that with oxalacetate. However, oxalacetate was the only keto acid acceptor which was easily assayed spectrophotometrically.

The evidence for nonenzymic transamination between L-DBA and oxalacetate is shown in Figure 18. The reaction was not affected by the presence of crude transaminases. The reaction rate was not significantly affected by pyridoxal-5-phosphate, but did increase when phosphate buffer was absent (Figure 18).

Some Possible Nonenzymic Transaminations

For meaningful measurements of enzymic transamination rate, the possible nonenzymic transaminations involved in an enzymic assay must be considered. Figure 19 shows these possible transaminations. No nonenzymic transaminations were detected between oxalacetate and glutamate or α-alanine, or between α-ketoglutarate and aspartate. Thus, they should not interfere with the enzymic assay.

There were nonenzymic transaminations between L-DBA and oxalacetate or pyruvate, and between D-DBA and oxalacetate (Figure 19). The millimolar extinction coefficients of α-ketoglutarate and pyruvate are 0.0213

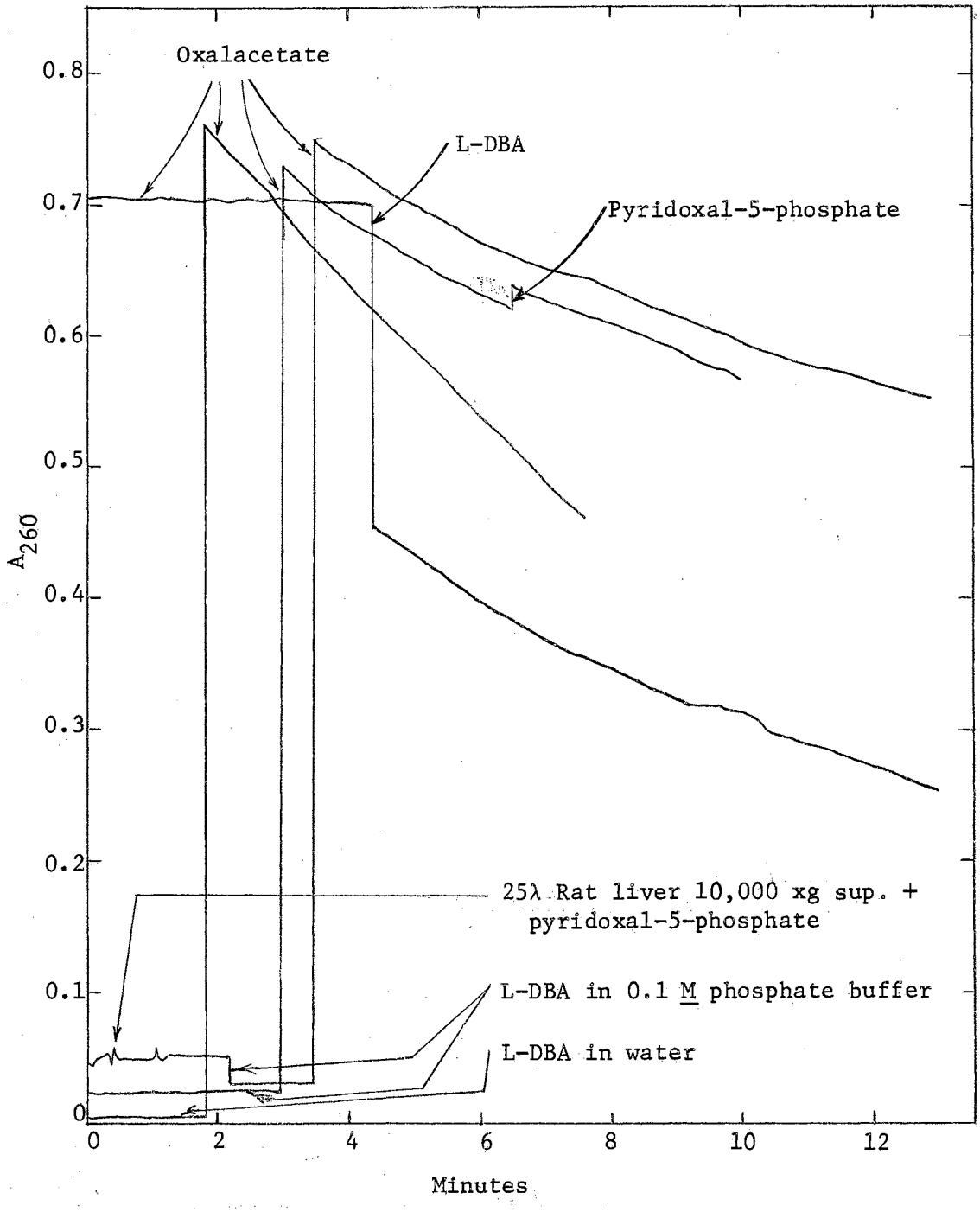


Figure 18. Nonenzymic Transamination Between L-DBA and Oxalacetate.

The incubation media consisted of L-DBA, 20 mM; oxalacetate, 0.8 mM; and pyridoxal-5-phosphate, 20 µg/ml. Assayed at pH 7.4 and 23°, and followed with the Cary 14 at 260 mµ.

