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THE CHEMICAL SYNTHESIS OF  
MODEL CATALYSTS RELATED TO  
ENZYMES

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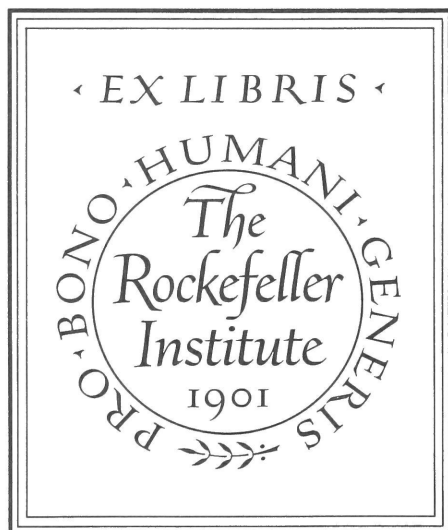
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JOHN WILLIAM BAKER HERSHEY, B.A.

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THE CHEMICAL SYNTHESIS OF MODEL CATALYSTS

RELATED TO ENZYMES

A thesis submitted to the Faculty of The Rockefeller Institute  
in partial fulfillment of the requirements  
for the degree of Doctor of Philosophy  
by

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Biochemistry  
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acceptable for publication*

15 April 1963

The Rockefeller Institute

New York, New York

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I wish to thank all those who aided in the preparation of this thesis: Miss Ruth Mandelbaum, who drew the figures; Miss Sonia Wohl and Miss Daphne Morse, who checked the bibliography, and Miss Elizabeth Bowman, who typed the manuscript.

## ABSTRACT

The chemical synthesis of highly specialized peptides that might possess catalytic action provides one of the approaches to an understanding of the mechanism of enzyme action. The experiments reported in this thesis involve the chemical synthesis of model compounds designed to explore how trypsin and chymotrypsin may function. Since several lines of evidence indicate a catalytic role for histidine residues in these enzymes, it was proposed to prepare imidazole derivatives with carefully designed side chains which might attract and orient similarly shaped substrates. A histidine with a lysine-like side chain attached to the imidazole ring was proposed as a model catalyst to mimic the specific action of trypsin. cyclo-D-Histidyl-L-phenylalanyl was proposed for a study of chymotrypsin-like activity. A third project concerned the determination of whether, in the reaction of 2-benzylimidazole with p-nitrophenyl phenylacetate, the two benzyl groups might attract each other and cause an increase in the rate of hydrolysis of the ester.

The work in this thesis involved primarily the synthesis of these model compounds. The "lysohistidine derivative" is a complicated 2- $\alpha$ -aminoalkyl-histidine and the classical imidazole syntheses routinely used to make 2,4-dialkylimidazoles failed when applied to this problem. The "lysohistidine derivative" with the various reactive groups protected was successfully prepared only through the development of a little-known reaction which involved the condensation of amidines with  $\alpha$ -haloketones. The reactants required were ethyl 2-carbobenzoxamido-4-oxo-5-chlorovalerate and  $N^{\omega}$ -acetyl- $N^{\epsilon}$ -tosyl-lysine amidine, both previously unknown. The chloromethylketone was synthesized from  $\alpha$ -ethyl carbobenzoxy-aspartic acid by conversion of the  $\beta$ -carboxyl group to an acid chloride and treatment with diazomethane followed by hydrogen chloride. As a pilot synthesis, the chloromethylketone was caused to react with *N*-benzylphenylacetamidine to produce  $N^{\omega}$ -carbobenzoxy-1,2-dibenzylhistidine, which was purified by counter-current distribution and silicic acid column chromatography.

$N^{\alpha}$ -Acetyl- $N^{\epsilon}$ -tosyl-lysine amidine was prepared from  $N^{\alpha}$ -acetyl- $N^{\epsilon}$ -tosyl-lysine by conversion of the carboxylic acid to an amide by the mixed anhydride method. Dehydrations of  $\alpha$ -acylamino carboxamides to nitriles had never been reported in the chemical literature. The usual reagents for amide dehydrations failed; only phosphorous oxychloride in pyridine could be used successfully. The nitrile was then treated with dry hydrogen chloride in ethanol and the resulting ethyl imidate ester hydrochloride was converted into the amidine with alcoholic ammonia. It was necessary to use countercurrent distribution in order to purify the  $N^{\alpha}$ -acetyl- $N^{\epsilon}$ -tosyl-lysine amidine hydrochloride. This amidine was then caused to react with ethyl 2-carbobenzox-amido-4-oxo-5-chlorovalerate and the desired pure "protected lysohistidine derivative" was obtained. Countercurrent distribution and silicic acid column chromatography were employed to obtain the material in analytically pure condition.

cyclo-Histidyl-phenylalanyl was synthesized by coupling  $N^{\alpha}, N^{Im}$ -dicarbobenzoxy-histidine with phenylalanine ethyl ester by the carbodiimide method. The carbobenzoxy groups were removed by catalytic hydrogenation and the diketopiperazine ring was caused to form in a refluxing ethanolic solution of the dipeptide ester free base. Both amino acids underwent racemization during this condensation. 2-Benzylimidazole and 2-isobutylimidazole were prepared from the corresponding imidazolines by catalytic dehydrogenation at  $245^{\circ}$ . In the course of the synthetic work, nineteen new compounds were synthesized, purified and characterized.

Preliminary kinetic studies were made in order to assay the model compounds for their catalytic properties. The addition of the "protected lysohistidine derivative" to reaction solutions of  $N^{\alpha}$ -acetyl- $N^{\epsilon}$ -tosyl-lysine ethyl ester caused no increase in the rate of hydrolysis. Similarly, no catalytic effect of cyclo-histidyl-phenylalanyl could be detected on the rates of hydrolysis of acetyl-phenylalanine ethyl ester and acetyl-phenylalanine p-methoxyphenyl ester. Also no specific enhancement of the rate of hydrolysis of p-nitrophenyl phenylacetate with 2-benzylimidazole could be demonstrated.

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## PART I

### INTRODUCTION

Enzymes are able to select from many possibilities only particular substrates on which they act, thus displaying what is called "specificity." Although the requirements, or allowable deviations, in structure are known for specific substrate molecules of some enzymes, there is still much to be learned about the basic nature of the enzyme-substrate interaction, or of which groups in the enzyme are most intimately involved or responsible for the specificity. An adequate theory of the mechanism of action of enzymes will have to explain why the reactions of certain substrates are catalyzed at a high rate, while the reactions of other similar molecules, e.g., stereoisomers, are not catalyzed at all. Such theories must therefore deal effectively with the problem of the source and nature of specificity, since specificity and catalytic action are so intimately related.

In this thesis the mechanisms of action of some proteolytic enzymes were considered with special regard to the problem of specificity. The experimental approach selected was the chemical synthesis of model compounds which might be expected to mimic the specific actions of the enzymes trypsin and chymotrypsin. Such models, if successful, could be thought to reflect the manner in which specificity arises in these enzymes, and could be used profitably in a study of the nature of the forces involved in enzyme-substrate interactions. In order to introduce the ideas associated with this work, it will be necessary to review in some detail what is known about trypsin and chymotrypsin.

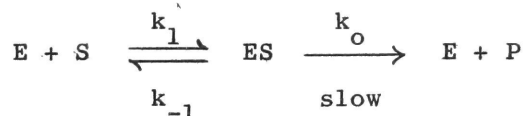
### Enzymology

Chymotrypsin and trypsin are members of a class of enzymes known as endopeptidases, which catalyze the hydrolysis of "internal" peptide bonds of proteins. They are elaborated by the acinous cells of pancreas as inactive zymogens, called chymotrypsinogen and trypsinogen. Activation of the zymogens can be complex (43), and in the case of chymotrypsinogen a number of closely related enzymes are formed, the most common being  $\alpha$ -chymotrypsin. Chymotrypsin rather specifically catalyzes the hydrolysis of amides and esters of the aromatic amino acids, while trypsin acts specifically on the corresponding carboxylic acid derivatives of the basic amino acids.

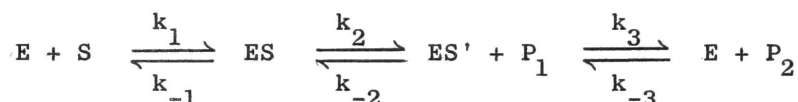
As with many enzymes, the catalytic action follows so-called Michaelis-Menten kinetics. The rate equation,

$$\text{rate} = \frac{k_o (E) (S)}{K_M + (S)}, \quad \text{where } K_M = \frac{k_{-1} + k_o}{k_1},$$

can be derived from the following simple mechanism by assuming that the concentration of ES, the enzyme-substrate complex, reaches a steady state.



It is suggested by the kinetics, and is generally thought, that the catalytic activity of hydrolytic enzymes is the result of three consecutive basic reactions: a. adsorption of the substrate; b. catalysis of the hydrolysis; and c. ejection of the final products. In the initial adsorption, or formation of the enzyme-substrate complex, the binding is not covalent and is therefore to be distinguished from the acyl-enzyme intermediates that are thought to be formed in the complex hydrolytic steps which follow. There are a number of more complicated mechanisms which also can be expressed by the same rate equation. The example given below will be encountered again in the development of the enzyme problem.



The symbols mean the following: E, enzyme; S, substrate; ES, enzyme-substrate complex; ES', acyl-enzyme; P<sub>1</sub> and P<sub>2</sub>, products. Although in the case of the more complicated mechanism the same kinetic parameters, k<sub>o</sub> and K<sub>M</sub>, are measured in initial steady state studies, it is often more difficult to interpret them, for each may be composites of a number of individual rate constants. Comparisons of k<sub>o</sub> and K<sub>M</sub> derived from studies of the reactions of different substrates can therefore be misleading. For a detailed development of enzyme kinetics, and a discussion of these problems, the reader is referred to the many excellent reviews available (44,74,154).

α-Chymotrypsin and trypsin are pure, crystalline proteins (obtained by Kunitz and Northrop (107)) with molecular weights of 24,800 (183) and 24,000 (95), respectively. The absence of prosthetic groups or coenzymes

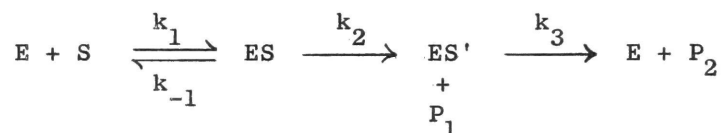
means that the catalytic activity must be associated with the amino acid components of the enzyme alone. However, it is unlikely that all the amino acids are directly involved in the catalytic action. In the enzyme papain, for example, a large part of the molecule can be removed without loss of activity (80,135). A relatively small area on the surface of the protein, called the "active site," is regarded as being directly responsible for the catalytic action of the whole enzyme. Evidence from studies of inactivation of  $\alpha$ -chymotrypsin and trypsin indicates that they contain only one active site per molecule (13). The meaning of the term "active site" is intended here to include the amino acid groups concerned with the bond making and breaking reactions, as well as those involved in substrate binding and in specificity. It may be profitable to consider these functions separately, however.

In order to understand completely the mechanism of action of an enzyme, one needs to know which amino acids are present in the active site and how these amino acids are arranged in space. The primary structure of the enzyme, ribonuclease, is known (81,160,164), and the amino acid sequences of chymotrypsin (69,96) and trypsin (116,117) are currently almost completely established. In addition, the tertiary structures of ribonuclease and  $\alpha$ -chymotrypsin in the crystalline state are being determined by X-ray diffraction studies. With sequence studies of the enzymes labeled chemically at the active site (to be discussed below), and with the likely assumption that the tertiary structure of the protein in the crystalline state resembles reasonably closely the structure in solution, it should be possible to identify the general area of the active site in the protein structures being determined. By inspection of such a structural model, the groups composing the active site could be identified, but it is doubtful that a complete understanding of the mechanism of action of an enzyme will be achieved in this way. However, it is exciting to anticipate knowledge of the primary and tertiary structures of ribonuclease and chymotrypsin.

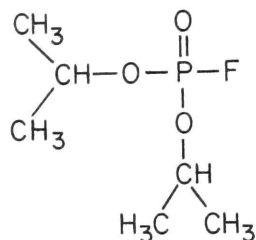
Long before sequential analyses were started on chymotrypsin and trypsin, other approaches to the problem of which amino acids contribute directly to the catalytic function of the enzymes were studied. One of the most fruitful of these has been chemical. A reagent is caused to react with an enzyme and the kinetic properties and chemical composition of the

modified protein are analyzed. For example, both  $\alpha$ -chymotrypsin and trypsin were shown by Balls and co-workers to react vigorously with just one equivalent of diisopropylfluorophosphate (DFP) (Figure 1,a), thereby generating an inactive protein (83,84). The rate of inactivation was retarded by the same competitive inhibitors which retard the rates of hydrolysis of specific substrates of the enzyme. It is thought that the inhibitor competes with substrate molecules for the binding position located at the active site. It is therefore reasonable to assume that the DFP reacted with a group near the active site which could be partially shielded by a bound inhibitor. From an acid hydrolysate of the inactivated enzyme, a phosphorylated serine residue was isolated (147), thus implicating serine as an important component of the active site. The sequence of amino acids around this unique serine, viz., glycyl-aspartyl-seryl-glycyl, is common for  $\alpha$ -chymotrypsin, trypsin and a number of other hydrolytic enzymes (43). It is surprising that many enzymes of similar function possess the same amino acid sequence around their unique serine residue and it is suggestive that the serine and possibly also the aspartic acid residue play important roles in the catalysis. The actual significance of the common sequence of amino acids is yet to be appreciated, however.

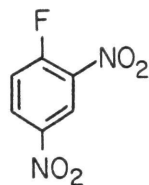
Additional evidence for the involvement of serine in the active site has been accumulated. Hartley and Kilby (70,71) first showed that when the labile, non-specific substrate ester, p-nitrophenyl acetate, is incubated with an equivalent amount of  $\alpha$ -chymotrypsin, p-nitrophenol is liberated more rapidly than is acetate. The kinetics were studied by Gutfreund and Sturtevant (63) in a stopped-flow apparatus (a device designed for the study of fast reactions), and were found consistent with the following mechanism:



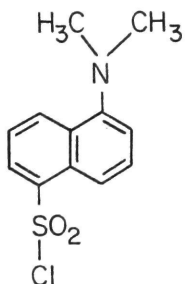
where ES is the so-called Michaelis-Menten complex, ES' is acetyl-chymotrypsin, P<sub>1</sub> is p-nitrophenol and P<sub>2</sub> is acetate. Balls and Wood (6) were actually able to isolate the acetyl-chymotrypsin by carrying out the reaction at a lower pH (pH 5). Oosterbaan and van Adrichem (126) degraded the acetyl-enzyme with pepsin and pancreatin and obtained peptides containing an



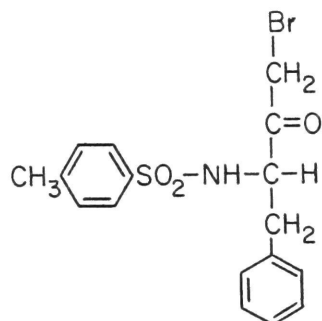
a. diisopropyl fluorophosphate



b. 2,4-dinitrofluorobenzene



c. 1-(dimethylaminonaphthalene-5-sulfonyl chloride



d. L-N-tosylphenylalanyl-bromomethane

**Figure 1.** Reagent Inhibitors of Chymotrypsin.

O-acetylserine residue surrounded by the same amino acids found in the vicinity of the phosphorylserine discussed above. It is therefore clear that the same serine residue is involved in both these reactions (DFP inactivation and p-nitrophenyl acetate hydrolysis) and that it is present at or near the active site.

In addition, serine has been directly implicated in the active sites of other enzymes, namely phosphoglucomutase (98), alkaline phosphatase (151), and a variety of proteolytic enzymes (68). It therefore seems safe to say that the presence of serine at the active site is not fortuitous, but that it probably plays some kind of important role in the enzymic action.

There is considerable evidence that a histidine residue is also intimately involved in the active site. The rate of inactivation of  $\alpha$ -chymotrypsin by photooxidation with methylene blue (180) was shown to parallel the rate of oxidation of a histidine residue in the enzyme. However, the data did not rule out the possibility that an alteration of tryptophan was responsible for the inactivation (181). Chemical studies with 2,4-dinitrofluorobenzene (112,181) (Figure 1,b) and 1-dimethyl-aminonaphthalene-5-sulfonyl chloride (72) (Figure 1,c) on  $\alpha$ -chymotrypsin showed that the enzyme is inhibited by these reagents when one of the two histidine residues has reacted at the imidazole ring. Recently, Schoellmann and Shaw (150) devised an alkylating reagent, L-N-tosylphenylalanyl-bromomethane (Figure 1,d), which specifically inactivated  $\alpha$ -chymotrypsin, but did not affect trypsin or acetylcholinesterase. Since this reagent resembles the structure of specific substrates of chymotrypsin, it appears likely that the greater reactivity shown toward chymotrypsin was due to specific binding of the reagent at the active site. This argument is supported by the fact that a competitive inhibitor of the hydrolysis of specific substrates of chymotrypsin protected the enzyme against inactivation. When the inactivated protein was analyzed, it was found that one equivalent of L-N-tosylphenylalanyl-bromomethane had reacted with the enzyme, and that one of the two histidine residues had been destroyed or altered (155).

Further support for the importance of histidine can be derived from enzyme kinetic studies. Hammond and Gutfreund (65) studied the effect of pH on the kinetic parameters of the Michaelis-Menten mechanism ( $K_M$  and  $k_O$ ) for the reaction of acetyl-L-phenylalanine ethyl ester and  $\alpha$ -chymotrypsin

and found that the catalytic rate constant,  $k_o$ , could be expressed as being dependent on the basic form of a group with an apparent pKa of 6.85. Subsequent studies with other specific esters and with acetyl-L-tyrosine amide confirmed this finding (40,64). A group with the same apparent pKa is involved in the hydrolysis of the labile p-nitrophenyl esters also. The apparent pKa agrees well with the pKa of the imidazole of a histidine. However, other possibilities such as a mercapto group or an alpha amino group cannot be ruled out by the kinetic data alone. The pKa's of amino acid residues can vary appreciably, depending on their exact molecular environment as determined by the protein molecule and solvent. In addition, extreme care must be exercised in interpreting apparent pKa's from kinetic data, because equilibrium reactions occurring before the rate determining step of a complex reaction can influence the observed pH effects (34). However, the observed effects of pH on the rates of  $\alpha$ -chymotrypsin catalyzed reactions are certainly entirely consistent with the view that a histidine residue in its basic form is involved in the catalytic action. The kinetic and chemical studies combined therefore provide convincing evidence that histidine is located in the active site.

In addition, histidine has been shown to be present and to possess unusual reactivity in the active site of ribonuclease. Treatment of the enzyme with bromoacetate (8) or iodoacetate (60,61) results in inactivation, a single histidine having been carboxymethylated. By degradation of the inactivated enzyme, it has been possible to determine which histidine of the possible four actually reacted. If the enzyme is first denatured with urea, the unusual reactivity of the unique histidine is lost. Ribonuclease represents the best documented case of the involvement of histidine in the active site of a hydrolytic enzyme.

There are other amino acid residues which also seem to be involved at or near the active site of chymotrypsin. When either one or both methionine residues of  $\alpha$ -chymotrypsin were oxidized with hydrogen peroxide, Koshland and co-workers (103) found partial inhibition of the enzyme. By treatment of  $\alpha$ -chymotrypsin with horse-radish peroxidase, Wood and Balls (188) obtained a crystalline enzyme with one less tryptophan, but with only 50% of the original activity. These, and other amino acids, might merely be important in preserving the conformation of the active site since the altered

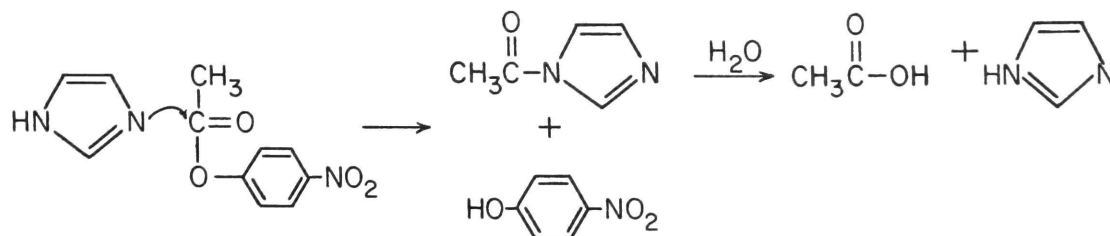
enzymes retained a significant amount of activity, but their real significance remains to be determined. The possibility that special peptide linkages of the protein chain might play some role must also be entertained. Koshland et al. (103) have written a recent comprehensive review of the evidence for the involvement of different amino acid groups in the action of chymotrypsin.

The experiments described above lead to the conclusion that both histidine and serine are important in the catalytic action of chymotrypsin. The fact that inactivation with the various reagents can be reduced by the inhibitors of specific substrates suggests that both groups are very near to the specificity site and thus are probably also in close proximity to each other. However, it is not known exactly how the histidine and serine groups contribute to the catalytic action of the enzyme. It should be mentioned that some of the evidence just cited is very recent and was not in existence at the time that the plan of experimentation described in this thesis was laid down.

The work to be mentioned now is primarily concerned with the elucidation of the roles played by histidine and serine in the mechanism of action of chymotrypsin. Studies of imidazole derivatives as model non-enzymic catalysts of ester hydrolysis have led to a better understanding of how histidine might be acting in the enzyme. There are two possible ways by which imidazole can function as a base catalyst: by nucleophilic, or by general, base catalysis (9). Nucleophilic base catalysis of ester hydrolysis involves attack by the imidazole nitrogen at the carbonyl of the ester, expulsion of the alcohol anion, and formation of an acyl-imidazole as an intermediate in the hydrolysis. The acyl-imidazole is subsequently hydrolyzed. General base catalysis involves the transfer of a proton by the imidazole, either from a species (e.g., water) which is simultaneously attacking the carbonyl group of the ester, or to the leaving alcoholate as the ester carbonyl is being attacked by hydroxide ion. In both cases the catalyst is involved in the transition state of the slow step of the reaction, but in nucleophilic catalysis the imidazole attacks a carbon atom, while in general base catalysis it reacts with a hydrogen atom.

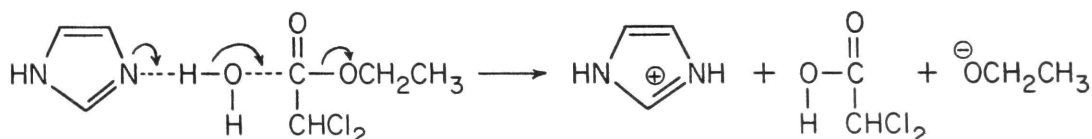
Following Hartley's report on the ability of N-benzoyl-L-histidine methyl ester to catalyze the hydrolysis of p-nitrophenyl acetate (182),

Bender and Turnquest (17) and Bruice and Schmir (33) simultaneously demonstrated that imidazole was a very effective catalyst of the hydrolysis of this labile ester. The reaction was shown to proceed through an N-acetyl-imidazole intermediate and the catalysis was therefore of the nucleophilic type.



Imidazole has been found to catalyze the hydrolysis of a variety of phenyl ester derivatives by nucleophilic base catalysis (33). However, hydrolysis of the less reactive ester, ethyl acetate, is not catalyzed at all by imidazole (17).

Imidazole catalysis of the hydrolysis of alkyl esters can be demonstrated if the acyl portion of the ester derivative is activated (e.g., the methyl or ethyl esters of fluoroacetate, mono-, di-, and tri-chloroacetate or glycine (87)). Likewise, catalysis will occur if the pK<sub>a</sub> of the leaving aliphatic alcohol derivative of an acetate ester is less than the pK<sub>a</sub> of water (e.g., 1,1,1-trichloroethanol (32) or N-acetylserine amide (3)). These reactions do not proceed by nucleophilic base catalysis, but rather by general base catalysis. A likely mechanism involves the removal by imidazole of a proton from a water molecule which is simultaneously attacking an ester carbonyl. When these reactions were run in deuterium oxide instead of water, the rates of catalysis were 2 to 3 times slower, as could be expected in the



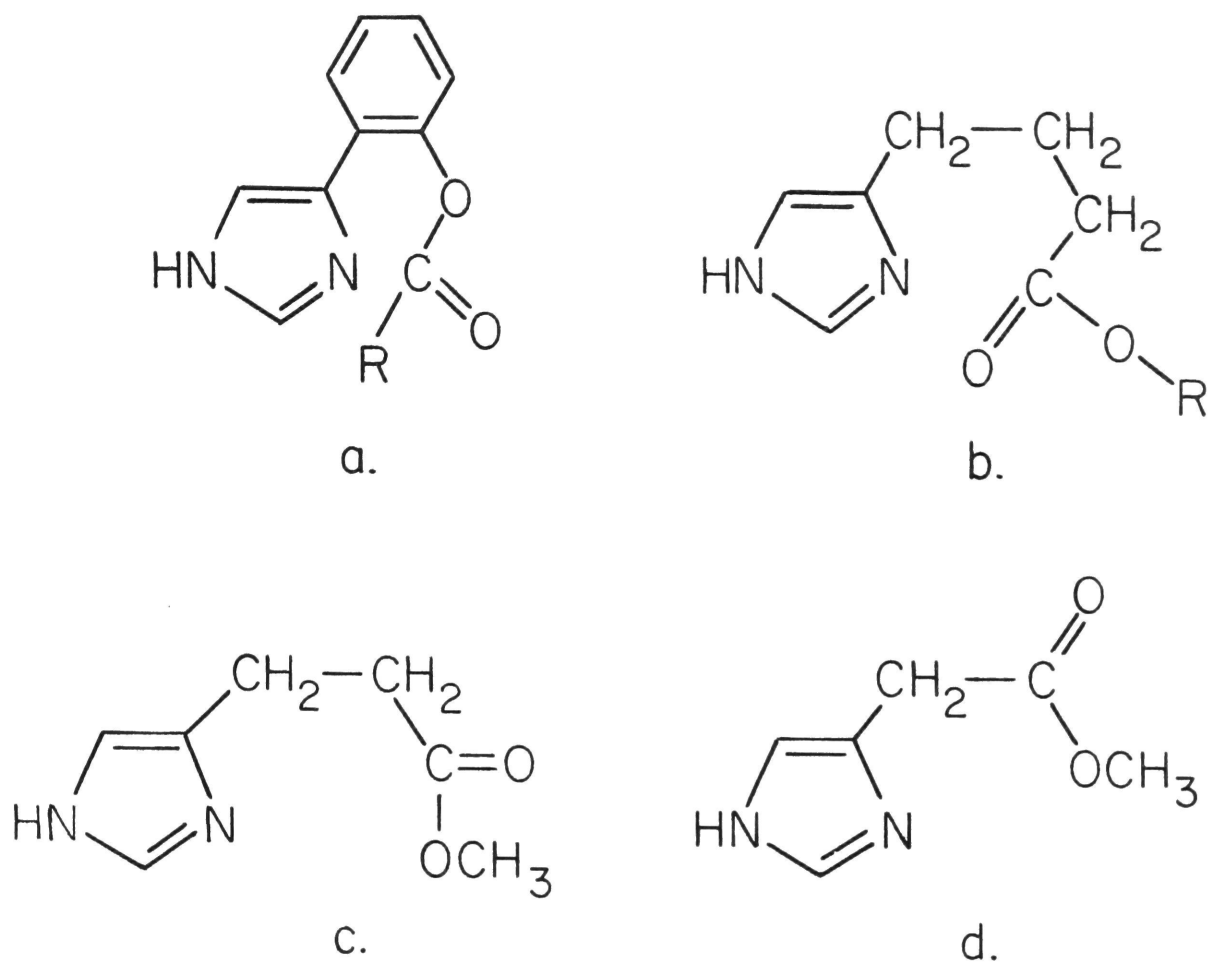
reactions where a hydrogen (or deuterium)-oxygen bond was being broken in the rate-limiting step (182). The difference in rate is called a deuterium isotope effect. On the other hand, all known cases of nucleophilic base

catalysis by tertiary amines have been observed to occur without an appreciable isotope effect (12). The observation of a deuterium isotope effect is considered good evidence that the catalysis occurring is of the general base type.

Bruice (30) has pointed out the interesting fact that imidazole is as good a general base catalyst as could be found for reactions at neutral pH. The effectiveness of a general base catalyst is determined by its basicity and not by its nucleophilicity. If the  $pK_a$  of the catalyst were more basic than 7, it would exist primarily in its inactive, protonated form, while if it were less than 7, it would be a weaker catalyst. Therefore histidine, with its  $pK_a$  of about 7, is ideal.

In enzymic catalyses, the substrate first is bound to the enzyme, thus forming a complex, and then the bond breaking (and making) processes occur within the confines of this complex. In this respect, enzymic reactions are similar to intramolecular reactions, and for this reason, the latter have been extensively investigated recently as model enzymic reactions. Some interesting cases of very efficient intramolecular imidazole catalysis are known. The first examples of this kind of catalysis, described by Bruice and co-workers (130,149), involved acetyl and benzoyl esters of 2-(4-imidazolyl)-phenol (Figure 2,a). Later, even more efficient intramolecular nucleophilic base catalysis was observed for the reactions of various phenyl esters of  $\gamma$ -(4-imidazolyl)-butyric acid (Figure 2,b) (31,35,36). With the p-nitrophenyl ester of the latter compound, the rate of liberation of p-nitrophenol (*i.e.*, the rate of acyl-imidazole formation) was slightly greater ( $200 \text{ min.}^{-1}$ ) than the rate of liberation of p-nitrophenol observed ( $180 \text{ min.}^{-1}$ ) in the reaction of  $\alpha$ -chymotrypsin and p-nitrophenyl acetate (*i.e.*, the rate of acyl-enzyme formation from the "intramolecular" enzyme-substrate complex (165)). Thus intramolecular nucleophilic attack by imidazole alone can equal the rate of acyl-enzyme formation when p-nitrophenyl esters are involved. The deacylation rate for the acyl-enzyme is considerably faster than for the acyl-imidazole, however.

Although hydrolysis of the phenyl esters of  $\gamma$ -(4-imidazolyl)-butyric acid is catalyzed very efficiently by the imidazole portion of the molecule, no detectable catalysis could be measured when the corresponding methyl ester was studied (36). Very recently, Koshland and Lukton (102) showed



**Figure 2.** Intramolecular Imidazole Catalysis of Ester Hydrolysis.

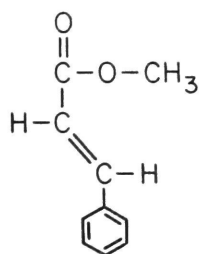
that the rates of hydrolysis of the methyl esters of the two lower homologs,  $\beta$ -(4-imidazolyl)-propionic acid (Figure 2,c) and 4-imidazolyl-acetic acid (Figure 2,d) are indeed catalyzed by the attached imidazole. These reactions exhibit a deuterium isotope effect and therefore likely proceed by general base catalysis. Pseudo 6- and 7-membered rings can form with water in the transition states of these reactions, while in the case of the uncatalyzed butyrate derivative a pseudo 8-membered ring, which seems to form reluctantly in most chemical reactions, would be required. These esters are involved in one of the few instances of imidazole catalysis of the hydrolysis of aliphatic ester bonds which are electronically only slightly different from that of ethyl acetate.

Two points emerge from the experiments cited. The first is that the intramolecular catalyses are much more efficient than the intermolecular ones. (Unfortunately it is difficult to compare quantitatively the rate constants of the two kinds of reactions, since each is expressed in different units). The second point is that the mechanism by which a given kind of ester is hydrolyzed appears not to be changed in going from an intermolecular to an intramolecular imidazole catalyzed reaction. Empirically, it is possible to say that nucleophilic base catalysis of ester hydrolysis by imidazole seems restricted to phenyl and thiol esters, while general base catalysis occurs, although less efficiently, for nearly all kinds of esters. The results of these studies on model imidazole catalysts strongly suggest that the histidine involved in the active site of chymotrypsin might act as a general base catalyst.

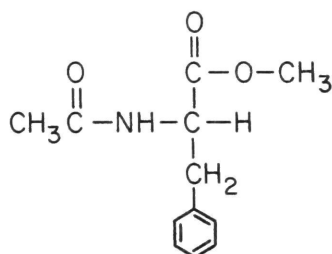
Serine shall now be considered from the point of view of how the group contributes to the catalytic function of the enzyme. In this respect, the evidence already presented for the involvement of serine can be justly criticized in three ways. The reagents used to label the enzyme (diisopropylfluorophosphate, p-nitrophenyl acetate) are not specific substrates of chymotrypsin and may have reacted in some other way. In the case of p-nitrophenyl acetate, the substrate is very reactive and the catalysis observed could have been due to a different mechanism. Also, all of the techniques used to isolate the acylated serine residues or peptides, including the enzymic degradation, involved acidic conditions, which are dif-

ferent from the neutral or slightly alkaline conditions encountered during the normal hydrolytic process. The acid conditions therefore could have caused an acyl migration from some other group to the serine hydroxyl.

Bender and co-workers (14,18) have used the substrates, methyl, benzyl and *p*-nitrophenyl trans-cinnamate, in order to overcome some of these objections. The acyl portion of these substrates resembles somewhat more closely the natural substrates of  $\alpha$ -chymotrypsin (e.g., acetyl-L-phenylalanine methyl ester) than does the acetyl group, and therefore might be



methyl trans-  
cinnamate



acetyl-L-phenylalanine  
methyl ester

expected to be bound and be hydrolyzed like a specific substrate. This argument is supported by the fact that the hydrolysis of non-labile alkyl esters of trans-cinnamic acid are in fact catalyzed by  $\alpha$ -chymotrypsin, while those of acetic acid are not appreciably. The trans-cinnamate esters were chosen because the cinnamoyl group absorbs light and any acyl intermediates formed during the enzymic reaction might therefore be detectable spectrophotometrically.

