AMINO ACID ANTAGONISM IN THE TETRAZOLE SERIES

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James Kenneth Elwood

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This is to certify that the

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IN THE TETRAZOLE SERIES

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James K. Elwood

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R. M. Herbst by A. Timmis

Major professor

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ABSTRACT

AMINO ACID ANTAGONISM IN THE TETRAZOLE SERIES

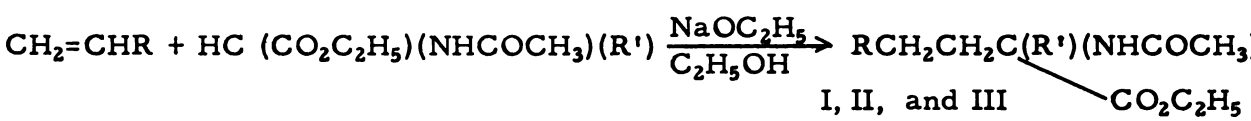
by James Kenneth Elwood

The occurrence of metabolic antagonism between a metabolite and a structurally related non-metabolite or "antimetabolite" is well-known. One of the direct methods of antagonism involves a competition between the metabolite and the structurally related antagonist for the active site of an enzyme. As a consequence of this phenomenon, a large number of compounds which closely resemble essential metabolic intermediates sterically and/or chemically have been prepared as potential antimetabolites.

The similarity of 5-aminoalkyltetrazoles to the amino acids has been well-established (1). The initial phase of this work is primarily concerned with extending the number of C-substituted 5-aminomethyltetrazoles by attempting to condense aromatic aldehydes with 5-acetamidomethyltetrazole and 5-benzamidomethyltetrazole in acetic anhydride. The ease with which both aceturic and hippuric acids condense with aromatic aldehydes in the Erlenmeyer azlactone synthesis made this approach seem feasible. Unfortunately, no interaction was observed between the 5-acylaminomethyltetrazoles and the aromatic aldehydes under experimental conditions mild enough to avoid interaction of the acetic anhydride with the tetrazole ring system. Under more vigorous reaction conditions the acetic anhydride underwent the Perkin condensation with the aldehyde present and 2, 5-disubstituted 1, 3, 4-oxadiazole formation with the 5-acylaminomethyltetrazole.

The new compound 2-methyl-5-benzamidomethyl-1, 3, 4-oxadiazole was prepared, and its structure was proven by degradation and elemental analysis. In order to achieve some insight into the mechanism of the interaction of tetrazoles with acetic anhydride, 5-phenyltetrazole enriched in N^{15} in the 1-(or 4-) position of the ring was subjected to refluxing acetic anhydride. The resulting 2-methyl-5-phenyl-1, 3, 4-oxadiazole was found to contain only half the nitrogen label corresponding to a loss of the 1, 2-(or 3, 4-) nitrogens of the tetrazole ring.

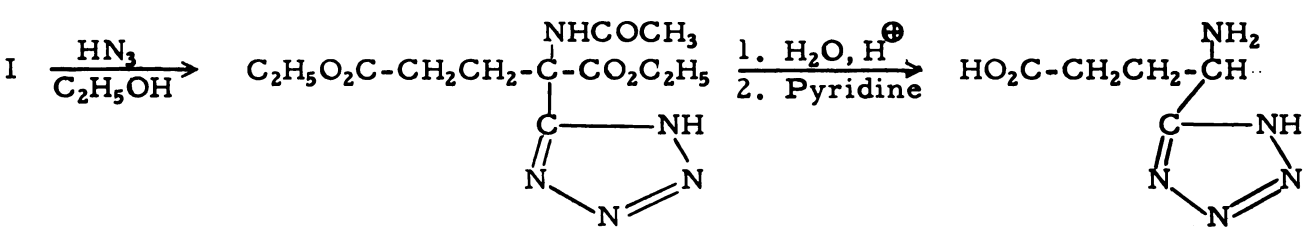
The preparation of the three tetrazole analogues of DL-glutamic acid is described. These analogues result from the substitution of tetrazole groups for the alpha and/or gamma carboxyl groups of DL-glutamic acid. The syntheses of these analogues were achieved by effecting Michael additions between ethyl acrylate or acrylonitrile and diethyl acetamidomalonate or ethyl acetamidocyanoacetate. The resulting nitriles were converted into the corresponding 5-substituted tetrazoles by refluxing with hydrazoic acid in various alcohols. Acidic hydrolysis and decarboxylation of the tetrazole intermediates furnished the amino acid analogues.

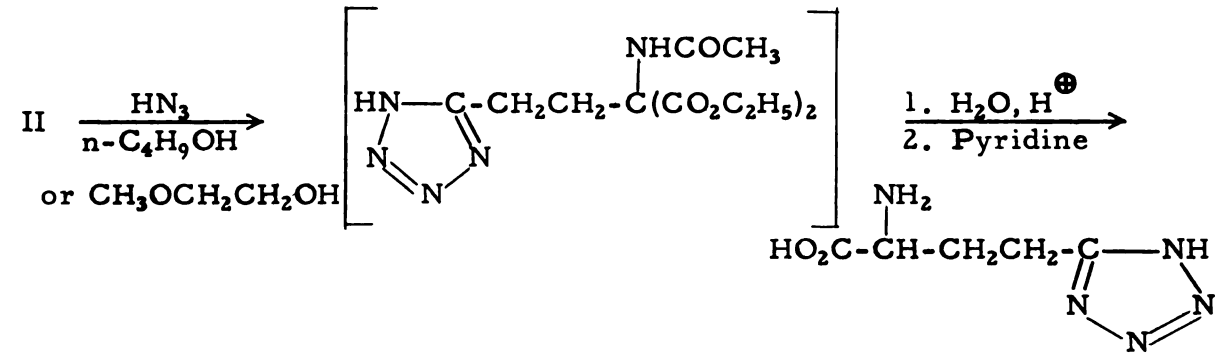


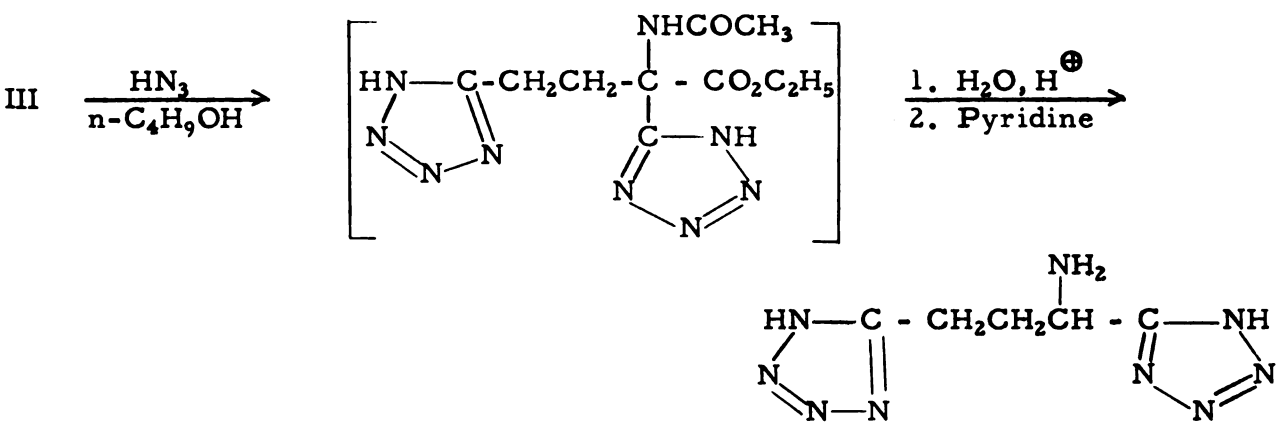
I: R = $-CO_2C_2H_5$, R' = $-CN$

II: R = $-CN$, R' = $-CO_2C_2H_5$

III: R = $-CN$, R' = $-CN$







The following derivatives of the tetrazole analogues of DL-glutamic acid were also prepared.

1. 2-acetamido-4-(5'-tetrazolyl)butanoic acid
2. 4-acetamido-4-(5'-tetrazolyl)butanoic acid
3. 1-acetamido-1,3-di(5-tetrazolyl)propane
4. ethyl 2-amino-4-(5'-tetrazolyl)butanoate hydrochloride
5. ethyl 2-amino-4-(5'-tetrazolyl)butanoate
6. 5-(5'-tetrazolyl)-2-pyrrolidone
7. ethyl 4-acetamido-4-(5'-tetrazolyl)butanoate
8. 2-amino-4-(5'-tetrazolyl)butanoic acid hydrochloride
9. 4-amino-4-(5'-tetrazolyl)butanoic acid hydrochloride

The three tetrazole analogues were further characterized by determining their pK_1, pK_2, and pK_3 values from the titrations of aqueous solutions of the analogues with standard acid and base. In addition, the Rf and Mf values of the two monotetrazole analogues were determined by paper chromatography using 80% phenol in water and a water saturated mixture of 1-butanol in 1,4-dioxane (4/1) as the moving phases.

Each of the three analogues was tested for its ability to function as either a substrate or inhibitor with L-glutamic acid dehydrogenase. Only 4-amino-4-(5'-tetrazolyl)butanoic acid was active as a substrate for the enzyme, however, the reaction rate was very slow when compared with the rate of L-glutamic acid. Clearly competitive inhibition of the oxidative deamination of L-glutamic acid was found for both 2-amino-4-(5'-tetrazolyl)butanoic acid and 1-amino-1, 3-di(5'-tetrazolyl)-propane. Michaelis constants, inhibition constants, and equilibrium constants were determined for the tetrazole analogues and for L-glutamic acid.

Two additional enzymatic studies indicated that the three tetrazole analogues were totally inactive toward D-amino acid oxidase and that their N-acetyl derivatives were equally unaffected by acylase I.

REFERENCE CITED

1. J. McManus and R. Herbst, J. Org. Chem., 24, 1643 (1959).

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INTRODUCTION

A large number of compounds have been produced which possess high degrees of similarity to various essential metabolites of the cell. Such compounds may differ from the corresponding metabolites in a wide variety of ways; for example, they may possess such variations as an additional methylene group, a sulfonic acid moiety in place of the carboxyl group (1), or a fluorine in place of one of the hydrogens (2). These modified compounds, when they are administered to living organisms, frequently lead to the appearance of symptoms associated with a deficiency of the corresponding metabolite. Analogues which exhibit this behavior are known as antimetabolites.

This phenomenon of metabolic antagonism has generally been attributed to a competition between the metabolite and the structurally related antimetabolite for the reactive site of an enzyme. Such antimetabolites are known as competitive inhibitors. When the antimetabolites are capable of combining with either the free enzyme or enzyme-substrate complex with the subsequent destruction of catalytic activity, the phenomenon is known as noncompetitive inhibition.

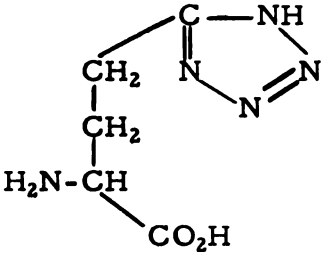
A striking similarity is known to exist between 5-substituted tetrazoles and carboxylic acids. It has been well-established (3) that 5-substituted tetrazoles dissociate in aqueous solution and that the pK values of these tetrazoles are comparable to those of the corresponding carboxylic acids. In addition, the tetrazole and carboxyl groups may be expected to have somewhat similar steric requirements. In light of these observations, McManus and Herbst (4), and also Sterken and Herbst (5), have synthesized a number of tetrazole analogues of biologically important amino acids as potential antimetabolites. The former

pair of workers successfully synthesized the tetrazole analogues of glycine, DL-alanine, β-alanine, DL-phenylalanine, and DL-tryptophan, while the latter pair prepared tetrazole analogues of DL-α-aminobutyric acid, DL-valine, DL-leucine, α-aminoisobutyric acid, DL-C-phenylglycine, and γ-aminobutyric acid.

A bacteriological study of the five tetrazole amino acid analogues prepared by McManus and Herbst has recently been conducted by Zygmunt (6). However, only the tetrazole analogue of DL-alanine exhibited any appreciable inhibition. Nevertheless, it seemed desirable to prepare additional analogues for study as antimetabolites since so little is known of the mechanisms through which the enzymes of living organisms render their catalytic effects.

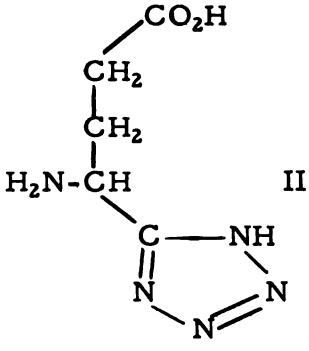
The present work was initially concerned with developing a route to the tetrazole analogues of aromatic α-amino acids through a direct condensation analogous to the Erlenmeyer azlactone synthesis. In view of the fact that tetrazole ring opening occurred under the reaction conditions, it was necessary to abandon this approach. This seemingly unusual behavior is explained on the basis of work conducted by Huisgen, Sauer, Sturm, and Markgraf (7).

In PART II of the Results, a description is given for the syntheses of the three tetrazole analogues of DL-glutamic acid (I, II, and III) and



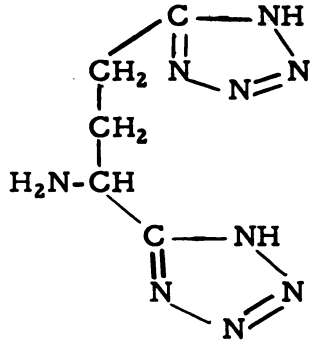
I

2-amino-4-(5'-tetrazolyl)butanoic acid (γ-tetrazole analogue)



II

4-amino-4-(5'-tetrazolyl)butanoic acid (α-tetrazole analogue)



III

1-amino-1,3-di-(5'-tetrazolyl)propane (ditetrazole analogue)

a number of their derivatives. The three pK values of each of the analogues were determined and compared with those for glutamic acid. Molecular flow (Mf) values of the monotetrazole analogues obtained by paper chromatography were also compared with those obtained for glutamic acid. In both cases a striking similarity was noted between the value of the physical property for glutamic acid and the corresponding value for each of the analogues.

The final section of this work describes the results of an enzymatic study of the three tetrazole DL-glutamic acid analogues with L-glutamic acid dehydrogenase (GAD). Two of the analogues were found to function as competitive inhibitors, although the inhibition observed was rather low. This is the first enzymatic study that has been made of a tetrazole α-amino acid analogue in which a single enzyme was employed. In fact, 5-(1'-hydroxyethyl)tetrazole was the only tetrazole that has been studied as a substrate for a specific enzyme (8).

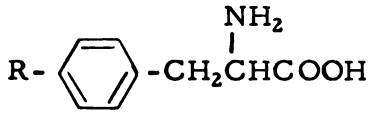
It was interesting to find that 4-amino-4-(5'-tetrazolyl)butanoic acid, the alpha-tetrazole analogue, was capable of functioning as a substrate for L-glutamic acid dehydrogenase. This enzyme is known to be very specific, and this is the first example of a glutamic acid analogue that functions as a substrate. In addition, each of the three tetrazole analogues was found to be inactive as a substrate for both acylase I and D-amino acid oxidase.

GENERAL HISTORICAL

The phenomenon of antagonism between structurally related metabolites has been known to exist in nature for several decades. Such antagonism has been rationalized to involve a competition between the similar species for an enzyme with which both are capable of associating. This phenomenon is exemplified in the antagonism between adenosine and cytidine in Neurospora mutants (9) and between glycine and alanine in Streptococcus fecalis (10) as well as in many other cases.

This same phenomenon has been extended to include antagonism between a metabolite and a structurally similar non-metabolite or "antimetabolite." These antimetabolites can exhibit antagonism by means of either a competitive or a non-competitive inhibition. Competitive inhibition of the utilization of the metabolite occurs in the instances where there is a competition between the metabolite and the antagonist for the reactive site of the enzyme. Under such conditions a large excess of the metabolite over the antimetabolite can largely offset the inhibition of the latter. In contrast, noncompetitive inhibition results when the antagonist combines with both the free enzyme and the enzyme-metabolite complex with subsequent destruction of catalytic activity. A large excess of the metabolite, in such cases, would have little effect in offsetting the actions of the antimetabolite which has essentially effected a permanent inactivation of a portion of the enzyme system. In light of this phenomenon of inhibition, a number of compounds which are isosteric with biologically active materials or show similar chemical reactivities have been investigated for activities as metabolic antagonists.

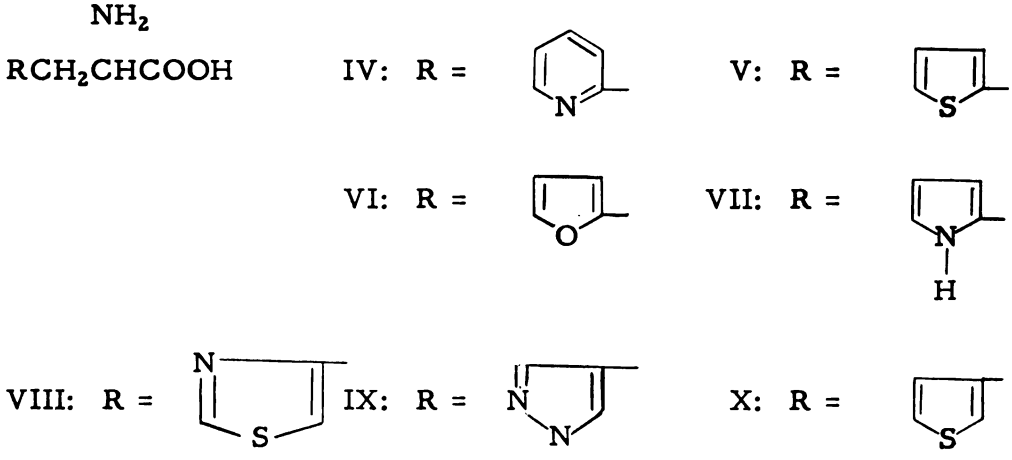
As a consequence of the role the α-amino acids play as building blocks of proteins, the very substances of life, a number of structurally related compounds have been synthesized for study as potential antimetabolites. One of the most thoroughly investigated classes of potential antimetabolites comprises ring-substituted phenylalanine (I). Various



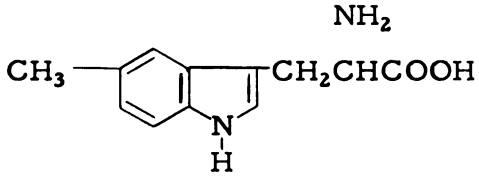
I: R = H
II: R = NH_2
III: R = F

substitutions in the phenyl ring have been found to transform the α-amino acid into antagonists of the naturally occurring phenylalanine in bacterial growth. The introduction of an amino group (II) (11) or a fluoro group (III) (2) para to the alanine moiety is sufficient to convert the nutritional α-amino acid into an antagonist. Various other substituted phenylalanines have been studied which were prepared by the introduction into the phenyl ring of one or more of the following groups: chloro, methyl, ethoxy, hydroxy, and nitro (11). In addition, a number of less common functional groups have been introduced into the phenyl ring (11).

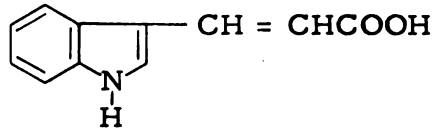
Various heterocyclic ring systems have been substituted for the phenyl group in phenylalanine, and a number of these analogues have specific antagonism for phenylalanine. Some of the analogues which were found to be antagonists are 2-pyridyl (IV) (12), 2-thienyl (V) (13), 2-furyl (VI) (14), 2-pyrryl (VII) (15), 4-thiazolyl (VIII) (12), 4-pyrazolyl (IX) (12), and 3-thienyl (X) (16) analogues.



Antagonists for tryptophan are found in 5-methyltryptophan (XI) (17) and β-indoleacrylic acid (XII) (18). Tryptophan is one of the essential amino acids for man.

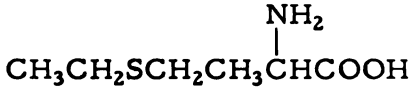


XI

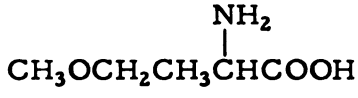


XII

Antimetabolites for methionine are found in ethionine (XIII) (19). DL-Ethionine has been found to inhibit amylase production in pigeon pancreas (20), but to accelerate amylase formation in other organisms (21). Another analogue, methoxinine (XIV) (22), has also been prepared and studied.



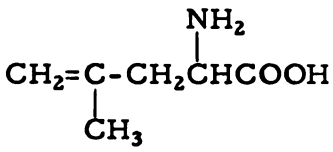
XIII



XIV

Analogues of glycine, valine, alanine, and leucine have been prepared by replacement of the carboxyl group by a sulfonic acid residue (1). In some cases specific inhibition of the use of the corresponding amino acids of which they were analogues was observed (1).

A variety of miscellaneous analogues have been prepared and studied. The 1-naphthyl and 2-naphthyl derivatives of phenylalanine (23) were found to be inactive as inhibitors. DL-Methallylglycine (XV), γ-chloro-DL-allylglycine (XVI), DL-propargylglycine (XVII), and 6-trifluoro-2-aminohexanoic acid all were antagonists of amylase production in pigeon pancreas (20). O-Diazo acetyl-L-serine (XVIII) has been found



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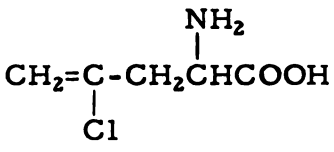
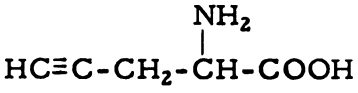
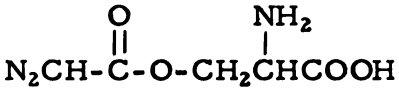


XVI



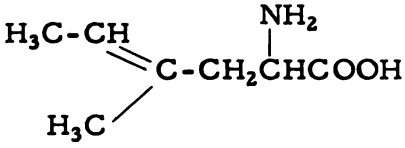
XVII



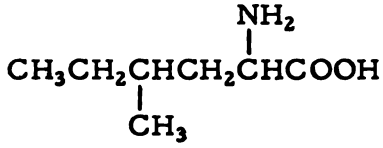
XVIII

to cause temporary remissions in an untreated patient with acute leukemia (24).

Recent studies have shown that 2-amino-4-methyl-4-hexenoic acid (XIX) with cis methyl groups is an antagonist of phenylalanine and leucine for Leuconostoc dextranicum 8086 (25). 2-Amino-4-methyl-hexanoic acid (XX) is an antagonist of leucine but not phenylalanine for this organism (25). However, 2-amino-4-methyl-4-pentenoic acid (XXI) does not show antimetabolite activity for this organism, but is a leucine antagonist for certain other microorganisms (25).



XIX



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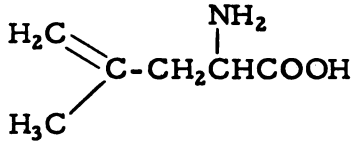
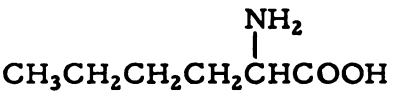


XXI

It has been found that, in general, inhibition of cellular multiplication by analogues is not due to blocking of synthesis of proteins but to syntheses of proteins abnormal in structure and activity (26). As examples, it has been found that p-fluorophenylalanine and β-(2-thienyl)alanine are incorporated in place of phenylalanine into the protein of Escherichia coli (27). Also norleucine (XXII) can replace methionine in E. coli protein (27).



XXII

The following discussion outlines the development of tetrazole chemistry to the preparation of tetrazole analogues of amino acids as potential antimetabolites.

HISTORICAL DISCUSSION

The first compound possessing the tetrazole ring system was prepared in 1885 by the Swedish chemist, Bladin (28). This compound which he had synthesized was shown to be 2-phenyl-5-cyanotetrazole (29, 30), however, Bladin believed that he had obtained the 1-phenyl isomer. It remained for Bamberger and De Gruyter (29) and independently for Widman (30) to establish the correct structure eight years later. It was Bladin who in 1886 first suggested the name "tetrazole" for the ring system (31).

Bladin prepared the parent compound, tetrazole, in 1892 from 2-phenyl-5-carboxytetrazole by means of a nitration, reduction, and oxidation (32). Considerable inherent ring stability was shown by the fact that the tetrazole ring system was able to withstand such a variety of chemical reactions. Recent independent studies (33, 34) have shown that the ring system of tetrazole possesses 60 ± 5 kcal/mole of resonance energy. In 1910 Dimroth and Fester succeeded in developing a direct synthesis of tetrazole by the combination of hydrogen cyanide with hydrazoic acid (35).

It was not long after tetrazole and a number of its 5-substituted derivatives had been synthesized that it was recognized that the compounds possessed considerable acidity (36-39). This acidity was attributed to the resonance stabilization of the anion which led to considerable ionization in solution of the hydrogen on the ring nitrogen. Acidic dissociation constants are routinely determined for 5-substituted tetrazoles in recent work (4, 5), and correlations have been successfully made between the acidities of the tetrazoles and the electron withdrawing power of the 5-substituents (40). In general, tetrazoles have been found

to parallel closely carboxylic acids in acidity as has been shown by a comparison of apparent dissociation constants of a group of 5-substituted tetrazoles with the corresponding acids (3).

Numerous methods have been developed for the synthesis of tetrazoles. In 1947 Benson reviewed the chemistry of tetrazoles and the known methods for the synthesis of the various mono- and disubstituted compounds (41). Since then a large number of additional methods have been used. Various syntheses have been developed for the preparation of the disubstituted and for the nitrogen monosubstituted tetrazoles (42-49), however, since this thesis is concerned exclusively with monosubstituted tetrazoles with the substituent in the 5 position, general methods of synthesis of only these compounds will be mentioned.

Pinner (50) developed a stepwise conversion of aryl cyanides to 5-aryltetrazoles by way of the iminoethers, amidrazones, and imide azides. A direct synthesis of either 5-alkyl or 5-aryl tetrazoles was developed by Mihina and Herbst (38). In the direct synthesis the nitriles were heated in a sealed tube with a benzene solution of hydrazoic acid. Behringer and Kohl (51) found that a tetrahydrofuran solution of aluminum azide prepared in situ from aluminum chloride and sodium azide would react conveniently with nitriles to give yields of around 80% of the tetrazole. Huisgen (7) and his co-workers prepared 5-phenyltetrazole by utilizing an ethylene glycol monomethyl ether solution of lithium azide prepared in situ from lithium chloride and sodium azide. One of the simplest procedures, which was developed by Herbst and Wilson (39) and used extensively during the course of this work, involved refluxing the nitrile in 1-butanol containing sodium azide and acetic acid. In effect, the nitrile was refluxed with an alcoholic solution of hydrazoic acid. This procedure, while excellent for "activated" nitriles, is rather poor for alkyl cyanides. Finnegan, Henry, and Lofquist (52) found that an

ammonium azide solution in dimethylformamide led to improved yields and shorter reaction times in the preparation of 5-alkyltetrazoles. A number of other workers have prepared 5-substituted tetrazoles by modifications of one of the methods previously described or by rather unique but specialized reactions (52-58).

The first 5-aminoalkyltetrazole was prepared by Ainsworth (59) in 1953 when he synthesized 5-(2'-aminoethyl)tetrazole by three independent methods. The first method involved the quantitative conversion of the corresponding nitrile to an iminoether hydrochloride by the method of McElvain and Nelson (60). This hydrochloride was then converted to 5-(2'-benzamidoethyl)tetrazole by a procedure which Oberhummer (61) had used in 1933 to prepare 5-methyltetrazole. The hydrochloride of the 5-aminotetrazole was obtained by hydrolyzing the amide. Oberhummer's procedure, which was the earliest for the preparation of 5-alkyltetrazoles, was a modification of the method Pinner had developed in 1897 for the syntheses of 5-aryltetrazoles. Oberhummer modified the procedure of Pinner only to the extent that he used amyl nitrite in place of sodium nitrite in the diazotization.

Ainsworth's second method for the preparation of 5-(2'-aminoethyl)tetrazole involved refluxing β-benzamidopropionitrile with hydrazoic acid in xylene. The resulting 5-(2'-benzamidoethyl)tetrazole was hydrolyzed with hydrochloric acid to give the product. This procedure was a modification of the method of Mihina and Herbst (38) in which the nitrile was heated with a benzene solution of hydrazoic acid in a sealed tube.

A final method of Ainsworth's involved interaction of ethyl β-benzamidopropionimidate hydrochloride with a solution of hydrazoic acid in glacial acetic acid. The 5-aminoalkyltetrazole resulted upon acid hydrolysis.

In 1956 Behringer and Kohl (51) were able to improve greatly upon Ainsworth's yields, which averaged about 10%, by refluxing the aminonitriles with a threefold excess of aluminum azide in tetrahydrofuran. Yields of about 80% were frequently obtained by this procedure which was used in the syntheses of 5-(2'-aminoethyl)tetrazole and 5-aminomethyltetrazole.

Finnegan, Henry, and Lofquist (52) found that yields comparable to those of Behringer and Kohl could be obtained by heating the nitriles with a dimethylformamide solution of ammonium chloride and sodium azide. The main advantage over the aluminum azide method was in the shorter reaction times that were required.

A number of tetrazole analogues of naturally occurring amino acids have been synthesized. In 1959 McManus and Herbst (4) were successful in preparing the tetrazole analogues of glycine, DL-alanine, β-alanine, DL-phenylalanine, and DL-tryptophan along with a number of tetrazole analogues of other physiologically important acids. However, in 1956 Berhinger and Kohl (51) reported syntheses for the glycine and β-alanine analogues. During the course of their work, McManus and Herbst utilized three schemes of synthesis. With the exception of DL-tryptophan, all the analogues were synthesized by two independent methods.

The first scheme employed by McManus and Herbst involved the initial reaction of an α-haloacyl halide with benzylamine forming the N-benzyl-α-haloamide. Subsequent conversion of the α-haloamide with phosphorus pentachloride to the imidyl chloride followed by treatment with hydrazoic acid gave rise to a 1-benzyl-5-α-haloalkyltetrazole. The resulting haloalkyltetrazole was then treated with potassium phthalimide to form the 1-benzyl-5-α-phthalimidoalkyltetrazole. Since the α-haloalkyltetrazoles were irritating to mucous membranes, alternately the α-phthalimidoamide was first prepared from potassium phthalimide and the α-haloamide. This product was then treated in turn

with phosphorus pentachloride and hydrazoic acid to give the 1-benzyl-5-α-phthalimidoalkyltetrazole. The phthalyl moiety was subsequently removed from the 1-benzyl-5-α-phthalimidoalkyltetrazole by treatment with hydrazine. Debenzylation of the resulting 1-benzyl-5-α-aminoalkyltetrazole hydrochloride was effected by reduction with hydrogen using palladium on charcoal. The free 5-α-aminoalkyltetrazole was released by treating the hydrochloride with pyridine.

A second scheme employed by McManus and Herbst for the synthesis of 5-α-aminoalkyltetrazoles consisted in the reaction of the phthalyl derivative of an α-amino acid with thionyl chloride followed by treatment with ammonia gas or ammonium hydroxide solution. The resulting α-phthalimidoamide was dehydrated to the α-phthalimidonitrile which was in turn converted to the 5-α-phthalimidotetrazole by an adaptation of the aforementioned method of Behringer and Kohl (51). Hydrazine was again utilized to remove the phthalyl moiety.

The last scheme employed consisted in the alkylation of ethyl acetamidocyanoacetate followed by tetrazole formation from the nitrile by the method of Behringer and Kohl (51). Acid hydrolysis and decarboxylation resulted in the formation of the α-aminoalkyltetrazole.

A number of 5-aminoaryltetrazoles and tetrazole analogues of physiologically and pharmacologically active carboxylic acids have been prepared by Van de Westerringh, Veldstra and co-workers (62, 63). They employed the methods of Mihina and Herbst (38) to obtain tetrazole analogues of p-aminobenzoic acid, 2-hydroxy-4-aminobenzoic acid, picolinic acid, nicotinic acid, isonicotinic acid, 2,4-dichlorophenoxyacetic acid, and 3-indoleacetic acid. However, all of these analogues had been prepared previously by McManus and Herbst (4) using the procedure of Herbst and Wilson (39).

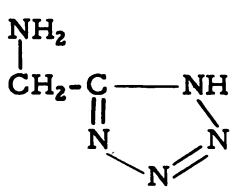
Recently Sterken and Herbst (5) have extended the work of McManus and Herbst (4) by preparing six additional tetrazole analogues of amino

acids. 5-(1'-aminopropyl)tetrazole, 5-(1'-amino-1'-methylethyl)tetrazole, 5-(α-aminobenzyl)tetrazole, and 5-(3'-aminopropyl)tetrazole were synthesized along with the analogues of valine and leucine. In addition 1,3-di(5'-tetrazolyl)propane was prepared as an analogue of glutaric acid. Sterken and Herbst (5) prepared each of the tetrazoles by two independent methods.

In the first method the corresponding α-amino acid was converted to the α-phthalimidonitrile by way of its acid chloride and amide by a scheme developed by McManus and Herbst. The α-phthalimidonitrile was then converted to the corresponding tetrazole by reaction with a salt of hydrazoic acid. Hydrazinolysis was employed to remove the phthalyl moiety leaving the 5-(1'-aminoalkyl)tetrazole.

Sterken and Herbst also prepared 5-aminoalkyltetrazoles by interaction of aldehydes and ammonium cyanide to give α-amino alkylnitriles. After acylation of the amino group, the corresponding 5-(1'-acylaminoalkyl)tetrazoles were obtained by reaction of the nitriles with salts of hydrazoic acid. The tetrazole analogues of the amino acids were obtained upon acid hydrolysis of the amides.

A bacteriological study of the five tetrazole amino acid analogues prepared by McManus and Herbst has recently been conducted by Zygmunt (6). The tetrazoles studied were 5-aminomethyltetrazole (IV), 5-(1'-aminoethyl)tetrazole (V), 5-(2'-aminoethyl)tetrazole (VI), 5-(1'-aminopropyl)tetrazole (VII), and 5-(1'-amino-2'-phenylethyl)tetrazole (VIII), which are the analogues of glycine, DL-alanine, β-alanine, DL-α-aminobutyric acid, and DL-phenylalanine, respectively.



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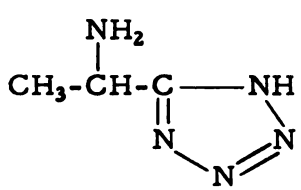

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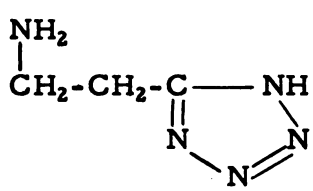

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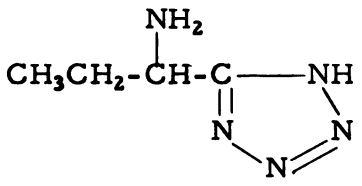
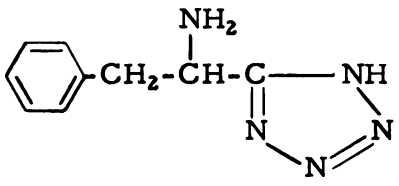


VII



VIII

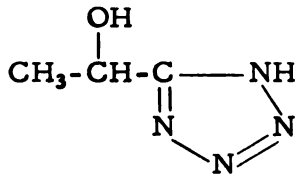
Each of the compounds was tested with a microorganism which synthesized its own amino acids, and certain compounds with microorganisms which require specific amino acids for growth. All the compounds were tested for their ability to inhibit the growth of Escherichia coli W in the synthetic medium of Davis and Mingioli (64). In addition, the analogues of glycine and phenylalanine were tested for their ability to inhibit utilization of the corresponding amino acids in an amino acid basal medium using Leuconostoc mesenteroides P60, an organism requiring these amino acids. The DL-alanine analogue was also tested for antagonism of DL-alanine using Pediococcus cervisiae (ATCC No. 8081), an alanine-dependent organism.

Zygmunt (6) found that all of the tetrazole analogues studied failed to inhibit the growth of E. coli in twenty-four hours. Thus, these five analogues are not potent inhibitors of the synthesis and/or utilization of amino acids. The glycine analogue when studied with L. mesenteroides showed only a low order of activity, while the DL-phenylalanine analogue failed completely to show any inhibition of growth with this organism at the concentrations of tetrazole analogue employed.

The only substantial inhibition observed during the course of the study was that of the DL-alanine analogue toward utilization of DL-alanine in P. cervisiae. However, this compound was a relatively weak inhibitor requiring approximately a 200-400 to 1 molar ratio of antagonist to metabolite to obtain a 50-90% inhibition of growth. This antagonism was readily reversed by DL-alanine.

In addition, 5-aminomethyltetrazole (IV), 5-(1'-aminoethyl)tetrazole (V), and 5-(1'-amino-2'-phenylethyl)tetrazole (VIII) with L. mesenteroides and P. cervisiae which require glycine, phenylalanine, or alanine, respectively, showed that the analogues did not support growth in place of the corresponding amino acids.

No tetrazole analogues of amino acids had been studied in isolated enzyme systems prior to the work reported in PART III of this thesis. In fact, only one example has been reported of the study of a tetrazole with a specific enzyme. In this case Fisher, Tomson, and Horwitz (8) found that 5-(1'-hydroxyethyl)tetrazole (IX), the analogue of lactic acid, was an acceptable substrate for lactic dehydrogenase.



IX

In spite of the unfavorable results obtained by Zygmunt (6) in the initial bacteriological study of the five tetrazole amino acid analogues prepared by McManus and Herbst (4), it was desirable to prepare additional analogues since detailed mechanisms by which enzymes carry out their catalysis are unknown. Whereas substitution of the tetrazolyl moiety for the carboxyl group of a substrate of an enzyme may completely inactivate the material in one case, this does not necessarily mean that the same substitution will inactivate a substrate of another enzyme for which the geometry and/or electronic effects of the carboxyl group may be less important. This is exemplified by the aforementioned results of Fisher and co-workers (8) and by work reported in PART III of this thesis.

As an extension of the work on the syntheses of tetrazole analogues of amino acids as potential antimetabolites, it was desirable to exploit new approaches to the syntheses while at the same time acquiring further knowledge into the chemistry of the tetrazole ring system. With these goals in mind it looked promising to try and extend the field of the tetrazole analogues of aromatic α-amino acids by means of a condensation of an aromatic aldehyde with either 5-benzamidomethyltetrazole or 5-acetamidomethyltetrazole in acetic anhydride. This reaction is known as the Erlenmeyer azlactone synthesis when hippuric acid or aceturic acid is used in place of the aforementioned tetrazoles, respectively. Success of this method would not only have resulted in a convenient approach to the aromatic α-amino acid analogues, but would have indicated the effectiveness of the tetrazole ring system as an activator of the methylene hydrogens adjacent to the tetrazole ring toward condensation under these conditions. This approach, unfortunately, proved to be unfruitful as the tetrazole ring system was destroyed by reaction with the acetic anhydride under the experimental conditions to yield 1,3,4-oxadiazoles. This reaction with acetic anhydride was not unknown, however, as Huisgen (7) and his co-workers had prepared 1,3,4-oxadiazoles by this method in 1960. It was hoped that condensation might occur under conditions milder than those necessary for ring opening.

Further work in the preparation of tetrazole analogues of α-amino acids was carried out by synthesizing the three glutamic acid analogues. A variety of conditions were used, however, in all cases the tetrazoles were prepared from the corresponding nitriles. Various intermediate compounds were also prepared and characterized.

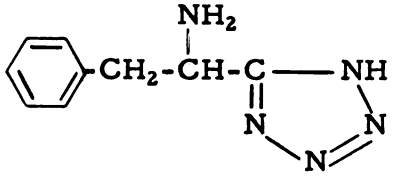
The final phase of the work discussed in this thesis involves an enzymatic study of L-glutamic acid and its three DL-tetrazole analogues with L-glutamic dehydrogenase. Activity was found with one of the analogues while competitive inhibition was observed for the other two.

The results of these three areas of work are discussed in the following sections.