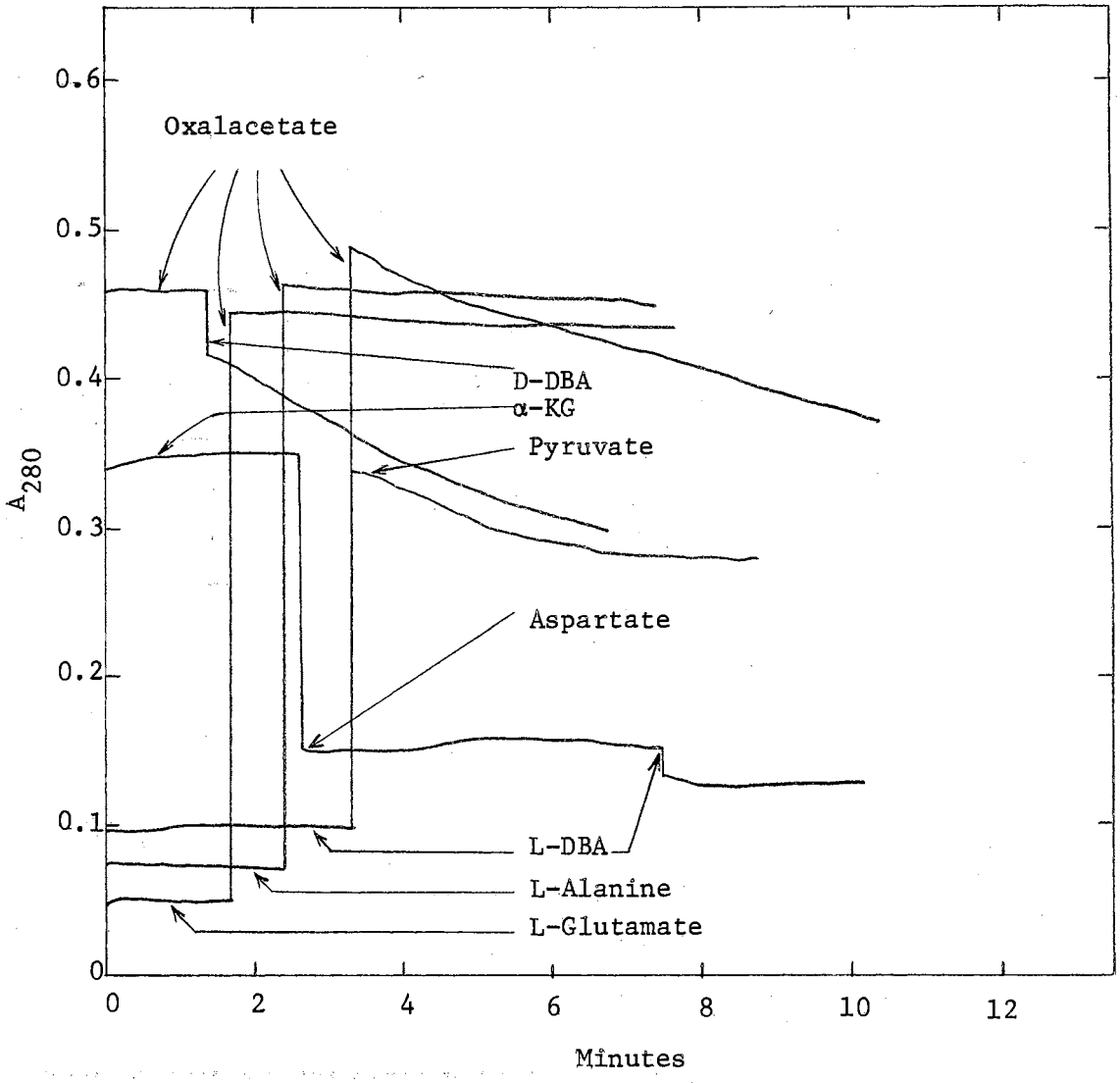


Figure 19. Some Possible Nonenzymic Transaminations Involved in Enzymic Assays.

The incubation media consisted of amino acids, 20 m$\underline{M}$; keto acids (except oxalacetate, 1.6 m$\underline{M}$), 20 m$\underline{M}$; and 0.1 $\underline{M}$ phosphate buffer, pH 7.4, to make up a volume of 1 ml. Assayed at 23°, and followed with the Cary 14 at 280 mμ.

and 0.0215, respectively, in phosphate buffer, pH 7.4, at 280 mμ. Therefore, a small rate of nonenzymic transamination between L-DBA and pyruvate or α-ketoglutarate would not be detected under our conditions of assay.

The Optimum pH of Nonenzymic Transamination

The nonenzymic transamination was pH-dependent, at least in the case of L-DBA and oxalacetate. The optimum pH for L-DBA and oxalacetate is 6.0-8.0 (Figure 20). The reaction rates were expressed in terms of μmoles/min by the equation:

$$\frac{-d[OAA]}{dt} = \left(\frac{-dD}{dt}\right)\left(\frac{1}{k_{OAA} + k_{DBA} - k_{prod}}\right)$$

as derived by Cohen (131), where -dD/dt is the change in absorbance, and k is the millimolar extinction coefficient, in which it is assumed that:

$$k_{OAA} + k_{DBA} - k_{prod} \approx k_{OAA} + k_{DBA}$$

where $k_{OAA} = 0.47$ and $k_{DBA} = 0.0013$ as measured at 280 mμ.

Effect of Buffers on the Nonenzymic Transamination Between L-DBA and Oxalacetate

Table XIV shows the effect of phosphate buffer, pH 7.4, and EDTA buffer, pH 7.4, on the nonenzymic transamination between L-DBA and oxalacetate. The reaction was strongly inhibited by phosphate buffer. An amount of 100 mM phosphate buffer, pH 7.4, gave an inhibition of up to 72%. Surprisingly, EDTA had no effect on the reaction. Pyridoxal-5-phosphate (20 μg/ml) had a slight inhibitory effect (Table XIV).