With the non-labile methyl and benzyl cinnamate esters, these workers were able to demonstrate an acyl-enzyme intermediate spectrophotometrically and to show that the kinetics were also consistent with the same mechanism proposed by Gutfreund and Sturtevant for *p*-nitrophenyl acetate hydrolysis (63). With the labile *p*-nitrophenyl ester (14), a trans-cinnamoyl- $\alpha$ -chymotrypsin intermediate was also demonstrated spectrophotometrically and was actually isolated. The difference spectrum of the acylated and deacylated enzyme was claimed to resemble the spectrum of O-cinnamoyl-N <sup>$\alpha$</sup> -acetylserine amide ( $\lambda_{\text{max}} = 281.5$ ), but the actual value of the difference spectrum ( $\lambda_{\text{max}} = 292$ ) lay between that of the serine derivative and that of N-cinnamoyl-imidazole ( $\lambda_{\text{max}} = 307$ ). The following experiments are more convincing.

Trans-cinnamoyl- $\alpha$ -chymotrypsin was prepared at pH 7.22 and was quickly transferred to an 8 M urea solution where the kinetics of deacylation at higher pH values were measured. Denaturation of the protein in the high concentration of urea caused the reaction to resemble an ordinary non-enzymic ester hydrolysis (pH dependent) rather than the enzymic deacylation one (which had been shown to be pH independent above pH 8.0). A comparison of the measured non-enzymic rate with the rates of alkaline hydrolysis of other trans-cinnamate esters of various types led these workers to conclude that the acyl-enzyme intermediate was an ester of serine or tyrosine, but was definitely not an acyl-imidazole. Since it is rather unlikely that an acyl migration would have been induced by urea denaturation, this work constitutes rather good evidence that the acyl group of the acyl-enzyme is in fact attached to a serine hydroxyl.

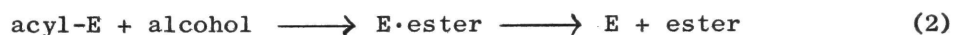
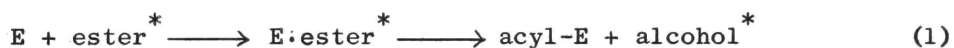
Although some of the enzyme kinetic studies to be presented below do not measure a property of the acyl-enzyme directly, they do measure a property of the system related to the acyl-enzyme. The following evidence, therefore, is consistent with the acyl-enzyme hypothesis and thereby supports it, but it is not useful in distinguishing which amino acid residue is involved. With the reactive esters of the "specific" substrates of chymotrypsin, N-carbobenzoxy-L-tyrosine p-nitrophenyl ester (62) and N-acetyl-L-tryptophan p-nitrophenyl ester (192), it was possible to measure the initial rapid liberation of p-nitrophenol (the pre-steady state) using the stopped-flow method. The subsequent slow reaction (the steady state) was also measured. The rate constants determined from these measurements are consistent with the hypothesis that the initial fast reaction is the acylation of the enzyme, and that the steady state reaction is the overall rate-controlling deacylation reaction.

Another argument for the existence of an acyl-enzyme intermediate is the observation that the hydrolysis of many different esters possessing the same acyl structure are catalyzed by trypsin or chymotrypsin at the same rate. For example, trypsin catalyzes the hydrolysis of the methyl, ethyl, isopropyl, benzyl and cyclohexyl esters of benzoyl-L-arginine with the same maximal velocity (152). The chymotrypsin catalyzed hydrolyses of the methyl, ethyl and p-nitrophenyl esters of N-acetyl-L-tryptophan also proceed at similar overall rates (192). It is reasonable to conclude that in both

studies a common intermediate, an acyl-enzyme, was formed with each substrate, and that the decomposition of the intermediate to products was the rate-controlling step.

The variation of rate with pH has been studied with  $\alpha$ -chymotrypsin and its specific substrates. The classical Michaelis constant,  $K_M$ , has been shown (65) to be essentially independent of pH from pH 6 to 8 (with acetyl-L-phenylalanine ethyl ester). The pH dependence of the overall catalytic rate,  $k_o$ , indicates that a group (in its basic form) with an apparent pKa of about 7 is necessary for catalysis. The rate of acylation of  $\alpha$ -chymotrypsin by acetyl-L-tryptophan amide exhibits a bell-shaped pH-rate profile, which can be interpreted to mean that a basic group (apparent pKa = 7) and an acidic group (apparent pKa = 8.5) are involved (10). The deacylation step in the reaction of acetyl-L-tryptophan esters, however, exhibits a sigmoid pH-rate profile involving a basic group (apparent pKa = 7) only. The deacylation of trans-cinnamoyl- $\alpha$ -chymotrypsin was shown to be pH independent from pH 9 to 13 (15). The experiments seem to indicate that the acylation reaction proceeds by general base and general acid catalysis, while the deacylation reaction proceeds only by general base catalysis. However, the following argument must be taken into account.

Chymotrypsin catalyzes the isotopic exchange of methyl-C<sup>14</sup> esters with methanol and carboxylic acids with H<sub>2</sub>O<sup>18</sup>. It is reasonable to assume that these reactions proceed through the same steps as the hydrolytic reactions and are essentially unaffected by the isotope. In reaction (1), an acyl-enzyme and labeled alcohol are formed. In reaction (2), the unlabeled al-



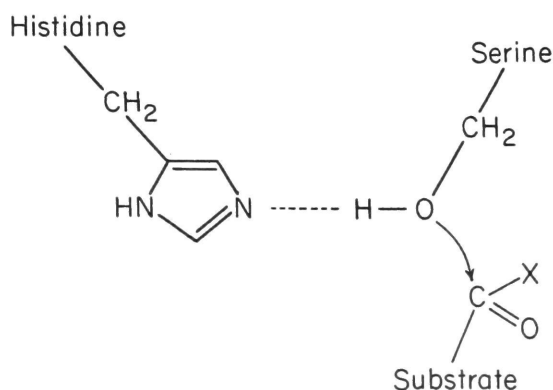
cohol reacts with the acyl-enzyme to generate unlabeled ester, and exchange is effected. If in equation (2) the alcohol were replaced by water, an acid would be produced and the sum of the equations would represent the hydrolytic pathway, equation (1) being the acylation step and equation (2) being the deacylation step. It can be seen that in the exchange reactions the two equations are symmetrical about the acyl-enzyme intermediate and can be written as an equilibrium reaction.



The principle of microscopic reversibility states that the transition states of the forward and reverse reactions of an equilibrium reaction must be identical. Therefore if groups displaying general base and general acid catalysis are involved in the acylation step, they must also be involved in the deacylation step. Bender et al (10) have pointed out that since there appears to be general base catalysis, but no general acid catalysis (at least, the  $pK_a$  of the group must be greater than 13.9) in the deacylation reactions of chymotrypsin, general base-general acid catalysis in the acylation step must be ruled out. The one ionizable group in the acylation reaction is either not present in the deacylation reaction or is not involved in the bond-changing process. These considerations impose a great limitation on any postulated mechanism.

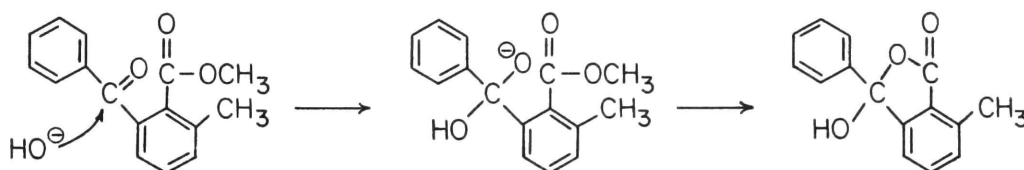
It is instructive to consider the effect of deuterium oxide on the rates of the acylation and deacylation reactions. Bender and Hamilton (11) showed that the rate of acylation of  $\alpha$ -chymotrypsin with *p*-nitrophenyl trimethylacetate, and the rate of deacylation of *trans*-cinnamoyl- $\alpha$ -chymotrypsin, both proceeded 2 to 3 times faster in water than in deuterium oxide. At enzyme saturating concentrations of acetyl-L-tryptophan methyl ester, the catalytic rate constant,  $k_o$ , was 2.83 times greater in water than in deuterium oxide. The demonstration of a deuterium isotope effect constitutes good evidence that the rate-controlling step in each of these enzymic hydrolyses involves the transfer of a proton.

The facts presented so far would seem to be in essential agreement with the diagram below. The histidine, acting as a general base catalyst, helps



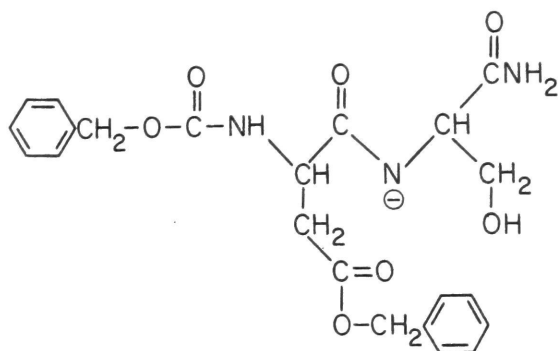
to activate the serine hydroxyl, which reacts with the substrate by nucleophilic attack at the carbonyl. Thus an acyl-serine enzyme is formed, a proton is transferred in the acylation step, and a basic group (imidazole) with a pKa of 7 is involved. A serious objection to this mechanism has been raised by Bernhard (22). When the initial rapid reaction of 2,4-dinitrophenyl acetate with chymotrypsin was studied between pH 7.4 and pH 9.1, no release of protons could be detected. (The pKa of 2,4-dinitrophenol is 3.9, so it should remain as an anion under the reaction conditions). If serine were acylated, the proton of the hydroxyl group should have been released and detected. The data indicate that either an intermediate other than an acyl-serine enzyme is formed, or that an anion attacks the ester.

Interest in alkoxide ions as nucleophilic base catalysts of ester hydrolysis has been greatly stimulated recently by the observation by Newman and Hishida (123) that the alkaline hydrolysis of methyl 2-benzoyl-6-methylbenzoate proceeds at an unexpectedly high rate. The facile hydrolysis seems to be interpreted best by initial attack of hydroxide ion on the ketonic carbonyl followed by intramolecular expulsion of the methoxide ion.

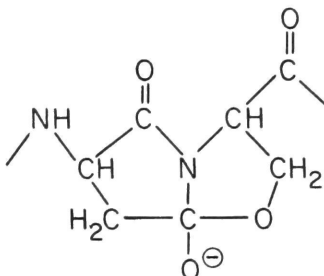


Bender and Silver (16) studied a similar kind of reaction, the alkaline hydrolysis of methyl o-formylbenzoate and found that the rate proceeded more than 100,000 times faster than was expected (from a calculation based on electronic and steric effects).

Related studies by Bernhard et al (24) have demonstrated an unusually rapid rate of alkaline hydrolysis of the benzyl ester of  $\beta$ -benzyl-carbo-benzoxypartylseryl amide. It was proposed that the reaction proceeded by intramolecular attack by the anion of the aspartyl-serine peptide nitrogen since a succinimide derivative was detected as an intermediate.



Bernhard (23) has suggested that the oxygen anion in the structure below (derived from aspartic acid and serine) might be the nucleophile in enzymic reactions.

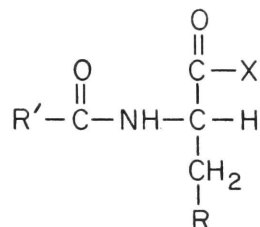


The important point to be learned from the numerous studies cited above is that a clear understanding has not yet been reached of the roles played by histidine, serine and other groups in the bond making and breaking reactions of chymotrypsin. It is therefore possible that extensive revisions in our thinking may be necessary before a satisfactory mechanism for the hydrolysis of specific substrates of  $\alpha$ -chymotrypsin is found.

The concepts discussed above are thought to apply to trypsin as well as to chymotrypsin, although most of the evidence collected has been from studies of the latter enzyme. It is certainly true that the two enzymes resemble each other very closely. Yet in one respect they are completely different. Trypsin catalyzes efficiently the hydrolysis of esters and amides of the basic amino acids, lysine and arginine, while chymotrypsin can best cleave derivatives of the aromatic amino acids, tryptophan, tyrosine and phenylalanine. Thus they exhibit different "specificity."

In 1937, Bergmann and Fruton (19) showed that chymotrypsin and trypsin possessed different structural requirements of their substrates. Neurath found that the enzymes could catalyze the hydrolysis of esters as well as amides (93,153). Niemann and co-workers and Neurath and colleagues have continued Bergmann's early work and have attempted to arrive at an understanding of the specificity site of these enzymes by synthesizing a great variety of substrates and inhibitors and measuring their kinetic parameters. The existence of a number of comprehensive reviews (19,76,121,122) of this work makes it unnecessary to describe the results in detail.

The structural requirements can most easily be discussed in terms of the groups attached to the asymmetric carbon atom of the amino acid substrate:



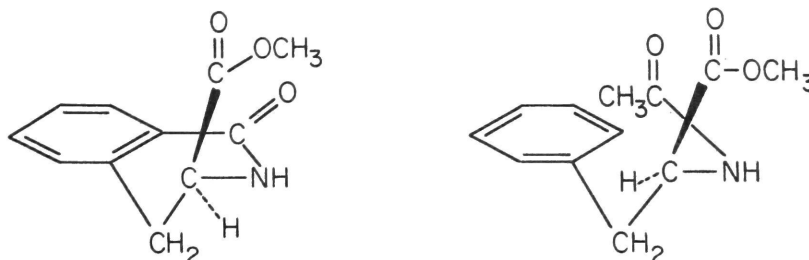
For trypsin, R should be an alkyl chain with a positively charged basic group at the end, e.g.,  $-\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_3^+$  (lysine) or  $-\text{CH}_2\text{CH}_2-\text{NH}-\underset{\text{NH}_2^+}{\overset{\parallel}{\text{C}}}-\text{NH}_2$  (arginine).

For chymotrypsin, R should be an aromatic group, although cyclohexane works equally well as benzene (88). The specificity of chymotrypsin is broader than trypsin, and alkyl groups in this position (e.g., isoleucine, valine) are sufficient to cause appreciable rates of hydrolysis. The distance of the R group from the substrate carbonyl is important. If a methylene group is added or subtracted, the rate of hydrolysis diminishes.

The acylamino group is also important and substitution of it for a carboxamide group (thus producing a malonic acid derivative) results in an inactive substrate. The formamido group ( $\text{R}' = \text{H}$ ) is adequate, indicating that only the amide portion of the group is sufficient for binding.

If the hydrogen attached to the asymmetric carbon is replaced by a methyl group, the substrate will bind to the enzyme but will not react (2). If instead of an L configuration, a D configuration is used, the substrate

binds, but is not hydrolyzed. An interesting exception to this has been found by Niemann and co-workers (75). Thus D-3-carbomethoxydihydrocarbo-styryl was bound to and hydrolyzed efficiently by chymotrypsin while the L isomer reacted much more slowly.



Awad et al. (4) have pointed out that there exists a conformation of this "locked" D configuration which resembles some conformations of normal L-substrates. If their explanation is correct, the relatively more rigid conformation of the carbostyryl derivative should reflect normal substrates in their binding conformations and thus should define to some extent the nature of the specificity site in the enzyme.

Many of the studies referred to above were designed to elucidate the topography of the "specificity site." A rather rigid hole in the enzyme was imagined into which specific substrates of complementary structure could fit, while those possessing the wrong shape would be excluded. This is essentially the lock and key analogy of Emil Fischer (52), as propounded in 1894. With this hypothesis, it has not been possible to deduce much about the enzyme from the hundreds of substrates and inhibitors tested. More recently, Niemann has collected and summarized a great deal of data in terms of hypothetical binding sites on the enzyme (76). He has correlated the available information in terms of the interactions of four enzyme binding modes with the four substituents attached to the asymmetric carbon atom of substrates. By studying good substrates with rather rigid conformations, it should be possible to determine the relative positions in space of the binding modes in the enzyme. The groups of the enzyme actually involved in the binding have not yet been given serious, critical consideration, however.

Another theory of substrate binding and specificity, called the induced-fit theory by Koshland (101), postulates that a specific substrate is bound

to a flexible active site and induces conformational changes in the site leading to the correct alignment of the groups necessary for catalysis. Although non-substrates may bind to the enzyme, the required conformational changes are not induced and therefore no reaction takes place. The observation that indole causes an increase in the rate of solvolysis of acetylchymotrypsin (54) can be interpreted as evidence in support of the induced-fit theory for this enzyme. Also, Hess and co-workers (73,190) have reported spectral evidence for structural changes in chymotrypsin when the enzyme reacts with p-nitrophenyl acetate or diisopropylfluorophosphate. Although the hypothesis is both interesting and useful in explaining certain phenomena, it does not consider in detail what groups or forces are responsible for the substrate binding and the changes in conformation envisioned.

#### Working Hypothesis and Experimental Approach

Before the ideas basic to the work of this thesis are developed, it may be helpful to summarize in general terms what is known about the active site of chymotrypsin, so that a clear concept of the problem at hand can be appreciated. In the enzyme is an area called the active site, which might be composed most simply of serine, histidine and a specificity site. The groups are thought to be very close to each other. It is known that the serine and histidine are not neighbors in a single peptide chain, so their proximity in space must be determined by the tertiary structure of the protein. The exact nature of the nucleophilic attack by the enzyme on a substrate, and the nature of the specificity site are the major problems that remain unsolved. From a consideration of model compounds and the chemistry of serine, it is difficult to account for the great reactivity of  $\alpha$ -chymotrypsin and trypsin on the basis of a favorable positioning of the imidazole and serine hydroxyl alone. Although mechanisms can be written, it still is not understood why these enzymes are so effective. Perhaps an extremely reactive nucleophile is not necessary if the substrate is held and oriented in a position favorable for attack. The necessary catalytic "power" may be derived in part from the fact that the substrate is in a bound form, thus making the reaction comparable to intramolecular catalyses, which have been shown to be extraordinarily efficient. Although an adequate description of the nucleophilic reaction is still lacking, the major problem then becomes

that of defining the nature of the specificity site and of showing that proper substrate binding can cause a large enhancement in a reaction rate.

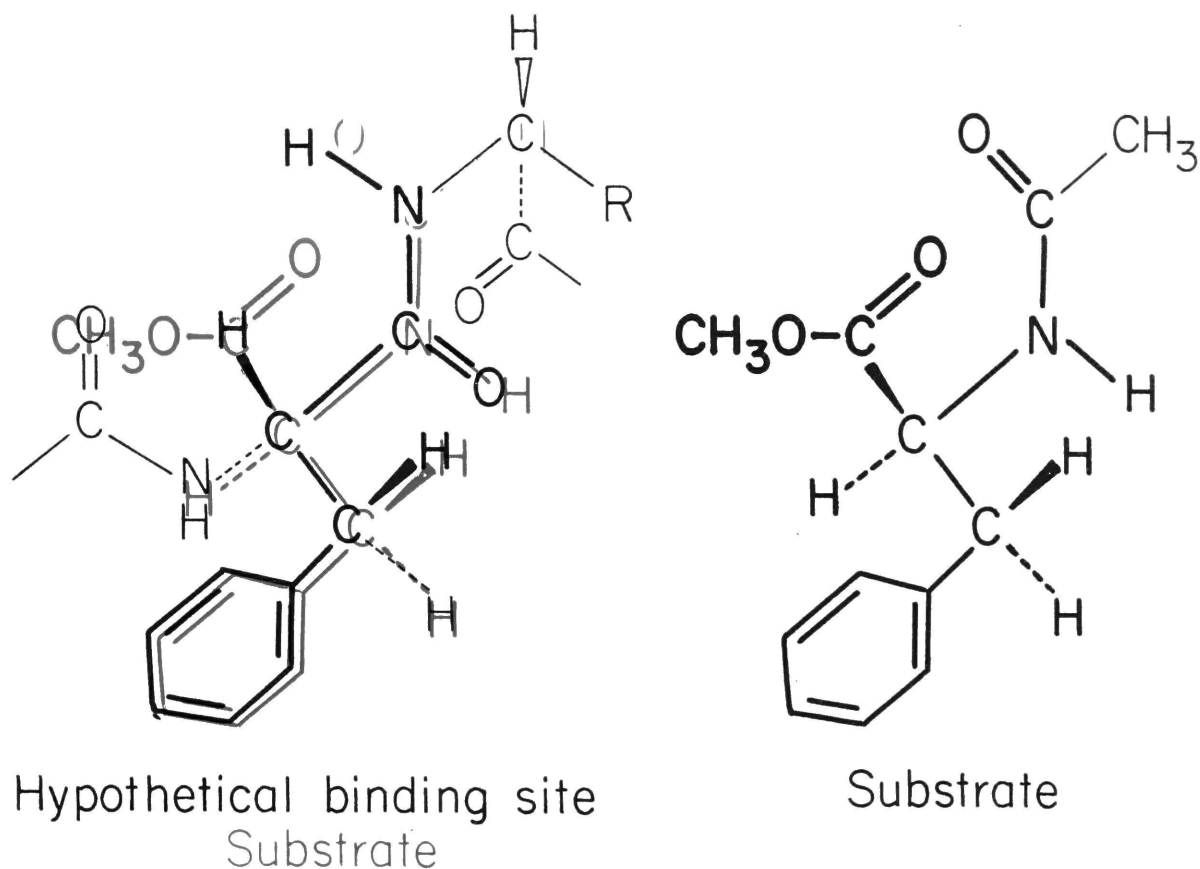
It is possible to make plausible postulates about the general requirements of the active site of an enzyme such as  $\alpha$ -chymotrypsin or trypsin. Unless long-range forces are postulated, the groups responsible for the binding of the substrate, and also the histidine and serine residues, probably must all be essentially in direct contact with the substrate and must therefore be very near to one another. Many specific substrate molecules are quite small (e.g., formyl-L-phenylalanine methyl ester, M.W. = 207), so the active site itself cannot be very large. On the other hand, it must not be too crowded, for the enzymes are endopeptidases and must also be able to accommodate as substrates the specific amino acids situated in peptide chains. What is required for  $\alpha$ -chymotrypsin, for example, is a hydrophobic region to bind the aromatic side chain of the substrate, a region to bind the acylamino group, and the groups composing the nucleophile. Two peptide chain portions are required to position the serine and histidine, and others may be necessary to account for the two binding modes, particularly if "holes" or "slits" are envisioned. That space may indeed be at a premium is suggested by the fact that the amino acids found around the unique aspartyl-seryl group are glycines, which are very small. If a binding "surface" is postulated, the structural requirements are somewhat simplified.

Woolley, Hershey and Koehelik (189) have advanced the hypothesis that enzymic specificity is due to an attraction between like molecules. In chymotrypsin for example, an aromatic amino acid (phenylalanine, tyrosine, or tryptophan) is thought to be located in the active site, while in trypsin, either lysine or arginine is involved. As the substrate diffuses into the vicinity of the active site, attractive forces may tend to hold the substrate to this amino acid and orient it so that the carbonyl group is in precisely the correct position to be attacked by the nucleophile. Two major groups are therefore envisioned: the nucleophile and the binding amino acid. Their proper location on the enzyme surface relative to one another is presumed to be determined by the tertiary structure of the entire enzyme, and at least two or three portions of peptide chains may be involved.

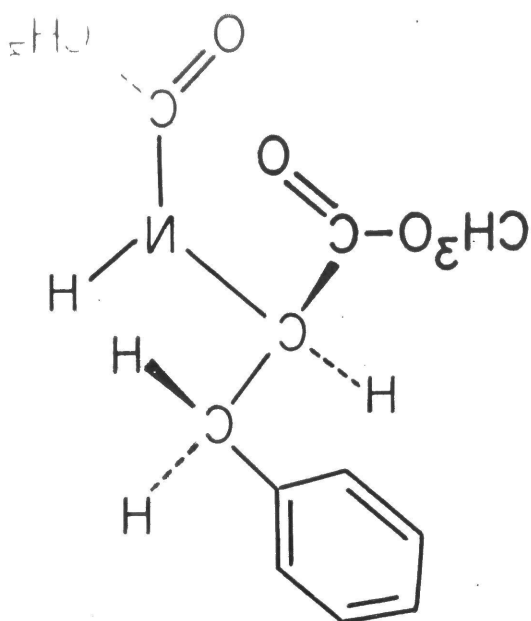
There is growing evidence for the existence and importance of hydrophobic bonding and attractive van der Waals or dispersion forces between

side chains of amino acids in proteins and peptides (94,171,178). They are thought to play an important role in determining the tertiary structure of these molecules. For example, Kendrew (97) reports that in crystalline myoglobin, X-ray analysis indicates that non-polar interactions are of paramount importance in stabilizing the tertiary structure of the molecule, the number of interactions exceeding polar interactions by a factor of ten. Although the nature and strength of these forces have not yet been demonstrated adequately experimentally, a firm theoretical treatment of dispersion forces has been worked out both with classical and with quantum mechanics (47). The strength of dispersion forces between two molecules the approximate size of amino acid side chains was crudely estimated by Jehle et al (85) to be about  $0.6 \text{ kcal. mole}^{-1}$ . More recently, Salem (146) calculated that the attractive energy of interaction of two long saturated fatty acid chains  $4.8 \text{ \AA}$  apart was approximately  $0.5 \text{ kcal. mole}^{-1}$  per methylene group, i.e.,  $8.4 \text{ kcal. mole}^{-1}$  for two stearic acid molecules. In the latter work it was pointed out that the dispersion forces can be very strong when the two interacting species are able to approach each other closely, but fall off rapidly at greater distances since the energy of attraction varies inversely with the fifth or sixth power of the distance. Thus those molecules which can approach each other at many points very closely can experience large energies of attraction, while those which cannot come into close contact will be attracted by dispersion forces only weakly. It is our contention that these forces play an important if not dominating role in the binding of the substrate to the enzyme. Hydrogen bonding or dipole interactions of the amide portion of the substrate may also be involved.

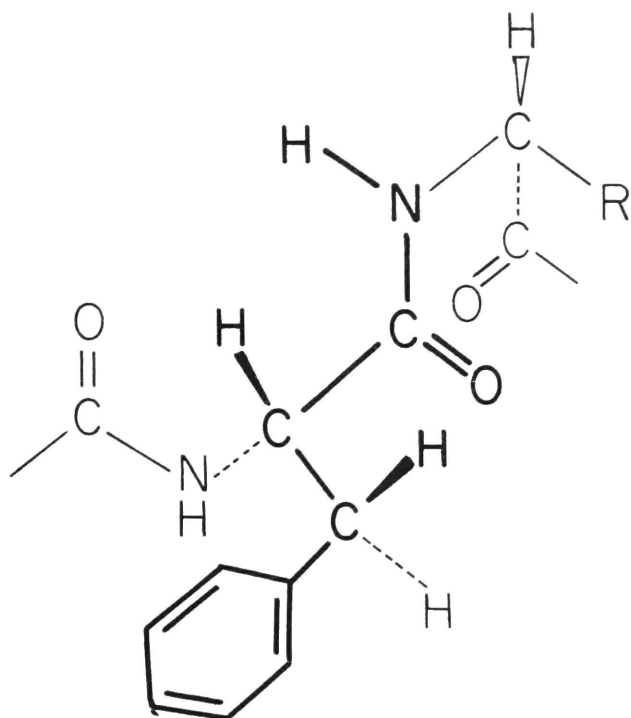
The hypothesis can be considered in more exact detail. Imagine a non-helical portion of a peptide chain of L-amino acids which contains an aromatic residue, e.g., phenylalanine. It is possible to construct a conformation of the phenylalanine portion of the peptide where the aromatic side chain and the carbonyl group of the same amino acid lie in approximately the same plane. When this is illustrated graphically (Figure 3), the alpha hydrogen is directed above the plane of the paper, while the amino group of the phenylalanine residue lies below the plane of the paper. Acetyl-L-phenylalanine methyl ester, for example, can be placed on this model in such a way that the phenyl rings lie directly on top of each other and the acetamido group lies on top of the peptide-bound phenylalanine carboxamide group in such



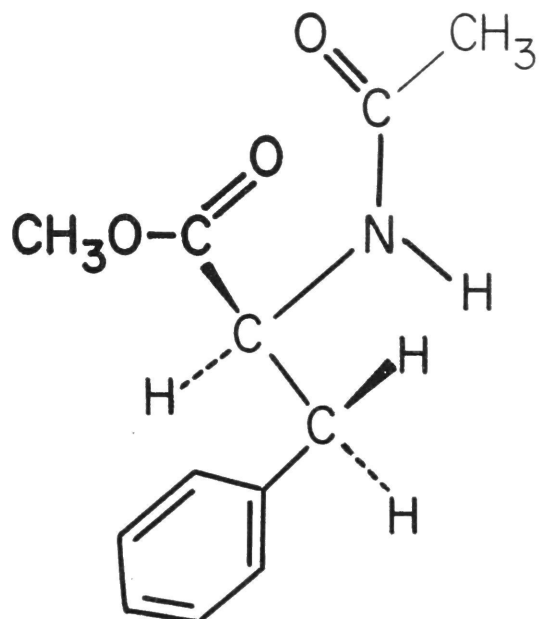
**Figure 3.** When the specific chymotrypsin, acetyl-L-phenylalanine methyl ester, is placed on the hypothetical binding site, it is noticed that corresponding phenyl-phenyl and amide-amide interactions are possible. With substrates of the L configuration, the ester group stands away from the complex toward the viewer, while the hydrogens on the alpha carbons of both amino acid derivatives interact only negligibly.



Substrate



Hypothetical binding site



Substrate

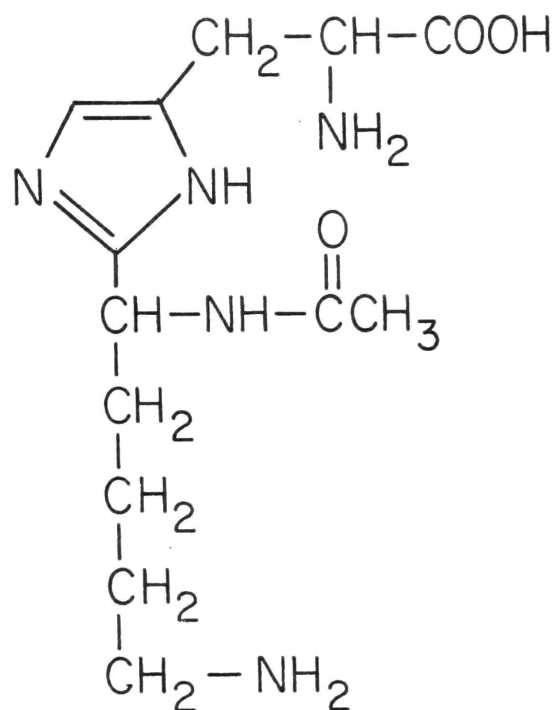
Figure 3. When the specific chymotrypsin, acetyl-L-phenylalanine methyl ester, is placed on the hypothetical binding site, it is noticed that corresponding phenyl-phenyl and amide-amide interactions are possible. With substrates of the L configuration, the ester group stands away from the complex toward the viewer, while the hydrogens on the alpha carbons of both amino acid derivatives interact only negligibly.

a way that dipole-dipole attraction could take place. It then follows necessarily that with L substrates, the carboxylic acid derivative to be hydrolyzed stands up from the complex in an unhindered position, while the two alpha hydrogens (one from the substrate and one from the binding amino acid) interact only negligibly because of their small size. If a substrate of the D configuration is used, the carboxylic acid derivative of the substrate is forced into the position occupied by the alpha hydrogen of the binding amino acid and is hindered. This might therefore be a position unfavorable for catalysis.

The simple model can make understandable: a. binding of the aromatic side chain; b. binding of the acylamino portion; c. optical specificity; and d. the fact that if a methyl group is substituted at the alpha carbon, the reaction rate is decreased. Also, the hypothetical site is extremely simple and meets the requirement of restricted size. Exceptional rigidity of the tertiary structure of the protein is not necessary in order to explain specificity of selection. It is fully realized, of course, that merely building an adequate model of the binding site does not in itself indicate that such a binding site exists in these enzymes.

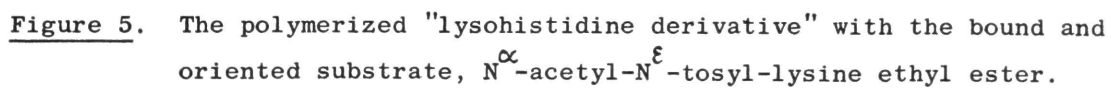
One of the attractive features of the general hypothesis is that it can be tested experimentally. The purpose of the work described in this thesis was to demonstrate a specific enhancement of a reaction rate due to the attraction of a substrate to a catalyst containing a similar structural unit. It was proposed to synthesize the "lysohistidine derivative" (2-(1-acetamido-5-aminopentyl)-histidine) shown in Figure 4, and to test the catalyst with esters of N<sup>α</sup>-acetyl-lysine. This particular catalyst was chosen for the following reasons. The lysine-like side chain at the 2-position of the imidazole should resemble the possible binding site of trypsin and thus should confer trypsin-like specificity on the catalyst. Attachment of the lysine-like side chain at the 2-position of imidazole was proposed so that when the substrate was bound to the side-chain (Figure 5), the carbonyl group would be in a favorable position to be attacked by the imidazole nitrogen.

The idea that the epsilon amino group of the lysine substrate and the corresponding one in the catalyst may actually attract each other at pH 8 may not be appealing. At that pH, both groups should be predominantly in



"Lysohistidine derivative"

Figure 4. The "Lysohistidine Derivative".



the positively charged ammonium form and would be expected to repel each other. It was hoped to find a pH at which attraction would occur between the model and substrate. Since the presence of one charge will tend to suppress the formation of a nearby like charge, this was not considered too unlikely. Although these considerations might apply to the alkylamino group of a lysine residue, they probably do not apply to the more basic guanidino group of an arginine residue. Therefore, such a picture would be more compatible with a lysine than with an arginine residue in the specificity site of trypsin. If no binding to the model compound could be demonstrated, however, it would still be possible to test the catalyst with a substrate containing a suitably situated negative carboxylate group (or tosylamino group, which ionizes at high pH) and thus test the binding effectiveness of the salt linkage which could be expected to form between the oppositely charged groups.

Rather than a simpler "lysoimidazole derivative", the "lysohistidine derivative" was proposed so that the entire molecule might eventually be incorporated into polypeptides. In this way it would be possible to test the effects of high molecular weight on the ability of two molecules to bind to each other and react. In addition to polymers composed only of the "lysohistidine derivative", a great variety of mixed polymers could be made by co-polymerization with other amino acids. It is evident that a histidine derivative enables a more flexible experimental approach to be made.

