I. THE SYNTHESIS OF PYRIDINE AND PYRIDINE 1-OXIDE SUBSTITUTED ALANINE DERIVATIVES

II. PRELIMINARY INVESTIGATIONS OF ALPHA-CHYMOTRYPSIN CATALYZED HYDROLYSES OF ACYLATED AMINO ACID ESTERS AT LOW ENZYME CONCENTRATIONS-- STUDIES OF SURFACE EFFECTS

III. THE SYNTHESIS AND RATE OF ACETOLYSIS OF 1-BICYCLO[2.2.1]HEPTYLMETHYL TOSYLATE

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This thesis is dedicated to my wife, whose efforts have provided much of the material and spiritual necessities associated with a comfortable home and a happy family life, and also to Linda Kay, whose small contribution has been of immeasurable importance.

ABSTRACT

Methods for the preparation of pyridine and pyridine 1-oxide substituted alanines for use as substrates for the enzyme alpha-chymotrypsin were studied. As a consequence of this work, N-benzoyl-L-4-pyridylalanine 1-oxide methyl and ethyl esters, N-acetyl-DL-4-pyridylalanine 1-oxide methyl ester, and N-(nicotinyl 1-oxide)-L-phenylalanine methyl ester were prepared. The method for the synthesis of 4-pyridylalanine via a malonic ester condensation has been improved to the point where it is preparatively useful.

It has been demonstrated that the catalytic reduction of pyridine 1-oxides in water with a platinum catalyst is nonspecific, the pyridine and pyridine 1-oxide groups being reduced at comparable rates.

A kinetic study of the effect of surface on alpha-chymotrypsin catalyzed hydrolyses of acylated amino acid esters was carried out. It was shown that there was no effect of surface on the kinetics, varying the surface by a factor of 3000. However, it was shown that serious losses of enzyme can occur during transfer operations at an enzyme to surface ratio of 6×10^8 cm.^{2}/mole, or greater. The possibility of avoiding these losses of enzyme during transfer by adding a "carrier" for the enzyme, in the form of colloidal silica, was investigated, and shown to have promise. It was shown that the enzyme was adsorbed on colloidal silica.

Bicyclo[2.2.1]heptylmethyl tosylate has been prepared, and its rate of acetolysis at 99°C. determined.

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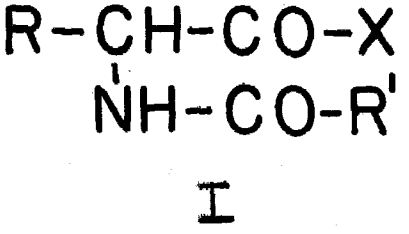
PART I

THE SYNTHESIS OF PYRIDINE AND PYRIDINE 1-OXIDE SUBSTITUTED ALANINES

A. INTRODUCTION

A large number of substrates for the enzyme alpha-chymotrypsin have the structure I. Variations of this



I

structure have included cases where R = benzyl, p-hydroxybenzyl, cyclohexylmethyl, 3-indoylmethyl, hydrogen; where R' = phenyl, methyl, chloromethyl, hydrogen, trifluoromethyl, 3- and 4-pyridyl; and -COX being an ester (aliphatic or aromatic), hydroxamide, amide, or hydrazide group. Foster and Niemann (1), in a compilation of previous work by Niemann and coworkers, noted that in the case of acylated tyrosine amides, there was an apparent linear relationship between the group refractivities of the acyl groups (R'CO-) and k_3, except where R' had a structural similarity to R. The exceptions showed lower values of k_3 than those expected from the linear relationship observed where R' was dissimilar to R.

The three-point attachment theory proposes that there exist at the active center of the enzyme three loci, each of which has a particular affinity for a portion of the substrate. Thus the locus β_1 may be associated with group R, β_2 with group R', and β_3 with X. A natural consequence of

this theory is the assumption that an alignment of the substrate molecule at the active center of the type R- ρ_1, R'- ρ_2, X- ρ_3, should lead to hydrolysis, while an alignment such as R'- ρ_1, R- ρ_2, X- ρ_3 should not be a productive mode of combination. When R' is different (presumably only structurally) from R, one would expect little interaction of the type R'- ρ_1. However, when R' is similar to R, it would be expected that combinations of the type R'- ρ_1 and R- ρ_2 could occur much more frequently. While it would be difficult to predict the effect these latter combinations would have on K_s, which is frequently assumed to be a measure of the extent of combination of the substrate with the active site, it would be predicted that for any apparent relationship between k_3 and R' where R' and R are quite different, lower values of k_3 than would follow from this relationship would be observed for cases where R and R' are similar, due to the fact that while more modes of combination of the substrate and enzyme are likely, there is a lesser chance they will be productive and lead to hydrolysis.

It would be of interest to consider cases where X is held constant and R and R' were interchanged. Thus one could study the kinetic effect of the combinations of R- ρ_1, R- ρ_2, R'- ρ_1, and R'- ρ_2. In considering substrates useful for this purpose, it was decided that the case where R was 4-pyridylmethyl or benzyl and R' was 3-pyridyl or phenyl would be of the most use. In the one

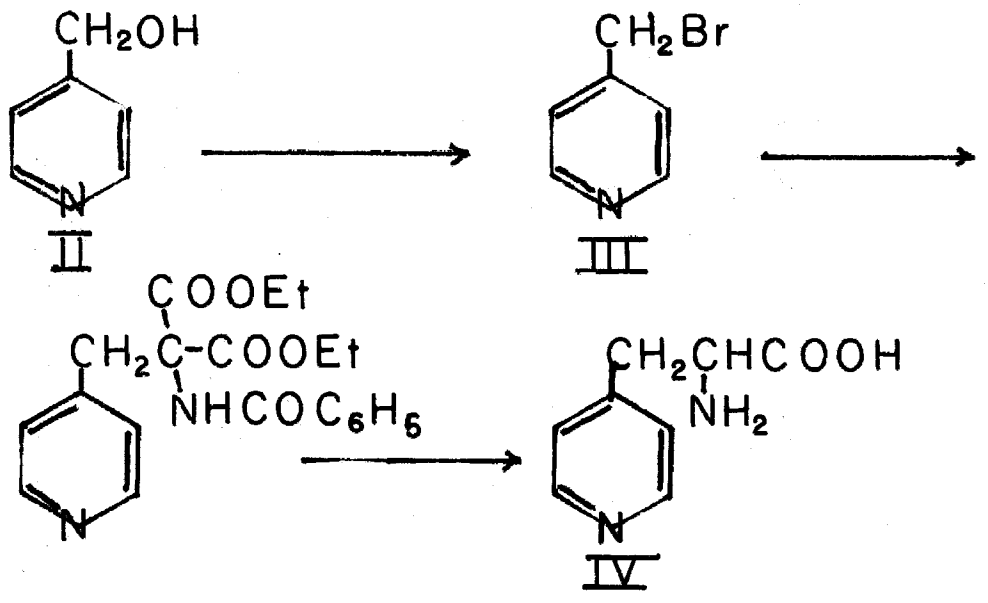
case the bonded distance from the asymmetric carbon atom to the nitrogen atom would be the same, i.e., five bonds. It was hoped that, at least in the cases of derivatives of 4-pyridylalanine, the compounds would have a relatively high water solubility, and should this value be such that the maximum value of $(S)/K_s$ obtainable (see Part III) was much greater than 20, a case where substrate inhibition might occur, could be examined kinetically.

It was found that 4-pyridylalanine was not very soluble in water, while its 1-oxide was very soluble, so attention was directed to the synthesis of 4-pyridylalanine 1-oxide derivatives and phenylalanine derivatives acylated with a 3-nicotinyl 1-oxide group.

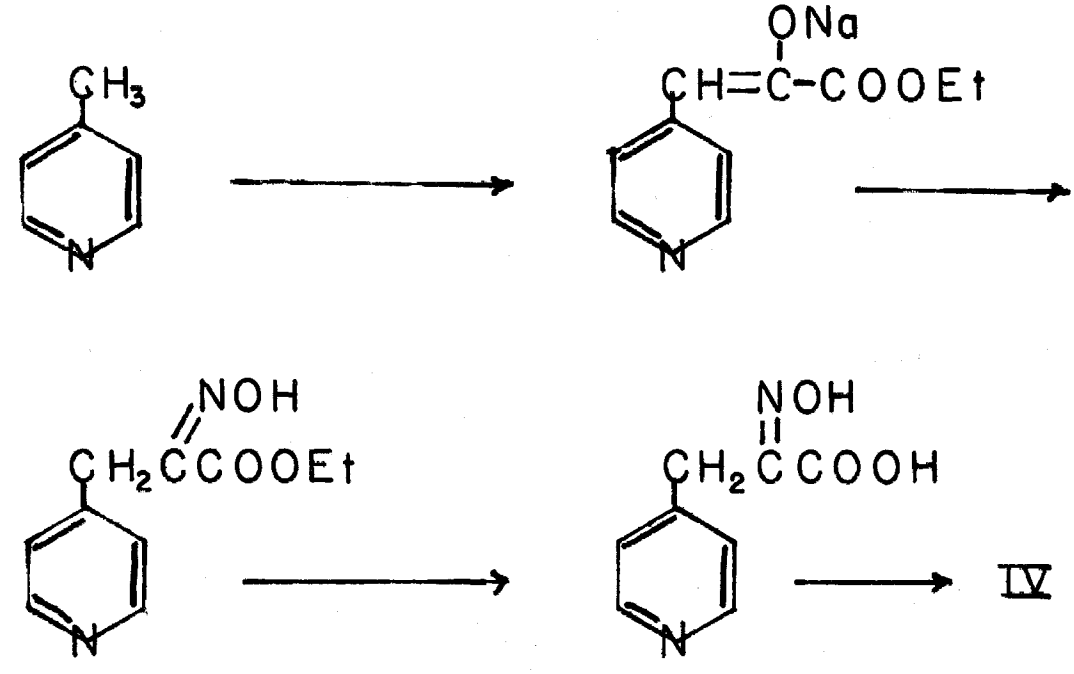
For purposes of continuity, compounds of type I where R is 4-pyridyl are discussed in Section B, where R is 4-pyridyl 1-oxide in section C, and where R is phenyl in section D.

B. 4-PYRIDYLALANINE AND DERIVATIVES

Two methods have been reported for the synthesis of 4-pyridylalanine. The first, by Niemann, Lewis and Hayes (2), utilized a malonic ester condensation as the key step in the series of reactions II ⟶ IV.



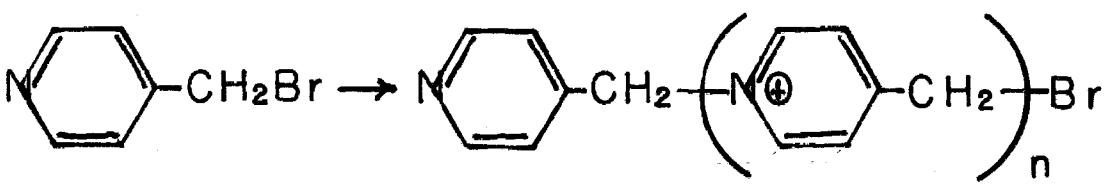
The second synthesis, by Elliott, Fuller and Harington (3), utilized a Claisen Condensation as the key step in the series of reactions.



Unfortunately, neither synthesis, as reported, was preparatively useful, for the malonic ester condensation took place in 4% yield, and the Claisen Condensation in 12% yield. It was decided to study the malonic ester condensation with the aim of raising the yield.

The starting material for the malonic ester condensation, 4-pyridylmethyl bromide hydrobromide III, was obtained consistently in 85% yields from 4-pyridylcarbinol. This hydrobromide, and its parent amine, are severe vesicants, even in trace quantities.

The competing side reaction in the malonic ester condensation was the polymeric quaternization of the neutralized hydrobromide, as below:



This polymeric quaternization has been reported by Sorm and Sedivy (4), who observed that catalytic reduction of the red viscous oil obtained by treatment of the hydrobromide with a small excess of base gave only an amorphous polymer. It was also observed that 2-pyridylmethyl bromide was quite slow to quaternize. The quaternization of 4-bromopyridine is much faster than that of 2-bromopyridine (5), the differences in rate being attributed to steric effects (6).

Presumably the same steric effect operates in the case of 2-pyridylmethyl bromide. Study of various modifications of the malonic ester condensation (7), such as using 4-pyridylmethyl tosylate, offered no improvement.

Considering the nature of the competing reactions, it would be predicted that use of a less polar solvent (e.g., benzene instead of ethanol) would favor the rate of condensation and retard the rate of quaternization (8).

Quaternization:

$$Y + RX \longrightarrow RY^{\oplus} + X^{\ominus}$$

Strong acceleration in more polar solvent.

Condensation:

$$Z^{\ominus} + RX \rightarrow RZ + X^{\ominus}$$

Weak retardation in more polar solvent.

It was observed experimentally that approximately 50% benzene in ethanol was the best solvent for this condensation.

Also, it was necessary to neutralize the hydrobromide salt of 4-pyridylmethyl bromide. Due to the fact that the quaternization of the free base was so rapid, the best method was to use an excess of the sodium salt of the acylamidomalonic ester as the base, and carry out the neutralization by adding the hydrobromide salt to a solution of the sodium salt. This had the added effect of providing a higher concentration of the attacking group in the S_n2 condensation step during the early part of the reaction, and also minimized the possibility of ether formation due to attack on the halide by alkoxide ion.

One would expect the rate of condensation of 4-pyridylmethyl bromide with the malonic ester to be reasonably fast, and to produce reasonable yields, if one uses the usual comparison of the nitrophenyl group to the pyridyl group (9). Dornow and Winter (10) obtained at least a 61% yield of product from the condensation of p-nitrobenzyl chloride and diethyl formamidomalonate, and it has been shown that p-nitrobenzyl bromide is about 400 times faster than n-butyl bromide in S_n2 reactivity (11). On the other hand, considering the quaternization reaction, p-nitrobenzyl bromide showed the slowest rate of several p-substituted benzyl bromides in reacting with triethylamine (12). Unfortunately, there is no data available that affords a comparison of the rate of S_n2 reaction with that of quaternization.

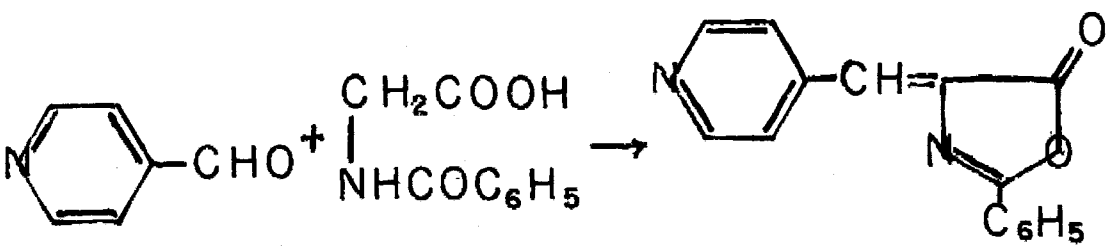
Addition of the hydrobromide salt in the dry state to the sodio acylamidomalonic ester solution was abandoned for two reasons. One, the salt was somewhat hygroscopic, and this complicated its addition; two, large local concentrations of 4-pyridylmethyl bromide formed when the addition was carried out in the dry state, favoring quaternization. Since the hydrobromide was soluble only in water, it was necessary to add it as a slurry in benzene-ethanol. This was accomplished by means of the equipment described in the experimental section.

As a result of studying the effect on the yield of the variables of time, temperature, solvent, mode of addition of

the hydrobromide, and concentration, in sixteen preparations, a procedure was devised which gave consistent 60-70% yields of diethyl acetamido-(4-pyridylmethyl)malonate on a 0.02 mole scale. However, when scaled up to 0.08 mole quantities, the yield dropped to 30%, presumably due to slurrying problems on this scale.

The diethyl acetamido-(4-pyridylmethyl)malonate was hydrolyzed and decarboxylated by heating with 48% hydrobromic acid to yield 76% of the dihydrobromide of IV. Treatment of the latter with "Amberlite IR-4B" resin gave 4-pyridylalanine in 90% yield. The amino acid reacted rapidly in the cold with ninhydrin to give a red color (2,3), and was soluble in water to the extent of 3.4 g. per 100 ml. at room temperature.

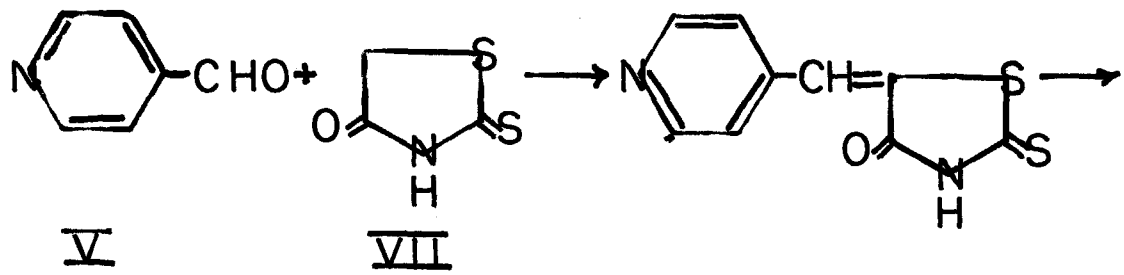
Another route to 4-pyridylalanine considered was via the azlactone VI, from isonicotinaldehyde V. However, the



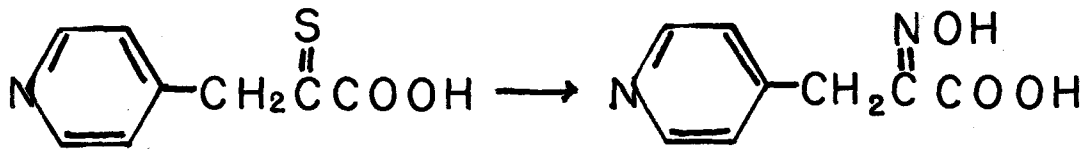
V VI

aldehyde is stable only as a hydrate, the anhydrous material being very sensitive to air oxidation (13), and it also readily undergoes the Cannizzaro reaction in the presence of air (14). No product could be isolated from

a standard Erlenmeyer synthesis (15), using the anhydrous aldehyde (or its diacetate) prepared from the hydrate and acetic anhydride. Attempts to use a modification involving rhodanine VII (16), as below, gave only non-characterizable products.

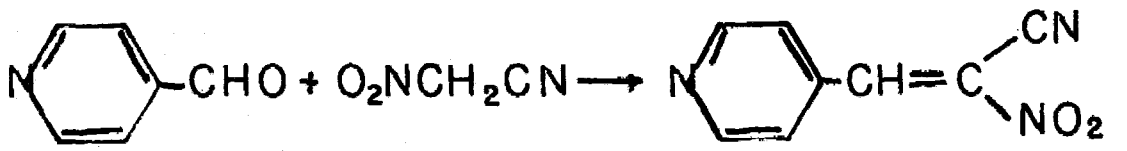


V VII



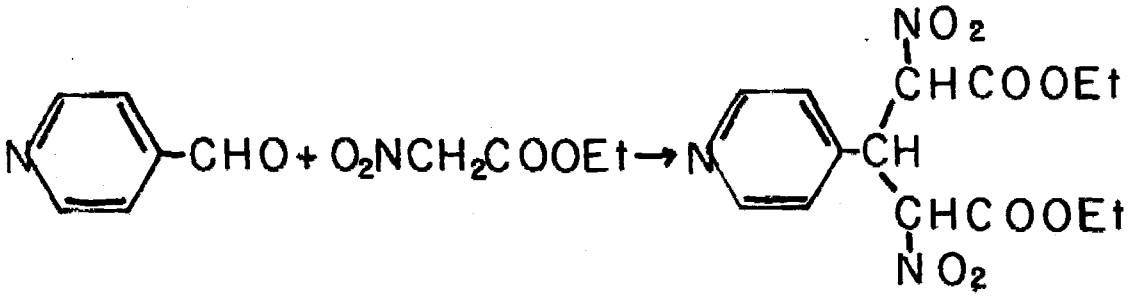
These failures parallel those observed by Niemann, Lewis and Hays (2) for the reaction of picolinaldehyde with hippuric acid or with diketopiperazine. Other attempts to synthesize 4-pyridylalanine were based on pyridine 1-oxide derivatives, and are discussed in Section C.

One possible route that was not investigated was the Knoevenagel condensation of isonicotinaldehyde with nitroacetonitrile.



V VIII

The yield of VIII has been reported as 35% (17). The use of ethyl nitroacetate with picolinaldehyde gave an 81% yield of IX (18) and could probably be controlled to yield a 1:1 condensation product. It would seem possible to reduce the double bond and nitro group of VIII without reducing the pyridine ring on the basis of work done by Walter on the reduction of pyridine acrylic acids (19).



V IX

Attempts to benzoylate 4-pyridylalanine with benzoyl chloride under Schotten-Baumann conditions gave only non-characterizable oils. However, benzoylation was successful with benzoic anhydride in the presence of triethylamine to give N-benzoyl-4-pyridylalanine. This compound was very insoluble in water.

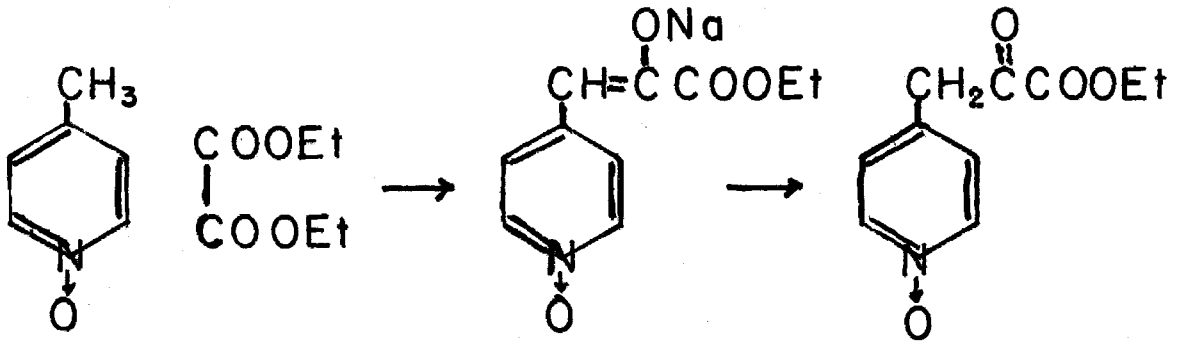
Due to the relatively low solubilities of 4-pyridyl-

alanine and its N-benzoyl derivative, and to the much higher solubilities of their 1-oxide derivatives, no further work was done on these compounds.

C. 4-PYRIDYLALANINE 1-OXIDE AND DERIVATIVES

While the aim of raising the yield in the series of reactions leading to 4-pyridylalanine was successful, the synthesis was not preparatively useful for large quantities of materials. The synthesis of Elliott, Fuller, and Harrington (3) probably was carried out in its best yield, and little improvement seemed possible there.

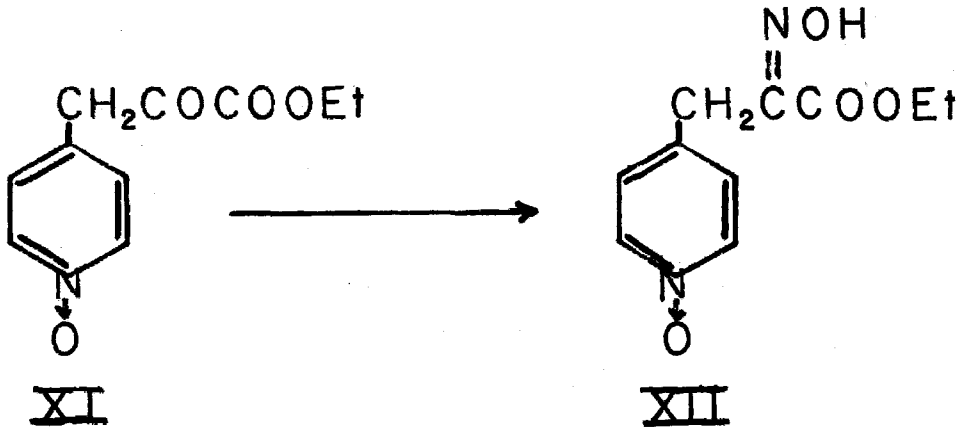
However, work done during World War II by the Japanese indicated that the electron deficiency at the methyl group of 4-picoline was greatly enhanced in 4-picoline 1-oxide due to the strong polarization of the N-O bond reinforcing the inherent electron-attracting nature of the ring nitrogen atom (20). This fact implied that a Claisen condensation on 4-picoline 1-oxide should be considerably improved over the condensation on 4-picoline. This was shown to be the case by Adams and Miyano (21). These authors reported a 48% yield of ethyl 4-pyridylpyruvate 1-oxide XI from the condensation of ethyl oxalate and 4-picoline 1-oxide, using potassium ethoxide as the condensing agent. The α-keto ester XI was isolated by aqueous hydrolysis of its potas-



X XI

sium salt, and extraction into chloroform. A modification of this reaction, carried out in this laboratory, involved aqueous hydrolysis of the sodium salt of the keto ester X, and, after cooling, removing the crystalline solid which had formed. This product was quite impure, and was found to contain an impurity which was insoluble in chloroform. Recrystallization of the impure material from water gave a 48% yield of the α-keto ester XI, and concentration of the filtrate gave a yellow crystalline solid, m.p. 186-187, of unknown constitution, which was light sensitive. This unknown compound was insoluble in chloroform, and would not form a water insoluble oxime, as the α-keto ester or α-keto acid would.

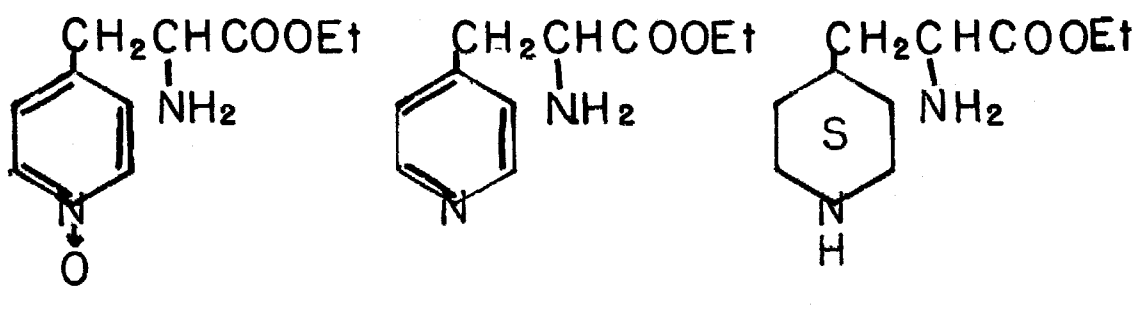
The oxime of the α-keto ester XII was prepared by Adams and Miyano (21) in 57% yield, and this yield was obtained in this laboratory. A modification of the method for the oxime, not involving isolation of the α-keto ester, gave consistent 55-60% yields of recrystallized ethyl



XI XII

4-pyridyl-(α-oximino)propionate 1-oxide, directly from 4-picoline 1-oxide.

