

NEWER METHODS IN PEPTIDE CHEMISTRY

by

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B.A. University of Kansas
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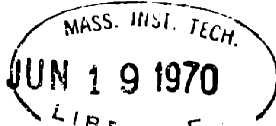
June, 1970

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Department of Chemistry, June 4, 1970

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Chairman, Department Committee on Graduate Students

Archives



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ABSTRACT

CHAPTERS I AND II

A new method for measuring the primary kinetic isotope effect (k_H/k_D) is described. The method is applied to distinguish between two mechanisms (enolization and oxazolone formation) for racemization during peptide synthesis with two model systems. Racemization during the synthesis of the Young model peptide via the acyl azide, mixed anhydride or two benzisoxazole-derived phenyl esters is shown to be oxazolone mediated under all conditions used. Racemization during the synthesis of the Anderson model peptide via the mixed anhydride or the Kemp-Chien reagent is best interpreted as oxazolone mediated. The acyl azide is racemized via an enolization mechanism in the presence of tertiary amines in nonpolar solvents. Under the same conditions in polar aprotic media, enolization and oxazolone formation are shown to be competitive.

CHAPTERS III AND IV

Factors influencing the design of effective peptide reagents are described. The interconversion between a catechol monoester and the corresponding oxazolone is elaborated using double radio-tracer techniques. The equilibrium constant for the ester-oxazolone reaction under basic conditions is defined. Rate constants for amino acid coupling via the catechol ester derived from the Kemp-Chien reagent are measured. The effects of amino acid side chains, temperature, basicity and solvent are described.

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TO MY FATHER

ACKNOWLEDGEMENTS

It has been a distinct pleasure to be associated with Professor Daniel Kemp these few years. For the benefits of his wisdom, generosity and many other expressions of his friendship, I am deeply indebted.

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INTRODUCTION

Exciting advances in peptide chemistry have appeared during the last decade. Substances exhibiting enzymatic activity have been synthesized and new hope prevails that life-sustaining chemical reactions might soon be understandable in terms of structure-function relationships. Before this goal can be achieved, convincing methods of structure proof for synthetic macromolecules are necessary. The absolute methods (X-ray crystallography, neutron diffraction) are presently so impractical that their routine use in the near future appears unlikely. Other spectroscopic techniques lack the requisite analytical power.

Biological assay cannot be employed for structure proof until the structure-function relationship has been shown to be specific in a predictable sense. Since one purpose of future synthesis will be to prove (or disprove) this very specificity, the current use of bioassay as a structure-determining method is groundless.

Peptide chemists agree that the structure problem will yield most readily to a statistical attack: by improving synthetic techniques, confidence in the structure assigned the synthetic product might be raised to near surety. The methodology to be used for these improvements is presently an intensely debated subject, with opponents in clearly distinguishable camps. One group champions a synthetic strategy in which single amino acids are added to a growing peptide chain; the

other schemes to combine peptide fragments of increasing size. A few chemists change allegiances in mid-synthesis.

There are no panaceas; both schemes have unique advantages and disadvantages. The linear scheme offers apparent freedom from racemization but suffers the problem that intermediates cannot be efficiently purified. Its proponents face the task of driving all reactions to completion. The pyramidal schemes provide readily purifiable intermediates but encounter the problem of racemization. Its proponents search for efficient methods of coupling large peptides.

The research program pursued in these Laboratories is allied to the latter methodology. With the premise that advances in this camp are critically dependent on a detailed understanding of the racemization problem, racemization studies have been our major concern. The first phase, in which a new and supersensitive method for the detection of racemization was developed, is now near completion. This thesis describes the second phase - a mechanistic study of racemization - and hopefully provides the basis for the most rewarding phase: the rational design of effective peptide reagents.

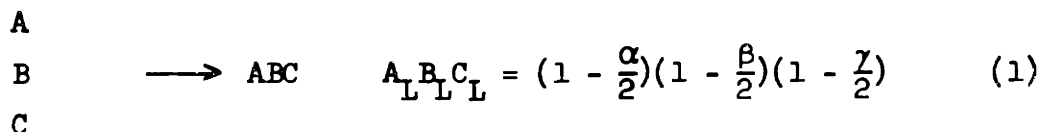
CHAPTER I

When faced with a choice between two mechanistic possibilities, I am happier with the one with the larger number of intermediates, since that one will be the easier to prove or disprove.

D. S. Kemp

ASPECTS OF THE RACEMIZATION PROBLEM

The dimensions of the racemization problem are most easily grasped by considering the synthesis of a substance with three chiral sites, i.e., a tripeptide. Assuming that amino acids A, B and C are available in optically pure form and racemization occurs only when the amino acids are introduced into the sequence ABC, the fraction of desired product ($A_L B_L C_L$) is given by (1), wherein α , β and γ represent the



percent racemization suffered by A, B and C respectively. From the illustration (Fig. 1) for the cases $\alpha = \beta = 20\%$, $\gamma = 30\%$ and $\alpha = \beta = \gamma = 100\%$, it can readily be appreciated that syntheses in which racemization occurs require purification at each step if isolation of the desired product is to be achieved.

Lest the reader cavil at the significance of much lower levels of racemization, Table 1 has been included. The calculations assume that the racemization level for each amino acid is constant and are based on the formula $(1 - \frac{\%}{2})^n$, where n represents the number of chiral sites in the peptide. The figures refer to the fractional yield of the chirally homogenous product.

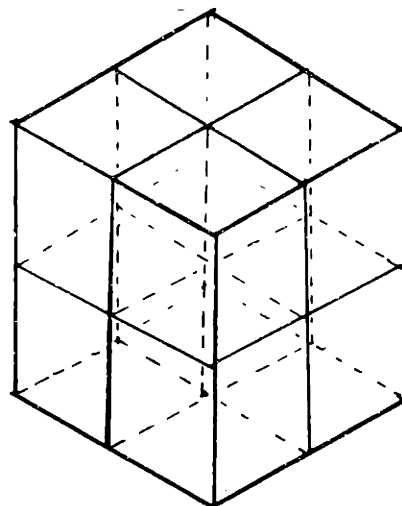
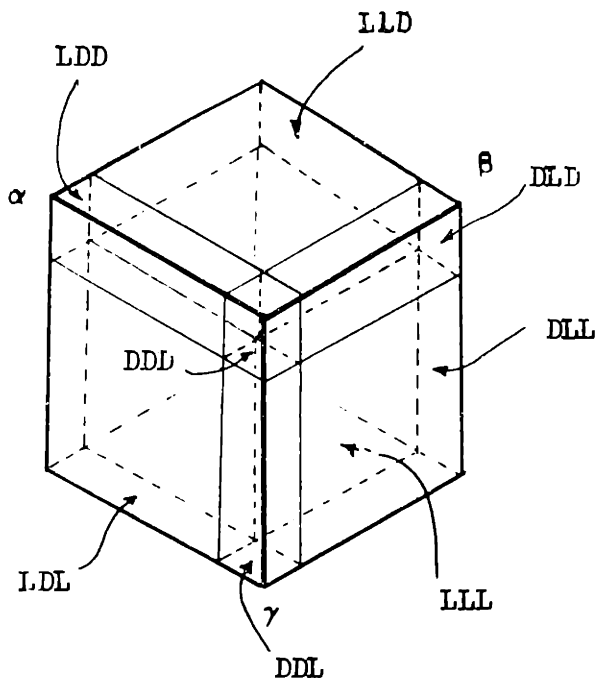


Fig. 1

Table 1¹

n	% enantiomer			
	10	1	0.1	
10	0.35	0.90	0.99	oxytocin insulin, ACTH, ferridoxin
100	3×10^{-5}	0.37	0.91	ribonuclease, lysozyme
1000	----	4×10^{-5}	0.37	hemoglobin

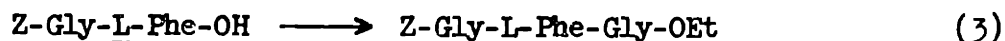
(1) J. P. Greenstein and M. Winitz, "Chemistry of Amino Acids," John Wiley and Sons, Inc., New York, N.Y., 1961, p. 947.