RESULTS

PART I

The purpose of this chemical investigation was to extend the initial work of McManus and Herbst (4) and of Sterken and Herbst (5) on the preparations of the tetrazole analogues of amino acids. A synthetic route involving a condensation reaction was to be attempted to prepare a number of aromatic analogues. McManus and Herbst were successful in preparing two aromatic tetrazole analogues, namely, those of DL-phenylalanine (I) and DL-tryptophan (II); Sterken and Herbst synthesized one additional aromatic amino tetrazole, 5-(α-aminobenzyl)tetrazole (III). A number of aliphatic tetrazole analogues of amino acids were also synthesized by these workers. It was desired to prepare additional analogues as potential antimetabolites of the corresponding amino acids, and to



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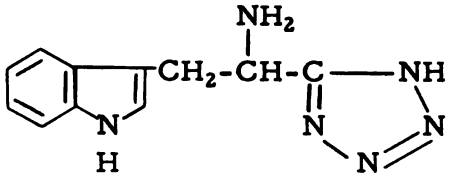

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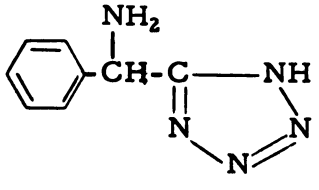
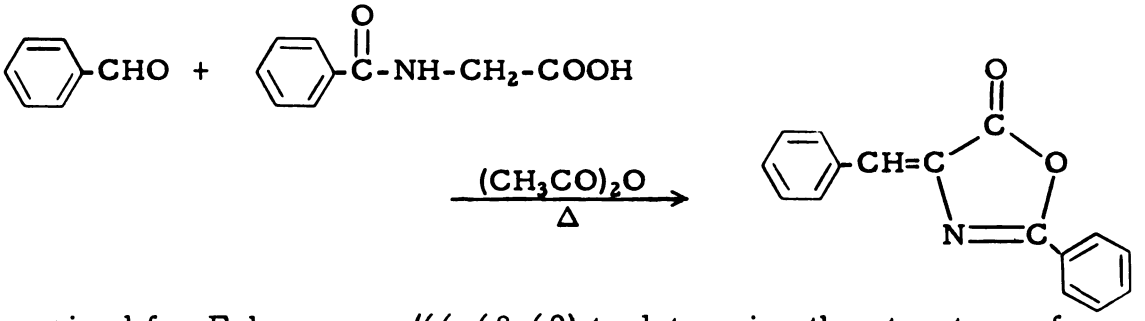


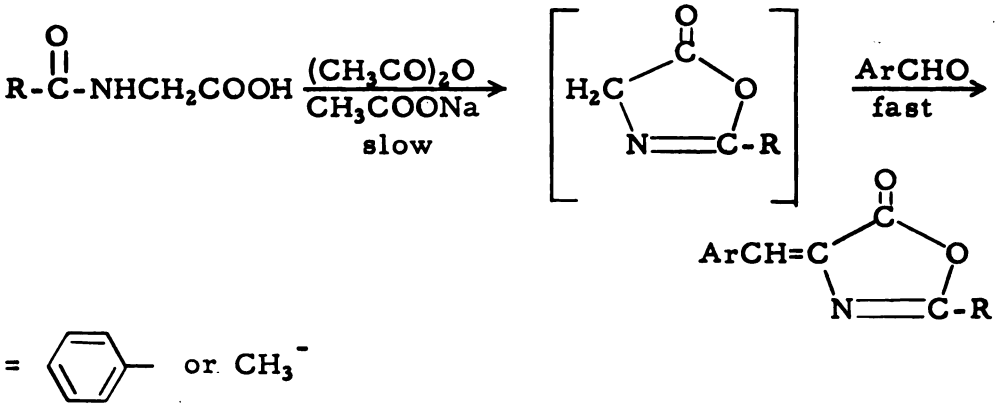
III

determine the characteristic physical and chemical properties of these compounds for comparison with the corresponding amino acids.

A new approach to the synthesis of aromatic tetrazole analogues of amino acids was attempted which, if successful, would be similar to the classical condensation of acylglycines with aromatic aldehydes in acetic anhydride. The first condensation of this type was carried out by Plöchl (65) in 1883 between benzaldehyde and hippuric acid, however,



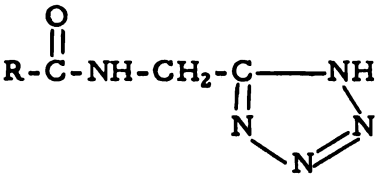
it remained for Erlenmeyer (66, 68, 69) to determine the structure of the product, to extend the reaction to other aldehydes, and to establish the usefulness of the synthesis in the preparation of α-keto and α-amino acids. Erlenmeyer (67) gave the name of "Azlactone" to the heterocyclic ring system formed, however the name "oxazolone" is preferred in accord with current nomenclature. This reaction has come to be known as the Erlenmeyer azlactone synthesis. Johnson (70) has discussed the synthesis briefly in the course of his discussion of the Perkin Reaction, however, Carter (71) has given a detailed review of the Azlactones. There is considerable evidence (72-75) that azlactone formation occurs prior to condensation as is indicated. In 1955 Baltazzi (72) brought the



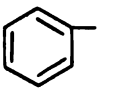
R = or CH_3^-

work up to date in an extensive review on azlactone chemistry.

It seemed desirable to attempt a condensation utilizing 5-acylamidomethyltetrazoles (X-XI) in place of the acylated glycines in light of the similarity of the tetrazolyl moiety to the carboxyl group. The original goal of extending the aromatic tetrazole analogues of amino acids

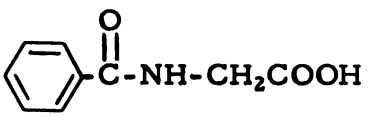


R = CH_3- X

R =  XI

might then be augmented by the acquisition of insight into the activating effects of the tetrazole ring system.

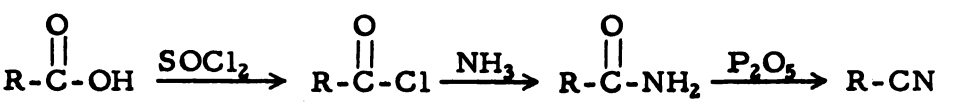
Since the preparative techniques cited in the literature for the 5-substituted tetrazoles utilized the interaction between hydrazoic acid, or one of its salts, and a nitrile, it was necessary to develop a synthetic route to the required nitriles. The acylated glycines most commonly used in the azlactone synthesis were hippuric acid (XII) and aceturic acid (XIII), therefore the corresponding nitriles of these acids were required as precursors to the tetrazole analogues.



XII

XIII

A plausible route to the preparation of nitriles from the corresponding acids involves use of the classical scheme of first preparing the acid chlorides and then subsequently allowing the acid chlorides to react with ammonia or concentrated ammonium hydroxide solution to furnish the amides. Dehydration to the nitriles can then be effected with a reagent such as benzenesulfonyl chloride or phosphorus pentoxide.



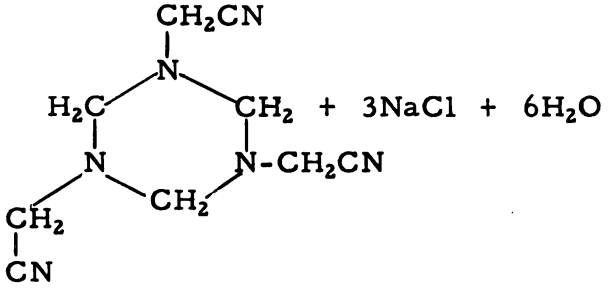
Attempts to prepare the acid chloride of aceturic acid by use of this approach met with failure. Interaction of the acid with thionyl chloride in benzene resulted in the formation within two hours at 100° C. of a dark brown insoluble material. When aceturic acid was allowed to interact with pure thionyl chloride at room temperature, a dark brown gummy mixture appeared within ninety minutes. By substituting phosphorus pentachloride for thionyl chloride it was possible to avoid much darkening if the reaction was run in toluene and cooled in an ice bath, however, under these conditions, it appeared that little if any reaction had occurred. When a mixture of aceturic acid with phosphorus pentachloride was stirred in toluene at room temperature, darkening gradually occurred, and the next day an insoluble reddish-brown polymeric gum separated. In view of the various reactions which can take place between amides and thionyl chloride or phosphorus pentachloride as discussed by Leonard and Nommensen (76), it seemed decidedly more expedient to search for an alternate route to the nitriles.

The tetrazole analogue of aceturic acid was not an unknown compound but had been reported by McManus and Herbst (4) in 1959. However, these workers prepared the analogue from 5-aminomethyltetrazole by acetylation. It was hoped that a more direct synthesis could be achieved which did not require the initial synthesis of the amino tetrazole.

An entirely different approach was finally utilized in the preparation of the nitriles of both aceturic and hippuric acids. The first step in this scheme involved the preparation of methyleneaminoacetonitrile by the gradual addition of an aqueous sodium cyanide solution to a cooled, stirred mixture of formaldehyde solution and ammonium chloride. After half of the sodium cyanide solution had been added, glacial acetic acid addition was commenced. The two were added concurrently at such a rate that both were completely added at about the same time. This reaction, described by Adams and Langley (77), is believed to result in

the formation of a methyleneaminoacetonitrile trimer possessing an N-trisubstituted saturated s-triazine structure (XIV) (78). The absence of any bands in the six micron region of the infra-red is in agreement with this assignment.

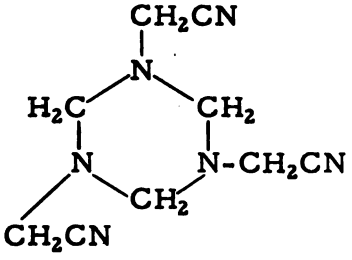
$$6HCHO + 3NaCN + 3NH_4Cl \xrightarrow[\text{added}]{HOAc}$$



$$+ 3NaCl + 6H_2O$$

XIV

Cleavage of the trimer was achieved by a procedure of Anslow and King (79) by the addition of a warm ethanol solution containing the calculated amount of sulfuric acid. The aminoacetonitrile hydrogen sulfate which crystallized from the lower layer was washed with ice cold ethanol and dried. In place of sulfuric acid it was found that the trimer could be successfully cleaved by allowing a suspension of the compound in ethanol at 50° C. to react with anhydrous hydrogen chloride gas. Careful control



$$+ 6C_2H_5OH + 3HA \longrightarrow 3A^{\ominus}H_3\overset{\oplus}{N}\text{-}CH_2\text{-}CN + 3CH_2(OC_2H_5)_2$$

$$A^{\ominus} = \overset{\ominus}{Cl},\ \overset{\ominus}{HSO_4}$$

of both the temperature and the amount of hydrogen chloride gas was

required since excess hydrogen chloride resulted in some imidoether formation as seen by the band at 6.05 microns in the infrared of some of the original product. Neither the triazine nor aminoacetonitrile hydrochloride absorb in this region. The necessary control was successfully achieved by maintaining the temperature at 50° C. and by vigorously stirring the reaction mixture during the addition of hydrogen chloride. The triazine was only sparingly soluble in ethanol at 50° C., however, aminoacetonitrile hydrochloride was quite soluble. The addition of hydrogen chloride was, therefore, terminated promptly as soon as the solid dissolved; aminoacetonitrile hydrochloride crystallized readily upon cooling. If the temperature was permitted to drop below 50° C., the product would tend to separate prior to the complete reaction of the triazine and, hence, it was impossible to visually control the hydrogen chloride addition. This procedure gave yields of 85% when carried out on a small scale (0.1-0.2 mole); larger scale reactions gave somewhat lower yields due to increased difficulty in regulating the hydrogen chloride addition.

The nitriles of aceturic and hippuric acids were prepared, respectively, by the acetylation and benzoylation of aminoacetonitrile hydrochloride. When an attempt was made to acetylate aminoacetonitrile hydrogen sulfate by warming with acetic anhydride, the sulfuric acid liberated attacked the nitrile, and, therefore, the aforementioned procedure utilizing hydrogen chloride was developed to effect the ethanolysis of the trimer. Acetylation of the resulting aminoacetonitrile hydrochloride proceeded smoothly and the hydrogen chloride was evolved. Benzoylation was carried out by the concurrent addition of benzoyl chloride in benzene and sodium hydroxide in water to a stirred aqueous solution of aminoacetonitrile hydrochloride. This Schotten-Baumann reaction proceeded smoothly to give yields of 64% of the nitrile of hippuric acid. In addition, either the hydrochloride or hydrogen sulfate of

aminoacetonitrile could be utilized since base was provided to neutralize the acid.

$$Cl^{\ominus}\ H_3N^{\oplus}\text{-}CH_2CN + (CH_3CO)_2O \xrightarrow{\Delta} CH_3CONHCH_2CN + CH_3COOH + HCl$$

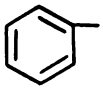
$$A^{\ominus}\ H_3N^{\oplus}\text{-}CH_2CN + \text{C}_6\text{H}_5\text{-COCl} \xrightarrow[\text{(benzene)}]{2NaOH} \text{C}_6\text{H}_5\text{-CONHCH}_2\text{CN} + NaCl + NaA + 2H_2O$$

$A^{\ominus} = Cl^{\ominus}, HSO_4^{\ominus}$

The tetrazole analogues of aceturic and hippuric acids were prepared from the nitriles by interaction with hydrazoic acid in 1-butanol. The reactions proceeded smoothly during a three day reflux period to provide yields of around 70% of the aceturic acid analogue and yields of 85-90% with the nitrile of hippuric acid.

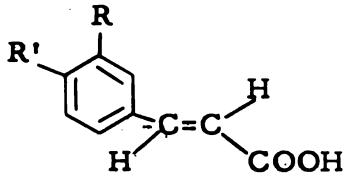
$$R\text{—}CONHCH_2CN + HN_3 \xrightarrow{\text{1-Butanol}} R\text{—}CONHCH_2\text{-}C_2N_3H$$

R = CH_3- XV

R =  XVI

The initial Erlenmeyer type condensation was attempted by heating 5-benzamidomethyltetrazole (XVI), with m-nitrobenzaldehyde in acetic anhydride containing sodium acetate. After a total reaction time of five hours at 130-140° C., the reaction was terminated and the solid sodium acetate was filtered. The filtrate was heated in vacuo to remove the acetic anhydride prior to treatment with 0.1N sodium hydroxide solution. After removal of the polymeric material, the solution was acidified and the resulting precipitate recrystallized from a mixture of water and ethanol. This material was found to be m-nitrocinnamic acid (XVII) through its melting point, its strong acidity, its rapid reaction with

potassium permanganate, its negative reaction toward bromine in carbon tetrachloride, and the presence of nitrogen in the compound. A small amount of benzoic acid, which would only have resulted from hydrolysis of the benzamido group, was obtained by ether extraction of the acidic filtrate.



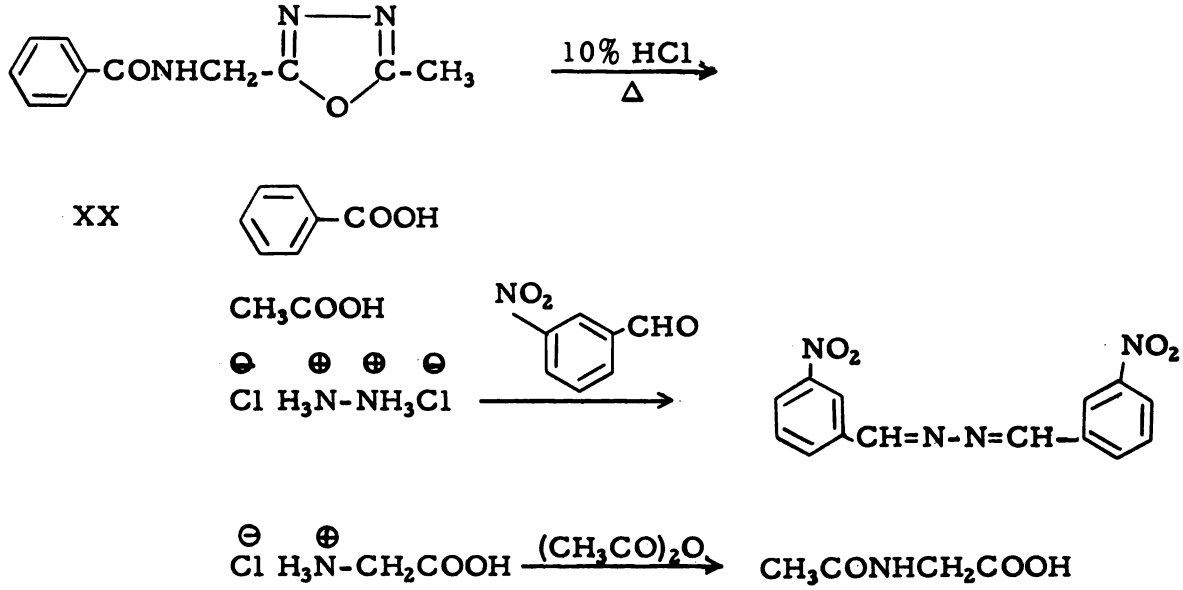
R = $-NO_2$, R' = -H	XVII
R = R' = -H	XVIII
R = -H, R' = $-OCH_3$	XIX

The absence of any compound possessing the tetrazole ring was of interest. Any such material should have partially precipitated upon acidification of the basic solution along with unreacted 5-benzamidomethyltetrazole. The presence of a small amount of benzoic acid did, however, indicate that some cleavage of the amide linkage had occurred, and to this extent the tetrazole would not be expected to precipitate under the alternately basic and acidic conditions.

Several repetitions of this reaction were carried out with benzaldehyde and anisaldehyde under varying conditions. When benzaldehyde was heated with 5-benzamidomethyltetrazole in acetic anhydride with sodium acetate for 22 hours, benzoic acid was again obtained along with cinnamic acid (XVIII). Likewise p-methoxycinnamic acid (XIX) resulted from a reaction utilizing anisaldehyde. Only in this latter reaction under the relatively mild conditions of three hours at 90 C. was any 5-benzamidomethyltetrazole recovered from the reaction mixture. It appeared that the conditions under which the reaction was conducted were sufficient to effect destruction of the tetrazole ring system, and that only a normal Perkin condensation had occurred between the aldehyde and the acetic anhydride. Similarly, reactions involving 5-acetamidomethyltetrazole were equally unsuccessful leading to only Perkin condensation products.

An investigation of the effects of acetic anhydride alone on 5-benzamidomethyltetrazole was then undertaken. At temperatures in excess of 90° C. none of the tetrazole could be recovered after several hours heating. The interaction of 5-benzamidomethyltetrazole with acetic anhydride was then employed on a preparative scale by refluxing the tetrazole in pure acetic anhydride for three hours. After removal of the anhydride by warming in vacuo, ethanol was added along with sufficient ether to induce slow crystallization of the product. Recrystallization from ethyl acetate yielded a nitrogen containing compound of m.p. 133.5-134.5° C; analysis indicated the empirical formula $C_{11}H_{11}N_3O_2$.

In light of the recent work of Huisgen and his co-workers (7, 80), it was likely that the compound obtained was 2-methyl-5-benzamidomethyl-1, 3, 4-oxadiazole (XX), and this assignment was confirmed by acidic hydrolysis of the compound. A four hour reflux period in 10% hydrochloric

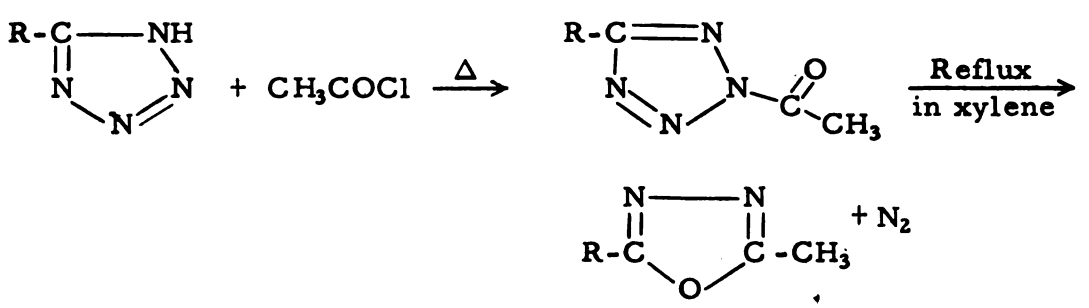


acid was sufficient to completely hydrolyze the oxadiazole, and upon allowing the hydrolysis mixture to stand overnight, crystals of benzoic acid were formed. After evaporation of an ethereal extract of the filtrate,

the odor of acetic acid could easily be detected. The filtrate was then evaporated to dryness and extracted with boiling ethanol to separate glycine hydrochloride from the hydrazine dihydrochloride which was only sparingly soluble in the hot ethanol. The glycine was freed from its hydrochloride by heating in pyridine. The resulting glycine was converted by acetylation to aceturic acid which served as a derivative since the melting point of glycine is not very characteristic. Excess m-nitrobenzaldehyde was added to an aqueous-ethanolic solution of the hydrazine dihydrochloride, and the resulting azine served as a derivative of the hydrazine.

It was mentioned that no tetrazoles were recovered during the work-ups of the attempted condensation reactions. In light of an understanding of the course taken by the interaction of acetic anhydride with 5-benzamidomethyltetrazole, it was interesting to note that no 2-methyl-5-benzamidomethyl-1,3,4-oxadiazole was obtained. However, under the alternately basic and acidic conditions of the work-up, considerable degradation must have occurred leading to benzoic and acetic acids which were already present to some extent in the reaction mixture. The hydrazine, presumably formed by hydrolysis, may have partially passed into the ethereal extract of the basic reaction mixture along with aromatic aldehydes and dark condensation products. Azine formation could have been masked by these condensation products. Due to the amphoteric nature of glycine it could not have been obtained by either precipitation or extraction of the acidic or basic mediums. During the course of one work-up, the aqueous solution obtained, after the ether extractions, was evaporated to dryness. The residual salt was then extracted with hot 1-butanol, and a small amount of a material containing nitrogen and chlorine was recovered which probably consisted of glycine hydrochloride.

The degradation of 5-substituted tetrazoles by interaction with acyl halides in pyridine was first observed by Huisgen, Sauer, and Sturm in 1958 (80). The 2,5-disubstituted 1,3,4-oxadiazoles occurred in good yields under mild conditions. In 1960 Huisgen and his co-workers (7) observed that 5-substituted tetrazoles would gradually dissolve in boiling acetyl chloride with evolution of hydrogen chloride, and further that upon evaporation of the acetyl chloride, crystals of the corresponding N-acetyl-tetrazole (XXI) could be obtained. Huisgen believed that the acetyl group was in the 2-position of the ring, however, this was not proven. The N-acetyl-tetrazoles were found to undergo ring opening to the corresponding 1,3,4-oxadiazoles at temperatures between 110-140° C., which is higher, in general, than the temperatures required for ring opening of the benzoyl derivatives. Further, it was observed that 5-aryltetrazoles would undergo oxadiazole formation by merely refluxing in acetic anhydride. However, 5-benzamidomethyltetrazole underwent oxadiazole formation during the attempted condensation reactions even though a number of these reactions were carried out under considerably milder conditions.

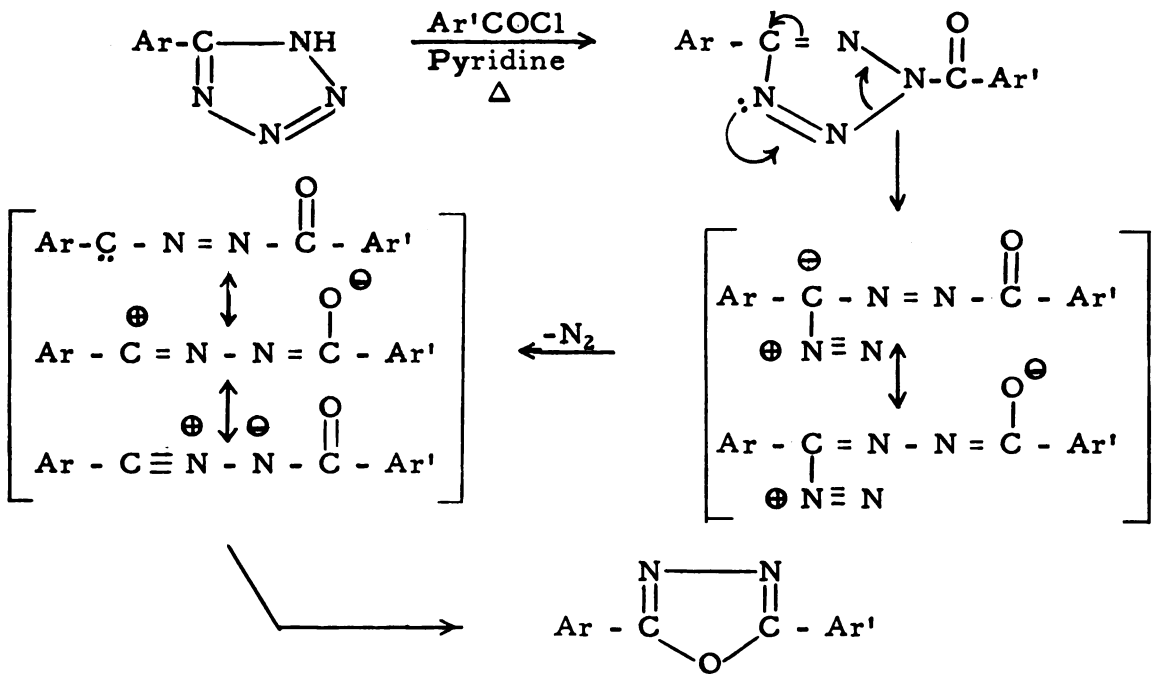


R = Alkyl, Aryl

XXI

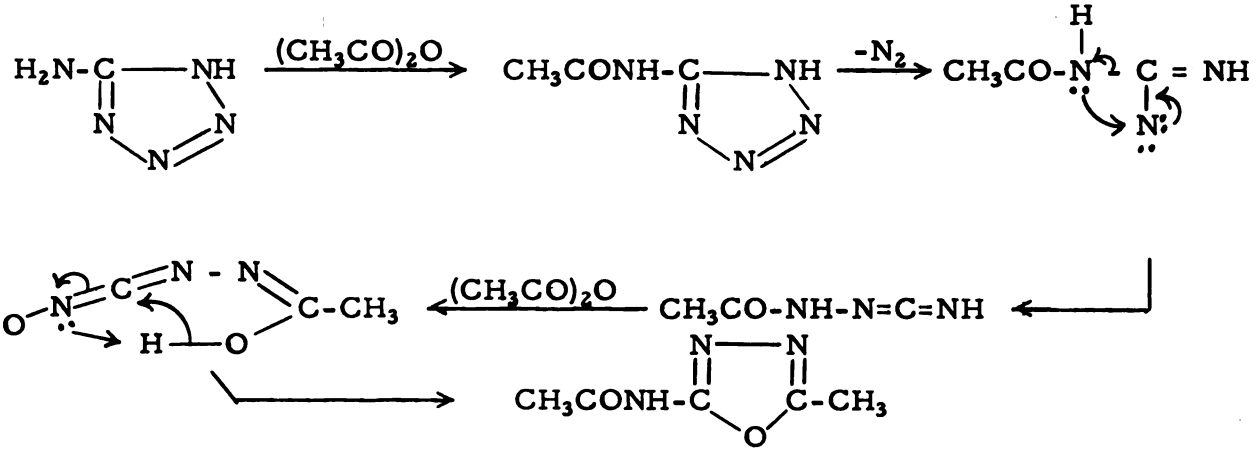
Mechanisms through which the 1,3,4-oxadiazoles are formed have been postulated independently by several groups of workers. Huisgen, Sauer, and Sturm (7) believed that acylation occurred in the 2-(or 3-)-position of the tetrazole ring, and that subsequent cleavage resulted

between the 2, 3 nitrogens of the ring. Supposedly, elimination of the 3, 4 (or 1, 2) nitrogens was followed by recyclization through the acyl oxygen to form the 2, 5-disubstituted 1, 3, 4-oxadiazole.

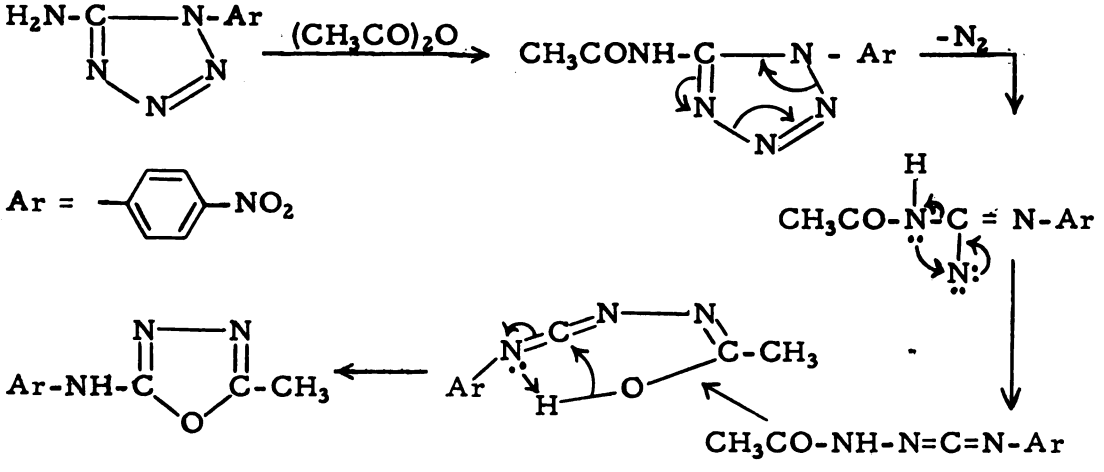


The ease with which the ring opening occurs depends upon the ability of the transition state to compensate for loss of the resonance energy of the tetrazole ring. Furthermore, substituent mesomeric resonance effects which are diminished in the ground state become important to resonance stabilization of the transition state. The acylation of the tetrazole ring, in addition, acts to decrease the aromaticity of the ring, and hence its stability, by decreasing the density of the pi electron cloud responsible for the resonance stabilization. The reluctance of the tetrazole ring to supply electrons to the acyl group is exemplified by the high wave number of the carbonyl (1780-1800 cm^{-1}) (7) of 5-substituted N-acetyl tetrazoles. A comparison of these values with the carbonyl stretching of anhydrides (1820 and 1760 cm^{-1}) further exemplifies the similar electronic effects of the tetrazole ring and carboxyl group.

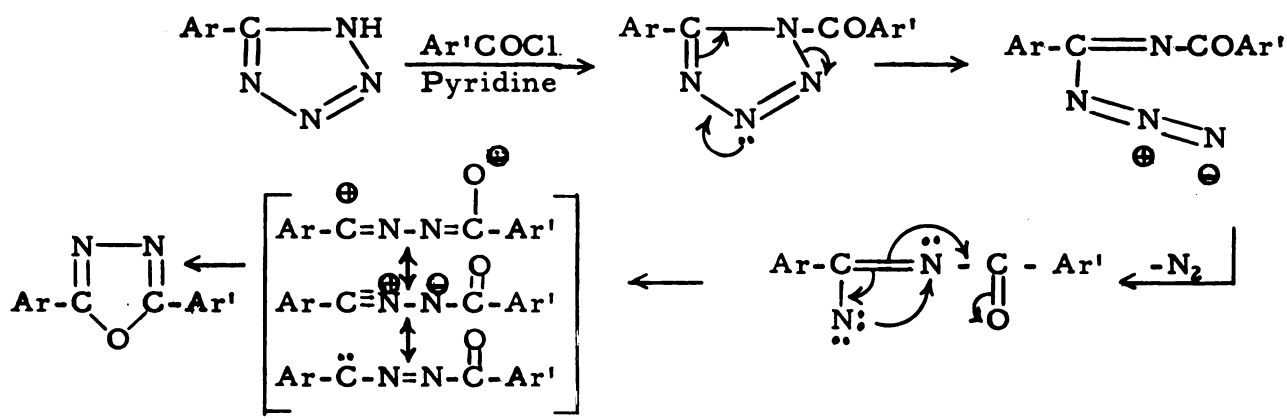
An alternate mechanism was one that had been originally suggested by Stollé (81) for the degradation of 5-aminotetrazole on prolonged heating with acetic anhydride to 2-acetamido-5-methyl-1, 3, 4-oxadiazole. Stollé envisioned the reaction as proceeding through an initial acetylation of the amino group followed by a double ring cleavage between the 1, 2 and 3, 4 nitrogens. Migration of the acetamido group from carbon to nitrogen was then supposedly followed by a second acetylation and recyclization to 2-acetamido-5-methyl-1, 3, 4-oxadiazole.



Herbst and Klingbeil (82) observed that when 1-p-nitrophenyl-5-aminotetrazole was acylated with acetic anhydride, 2-methyl-5-p-nitrophenylamino-1, 3, 4-oxadiazole resulted. The formation of this product could most easily be explained by Stollé's mechanism.



It was also suggested that the degradative acylation of 5-substituted tetrazoles could be explained by assuming an initial attack of the acyl group on the 1-(or 4-)position of the tetrazole ring followed by ring opening at the 1, 2-(or 3, 4-)positions. The oxadiazole could then be formed by elimination of nitrogen from the resulting azido group, by migration of the acylimido group from carbon to nitrogen, and by recyclization.



Ar and Ar' = Aryl

The mechanism proposed by Huisgen and his co-workers (7) involved elimination of the nitrogens at the 3, 4-(or 1, 2-)positions while the mechanisms suggested by Stollé (81) and by Herbst and Klingbeil (82) necessitated loss of the 2, 3 nitrogens. In order to acquire some insight into the mechanism, Herbst (83) prepared 5-phenyltetrazole enriched with N^{15} in the 1-(or 4-)position. Subsequent reaction with benzoyl chloride in pyridine furnished 2, 5-diphenyl-1, 3, 4-oxadiazole in which half of the label was left. Since half of the isotope was lost during acylation of the tetrazole, it was concluded that the elimination occurred from the 3, 4-(or 1, 2-) positions, and that, therefore, the pathway suggested by Huisgen, Sauer, and Sturm more accurately represented the course of the reaction.

It should be noted, however, that the formation of 2-methyl-5-p-nitrophenylamino-1, 3, 4-oxadiazole from the reaction of acetic anhydride

with 1-p-nitrophenyl-5-aminotetrazole as recorded by Herbst and Klingbeil (82) may have proceeded with elimination of the nitrogens in the 2, 3-position. However, an isotopic study of this compound has not been made.

In view of the mechanistic paths available for the conversion of tetrazoles into 1, 3, 4-oxadiazoles, it was of interest to study the reaction of 5-phenyltetrazole labeled in the 1-(or 4-)positions with acetic anhydride. The original labeling experiment conducted by Herbst (83) involved a benzoylation in the basic medium of pyridine, and it would be presumptuous to assume that the reaction in acetic anhydride would necessarily proceed by the same mechanism. Samples of 5-phenyltetrazole labeled to the extent of 4.4 at. percent N^{15} were heated with acetic anhydride at reflux temperature for nearly three hours. The 2-methyl-5-phenyl-1, 3, 4-oxadiazole obtained from the reaction was found to contain 4.06 at. percent of the label which could only have resulted from elimination of nitrogen from the 3, 4-(or 1, 2)positions on the ring. Hence, the reaction with acetic anhydride as well as the reaction of benzoyl chloride in pyridine are best described by the pathway suggested by Huisgen and co-workers (7).

EXPERIMENTAL

PART I

The Preparation of 5-Acetamidomethyltetrazole

Scheme A. (Unsuccessful)

(a) Acetylglycine. Glycine (150 g., 2.0 moles) was stirred with 600 ml. of water until it was nearly all dissolved. Acetic anhydride (415 g., 384 ml., 4 moles) was then added in one portion. Vigorous stirring was continued for about twenty minutes during which time the solution became hot and acetylglycine began to crystallize. The solution was placed in the refrigerator overnight to effect complete crystallization.

The crude product after filtering, washing with ice cold water, and drying at 110° C., weighed 166.2 g. The combined filtrates and washings were evaporated to dryness under reduced pressure at 60-90° C. The residue on recrystallization from 150 ml. of boiling water yielded a second fraction of 36 g. of product.

Both fractions were recrystallized from water to give 189.9 g. (81.2% of theory) of acetylglycine, m.p. 205-206° C.

This compound is reported by Herbst and Shemin to have a melting point of 207-208° C. (84).

(b) Attempted Preparation of the Acid Chloride of Acetylglycine. Acetylglycine (39.03 g., 0.33 mole) in benzene (300 ml.) was stirred with thionyl chloride (45.3 g., 27.4 ml., 0.38 mole) on a steam bath. After two hours the mixture became a dark brown in color. Solid

remained in the reaction flask throughout the course of the reaction period. The material was discarded.

A second attempt was conducted using acetylglycine (3.4 g.) and thionyl chloride (2.5 ml.). After allowing the mixture to stand for fifteen minutes at room temperature, the mixture became yellow and after ninety minutes it was brown.

Phosphorous pentachloride was utilized in a third attempt. By allowing acetylglycine (11.7 g., 0.1 mole) and phosphorous pentachloride (25 g., 0.12 mole) in toluene (100 ml.) to stir at room temperature for two hours, a considerable amount of darkening was observed. No reaction was observed to occur when the experiment was repeated at ice bath temperatures.

Scheme B.

(a) Methyleneaminoacetonitrile. Formaldehyde (810 g., 750 ml., 9.5 moles) and ammonium chloride (270 g., 5 moles) were stirred with cooling to 0-5° C. Then with vigorous stirring a solution of sodium cyanide (245 g., 4.9 moles) in 425 ml. of water was added at such a rate that 20-30 minutes were required for the addition of one-half of the solution. It was necessary to keep the temperature below 5° C. at all times. When one-half of the sodium cyanide solution had been added, the addition of glacial acetic acid (190 ml.) was commenced. The remaining sodium cyanide solution and the glacial acetic acid were added simultaneously at such a rate that 30-40 minutes were required for the complete addition of both.

The methyleneaminoacetonitrile began to separate as fine white crystals borne up by a foam shortly after the addition of acetic acid was started. After all solutions had been added, the mixture was stirred for an additional two hours. The product was washed first in 750 ml. and

then in 250 ml. of water and allowed to dry in the open at room temperature. The dried product weighed 216 g. (67% of theory), m.p. 126-127° C.

Adams and Langley report a melting point of 129° C. for this compound (77).

(b) Aminoacetonitrile hydrogen sulfate. To a solution of sulfuric acid (51.5 g., 28 ml. of 95%, 0.5 mole) in ethanol (125 ml. of 95%) at 45-50° C. was added 34 g. (0.5 mole) of methyleneaminoacetonitrile. The flask was closed and the mixture was vigorously shaken by hand. Solution took place and the mixture separated into two layers within 30 minutes. The mixture was maintained at about 50°C. The lower layer of aminoacetonitrile hydrogen sulfate gradually crystallized, and it was necessary to shake the mixture frequently to prevent the formation of a solid cake. After refrigerating the mixture overnight, the crude product was filtered and washed with 20 ml. of ice cold alcohol. The aminoacetonitrile hydrogen sulfate weighed 60.3 g. (78.3% of theory) (79).

(c) Aminoacetonitrile hydrochloride. (1) Aminoacetonitrile hydrogen sulfate (180 g., 1.17 moles) was finely powdered, covered with methanol (150 ml.) containing a trace of phenolphthalein, and neutralized by adding a solution of 32 g. (1.39 moles) of sodium in methanol (800 ml.) to the stirred mixture during the course of one hour. The reaction was carried out in a nitrogen atmosphere. The solution should remain neutral enough to keep the phenolphthalein colorless. The sodium sulfate was filtered off, and the filtrate was evaporated at room temperature in vacuo. The methanol removed was then replaced by ether.

The ethereal solution was treated with anhydrous hydrogen chloride gas while cooling in an ice bath. The aminoacetonitrile hydrochloride was removed by filtration. Further treatment of this filtrate

with hydrogen chloride gas yielded a small additional amount of the product. After drying in the open the aminoacetonitrile hydrochloride weighed 36.2 g. (33.6% of theory).

(2) Methyleneaminoacetonitrile (3.4 g., 0.05 mole) was added to 12.5 ml. of 95% ethanol, and the mixture was warmed to 50° C. Anhydrous hydrogen chloride gas was bubbled through the mixture with swirling until the solid just went into solution. Upon allowing the mixture to cool, aminoacetonitrile hydrochloride began to precipitate. Frequent swirling was advantageous at first to prevent the formation of a hard cake. The product was refrigerated overnight and filtered the next day. After washing the product with 10 ml. of ice cold ethanol and allowing it to dry, 4.5 g. (85% of theory) of aminoacetonitrile hydrochloride was obtained.

(d) Acetaminoacetonitrile. Aminoacetonitrile hydrochloride (30.g., 0.325 mole) was carefully warmed with 150 ml. of fresh acetic anhydride until the solution became clear and the hydrogen chloride was lost. After allowing the mixture to stand for a few minutes, the solution was diluted with benzene and evaporated to a syrupy consistency on a hot plate. The thick solution was then cooled and seeded; after crystallization was complete, the product was washed with ether. The crude product weighed 16 g. (50.3% of theory), and was purified by dissolving in chloroform, diluting with ether, and cooling. The purified product had a melting point of 76° C.

Acetoaminoacetonitrile is reported by Johnson and Gatewood to melt at 77° C. (85).

(e) 5-Acetamidomethyltetrazole. Acetaminoacetonitrile (5.88 g., 0.06 mole) and sodium azide (5.85 g., 0.09 mole) were added to a solution of 5.14 ml. (0.09 mole) of glacial acetic acid in 90 ml. of

1-butanol. The mixture was then refluxed for three days during which time most of the solid material went into solution.

After three days the refluxing was interrupted and the hot solution was carefully acidified by the dropwise addition of concentrated hydrochloric acid. The acidified solution was heated to near boiling and was filtered to remove the sodium chloride. After a five minute treatment of the filtrate with Norite, the filtered solution was evaporated in vacuo to one-half of its original volume. Upon refrigeration overnight the product crystallized. The filtrate was concentrated and cooled to yield additional product. The total crude product was recrystallized from 1-butanol, filtered, and washed with a little cold 1-butanol. The dried product gave 5.76 g. (68% of theory) of 5-acetamidomethyltetrazole, m.p. 157-158° C.

This compound is reported by McManus and Herbst to melt at 159.5-160° C. (4).