The reduction of the α-oximino ester XII would conveniently yield the α-amino ester XIII or XIV, intermediates which would be very useful, or perhaps XV, which compound would be of interest eventually.



XIII XIV XV

Oximes are readily reduced to amines, but α-oximino esters require rather strenuous conditions. The presence of a pyridine nucleus in XII requires, if the pyridine ring is not to be reduced, a selective reduction. Hartung and coworkers (see (22) for leading references) have done con-

siderable work on α-oximino ester reductions, and the following presents a summary of their results:

Solvent: ethyl alcohol

Catalyst: Pd on charcoal plus palladium chloride

Pressure: 10 atmospheres

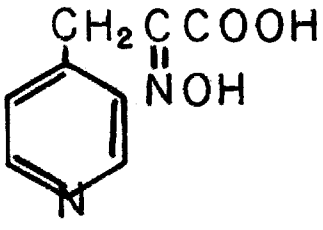
Temperature: room

Generally, five grams of palladium on charcoal plus one to five grams of palladium chloride is used for 0.15 mole of oximino ester. In addition, a three-fold excess of concentrated hydrochloric acid is necessary to prevent formation of secondary amines. Hartung and Waters (23) have shown that α-amino acids poison the catalytic reduction of α-oximino acids. In the case of oximinomalonic ester, 1500 psi were necessary to obtain reduction with the palladium catalyst (24). Reductions using Adam's catalyst are poor. Thus, it has been reported that the two atmosphere reduction of 3-oximino-1-dimethylaminobutane at 60°C. gave a 25% yield of diamine, with extensive cleavage (25).

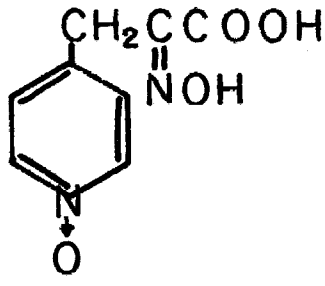
Shivers and Hauser (26) reported the reduction of α-oximino esters with Raney nickel at high pressures, 70-75°C., in absolute ethanol, to give 85% yields of amino esters. Reid and Schiller (27) reported the use of Raney nickel in glacial acetic acid for the reduction of ethyl 2-quinolyl-(α-oximino)propionate at 1500 psi and 70-80°C. Considerable amounts of tetrahydroquinolylalanine were

isolated, and frequently, in other cases, only the ∝ -hydroxylamino ester could be isolated (28).

In all of the above cases, it would be expected that concurrent reduction of a pyridine nucleus would occur, and this has been shown to be the case for 4-pyridyl-(∝-oximino)-pyruvic acid XVI by Elliott (3), and for its 1-oxide XVII in this work. In every case, only non-characterizable oils, presumably containing partially reduced pyridine nuclei and polymeric substances, were isolated. Adkins (29) has discussed some of the problems connected with catalyst poisoning by pyridine compounds. Other methods used for the reduction



XVI



XVII

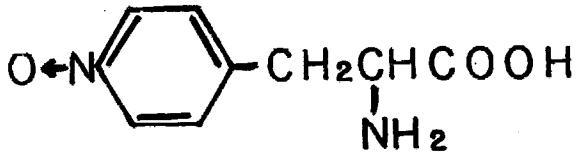
of oximes, such as lithium aluminum hydride, or sodium amalgam, are not applicable here due to the presence of a carboxyl group. The use of sodium borohydride was investigated, but no reduction was obtained.

Elliott, Fuller, and Harington (3), found that the oximino acid XVI could be reduced with stannous chloride in concentrated hydrochloric acid. The tin was removed by hydrogen sulfide after dilution to the correct pH. The

oximino acid XVII was prepared in consistent 80% yields from the oximino ester XII. Treatment of XVII in concentrated hydrochloric acid with stannous chloride gave an exothermic reaction. It was not feasible to use the dilution technique of Elliott to remove the tin salts, and the use of inorganic bases to raise the pH would lead to the formation of water soluble salts, difficult to separate from the product. After removal of as much hydrochloric acid as possible in vacuum, extreme dilution and frequent saturations with hydrogen sulfide, no product could be isolated. It had been presumably coprecipitated with the tin sulfides. Reduction of the oximino acid was shown to occur, however, since a portion of the acid solution, after neutralization, gave a ninhydrin test for α-amino acids.

The best scheme for removal of tin ions from the acid solution required a neutralization to pH 6.7 with ammonium hydroxide, after vacuum stripping to remove as much hydrochloric acid as possible. The precipitation of stannous and stannic hydroxides was so complete that no further precipitate could be obtained with hydrogen sulfide. The aqueous filtrate from the removal of tin hydroxides was evaporated to dryness to leave a mixture of ammonium chloride and amino acid, in a ratio of about four to one. The ammonium chloride was removed by extraction of the powdered mixture with methanol, the salt being soluble in methanol

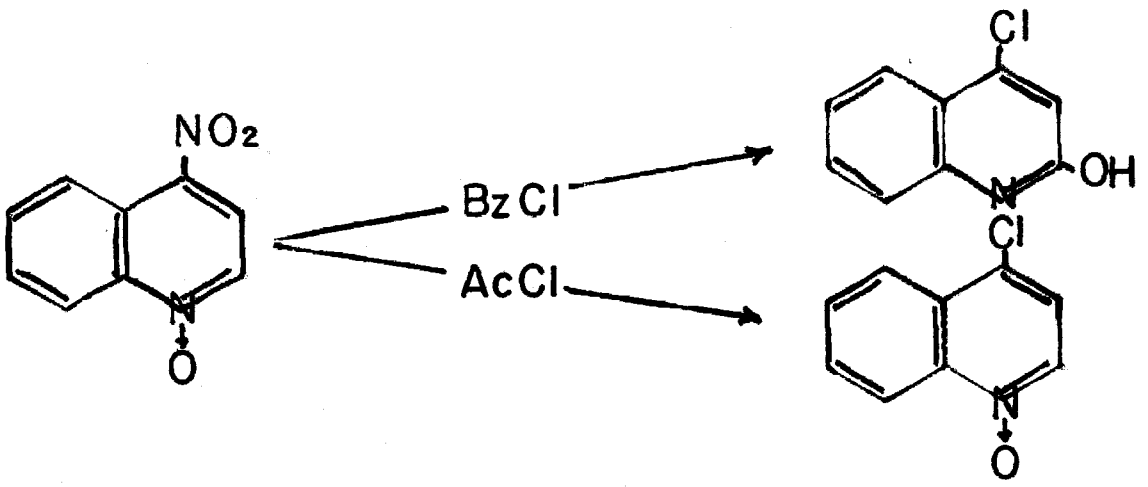
to the extent of 3.35 g. per 100 g. at 19°C. (30). The residue analyzed correctly for 4-pyridylalanine 1-oxide XVIII.



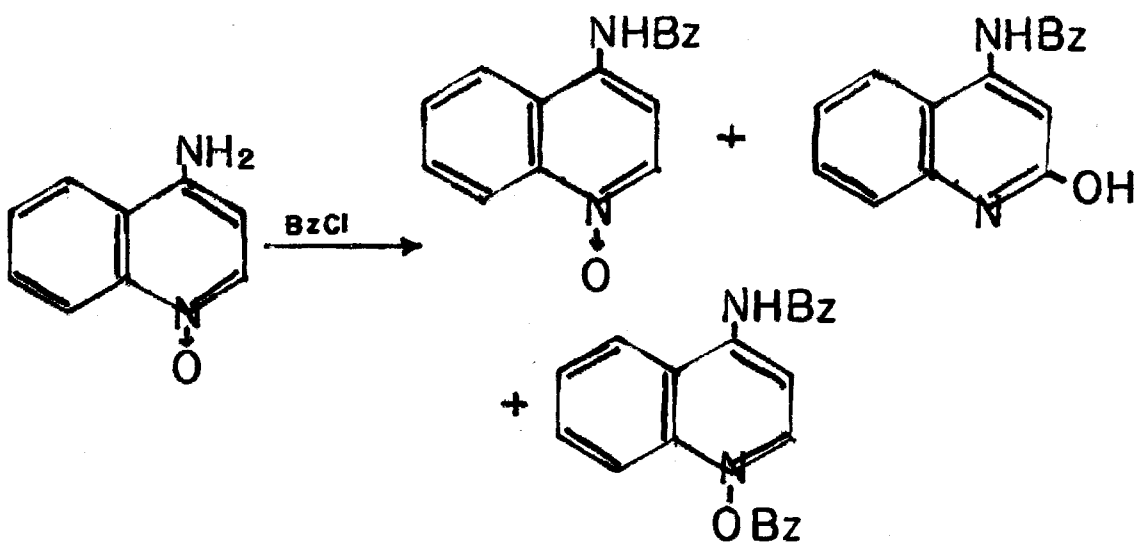
XVIII

This amino acid XVIII reacted very slowly at room temperature with an aqueous solution of ninhydrin to give an orange color. The amino acid was soluble in water to the extent of 47 g. per 100 ml. at room temperature, and essentially insoluble in the usual organic solvents.

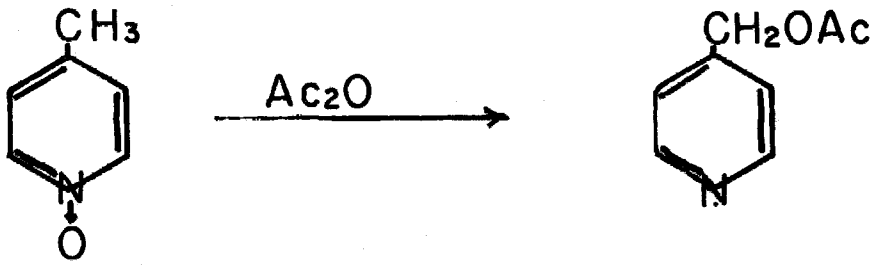
In considering the preparation of acylated 4-pyridylalanine 1-oxides, one potentially complicating factor had to be considered. This was the fact that amine oxides occasionally react with anhydrides and acid chlorides. In the case of heterocyclic aromatic amine oxides, the literature, while consistent, is rather difficult to explain. Thus, 4-nitroquinoline 1-oxide reacted with acetyl chloride at room temperature to yield 4-chloroquinoline 1-oxide, while reaction with benzoyl chloride gave 4-chlorocarbostyril (31).



The reaction of 4-aminopyridine 1-oxide with benzoyl chloride under Schotten-Baumann conditions gave a dibenzoyl derivative, which yielded 4-benzamidopyridine 1-oxide after recrystallization (32). Acylation of 4-aminoquinoline 1-oxide with benzoyl chloride under Schotten-Baumann conditions gave a dibenzoate, a monobenzoate and a carbostyril (33). Treatment of the same amine with acetic anhydride or benzoic anhydride at room temperature gave only 4-acylamidoquinoline 1-oxides (33); both anhydrides reacted with pyridine 1-oxide at temperatures near 140° to give α-pyridone (34).

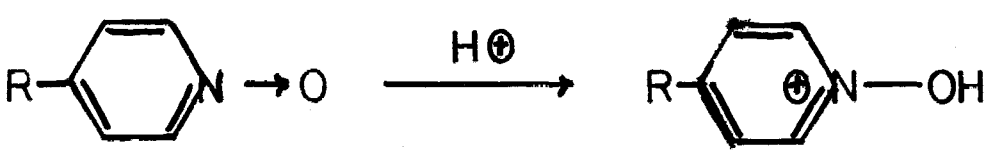


In the case of α- and γ-picoline 1-oxides, reaction with acetic anhydride at room temperature gave pyridylcarbinol acetates (35).



However, the reaction of acetyl chloride with 4-nitro-2-picoline 1-oxide gave 4-chloro-2-picoline 1-oxide (36), with no evidence of rearrangement.

Attempted acylations of 4-pyridylalanine 1-oxide with benzoyl chloride under Schotten-Baumann conditions gave only red oils, from which no single product could be isolated. The use of benzoic anhydride in the presence of triethylamine however, gave the N-benzoyl-4-pyridylalanine 1-oxide in 74% yield. The product was separated from benzoic acid by acidifying the reaction mixture so it was 2 N in hydrochloric acid. The acylated amino acid was soluble at this acid concentration, due to formation of the 1-hydroxypyridinium ion.



XIX

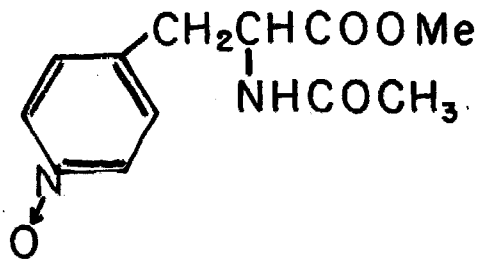
After removal of the precipitated benzoic acid and adjustment of the solution to pH 2.3, the product precipitated. N-Benzoyl-4-pyridylalanine 1-oxide was also obtained in 70% yield from the oximino acid XVII by reduction with stannous chloride, precipitation of the tin with sodium hydroxide, and benzoylation of the filtrate after removal of the tin hydroxides (versus a 63% yield from the oximino acid XVII via the route involving isolation of the amino acid). The pKa of the 1-hydroxypyridinium ion (XIX, R = $HOOCCH(NHCOC_6H_5)CH_2$-) was determined to be 1.15 by the spectrophotometric method outlined by Flexser, Hammett, and Dingwall (37). This value agrees well with the values for this type of ion obtained by Jaffe and Doak (38), and assuming a pKa of 3.5 for the carboxyl function (39), was used to calculate the approximate pH for minimum solubility to be at pH 2.3.

It was found that 4-pyridylalanine 1-oxide could be readily acylated with acetic anhydride under Schotten-Baumann conditions in 87% yield. One unique feature of this acylation was the fact that no excess of the anhydride was required. In fact, an excess led to the immediate formation of colored complexes, presumably involving reaction between the anhydride and the N-oxide group. This fact implies that the amino group is more nucleophilic than the N-oxide oxygen with its formal negative charge. The N-acetyl-4-pyridylalanine 1-oxide was found to be so soluble in water (about 50 g./100 ml.) and so insoluble in organic solvents

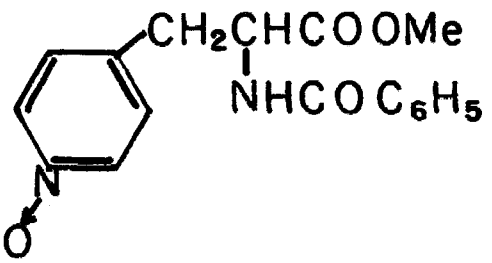
that it could not be separated from sodium chloride. This led to the use of triethylamine as the base in the Schotten-Baumann acylation, for after acidification of the solution to pH 2 and evaporation to dryness, the triethylamine hydrochloride formed could be removed by extraction of the solid residue with chloroform (its solubility being 17.4 g./100 g. at 25° (40)). No free amino acid could be detected in the extracted residue, indicating the acylation must have been quantitative.

In order to see how far one could extend the apparent low reactivity of anhydrides toward the N-oxide function, equimolar amounts of 4-pyridylalanine 1-oxide and phthalic anhydride were heated together in the dry state at 140°. N-Phthaloyl-4-pyridylalanine 1-oxide was isolated in 64% yield. Since there were several structures which would fit the analysis, the compound was characterized by its strong U-V absorption at 260 mμ, characteristic of N-oxide compounds (41), failure to give a positive ninhydrin reaction, and positive ferric hydroxamate test (42).

The methyl esters of N-benzoyl- and N-acetyl-4-pyridylalanine 1-oxide, as well as the ethyl ester of the former, were prepared by the thionyl chloride method described by Brenner and Huber (43). In all cases the product isolated was the 1-hydroxypyridinium chloride salt, which was converted to the free ester by the use of ammonia in chloroform at ice temperature (44). XX was soluble in water at room



XX



XXI

temperature to the extent of 50 g./100 ml., and XXI to the extent of 8 g./100 ml.

In order to obtain acylated L-4-pyridylalanine 1-oxide derivates a resolution was necessary. An enzymatic resolution seemed the most straightforward. In the case of the resolution of N-benzoyl-DL-4-pyridylalanine 1-oxide methyl ester by alpha-chymotrypsin, the resolution was carried out at pH 7.9, the solution acidified to pH 2.2, and after standing for one hour at 4°C., the N-benzoyl-L-4-pyridylalanine 1-oxide was removed by filtration. The filtrate was neutralized to pH 7 and saturated with salt, whereupon the N-benzoyl-D-4-pyridylalanine 1-oxide methyl ester precipitated. The L-acid was purified by dissolving in sodium bicarbonate solution at pH 7 and filtering from denatured enzyme, precipitation at pH 2.2, and recrystallization from water. This compound never gave good analyses, but most of them fitted a monohydrate of the L-acid. Esterification of the L-acid with methanol via the thionyl chloride method gave N-benzoyl-L-4-pyridylalanine 1-oxide methyl ester, with the

same melting point and same rotation (of opposite sign) as the D-ester.

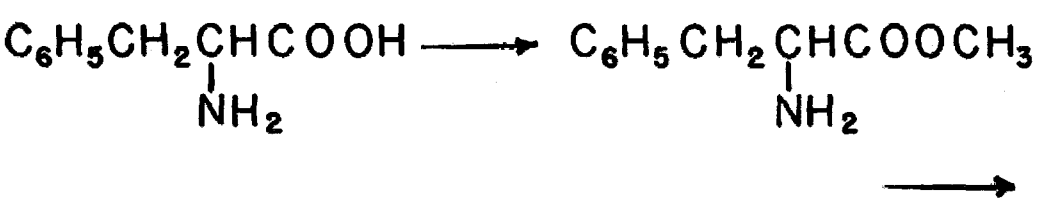
In the case of the alpha-chymotrypsin resolution of N-acetyl-DL-4-pyridylalanine 1-oxide methyl ester, solubility problems appeared. Due to the high water solubility of both the ester and the parent acid, separations attempted were based on differing solubilities in organic solvents. Both compounds had rather low solubilities in methanol or ethanol but the ester was soluble in chloroform, while the acid had a very low solubility in chloroform. Since the resolutions were carried out in aqueous solution at pH 7.9, it was necessary to add a base to hold the pH constant. Two choices were available, sodium hydroxide or triethylamine. In the case where sodium hydroxide was used, the sodium salt of the L-acid was generated, and this compound had a low solubility in methanol or ethanol, as well as being slightly hygroscopic. When the solution containing the D-ester and L-acid salt was acidified, sodium chloride was generated, and it was not possible to separate the salt from the L-acid. Using triethylamine as the base in the resolution, the solution had to be acidified before working up, for the triethylammonium salt of the L-acid was soluble in chloroform. Acidification of the solution produced triethylamine hydrochloride, which is soluble in chloroform. Evaporation of the acidified solution to dryness in vacuo gave a dry powder of which only a small portion would dissolve in chloroform. The low solu-

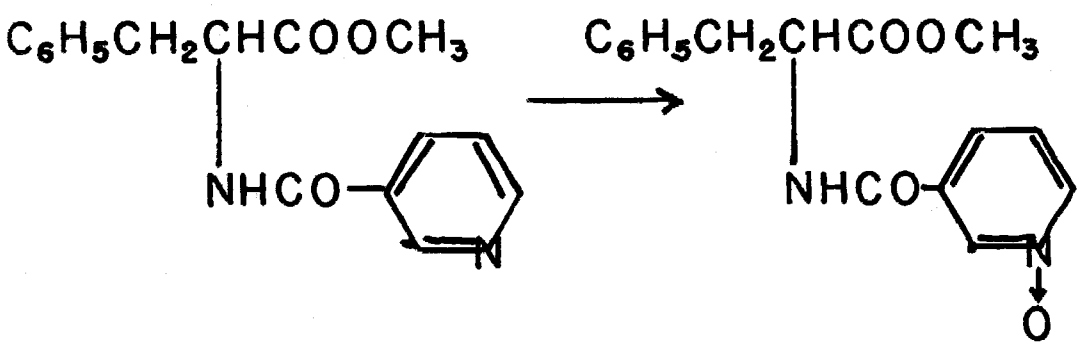
bility in chloroform was due to the fact that enzyme was present in the dry powder. The dry enzyme is very hygroscopic, absorbing water rapidly. Also, triethylamine hydrochloride is very hygroscopic in the presence of an excess of hydrochloric acid. This combined absorption of water led to wetting of the methyl ester, which then was insoluble in chloroform.

A method considered for the resolution of N-acetyl-DL-4-pyridylalanine 1-oxide was via a papain catalyzed synthesis of the phenylhydrazide, but this method depends on the formation of a water-insoluble phenylhydrazide, which for the case at hand would not be likely, since N-acetyl-DL-4-pyridylalanine 1-oxide amide was prepared and found to be very water soluble.

D. PHENYLALANINE DERIVATIVES

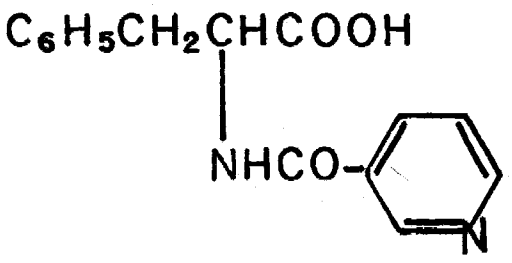
It was desired to synthesize N-(nicotinyl-1-oxide)-L-phenylalanine methyl ester XXII. The most straightforward route to this compound would be as below.



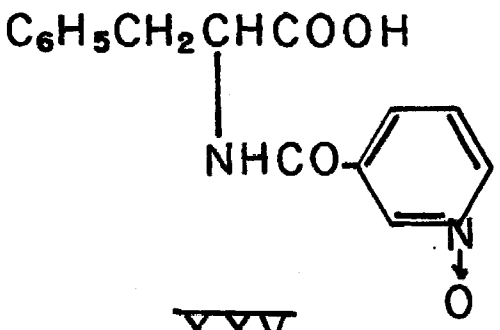


XXIII XXII

Phenylalanine methyl ester was prepared via the thionyl chloride method (43) and acylated with nicotinyl azide to give N-nicotinylphenylalanine methyl ester XXIII. Attempted oxidations of this compound with hydrogen peroxide in glacial acetic acid gave only oils, however, so this route was abandoned. Instead, the ester XXIII was hydrolyzed by base to the acid XXIV, and the acid oxidized to N-(nicotinyl 1-oxide)phenylalanine XXV with hydrogen peroxide in glacial acetic acid. The N-oxide compound XXV could be separated from XXIV by means of the much higher solubility of the latter in acid solutions. Esterification of the acid XXV with methanol gave the DL-methyl ester XXII.



XXIV



XXV

An enzymatic hydrolysis of the DL-ester XXII by alpha-chymotrypsin gave N-(nicotinyl 1-oxide)-L-phenylalanine and N-(nicotinyl 1-oxide)-D-phenylalanine methyl ester. Esterification of the L-acid with methanol and thionyl chloride gave N-(nicotinyl 1-oxide)-L-phenylalanine methyl ester.

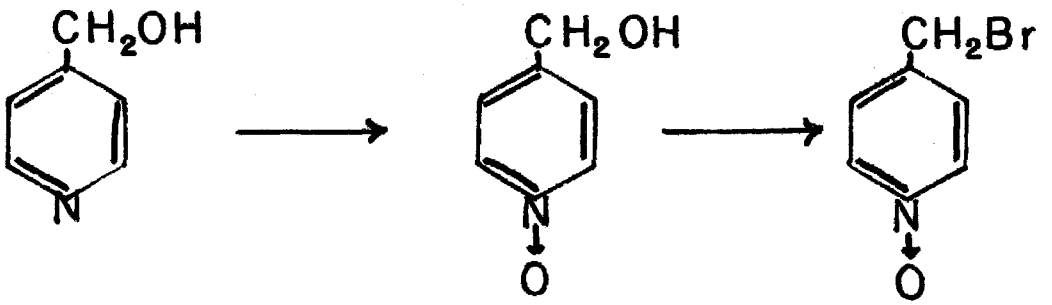
This latter ester was also prepared by esterification of L-phenylalanine, acylation of the ester with nicotinyl chloride, enzymatic hydrolysis to N-nicotinyl-L-phenylalanine, oxidation and esterification. It is interesting to note that the acid hydrolysis of XXIII is extremely slow, presumably due to the presence of a cationic center near the carbomethoxy group.

E. MISCELLANEOUS

Several compounds were synthesized and a few investigations of the catalytic reduction of heterocyclic aromatic amine oxides were carried out in conjunction with the work reported in Sections C and D.

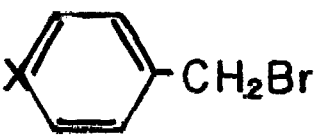
One of the principal hindrances in preparing 4-pyridylalanine via the malonic ester route was the quaternization of 4-pyridylmethyl bromide. It would be expected that 4-pyridylmethyl bromide 1-oxide XXVII would show a marked decrease in rate of quaternization, due to the greater electronegativity of oxygen with respect to nitrogen (3.5 and 3.0 respectively) (45). Jaffe and Doak (38) recently reported the pKa's of a series of substituted pyridines and pyridine 1-oxides, and, as a rule, the pKa's of the pyridines were found to be reduced by about five pK units after N-oxidation. This marked decrease in basicity would also contribute to reduction of quaternization. Quaternary salts of heterocyclic aromatic amine oxides have been prepared (46).

4-Pyridylcarbinol 1-oxide was prepared essentially by the general method described by Ochiai (20). Refluxing the carbinol XXVI in 48% hydrobromic acid for one hour gave only 4-pyridylcarbinol 1-oxide hydrobromide, but extending the time to eight hours gave 4-pyridylmethyl bromide 1-oxide hydrobromide. Treatment of a water solution of the hydrobromide with sodium bicarbonate and extraction with chloroform gave 4-pyridylmethyl bromide 1-oxide XXVII. This compound was quite slow to develop color in methanol solution, in sharp contrast to 4-pyridylmethyl bromide.