As a second approach to the same problem, it was proposed to synthesize the cyclic dipeptide, cyclo-D-histidyl-L-phenylalanyl. This compound might be expected to mimic the specificity of chymotrypsin. It is evident from Figure 6 that the same sort of planar binding site can be envisioned at the phenylalanine residue as was proposed for the hypothetical enzyme binding site (Figure 3). Histidine of the D configuration was chosen so that the imidazole would be properly located near the protruding carbonyl group of the bound substrate.

Both proposed models involve an imidazole as the catalytic entity, while in the enzymes both serine and imidazole are thought to be the active species. Histidine or imidazole derivatives were chosen for a number of reasons. First, it is known rather well from kinetic studies on model compounds what imidazole can do and how it acts. On the other hand, our understanding of exactly how the serine hydroxyl is involved in the enzymic mechanism is inadequate, and no effective model compounds involving the

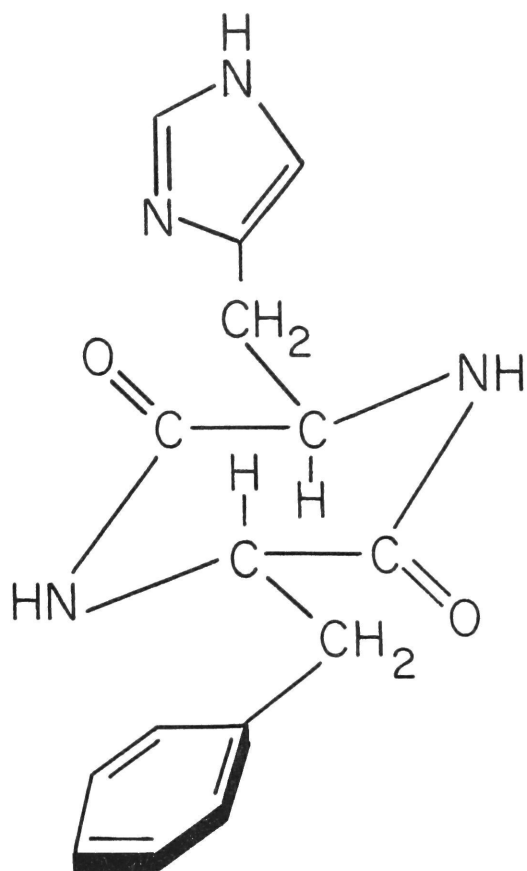


Figure 6. cyclo-D-Histidyl-L-phenylalanyl.

serine hydroxyl as a nucleophile are known.\* The best chance of synthesizing an active catalyst therefore appeared to lie in imidazole derivatives.

As a simpler study, it was also proposed to synthesize 2-benzylimidazole and 2-isobutylimidazole and compare their catalytic properties in the hydrolysis of p-nitrophenyl acetate and p-nitrophenyl phenylacetate (Figure 7). If the phenyl group of the latter substrate and the phenyl group of the 2-benzylimidazole were to attract each other and thus stabilize the transition state, a specific enhancement of rate might be observed. These simple compounds could then be used in a detailed study of the nature of such interactions.

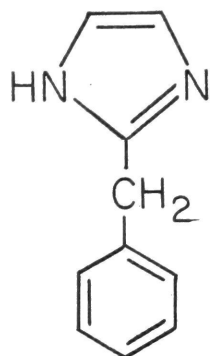
### Chemistry

As one approach to testing the hypothesis concerning the nature of enzyme-substrate interactions, it was proposed above to synthesize the "lyso-histidine derivative" (2-(1-acetamido-5-aminopentyl)-histidine) shown in Figure 4, and to polymerize it. The chemical literature contains no precedents for the synthesis of such compounds. The successful synthesis of the "protected lysohistidine derivative" (2-(1-acetamido-5-p-toluenesulfonamidopentyl)-4-(2-carbethoxy-2-carbobenzoxamidoethyl)-imidazole), shown in Figure 8, will be described in Part II. In the following discussion will be presented various possible approaches to the synthesis of such imidazole derivatives and the reasons why the "protected lysohistidine derivative" was prepared as an appropriate precursor of the polymerized "lyso-histidine derivative."

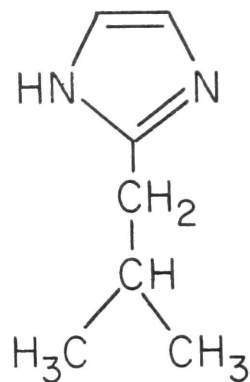
Amino acid derivatives are usually polymerized by the N-carboxy- $\alpha$ -amino acid anhydride method (57,92). When an N-carboxy- $\alpha$ -amino acid anhydride (Leuchs' anhydride) reacts with an initiator (e.g., an amine or sodium methoxide) an amide or ester of the amino acid is formed with the concomitant liberation of carbon dioxide and the exposure of the amino group of the amino acid derivative. This amino group is capable of reacting with another N-carboxy anhydride in the same way, and as the process is repeated a polypeptide is formed. The cyclic anhydrides are commonly prepared by

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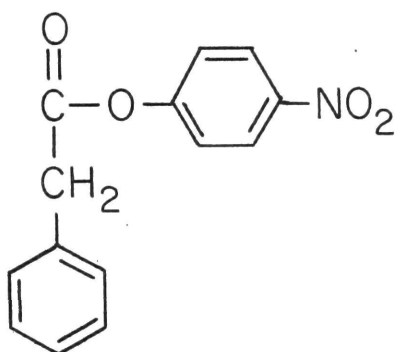
\* The only comparable case is catalysis of the hydrolysis of labile phenyl esters by tris-(hydroxymethyl)-aminomethane buffer where the attack is thought to be by a hydroxyl oxygen hydrogen bonded to the amine nitrogen (86).



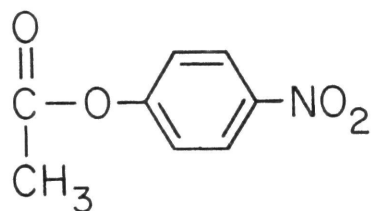
2-benzylimidazole



2-isobutylimidazole



p-nitrophenyl phenylacetate



p-nitrophenyl acetate

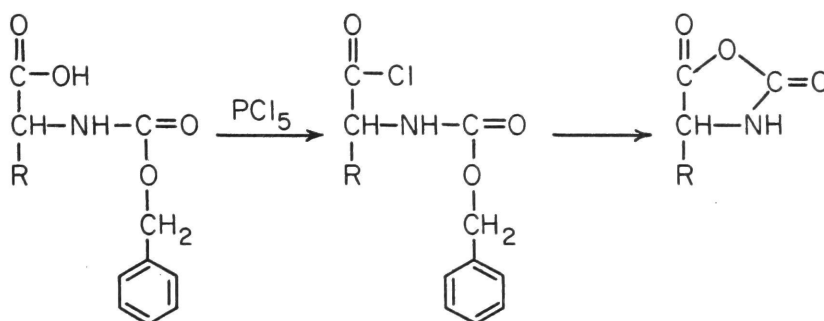
Figure 7.



**Figure 8.** The "Protected Lysohistidine Derivative".

two methods (57): a. the action of heat on a carboalkoxylated  $\alpha$ -amino acid chloride, or b. the phosgenation of a free amino acid. The latter method is now generally preferred because the product is more easily purified. A highly purified N-carboxy- $\alpha$ -amino acid anhydride is required if polypeptides of large molecular weight are desired (51).

The preparation of poly-L-histidine was first reported in 1957 by Patchornik, Berger and Katchalski (131). They prepared the acid chloride of N<sup>ε</sup>-carbobenzoxy-N<sup>Im</sup>-benzyl-L-histidine, which cyclized at 25° to the corresponding N-carboxy anhydride and benzyl chloride. The N-carboxy anhydride

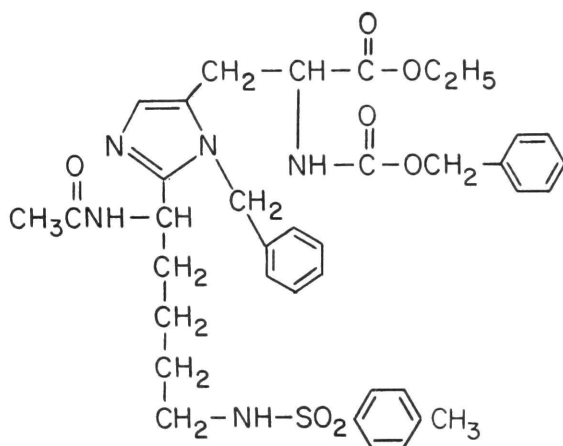


was caused to polymerize in solution by adding either di- or tri-ethylamine as initiator. It was necessary to protect the imidazole ring during these reactions, and a benzyl group was employed. The group was removed after the polymerization by treatment with sodium in liquid ammonia (45) to yield the desired poly-L-histidine.

In order to polymerize the "lysohistidine derivative", it would be necessary to prepare the corresponding N-carboxy anhydride with an acylated  $\epsilon$ -amino group on the 2-pentyl (lyso) side chain and with a protecting group, such as the benzyl group, on the nitrogen of the imidazole ring. However, these protecting groups would have to be cleaved later in such a way that the acetamido group on the alpha carbon of the 2-pentyl side chain and also the peptide bonds of the polypeptide would not be hydrolyzed. Since sodium in liquid ammonia is necessary for the removal of the benzyl group from N-benzylimidazole derivatives (45), the *p*-toluenesulfonyl (tosyl) group, which is also cleaved by this reaction, was chosen to protect the  $\epsilon$ -amino group. Tosylamino groups are quite stable under acidic and basic conditions and are ideal as protecting groups if a cleavage step using sodium in liquid ammonia is necessary anyway.

The synthesis of the histidine derivative required that all reactive groups be masked, so the  $\alpha$ -amino and carboxylate groups on the histidine side chain of the "lysohistidine derivative" had to be protected also. The carbobenzoxy group and the ethyl ester were chosen so that the N-carboxy anhydride derivative could be most readily formed. It was envisioned that the ethyl ester could be hydrolyzed to the corresponding carboxylic acid, an acid chloride prepared and the N-carboxy anhydride formed in the same manner as described for the polymerization of L-histidine above. If the N-carboxy anhydride derivative were to resist formation through the acid chloride, the carbobenzoxy group could be selectively cleaved by catalytic hydrogenation and the N-carboxy anhydride could then be prepared by phosgenation.

The fully protected "lysohistidine derivative" desired, therefore, is shown below:



There are two basically different approaches to the synthesis of this derivative. The compound might be prepared by coupling an appropriate side chain at the 2-position of the imidazole ring of a suitable histidine derivative. Alternatively, the imidazole ring with the desired side chains attached could be synthesized de novo from appropriate precursors.

It was appealing to consider ways to couple an alkyl side chain at the 2-position of the imidazole ring of histidine, because fewer preparative steps would likely be necessary in such a synthesis. Carbon alkylations of imidazoles are rare, and imidazole derivatives fail to undergo the Friedel-Crafts acylation reaction (Hofmann (82), p. 59). When 1-methyl-imidazole

is passed through a red-hot tube, 2-methylimidazole is formed (177,184) but the severe conditions of the reaction rule out the possibility of its use here. The only other alkylation reaction known is the coupling of an aldehyde to imidazole; various imidazole derivatives have been hydroxymethylated with formaldehyde (16,77,59,185). A number of attempts to synthesize 2- $\alpha$ -hydroxyalkylimidazole derivatives with aliphatic aldehydes will be described in Part II, but it was not found possible to add large alkyl groups to the position by this method.

The second general approach was the de novo synthesis of the imidazole ring with the histidine and "lyso" side chains already attached, or with simpler side chains from which the histidine or "lyso" chains could be developed. It is instructive to consider the classical histidine syntheses in this regard. Pyman (137), in 1911, first synthesized histidine via the following pathway. Diaminoacetone was condensed with potassium thiocyanate to give 2-thiol-4-aminomethylimidazole, which was oxidized to 4-aminomethylimidazole with nitric acid. This derivative was converted to 4-hydroxymethylimidazole with nitrous acid, and then to 4-chloromethylimidazole with phosphorous pentachloride. The latter was condensed with diethyl sodiochloromalonate to give a product which on hydrolysis, decarboxylation and amination formed racemic histidine. The reactions are included in Figure 9.

A more practical preparation of 4-hydroxymethylimidazole from fructose, ammonia and formaldehyde has been described by Totter and Darby (174). The method is essentially a modification of the so-called Weidenhagen imidazole synthesis (Hofmann (82), p. 38). A number of improved pathways from this imidazole derivative to histidine also have been developed. Pyman oxidized 4-hydroxymethylimidazole to 4-formylimidazole and then condensed the aldehyde with hippuric acid in the presence of acetic anhydride to form a product which, on cleavage of the oxazolone ring, reduction of the double bond and hydrolysis, gave histidine (138). More recently Davis and Levy (41a) condensed 4-formylimidazole with 2-mercaptothiazol-5-one and treated the product with red phosphorous and hydrogen iodide to obtain histidine in excellent yield. It is also more common now to treat 4-chloromethylimidazole with diethyl sodioacetamidomalonate (rather than diethyl sodiochloromalonate) in order to prepare the  $\alpha$ -amino acid side chain. The reactions are shown in Figure 9.



By these syntheses it is demonstrated that histidine derivatives can be obtained readily from 4-hydroxymethylimidazole derivatives. For example, 2-methyl-L-histidine has been prepared (125) from 2-methyl-4-hydroxymethylimidazole by the pathway involving diethyl sodioacetamidomalonate. The 2-methyl-4-hydroxymethylimidazole was successfully synthesized (125) by substituting acetaldehyde for formaldehyde in the Weidenhagen synthesis with fructose, although it has been stated that larger aliphatic aldehydes will not react in this method (Hofmann (82), p. 39). A description of our own unsuccessful attempts to react larger aliphatic aldehydes in this way is given in Part II.

Pyman's original synthesis, in which the number 2 carbon of the imidazole ring was derived from thiocyanate, cannot be used to prepare 2-substituted imidazoles, and the Weidenhagen synthesis likewise could not be employed successfully in the synthesis of the complicated 2-( $\alpha$ -aminoalkyl)-imidazole derivatives required here. Although a few simple 2-( $\alpha$ -aminoalkyl)-imidazoles are otherwise known (56,162), the methods used in their syntheses also could not be applied to this problem. It was necessary to examine other known procedures and find a general synthetic route to such imidazole derivatives.

A rather poorly characterized reaction of benzamidines with phenacyl halides, first reported by Kunckell (105), seemed attractive for a number of reasons. If the reaction could be successfully executed with aliphatic reactants, amidine and  $\alpha$ -haloketone derivatives could possibly be used which would form an imidazole with completed side chains, rather than an incomplete imidazole derivative. This route would also make possible the synthesis of the compound with the configurations of the two asymmetric carbons of the "lysohistidine derivative" determined, if the ring forming reaction could be carried out without causing racemization. In addition, by using an N-benzylamidine derivative, a 1-benzylimidazole derivative could be formed directly. The reaction of amidines with  $\alpha$ -haloketones is discussed in detail in Part II and was the only route which led successfully to the desired "protected lysohistidine derivative."

Some other general procedures were also considered: a. The preparation of 2-imidazolines (Hofmann (82), p. 213) from derivatives of ethylenediamine and esters is rather simple and has found general use. However, conversion

of the 2-imidazolines to imidazoles has been effected only by catalytic dehydrogenation at temperatures above  $200^{\circ}$  (Hofmann (82), p. 42). It was thought that such conditions were too severe to be used successfully in the synthesis of the "protected lysohistidine derivative", although the method served well in the preparation of 2-benzylimidazole and 2-isobutylimidazole. b. A number of 2-alkyl-4,5,-imidazolecarboxylic acid derivatives have been prepared from dinitrotartaric acid, ammonia and an aldehyde (111,161). An unsuccessful attempt is described in Part II to selectively reduce one of the carboxylic acid groups to a hydroxymethyl group, and to decarboxylate the other. c. The recent method of Brederick (27) of preparing imidazoles from oxazoles with formamide was not considered because the complicated oxazole derivatives are also difficult to prepare. d. Imidazoles are converted to diacylamido-ethylene derivatives with a number of acyl chlorides in a reaction known as the Bamberger imidazole ring cleavage (7). Windaus (187) showed that the imidazole ring will form again in the presence of carboxylic acid anhydrides. The resulting imidazole derivative is substituted at the 2-position, acetic anhydride giving 2-methylimidazoles and propionic anhydride giving 2-ethylimidazoles. This route was also probed and is discussed in Part II. With the complicated acid anhydride required to introduce the desired 2-alkyl derivatives, the reaction failed.

As a second approach to the problem of the nature of enzyme-substrate binding, it was proposed to synthesize the diketopiperazine, cyclo-D-histidyl-L-phenylalanyl, as mentioned above. The first 2,5-diketopiperazine, leucine anhydride, was described by Bopp (25) in 1849 and was synthesized by Hesse and Limpricht (78) in 1860. Therefore such derivatives have been known for a long time. Rather than synthesizing the cyclic dipeptide from the free amino acids, Curtius and Goebel (41) developed an alternative route which depended on the more facile reactions of amino acid esters. All of these reactions gave rise to symmetrical or homogeneous 2,5-diketopiperazines, i.e., ones containing the same two amino acid residues.

The first unsymmetrical or mixed diketopiperazine, cyclo-alanyl-glycyl, was prepared by Fischer and Otto (53) in 1903 from chloroacetylalanine ethyl ester by the action of alcoholic ammonia. Such derivatives are now routinely prepared by the intramolecular condensation of dipeptide esters (Greenstein and Winitz (57), p. 799). Therefore the appropriate dipeptide ester, histidyl-

phenylalanine ethyl ester was prepared and was caused to condense to cyclo-histidyl-phenylalanyl. The chemistry is described and discussed in detail in Part II.

The "protected lysohistidine derivative" (Figure 8), but not the intended polymerized form of the "lysohistidine derivative" (Figure 4), was synthesized, as was cyclo-histidyl-phenylalanyl (Figure 6), 2-benzylimidazole and 2-isobutylimidazole. In order to determine whether these compounds possessed any unusual catalytic properties, it was necessary to assay them kinetically. The kinetic studies designed to measure any enzyme-like specificity or enhanced catalytic "power" due to the peculiar structure of the catalysts are described in detail in Part III. In no instance could any unusual catalytic property be demonstrated.

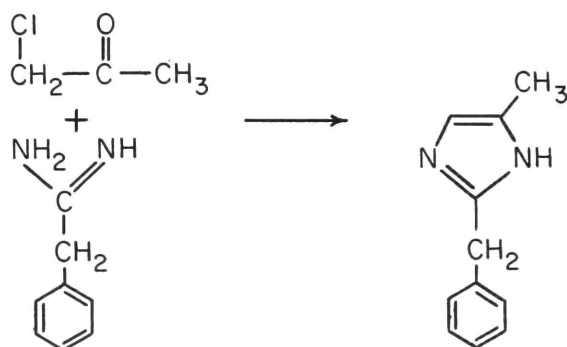
**PART II****CHEMICAL SYNTHESSES**

### A. The Synthesis of the "Protected Lysohistidine Derivative"

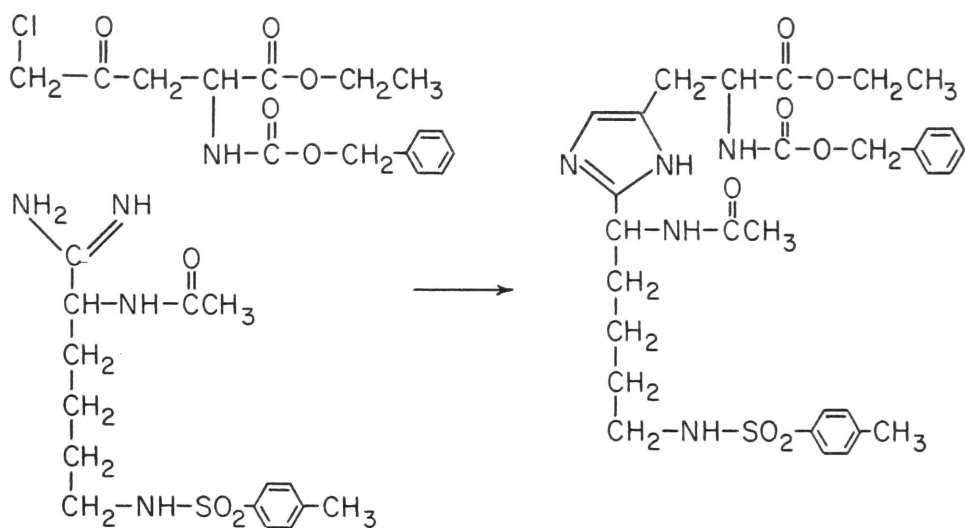
The "protected lysohistidine derivative", Figure 8, was synthesized from ethyl L-2-carbobenzoxamido-4-oxo-5-chlorovalerate and 2-acetamido-6-*p*-toluenesulfonamidocaproamide. The preparative reaction is shown in Figure 10. Since the amidine and chloromethylketone are both complicated compounds which were previously unknown and which proved to be difficult to prepare, it was first desirable to test the applicability of this little-known imidazole ring synthesis by making simpler imidazole and histidine derivatives with it.

#### 1. The amidine- $\alpha$ -haloketone reaction.

Kunckell (105) reported that phenacyl bromide reacted with excess benzamidine in refluxing chloroform to give a large yield of 2,4-diphenylimidazole. In other instances, a variety of benzamidines were caused to react with phenacyl chloride (37), and 3-bromo-3-acetopropan-1-ol was treated with formamidine to give a very poor yield of 4- $\beta$ -hydroxyethyl-5-methylimidazole (48). However, in no cases have other aliphatic amidines been used as reactants. Since phenacyl halides are more reactive than aliphatic  $\alpha$ -haloketones, and since benzamidines and formamidine might also be exceptionally reactive, it was not known whether the reaction could be successfully carried out with aliphatic reactants. In order to determine this, phenylacetamidine was treated with chloroacetone in refluxing chloroform. The desired 2-benzyl-



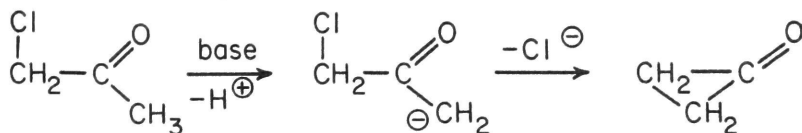
4-methylimidazole was isolated as a pure bioxalate salt in 45% yield, thus demonstrating that the reaction might be successfully applied to the more complicated aliphatic reactants. A detailed description of the experimental procedures used in the preparation of phenylacetamidine and the imidazole is given in Preparations 1, 2 and 3.



Synthesis of the "protected lysohistidine derivative"

**Figure 10.** Synthesis of the "Protected Lysohistidine Derivative".

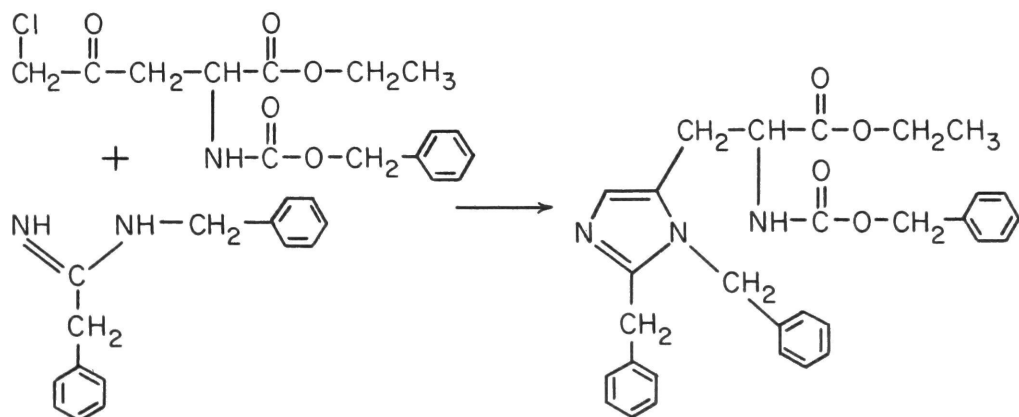
There are a number of likely side reactions which can cause complications and lower yields. Amidines are strong bases and might catalyze reactions involving the two active methylene groups adjacent to the ketone. For example, a cyclopropanone derivative could form from the chloromethylketone, or the



familiar Favorskii reaction could occur to yield acid derivatives. The amidine could hydrolyze to an amide if conditions were not kept strictly anhydrous (water is produced in the ring forming reaction). Also an amidine might be alkylated a second time before ring closure could occur, or the imidazole product itself could be alkylated on the nitrogen by the chloromethylketone. The technique of slowly dropping the  $\alpha$ -haloketone into an excess of amidine was used to minimize the alkylation side reactions.

## 2. N<sup>+</sup>-Carbobenzoxy-1,2-dibenzylhistidine.

In order to gain more experience in the synthesis of complicated histidine derivatives, it was proposed to react a simple amidine with ethyl-L-2-carbobenzoxamido-4-oxo-5-chlorovalerate. The reaction was regarded as a pilot synthesis of the one involving the amidine derived from lysine, which proved to be difficult and expensive to prepare. The amidine of choice was N-benzylphenylacetamidine.



This N-alkylamidine however would be expected to generate an N-substituted imidazole which, it will be recalled, is desirable if the histidine derivative is to be polymerized. Reactions involving N-alkylamidines and  $\alpha$ -halo-ketones have never been described before.\* N $^{\alpha}$ -Carbobenzoxy-1,2-dibenzyl-histidine was prepared in this way.

The amidine. The desired N-benzylphenylacetamidine was first described by Neber and Uber (120), who isolated it as a benzenesulfonate salt from a complicated mixture of compounds resulting from the interaction of O-benzene-sulfonyldibenzylketoxime and ammonia. They were unable to prepare the compound by treating ethyl phenylacetimidate with benzylamine; the method yielded only N,N-dibenzylphenylacetamidine. However, by using one equivalent of benzylamine, we were able to collect a 55% yield of pure N-benzylphenylacetamidine as the hydrochloride salt. Experimental details are described in Preparation 4. An attempt to synthesize N-benzylphenylacetamidine by heating benzylamine, *p*-toluenesulfonic acid and phenylacetonitrile at 260-270 $^{\circ}$ , according to the general method of Oxley and Short (129), was less successful and a poor yield was obtained.

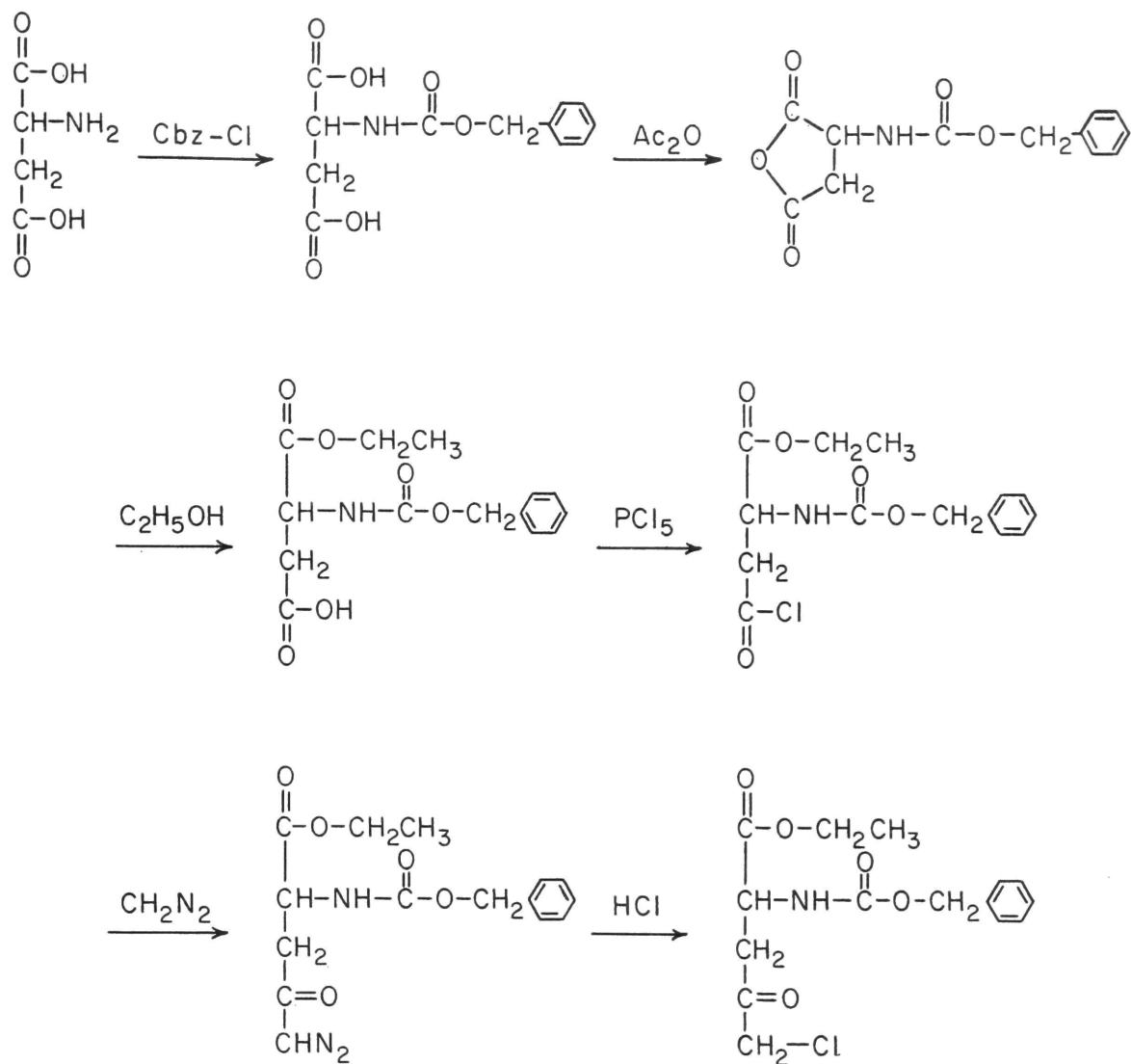
The chloromethylketone which would yield the desired histidine side chain is ethyl L-2-carbobenzoxamido-4-oxo-5-chlorovalerate. The synthetic reaction steps leading to the compound are shown in Figure 11. Experimental details are described in Preparations 5 through 9.

The esters. A satisfactory procedure for the preparation of N-carbobenzoxy-L-aspartic acid has been described by Bergmann and Zervas (20), and yields up to 85% were obtained. A description of the reaction is given in Preparation 5.

The preparation of the  $\alpha$ -ethyl and  $\alpha$ -benzyl esters of N-carbobenzoxy-L-aspartic acid has also been described previously. Thus Bergmann, Zervas and Salzmann (21) prepared N-carbobenzoxy-L-aspartic anhydride by heating briefly N-carbobenzoxy-L-aspartic acid in acetic anhydride. The  $\alpha$ -benzyl ester was then made by treatment of the anhydride with benzyl alcohol at 100 $^{\circ}$ . The ester which they isolated was shown to be that of the  $\alpha$ -carboxyl group, because subsequent treatment with phosphorous pentachloride and

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\* Schipper and Day (148) mention the use of N-alkylbenzamidines in this connection, but the reference cited (37) does not describe any such reactions.



Synthetic route to the chloromethylketone

**Figure 11.** Synthetic Route to the Chloromethylketone.

ammonia, and hydrogenation, yielded L-asparagine. That racemization did not occur during the formation of the anhydride (as might be anticipated if for example appreciable amounts of oxazolone were formed) was shown by regeneration of the N-carbobenzoxy-L-aspartic acid and measurement of its optical activity. The success of the method depends on a very brief heating time. When larger amounts of N-carbobenzoxy-L-aspartic acid are used, it is necessary to heat longer in order to bring the acetic anhydride solution to its boiling point, and partial racemization occurs.

Similarly, Le Quesne and Young (109) prepared  $\alpha$ -ethyl N-carbobenzoxy-L-aspartic acid, except that the N-carbobenzoxy-L-aspartic anhydride was made at room temperature for greater lengths of time, as recommended by Miller et al (118). Ammonolysis of the product yielded N-carbobenzoxy-L-isoasparagine, thus indicating that the  $\alpha$ -ethyl ester was formed. Pure  $\alpha$ -ester could be obtained only by preferential extraction of the contaminating  $\beta$ -ester from ether with small amounts of sodium bicarbonate solution. This was possible because the  $\alpha$ -carboxylic acid is a stronger acid than the  $\beta$ -acid, and is extracted first.

Thus small quantities of  $\alpha$ -benzyl N-carbobenzoxy-L-aspartic acid were prepared by the method of Bergmann et al (21) (Preparation 7). This method could not be used to prepare the large amounts of  $\alpha$ -ethyl N-carbobenzoxy-L-aspartic acid required, because of the racemization resulting from prolonged heating. Adequate yields (about 50%) of the  $\alpha$ -ethyl ester were obtained by following the directions of Le Quesne and Young (109) (Preparation 6).

It is interesting, and not very obvious, that unsymmetrical acyl-aspartic anhydrides should give so predominantly the  $\alpha$ -carboxylic acid esters when reacted with alcohols or alkoxides. Other examples include N-benzoyl-L-aspartic anhydride and methanol (134), and N-tosyl-L-aspartic anhydride and sodium methoxide (66). In contrast, the treatment of N-phthalyl-L-aspartic anhydride with ammonia in an anhydrous solvent yields mostly the  $\beta$ -carboxamide derivative (99), while in aqueous solutions a mixture of the  $\alpha$ - and  $\beta$ - (or predominantly the  $\alpha$ -) carboxamide derivative is formed (173). In addition to the solvent, the acyl group may also influence the direction of ring opening (20,38). Neglecting steric factors, base catalyzed nucleophilic attack might be expected to occur more readily at the  $\alpha$ -carboxyl group (the stronger acid) of the anhydride, while acid catalysis in aqueous solvents might be expected to favor the formation of  $\beta$ -derivatives.