Analysis. Calculated for $C_4H_7N_5O$: C, 34.0; H, 5.0; N, 49.6
Found: C, 34.2; H, 4.9; N, 49.6.

The Preparation of 5-Benzamidomethyltetrazole

Scheme B.

(a) Benzamidoacetonitrile. Aminoacetonitrile hydrochloride (23.13 g., 0.25 mole) was dissolved in 100 ml. of distilled water, and benzoyl chloride (52.73 g., 42.2 ml., 0.375 mole) in 100 ml. of benzene and sodium hydroxide (30 g., 0.75 mole) in 75 ml. of water were added simultaneously to the stirred and cooled aminoacetonitrile hydrochloride solution. A few drops of methyl red indicator were also added to assure basicity at all times. The concurrent addition of the two solutions was carried out over a half hour period. After the solutions had all been added, the mixture was stirred for an additional fifteen minutes.

The precipitate of crude product was thoroughly washed with distilled water and was recrystallized from 95% ethanol after a treatment with Norite. The dried benzamidoacetonitrile weighed 25.5 g. (64% of theory), m.p. 139-140° C.

This compound is reported by Haack to have a melting point of 144° C. (86).

(b) 5-Benzamidomethyltetrazole. Benzamidoacetonitrile (8.00 g., 0.05 mole) and sodium azide (5.20 g., 0.08 mole) were added to a solution of 4.57 ml. (0.08 mole) of glacial acetic acid in 100 ml. of 1-butanol. The mixture was then refluxed for three days. Sometimes solid material, probably the sodium salt of the product, comes out of solution.

After three days the refluxing was interrupted and the hot solution was carefully acidified by the dropwise addition of concentrated hydrochloric acid. Additional 1-butanol was added to the mixture prior to acidification whenever material had come out of solution during the reaction. The acidified solution was heated to near boiling and was filtered to remove the sodium chloride. Crystals of product were formed upon refrigeration of the hot solution. The filtrate was concentrated and cooled to yield a small additional amount of product. The total crude product was recrystallized from water to give 8.6 g. (85% of theory) of pure 5-benzamidomethyltetrazole, m.p. 228-229° C.

This compound is reported by McManus and Herbst to melt at 229.5-230° C. (4).

Analysis. Calculated for $C_9H_9N_5O$: C, 53.2; H, 4.5; N, 34.5
Found: C, 53.4; H, 4.6; N, 34.3.

Attempted Condensation Reactions

Attempted Reaction of 5-Benzamidomethyltetrazole with Acetic Anhydride and m-Nitrobenzaldehyde. 5-Benzamidomethyltetrazole (1.02 g., 0.005 mole) was heated for five hours at 130-140° C. with acetic

anhydride (10 ml.), sodium acetate (0.41 g., 0.005 mole), and m-nitrobenzaldehyde (0.76 g., 0.005 mole). The reaction mixture was then refrigerated overnight. Sodium acetate was removed by filtration of the cold mixture. The filtrate was evaporated nearly to dryness, and the residue was treated with sodium hydroxide solution (0.1 N, 100 ml.) at room temperature. The resulting polymeric material was removed by filtration. The basic filtrate was acidified with concentrated hydrochloric acid, and the precipitate was removed. This solid was treated with Norite in a water-ethanol solution. Slow crystallization of the product from water-ethanol yielded pale cream colored needles of m-nitrocinnamic acid, m.p. 199-201° C. This compound is reported to melt at 199-200° C. (87).

The acidic filtrate was extracted with ether to yield a solid which, after recrystallization from water, proved to be benzoic acid.

Similar attempted condensations using benzaldehyde and anisaldehyde furnished only benzoic acid and the corresponding cinnamic acids from the Perkin condensation. Under the milder reaction condition of 3 hours at 90° C. a considerable amount of 5-benzamidomethyltetrazole was recovered.

The Reaction of 5-Substituted Tetrazoles with Acetic Anhydride

(a) 2-Methyl-5-benzamidomethyl-1,3,4-oxadiazole. 5-Benzamidomethyltetrazole (2.03 g., 0.01 mole) was refluxed with 40 ml. of acetic anhydride for 2½ hours. The mixture was then evaporated on a steam bath *in vacuo*. Ethanol (50 ml.) was added and removed on a steam bath as before. Again ethanol (50 ml.) was added and the mixture was treated with Norite. The clear ethanolic filtrate was concentrated to 5 ml., and ether (30 ml.) was added to precipitate the product. This crude product

was purified by recrystallization from ethyl acetate and gave 0.98 g. (45.2% of theory) of 2-methyl-5-benzamidomethyl-1, 3, 4-oxadiazole, m.p. 133.5-134.5° C.

Analysis. Calculated for $C_{11}H_{11}N_3O_2$: C, 60.82; H, 5.10; N, 19.34.
Found: , , , . . C, 60.76; H, 5.09; N, 19.11.

(b) Degradation of 2-methyl-5-benzamidomethyl-1, 3, 4-oxadiazole. The following degradation was carried out as a proof of structure of the oxadiazole:

2-Methyl-5-benzamidomethyl-1, 3, 4-oxadiazole (0.5 g., 0.0023 mole) was refluxed for $4\frac{1}{2}$ hours with 10% hydrochloric acid (5 ml.) and allowed to stand at room temperature overnight. The next day the crystallizate of benzoic acid was filtered, washed, and dried; the yield was 0.25 g. (89% of theory) of pure product, m.p. 122-123° C., mixture m.p. 122-123° C.

The filtrate was combined with the small amount of wash water, and the mixture was concentrated to the original volume. The aqueous solution was then extracted with ether (7 ml.) in a small separatory funnel. Upon evaporation of the ether the odor of acetic acid could easily be detected. In addition benzoic acid (0.01 g.) was observed as a small residue.

The aqueous layer from the extraction was evaporated to dryness by warming in vacuo. The resulting residue was then boiled with absolute ethanol (25 ml.) for several minutes. A white residue remained on the filter paper after filtration of the hot ethanolic mixture. When the ethanolic mixture was allowed to cool, a further yield of material was obtained which proved to be identical with the material on the filter paper. The combined fractions were carefully washed with a little cold ethanol and allowed to dry to give 0.16 g. (67.5% of theory) of hydrazine dihydrochloride, m.p. 199-202° C.

This compound is reported to melt at 198° C. (88).

The hydrazine dihydrochloride was treated with an excess of m-nitrobenzaldehyde in ethanol-water mixture to form the corresponding azine, m.p. 196-198° C.

This compound is reported by Knöpfer to melt at 196-197° C. (89).

The alcoholic filtrate from above was evaporated to dryness by heating in vacuo. To the residue was added pyridine (15 ml.) and the mixture was heated several minutes on a steam bath. Filtration of this mixture left a residue of crude glycine on the filter paper. This crude glycine was boiled several minutes with 15 ml. of absolute ethanol. Filtration of the mixture left a residue of purer glycine which weighed 0.13 g. (50.6% of theory), m.p. 225-230° C.

Glycine is reported to melt at 225-233° C. (90).

The glycine was warmed with acetic anhydride in water, and from the cooled mixture crystals of acetylglycine were removed, m.p. 206-208° C., mixed m.p. 206-208° C.

This compound is reported by Herbst and Shemin to have a melting point of 207-208° C. (84).

(c) Formation of 2-Methyl-5-phenyl-1,3,4-oxadiazole from N^{15} Labeled 5-Phenyltetrazole. 5-Phenyltetrazole (0.146 g., 0.001 mole) labeled with N^{15} in the 1-(or 4-)position of the ring to the extent of 4.4 at. percent was refluxed with acetic anhydride (4 ml.) for 2½ hours. The excess acetic anhydride was then removed by heating gently in vacuo. The resulting syrup solidified and was purified by sublimation at 56° C. under vacuum pump pressure. The resulting 2-methyl-5-phenyl-1,3,4-oxadiazole was found to contain N^{15} to the extent of 4.06 at. percent, m.p. 68° C.

This compound is reported by Stollé (91) to have a melting point of 67° C.

All the mass spectra were run on a Type 21-103 C Mass Spectrometer (Consolidated Electrodynamics Corp.). The extent of labeling of 5-phenyltetrazole could not be obtained directly due to decomposition, but the compound was methylated by the procedure of Henry (92) to give a mixture of 1-methyl-5-phenyltetrazole and 2-methyl-5-phenyltetrazole which were found to contain 4.4 and 4.5 at. percent N^{15}, respectively. The resulting 2-methyl-5-phenyl-1,3,4-oxadiazole contained 4.06 at. percent N^{15} which corresponded to a loss of half the label.

RESULTS

PART II

In PART I a number of tetrazole analogues of amino acids were mentioned which had been prepared by McManus and Herbst (4) and by Sterken and Herbst (5) as potential antimetabolites. In addition, the results of an attempt to extend the preparation of tetrazole amino acid analogues by Erlenmeyer type condensations were discussed, and the failures were rationalized in light of the interaction of the 5-substituted tetrazoles with acetic anhydride to form 2, 5-disubstituted 1, 3, 4-oxadiazoles. The complete list of tetrazole analogues which have been prepared includes the analogues of glycine, DL-alanine, β-alanine, DL-phenylalanine, and DL-tryptophan whose preparation has been described by McManus and Herbst and also the analogues of DL-α-amino-butyric acid, DL-valine, Dl-leucine, α-aminoisobutyric acid, and DL-C-phenylglycine as prepared by Sterken and Herbst.

In this section a description is given for the syntheses of the three tetrazole analogues of DL-glutamic acid as potential antimetabolites. These analogues result from the substitution of tetrazolyl groups for the alpha and/or gamma carboxyl groups of DL-glutamic acid.

L(+)Glutamic acid itself is biologically one of the most important of the amino acids since it is known to participate in many important chemical processes in plants, animals and microorganisms. In addition to being an important constituent of proteins, glutamic acid is involved in such biochemical processes as transamination, deamination, glutamine and glutathione formation, and nitrogen fixation as well as in others.

Glutamic acid was first synthesized by Wolff (93) in 1890 from levulinic acid through a long series of classical reactions. This scheme

possessed no value as a preparative method, but rather served as a proof of structure of DL-glutamic acid. In 1925 Keimatsu and Sugasawa (94) developed a synthesis of the acid from acrolein. More recently it has been found that DL-glutamic acid can be conveniently prepared by acidic

$$CH_2 = CH\text{-}CHO \xrightarrow[C_2H_5OH]{HCl} Cl\text{-}CH_2CH_2CH(OC_2H_5)_2 \xrightarrow[HI]{KCN}$$

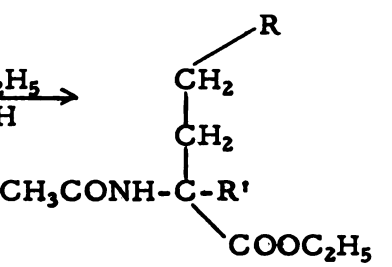
$$(C_2H_5O)_2CHCH_2CH_2CN \xrightarrow[C_2H_5OH]{KOH} (C_2H_5O)_2CHCH_2CH_2COOH \xrightarrow{H_2SO_4}$$

$$HO_2C\text{-}CH_2CH_2CHO \xrightarrow{KCN} HO_2C\text{-}CH_2CH_2CH(OH)CN \xrightarrow{NH_4Cl}$$

$$HO_2C\text{-}CH_2CH_2CH(NH_2)CN \xrightarrow[HCl]{H_2O} HO_2CCH_2CH_2CH(NH_2)CO_2H$$

hydrolysis of the products resulting from the Michael addition of acrylonitrile or ethyl acrylate with diethyl acetamidomalonate or ethyl acetamidocyanoacetate (95-96).

$$H_2C = CH\text{-}R + HC(NHCOCH_3)(COOC_2H_5)(R') \xrightarrow[C_2H_5OH]{NaOC_2H_5}$$



R = -CN, $-COOC_2H_5$

R' = -CN, $-COOC_2H_5$

Since 5-substituted tetrazoles are most conveniently prepared from the corresponding nitriles, an obvious approach to the tetrazole analogues of DL-glutamic acid would be through interaction of the nitriles formed in the Michael additions with either hydrazoic acid or one of its salts. By subsequent hydrolysis and decarboxylation the analogues could be obtained.

This approach was utilized in the preparation of 4-amino-4-(5'-tetrazolyl)butanoic acid, the alpha-tetrazole analogue of DL-glutamic acid. The interaction of ethyl acetamidocyanoacetate with ethyl acrylate in ethanol containing sodium ethoxide furnished yields of the Michael addition product which were consistently in the range of 75-80%.

The correspodning 5-substituted tetrazole was easily prepared by refluxing the alpha-nitrile for three days in an ethanolic solution containing sodium azide and acetic acid. This procedure was a modification of the method developed by Herbst and Wilson (39). After removal of the ethanol, the resulting syrup was dissolved in a minimum of water, the solution was cooled, and concentrated hydrochloric acid was added. Ethyl 2-acetamido-2-(5'-tetrazolyl)-4-carboethoxybutanoate crystallized in 79% yield upon refrigeration.

The alpha-tetrazole analogue of DL-glutamic acid was obtained by hydrolyzing ethyl 2-acetamido-2-(5-tetrazolyl)-4-carboethoxybutanoate with 10% hydrochloric acid. The dilute hydrochloric acid was removed and the resulting syrup was dissolved in a minimum of water. A 70% yield of 4-amino-4-(5'-tetrazolyl)butanoic acid was obtained after recrystallizing the crude product that separated upon adjusting the solution to a pH of 5.

The same approach proved to be satisfactory for the preparation of 1-amino-1,3-di(5'-tetrazolyl)propane, the ditetrazole analogue of DL-glutamic acid. A Michael addition of ethyl acetamidocyanoacetate with acrylonitrile was done in a manner analogous to that previously described. The resulting ethyl 2-acetamido-2,4-dicyanobutanoate was obtained in 62% yield by merely refrigerating the reaction mixture. Additional dinitrile could be recovered by first removing the ethanol, dissolving the product in chloroform, washing with water, and evaporating the chloroform to a syrup. An additional 10% of the dinitrile resulted upon refrigeration of an ethanolic solution of the syrup containing isopropyl ether.

The corresponding ditetrazole was prepared by refluxing the dinitrile for three to five days in 1-butanol containing sodium azide and acetic acid. Concentrated hydrochloric acid was added to the hot alcoholic solution to remove the sodium ions as sodium chloride. When the hot solution was distinctly acidic (pH of 1-3), the salt was filtered and the filtrate was evaporated to a syrup.

All efforts to induce crystallization of the crude ethyl 2-acetamido-2, 4-di(5'-tetrazolyl)butanoate met with failure. The product would not separate from cold aqueous solution containing hydrochloric acid even upon refrigeration for several days at -10°C. Repeated treatment of the syrup with boiling cyclohexane effected a gradual increase in its viscosity until eventually the ditetrazole was nearly rigid in boiling cyclohexane. However, this material was only an amorphous solid possessing no definite melting point.

A small sample of the amorphous solid was chromatographed on an alumina column prepared with acetone. Elution was carried out successively with acetone, acetone-ethanol mixtures, ethanol, ethanol-water mixtures, and water. Four products were obtained and fractions of the same product were combined to give four fractions. Only the latter two of the four fractions were obtained as crystalline materials and identified, however, the second and major fraction contained the desired ethyl 2-acetamido-2,4-di(5'-tetrazolyl)butanoate. The first fraction, which was present only as a small amount of syrup, was not identified but may have contained some butyl ester of the product formed by interaction with the solvent. The third and fourth fractions were found to be 1-acetamido-1,3-di(5'-tetrazolyl)propane and aluminum acetate, respectively. Fraction three, which resulted from partial hydrolysis of the product, was identified by comparison with an authentic sample whose preparation will be described in a later section. In spite of all efforts at purification the ethyl 2-acetamido-2,4-di(5'-tetrazolyl)butanoate remained a clear, colorless syrup which defied crystallization.

The ditetrazole analogue of DL-glutamic acid was obtained by hydrolyzing the syrup containing ethyl 2-acetamido-2,4-di(5'-tetrazolyl)-butanoate with 10% hydrochloric acid. The hydrochloric acid was subsequently removed by heating the hydrolysis mixture in vacuo, and the resulting syrup was dissolved in a minimum of water. The ditetrazole analogue readily separated upon scratching the walls of the flask with a glass rod after first adjusting the solution to a pH of 5 with pyridine. 1-Amino-1,3-di(5'-tetrazolyl)propane was obtained as a clear crystalline material in 50-55% yield after two recrystallizations from water. It was found that the analogue possessed water of crystallization in the ratio of three molecules of water per two molecules of the ditetrazole analogue. DL-glutamic acid, by comparison, crystallizes with water in a one to one mole ratio.

The preparation of 2-amino-4-(5'-tetrazolyl)butanoic acid, the gamma-tetrazole analogue of DL-glutamic acid, was achieved by a route analogous to that utilized in the preparation of the other two analogues. The Michael addition of diethyl acetamidomalonate with acrylonitrile (96) afforded large crystals of ethyl 2-acetamido-2-carboethoxy-4-cyano-butanoate (also referred to as the gamma-nitrile) in 90% yield by merely refrigerating the reaction mixture.

A considerable amount of difficulty was encountered in attempting to prepare the corresponding 5-substituted tetrazole from the gamma-nitrile in a manner similar to that employed during the preparations of the other two analogues. Initially, the gamma-nitrile was refluxed for a week in ethanol containing sodium azide and acetic acid. The presence in the flask of solid sodium azide throughout the reaction period was evidence that the reaction was not proceeding. On the other hand, a one day reflux period was sufficient to effect solution of all the azide during reaction with the alpha-nitrile isomer. Upon evaporation of the ethanol a residue resulted which upon recrystallization from water furnished the original gamma-nitrile in 65% yield.

In order to overcome the reluctance of the gamma-nitrile to react with the hydrazoic acid, a twelve day reflux period was carried out in 1-butanol with sodium azide and acetic acid. After termination of the reaction period, the hot alcoholic solution was acidified to a pH of 1 with concentrated hydrochloric acid, and the resulting sodium chloride was filtered. Evaporation of the filtrate left a syrupy residue which was dissolved in dilute hydrochloric acid and refrigerated, however, no material crystallized. Since ethyl 2-acetamido-2,4-di(5'-tetrazolyl)butanoate failed to crystallize under similar conditions, it was not too surprising that the ethyl 2-acetamido-2-carboethoxy-4-(5'-tetrazolyl)butanoate, which presumably was present in the solution, did not separate. Therefore, the intermediate was hydrolyzed by refluxing overnight in 15% hydrochloric acid. The dilute hydrochloric acid was removed by heating in vacuo, and the residue was dissolved in a minimum of water. After adjusting the mixture to a pH of 5 with ammonium hydroxide, the solution was cooled while the walls of the flask were scratched with a glass rod. Unlike the alpha- and ditetrazole analogues, the gamma-tetrazole analogue failed to crystallize under these conditions.

Since it was believed that the failure of the gamma-tetrazole analogue to separate may have been due to water solubility, a procedure was employed which was developed by Finnegan, Henry, and Lofquist (52) for the isolation and purification of water soluble tetrazoles. To the neutralized hydrolysis mixture containing the product was added an excess of saturated cupric acetate solution. The insoluble cupric salt of the gamma-tetrazole analogue was filtered and washed carefully with water to remove all by-products. Fortunately, the cupric salt of glutamic acid was soluble, since glutamic acid would result from the hydrolysis of any unreacted nitrile and would, therefore, constitute an important impurity. Hydrogen sulfide gas was bubbled into a stirred aqueous suspension of the cupric salt of the gamma-tetrazole analogue at 50° C. Careful

filtration of the cupric sulfide left a clear solution from which 2-amino-4-(5'-tetrazolyl)butanoic acid crystallized upon concentration and refrigeration. Unfortunately, the gamma-tetrazole analogue was obtained in yields of only 15-20% after recrystallization from water.

In order to try and achieve better yields of the analogue, a number of other procedures were utilized which had been developed for converting nitriles into 5-substituted tetrazoles. The first of these alternate methods considered involved interaction of the nitrile in dimethylformamide with ammonium azide prepared by double decomposition of sodium azide and ammonium chloride. This alternate method, which was developed by Finnegan, Henry, and Lofquist (52), was utilized in view of its successful application by these workers in the preparation of 5-alkyl tetrazoles from the corresponding alkyl cyanides. The following three sets of reaction conditions were employed: three days at 95° C., one day at 125° C., two days at 125° C. In all cases the reaction mixture turned a chocolate brown while reaction with the azide remained incomplete as evidenced by a considerable amount of sublimed ammonium azide in the condenser throughout the reaction period. After terminating the reaction, the dimethylformamide was removed from the brown solution by heating in vacuo. The residue was dissolved in water and treated twice with Norite to yield a pale yellow solution which was subsequently hydrolyzed by adding hydrochloric acid to give a 10% solution and refluxing overnight. After evaporating the hydrolysis mixture to dryness, water was added to dissolve the residue and the pH was adjusted to 5 with sodium hydroxide solution. Only a deep blue solution resulted upon addition of cupric acetate, and since a control solution containing 2-amino-4-(5'-tetrazolyl)butanoic acid along with ammonium chloride and sodium chloride furnished a precipitate at a pH of 5 with cupric acetate solution, it was concluded that no appreciable amount of the desired product had been formed. An interaction between the gamma-nitrile and ammonium azide

in methyl cellosolve was also found to be unsuccessful in producing any product by a similar procedure.

Another method attempted to effect the desired conversion was one developed by Behringer and Kohl (51). These workers treated nitriles in tetrahydrofuran solution with aluminum azide prepared in situ from aluminum chloride and sodium azide. Applications of this procedure with the gamma-nitrile resulted in the formation (in the reaction mixture) of a yellow solid which was found to contain only sodium chloride, aluminum azide, gamma-nitrile, and decomposition products. The liquid portion of the reaction mixture contained unreacted gamma-nitrile along with aluminum azide and decomposition products. Again no product could be obtained by working up the reaction mixture by the procedure of Finnegan, Henry, and Lofquist (52) subsequent to the removal of the sodium chloride and aluminum as its hydroxide.

A direct interaction between the gamma-nitrile and hydrazoic acid was attempted by heating the gamma-nitrile in a sealed tube with a standardized solution of hydrazoic acid in xylene. This procedure was a modification of a method developed in 1950 by Mihina and Herbst (38) in which the nitrile was heated in a sealed tube with a benzene solution of hydrazoic acid. The reaction was interrupted after heating at 132° C. for a period of ten days. After opening the tube, the golden-brown xylene solution was removed and the residual syrup was taken up in hot ethanol and combined with the xylene solution. After treatment with Norite, the solvent was replaced by 12% hydrochloric acid and the mixture was refluxed overnight. Treatment of the concentrated, neutralized reaction mixture with cupric acetate solution afforded only a very small amount of precipitate from which no product could be obtained.

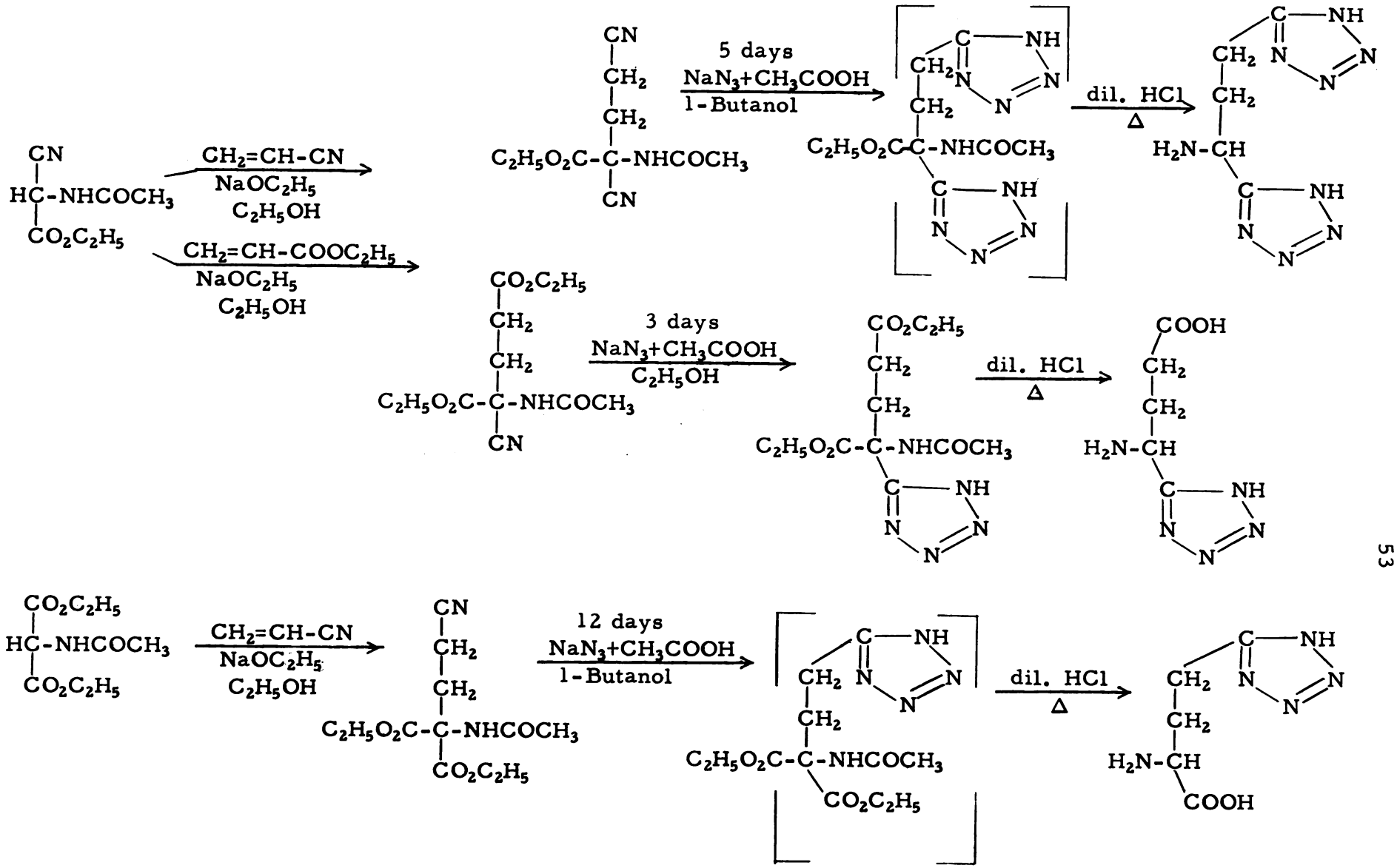
Only two procedures other than the original reaction of hydrazoic acid in 1-butanol afforded any 2-amino-4-(5'-tetrazolyl)butanoic acid. The first of these was a modification of the method of Herbst and Wilson (39)

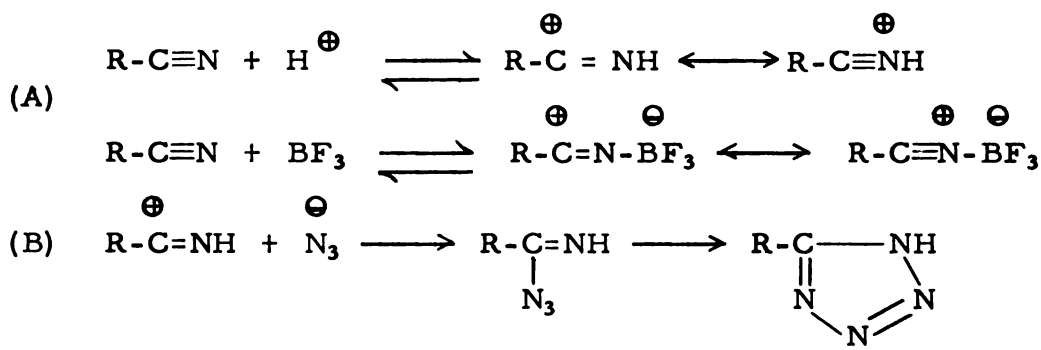
in which methyl cellosolve was used in place of 1-butanol. The modified method furnished yields of 21-24% of the gamma-tetrazole analogue which was slightly higher than those obtained with 1-butanol. The other, less successful, alternate method involved the interaction of lithium azide with the gamma-nitrile in methyl cellosolve. The lithium azide was prepared in situ from sodium azide and lithium chloride as Huisgen and his co-workers (7) had done. Isolation of the product by the use of cupric acetate furnished 7% of 2-amino-4-(5'-tetrazolyl)butanoic acid.

The following scheme summarizes the reactions involved in the preparation of the three tetrazole analogues of DL-glutamic acid (page 53). Each of the three tetrazole analogues is known to exist in its dipolar form as do the amino acids.

The large difference in yields and rates of reaction between the alpha-nitrile and gamma-nitrile isomers was not too surprising in view of the work conducted by Finnegan, Henry, and Lofquist (52). These workers found that in the preparation of 5-alkyltetrazoles the use of ammonium azide in dimethylformamide led to improved yields and shorter reaction times. The other methods were known to be more applicable to the preparations of 5-aryltetrazoles or 5-alkyltetrazoles containing electronegative substituents on the alpha-carbon. Unfortunately, the use of ammonium azide with the gamma-nitrile led to a large amount of decomposition.

The rates of tetrazole formation with the various methods are dependent upon several factors, i.e., the temperature, the nitrile used, the solvent polarity, and the Lewis acid strength. Of course, a higher reaction temperature would lead to an increased rate of reaction. The effects of the solvent polarity and Lewis acid strength can be rationalized in light of a mechanism involving an initial reaction with the Lewis acid (A). The azide ion subsequently attacks the relatively positive carbon, and the resulting imide azide cyclizes to the tetrazole (B).


CN
HC-NHCOCH3
CO2C2H5
CH2=CH-CN
NaOC2H5
C2H5OH
CH2=CH-COOC2H5
NaOC2H5
C2H5OH
C2H5O2C-C-NHCOCH3
5 days
NaN3+CH3COOH
1-Butanol
dil. HCl
Δ
3 days
NaN3+CH3COOH
C2H5OH
12 days
NaN3+CH3COOH
1-Butanol
COOH
H2N-CH
CH2
NH
N



Increasing Lewis acid strength would tend to shift step (A) to the right and, hence, to increase the overall reaction rate. Increased solvent polarity would tend to act in the same direction by effecting greater solubility of the various azides and higher concentrations of azide ions.

In accord with the theory that the reaction rates are dependent upon the extent of positive charge on the attacked carbons, it is not surprising that the reaction rates vary greatly from one nitrile to another. Electronegative groups attached to the nitrile group or to the alpha-carbon would be expected to accelerate the reaction by increasing the extent of positive charge at the reaction site. This has been observed by Finnegan, Henry, and Lofquist (52) who found that the rates of formation of substituted 5-phenyltetrazoles followed Hammett's equation (97). Further these workers found that ethyl cyanoacetate is converted to its tetrazole in better yields, at lower temperatures, and with shorter reaction times than is butanenitrile when the reactions were carried out with ammonium azide in dimethylformamide. The presence of the carboethoxy and acetamido groups on the alpha-carbon of ethyl 2-acetamido-2-cyano-4-carboethoxybutanoate should tend to increase the extent of positive charge at the reaction site and, hence, the rate of reaction.

The low yields of 2-amino-4-(5'-tetrazolyl)butanoic acid can be attributed to several factors. The gamma-nitrile was essentially an alkyl nitrile and, therefore, would not be expected to react rapidly with hydrazoic acid in alcohol. The presence of two carboethoxy groups and an acetamido group at the end of the molecule removed from the nitrile

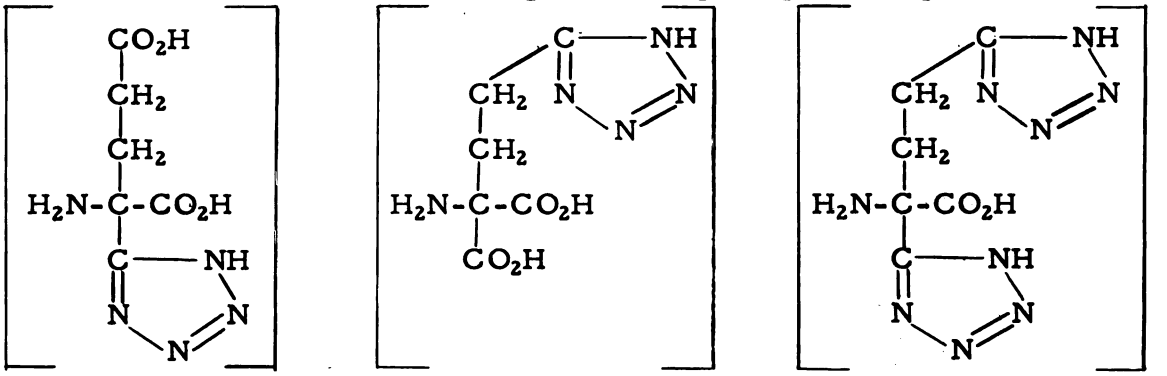
group could conceivably render a retarding effect by attracting azide ion toward the relatively positive carbonyl carbons and, hence, away from the nitrile. In addition, the extent of correlation between reaction rates and yields depends upon the importance of side reactions and the stability of the product. In the case of the gamma-nitrile, a considerable amount of ammonium azide collected in the condenser after several days of refluxing in 1-butanol or methyl cellosolve with sodium azide and acetic acid. This suggested that interaction of the nitrile and the solvent had occurred under the acidic conditions. It was well-known that iminoethers are formed from nitriles, alcohols, and mineral acids at room temperature, and further that conversion to the ortho esters occurs with the release of ammonia on heating. It is conceivable that upon prolonged heating the nitrile reacted under the acidic conditions with the solvent and/or small amounts of water impurity to furnish ammonia which was observed as ammonium azide. To the extent that this occurred, the yield of tetrazole analogue would be reduced.

The inability to recover all of the gamma-tetrazole analogue formed also contributed to the low overall yield. The necessity of isolating the material as its cupric salt undoubtedly resulted in some loss since the cupric salt was somewhat water soluble and had to be washed carefully. In addition, 2-amino-4-(5'-tetrazolyl)butanoic acid was more water soluble than any of the other analogues and, hence, more loss would occur in its recrystallization than with the others.

The intermediate position occupied by 1-amino-1,3-di(5'-tetrazolyl)-propane, the ditetrazole analogue, is in good agreement with the preceding arguments. The formation of the ditetrazole analogue would not be expected to occur to the extent of the alpha-tetrazole analogue. However, formation somewhat in excess of that of the gamma-tetrazole analogue might have occurred through some unknown effect rendered by

the alpha-tetrazole moiety upon the formation of the second tetrazole ring. Probably more important was the fact that the ditetrazole analogue was very water insoluble in the cold, and, therefore, it was readily recovered from the reaction mixture and purified with minimum loss.

The three tetrazole analogues of DL-glutamic acid were formed by acidic hydrolysis of the intermediate tetrazoles which possessed an acetamido group and either one or two carboethoxy groups. Under the conditions of refluxing in 10-20% hydrochloric acid solution decarboxylation was complete. An attempt was made to isolate the intermediate acids, (I-III), which must have resulted from hydrolysis of the ester and amide linkages, prior to decarboxylation by the utilization of basic hydrolysis. The three intermediate tetrazoles were refluxed with aqueous solutions of sodium hydroxide, potassium hydroxide, and barium hydroxide to effect the hydrolysis. Upon neutralization with either mineral acid or acetic acid a gelatinous precipitate separated.

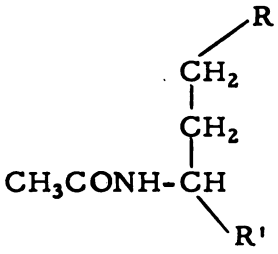


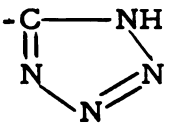
I II III

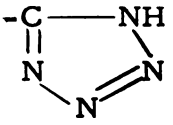
When the basic hydrolysate was diluted with water prior to acidification, only a clear solution resulted upon the addition of acid. A gradual concentration of this solution by means of an air jet at room temperature caused the solution to set to a transparent gel. All the gelatinous precipitates formed from the various tetrazole intermediates, when thoroughly washed

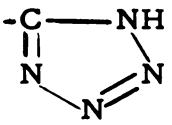
and allowed to dry in the air, yielded white infusible powders which were insoluble in organic solvents, water, concentrated acids, and weak bases. Only strong bases would dissolve the materials. In addition, the white powders were found to contain the metal ions of the base used in the hydrolysis. By using only a three to one mole ratio of base to intermediate tetrazole, it was possible upon acidification to isolate small amounts of the tetrazole glutamic acid analogues along with moderate yields of the corresponding acetylated analogues. At no time were any of the acid intermediates (I-III) isolated.

A number of reactions have been carried out with the three tetrazole glutamic acid analogues which have led to nine new compounds. The three acetyl derivatives (IV-VI) have been prepared by allowing aqueous solutions of the analogues to interact at room temperature with acetic anhydride in acetic acid.

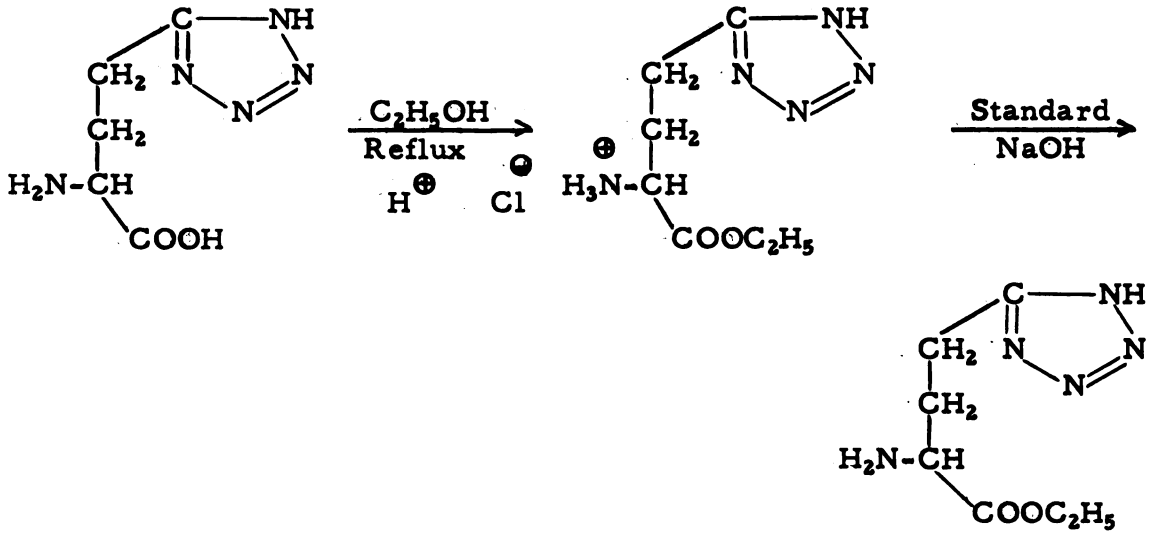


IV: R = $-CO_2H$; R' = 

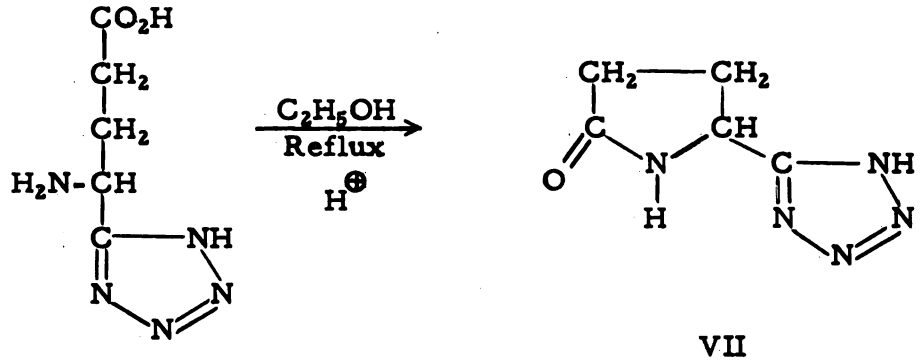
V: R =  ; R' = $-CO_2H$

VI: R =  = R'

Esterification of 2-amino-4-(5'-tetrazolyl)butanoic acid was accomplished by refluxing the analogue in acidic ethanol. The resulting hydrochloride was isolated, analyzed, and converted into the free amino ester by the addition of an equivalent amount of standard sodium hydroxide solution. An attempt to esterify 4-amino-4-(5'-tetrazolyl)butanoic acid



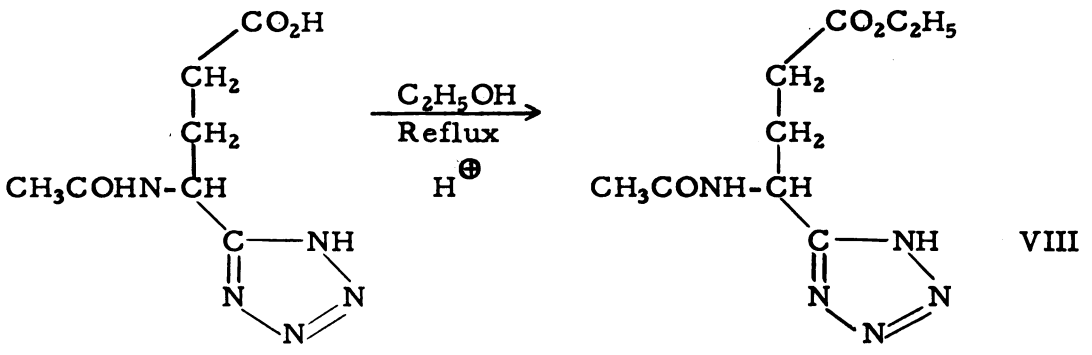
resulted in the formation of a lactam (VII). Under the nearly anhydrous conditions of absolute ethanol containing a small amount of hydrogen



VII

chloride, it was not too surprising that cyclization to 5-(5'-tetrazolyl)-2-pyrrolidone occurred. This structure was readily confirmed by infrared and elemental analysis.