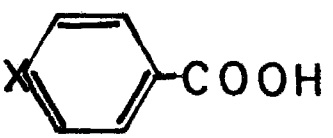


II XXVI XXVII

The halide XXVII should be quite reactive in an S_N2 type reaction, due to the strong inductive effect of the N-oxide group. A measure of the electron attracting ability of X in XXVIII can be made by comparing the pKa's of the corresponding acids XXIX. Thus it would be predicted that there would be increasing S_N2 activity of the halide XXVIII



XXVIII



XXIX

X	CH	C-NO_2	N	NO	$NH^{\oplus}$
pKa	4.20	3.44	3.20	2.86	1.80
Ref.	47	47	*	48	48

*This value was calculated by means of the Hammett equation $\log (K_{\text{isonicotinic acid}}/K_{\text{benzoic acid}}) = \rho\sigma$ (49), where ρ, the reaction constant, equals one for acid ionization (49), and σ, the substituent constant, was taken as one for the 4-position of pyridine, the average of the value 0.93 calculated by Jaffe (50), and the value 1.07 obtained by Kinder for the alkaline saponification of methyl isonicotinate (51).

as the pKa of the corresponding acid decreased. No attempt was made to use the halide in a malonic ester condensation, due to the fact that no method for selective reduction of the amine oxide group could be found (see further).

Since the original aim of this synthesis work was an improved route to 4-pyridylalanine, the problem of removing the N-oxide group presented itself immediately. Aliphatic amine oxides or those typified by dimethylaniline N-oxide are readily reduced to the corresponding tertiary amine by even mild reducing agents. On the other hand, aromatic heterocyclic amine oxides are characterized by a marked resistance to reduction. The reduction potentials of some amine oxides have been determined polarographically by Ochiai (52). A few of these are indicated below:

Compound	Red. Pot. (v.)*
Matrine N-oxide	-0.4562
Dimethylaniline N-oxide	-0.7047
Pyridine N-oxide	-1.2786
2,4,6-Collidine N-oxide	-1.3924
4-Nitropyridine N-oxide	-0.2890, -1.6622
4-Ethoxyquinoline N-oxide	-1.1802

The oxide oxygen of pyridine and quinoline N-oxide is not readily removed by catalytic hydrogenation at ordinary temperatures and pressures (53). Even 4-benzyloxypyridine 1-oxide can be hydrogenolyzed to 4-hydroxypyridine 1-oxide (32). There is some tendency to remove the N-oxide oxygen in acid solution, while the use of acetic acid-acetic

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*Relative to the saturated calomel electrode and at pH 3.5.

anhydride as a solvent system apparently allows complete reduction of the N-oxide group (54). The use of Raney nickel at elevated temperatures and pressures allows a fair amount of tertiary amine to be obtained, but a large amount of by-products, pyridones and nickel salts, are formed (55). Of the many chemical reducing agents investigated, few have proved successful. One of the most useful reducing agents, phosphorus trichloride (56), has obvious limitations. Hertog has reported the use of iron and glacial acetic acid (57).

The most desirable method of reduction would be a catalytic one. One is severely handicapped, however, by the ease of reduction of the pyridine nucleus itself. Initial studies were made on the reduction of 4-picoline 1-oxide using platinum and palladium on charcoal. The extent of reduction was followed by means of pressure-drop in the low pressure hydrogenation apparatus at room temperature. The presence of secondary amine was determined by means of the nickel chloride-carbon bisulfide reagent described by Shriner and Fuson (58). Using 5% palladium on norite, no reduction was obtained using absolute ethanol or 1 N hydrochloric acid in absolute ethanol; using glacial acetic acid plus one mole equivalent of acetic anhydride, ring reduction was extensive. With Adams catalyst, and the three solvents mentioned above, ring reduction was concomitant with N-oxide reduction.

It was decided to study the catalytic reduction spectrophotometrically, using the method outlined by Friedel and Orchin (59). The platinum catalyzed hydrogenation of 4-pyridylalanine and 4-pyridylalanine 1-oxide at 40 psig and room temperature in water was studied. The results are shown in Figure I. Although the data are somewhat crude, there is good evidence that there is concomitant reduction of 4-pyridylalanine 1-oxide and its hydrogenated product, 4-pyridylalanine, under these conditions.

In conjunction with this work, a sample of 4-pyridylalanine 1-oxide was hydrogenated at 40 psig and room temperature over a platinum catalyst, allowing 2.5 equivalents of hydrogen to be absorbed (versus 4 equivalents for complete reduction). This reaction should lead to a 50-50 mixture of 4-pyridylalanine and 4-piperidylalanine. A crude sample of 4-piperidylalanine had been prepared and found to be quite soluble in absolute methanol. The reduction product was isolated, dried, and extracted with methanol, in hopes of removing the 4-piperidylalanine. The U-V absorption spectra of the dried product showed about one-half of the theoretical molar absorption for 4-pyridylalanine, and gave a blue-purple ninhydrin reaction, in contrast to the red color given by 4-pyridylalanine. The same product was isolated after another methanol extraction. The only reasonable structure for this product would be the 4-piperidiniumalanine salt of 4-pyridylalanine.

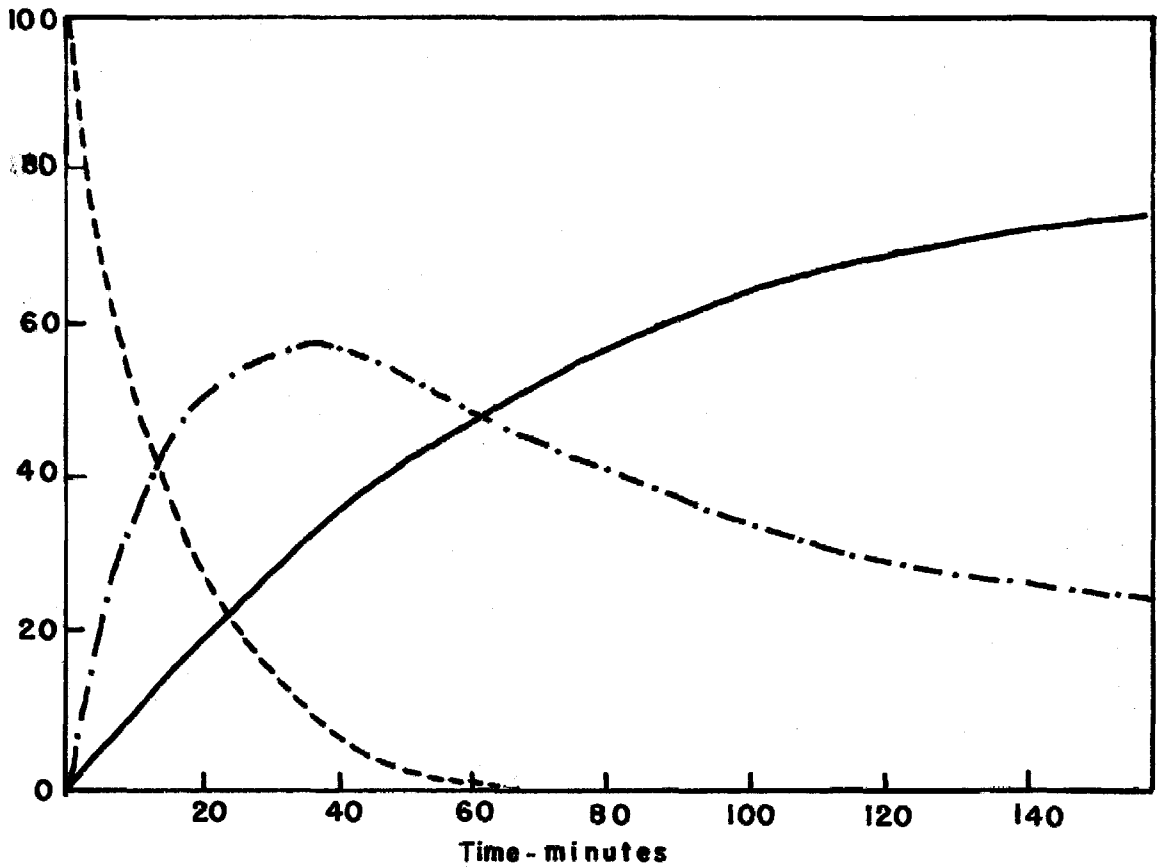
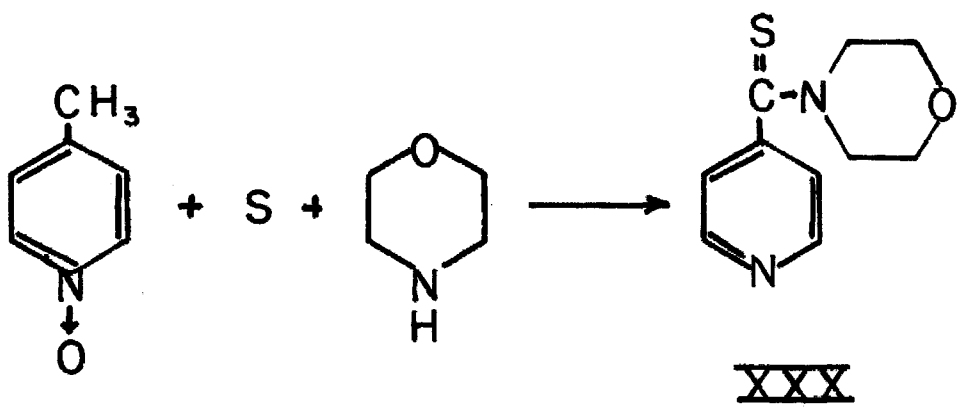


Figure 1.--The catalytic hydrogenation of 4-pyridylalanine 1-oxide: ---- mole percent 4-pyridylalanine 1-oxide; -·- mole percent 4-pyridylalanine; —— mole percent 4-piperidylalanine.

Other methods investigated for the reduction of the heterocyclic aromatic amine oxide group involved the use of hypophosphorous acid and also that of triphenyl phosphine, but in both cases no reduction was obtained. Horner and Hoffmann (60) recently reported the failure of attempted reductions of pyridine and quinoline 1-oxides with triethyl- and triphenylphosphine. Due to the similarity of the semi-polar N—O bond in heterocyclic aromatic amine oxides to that of the N—O bond in aromatic nitro compounds, it was hoped that the use of hydrazine and Raney nickel in alcohol for the reduction, a method which works well for aromatic nitro compounds (61), would apply in the case of the N-oxide compounds. No reduction was obtained in the case of 4-picoline 1-oxide. A reduction was attempted using sulfur under the conditions of the Willgerodt reaction on 4-picoline 1-oxide in morpholine. The product obtained was the thioisonicotinyl morpholine XXX. These conditions were too strenuous for application to more complex compounds.



XXX

In considering methods for the preparation of N-(nicotinyl 1-oxide)phenylalanine, it was hoped that some method of acylation in which the nicotinyl 1-oxide group would be introduced in one step could be devised. The most obvious route would involve the acylation of phenylalanine methyl ester. For the introduction of nicotinyl groups, nicotinyl azide or nicotinyl chloride has been used. It was expected that nicotinyl chloride 1-oxide would not be stable, due to interaction between the N-oxide and acid chloride groups, so attention was directed to the preparation of nicotinyl- and isonicotinyl azide 1-oxide. Methyl isonicotinate 1-oxide was prepared by the general method of Ochiai (20), and converted to the hydrazide. Treatment of the hydrazide in acid solution with nitrous acid gave only isonicotinic acid 1-oxide, presumably through the azide, which being soluble in water, reacted readily to give the acid. The same results were obtained with nicotinic acid hydrazide 1-oxide.

Another type of acylating agent considered was active esters. Schwyzer has reported the use of cyanomethyl esters as being quite satisfactory (62). The cyanomethyl ester of nicotinic acid 1-oxide was prepared via a modification of the method reported by Schwyzer (63), but did not react with phenylalanine methyl ester under the conditions employed, only starting material being isolated.

F. EXPERIMENTAL*

4-Pyridylmethyl Bromide Hydrobromide -- A solution of 20 g. of 4-pyridylcarbinol (Aldrich Chemical Co.) in 180 ml. of 48% hydrobromic acid was held at reflux for four hours, then concentrated *in vacuo* to a thick paste, which was diluted with 100 ml. of absolute ethanol. The resulting suspension was filtered at ice temperature and the residue washed with 20 ml. of absolute ethanol. The product, white needles, was dried *in vacuo* to yield 38.7 g. (84%), m.p. 185-187° dec. (lit. (2) 145-150°, (4) 187°).

Diethyl Acetamido-(4-pyridylmethyl)malonate -- As a result of sixteen preparations of this compound under varying conditions, the procedure below was found to be optimal on an 0.02 mole (of hydrobromide) scale.

A 500 ml. three-necked round-bottomed flask, equipped with a Teflon-bladed Trubore stirrer, reflux condenser, drying tube, and slurrying equipment (see below), was purged with nitrogen. After 0.92 g. (0.04 g. atom) of sodium had been dissolved in 70 ml. of 50% benzene-50% ethanol, 8.46 g. (0.04 mole) of diethyl acetamidomalonate (Winthrop-Stearns Co.) was added and the resultant solution was heated to

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*All melting points are corrected. All boiling points are uncorrected. The microanalyses were done by Dr. A. Elek, Los Angeles 16, California. All solubilities reported are approximate and should be considered minimum values.

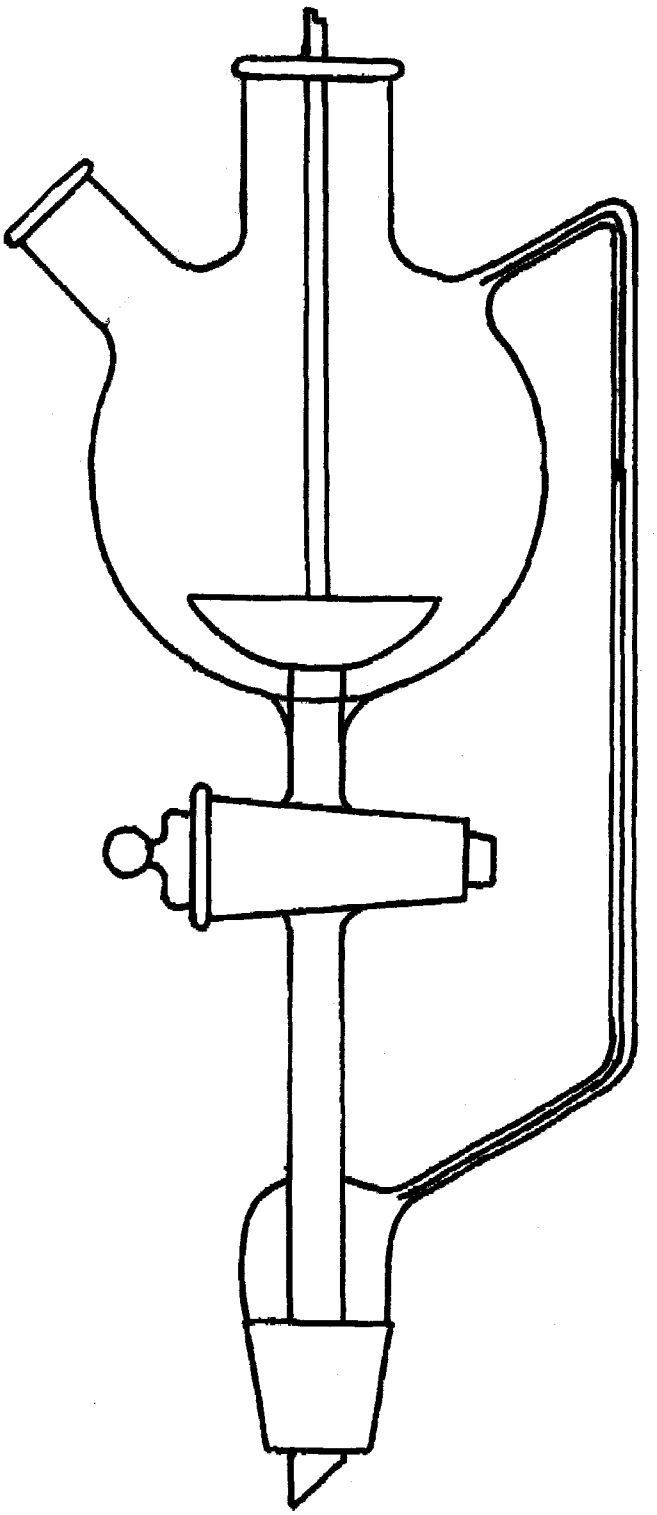

Figure 2.--Slurrying apparatus.

reflux. With the stopcock on the slurrying apparatus closed, 100 ml. of 50% benzene-50% ethanol was added to the bulb with the stirrer on, followed by 5.06 g. (0.02 mole) of 4-pyridylmethyl bromide hydrobromide. With the stirrer on in the reaction flask, the stopcock on the slurrying apparatus was opened. The slurry was added to the reaction flask over 40 minutes, the rate of addition being controlled by the stirring rate in the slurrying apparatus. The resultant red solution was held at reflux for two hours, then allowed to stand overnight at room temperature. The solvent was removed by evaporation under a stream of air, leaving a brick-red solid. The solid was extracted with 100 ml. of dry chloroform, and the chloroform solution extracted with 40 ml. of 4 N hydrochloric acid. The acid phase was neutralized with 30% sodium hydroxide in the cold to pH 6. The resultant precipitate was removed by filtration, and combined with a small amount of material obtained by chloroform extraction of the filtrate to give about 4 g. of product (70%). The product was recrystallized from water m.p., 121.7-122.0°.

Anal. Calc. for $C_{15}H_{20}O_5N_2$ (308.3): C, 58.43; H, 6.54; N, 9.09. Found: C, 58.52; H, 6.47; N, 9.06.

This procedure gave only 30% yields of product when 0.08 mole quantities were used, presumably due to slurrying problems. A drawing of the slurrying apparatus is shown

in Figure 2. For the 0.02 mole scale, the bulb was prepared from a 250 ml. flask, and the exit tube from the bulb projected 1/4" into the bulb. The stirrer must operate on the top of the exit tube.

DL-4-Pyridylalanine -- A solution of 15 g. of recrystallized diethyl acetamido-(4-pyridylmethyl)malonate in 75 ml. of 40% hydrobromic acid was held at reflux for six hours. The solvent was removed under vacuum until a dense white solid separated. The crystalline material was removed by filtration and washed with 20% hydrobromic acid. The filtrates were concentrated to 10 ml., and another batch of crystals obtained. The combined solids were dried in vacuo to yield 12.2 g. (76%) of DL-4-pyridylalanine dihydrobromide, m.p. 250-252° dec.

Anal. Calc. for $C_8H_{12}O_2N_2Br_2$ (330.0): C, 29.0; H, 3.66; N, 8.49; Br, 48.4. Found: C, 29.1; H, 3.63; N, 8.70; Br, 48.5.

A water solution of 11.2 g. of dihydrobromide was shaken with water-washed Amberlite IR-4B until the solution was neutral. The resin was removed by filtration, and the yellow solution treated with norite to yield a colorless solution. The water was removed under vacuum to yield after recrystallization from water, 5.11 g. (90%) of DL-4-pyridylalanine, m.p. 234-235° dec. (lit. (2) 235-236°).

Anal. Calc. for $C_8H_{10}O_2N_2$ (166.2): C, 57.8; H, 6.1; N, 16.9. Found: C, 57.5; H, 6.1; N, 16.2; Br, 0.3.

The addition of ninhydrin to an aqueous solution of the amino acid resulted in the formation of a red color, as previously reported (2,3). The amino acid is soluble in water to the extent of 3.4 g. per 100 ml., and is slightly soluble in pyridine.

N-Benzoyl-DL-4-pyridylalanine -- To 0.5 g. (3.02 mole) of 4-pyridylalanine dissolved in 20 ml. of water at 10° was added 0.835 ml. of triethylamine (6.04 mmole) and 0.75 g. (3.32 mmole) of benzoic anhydride. The suspension was stirred for 18 hours, after which most of the anhydride had disappeared. The solution was filtered from the residual anhydride, acidified to pH 1 with concentrated hydrochloric acid, and filtered from the precipitated benzoic acid. The filtrate was then neutralized to pH 4.3 with sodium bicarbonate. After standing overnight at 4°, 0.72 g. of product (89%), needles, were obtained. Recrystallization from 50% aqueous methanol, gave 0.67 g. of product, m.p. 246° dec.

Anal. Calc. for $C_{15}H_{14}O_3N_2$ (270.3): C, 66.65; H, 5.22; N, 10.37. Found: C, 66.44; H, 5.30; N, 10.30.

Ethyl 4-Pyridylpyruvate 1-Oxide -- To a solution of 28 g. (1.22 g. atom) of sodium in 300 ml. of absolute ethanol was added 109.2 g. (1.00 mole) of 4-picoline 1-oxide (Reilly Tar and Chemical Co.) dissolved in 300 ml. of absolute

ethanol. The solution was stirred at reflux for 15 minutes to give a clear red solution. Then 146.2 g. (1.00 mole) of ethyl oxalate was added over a period of five minutes. After about two minutes, a yellow precipitate of the sodium salt of the keto ester appeared. The heat was removed, and the resultant yellow paste was stirred for two hours. The solvent was removed *in vacuo* to leave a damp yellow paste, which was dissolved in water to give a dark red solution. The solution was neutralized with 12 *N* hydrochloric acid, and the precipitate which formed was removed and recrystallized from water to yield 100 g. of yellow needles, m.p. 140.2-141.5°(A). Concentration of the filtrate from the recrystallization gave 20 g. of yellow prisms, m.p. 182.8-183.0°(B). Analyses of both samples showed neither to be pure keto ester. (B) would not dissolve in chloroform, while (A) dissolved nearly completely. Recrystallization of (A) twice from water after dissolving in chloroform and removing insoluble material (B) gave 95 g. of α-keto ester, m.p. 129° dec. (lit. (21) 122-123°). Another 5.0 g. of product was obtained from the chloroform extraction of (B) to give a total 48% yield.

Recrystallization of (B), after chloroform extraction, from water gave a pale yellow solid, m.p. 187-188° dec. This compound was an acid, molecular weight *ca.* 300 from the U-V spectra assuming one N-oxide group, and from base titration. The compound would not form an oxime. When

heated, the compound would suddenly decompose and evolve a purple vapor. The compound would turn bright yellow after prolonged exposure to light. No consistent analyses could be obtained.

Anal. a) C, 56.57; H, 4.40; N, 6.20.

b) C, 57.24; H, 4.57; N, 6.92.

Ethyl 4-Pyridyl-(α-oximino)propionate 1-Oxide (from 4-picoline 1-oxide) -- The condensation of 4-picoline 1-oxide and ethyl oxalate was carried out as in the procedure above. However, the solvent was not removed in vacuo. The yellow paste was washed out of the reaction flask with the aid of about 2 l. of 50% aqueous ethanol into a 4-l. beaker, the resultant red solution heated to about 60°, and 85 g. (1.22 mole) of hydroxylamine hydrochloride and 100 g. (1.22 mole) of sodium acetate added. The resultant solution was allowed to cool overnight, the crystals which had formed removed by filtration, the filtrate concentrated to one-half its original volume, and another crop of crystals removed. The combined solids were combined and recrystallized from water to yield 136 g. (60%) of oximino ester, m.p. 210° dec. (lit. (21) 221-222°). Despite the fact that the oximino ester was dried at 65° over phosphous pentoxide and at 100 mm., the low melting point and the analysis indicated that a hydrate was obtained.

Anal. Calc. for $C_{10}H_{12}O_4N_2.H_2O$ (242.2): C, 49.58; H, 5.83; N, 11.57. Found: C, 49.68; H, 5.61; N, 11.68.

The same product was obtained from ethyl 4-pyridyl-pyruvate 1-oxide. To a hot solution of 15 g. (0.0702 mole) of keto ester in 100 ml. of water, 4.9 g. (0.0702 mole) of hydroxylamine hydrochloride, 2.8 g. (0.0702 mole) of sodium hydroxide and 5.75 g. (0.0702 mole) of sodium acetate were added. A white flocculent precipitate formed immediately as the solution cooled. The precipitate was recrystallized from water to give 8.2 g. (52%) of fibrous needles, m.p. 210° dec.

4-Pyridyl-(α-oximino)pyruvic acid 1-Oxide -- A solution containing 20 g. (0.0893 mole) of ethyl 4-pyridyl-(α-oximino)-pyruvate 1-oxide and 7.15 g. (0.1786 mole) of sodium hydroxide in 180 ml. of water was held at the boiling point for five minutes. The solution was cooled in an ice bath to about 50°, and neutralized with 14.9 ml. of concentrated hydrochloric acid dissolved in 25 ml. of water. After standing in an ice bath for four hours, the white solid which had formed was removed by filtration and washed with a small amount of cold water. The product was dried in vacuo to give 14.3 g. (82%) of product with m.p. 140-141°. Recrystallization from water (7 ml. per g.) gave clusters of fine needles, m.p. 139.8-140° dec.

Anal. Calc. for $C_8H_8O_4N_2$ (196.2): C, 48.98; H, 4.11; N, 14.28. Found: C, 49.22; H, 4.23; N, 14.41.

DL-4-Pyridylalanine 1-Oxide -- To 1 l. of concentrated hydrochloric acid containing 260 g. (1.15 mole) of stannous chloride dihydrate, 100 g. (0.51 mole) of 4-pyridyl-(α-oximino)pyruvic acid 1-oxide was added in portions. The solution was exothermic. The clear solution was allowed to stand at room temperature overnight, then concentrated in vacuo to a thick paste. The paste was dissolved in 750 ml. of water and the solution neutralized to pH 6.8 with about 375 ml. of 28% aqueous ammonia. It was found best to keep stirring at a minimum during this neutralization, in order to produce a precipitate more amiable towards filtration. The white precipitate of tin hydroxides was removed by filtration, the residue being washed with 200 ml. of water. The filtrate gave no precipitate when a sample was saturated with hydrogen sulfide. The filtrate was evaporated to dryness in vacuo, and the granular mixture of product and ammonium chloride ground so as to pass an 80 mesh screen. The fine powder was extracted with 6 l. of absolute methanol for one week to yield 90 g. of crude product. Further extraction with 2-500 ml. portions of methanol gave 78 g. (84%) of pure DL-4-pyridylalanine 1-oxide, m.p. 238.2° dec. An aqueous solution of the product did not give a halide ion test with silver nitrate.