The chloromethylketone. The synthesis of chloromethylketones from diazoketones and hydrogen chloride is well known (175). Diazoketones are intermediates in the Arndt-Eistert synthesis (5) and can be readily prepared by treatment of an acid chloride with excess diazomethane. The method was applied by Harington and Overhoff (67) in the preparation of a related compound, ethyl 2-acetamido-4-oxo-5-chlorovalerate, from  $\alpha$ -ethyl N-acetyl-aspartate. When their procedure was applied to  $\alpha$ -benzyl N-carbobenzoxy-L-aspartate, a low yield of product was obtained. By preparing the acid chloride in ether (21) rather than in acetyl chloride, and by making a few alterations in the procedure with diazomethane, 70% yields could be obtained in the synthesis of ethyl L-2-carbobenzoxamido-4-oxo-5-chlorovalerate. Furthermore, the optical activity of the product was retained. Experimental details are given in Preparations 8 and 9.

The histidine derivative. A solution of ethyl L-2-carbobenzoxamido-4-oxo-5-chlorovalerate was added dropwise to N-benzylphenylacetamidine in dioxane, as described for the synthesis of 2-benzyl-4-methylimidazole. In order to assay the amount of histidine derivative formed, an aliquot of the crude reaction mixture was removed and hydrolyzed with acid to 1,2-dibenzylhistidine. The hydrolysate was subjected to paper electrophoresis at pH 5 and the relative intensities of the ninhydrin positive spots were compared. The spot corresponding to 1,2-dibenzylhistidine was determined by running along side an authentic sample of this compound prepared by a different route (189). From the reaction mixture above, only a faint spot corresponding to 1,2-dibenzylhistidine was observed. The procedure gave very poor yields.

Improved yields were obtained if instead the amidine solution was dropped slowly into the chloromethylketone solution, rather than vice versa. In this case there was no danger of alkylating the product since there was already an alkyl group attached to the imidazole ring nitrogen. Separation of N $^{\alpha}$ -carbobenzoxy-1,2-dibenzylhistidine ethyl ester from excess reagents and side products was extremely difficult. Since the free acid derivative was eventually required, a crude extract of the product was saponified to N $^{\alpha}$ -carbobenzoxy-1,2-dibenzylhistidine. This was then purified by counter-current distribution and silicic acid chromatography. Attempts to separate the desired compound from impurities by ion-exchange chromatography were not successful. The pure product, N $^{\alpha}$ -carbobenzoxy-1,2-dibenzylhistidine hydro-

chloride, was obtained as an oil in 11% yield. Details of the preparation and purification are given in Preparation 10.

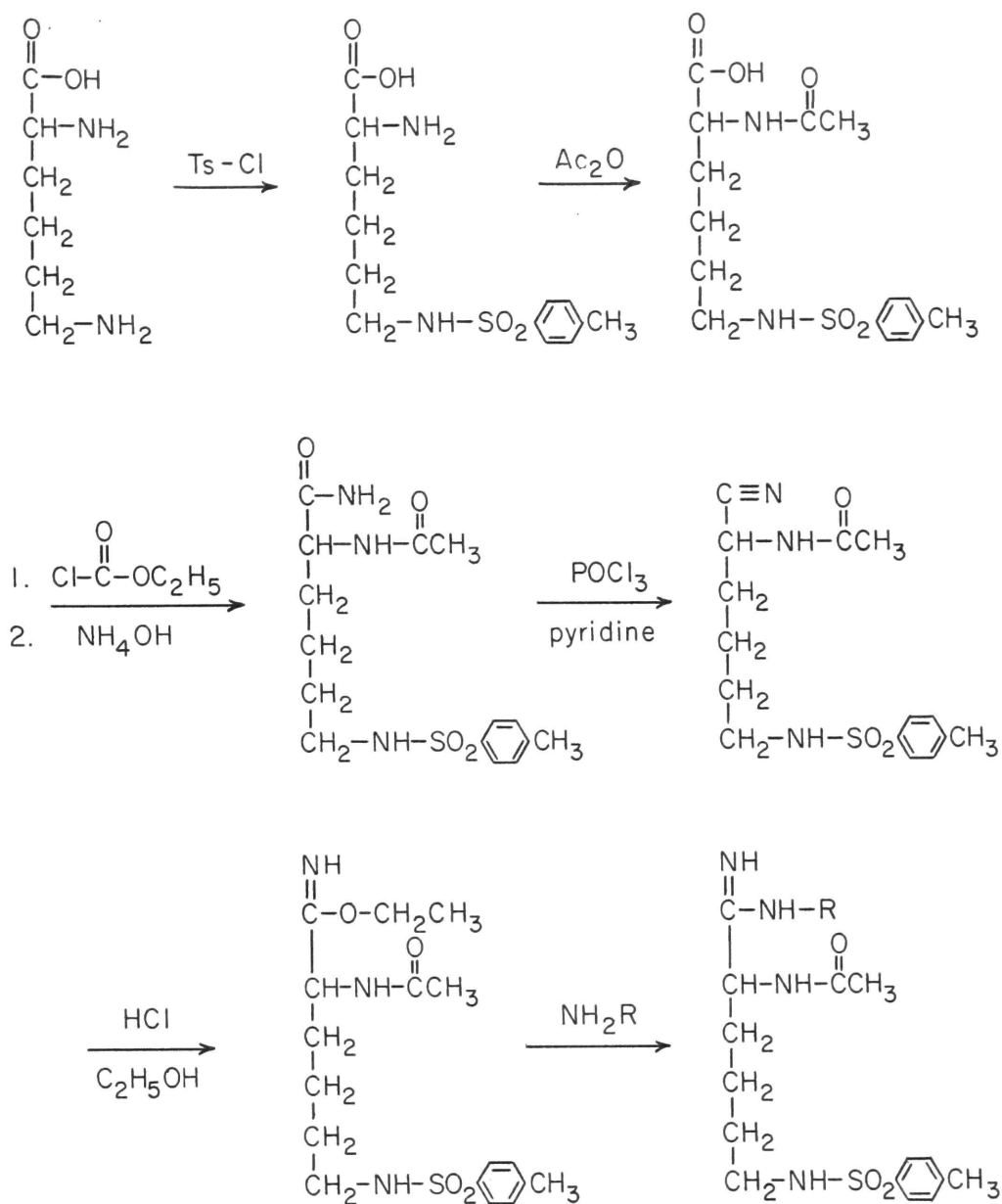
### 3. The "protected lysohistidine derivative."

The reaction involved in the synthesis of the "protected lysohistidine derivative" is shown in Figure 10. The preparation of the one precursor, ethyl L-2-carbobenzoxamido-4-oxo-5-chlorovalerate, has already been discussed, so there remains to be considered the synthesis of the other, the amidine derived from the amino acid, lysine.

The lysine amidines. Of the reactions known to form unsubstituted amidines, nearly all involve the corresponding nitrile.  $\alpha$ -Amino nitriles have been known for some time because they are intermediates in the Strecker synthesis (Greenstein and Winitz (57), p. 698) of  $\alpha$ -amino acids from aldehydes and ammonium cyanide. However, they are racemic mixtures, while for the problem at hand the optically active L-isomer was preferred. It was thought that dehydration of the primary carboxamido group, readily obtainable from an acylated L-lysine, would be the simplest route to the nitrile. The reactions are discussed below, and are shown in Figure 12. The experimental details can be found in Preparations 11 through 20.

$N^{\epsilon}$ -Tosyl-L-lysine was prepared by the method of Roeske et al (144) in 75% yield (Preparation 11). The procedure involves protection of the  $\alpha$ -amino group by formation of a copper salt, and then tosylation with an acetone solution of p-toluenesulfonyl chloride. The Schotten-Baumann technique for the tosylation did not succeed well, but it was very effective when applied in the subsequent synthesis of  $N^{\omega}$ -acetyl- $N^{\epsilon}$ -tosyl-L-lysine (Preparation 12) with acetic anhydride, giving essentially quantitative yields.

In an effort to prepare  $N^{\omega}$ -acetyl- $N^{\epsilon}$ -tosyl-L-lysine amide,  $N^{\omega}$ -acetyl- $N^{\epsilon}$ -tosyl-L-lysine ethyl ester (Preparation 13) was synthesized. A number of attempts to convert the ester to the amide with ethanolic ammonia failed, the pure ester being recovered each time. However, the amide (Preparation 14) was obtained in excellent yields when the acid group was first converted to a mixed anhydride with ethyl chloroformate, and the anhydride was caused to react with concentrated aqueous ammonia. The lysine amide derivative crystallized with great difficulty until seed crystals were obtained. Unexpectedly, however, this reaction caused racemization of the optical center.



Synthetic route to the lysine amidines

**Figure 12.** Synthetic Route to the Lysine Amidines.

The conversion of carboxamide groups to nitriles is well known (176), and a great number of reagents are available. However, no reference to the dehydration of  $\alpha$ -acylamino-carboxamides could be found in the literature. With the lysine amide derivative, and in parallel studies with other acyl-amino acid amides in this laboratory, the usual reagents such as phosphorous pentoxide and phosphorous pentachloride were completely ineffective. Attempts to dehydrate with *p*-toluenesulfonyl chloride (170,191) and boron trifluoride (163) were also unsuccessful. The only method which succeeded was the one introduced by Delaby et al (42) which involves the use of pyridine.  $N^{\alpha}$ -Acetyl- $N^{\epsilon}$ -tosyl-lysine amide was treated with dry pyridine and phosphorous oxychloride in the cold. The experimental conditions are given in Preparation 15.  $N^{\alpha}$ -Acetyl- $N^{\epsilon}$ -tosyl-lysine nitrile could not be crystallized, but was purified from a small amount of unreacted amide by countercurrent distribution.

$N^{\alpha},N^{\epsilon}$ -dibenzoyl-lysine was prepared by the Schotten-Baumann method for the acylation of amino acids (Preparation 18), and  $N^{\alpha},N^{\epsilon}$ -dibenzoyl-lysine amide was obtained smoothly by the mixed anhydride method (Preparation 19). However, all attempts to convert the carboxamide to a nitrile, including treatment with phosphorous oxychloride in pyridine, were unsuccessful.

The amidine and *N*-benzylamidine of  $N^{\alpha}$ -acetyl- $N^{\epsilon}$ -tosyl-lysine were both prepared from the corresponding nitrile by way of a common intermediate, ethyl  $N^{\alpha}$ -acetyl- $N^{\epsilon}$ -tosyl-lysine imidate.  $N^{\alpha}$ -Acetyl- $N^{\epsilon}$ -tosyl-lysine nitrile and ethanol were treated with anhydrous hydrogen chloride according to the general method of Pinner (136). The resulting imidate ester hydrochloride was not isolated and characterized but was caused to react with ethanolic ammonia or benzylamine. A detailed description of the synthesis and purification of  $N^{\alpha}$ -acetyl- $N^{\epsilon}$ -tosyl-lysine amidine hydrochloride and  $N^{\alpha}$ -acetyl- $N^{\epsilon}$ -tosyl-lysine *N*-benzylamidine acetate salt is given in Preparations 16 and 17, respectively. The amidines were extremely difficult to crystallize, but could be purified by countercurrent distribution. Reagents more recently developed for amidine syntheses, *e.g.*, aluminum chloride (128), ammonium benzenesulfonate (129), and benzylammonium benzenesulfonate (129), failed when applied to these compounds. Reactions involving the alkali amides, *e.g.*, sodamide, would likely lead to racemization and side reactions at the  $\alpha$ -carbon, while those involving imino chloride intermediates cannot be used to make aliphatic amidines (159).

In order to avoid hydrolysis of the acetamido group, it was important to maintain strictly anhydrous conditions during the treatment with strong acid. Also during concentration of aqueous solutions of the amidine, it was necessary to neutralize any excess acid present. In some preparations of  $N^{\alpha}$ -acetyl- $N^{\epsilon}$ -tosyl-lysine amidine, a substantial amount of  $N^{\epsilon}$ -tosyl-lysine amidine dihydrochloride was isolated. The apparent ease with which the acetamido group undergoes hydrolysis stands in direct contrast to the stability reported for  $\alpha$ -p-toluenesulfonamido amidines and  $\alpha$ -carbobenzoxamido amidines (115).

The "protected lysohistidine derivative." The "protectec lysohistidine derivative" was prepared by slowly dropping a solution of ethyl L-2-carbobenzoxamido-4-oxo-5-chlorovalerate into an excess of  $N^{\alpha}$ -acetyl- $N^{\epsilon}$ -tosyl-lysine amidine in refluxing chloroform. Descriptions of the reaction, assay procedure and purification are given in Preparation 20. The purification procedure used for  $N^{\alpha}$ -carbobenzoxy-1,2-dibenzylhistidine had to be modified for the purification of this compound. Instead of the extraction step with chloroform from aqueous hydrochloric acid, the crude reaction mixture was subjected to countercurrent distribution and the appropriate fraction was chromatographed on a silicic acid column. During the latter purification step, thin layer chromatography was invaluable as a means of assaying the presence and purity of the compound. Pure 2-(1-acetamido-5-p-toluenesulfonamidopentyl)-4-(2-carbethoxy-2-carbobenzoxamidoethyl)-imidazole hydrochloride was obtained as an amorphous solid in 12% yield, as based on the chloromethylketone.

A number of attempts were made to condense  $N^{\alpha}$ -acetyl- $N^{\epsilon}$ -tosyl-lysine N-benzylamidine with ethyl L-2-carbobenzoxamido-4-oxo-5-chlorovalerate to form the "protected lysohistidine derivative" with the imidazole ring already benzylated. Unfortunately, no evidence could be found that the desired product had formed.

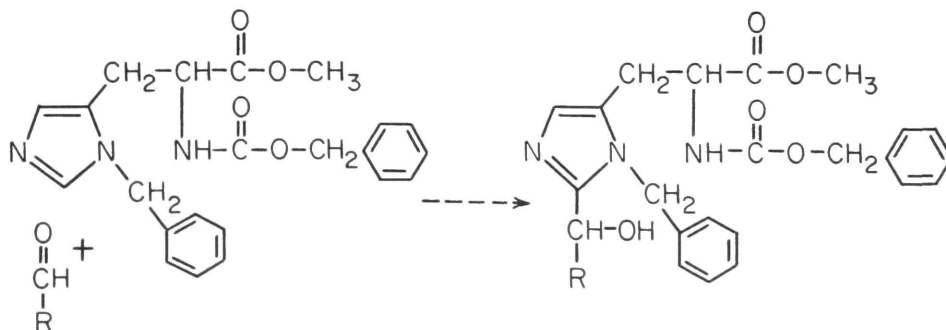
## B. Other Attempts to Synthesize the "Lysohistidine Derivatives"

Before and during the attempts to prepare the "protected lysohistidine derivative" by the amidine -  $\alpha$ -haloketone route, a great variety of other approaches was explored. The standard imidazole syntheses studied, although useful in the preparation of many imidazole derivatives, failed when applied to the synthesis of the 2- $\alpha$ -aminoalkyl-histidine derivatives. However, part of the research involved some interesting chemistry, so a number of the reactions studied will be discussed briefly.

### 1. Alkyl substitution at the 2-position of imidazole.

The ability of imidazoles to undergo hydroxymethylation has been known for some time (185). This typical electrophilic substitution reaction is facilitated by the electron-releasing methyl group, while electronegative nitro and phenyl groups inhibit it. 1-Alkylimidazoles tend to form 2-hydroxymethyl derivatives (59). However, 1,4-dimethylimidazole yields 1,4-dimethyl-5-hydroxymethylimidazole (59).

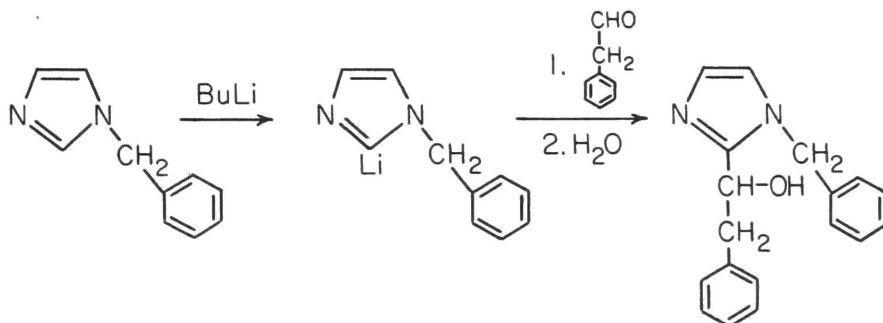
It was not known whether histidine derivatives would undergo this reaction.  $N^{\alpha}$ -carbobenzoxy- $N^{\text{Im}}$ -benzyl-L-histidine methyl ester was prepared (Preparation 21) and was treated with a ten fold excess of 39% aqueous formaldehyde at 125° for eight hours. The mixture was hydrolyzed in refluxing 6 *N* hydrochloric acid for two hours, and samples, freed from excess acid, were examined by ascending paper chromatography using sec-butanol, water ammonium hydroxide, 25:9:1, as solvent. Development with ninhydrin showed a spot ( $R_f = .48$ ) which moved slightly faster than benzylhistidine ( $R_f = .42$ ). Thus it appeared that hydroxymethylation might have taken place, but the preparation, isolation, and proof of the structure of the new histidine derivative was not considered worthwhile unless the method proved effective for larger aliphatic aldehydes.



Attempts to couple acetaldehyde, propionaldehyde and benzaldehyde to  $N$ -carbobenzoxy- $N^{\text{Im}}$ -benzyl-L-histidine methyl ester were made using essentially the same conditions as described for formaldehyde. The base, piperidine, or the Lewis acid, zinc chloride, were added as catalysts to some of the runs. Each reaction was hydrolyzed and analyzed by paper chromatography, using the systems sec-butanol, water, ammonium hydroxide, 25:9:1, and sec-butanol,  $N$  hydrochloric acid, 10:4. From all runs a single ninhydrin positive spot was obtained which corresponded exactly to the benzylhistidine standard employed. It was concluded that no condensation had taken place.

Another possible way to substitute imidazole at the 2-position is to prepare a 2-lithio-imidazole derivative (158) and react it with an aldehyde or an acyl chloride. Such reactions are not well known in imidazole chemistry, but may represent a powerful synthetic technique for substitution on the imidazole ring. 1-Benzylimidazole has been prepared before by other routes (89,166,177), but was synthesized here from the sodium salt of imidazole and benzyl chloride, as described in Preparation 22, and the 2-lithio derivative was prepared with butyl lithium as described by Shirley and Allan (158).

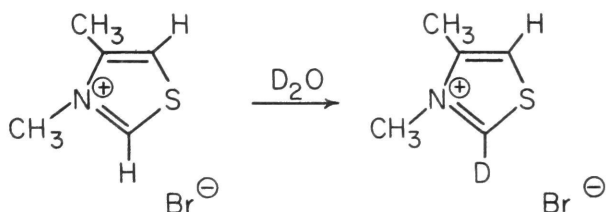
Preliminary experiments, where 1-benzyl-2-lithioimidazole (158) was treated with phenylacetyl chloride, indicated that a ketone was formed (phenylhydrazine test) but the material has not been crystallized and identified. Since this work was done, others in this laboratory (90) have caused 1-benzyl-2-lithioimidazole to react successfully with phenylacetaldehyde to give a 39% yield of 1-benzyl-2-( $\alpha$ -hydroxy- $\beta$ -phenylethyl)-imidazole.



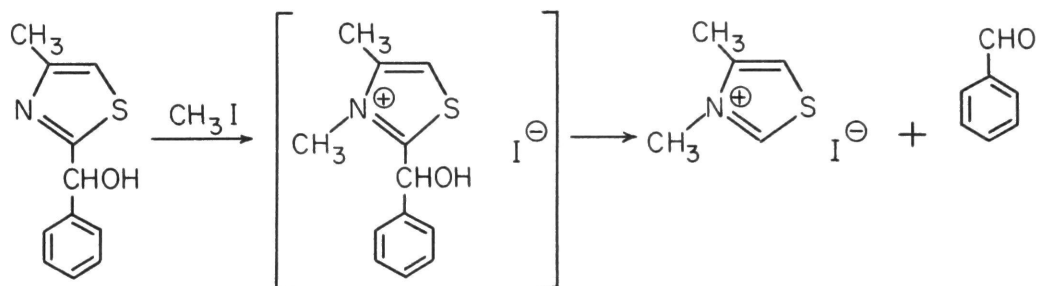
The work with lithioimidazoles was interrupted before satisfactory reactions were developed. However, this area seems particularly worthy of additional research.

## 2. Activation of the 2-position of imidazoles.

The chemistry of imidazole and other heteroaromatic ring compounds such as thiazole is strikingly similar. Thus thiazole derivatives can react with formaldehyde or benzaldehyde to form 2-( $\alpha$ -hydroxyalkyl)-thiazoles (49). From studies of the mechanism of action of thiamine, an even greater reactivity at the 2-position of thiazolium salts has been demonstrated. Deuterium exchange studies showed that the hydrogen on the 2-carbon of 3,4-dimethylthiazolium bromide is acidic (28).

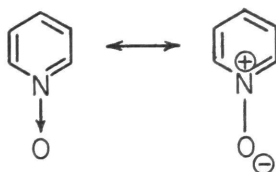


When 2-( $\alpha$ -hydroxybenzyl)-4-methylthiazole was treated with methyl iodide, the compound decomposed to benzaldehyde and 3,4-dimethylthiazolium iodide (28).

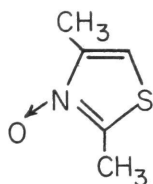


Alkylation of the ring nitrogen thus enhances the reactivity of the 2-position. N,N'-Dialkylimidazolium salts have been prepared but they are very unstable to aqueous alkali and heat, and otherwise have not been well characterized (Hofmann (82), p. 50).

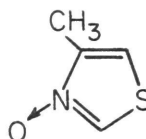
A similar type of activation can be effected in pyridine derivatives (156), for example, by preparing the amine oxide. The dipolar representation results in the placement of a formal positive charge on the nuclear nitrogen, thus making the amine oxide analogous to alkyl ammonium salts.



Amine oxides of 4-methylthiazole and 2,4-dimethylthiazole have been prepared (124) and isolated as picrate salts in 27% and 58% yields, respectively.



2,4-dimethylthiazole N-oxide

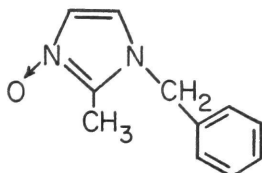


4-methylthiazole N-oxide

The 4-methylthiazole N-oxide is unstable in aqueous alkali, but other properties of these compounds have not yet been elucidated. No amine oxide derivatives of imidazoles have ever been reported.

It was decided to try to synthesize an imidazole N-oxide because the expected properties of such derivatives would be both interesting and useful synthetically, and because their stability might be greater than the N,N'-dialkylimidazolium salts. Per-acids are usually employed to prepare N-oxides (156). It is known, however, that aqueous peroxide causes complete decomposition of imidazole, oxamide and formic acid being typical reaction products (140). A non-aqueous system would therefore be preferred and a per-acid such as perbenzoic acid could be readily used. Since the amine oxide of the 2-methyl derivative in the thiazole series was produced in better yields (124), 1-benzyl-2-methylimidazole was selected as an appropriate imidazole derivative. Possibly ionization of the 2-hydrogen of imidazole, which could be expected after activation, would cause ring cleavage; the 2-methyl group would exclude such possibilities. However, rather than activating the 2-position of the imidazole, this N-oxide derivative would be expected to activate the 2-methyl group in the same way that -picoline N-oxides are activated (156).

1-Benzyl-2-methylimidazole was prepared by benzylation of 2-methylimidazole (Preparation 23). When the imidazole derivative was treated with perbenzoic acid in dry chloroform (Preparation 24), a compound was isolated in low yield, the melting point and infra-red spectrum of which differed appreciably from starting material. An elemental analysis was near to that required for the N-oxide,  $C_{11}H_{12}N_2O$ , but was not entirely satisfactory. The formula can be expressed graphically as:



Placement of the oxygen at the 3-nitrogen seems most reasonable since resonance can still occur in the ring, while the formation of an amine oxide on the nitrogen already containing the benzyl group would necessarily result in the loss of aromaticity of the imidazole ring. Preliminary attempts to reduce the compound back to 1-benzyl-2-methylimidazole with iron in acetic acid (77) or with thiourea (143) were unsuccessful, the presumed N-oxide always having been recovered.

Further work on this interesting imidazole derivative could not be carried out at this time, but we hope to investigate its properties and determine its structure in the near future.

### 3. Imidazole ring cleavage and reclosure.

In 1893, Bamberger (7) showed that imidazole treated with benzoyl chloride and aqueous alkali at low temperatures is cleaved to 1,2-dibenzamidoethylene and formic acid. The reaction is general for acyl chlorides and imidazoles; exceptions include 1-alkylimidazoles and derivatives substituted with electron-withdrawing groups. The imidazole of histidine is cleaved only when the carboxyl group is masked, for example as an ester. Windaus (187) showed that if 1,2-dibenzamido-1-propene is treated with acetic anhydride or propionic anhydride, 2,4-dimethylimidazole and 2-ethyl-4-methylimidazole are formed, respectively. If the cleavage product of histidine would also undergo ring closure with anhydrides of lysine derivatives, this method would constitute a simple route to the "lysohistidine derivative."

Histidine methyl ester was treated with benzoyl chloride to yield methyl 2,4,5-tribenzamido-4-pentenoate (186) (Preparation 25). This derivative was caused to react with the mixed anhydride formed from  $N^{\alpha}, N^{\epsilon}$ -diacetyl-L-lysine and ethyl chloroformate. The reaction mixture was hydrolyzed in refluxing 6  $N$  hydrochloric acid overnight and was examined by ascending paper chromatography in four systems: sec-butanol, water, formic acid, 10:9:1; sec-butanol, water, ammonium hydroxide, 10:9:1; phenol, 0.1  $N$  hydrochloric acid, 5:1; and phenol, 0.1  $N$  ammonium hydroxide, 5:1. The major ninhydrin positive spots corresponded to lysine and the degraded form of the cleaved histidine derivative, *i.e.*, 2,5-diamino-4-ketovaleric acid. There was no indication that the desired histidine derivative had formed. More extensive work by others in this laboratory led to the conclusion that the method was not suitable for the synthesis of the histidine derivatives required.

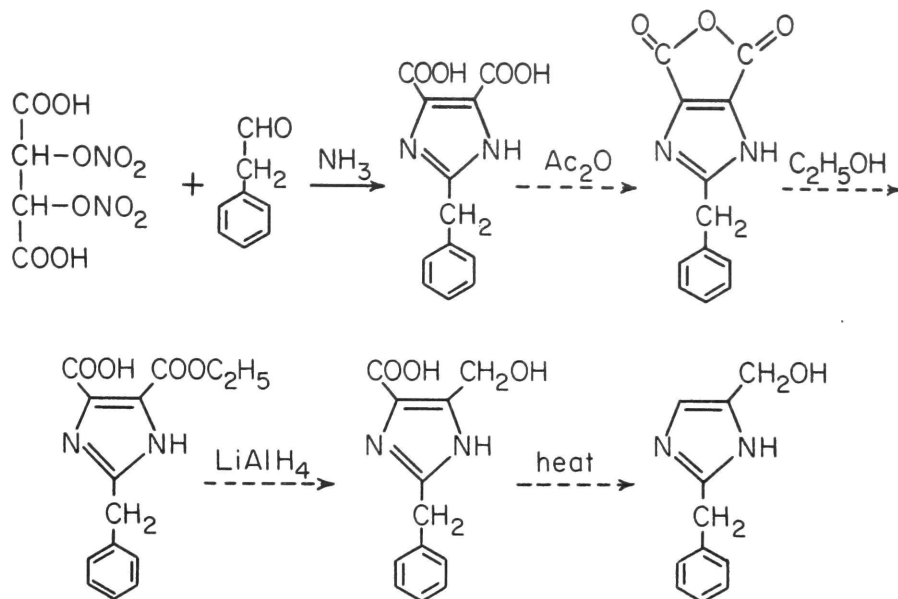
#### 4. Glyoxals, ammonia and aldehydes.

An example of this general procedure for the preparation of imidazoles, called the Weidenhagen synthesis, is found in Organic Syntheses (174); 4-hydroxymethylimidazole is prepared from fructose, basic cupric carbonate, ammonium hydroxide and formaldehyde. The detailed directions for the reaction were followed, except that butyraldehyde was substituted in place of formaldehyde. This would be expected to yield 2-propyl-4-hydroxymethylimidazole. A small yield of a product was isolated as a picrate salt. Paper chromatography in sec-butanol, water, ammonium hydroxide, 25:9:1, developed with the Pauly spray or a silver nitrate reagent, indicated that only 4-hydroxymethylimidazole had been formed. The melting point of the picrate salt and the mixed melting point with authentic 4-hydroxymethylimidazole picrate, confirmed this conclusion. It is likely that large aliphatic aldehydes are too unreactive to be used in this procedure. Apparently formaldehyde was formed by degradation of the fructose molecule and was able to compete effectively against the higher concentration of butyraldehyde added.

#### 5. Dinitrotartaric acid, ammonia and aldehydes.

Related formally to the previous general method is the reaction of dinitrotartaric acid with ammonia and formaldehyde or other aldehydes. In this reaction, higher aliphatic aldehydes can be used, and 2-benzyl-4,5-imidazoledicarboxylic acid actually was prepared in rather poor yield according to the directions of Sonn and Greif (161). It had been hoped to

form the cyclic anhydride, open it with ethanol, reduce the resulting mono-ester with lithium aluminum hydride and decarboxylate the free acid, thus giving 2-benzyl-4-hydroxymethylimidazole. However, preliminary attempts



to make the cyclic anhydride with acetic anhydride failed. This and the difficulty in preparing dinitroterephthalic acid, caused the abandonment of the project.

## 6. Miscellaneous procedures.

There are a number of other synthetic routes to 2-alkylimidazoles which have been or are being pursued by others in this laboratory. The reaction of amidines with aminoketones was studied and 2-benzylhistidine was actually prepared by this method using glucosamine and phenylacetamidine (189). The reaction of aminoketones and ethyl imidate esters is also being investigated, and 2-benzyl-4-methylimidazole has been synthesized in this way (90). Attempts to cyclize benzoylphenylalanylserine amidine to a 4-aminoimidazole derivative by the method developed by Shaw and Woolley (157), were thwarted by an unusual rearrangement during the preparation of the amidine.

### C. Other Syntheses

#### 1. Protecting groups on the imidazole nitrogen.

In order to polymerize the "protected lysohistidine derivative", the imidazole ring nitrogen must be substituted before the compound is converted into an N-carboxy anhydride derivative. Since the synthetic route which would have given rise to an N<sup>Im</sup>-benzyl derivative failed, the substitution must be performed on the monomeric "protected lysohistidine derivative" actually synthesized. The conventional way to protect the imidazole ring is to react the imidazole derivative with metallic sodium in liquid ammonia, and then to treat the sodium salt with benzyl chloride. However, the technique causes reductive cleavage of carbobenzoxy and tosyl groups during the first step, and these groups would have to be replaced before the polymerization reaction. The synthetic problem therefore was to devise a method to substitute the imidazole ring nitrogen without causing appreciable side reactions or cleavage of the protecting groups. The experiments were performed with N<sup>α</sup>-carbobenzoxy-L-histidine, which resembles the histidine-like portion of the saponified "protected lysohistidine derivative." The former compound was used in preference to the "protected lysohistidine derivative" because it was much more readily available.

The powerful reducing properties of metallic sodium in liquid ammonia make this technique unacceptable for the problem at hand. Sodium hydride, on the other hand, is a very poor reducing agent, but should readily form a sodium salt with imidazole. Dr. Kadin (90) in this laboratory was able to synthesize 1-benzylimidazole in 72% yield by causing a solution of imidazole in dimethylformamide (DMF) to react with a suspension of sodium hydride in DMF at 0°, and then by treating the solution with benzyl chloride at 0°. Since the reaction could be carried out with sodium hydride and imidazole, it was desirable to determine whether the carbobenzoxy group of N<sup>α</sup>-carbobenzoxy-L-histidine could withstand the treatment and thereby allow the appropriate substitution to occur.

N<sup>α</sup>-Carbobenzoxy-L-histidine was first reported by Bergmann and Zervas (20), but Patchornik et al. (132) showed that the compound was racemic. The optically active derivative was prepared by Patchornik et al. by the following route. N<sup>α</sup>,N<sup>Im</sup>-Dicarbobenzoxy-L-histidine was prepared from L-

histidine and carbobenzoxy chloride by the Schotten-Baumann technique and the  $N^{\text{Im}}$ -carbenzoxy group was selectively removed with aniline at  $100^{\circ}$  for 30 minutes in good yield.

When  $N^{\alpha}$ -carbenzoxy-L-histidine was treated with sodium hydride and benzyl chloride, as described in Preparation 26, a 41% yield of  $N^{\alpha}$ -carbenzoxy- $N^{\text{Im}}$ -benzyl-L-histidine was obtained. Examination of the fractions from the reaction mixture indicated that no cleavage of the  $N^{\alpha}$ -carbenzoxy-L-histidine had occurred. Since the carbobenzoxy group was stable during the reaction, it can be reasonably expected that this procedure could be used successfully with the saponified "protected lysohistidine derivative." The sodium hydride method appears to have a number of advantages over the one using metallic sodium in liquid ammonia. The reaction is not so sensitive to moisture, the experimental technique is simpler and the possibility of reductive side reactions is reduced, while the yields are at least comparable.

A number of other attempts to protect the imidazole ring nitrogen of  $N^{\alpha}$ -carbenzoxy-L-histidine will be mentioned briefly. The imidazole nitrogen can be acylated successfully with carbobenzoxy chloride to regenerate  $N^{\alpha}, N^{\text{Im}}$ -dicarbenzoxy-L-histidine (Preparation 27). The acid chloride of this compound will not form an N-carboxy anhydride (132), but it was thought that the acid bromide might. Attempts to make the corresponding acid bromide with phosphorous tribromide or thionyl bromide resulted in the isolation of amorphous solids which could not be characterized satisfactorily.