Ethyl 4-acetamido-4-(5'-tetrazolyl)butanoate (VIII) was prepared from the corresponding acid by refluxing in acidic ethanol. This compound was found to be very water soluble and somewhat difficult to crystallize. All attempts to prepare and isolate its isomer in crystalline



VIII

form by esterification of 2-acetamido-4-(5'-tetrazolyl)butanoic acid or by acetylation of ethyl 2-amino-4-(5'-tetrazolyl)butanoate met with failure.

The hydrochlorides of the two monotetrazole analogues were readily prepared by the evaporation of solutions of the analogues in hydrochloric acid. The hydrochlorides were found to be more soluble in concentrated hydrochloric acid than is the hydrochloride of glutamic acid. The ditetrazole analogue failed to yield a crystalline hydrochloride.

An interesting phenomenon was observed during the thermal decomposition of 1-amino-1,3-di(5'-tetrazolyl)propane. At its melting point the analogue decomposed with much darkening and foaming, and in addition, ammonium azide sublimed from the mixture and collected in the cooler portions of the tube. By conducting the thermal decomposition on a weighed sample, it was found that two moles of the ditetrazole were required to furnish one mole of ammonium azide. No crystalline products could be recovered from the dark decomposed residue. Neither the alpha-tetrazole analogue nor the gamma-tetrazole analogue exhibited similar behavior. However, the alpha-tetrazole analogue melted with evolution of water which is attributed to lactam formation and is in agreement with the dependence of the melting point upon the rate of heating.

The apparent pK values of glutamic acid and its three tetrazole analogues were determined by titration of weighed samples of the tetrazoles in aqueous solution with standard sodium hydroxide solution.

The determinations were conducted at 25 ± 2° C. using a Beckman Model G pH meter.

The titration curves were obtained in each case by plotting the equivalents of sodium hydroxide added per mole of sample on the ordinate against the pH on the abscissa. The three apparent pK values were then obtained by taking the pH of the solution at readings of 0.5, 1.5, and 2.5 on the ordinate. The end points were observed as breaks in the curves at ordinate readings of 1.0, 2.0, and 3.0.

The titrations were conducted upon the pure hydrochlorides of the analogues with one exception. The ditetrazole analogue of DL-glutamic acid could not be converted to a crystalline hydrochloride, and, therefore, the calculated amount of standard hydrochloric acid was added to a weighed sample of the analogue. This solution of the hydrochloride was then titrated.

The following conclusions can be drawn from an examination of the apparent pK values as listed in Table 1.

1. The tetrazole moiety lowers the basicity of the amine group by 1.23 to 1.44 pK units when alpha to the amine and by 0.0 to 0.21 pK units when gamma to the amine.

2. The presence of the tetrazole moiety alpha to the amino group increases the acidity of the gamma substituent whether carboxyl or tetrazolyl.

3. The presence of the tetrazole moiety gamma to the amino group lowers the acidity of the alpha substituent whether carboxyl or tetrazolyl.

4. The gamma-tetrazole moiety is less acidic than the gamma-carboxyl group by 0.60 pK units when the alpha substituent remains the same.

5. The alpha-tetrazole moiety is comparable in acidity to the alpha-carboxyl group when the gamma substituent remains the same.

Table 1. Apparent pK values of glutamic acid and its three tetrazole analogues in aqueous solution at 25° C.

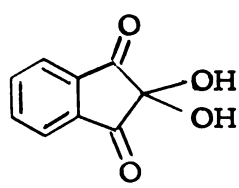
Compound	pK_1	pK_2	pK_3
Glutamic acid	2.16 (2.19)*	4.23 (4.28)*	9.46 (9.66)*
α-Tetrazole analogue	2.08	3.97	8.23
γ-Tetrazole analogue	2.33	4.83	9.46
Ditetrazole analogue	2.42	4.57	8.02

*These values in parentheses are those given by West and Todd (98) for glutamic acid.

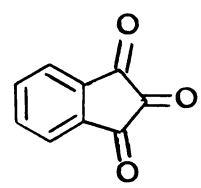
Rf (rate of flow) values for the two monotetrazole analogues were determined along with those for glutamic acid and glycine when it was observed that these analogues would give color reactions with ninhydrin. The two solvent systems which were employed gave Rf values for the monotetrazole analogues which differed considerably from those of glutamic acid. An eighty percent solution of phenol in water was used as the first system, while a water saturated mixture of 1-butanol in 1,4-dioxane in a ratio of four to one was used as the second. The temperatures were recorded for the individual determinations. The Rf value is defined as the ratio of the distances traveled by the sample to that of the solvent system. Mf (molecular flow) values are calculated by dividing the molecular weight of the sample by one hundred Rf (99). Increasing the length of the alkyl chain of an amino acid causes an increase in the Rf value, however, if Mf values for a particular series are compared, they are found to be similar. Hence, the Mf value may be thought of as expressing the relative amounts of hydrophilic and lipophilic character of a given amino acid.

The Rf and Mf values for the four compounds are listed in Table 2. As expected, the Mf values of the monotetrazole analogues are similar to those of glutamic acid.

The color reaction observed when ninhydrin (IX) reacts with amines, amino acids, amino alcohols, 5-aminoalkyltetrazoles, etc., has been the subject of much controversy, and a number of mechanisms (100) have been suggested to account for the observed colors and products. Recently McCaldin (100) reviewed the chemistry of ninhydrin and suggested



IX

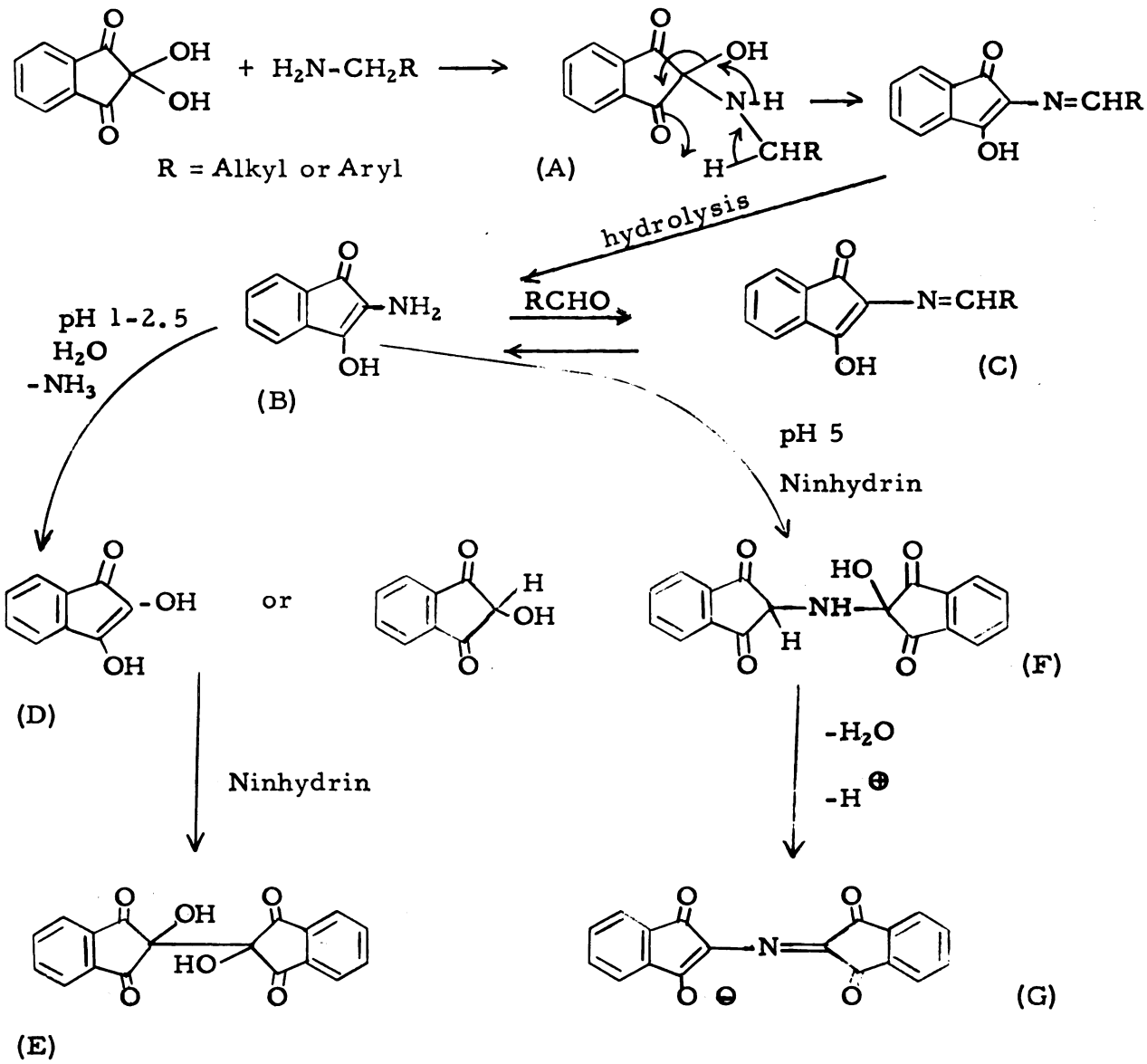


X

Table 2. Rf and Mf values for glutamic acid, glycine and the two mono-tetrazole analogues with two solvent systems.

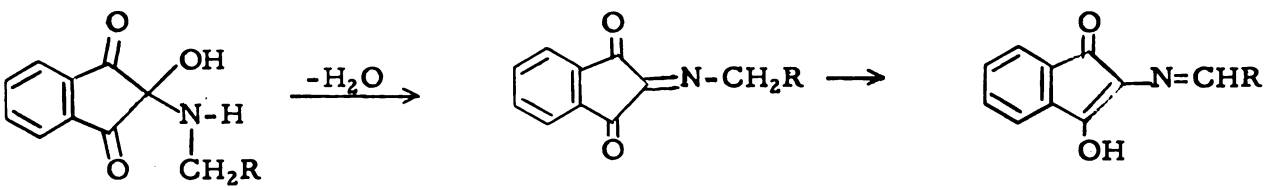
Compound	Phenol-Water System		Butanol-Dioxane-Water System	
	Rf	Mf (Average)	Rf	Mf (Average)
Glutamic acid	0.335±.02	4.4	0.16±.015	9.15
Glycine	0.42±.015	1.8	0.16±.015	4.7
γ-Tetrazole analogue	0.42±.015	4.05	0.22±.015	7.8
α-Tetrazole analogue	0.345±.02	4.95	0.24±.015	7.1

the following reaction path as being consistent with the known facts. A number of the intermediates indicated (B, C, E) in the sequence have been isolated. The purple color of the reaction is due to compound G which is known as Ruhemann's Purple. Schiff's bases of the type C have been isolated and are found to be colored (100). These substances

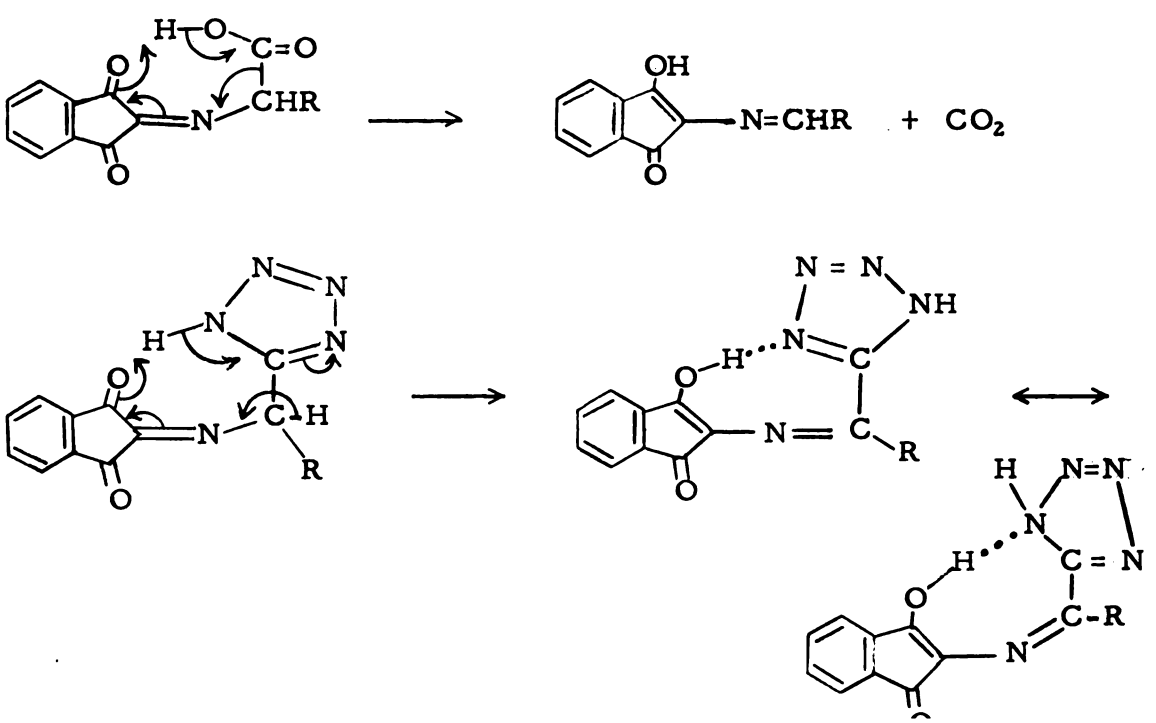


are responsible for the various colors which have been observed. The absence of the formation of Ruhemann's Purple at low pH values is consistent with the formation of hydrindantin (E) as indicated.

Neuzil, Josselin, and Breton (101) have suggested a mechanism which differs from that of McCaldin in only two points. Neuzil and co-workers suggested that triketoindane (X) could condense directly with the amino compound to give the ketimine prior to tautomerizing to the aldimine. In addition, Neuzil and co-workers believed that condensation with ninhydrin to give A, as suggested by McCaldin, was followed by elimination of water to form the ketimine as a precursor to the aldimine.



In general, the color reaction with ninhydrin did not proceed with amines possessing only one alpha hydrogen (101). However, if a carboxyl or tetrazole group was present on the carbon alpha to the amine, the reaction was observed by Neuzil and co-workers to precede neatly. Participation of the acidic functional groups in converting the ketimine to the required aldimine was considered responsible. The reason for the failure of 1-amino-1,3-di(5'-tetrazolyl)propane to give a color reaction with ninhydrin is unknown.



EXPERIMENTAL

PART II

The Preparation of 4-Amino-4-(5'-tetrazolyl)butanoic Acid (alpha-Tetrazole Analogue of DL-Glutamic Acid)

(a) Ethyl 2-acetamido-2-cyano-4-carboethoxybutanoate. Ethyl acetamidocyanoacetate (51.0 g., 0.30 mole) was suspended in 200 ml. of absolute ethanol in which 0.25 g. of sodium had been dissolved and cooled with stirring. Ethyl acrylate (33.0 g., 0.33 mole) was added dropwise over a thirty minute period to the stirred slurry. The resulting mixture was then allowed to stir for an additional hour at room temperature. The reaction mixture was filtered to remove traces of insoluble impurities. The ethanol was then removed by warming the reaction mixture in vacuo, and the residual oil was subsequently taken up in 100 ml. of chloroform. The chloroform solution was washed with 50 ml. of water and dried with anhydrous sodium sulfate after which the chloroform was removed by warming the mixture in vacuo.

The resulting oil was dissolved in 100 ml. of benzene, and 100 ml. of cyclohexane was added. After treating this solution with Norite, the filtrate was refrigerated. Product separated upon cooling and formed a solid cake in the bottom of the flask. The cake was broken up, removed, washed with benzene-cyclohexane mixture, and dried in a vacuum desiccator for two days. Concentration of the filtrate yielded a small addition amount of product. The combined weight of the fractions was 60.5 g. (75% of theory), m.p. 68-70° C.

This compound is reported by Herbst and Sterken to melt at 62-64° C. (5).

Analysis: Calculated for $C_{12}H_{18}N_2O_5$: C, 53.34; H, 6.71; N, 10.36.
Found: C, 53.17; H, 6.62; N, 10.34.

(b) Ethyl 2-acetamido-2-(5'-tetrazolyl)-4-carboethoxybutanoate. Ethyl 2-acetamido-2-cyano-4-carboethoxybutanoate (5.4 g., 0.02 mole) was mixed with sodium azide (1.43 g., 0.022 mole) and ethanol (70 ml.) to which 2 ml. of acetic acid had been added. The mixture was refluxed for three days after which the ethanol was removed by heating in vacuo. A syrup resulted which was dissolved in 20 ml. of warm water. After cooling to room temperature 8 ml. of concentrated hydrochloric acid were added, and the mixture was refrigerated for a period of four hours. The product was filtered, washed with a little cold dilute hydrochloric acid, and dried to give 4.9 g. (79% of theory) of the tetrazole, m.p. 79-80° C.

This tetrazole is reported by Herbst and Sterken to melt at 81° C. (5).

Analysis. Calculated for $C_{12}H_{19}N_5O_5$: C, 46.00; H, 6.11; N, 22.36.
Found: C, 45.74; H, 5.87; N, 22.41.

(c) 4-Amino-4-(5'-tetrazolyl)butanoic acid. Ethyl 2-acetamido-2-(5'-tetrazolyl)-4-carboethoxybutanoate (6.26 g., 0.02 mole) was refluxed for 24 hours with 10% hydrochloric acid (50 ml.). The hydrolysis mixture was then evaporated to dryness by heating in vacuo. Ten milliliters of water were added and again the mixture was evaporated to dryness as before. A second addition of water (10 ml.) was followed by warming and the dropwise addition of pyridine. When the pH reached 5, the mixture was cooled and the walls of the flask were scratched with a glass rod to induce crystallization of the product. The mixture was cooled for several hours to complete crystallization. The crude product was filtered, washed with a little cold water, and dried to give 2.8 g.

(82% of theory) of the tetrazole. Purification was effected by recrystallization from water to give pure product, m.p. 194° C. upon rapid heating. The product could be made to melt as low as 165° C. by very slowly raising the temperature. This was probably due to lactam formation. The pure product weighed 2.0 g. (59% of theory).

Analysis. Calculated for $C_5H_9N_5O_2$: C, 35.08; H, 5.30; N, 40.92.
Found: C, 35.09; 35.34; H, 5.19, 5.43; N, 41.15.

Derivatives of 4-Amino-4-(5'-tetrazolyl)butanoic Acid

(a) 4-Amino-4-(5'-tetrazolyl)butanoic Acid Hydrochloride. 4-Amino-4-(5'-tetrazolyl)butanoic acid (0.86 g., 0.005 mole) was added to 10 ml. of concentrated hydrochloric acid. The mixture was warmed until the solid dissolved in the acid. Gradual evaporation of the mixture with an air jet resulted in crystallization of the hydrochloride. This hydrochloride is more soluble in concentrated hydrochloric acid than is glutamic acid hydrochloride and does not crystallize from the solution on mere cooling. The hydrochloride was filtered, washed with a little concentrated hydrochloric acid, and dried to give 0.7 g. (70% of theory) of pure product, m.p. 153-155° C.

Analysis. Calculated for $C_5H_{10}ClN_5O_2$: C, 28.92; H, 4.85; Cl, 17.08; N, 33.73.
Found: C, 28.92; H, 4.87; Cl, 17.04 N, 33.59.

(b) 4-Acetamido-4-(5'-tetrazolyl)butanoic acid. 4-Amino-4-(5'-tetrazolyl)butanoic acid (1.71 g., 0.01 mole) was dissolved in 10 ml. of water containing 10 ml. of glacial acetic acid. After allowing the solution to cool, 20 ml. of acetic anhydride was added, and the solution was allowed to stand overnight.

The next day the mixture was evaporated to dryness by warming in vacuo. Water was added and again the mixture was evaporated as before. Product crystallized upon evaporation. The crude product was recrystallized from water to give 1.75 g. (82% of theory) of pure product, m.p. 195-196° C.

Analysis. Calculated for $C_7H_{11}N_5O_3$: C, 39.43; H, 5.20; N, 32.85.
Found: C, 39.29; H, 5.26; N, 32.94.

(c) 5-(5'-Tetrazolyl)-2-pyrrolidone. 4-Amino-4-(5'-tetrazolyl)-butanoic acid (1.71 g., 0.01 mole) was refluxed for three days with 60 ml. of absolute ethanol containing 5 drops of concentrated hydrochloric acid. The reaction mixture was then evaporated to a syrup by heating in vacuo to remove the ethanol and hydrochloric acid. The syrup was redissolved in a minimum of absolute ethanol, and ethyl acetate was added in equal volume. When this mixture was concentrated and cooled by means of an air jet, crude product separated. The crude pyrrolidone was washed with a mixture of ethanol and ethyl acetate and allowed to dry. It was recrystallized from acetone to give 0.77 g. (50% of theory) of pure product, m.p. 158° C. A small amount of syrup remained when the ethanol-ethyl acetate filtrate was evaporated, however, it could not be induced to crystallize.

Analysis. Calculated for $C_5H_7N_5O$: C, 39.21; H, 4.61; N, 45.73.
Found: C, 39.27; H, 4.85; N, 45.58.

(d) Ethyl 4-acetamido-4-(5'-tetrazolyl)butanoate. 4-Acetamido-4-(5'-tetrazolyl)butanoic acid (1.71 g., 0.008 mole) was mixed with ethanol (25 ml.), ethyl acetate (25 ml.), and 5 drops of concentrated hydrochloric acid. The mixture was refluxed for two days followed by evaporation to dryness by warming in vacuo. The syrup was dissolved in a minimum of water, and a very little pyridine was added to neutralize excess

hydrochloric acid. Again the mixture was evaporated to dryness as before. The resulting syrup was treated repeatedly with boiling cyclohexane to remove solvent and soluble impurities. Gradually the product became nearly rigid in boiling cyclohexane. After the final treatment with cyclohexane, the product was dissolved in water and treated with Norite. The clear aqueous filtrate upon evaporation with an air jet crystallized when the mixture had been concentrated nearly to a syrup. In some cases it was necessary to scratch the bottom of the flask with a glass rod to induce crystallization. Filtration of the crystals yielded 1.7 g. (88% of theory) of crude dry product. The material was recrystallized from a very small amount of water to yield 0.8 g. (41% of theory) of pure product, m.p. 107-108° C.

Analysis. Calculated for $C_9H_{15}N_5O_3$: C, 44.80; H, 6.27; N, 29.03.
Found: C, 44.75; H, 6.24; N, 28.90.

The Preparation of 1-Amino-1, 3-di(5'-tetrazolyl)propane
(Ditetrazole Analogue of DL-Glutamic Acid)

(a) Ethyl 2-acetamido-2, 4-dicyanobutanoate. Ethyl acetamidocyanoacetate (34.0 g., 0.20 mole) was suspended in 200 ml. of absolute ethanol and cooled with stirring. A solution of sodium ethoxide (0.25 g., 0.01 mole of sodium in 50 ml. of absolute ethanol) was added to the cold slurry followed by the dropwise addition of freshly distilled acrylonitrile (10.6 g., 0.2 mole) in 50 ml. of absolute ethanol. The addition was carried out over a period of two hours after which the mixture was allowed to stand with stirring and cooling for an additional five hours. Acetic acid was then added to the reaction mixture to lower the pH to 8.5. The ethanol was then removed in vacuo with warming. An oily residue remained.

After the oily residue solidified, it was dissolved in 200 ml. of hot water. The aqueous solution was extracted thrice with 75 ml. portions

and thrice with 50 ml. portions of chloroform. The combined chloroform extracts were washed with 50 ml. of water and dried over anhydrous sodium sulfate. After removal of the chloroform, the product solidified. The crude product was dissolved in ethanol and treated with Norite. To the clear ethanolic filtrate was added isopropyl ether, and upon refrigeration the product crystallized. Additional product can be obtained by concentrating the filtrate and cooling. The total weight of ethyl 2-acetamido-2,4-dicyanobutanoate was 32.1 g. (72% of theory), m.p. 100-101° C.

This compound is reported by Hanby, Waley, and Watson to melt at 101-102° C. (95).

(b) Synthesis and Attempted Chromatographic Isolation of Ethyl 2-acetamido-2,4-di(5'-tetrazolyl)butanoate. Ethyl 2-acetamido-2,4-dicyanobutanoate (4.46 g., 0.02 mole) was mixed with sodium azide (3.90 g., 0.06 mole) and a solution of 6 ml. of acetic acid in 100 ml. of 1-butanol was added. The mixture was refluxed for five days after which the hot solution was acidified with concentrated hydrochloric acid to pH 4. The sodium chloride was filtered and the filtrate was evaporated to a syrup by heating in vacuo. The syrup was dissolved in absolute ethanol, and again the solvent was removed by evaporation in vacuo. This time the syrup was treated with ethyl ether until the product formed as a white amorphous slightly tacky solid, (2.65 g., 43% of theory).

The amorphous material (1.53 g.) was chromatographed on alumina (37.5 g.) using acetone in the column. Four substances were obtained from the chromatography. The first four fractions from the column (elution with 100 ml. of acetone) were combined to give a small amount of an unidentified amorphous material. The next nine fractions (elution with acetone-ethanol mixtures from 90% acetone to 60% acetone by volume; total volume of 650 ml.) were combined to give the major product as an amorphous material which could not be made to crystallize. The third

group of five fractions (elution with acetone-ethanol mixtures from 50% acetone to 0% acetone by volume; total volume of 500 ml.) were combined to yield a material which crystallized from acetone. It was found to be 1-acetamido-1,3-di(5'-tetrazolyl)propane resulting from partial hydrolysis of the ditetrazole. The last two fractions resulting from elution with excess aqueous ethanol gave only aluminum acetate on crystallization.

(c) 1-Amino-1,3-di(5'-tetrazolyl)propane hydrate. Ethyl 2-acetamido-2,4-dicyanobutanoate (2.23 g., 0.01 mole) was mixed with sodium azide (1.95 g., 0.03 mole) in 50 ml. of 1-butanol to which 3 ml. of acetic acid was added. The mixture was then refluxed for three days. The hot mixture was then carefully acidified with concentrated hydrochloric acid until the pH was 3. The sodium chloride which precipitates was removed by filtration. The clear filtrate did not yield any product on cooling. Evaporation with heating in vacuo yielded a syrup which could never be made to crystallize. Several repetitions of the experiment gave in every case the syrup which could not be induced to crystallize in any common solvent system.

The crude material, which is presumably ethyl 2-acetamido-2,4-di(5'-tetrazolyl)butanoate, was refluxed with 50 ml. of 10% hydrochloric acid for several hours. The acid was removed by heating in vacuo, and a syrup remained. Water was added and subsequently removed in the same manner as the acid. Again the syrup was dissolved in a minimum of water, and pyridine was carefully added dropwise until the pH was just 5. At this point the product may crystallize immediately otherwise scratching with a stirring rod may be necessary. The mixture was cooled overnight in the refrigerator to complete crystallization. The crude product weighed 1.55 g. (79.5% of theory). The crude product was recrystallized twice from water to yield 1.10 g. (50% of theory) of pure 1-amino-1,3-di-(5'-tetrazolyl)propane hydrate, m.p. 264° C.

The hydrate was found to contain three molecules of water per two molecules of the tetrazole. The compound loses its water of crystallization rapidly on heating over 200° C. Analyses were run for carbon, hydrogen, and nitrogen on both the hydrate and anhydrous tetrazoles.

Analyses. Calculated for $C_5H_9N_9$: C, 30.76; H, 4.65; N, 64.59.
Found: C, 30.75; H, 4.53; N, 64.76.

Calculated for $(C_5H_9N_9)_2 \cdot (H_2O)_3$: C, 27.02; H, 5.44; N, 56.74.
Found: C, 27.24; H, 5.40; N, 56.94.

Derivative of 1-Amino-1, 3-di(5'-tetrazolyl)propane

1-Acetamido-1, 3-di(5'-tetrazolyl)propane. 1-Amino-1, 3-di(5'-tetrazolyl)propane hydrate (0.89 g., 0.004 mole) was dissolved in 50 ml. of boiling water. After allowing the solution to cool somewhat, a mixture of acetic acid (10 ml.) and acetic anhydride (15 ml.) was added, and the solution was heated on a steam bath for thirty minutes. After allowing the mixture to stand until the next day, the water and acetic acid were removed by heating in vacuo. To the residual syrup was added a warm mixture of water and ethanol to effect solution. This solution was then slowly evaporated on a steam bath with an air jet to aid in evaporation. Product gradually crystallizes during the concentration. The filtered, dry, crude product weighed 0.8 g. (84% of theory). Recrystallization from acetone yielded 0.5 g. (53% of theory) of the ditetrazole, m.p. 187° C.

Analysis. Calculated for $C_7H_{11}N_9O$: C, 35.44; H, 4.68; N, 53.15.
Found: C, 35.66; H, 4.90; N, 53.34.

Thermal Decomposition of 1-Amino-1, 3-di(5'-tetrazolyl)propane. It was observed that this compound decomposed upon melting at 264° C. to yield ammonium azide in the upper portions of the capillary tube.

The following procedure was employed to determine the amount of ammonium azide produced.

To an analytically weighed capillary tube was introduced a small amount of the ditetrazole. The tube was then weighed again to determine the weight of compound which had been added. The compound was thermally decomposed in a bath at about 270° C., and the ammonium azide sublimed and condensed in a narrow region in the cooler portions of the tube. After allowing the tube to cool thoroughly, it was cut off between the dark decomposed material in the bottom of the tube and the clear crystalline ammonium azide band. The tube and ammonium azide were weighed after which the ammonium azide was washed out and the tube was thoroughly cleaned and dried. Again the tube was weighed to determine the weight of ammonium azide produced.

It is important to use the proper amount of compound so as not to have too small a weight of ammonium azide or so much ammonium azide that some is lost from the tube. Use of an excessive amount of compound also results in so much foaming of the decomposing mixture in the bottom of the tube that it foams up into the band of ammonium azide.

The experiment was carried out twice: first, an ordinary analytical balance was used weighing to four decimal places, and second, a micro-analytical balance was used weighing to six decimal places. It was necessary to weigh to six places to obtain conclusive results.

Results of Reaction Using Analytical Balance

Weight of capillary tube and ditetrazole	0.1871 g.
Weight of capillary tube	0.1822 g.
Weight of ditetrazole	0.0049 g.
Weight of cut tube and ammonium azide	0.0744 g.
Weight of cut tube	0.0737 g.
Weight of ammonium azide	0.0007 g.

These results calculate to 0.46 moles of ammonium azide per mole of ditetrazole decomposed.

Results of Reaction Using Microanalytical Balance

Weight of capillary tube and ditetrazole	0.187711 g.
Weight of capillary tube	0.187090 g.
Weight of ditetrazole	0.000621 g.
Weight of cut tube and ammonium azide	0.131354 g.
Weight of cut tube	0.131268 g.
Weight of ammonium azide	0.000086 g.

These results calculate to 0.45 moles of ammonium azide per mole of ditetrazole decomposed.

It appears that two moles of the ditetrazole are required to produce one mole of ammonium azide.

The Preparation of 2-Amino-4-(5'-tetrazolyl)butanoic Acid (gamma-Tetrazole Analogue of DL-Glutamic Acid)

(a) Ethyl 2-acetamido-2-carboethoxy-4-cyanobutanoate. Diethyl acetamidomalonate (65.1 g., 0.3 mole) was suspended in 200 ml. of absolute ethanol containing 0.25 g. of sodium and cooled with stirring. Acrylonitrile (18 g., 0.34 mole) was added dropwise over a twenty minute period to the stirred slurry. The resulting mixture was then allowed to stir for an additional three hours at room temperature. Since an appreciable amount of product generally crystallizes during the reaction, the mixture was warmed to effect solution, filtered, and the product then allowed to crystallize in the refrigerator overnight. The resulting beautiful crystals of product weighed 72.3 g. (89.4% of theory), m.p. 92-94° C.

This compound is reported by Albertson and Archer to melt at 92-94° C. (96).

(b_1) 2-Amino-4-(5'-tetrazolyl)butanoic acid. Ethyl 2-acetamido-2-carboethoxy-4-cyanobutanoate (5.4 g., 0.02 mole) was mixed with sodium azide (1.56 g., 0.024 mole) and a solution of acetic acid (4 ml.) in 1-butanol (70 ml.) was added. The mixture was refluxed for twelve

days and then acidified to pH 4 with conc. hydrochloric acid. The sodium chloride which separated was filtered, and the filtrate was evaporated to a syrup by heating in vacuo. Since this syrup could not be made to crystallize, it was hydrolyzed as such.

The crude syrup, which is presumably ethyl 2-acetamido-2-carboethoxy-4-(5'-tetrazolyl)butanoate, was refluxed with 50 ml. of 6N hydrochloric acid for several hours. The hydrolysis mixture was boiled for a few minutes to remove excess 1-butanol and then treated with Norite. The filtrate was then concentrated to about thirty milliliters, the pH was adjusted to 5 with pyridine, and the product was isolated by a special procedure.

The following method of product isolation is similar to that employed by Finnegan, Henry and Lofquist (52). To the concentrated filtrate from above was added a saturated solution of cupric acetate in water at room temperature. The cupric salt of the tetrazole precipitated and was filtered and washed with water. This light blue precipitate was then suspended in 100 ml. of pure water at 50° C., and hydrogen sulfide gas was bubbled into the suspension until all the copper had been precipitated as the sulfide. Filtration removed the cupric sulfide and left a colorless solution of product. This solution was then concentrated to about 10 ml., and refrigeration resulted in the crystallization of 0.6 g. (17.6% of theory)* of pure product, m.p. 241° C.

Analysis. Calculated for $C_5H_9N_5O_2$: C, 35.08; H, 5.30; N, 40.92.
Found: C, 35.14; H, 5.32; N, 40.95.

(b_2) 2-Amino-4-(5'-tetrazolyl)butanoic Acid. Ethyl 2-acetamido-2-carboethoxy-4-cyanobutanoate (13.5 g., 0.05 mole) was mixed with sodium azide (4.0 g., 0.06 mole) and lithium chloride (4.7 g., 0.06 mole)

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*Yields of 21-24% of theory can be obtained by using ethylene glycol monomethyl ether as solvent, however, the product obtained is somewhat less pure.

in methyl cellosolve (50 ml.). The mixture was then refluxed for three days during which time the solution became dark brown. The reaction mixture was then acidified with hydrochloric acid, filtered, treated with Norite, and evaporated to a syrup. The product was then hydrolyzed by refluxing with 10% hydrochloric acid for a day. After treating with Norite the solution was concentrated and neutralized with sodium hydroxide solution.

A saturated solution of cupric acetate was added to the neutralized reaction mixture, and the product was isolated from the precipitate by the method of Finnegan and co-workers (52) as described previously. A 7% yield of the gamma-tetrazole analogue was recovered, m.p. 239° C.

Attempted Preparations of 2-Amino-4-(5'-tetrazolyl)-butanoic Acid

(a) Attempted Preparation of 2-Amino-4-(5'-tetrazolyl)butanoic Acid Using Aluminum Azide Procedure. Ethyl 2-acetamido-2-carboethoxy-4-cyanobutanoate (13.5 g., 0.05 mole) was mixed with sodium azide (3.6 g., 0.055 mole) and aluminum chloride (2.5 g., 0.018 mole) in tetrahydrofuran (80 ml.). The mixture was refluxed for three days, and during the reaction period some yellow solid separated upon the sides of the flask. The solid was filtered off after which the filtrate was evaporated to a syrup. When this syrup was dissolved in ethanol and refrigerated, a 20% yield of unreacted nitrile was recovered.

The yellow solid was placed in a beaker, and a little water was added to form a thin paste. After acidification with concentrated hydrochloric acid, the mixture was evaporated to dryness. The dry solid was treated with absolute ethanol and a brown filtrate was obtained upon filtration of the sparingly soluble sodium chloride. After replacing the ethanol with water, sodium hydroxide solution was added to precipitate the aluminum as its hydroxide. The resulting filtrate was acidified to 10%

acid and hydrolyzed for a day. Adjusting the pH to 5 and adding cupric acetate solution furnished a blue solution along with traces of dark decomposition materials. No cupric salts separated.

(b) Attempted Preparation of 2-Amino-4-(5'-tetrazolyl)butanoic Acid Using Ammonium Azide Procedure. Ethyl 2-acetamido-2-carboethoxy-4-cyanobutanoate (13.6 g., 0.05 mole) was mixed with sodium azide (3.58 g., 0.055 mole), ammonium chloride (2.95 g., 0.005 mole), and lithium chloride (0.5 g.) in dimethylformamide (100 ml.). The mixture was heated at 125° C. with stirring for two days. A chocolate brown solution resulted and even after two days a rather large amount of ammonium azide remained sublimed in the condenser. The resulting mixture was evaporated to a brown syrup by heating in vacuo, dissolved in water, and treated with Norite. Sufficient hydrochloric acid was then added to the solution to furnish a 10% acid solution. After refluxing for a day the solution was evaporated to dryness, water was added to dissolve the products, and the solution was adjusted to a pH of 5 with sodium hydroxide solution.

The addition of a saturated solution of cupric acetate to the neutralized reaction mixture furnished only a deep blue solution. It therefore appeared that no desired product had been formed. The effect of the presence of ammonium chloride on the ability of the product to separate as its cupric salt was tested by dissolving some of the gamma-tetrazole analogue prepared by another method in water containing ammonium chloride. After adjusting the solution to a pH of 5, the cupric salt of the tetrazole separated upon the addition of a solution of cupric acetate.

(c_1) Hydrazoic Acid in Xylene. Sodium azide (95 g., 1.46 moles) was dissolved in 200 ml. of water, and 600 ml. of xylene was added. The mixture was stirred vigorously and the temperature was maintained between 15-20° C. Through a dropping funnel, whose stem extended below the surface of the stirred mixture, was added 40 ml. of concentrated

sulfuric acid. The rate of addition of acid was such that the temperature could be maintained within the aforementioned range. After all of the acid had been added, the mixture was allowed to stir at room temperature for an hour. The xylene layer was then removed and dried over anhydrous sodium sulfate.

Standardization of the Hydrazoic Acid.

	Run 1	Run 2	Run 3
Volume of acid	2.0 ml.	2.0 ml.	2.0 ml.
Volume of base	16.25 ml.	16.70 ml.	16.45 ml.
m.e. of acid	3.31	3.40	3.35
m.e. of acid per 100 ml.	165.5	170.0	167.5
g. acid per 100 ml.	7.12	7.31	7.20

The sodium hydroxide solution used was 0.2038 N.

(c_2) Attempted Preparation of 2-Amino-4-(5'-tetrazolyl)butanoic Acid Using a Sealed Tube Reaction. Ethyl 2-acetamido-2-carboethoxy-4-cyanobutanoate (13.5 g., 0.05 mole) was placed in a thick walled glass pressure tube with a standardized solution of hydrazoic acid in xylene (48 ml., 0.08 mole of HN_3). After sealing the tube with a torch the mixture was heated in an oven at 132° C. for ten days. After cooling the oven the tube was carefully opened by heating the tip with a torch. Heavy gloves were worn and a safety shield was used. When all the gases had escaped, the end of the tube was cut off and the golden-brown solution was poured out. A considerable amount of syrup was also present in the bottom of the pressure tube. This was removed by dissolving the syrup in hot ethanol. The combined solutions were evaporated to a syrup, dissolved in ethanol, treated with Norite, and refrigerated. No product separated. The mixture was then hydrolyzed by replacing the ethanol with 12% hydrochloric acid and refluxing overnight. The acid was removed by heating in vacuo and the residue was dissolved in a minimum of water. After adjusting the pH of

the solution to 5, cupric acetate solution was added. Only trace amounts of precipitate were obtained.

Derivatives of 2-Amino-4-(5'-tetrazolyl)butanoic Acid

(a) 2-Amino-4-(5'-tetrazolyl)butanoic Acid Hydrochloride. 2-Amino-4-(5'-tetrazolyl)butanoic acid (0.43 g., 0.0025 mole) was dissolved in about 8 ml. of concentrated hydrochloric acid by warming. Gradual evaporation of the mixture with an air jet resulted in crystallization of the hydrochloride. The crude product, which crystallizes readily, was filtered, washed with concentrated hydrochloric acid, and dried to give 0.5 g. (96.5% of theory) of product. The crude product was recrystallized from concentrated hydrochloric acid to give 0.35 g. (68% of theory) of pure product, m.p. 220-222° C. d.