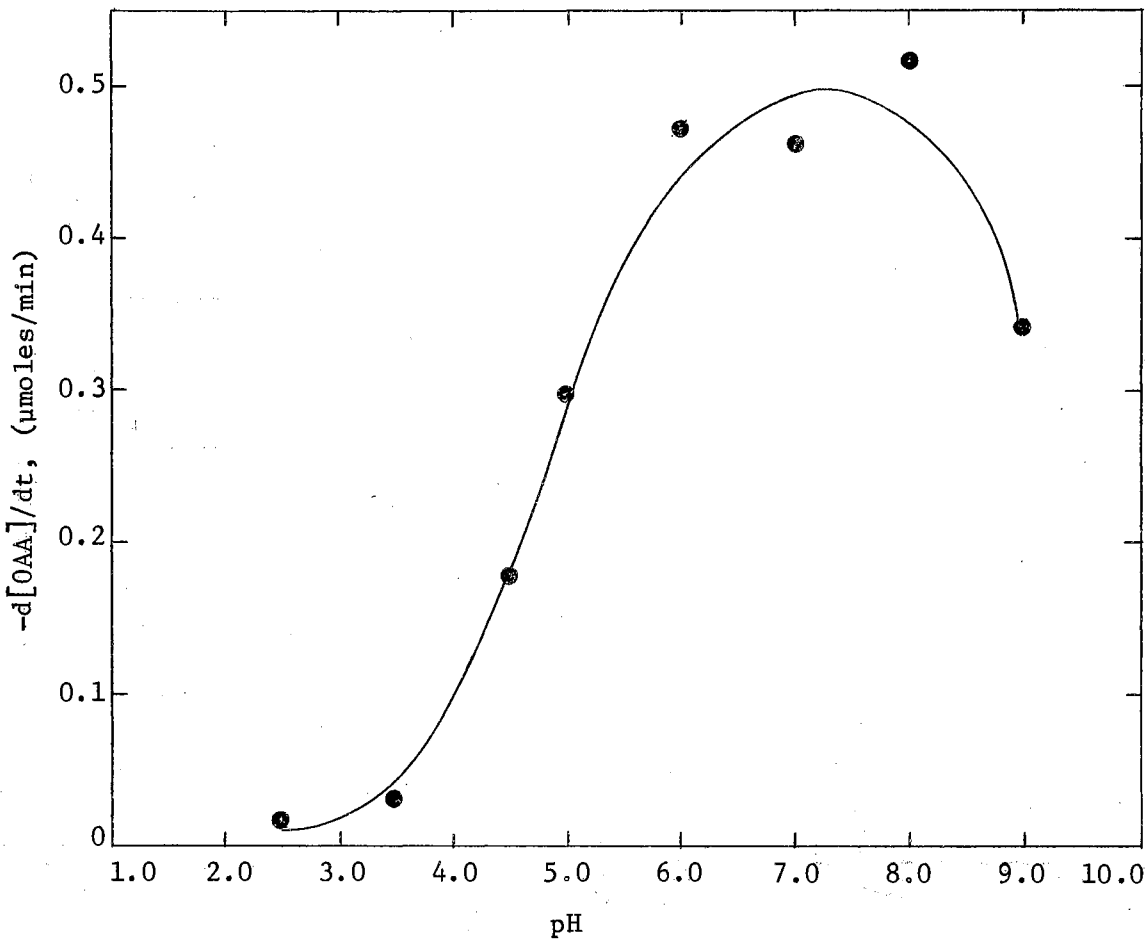


Figure 20. Optimum pH of Nonenzymic Transamination Between L-DBA and Oxalacetate in Aqueous Solution.

TABLE XIV

EFFECT OF BUFFERS ON THE NONENZYMIC TRANSAMINATION OF L-DBA WITH OXALACETATE

Phosphate Buffer pH 7.4	EDTA Buffer pH 7.4	[a] Rate $(\frac{-dD}{dt})$	Per Cent of Rate
mM	mM	A_{280}/min	
-	-	.071	100
-	2.0	.074	104
-	5.0	.073	103
-	10.0	.073	103
2.5	-	.049	69
2.5	10.0	.038	54
27.5	72.5	.022	31
40.0	60.0	.022	31
80.0	20.0	.020	28
100.0	-	.020	28
90.0 (20 µg pyridoxal)	-	.019	27

[a] Measured at 280 mµ. Media consisted of L-DBA, 20 mM and oxalacetate, 2 mM, pH 7.4.

Effect of Metals on the Nonenzymic Transamination Between L-DBA and Oxalacetate

Within a range of 7.5-300 μM of $CuSO_4$, of 4-150 μM of $BaCl_2$, and of 2-10 μM of $FeSO_4$ examined, there was no significant effect on the rate of transamination between L-DBA and oxalacetate with or without 10 mM of EDTA, pH 7.0, whereas 36 μM of $FeSO_4$ gave approximately 20% inhibition. No assay could be made with EDTA in the presence of $FeSO_4$, because the addition of EDTA caused a slow decrease in absorbance.

The Nonenzymic Transamination Between L-DBA and Oxalacetate in the Presence of Crude Transaminases, Coenzyme and Glutamate

Pyridoxal-5-phosphate had a small depressive effect (about 10%) on the rate of nonenzymic transamination between L-DBA and oxalacetate. The addition of crude transaminases from the liver 10,000 xg pellet treated with 0.2% Triton X-100 or liver 10,000 xg supernatant had no effect.