Coincidentally these figures can also be applied to another important aspect of peptide synthesis, the yield (n = the number of steps and $\%$ = the incompleteness of reaction for each step). The synthesis² of a substance with ribonucleic activity, in which $\gg 1100$ steps were involved without purification of intermediates, would make an interesting example if the average $\%$ incompleteness were known. The steps most vulnerable to incomplete reaction are the coupling steps ($n = 128$), which have recently been examined for solid phase peptide synthesis.³ Results for the synthesis of a dodecapeptide indicate the incompleteness of each coupling step varies between 0.5% and 50%!

In the light of the important consequences of racemization levels between 0.1 and 1%, it is remarkable that only in the present year has a technique been developed that can accurately measure racemate levels in this range. Kemp⁴ has assessed the extent of racemization during the preparation of the Young⁵ (2) and Anderson⁵ (3) model

-
- (2) B. Gutte and R. B. Merrifield, J. Am. Chem. Soc., 91, 501 (1969).
 - (3) H. Hagenmaier, Tet. Lett., 283 (1970).
 - (4) D. S. Kemp, S. W. Wang, G. Busby, III, and G. Hugel, J. Am. Chem. Soc., 92, 1043 (1970).
 - (5) G. W. Anderson and F. M. Callahan, ibid., 80, 2902 (1958).
 - (6) M. W. Williams and G. T. Young, J. Chem. Soc., 881 (1963).

peptides* by a variety of commonly used peptide reagents.



The results (Table 2), which show the racemate observed under optimal conditions for each method, indicate that even the best peptide coupling methods presently in use are not free of racemization.

* The use of model peptides is dictated by the lack of methods for detecting a single racemized site in a large peptide. A recently explored possibility,⁷ involving hydrolysis, esterification, and vpc analysis on capillary columns coated with optically active substances appears to have few advantages over the classical method of enzymatic digestion.

(7) E. Bayer, et al., J. Am. Chem. Soc., 91, 1735, 1738 (1970).

† Abbreviations are according to standard peptide nomenclature.

TABLE 2⁴

Method	Anderson $\%_{\text{rac}}$	Young $\%_{\text{rac}}$
Acyl Azide	0.01	0.15
Kemp-Chien Reagent ⁸	0.01	0.15
p-Nitrophenyl Ester	0.1	----
Woodward's Reagent K ⁹	2.1	8.5
Mixed Anhydride ¹⁰	0.01	0.4

It must be borne in mind that these are model systems; information concerning racemization during the coupling of larger peptide fragments is not available. The definition of fraction racemate (4)

$$\text{fraction rac} = \frac{\text{DL}}{\text{L} + \text{DL}} = \frac{\text{rate of rac}}{\text{rate of coupling} + \text{rate of rac}}$$

suggests that for couplings involving larger peptides the racemate level would increase since the coupling rate should show sensitivity

(8) D. S. Kemp and S. W. Chien, J. Am. Chem. Soc., 89, 2743 (1967).

(9) R. B. Woodward, R. A. Olofson and H. Mayer, ibid., 83, 1010 (1961).

(10) G. W. Anderson, et al., ibid., 89, 5012 and 7152 (1967). These results are described in the experimental sections of this thesis.

to the steric bulk of the peptide components. The effect of size on the racemization rate has yet to be explored. Indeed, little information regarding the racemization rate can be found in the peptide literature; only scattered reports of the effect of solvent and temperature¹¹ on racemization levels have appeared.

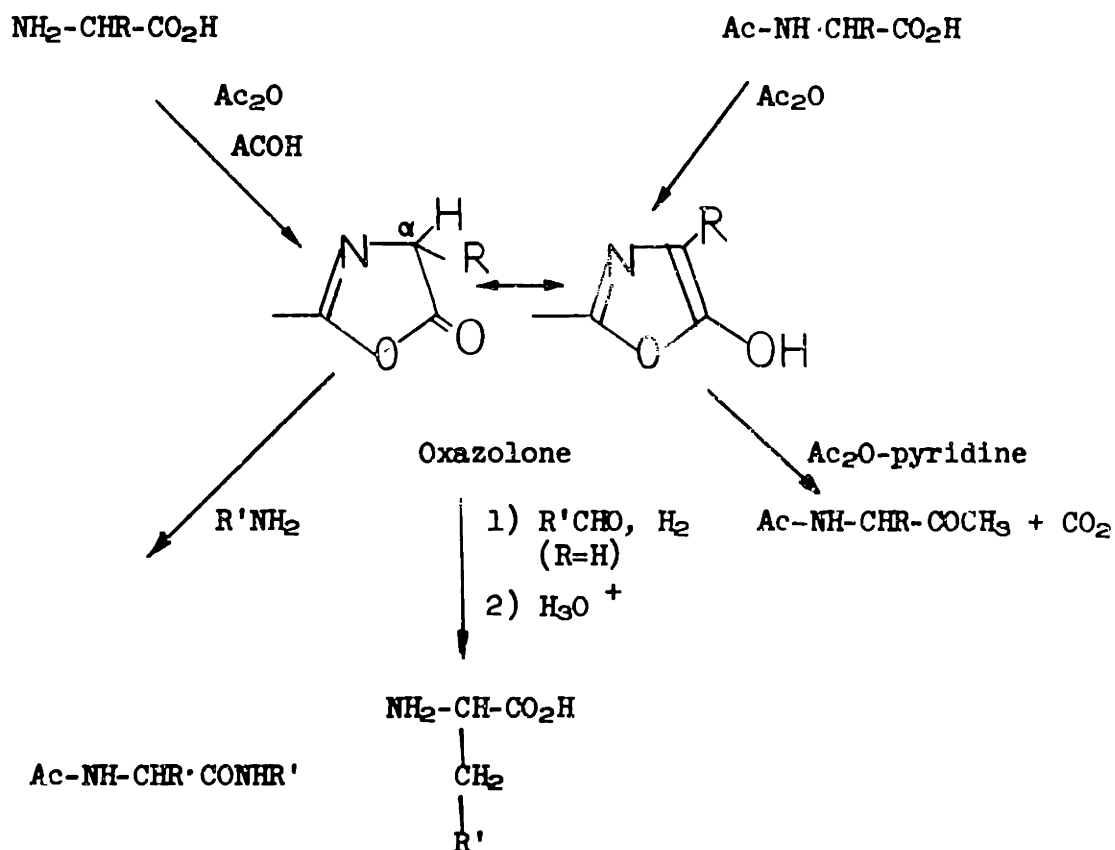
In order to define the factors that influence the racemization rate, we felt it was essential to understand the racemization process in great detail, and to this end a study of the racemization mechanism was undertaken.

(11) F. Weygand, A. Prox, L. Schmidhammer and W. König, Angew. Chem., 75, 282 (1963).

The Mechanisms of Racemization

Any study of racemization mechanisms must include consideration of the well-known oxazolone or azlactone pathway. These substances (Fig. 2), which have always played an integral role in the chemistry of amino acids, can be generated from acyl amino acids by a variety of dehydrating agents.