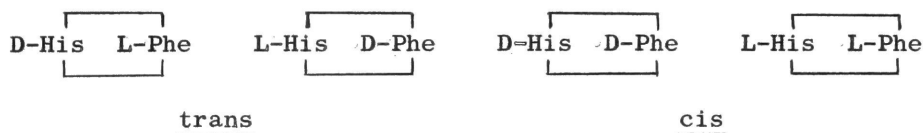
It was possible to prepare  $N^{\alpha}$ -carbenzoxy- $N^{\text{Im}}$ -benzenesulfonyl-L-histidine in anhydrous pyridine, as described in Preparation 28. It has been known that N-arylsulfonyl-imidazoles are less readily hydrolyzed than are N-acetyl- or N-benzoyl-imidazole derivatives (133,166). The benzenesulfonyl or tosyl groups might therefore possibly find use as protecting groups on the imidazole nitrogen. Unfortunately, it has not yet been possible to determine whether the acid chloride of  $N^{\alpha}$ -carbenzoxy- $N^{\text{Im}}$ -benzenesulfonyl-L-histidine will react to form an N-carboxy anhydride which could subsequently polymerize. If it will not, it should be possible to selectively cleave the  $N^{\alpha}$ -carbenzoxy group by catalytic hydrogenation (166), and the N-carboxy anhydride might then be synthesized by phosgenation of  $N^{\text{Im}}$ -benzenesulfonyl-L-histidine.

## 2. cyclo-Histidyl-phenylalanyl.

It was proposed to synthesize the trans-2,5-diketopiperazine, cyclo-D-histidyl-L-phenylalanyl. Either D-histidyl-L-phenylalanine ester or L-phenylalanyl-D-histidine ester could serve as the appropriate intermediate dipeptide ester. The route involving the former dipeptide was chosen primarily for two reasons. A protected form of histidine,  $N^{\alpha}, N^{\text{Im}}$ -dicarbobenzoxy-L-histidine, had previously been made frequently in our laboratory. Secondly, L-phenylalanine ethyl ester was commercially available while D-histidine esters were not.

It was decided to couple  $N^{\alpha}, N^{\text{Im}}$ -dicarbobenzoxy-D-histidine (Preparation 29) and L-phenylalanine ethyl ester by the carbodiimide method, which had been used successfully in syntheses with  $N^{\alpha}, N^{\text{Im}}$ -dicarbobenzoxy-L-histidine (1). The protected dipeptide intermediate was not purified. The carbobenzoxy groups were removed by catalytic hydrogenation with palladium on carbon and the dipeptide ester was caused to cyclize to the 2,5-diketopiperazine derivative by refluxing an ethanolic solution of its free base. A 16% yield of cyclic dipeptide was obtained in analytically pure condition. The reactions are described in detail in Preparation 30.

When a portion of the 2,5-diketopiperazine derivative was hydrolyzed in acid to the amino acids, both histidine and phenylalanine were demonstrated. The two amino acids were separated chromatographically on a Dowex 50 column and the optical rotation of each was measured. Both amino acids were racemic. Therefore, it is possible that the cyclic dipeptide isolated is composed of four isomeric forms, two of them cis on the ring and two of them trans:



## 3. 2-Alkylimidazoles.

2-Benzylimidazole has been described previously by Sonn and Greif (161), who prepared it by decarboxylating 2-benzyl-4,5-imidazoledicarboxylic acid. Kyrides et al (108) describe the synthesis of this imidazole by dehydrogenation of 2-benzyl-2-imidazoline with nickel catalyst. Since 2-benzyl-

2-imidazoline was commercially available, the latter method was selected in as much as only one reaction step was necessary. The dehydrogenation with Raney nickel was carried out at  $245^{\circ}$  and the desired compound was isolated as a bioxalate salt, from which 2-benzylimidazole free base was readily obtained. The details of the synthesis are described in Preparation 31.

2-Isobutyylimidazole was synthesized by Radziszewski and Szul (141) by the treatment of glyoxal with ammonia and isovaleraldehyde, and by Maquenne (111) by the decarboxylation of 2-isobutyl-4,5-imidazoledicarboxylic acid. It was decided, however, to prepare this derivative by dehydrogenation of 2-isobutyl-2-imidazoline, as described for the preparation of 2-benzylimidazole above. The imidazoline was readily prepared by treatment of ethyl isovalerate with an excess of ethylenediamine to form the mono-isovaleryl-ethylenediamine, and then by the action of heat on the amide in order to cyclize it to the 2-imidazoline derivative. These kinds of reactions were developed by Hill and Aspinall (79) as a general method for the synthesis of 2-alkyl-2-imidazolines. A crude fraction of 2-isobutyl-2-imidazoline was obtained by distillation but the compound was not purified and characterized. It was treated with Raney nickel according to the method of Kyrides et al (108) and the resulting 2-isobutyylimidazole was isolated as an oxalate salt. The 2-isobutyylimidazole free base was then readily obtained from the oxalate salt. A description of the reactions is given in Preparation 32.

#### 4. Esters.

Acetyl-L-phenylalanine was made by the Schotten-Baumann procedure, as described by du Vigneaud and Meyer (46) (Preparation 34). The p-methoxy-phenyl ester was prepared in 58% yield by treatment of the mixed anhydride obtained from acetyl-L-phenylalanine and ethyl chloroformate with p-methoxy-phenol. The reaction is described in Preparation 35. The carbodiimide procedure which is routinely used to make p-nitrophenyl esters of acylamino acids was not selected because it was thought that the less acidic p-methoxy-phenol would react more slowly than the p-nitrophenol and thus increase the probability that side reactions such as acylurea formation would occur. The ester had no optical activity in a 3% ethanol solution, and therefore may have racemized during the synthesis.

p-Nitrophenyl phenylacetate was made simply by treatment of p-nitro-phenol with phenylacetyl chloride and triethylamine, as described in detail in Preparation 33.

## D. Experimental

### Separatory and Analytical Methods

#### 1. Countercurrent distribution (39).

An automatic, 100 tube, Craig-type apparatus, with a tube capacity of 10 ml. per phase, was used. The sample was routinely dissolved in a minimum amount of upper and lower phase and was added to the first tubes. If the compound was very soluble in the phases, up to 8 g. could be distributed satisfactorily.

#### 2. Column chromatography.

The ion-exchange columns were prepared from sulfonated cross-linked polystyrene (Dowex 50W X4) according to the directions of Moore and Stein (119).

The silicic acid columns were made as follows. The silicic acid (Mallinckrodt, 100 mesh) was dried by heating in an oven overnight at 110° or by washing with acetone. It was suspended in chloroform and poured into the column so that no air bubbles were trapped. The column was then washed with the appropriate solvent overnight.

#### 3. Paper electrophoresis.

Strips of Whatman number 1 filter paper, 11.4 by 56 cm., were cut, wetted with buffer, blotted, and the materials were applied on a center line, as described by Kunkel and Tiselius (106). The paper was clamped between two glass plates 45.7 cm. long, and was placed in the apparatus so that the ends of the paper dipped into the two buffer solutions. An electric field of 14.6 volts per centimeter for one hour was routinely used. The paper was removed, dried in the air or in an oven, and developed with an appropriate spray (ninhydrin or Pauly reagents). The buffer employed was 0.1 M pyridine-acetate, pH 5.0, made by mixing 32.2 ml. pyridine, 22.8 ml. glacial acetic acid, and 4 liters of water.

#### 4. Ascending paper chromatography.

Samples were applied by capillary tubes on a line 3 cm. from the bottom of pieces of Whatman number 1 filter paper. The paper was bent into a cylinder, clipped, and placed in large chromatography jars, in which about 75 to

100 ml. of the solvent had been allowed to equilibrate with the atmosphere of the jar. When the solvent rose near to the top of the paper, the paper was removed, dried, and developed with an appropriate reagent.

5. Thin layer chromatography (167,168).

Glass plates covered with a uniform 250 micron layer of Silica Gel G were prepared with the Model A-200 Assembly of Research Specialties Company. The plates were dried at 110° and used in the manner of ascending paper chromatography. The technique was used as a criterion of purity and as a guide to the selection of solvent systems for silicic acid column chromatography.

6. Polarimetry.

A Schmidt-Haensch polarimeter with a sodium lamp was used. Solutions of samples were placed in one decimeter tubes for analysis. The reported reading was the average of 10 separate readings.

7. Detecting reagents for chromatography and electrophoresis.

The ninhydrin spray for amino groups was made by dissolving 100 mg. of ninhydrin in 100 ml. of n-butanol.

The Pauly spray for imidazole was prepared by freshly mixing in the cold three parts of 5% aqueous sodium nitrite solution with one part of 0.5% sulfanilic acid in 0.6 N hydrochloric acid. This solution was sprayed on the paper and was followed immediately with a spray of 10% sodium carbonate. A red-orange color indicated the position of the imidazole. When the Pauly reagents were used for quantitative determinations of imidazoles in solution, 1 ml. of an appropriately diluted sample was combined with 1 ml. of the diazotized sulfanilic acid, followed directly with 1 ml. of 10% sodium carbonate. The optical densities of the solutions were read immediately at 540 mμ and compared with standard solutions of histidine.

A spray positive for amide groups was made according to Mazur et al (114); 0.5 ml. of t-butyl hypochlorite was mixed with 10 ml. of cyclohexene. This solution was sprayed on the paper, the paper was heated in a steam oven for 2 to 5 minutes, and a 1% potassium iodide - 1% starch solution was sprayed on the paper. A blue color developed immediately and indicated amide or amine groups. Aqueous solutions of chlorine were also used with potassium

iodide - starch (145) or with toluidine (58,142) for the detection of amide derivatives, but results were less consistently reproducible than the method of Mazur et al.

Concentrated sulfuric acid was occasionally used to detect spots on the silicic acid in thin layer chromatography.

### Preparations

#### 1. Ethyl phenylacetimidate hydrochloride.

This compound was prepared by the method of Luckenbach (110). A solution of phenylacetonitrile (10.0 ml., 86.7 mmoles) and absolute ethanol (10.0 ml.) was saturated at 0° with dry hydrogen chloride. After 24 hours at 0°, the imidate ester hydrochloride was precipitated with 200 ml. of dry ether, filtered, and dried over solid potassium hydroxide in a vacuum desiccator. The yield was 16.2 g., or 95%, m.p. 96-97° (lit. (110), 85°).

#### 2. Phenylacetamidine hydrochloride.

Ethyl phenylacetimidate hydrochloride (14.5 g., 72.5 mmoles) was dissolved in 200 ml. of absolute ethanol previously saturated with ammonia at 0°. The solution was held at room temperature for three days in a stoppered flask. The solvent was evaporated under reduced pressure and the residue was dissolved in about 20 ml. of ethanol. Dry ether was added until the solution just became turbid. After two hours at room temperature, the light precipitate of ammonium chloride was filtered off and discarded. Cautious addition of more ether caused the precipitation of 11.8 g. of phenylacetamidine hydrochloride, m.p. 147-149° (lit. (50), 151-153°). Yield, 95%. Sometimes the amidine hydrochloride could not be crystallized in the manner described, but separated as an oil. Trituration of the oil with 3 ml. of absolute ethanol was successful in inducing crystallization.

#### 3. 2-Benzyl-4-methylimidazole.

Phenylacetamidine hydrochloride (10.0 g., 58.5 mmoles) was suspended in 100 ml. of dry chloroform and 38 ml. of 1.50 N sodium ethoxide (57.0 mmoles) was added to neutralize the hydrochloride. The sodium chloride was separated by filtration and the solution was brought to reflux temperature. Chloroacetone (1.85 g., 20 mmoles), diluted with 25 ml. of dry chloroform,

was dropped in slowly, with stirring, for two hours. The reaction mixture was stirred and refluxed an additional four hours, and was held overnight at room temperature. The solvent was evaporated and the excess amidine was hydrolyzed by treating the reaction mixture in 50% aqueous ethanol with dilute alkali. The solution was acidified, concentrated to remove most of the ethanol, and extracted with ether. The aqueous fraction was brought to pH 10 and was extracted three times with ether. A saturated solution of oxalic acid in acetone was added to the ethereal phase until no further precipitation was observed. The imidazole precipitated out as a bioxalate salt which was filtered off and recrystallized twice from water-acetone to yield 2.36 g., or 45%, m.p. 151-152°.

Anal. Calcd. for  $C_{11}H_{12}N_2 \cdot C_2H_2O_4$ : C, 59.56; H, 5.34; N, 10.68

Found: C, 59.59; H, 5.35; N, 10.79

The free base was separated from the oxalate by shaking with chloroform and dilute aqueous sodium hydroxide. Evaporation of the chloroform gave crystals of 2-benzyl-4-methylimidazole, m.p. 95-97°.

#### 4. N-Benzylphenylacetamidine hydrochloride.

Ethyl phenylacetimidate hydrochloride (17.7 g., 88 mmoles) was dissolved in 50 ml. of absolute ethanol, and 9.5 ml. of benzylamine (88 mmoles) were added at 0°. The solution was held three days at room temperature, and the product was isolated as described for phenylacetamidine hydrochloride (Preparation 2). After three crystallizations from ethanol-ether, 12.6 g. of N-benzylphenylacetamidine hydrochloride were obtained, m.p. 156-157°, yield, 55%. Although the melting point was the same as for phenylacetamide (156°), the compound isolated was not the amide because when mixed with amide, the melting point was depressed.

Anal. Calcd. for  $C_{15}H_{16}N_2 \cdot HCl$ : C, 69.09; H, 6.53; N, 10.74

Found: C, 68.90; H, 6.51; N, 10.80

#### 5. N-Carbobenzoxo-L-aspartic acid.

The compound was readily prepared following the directions of Bergmann and Zervas (20). L-Aspartic acid (10.0 g., 75.2 mmoles), magnesium oxide (10.0 g., 250 mmoles), 130 ml. of water and 45 ml. of ether were mixed well and cooled to 0°. Carbobenzoxychloride (26 ml., 183 mmoles) was added drop-

wise over one hour with vigorous mechanical stirring. The mixture was stirred two hours at room temperature and extracted with ether, and the extract was discarded. After acidification of the aqueous phase to pH 1 with 6 N hydrochloric acid, the solid was extracted with ethyl acetate. The ethyl acetate solution was washed with N hydrochloric acid and water, dried with magnesium sulfate and evaporated to an oil. Trituration with dry hexane caused the oil to crystallize, yielding 17 g., m.p. 114-116° (lit. (20), 116°). Yield, 85%.

6. α-Ethyl N-carbobenzoxy-L-aspartate.

N-Carbobenzoxy-L-aspartic acid (25.0 g., 93.5 mmoles) was shaken at room temperature with 125 ml. of freshly distilled acetic anhydride until a clear solution was obtained (about one hour). The acetic acid and excess acetic anhydride were rapidly evaporated under reduced pressure below 40° and the resulting glass was dissolved in 110 ml. of absolute ethanol. The solution was heated in a stainless steel bomb at 130° for four hours, concentrated to a syrup and dissolved in ether. The ether solution was repeatedly extracted with 20 ml. portions of 0.5 M sodium bicarbonate until acidification of the aqueous extracts gave no more precipitate. The first three extracts, containing acetic acid and the β-ethyl ester, were discarded. Subsequent ones were combined and left overnight at 4° to crystallize. The product was filtered and recrystallized from ether-hexane to yield 13 g., m.p. 85-86° (lit. (118), 85°). Yield, 50%.

7. α-Benzyl N-carbobenzoxy-L-aspartate.

N-Carbobenzoxy-L-aspartic anhydride, prepared from 4.89 g. of N-carbobenzoxy-L-aspartic acid (18.3 mmoles), was caused to react with 2.8 ml. (27.4 mmoles) of freshly distilled benzyl alcohol at 100° for 3½ hours. Ether was added and the mono-ester derivative was extracted with aqueous sodium bicarbonate. Upon acidification of the aqueous phase with hydrochloric acid, an oil formed which crystallized overnight in the cold. The product was recrystallized once from ether-hexane to yield 2.0 g., m.p. 80-82° (lit. (21), 84-85°).

8. Benzyl 2-carbobenzoxamido-4-oxo-5-chlorovalerate.

α-Benzyl N-carbobenzoxy-L-aspartate (1.53 g., 4.5 mmoles) in acetyl chloride was treated with phosphorous pentachloride, as described by Haring-

ton and Overhoff (67). This method gave poor yields and is inferior to the one described in Preparation 9. The acid chloride was caused to react with diazomethane and hydrogen chloride essentially as described for the ethyl ester (Preparation 9). The product crystallized from ether-hexane to yield .31 g., m.p. 105-110°.

9. Ethyl L-2-carbobenzoxamido-4-oxo-5-chlorovalerate.

$\alpha$ -Ethyl N-carbobenzoxy-L aspartate (9.7 g., 34.8 mmoles), suspended in 100 ml. of dry ether, was cooled to 0°, treated with 7.6 g. (36.5 mmoles) of phosphorus pentachloride, and shaken occasionally until everything dissolved. The ether was evaporated in vacuo through a calcium chloride drying tube without heating and the residue was triturated at -70° with dry hexane. The crystalline acid chloride was separated, washed with cold dry hexane and dissolved in 100 ml. of ether at 0°. It was used immediately in the next step.

An excess of diazomethane was prepared from 47.8 g. of Dupont EXR-101 (N,N'-dinitroso-N,N'-dimethylterephthalamide, 70%) and 404 ml. of 40% NaOH, and was slowly distilled (bath temperature must not exceed 40°) with 450 ml. of ether. The yellow distillate was dried with potassium hydroxide pellets at -70°.

The acid chloride solution above was poured slowly into the ethereal diazomethane at -70°. After an hour, during which the solution was shaken frequently, the mixture was allowed to warm to -10° for two more hours. The excess diazomethane was removed by concentration under reduced pressure to half the volume. Dry ethereal hydrogen chloride (19 ml., 4.5 M) was added, the contents were mixed for 10 minutes and the solvents were evaporated under reduced pressure. The residue was dissolved in warm ethanol, from which it crystallized easily when cooled to give 7.8 g., m.p. 93-95°,

$\frac{25}{D} + 28.4^\circ$  (c = 3, CHCl<sub>3</sub>). Yield, 68%.

D

Anal. Calcd. for C<sub>15</sub>H<sub>18</sub>ClNO<sub>5</sub>: C, 54.96; H, 5.55; N, 4.27; Cl, 10.83

Found: C, 54.55; H, 5.56; N, 4.27; Cl, 11.27

10. N<sup>α</sup>-Carbobenzoxy-1,2-dibenzylhistidine (1,2-dibenzyl-4(or 5)-(2-carboxy-2-carbobenzoxamidoethyl)-imidazole).

Ethyl L-2-carbobenzoxamido-4-oxo-5-chlorovalerate (4.66 g., 14.2 mmoles) was dissolved in 50 ml. of dry dioxane. The solution was heated to 100°, stirred, and treated anhydrously with a solution composed of N-benzylphenylacetamidine hydrochloride (9.19 g., 35.3 mmoles), 30 ml. of absolute ethanol, 100 ml. of dioxane, and 19.5 ml. of sodium ethoxide (1.81 M, 35.3 mmoles). The dropwise addition of the amidine free base required seven hours and the reaction mixture was stirred and refluxed for four additional hours. Ethanollic 3.7 M hydrogen chloride (9.6 ml., 35.5 mmoles) was added, the solvents were evaporated under reduced pressure, and the residue was dissolved in 500 ml. of 0.1 N hydrochloric acid and 500 ml. of ether-hexane (2:1). After shaking, the organic phase containing unreacted chloromethylketone was separated and the aqueous phase was extracted three times with chloroform. The combined chloroform extracts contained the desired histidine derivative while most of the unreacted amidine remained in the aqueous phase. It is unusual that chloroform should be able to extract a hydrochloride salt from aqueous acid; the presence of three benzene rings in the molecule is probably mainly responsible. The chloroform was evaporated under reduced pressure and the ethyl ester of the histidine derivative was saponified at 25° by dissolving the syrup (3.7 g.) in 75 ml. of 95% ethanol and 15 ml. of N sodium hydroxide. After two hours the alkali was neutralized and the solvents were evaporated under reduced pressure.

The presence of the desired product was assayed by hydrolyzing aliquots in 6 N hydrochloric acid for six hours. The resulting 1,2-dibenzylhistidine was demonstrated by paper electrophoresis at pH 5, developed with ninhydrin. Authentic 1,2-dibenzylhistidine (189) traveled 8.5 cm. toward the cathode when histidine traveled 14 cm. A rough estimate of the amount of dibenzylhistidine present was made by comparison of the relative intensities of the ninhydrin spots from appropriate dilutions of the known and unknown.

The N<sup>α</sup>-carbobenzoxy-1,2-dibenzylhistidine was purified as follows. The material from the saponification reaction was subjected to countercurrent distribution (98 transfers) in methanol, chloroform, hexane, 0.1 N hydrochloric acid, 3:3:1:3. The tube fractions were analyzed by measuring the optical density at 258 mμ, and a peak (maximum, tube 60) was found which

contained all of the desired product. However, the assay indicated that a major and a minor contaminant were still present. The desired material was recovered (1.14 g.) by evaporation of the solvents and was chromatographed on a silicic acid column (4 x 36 cm.) with chloroform-methanol (1:1) as eluant. Nearly all of the contaminants were separated and re-chromatography on a 2 x 103 cm. silicic acid column with chloroform-methanol (19:1) gave a single peak (from 150 to 200 ml. eluate). The amorphous product, N<sup>α</sup>-carbo-benzoxo-1,2-dibenzylhistidine hydrochloride, was recovered by evaporation of the solvents to give 0.78 g., or 11% overall yield based on the chloromethylketone. On hydrolysis and electrophoresis, only one ninhydrin positive spot, corresponding to authentic 1,2-dibenzylhistidine (189), was observed.

Anal. Calcd. for C<sub>28</sub>H<sub>27</sub>N<sub>3</sub>O<sub>4</sub>·HCl: C, 66.46; H, 5.58; N, 8.30

Found: C, 65.88; H, 5.63; N, 8.02

11. N<sup>ε</sup>-Tosyl-L-lysine (L-2-amino-6-p-toluenesulfonamidocaproic acid).

L-Lysine hydrochloride (25 g., 137 mmoles) and basic cupric carbonate (40 g.) were refluxed in one liter of water for two hours, filtered hot, and washed with hot water. After the filtrate cooled to 25<sup>o</sup>, sodium bicarbonate (42 g., 500 mmoles) was added and a solution of p-toluenesulfonyl chloride (39 g., 205 mmoles) in 1.2 liters of acetone was added dropwise during one hour with vigorous stirring. The solution was stirred an additional 10 hours at room temperature. The light blue precipitate was filtered through filter-cell, re-suspended in water, filtered again, and washed with acetone and ether. The precipitate and filter-cell were re-suspended in water, the pH was adjusted to about 3 with hydrochloric acid, and hydrogen sulfide was bubbled through until no more black precipitate formed. The solid was filtered off and the filtrate was boiled until free of hydrogen sulfide. On cooling the acid solution was extracted with ethyl acetate to remove any di-tosyl-lysine. The pH was carefully adjusted to 6 and the resulting white precipitate was collected, 29.3 g., m.p. 216-220<sup>o</sup>. Yield 75%. Paper electrophoresis at pH 5 showed a single neutral spot positive to ninhydrin, but no spot corresponding to lysine.

12. N<sup>α</sup>-Acetyl-N<sup>ε</sup>-tosyl-L-lysine (L-2-acetamido-6-p-toluenesulfonamidocaproic acid).

A solution of N<sup>ε</sup>-tosyl-L-lysine (25 g., 83 mmoles) in 175 ml. of water and 16.7 ml. of 5 N sodium hydroxide was cooled to a sludge in an ice-salt bath. Acetic anhydride (9.2 ml., 98 mmoles) was added in ten portions with vigorous manual shaking and with cooling. Sufficient sodium hydroxide was added to maintain the pH above 9. The contents were then shaken 90 minutes at room temperature, cooled, and acidified to pH 2 with hydrochloric acid. The precipitate was filtered off and the filtrate was extracted three times with ethyl acetate. The solid formed on evaporation of the ethyl acetate was combined with the first to yield 27.4 g.; m.p. 142-144°. Yield 96%. The compound was recrystallized from water; m.p. 145-146°.  $[\alpha]_D^{25} + 6.1^\circ$  (c = 3, ethanol).

Anal. Calcd. for C<sub>15</sub>H<sub>22</sub>N<sub>2</sub>O<sub>5</sub>S: C, 52.60; H, 6.48; N, 8.18

Found: C, 52.52; H, 6.40; N, 8.19

A part of the product was hydrolyzed in refluxing 6 N hydrochloric acid for 3 hours to N<sup>ε</sup>-tosyl-L-lysine,  $[\alpha]_D^{22} + 13.1^\circ$  (c = 3, 2 N hydrochloric acid), (lit. (144) + 13.6°).

13. N<sup>α</sup>-Acetyl-N<sup>ε</sup>-tosyl-L-lysine ethyl ester (ethyl L-2-acetamido-6-p-toluene-sulfonamidocaproate).

To a solution of N<sup>α</sup>-acetyl-N<sup>ε</sup>-tosyl-L-lysine (5.06 g., 14.4 mmoles) in 50 ml. of absolute ethanol was added 10 ml. of absolute ethanol saturated with dry hydrogen chloride. The solution was held at room temperature overnight and the solvents were evaporated under reduced pressure. The residue was dissolved in absolute ethanol and treated twice more as above. The resulting residue was dissolved in ethyl acetate and the solution was extracted with cold aqueous sodium bicarbonate and water, dried, and concentrated under reduced pressure to about 10 ml. Hexane was added cautiously until the solution became turbid. The mixture was held at -15° overnight and the crystals were filtered off to give 3.2 g., or 60% yield, m.p. 65-70°.

Anal. Calcd. for C<sub>17</sub>H<sub>26</sub>N<sub>2</sub>O<sub>5</sub>S: C, 55.11; H, 7.07; N, 7.56

Found: C, 55.26; H, 7.12; N, 7.52

14. N<sup>α</sup>-Acetyl-N<sup>ε</sup>-tosyl-DL-lysine amide (DL-2-acetamido-6-p-toluenesulfonamidocaproamide).

A solution of N<sup>α</sup>-acetyl-N<sup>ε</sup>-tosyl-L-lysine (21.1 g., 61.7 mmoles) and triethylamine (17.25 ml., 123.4 mmoles) in 500 ml. of dry tetrahydrofuran (THF) was cooled to -10°. Ethyl chloroformate (6.5 ml., 67.9 mmoles) was added dropwise during 5 minutes with vigorous mechanical stirring. The solution was stirred an additional 10 minutes at -10°, and 13 ml. of concentrated ammonium hydroxide were added. The cooling bath was removed and the contents were stirred 30 minutes. Water (200 ml.) was added and the solution was concentrated under reduced pressure to about 150 ml. and stored overnight at 4°. When the amide crystallized it was filtered off and recrystallized from ethanol-water to yield 18.7 g. (89%), m.p. 167-167.5°.

Anal. Calcd. for C<sub>15</sub>H<sub>23</sub>N<sub>3</sub>O<sub>4</sub>S: C, 52.74; H, 6.79; N, 12.30

Found: C, 52.53; H, 6.75; N, 12.15

Acid hydrolysis of the product to N<sup>ε</sup>-tosyl-lysine and measurement of its optical rotation indicated that the amide was completely racemized. Apparently either the excess triethylamine or the ammonium hydroxide was responsible for this surprising result.

15. N<sup>α</sup>-Acetyl-N<sup>ε</sup>-tosyl-DL-lysine nitrile (DL-2-acetamido-6-p-toluenesulfonamidocapronitrile).

N<sup>α</sup>-Acetyl-N<sup>ε</sup>-tosyl-DL-lysine amide (10.0 g., 29.3 mmoles) was dissolved in 200 ml. of dry pyridine (freshly distilled from sodium) and cooled to -5°. Phosphorous oxychloride (2.69 ml., 29.3 mmoles) was added dropwise with vigorous stirring at a rate which maintained the temperature at -5°. After 10 minutes of additional stirring the pyridine was removed under reduced pressure below 40° and 100 ml. of water were added. The pH was adjusted to 3 with hydrochloric acid and the product was extracted four times with 150 ml. portions of ethyl acetate. The combined ethyl acetate solutions were washed with 0.3 N hydrochloric acid, 0.1 M sodium bicarbonate and water, and dried overnight with sodium sulfate. The ethyl acetate was evaporated under reduced pressure and the crude product (7.5 g., yield 79%) was finally purified by countercurrent distribution (60 transfers) in ethanol 460 ml., ethyl acetate 675 ml., hexane 225 ml., and water 440 ml. A symmetrical nitrile peak showed a maximum in tube 32, and a very small amount of amide

with a peak maximum in tube 8 was removed. Infra-red spectroscopy (Perkin-Elmer Model 137) showed nitrile absorption at  $2235\text{ cm}^{-1}$ . All attempts to crystallize this compound were unsuccessful.

Anal. Calcd. for  $\text{C}_{15}\text{H}_{21}\text{N}_3\text{O}_3\text{S}$ : C, 55.69; H, 6.55; N, 12.98

Found: C, 55.35; H, 6.67; N, 12.52

16.  $\text{N}^{\alpha}$ -Acetyl- $\text{N}^{\xi}$ -tosyl-DL-lysine amidine hydrochloride (DL-2-acetamido-6-p-toluenesulfonamidocaproamidinium hydrochloride).

$\text{N}^{\alpha}$ -Acetyl- $\text{N}^{\xi}$ -tosyl-DL-lysine nitrile (7.0 g., 21.5 mmoles) was dissolved in 1.5 ml. of absolute ethanol (25 mmoles) and 50 ml. of dry chloroform. Dry hydrogen chloride was bubbled through the solution for 20 minutes at  $0^{\circ}$ . The flask was tightly stoppered and placed at  $4^{\circ}$  overnight. The solvents were removed under reduced pressure and the residue was dissolved in cold absolute ethanol saturated with ammonia (150 ml.). The solution was kept at room temperature for three days. The solvents were evaporated and the residue was dissolved in chloroform, methanol and 0.01  $\text{N}$  aqueous hydrochloric acid (3:2:2), acidified to pH 2 and subjected to countercurrent distribution (100 transfers). The amidine hydrochloride was in tubes 75 to 95, as determined by measurement of absorption at  $260\text{ m}\mu$ , while unchanged nitrile showed a maximum concentration in tube 15. The amidine fraction was brought to pH 5 with ammonium hydroxide, the solvents were evaporated, and the residue was freed of ammonium chloride by fractional precipitation with ether from absolute ethanol. Careful addition of more ether to the ethanol solution gave crystals of  $\text{N}^{\alpha}$ -acetyl- $\text{N}^{\xi}$ -tosyl-DL-lysine amidine hydrochloride, m.p.  $171\text{--}172^{\circ}$ . Yields from different experiments varied from 30 to 50%.

Anal. Calcd. for  $\text{C}_{15}\text{H}_{24}\text{N}_4\text{O}_3\text{S}\cdot\text{HCl}$ : C, 47.77; H, 6.68; N, 14.86

Found: C, 47.30; H, 6.64; N, 14.54

The amidinium picrate was also prepared and was crystallized from water, m.p.  $172\text{--}174^{\circ}$ .

Anal. Calcd. for  $\text{C}_{15}\text{H}_{24}\text{N}_4\text{O}_3\text{S}\cdot\text{C}_6\text{H}_3\text{N}_3\text{O}_7$ : N, 17.22

Found: N, 17.23

17.  $N^{\alpha}$ -Acetyl- $N^{\epsilon}$ -tosyl-DL-lysine N-benzylamidine (N-benzyl-2-acetamido-6-p-toluenesulfonamido-DL-caproamidine).

$N^{\alpha}$ -Acetyl- $N^{\epsilon}$ -tosyl-DL-lysine nitrile (6.37 g., 19.7 mmoles) was converted to the ethyl imidate ester hydrochloride as described in the preparation of the unsubstituted amidine (Preparation 16). The ethyl imidate ester was caused to react with benzylamine (3.26 ml., 30 mmoles) in absolute ethanol for three days at room temperature. The solvents were evaporated under reduced pressure and the residue was subjected to countercurrent distribution (96 transfers) in *n*-butanol, water, acetic acid, 15:18:2. This gave the benzylamidine as the acetate salt with a peak maximum in tube 84. The oily salt was purified further by precipitation from alcohol with ether, but it never crystallized.

Anal. Calcd. for  $C_{22}H_{30}N_4O_3S \cdot C_2H_4O_2$ : C, 58.74; H, 6.99; N, 11.42

Found: C, 58.43; H, 6.52; N, 11.28

18.  $N^{\alpha},N^{\epsilon}$ -dibenzoyl-lysine (2,6-dibenzamidocaproic acid).

L-Lysine monohydrochloride (11.7 g., 64.2 mmoles) was dissolved in 40 ml. of water and 25.7 ml. of 5 *N* sodium hydroxide (128.5 mmoles). Sodium bicarbonate (32.4 g., 385 mmoles) was added, the solution was cooled to a sludge in an ice-salt bath, and benzoyl chloride (22.2 ml., 193 mmoles) was added in 10 parts, with vigorous shaking. After stirring two hours at  $-10^{\circ}$ , the solution was acidified and the solid was collected. Benzoic acid was removed by boiling the solid in hexane and filtering hot. The solid was recrystallized from ethanol-water to yield 12.7 g. (56%), m.p.  $143-145^{\circ}$  (lit. (91),  $149-150^{\circ}$ ).

19.  $N^{\alpha},N^{\epsilon}$ -dibenzoyl-lysine amide (2,6-dibenzamidocaproamide).

$N^{\alpha},N^{\epsilon}$ -Dibenzoyl-L-lysine (2.45 g., 6.92 mmoles) was dissolved in 25 ml. of anhydrous tetrahydrofuran, and 0.96 ml. of triethylamine (6.92 mmoles). The solution was cooled to  $-10^{\circ}$ , ethyl chloroformate (0.73 ml., 7.6 mmoles) was added, and the mixture was shaken vigorously for 10 minutes. Concentrated ammonium hydroxide (1.7 ml., 25 mmoles) was added and the solution was stirred for 30 minutes at room temperature, and concentrated under reduced pressure to about 10 ml. The solid was collected, triturated with

hot water, and recrystallized from dimethylformamide-water to yield 1.15 g., or 60%, m.p. 199-200°.