Anal. Calc. for $C_8H_{10}O_3N_2$ (182.2): C, 52.74; H, 5.53; N, 15.38. Found: C, 52.67; H, 5.61; N, 15.41; Cl, 0.03.

The product could be obtained more rapidly, but in a

lower yield (about 45%) by vacuum stripping the filtrate from the neutralization until solid appeared, cooling the suspension, filtering off the solid, then continuing the stripping on the filtrate, stopping only to remove solid whenever it appears, until the volume of the filtrate is about 150 ml. The filtrate is then evaporated to dryness and the residue, mainly product, extracted with methanol to remove ammonium chloride.

This amino acid gave an orange color, preceded by a violet-blue color, with ninhydrin solution. The reaction was very slow. The amino acid was soluble in water to the extent of 45 g. per 100 ml. of water, and slightly soluble in pyridine.

N-Benzoyl-DL-4-pyridylalanine 1-Oxide -- To a solution of 61.0 g. (0.33 mole) of 4-pyridylalanine 1-oxide in 600 ml. of ice cold water containing 92 ml. (0.66 mole) of triethylamine was added 82.2 g. (0.363 mole) of finely ground benzoic anhydride. The mixture was stirred for 18 hours. The resultant clear solution was filtered from a small amount of unreacted anhydride and acidified with 246 ml. of 12 N hydrochloric acid, enough to make the solution 2 N in hydrochloric acid. The precipitated benzoic acid was removed by filtration, washed with a small amount of 2 N hydrochloric acid, and the combined filtrate and wash adjusted to pH 2.3 with 91.2 g. of sodium hydroxide in 200 ml. of

water. The resultant paste was cooled to 4°C., the product removed by filtration, washed with 250 ml. of water, and air dried to yield 73 g. (77%) of product, m.p. 228.5-229.2° dec. The acid was recrystallized from 60% ethanol-water (8 ml. per g.) to give 70 g. (74%), m.p. 229.5° dec.

Anal. Calc. for $C_{15}H_{14}O_4N_2$ (286.3): C, 62.93; H, 4.93; N, 9.79. Found: C, 62.89; H, 5.02; N, 9.77.

The compound was soluble in water to the extent of 0.5 g./100 ml. at room temperature.

N-Acetyl-DL-4-pyridylalanine 1-Oxide -- To 30.0 g. (0.165 mole) of DL-4-pyridylalanine 1-oxide dissolved in 165 ml. of water was added 23.1 ml. (0.165 mole) of triethylamine. The solution was cooled to about 0°C. by means of an ice-salt bath and, with vigorous stirring, portions, each of 5.8 ml. (0.041 mole) triethylamine and 3.85 ml. (0.041 mole) of acetic anhydride, in that order, were added at 10 minute intervals. When a few drops of the fifth portion of acetic anhydride were added, the solution became bright orange, and no further addition was made. The solution was acidified with 100 ml. of 4 N hydrochloric acid and concentrated in vacuo. The residual paste was dried in vacuo over potassium hydroxide and phosphorus pentoxide for two days. The dried material was rapidly powdered (hygroscopic) and shaken with 400 ml. of dry chloroform for 20 minutes to remove triethylamine hydrochloride, then fil-

tered to give 32.9 g. of crude material. An additional 3.6 g. of product separated from the filtrate on standing a few hours, for a total yield of 36.5 g. The crude material was stirred with 100 ml. of dry chloroform for 10 minutes, filtered off and dried to give 32.3 g. (87%) of chloride-free product, giving no ninhydrin reaction. The acid was recrystallized from toluene* or absolute ethanol,** m.p. 210.2° dec.

Anal. Calc. for $C_{10}H_{12}O_4N_2$ (224.2): C, 53.57; H, 5.39; N, 12.50. Found: C, 53.47; H, 5.07; N, 12.51.

The acylated acid was soluble in water at room temperature to the extent of 60 g./100 ml.

N-Phthaloyl-DL-4-pyridylalanine 1-Oxide -- DL-4-pyridylalanine 1-oxide (1.0 g., 5.5 mmole) and phthalic anhydride (0.816 g., 5.5 mmole) were mixed thoroughly in the dry state, then heated in a test tube in a xylene bath (140°) for one hour. A yellowish paste formed, but the mixture never became transparent. Water was evolved. The paste was cooled and treated with a saturated sodium bicarbonate solution. The

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*Once the amorphous material had been crystallized in toluene, it could not be redissolved.

**The first sample, recrystallized from ethanol, had m.p. 190.5-191° dec. After the appearance of the higher melting form, the lower melting form was never obtained. The lower melting form gave a correct analysis.

residual anhydride was removed by filtration, and the filtrate acidified with hydrochloric acid to yield an oil which crystallized slowly after rubbing with water to give 1.06 g. (64%) of product, m.p. 253-254° dec. The product, which was insoluble in all of the usual solvents, could be recrystallized from 200 ml. of water to yield rhombs, m.p. 254.5-255.0° dec. The U-V spectra of the compound showed a characteristic N-oxide absorption at 260 mμ in water, the compound would not give a ninhydrin test, and reacted readily to give a ferric hydroxamate test (red color), similar to N-phthaloylphenylalanine.

Anal. Calc. for $C_{16}H_{12}N_2O_5$ (312.3): C, 61.54; H, 3.87; N, 8.97. Found: C, 61.50; H, 4.03; N, 9.08.

N-Benzoyl-Dl-4-pyridylalanine 1-Oxide Methyl Ester -- To 200 ml. (4.88 mole) of absolute methanol, cooled in a 3:1 ice-salt bath and magnetically stirred, was added over a period of 15 minutes 26.7 ml. (0.366 mole) of thionyl chloride. Then 70.0 g. (0.244 mole) of N-benzoyl-DL-4-pyridylalanine 1-oxide was added in portions, over a period of 20 minutes. The resultant clear solution was warmed to 40°, and after about 15 minutes, a white solid began to precipitate. The slurry was stirred overnight at room temperature, filtered, and the filtrate stripped to dryness. The residues from the filtration and stripping were combined and dried in vacuo to yield 77 g. (94%) of ester hydrochloride.

The dry hydrochloride was suspended in 600 ml. of ice-cold dry chloroform and treated with 150 ml. of 1.8 N ammonia in chloroform (0.270 mole). The ammonium chloride that formed was removed by filtration, and the filtrate evaporated to dryness. The dry residue was washed with ligroin, then dried in vacuo to yield 63.5 g. (86%) of ester, m.p. 190.0-190.5°, m.p. not raised by recrystallization from chloroform. The ester was soluble in water to the extent of 8.0 g./100 ml. at room temperature.

Anal. Calc. for $C_{16}H_{16}O_4N_2$ (300.3): C, 63.99; H, 5.37; N, 9.33. Found: C, 63.90; H, 5.42; N, 9.46.

N-Benzoyl-DL-4-pyridylalanine 1-Oxide Ethyl Ester -- This ester was prepared from N-benzoyl-DL-4-pyridylalanine 1-oxide by the same method used for the methyl ester, only substituting absolute ethanol for methanol, m.p. 141-143°.

Anal. Calc. for $C_{17}H_{18}O_4N_2$ (314.3): C, 64.95; H, 5.77; N, 8.91. Found: C, 65.20; H, 5.64; N, 9.01.

N-Acetyl-DL-4-pyridylalanine 1-Oxide Methyl Ester -- This ester was prepared in 75% yield from N-acetyl-DL-4-pyridylalanine 1-oxide in a manner identical to that for the N-benzoyl- compound. The ester was very soluble in chloroform and water (about 50 g./100 ml. in the latter), soluble in methanol, less soluble in ethanol and isopropanol, and insoluble in hot ethyl acetate or toluene. The ester was recrystallized from absolute ethanol (4 ml. per g.) to

form clusters of slightly hygroscopic needles, m.p. 193.5-194.2° dec.

Anal. Calc. for $C_{11}H_{14}O_4N_2$ (238.2): C, 55.45; H, 5.92; N, 11.76. Found: C, 55.41; H, 6.03; N, 11.70.

This ester was also obtained in a 47% yield from 4-pyridyl-(α-oximino)pyruvic acid 1-oxide as follows: the oximino acid was reduced with stannous chloride and the tin salts removed by the addition of sodium hydroxide and filtration. After concentration, the filtrate was acetylated with acetic anhydride and sodium hydroxide. The resultant solution was acidified to pH 2.0 and evaporated to dryness. Treatment of the residue via the thionyl chloride-methanol method gave a solution of the ester hydrochloride and a precipitate of sodium chloride. After filtration, the solution was treated as in the above paragraph.

The Determination of the pKa of the 1-Hydroxy-N-benzoyl-DL-4-pyridylalanine Ion -- A stock solution, 0.930 X 10^{-2} M in N-benzoyl-DL-4-pyridylalanine 1-oxide in ca. 0.3 N hydrochloric acid was prepared. The U-V spectra of 1.00 ml. of the stock solution, diluted to 50.00 ml. with 4 N hydrochloric acid, was taken to obtain A_{BH}. The U-V spectra of 1.00 ml. of the stock solution, diluted to 50.00 ml. with 0.3 N hydrochloric acid, was obtained to give A and the pH of the solution measured to give (H). The U-V spectra of 1.00 ml. of the stock solution, made basic with sodium hydroxide and

diluted to 100.0 ml. with water (pH = 10.6), was obtained to give A_B.

Using the scheme of Flexser, Hammett, and Dingwall (37), the total concentration of material C at any given pH is the sum

$$C = C_B + C_{BH},$$

the concentrations of the unprotonated species C_B and the protonated species C_{BH}. The optical density A for C is given by the expression

$$A = -\log T = (k_B C_B + k_{BH} C_{BH})l,$$

where $k = A/Cl$. Substituting $k' = kl$, we get

$$A = k'_B C_B + k'_{BH} C_{BH} = k'C.$$

This leads to

$$\frac{C_{BH}}{C_B} = \frac{k'_B - k'}{k' - k'_{BH}} = \frac{C_H}{K_A},$$

where C_H is measured at concentration C. The data below are for = 260 mμ.

$A_{BH} = 1.013$ $C_{BH} = 1.86 \times 10^{-4}$ M $k'_{BH} = 0.545 \times 10^4$ cm. M^{-1}

$A = 1.438$ $C = 1.86 \times 10^{-4}$ M $k' = 0.773 \times 10^4$ cm. M^{-1}

$A_B = 1.648$ $C_B = 0.93 \times 10^{-4}$ M $k'_B = 1.772 \times 10^4$ cm. M^{-1}

$C_H = 0.309$ M $C_{BH}/C_B = 4.37$ $K_A = 0.0707$

pKa = 1.15

DL-4-Pyridylalanine 1-Oxide Methyl Ester -- This compound was prepared by suspending DL-4-pyridylalanine 1-oxide in absolute methanol and saturating with dry hydrogen chloride,

allowing the temperature to rise to about 60°C. The resultant clear solution was stripped *in vacuo*, and the residue treated with sodium methoxide in methanol. The sodium chloride was removed by filtration, the filtrate concentrated, filtered again, and the filtrate evaporated to dryness in a hard vacuum. The product, a hard glass, was soluble in chloroform if initially wet with methanol, but was completely insoluble in anhydrous chloroform once exposed to a trace of moisture. The product was deliquescent in air, and it was impossible to obtain a pure sample, even working in a dry box.

DL-4-Pyridylalanine 1-Oxide Hydrazide -- Approximately 1.0 g. of crude ester (see above) was dissolved in 5 ml. of absolute methanol and 0.5 ml. of anhydrous hydrazine was added. The solution was held at reflux for one hour, then evaporated in a dry nitrogen atmosphere to a viscous oil, which crystallized after rubbing with chloroform. The suspension was filtered and the product dried *in vacuo* to give 0.71 g. (70%) of product, quite hygroscopic, which, after recrystallization from a concentrated ethanol solution, gave large rhombs, m.p. 147-148°.

Anal. Calc. for $C_8H_{12}O_2N_4$ (196.2): C, 48.97; H, 6.16; N, 28.56. Found: C, 48.87; H, 6.04; N, 28.43.

N-Acetyl-DL-4-pyridylalanine 1-Oxide Amide -- N-Acetyl-DL-4-pyridylalanine 1-oxide methyl ester (5.0 g.) was dissolved in 120 ml. of dry methanol saturated with dry ammonia

at room temperature (16.5% ammonia) and allowed to stand overnight. The solution was evaporated to dryness *in vacuo*, suspended in absolute ethanol and filtered to yield 4.0 g. of product (86%). The amide was recrystallized from 300 ml. of absolute ethanol to give 3.4 g., m.p. 235-236° dec. The amide was soluble in water to the extent of about 50 g./100 ml.

Anal. Calc. for $C_{10}H_{13}O_3N_3$ (223.2): C, 53.80; H, 5.87; N, 18.83. Found: C, 53.76; H, 5.75; N, 18.81.

N-Benzoyl-L-4-pyridylalanine 1-Oxide -- *alpha*-Chymotrypsin, 20 mg., was added to a solution of 10 g. of N-benzoyl-DL-4-pyridylalanine 1-oxide methyl ester in 100 ml. of water at pH 7.9 by the addition of 1 *N* sodium hydroxide. The resolution was complete in one hour, and the clear solution was acidified to pH 2.3 by the addition of 6 *N* hydrochloric acid. After standing for one hour at 4°C., the precipitated L-acid was removed by filtration, dissolved in the theoretical amount of saturated sodium bicarbonate solution, filtered from denatured enzyme, the solution acidified to pH 2.3, and the precipitated L-acid collected. The L-acid was recrystallized from water to give 2.73 g. (58%), $(\alpha)_D^{25^\circ}$ = -45.1° to -43.4° (various preparations) (C, 1.5% in acetic acid), m.p., 216-220°, with oil formation at 145-50°. Analyses (C,H,N) for this compound were never good, despite many recrystallizations. The samples were dried *in vacuo* over phosphorus pentoxide

at 60°C. Samples dried at higher temperatures (about 140°C.) suffered partial decomposition.

Anal. Calc. for $C_{15}H_{14}O_4N_2 \cdot H_2O$ (316.3): C, 60.7; H, 5.10; N, 8.86. Found: C, 59.5; H, 5.24; N, 10.1

The identical procedure was carried out on N-benzoyl-DL-4-pyridylalanine 1-oxide ethyl ester to give the same product.

N-Benzoyl-D-4-pyridylalanine 1-Oxide Methyl Ester -- The acidified filtrate from the DL-methyl ester resolution was adjusted to pH 7.0 by the addition of saturated sodium bicarbonate solution, and the solution saturated with sodium chloride. The D-methyl ester precipitated. The crude ester was air dried, then dissolved in chloroform and the solution filtered from a small amount of sodium chloride. The filtrate was concentrated to yield 2.82 g. (56%) of D-methyl ester, m.p. 207° dec., $(\alpha)_D^{25°}$ = +95.5° (C, 3.3% in methanol).

Anal. Calc. for $C_{16}H_{16}O_4N_2$ (300.3): C, 63.99, H, 5.37; N, 9.33. Found: C, 63.87; H, 5.34; N, 9.24.

N-Benzoyl-D-4-pyridylalanine 1-Oxide Ethyl Ester -- This ester was recovered from the resolution of the DL-ester as carried out for the D-methyl ester to yield 3.7 g. (74%) of product, m.p. 181.0-182.0°, after recrystallization from 300 ml. of ethyl acetate 99%-water 1%, $(\alpha)_D^{25°}$ = +87.6° (C, 1.5% in methanol).

Anal. Calc. for $C_{17}H_{18}O_4N_2$ (314.3): C, 64.95; H, 5.77; N, 8.91. Found: C, 65.01; H, 5.86; N, 8.82.

N-Benzoyl-L-4-pyridylalanine 1-Oxide Methyl Ester -- This ester was prepared from the N-benzoyl-L-4-pyridylalanine 1-oxide described previously via the thionyl chloride method described for the DL-methyl ester to yield 88%, m.p. 208°, $(\alpha)_D^{25^\circ}$ = -95.5° (C, 4% in methanol).

Anal. Calc. for $C_{16}H_{16}O_4N_2$ (300.3): C, 63.99; H, 5.37; N, 9.33. Found: C, 63.87; H, 5.43; N, 9.33.

N-Benzoyl-L-4-pyridylalanine 1-Oxide Ethyl Ester -- This ester was prepared essentially as described for the DL-ester, using the N-benzoyl-L-4-pyridylalanine 1-oxide described previously to give 86%, m.p. 181.0-182.5°, after recrystallization from ethyl acetate 95%-water 5%, $(\alpha)_D^{25^\circ}$ = -87.3° (C, 1.5% in methanol).

Anal. Calc. for $C_{17}H_{18}O_4N_2$ (314.3): C, 64.95; H, 5.77; N, 8.91. Found: C, 64.89; H, 5.86; N, 8.89.

N-Nicotinyl-DL-phenylalanine -- To a solution of 22 g. (0.0775 mole) of N-nicotinyl-DL-phenylalanine methyl ester (64) in 500 ml. of methanol was added 39 ml. of 2 N sodium hydroxide (0.0775 mole) and the solution warmed for one hour at 50°. The methanol was removed by evaporation, and the resultant solid dissolved in water and acidified to pH 4.3. An oil formed which slowly crystallized to give, after recrystallization from water, 20.0 g. (95%) of acid,

m.p. 198-199° dec. (lit. (65) 198°).

N-(Nicotinyl 1-Oxide)-DL-Phenylalanine -- 20 g. of acid (from above) was dissolved in 75 ml. of glacial acetic acid and 17 ml. of 30% hydrogen peroxide at 90°C. The solution was held at 90° for 16 hours, cooled, diluted with 150 ml. of water, and filtered. The product after air drying, weighed 15.0 g. (71%) and had m.p. 230° dec. After suspending in 400 ml. of 0.5 N hydrochloric acid and filtering, the residue was recrystallized from 1.5 l. of water to give 12.0 g. (57%), m.p. 232.0-232.5° dec.

Anal. Calc. for $C_{15}H_{14}O_4N_2$ (286.3): C, 62.93; H, 4.93; N, 9.79. Found: C, 62.98; H, 4.97; N, 9.84.

N-(Nicotinyl 1-Oxide)-DL-phenylalanine Methyl Ester -- This ester was prepared by a thionyl chloride esterification of the parent acid in 93% yield. The ester was obtained as an oil by addition of pentane to a concentrated chloroform solution. The oil was crystallized by rubbing with water. After recrystallization from water, it had m.p. 158.5-160° dec.

Anal. Calc. for $C_{16}H_{16}O_4N_2$ (300.3): C, 63.99; H, 5.37; N, 9.33. Found: C, 64.10; H, 5.29; N, 9.51.

N-(Nicotinyl 1-Oxide)-L-phenylalanine -- To a suspension of 8.0 g. of the above ester in 200 ml. of water was added 40 mg. of α-chymotrypsin and the pH of the solution maintained between 7-8 by the addition of 1 N sodium

hydroxide. The hydrolysis appeared to finish in 20 minutes. The resultant clear solution (the solubility of the ester in water is about 2 g./100 ml.) was acidified to pH 2.0 with 12 N hydrochloric acid and allowed to stand for 30 minutes. The acid which had formed was removed by filtration and recrystallized from 200 ml. of water to give 2.8 g. (73%), m.p. 218° dec., $(\alpha)_D^{25} = -83.5°$ (C, 1.6% in dimethylformamide).

Anal. Calc. for $C_{15}H_{14}O_4N_2$ (286.3): C, 62.93; H, 4.93; N, 9.79. Found: C, 62.95; H, 4.86; N, 9.88.

This product was identical to one prepared by the hydrogen peroxide-acetic acid oxidation of N-nicotinyl-L-phenylalanine.

N-(Nicotinyl 1-Oxide)-L-phenylalanine Methyl Ester -- Esterification of the L-acid was carried out as for the DL-acid to give after recrystallization from water, 74%, m.p. 161.0-161.2°, $(\alpha)_D^{25} = -57.7°$ (C, 1.7% in methanol).

Anal. Calc. for $C_{16}H_{16}O_4N_2$ (300.3): C, 63.99; H, 5.37; N, 9.33. Found: C, 63.78; H, 5.55; N, 9.25.

N-(Nicotinyl 1-oxide)-D-phenylalanine Methyl Ester -- The acid filtrate from the resolution of the DL-mixture of the above compound was neutralized to pH 7 and saturated with salt. An oil formed, which after standing in the solution for two days, crystallized in needles. The crystals of D-ester were removed by filtration, washed with water,

and dried in vacuo to give 2.8 g. (70%), m.p. 160-161°, $(\alpha)_D^{25} = 58.2°$ (C, 2.1% in methanol).

Anal. Calc. for $C_{16}H_{16}O_4N_2$ (300.3): C, 63.99; H, 5.37; N, 9.33. Found: C, 63.82; H, 5.20; N, 9.20.

N-Nicotinyl-L-phenylalanine -- To a clear solution of the triethylammonium salt of L-phenylalanine methyl ester, prepared by adding 16.3 ml. (0.112 mole) of triethylamine to a suspension of 24.3 g. (0.112 mole) of L-phenylalanine methyl ester hydrochloride (65) in 150 ml. of dry chloroform, was added a solution (red colored) prepared from 20.0 g. (0.112 mole) of nicotinyl chloride hydrochloride and 31.2 ml. (0.224 mole) of triethylamine in 250 ml. of dry chloroform. The temperature of the mixed solutions rose to 50°C., and the red solution was allowed to stand for 1½ hours, after which it was a pale yellow. The chloroform solution was extracted with 2-150 ml. portions of water, 75 ml. of a saturated sodium bicarbonate solution, and 75 ml. of water, then dried with magnesium sulfate, and the solvent removed in vacuo. The residual oil was partially dissolved in 100 ml. of 30% methanol-water, 50 mg. of α-chymotrypsin added, and the pH of the solution maintained between pH 7-8 by the addition of 1 N sodium hydroxide. The methanol was removed by a stream of air, and the solution acidified to pH 4.5. The resulting precipitate was recrystallized from water to give 27.2 g. (90%) of acid, m.p. 176-177° dec., (lit. (66) 177-178°).

4-Pyridylcarbinol 1-Oxide -- To 23 g. of 4-pyridylcarbinol (Aldrich Chemical Co.) dissolved in 200 ml. of glacial acetic acid was added 30 ml. of 30% aqueous hydrogen peroxide and the solution held at 70° for three hours, another 30 ml. of hydrogen peroxide solution added, and the solution held at 70° overnight. The solvent was removed *in vacuo* and the residue recrystallized from ethanol-ethyl acetate to give 19.6 g. (74%) of fine needles, m.p. 111.5-112.0°.

Anal. Calc. for $C_6H_7NO_2$ (125.1): C, 57.59; H, 5.64; N, 11.20. Found: C, 57.56; H, 5.63; N, 11.08

4-Pyridylmethyl Bromide 1-Oxide Hydrobromide -- 10.0 g. of 4-pyridylcarbinol 1-oxide was dissolved in 50 ml. of 48% hydrobromic acid and the solution was heated twice to the boiling point, then allowed to stand overnight. The acid was removed *in vacuo*, 50 ml. of absolute ethanol added, and the solution cooled in ice water to yield a paste, which when recrystallized from absolute ethanol yielded 8.2 g. of white needles, m.p. 93-95°, and which analyzed approximately for the hydrobromide of 4-pyridylcarbinol 1-oxide.

The above material, 6.53 g., was dissolved in 25 ml. of hydrobromic acid and held at reflux for 18 hours. The acid was removed *in vacuo*. Addition of absolute ethanol to the residue caused the formation of a white crystalline solid, which was separated by filtration, washed with

alcohol, and dried in vacuo to yield 7.2 g. of product (88%) m.p. 170.5-171.8°, somewhat hygroscopic.

Anal. Calc. for $C_6H_6ONBr \cdot HBr$ (268.9): C, 26.79; H, 2.62; N, 5.21; Br, 59.43. Found: C, 26.88; H, 2.56; N, 5.18; Br, 59.38.

4-Pyridylmethyl Bromide 1-Oxide -- 5.0 g. of 4-pyridylmethyl bromide 1-oxide hydrobromide was dissolved in the minimum amount of water and solid sodium bicarbonate was added until the solution was at pH 7.0. The solution was saturated with salt and extracted with chloroform. The extracts were dried and the solvent removed to give the theoretical amount of solid, m.p. 138-138.5°. No analysis was obtained as the compound becomes colored and degrades after one day.

The Catalytic Hydrogenation of 4-Pyridylalanine 1-Oxide and 4-Pyridylalanine-Spectra Studies -- The reductions were carried out in the same manner for both compounds. A sample of compound was weighed out, dissolved in 25 ml. of water, the weighed catalyst, platinum dioxide (Baker and Co., Inc.), was added, and the mixture hydrogenated at 40 psig. and room temperature. At given time intervals, the hydrogenation was interrupted, a 2.5 ml. aliquot removed, filtered from catalyst, and a 1.0 ml. aliquot of the filtrate diluted 1:10 for 4-pyridylalanine, 1:100 to 1:5 for 4-pyridylalanine 1-oxide, and the U-V spectra taken at

λ = 260 mμ and λ = 250 mμ. The data and results are tabulated below.