The reaction rate of enzymic transamination between glutamate and oxalacetate with the crude transaminases was slightly lower in the presence of L-DBA (Table XV). The depression was up to 30% with a liver 10,000 xg supernatant or kidney 10,000 xg pellet treated with 0.2% Triton X-100. This could be due to L-DBA competing with glutamate for either the enzyme or oxalacetate, probably the latter.

Enzymic Transamination of L-DBA by Homogenates of Rat Liver and Kidney

Paper chromatography revealed that the reactions with the crude enzymes from liver and kidney were essentially the same, except the liver homogenate worked better. The oxalacetate was the best keto acid accep-

TABLE XV

THE ENZYMIC TRANSAMINATION OF GLUTAMATE WITH OXALACETATE IN THE PRESENCE OF L-DBA

Tissue Fractionations	Glu + OAA $\xrightarrow{E}$ αKG + Asp (pyr-5-phosphate)		Glu + OAA $\xrightarrow[(1)]{E}$ αKG + Asp + ??? (pyr-5-phosphate) (2) (L-DBA present)				Per Cent* of Rate
	$\frac{-dD}{dt}$	$\frac{-d[OAA]}{dt}$	$\frac{-dD}{dt})_1$	$\frac{-dD}{dt})_2$	$\frac{-dD}{dt})_2 - \frac{-dD}{dt})_1$	$\frac{-d[OAA]}{dt})_{net}$	
	A_{280}/min	μmoles/min/g		A_{280}/min		μmoles/min/g	
Liver Homogenate	0.14	110	0.03	0.16	0.13	103	94
Homogenate + 0.2% Triton x100	0.16	140	0.03	0.15	0.12	101	72
10,000 xg Supernatant	0.09	66	0.04	0.10	0.06	46	70
10,000 xg Pellet + 0.2% Triton x100	0.21	94	0.04	0.22	0.18	82	88
Kidney Homogenate	0.05	38	0.03	0.06	0.03	30	80
Homogenate + 0.2% Triton x100	0.14	124	0.03	0.13	0.10	88	71
10,000 xg Supernatant	0.04	32	0.03	0.07	0.04	28	87
10,000 xg Pellet + 0.2% Triton x100	0.11	49	0.03	0.11	0.08	34	70

*Based on the rate of L-DBA absent as 100%.

tor, visible results being seen by paper chromatography after 1 hr incubation, whereas with pyruvate or α-ketoglutarate the α-alanine or glutamic acid formed, respectively, were observed only after a 3 hr incubation period. In spite of the fact that the transamination was fastest between L-DBA and oxalacetate, α-alanine was formed instead of asparate. Paper chromatography showed that qualitatively the major products after 20 hrs incubation were the same as those after 1 hr incubation. The longer incubation gave much higher product concentrations. The mixture remained clear during the 20 hr incubation; no microbial growth was observed. At 20 hrs after incubation of L-DBA with oxalacetate or pyruvate in the presence of liver enzyme, a yellow spot developed (R_f = 0.22 in 76% ethanol), which gave β-alanine in the presence of hydrogen peroxide, and was identified as KABA. The analysis of this 20 hrs incubation solution in the amino acid analyzer revealed that there were more than 20 amino acids detected (Figure 21), among them α-alanine was the major component followed by glycine. Two compounds showed very high intensity at 440 mμ. Most of the compounds were acidic.

Product of Nonenzymic Transamination Between L-DBA and Oxalacetate

When L-DBA and oxalacetate were mixed together at pH 6.0, bubbles were formed immediately. Nitrogen gas slowed down the bubbling, but did not stop it. Apparently, there was a decarboxylation during transamination.

At 20 hrs after the incubation of L-DBA with oxalacetate in the presence of nitrogen gas, the pH of the solution was 7.2. The solution was decolorized during reduction with sodium borohydride. Paper chromatography failed to identify the amino acids in both solutions, with and