Anal. Calcd. for  $C_{20}H_{23}N_3O_3$ : C, 67.97; H, 6.56; N, 11.88

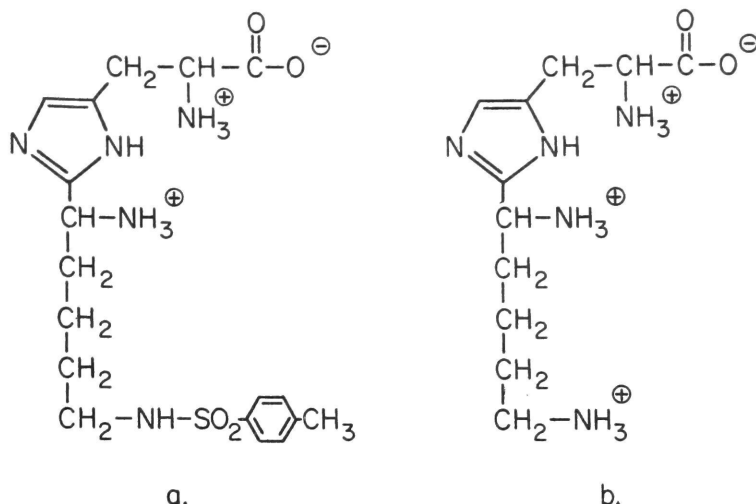
Found: C, 67.93; H, 6.53; N, 11.78

20. 2-(1-acetamido-5-p-toluenesulfonamidopentyl)-4-(2-carbethoxy-2-carbo-benzoxamidoethyl)-imidazole (the "protected lysohistidine derivative").

Ethyl L-2-carbobenzoxamido-4-oxo-5-chlorovalerate (0.525 g., 1.60 mmoles) was dissolved in 25 ml. of dry chloroform and heated to refluxing. A solution of  $N^\alpha$ -acetyl- $N^\epsilon$ -tosyl-DL-lysine amidine hydrochloride (1.53 g., 4.06 mmoles) and sodium ethoxide (4.00 mmoles) in 10 ml. of absolute ethanol and 20 ml. of dry chloroform was slowly dropped into the refluxing chloromethylketone solution during 2½ hours. The reaction mixture was refluxed 9 hours more, cooled, and neutralized with an equivalent of hydrochloric acid.

In order to assay for the desired histidine derivative, an aliquot was hydrolyzed in refluxing 6 N hydrochloric acid for 3 hours. Paper electrophoresis of the hydrolysate at pH 5 was performed and the paper was developed with ninhydrin. The crude reaction mixture showed two intense spots, one neutral, and one traveling 7.2 cm. toward the cathode when histidine traveled 11.8 cm. The 7.2 cm. spot had a characteristic gray color. Two faint spots at 11.8 and 14.2 cm. were also observed. When the material running 7.2 cm. was eluted from the paper, re-hydrolyzed overnight in refluxing 6 N hydrochloric acid, and then subjected again to electrophoresis, the intensity of the 7.2 cm. spot was reduced, while material at 14.2 cm. appeared. It is likely that the neutral spot represented  $N^\epsilon$ -tosyl-lysine and 2-amino-4-oxo-5-chlorovaleric acid; the 7.2 cm. spot, the "lysohistidine derivative" with the ethyl ester, carbobenzoxo and acetyl groups hydrolyzed but the tosyl group still intact (a); the 11.8 cm. spot, lysine; and the 14.2 cm. spot the completely hydrolyzed "lysohistidine derivative" (b).

The "protected lysohistidine derivative" was believed to be present when the 7.2 cm. gray spot was observed on treatment of an aliquot by this procedure.



The solvents of the neutralized reaction mixture were evaporated under reduced pressure and the residue was subjected to countercurrent distribution (200 transfers) in chloroform, methanol, 0.01 *N* hydrochloric acid, 3:2:2. A distribution profile was obtained by reading the optical density of the tube fractions at 260 mμ. The desired compound was present in tubes 140 to 180, but was contaminated with amidine. A second countercurrent distribution (98 transfers) in chloroform, methanol, 0.01 *N* hydrochloric acid, 2:2:1, gave a nearly isolated peak with a maximum at tube 65. Electrophoresis of a hydrolyzed aliquot still showed contamination by a compound neutrally charged at pH 5.

To purify further the "protected lysohistidine derivative", the compound was chromatographed on a silicic acid column (2 x 24 cm.) using 10% methanol in chloroform as the eluant. A single peak was eluted directly following some material coming off at one column volume. The position of the peaks from the column were determined by measurement of the optical density at 260 mμ, and the recognition of the desired peak was made by thin layer chromatography of the unhydrolyzed derivative. Evaporation under reduced pressure of the solvents from the fractions containing the desired "protected lysohistidine derivative" gave 123 mg. of an amorphous solid, or 12% yield as calculated from the chloromethylketone.

Anal. Calcd. for  $C_{30}H_{39}N_5O_7S \cdot HCl$ : C, 55.42; H, 6.20; N, 10.77

Found: C, 54.85; H, 5.95; N, 10.58

In order to determine the purity of the compound, a portion was acid hydrolyzed and subjected to paper electrophoresis at pH 5. An intense ninhydrin positive spot at 7.2 cm. (tosyl-lysohistidine), a less intense spot at 14.2 cm. (lysohistidine) and an extremely faint neutral spot were observed. Thin layer chromatography of the unhydrolyzed derivative showed a single spot, as developed for amides with t-butylhypochlorite-potassium iodide-starch, with an  $R_f$  of .70 in 30% ethanol in chloroform.

The ethyl ester of the "protected lysohistidine derivative" was hydrolyzed by dissolving 40 mg. of the material in 10 ml. of 95% ethanol and adding 1.0 ml. of N sodium hydroxide. After two hours at room temperature the alkali was neutralized and the solvents were removed under reduced pressure. Thin layer chromatography with 30% ethanol in chloroform showed a faint spot at  $R_f$  .7 (starting material) and an intense spot at  $R_f$  .20 (saponified material).

21.  $N^\alpha$ -Carbobenzoxy- $N^{Im}$ -benzyl-L-histidine methyl ester.

$N^\alpha$ -Carbobenzoxy- $N^{Im}$ -benzyl-L-histidine (127) (5.0 g., 13.2 mmoles) was suspended in 200 ml. of anhydrous methanol and 9.8 ml. of 1.56 N methanolic hydrogen chloride (15.3 mmoles) were added. The mixture was shaken and then held at room temperature overnight. The solvent was removed under reduced pressure and the residue was cooled to 4°. The oil crystallized on standing several months in the cold. It was recrystallized from ethanol-hexane, m.p. 82-84°.

Anal. Calcd. for  $C_{22}H_{23}N_3O_4$ : C, 67.17; H, 5.89; N, 10.68

Found: C, 67.14; H, 5.76; N, 10.82

22. 1-Benzylimidazole.

Imidazole (10 g., 147 mmoles) was dried thoroughly and dissolved in 200 ml. of anhydrous liquid ammonia. Metallic sodium was added until the deep blue color of the solution persisted more than five minutes. Benzyl chloride (20 ml., 175 mmoles) was dropped in slowly with vigorous stirring. After two hours ammonium chloride was added and the ammonia was allowed to distill. The residue was dissolved in water, acidified, and extracted twice with ether. The aqueous phase was brought to pH 10 with sodium hydroxide and extracted three times with ether. The ether solution was dried and

crystals were obtained by cautious addition of hexane. The yield was 8.72 g., or 37%, m.p. 70-72° (lit. (89), 71-72°).

23. 1-Benzyl-2-methylimidazole.

2-Methylimidazole (10.8 g., 132 mmoles) was dried thoroughly and dissolved in 250 ml. of anhydrous liquid ammonia under nitrogen. Metallic sodium was added until the solution remained dark blue for five minutes (3.0 g., 130 mmoles). The solution was stirred with a magnetic stirring bar and benzyl chloride (16.6 ml., 144 mmoles) was added dropwise. After two hours of stirring ammonium chloride was added and the ammonia was allowed to distill off through a drying tube. The residue was dissolved in chloroform and the solution was extracted with water, dried, and evaporated to an oil. The crude oil was distilled at 16 mm. Hg and the fraction from 148-150° was collected to yield 12.1 g., or 56% (lit. (89), b.p. 125-127°, 3 mm.).

24. 1-Benzyl-2-methylimidazole N-oxide.

1-Benzyl-2-methylimidazole (1.5 ml., 9.18 mmoles) was dissolved in 30 ml. of dry ether, cooled to 0°, and treated with a solution of perbenzoic acid (26) (33 ml., 10.0 m. eq. active oxygen) in chloroform. After 24 hours at 4° and then 24 hours at room temperature, no active oxygen could be detected by titration with potassium iodide. The solvents were evaporated and the residue was dissolved in N hydrochloric acid and ether. The aqueous phase was washed again with ether and was brought to pH 10 with sodium hydroxide. The cloudy solution was quickly extracted twice with ether and then twice with chloroform. The organic extracts were dried over sodium sulfate and evaporated separately. The ether solution yielded 1.15 g., or 73%, of the starting material. The chloroform extract yielded 180 mg. of an oil which could be crystallized from hot benzene-hexane, m.p. 139-140°.

Anal. Calcd. for  $C_{11}H_{12}N_2O$ : C, 70.19; H, 6.43; N, 14.89

Found: C, 69.56; H, 6.24; N, 14.26

25. Methyl 2,4,5-tribenzamido-4-pentenoate.

This compound was prepared essentially by the method of Kossel and Edlbacher (104). Histidine methyl ester dihydrochloride (2.0 g., 8.33 mmoles) was dissolved in 50 ml. of water, and sodium carbonate (20.0 g., 188 mmoles) and 50 ml. of acetone were added. The solution was cooled to

0° and benzoyl chloride (8.2 ml., 7.14 mmoles) was added dropwise with vigorous stirring. After standing overnight at 4°, the mixture was concentrated under reduced pressure to a sludge. The residue was dissolved in 200 ml. of chloroform and 200 ml. of water. The chloroform phase was separated and was extracted with N hydrochloric acid until the organic phase no longer gave a positive Pauly test for imidazole. The chloroform phase was washed with dilute aqueous sodium bicarbonate and water and the solvent was evaporated under reduced pressure. The yield of product was 1.89 g., or 48%. The solid was recrystallized from ethanol, m.p. 216-219° (lit. (104), 219°).

26. N<sup>α</sup>-Carbobenzoxy-N<sup>Im</sup>-benzyl-L-histidine.

A suspension of sodium hydride (186 mg., 7.74 mmoles) in 3 ml. of dimethylformamide (DMF) was added to a solution of N<sup>α</sup>-carbobenzoxy-L-histidine (132) (1.12 g., 3.87 mmoles) in 5 ml. of DMF at 0°. The mixture was stirred until the sodium hydride dissolved. Then benzyl chloride (.215 ml., 3.87 mmoles) was added all at once and the solution was stirred at 0° for 10 minutes. About 25 ml. of water were added and the solution was adjusted to pH 2 to 3 with 6 N hydrochloric acid, washed with ether and brought to pH 6 with 5 N sodium hydroxide. On cooling, N<sup>α</sup>-carbobenzoxy-N<sup>Im</sup>-benzyl-L-histidine precipitated out and was collected by filtration to yield 0.6 g., m.p. 208°, dec. (lit. (127), 216°, dec.), or 41% yield.

The crude product was hydrolyzed to N<sup>Im</sup>-benzyl-L-histidine in refluxing 6 N hydrochloric acid for 4 hours and then subjected to paper electrophoresis. A dense spot corresponding to an authentic sample of N<sup>Im</sup>-benzyl-L-histidine was observed. In addition, a very faint histidine spot was present. Various fractions of the reaction and purification steps were subjected to paper electrophoresis at pH 5 in pyridine-acetate buffer. There was no indication that the carbobenzoxy group was cleaved to yield either histidine or 1-benzyl-histidine.

27. N<sup>α</sup>,N<sup>Im</sup>-dicarbobenzoxy-L-histidine.

N<sup>α</sup>-Carbobenzoxy-L-histidine (132) (2.95 g., 10.2 mmoles) was dissolved in 10.2 ml. of N sodium hydroxide and 20 ml. of water. The solution was cooled to a sludge in an ice-salt bath and carbobenzoxy chloride (1.71 ml., 12.0 mmoles) was added with shaking in 8 portions, along with enough 2 N

sodium hydroxide to keep the pH of the solution above 9. Five minutes after the last addition, the cold solution was adjusted to pH 3 with 6 N hydrochloric acid and the aqueous phase was decanted. The oily residue was dissolved in 50 ml. of methanol and cooled. After 2 hours the crystals were collected by filtration to yield 2.15 g., or 46%, of crude product. The solid was then suspended in warm methanol, cooled, and filtered to give  $N^{\alpha}$ ,  $N^{Im}$ -dicarbobenzoxy-L-histidine as crystals with one molecule of methanol, m.p. 93-96°. A mixed melting point with authentic material (m.p. 98-100°) was not depressed.

28.  $N^{\alpha}$ -Carbobenzoxy- $N^{Im}$ -benzenesulfonyl-L-histidine.

Benzenesulfonyl chloride (0.64 ml., 5.0 mmoles) was added to a suspension of  $N^{\alpha}$ -carbobenzoxy-L-histidine (1.37 g., 4.74 mmoles) in 10 ml. of dry pyridine at room temperature. The mixture became clear on stirring. After 3 hours, the pyridine was evaporated under reduced pressure at 35° and the residue was dissolved in 25 ml. of ice-cold 0.3 N sodium bicarbonate and 25 ml. of ethyl acetate. More sodium bicarbonate solution was added until the pH of the aqueous phase was about 7. The organic phase was separated and washed once with ice-cold 0.1 N sodium bicarbonate. The aqueous fractions were combined, acidified to pH 3 with cold 6 N hydrochloric acid and extracted 3 times with chloroform. The chloroform solution was dried with sodium sulfate and evaporated to a dry powder, .63 g., or 31% yield. The  $N^{\alpha}$ -carbobenzoxy- $N^{Im}$ -benzenesulfonyl-L-histidine was recrystallized from methanol-water, m.p. 94-96°. The compound was ninhydrin negative and gave a single spot ( $R_f = .2$ ) on silicic acid thin layer chromatography with methanol.

Anal. Calcd. for  $C_{20}H_{19}N_3O_5S$ : C, 55.93; H, 4.46; N, 9.76

Found: C, 56.13; H, 4.61; N, 9.66

29.  $N^{\alpha}$ ,  $N^{Im}$ -Dicarbobenzoxy-D-histidine.

This compound was prepared according to the directions of Patchornik et al (132) for the L-isomer. D-Histidine hydrochloride monohydrate (10.5 g., 50 mmoles) was dissolved in 50 ml. of 2 N sodium hydroxide and 25 ml. of water. The solution was cooled in ice to 0° and carbobenzoxy chloride (17 ml., 120 mmoles) was added with vigorous shaking in ten portions with enough 5 N sodium hydroxide to maintain the pH above 9. After the addition was completed the thick white mixture was acidified to pH 3 with 6 N hydro-

chloric acid, the water was decanted and discarded, and the residue was dissolved in 50 ml. of methanol. On cooling and shaking crystals of  $N^{\alpha}, N^{Im}$ -dicarbobenzoxy-D-histidine (containing one molecule of methanol) formed and were removed by filtration. The crystals were suspended in warm methanol, cooled, and filtered again to give 9.9 g., m.p. 96-100°. The corresponding L-isomer, as prepared by the method of Patchornik et al (132), melted at 98-100°.

### 30. cyclo-Histidyl-phenylalanyl.

The free base of L-phenylalanine ethyl ester hydrochloride (2.0 g., 8.7 mmoles) was prepared as follows. The ester hydrochloride salt was dissolved in about 4 ml. of water, 50 ml. of chloroform were added and the mixture was cooled in ice. Then 5  $N$  sodium hydroxide (1.74 ml., 8.7 mmoles) was added, the mixture was shaken for 30 seconds, and anhydrous potassium carbonate was added to remove the water. The chloroform solution was decanted, dried thoroughly with anhydrous magnesium sulfate, and concentrated to an oil. The residue of phenylalanine ethyl ester weighed 1.24 g. (6.4 mmoles).

The phenylalanine ethyl ester (6.4 mmoles) was dissolved in 5 ml. of methylene chloride and was cooled to 0°. A cold, concentrated methylene chloride solution of  $N^{\alpha}, N^{Im}$ -dicarbobenzoxy-D-histidine (Preparation 29, 2.91 g., 6.4 mmoles) and another of dicyclohexylcarbodiimide (1.32 g., 6.4 mmoles) were added quickly and the mixture was allowed to stand 1½ hours at 0°. At this time, 0.2 ml. of glacial acetic acid were added to react with any remaining carbodiimide. After 40 minutes, the solution was separated from the insoluble dicyclohexylurea by filtration, the methylene chloride was evaporated under reduced pressure and the oil was dissolved in 25 ml. of ethyl acetate. The solution was held at 0° for one hour and more insoluble dicyclohexylurea was removed by filtration. The solvent was evaporated again and the residue, presumably  $N^{\alpha}, N^{Im}$ -dicarbobenzoxy-D-histidyl-L-phenylalanine ethyl ester, was dissolved in 50 ml. of absolute ethanol and 2 ml. of glacial acetic acid. Without further purification the mixture was hydrogenated with palladium on carbon at 35 lbs per square inch for 14 hours at room temperature. The suspension was filtered through filter cell to remove the palladium catalyst and the filtrate was concentrated under reduced pressure to a syrup.

The syrup, presumably mostly D-histidyl-L-phenylalanine ethyl ester diacetate, was dissolved in 150 ml. of absolute ethanol, and sodium ethoxide was added carefully until the solution became basic, as determined by spot tests in water with phenolphthalein as indicator. The ethanolic solution was refluxed 8 hours, as suggested by Kopple and Nitecki (100). After the solution had cooled the ethanol was evaporated under reduced pressure and the residue was dissolved in 10 ml. of N hydrochloric acid. The acid solution was filtered and the pH of the filtrate was adjusted to 8 to 9. On standing, a precipitate formed, which was filtered off and dried to yield 265 mg., or 16%, m.p. 255-258°. The cyclo-histidyl-phenylalanyl gave a positive Pauly reaction, was negative to ninhydrin, and was homogeneous on electrophoresis at pH 5 in pyridine-acetate buffer.

Anal. Calcd. for  $C_{15}H_{16}N_4O$ : C, 63.37; H, 5.67; N, 19.74

Found: C, 63.30; H, 5.55; N, 19.57

A 175 mg. sample of the product was hydrolyzed 8 hours in refluxing 6 N hydrochloric acid, and an aliquot was subjected to paper electrophoresis. Only spots corresponding to phenylalanine and histidine were observed. The histidine and phenylalanine in the remaining portion of the hydrolysate were separated on a 2 x 63 cm. Dowex 50W X 4 column with 4 N hydrochloric acid, as described by Stein and Moore (169). The optical rotations of each isolated amino acid were measured and both were inactive. Therefore the cyclic dipeptide was a mixture of diastereoisomers.

### 31. 2-Benzylimidazole.

2-Benzyl-2-imidazoline (10.0 g., 62.5 mmoles) was heated under nitrogen at 245° for 30 minutes with 1 g. of Raney nickel (washed with water and absolute ethanol). After cooling the residue was extracted with 50 ml. of N hydrochloric acid and separated from the Raney nickel by centrifugation. The clear acid solution was brought to pH 10 with 5 N sodium hydroxide and was extracted three times with chloroform. The dried chloroform solution was evaporated to an oil and the residue was dissolved in 160 ml. of acetone and a saturated solution of oxalic acid in acetone was added until no further precipitation was observed. The crystals of the salt were filtered off and recrystallized from acetone-water to yield 3.0 g., m.p. 150-152°. The free base was obtained by adjustment of an aqueous solution of the salt

to pH 10, and extraction with chloroform. The chloroform solution was dried and evaporated, under reduced pressure. The residue was recrystallized three times from benzene-hexane to yield 0.80 g., m.p. 124-125<sup>o</sup> (lit. (161), 125-126<sup>o</sup>). Yield, 8%.

### 32. 2-Isobutylimidazole.

Anhydrous ethylenediamine (20 ml., .30 mole) and ethyl isovalerate (15 ml., .10 mole) were refluxed for 40 hours. The excess ethylenediamine was evaporated off (29 mm. pressure) below 50<sup>o</sup> and the residue was heated at atmospheric pressure for 8 hours at 160<sup>o</sup>. The syrup was vacuum distilled at 29 mm. Hg and the crude fraction coming over from 140-170<sup>o</sup> was collected (about 7 g.). Raney nickel (1 g., washed thoroughly with water and absolute ethanol) was added and the syrup was heated to 245<sup>o</sup> and stirred under nitrogen for 30 minutes. On cooling, the residue was extracted with 25 ml. of absolute ethanol and the Raney nickel was separated by centrifugation. The clear ethanol solution was evaporated to an oil, which was dissolved in 150 ml. of acetone. A saturated acetone solution of oxalic acid was added until no more precipitation was observed. The yield of the crude crystalline oxalate salt was 3.36 g., m.p. 184-189<sup>o</sup> (lit. (141), 196<sup>o</sup>). The free base of 2-isobutylimidazole was obtained by adjustment of an aqueous solution of the salt to pH 10 with sodium hydroxide and extraction three times with chloroform. The chloroform solution was dried with sodium sulfate and evaporated under reduced pressure. The residue was crystallized from benzene by cautious addition of hexane. The yield was 1.92 g., or 16%; m.p. 122-123<sup>o</sup> (lit., 120-121<sup>o</sup> (141) or 127<sup>o</sup> (111)).

### 33. p-Nitrophenyl phenylacetate.

A solution of p-nitrophenol (5.56 g., 40 mmoles) and triethylamine (6.18 g., 61 mmoles) in 75 ml. of dry dioxane was cooled to 0<sup>o</sup>, and phenylacetyl chloride (6.18 g., 40 mmoles) was added dropwise during fifteen minutes. The solution was stirred 30 minutes at 0<sup>o</sup> and two hours at room temperature. The triethylamine hydrochloride was filtered off and the solvent was evaporated under reduced pressure. The residue was dissolved in benzene and the solution was cooled to 0<sup>o</sup>, washed with cold N hydrochloric acid, 0.5 M sodium bicarbonate and water, and dried with magnesium sulfate. The benzene was evaporated under reduced pressure and the resulting oil was triturated

with a small amount of cold ethanol. Crystals appeared immediately and the crude product, 7.3 g. or 71% yield, was filtered. The ester was recrystallized from ethanol, m.p. 63-64°.

Anal. Calcd. for  $C_{14}H_{11}NO_4$ : C, 65.37; H, 4.31; N, 5.44

Found: C, 65.61; H, 4.33; N, 5.53

#### 34. Acetyl-L-phenylalanine.

The compound was made by the directions of du Vigneaud and Meyer (46). L-Phenylalanine (15.0 g., 91 mmoles) was dissolved in 91 ml. of N sodium hydroxide and the solution was cooled to 0°. Acetic anhydride (9.4 ml., 100 mmoles) was added with vigorous shaking in six portions along with sufficient 5 N sodium hydroxide to maintain the pH above 9. After one hour at room temperature, the solution was brought to pH 2 with 6 N hydrochloric acid and was cooled to 0°. The solid was filtered and dried to give 17.2 g., or 91%, m.p. 168-169° (lit. (46), 171°).

#### 35. Acetyl-phenylalanine p-methoxyphenyl ester.

Acetyl-L-phenylalanine (3.0 g., 14.5 mmoles) was dissolved in 30 ml. of tetrahydrofuran (dried and freshly distilled) and triethylamine (2.03 ml., 14.5 mmoles). The solution was cooled to -10° and stirred vigorously as ethyl chloroformate (1.53 ml., 16.0 mmoles) was dropped in slowly. The mixture was stirred 10 minutes at -10° and a solution of p-methoxyphenol (1.80 g., 14.5 mmoles) in 10 ml. of dry tetrahydrofuran was added dropwise. The mixture was allowed to warm to room temperature, was stirred one hour, and the solvents were evaporated under reduced pressure. The residue was dissolved in 100 ml. chloroform and the solution was extracted with water adjusted to pH 7 with sodium bicarbonate. The chloroform phase was dried with sodium sulfate and concentrated to half its volume. The desired product precipitated upon addition of hexane to yield 1.2 g., m.p. 116-118°. A crude second crop (1.44 g.) was obtained, giving an overall yield of 58%.

Anal. Calcd. for  $C_{18}H_{19}NO_4$ : C, 68.98; H, 6.11; N, 4.47

Found: C, 68.93; H, 5.97; N, 4.39

### PART III

### KINETIC STUDIES

### A. Introduction

Preparations of the "protected lysohistidine derivative", the cyclo-histidyl-phenylalanyl and 2-benzylimidazole have been described. It was hoped that these compounds would possess unusual catalytic "power" and "specificity" which could be related to their peculiar structure. Therefore, it was necessary to assay their catalytic properties, so kinetic studies were made of the effect of these derivatives on the rates of hydrolysis of appropriate esters.

It was intended to study the catalytic properties of the "lysohistidine derivative" after it had been incorporated into a polypeptide, but synthetic difficulties have postponed the realization of this goal. Instead, the assays were performed on the monomeric "protected lysohistidine derivative" (Figure 8), and on this same derivative except that the ethyl ester was hydrolyzed to an acid.  $N^{\alpha}$ -Acetyl- $N^{\epsilon}$ -tosyl-L-lysine ethyl ester was chosen as the appropriate substrate to be hydrolyzed, because its side chain corresponds to the 2-alkyl side chain on the "protected lysohistidine derivative." It should be pointed out that the model catalyst does not strictly resemble the hypothetical binding site of trypsin, because the  $N^{\epsilon}$ -tosyl group has not been removed. Its expected action therefore will not be the same as that of trypsin, and hydrolysis of the selected substrate ester for the model catalyst is in fact not catalyzed by trypsin. No specific substrates of trypsin, such as  $N^{\alpha}$ -acetyl-L-lysine ethyl ester, have been used in the assays of the "protected lysohistidine derivative."

Studies of imidazole catalysis indicate that the rate of hydrolysis of  $N^{\alpha}$ -acetyl- $N^{\epsilon}$ -tosyl-L-lysine ethyl ester, which is a rather stable ester, should not be affected by imidazole or histidine. Control experiments have confirmed this. Therefore any detectable catalysis of the hydrolysis of this ester by the "protected lysohistidine derivative" would be unusual, and could be considered as suggestive evidence that some sort of binding of catalyst and substrate was involved in the catalysis. The assay procedure used was designed to measure the looked-for enhancement in rate of hydrolysis of the ester due to the model catalyst. If catalysis were found, a more sophisticated kinetic study of the reaction would then be initiated. A description

of the attempts to detect catalysis by the "protected lysohistidine derivative" are given in the next section below.

The ethyl ester of  $N^{\alpha}$ -acetyl- $N^{\epsilon}$ -tosyl-L-lysine (LEE) was chosen because it is rather unreactive. The hydrolysis of more reactive esters, such as p-nitrophenyl esters, are catalyzed by nearly any imidazole or histidine derivative, and it seemed possible that the high reactivity of such esters would decrease the expected selectivity of the reaction. For example, trypsin has been shown to catalyze the hydrolysis of the "specific" chymotrypsin substrate, N-carbobenzoxy-L-tyrosine p-nitrophenyl ester, nearly as well as chymotrypsin itself (113), whereas the corresponding ethyl ester is not hydrolyzed by trypsin. In many organic reactions it has been observed that a group of related reactions will show larger differences in the reaction rates of the individual members if the inherent "reactivity" of these reactions is smaller (9). In aromatic substitution reactions, Brown and co-workers (29) have been able to correlate quantitatively the selectivity (position of substitution on the benzene ring) displayed by the reaction and the reactivity of the reagent (e.g., nitrating or sulfonating reagents). The reactions of 2,4-dinitrophenyl acetate, p-nitrophenyl acetate and acetic anhydride with a family of pyridines also display a correlation between selectivity and reactivity (9). It was thought that such an effect might also operate in some way in the ester hydrolyses being studied here.

In order to test the catalytic abilities of the cyclic dipeptide, cyclo-histidyl-phenylalanyl (Figure 6), acetyl-L-phenylalanine ethyl ester (APEE) was selected as the substrate. This ester is a specific substrate of chymotrypsin, and the model catalyst could be expected to mimic the specific action of this enzyme. It had been postulated above that the model catalyst might be capable of binding the substrate in such a way that the L isomer would be in a more favorable position to be acted on by the imidazole of the histidine residue than would be the corresponding D isomer. Therefore a demonstration that the L isomer of the substrate is hydrolyzed at a faster rate than the D isomer in the presence of the cyclic dipeptide would constitute evidence for specific interaction of the substrate and catalyst. A somewhat more reactive ester, acetyl-phenylalanine p-methoxyphenyl ester (APME), was also used. The studies are described in detail below.

The last group of model catalysts to be considered are 2-benzylimidazole and 2-isobutylimidazole (Figure 7). It was intended to use these simple derivatives in detailed kinetic studies of the catalysis of ester hydrolysis if any unusual reactivity could be demonstrated. The rates of hydrolysis of p-nitrophenyl phenylacetate and p-nitrophenyl acetate were measured in the presence of both imidazoles. A specific rate enhancement could be expected in the case of 2-benzylimidazole and p-nitrophenyl phenylacetate if an interaction of the two phenyl groups were to cause a stabilization of the transition state. The kinetic studies are presented in Section D.

## B. The "Protected Lysohistidine Derivative".

### 1. Methods.

The rate of hydrolysis of  $N^{\alpha}$ -acetyl- $N^{\epsilon}$ -tosyl-L-lysine ethyl ester was measured by titrating the liberated carboxylate group with sodium hydroxide solution. A Radiometer Titrator (Type TTT-1c) and Titrigraph (Type SBR 2C) were employed with a 1 ml. syringe. The pH-stat maintained a constant pH ( $\pm 0.02$  pH units) in the reaction solution by adding appropriate amounts of alkali, and recorded the amount of alkali added versus time on a chart. The volume of titrant added at a given time could be read from the chart with an accuracy of  $\pm .001$  ml. The titrator was calibrated with buffers before and after each experiment, and runs were discarded if the final buffer reading deviated by more than 0.02 pH units from the correct reading.

A solution of carbonate-free sodium hydroxide was prepared by addition of a clear, saturated sodium hydroxide solution to twice distilled water which had been boiled to remove carbon dioxide. A reservoir of this solution, protected from the atmosphere with a potassium hydroxide tube, was connected directly to the syringe of the pH-stat by means of a tube and a 2-way stopcock. In this way the syringe could be filled and the alkali could be added to the reaction mixture without exposure to the atmosphere. The concentration of alkali before and after the experiments was determined by titration against standard potassium acid phthalate, and was found to be 0.0540 N.

The reaction vessel was a jacketed beaker with connections to a constant temperature bath. A temperature of  $39.5^{\circ} \pm 0.1^{\circ}$  was maintained by circulation of water from the constant temperature bath. A polyethylene sheet was stretched across the top of the beaker in order to decrease the rates of solvent evaporation and carbon dioxide uptake. Appropriate holes for the glass electrode (Radiometer, Type G 202 C), the calomel electrode (Radiometer, Type K 401) and the alkali inlet tube were made in the membrane with a hot spatula. The beaker was then raised into position so that the electrodes were properly immersed in the solution. A magnetic stirrer and small stirring bar provided adequate mixing and stirring. Nitrogen gas was washed with dilute alkali, was saturated with the solvent being used by bubbling it through a solution at the reaction temperature, and then was blown across the surface of the reaction solution by means of a hypodermic needle inserted through the polyethylene membrane. A gas flow-rate of 75 to 100 bubbles per minute was necessary to eliminate effects due to carbon dioxide or acids in the atmosphere.

The reaction solution was routinely prepared by addition by pipette of 7.00 ml. of water, 1.00 ml. of M potassium chloride, 1.00 ml. of ethanol and 1.00 ml. of 0.1 M  $N^{\alpha}$ -acetyl- $N^{\epsilon}$ -tosyl-L-lysine ethyl ester (Preparation 13) in ethanol to the jacketed beaker. The resulting 10.00 ml. of solution was 20% in ethanol, 0.1 M in potassium chloride, and 0.01 M in substrate ester. After the basal rate of hydrolysis of the substrate (due to hydroxide ion) was measured at a desired pH, a solution of catalyst was injected into the beaker through the polyethylene membrane by means of a graduated syringe and the rate was measured again.

The primary data were treated in the following way. Since there were 0.100 mmole of ester contained in the reaction mixture, total hydrolysis would theoretically require 1.853 ml. of 0.0540 N sodium hydroxide to neutralize the acid produced. Therefore, the initial ester concentration can be expressed as "a", which in arbitrary units is 1.853 ml. The ester concentration at any given time is equal to "a" minus the total amount "x" (in ml.) of titrant added at that time. The data were plotted as  $\log \frac{a}{a-x}$  versus t (time), and a straight line was drawn through the points by visual

inspection. The observed first order rate constant was calculated from the slope of the line (55).

$$k_{\text{obs.}} = 2.303 \times \frac{\log\left(\frac{a}{a-x}\right)}{t} = 2.303 \times \text{slope}$$

## 2. Results.

As a control, the rate of hydrolysis of  $N^{\alpha}$ -acetyl- $N^{\epsilon}$ -tosyl-lysine ethyl ester (LEE) was measured at different pH values in order to determine the catalytic rate constant due to hydroxide ion. The results of a typical experiment are shown graphically in Figure 13. From the slopes of the best straight lines which could be drawn through the points, the observed first order rate constants were calculated and the results from a number of different experiments are listed in the table below.

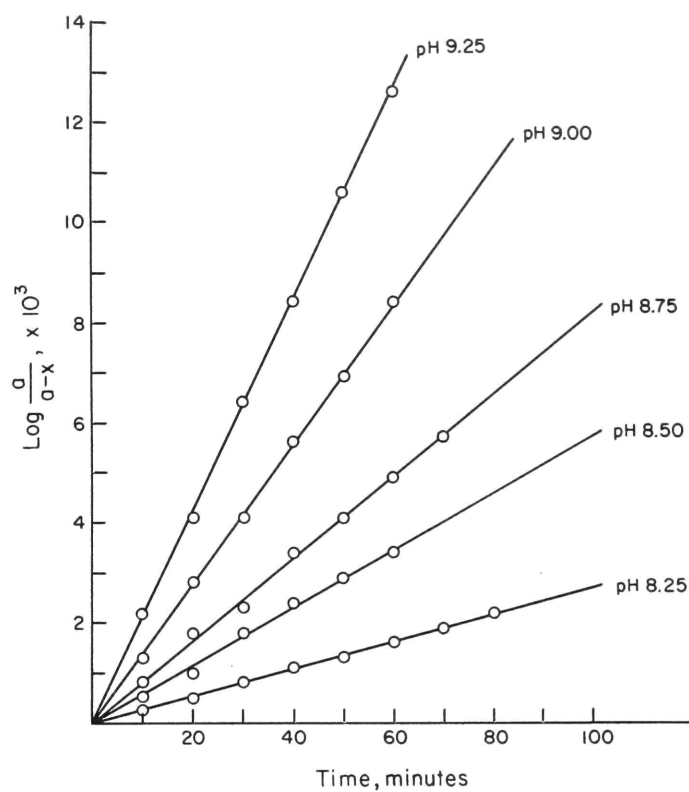
Table 1

Observed First Order Rate Constants for the Alkaline Hydrolysis of LEE.

pH	8.25	8.50	8.75	9.00	9.25
$k_{\text{obs.}} \times 10^6,$ $\text{sec}^{-1}$			3.63	5.25	8.07
	1.04	2.24	3.16	5.25	
	1.49				
		2.55	3.52	5.12	8.04
	1.36	2.31	3.19	5.57	8.80
Solution: 20% ethanol, $\mu = 0.1 \text{ M}$ (KCl), $39.5^{\circ}$ .					