OPTICAL DENSITY VS. CONCENTRATION

4-Pyridylalanine			4-Pyridylalanine 1-Oxide		
Conc. M X 10^{-3}	O.D.$_{260}$	O.D.$_{250}$	Conc. M X 10^{-4}	O.D.$_{260}$	O.D.$_{250}$
0.120	0.258	0.246	0.209	0.328	0.213
0.240	0.526	0.498	0.418	0.660	0.434
0.480	1.042	0.981	0.835	1.343	0.878
0.959	2.056	1.947	1.044	1.692	1.106
1.199	2.619	2.416	1.225	2.053	1.402

OPTICAL DENSITY VS. TIME

4-Pyridylalanine			4-Pyridylalanine 1-Oxide		
C_o = 1.199 X 10^{-2} M Pt = 0.265 g./l.			C_o = 1.074 X 10^{-2} M Pt = 0.265 g./l.		
Time (Min.)	O.D.$_{260}$ X 10^{-1}	O.D.$_{250}$ X 10^{-1}	Time (Min.)	O.D.$_{260}$ X 10^{-2}	O.D.$_{250}$ X 10^{-2}
0	2.619	2.416	0	1.724	1.185
5	2.323	2.147	10	0.983	0.672
10	2.167	2.010	23	0.504	0.362
21	2.010	1.838	39	0.263	0.213
46	1.767	1.648	64	0.1250	0.118
63	1.672	1.605	109	0.0730	0.0730
91	1.616	1.477	155	0.0581	0.0578
298	1.010	0.990	1130	0.0149	0.0151

Calculated calibration curves:

λ = 260 mμ	λ = 250 mμ
$\frac{O.D._{PAO}}{C_{PAO}}$ = 1.604 X 10^4 M^{-1}	$\frac{O.D._{PAO}}{C_{PAO}}$ = 1.058 X 10^4 M^{-1}
$\frac{O.D._{PA}}{C_{PA}}$ = 2.183 X 10^3 M^{-1}	$\frac{O.D._{PA}}{C_{PA}}$ = 2.017 X 10^3 M^{-1}

MOLE PERCENT VS. TIME

4-Pyridylalanine		4-Pyridylalanine 1-Oxide			
Time (Min.)	Mole % PA	Time (Min.)	Mole % PAO	Mole % PA	Mole % PIA
0	100	0	100	0	0
5	89	10	51	44	5
10	82	23	22	49	29
21	76	39	6.5	63.5	30
46	68	64	1	46	53
63	65	109	0	32.5	67.5
91	61	155	0	25.5	74.5
298	40	1130	0	6.5	93.5

The data for the mole percentage of 4-pyridylalanine and 4-piperidylalanine in the reduction of 4-pyridylalanine 1-oxide have error in them estimated at $\pm$ 10 mole percent through the fifth point, and about $\pm$ 2% in the last points. This is due to the fact there is approximately a factor of 10 between the molar extinction coefficients of 4-pyridylalanine 1-oxide and 4-pyridylalanine, and small errors in the concentration of 4-pyridylalanine 1-oxide are reflected by ca. 10 times that error in the concentration of pyridylalanine. 4-Piperidylalanine has no U-V absorption in this region.

The Catalytic Hydrogenation of 4-Pyridylalanine 1-Oxide -- After the addition of 0.5 g. of platinum dioxide (Baker and

Co., Inc.) to a solution of 5.0 g. (.0275 mole) of 4-pyridylalanine 1-oxide in 25 ml. of water, the mixture was hydrogenated at 40 psig. and room temperature until 0.069 mole of hydrogen had been absorbed (as measured by pressure-drop). The catalyst was removed and the solution evaporated to dryness in vacuo. The residue was extracted with 25 ml. of dry methanol, the residuum (2.82 g.) was dried in vacuo. Only an oily solid was isolated from the methanol extracts (4-piperidylalanine is very hygroscopic). A determination of the U-V spectra at λ = 256 mμ gave a molecular weight for the solid (based on no 4-pyridylalanine 1-oxide, which is reasonable, for the time required for the hydrogen uptake was 4 hrs.) of 311 ± 6 and after another extraction, a value for the molecular weight of 380 ± 10. Theory for 4-piperidiniumalanine 4-pyridylalanate is 338. The compound gave no sharp melting point, but decomposed in the range 250-280°. From the previous study, it would be predicted that the yield of this salt should be ca. 2.5 g. for the time involved. It is doubtful if the pressure-drop reading was accurate to better than .0275 mole. Reaction of the compound with ninhydrin gave a blue-purple color.

Thioisonicotinyl Morpholine -- A suspension of 9.6 g. (0.3 g. atom) of sulfur in a mixture of 10.9 g. (0.1 mole) of 4-picoline 1-oxide and 13.1 g. (0.15 mole) of morpholine was heated at 170° for 12 hours. The reaction mixture was

cooled, diluted with 50 ml. of absolute ethanol, the precipitated product filtered off, and recrystallized twice from ethanol to give 11.4 g. of product (55%) m.p. 150-152° (lit. (67) 150-151°).

Anal. Calc. for $C_{10}H_{12}N_2OS$ (208.3): C, 57.67; H, 5.81; N, 13.45. Found: C, 57.61; H, 5.82; N, 13.39.

Methyl Isonicotinate 1-Oxide -- This ester was prepared by the hydrogen peroxide oxidation of methyl isonicotinate (Reilly Tar and Chemical Corp.) according to the method of Ochiai (20). The product was recrystallized from ligroin (60-70°)-chloroform to give 67% of white needles, m.p. 121.0-121.5°.

Anal. Calc. for $C_7H_7O_3N$ (153.1): C, 54.90; H, 4.61. Found: C, 54.94; H, 4.58.

Isonicotinyl Hydrazide 1-Oxide -- This was prepared by treatment of the above ester with a two-fold excess of hydrazine hydrate in methanol while warm to give a 90% yield, m.p. 229° dec. (lit., 227° dec. (68), 233-234°(69)).

Cyanomethyl Nicotinate 1-Oxide -- Nicotinic acid 1-oxide was prepared by the method of Ochiai (20) to give 75% of product, m.p. 250-251° (lit., 249° (70)).

To a suspension of 10 g. (.072 mole) of the above acid in 100 ml. of dry ethyl acetate was added 15 ml. (.108 mole) of triethylamine and 8.16 g. (.108 mole) of

chloroacetonitrile. The gummy mixture from the addition of triethylamine dissolved upon the addition of the chloroacetonitrile. The resultant clear yellow solution was held at reflux for 1½ hours with stirring in order to break up the precipitate which formed. The suspension was cooled in an ice bath while stirring, then filtered, washing the residue with 10 ml. of ethyl acetate. The residue was air dried a few minutes, then suspended in 85 ml. of cold absolute methanol (to remove triethylamine hydrochloride), stirred vigorously for two minutes, filtered and washed with 25 ml. of cold methanol, and dried *in vacuo*. The product was recrystallized from ethyl acetate to give 10.0 g. (78%), m.p. 160-161°.

Anal. Calc. for $C_8H_6O_3N_2$ (178.1): C, 53.93; H, 3.40; N, 15.73. Found: C, 53.66, 53.87; H, 3.60, 3.40; N, 15.81, 15.73.

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PART II

PRELIMINARY INVESTIGATIONS OF ALPHA-CHYMOTRYPSIN CATALYZED HYDROLYSES OF ACYLATED AMINO ACID ESTERS AT LOW ENZYME CONCENTRATIONS -- STUDIES OF SURFACE EFFECTS

A. INTRODUCTION

Since the demonstration by Bergmann and coworkers that synthetic peptides can take the place of more complex proteins as substrates for proteolytic enzymes, and the subsequent successful formulation of the kinetics of these proteolytic reactions in terms of an intermediate enzyme-substrate complex suggested by Michaelis and Menton, as in equation 1, a considerable number of kinetic investigations

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P_1 + P_2 \qquad (1)^*$$

have been carried out with the aim of understanding the mode of actions of these enzymes. In the case of alpha-chymotrypsin (to which this discussion will be limited), it has been shown that amides (1), hydroxamides (2), hydrazides (3), and esters (4) can be used as substrates for this enzyme. As these kinetic investigations became more quantitative in nature, many effects that influenced the rates of the enzymatic reactions, other than the obvious one of enzyme and substrate concentration, became apparent.

It has been shown that alpha-chymotrypsin will dimerize in rather concentrated solutions, but at concentrations of about 10^{-5} M and in the pH range 7-8, this dimerization is

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*The terminology and symbols used are those of ref. 17.

of no kinetic consequence (5-8). When buffers are present in the reaction mixture, the rates of the reactions are frequently altered, but this effect is not well understood (9,10). The addition of mono- or divalent ions, or such non-polar substances as sucrose to the reaction mixture increases the rate of the reaction (11,12). On the other hand, the rates of reactions are lowered by the addition of alcohols, as in the case of a mixed solvent of alcohol-water (13,14).

The product which is generated during the enzymatic hydrolysis will frequently combine with the enzyme as in equation 2, and essentially lower the effective enzyme

$$E + P \rightleftharpoons EP \qquad (2)$$

concentration available for enzyme-substrate complex formation (15). In addition, kinetics should be determined on the L-optical isomer of the substrate, for, while the D-antipode is usually not a substrate, it is generally a strong inhibitor of the reaction, as in equation 3. The re-

$$E + I \rightleftharpoons EI \qquad (3)$$

producibility of various alpha-chymotrypsin preparations has been shown kinetically (16) and the stability of aqueous solutions of the enzyme with time, as measured by kinetics, has been shown to be excellent over at least a 24 hour period (17), in sharp contrast to trypsin solutions (18). The velocity of the enzymatic reaction is very markedly tempera-

ture and pH dependent.

Considering the usual Michaelis-Menton formulation of these enzymatic hydrolyses (equation 1), it has been shown that the velocity of the reaction can be given by equation 4, based on the assumptions $d(ES)/dt \doteq 0$, $(S) \doteq (S_f) \gg (ES)$ (zone A assumptions (18,19)), where $K_s = (k_2 + k_3)/k_1$.

$$v = -\frac{d(S)}{dt} = \frac{k_3(E)(S)}{K_S + (S)} \qquad (4)$$

For the evaluation of the kinetic constants k_3 and K_S, a Lineweaver-Burk plot (20) of $1/v_o$ vs. $1/(S)_o$, or a modification thereof, is generally used, based on a rearranged form of equation 4 at zero time, as in equation 5. Due to the

$$\frac{1}{v_o} = \frac{K_S}{k_3(E)} \cdot \frac{1}{(S)_o} \qquad \frac{1}{k_3(E)} \qquad (5)$$

error inherent in the calculated value of K_S, certain restrictions must be placed upon the experimental conditions used to determine K_S and k_3. For an experimental error of $\pm$ 5%, the values of $(S)_o$ one can use are limited to $20 \leqslant (S)_o/K_S \geqslant 0.05$. In order that zone A assumptions are approximated, $(E)/K_S$ must be < 0.1 for a 1% error, and < 0.6 for a 5% error.

It has been shown that the assumption that alpha-chymotrypsin catalyzed hydrolyses proceed entirely in solution is valid, and that wall effects, arising from the interaction of the reactants, especially the enzyme, are unimportant,

for an enzyme concentration of 7.5×10^{-6} M (21).*

For kinetic studies involving amides and hydrazides, enzyme concentrations of about 5×10^{-5} M give convenient rates of hydrolysis for an aliquot type of analysis, and for hydroxamides, enzyme concentrations of about 6×10^{-6} M are sufficient. However, due to the extreme susceptibility to hydrolysis catalyzed by alpha-chymotrypsin of most amino acid esters, especially those based on amino acids having an aromatic nucleus, the use of enzyme concentrations as low as 5×10^{-9} M is required to give a modest degree of hydrolysis in a reasonable length of time.

The alpha-chymotrypsin catalyzed hydrolysis of many esters has been studied qualitatively, and a few esters have been examined quantitatively. These latter, and the conditions under which they have been studied are summarized in Table 1. In several cases the solvent used was partially organic, due to the low water-solubility of the substrate ester. Values obtained for the same substrate with different buffer systems, solvents, inorganic salts present, or ionic strengths cannot be compared for the reasons outlined previously. Cases where DL-mixtures, or substrates not based on amino acids, were used are not included in the table, since the former have doubtful significance, and the

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*Based on a nitrogen content of 16% and a molecular weight of 22,000 for alpha-chymotrypsin.

latter are not to be considered here.

Before undertaking the kinetic study of alpha-chymotrypsin catalyzed hydrolyses of esters, it was decided to investigate the kinetic effect of lowering the enzyme concentration to regions where the enzyme concentration is 1/100 to 1/1000 times, and the available surface area 10 to 100 times, that where it had been shown there was no surface effect (21).

Preliminary evidence of the effect of surface on the velocity of the enzymatic reaction was observed by Applewhite (22) in the case of the alpha-chymotrypsin catalyzed hydrolysis of N-acetyl-L-phenylalanine glycolamide ester at an enzyme concentration of 2.1×10^{-8} M. The values of v_o obtained for a constant value of $(S)_o$ varied widely with little pattern. Treatment of titration cell components with "Dessicote" (23) helped to reduce this variation, but did not eliminate it. The use of an acid wash (3 N hydrochloric acid) of the cell components prior to each run allowed a reasonable degree of reproducibility, but only if the same stock enzyme solution was used for all runs. When individual enzyme stock solutions were used, the initial velocities showed wide variations.

The next section describes work designed to discover if there is a surface effect on enzyme kinetics carried out in solution under usual conditions, and if there is an effect, at what enzyme concentrations it appears.

B. THE PROCEDURE FOR THE INVESTIGATION OF SURFACE EFFECTS

When discussing the effect of surface on enzymatic reactions, a criteria for comparison between cases where there is no effect and cases where there is an effect must be established. Since these studies are kinetic in nature, it was decided that a proper criteria would be the initial velocity, v_o, of the enzymatic hydrolysis of the ester substrate studied. As can be seen from equation 4, the initial velocity v_o is linearly dependent on the enzyme concentration. However, v_o is not linearly dependent on $(S)_o$, and since it was not desired to assume any values (known or unknown) for k_3 and K_S, all comparisons of v_o's for a given substrate are based on reactions carried out at the same initial substrate concentration.

In order for a surface effect to be apparent in an enzymatic reaction, one or more of the components of the reaction mixture must interact with the surface. In the case at hand, this must be either or both the substrate and enzyme. Since there is little evidence for significant absorption of low-molecular-weight compounds on glass surfaces at concentrations of about 10^{-3} M, the possibility of substrate absorption effecting the rate of the reaction was ruled out. On the other hand, there is good evidence that enzymes are significantly absorbed on porous surfaces, even at concentrations as high as 10^{-4} M (24-26). Thus it was decided that the surface effect to be looked for

would be one involving the absorption of enzyme on surfaces.

Another requirement for a study of this type is a base-line for comparison, i.e., an enzymatic reaction or region of enzyme concentration in which it is known there is no surface effect. A tentative standard adopted for these studies was the region of enzyme concentration 4×10^{-6} M or above, based on work done by Bernhard (21).

The method used for following the enzymatic hydrolyses of esters was by titration of the liberated acid at a constant pH, using a recording titrimeter of the type described by Neilands and Cannon (27). The essentials of operation have been described by Applewhite (22). No buffers were used in the reaction mixture.

For the ideal case, a study of this type would involve the measurement of the initial velocities of hydrolysis of a given substrate at varying enzyme concentrations and constant substrate concentration. Under these conditions the ratio $v_o/(E)$ should be a constant if there is no surface effect. If one is willing to limit the enzyme concentrations used to a range of 1-10 units, this is feasible, but the range to be investigated here was 1-1000 units, and no single substrate is useful over this range of enzyme concentrations. There are two reasons for this limitation. One, the rate of non-enzymatic hydrolysis, remaining constant at a constant substrate concentration and pH, becomes more significant as the rate of the enzymatic hydrolysis decreases, and two, a lower base concentration is required to accurately

follow a slower reaction. When the base concentration is below 10^{-3} M, and the volume of the sample being titrated is 10 ml., the volume of base required to affect a pH change in the sample solution to which the pH controlling mechanism would be sensitive becomes large, and the liberated acid is constantly over- or under-titrated. Thus it was necessary to use a series of substrates having a progressively lower rate of enzymatic hydrolysis for a given enzyme concentration.

As a guide for the selection of suitable substrates, the values of k_3 and K_S reported in Table 1 were considered. The range of enzyme concentration to be studied was established at 10^{-6} to 10^{-9} M. For enzyme concentration in the range of 4×10^{-5} M methyl hippurate would work well, and could also be used for enzyme concentrations in the range of 4×10^{-6} M. However, at (E) = 4×10^{-7} M, the non-enzymatic hydrolysis is about 50% of the total hydrolysis measured. Although N-acetyl-L-tyrosine ethyl ester would presumably be a useful substrate for the region 10^{-7} M, it was found that N-acetyl-L-tyrosine methyl ester required an enzyme concentration of only 4×10^{-8} M and the rate of hydrolysis of this latter ester could also be followed at enzyme concentrations of about 10^{-9} M. It was found that N-benzoyl-L-4-pyridylalanine 1-oxide ethyl ester had a reasonable rate of hydrolysis at 4×10^{-7} M enzyme, and the rate could also be followed at 4×10^{-8} M enzyme.

This substrate was selected in preference to one such as N-benzoyl-L-phenylalanine methyl ester, which would presumably be useful in this range, for the constants obtained for this latter substrate were for alcohol solutions, and k_3 would be expected to be higher in an entirely aqueous media (13). No substrate appeared to be available in the literature for measurements in the enzyme concentration range 10^{-6} to 10^{-7} M. It was observed, however, that the hydrolysis of N-acetyl-DL-4-pyridylalanine 1-oxide methyl ester had a rate which was reasonable in this range of enzyme concentration. The substrates selected for use and their useful range are listed below.

Substrate	(E) range
N-Acetyl-DL-4-pyridylalanine 1-oxide methyl ester	10^{-6} - 10^{-7} M
N-Benzoyl-L-4-pyridylalanine 1-oxide ethyl ester	10^{-7} - 10^{-8} M
N-Acetyl-L-tyrosine methyl ester	10^{-8} - 10^{-9} M

The usual procedure for the preparation of solutions having low enzyme concentrations is to prepare a rather concentrated solution (ca. 10^{-5} M), determine the enzyme concentration spectrophotometrically and then perform a serial dilution to the desired concentration. This procedure has the disadvantage of exposing the enzyme to a number of extensive surfaces, and if the enzyme is adsorbed on these surfaces, some loss will be concomitant with transfer. In

order to minimize this effect, dilute enzyme solutions were prepared by dissolving a known weight of dry enzyme in enough water to give ten times the concentration required for the enzymatic reaction. An aliquot of this solution was then diluted ten-fold by addition to the substrate solution. In extreme cases this required the solution of 10 mg. of enzyme in 1 l. of water (for 4×10^{-8} M). For the case of (E) = 10^{-9} M, a 10:1 dilution of 10^{-8} M solution was carried out before addition to the substrate solution.

It was not found possible to accurately weigh 10.0 mg. of alpha-chymotrypsin as a powder. This was due to the hygroscopicity of the dry enzyme, which would adsorb water at the rate of about 1 percent/min. It was not possible to weigh directly into the flask containing water, for the weight of the volumetric flask prohibited this. The procedure adopted was to place dry enzyme in a capped weighing bottle, determining the weight, removing one sample, approximately 10 mg., and determining the loss in weight of the weighing bottle. This procedure gave a minimum exposure of the material being weighed to moist air, but also led to the use of different enzyme concentrations for each set of runs. It should be mentioned that the enzyme concentrations referred to in this work are apparent enzyme concentrations, based entirely on a weighed sample of enzyme in a given amount of water, in contrast to effective enzyme concentrations, which will be defined as the kinetic activity of an apparent enzyme concentration.

No special treatment was given to any of the surfaces involved, all glassware being washed in detergent, rinsed with distilled water and air dried between runs. To minimize the effect of residual enzyme on the electrodes in the titration cell, the electrodes were washed with stirring for one minute with 1 N hydrochloric acid, to deactivate the residual enzyme, rinsed with water, and dried between runs. In one case where the electrodes had been previously treated with a 10^{-5} M enzyme solution, washed with water for one minute, rinsed with water, and dried, the residual activity on the electrodes was equivalent to a 10^{-9} M enzyme solution (see Table 6, runs no. 3-1-10 and 4-1-10).

The method used to detect a surface effect was the following. The initial velocity v_o for the alpha-chymotrypsin catalyzed hydrolysis of N-acetyl-DL-4-pyridylalanine 1-oxide methyl ester was determined at a given value of $(S)_o$ and with $(E) \sim 4 \times 10^{-6}$ M. It was assumed there was no loss of enzyme during transfers, or due to wall reactions in the reaction vessel. Then the initial velocity v_o' for the enzyme catalyzed hydrolysis of this same substrate at $(S)_o$, but with $(E') \sim 4 \times 10^{-7}$ M, was determined. Theoretically, for no loss in activity in either case, $v_o/(E) = v_o'/(E')$, where $v_o/(E)$ will be called the initial "specific" velocity. If $v_o'/(E') < v_o/(E)$, then a loss of activity has occurred, and it is possible to calculate the percentage loss P. Then, the enzyme catalyzed hydrolysis

of N-benzoyl-L-4-pyridylalanine 1-oxide ethyl ester was studied at a given value of $(S')_o$ at enzyme concentrations $(E'') \sim 4 \times 10^{-7}$ M and $(E'') \sim 4 \times 10^{-8}$ M, and the respective initial specific velocities $v_o''/(E'')$ and $v_o'''/(E''')$ calculated. The percentage loss of activity over this range will be P'. Assuming the effective initial specific velocity $v_o''/(E'')$ is already P percent lower than the apparent specific velocity, the total loss of activity over the range E - E''' will be P X P' percent. This same type of reasoning was applied to the enzymatic hydrolysis of N-acetyl-L-tyrosine methyl ester at enzyme concentrations of about 4×10^{-8} M and 4×10^{-9} M and at a given value of $(S'')_o$. The results of this procedure are discussed in the next section.

C. THE EFFECT OF SURFACE

After obtaining the primary data (a plot of $(S_o - S_t)$ vs. time), a method for evaluating initial velocities had to be chosen. For this purpose, the method recently developed by Booman and Niemann (28), based on orthogonal polynomials, was used. This method has the advantage of an entirely mathematical treatment, and involves no assumptions pertaining to the kinetics.

Preliminary investigations of the alpha-chymotrypsin catalyzed hydrolysis of N-benzoyl-L-4-pyridylalanine 1-oxide methyl ester indicated that there was an impurity present in this substrate, which was hydrolyzed much faster than the

main body of the material. This substrate was recrystallized two more times, and analyzed each time correctly, and had the same optical rotation each time. The material was paper chromatographed in butanol-acetic acid-water, and gave only one spot when the paper was developed according to a ferric hydroxamate test (29). It was suspected that perhaps the fast component was the ethyl ester instead of the methyl ester, but the ethyl ester showed the same behavior. It was found that N-acetyl-DL-4-pyridylalanine 1-oxide methyl ester had this same behavior. The only obvious possibilities for these impurities are the analogous compounds without the N-oxide group. These compounds would be expected to be hydrolyzed faster by alpha-chymotrypsin, since the affinity of the catalytically active site for the phenyl group (30) is greater than that for the more hydrated pyridine group (39), and a pyridine 1-oxide group would be expected to be more heavily hydrated than a pyridine group. This impurity was present to the extent of 1-3%.

Since the impurities were present to such a small extent, and were hydrolyzed nearly completely in the first few minutes, the effect of their hydrolysis on the total hydrolysis curves was corrected for in the following manner. The quantity $(S_o - S_t) - (S_o - S_t)$ was plotted vs. time, where t_1 and t_2 were at one minute intervals. These curves showed a marked initial curvature, followed by a straight line after 2-3 minutes. The straight line portion of the curve was used to extrapolate back to the points

for the first few minutes due to the substrate hydrolysis. This corrected curve was used to calculate v_o values.

In the case of the alpha-chymotrypsin catalyzed hydrolysis of N-acetyl-L tyrosine methyl ester, other complications developed. Although the extent of hydrolysis measured was high, and some curvature of the $(S_o - S_t)$ vs. time plot was expected, the actual curvature found was extreme. There was no evidence of impurities here. The curvature was so marked that a fifth order polynomial would frequently not give a good fit to the curve. It was suspected that this curvature was due to enzyme being removed from solution by adsorption on the walls of the reaction vessel in a time dependent way. Some evidence for this is given by the work done with "Ludox" in the reaction mixture (see Section D). Initial velocities were determined from the $(S_o - S_t)$ vs. time plots for this substrate by determining the slope of the tangent to the curve at zero time, an admittedly poor procedure, but in this case, about as good a one as the orthogonal polynomial method.

One factor that could make the values of $v_o/(E)$ obtained for a given substrate at two different enzyme concentrations, but the same substrate concentration, noncomparable is the extent of hydrolysis. This applies, however, only when the product is a good inhibitor for the enzymatic reaction. The rate expression for the case of an enzymatic hydrolysis where the product which is being generated is an inhibitor is given in equation 6. The

$$v_o = -\frac{d(S)}{dt} = \frac{k_3(E)(S)}{K_S(1 + \frac{(P)}{K_P}) + (S)} \qquad (6)$$

assumption that $(P) \doteq (P_f) \gg (EP)$ is required in addition to the ones discussed in Section A, and $K_P = k_5/k_4$ in equation 2. It can be seen that in the case of an inhibitor

$$E + P \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EP \qquad (2)$$

whose K_P is approximately 0.100 M, an inhibitor concentration of approximately 0.005 M would be required at the minimum (or $(P)/K_P$ 0.05) for a 5% error in order for the inhibitor to have a measurable effect on the kinetics. For the cases at hand, $(S)_o$ values were all in the range of 1×10^{-3}, and thus even 100% hydrolysis should show no effect of product inhibition, if the products have K_P's of about 0.10 M. N-Acetyl-L-tyrosinate ion has been shown to have a K_P of about .080 M (15), and in an attempted evaluation of K_I value (in this case identical to the K_P in question) of the N-benzoyl-L-4-pyridylalaninate ion against N-benzoyl-L-valine methyl ester, at $(I) \sim 10^{-3}$ M, no inhibition was observed. Since N-acetyl-amino acids have as a rule higher values of K_I (or K_P) than their analogs acylated with groups having aromatic properties (15), it would not be expected that N-acetyl-L-4-pyridylalaninate ion would be a good inhibitor either in concentrations of

about 10^{-3} M. Thus it can be concluded that for these studies, product inhibition is of no consequence.

The results of kinetic studies of ester hydrolyses at enzyme concentrations ranging from 4.7×10^{-6} M to 4.5×10^{-9} M are summarized in Tables 4-6, and in condensed form, in Tables 7-9. There is an approximate drop in effective enzyme concentration of 15-25% in going from 4.7×10^{-6} M to about 4.7×10^{-7} M apparent enzyme concentration; there is an approximate 50-60% drop in effective enzyme concentration in going from 4.7×10^{-7} M to 6.5×10^{-8} M apparent enzyme concentration; and there is an approximate 80-90% drop in effective enzyme concentration in going from 6.5×10^{-8} M to 4.7×10^{-9} M apparent enzyme concentration. This leads to the values summarized below in Table 1.

Table 1. -- Loss of Enzymatic Activity

Apparent (E)	% of Theoretical Effective (E)	
	Max.	Min.
4.7×10^{-6} M	100*	100*
4.7×10^{-7} M	85	75
6.5×10^{-8} M	43	30
4.7×10^{-9} M	9	3

*Assumed.