Fig. 2

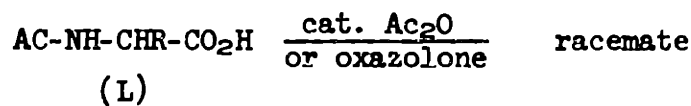


The rich chemistry of oxazolones,¹² which includes amino acid

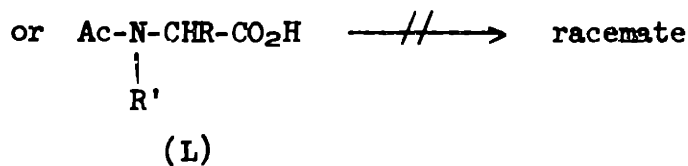
(12) For reviews see J. Cornforth in "The Chemistry of Penicillins," H. Clarke, Ed., Princeton Univ. Press, Princeton, N.J., 1945, p. 730 ; and H. E. Carter in "Organic Reactions," Vol. III, John Wiley and Sons, Inc., N.Y., 1946, Chapter 5.

syntheses, acylamino ketone syntheses as well as peptide synthesis is mostly a consequence of the lability of the C_α-H bond. It is just this feature of oxazolones that made them attractive intermediates for the racemization mechanism. The actual participation of oxazolones in the racemization of acylamino acids was suggested by Bergmann and collaborators* in 1926.^{13,14} (Fig. 3).

FIG. 3



but

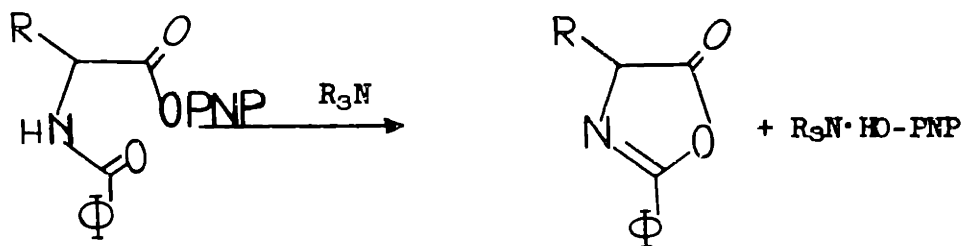


(13) M. Bergmann and H. Koster, Z. physiol. Chem., 159, 179 (1926).

(14) M. Bergmann and L. Zervas, Biochem. Z., 203, 280 (1928).

* The experiments outlined above bore fruit in the form of the immeasurably valuable benzyloxycarbonyl(carbobenzoxy, Z) protective group. Urethane groups had previously been used by Fischer¹⁵ but had been abandoned due to the fact that they could not be selectively removed. The benzyloxycarbonyl function, introduced by Bergmann and Zervas in 1932,¹⁶ became the first and foremost selectively removable blocking group in peptide synthesis since it could be removed by catalytic hydrogenolysis.

Inasmuch as acylamino acids are model peptides, it was inevitable that the oxazolone mechanism would be expanded to account for the racemization of all activated peptide derivatives, especially active esters, when these latter substances appeared in the peptide chemistry scenerio. The p-nitrophenyl (OPNP) esters are the most extensively studied and oxazolones can be isolated when these esters are treated with tertiary amines.¹⁷



In an elegant investigation Young¹⁸ was able to show that the intermediacy of oxazolones was both sufficient and necessary to account for the racemization of

(15) E. Fischer, Ber., 36, 2094 (1903).

(16) M. Bergmann and L. Zervas, Ber., 65, 1192 (1932).

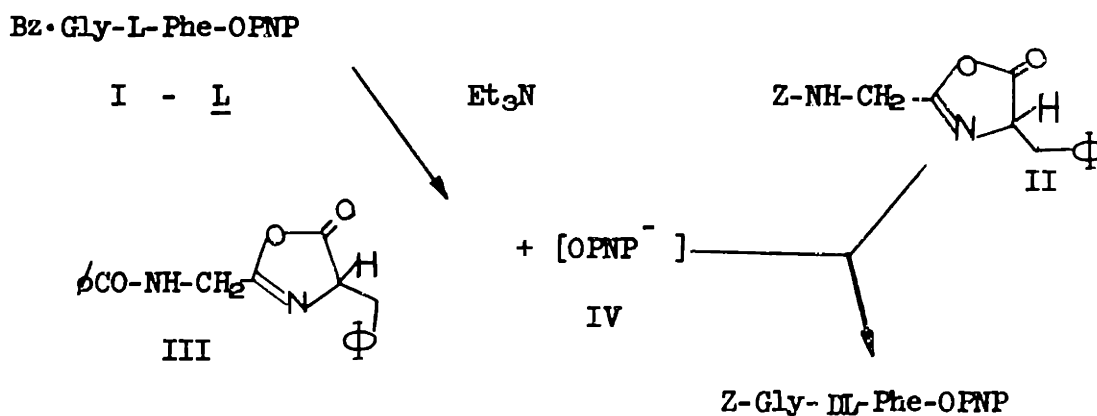
(17) M. W. Williams and G. T. Young, J. Chem. Soc., 3701 (1964).

(18) I. Antonovics and G. T. Young, Chem. Comm., 398 (1965).

p-nitrophenyl esters under these conditions. Since this beautiful experiment alone provides the basis for the argument that racemization of peptide-activated species proceeds via oxazolone intermediates, it will be described in some detail (Fig. 4).

The p-nitrophenyl ester of Benzoyl-Gly-L-Phe (I) was treated with triethylamine in the presence of a ten-fold excess of the oxazolone (II). This latter reagent was used to trap any phenol (generated by the collapse of the ester (I) into the corresponding oxazolone (III)) and thus preclude the formation of racemic ester by the return reaction (III) and (IV) \longrightarrow DL (I). When the optical rotation of the system

FIG. 4



had fallen to half its initial value, the reaction was quenched by acids and the recovered ester I was found to be "scarcely changed in rota-

tion". Thus Young concluded that the oxazolone was responsible for the racemization.

The control experiments (unfortunately not described) indicated that as much as 10% racemate in the recovered ester I could have eluded detection by the analytical procedure used (polarimetry). Among tertiary bases, triethylamine has been shown to be among the least effective catalysts for the racemization of p-nitrophenyl esters by mechanisms which do not involve oxazolones.* It would seem that generalizations toward the racemization behavior of other systems should be made with caution, yet this study - which lacks a single peptide coupling - is entitled "The Mechanism of Racemization during the Coupling of Acyl Peptides." Despite the minor blemishes of this study, there is little doubt that oxazolones can sufficiently account for the racemization of activated peptides by strong bases.¹⁹

Evidence also exists that substances capable of forming oxazolones racemize more readily than nearly identical substances which, by virtue of their blocking groups, are incapable of oxazolone formation.

* Ref. 28 (also discussed in This Thesis).

(19) Goodman and Stuben have reached similar conclusions for the reaction of a peptide p-nitrophenyl ester with hydroxide ion, J. Org. Chem., 27, 3409 (1962).

active ester	relative racemization rate† (Et ₃ N in CHCl ₃)
Z- <u>L</u> -Phe-OPNP	1
Phth * <u>L</u> -Phe-OPNP	10
Z-Gly- <u>L</u> -Phe-OPNP	50

The argument for oxazolone-catalyzed racemization rests on the evidence summarized as follows:

1. Through the action of dehydrating agents, acylamino acids can be completely racemized by conversion to oxazolones.
2. Peptide-activated species can be converted to oxazolones by treatment with strong bases.
3. Oxazolone-catalyzed racemization appears to be a more facile process than other mechanistic pathways, ceteris paribus.

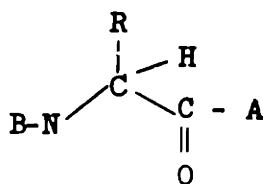
Since all of this evidence has been produced under conditions in which large (10-100%) levels of racemization are observed, the

* Phth = phthaloyl.

† Calculated from polarimetric data given by G. T. Young and B. Liberek in Proc. 8th Eur. Pept. Symp., Noordwijk, 55 (1967).

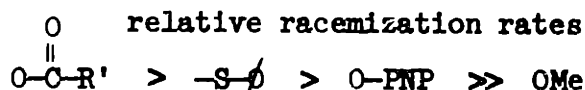
oxazolone argument, when applied to peptide coupling conditions, is clearly one of analogy. Yet other mechanistic possibilities exist which can also be applied by the same argument.