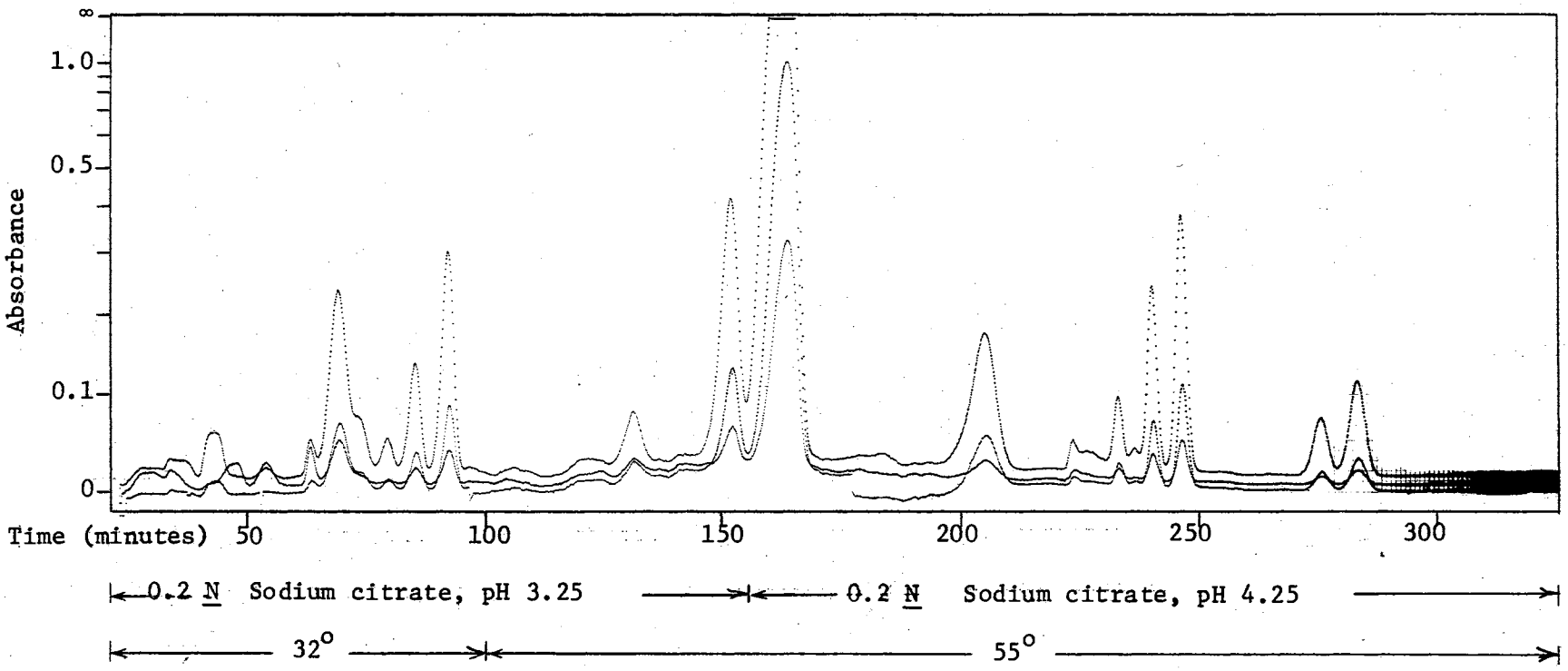


Figure 21. Amino Acid Analyses of the Transamination Products of L-DBA and Oxalacetate in the Presence of Crude Rat Liver Transaminases.

without reduction. Too many ninhydrin-positive components were present. Assay of the reaction mixture (without $NaBH_4$) in the amino acid analyzer revealed that at least 7 amino acids were formed during the nonenzymic transamination (Figure 22). Five of them were very acidic, having retention times between 20 and 60 minutes; the other two appeared at 122 and 134 minutes, respectively. Apparently, none of these seven compounds was one of the common amino acids.

Analysis of the reduced solution showed 6 major peaks along with several very small peaks. The major peaks had retention times between aspartic acid and glutamine (Figure 23).

GLC of N-acetyl ethyl esters and N-trifluoroacetyl n-butyl esters of the nonenzymic transamination mixture reduced with sodium borohydride are shown in Figure 24. With temperature programming from 100^{o} to 240^{o}, the N-acetyl ethyl esters gave 5 major peaks on OV-1 (8 ft, 1% column, whereas N-trifluoroacetyl n-buty esters gave 4 major peaks on SE-30 (10 ft, 1%) column. These showed the presence of many products produced by simply mixing L-DBA with oxalacetate at pH 6.0. Unfortunately, scanning in the mass spectrometer showed that none of the peaks was a pure amino acid derivative, and almost every peak consisted of at least 2 compounds. Identification via thin-layer chromatography is unsuccessful to date.

Discussion

Although the identification of the products of nonenzymic transamination of L-DBA with oxalacetate was unsuccessful, evidence for the reaction are the spectrophotometric assay (Figures 18 and 19), the amino acid analyses (Figures 22 and 23) and the GLC of the N-acetyl ethyl esters and N-trifluoroacetyl n-butyl esters of the reaction mixture