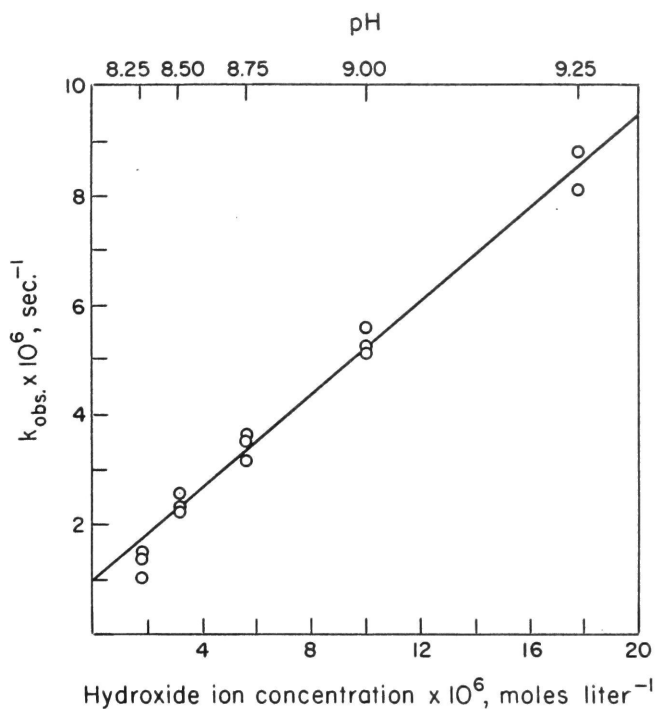
These values were plotted versus the hydroxide ion concentration (Figure 14) and from the slope of the best straight line, the second order catalytic rate constant for hydroxide ion was obtained.

$$k_{2(\text{OH}^-)} = \text{slope} = 0.43 \text{ liters mole}^{-1} \text{ sec}^{-1}$$



**Figure 13.** Alkaline Hydrolysis of LEE.

The initial rates of hydrolysis were measured at  $39.5^{\circ}$  in 20% ethanol,  $\mu = 0.1 \text{ M}$  (KCl).



**Figure 14.** Alkaline Hydrolysis of LEE.

The observed first order rate constants, obtained in 20% ethanol,  $\mu = 0.1 \text{ M}$  (KCl), at  $39.5^\circ$ , versus the hydroxide ion concentration, as calculated from the pH. The second order rate constant for hydroxide ion catalysis was obtained from the slope of the line.

In the same way, a second order catalytic rate constant for the alkaline hydrolysis of LEE in 10% ethanol (rather than 20% ethanol) and ion strength 0.1 M (potassium chloride) at 39.5° was determined and a value of 0.91 liters mole<sup>-1</sup> sec<sup>-1</sup> was obtained.

It must be stressed that the observed rate constants above were obtained by measurement of initial rates of reaction where the reaction was seldom followed to more than 5% completion. In order to follow the reactions at lower pH values to 50% or more completion, a great deal of time would be necessary (in the order of magnitude of days), and effects such as evaporation of the solvents would cause gross deviations from first order kinetics. Therefore it was necessary to assume that the kinetics were first order with respect to ester concentration. The initial rates could not be expected to show any deviations from straight lines by the graphical analysis if another reaction order were actually operating. Assumption of first order kinetics, however, is highly reasonable for the alkaline hydrolysis of carboxylic esters.

It was found that a number of reaction rates could be studied with one preparation of the reaction solution, by merely changing the pH of the reaction at various times. The new initial concentration of ester for a given run was readily calculated from the knowledge of how much ester had been hydrolyzed in all previous runs. This method gave reproducible results and conserved both time and materials.

When 0.10 ml. of a  $1.3 \times 10^{-2}$  M ethanolic solution of the "protected lysohistidine derivative" (Preparation 20) was injected into the reaction mixture at pH 8.50, a very small increase in rate over the blank reaction, which had just been measured, was observed. However, hydrolysis of the ethyl ester of the catalyst could explain the small increase in rate, so solutions of the catalyst with the ester group already hydrolyzed (see Preparation 20) were used in subsequent studies. All attempts to demonstrate an unequivocal catalytic effect due to the saponified "protected lysohistidine derivative" at reaction concentrations of catalyst ranging from  $1.6 \times 10^{-4}$  M to  $9.6 \times 10^{-4}$  M were unsuccessful at pH 8.0, 8.25 and 8.50.

The sensitivity of the assay is not very great. If it is assumed that a 20% increase in rate could be detected unequivocally, then the observed first order catalytic rate constant would be about  $2 \times 10^{-7}$  sec<sup>-1</sup>, since

the basal rate constant is about  $10^{-6} \text{ sec}^{-1}$ . Since the highest concentration of catalyst employed was about  $10^{-3} \text{ M}$ , it follows that the second order catalytic rate constant would have to have been at least  $2 \times 10^{-4} \text{ liters mole}^{-1} \text{ sec}^{-1}$  in order to be detected. Unfortunately, there are a number of

$$k_{\text{obs.}} = k_2(\text{catalyst}) + k_2(\text{OH}^-) + k_w$$

restrictions which limit the sensitivity of the assay. The concentration of the catalyst could not be increased appreciably in 20% ethanol because of its low solubility. Although the solubility would be increased at lower pH values due to salt formation, the salt would not be expected to be active as a catalyst. Solubility could have been increased by increasing the percentage of organic solvent, but then the looked-for effects of hydrophobic bonding would be reduced. If higher temperatures were used, evaporation of the solvent would become appreciable. Lower pH values in the range of pH 7 to 8 were impractical because the observed hydrolytic reaction proceeded too slowly to be measured accurately by the pH-stat.

### C. cyclo-Histidyl-phenylalanyl.

#### 1. Methods.

The same physical apparatus was used as was described above in the studies on the "protected lysohistidine derivative". The specific chymotrypsin substrate, acetyl-L-phenylalanine ethyl ester\* (APEE), was studied in the same way as LEE. The reaction conditions with acetyl-phenylalanine p-methoxyphenyl ester (APME) (Preparation 35), however, had to be changed. A 0.01 M solution of APME could not be made in 20% ethanol and 0.1 M potassium chloride; the material precipitated on the glass electrode. A stable solution was obtained with  $2.0 \times 10^{-3} \text{ M}$  ester in 30% ethanol and 0.05 M potassium chloride at  $39.5^\circ$ . The strength of the titrant was changed to 0.0255 N sodium hydroxide, carbonate free. Therefore, in these studies the initial ester concentration, "a", was equal to 78.4 ml. and the data were processed accordingly in the manner described above.

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\* Kindly supplied by Dr. Woolley, m.p.  $90-92^\circ$ .

## 2. Results.

Initial rates of hydrolysis of acetyl-L-phenylalanine ethyl ester (APEE) were determined at various pH values in the absence of catalyst. The observed first order rate constants as determined by graphical analysis, are given in the table below.

Table 2

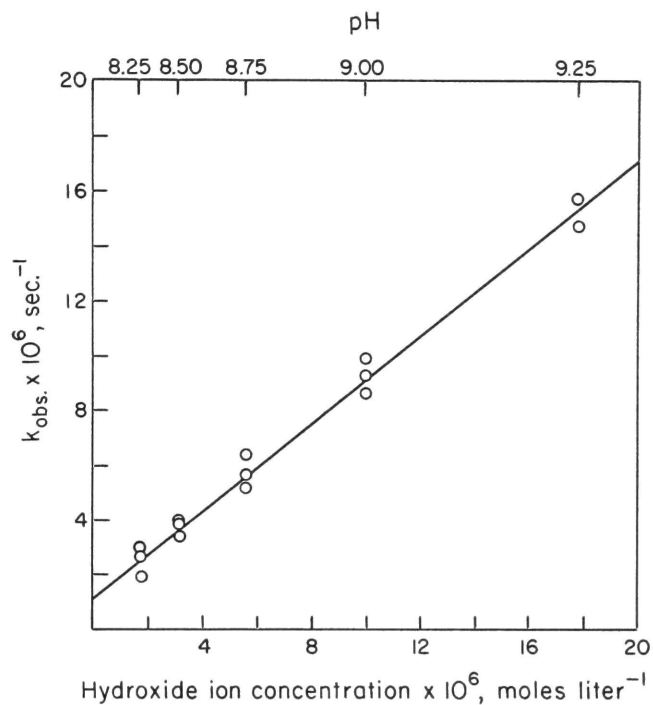
Observed First Order Rate Constants for the Alkaline Hydrolysis of APEE.

pH	8.25	8.50	8.75	9.00	9.25
$k_{\text{obs.}} \times 10^6,$ $\text{sec}^{-1}$		4.00	5.19	8.60	
	1.92	3.42	5.19	8.60	14.7
	2.69	4.00	5.65	9.87	
	2.69	4.00	6.42	9.27	15.7
	2.96				
	2.22				
Solution: 20% ethanol; 0.1 M KCl, 39.5°.					

When the values in the table were plotted against the concentration of hydroxide ion, a straight line was obtained from which the second order rate constant was calculated (Figure 15). A value of  $0.80 \text{ liters mole}^{-1} \text{ sec}^{-1}$  was observed.

After the rate of alkaline hydrolysis of APEE at pH 8.25 was determined, 0.20 ml. of 0.20 M cyclo-histidyl-phenylalanyl in 56% ethanol was injected into the solution. No increase in rate was detectable. By the injection of more catalyst, the concentration of catalyst in the reaction solution was varied from  $4 \times 10^{-4} \text{ M}$  to  $12 \times 10^{-4} \text{ M}$ , but no catalysis was observed. Injection of comparable amounts of the solvent without the catalyst dissolved in it also caused no change in rate.

It was decided to test the catalytic "power" of the cyclic dipeptide with the more labile ester, acetyl-phenylalanine p-methoxyphenyl ester (APME). The initial rates of alkaline hydrolysis of this ester at various pH values



**Figure 15.** Alkaline Hydrolysis of APPEE.

The observed first order rate constants, obtained in 20% ethanol,  $\mu = 0.1 \text{ M}$  (KCl), at  $39.5^\circ$ , versus the hydroxide ion concentration, as calculated from the pH. The second order constant for hydroxide ion catalysis was obtained from the slope of the line.

were measured and the observed first order rate constants are listed in the table below. In one run, the hydrolysis at pH 8.00 was followed to 65% completion, and the reaction was found to obey first order kinetics (Figure 16). The rate constants in the table were plotted versus the concentration of hydroxide ion, and the value,  $k_2 = 9.9 \text{ liters mole}^{-1} \text{ sec}^{-1}$ , was obtained from the slope of the line (Figure 17).

Table 3

Observed First Order Rate Constants for the Alkaline Hydrolysis of APME.

pH	7.75	8.00	8.25	8.50	8.75
$k_{\text{obs.}} \cdot 10^5, \text{ sec}^{-1}$	0.93	1.59	2.53	3.82	6.19
			2.20	3.48	5.25
		1.52			
		1.31			
		1.42			

When reaction concentrations of the cyclo-histidyl-phenylalanyl were varied from  $4 \times 10^{-4} \text{ M}$  to  $12 \times 10^{-4} \text{ M}$  by injection of appropriate amounts of the catalyst in 56% ethanol, the rate of hydrolysis of APME did not increase. There was also no increase in rate over the blank reaction detected in a run where  $2 \times 10^{-3} \text{ M}$  catalyst was employed by direct incorporation of it in the original reaction solution rather than by injection.

A limit of the sensitivity of this assay can be calculated also. The value of the catalytic second order rate constant,  $k_2$ , which would cause a 20% increase in the value of  $k_{\text{obs.}}$ , should be about  $10^{-3} \text{ liters mole}^{-1} \text{ sec}^{-1}$  when  $k_{\text{obs.}}$  is about  $10^{-5} \text{ sec}^{-1}$  and the concentration of catalyst is  $2 \times 10^{-3} \text{ M}$ . Since such an increase was not observed, the catalytic rate constant,  $k_2$ , must be less than  $10^{-3} \text{ liters mole}^{-1} \text{ sec}^{-1}$ , if it exists at all. Again, the sensitivity of the assay was limited by the solubility of the catalyst in the solvents employed and by the other factors mentioned above.

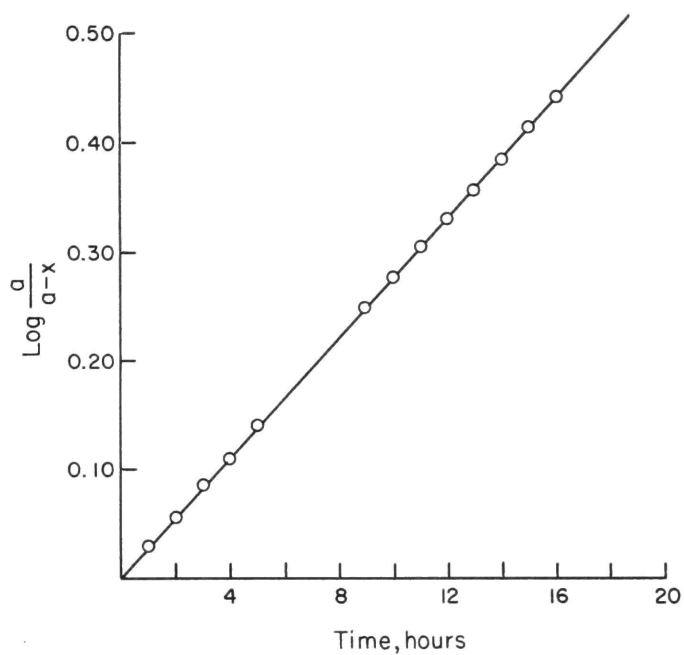
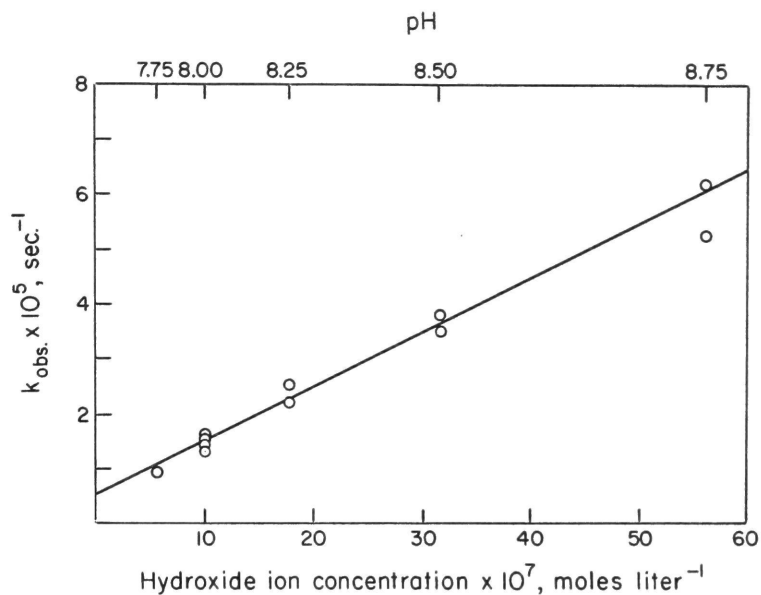


Figure 16. Alkaline Hydrolysis of APME at pH 8.00.

The reaction was followed to about 65% completion in 30% ethanol,  $\mu = 0.05 \text{ M}$  (KCl), at  $39.5^{\circ}$ .



**Figure 17.** Alkaline Hydrolysis of APME.

The observed first order rate constants, obtained in 30% ethanol,  $\mu = 0.05 \text{ M KCl}$ , at  $39.5^\circ$ , versus the hydroxide ion concentration, as calculated from the pH. The second order rate constant for hydroxide ion catalysis was obtained from the slope of the line.

D. 2-Alkylimidazoles.1. Methods.

Stock solutions of imidazole and 2-benzylimidazole (Preparation 31) were half-neutralized with hydrochloric acid and were brought to 0.12 M in 28.5% aqueous ethanol. Likewise,  $1.5 \times 10^{-4}$  M p-nitrophenyl acetate\* and p-nitrophenyl phenylacetate (Preparation 33) solutions were prepared in 28.5% ethanol just before use. Each reaction solution was prepared as follows. The catalyst solution was mixed with water, 0.06 M sodium chloride solution and ethanol, brought to 25° and the substrate solution at 25° was added quickly, shaken, and placed in a quartz cuvette. The resulting solution (3 ml.) was  $5 \times 10^{-5}$  M in substrate ester, .01, .02, .03 or .04 M in total imidazole catalyst, 28.5% in ethanol (v./v.), and the ionic strength was maintained at 0.02 M with sodium chloride. The half-neutralized imidazole catalyst also acted as the buffer in these reactions. The cuvette was placed in a thermally jacketed Beckmann DU spectrophotometer at  $25.0^{\circ} \pm 0.1^{\circ}$  and the liberation of p-nitrophenolate was measured at 400 mμ. The reactions were followed at least to 60% completion. The value at infinite time was taken after 3 days.

The kinetics were analyzed by plotting  $\log(\frac{a}{a-x})$  vs.  $t$ , where "a" is the final (infinite time) reading and "x" is the reading at time "t". From the slope of the line, the first order rate constant was obtained.

$$k_{\text{obs.}} = \frac{\log(\frac{a}{a-x})}{t} \times 2.303$$

The second order rate constant,  $k_2$ , was obtained from the slope of a plot of the  $k_{\text{obs.}}$ , for various concentrations of catalyst versus the concentration of catalyst in the free base form (i.e., half of the total catalyst concentration).

$$k_{\text{obs.}} = k_2(\text{catalyst free base})$$

The series of studies with 2-benzylimidazole and 2-isobutylimidazole were made in the same way, except that the reaction solutions were .004, .008, .014 and .020 M in total catalyst at half neutralization, only 6.3% in ethanol, and the ionic strength was maintained at .05 M with potassium chloride. The p-nitrophenyl ester concentrations remained at  $5 \times 10^{-5}$  M.

## 2. Results.

The observed first order rate constants for the hydrolysis of p-nitrophenyl acetate (NPA) and p-nitrophenyl phenylacetate (NPPA) with imidazole and 2-benzylimidazole in 28.5% aqueous ethanol are recorded in Table 4.

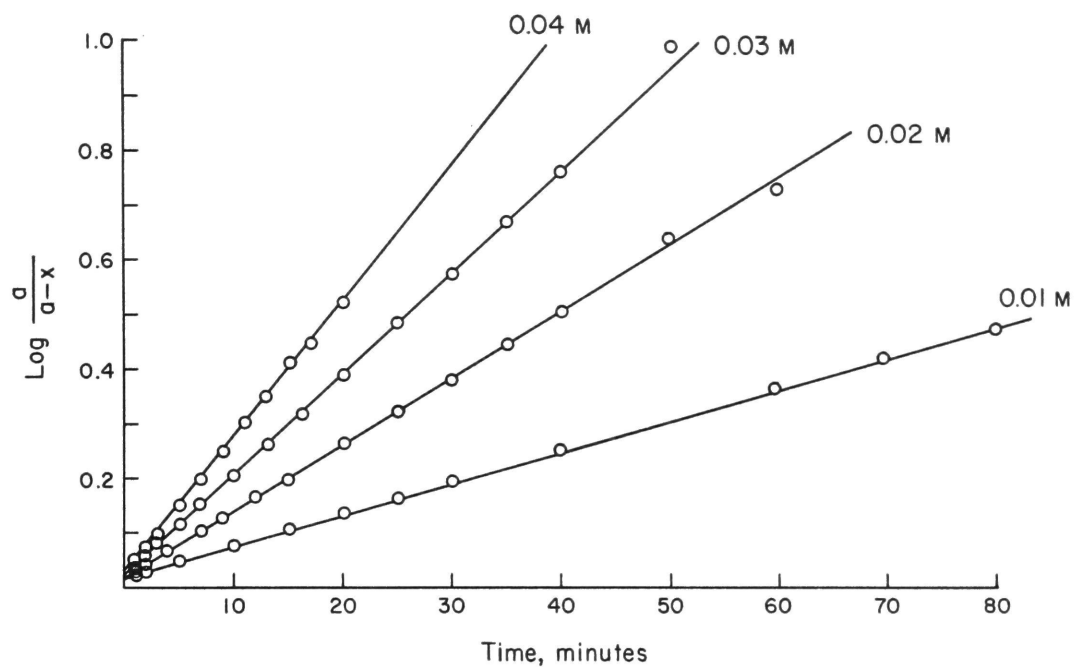
Table 4

Observed First Order Rate Constants

Catalyst	Total catalyst conc., moles/l	$k_{\text{obs.}}, \text{sec}^{-1}$	
		<u>p</u> -nitrophenyl acetate	<u>p</u> -nitrophenyl phenylacetate
Imidazole	.040	$5.43 \times 10^{-3}$	$10.57 \times 10^{-3}$
	.030	$3.87 \times 10^{-3}$	$7.67 \times 10^{-3}$
	.020	$2.67 \times 10^{-3}$	$4.97 \times 10^{-3}$
	.010	$1.32 \times 10^{-3}$	$2.64 \times 10^{-3}$
2-Benzyl-imidazole	.040	$2.28 \times 10^{-4}$	$9.61 \times 10^{-4}$
	.030	$1.66 \times 10^{-4}$	$6.40 \times 10^{-4}$
	.020	$1.18 \times 10^{-4}$	$4.70 \times 10^{-4}$
	.010	$.59 \times 10^{-4}$	$2.18 \times 10^{-4}$
Ester, $5 \times 10^{-5} \text{ M}$ ; $T = 25^{\circ}$ ; 28.5% ethanol; $\mu = 0.02 \text{ M}$ (NaCl).			

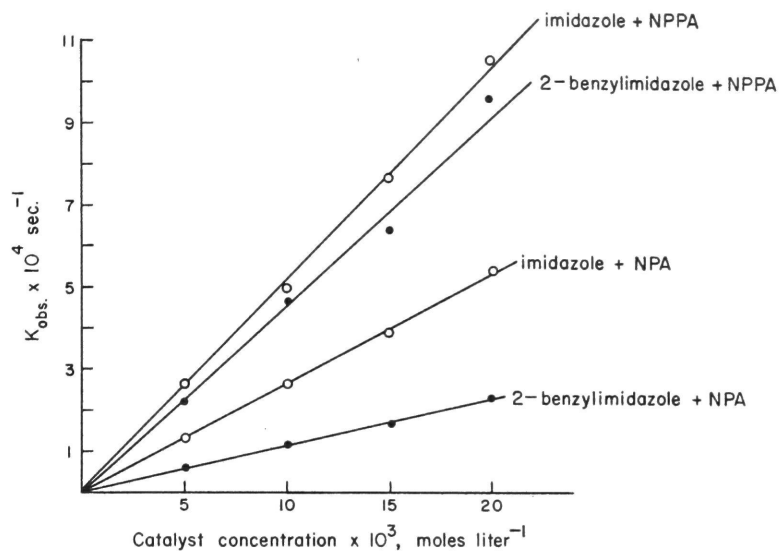
A typical plot of the kinetic data, from which the observed rate constants were obtained, is shown in Figure 18. All the reactions followed pseudo first order kinetics as far as they were measured (from 60 to 90% completion). Since all points lay very close to a straight line, the slope could be obtained by simple graphical analysis, and a statistical analysis, such as the method of "least squares", was considered unnecessary.

A plot of the observed first order rate constants versus the concentration of imidazole catalyst in the free base form for the various reaction series (Figure 19) showed that the rate of hydrolysis was proportional to



**Figure 18.** 2-Benzylimidazole Catalyzed Hydrolysis of NPPA.

The hydrolyses were conducted in 28.5% ethanol,  $\mu = 0.02 \text{ M}$  (NaCl), at  $25^{\circ}$ , with total 2-benzylimidazole concentrations, half-neutralized, of .01, .02, .03 and .04 M, and an initial ester concentration of  $5 \times 10^{-5} \text{ M}$ .



**Figure 19.** Imidazole and 2-Benzylimidazole Catalyzed Hydrolyses of NPA and NPPA.

The observed first order rate constants, obtained in 28.5% ethanol,  $\mu = 0.02 \text{ M}$  (NaCl), at  $25^\circ$ , versus the concentration of the catalyst free base. The second order catalytic rate constants were obtained from the slopes of the lines.

the concentration of the active catalyst. By analogy to similar studies (17), it was assumed that the hydrochlorides of these imidazoles were not reactive. The second order rate constants for imidazole were higher than for 2-benzylimidazole with the two esters, even though the basicity of 2-benzylimidazole (apparent  $pK_a = 6.98$ ) is slightly greater than imidazole (apparent  $pK_a = 6.75$ ). The apparent  $pK_a$ 's were determined by half-neutralization in 28.5% ethanol. Apparently steric hindrance by the 2-benzyl group plays a significant role in the catalysis of hydrolysis of even the smaller p-nitrophenyl acetate. Our value for the second order rate constant of imidazole and p-nitrophenyl acetate ( $0.23 \text{ liters mole}^{-1} \text{ sec}^{-1}$ ) compares favorably with the value of Bruice and Schmir (33), obtained in 28.5% ethanol at  $25^\circ$  ( $0.21 \text{ liters mole}^{-1} \text{ sec}^{-1}$ ).

If we assume that the ratio,  $k_2$ , of p-nitrophenyl phenylacetate over  $k_2$  of p-nitrophenyl acetate, for imidazole catalysis represents a crude measure of the reactivity of p-nitrophenyl phenylacetate relative to p-nitrophenyl acetate, then deviations from this value for other imidazoles would indicate that factors other than ester reactivity are operating. The ratio for imidazole is 2 while for 2-benzylimidazole it is 4, indicating that a very small enhancement in rate has been observed for 2-benzylimidazole and the phenylacetate ester. Unfortunately, the "effect" is so small that any hypotheses concerning its cause cannot be convincing.

Since the catalysis of p-nitrophenyl acetate by 2-benzylimidazole is considerably less than would be anticipated, from knowledge of its basicity, it was assumed that steric hindrance by the bulky 2-benzyl group was responsible. Interaction with the larger p-nitrophenyl phenylacetate might therefore be expected to show even greater steric hindrance, and it was possible that the small effect expressed by the ratios above was due to steric hindrance which would tend to make the "effect" smaller. Therefore it was decided to compare 2-isobutylimidazole and 2-benzylimidazole, on the assumption that the isobutyl group would exert at least as much steric interference as the benzyl group. At the same time, it was decided to decrease the ethanol concentration to 6.3% in order to enhance the possibility of hydrophobic bonding interactions. The observed first order rate constants obtained from these studies are given in Table 5, and the typical rate plots

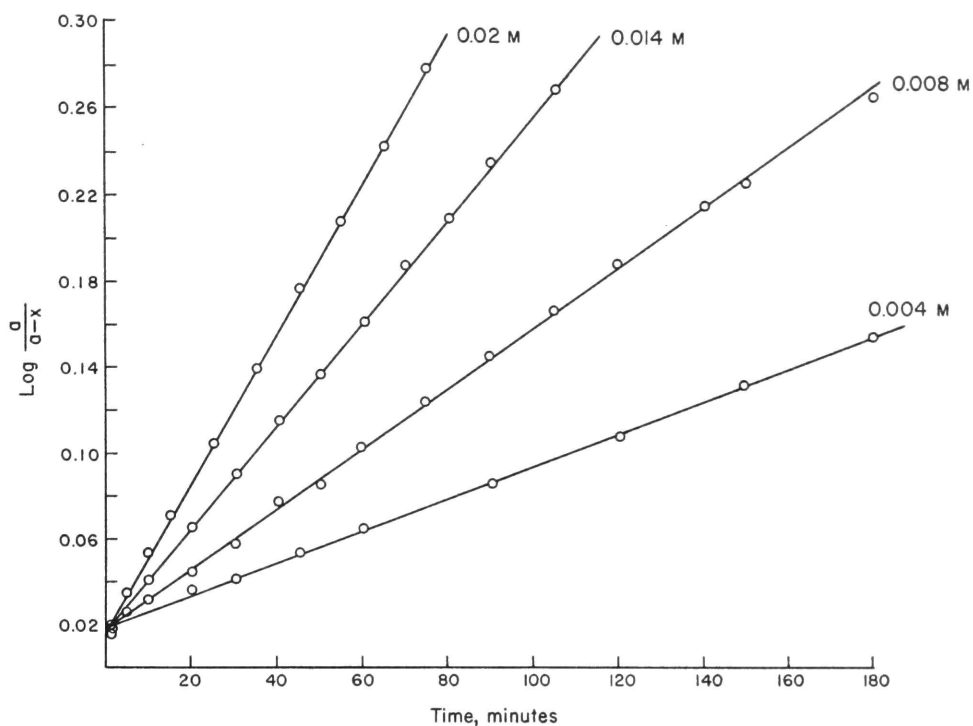
are shown in Figure 20. The second order constants were obtained from a graphical analysis of Figure 21, where  $k_{\text{obs}}$  versus active catalyst concentration was plotted.

Table 5

Observed First Order Rate Constants

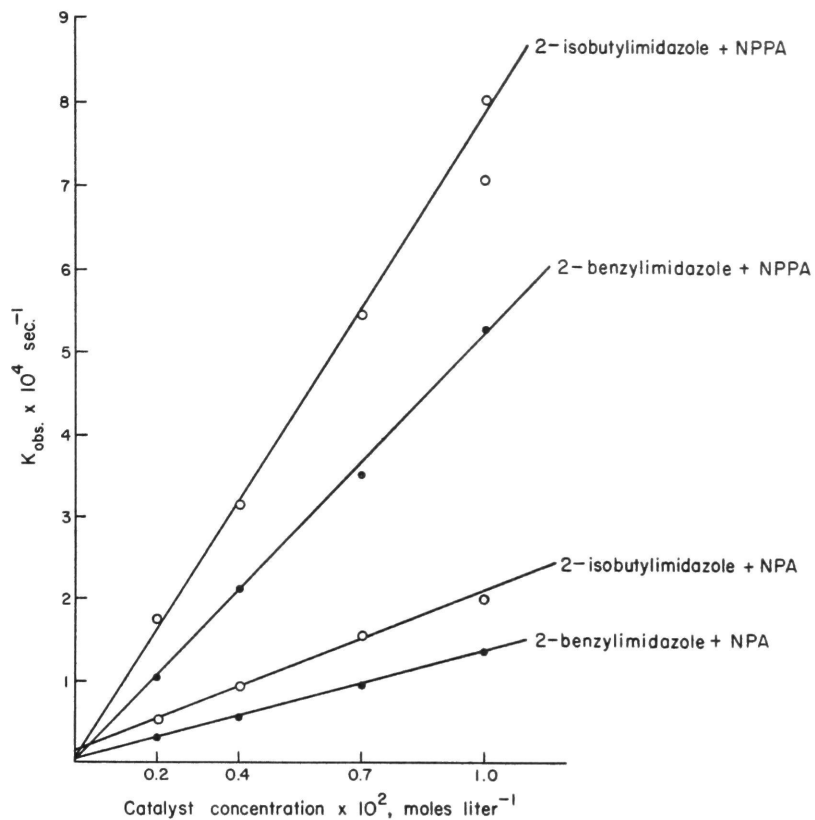
Catalyst	Total catalyst conc. moles/l	$k_{\text{obs}}$	
		p-nitrophenyl acetate	p-nitrophenyl phenylacetate
2-Benzyl-imidazole	.020	$1.34 \times 10^{-4}$	$5.30 \times 10^{-4}$
	.014	$.92 \times 10^{-4}$	$3.52 \times 10^{-4}$
	.008	$.54 \times 10^{-4}$	$2.12 \times 10^{-4}$
	.004	$.29 \times 10^{-4}$	$1.05 \times 10^{-4}$
2-Isobutyl-imidazole	.020	$2.04 \times 10^{-4}$	$8.06 \times 10^{-4}$
	.014	$1.58 \times 10^{-4}$	$5.45 \times 10^{-4}$
	.008	$.94 \times 10^{-4}$	$3.15 \times 10^{-4}$
	.004	$.52 \times 10^{-4}$	$1.76 \times 10^{-4}$
Ester, $5 \times 10^{-5}$ M; $T = 25^{\circ}$ ; 6.3% ethanol; $\mu = .05$ M (KCl).			

In these studies, the ratio,  $k_2$ , of p-nitrophenyl phenylacetate over  $k_2$  of p-nitrophenyl acetate, is approximately 4 for both 2-isobutylimidazole and 2-benzylimidazole. Thus, the benzyl group shows no "effect" due to selective binding in relation to the isobutyl group. Although the basicity of the 2-benzylimidazole group (apparent  $\text{pK}_a = 7.20$ , measured in 6.3% ethanol, 0.05 M potassium chloride, by half-neutralization) is considerably less than that of 2-isobutylimidazole (apparent  $\text{pK}_a = 7.85$ , measured as above), expression of the "effect" by a ratio should have cancelled out deviations due to such differences in the catalysts.



**Figure 20.** 2-Benzylimidazole Catalyzed Hydrolyses of NPA.

The hydrolyses were conducted in 6.3% ethanol,  $\mu = 0.05$  M (KCl), at  $25^{\circ}$ , with total 2-benzylimidazole concentrations, half-neutralized, of .004, .008, .014 and .020 M and an initial ester concentration of  $5 \times 10^{-5}$  M.