Analysis. Calculated for $C_5H_{10}ClN_5O_2$: C, 28.92; H, 4.85; Cl, 17.08; N, 33.73.

Found: C, 29.00; H, 4.93; Cl, 17.30; N, 33.98.

(b) 2-Acetamido-4-(5'-tetrazolyl)butanoic acid. 2-Amino-4-(5'-tetrazolyl)butanoic acid (1.71 g., 0.01 mole) was dissolved in 20 ml. of water. After cooling the solution to room temperature, 20 ml. of acetic acid and 20 ml. of acetic anhydride were added. The solution was allowed to stand overnight, and the next day it was boiled for several minutes followed by evaporation to dryness by heating in vacuo.

The crude product was recrystallized from water to yield 2.0 g. (94% of theory) of the amide, m.p. 189° C.

Analysis: Calculated for $C_7H_{11}N_5O_3$: C, 39.42; H, 5.20; N, 32.85.

Found: C, 39.44; H, 5.40; N, 33.02.

(c) Ethyl 2-amino-4-(5'-tetrazolyl)butanoate hydrochloride. 2-Amino-4-(5'-tetrazolyl)butanoic acid (1.72 g., 0.01 mole) was added to 50 ml. of absolute ethanol. Hydrogen chloride gas was bubbled into the solution until all the tetrazole dissolved. The resulting solution was refluxed for a day, and the mixture was evaporated to dryness by heating in vacuo. Ethanol was added to the crude product and removed as before to assure the removal of excess hydrogen chloride. The resulting syrup was dissolved in a minimum of ethanol. This solution was then treated with acetone and cooled with scratching to induce the product to crystallize. The product was washed with acetone and dried to give 1.1 g. (47% of theory) of the hydrochloride, m.p. 157-159° C. Additional product may be obtained by concentration of the acetone-ethanol filtrate, however, the purity in lower, (0.5 g., m.p. 145-152° C.)

Analysis. Calculated for $C_7H_{14}N_5O_2Cl$: C, 35.67; H, 5.99; N, 29.72; Cl, 15.04.

Found: C, 35.78; H, 6.02; N, 29.80; Cl, 14.98.

(d) Ethyl 2-amino-4-(5'-tetrazolyl)butanoate. Ethyl 2-amino-4-(5'-tetrazolyl)butanoate hydrochloride (1.1784 g., 0.005000 mole) was dissolved in a little water, and standard sodium hydroxide solution (52.0 ml. of 0.09615 N, 0.005000 mole) was added. The solution was concentrated by boiling to 5-10 ml. Upon cooling and scratching the walls of the flask, product began to crystallize. The product was filtered and washed with a little water. The filtrate and wash water were combined and concentrated to obtain a second crop of product. In the same manner a third crop was obtained. The combined washed products were dried to give 0.82 g. (82% of theory) of the tetrazole, m.p. 181-182° C. Recrystallization from water gave m.p. 181-182° C.

Analysis. Calculated for $C_7H_{13}N_5O_2$: C, 42.20; H, 6.58; N, 35.16.

Found: C, 42.48; H, 6.82; N, 34.96.

Basic Hydrolysis of the Tetrazole Intermediates

(a) Reactions of Ethyl 2-acetamido-2-(5'-tetrazolyl)-4-carboethoxybutanoate, amorphous Ethyl 2-acetamido-2-carboethoxy-4-(5'-tetrazolyl)-butanoate, and amorphous Ethyl 2-acetamido-2,4-di(5'-tetrazolyl)-butanoate with aqueous sodium hydroxide.

The basic hydrolysis of the three substances are discussed together since similar phenomena were encountered with each compound.

Ethyl 2-acetamido-2-(5'-tetrazolyl)-4-carboethoxybutanoate (6.26 g., 0.02 mole) was refluxed with sodium hydroxide (3.2 g., 0.08 mole) in 75 ml. of water for one day. The hydrolysis mixture was evaporated to dryness, and the residue was dissolved in 8 ml. of water. This solution was cooled while concentrated hydrochloric acid was slowly added until the pH was 5. During the addition of the acid a gelatinous material continued to separate in the solution. This material was filtered, washed well with water, and allowed to dry.

The gelatinous product was found to be soluble in sodium hydroxide solution but insoluble in water and acids. The material would not even dissolve in refluxing concentrated hydrochloric acid. The material was infusible and contained sodium since a basic residue remained after heating the material at red heat for several minutes. The gelatinous material seemed to have a minimum solubility around pH 7. With increasing acidity the solubility increased very slightly down to pH 2, but lower pH values again resulted in decreased solubility. This effect was not very pronounced and was only observed qualitatively. When the basic hydrolysis mixture was diluted greatly with water prior to acidification, no precipitate formed. However, allowing the solution to concentrate resulted in formation of a gel.

It was found that acidifying with acetic acid in place of hydrochloric acid led to the same result. In addition, potassium hydroxide hydrolysis also gave a gelatinous material upon acidification.

In one reaction only three moles of sodium hydroxide were used per mole of tetrazole. The solution (1N in NaOH) was refluxed for 1.5 hours. Upon acidification no precipitate was formed. A small amount of scum was filtered and the solution was evaporated to dryness by means of an air jet at room temperature. The product was extracted from the sodium chloride* with acetone, and the acetone was evaporated with an air jet. The resulting syrup was dissolved in a minimum of water, and 4-acetamido-4-(5'-tetrazolyl)butanoic acid (0.5 g., 24% of theory) crystallized upon refrigeration.

The amorphous ethyl 2-acetamido-2, 4-di(5'-tetrazolyl)butanoate formed from ethyl 2-acetamido-2, 4-dicyanobutanoate (0.01 mole) was hydrolyzed by refluxing with sodium hydroxide solution (24 g., 0.6 mole in 50 ml. of water) overnight. Again a gelatinous material forms upon acidification. The material was redissolved in base, diluted with water, and reacidified to a pH of 3. Cupric acetate was added and the blue gelatinous complex material was filtered, washed, and treated with hydrogen sulfide gas to precipitate the copper as its sulfide. The filtrate was evaporated with an air jet at room temperature to a syrup. A certain amount of crystalline material was also present in the syrup. A minimum of water was added to dissolve the syrup, and the crystalline material was filtered to yield 2.3 g. (11.8% of theory) of 1-amino-1, 3-di(5'-tetrazolyl)propane hydrate.

The filtrate containing the syrup was again evaporated with an air jet, and the clear syrup was treated with acetone to give a small amount of a material which contained sodium but which was not identified.

Repeated acetone treatment and evaporation finally resulted in the crystallization of considerable material which was recrystallized from acetone to give 9.0 g. (38% of theory) of 1-acetamido-1, 3-di(5'-tetrazolyl)-propane, m.p. 185-187° C.

The remainder of the product occurred as a gelatinous material along with the copper sulfide on the filter.

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*Undoubtedly gelatinous material was also present with the sodium chloride and accounts for the rest of the product.

The amorphous ethyl 2-acetamido-2-carboethoxy-4-(5'-tetrazolyl)-butanoate formed from ethyl 2-acetamido-2-carboethoxy-4-cyanobutanoate (0.01 mole) was isolated via its copper salt as discussed above. Hydrogen sulfide treatment, filtration of the copper sulfide, and evaporation of the filtrate yielded a syrup of crude tetrazole (8.5 g., 27% of theory).

The syrup was hydrolyzed by refluxing for two days with sodium hydroxide solution (12 g., 0.3 mole in water). When the basic solution was acidified with cooling, a gelatinous precipitate again formed. As was observed in the case of the isomer, the gel seemed to possess a minimum solubility around pH 7. With increased acidity solubility again increased very slightly down to a pH of about 1. Very strongly acidic solutions tend to decrease this solubility slightly.

When the hydrolysis mixture is diluted greatly with water prior to acidification, no gelatinous precipitate formed. Concentration of the clear solution by gradual evaporation at room temperature caused the entire solution to set to a gel.

(b) Reaction of Ethyl 2-acetamido-2-(5'-tetrazolyl)-4-carboethoxybutanoate with Barium Hydroxide. Ethyl 2-acetamido-2-(5'-tetrazolyl)-4-carboethoxybutanoate (3.13 g., 0.01 mole) was refluxed for a day under nitrogen with a clear barium hydroxide solution (11 g. in sufficient water to dissolve the hydroxide when hot). A little barium carbonate separated along with a small amount of other barium salts--probably of the tetrazole. The reaction mixture was filtered and 30% sulfuric acid was added until there was neither an excess of barium ions nor of sulfate ions in the solution. The barium sulfate was filtered and the filtrate was evaporated to dryness at room temperature with an air jet. A white amorphous solid remained.

When this amorphous solid was treated with acetone, part of the material dissolved. After filtering the acetone solution and allowing the

filtrate to stand overnight, crystals formed in it. When the amorphous material which did not go into the acetone was treated with more acetone, additional material dissolved and again yielded crystals on standing. Concentration of the acetone filtrates yielded more of the same crystals. All of the fractions were combined and recrystallized from water-acetone mixture to give 1.2 g. (57% of theory) of 4-acetamido-4-(5'-tetrazolyl)-butanoic acid, m.p. 195-196° C.

(c) Reaction of Crude Amorphous Ethyl 2-acetamido-2,4-di(5'-tetrazolyl)butanoate with Barium Hydroxide. Ethyl 2-acetamido-2,4-dicyanobutanoate (11.2 g., 0.05 mole) was mixed with sodium azide (10 g., 0.15 mole) and a solution of 15 ml. of acetic acid in 300 ml. of 1-butanol was added. The mixture was refluxed for three days after which the hot solution was acidified with concentrated hydrochloric acid to pH 4. The sodium chloride was filtered and the filtrate was evaporated to a syrup by heating in vacuo. The syrup was dissolved in about 75 ml. of water.

An aqueous solution of barium hydroxide (50 g. in 130 ml. of water) was prepared and filtered to remove barium carbonate. The clear barium hydroxide solution was added to the aqueous solution of the crude product, and the mixture was refluxed under nitrogen for one day.

After the hydrolysis period the mixture was filtered to remove a small amount of barium carbonate which had precipitated. The clear filtrate was then cooled in an ice bath while dilute sulphuric acid was added until there was neither an excess of barium ions nor sulfate ions. At this point the pH of the solution is about 2. The large amount of barium sulfate was filtered, and the clear filtrate was allowed to evaporate slowly at room temperature by use of an air jet playing upon the surface of the solution. After two days the solution became very concentrated, and the last of the water was removed in vacuo at room temperature.

The resulting syrup was dissolved in a minimum of cold water and an equal volume of acetone was added followed by ether until the cloud point was nearly reached. Scratching caused the separation of a white solid which proved to be 1-amino-1,3-di(5'-tetrazolyl)propane hydrate (1 g., 9% of theory). Concentration of the filtrate resulted in a syrup which could not be made to crystallize. This syrup probably consisted of unhydrolyzed and partially hydrolyzed materials.

Determination of Apparent pK Values for Glutamic Acid Hydrochloride and the Three Tetrazole Analogues of Glutamic Acid Hydrochloride

Weighed samples of the four hydrochlorides* were titrated in aqueous solution with standard sodium hydroxide solution. A Beckman Model G. pH meter was used. All the data obtained are tabulated in Appendix I, and titration curves are plotted for all the compounds. The following apparent pK values were observed for the compounds:

D (-)-Glutamic Acid Hydrochloride

pK_1'	2.16	Values of West and Todd (98)	2.19
pK_2'	4.23		4.28
pK_3'	9.46		9.66

4-Amino-4-(5'-tetrazolyl)butanoic Acid Hydrochloride

pK_1'	2.08
pK_2'	3.97
pK_3'	8.23

2-Amino-4-(5'-tetrazolyl)butanoic Acid Hydrochloride

pK_1'	2.33
pK_2'	4.83
pK_3'	9.46

(1-Amino-1, 3-di(5'-tetrazolyl)propane Hydrochloride)*

pK_1'	2.38, 2.46
pK_2'	4.53, 4.60
pK_3'	8.00, 8.04

Reactions of Ninhydrin on Glutamic Acid and the Three Tetrazole Analogues of Glutamic Acid

Ninhydrin tests were carried out by treating approximately 50 mg. of compound with about 10 ml. of reagent (0.25% ninhydrin in water) freshly prepared. The results are summarized as follows:

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*The hydrochloride of the ditetrazole analogue could not be obtained in crystalline form. In forming a solution of the hydrochloride it was necessary to add the equivalent amount of standard hydrochloric acid solution to a weighed sample of the ditetrazole.

LEGEND

G = D(-) Glutamic Acid (practical grade)
α = 4-Amino-4-(5'-tetrazolyl)butanoic Acid
γ = 2-Amino-4-(5'-tetrazolyl)butanoic Acid
D = 1-Amino-1, 3-di(5'-tetrazolyl)propane Hydrate

A = Strong reaction within a minute
B = Mild reaction within a minute
C = Weak reaction within five minutes
D = Just detectable reaction within five minutes
O = No reaction in five minutes

Temperature °C.	G	γ	α	D
40	A	A	O	O
50			D	O
70			C	O
90			B	O
100			A	O
100	(one-half hour of boiling)			slight yellowing

Comments on Color, Intensity, and Stability of Ninhydrin Products.

Compound	Thirty minutes	Two days
G	Maintains original violet color	Violet color is lightened considerably
γ	Original violet color darkens to very deep violet on standing	Remains very deep violet
α	The violet color lightens and acquires some reddish-brown	Becomes light yellow
D	Slight yellowing on prolonged boiling	Remains light yellow

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Determination of the Rf and Mf Values of Glycine, Glutamic Acid, and the Monotetrazole Analogues of Glutamic Acid

The following procedure was employed in the paper chromatographic determination of the Rf and Mf values of glycine, glutamic acid, and the monotetrazole analogues of glutamic acid. Strips of Whatman No. 1 paper were used in all cases. The studies were conducted with two solvent systems--80% phenol in water and 1, 4-dioxane in 1-butanol in a 1 to 4 volume ratio saturated with water.

Preparation of the Strips. Whatman No. 1 paper was used in all the work to be discussed. Rubber gloves were worn at all times in order to avoid contaminating the strips with materials from the skin. The paper was cut into strips $\frac{1}{2}$ inch wide and about 17 cm. long. Lines were ruled across the strips at four intervals--about 2 cm. from one end, $1\frac{1}{2}$ cm. from the first line, exactly 10 cm. from the second line, and $1\frac{1}{2}$ cm. from the third line. The fourth line is then approximately 2 cm. from the other end of the strip. The 2 cm. lengths on each end of the strip function as handles and can be touched with the bare hands. A small hole was punched between the first two line in the center of the strip crosswise in order to hang the strips for drying. A letter indicative of the compound being chromatographed was written on the paper between the third and fourth line. The strip was then folded in half along its length and creased. This enables the strips to be inserted in a test tube and to make contact with the glass walls of the tube only at the top and bottom of the strip.

Preparation of Solutions and Application. The solutions of glycine and the monotetrazole analogues of glutamic acid used were 0.1 molar. The glutamic acid solution was saturated at room temperature. Small wooden sticks were sharpened to fine points and allowed to soak in the

test solutions. These sticks were used as applicators. To apply the test compound one of the prepared strips was taken and marked with the appropriate letter of the compound. The application stick was removed from the solution and touched on one handle of the strip several times to remove excess solution. Then the stick was very carefully touched to both edges of the strip precisely at the third line. After allowing the strips to dry for a couple minutes, both handles were cut off.

Chromatographic Procedure. In the bottom of six inch test tubes was placed 0.2 ml. of the solvent system to be used. The test tubes were placed in Erlenmeyer flasks so as to allow them to be held at about an 80^{o} angle off the horizontal. Each tube was fitted with a tight rubber stopper. When one of the prepared strips was dry, it was carefully placed in one of the test tubes such that the punched hole was at the top and the rubber stopper was replaced. The strip should touch the tube only at the bottom and at the two corners of the top. The creased edge of the strip should be up relative to the two side edges of the strip. Therefore, the paper strip will tilt more from the vertical than does the tube.

After two or three hours the solvent system was seen to have nearly reached the second line. The strip was then carefully removed from the test tube with forcepts and marked with a pencil on each edge at the solvent front. It was then washed with a stream of acetone and hung to dry. When the strip was dry, it was sprayed with a freshly prepared ninhydrin reagent (0.8 g. of ninhydrin, 1.5 ml. of s-collidine, and 100 ml. of 95% ethanol) and hung up to dry and develop. Within a few minutes a spot of color was seen on either side of the strip. The distance from line three to the front of the spot was carefully measured; also the distance from line three to the pencil marks of the solvent front were carefully measured. The Rf values were calculated by dividing the distance to the spot by the distance to the solvent front. The Mf values were calculated by dividing the molecular weight of the compound by 100xRf.

Tabulation of the Rf and Mf Values Obtained from the Monotetrazole Analogues of Glutamic Acid, Glycine, and Glutamic Acid.

Part I

The following Rf and Mf values were obtained using 80% phenol in water as the solvent system and Whatman No. 1 paper for the strips.

L(+)Glutamic Acid

Temperature °C.		Rf	Mf
26		0.325	
26		0.335	
24		0.335	
23		0.345	
23		0.355	
23		0.335	
23		0.34	
23		0.32	
	Average	0.335 ± .02	4.4

Glycine

Temperature °C.		Rf	Mf
26		0.41	
25		0.42	
25		0.415	
25		0.425	
26		0.435	
	Average	0.42 ± .015	1.8

2-Amino-4-(5'-tetrazolyl)butanoic Acid

Temperature °C.		Rf	Mf
26		0.41	
26		0.405	
25		0.43	
25		0.43	
25		0.43	
25		0.435	
25		0.425	
	Average	0.42 ± .015	4.05

4-Amino-4-(5'-tetrazolyl)butanoic Acid

Temperature °C.		Rf	Mf
26		0.36	
26		0.325	
26		0.35	
26		0.355	
26		0.35	
26		0.35	
26		0.33	
	Average	0.345 ± .02	4.95

Part II

The following Rf and Mf values were obtained using 1,4-dioxane in 1-butanol in a 1 to 4 volume ratio saturated with water. Whatman No. 1 paper was used for the strips.

L(+)Glutamic Acid

Temperature °C.		Rf	Mf
25		0.175	
25		0.165	
24		0.155	
26		0.16	
26		0.15	
	Average	0.16 = .015	9.15

Glycine

Temperature °C.		Rf	Mf
25		0.165	
25		0.175	
24		0.15	
26		0.15	
26		0.155	
	Average	0.16 ± .015	4.7

2-Amino-4-(5'-tetrazolyl)butanoic Acid

Temperature °C.		Rf	Mf
25		0.215	
25		0.235	
26		0.22	
26		0.225	
26		0.21	
26		0.21	
26		0.215	
26		0.225	
	Average	0.22 ± .015	7.8

4-Amino-4-(5'-tetrazolyl)butanoic Acid

Temperature °C.		Rf	Mf
25		0.25	
25		0.255	
26		0.23	
26		0.235	
26		0.235	
26		0.24	
26		0.245	
26		0.25	
	Average	0.24 ± .015	7.1

RESULTS

PART III

In PART III the syntheses of the three tetrazole analogues of DL-glutamic acid were described and a number of their physical properties were determined. The dissociation constants of the three acidic groups were found for each analogue, and in addition, Rf and Mf values were obtained for the two monotetrazole analogues employing two different organic systems as the moving phases. The marked similarities in dissociation constants and molecular flow (Mf) values between glutamic acid and its tetrazole analogues suggested the possibility that some of the analogues might well function as substrates or inhibitors in enzyme systems specific for glutamic acid. L-glutamic acid dehydrogenase (GAD) was chosen as the enzyme for the initial study.

L-glutamic acid dehydrogenase has been found to be largely specific for L-glutamic acid, in fact, no glutamic acid analogue prior to this study has been shown to function as a substrate (102-104). The structural requirements for inhibition of this enzyme have also been studied in detail (105) and will be presented in connection with the discussion on the reactivity and inhibition of the three tetrazole analogues with the enzyme.

In nature L-glutamic acid dehydrogenase functions as a catalyst for the reversible reductive amination of ketoglutaric acid to L-glutamic acid. Diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN) serves as the oxidizing coenzyme. At nearly neutral pH both the glutamate and ammonia exist almost entirely in the ionized

form indicated, and the over-all reaction can be represented as follows:

$$\text{Glutamate}^{\ominus} + \text{DPN}^{\oplus} + H_2O \rightleftharpoons \underline{\text{alpha}}\text{-Ketoglutarate}^{\ominus} + NH_4^{\oplus} + \text{DPNH} + H^{\oplus}$$

Originally it was suggested (103) that the enzymatic reaction led to the formation of iminoglutaric acid which was then subsequently hydrolyzed nonenzymatically to furnish the alpha-ketoglutarate. However, Strecker (104) found that there was no evidence for a spontaneous, nonenzymatic formation of iminoglutaric acid in the reverse system.

Throughout all the subsequent discussions in PART III the values calculated for the various binding and equilibrium constants are only apparent values obtained from the racemic analogues. Without resolving the various analogues, it was impossible to determine the specific amounts of activity or inhibition (if any) of the various enantiomers. Only the combined effects of the enantiomeric pairs could be physically observed. The values which were obtained are sometimes given as a function of the actual binding constant for each enantiomer of the pair. The meanings of the apparent constants are given as they appear.

Each of the three tetrazole analogues of DL-glutamic acid was tested for activity as a substrate of L-glutamic acid dehydrogenase. The change of absorption at 340 mμ was used to determine the concentration of DPNH formed on the basis of an extinction coefficient of 6.22×10^6 cm^2 $mole^{-1}$. Neither the ditetrazole nor the gamma-tetrazole analogue showed any appreciable reaction even after several hours. In some of the determinations a slight initial reaction occurred with the gamma-tetrazole analogue, however, this was attributed to trace amounts of glutamic acid since an absorption of only about 0.03 optical density units occurred after several hours. In all cases the ditetrazole analogue failed to react.

The alpha-tetrazole analogue of DL-glutamic acid was unique among all analogues which have been studied with GAD in that it was capable of functioning as a substrate for the enzyme. The relative initial zero order rates of oxidation of L-glutamate/DL-alpha-tetrazole analogue were roughly 150/1 under identical reaction conditions; however, the equilibrium for the reaction with the tetrazole analogue lay farther toward the keto product. Since the analogue was not resolved into its optically active enantiomers, a direct comparison between the active forms could not be made, and the actual rate ratio of the active forms is most certainly smaller than 150/1.

The combined abilities of the two alpha-tetrazole enantiomers to complex with GAD were determined by a modification of the treatment first developed by Michaelis and Menten (106). These workers were successful in mathematically treating the theory of the enzyme-substrate complex which was suggested by Henri and independently by Brown. The enzyme (E) was presumed to complex with the substrate (S) in a rapid equilibrium, and the resulting complex (ES) was then gradually converted into the products (P) as indicated. Although this theory had been widely

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} P + E$$

accepted, no proof was available until 1943 when Chance (107) spectroscopically demonstrated the existence of the peroxidase-H_2O_2 complex. Michaelis and Menten derived an equation based upon the theory of the enzyme-substrate complex. The equilibrium constant for the initial rapid equilibrium has become known as the Michaelis constant (Km). The Michaelis-Menten equation may be written as equation I, where v signifies the initial reaction rate and V denotes the maximum velocity corresponding to enzyme saturation. The extent to which Km is representative of the true equilibrium constant for the binding of the substrate to the

enzyme is determined by the relative sizes of k_2 and k_3. Only if $k_3 << k_2$ is Km truly representative of the equilibrium since $Km = (k_2 + k_3)/k_1$. The very slow rate of product formation with the alpha-tetrazole analogue indicated that $k_3 << k_2$ and that, therefore, the experimental value of Km was a good approximation of the true equilibrium constant. When equation I is reciprocated, it is known as the Lineweaver-Burk equation (II) (108). A plot of reciprocal velocity on the ordinate against

$$v = \frac{V\,(S)}{Km + (S)} \qquad \text{I}$$

$$\frac{1}{v} = \frac{Km}{V}\left[\frac{1}{(S)}\right] + \frac{1}{V} \qquad \text{II}$$

reciprocal substrate concentration on the abscissa furnishes a straight line with an intercept on the abscissa at -1/Km. When a similar plot is made using the DL-alpha-tetrazole analogue, the value obtained as the intercept on the abscissa is not truly -1/Km since the substrate is racemic. If the inactive enantiomer is also noninhibiting and if the substrate concentration is taken as half the concentration of the racemic analogue, then the intercept will be -1/Km.

The actual meaning of the intercept on the abscissa can be determined by modifying the Lineweaver-Burk equation for competitive inhibition. When both the substrate and inhibitor compete reversibly for the same reaction site on the enzyme, equation III is valid, where Ki denotes the constant for the complexing of the inhibitor to the enzyme. When used in the usual manner with constant inhibitor concentration, a plot of reciprocal

$$\frac{1}{v} = \frac{1}{V}\left[Km + \frac{Km\,(I)}{Ki}\right]\frac{1}{(S)} + \frac{1}{V} \qquad \text{III}$$

velocity against reciprocal substrate concentration gives an abscissa intercept as expressed by IV. However, in the case of the racemic alpha-tetrazole analogue, the inhibitor concentration equals the substrate concentration at all times. Equation III then takes the form of V. A mathematical examination of equation V shows the impossibility of determining any of the three unknowns (Km, Ki, and V) numerically. The intercept on the abscissa is found to be $-(1/Km + 1/Ki)$, thus if no

$$-\frac{1}{Km}\left[\frac{Ki}{Ki + (I)}\right] \qquad \text{IV}$$

$$\frac{1}{v} = \frac{Km}{V}\left[\frac{1}{(S)}\right] + \frac{Km + Ki}{VKi} \qquad \text{V}$$

inhibition occurred (i.e. $Ki = \infty$) the intercept would be $-1/Km$ as mentioned previously.

The initial rates for the enzymatic oxidation of the alpha-tetrazole analogue of DL-glutamic acid were determined at six different concentrations of analogue. When the data were plotted in the manner of Lineweaver and Burk (Figure I), values in independent runs ranging from 64 to 76 were obtained for $1/Km + 1/Ki$, considering the substrate concentration equal to half the concentration of the racemic tetrazole. Apparent Michaelis constants (Km') of $(2.8 \pm .3) \times 10^{-2}$ were found as the reciprocals of the intersections on the abscissa using total concentration of racemic tetrazole in the calculations. The Km' value bears the same mathematical relationship to Ki and Km as the reduced mass does to the two individual masses. Only if both the enantiomers bind to the enzyme with equal facility would Km' represent the true Michaelis constant.

The equilibrium constant for the reversible oxidative deamination of the alpha-tetrazole analogue was determined by incubating known concentrations of the analogue with DPN, ammonium chloride, and GAD in phosphate buffer. After twelve hours the change in absorption at 340 mμ

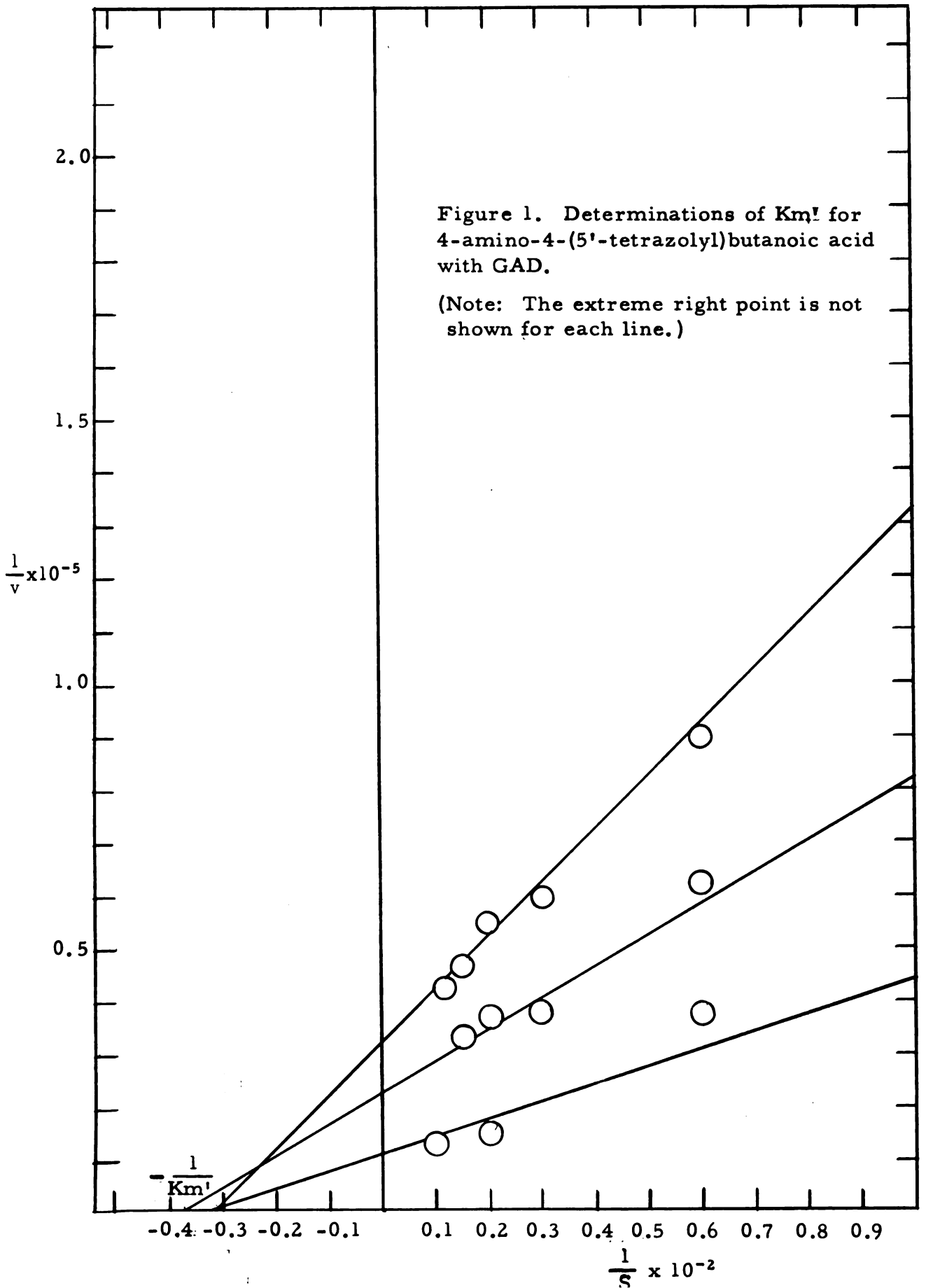

Figure 1. Determinations of Km' for 4-amino-4-(5'-tetrazolyl)butanoic acid with GAD.

(Note: The extreme right point is not shown for each line.)

was very slow (Figure II). Based upon the relative rates of reaction of the alpha-tetrazole analogue and L-glutamic acid and upon the rate at which L-glutamic acid reached equilibrium under identical conditions, it was estimated that in twelve hours the reaction had proceeded better than 80% of the way to equilibrium. The equilibrium constant for the alpha-tetrazole analogue was calculated on the basis of equation VI (104) using 55.5 moles per liter as the concentration of water. A value of 3.65×10^{-14} was calculated considering the substrate concentration

$$K = \frac{(\text{alpha-Ketotetrazole}^{\ominus})\ (NH_4^{\oplus})\ (\text{DPNH})\ (H^{\oplus})}{(\text{alpha-Tetrazole}^{\oplus})\ (\text{DPN}^{\oplus})\ (H_2O)}$$

VI

equivalent to the total concentration of the racemic analogue. Basing the calculation upon the assumption that only one enantiomer was active gives a value of 7.3×10^{-14}. Since the constants were determined at a pH of 6.8, it was not necessary to make corrections. At this pH the alpha-tetrazole analogue exists almost exclusively in the ionized form expressed in equation VI. The same is also true for the ammonium ion. Any effect upon the equilibrium associated with the presence of an inactive enantiomer is indeterminable, and therefore the equilibrium constant is valid only in the absence of such effects.

To test the precision of the method, the equilibrium constant for the reversible oxidative deamination of L-glutamic acid was determined and found to be 3.14×10^{-15} (Figure III). By multiplying this value by 55.5 a new constant of 1.74×10^{-13} is obtained which can be compared directly with the values of 1.98×10^{-13} at a pH of 6.74 and 1.74×10^{-13} at a pH of 7.38 as reported by Strecker (104).

It was found that both the gamma- and ditetrazole analogues of DL-glutamic acid were inactive as substrates for GAD, however, these analogues might well be expected to function as competitive inhibitors in

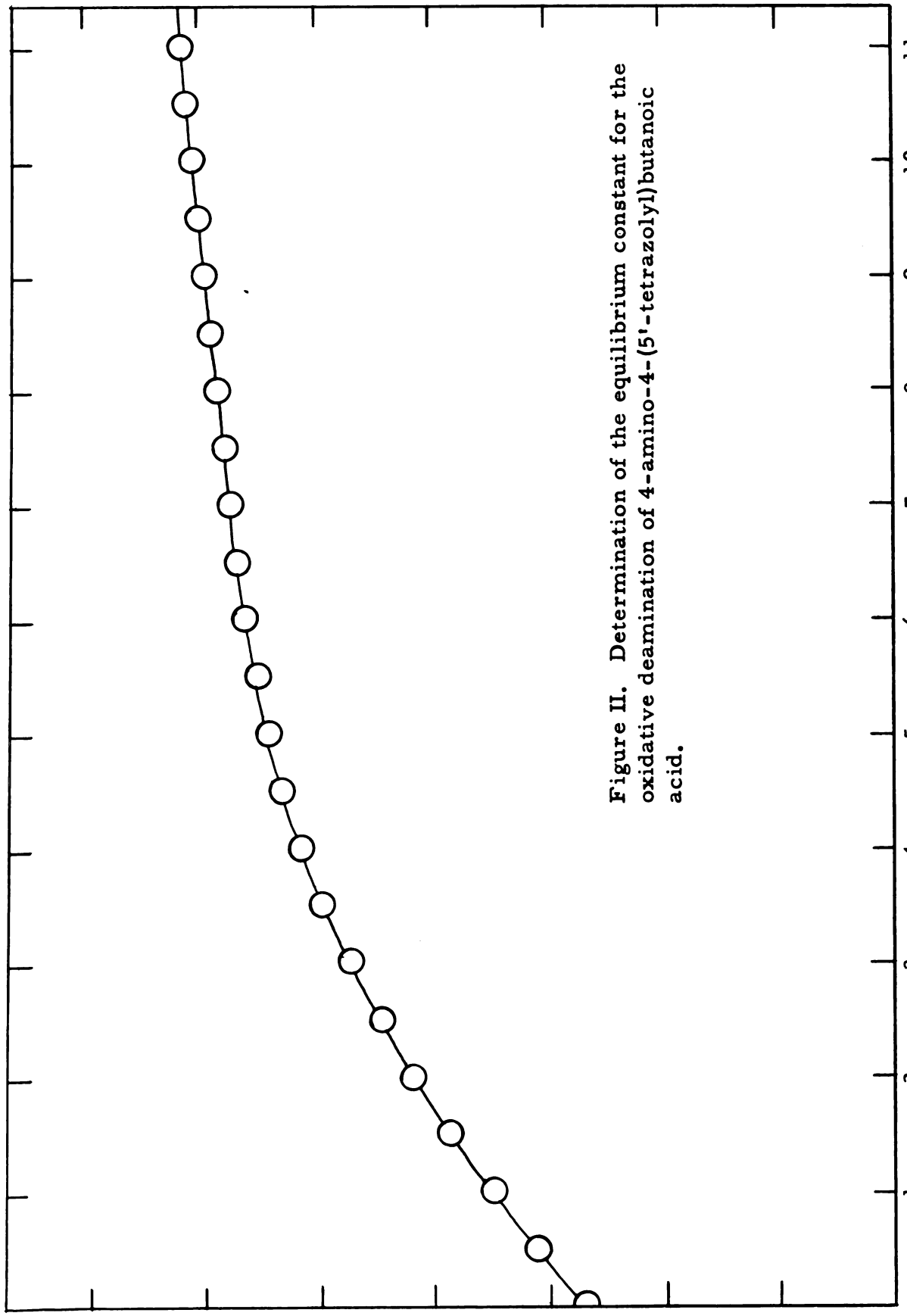

Figure II. Determination of the equilibrium constant for the oxidative deamination of 4-amino-4-(5'-tetrazolyl)butanoic acid.

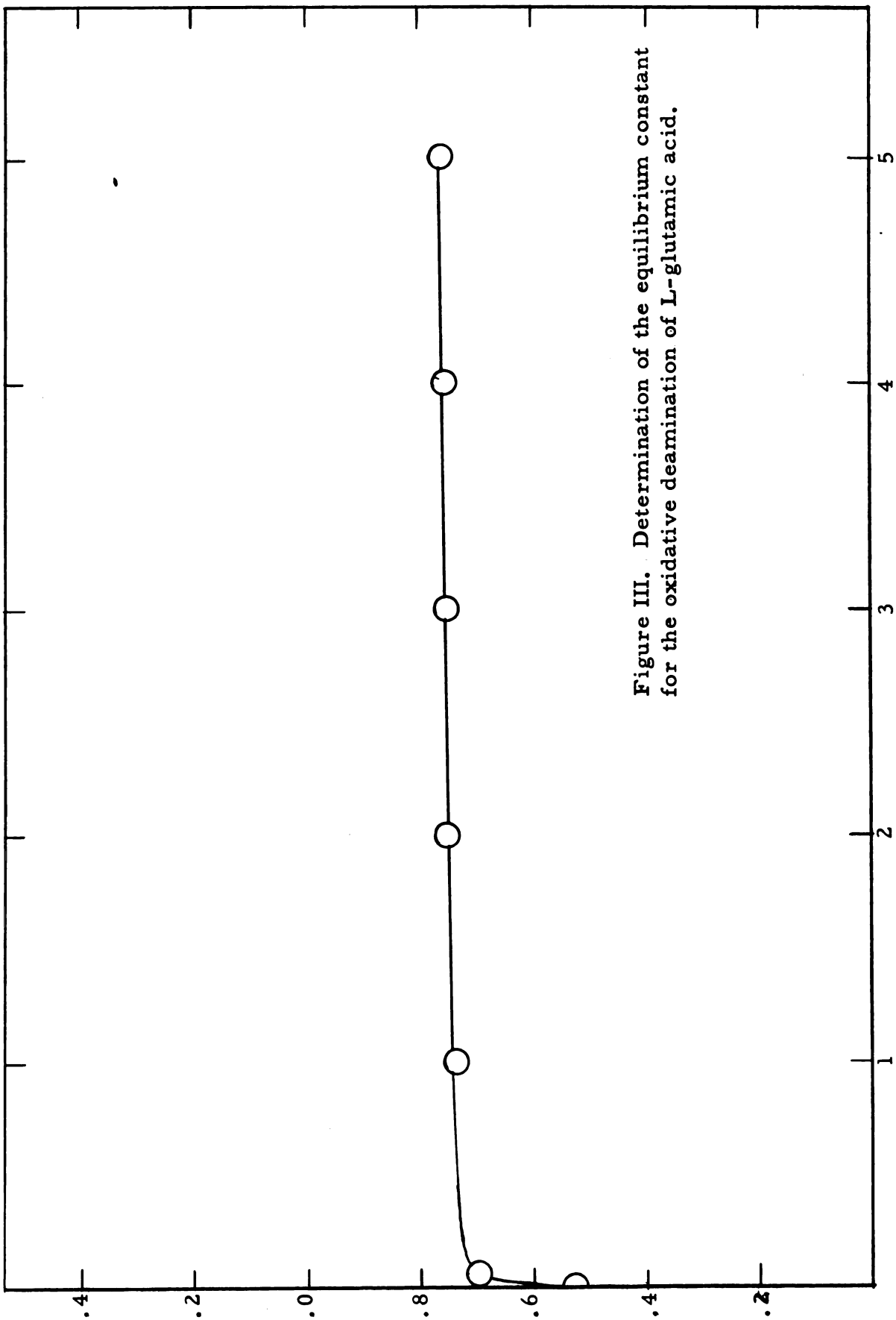

Figure III. Determination of the equilibrium constant for the oxidative deamination of L-glutamic acid.

the enzymatic oxidative deamination of L-glutamic acid. Indeed, a definite inhibition was observed when either of these analogues was included in the enzymatic reaction mixture. In order to determine the inhibition constants (Ki) three series of runs were made for each of the two inhibiting analogues. In the first series six different concentrations of L-glutamic acid were used but no analogue was included. In the second series a constant amount of one of the analogues was also included in each run. A third series was run with another constant amount of the inhibitor included. The concentrations of the analogues were taken as total concentration of the racemic tetrazoles.

A plot of reciprocal initial velocity against reciprocal substrate concentration in the manner of Lineweaver and Burk furnished a straight line for each of the three series of runs. The three lines intersected the ordinate at nearly the same point which indicated that the inhibition was clearly competitive. Both the gamma- and the ditetrazole analogues gave lines which showed the clearly competitive nature of the inhibition (Figures IV-V).