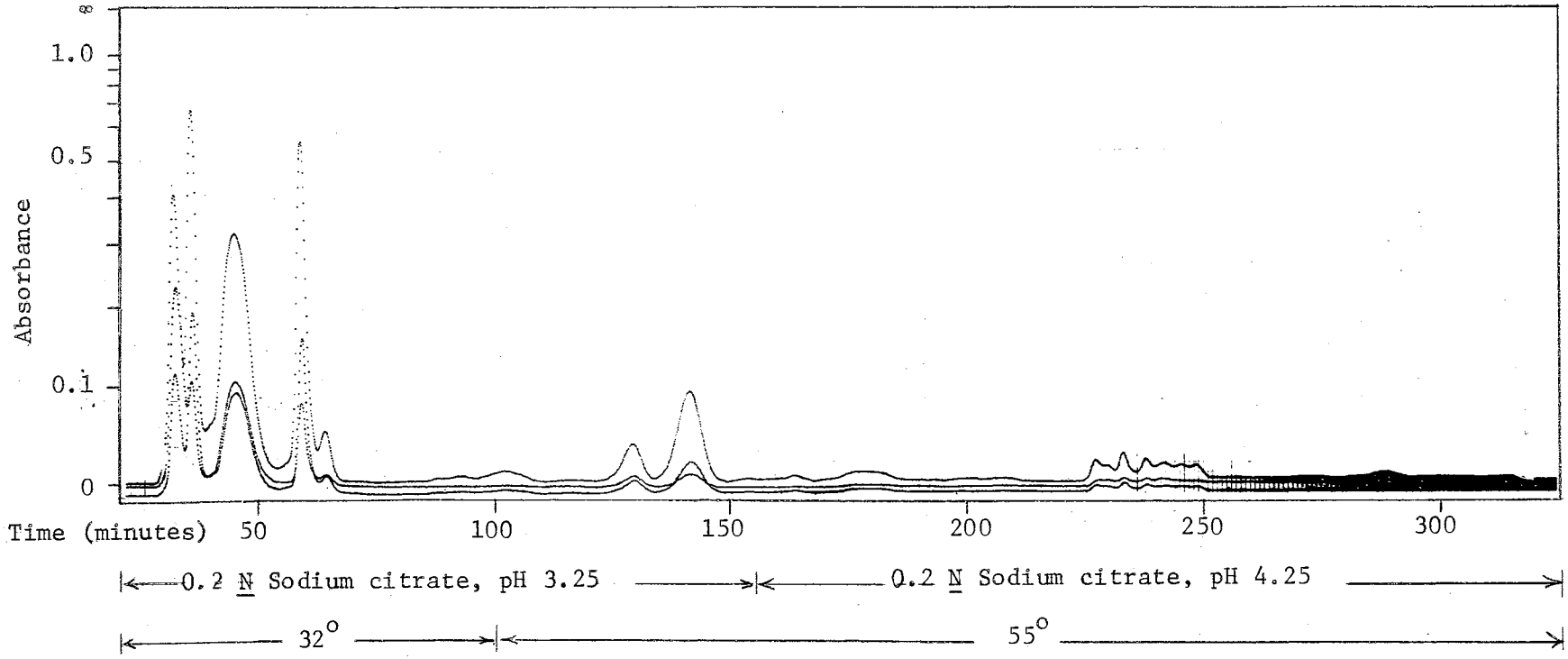


Figure 22. Amino Acid Analyses of Nonenzymic Transamination Products of L-DBA with Oxalacetate (No $NaBH_4$ Added).

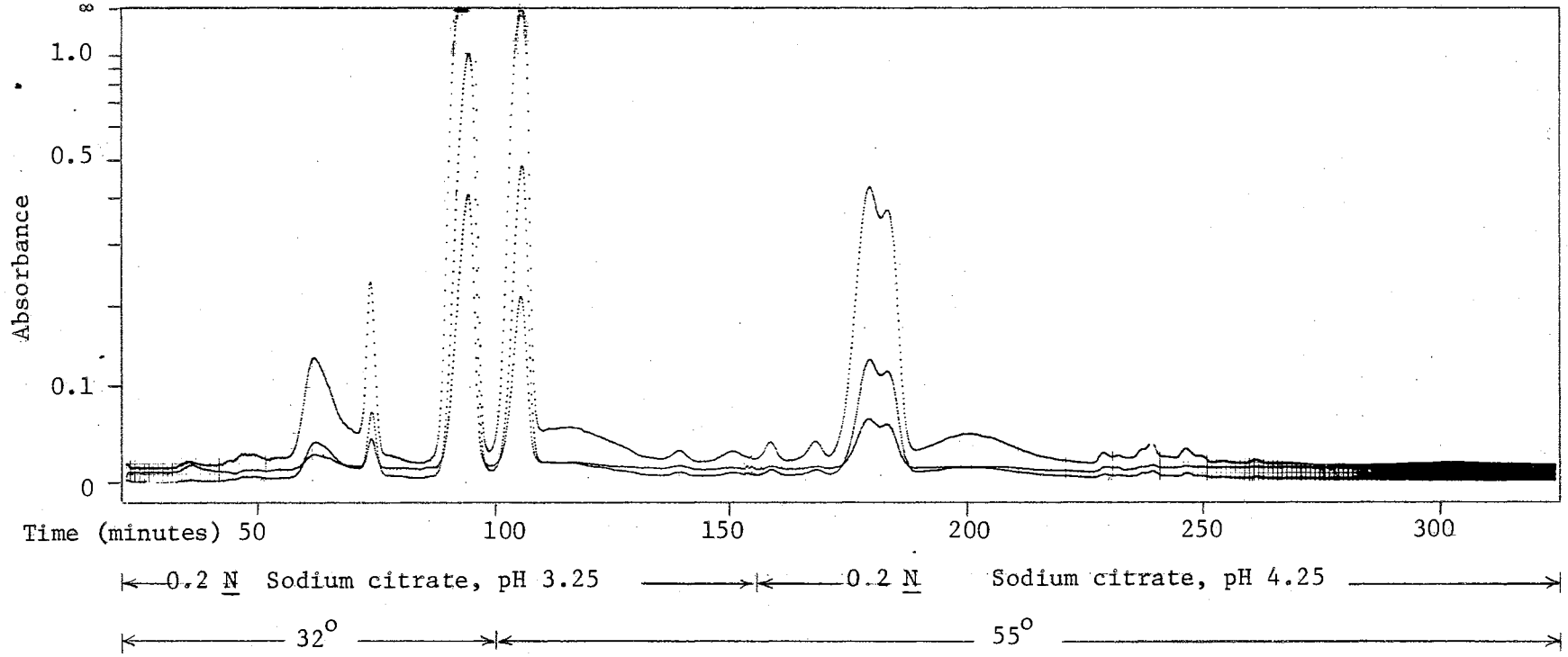


Figure 23. Amino Acid Analyses of Nonenzymic Transamination Products of L-DBA with Oxalacetate ($NaBH_4$ Added).