Direct enolization (in its various forms)* is a mechanistic possibility which has, in fact, been explored for amino acid derivatives. Liberek²⁰ has studied the effect of blocking and activating groups on the triethylamine-catalyzed racemization of compounds of the type (V) (incapable of oxazolone formation).



V

A = Activating group.



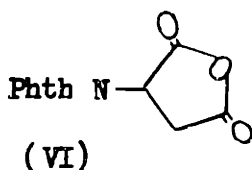
B = Blocking group.

Phthaloyl > Benzyloxycarbonyl

(20) B. Liberek, Tet. Lett., 925, 1103 (1963).

* Ketene formation is also considered herein as an enolization pathway.

The extreme cases exemplified by (VI) and (VII) below indicate a wide (ca. 10^4) range of racemization rates.



Time required for rotation to reach zero or nearly zero (triethylamine in acetone)

2 min ; $k_{\text{rac}} \doteq 20 \underline{\text{M}}^{-1} \text{min}^{-1}$. *

Z-L-Phe-OPNP

11 days ; $k_{\text{rac}} \doteq 2 \times 10^{-3} \underline{\text{M}}^{-1} \text{min}^{-1}$.

(VII)

The important conclusion to be derived from such studies is that these enolization rates are sufficient to account for the level of racemate observed during peptide coupling. The reaction of Z-Gly-L-Phe-OPNP with ethyl glycinate to give Anderson tripeptide proceeds with an aminolysis rate of about $0.5 \underline{\text{M}}^{-1} \text{min}^{-1}$.²¹ and yields ca. 0.1% racemate. The racemization rate can be approximated to be

$$\text{fraction rac} = \frac{k_{\text{rac}}}{k_{\text{coup}}} = 1 \times 10^{-3}; k_{\text{rac}} \doteq 2 \times 10^{-3} \underline{\text{M}}^{-1} \text{min}^{-1} \quad (4)$$

* Liberek's method of reporting data does not allow precise calculation of the rate constants. The figures reported here are based on the assumption that "zero or nearly zero" implies 98% racemization.

(21) D. S. Kemp and R. Sitrin, unpublished.

of the proper order of magnitude expected for an enolization mechanism for racemization (4).

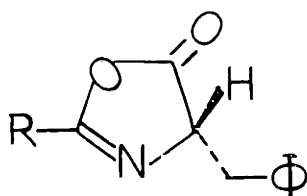
The two possible modes of racemization (oxazolone formation and direct enolization) have now been outlined. No evidence exists that allows a distinction between the two mechanisms for the extremely low levels of racemization observed under optimal peptide coupling conditions. Nor, in fact, could there be such evidence for the simple reason that in order to study racemization one must be able to detect it. Earlier students of the racemization problem were unaware that even under optimal conditions the best peptide reagents (those of Table II) yield detectable amounts of racemate. The techniques in use before 1970 (polarimetry, fractional crystallization) were far too insensitive to detect less than 1% racemization, consequently the studies previously described were characterized by the gross perturbations (excess tertiary bases) required to observe racemization.

The availability of the isotopic dilution microscope made it possible to study racemization phenomenon under real peptide coupling conditions for the first time. Almost dutifully, then, with the aid of our unique method, we addressed the problem of distinguishing between the two mechanistic possibilities.

In order to penetrate the mechanistic problem, an understanding of the kinetics governing the two racemization pathways is necessary.

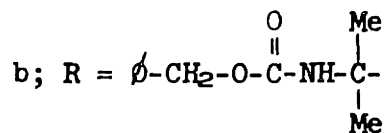
The essential features of the oxazolone mechanism - the optical fragility of oxazolones and the kinetics of oxazolone formation - will therefore be described in some detail.

1. Goodman²² has measured the racemization and coupling rates for a number of optically-active oxazolones. When the oxazolone VIII-a was allowed to react with ethyl glycinate (or any amino acid ester) completely racemic peptide products resulted. The relative



VIII

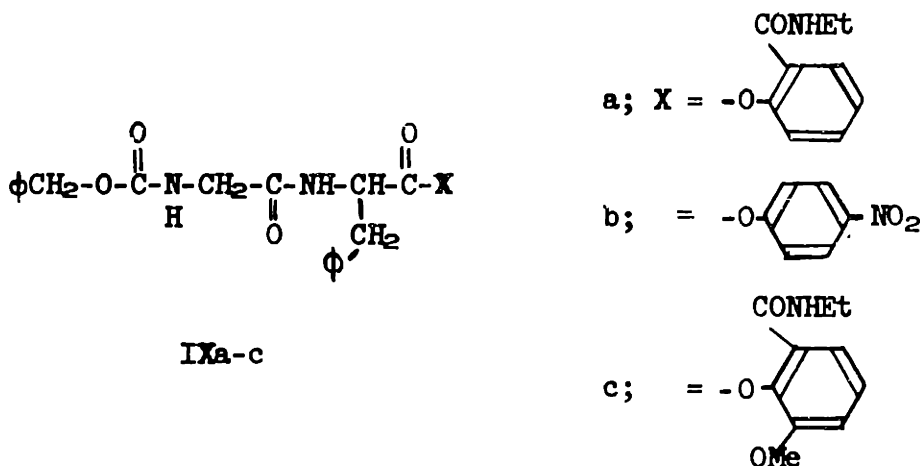
a; R = ϕ



rates for coupling and racemization were estimated to be 1 and 200 respectively, i.e., racemization proceeds much faster than ring opening. For the oxazolone VIII-b the reaction with ethyl glycinate gave product that was only partially racemic, thus the coupling and racemization rates were of the same order of magnitude. Both oxazolones are instantly racemized by tertiary amines.

(22) M. Goodman and C. Glaser in "Peptides: Chemistry and Biology," B. Weinstein and S. Lande, Eds., M. Dekker, New York, 1970, p. 267. (Proceedings of the First American Peptide Symposium, Yale, 1968).

2. Although a lactone formation from peptide-active esters has long been known to be base-catalyzed, the exact nature of the base catalysis was defined only in 1967.²³ The rates of racemization for a variety of phenyl esters of benzyloxycarbonylglycyl-L-phenylalanine (IX-a-c) by triethylamine in the presence of Et_3NHF_4 were found to

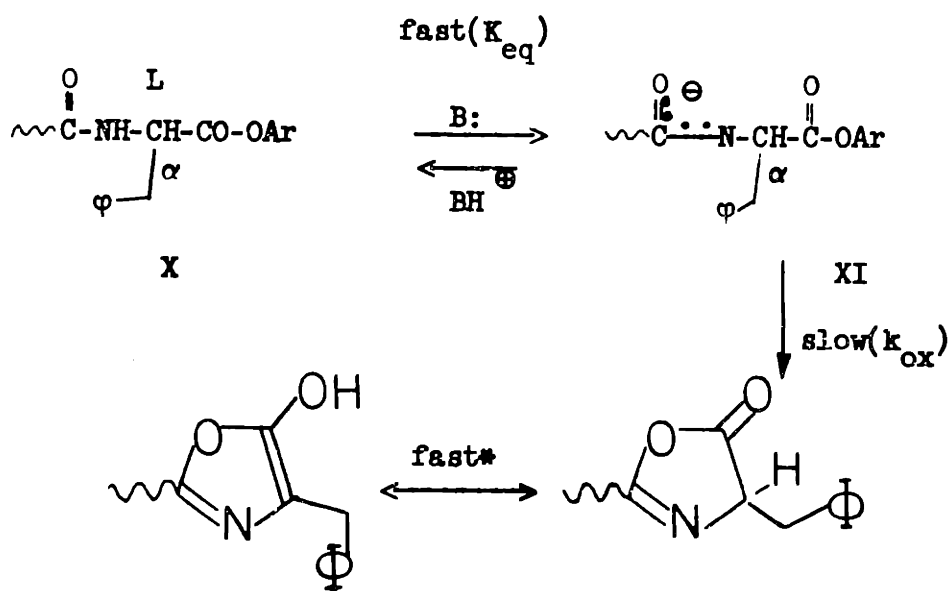


be (1) proportional to the N/NH^\oplus ratio at constant amine concentration and (2) independent of the amine concentration at a constant N/NH^\oplus ratio. These phenomena are consistent only with a specific-base catalyzed process, (Fig. 5) and the rate of racemization is given by (5).