**Figure 21.** 2-Alkylimidazole Catalyzed Hydrolyses of NPA and NPPA.

The observed first order rate constants, obtained in 6.3% ethanol,  $\mu = 0.05$  M (KCl), at 25°, versus the concentration of the catalyst free base. The second order rate constants were obtained from the slopes of the lines.

It is therefore necessary to conclude that no unusual specific effects on the rate of hydrolysis due to hydrophobic bonding or van der Waals attraction were demonstrated in this experimental system.

## **PART IV**

### **DISCUSSION**

In Part II has been described in detail a number of reactions of  $\alpha$ -haloketones and amidines. The general usefulness of this reaction in the preparation of 2,4-dialkylimidazoles has therefore been established. The unalkylated amidines underwent the reaction much more readily than the alkylated amidines, so the preparation of 1,2,4-trialkylimidazoles in this way is not always practical. The rather small yields of products from the reactions of the  $\alpha$ -acetamido-amidines may have been due in part to the less reactive properties of the amidines in comparison to unsubstituted alkyl-amidines. However, it should be pointed out that the reported yields of isolated products were considerably lower than actually produced in the reaction, because much material was lost during the numerous operations performed in attempts to find a way to purify them.

The synthesis of ethyl L-2-carbobenzoxamido-4-oxo-5-chlorovalerate was carried out in rather good overall yield, 30% from L-aspartic acid in five steps. The major difficulty in this synthesis was the preparation of the  $\alpha$ -ethyl ester of carbobenzoxy-L-aspartic acid. One of the important side reactions was the formation of the diester, diethyl N-carbobenzoxy-L-aspartate. Also the desired mono- $\alpha$ -ethyl ester crystallized reluctantly and could be purified only by the laborious fractional extraction with bicarbonate solution as has been described. The chloromethylketone, on the other hand, crystallized with great ease and was more easily purified.

The preparation of the amidines derived from  $N^{\alpha}$ -acetyl- $N^{\epsilon}$ -tosyl-lysine was more difficult. The amidines were obtained in 20 to 25% overall yield from L-lysine in seven steps. In the synthesis of the amide, complete racemization unexpectedly occurred. The mixed anhydride method of amide or peptide synthesis normally does not produce racemization, particularly in tetrahydrofuran (Greenstein and Winitz (57), p. 981). The excess ammonium hydroxide used may have been the cause of the difficulty, but it has not yet been possible to determine this. In the next step, the dehydration of the amide to the nitrile, it is noteworthy that only one of many methods was successful. Phosphorous oxychloride alone failed, but in the presence of dry pyridine succeeded in converting the amide to the nitrile. It is not unlikely that steric effects play an important role in all of these reactions at the 1-carbon of the lysine derivatives.

The preparation of pure amidines was particularly difficult because they crystallized as hydrochloride salts only reluctantly. In all cases it was necessary to use countercurrent distribution to purify the compounds. The imidate ester intermediate was sensitive to moisture and hydrolyzed readily to an amide, while the amidine derivative was labile to rather strong acid, the  $\alpha$ -acetamido group being hydrolyzed easily to an amine.

The synthesis of  $N^\alpha$ -carbobenzoxy-1,2-dibenzylhistidine and the "protected lysohistidine derivative" proceeded in small yields and the compounds could not be crystallized, even when purified. Without the techniques of separation such as countercurrent distribution and column chromatography recently developed in the field of biochemistry, it would have been impossible to isolate the desired products from the excess reagents and contaminants of the crude reaction mixtures. It is probably true that these preparations could not have been performed successfully twenty years ago. The powerful separatory techniques have made it possible for the organic chemist to synthesize and purify unusual compounds which can be obtained only in poor yields and which are difficult to crystallize.

It is a general characteristic of reactions and preparations of even simple imidazoles that by-products are formed which are difficult to separate from the desired products and which cause the reaction solutions to be deeply colored. The simpler imidazoles were converted into insoluble oxalate or bioxalate salts in order to isolate them, a common practice in the early work with imidazoles. The technique therefore complemented countercurrent distribution and column chromatography as an effective means of purification of these derivatives. The salts crystallized easily and the imidazole free base could be readily regenerated.

The ultimate synthetic goal involving the "lysohistidine derivative" was the formation of polypeptides of this compound. The corresponding N-carboxy- $\alpha$ -amino acid anhydride could not be prepared, however, until its imidazole nitrogen was protected. The attempt to prepare the "protected lysohistidine derivative" with a 1-benzyl group attached to the imidazole ring nitrogen by the use of the N-benzylamidine derived from  $N^\alpha$ -acetyl- $N^\epsilon$ -tosyl-lysine was not successful. The "protected lysohistidine derivative" without the benzyl group was synthesized instead. Since it was shown in Part II that  $N^\alpha$ -carbobenzoxy-L-histidine could be benzylated with sodium

hydride and benzyl chloride without reductive cleavage of the carbobenzoxy group, it seems reasonable to expect that the reaction can be performed successfully with the saponified "protected lysohistidine derivative." Due to a lack of material and time, however, the reactions have not been carried out yet.

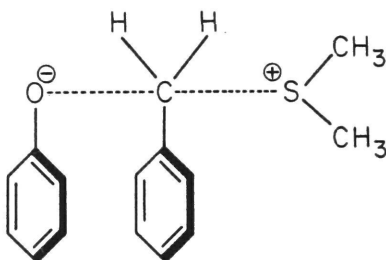
All three preliminary kinetic studies reported in Part III failed to show any unusual catalytic effect which could be attributed to an interaction of the side chains of the catalyst and substrate. The assays involving the "protected lysohistidine derivative" and cyclo-histidyl-phenylalanyl were particularly insensitive to the detection of any catalysis by these compounds, since the concentration of catalysts, as dictated by their solubilities, was necessarily low. Second order catalytic rate constants could exist below the level of the sensitivity (about  $10^{-3}$  to  $10^{-4}$  liters mole<sup>-1</sup> sec<sup>-1</sup>) which would still be very meaningful with respect to the working hypothesis. It is clear that the rates of the catalyzed reactions are small, if they exist at all, and therefore the catalysts do not resemble their respective enzymes in this respect. What is not yet clear is whether the model catalysts may be capable of catalyzing the hydrolysis of their "specific" substrates, but not others, albeit at rather low rates. It is therefore necessary to devise additional kinetic experiments to determine whether these catalysts can at least mimic the "specific" action of trypsin and chymotrypsin, but not the efficiency. In the studies with 2-benzylimidazole, however, the catalytic rate constants were actually measured, but no looked-for effect due to the phenyl rings was observed.

There are a number of steps which could be taken to help increase the sensitivity of the assay. The basic problem is to reduce the alkaline catalyzed reaction in relation to the looked-for imidazole catalyzed reaction. This might be done by lowering the pH from 8.0 to 7.0 or 7.5, where the imidazole is still largely unprotonated. The actual rate might then be too small to measure, but could be increased by carrying out the hydrolyses at higher temperatures. However, hydrophobic bonding, which may contribute considerably to substrate binding, is thought to be diminished as the temperature is increased. Perhaps a different solvent system could be found (e.g., dimethylsulfoxide-water) in which the catalyst would be more soluble, but which still would have a rather high dielectric constant and

water content. The ratio of the concentration of ester to catalyst might be lowered, also. In addition, a slightly more reactive aliphatic ester could be employed as the substrate, e.g.,  $\beta,\beta$ -dichloroethyl esters.

The solubility problem encountered with the "protected lysohistidine derivative" will be considerably reduced when the originally proposed "lysohistidine derivative" is finally made. Problems of solubility of the polymer should be capable of resolution by co-polymerization of the "lysohistidine derivative" with, for example, glutamic or aspartic acid. These amino acids could be expected to confer solubility on the polymer in aqueous solutions.

In view of the experimental results described here, it is valid to question whether the attraction of a substrate to a catalyst can ever effect an enhancement in reaction rate. It is necessary that the interaction lower the energy of the transition state in the rate-determining step. Through ideal binding the substrate is correctly oriented with respect to the catalyst, and thus the entropy of the transition state should be lower as the catalyst acts within the complex, than when the catalyzed reaction occurs freely by collision in solution. Swain (172) believes that such an effect is operating in the nucleophilic substitution reaction of benzyldimethylsulfonium chloride with hydroxide and phenoxide ions. Phenoxide ion is normally twice as poor a nucleophile as hydroxide ion, while in this case its rate of reaction was three times greater than that of the hydroxide. Swain suggests that a phenyl-phenyl interaction in the transition state is responsible for the 6-fold enhancement over the expected rate.



Although the reaction has not been studied carefully in different solvent systems and under various conditions, and thus the conclusions may be premature, it is nevertheless interesting and does suggest that rate enhancements of this kind are possible when the proper conditions are met.

It was not expected that the monomer of the "protected lysohistidine derivative" would possess exceptional catalytic activity. Rather, it was hoped that some sort of catalytic effect would be observed with the proposed polymers containing the "lysohistidine derivative", but these have not yet been prepared and tested. However, the other two approaches to the problem of specificity and enzyme action were studied as originally intended. These were the cyclo-histidyl-phenylalanyl and the 2-alkylimidazoles, but as has been mentioned before, they exhibit no effect due to substrate-catalyst side chain interaction. It is therefore appropriate to consider possible rationalizations of why these derivatives were not active in the manner anticipated.

The binding energy might not have been sufficient to allow the reaction to take place. There is little quantitative data from which an answer can be formed to this question. Dispersion forces, hydrophobic bonding and hydrogen bonding are certainly relatively weak interactions which can be disrupted by thermal energy. It may be that some little understood effect of large size of at least one of the molecules may play an important role in this regard.

If binding had taken place, the substrate might have been oriented more often incorrectly than correctly. Such a criticism could be used in the case of 2-benzylimidazole and p-nitrophenyl phenylacetate, since the benzene rings could be attracted to each other equally well with the ester portion of the substrate extending away from the imidazole portion of the catalyst. The argument is less valid for two-point binding, as envisioned in the case of the "protected lysohistidine derivative" and cyclo-histidyl-phenylalanyl. It was anticipated that with both catalysts the substrate would bind along the alkyl side chain and the acetamido groups. If such 2-point binding did occur, the orientation with respect to the catalyst would necessarily be relatively favorable.

The conformation of the catalyst might have been unfavorable for maximal binding and correct orientation. The catalysts synthesized in this study were designed in particular to eliminate such conformational or configurational uncertainty. Rather than to synthesize polymers of random or uncertain conformation with histidine and phenylalanine or lysine, it was decided on the one hand to attach the lysine portion directly to the imidazole ring and, on the other, to bring phenylalanine into a relatively fixed

position with respect to the imidazole of histidine by forming a rather rigid diketopiperazine derivative. In other words, "tertiary structure" is not necessary to hold the specificity site and the imidazole close to each other in these model compounds, as it is believed to be in the case of the enzymes. However, the lysine-like side chain in particular is still capable of rotating freely and might have assumed a conformation unfavorable to binding. In the case of the cyclic peptide, the amide bonds which are envisioned as the site of binding of the acetamide portion of the substrate are cis,\* while the substrate acetamido groups in solution is probably predominantly in the more stable trans conformation. In order to bind, therefore, the substrate acetamido group must assume an energetically less favorable cis conformation.

Even if the substrate had been attracted ideally to the specificity site of the model catalyst, the geometry may not have been sufficiently precise for the imidazole to act efficiently. When the "lysohistidine derivative" was proposed as a suitable catalyst, it was believed that the imidazole in chymotrypsin and trypsin acted by nucleophilic attack on the ester carbonyl carbon. Recent evidence, some of which was described in Part I, indicates that a general base mechanism of imidazole catalysis might more likely be involved in the hydrolysis of the ethyl esters selected here as substrates. It is quite possible that when the  $N^{\alpha}$ -acetyl- $N^{\epsilon}$ -tosyl-lysine ethyl ester is bound to the "protected lysohistidine derivative", the imidazole is not located in precisely the correct position for general base catalysis to occur. Likewise the more flexible imidazole group in the cyclo-histidyl-phenylalanyl model may be poorly located with respect to the ester group in the bound substrate. It will be recalled that intramolecular general base catalysis by imidazole occurred when pseudo 6- and 7-membered rings could form with water, but did not occur measurably when a pseudo 8-membered ring was required. There appears to be a very high sensitivity to the geometry of the transition states in these reactions, and therefore most likely in the ones being studied here. Molecular models (Stuart-Briegleb space-filling

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\* The conformation of peptide bonds of proteins, as determined by X-ray crystallographic studies on myoglobin, seem to be invariably trans, even in non-helical regions. Because the six atoms involved in the amide bond, C-NH-CO-C, were found to be co-planar, the cis-trans terminology is justifiable. The peptide bond is called trans or cis with respect to the two adjoining carbon atoms.

models) of these compounds were built to be certain that substrate binding and subsequent reactions were at least sterically possible.

The inability to demonstrate the desired effect of rate enhancement due to specific attraction of similar side chains on the catalyst and substrate does not disprove the working hypothesis concerning the nature of the specificity site in enzymes. There are too many possible imperfections in the model catalysts actually synthesized to account for the negative result. It was recognized when this work was begun that only a positive demonstration of the looked-for rate enhancement could be meaningful with respect to the enzyme problem. Although the great risks involved in the undertaking were appreciated, the potential gain was considered sufficiently high to warrant the experiments.

## BIBLIOGRAPHY

1. Akabori, S., K. Okawa and F. Sakiyama, Nature, 181, 772 (1958).
2. Almond, H.R., Jr., D.T. Manning and C. Niemann, Biochemistry, 1, 243 (1962).
3. Anderson, B.M., E.H. Cordes and W.P. Jencks, J. Biol. Chem., 236, 455 (1961).
4. Awad, E.S., H. Neurath and B.S. Hartley, J. Biol. Chem., 235, P.C. 35 (1960).
5. Bachmann, W.E. and W.S. Strune, in Organic Reactions, John Wiley, New York, 1942, v. 1, p. 38.
6. Balls, A.K. and H.N. Wood, J. Biol. Chem., 219, 245 (1956).
7. Bamberger, E., Ann. Chem., 273, 267 (1893).
8. Barnard, E.A. and W.D. Stein, Biochem. J., 71, 19P (1959).
9. Bender, M.L., Chem. Rev., 60, 53 (1960).
10. Bender, M.L., G.E. Clement, F.J. Kézdy and B. Zerner, J. Am. Chem. Soc., 85, 358 (1963).
11. Bender, M.L. and G.A. Hamilton, J. Am. Chem. Soc., 84, 2570 (1962).
12. Bender, M.L., E.J. Pollock and M.C. Neveu, J. Am. Chem. Soc., 84, 595 (1962).
13. Bender, M.L., G.R. Schonbaum and G.A. Hamilton, J. Polymer Sci., 49, 75 (1961).
14. Bender, M.L., G.R. Schonbaum and B. Zerner, J. Am. Chem. Soc., 84, 2540 (1962).
15. Bender, M.L., G.R. Schonbaum and B. Zerner, J. Am. Chem. Soc., 84, 2562 (1962).
16. Bender, M.L. and M.S. Silver, J. Am. Chem. Soc., 84, 4589 (1962).
17. Bender, M.L. and B.W. Turnquest, J. Am. Chem. Soc., 79, 1652, 1656 (1957).
18. Bender, M.L. and B. Zerner, J. Am. Chem. Soc., 84, 2550 (1962).
19. Bergmann, M. and J.S. Fruton, Advan. Enzymol., 1, 63 (1941).
20. Bergmann, M. and L. Zervas, Ber. deut. chem. Ges., 65, 1192 (1932).

21. Bergmann, M., L. Zervas and L. Salzmann, Ber. deut. chem. Ges., 66, 1288 (1933).
22. Bernhard, S.A., Brookhaven Symp. Biol., 15, 128 (1962).
23. Bernhard, S.A., J. Cell. Comp. Physiol., 54, Suppl. 1, 195 (1959).
24. Bernhard, S.A., A. Berger, J.H. Carter, E. Katchalski, M. Sela and Y. Shalitin, J. Am. Chem. Soc., 84, 2421 (1962).
25. Bopp, H., Ann. Chem., 69, 28 (1849).
26. Braun, G., in Organic Syntheses, ed. 2, John Wiley, New York, 1941, coll. v. 1, p. 431.
27. Bredereck, H., R. Gompper, H.G.v. Schuh and G. Theilig, Angew. Chem., 71, 753 (1959).
28. Breslow, R., J. Am. Chem. Soc., 80, 3719 (1958).
29. Brown, H.C. and C.W. McGary, Jr., J. Am. Chem. Soc., 77, 2300, 2306 (1955).
30. Bruice, T.C., Brookhaven Symp. Biol., 15, 52 (1962).
31. Bruice, T.C., J. Am. Chem. Soc., 81, 5444 (1959).
32. Bruice, T.C., T.H. Fife, J.J. Bruno and P. Benkovic, J. Am. Chem. Soc., 84, 3012 (1962).
33. Bruice, T.C. and G.L. Schmir, J. Am. Chem. Soc., 79, 1663 (1957).
34. Bruice, T.C. and G.L. Schmir, J. Am. Chem. Soc., 81, 4552 (1959).
35. Bruice, T.C. and J.M. Sturtevant, Biochim. et Biophys. Acta, 30, 208 (1958).
36. Bruice, T.C. and J.M. Sturtevant, J. Am. Chem. Soc., 81, 2860 (1959).
37. Buu-Hoï and J. Lecocq, Bull. Soc. Chim. France, ser. 5, 13, 139 (1946).
38. Chambers, R.W. and F.H. Carpenter, J. Am. Chem. Soc., 77, 1522 (1955).
39. Craig, L.C. and D. Craig, in Weissberger, A., ed., Separation and Purification, Interscience, New York, 1956, p. 149.
40. Cunningham, L.W. and C.S. Brown, J. Biol. Chem., 221, 287 (1956).
41. Curtius, T. and F. Goebel, J. prakt. Chem., n. 7, 37, 173 (1888).
- 41a. Davis, A.C. and A.L. Levy, J. Chem. Soc., 2179 (1949).
42. Delaby, R., G. Tsatsas and X. Lusinchi, Bull. Soc. Chim. France, 409 (1958).

43. Dixon, G.H., H. Neurath and J.F. Pechère, Ann. Rev. Biochem., 27, 489 (1958).
44. Dixon, M. and E.C. Webb, Enzymes, Academic Press, New York, 1958, p. 62.
45. du Vigneaud, V. and O.K. Behrens, J. Biol. Chem., 117, 27 (1937).
46. du Vigneaud, V. and C.E. Meyer, J. Biol. Chem., 98, 295 (1932).
47. Dzyaloshinskii, I.E., E.M. Lifshitz and L.P. Pitaevskii, Advan. Phys., 10, 165 (1961).
48. Erlenmeyer, H., D. Waldi and E. Sorkin, Helv. Chim. Acta, 31, 32 (1948).
49. Erne, M. and H. Erlenmeyer, Helv. Chim. Acta, 31, 652 (1948).
50. Fanta, P.E. and E.A. Hedman, J. Am. Chem. Soc., 78, 1434 (1956).
51. Fasman, G.D., M. Idelson and E.R. Blout, J. Am. Chem. Soc., 83, 709 (1961).
52. Fischer, E., Ber. deut. chem. Ges., 27, 2985 (1894).
53. Fischer, E. and E. Otto, Ber. deut. chem. Ges., 36, 2106 (1903).
54. Foster, R.J., J. Biol. Chem., 236, 2461 (1961).
55. Frost, A.A. and R.G. Pearson, Kinetics and Mechanism, ed. 2, John Wiley, New York, 1961, p. 14.
56. Goldberg, A.A., S. Mallet and L.P. Ellinger, U.S. Patent No. 2532547 (1950).
57. Greenstein, J.P. and M. Winitz, Chemistry of the Amino Acids, John Wiley, New York, 1961.
58. Greig, C.G. and D.H. Leaback, Nature, 188, 310 (1960).
59. Grindley, R. and F.L. Pyman, J. Chem. Soc., 3128 (1927).
60. Gundlach, H.G., S. Moore and W.H. Stein, J. Biol. Chem., 234, 1761 (1959).
61. Gundlach, H.G., W.H. Stein and S. Moore, J. Biol. Chem., 234, 1754 (1959).
62. Gutfreund, H. and B.R. Hammond, Biochem. J., 73, 526 (1959).
63. Gutfreund, H. and J.M. Sturtevant, Biochem. J., 63, 656 (1956).
64. Gutfreund, H. and J.M. Sturtevant, Proc. Nat. Acad. Sci. U.S., 42, 719 (1956).

65. Hammond, B.R. and H. Gutfreund, Biochem. J., 61, 187 (1955).
66. Harington, C.R. and R.C.G. Moggridge, J. Chem. Soc., 706 (1940).
67. Harington, C.R. and J. Overhoff, Biochem. J., 27, 338 (1933).
68. Hartley, B.S., Ann. Rev. Biochem., 29, 45 (1960).
69. Hartley, B.S., Brookhaven Symp. Biol., 15, 85 (1962).
70. Hartley, B.S. and B.A. Kilby, Biochem. J., 50, 672 (1952).
71. Hartley, B.S. and B.A. Kilby, Biochem. J., 56, 288 (1954).
72. Hartley, B.S. and V. Massey, Biochim. et Biophys. Acta, 21, 58 (1956).
73. Havsteen, B.H. and G.P. Hess, J. Am. Chem. Soc., 84, 491 (1962).
74. Hearon, J.Z., S.A. Bernhard, S.L. Friess, D.J. Botts and M.F. Morales, in P.D. Boyer, H.A. Lardy and K. Myrbäck, eds., The Enzymes, ed. 2, Academic Press, New York, 1959, v. 1, p. 49.
75. Hein, G.E., R.B. McGriff and C. Niemann, J. Am. Chem. Soc., 82, 1830 (1960).
76. Hein, G.E. and C. Niemann, J. Am. Chem. Soc., 84, 4495 (1962).
77. Hertog, H.J. den, C.R. Kolder and W.P. Combé, Rec. Trav. Chim., 70, 591 (1951).
78. Hesse, O. and H. Limpricht, Ann. Chem., 116, 201 (1860).
79. Hill, A.J. and S.R. Aspinall, J. Am. Chem. Soc., 61, 822 (1939).
80. Hill, R.L. and E.L. Smith, J. Biol. Chem., 235, 2332 (1960).
81. Hirs, C.H.W., S. Moore and W.H. Stein, J. Biol. Chem., 235, 633 (1960).
82. Hofmann, K., Imidazole and Its Derivatives, Interscience, New York, pt. 1, 1953.
83. Jansen, E.F., M.-D.F. Nutting and A.K. Balls, J. Biol. Chem., 179, 201 (1949).
84. Jansen, E.F., M.-D.F. Nutting, R. Jang and A.K. Balls, J. Biol. Chem., 179, 189 (1949).
85. Jehle, H., J.M. Yos and W.L. Bade, Phys. Rev., ser. 2, 110, 793 (1958).
86. Jencks, W.P. and J. Carriuolo, J. Am. Chem. Soc., 82, 1778 (1960).
87. Jencks, W.P. and J. Carriuolo, J. Am. Chem. Soc., 83, 1743 (1961).

88. Jennings, R.R. and C. Niemann, J. Am. Chem. Soc., 75, 4687 (1953).
89. Jones, R.G., J. Am. Chem. Soc., 71, 383 (1949).
90. Kadin, S., personal communication.
91. Karrer, P. and M. Ehrenstein, Helv. chim. Acta, 9, 323 (1926).
92. Katchalski, E., Adv. Prot. Chem., 6, 123 (1951).
93. Kaufman, S., G.W. Schwert and H. Neurath, Arch. Biochem., 17, 203 (1948).
94. Kauzmann, W., Adv. Prot. Chem., 14, 1 (1959).
95. Kay, C.M., L.B. Smillie and F.A. Hilderman, J. Biol. Chem., 236, 118 (1961).
96. Keil, B., B. Meloun, J. Vaněček, V. Kostka, Z. Prusík and F. Šorm, Biochim. et Biophys. Acta, 56, 595 (1962).
97. Kendrew, J.C., Brookhaven Symp. Biol., 15, 216 (1962).
98. Kennedy, E.P. and D.E. Koshland, Jr., J. Biol. Chem., 228, 419 (1957).
99. King, F.E. and D.A.A. Kidd, J. Chem. Soc., 2976 (1951).
100. Kopple, K.D. and D.E. Nitecki, J. Am. Chem. Soc., 84, 4457 (1962).
101. Koshland, D.E., Jr., Proc. Nat. Acad. Sci. U.S., 44, 98 (1958).
102. Koshland, D.E., Jr., and A. Lukton, personal communication.
103. Koshland, D.E., Jr., D.H. Strumeyer and W.J. Ray, Jr., Brookhaven Symp. Biol., 15, 101 (1962).
104. Kossel, A. and S. Edlbacher, Z. physiol. Chem., 93, 396 (1915).
105. Kunckell, F., Ber. deut. chem. Ges., 34, 637 (1901).
106. Kunkel, H.G. and A. Tiselius, J. Gen. Physiol., 35, 89 (1951).
107. Kunitz, M. and J.H. Northrop, J. Gen. Physiol., 19, 991 (1936).
108. Kyrides, L.P., F.B. Zienty, G.W. Steahly and H.L. Morrill, J. Org. Chem., 12, 577 (1947).
109. Le Quesne, W.J. and G.T. Young, J. Chem. Soc., 24 (1952).
110. Luckenbach, G., Ber. deut. chem. Ges., 17, 1421 (1884).
111. Maquenne, Ann. Chim. Phys., ser. 6, 24, 522 (1891).

112. Massey, V. and B.S. Hartley, Biochim. et Biophys. Acta, 21, 361 (1956).
113. Martin, C.J., J. Golubow and A.E. Axelrod, Biochim. et Biophys. Acta, 27, 430 (1958).
114. Mazur, R.H., B.W. Ellis and P.S. Cammarata, J. Biol. Chem., 237, 1619 (1962).
115. Mengelberg, M., Chem. Ber., 89, 1185 (1956).
116. Mikeš, O., V. Holeyšovský, V. Tomášek, B. Keil and F. Šorm, Coll. Czec. Chem. Comm., 27, 1964 (1962).
117. Mikeš, O., V. Holeyšovský, V. Tomášek and F. Šorm, Coll. Czec. Chem. Comm., 26, 1048 (1961).
118. Miller, G.L., O.K. Behrens and V. du Vigneaud, J. Biol. Chem., 140, 411 (1941).
119. Moore, S. and W.H. Stein, J. Biol. Chem., 192, 663 (1951).
120. Neber, P.W. and A. Uber, Ann. Chem., 467, 52 (1928).
121. Neurath, H. and B.S. Hartley, J. Cell. Comp. Physiol., 54, Suppl. 1, 179 (1959).
122. Neurath, H. and G.W. Schwert, Chem. Rev., 46, 69 (1950).
123. Newman, M.S. and S. Hishida, J. Am. Chem. Soc., 84, 3582 (1962).
124. Ochiai, E. and E. Hayashi, J. Pharm. Soc. Japan, 67, 34 (1947); (per Chem. Abst., 45, 9533 (1951)).
125. Ono, T. and R. Hirohata, Z. physiol. Chem., 304, 77 (1956).
126. Oosterbaan, R.A. and M.E. van Adrichem, Biochim. et Biophys. Acta, 27, 423 (1958).
127. Overell, B.G. and V. Petrow, J. Chem. Soc., 232 (1955).
128. Oxley, P., M.W. Partridge and W.F. Short, J. Chem. Soc., 1110 (1947).
129. Oxley, P. and W.F. Short, J. Chem. Soc., 147 (1946).
130. Pandit, U.K. and T.C. Bruice, J. Am. Chem. Soc., 82, 3386 (1960).
131. Patchornik, A., A. Berger and E. Katchalski, J. Am. Chem. Soc., 79, 5227 (1957).
132. Patchornik, A., A. Berger and E. Katchalski, J. Am. Chem. Soc., 79, 6416 (1957).
133. Pauly, H., Z. physiol. Chem., 42, 508 (1904).

134. Pauly, H. and J. Weir, Ber. deut. chem. Ges., 43, 661 (1910).
135. Perlmann, G.E., Nature, 173, 406 (1954).
136. Pinner, A. and F. Klein, Ber. deut. chem. Ges., 10, 1889 (1877).
137. Pyman, F.L., J. Chem. Soc., 99, 668, 1386 (1911).
138. Pyman, F.L., J. Chem. Soc., 109, 186 (1916).
139. Radziszewski, B., Ber. deut. chem. Ges., 15, 2706 (1882).
140. Radziszewski, B., Ber. deut. chem. Ges., 17, 1289 (1884).
141. Radziszewski, B. and L. Szul, Ber. deut. chem. Ges., 17, 1291 (1884).
142. Reindel, F. and W. Hoppe, Chem. Ber., 87, 1103 (1954).
143. Relyea, D.I., P.O. Tawney and A.R. Williams, J. Org. Chem., 27, 477 (1962).
144. Roeske, R., F.H.C. Stewart, R.J. Stedman and V. du Vigneaud, J. Am. Chem. Soc., 78, 5883 (1956).
145. Rydon, H.N. and P.W.G. Smith, Nature, 169, 922 (1952).
146. Salem, L., J. Chem. Phys., 37, 2100 (1962).
147. Schaffer, N.K., S.C. May, Jr. and W.H. Summerson, J. Biol. Chem., 202, 67 (1953); 206, 201 (1954).
148. Schipper, E.S. and A.R. Day, in Elderfield, R.C., ed., Heterocyclic Compounds, John Wiley, New York, 1957, v. 5, p. 194.
149. Schmir, G.L. and T.C. Bruice, J. Am. Chem. Soc., 80, 1173 (1958).
150. Schoellmann, G. and E. Shaw, Biochem. Biophys. Res. Comm., 7, 36 (1962).
151. Schwartz, J.H. and F. Lipmann, Proc. Nat. Acad. Sci. U.S., 47, 1996 (1961).
152. Schwert, G.W. and M.A. Eisenberg, J. Biol. Chem., 179, 665 (1949).
153. Schwert, G.W., H. Neurath, S. Kaufman and J.E. Snoke, J. Biol. Chem., 172, 221 (1948).
154. Segal, H.L., in P.D. Boyer, H.A. Lardy and K. Myrbäck, eds., The Enzymes, ed. 2, Academic Press, New York, 1959, v. 1, p. 1.
155. Shaw, E., Brookhaven Symp. Biol., 15, 123 (1962).
156. Shaw, E., in Klingsberg, E., ed., Pyridine and Its Derivatives, Interscience, New York, 1961, pt. 2, p. 97.

157. Shaw, E. and D.W. Woolley, J. Biol. Chem., 181, 89 (1949).
158. Shirley, D.A. and P.W. Alley, J. Am. Chem. Soc., 79, 4922 (1957).
159. Shriner, R.L. and F.W. Neumann, Chem. Rev., 35, 351 (1944).
160. Smyth, D.G., W.H. Stein and S. Moore, J. Biol. Chem., 237, 1845 (1962).
161. Sonn, A. and P. Greif, Ber. deut. chem. Ges., 66, 1900 (1933).
162. Sonn, A., E. Hotes and H. Sieg, Ber. deut. chem. Ges., 57, 953 (1924).
163. Sowa, F.J. and J.A. Nieuwland, J. Am. Chem. Soc., 59, 1202 (1937).
164. Spackman, D.H., W.H. Stein and S. Moore, J. Biol. Chem., 235, 648 (1960).
165. Spencer, T. and J.M. Sturtevant, J. Am. Chem. Soc., 81, 1874 (1959).
166. Staab, H.A. and K. Wendel, Chem. Ber., 93, 2902 (1960).
167. Stahl, E., Angew. Chem., 73, 646 (1961).
168. Stahl, E., ed., Dünnschicht-Chromatographie, Springer-Verlag, Berlin 1962.
169. Stein, W.H. and S. Moore, Cold Spring Harbor Symp., 14, 179 (1950).
170. Stephens, C.R., E.J. Bianco and F.J. Pilgrim, J. Am. Chem. Soc., 77, 1701 (1955).
171. Stockmayer, W.H., in Biophysical Science - A Study Program, John Wiley, New York, 1955, p. 103.
172. Swain, C.G. and L.J. Taylor, J. Am. Chem. Soc., 84, 2456 (1962).
173. Tanenbaum, S.W., J. Am. Chem. Soc., 75, 1754 (1953).
174. Totter, J.R. and W.J. Darby, in Organic Syntheses, John Wiley, New York, 1955, coll. v. 3, p. 460.
175. Wagner, R.B. and H.D. Zook, Synthetic Organic Chemistry, John Wiley, New York, 1953, p. 95.
176. Wagner, R.B. and H.D. Zook, Synthetic Organic Chemistry, John Wiley, New York, 1953, p. 596.
177. Wallach, O., Ber. deut. chem. Ges., 16, 534 (1883).
178. Warner, D.T., Nature, 190, 120 (1961).
179. Weidenhagen, R. and R. Herrmann, Ber. deut. chem. Ges., 68, 1953 (1935).

180. Weil, L., S. James and A.R. Buchert, Arch. Biochem. Biophys., 46, 266 (1953).
181. Whitaker, J.R. and B.J. Jandorf, J. Biol. Chem., 223, 751 (1956).
182. Wiberg, K.B., Chem. Rev., 55, 713 (1955).
183. Wilcox, P.E., J. Kraut, R.D. Wade and H. Neurath, Biochim. et Biophys. Acta, 24, 72 (1957).
184. Windaus, A., Ber. deut. chem. Ges., 39, 3886 (1906).
185. Windaus, A., Ber. deut. chem. Ges., 42, 758 (1909).
186. Windaus, A., W. Dörries and H. Jensen, Ber. deut. chem. Ges., 54, 2745 (1921).
187. Windaus, A. and W. Langenbeck, Ber. deut. chem. Ges., 55, 3706 (1922).
188. Wood, H.N. and A.K. Balls, J. Biol. Chem., 213, 297 (1955).
189. Woolley, D.W., J.W.B. Hershey and I.H. Koehelik, Proc. Nat. Acad. Sci. U.S., 48, 709 (1962).
190. Wootton, J.F. and G.P. Hess, J. Am. Chem. Soc., 84, 440 (1962).
191. Zaoral, M. and J. Rudinger, Coll. Czec. Chem. Comm., 24, 1993 (1959).
192. Zerner, B. and M.L. Bender, J. Am. Chem. Soc., 85, 356 (1963).

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