It thus appears there is a surface effect. The word "appears" is used, since an effect of this type could also be attributed to a non-linear dependence of v_o on (E). The only investigation in the literature that reports any consideration of the effect of varying enzyme concentration involved work done at 2-10.5 X 10^{-7} M* enzyme (14), and the authors apparently observed no effect in this region, although all that was reported was that enzyme concentration regions were overlapped. The question of a non-linear dependence of v_o on (E) will be deferred until later, and for the present the surface will be held responsible for the decreased activity. In order to determine whether interactions with the surface of the reaction vessel was the cause of the lowered activity, a suspension of colloidal silica, having a surface area of 350 $m.^2/g.$ of silica, was added to the reaction mixture. In this case, the surface area of the colloidal silica was approximately 3000 times that of the reaction vessel. There was no change in the value of v_o compared to the case where there was no silica, within experimental error. Thus it appeared that the surface of consequence was that contacted by the enzyme prior to the reaction vessel.

To provide a basis for comparison, the "specific surface areas" were calculated for each set of runs. Specific

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*Calculated from 7.45-37.5 X 10^{-3} mg. P-N/ml., assuming 16% P-N and a molecular weight of 22,000 for the enzyme.

surface area will be defined here as the surface area/ml. contacted by the enzyme solution, or A/(E). In order to illustrate the difference in the effect of surface contacted by the enzyme prior to the reaction mixture (A_p at $(E)_p$) and the surface associated with the reaction vessel (A_r at (E)), the specific surface areas $A_p/(E)_p$ and $A_r/(E)$ were calculated. The values are given in Tables 7-9. The specific surface area varies from 5.6×10^8 cm.^{2}/mole at 4.7×10^{-6} M enzyme up to 5.8×10^{10} cm.^{2}/mole at 4.7×10^{-9} M enzyme for the reaction vessel, and prior to dilution in the reaction vessel, 6.0×10^8 cm.^{2}/mole to 6.0×10^{12} cm.^{2}/mole for respective enzyme concentrations of 4.7×10^{-5} M and 4.7×10^{-8} M. In one case, it was possible to vary the specific surface area prior to dilution by a change in technique (compare runs 8-1-2 and 6 through 8-1-14, Table 5). It would be predicted that the values of $v_o/(E)$ obtained would have some inverse relationship to the values of $A_p/(E)_p$. At a final enzyme concentration of about 5×10^{-7} M, changing the value of $A_p/(E)_p$ from 6×10^9 cm.^{2}/mole to 2.7×10^8 cm.^{2}/mole allowed a 23% increase in the value of $v_o/(E)$ obtained, actually nearly the theoretical increase.

Although the data reported here are necessarily somewhat approximate, there is good evidence that enzyme is lost by serial dilution and transferring in these regions of concentration considered. Also, it appears that surface absorption in the reaction vessel has little effect on the rate of the reaction. It should be mentioned that

data of the type obtained here is difficult to reproduce quantitatively, since the nature of the surfaces involved can vary widely from one set of runs to another. This is evidenced by the data obtained here, despite careful control of technique in the sense of reproducing the procedure used to carry out the runs.

It is interesting to consider the effect these surface effects would have on values of k_3 and K_S. Since a plot of the type of Lineweaver and Burk (20) allows an evaluation of K_S which is independent of enzyme concentration, it would be predicted that K_S values would be reproducible despite the surface effect. On the other hand, k_3 cannot be evaluated independent of enzyme concentration, and it would be predicted that k_3 values would not be very reproducible at low enzyme concentrations.

D. THE EFFECT OF COLLOIDAL SILICA

Several methods were considered to avoid the loss of enzyme on surfaces. In the case where all of the equipment was treated so that water would not wet the surface, no improvement was observed. As mentioned previously, some change in the available specific area could be made, but this was with a loss in convenience. It was decided to investigate the effect of adding a "portable" surface to the enzyme solution. The principle here was to adsorb the enzyme on fine particles suspended in solution, and

having a total specific surface area much larger than that involved in the container and various transfer operations. The ideal material for this purpose would be inert, and of such a particle size that sedimentation would be of no consequence. Stable colloidal silica sols are commercially available with particle sizes down to 7 mμ under the trade name of "Ludox."* This material was available as a stable sol containing 17.3% silica with a surface area of 422 $m.^2/g.$ of silica, and an average particle size of 7 mμ. Further references to "Ludox" will refer to this material.

Before using "Ludox" in the enzyme solution, a standard scheme for its introduction had to be adopted. In order to minimize any time effects involved in the equilibration of the enzyme between the "Ludox" surface and the surface of the container, the enzyme was dissolved in the "Ludox" solution first, then diluted to the desired volume. In order to determine at what pH to perform this dilution, some of the properties of silica sols had to be considered. Although the literature is extensive on this subject, most of the material is descriptive only. The major problem involved with the use of silica sols is avoidance of gel formation. Holmes (32) observed that between hydrochloric acid concentrations of 0.1 N and about 6 N, gel time increased with increasing acid concentration. Also, increasing

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*From E. I. du Pont de Nemours and Co., Inc., Wilmington, Delaware.

the temperature decreased the gel time. It has been shown that there is a linear relationship between hydrogen ion concentration and gel time between pH 4 and 6, and that increased ionic strength of the sol gives a decreased gel time (33). Treadwell reported a minimum gel time between pH 5.8-6.2, with marked increases in gel time on either side, for concentrated sols (34). A high silica content in the sol reduces the gel time, and Hurd reported a linear decrease in gel time with increasing hydroxyl ion concentration (35). Hurd (35) also makes the statement that gel time vs. pH is linear down to pH 8, but cites only the work of Hurd, Raymond and Miller (33). These latter authors reported no studies above pH 6. Some evidence of polymerization of the sol was obtained when the pH of an enzyme solution containing "Ludox" was changed from 8.0 to 3. The initial velocity of a standard reaction decreased steadily with time from the value obtained with enzyme-"Ludox" solution at pH 8.0, after adjustment of the enzyme-"Ludox" solution to pH 3.0. No problems of this type were observed at pH 7.2. As a consequence, the enzyme-"Ludox" solutions were made up at pH 8.0, and used for no more than two hours. There was no apparent loss in activity in the solution during this period at pH 7.2 or pH 8.0.

The same problem of lack of reproducibility of the data existed with the runs involving "Ludox." In general, however, marked increases in rate over cases where no "Ludox" was used were observed. The results are summarized below

in Table 2. The ratios of the initial specific velocities with "Ludox," $v_o^L/(E)$, and without "Ludox," $v_o/(E)$, are presented, along with the ratios of the specific surface area of the added "Ludox," $A_p^L/(E)_p$, and the specific surface area of the prior equipment, $A_p/(E)_p$. The ranges of ratios of initial specific velocities were determined from the possibilities in the data. The ratios of initial specific velocities should be compared with the theoretical values for complete absence of a surface effect, calculated from the data in Table 1.

Table 2. -- The Effect of "Ludox"

Apparent (E)	Substrate	$\frac{v_o^L/(E)}{v_o/(E)}$		$\frac{A_p^L/(E)p}{A_p/(E)_p}$
		Observed	Theoretical	
4.7×10^{-7} M	APAOME	1.16-1.30	1.18-1.34	1550
	BPAOEE	1.48-1.70		1350
4.7×10^{-8} M	BPAOEE	2.74-2.85	2.32-3.33	320
	ATME	0.93-1.70		148-320
4.7×10^{-9} M	ATME	2.41-4.03	11.1-33.3	2.3

It can be seen that when the "Ludox" specific area is about 1500 times that of the prior specific surfaces, the values of v_o obtained approach the theoretical value fairly closely. The notable case is that of N-benzoyl-L-4-pyridylalanine 1-oxide ethyl ester. The v_o's for this compound

show a much greater enhancement with "Ludox" than do the others. In general, it appears that small changes in the amount of "Ludox" added have little effect. One striking effect of "Ludox" on the kinetics was to remove a large amount of the curvature present in the runs involving N-acetyl-L-tyrosine methyl ester. This might have been due to effectively keeping the enzyme in the reaction mixture out in the main body of the reaction mixture, rather than being gradually adsorbed on the walls.

It is interesting to consider here studies by McLaren (24-26) of the alpha-chymotrypsin catalyzed hydrolysis of heat-inactivated lysozyme with kaolinite suspended in the reaction mixture. He found that the reaction took place both in solution and on kaolinite, the reaction on kaolinite being due to the formation of an enzyme-substrate complex on the kaolinite from adsorbed substrate and adsorbed enzyme, and that in solution due to dissolved (or desorbed) enzyme and substrate. The reaction on kaolinite had a rate approximately two-thirds of the rate in solution. The adsorption of alpha-chymotrypsin on kaolinite was found to be an equilibrium process, and his adsorption isotherm was markedly dependent on both pH and the buffer present in the solution. It was found that 90% of the equilibrium amount of enzyme was adsorbed in 2-3 minutes.

It is doubtful that much of the above work is applicable here. Kaolinite particles are generally about 1 μ

across and are rather flat (36), whereas the silica particles are about 7 mμ across and are spherical. Also, adsorptive properties could be quite different, since the kaolinite surface consists of a mixture of Al-O-Al bonds and Si-O-Si bonds, whereas the silica surface consists of pure Si-O-Si bonds. Also, there is no reason to suspect that enzyme adsorbed upon a surface is more or less active than enzyme in solution. Arguments can be presented for both cases. However, the rate of adsorption is probably quite fast for alpha-chymotrypsin on silica , and is probably pH dependent, although these are points which need better definition.

It was considered of interest to calculate an approximate degree of coverage of the silica surface. Assuming the average diameter of a silica particle is 70 Å, its surface will be 1.6×10^{-12} cm.^{2} For a preparation having a surface area of 400 m.^{2}/g., there will be 2.5×10^{18} particles/g. Assuming a spherical shape for the alpha-chymotrypsin molecule, and a molecular weight of 22,000, its volume per molecule can be calculated from its density, 1.28 (37). From the volume so calculated, 1.4×10^{-20} cm.^{3}, the diameter of an enzyme particle is 30 Å, and its cross section is 2.8×10^{-13} cm.^{2} Assuming the spherical enzyme molecules cover the surface of the spherical silica particle, it can be calculated that about 7-8 enzyme molecules will cover a silica particle. Considering a case where 1.38 g. of silica (corresponding to 8 ml. of "Ludox") is used for 10 mg. of enzyme, there are 3.4×10^{18} silica

particles and 2.7 X 10^{17} enzyme molecules. Thus, only one silica particle in 10 has an enzyme molecule attached, on the basis of this calculation.

Although it has been tacitly assumed that the enzyme was adsorbed on the silica particles, no evidence has been presented to substantiate this assumption. In order to show that enzyme is adsorbed on the silica surface, sedimentation studies were made on an enzyme solution containing "Ludox," on an enzyme solution alone, and on "Ludox" alone. The sedimentation constant for alpha-chymotrypsin has been estimated as 2.5 Svedberg units (38), and its diffusion constant D^{20° = 7.1 X 10^{-7} $cm.^2/sec.$ (1). Using the value of the diffusion constant, and assuming the particle meets the same resistance to diffusion and sedimentation, and a density of 1.28 (37), the sedimentation constant for the enzyme calculates (39) to be 1.8 Svedberg units. Assuming a diameter of 70 Å and a density of 2.3 for the silica, the sedimentation constant for the silica particles calculates to be 40 Svedberg units. Thus, it appears that enzyme and silica could be separated in an ultracentrifuge.

The enzyme-"Ludox" solution was prepared at approximately 4.5 X 10^{-5} M in enzyme and containing 1.38 g. of silica/1., then centrifuged for 2½ hours at 100,000 times gravity. Comparison enzyme solution and "Ludox" solution were also run. The enzyme concentration was measured spectrophotometrically on samples not centrifuged, and on the top 40% of the solution and the bottom 60% of the solution after

centrifuging. There was a 7.5% loss of enzyme in the top of the sample without "Ludox," and an 84% loss in the top of the enzyme-"Ludox" sample. The "Ludox" was visibly evident in the bottom of the centrifuge cones. The ratio of silica particles to enzyme molecules in these studies was 1.3/1.0. Thus, it seems safe to conclude that the enzyme is adsorbed on the silica.

Finally, the question must be considered of whether these results are compatible with a non-linear dependence of v_o with (E), instead of being a surface effect. Certainly there is a considerable amount of evidence in the literature that alpha-chymotrypsin catalyzed hydrolyses have a linear dependence of v_o on (E) over small ranges of enzyme concentration, although it must be admitted that in the case of esters, as given in Table 3, the evidence is not very impressive. However, it is felt that the effect of "Ludox," on increasing the effective enzyme concentration, is fairly indicative that the real problem at low enzyme concentrations is loss of enzyme by adsorption on surfaces prior to addition to the reaction mixture. Also, work by Booman (8) showed a linear dependence of v_o on (E) in the range of enzyme concentrations 9×10^{-4} M to 9×10^{-6} M.

In conclusion, it can be stated that there is good evidence that the kinetics of the alpha-chymotrypsin catalyzed hydrolysis of substrates are not effected by surface. This fact implies that for the case of enzyme adsorbed on silica, there is a negligible loss of activity. When the

mechanics of handling the enzyme solution prior to addition to the substrate solution allow contact with immobile surfaces having a total surface to enzyme ratio of greater than 6×10^8 cm.^{2}/mole, losses of enzyme will occur. Such losses occurred in this work at an enzyme concentration of about 4.6×10^{-6} M. The effect of adding a carrier for the enzyme in the form of colloidal silica has helped avoid these losses, but a considerable amount of study will be required to determine the optimum conditions for its use. It has been shown that the enzyme is adsorbed on colloidal silica.

E. THE RATE OF THE ALPHA-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF N-BENZOYL-L-4-PYRIDYLALANINE 1-OXIDE METHYL ESTER AND N-(NICOTINYL 1-OXIDE)-L-PHENYLALANINE METHYL ESTER

Although a suitable procedure for carrying out kinetic studies on acylated amino acid esters at low enzyme concentrations had not been established, it was decided to at least compare the rates of hydrolysis of N-benzoyl-L-4-pyridylalanine 1-oxide methyl ester (BPAOME) and N-(nicotinyl 1-oxide)-L-phenylalanine methyl ester (NOPAME). The reasons for interest in these compounds as substrates has been discussed in Part I, Section A. Since the values of K_S and k_3 were not known for these compounds, and it did not seem justified to attempt to determine these constants under the conditions available, some procedure was required that would yield a reasonable basis for comparison of these substrates.

Two procedures were available. In one case, by using an initial substrate concentration that is very high, on the order of 0.1 M, and assuming $(S)_o \gg K_S$, v_o approximates $k_3(E)$ (see Section A). While substrate concentrations of this order were obtainable with BPAOME they were not obtainable with NOPAME. On the other hand, by making $(S)_o \ll K_S$, v_o approximates $k_3(E)(S)_o/K_S$. Although it cannot be said with certainty that when $(S)_o = 1 \times 10^{-4}$, $(S)_o \ll K_S$, it is probably a fair approximation within 20% for the cases in consideration.

The kinetics of the enzyme catalyzed hydrolysis of the two substrates were studied where $(S)_o$ was about 1×10^{-4} M. In order to avoid the effect of loss of enzyme to prior surfaces on the comparison of rates, the same enzyme solution was used for both substrates, and at a concentration of 6.6×10^{-8} M. This concentration was the minimum at which the rate of hydrolysis of BPAOME could be followed, and the maximum at which the rate of hydrolysis of NOPAME could be followed. From the known values of (E) apparent and $(S)_o$, and the obtained values of v_o, the respective values of k_3/K_S were calculated. For BPAOME, $k_3/K_S = 9.9$, and for NOPAME, $k_3/K_S = 2100$, and the ratio for NOPAME/BPAOME = 200. Thus, it appears that for the case where R in $R\text{-}CH_2CH(NHCOR')COOCH_3$ is 4-pyridine 1-oxide, a much less labile substrate is produced than for the case where R is phenyl. This is probably due to lesser interaction between the enzyme and the 4-pyridine 1-oxide group.

F. EXPERIMENTAL

Standard Sodium Hydroxide Solutions -- These were prepared as described by Swift (40) and stored in polyethylene bottles. They were individually standardized against Merck "Primary Standard" potassium acid phthalate.

Substrate Solutions -- The required amounts of substrate necessary to provide suitable concentrations in the individual stock solutions were accurately weighed into G. S. Pyrex volumetric flasks. About 5 minutes before the start of a run, the substrate sample was dissolved in nearly the total volume of water, with warming if necessary. N-Benzoyl-L-4-pyridylalanine 1-oxide ethyl ester dissolved at 40° in 2 min., and N-acetyl-L-tyrosine methyl ester would dissolve in about 2 min. at 90°. Rapid cooling of the latter produced a supersaturated solution stable for at least one hour. After solution was complete, the flask was thermostated at 25 ± 0.1° for three minutes, the volume adjusted to the mark, and inverted gently ca. 10 times. An individual stock solution was used for three runs. It was determined by titration that the change in initial substrate concentration due to warming to obtain solution and standing for approximately one-half hour was less than 2%.

Enzyme Solutions -- The enzyme used was Armour salt-free alpha-chymotrypsin, Lot 3283, N (found) 14.52%, and Armour salt-free alpha-chymotrypsin, no Lot #, N (found) 14.54%.

The enzyme was weighed in the following manner: ca. 20 mg. of enzyme was spilled from the bottle into a weighing bottle which was capped immediately. The weight (tare + sample) was determined to the nearest 0.1 mg. The one sample was removed, ca. 10 mg., and placed in a suitable size G. S. Pyrex volumetric flask. The weight of the weighing bottle was again determined (tare). The sample weight was in the range of 10-15 mg. For (E)$\sim 1.5 \times 10^{-2}$ mg. P-N/ml., the sample was dissolved in 10 ml. of water to give a solution of pH 4. For (E)$\sim 1.5 \times 10^{-3}$ mg. P-N/ml., the sample was dissolved in 100 ml. of water to give a solution of pH 5. For (E)$\sim 1.5 \times 10^{-4}$ mg. P-N/ml., the sample was dissolved in 1 l. of water and 0.15 ml. of 1 N hydrochloric acid added to give a pH of 3. For (E)$\sim 1.5 \times 10^{-5}$ mg. P-N/ml., 5 ml. of the 1.5×10^{-4} mg. P-N/ml. solution was added to a 50 ml. G. S. volumetric flask and diluted to the mark to give a solution of pH 4. For the cases where the enzyme solution was prepared at pH 8, the enzyme was dissolved in the required amount of water containing sufficient base to give a final pH of 8. The solutions were thermostated before use, and were used for a maximum of six runs or three hours, whichever came first.

Colloidal Silica -- The material used was a dry powder, labeled "Highly porous Silica powder," designation 2249-27 A, surface area of 350 $m.^2/g.$, 96% SiO_2, 4% H_2O, kindly furnished by R. K. Iler, Grasselli Chemicals Department, E. I. du Pont

de Nemours and Co., Inc., Wilmington, Del. A suspension of this material in water, 0.221 g./10 ml. was used in runs number 6 and 9-1-2, Table 4. It was necessary to adjust this silica solution to pH 8.0 (from ca. 6) before use.

Enzyme-"Ludox" Solutions -- The "Ludox" used was "Special Ludox SM Colloidal Silica," a sol containing 17.3% SiO_2, SiO_2:Na_2O = 78:8, a surface area of 422 $m.^2/g.$ of silica, designation 2692-74, kindly furnished by R. K. Iler of du Pont. This solution, which had a slight turbidity, was at pH 9.4 when received. The general procedure used to prepare enzyme-"Ludox" solutions was as follows: the "Ludox" was adjusted to pH 8.0 by the addition of 1 N hydrochloric acid. Then a given volume of this solution 8, 5 or 2 ml., was added to the dry enzyme in the proper size volumetric flask (see above), and the solution diluted to the proper volume. The pH of the resultant solution was ca. 9. The enzyme-"Ludox" solution was adjusted to the desired pH by the addition of 1 N hydrochloric acid (generally 1-5 drops). This further dilution introduced a maximum 1% error in enzyme concentration. The solutions were thermostated before use, and were used for a maximum of six runs, or two hours, whichever came first. Solutions prepared at pH 7.2 and 8.0 gave consistant results over this period, while a solution at pH 3 did not.

Operation and Calibration of the pH-Stat-Titrimeter -- The instrument, its operation, and calibration, have been described by Applewhite (22). The same procedure was used, as well as the same titration cell.

Enzyme pH-Adjustment -- The same apparatus was used as described by Applewhite (22). For enzyme samples at pH 8, no adjustment was used. For enzyme samples at pH's lower than 7.9, the following procedure was used: a ca. 2.5 ml. aliquot of stock enzyme solution was pipeted from the stock solution, and drained into a 10 ml. beaker. This aliquot was adjusted to pH 7.9 by the addition of 0.1 N base for solutions containing 0.15 mg. P-N/ml., and by the addition of 0.04 N base for enzyme solutions of lower concentrations. The quantity of base required was less than 0.02 ml. The enzyme, at pH 7.9, was added to the reaction mixture at zero time by means of a weight-calibrated syringe in a two-stop holder. One ml. aliquots of enzyme were used in every case, except for runs 6 through 9-1-2, Table 4, where the enzyme aliquot was not adjusted to pH 7.9, and the aliquot was 0.1 ml. The data was corrected for the addition of an acid enzyme sample in this latter case.

Procedure for a Typical Run -- The procedure used was as described by Applewhite (22). In addition, between runs, the electrodes and stirrer in the reaction cell were washed with 1 N hydrochloric acid for one minute, rinsed with water,

and dried. Sodium chloride, 0.2 M, was added to every run to give a final sodium chloride concentration of 0.02 M. The total volume in each run was 10 ml. In cases where the enzyme did not need adjustment to pH 8, a 2.5 ml. aliquot was removed from the stock solution and placed in a 10 ml. beaker. The enzyme syringe was filled with this aliquot.

Calculation of Surface Areas -- The surface areas of the components were calculated from their physical dimensions. In the cases of the enzyme syringes, the effective areas are probably higher, since the surfaces are ground glass. The values used are given below as surface per ml. of solution contained, based on the total capacity of the equipment as used.

10 ml. volumetric flask	3.5 $cm.^2$/ml.
25 ml. volumetric flask	2.0 $cm.^2$/ml.
50 ml. volumetric flask	1.4 $cm.^2$/ml.
100 ml. volumetric flask	1.5 $cm.^2$/ml.
1000 ml. volumetric flask	0.7 $cm.^2$/ml.
0.2 ml. pipet	33 $cm.^2$/ml.
2.0 ml. pipet	15 $cm.^2$/ml.
5.0 ml. pipet	7.5 $cm.^2$/ml.
0.1 ml. syringe	10.0 $cm.^2$/ml.
1.0 ml. syringe	5.3 $cm.^2$/ml.
10 ml. beaker	3.2 $cm.^2$/ml.
reaction vessel	2.4 $cm.^2$/ml.

Substrates -- The N-benzoyl-L-4-pyridylalanine 1-oxide ethyl ester, N-acetyl-DL-4-pyridylalanine 1-oxide methyl ester, N-benzoyl-L-4-pyridylalanine 1-oxide methyl ester, and N-(nicotinyl 1-oxide)-L-phenylalanine methyl ester used were materials prepared in Part I of this thesis. N-Acetyl-L-tyrosine methyl ester was prepared by Mr. W. Brady. It

had m.p. 136-138°, $(\alpha)_D^{25^\circ} = +24.6^\circ$ (41).

Sedimentation Studies -- The effect of surface should be essentially zero here, so no loss of enzyme by transfer was anticipated. The physical data are reported in Table 10. The samples were centrifuged at 40,000 R.P.M. for $2\frac{1}{2}$ hours in a Model L Spinco Ultracentrifuge (42) with refrigeration. The top 40% was withdrawn with the aid of a syringe. The optical densities were determined in a Cary recording spectrophotometer. The O.D. values reported were corrected for water and "Ludox" where required. All solutions were at pH 8.

The K_I Value for N-Benzoyl-L-4-pyridylalanine 1-Oxide at pH 7.9 -- The substrate used was N-benzoyl-L-valine methyl ester, at $(S)_o = 2.04 \times 10^{-3}$ M. The enzyme concentration was 0.15 mg. P-N/ml. (Armour Lot #90492), the inhibitor concentration was $(I) = 0.877 \times 10^{-3}$ M, and (NaCl) = 0.02 M. The v_o calculated from the data was $v_o = 2.86 \times 10^{-5}$ M/min., and $(S)_o(E)/v_o = 10.7$ min mg. P-N/ml. For the same enzyme concentration and substrate concentration, $v_o = 2.96 \times 10^{-5}$ M/min., and $(S)_o(D)/v_o = 10.3$ min. mg. P-N/ml. Within experimental limits, there was no inhibition, and the lower limit (for a 5% error) of K_I is established as .020 M. All runs were carried out at pH 7.9 and 25°.

Table 3. -- alpha-Chymotrypsin Catalyzed Ester Hydrolyses at pH 7.8 and 25°C.

Substrate	k_3[a] $\times 10^3$	K_S $\times 10^3$ M	(E)[b]	Solvent[c]	$(S)_o$[d] $\times 10^3$ M	Buffer[e] M	Salts	Ref.
Methyl Hippurate[f]	2.2	6.5	0.21	H_2O	5-25	.02 THAM	--	9,43
	3.2	6.6	0.15	H_2O	4-24	--	0.02 F NaCl	22
L-Benzoyl-valine Methyl Ester[f]	0.55	4.2	0.15	H_2O	1-4	--	0.02 F NaCl	22
L-Acetyl-phenylalanine Ethyl Ester	2720	1.0	.0007	H_2O	0.2-1.2	0.005-0.05 M EDTA	0.1 F NaCl	44
L-Benzoyl-phenylalanine Ethyl Ester	390	5.7	0.05-.0018	30% MeOH 70% H_2O	?	0.045 M PHOS	--	45
L-Benzoyl phenylalanine Methyl Ester	550	4.6	.00115	20% MeOH 80% H_2O	1.2-4.4	0.01 M PHOS	--	46
L-Acetyl-tryptophan Ethyl Ester	740[h]	.093[h]	.00075	H_2O	.25-1.0	THAM	0.10 M $CaCl_2$	47

Table 3. -- continued

Substrate	k_3 [a] $\times 10^3$	K_S $\times 10^3$ M	(E) [b]	Solvent [c]	$(S)_o$ [d] $\times 10^3$ M	Buffer [e] M	Salts	Ref.
L-Benzoyl-tyrosine Ethyl Ester	820	3.9	.05-.001 [g]	30% MeOH 70% H_2O	5-25	.045 M PHOS	--	48
L-Acetyl-	4500	74	.05 .001 [g]	30% MeOH	8-23	.045 M PHOS	--	48
tyrosine	2150	30	.005-	70% H_2O	?	.010 M PHOS	--	49
Ethyl Ester	2460	1.0	.001 [gi]	water	?	.001 M EDTA	0.1 F NaCl	49
	2720	0.7	?	water	?	THAM	0.1 M $CaCl_2$	47

a. In units of M/min./mg. protein-nitrogen/ml. b. In units of mg. protein-nitrogen/ml. c. Percent by volume given if a mixture. d. Range of values used for the evaluation of k_3 and K_S. e. THAM = tris-(hydroxymethyl)aminomethane, EDTA = ethylene diamine tetracetic acid, PHOS = phosphate. f. Not corrected for a possible inhibition by product in determination of k_3 and K_S; values determined at pH 7.9. g. Only a range was reported. h. Extrapolated from values reported at pH 8.0. i. Value assumed from that reported in ref. 50.