The apparent inhibition constant (Ki) for the racemic ditetrazole analogue was determined from the slopes and intercepts of the lines in the Lineweaver-Burk plot and the mathematical expression (VII) for the slope as taken from equation III. A Michaelis constant of 1.25×10^{-3}

$$\text{slope} = \frac{1}{V}\left[Km + \frac{Km\,(I)}{Ki}\right] \qquad \text{VII}$$

was obtained for L-glutamic acid from the abscissa intercept for the series of runs without inhibitor. Adelstein and Mee (109) have recently reported the value of 1.0×10^{-3} for this constant. A maximum velocity (V) of 1.0×10^{-4} was obtained as the reciprocal of the ordinate intercept. From equation VII, Ki for the ditetrazole analogue was found to be

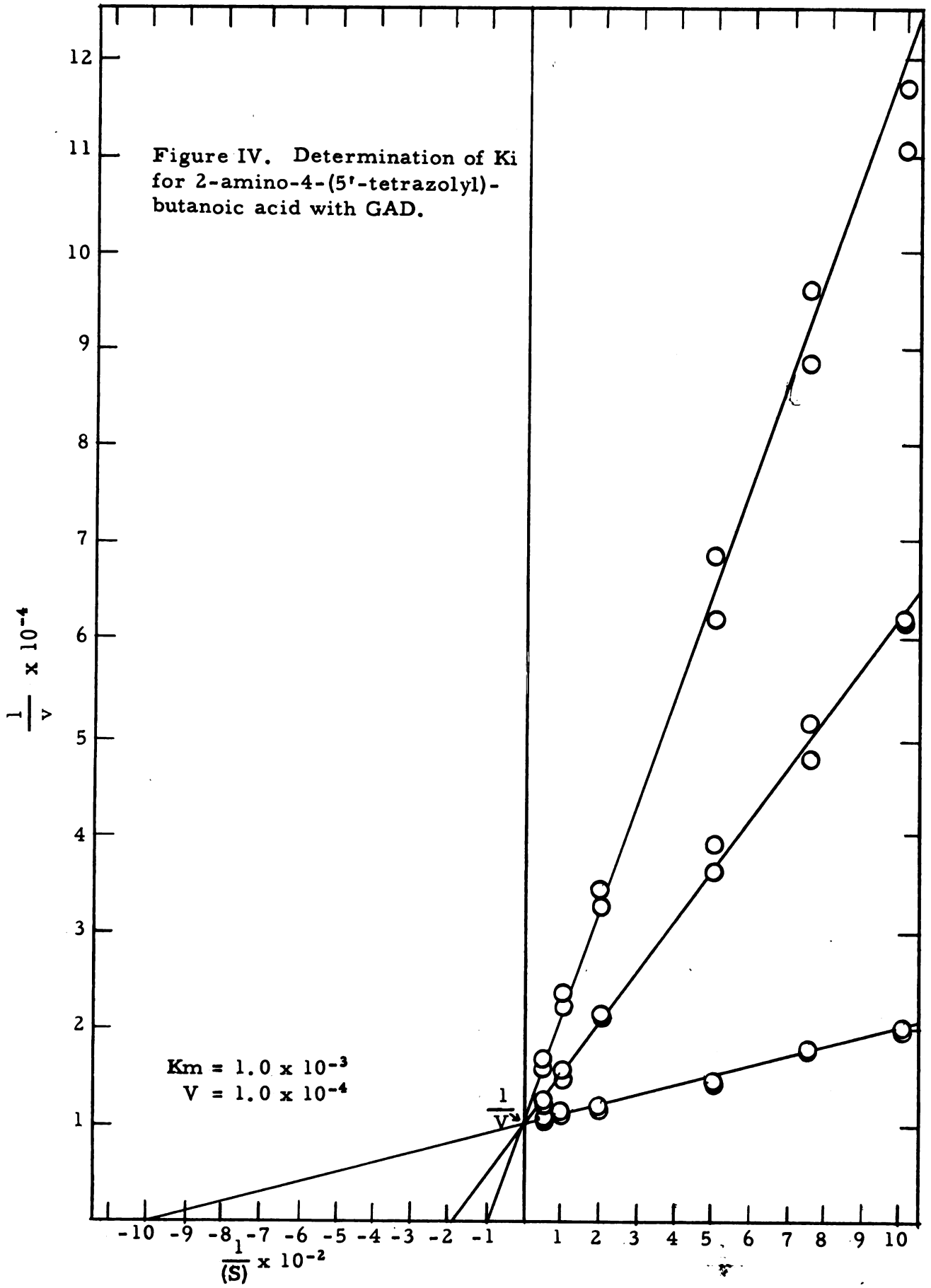

Figure IV. Determination of Ki for 2-amino-4-(5'-tetrazolyl)-butanoic acid with GAD.

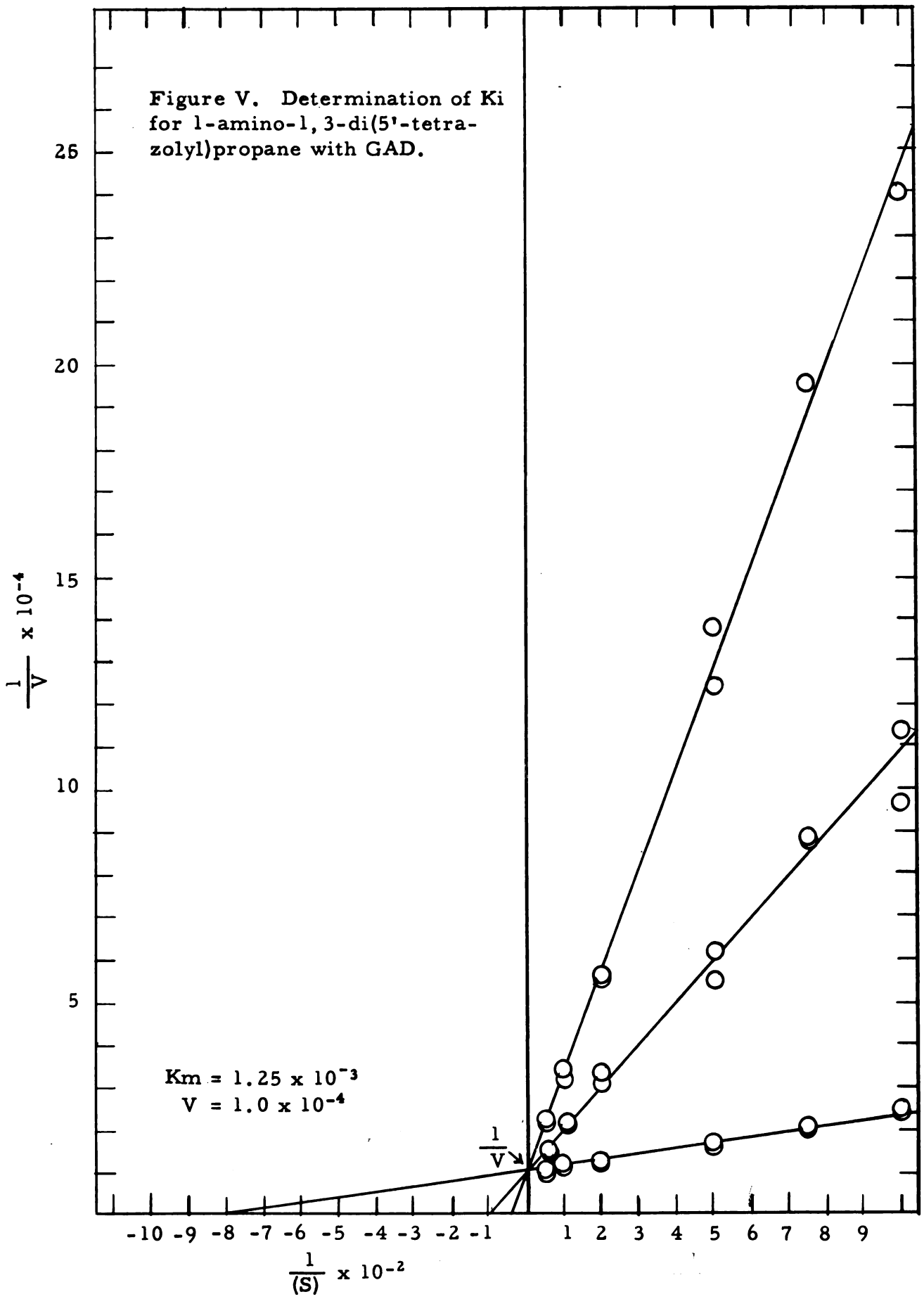

Figure V. Determination of Ki for 1-amino-1, 3-di(5'-tetrazolyl)propane with GAD.

1.4×10^{-3} in each of two independent runs. If the value of Km reported by Adelstein and Mee is used in the calculations, Ki becomes 1.1×10^{-3}.

The apparent inhibition constant for the racemic gamma-tetrazole analogue was determined in an identical manner. Values of 5.5×10^{-3} and 5.0×10^{-3} were obtained for Ki from the slopes of the two inhibition lines. A value of Km for L-glutamic acid was calculated from the intercept to be 1.0×10^{-3} in agreement with the results of Adelstein and Mee.

In 1957 Caughey, Smiley, and Hellerman (105) studied the structural requirements of compounds for substrate competition in the enzymatic oxidative deamination of L-glutamic acid. Their findings indicated that several factors were operative in determining the ability of a compound to competitively inhibit the reaction. Stereochemically, an extended length of about 7.5 A. units seemed to be optimum for inhibition. The necessity of having electronegative groups at the ends of the molecule was also demonstrated. Dipole moment and hydration effects were important, however the two effects were found to be opposed. Usually an increased dipole moment led to increased hydration and the effects nearly cancelled.

The three tetrazole analogues of DL-glutamic acid possessed the necessary requirements for inhibition, and so the ability of the analogues to bind to the enzyme was not surprising. An extended length somewhat greater than 7.5 A. (110) would be expected for the analogues, however, the distances between the centers of negative charge probably do not differ much from glutamic acid. A comparison of the inhibition constants and of the Michaelis constants for glutamic acid and the analogues indicates that the extent of binding decreases in the following order: L-glutamic acid > DL-ditetrazole analogue > DL-gamma-tetrazole analogue > DL-alpha-tetrazole analogue. Table III compares the binding constants of the tetrazoles with selected compounds studied by Caughey et al. (105).

Table III. Michaelis and inhibition constants for the tetrazole analogues and a number of selected compounds.

Compound	Average Ki x 10^4	Average Km x 10^4
Fumarate	68	
Trimesate*	40	
D-glutamate	20	
Glutarate	5.8	
5-Bromofuroate	0.6	
gamma-Tetrazole	53	
Ditetrazole	14	
alpha-Tetrazole		280
L-glutamate		10

*Reported as not clearly competitive.

A meaningful rationalization of these results cannot be made. It was surprising that the DL-ditetrazole analogue would bind to the enzyme about equally with DL-glutamic acid while the gamma-tetrazole was somewhat less efficient in binding and hence in functioning as an inhibitor. It was also surprising that the Km value for the alpha-tetrazole analogue was roughly a power of ten larger than the constants for the other analogues. Although the alpha-tetrazole was a relatively poor binder, it possessed the necessary structural requirements for the oxidative deamination. The slow rate of reaction of the alpha-tetrazole in the enzyme system was probably due to both a relatively slow rate of decomposition of the substrate to products and to a relatively fast rate of dissociation to substrate and enzyme. Also there may be a substantial amount of inhibition of an inactive enantiomer.

In order to study the enantiomeric specificity of action of the enzyme, GAD, toward the various analogues, it would be necessary to obtain the pure optical isomers. In view of the fact that N-acetyl-DL-glutamic acid is a substrate for acylase I(111)it was hoped that the N-acetyl tetrazole analogues would be capable of functioning as substrated for the enzyme. However, each of the three tetrazole analogues was found to be inactive toward acylase I under experimental conditions essentially the same as those of Greenstein (111). However, the N-acetyl derivatives of the alpha- and ditetrazole analogues were found to act as weak inhibitors of the enyzme when N-acetyl-DL-methionine was used as the substrate.

The ability of the three analogues to function as substrates for D-amino acid oxidase was tested by a modification of the procedure of Huennekens and Felton (112). These workers followed the reaction by measuring the oxygen uptake whereas in the following work the oxidation was followed by observing the fading of indophenol blue which was coupled to the flavin adenine dinucleotide (FAD) in an anaerobic system. Each of

the three analogues was incubated with the enzyme, FAD, and indophenol. Acceptable substrates for this enzyme are oxidized to the imino acids with subsequent reduction of the FAD. The indophenol then functions to oxidize $FADH_2$ while itself being converted into the colorless leuco-indophenol. Unfortunately, all three of the tetrazole analogues were found to be totally inactive as substrates for this enzyme.

EXPERIMENTAL

PART III

The following materials were used in the enzymatic reactions: L-glutamic acid dehydrogenase (GAD) obtained from Sigma Chemical Company as a crystalline suspension in 50% saturated ammonium sulfate; grade III beta-DPN obtained from the same source and assaying 95% and 98%; chromatographically pure L-glutamic acid supplied by Nutritional Biochemical Corporation; acylase I obtained from Sigma Chemical Company; D-amino acid oxidase prepared by the method of Huennekens and Felton (112), phosphate buffers of pH = 7.5 (0.5 M) and pH = 7.6 (0.05 M); and pyrophosphate buffer of pH = 8.3 (0.1 M).

The assay procedure used for L-glutamic dehydrogenase was essentially that of Strecker (104). All reactions were followed by measuring the absorption of 340 mμ corresponding to the formation of DPNH ($\epsilon = 6.22 \times 10^6$ cm^2 $mole^{-1}$). A DU spectrophotometer equipped with a log converter (Ledland Instrument Engineering Company) and recorder was used which furnished a direct plot of optical density at 340 mμ against time.

The assay for acylase I was essentially that of Greenstein (111). The reactions were followed spectrophotometrically using a Cary Model II spectrometer.

A modification of the procedure of Huennekens and Felton (112) was used in the assay for D-amino acid oxidase. Again the Cary Model II spectrometer was used to follow the reaction.

Testing the Three Tetrazole Analogues of DL-Glutamic Acid as Substrates for L-Glutamic Acid Dehydrogenase

Into each of four cuvettes of 3 ml. capacity was placed 2.4 ml. of phosphate buffer (pH = 7.6, 0.05 M), 0.1 ml. of DPN (0.01 M), 0.1 ml. GAD (0.3 mg. per 0.1 ml. of enzyme solution), and 0.2 ml. of pure water. To the first tube was added 0.2 ml. of alpha-tetrazole solution (0.5 M). To the second and third tubes were added an equivalent amount of the ditetrazole and gamma-tetrazole analogues, respectively. The fourth tube served as the blank.

The four cells were placed in the DU spectrophotometer, and after an hour a change in optical density of 0.24 was observed for the alpha-tetrazole analogue while the changes for the di- and gamma-tetrazole analogues were 0.00 and 0.02, respectively.

When the test was repeated using 2.4 ml. of phosphate buffer (pH = 7.5, 0.5 M), 0.1 ml. of DPN (0.1 M), 0.1 ml. of GAD (0.075 mg. per 0.1 ml. of enzyme solution), 0.2 ml. of pure water, and 0.2 ml. of test compound (0.005 M), the changes in optical density after 100 minutes were 0.05, 0.00, and 0.01 for the alpha-, di-, and gamma-tetrazole analogues, respectively.

Determination of the Apparent Michaelis Constant, Km', for 4-Amino-4-(5'-tetrazolyl)butanoic Acid with GAD

Into each of six absorption cells of 3 ml. capacity was placed 2.2 ml. of buffer (pH = 7.5, 0.5 M), 0.2 ml. of DPN (0.1 M), and 0.2 ml. of GAD (0.075 mg. per 0.2 ml. of enzyme solution). Into the first cell was pipetted 0.35 ml. of water and 0.05 ml. of alpha-tetrazole solution (0.5 M). After mixing, the change in optical density was plotted against time for about ten minutes. Similarly plots were made by adding 0.1, 0.2, 0.3, and 0.4 ml. of the analogue solution along with sufficient water

to give a total cell volume of 3 ml. to each of the next four cells. The sixth cell served as the blank. The initial slopes were determined for each curve, and from the initial rate of change of optical density and the extinction coefficient, an initial velocity was calculated in units of moles/liter/ten minutes. A plot of reciprocal velocity on the ordinate against reciprocal substrate concentration on the abscissa, in the manner of Lineweaver and Burk (108), furnished an abscissa intercept of $-1/Km'$ from which a value of 2.6×10^{-2} moles/liter was calculated for Km'.

A second determination using 1.9 ml. of buffer (pH = 7.5, 0.5 M), 0.3 ml. of DPN (0.1 M), and 0.3 ml. of GAD (0.15 mg. per 0.1 ml. of enzyme solution), and substrate concentrations of 0.1, 0.2, and 0.3 ml. of 0.05 M and 0.1, 0.3, and 0.5 ml. of 0.5 M solutions furnished a Km' of 3.0×10^{-2} moles/liter.

A value of 3.1×10^{-2} moles/liter was found for Km' under the following conditions: 2.1 ml. of buffer (pH = 7.6, 0.05 M), 0.2 ml. of DPN (0.01 M), 0.2 ml. of GAD (0.15 mg. per 0.1 ml. of enzyme solution), and 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 ml. of alpha-tetrazole analogue (0.5 M).

Determination of the Michaelis Constant, Km, for L-Glutamic Acid with GAD

The Michaelis constant for L-glutamic acid was determined in the same manner as for the alpha-tetrazole analogue. Each cell of 1 ml. capacity contained 0.5 ml. of buffer (pH = 7.5, 0.5 M), 0.1 ml. of DPN (0.1 M), 0.1 ml. of GAD (0.0125 mg. per 0.1 ml. of enzyme solution), and the proper amount of pure water to bring the total volume to 1 ml. upon the addition of the substrate. The following amounts of substrate were added: 0.2 ml. of 0.1 M, 0.1 ml. of 0.1 M, 0.1 ml. of 0.05 M, 0.2 ml. of 0.01 M, 0.13 ml. of 0.01 M, and 0.1 ml. of 0.01 M. Two runs

were made at each substrate concentration to give a total of twelve curves. A plot of the twelve points in the manner of Lineweaver and Burk (108) gave a value of 1.0×10^{-3} moles/liter for Km from the intercept. A repetition of the determination with identical amounts of all the substituents gave a Km of 1.25×10^{-3} moles/liter.

Determination of the Equilibrium Constant for the Oxidative Deamination of 4-Amino-4-(5'-tetrazolyl)butanoic Acid

Into a 1 ml. absorption cell was placed 0.6 ml. of buffer (pH = 7.5, 0.5 M), 0.1 ml. of DPN (0.1 M), 0.1 ml. of GAD (0.1 mg. per 0.1 ml. of enzyme solution), 0.1 ml. of ammonium chloride solution (0.5 M), and 0.1 ml. of the alpha-tetrazole analogue (0.05 M). A blank was prepared in which the substrate was replaced with 0.1 ml. of pure water. The optical density was plotted against time for twelve hours. The apparent equilibrium constant after twelve hours was calculated to be 3.65×10^{-14} moles/liter, (pH = 6.8) from a change in optical density of 0.67 corresponding to a DPNH concentration of 1.08×10^{-4} moles/liter. A value of 7.3×10^{-14} moles/liter is obtained by using half the concentration of racemic tetrazole as the substrate concentration. The ammonium chloride was added to repress the reaction and to stabilize the ammonium ion concentration. The pH was lowered to 6.8 by the addition of the ammonium chloride. The expression for the equilibrium constant is given as equation VI in PART III of the Results.

Determination of the Equilibrium Constant for the Oxidative Deamination of L-Glutamic Acid

The equilibrium constant for L-glutamic acid was determined by mixing identical amounts of all the substituents used in the determination of the equilibrium constant for the alpha-tetrazole analogue, except 0.1

ml. of L-glutamic acid (0.05 M) was used as substrate. After plotting the optical density against time for two hours, an equilibrium constant of 3.14×10^{-15} moles/liter (pH = 6.8) was calculated from a change in optical density of 0.20 corresponding to a DPNH concentration of 3.2×10^{-5} moles/liter. After five hours the optical density was essentially the same as at the end of two hours. A value of 55.5 moles/liter was used for the concentration of water. By multiplying the value of the equilibrium constant by 55.5 a new constant of 1.74×10^{-13} is obtained which can be compared directly with the values of 1.98×10^{-13} at a pH of 6.74 and 1.74×10^{-13} at a pH of 7.38 as reported by Strecker (104).

Determination of the Apparent Inhibition Constant, Ki, of 1-Amino-1, 3-di(5'-tetrazolyl)propane with GAD

Into each of six absorption cells of 1 ml. capacity was placed 0.5 ml. of buffer (pH = 7.5, 0.5 M), 0.1 ml. of DPN (0.1 M), and 0.1 ml. of GAD (0.0125 mg. per 0.1 ml. of enzyme solution). Into the first cell was pipetted 0.1 ml. of pure water. After mixing the solution, 0.2 ml. of L-glutamic acid (0.1 M) was added and again the solution was quickly mixed. The optical density was immediately plotted against time for several minutes, and from the initial rate of change of optical density with time and the extinction coefficient of DPNH an initial velocity was calculated in units of moles/liter/minute. A determination of initial velocity was made in a similar manner for cells into which the following amounts of substrate were added: 0.1 ml. of 0.1 M, 0.1 ml. of 0.05 M, 0.2 ml. of 0.01 M, 0.13 ml. of 0.01 M, and 0.1 ml. of 0.01 M. The calculated amount of water to give a total volume of 1 ml. was added to each cell prior to the addition of the substrate. The assay was run twice at each concentration of substrate to reduce error. A Lineweaver-Burk (108) plot of the twelve points furnished a straight line.

A second series of runs was made in which identical amounts of buffer, DPN, enzyme, and substrate was used, however, 0.02 ml. of the ditetrazole analogue (0.5 M) was also included in each cell, the volume being adjusted by reducing the amount of water added. From these runs twelve more points were calculated which gave a second straight line when plotted.

A third line was obtained in an identical manner for runs in which 0.05 ml. of ditetrazole analogue (0.5 M) had been added as inhibitor.

The three lines crossed at a single point on the ordinate from which a maximum velocity of 1.0×10^{-4} moles/liter/minute was calculated as the reciprocal of the intercept. From the slope of the lines a Ki value of 1.4×10^{-3} moles/liter was calculated from the following relationships: slope = Km/V[1 + I/Ki].

$$101 = 12.5\ (1 + 0.01/Ki),\ Ki = 0.125/88.5 = 1.4 \times 10^{-3}$$

and $$237 = 12.5\ (1 + 0.025/Ki),\ Ki = 0.312/224.5 = 1.4 \times 10^{-3}$$

Determination of the Apparent Inhibition Constant, Ki, of 2-Amino-4-(5'-tetrazolyl)butanoic Acid with GAD

The inhibition constant, Ki, for the gamma-tetrazole analogue was determined in exactly the manner described above for the ditetrazole analogue. All volumes and concentrations were identical with one exception--0.05 ml. and 0.10 ml. of gamma-tetrazole analogue (0.5 M) were used in the second and third series, respectively.

A Lineweaver-Burk plot of the reciprocal velocities against the reciprocal substrate concentrations again furnished three straight lines which mutually intersected at the ordinate. From the intersection a maximum velocity of 1.0×10^{-4} moles/liter/minute was calculated. From the slopes of the lines Ki values of 5.5×10^{-3} and 5.0×10^{-3} moles/liter were calculated as before.

$$55 = 10\ (1 + 0.025/Ki),\ Ki = 5.5 \times 10^{-3}$$

$$110 = 10\ (1 + 0.05/Ki),\ Ki = 5.0 \times 10^{-3}$$

Effects of the N-Acetyl Tetrazole Analogues Upon Acylase I

Each of the three N-acetyl tetrazole analogues was tested for its ability to function as a substrate for acylase I. To check for activity in the enzyme an assay was run with N-acetyl-DL-methionine as substrate. The reaction was followed by observing the increase in optical density at 238 mμ. Each of the three tetrazole analogues was tested in turn for substrate activity by following the formation of the free analogue at 238 mμ for the alpha- and ditetrazoles and at 230 mμ for the gamma-tetrazole analogue. The wavelengths used to follow each run were selected on the basis of the ultraviolet spectra of the analogue and its N-acetyl derivative. All three of the tetrazoles were found to be uniformly unaffected.

Each of the three N-acetyl tetrazole analogues was also tested for its ability to inhibit the catalytic action of the enzyme when N-acetyl-DL-methionine was used as substrate. Under identical experimental conditions and in the presence of a substantial amount of N-acetyl tetrazole analogue a slight inhibition was noted with the N-acetyl derivatives of both the alpha- and ditetrazoles. The N-acetyl derivative of the gamma-tetrazole appeared to be without effect.

Effects of the Three Tetrazole Analogues, L-Glutamic Acid, and DL-Methionine Upon D-Amino Acid Oxidase (DAAO)

DL-Methionine, L-glutamic acid, and each of the three tetrazole analogues were tested as substrates for D-amino acid oxidase. A modified Tunberg cell was used to conduct the anaerobic oxidation of the compounds. The reactions were followed by observing the reduction of indophenol at 560 mμ on a Cary Model II spectrophotometer.

As a test of the enzyme 1.0 ml. of pyrophosphate buffer (pH = 8.3, 0.1 M), 0.05 ml. of flavin adenine dinucleotide (FAD) solution, and 0.3 ml. of DAAO solution were placed in the absorption cell. In the side bulb were placed 0.5 ml. of DL-methionine (4.2 gm./120 ml.) and 0.3 ml. of indophenol (0.0012 M). The Tunberg cell was evacuated and the contents were mixed. The absorption was followed for several minutes and the optical density was found to change at the rate of 0.24 units per minute.

Each of the three tetrazole analogues and L-glutamic acid was tested as a substrate by placing 1.0 ml. of pyrophosphate buffer (pH = 8.3, 0.1 M), 0.10 ml. of FAD, and 0.5 ml. of DAAO in the absorption cell. In the side bulb were placed 1.0 ml. of substrate (0.05 M)* along with 0.10 ml. of indophenol (0.0012 M). After evacuating and mixing the solution as before, a plot of optical density against time indicated that no reduction was occurring. All three of the tetrazole analogues and L-glutamic acid were inactive as substrates.

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*For the ditetrazole analogue 1.0 ml. of 0.5 M solution was used.

SUMMARY

1. The interaction of 5-benzamidomethyltetrazole with acetic anhydride has been found to result in the formation of 2-methyl-5-benzamidomethyl-1, 3, 4-oxadiazole. The interaction of 5-phenyltetrazole, labeled with N^{15} in the 1-(or 4-) position of the ring, with acetic anhydride has been found to furnish 2-methyl-5-phenyl-1, 3, 4-oxadiazole in which half of the label was lost. This indicated that the 1, 2-(or 3, 4-) nitrogens of the ring were eliminated during the oxadiazole formation.

2. The three tetrazole analogues of DL-glutamic acid were prepared along with nine additional derivatives. These analogues result from the substitution of tetrazolyl groups for the alpha and/or gamma carboxyl groups of DL-glutamic acid.

3. The apparent pK_1, pK_2, and pK_3 values of the three tetrazole analogues of DL-glutamic acid were determined in aqueous solution. The three pK values of glutamic acid were also determined and compared with those for the analogues. The tetrazolyl groups were found to be comparable in acidity to the carboxyl groups.

4. The Rf and Mf values of glycine, glutamic acid and the two monotetrazole analogues were determined by paper chromatography using 80% phenol in water and a water saturated mixture of 1-butanol in 1, 4-dioxane as the moving phases. The Mf values of the monotetrazole analogues were similar to those of glutamic acid but differed greatly from those of glycine. This indicated a similarity in the relative amounts of hydrophilic and lipophilic character between glutamic acid and the monotetrazole analogues.

5. The three tetrazole analogues of DL-glutamic acid were tested for their ability to function as either substrates or inhibitors with L-glutamic acid dehydrogenase. The alpha-tetrazole analogue, 4-amino-4-(5'-tetrazolyl)butanoic acid, was found to be active as a substrate for the enzyme. No other analogue of glutamic acid has been found to be active as a substrate. The ditetrazole and gamma-tetrazole analogues were found to be inactive as substrates but effective as competitive inhibitors of the enzyme with L-glutamic acid as substrate.

6. The three tetrazole analogues were found to be totally inactive as substrates for D-amino acid oxidase. In addition, the three N-acetyl derivatives were found to be equally unaffected by acylase I.

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APPENDICES

APPENDIX I

(Titration Data and Curves)

POTENTIOMETRIC TITRATION DATA
for
D(-)-Glutamic Acid Hydrochloride
0.5238 g.

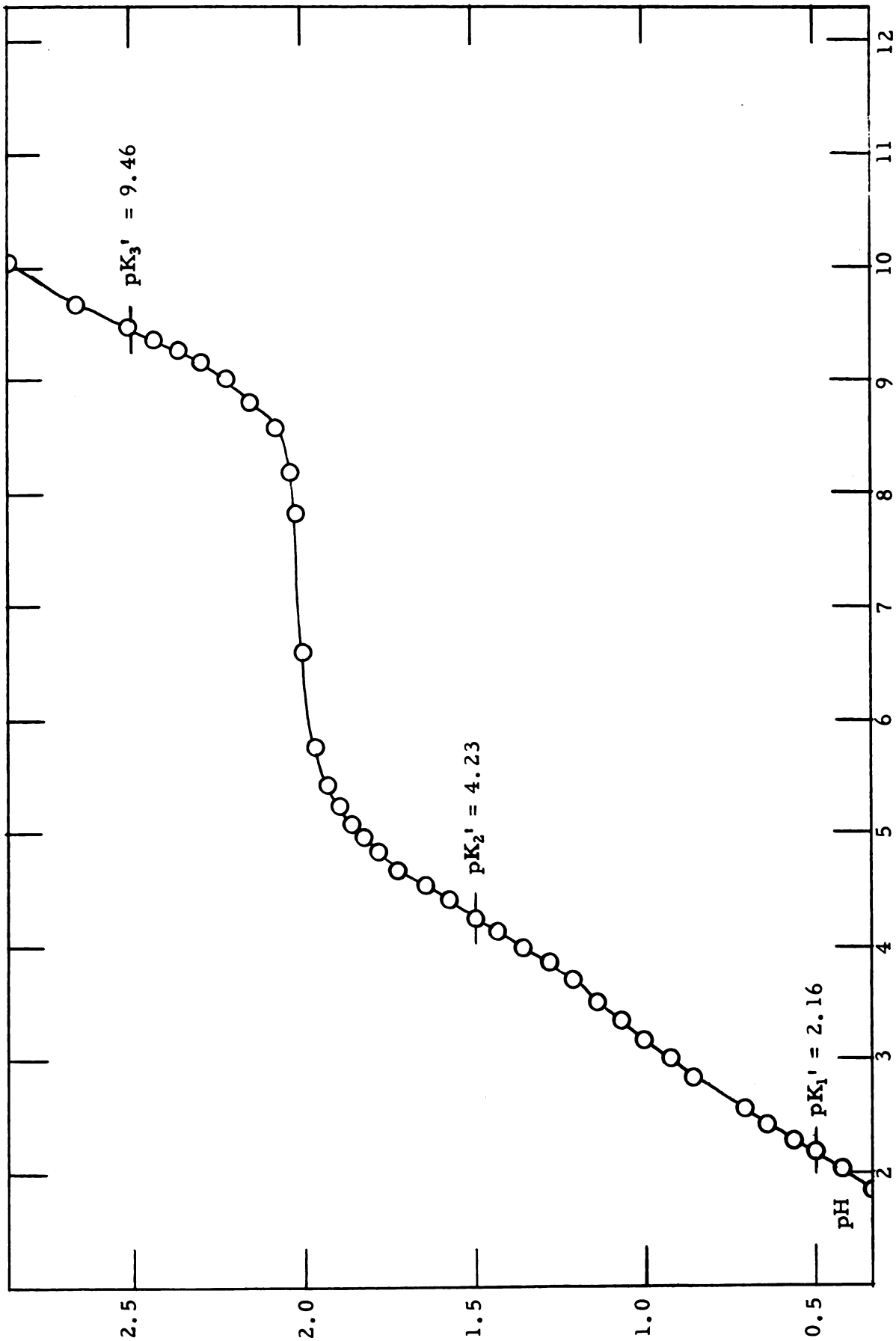
pH	Ml. of Aq. NaOH 0.2038 N	Equ. of NaOH per mole of compound	Apparent pK
1.24	0.00	0.000	
1.55	2.10	0.150	
1.67	3.10	0.221	
1.84	4.50	0.321	
2.02	6.00	0.424	
2.16	7.00	0.500	pK_1
2.28	8.00	0.571	2.16
2.41	9.00	0.643	
2.56	10.00	0.714	
2.83	12.00	0.857	
3.00	13.00	0.929	
3.15	14.00	1.000	EP_1
3.34	15.00	1.071	
3.50	16.00	1.143	
3.69	17.00	1.214	
3.84	18.00	1.286	
3.97	19.00	1.357	
4.10	20.00	1.429	
4.23	21.00	1.500	pK_2
4.38	22.00	1.571	4.23
4.51	23.00	1.643	
4.66	24.00	1.714	
4.83	25.00	1.786	
4.92	25.50	1.821	
5.06	26.00	1.857	
5.21	26.50	1.893	
5.41	27.00	1.929	
5.74	27.53	1.966	
6.59	28.00	2.000	EP_2
7.80	28.20	2.014	
8.17	28.45	2.032	
8.56	29.05	2.075	
8.78	30.00	2.143	
9.01	31.00	2.214	
9.13	32.00	2.286	
9.26	33.00	2.357	
9.36	34.00	2.429	

Continued

Potentiometric Titration Data for D(-)-Gluatmic Acid Hydrochloride 0.5238 g. - Continued

pH	Ml. of Aq. NaOH 0.2038 N	Equ. of NaOH per mole of compound	Apparent pK
9.46	35.00	2.500	pK_3
9.67	37.00	2.643	9.46
10.05	40.00	2.857	
10.24	41.00	2.929	
10.48	42.00	3.000	EP_3
10.72	43.00	3.071	
10.83	44.00	3.143	
10.90	45.00	3.214	

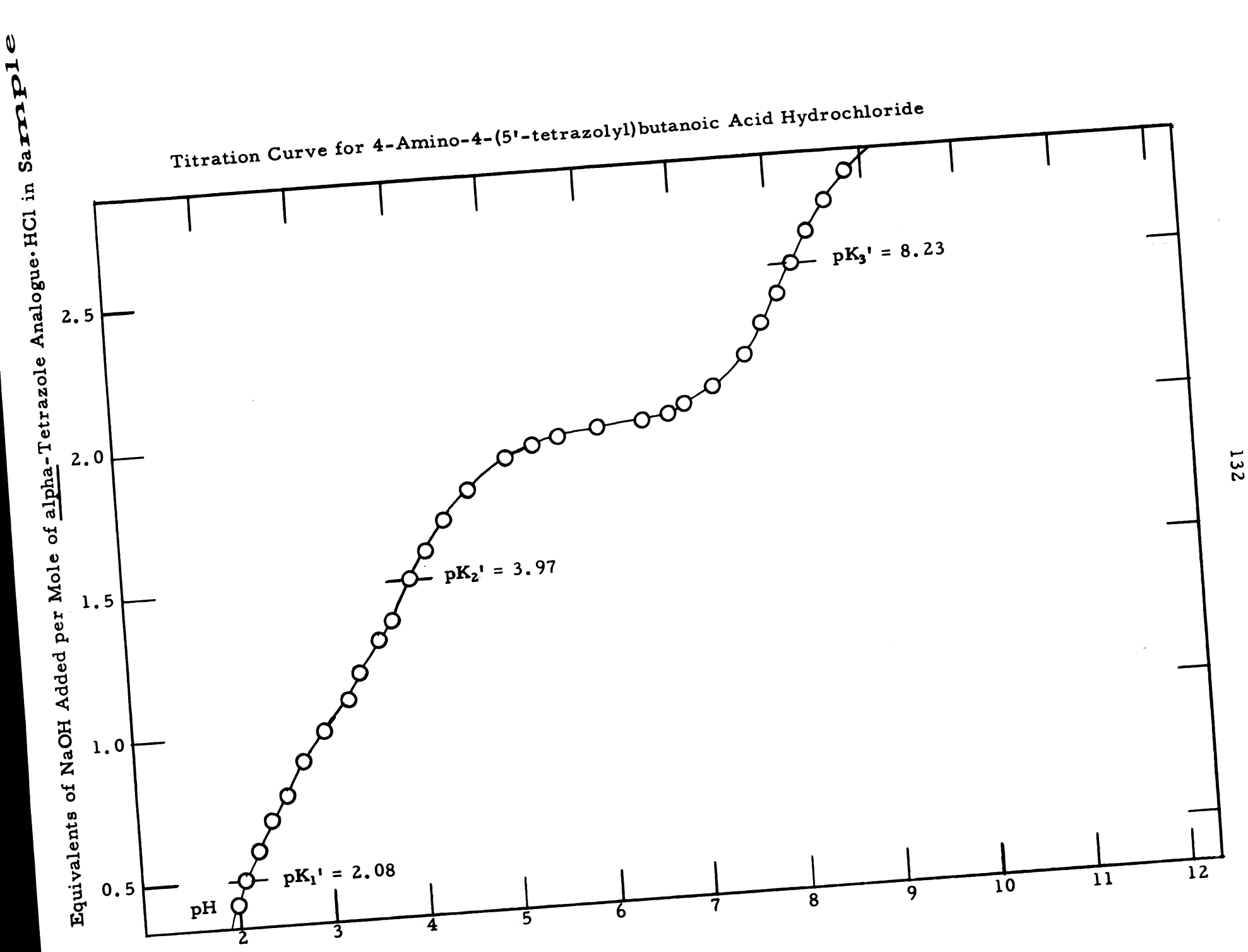
Titration Curve for D(-)-Glutamic Acid Hydrochloride



POTENTIOMETRIC TITRATION DATA
for
4-Amino-4-(5'-tetrazolyl)butanoic Acid Hydrochloride
0.2112 g.

pH	Ml. of Aq. NaOH 0.2038 N	Equ. of NaOH per mole of compound	Apparent pK
1.68	0.00	0.000	
1.76	0.50	0.100	
1.81	1.00	0.200	
1.84	1.50	0.301	
1.97	2.00	0.401	
2.08	2.50	0.501	pK_1
2.23	3.00	0.601	2.08
2.37	3.50	0.701	
2.55	4.00	0.801	
2.74	4.50	0.902	
3.00	5.00	1.002	EP_1
3.28	5.50	1.102	
3.37	6.00	1.202	
3.63	6.50	1.302	
3.77	7.00	1.403	
3.97	7.50	1.503	pK_2
4.17	8.00	1.603	3.97
4.38	8.50	1.703	
4.66	9.00	1.803	
5.07	9.50	1.904	
5.37	9.70	1.944	
5.64	9.80	1.964	
6.06	9.90	1.984	
6.52	10.00	2.004	EP_2
6.82	10.10	2.024	
7.00	10.20	2.044	
7.33	10.50	2.104	
7.66	11.00	2.204	
7.86	11.50	2.304	
8.05	12.00	2.404	
8.23	12.50	2.504	pK_3
8.40	13.00	2.605	8.23
8.60	13.50	2.705	
8.83	14.00	2.805	
9.21	14.50	2.905	
9.66	14.80	2.965	
9.94	14.90	2.986	
10.26	15.00	2.006	EP_3
10.70	15.50	3.106	