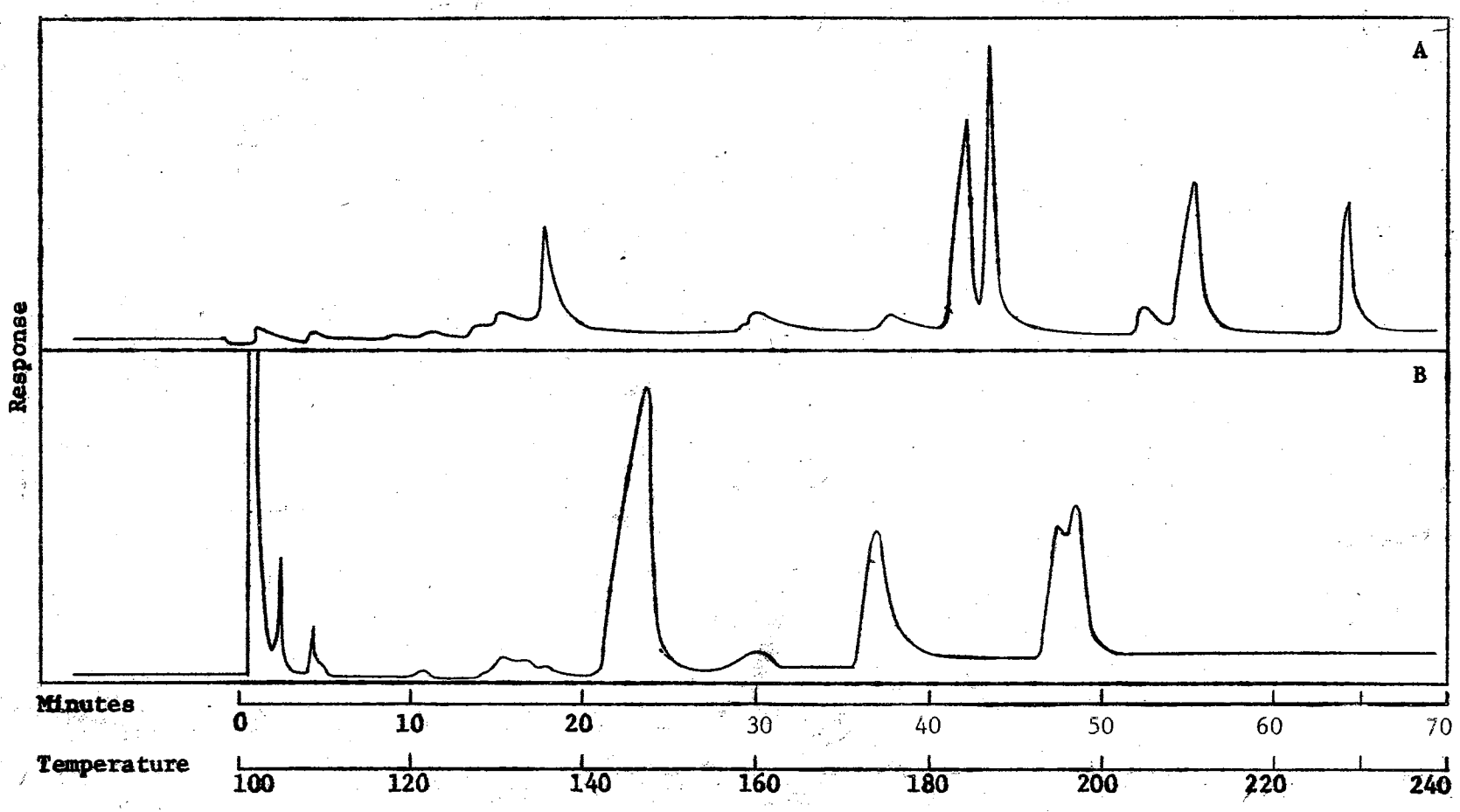


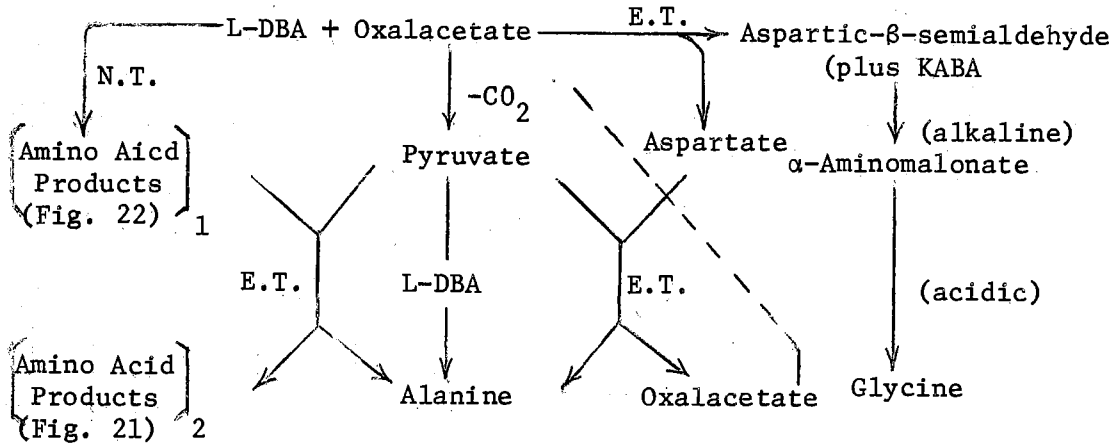
Figure 24. Gas-Liquid Chromatography of Amino Acid Derivatives of Nonenzymic Transamination Products.

A. N-acetyl ethyl esters (OV-1, 1% 8 ft. column) (solid injection).
B. N-trifluoroacetyl n-butyl esters (SE-30, 1% 10 ft. column).

(Figure 24). This nonenzymic transamination is not a simple reaction, because it gives at least 7 major ninhydrin-positive compounds. Most of them are very acidic.