Table 4. -- The alpha-Chymotrypsin Catalyzed Hydrolysis of N-Acetyl-DL-4-pyridylalanine 1-Oxide Methyl Ester at pH 7.9 and 25°.

$(S)_o = 2.407 \times 10^{-3}$ M, (NaCl) = 2.0×10^{-2} M.

Run No.[2]	8-12-13	1-1-16	2-1-16	3-1-16	2-1-14	3-1-14	4-1-14	5-1-14	12-1-28	14-1-28	16-1-28	18-1-28
(E)[b]	0	1.51	1.51	1.51	.145	.145	.145	.145	.203	.203	.158	.158
(NaOH)[c]	.1221	1.114	1.114	1.114	.1221	.1221	.1221	.1221	.1860	.1860	.4650	.4650
	S.R.[d]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]
t[f]												
0	0	0	0	0	0	0	0	0	0	0	0	0
1	2.6	38.0	37.5	38.0	28.5	28.5	27.0	26.5	23.0	23.0	9.1	9.6
2	5.3	74.0	73.0	74.0	56.5	55.5	56.0	52.5	46.0	46.0	18.2	19.2
3	8.1	108.5	107.0	108.5	83.5	82.5	80.0	78.0	69.0	69.0	27.3	28.4
4	10.9	142.0	140.5	142.0	110.0	109.0	104.0	101.5	91.5	92.0	36.4	37.4
5	13.6	173.5	171.5	173.5	136.0	136.5	126.0	125.0	114.5	114.5	46.0	47.0
6	16.4	203.5	201.0	203.5	161.5	163.5	153.5	147.5	136.5	137.0	55.1	56.7
7	19.1	233.5	230.0	233.0	187.0	190.0	178.0	171.5	159.5	160.0	64.2	66.2
8	21.6	259.5	256.0	260.5	210.0	216.5	203.5	194.5	182.5	182.5	73.8	75.8
H[g]	-	24	24	24	2.1	2.2	2.0	1.9	2.8	2.8	2.8	2.8
v_o[h]	-	4.28	4.21	4.28	.349	.340	.347	.340	.425	.430	.428	.439
$V_o/(E)$[i]	-	2.83	2.79	2.83	2.41	2.34	2.39	2.34	2.10	2.12	2.71	2.78
Notes		j	j	j	j	j	j	j	k	k	l	l

Table 4. -- continued

a. Run number-month-day (1956-57). b. Apparent enzyme concentration. In units of 10^{-2} mg. P-N/ml. c. In units of 10^{-2} M. d. S.R. = scale reading. Corrected to S.R. = 0 at t = 0. In units of 10^{-3} ml. e. As in note d. Corrected for the blank given in the first column. f. In units of minutes. g. Approximate percent hydrolysis of the L-isomer. h. Calculated by the orthogonal polynomial method. The data had been treated as described in the text. In units of 10^{-5} M/min. i. In units of 10^{-3} M ml./mg. P-N min. j. The enzyme solution was made up at pH 4.0 k. The enzyme solution was made up at pH 8.0. l. 14 mg. of enzyme was dissolved in 5 ml. of "Ludox" at pH 8, and diluted to 100 ml. for use as a stock enzyme solution after adjustment to pH 8.

Table 5. -- The alpha-Chymotrypsin Catalyzed Hydrolysis of N-Benzoyl-L-4-pyridylalanine 1-Oxide Methyl Ester at pH 7.9 and 25°.

$(S)_o = 1.051 \times 10^{-3}$ M, (NaCl) = 0.02 M.

Run No.[a]	1-1-28	2-1-28	3-1-28	6-1-14	7-1-14	8-1-14	1-1-29	2-1-29	3-1-29	9-1-14	10-1-14
(E)[b]	1.46	1.46	1.46	1.45	1.45	1.45	.209	.209	.209	.220	.220
(NaOH)[c]	1.162	1.162	1.162	1.221	1.221	1.221	.186	.186	.186	.1221	.1221
	S.R.[d]	S.R.[d]	S.R.[d]	S.R.[d]	S.R.[d]	S.R.[d]	S.R.[d]	S.R.[d]	S.R.[d]	S.R.[d]	S.R.[d]
t[e]											
0	0	0	0	0	0	0	0	0	0	0	0
1	25.0	24.5	24.5	26.5	25.0	26.6	10.0	10.0	10.0	15.5	15.5
2	49.0	48.5	49.0	51.7	49.0	52.5	20.0	20.0	20.0	30.0	30.2
3	72.5	72.0	72.5	75.8	72.1	77.7	29.5	29.5	29.5	43.8	44.2
4	95.5	95.5	95.5	98.5	94.3	102.2	39.0	39.0	39.0	56.8	57.5
5	118.0	118.0	118.5	120.0	115.6	126.0	48.5	48.5	48.0	69.0	70.0
6	140.0	140.0	140.5	140.2	136.0	149.1	57.5	57.5	57.0	80.5	81.5
7	161.5	162.0	162.0	159.2	155.4	171.5	66.5	67.0	65.5	91.2	92.2
8	182.5	183.0	184.0	177.0	173.9	193.2	75.5	76.0	74.0	101.2	102.2
H[f]	20	20	20	20	20	22	1.3	1.3	1.3	1.2	1.2
V_o[g]	2.91	2.88	2.89	3.31	3.10	3.29	.188	.189	.190	.193	.194
$V_o/(E)$[h]	1.99	1.97	1.98	2.28	2.14	2.27	.898	.908	.911	.878	.879
Notes	i	i	i	j	j	j	i	i	i	k	k

Table 5. -- continued

Run No.[a]	11-1-14	9-1-28	10-1-28	11-1-28	10-1-5	14-1-5	8-1-2	9-1-2	6-1-2	7-1-2	12-1-29
(E)[b]	.220	1.57	1.57	1.57	1.68	1.68	1.57	1.57	1.57	1.57	.155
(NaOH)[c]	.1221	1.162	1.162	1.162	1.221	1.221	1.221	1.221	1.221	1.221	.186
	S.R.[d]	S.R.[d]	S.R.[d]	S.R.[d]	S.R.[d]	S.R.[d]	S.R.[d]	S.R.[d]	S.R.[d]	S.R.[d]	S.R.[d]
t[e]											
0	0	0	0	0	0	0	0	0	0	0	0
1	15.2	46.0	44.0	44.0	43.0	46	34.6	36.8	36.5	37.1	20.5
2	29.8	89.5	85.5	86.5	84.2	90	67.6	72.7	72.0	72.6	40.5
3	43.5	131.0	125.0	127.0	123.8	132	99.2	107.7	106.4	106.5	60.5
4	56.5	170.0	161.5	166.0	161.2	172	129.2	141.9	139.6	138.6	80.0
5	68.8	207.5	196.0	203.5	197.0	210	151.7	175.3	171.7	169.1	99.5
6	80.2	242.5	228.0	239.5	231.0	246	184.8	207.9	202.7	198.0	119.0
7	91.0	275.5	258.0	273.5	263.2	280	210.4	239.8	232.7	225.3	139.0
8	101.0	306.5	285.5	306.0	293.7	312	234.4	270.8	261.7	251.0	159.0
H[f]	1.2	34	32	34	33	34	27	32	30	29	2.8
V_o[g]	.191	5.46	5.26	5.21	5.37	5.74	4.31	4.54	4.54	4.64	.386
$V_o/(E)$[h]	.865	3.48	3.36	3.32	3.20	3.41	2.75	2.90	2.90	2.98	2.49
Notes	k	l	l	l	m	m	n	no	nop	np	q

Table 5. -- continued

a. Run number-month-day (1957). b. Apparent enzyme concentration. In units of 10^{-3} mg. P-N/ml. c. In units of 10^{-2} M. d. There is no blank correction. S.R. = scale reading. Corrected to S.R. = 0 at t = 0. In units of 10^{-3} ml. e. In units of minutes. f. Approximate percent hydrolysis. g. Calculated by the orthogonal polynomial method. The data had been treated as described in the text. In units of 10^{-5} M/min. h. In units of 10^{-2} M ml./mg. P-N min. i. The enzyme solution was made up at pH 8.0 j. The enzyme solution was made up at pH 5.0. k. The enzyme solution was made up at pH 3.5. l. 10.8 mg. of enzyme was dissolved in 5 ml. of "Ludox" at pH 8, and diluted to 100 ml. for use as a stock enzyme solution after adjustment to pH 8. m. 11.6 mg. of enzyme was dissolved in 2 ml. of "Ludox" at pH 8, and diluted to 100 ml. for use as a stock enzyme solution after adjustment to pH 8. n. A 0.1 ml. syringe was used for introduction of the enzyme solution, was at pH 4. o. The reaction solution contained 0.0221 g./10 ml. of "highly porous" silica powder (see text), added before the enzyme. p. $(S)_o = 1.070 \times 10^{-3}$ M. q. 10.7 mg. of enzyme was dissolved in 8 ml. of "Ludox" at pH 8, and diluted to 1 l. for use as a stock enzyme solution after adjustment to pH 8.

Table 6. -- The alpha-Chymotrypsin Catalyzed Hydrolysis of N-Acetyl-L-tyrosine Methyl Ester at pH 7.9 and 25° $(S)_o = 1.581 \times 10^{-3}$ M, (NaCl) = 2.0×10^{-2} M.

Run No.[a]	1-12-4	13-1-14	14-1-14	15-1-14	1-1-3	2-1-3	3-1-3	4-1-29	5-1-29	6-1-29
(E)[b]	0	2.20	2.20	2.20	1.90	1.90	1.90	2.09	2.09	2.09
(NaOH)[c]	.4513	2.972	2.972	2.972	4.340	4.340	4.340	4.650	4.650	4.560
	S.R.[d]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]
t[f]										
0	0	0	0	0	0	0	0	0	0	0
1	1.5	67.0	66.0	79.0	36.0	37.5	36.0	50.0	48.5	49.5
2	3.0	108.5	106.0	127.0	60.5	62.0	58.5	84.0	81.5	80.5
3	4.5	141.0	137.0	164.0	80.5	81.5	75.5	111.0	106.5	104.5
4	6.0	166.0	161.0	194.0	97.5	98.0	89.5	132.0	128.0	123.0
5	7.5	187.0	181.0	219.0	111.5	111.5	102.0	150.5	146.0	139.0
6	9.0	205.0	198.5	239.5	124.0	123.0	112.5	166.5	161.5	153.5
7	10.5	221.0	214.0	259.5	136.5	134.0	122.5	181.0	175.5	165.0
8	12.0	235.5	228.0	277.0	148.0	144.0	131.0	194.0	188.5	176.5
H[g]	--	45	43	52	41	40	36	57	56	52
V_o[h]	--	1.99	1.96	2.23	1.69	1.50	1.50	2.74	2.55	2.60
$V_o/(E)$[i]	--	9.05	8.90	10.15	8.90	7.90	7.90	13.10	12.20	12.42
Notes		j	j	j	j	j	j	k	k	k

Table 6. -- continued

Run No.[a]	4-1-3	5-1-3	6-1-3	7-1-29	8-1-29	9-1-29	17-1-29		1-1-5	2-1-5	3-1-5
(E)[b]	2.15	2.15	2.15	1.55	1.55	1.55	1.47		2.18	2.18	2.18
(NaOH)[c]	4.340	4.340	4.340	4.650	4.650	4.650	4.650		4.340	4.340	4.340
	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]		S.R.[e]	S.R.[e]	S.R.[e]
t[f]								t[f]			
0	0	0	0	0	0	0	0	0	0	0	0
1	63	22	12	41.5	38.0	37.5	43.0	1/2	37.5	34.5	34.5
2	117	42	23	77.5	70.0	71.5	80.0	1	71.0	66.0	66.0
3	165	61	34	109.0	100.5	102.5	115.0	1-1/2	103.5	96.0	96.0
4	208	80	44	140.5	130.0	152.0	147.5	2	134.0	125.0	125.0
5	243	98	55	169.0	157.0	159.0	179.0	2-1/2	164.0	152.5	152.0
6	273	116	65	195.5	182.5	184.5	207.5	3	191.5	179.0	178.0
7	298	134	76	220.0	205.0	208.0	233.0	3-1/2	216.0	203.0	203.5
8	317	151	86	241.0	227.5	229.5	255.0	4	240.0	226.0	226.0
H[g]	87	43	24	70	67	68	75		66	65	65
V_o[h]				1.92	1.75	1.78	1.96		3.22	2.98	3.01
$V_o/(E)$[i]				12.40	11.30	11.50	13.32		14.80	13.70	13.80
Notes	l	m	n	o	o	o	o		p	p	p

Table 6. -- continued

Run No.[a]	7-12-6	9-12-6	10-12-6	3-1-10	4-1-10	5-1-10	14-1-29
(E)[b]	.145	.145	.145	.146	.146	.146	.155
(NaOH)[c]	.4513	.4513	.4513	.4513	.4513	.4513	.1860
	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]	S.R.[e]
t[f]							
0	0	0	0	0	0	0	0
1	8.0	6	2.0	26	4.5	2.1	30.0
2	14.0	11	4.5	49	9.0	4.0	56.5
3	18.0	15	7.0	70	13.5	6.1	79.0
4	21.5	18	8.5	89	17.6	8.2	101.5
5	24.0	21	10.0	106	21.9	10.3	121.0
6	26.5	24	11.0	122	26.2	12.4	141.5
7	28.5	27	12.5	137	30.5	14.3	163.0
8	30.5	29	13.5	152	34.6	16.2	181.0
H[g]	.87	.83	.39	4.3	.99	.46	2.1
V_o[q]	3.52	2.68	1.04	10.2	2.02	.92	6.25[u]
$V_o/(E)$[i]	2.43	1.85	.72	7.00	1.38	.63	4.03
Notes	r	r	r	s			t

Table 6. -- continued

a. Run number-month-day (1956-57). b. Apparent enzyme concentration in units of 10^{-4} mg. P-N/ml. c. In units of 10^{-2} M. d. S.R. = scale reading. Corrected to S.R. = 0 at t = 0. In units of 10^{-3} ml. e. As in note d. Corrected for the blank given in the first column. f. In units of minutes. g. Approximate percent hydrolysis. h. Calculated as the slope of the tangent to the curve at zero time. In units of 10^{-4} M/min. i. In units of 10^{-1} M ml./mg. P-N min. j. The enzyme solution was made up at pH 3.5. k. The enzyme solution was made up at pH 8.0. l. The enzyme solution was prepared by dissolving the enzyme in 8 ml. of "Ludox" solution at pH 8. The final pH was 9, after dilution to 1 l. m. The enzyme solution from l had been standing at pH 3 for 10 minutes. n. The enzyme solution from l had been standing at pH 3 for 20 minutes. o. The enzyme was dissolved in 8 ml. of "Ludox" solution at pH 8, and after dilution to 1 l. and adjustment to pH 8.0, was used as a stock solution. p. The enzyme was dissolved in 5 ml. of "Ludox" solution at pH 8 and after dilution to 1 l., was adjusted to pH 7.2 and used as a stock solution. q. Calculated as the slope of the tangent to the curve at zero time. In units of 10^{-6} M/min. r. (NaCl) = 0.10 M. s. The electrodes and stirrer were pretreated with a solution containing enzyme at 1.0 mg./ml., washed with water for one minute, rinsed once with water, and dried prior to the run. t. The enzyme solution was prepared as in note o, then diluted 10:1 for use as stock enzyme. u. V_o calculated by the orthogonal polynomial method.

Table 7. -- Initial Specific Velocities vs. Specific Surface Areas

Substrate: N-acetyl-DL-4-pyridylalanine 1-oxide methyl ester

Run No.[ab]	(E)[c]	$v_o/(E)$[d]	$A_p/(E)_p$[eh]	$A_r/(E)$[fh]
1-1-16 2-1-16 3-1-16	1.51	2.82	1.69	1.59
2-1-14 3-1-14 4-1-14 5-1-14	.145	2.37	17.2	16.5
12-1-28 14-1-28	.203	2.11	12.3	11.8
16-1-28 18-1-28	.158	2.74	$2.3X10^4$ g	$2.3X10^4$ g

a. Run number-month-day (1957). b. For details concerning runs, see Table 4. c. Apparent enzyme concentration. In units of 10^{-2} mg. P-N/ml. d. The average value. In units of 10^{-3} M ml./mg. P-N min. e. Specific surface of components prior to dilution. In units of 10^2 $cm.^2$/mg. P-N. f. Specific surface associated with the reaction cell. In units of 10^2 $cm.^2$/mg. P-N. g. Specific surface of added "Ludox." h. To convert to units of $cm.^2$/mole, multiply by 3.52×10^6.

Table 8. -- Initial Specific Velocities vs. Specific Surface Areas.

Substrate: N-benzoyl-L-4-pyridylalanine 1-oxide ethyl ester.

Run No.[ab]	(E)[c]	$v_o/(E)$[d]	$A_p/(E)_p$[e]	$A_r/(E)$[f]
1-1-28 2-1-28 3-1-28	1.46	1.98	17.1	16.5
6-1-14 7-1-14 8-1-14	1.45	2.23	17.2	16.6
1-1-29 2-1-29 3-1-29	.209	.906	116	115
9-1-14 10-1-14 11-1-14	.220	.872	110	109
9-1-28 10-1-28 11-1-28	1.57	3.37	2.3×10^4 g	2.3×10^4 g
10-1-5 14-1-5	1.68	3.30	8.7×10^3 g	8.7×10^3 g
6-1-2	1.57	2.90	.77	4.9×10^4 g
7-1-2		2.98	.77	15.3
8-1-2		2.75	.77	15.3
9-1-2		2.90	.77	4.9×10^4 g
12-1-29	.155	2.49	3.7×10^4 g	3.7×10^4 g

a. Run number-month-day (1957). b. For details concerning runs, see Table 5. c. Apparent enzyme concentration. In units of 10^{-3} mg. P-N/ml. d. The average value. In units of 10^{-2} M ml./mg. P-N min. e. Specific surface of

Table 8. -- continued

components prior to dilution. In units of 10^2 cm.^{2}/mg. P-N. f. Specific surface associated with the reaction cell. In units of 10^3 cm.^{2}/mg. P-N. g. Specific surface of added "Ludox." In units of 10^3 cm.^{2}/mg. P-N.

Table 9. -- Initial Specific Velocities vs. Specific Surface Areas.

Substrate: N-acetyl-L-tyrosine methyl ester

Run No.[ab]	(E)[c]	$v_o/(E)$[d]	$A_p/(E)_p$[e]	$A_r/(E)$[f]
13-1-14 14-1-14 15-1-14	2.20	9.37	110	109
1-1-3 2-1-3 3-1-3	1.90	8.23	127	126
4-1-29 5-1-29 6-1-29	2.09	12.6	116	115
7-1-29 8-1-29 9-1-29	1.55	11.7	3.7×10^{4} g	3.7×10^{4} g
17-1-29	1.47	13.3	3.9×10^{4} g	3.9×10^{4} g
1-1-5 2-1-5 3-1-5	2.18	14.1	1.7×10^{4} g	1.7×10^{4} g
7-12-6 9-12-6 10-12-6	.145	1.67	1.7×10^{4}	1.66×10^{3}
4-1-10 5-1-10	.146	1.00	1.7×10^{4}	1.66×10^{3}
14-1-29	.155	4.03	1.6×10^{4} 3.7×10^{4} g	1.55×10^{3} 3.7×10^{4} g

a. Run number-month-day (1956-57). b. For details concerning runs, see Table 6. c. Apparent enzyme concentration. In units of 10^{-4} mg. P-N/ml. d. The average

Table 9. -- continued

value. In units of 10^{-1} M ml./mg. P-N min. e. Specific surface of components prior to dilution. In units of 10^{2} cm.^{2}/mg. P-N. f. Specific surface associated with the reaction cell. In units of 10^{2} cm.^{2}/mg. P-N. g. Specific surface of added "Ludox." In units of 10^{2} cm.^{2}/mg. P-N.

Table 10. -- The Sedimentation of alpha-Chymotrypsin in the Absence and Presence of "Ludox" at pH 8.

Solution A: 0.128 mg. P-N/ml., 0.08 ml. "Ludox"/ml.

Solution B: 0.119 mg. P-N/ml.

Solution C: 0.08 ml. "Ludox"/ml.

Time: 150 min. R.P.M: 40,000

Temp: 25° λ = 281 mμ

Sol'n.	Not Centrifuged O.D.[a]	Centrifuged O.D.-T[b]	Centrifuged O.D.-B[c]	% Loss T	% Loss B
A	1.65	0.26	1.64	84	0.6
B	1.48	1.37	1.53	7.5	-3.4
C	0.08	0.05	0.06	--	--

a. Optical density units. b. O.D. measured on the top 40% of the solution. c. O.D. measured on the bottom 60% of the solution.

Table 11. -- The Rate of the alpha-Chymotrypsin Catalyzed Hydrolysis of N-Benzoyl-L-4-pyridylalanine 1-Oxide Methyl Ester and N-(Nicotinyl 1-oxide)-L-phenylalanine Methyl Ester at pH 7.9 and 25°.

BPAOME		NOPAME	
$(S)_o = 1.15 \times 10^{-4}$ M		$(S)_o = 1.10 \times 10^{-4}$ M	
$(NaOH) = 1.162 \times 10^{-3}$ M		$(NaOH) = 4.65 \times 10^{-3}$ M	
$(E)^a = 2.32 \times 10^{-4}$ mg. P-N/ml.		$(E)^a = 2.32 \times 10^{-4}$ mg. P-N/ml.	
(NaCl) = .02 M		(NaCl) = .02 M	
Run No.[b]	3-2-13	Run No.[b]	1-2-13
	S.R.[c]		S.R.[c]
t^d		t^d	
0	0	0	0
2	4.5	0.4	40.0
4	9.0	0.8	71.5
6	13.5	1.2	95.0
8	17.5	1.6	115.0
10	21.5	2.0	132.5
12	25.5	2.4	148.0
14	29.5	2.8	160.5
16	33.5	3.2	172.0
H^e	3.9	H^e	80
v_o^f	2.65×10^{-7}	v_o^f	5.43×10^{-5}
$v_o/(E)(S)_o^g$	9.93	$v_o/(E)(S)_o^g$	2130

a. Apparent enzyme concentration. b. Run number-month-day (1957). c. S.R. = scale reading. In units of 10^{-3} ml. No blank correction was necessary. d. In units of minutes. e. Approximate percent hydrolysis. f. In units of M/min. Calculated by the orthogonal polynomial method. g. In units of ml./min. mg. P-N.

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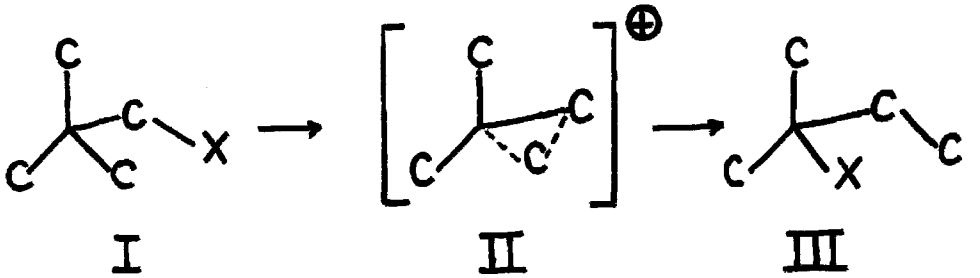
PART III

THE SYNTHESIS AND RATE OF ACETOLYSIS OF 1-BICYCLO[2.2.1]HEPTYLMETHYL TOSYLATE

A. INTRODUCTION

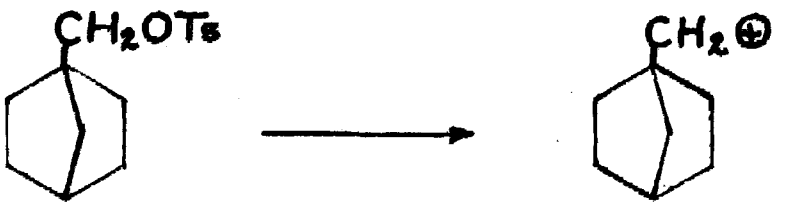
The relative inertness of such halides as neopentyl halides to S_N2 type substitutions is well known (1). This lack of reactivity has been attributed to steric shielding of the back side of the carbon atom bonded to the halide, thus decreasing the possibility for back-side attack by the incoming nucleophilic reagent. Neopentyl type halides are also quite slow to react via S_N1 type mechanisms, since the carbonium ion which would be formed would be a primary carbonium ion. However, neopentyl halides will react via a Wagner-Meerwein rearrangement at elevated temperatures (2).



I II III

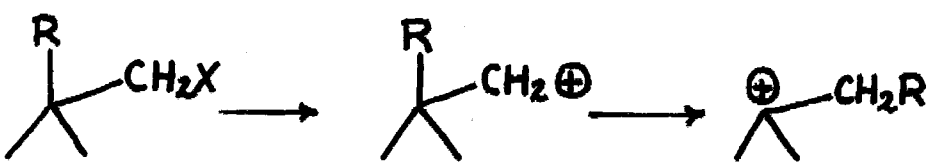
Studies aimed at elucidating the mechanism and driving force for this type of rearrangement have been made by Winstein and coworkers (3), and these authors concluded that the acetolysis of neopentyl type halides involved a minimum amount of solvent participation. Also, for their evaluation of driving forces, neopentyl derivatives were chosen as reference compounds, since they represented the minimum neighboring group effect, and steric effects, such as relief of steric strain via formation of the carbonium ion II, had no accelerating effect on the reaction.