$$v_{\text{rac}} = k_{\text{ox}}[K_{\text{eq}}][\text{X}][\text{B}/\text{BH}^\oplus]. \quad (5)$$

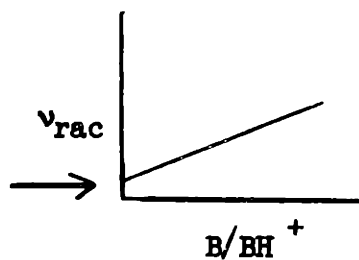
(23) D. S. Kemp and S. W. Chien, J. Am. Chem. Soc., 89, 2745 (1967).

FIG. 5



(* note that excess tertiary bases are present)

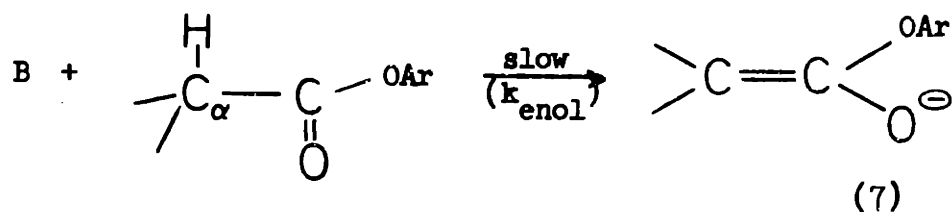
For several of these esters a plot v_{rac} vs. B/BH^+ resulted in a small but significant intercept:



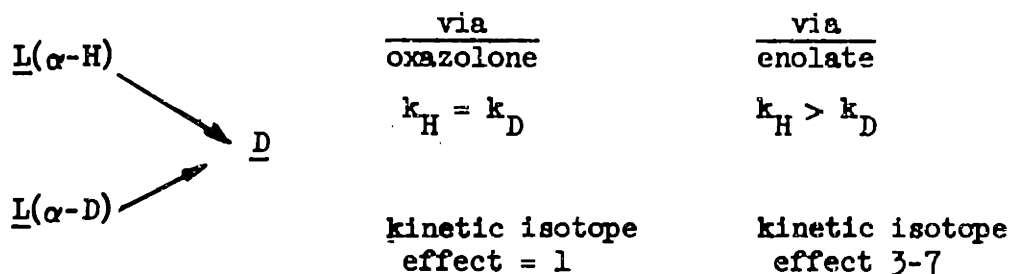
This intercept indicates a finite rate of racemization that is not specific base-catalyzed and is consistent with racemization processes of two types: (1) general base-catalyzed oxazolone formation (6)



or (2) enolization (7).



The tremendously important consequence of the oxazolone-mediated racemization process (5 or 6) is that the rate-determining step for the appearance of racemate does not involve the breaking of the C α -H bond! In sharp contrast, racemization by the enolization mechanism involves this bond in its rate-determining step. Herein lies the possible means of distinguishing between the two mechanisms: substitution of deuterium for the α -hydrogen will not affect the rate of racemization via the oxazolone mechanism (either (5) or (6),

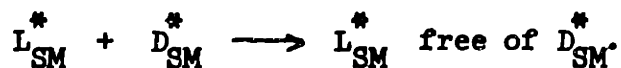


but should reduce the rate of racemization if an enolization mechanism is followed.

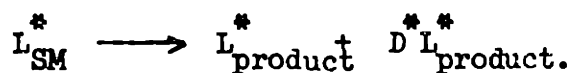
With the resolution of the theoretical aspect the practical problem of experimental design emerged. How could this kinetic isotope effect be measured for the cases of most interest; i.e., those coupling reactions for which the racemate accounts for only a few hundredths of a percent of a product mixture. The isotopic dilution assay clearly would be required to detect such levels of racemization and, since it was also used (in modified form) to measure the kinetic isotope effect it is briefly outlined below.

The method involves four distinct phases.

1. Preparation of a radiolabeled substance (bearing a single chiral site) that is free of any labeled enantiomer: (* denotes label.)



2. Submission of this material to a procedure whose stereospecificity is to be assessed:



3. Isolation of the labeled racemate:



4. Isotopic purification of this racemate in such a manner that the activity due to the D enantiomer may be measured:



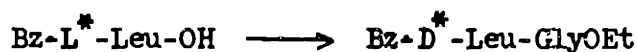
Thus the procedure measures the extent of the overall racemization process



and experimental details[†] have been established for the two cases:

[†] Although the actual mechanics of these operations need not concern us in the present discussion, the cogniscenti will avail themselves of the discussions and experimental design described in Ref. 4.

Young Model



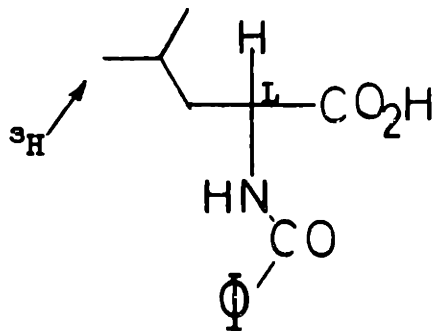
Anderson Model



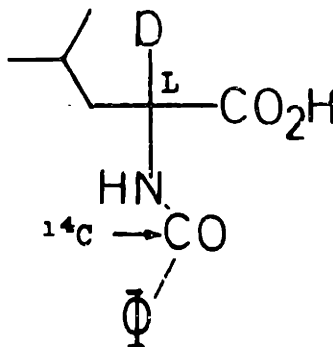
Let us return now to the problem of measuring the kinetic isotope effect for the racemization process. Quite obviously, the α -deuterated analog of one of the starting materials must be prepared, subjected to racemization assay described above and the results for any given coupling procedure compared with the corresponding results obtained with the α -protio substance. Ideally, however, the α -protio and α -deuterio species should be subjected to a competition experiment, in which both substances are simultaneously exposed to a given experimental procedure. This requirement could easily be met within the framework of Kemp's racemization assay if the α -protio and α -deuterio species were labeled with different radioisotopes.

Consequently the following experimental design was envisaged. Tritium labeled benzoyl-L-leucine (XII) would be combined with carbon-14 labeled, α -deuterated benzoyl-L-leucine (XIII) and the $^3\text{H}/^{14}\text{C}$ activity

ratio of this mixture would be assessed*. Inasmuch as all the tritium-marked molecules bear α -protium and all the ^{14}C -marked molecules bear α -D deuterium, the $^3\text{H}/^{14}\text{C}$ activity ratio represents a $\alpha\text{H}/\alpha\text{D}$ ratio



XII



XIII

Consider now a process that selects between hydrogen and deuterium (i.e., enolization) for the conversion $\underline{\text{L}} \longrightarrow \underline{\text{DL}}$ (racemization). For molecules that undergo the $\text{L} \longrightarrow \text{D}$ epimerization, a redistribution of radiolabel will result, since ^3H -marked (α -H) molecules will epimerize at a faster rate than ^{14}C -marked (α -D) molecules. The $^3\text{H}/^{14}\text{C}$ activity ratio of the $\underline{\text{D}}$ product will thus be different than that of the $\underline{\text{L}}$ starting materials.