Copper and Barium ions had no catalytic effect on the nonenzymic transamination while 36 μM of $FeSO_4$ exerted an inhibition. Metal ions will chelate to both amino groups of DBA (29). The lack metal ion catalyses at low concentrations of copper and barium ions, indicate that the metal chelates are not more active in nonenzymic transaminations here reported. The cause of the inhibition by $FeSO_4$ at 36 μM is not known to date.

The amino acid analyses revealed that most of the peaks observed after 20 hrs nonenzymic transamination of L-DBA with oxalacetate (no $NaBH_4$ added) were different from those observed when crude liver or kidney transaminases were present (Figures 21 and 22). The rate of enzymic transamination was very slow. It was surprising to find alanine as the major product (glycine next) instead of aspartic acid, with oxalacetate as the keto acid acceptor (Figure 21). The following is a plausible explanation for the finding of alanine as a major product,



where E.T. is enzymic transamination, and N.T. nonenzymic transamination. The formation of glycine indicated aspartic-β-semialdehyde as one of the products of enzymic transamination of L-DBA with oxalacetate, because aspartic-β-semialdehyde is very unstable at neutral or alkaline pH. It will enolize and be oxidized to give α-aminomalonate, which is stable in alkaline solution but forms glycine by decarboxlyation in acid.

The amino acid analyses also revealed two ninhydrin-positive compounds with high, and approximately equal, intensity at 440 mμ (Figure 21). The finding in the enzymic transamination mixture of KABA, by paper chromatography, and glycine, by amino acid analyses, suggested that the two 440 mμ peaks are KABA and aspartic-β-semialdehyde. KABA is rather stable under incubation conditions followed by 1 N HCl (storage). However, aspartic-β-semialdehyde forms glycine during similar treatment. Therefore, the sum of glycine plus aspartic-β-semialdehyde is a measure of gamma transamination while KABA formation represents alpha transamination. The results presented in Figure 21 indicate that most of the transamination of L-DBA with oxalacetate was gamma.

From the observations and discussions above concerning the transamination of L-DBA with oxalacetate the following is concluded: a) the nonenzymic transamination was superior to the enzymic reaction, b) the major product of enzymic transamination of L-DBA with oxalacetate is aspartic-β-semialdehyde while KABA is the exclusive product in the oxidative deamination by amino acid oxidases, and c) the main enzymic reaction path to give L-alanine is not known to date.

Although the pattern of increment in brain alanine and glycine concentrations of intraperitoneal L-DBA-treated rats (Table II) is similar

to that observed in the enzymic transamination of DBA with oxalacetate (Figure 21), it is not known whether a combination of nonenzymic and enzymic transamination of DBA accounts for these increments (in brain).

The finding of aspartic-β-semialdehyde as an enzymic transamination product of L-DBA agrees with the suggestion (41) that some L-DBA-2-^{14}C is converted to aspartate-2-^{14}C or to some closely related compound.

CHAPTER VI

SUMMARY

KABA has been synthesized by condensing diethyl oxalate with β-alanine ethyl ester in the presence of $EtO^- Na^+$ followed by refluxing with conc. HCl for 2 hrs. KABA was characterized by NMR and IR spectra.

KABA is the oxidation product with L- and D-DBA incubated snake venom L-amino acid oxidase and hog kidney D-amino acid oxidase, respectively, in the presence of catalase. When catalase was absent, the product was β-alanine. The study with crystalline hog kidney D-amino acid oxidase and Crotalus adamanteus venom L-amino acid oxidase herein reported, showed that V_{max} for L-DBA was 10 times higher than that for D-DBA, with approximately the same K_m values.

The study of rat kidney L-amino acid oxidase is unsuccessful to date. Unfortunately the enzymic activity is very low, if existent, and the amount of enzyme used was not sufficient to give a detectable reaction.

The enzymic transamination of L-DBA was very slow. Alanine was found as the major product (glycine next) instead of aspartic acid, with oxalacetate as the keto acid (acceptor). A plausible reaction scheme is presented to explain the finding of alanine as a major product. The sum of glycine plus aspartic-β-semialdehyde is a measure of gamma transamination of L-DBA. Most of the transamination of L-DBA with oxalacetate

was gamma.

DBA can transaminate with oxalacetate nonenzymically with an optimum pH between 6.0 and 8.0. With a reaction mixture consisting of 20 mM of L-DBA and 2 mM of oxalacetate, an amount of 100 mM of phosphate buffer, pH 7.4, gave an inhibition of up to 72%. Metals had no effect on the reaction, except 36 μM of $FeSO_4$ gave approximately 20% inhibition. At least 7 amino acids were formed during the nonenzymic transamination. Apparently, none of those 7 compounds was one of the common amino acids. The identification of nonenzymic transamination products is unsuccessful to date.

L-DBA was found to inhibit ornithine transcarbamylase of rat liver noncompetitively while D-DBA had no effect. The intraperitoneal administration of toxic doses of L-DBA to rats resulted in hyperirritability, tremors and convulsions in 12-20 hrs and increased the concentration of ammonia of blood and brain slightly, and the concentrations of brain glutamine and GABA 2-3 fold. Intraperitoneal doses of D-DBA, 3 times as high, had no toxic effects. Intracerebral treatment with small doses (6-7 mg) of D- and L-DBA resulted in the same toxic symptoms as those observed after intraperitoneal treatment with L-DBA. No severe modification in brain free amino acid concentrations was observed in the intracerebral treated rats. Thus, the neurotoxicity may be due to that DBA which penetrates brain.

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