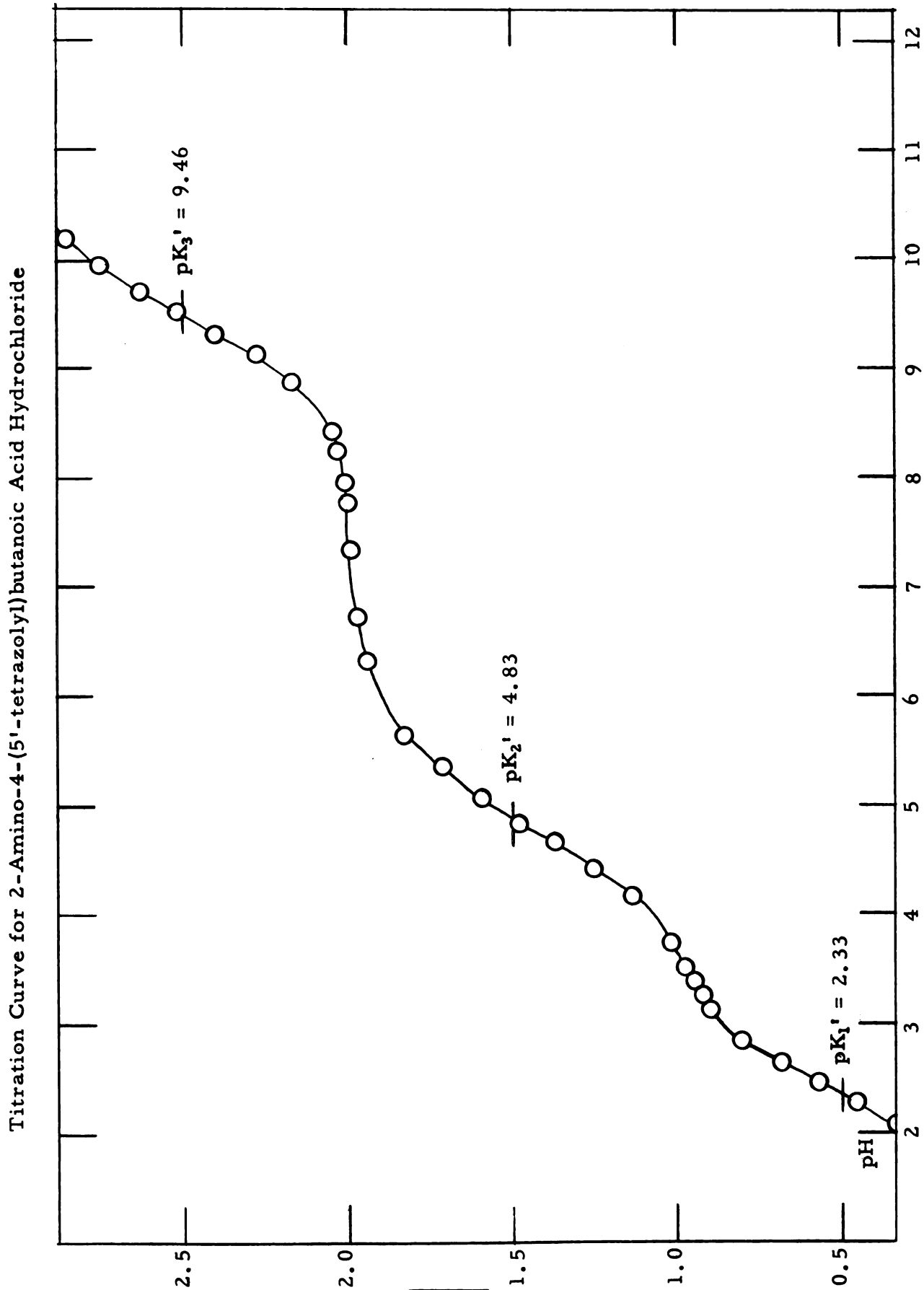
Titration Curve for 4-Amino-4-(5'-tetrazolyl)butanoic Acid Hydrochloride


Titration Curve for 4-Amino-4-(5'-tetrazolyl)butanoic Acid Hydrochloride
Equivalents of NaOH Added per Mole of alpha-Tetrazole Analogue·HCl in Sample
pK1' = 2.08
pK2' = 3.97
pK3' = 8.23
pH
0.5
1.0
1.5
2.0
2.5
2
3
4
5
6
7
8
9
10
11
12

POTENTIOMETRIC TITRATION DATA
for
2-Amino-4-(5'-tetrazolyl)butanoic Acid Hydrochloride
0.1847 g.

pH	Ml. of Aq. NaOH 0.2038 N	Equ. of NaOH per mole of compound	Apparent pK
1.70	0.00	0.000	
1.79	0.22	0.050	
1.88	0.50	0.115	
1.96	1.00	0.229	
2.03	1.50	0.344	
2.25	2.00	0.458	pK_1
2.44	2.50	0.573	2.33
2.61	3.00	0.687	
2.80	3.50	0.802	
3.12	4.00	0.916	
3.22	4.10	0.939	
3.36	4.20	0.962	
3.44	4.27	0.978	
3.71	4.50	1.031	EP_1
4.13	5.00	1.146	
4.38	5.50	1.260	
4.63	6.00	1.375	
4.81	6.50	1.489	pK_2
5.03	7.00	1.604	4.83
5.33	7.50	1.718	
5.61	8.00	1.833	
6.30	8.50	1.947	
6.69	8.60	1.970	
7.31	8.70	1.993	
7.75	8.74	2.002	EP_2
7.91	8.80	2.016	
8.23	8.90	2.039	
8.37	9.00	2.062	
8.87	9.50	2.176	
9.11	10.00	2.291	
9.29	10.50	2.406	pK_3
9.50	11.00	2.520	9.46
9.69	11.50	2.635	
9.92	12.00	2.749	
10.18	12.50	2.864	
10.70	13.00	2.978	
10.87	13.11	3.003	EP_3
11.17	13.50	3.093	
11.31	14.00	3.207	

Titration Curve for 2-Amino-4-(5'-tetrazolyl)butanoic Acid Hydrochloride


Titration Curve for 2-Amino-4-(5'-tetrazolyl)butanoic Acid Hydrochloride
Equivalents of NaOH Added per Mole of gamma-Tetrazole Analogue·HCl in Sample
pH
pK1' = 2.33
pK2' = 4.83
pK3' = 9.46
0.5
1.0
1.5
2.0
2.5
2
3
4
5
6
7
8
9
10
11
12

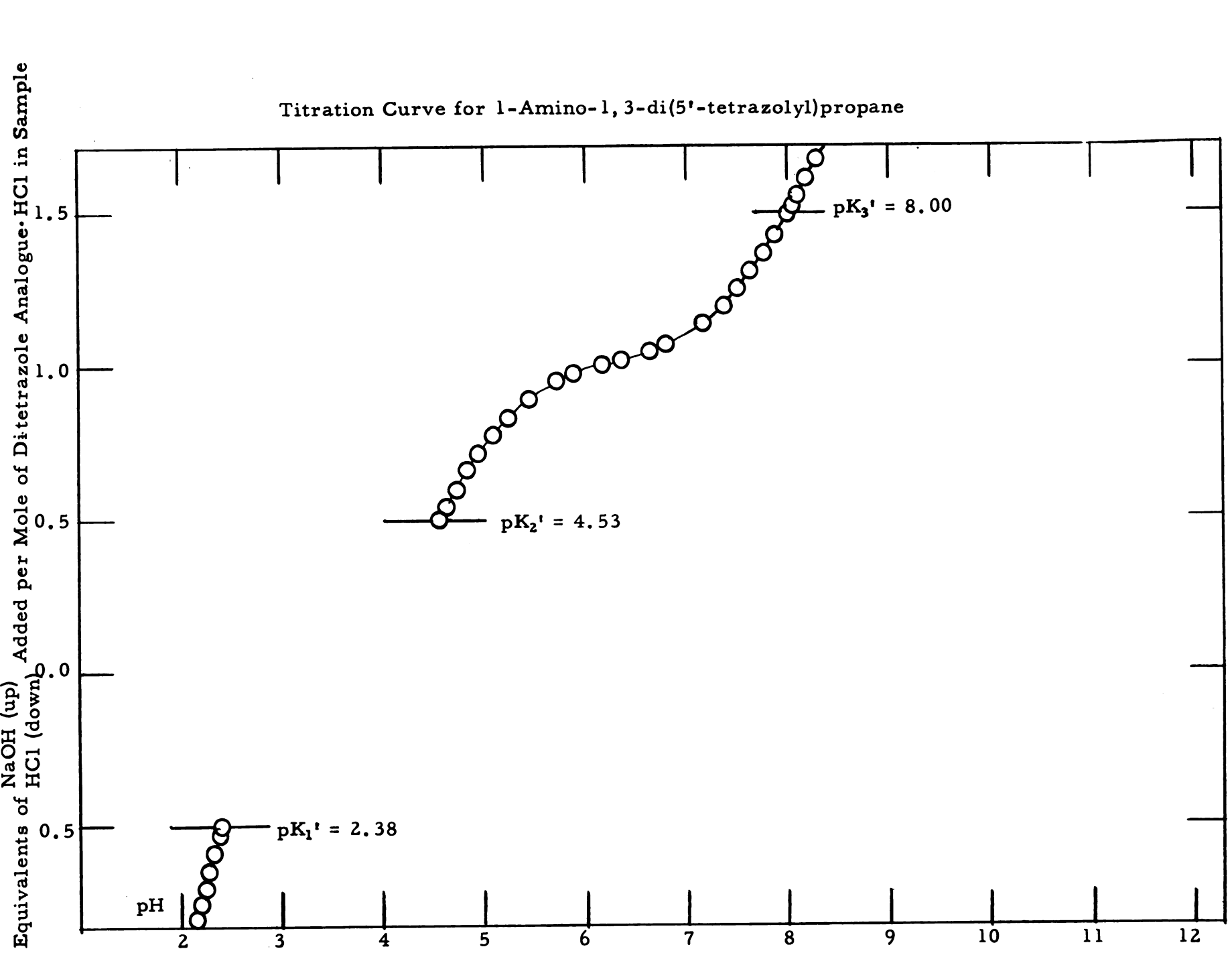
POTENTIOMETRIC TITRATION DATA
for
1-Amino-1, 3-di(5'-tetrazolyl)propane hydrate
0.3620 g.

pH	Ml. of Aq. HCl 0.1761 N	Equ. of HCl per mole of compound	Apparent pK
2.46	4.07	0.440	
2.41	4.50	0.486	
2.38	4.62	0.500	pK_1
2.35	5.00	0.540	2.38
2.30	5.50	0.595	
2.26	6.00	0.649	
2.22	6.50	0.703	
2.18	7.00	0.757	
2.13	7.50	0.811	
2.10	8.00	0.865	
2.07	8.50	0.919	
2.04	9.00	0.973	
2.02	9.25	1.000	EP_1
1.94	11.00	1.189	

POTENTIOMETRIC TITRATION DATA
for
1-Amino-1, 3-di(5'-tetrazolyl)propane hydrate
0.3810 g.

pH	Ml.of Aq. NaOH 0.2038 N	Equ. of NaOH per mole of compound	Apparent pK
4.53	4.21	0.500	pK_2
4.59	4.50	0.535	4.53
4.67	5.00	0.594	
4.81	5.50	0.654	
4.90	6.00	0.713	
5.05	6.50	0.773	
5.20	7.00	0.832	
5.42	7.50	0.891	
5.68	8.00	0.951	
5.83	8.20	0.975	
6.13	8.42	1.001	EP_2
6.33	8.62	1.022	
6.59	8.80	1.046	
6.77	9.00	1.070	
7.13	9.50	1.129	
7.33	10.00	1.189	
7.48	10.50	1.248	
7.61	11.00	1.307	
7.73	11.50	1.367	
7.85	12.00	1.426	
7.97	12.50	1.486	
8.00	12.63	1.501	pK_3
8.05	13.00	1.545	8.00
8.16	13.50	1.605	
8.27	14.00	1.664	
8.39	14.50	1.723	
8.51	15.00	1.783	
8.66	15.50	1.842	
8.84	16.00	1.902	
9.10	16.50	1.962	
9.17	16.60	1.973	
9.24	16.70	1.985	
9.43	16.84	2.002	EP_3
9.63	17.00	2.021	

Titration Curve for 1-Amino-1, 3-di(5'-tetrazolyl)propane


Titration Curve for 1-Amino-1, 3-di(5'-tetrazolyl)propane
Equivalents of NaOH (up) HCl (down) Added per Mole of Ditetrazole Analogue·HCl in Sample
1.5
1.0
0.5
0.0
0.5
pK3' = 8.00
pK2' = 4.53
pK1' = 2.38
pH
2
3
4
5
6
7
8
9
10
11
12

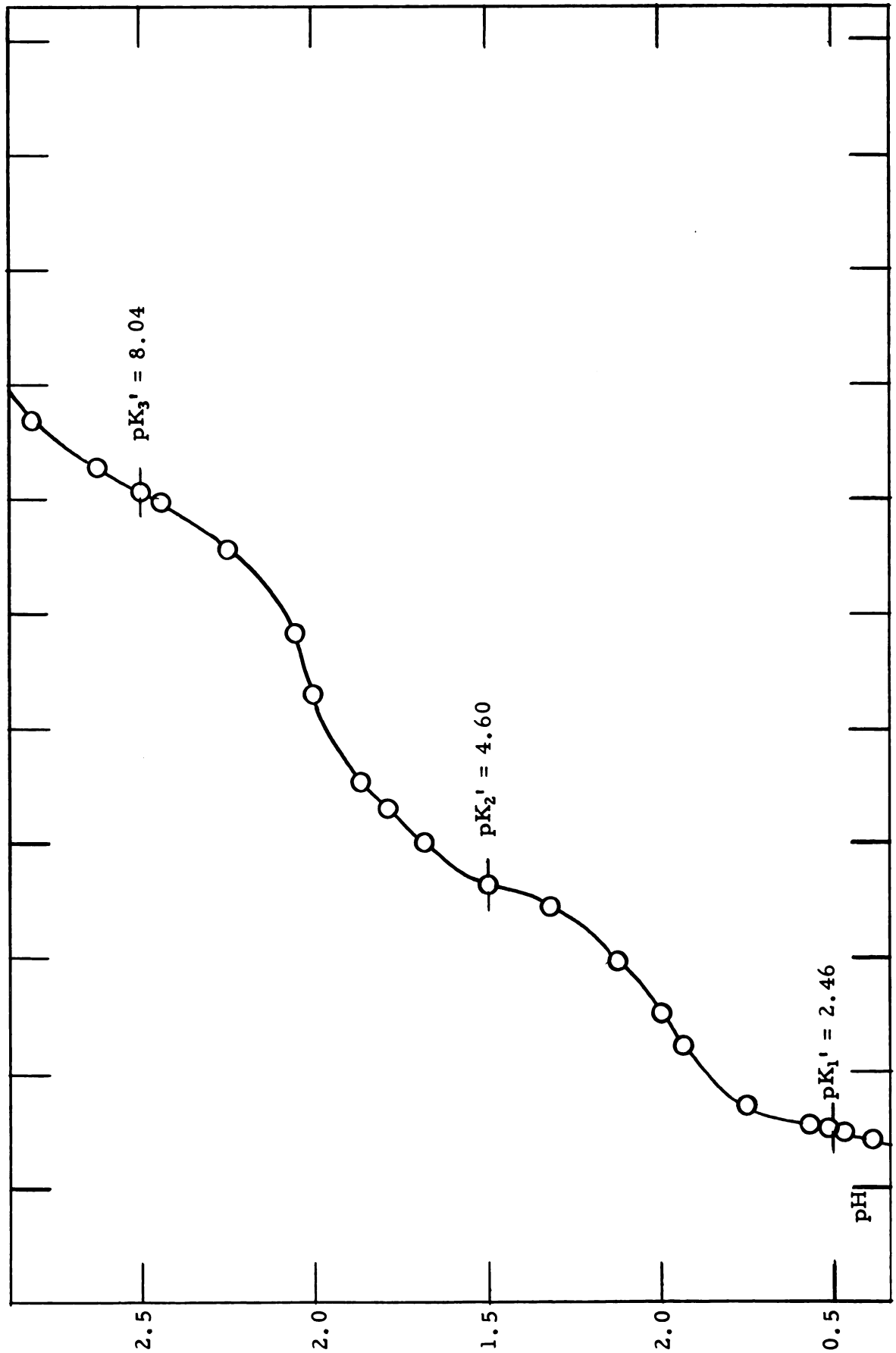
POTENTIOMETRIC TITRATION DATA
for
(1-Amino-1, 3-di(5'-tetrazolyl)propane hydrochloride)*
0.3620 g. of hydrate

pH	Ml. of Aq. NaOH 0.2038 N	Equ. of NaOH per mole of compound	Apparent pK
2.14	0.00	0.000	
2.25	1.00	0.187	
2.40	2.06	0.386	
2.45	2.50	0.468	
2.46	2.67	0.500	pK_1
2.53	3.00	0.562	2.46
2.67	4.00	0.749	
3.23	5.00	0.936	
3.50	5.34	1.000	EP_1
3.94	6.00	1.123	
4.42	7.00	1.311	
4.60	8.01	1.500	pK_2
5.01	9.00	1.779	4.60
5.51	10.00	1.872	
6.28	10.68	2.000	EP_2
6.80	11.00	2.056	
7.54	12.00	2.247	
7.95	13.00	2.434	
8.04	13.35	2.500	pK_3
8.25	14.00	2.621	8.04
8.66	15.00	2.808	
9.68	16.02	3.000	EP_3

*The solution of the hydrochloride was prepared by adding the calculated volume of standard hydrochloric acid solution (0.1761 N) to 0.3620 g. of the ditetrazole hydrate in water.

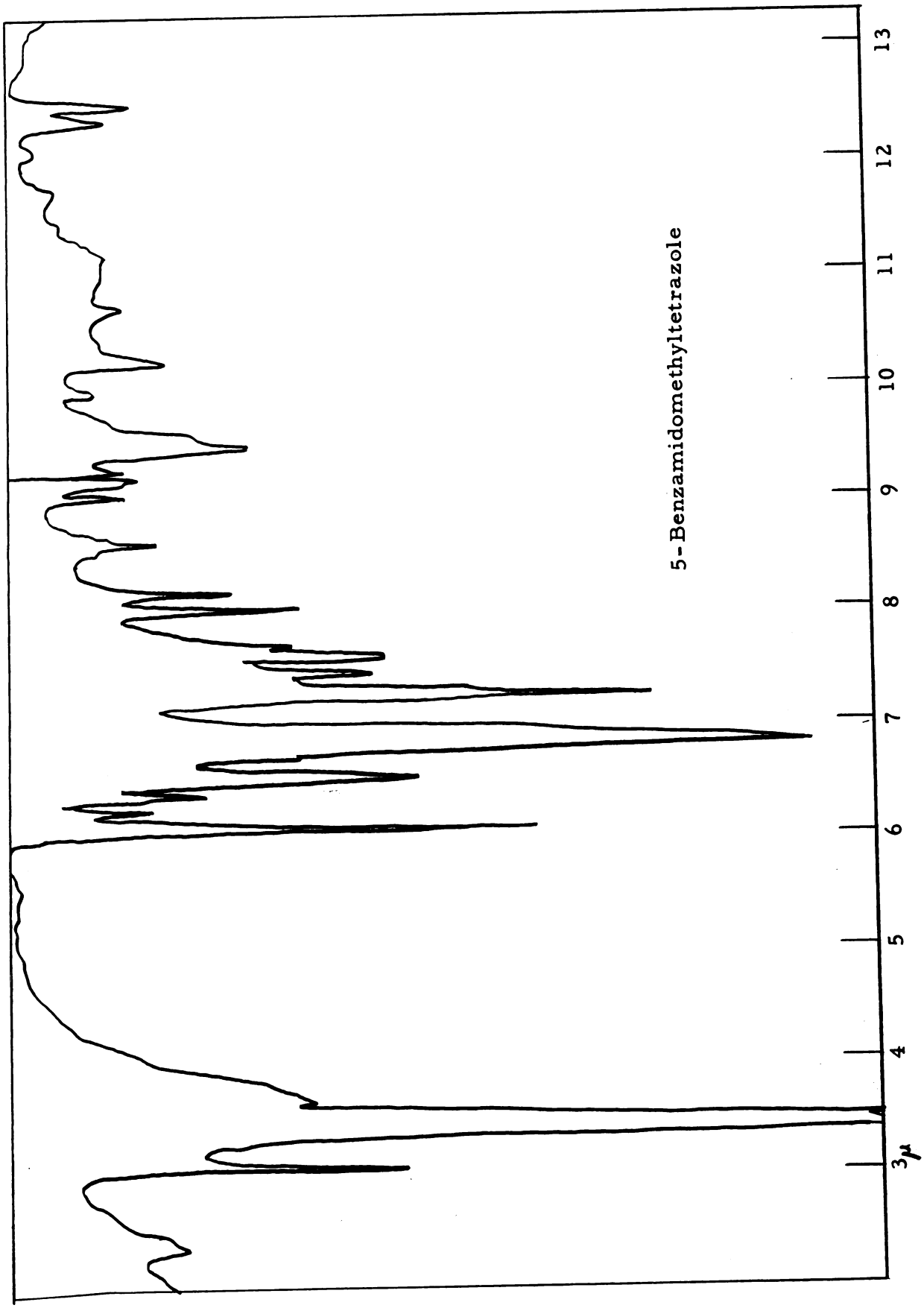
Titration curve for 1-Amino-1,3-di(5'-tetrazolyl)propane Hydrochloride Solution

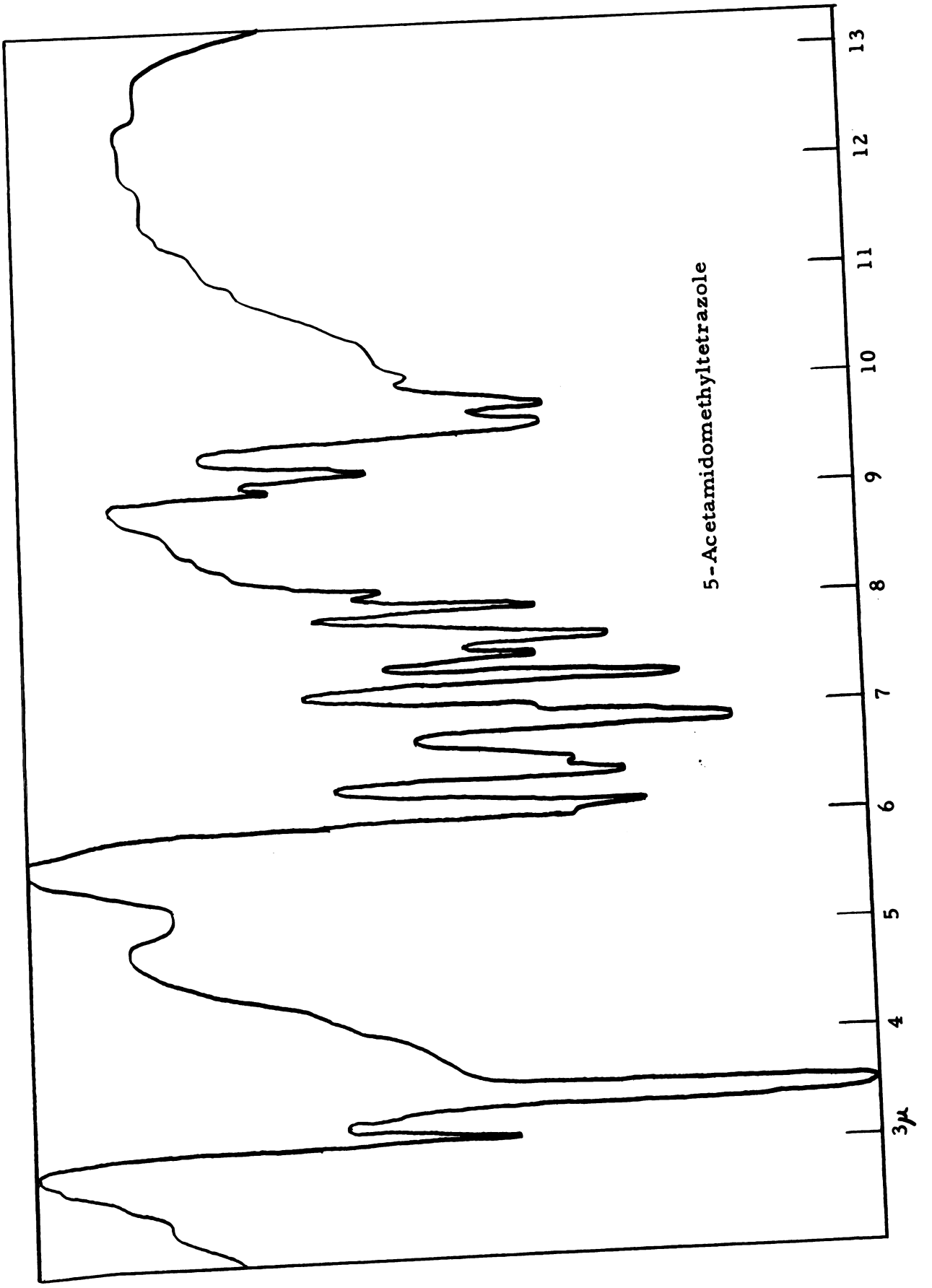
Titration Curve for 1-Amino-1, 3-di(5'-tetrazolyl)propane Hydrochloride Solution

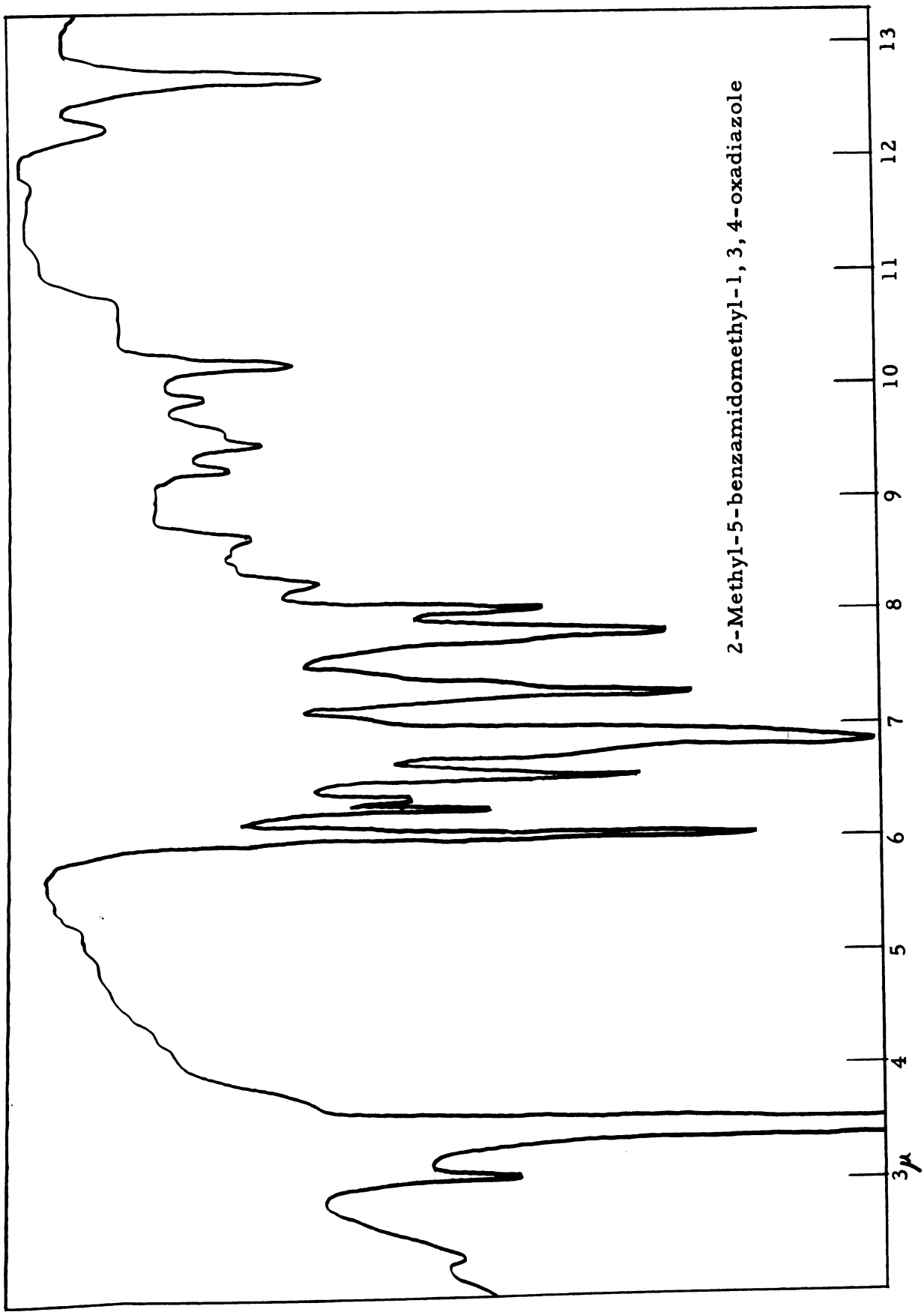


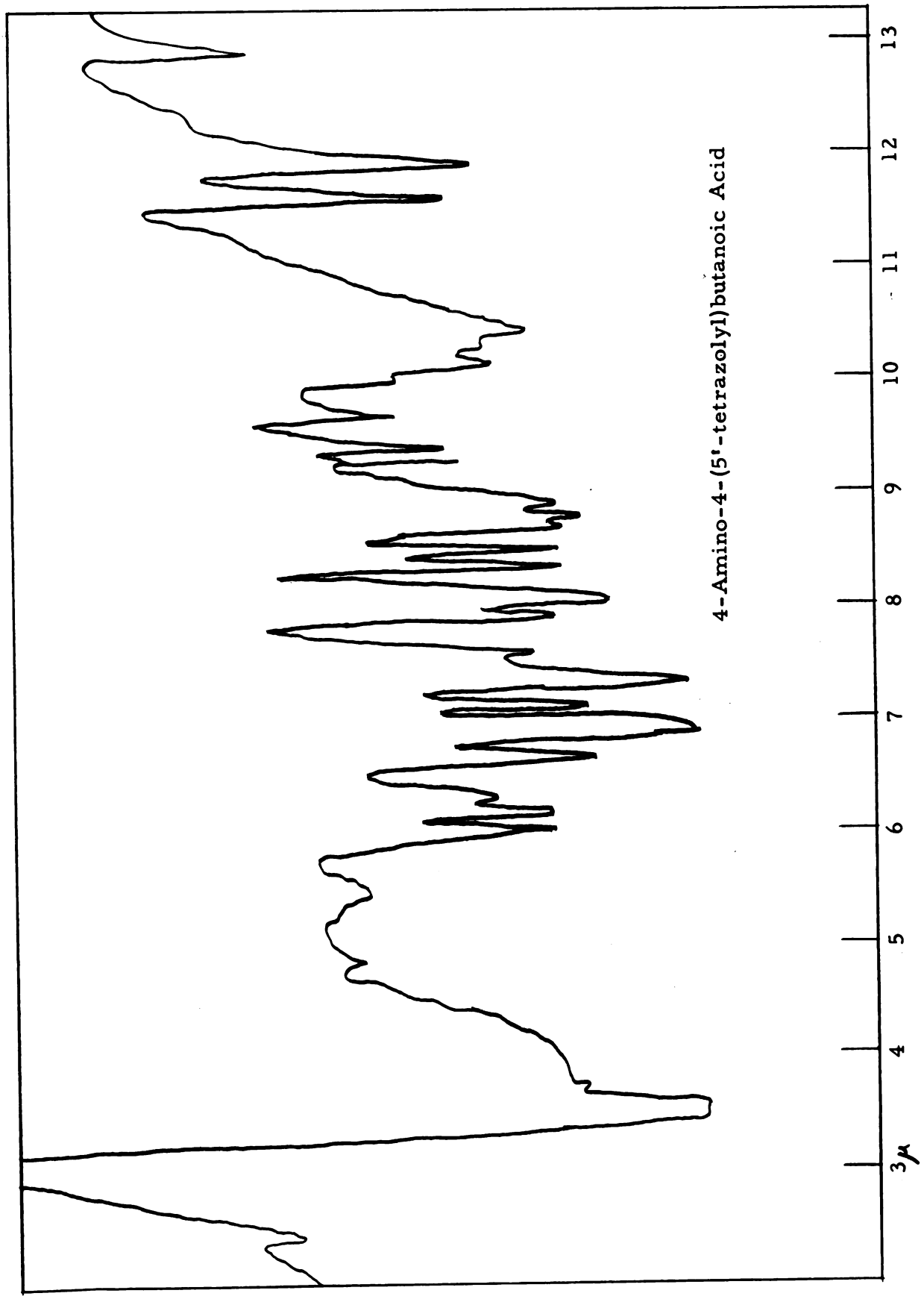
APPENDIX II

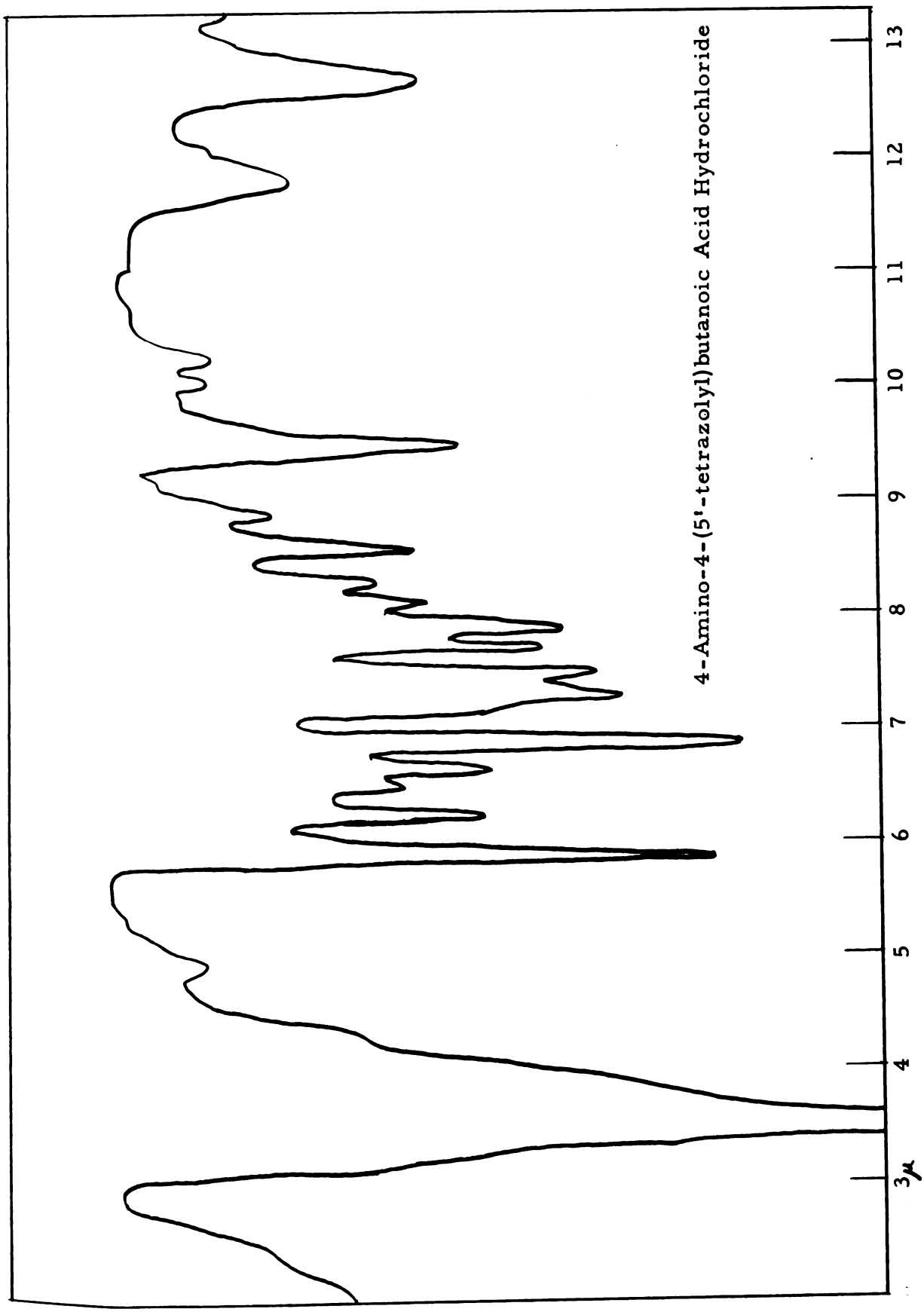
(Infrared Spectra)
(Nujol Mulls)