* Modern liquid scintillation counters are readily capable of distinguishing between ^3H and ^{14}C disintegrations when both sources of radioactivity are present in the same sample.

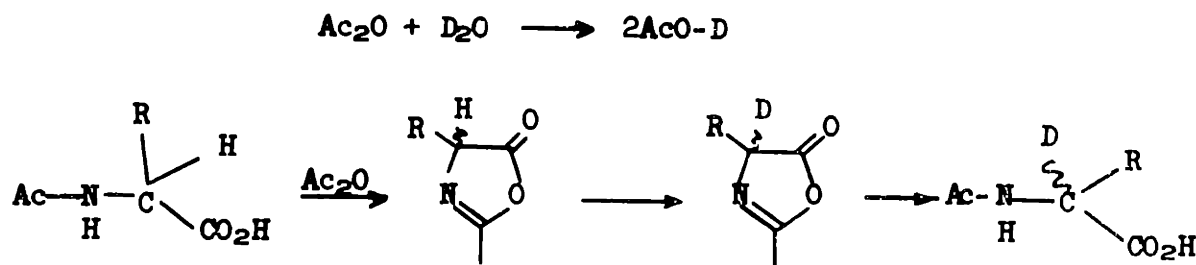
On the other hand, a process that does not select between hydrogen and deuterium for the L → DL conversion (oxazolone mediated racemization) will not change in label distribution, since the ¹⁴C-marked (α -D) molecules must undergo the L → D epimerization at the same rate as the ³H-marked (α -H) molecules. The ³H/¹⁴C activity ratio of the D product will be the same as that of the L starting material.

The ³H/¹⁴C activity ratio of the D enantiomer is thus a unique function of the mechanism of racemization! Since the racemization assay described by Kemp ultimately measures the activity due to the D enantiomer, the mechanistic problem could be solved within the framework of the existing technique.

It should be noted that the proposed technique for measuring the primary kinetic isotope effect, k_H/k_D , represents a novel method. The potential applications are limited only by the availability of radiolabeled substances. For our purposes, clearly an α -deuterated analog of L-leucine (Young model) or L-phenylalanine (Anderson model) was required. The Young model was selected since the oxazolone derived from benzoyl-L-leucine was of the sort (i.e., a 2-phenyl oxazolone)

that was sure to racemize many times faster than it acylated ethylglycinate (See p. 25) to give the Young peptide.

The necessary starting material, ^{14}C -labeled benzoyl-L-leucine- α -deuterated, was expected to present no synthetic difficulties. The introduction of deuterium could be accomplished by any method that racemizes leucine in protic medium. The classical method of racemizing amino acids in acetic acid-acetic anhydride appeared especially promising since (1) deuterium could be introduced by in situ generation of Ac-O-D from the reaction of heavy water with acetic anhydride:



and (2) acetyl-L-leucine has been used to resolve α -phenyl ethylamine.²⁴ If the resolution could be performed in the reverse sense (using optically-active α -phenyl ethylamine to resolve the deuterated

(24) H. DeWitt and A. Ingersoll, J. Am. Chem. Soc., 73, 5782 (1951).

acetyl-DL-leucine) the synthetic problem would be solved. The resulting acetyl-L-leucine could be converted to L-leucine and thence to benzoyl-L-leucine by well-established procedures.

The resolution could indeed be performed in the desired sense,* but the introduction of deuterium into the α -position of acetyl leucine could not be practically achieved. Treatment of acetyl leucine with acetic anhydride-heavy water mixtures not only exchanged the labile α -hydrogen, but the methyl hydrogens of the N-acetyl function as well. Fig. 6. This phenomenon is most easily rationalized by assuming that equilibration between the imine XIV and enamine XV is a facile process under these conditions. Although acetic acid is used as a reaction medium for peptide synthesis only in a few cases,²⁵ it is possible that this equilibration may occur under basic or neutral conditions. The awesome consequence for peptide synthesis should be clear. (Fig. 7). Any method that can generate oxazolones in the C-terminal (ultimate) amino acid residue is potentially capable of racemizing the penultimate amino acid as well. In this light, the

* A system in which "reverse resolution" led to nightmare-like difficulties is described in the total syntheses of Quinine, R. B. Woodward and W. E. von Doering, J. Am. Chem. Soc., 67, 860 (1945).

(25) F. Weygand and W. Steglich, Ber., 93, 2983 (1960).

Fig. 6

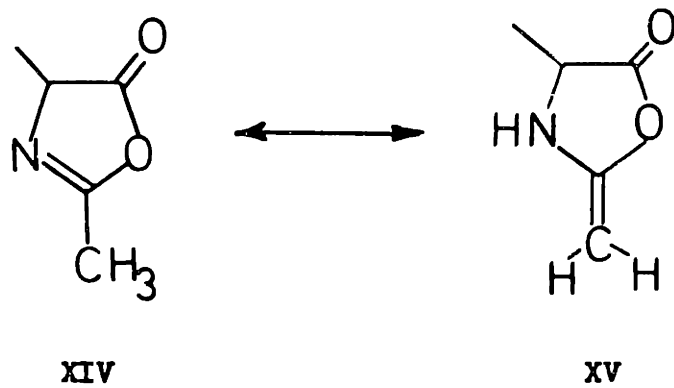
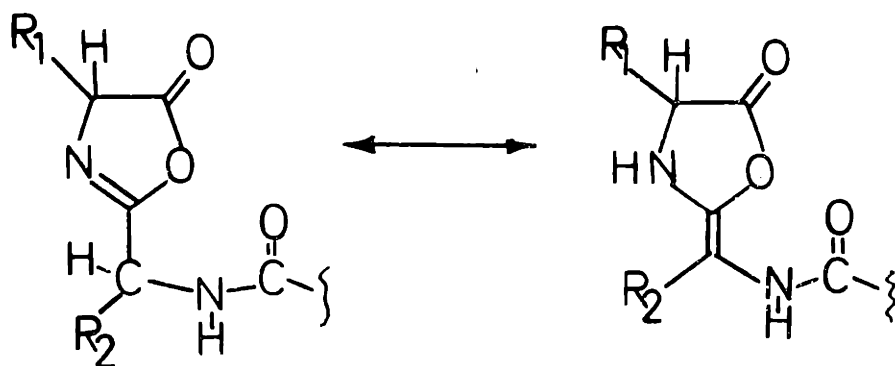


Fig. 7



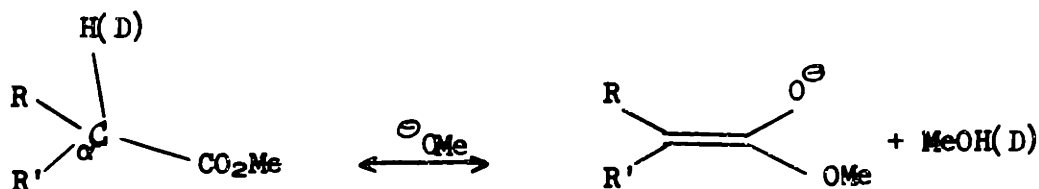
frequently made assumption - that couplings involving C-terminal glycine can be safely performed using methods that, in other cases, yield racemic products - is questionable.

Ironically, the intermediate that this mechanistic study was designed to prove (or disprove), proved to be the ideal intermediate for the deuteration. The oxazolone derived from benzoyl leucine is easily prepared and, unlike its acetyl counterpart, is a stable crystalline substance with only one labile hydrogen. The deuteration* proceeded without event and the resulting deuterated, racemic acid was cleanly resolved via the chincocaine salts. The overall procedure $[7-^{14}\text{C}]\text{benzoylDL-leucine} \rightarrow [7-^{14}\text{C}]\text{benzoyl-L-[2-}^2\text{H]leucine}$ could be accomplished in satisfactory yield. This material was combined with benzoyl-L-[4,5- ^3H]leucine and the mixture was washed free of radio-labeled D enantiomers by the technique described in Ref. 4 before use in the experiments outlined below.