Considering 1-bicycloheptylmethyl* tosylate IV, the acetolysis of the tosylate should also involve a minimum amount of solvent participation, and formation of the carbonium ion V should not have any different steric effect on the rate than in the case of neopentyl tosylate.



IV V

Should the migrating group R in VI not participate in the rate determining step of the rearrangement, then it would be expected that the rates of acetolysis of neopentyl tosylate and bicycloheptylmethyl tosylate would be nearly the same. However, should the group R participate in the



VI

rate controlling step, essentially through a nucleophilic displacement as below, it would be predicted that the acetolysis of bicycloheptylmethyl tosylate would be faster

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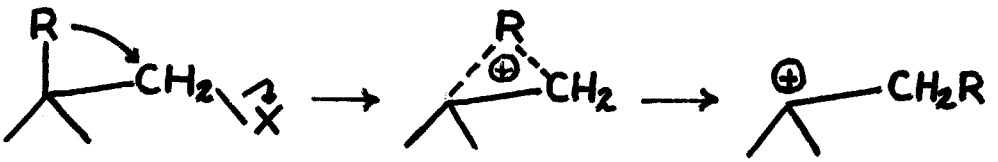
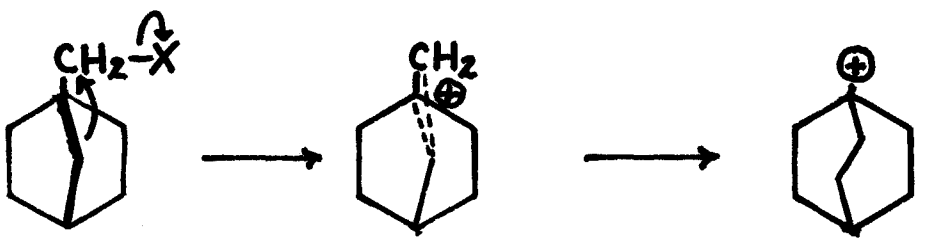
*Bicycloheptyl will be used for the bicyclo[2.2.1]heptane radical.



than that of neopentyl tosylate, for participation by the former would involve rearrangement to a bicyclo[2.2.2]-octyl radical VII, with relief of some steric strain, and concomitant increase in driving force.



IV VII

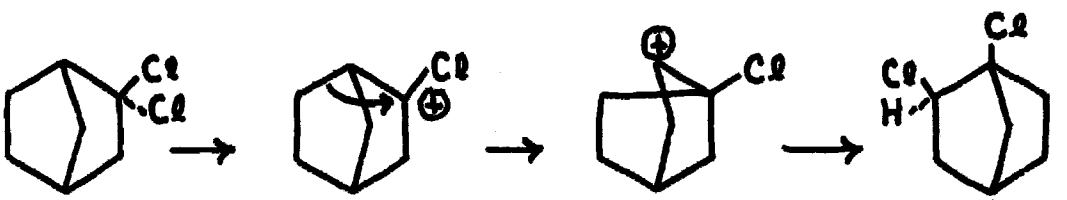
B. THE SYNTHESIS OF 1-BICYCLOHEPTYLMETHYL TOSYLATE

The preparation of 1-bicycloheptylmethyl derivatives has not been reported, but methods have been described in the literature which lead to a relatively simple scheme for their synthesis, involving bridgehead substituted bicyclo-heptanes. Many attempts to prepare bridgehead substituted bicycloheptanes have failed. Thus the trans-halogenation of bicycloheptane VIII with t-butyl chloride and aluminum chloride gave only exo-2-chloro-bicycloheptane IX (4), and the peroxide directed chlorination of bicycloheptane gave

the same product (5). On the other hand, the vapor phase nitration of bicycloheptane gave 1-nitrobicycloheptane (6), and 1-chlorobicycloheptane has been reported in the literature (7), although the method of preparation was not described, nor its physical properties.

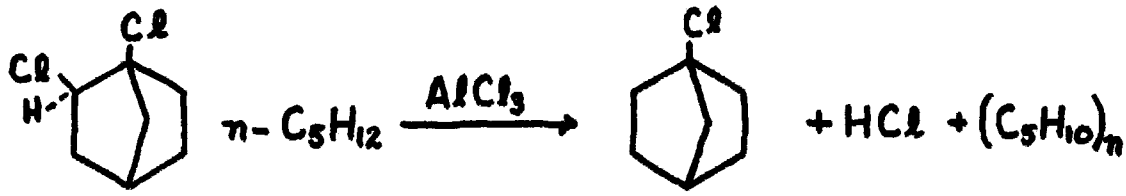
VIII IX

The Wagner-Meerwein rearrangement on 2-chlorobicycloheptane gives only the mirror image of the starting material, but rearrangement of 2,2-dichlorobicycloheptane X would give 1,2-dichlorobicycloheptane XI, as below.



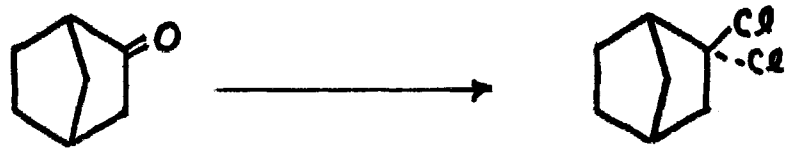
X XI

This reaction has been carried out in the camphane series by Houben and Pfankuch (8). The 2-chloro substituent can be removed selectively either by catalytic hydrogenation in base (9), or by means of a trans-halogenation reaction, first described in the aliphatic series by Bartlett (10).



XI XII

The dichloride X can be obtained from 2-ketobicycloheptane XIII using phosphorus pentachloride (11). XIII can be prepared by the method of Alder and Rickert (12).



XIII X

Thus 1-chlorobicycloheptane XII was synthesized via the ketone XIII, dichloride X, and 1,2-dichloride XI. XII was converted to 1-carboxybicycloheptane XIV via the lithium salt of bicycloheptane, the acid reduced to 1-hydroxymethylbicycloheptane XV, and the latter converted to its tosyl derivative IV. The entire scheme is summarized in Figure 1.

C. THE RATE OF ACETOLYSIS OF 1-BICYCLOHEPTYLMETHYL TOSYLATE

The rate of acetolysis of IV was measured in anhydrous acetic acid at 99.7°C., using the method outlined by Winstein, Grunwald and Ingraham (13). The first-order rate constant for the acetolysis was found to be $11.69 \pm 0.29 \times 10^{-6}$ sec.$^{-1}$,

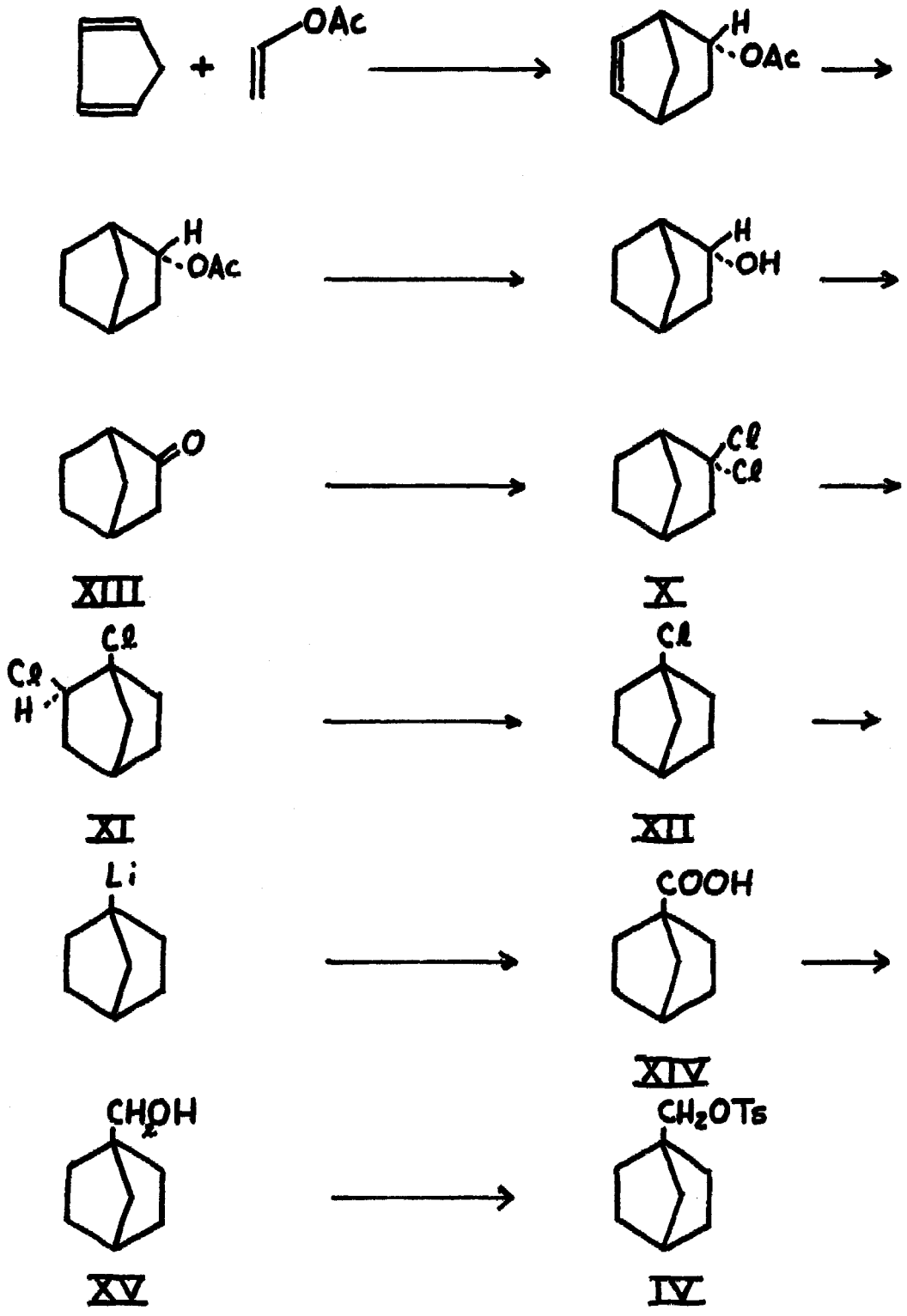


Figure 1. -- The synthesis of 1-bicycloheptylmethyl tosylate.

to be compared with the value given for neopentyl tosylate at 99.58°C., 1.66 ± 0.09 X 10^{-6} sec.$^{-1}$ (3). The results indicate that anchimeric assistance occurs in the solvolysis reaction. If the solvolysis of neopentyl derivatives involves no anchimeric assistance (see (3) and (14)), and if the carbonium ions derived from neopentyl tosylate and bicycloheptylmethyl tosylate have the same solvation energy, it would be possible to relate the increase in rate of the bicyclic derivative over the neopentyl derivative to relief of steric strain. However, at the present, there is no definite evidence that this is possible.

D. EXPERIMENTAL

Endo-2-hydroxybicycloheptane -- A total of 604 g. (9.15 mole) of freshly distilled cyclopentadiene and 906 g. (9.55 mole) of technical vinyl acetate was sealed in 20 pyrex tubes and heated at 185° for 10-15 hours. The contents of the tubes were aspirated for twenty hours, and the residue distilled *in vacuo* to give 568 g. (40%) of crude endo-2-acetoxybicycloheptene, b.p. 88.5-103°/33 mm. (lit. (14) 82-83°/17 mm.). This crude product was hydrogenated in a low-pressure apparatus using Adam's catalyst in methanol solution. The catalyst was removed by filtration, and the filtrate concentrated until the crude endo-2-acetoxybicycloheptane was dissolved in *ca.* 1250 ml. of methyl alcohol. Then 200 g. (3.58 mole) of potassium hydroxide was dissolved in the solution and the mixture held

at reflux for four hours. The solution was cooled and diluted with 2200 ml. of water; then the mixture was extracted with 3-1200 ml. portions of 30-60° petroleum ether, the extracts combined and dried over sodium sulfate. The petroleum ether solution was gravity fed to a chromatographing column packed with 3000 g. of alumina. Three fractions were collected from the bottom, and the I-R spectra taken of each. Fractions one and two had essentially no bicyclic alcohol, while fraction three had a considerable amount of bicyclic alcohol in it. The column was eluted with 4 l. of dry methanol, and the eluate combined with the third fraction above. The solvents were removed in vacuo to give an oily residue, which, after recrystallization from hexane, gave 253 g. (25% based on cyclopentadiene) of product, m.p. 151-151.5° (lit. (14) 152-153°).

2-Ketobicycloheptane -- To an ice-cooled 5-l. round-bottomed flask, fitted with a stirrer, were added 1500 ml. of water, 74 ml. (1.39 mole), of 96% sulfuric acid, 99.5 g. (0.338 mole) potassium dichromate, and 600 ml. of glacial acetic acid. After the solution was cooled, 115 g. (1.025 mole) endo-2-hydroxybicycloheptane was added and the solution stirred for six hours. The ice bath was allowed to melt, and the solution stood overnight. Then a cold solution of 500 g. of technical sodium hydroxide in 800 ml. of water was added slowly, keeping the temperature of the solution between 0-10°. The resulting chromium trihydroxide

slurry was steam distilled and about 1500 ml. of distillate collected. The distillate was saturated with sodium chloride and extracted with 3-500 ml. portions of ether. The ether extracts were combined and dried over calcium sulfate. The solution was filtered, and the ether removed by distillation. The residue was vacuum distilled to yield 85.4 g. (76%) of 2-ketobicycloheptane, b.p. 89-94°/60 mm.; 2,4-dinitrophenylhydrazone, m.p. 131.5-132.0° (lit. (15) 131.5-132.5°).

2,2-Dichlorobicycloheptane -- To a 500-ml. round-bottomed flask protected by a calcium chloride drying tube and containing 29 ml. of phosphorus trichloride cooled to 0° in an ice-salt bath was added 45.1 g. (0.41 mole) of 2-ketobicycloheptane. After the ketone had dissolved, 96.7 g. (0.463 mole) of phosphorus pentachloride was added portionwise over a period of one hour with vigorous shaking and cooling. The solution was allowed to slowly warm, and stood overnight. The solution was then poured onto 500 g. of ice, and hydrolysis allowed to proceed, keeping the temperature below 0° at all times. After 30 minutes the solution was allowed to come to room temperature. There were two phases. The mixture was extracted with 4-250 ml. portions of pentane, the pentane extracts washed with 2-300 ml. portions of water, then dried over magnesium sulfate. The pentane was distilled off, and the residue distilled *in vacuo* to give 1 g. of forerun, 47.8 g. (64%) of dichloride, b.p. 65.0-68.1°/12.0-12.4 mm., and 5 ml. of residue. The product solidified to

a low-melting solid.

Anal. Calc. for $C_7H_{10}Cl_2$ (165.1): C, 50.93; H, 6.11. Found: C, 51.02; H, 6.26.

1-Chlorobicycloheptane -- Aluminum chloride, 16.9 g. (0.127 mole), was added in four portions over a period of five hours to a solution of 43.9 g. (0.266 mole) of dichloride dissolved in 700 ml. of pentane (dried over aluminum chloride). Hydrochloric acid was slowly evolved and a red brown sludge formed. After six hours the walls of the flask were covered with sludge. The mixture stood for 43 additional hours. The clear pentane solution was decanted from the oil, and the oil washed with 100 ml. dry hexane. The combined wash and pentane solution was washed with 4-300 ml. portions of water until neutral, then dried over magnesium sulfate. The solvent was removed by distillation, and residue vacuum distilled to give 1.1 g. of forerun, 14.9 g. (43%) of 1-chlorobicycloheptane, and 5.6 g. of residue. The product b.p. 70-72°/54 mm., $n_D^{25.7} = 1.4722$ would solidify at ice temperature, and gave a slight test with an alcoholic silver nitrate solution at room temperature. This test may have been due to the presence of small amounts of either exo-2-chlorobicycloheptane or 1-chloro-exo-2-chlorobicycloheptane, or both. The I-R spectra showed no absorption at 14.85 μ or 14.61 μ in carbon disulfide, characteristic of exo- or endo-2-chlorobicycloheptane (16), and no absorption in the region 12.5 μ, characteristic of nortricyclene

derivatives (17). A redistilled sample, b.p. 70-71°/54 mm., $n_D^{25.7}$ = 1.4722, gave no halide test.

Anal. Calc. for $C_7H_{11}Cl$ (130.6): C, 64.36; H, 8.49; Cl, 27.15. Found: C, 64.12; H, 8.45; Cl, 27.30

1-Carboxybicycloheptane -- Lithium wire, 2.4 g. (0.345 mole), was placed under 20 ml. of #7 mineral oil in a 200-ml. round-bottomed three-necked flask equipped with a Trubore stirrer, separatory funnel, condensor (drying tube attached), and Y-inlet for carbon dioxide and nitrogen. The equipment was flamed, then purged with dry nitrogen for 30 minutes, before the lithium and oil were added. With vigorous stirring the oil was heated to boiling with a bare flame, and the suspension of lithium so obtained was allowed to cool. While purging with nitrogen, the mineral oil was pipetted off after removal of the stirrer, and the lithium sand washed with 3-20 ml. portions of dry cyclohexane. A glass-sealed magnetic stirring bar was introduced and the flask sealed. One half of a solution of 10.0 g. (0.0767 mole) of 1-chlorobicycloheptane in 40 ml. of dry cyclohexane was added to the lithium sand, and the suspension, while being stirred, was heated to 90°. An exothermic reaction soon started, and was maintained by the addition of the other half of the solution of the halide. The suspension was heated for one hour at reflux under a positive nitrogen pressure, then cooled, and 65 ml. of dry pentane added. Carbon dioxide, dried by passage through concentrated sulfuric

acid, was passed over the vigorously stirred suspension for two hours. To remove the excess lithium, 20 ml. of absolute ethanol, followed by 60 ml. of water, were added. After the addition of 50 ml. of ether, the aqueous phase was acidified with concentrated hydrochloric acid. The phases were separated, and the aqueous phase, after saturation with salt, was extracted with 3-100 ml. portions of ether. The combined ether extracts were extracted with 3-100 ml. portions of sodium carbonate solution. The sodium carbonate solution was acidified with 12 *N* hydrochloric acid, saturated with salt, and extracted with 3-100 ml. portions of ether. The ether extracts were dried and the solvent removed by distillation through a 12" column. The product was sublimed at 10 mm. and 80° to give 5.48 g. (51%) of 1-carboxybicycloheptane, m.p. 113.8-115.5°.

Anal. Calc. for $C_8H_{12}O_2$ (140.2): C, 68.54; H, 8.62. Found: C, 68.47; H, 8.52.

The reported m.p.'s for exo- and endo-2-carboxybicycloheptane are 48° (18) and 65° (19) respectively. The acid has a mild odor, similar to butyric acid.

1-Hydroxymethylbicycloheptane -- 2.19 g. (0.0156 mole) of the above acid was reduced in 130 ml. of ether with 3.0 g. (0.0790 mole) lithium aluminum hydride. After destroying the excess hydride by addition of water, the contents of the flask were poured into 100 ml. of 10% aqueous sulfuric acid. The phases were separated, and the aqueous phase

extracted with 2-25 ml. portions of ether. The combined ether phases were washed with water, saturated sodium bicarbonate solution, and water, then dried over magnesium sulfate. The dried ether extracts were stripped of ether by distillation through a 12" Vigreaux column to yield an oily residue. The residue was sublimed at 6 mm. and 65° to yield 1.76 g. (91%) of product, m.p. 59.0-60.2°, soft waxy needles.

Anal. Calc. for $C_8H_{14}O$ (126.2): C, 76.14; H, 11.18. Found: C, 75.91; H, 11.27.

1-Bicycloheptylmethyl Tosylate -- To an ice-cold solution of 0.70 g. (5.56 mmole) of the above alcohol dissolved in 6 ml. of dry pyridine was added 1.06 g. (5.56 mmole) of tosyl chloride in one portion. The solution was swirled for several minutes, then stood overnight at 4°. The cold solution was then added to 12 ml. of ice-cold 6 N hydrochloric acid. An oil formed which partially crystallized on scratching. The mixture was stirred with 25 ml. of carbon tetrachloride until all of the solid dissolved, the phases separated, and the aqueous phase extracted with 15 ml. of carbon tetrachloride. The organic phases were combined, dried, and the solvent removed by evaporation. The residue was recrystallized from 6 ml. of hexane to give 1.33 g. (86%) of tosyl derivative, m.p. 78.9-80.0°.

Anal. Calc. for $C_{15}H_{20}O_3S$ (280.4): C, 64.25; H, 7.19. Found: C, 64.19; H, 7.22.

The Rate of Acetolysis of 1-Bicycloheptylmethyl Tosylate -- The acetolysis was carried out in anhydrous acetic acid, according to the method published by Winstein, Grunwald, and Ingraham (13). Anhydrous acetic acid was prepared by adding enough acetic anhydride to react with the water present in glacial acetic acid (determined by freezing point), allowing the mixture to reflux for 3 hours, then distilling. The distillate was made approximately 0.5% in acetic anhydride, and held at reflux for 3 hours, cooled, and stored (stock acid).

Approximately 0.05 N perchloric acid in acetic acid was prepared by dilution of a 9 F aqueous solution with stock acid, and standardization against potassium acid phthalate to a brom phenol blue (0.1% solution in stock acid) end point.* Approximately 0.10 N sodium acetate in acetic acid was prepared by the addition of sodium carbonate to stock acid, and was standardized against the perchloric acid solution. The acetic anhydride in the stock acid was determined to be 0.50% by the method of Kilpi (20), i.e., the addition of anthranilic acid to the stock acid and titration with perchloric acid. In all titrations, 8 drops of brom phenol blue indicator solution were used per 5 ml. of volume.

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*Due to the high temperature coefficient of expansion of acetic acid, all solutions were at 25.000 just before standardization, or any volume measurement.

The acetolysis was carried out by placing 6 ml. aliquots of a tosyl derivative solution in stock acid in ampules, sealing the ampules, and placing them in an oil bath at 99.66 ± 0.02°, removing ampules at intervals, cooling immediately, opening, removing 5.00 ml. aliquots, and titrating with sodium acetate. The time was calculated from the time of opening of the ampules. The first aliquot was called zero time. The aliquots were titrated using a syringe burette (21), calibrated by titration of the perchloric acid with sodium acetate. The first-order rate constants were calculated using the expression $kt = \ln(a/a-x)$. The hydrolysis was followed to 64%. The results are summarized in the following table.

The Rate of Acetolysis of 1-Bicycloheptylmethyl Tosylate

Solvent: Acetic Acid (0.5% Ac_2O) Temp: 99.66 ± .02°C.

5 ml. samples 0.1035 N NaOAc,

a_o = 0.03547 M

Time (sec.)	Ml. base	a-x (M)	k_1 ($sec.^{-1} \times 10^{-6}$)
0	0.1933	0.03147	-----
9549	0.3529	0.02816	11.63
21860	0.5435	0.02472	11.97
34676	0.7000	0.0298	11.70
44576	0.8213	0.01847	11.95
74040	1.0938	0.01283	12.13
86384	1.1129	0.01243	10.76
95565	1.2147	0.01033	11.66

k_{mean} = 11.69 ± 0.29 X 10^{-6} $sec.^{-1}$

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PART IV

PROPOSITIONS

PROPOSITIONS

1. It is proposed that the incorporation of allyl glycidyl ether into epoxide polymers would lead to systems which could be cross-linked with sulfur monochloride (1).

2. There is evidence that increasing the size of the R and R' groups in $RCH_2CH(NHCOR')COZ$ leads to a more rapidly hydrolyzed substrate for alpha-chymotrypsin, in terms of its k_3 value (2). It is proposed that the greater the interaction between R and the active site of this enzyme, and the lesser the interaction between R' and the active site, for the case where R and R' are nearly the same size, the more rapidly the substrate will be hydrolyzed. Thus it is predicted that for the values of k_3 for $RCH_2CH(NHCOR')COOCH_3$, $R = C_5H_4N$ and $R' = C_5H_4N < R = C_5H_4N$ and $R' = C_6H_5 < R = C_6H_5$ and $R' = C_6H_5 < R = C_6H_5$ and $R' = C_5H_4N$.

3. It is proposed that the observed increase in pH after dilution of colloidal silica sols (3) is due to polymerization of the silica, and may account for the lack of complete restoration of enzymatic activity observed when enzyme-"Ludox" solutions are used.

4. It is proposed that 1-keto-3-carbomethoxytetrahydroisoquinoline be investigated as a potential substrate for alpha-chymotrypsin. This compound has all of the structural requirements for a substrate, and in addition, contains a fixed steric relationship between R and R' in $RCH_2CH(NHCOR')COOCH_3$

5. Treatment of 4-nitropyridine 1-oxide with various nucleophilic reagents gives 4-substituted pyridine and pyridine 1-oxide derivatives (4). A mechanism is proposed for these reactions.

6. Mayer and Freiling (5) have shown that solutions of α-hydroxy acids are useful as eluting agents for rare earths complexed on Dowex-50 cation exchange resin, and values are reported for residence times in exchange columns for several rare earths. It is proposed that a shortening of residence times is possible in the case of lactic acid, if the acid is purified to remove dimer prior to use.

7. It is proposed that the so-called methylate of trimethylamine oxide, $[(CH_3)_3N\text{-}OH]^+ OCH_3^-$ (6), does not exist as written, but is actually a solvated trimethylamine oxide molecule. The structure written above requires that the pKa of methyl alcohol < pKa of trimethylamine oxide conjugate acid, which is not the case.

8. Several empirical factors used in the engineering evaluation of the reaction rates of heterogeneous catalytic reactions are dependent on the surface area and pore radii of the catalyst particles. It is proposed that more effective values for these factors could be obtained by determining the apparent surface areas and pore radii using the materials being adsorbed on and desorbed from the catalyst, or similar materials.

9. It has been shown that trialkylaluminums will add to unsymmetrical disubstituted olefins, and that the reaction is reversible (7). It is proposed that information regarding the relative ease of migration of alkyl groups could be obtained from studies of this reaction.

10. It is proposed that the relative efficiencies of various blending systems with respect to particle size and time could be readily obtained with the use of radioactive tracers.

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