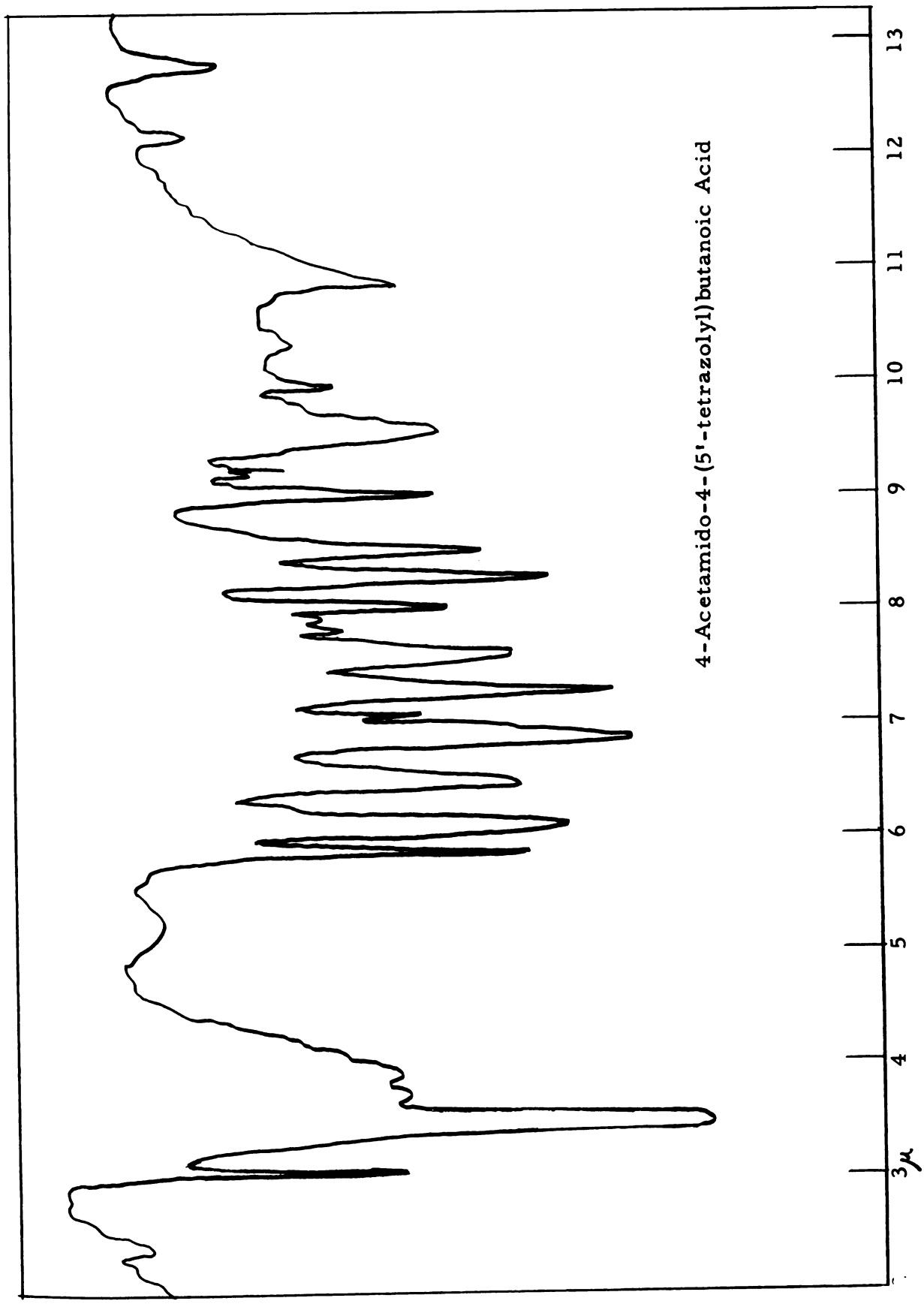

5-Benzamidomethyltetrazole
3μ
4
5
6
7
8
9
10
11
12
13

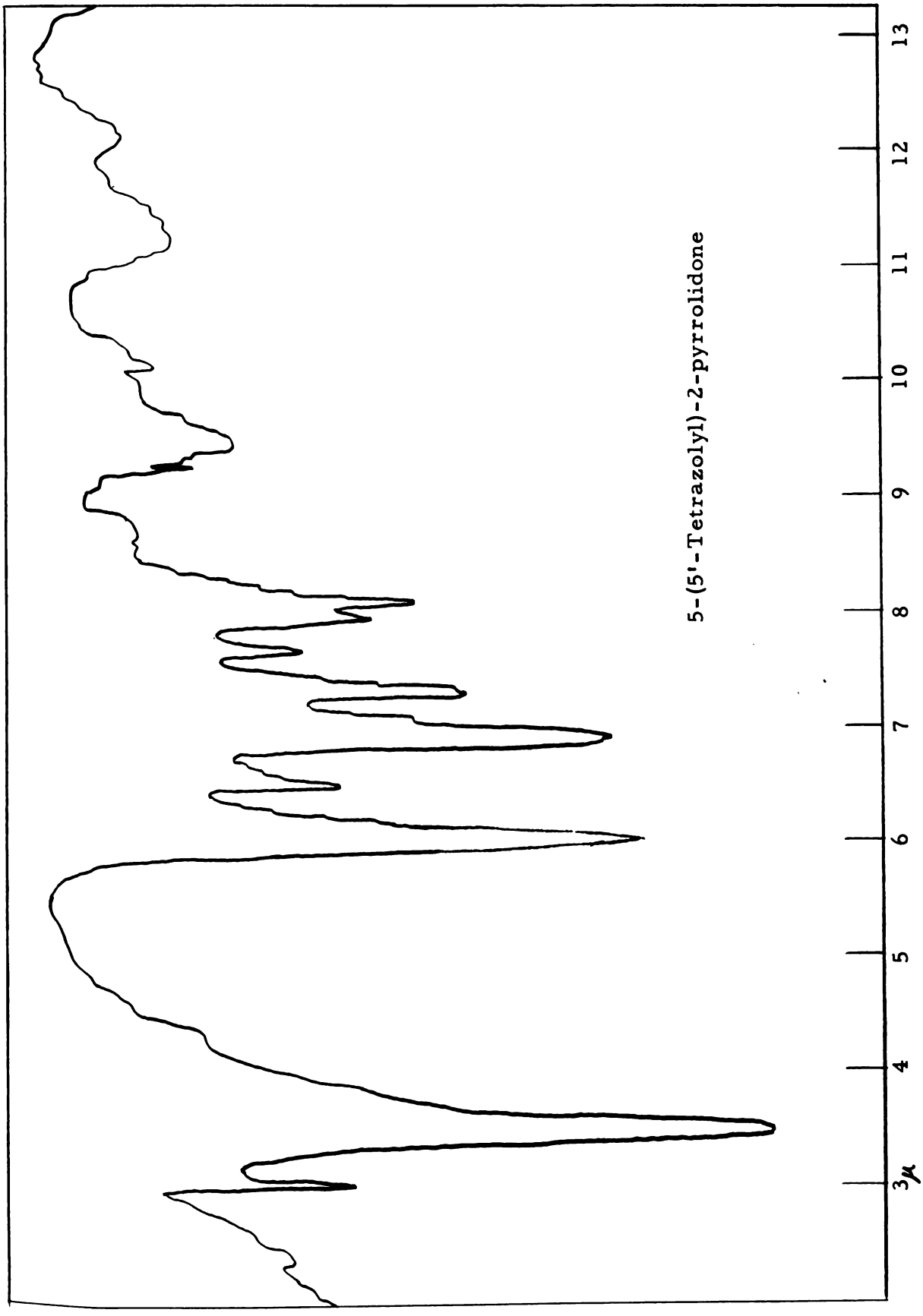

5-Acetamidomethyltetrazole
3μ
4
5
6
7
8
9
10
11
12
13

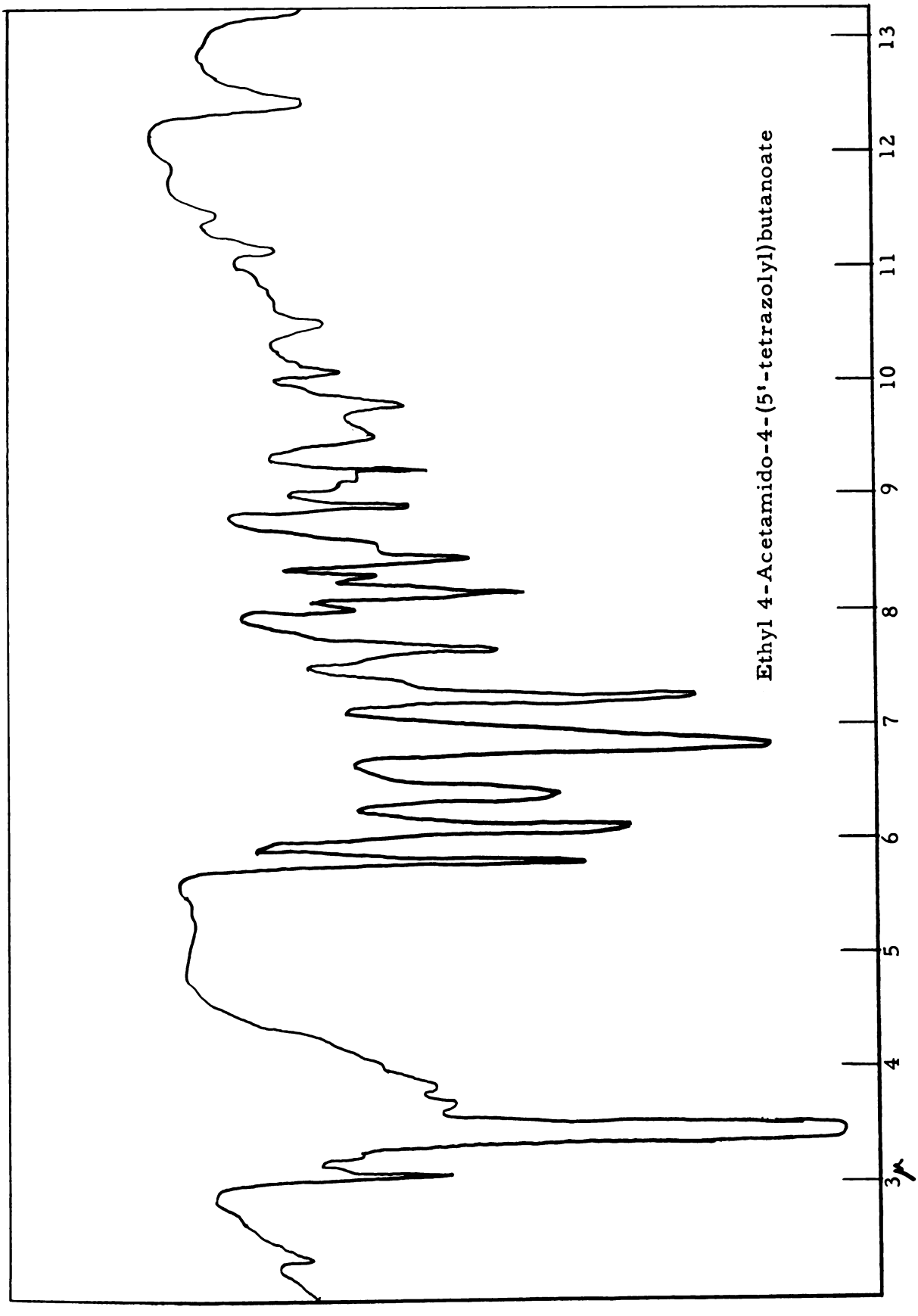

2-Methyl-5-benzamidomethyl-1, 3, 4-oxadiazole
3μ
4
5
6
7
8
9
10
11
12
13

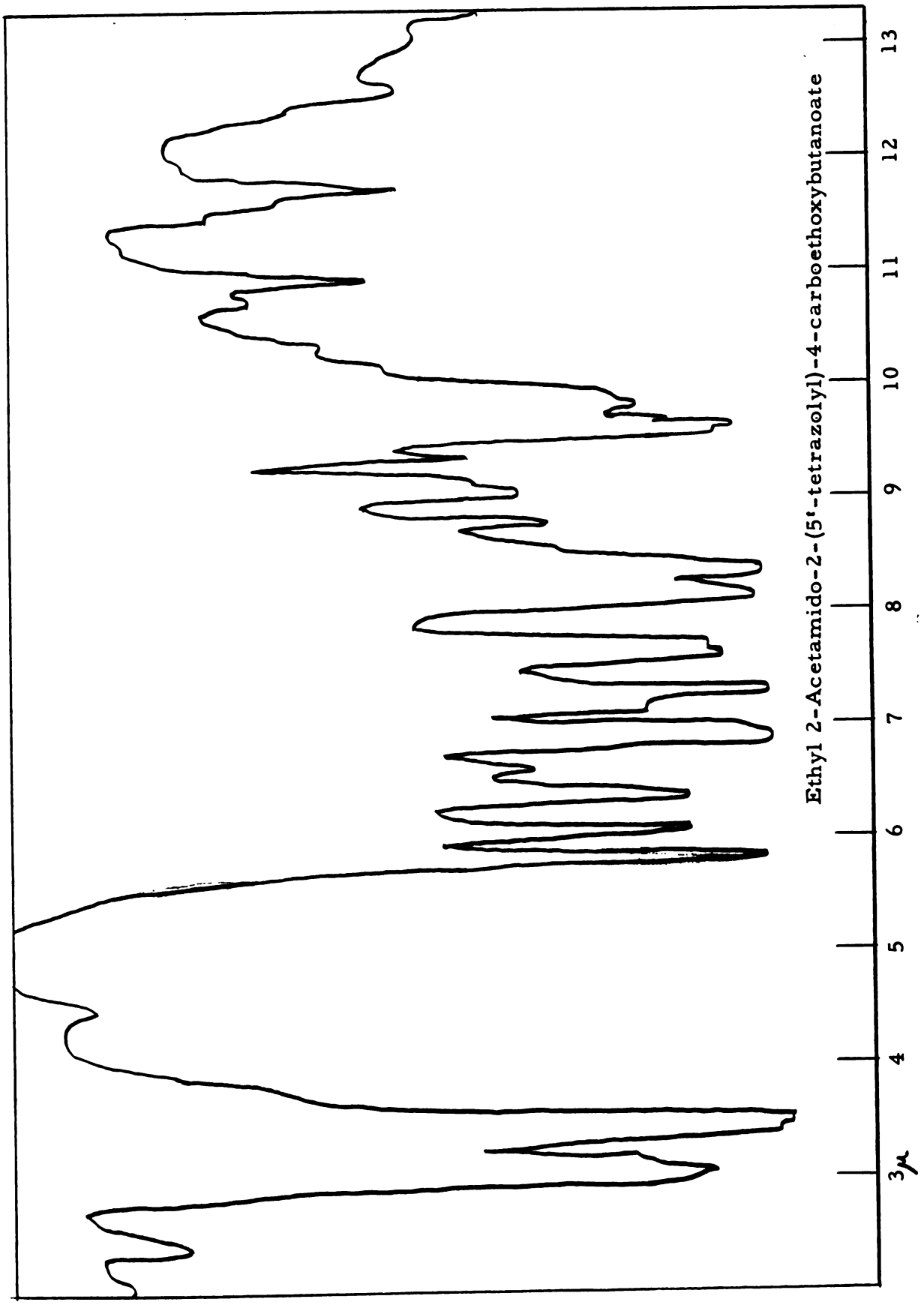

4-Amino-4-(5'-tetrazolyl)butanoic Acid
3μ
4
5
6
7
8
9
10
11
12
13

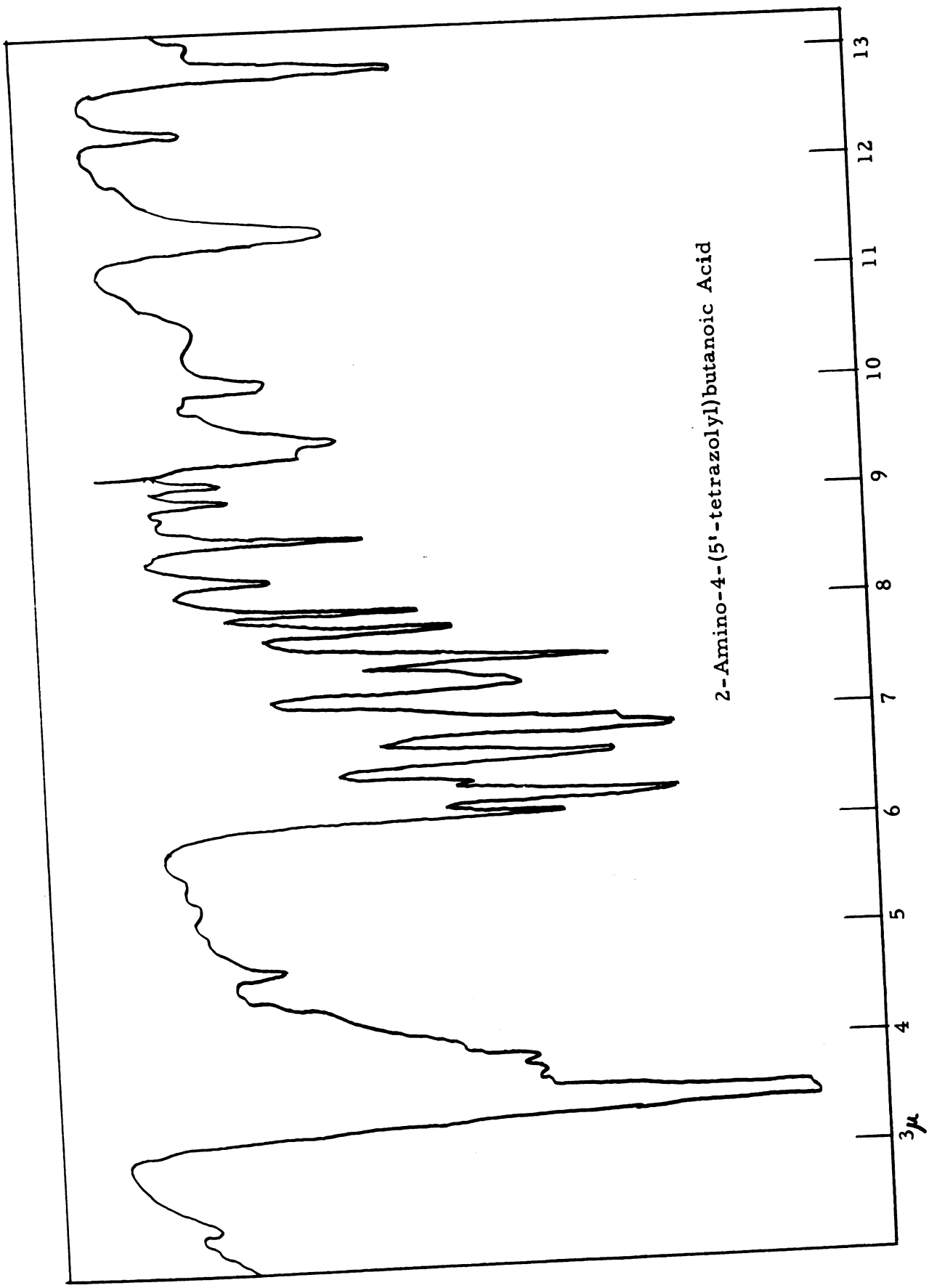

4-Amino-4-(5'-tetrazolyl)butanoic Acid Hydrochloride
3μ
4
5
6
7
8
9
10
11
12
13

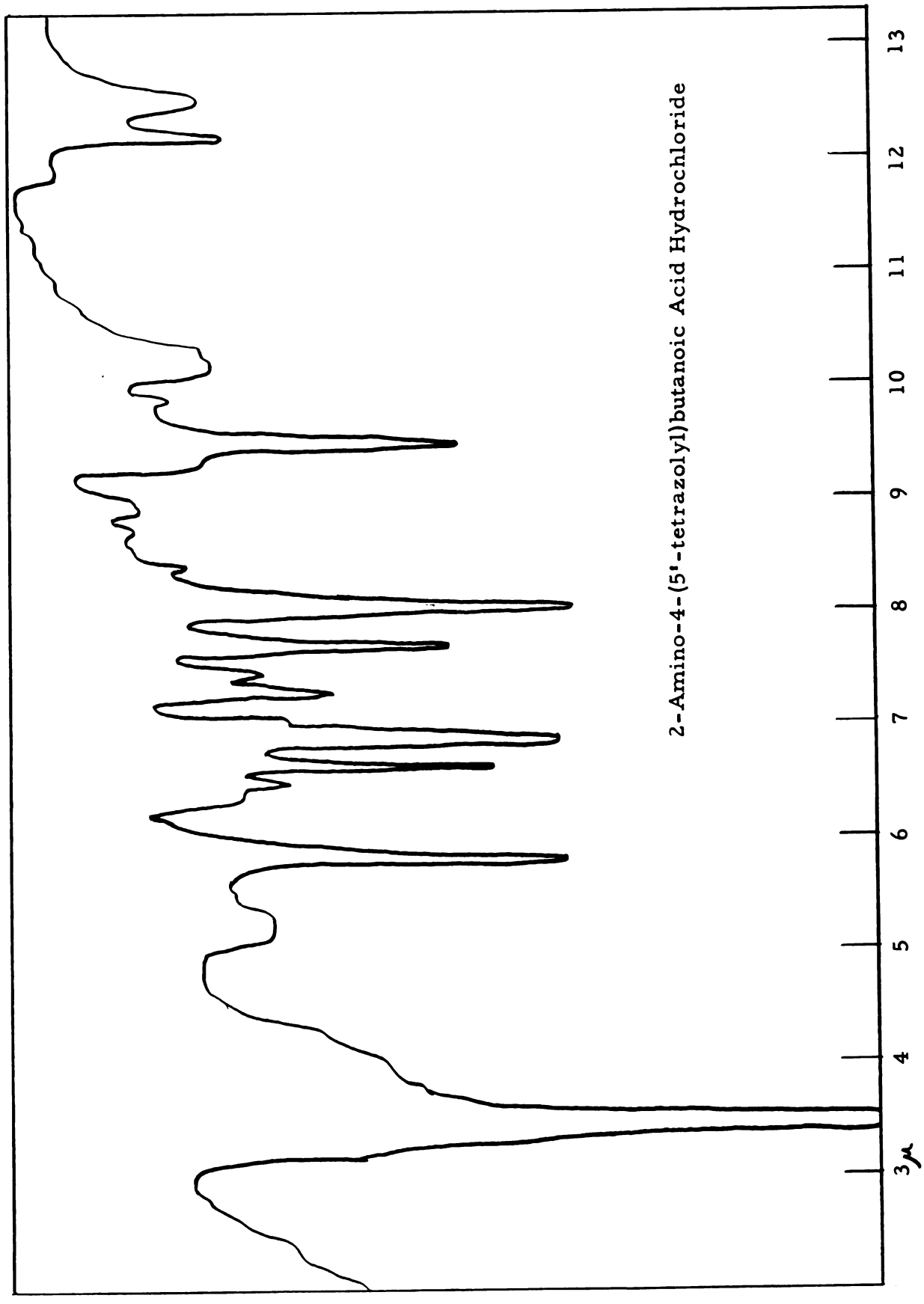

4-Acetamido-4-(5'-tetrazolyl)butanoic Acid
3μ
4
5
6
7
8
9
10
11
12
13

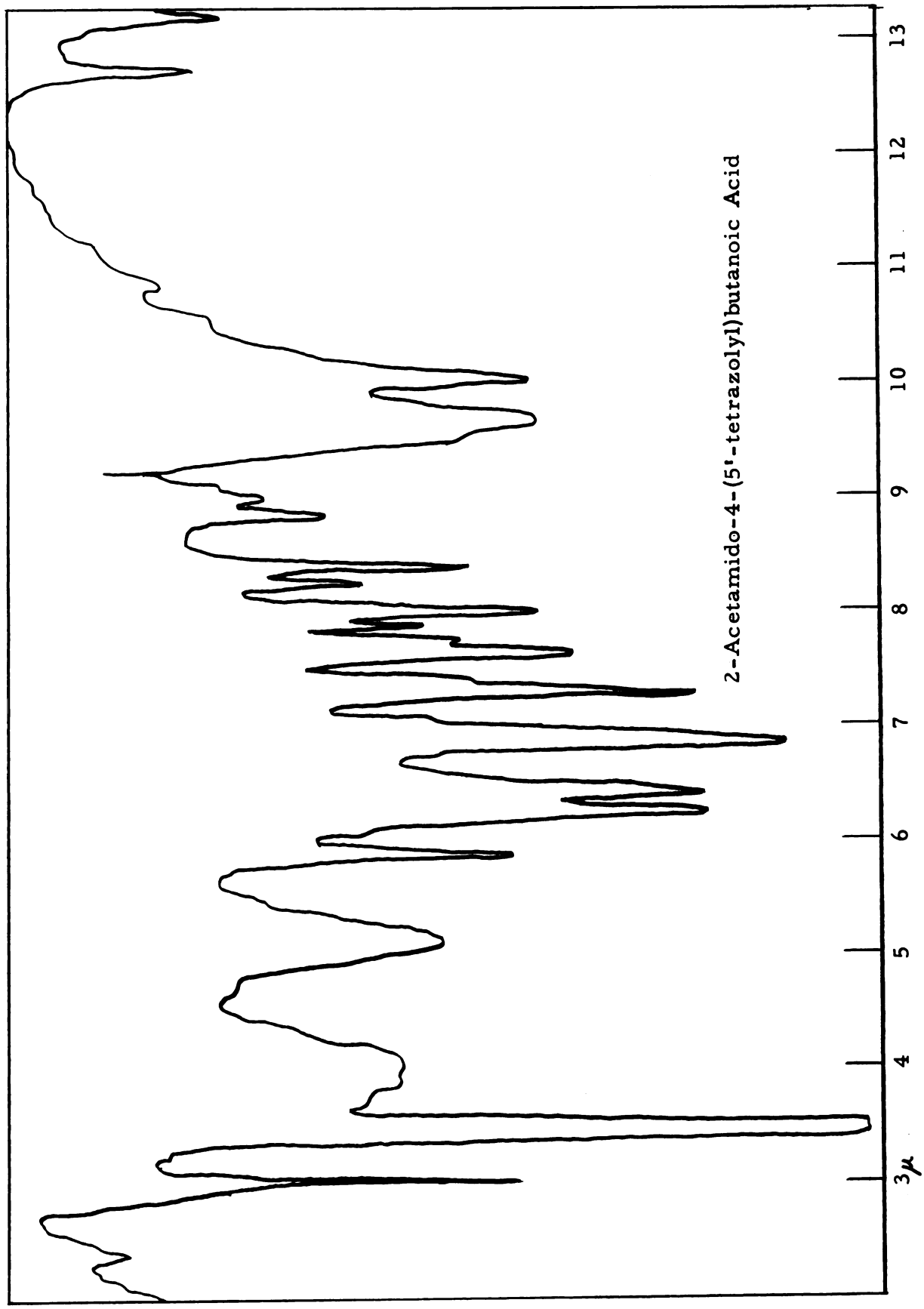

5-(5'-Tetrazolyl)-2-pyrrolidone
3μ
4
5
6
7
8
9
10
11
12
13

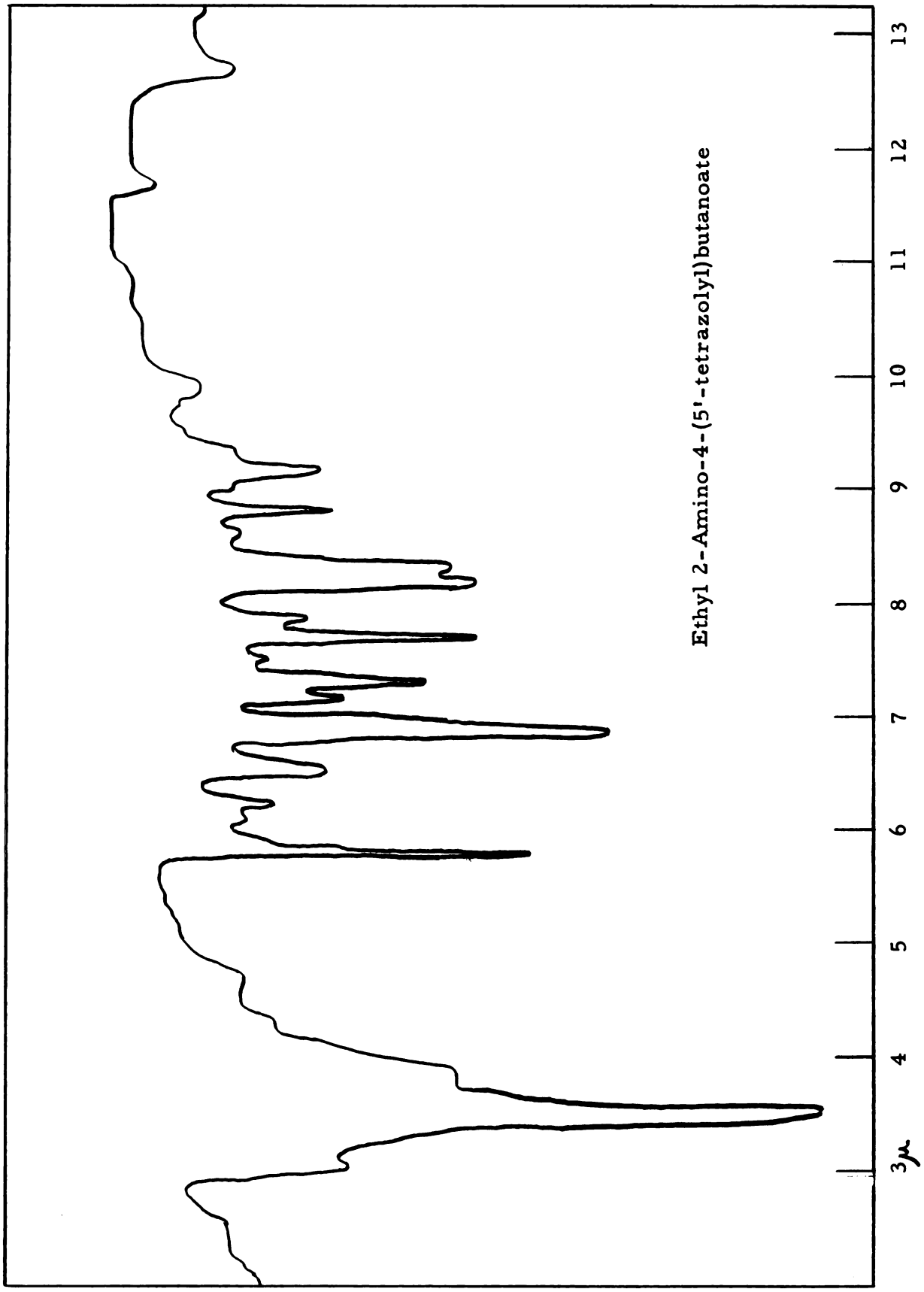

Ethyl 4-Acetamido-4-(5'-tetrazolyl)butanoate
3μ
4
5
6
7
8
9
10
11
12
13

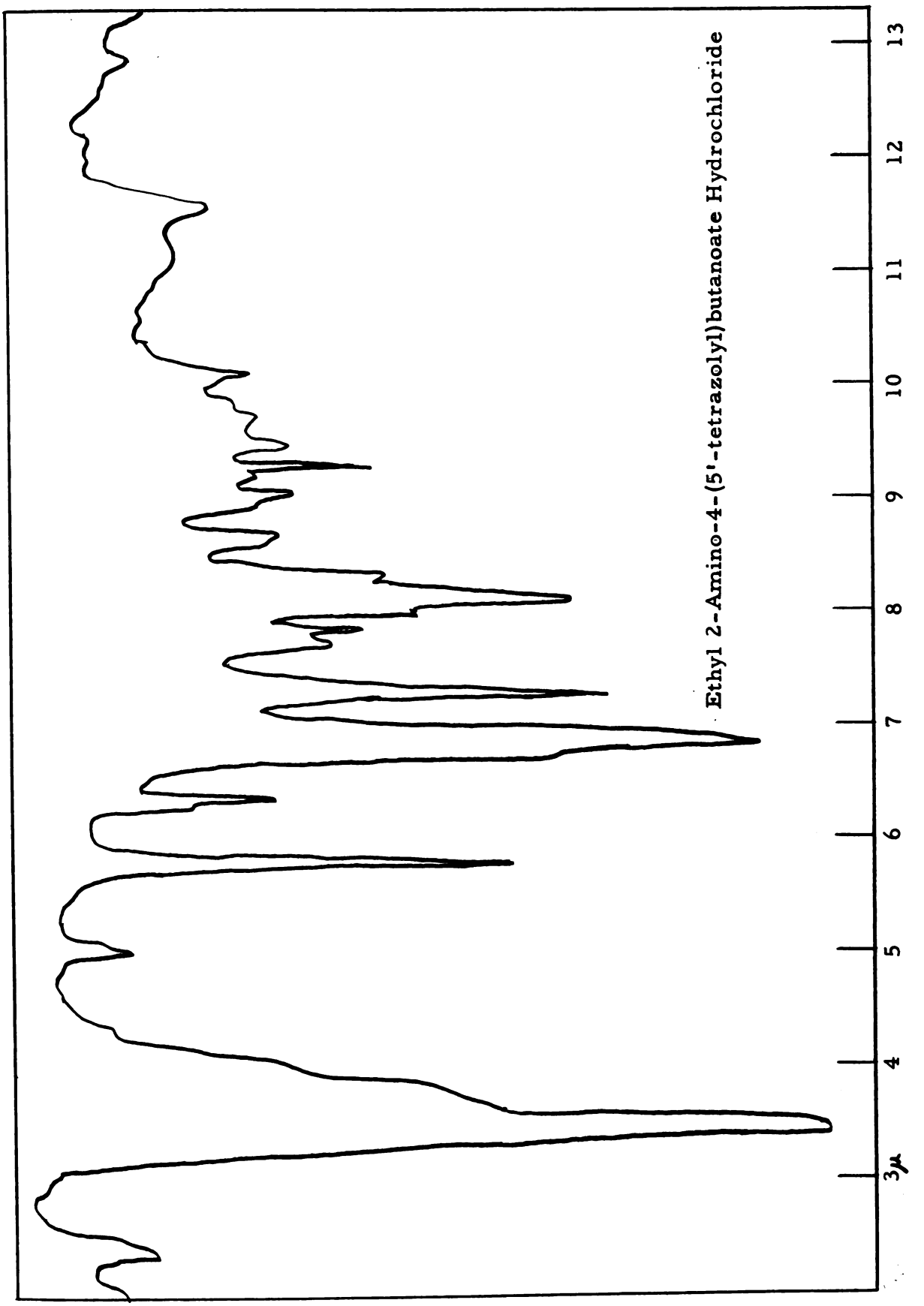

Ethyl 2-Acetamido-2-(5'-tetrazolyl)-4-carboethoxybutanoate
3μ
4
5
6
7
8
9
10
11
12
13

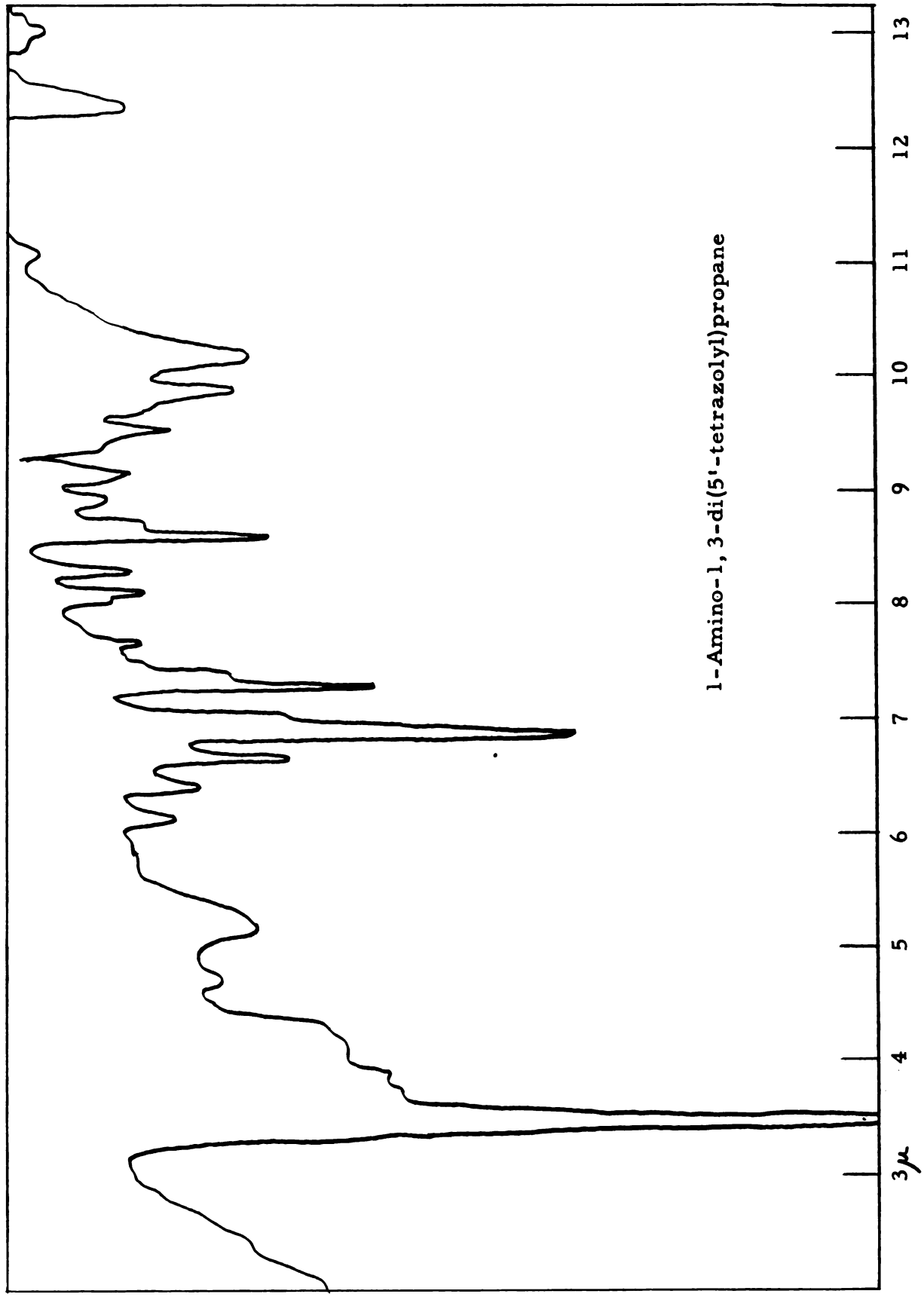

2-Amino-4-(5'-tetrazolyl)butanoic Acid
3μ
4
5
6
7
8
9
10
11
12
13

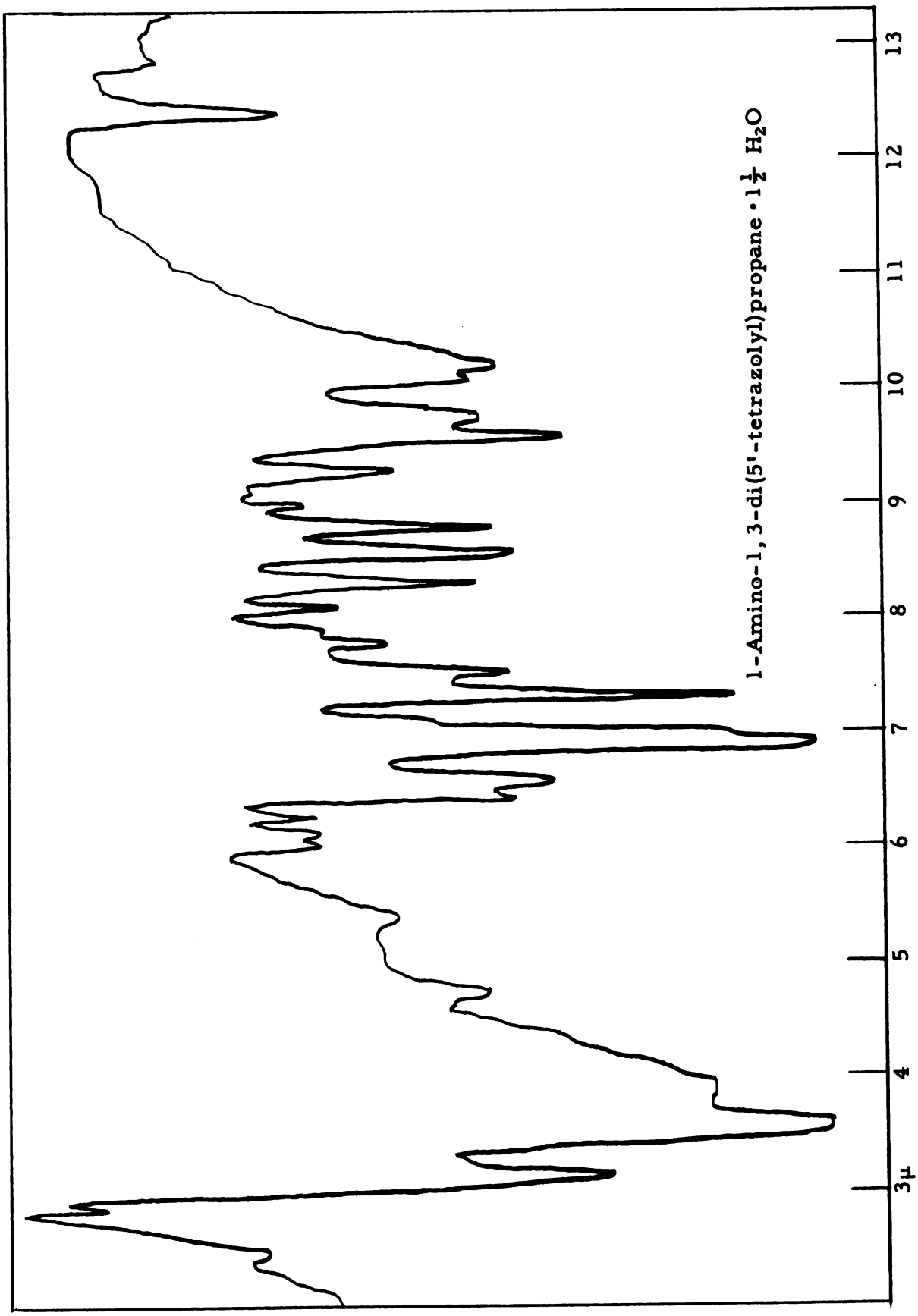

2-Amino-4-(5'-tetrazolyl)butanoic Acid Hydrochloride
3μ
4
5
6
7
8
9
10
11
12
13

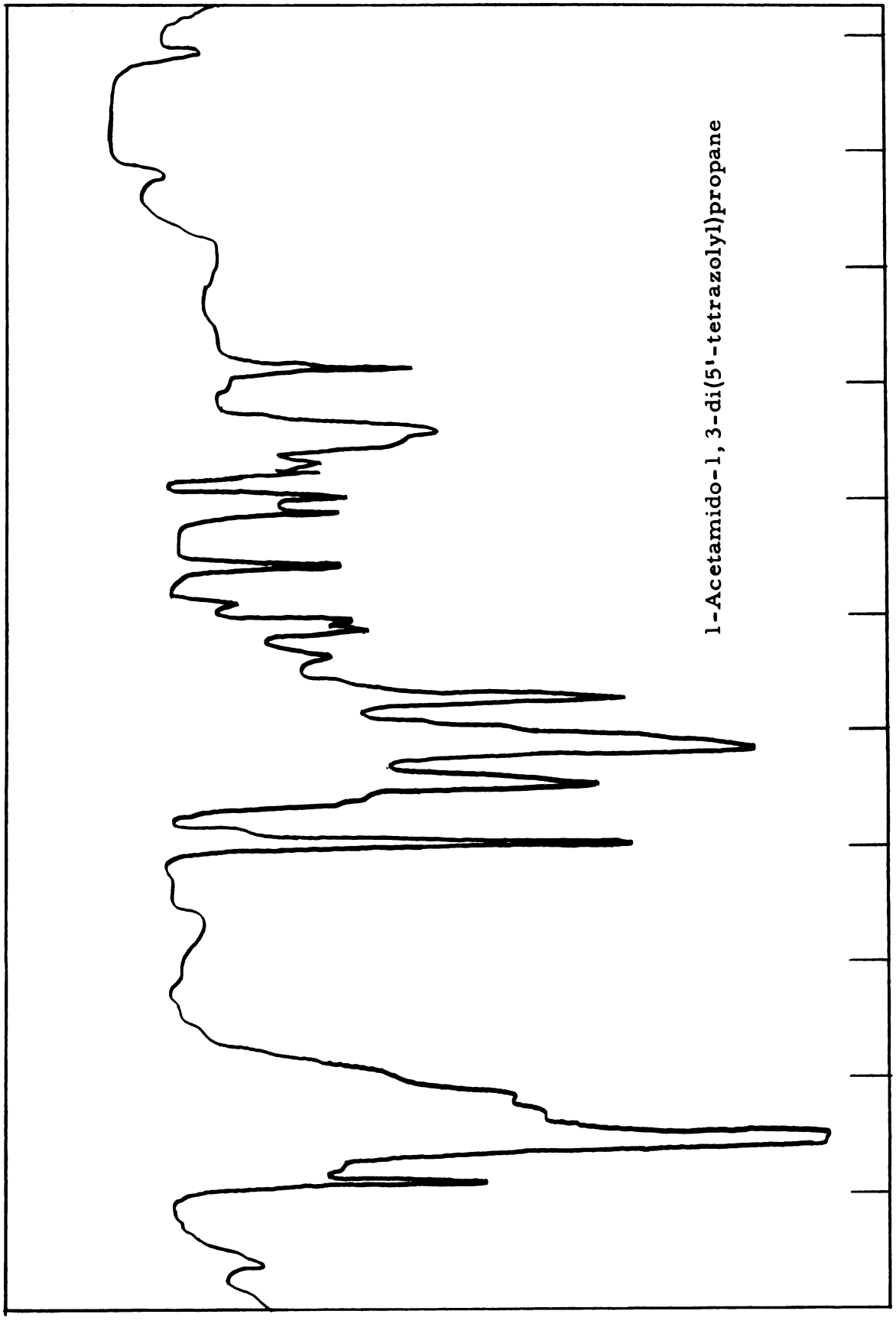

2-Acetamido-4-(5'-tetrazolyl)butanoic Acid
3μ
4
5
6
7
8
9
10
11
12
13


Ethyl 2-Amino-4-(5'-tetrazolyl)butanoate
3μ
4
5
6
7
8
9
10
11
12
13


Ethyl 2-Amino-4-(5'-tetrazolyl)butanoate Hydrochloride
3μ
4
5
6
7
8
9
10
11
12
13


1-Amino-1, 3-di(5'-tetrazolyl)propane
3μ
4
5
6
7
8
9
10
11
12
13


1-Amino-1,3-di(5'-tetrazolyl)propane · 1½ H2O
3μ
4
5
6
7
8
9
10
11
12
13


1-Acetamido-1,3-di(5'-tetrazolyl)propane
3μ
4
5
6
7
8
9
10
11
12
13