In order to establish that isotope effects could indeed be measured by this new technique, a series of control experiments were performed. The trebly-labeled benzoyl-L-leucine was converted to the methyl ester (diazomethane) and this substance was exposed to methoxide ion.

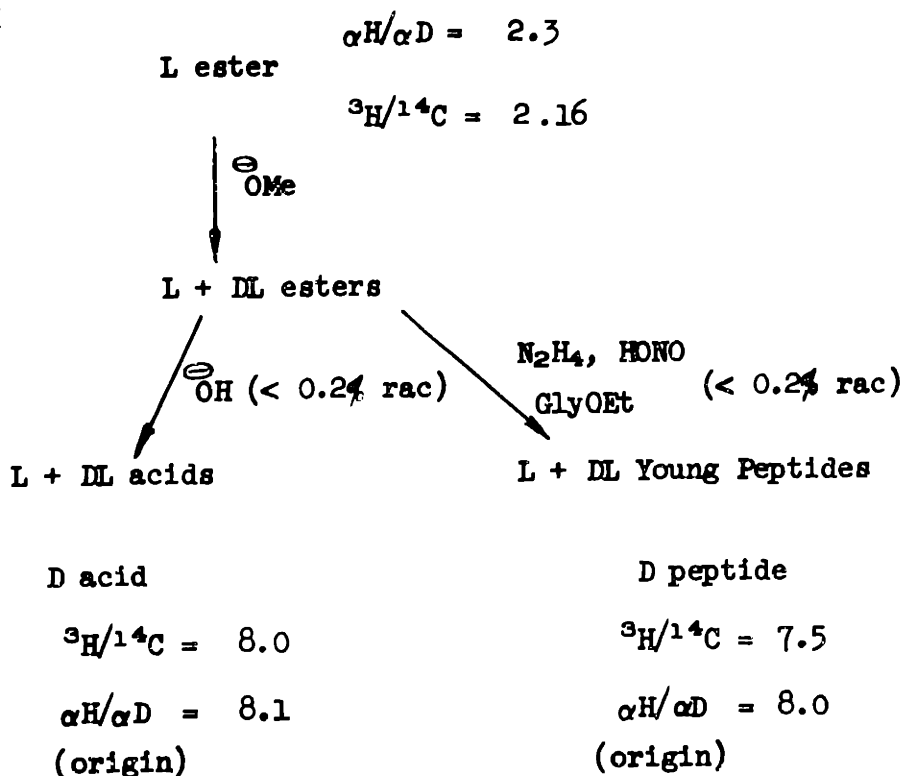
* The extent of deuteration of the unwanted enantiomer from the resolution was determined (by nmr using CAT techniques) to be > 97%.

After partial racemization had occurred, the reaction was quenched with



dilute acids and the recovered ester (in solution) was converted to the acid by saponification and to the Young peptide via the acyl azide coupling procedure. (Both saponification and peptide coupling proceed without significant racemization):

SCHEME I



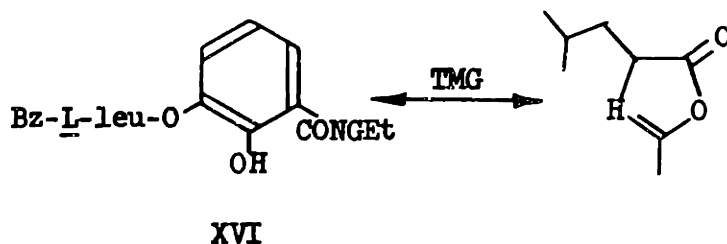
§ rac in Step (1)

αH	11.8	αH	11.8
αD	3.1	αD	3.3

These results, SCHEME I, in which the redistribution of radio-label for the process $\underline{\text{L}} \rightarrow \underline{\text{D}}$ reflects the selectivity of methoxide for αH vs. αD establish a new and general method of measuring the kinetic isotope effect, $k\text{H}/k\text{D}$. For our specific purposes, this experiment established that racemization via enolization will give an observable isotope effect.

To complete the control experiments, the other side of the argument (viz. an oxazolone mediated racimization shows no kinetic isotope effect) had to be shown valid.

The reaction of tetramethylguanidine (TMG) with the benzoyl-L-leucine ester of the Kemp-Chien reagent XVI, produces a system in which oxazolones have been shown to be present by independent proof (Chapter III). This experiment was performed on the corresponding



ester derived from trebly-labeled benzoyl-L-leucine. After one hour the reaction mixture was treated with ethyl glycinate and the resulting

Young peptides were taken through the isotopic dilution assay. The D peptide had the same $^3\text{H}/^{14}\text{C}$ ratio as the L starting material, demonstrating that no selectivity between $\alpha\text{-H}$ and $\alpha\text{-D}$ occurs in an oxazolone mediated racemization mechanism. ($k\text{H}/k\text{D} = 1$).

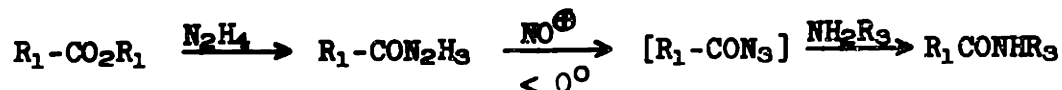
With the completion of the control experiments we turned to the selection of peptide coupling methods for testing by this new procedure. The general criteria for selection were:

1. Methods which show the lowest levels of racemization were the most important, since these are the most frequently employed in peptide coupling.
2. A variety of structural types was desirable, since any subsequent generalizations regarding closely related methods could be made with confidence.
3. Methods for which enolization seemed likeliest were attractive for the reason given in (2).
4. Methods in which the activated species could be prepared in optically pure form from the starting material were necessary because the mechanistic study was aimed at the racemization during peptide coupling.

The criteria were met by the acyl azide, and the mixed anhydride methods, and two isoxazole-derived phenyl esters; detailed discussion on how these procedures came to meet the specifications follows.

The Acyl Azide Method

Any study of racemization must begin with this classical peptide synthetic procedure. Introduced by Curtius²⁶ in 1902, its magnificent ability to mediate peptide bond formation without observable racemization has made it the reagent of choice for the condensation of peptide fragments, despite the number of steps and possible side reactions involved:



The first report of racemization during acylazide couplings was made by Anderson²⁷ in the preparation of his model peptide. Treatment of the freshly prepared benzyloxy carbonylglycyl-L-phenylalanyl azide with trimethylamine for 12 min before the addition of ethylglycinate gave rise to product which was > 1% racemic. Young,⁸ however, was unable to detect any artificially induced racemization in a similar treatment of benzoyl-L-leucylazide with triethylamine during the preparation of his own model peptide. The evidence accumulating from the isotopic dilution studies in these Laboratories,⁴ indicating

(26) T. Curtius, Ber., 35, 3226 (1902).

(27) G. W. Anderson, J. E. Zimmerman and F. M. Callahan, J. Am. Chem. Soc., 88, 1338 (1966).

that the Young model was approximately an order of magnitude more sensitive to racemization than the Anderson model, made the discrepancy concerning the two acyl azides notable, and required that a preliminary study of the acyl azides be executed to define optimal and racemizing conditions. Using the single-label Young test as the model, some critical variables in the acyl azide purification and coupling steps were determined.

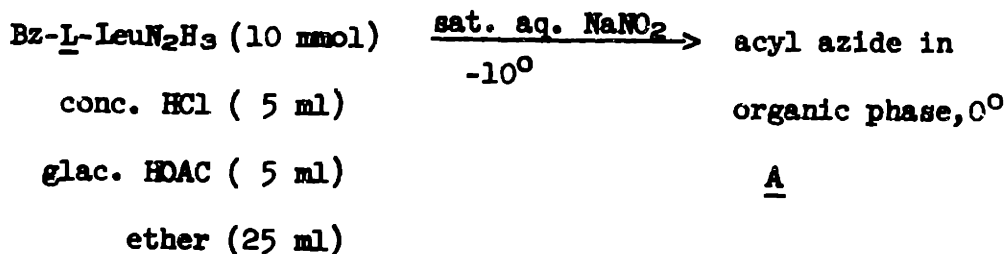


TABLE III

Racemization via Acyl Azides

<u>Conditions</u>	<u>% yield</u>	<u>% rac</u>
<u>A</u> Dried(MgSO ₄) then		
(1) 2 eq. GlyOEt, 0°	62	0.1
(2) 1 eq. Et ₃ N(15 min) then GlyOEt, 0°	66	0.1
<u>A</u> Extracted exhaustively with bicarbonate then		
(3) 2 eq. GlyOEt, 0°	73	0.35
(4) 1 eq. HOAc then 1 eq. GlyOEt, 0°	67	0.3
(5) 1 eq. Et ₃ N then GlyOEt, 0°	70	2.3
(6) 1 eq. GlyOEt, 1 hr 0°, then R.T.	62	0.3

An important observation which concilates Young's results with those of Anderson concerns the effect of the bicarbonate extractions. Young's conditions (2) show nearly minimal racemization, while Anderson's conditions (5)* correspond to maximum racemization. It is noteworthy that the acidic components in the diazotization mixture persist in the ethereal phase through three extractions with cold, saturated, sodium bicarbonate solution and a fourth extraction is required to bring the pH of the aqueous phase to ~ 8 . Since added acetic acid failed to reduce the level of racemization, the bicarbonate may be responsible for the racemization observed.

The trebly-labeled benzoyl-L-leucine was subjected to conditions corresponding to optimal (1) and racemizing (5) conditions.

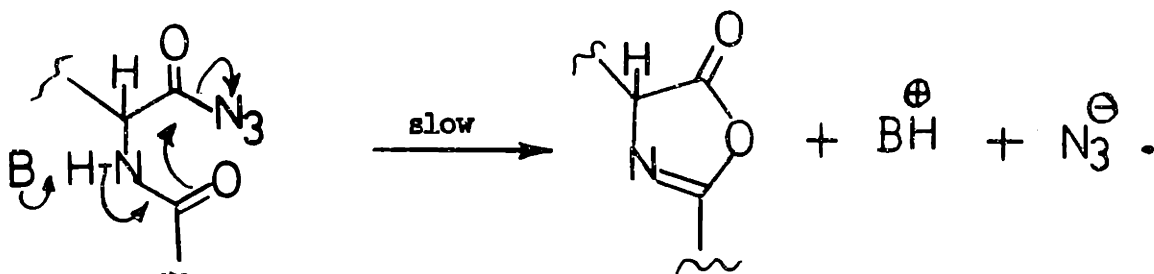
<u>Conditions</u>	<u>% rac</u>	<u>Isotope effect[†]</u>
Optimal	0.15	1.0
Racemizing	1.8	1.1

$$\dagger \frac{{}^3\text{H}/{}^{14}\text{C D product}}{{}^3\text{H}/{}^{14}\text{C L starting material}}$$

The results demonstrate that racemization is oxazolone mediated. Further, since ether is a poor solvent for the support of equilibrated

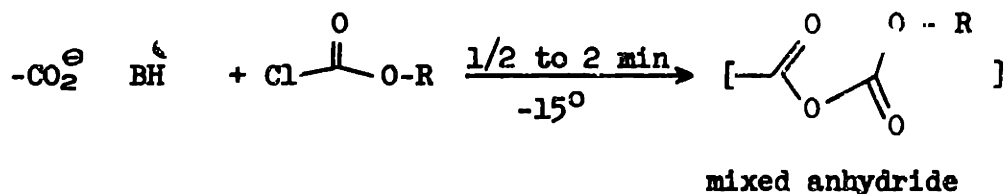
* Anderson's solvent was ethyl acetate, Young's was ether.

amide anions, a general base catalyzed oxazolone formation is probably indicated:



The Mixed Anhydride Method

The reinvestigation of the mixed anhydride method by Anderson²⁸, with his conclusion that under carefully defined conditions optical purity could be maintained, warranted its inclusion in the present study. The general procedure recommends activation of a tertiary amine salt of the peptide acid by a few moments exposure to a chloroformate (ethyl or isobutyl) at low temperature. As in the azide method,



(28) G. W. Anderson, J. E. Zimmerman and F. M. Callahan, J. Am. Chem. Soc., 89, 5012 (1967).

the mixed anhydrides are not isolated but treated in situ with amine nucleophiles. The observed effect of excess base on racemic content is of special relevance to the mechanistic question. (Table IV).

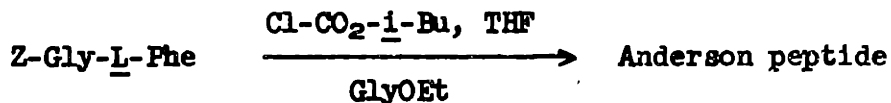
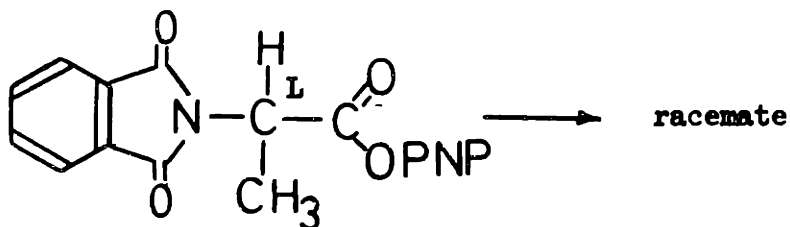


TABLE IV

<u>Base</u>	<u>% rac*</u>
Quinuclidine	100
Me ₃ N	90
Et ₂ NMe	25
N-Me-Morpholine	0

Anderson suggested that a steric effect was responsible for the order of racemization, since the rate of racemization of phthaloyl alanine *p*-nitro phenyl ester by these bases followed the order quinuclidine >



* Anderson reports yields of L and DL peptides; the figures above are calculated from Anderson's data using the formula

$$\% \text{ rac} = \frac{\text{yield DL}}{\text{total yield}}$$

Me₃N > Et₃N > N-Me Morpholine.

The anhydride results are inconsistent with the notion of specific base catalyzed racemization,* but are consistent with a general base catalyzed oxazolone mechanism, or direct enolization. The investigation of this system using the single-label Young model was undertaken and gave results which were in excellent agreement with those of Anderson (Table V).

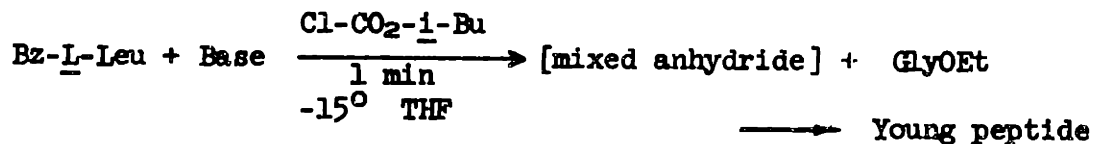


TABLE V

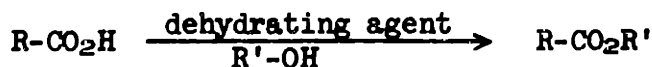
<u>Base</u>	<u>% yield</u>	<u>% rac</u>
N-Me Morph.		
1 eq.	60	0.4
2 eq.	60	15
Et ₃ N		
1 eq.	25	1.5
2 eq.	70	39

* Unless the solubilities of the corresponding amine hydrochlorides are both appreciable in THF and fortuitously follow the order N-MeMorpholine > Et₃N > Me₃N > Quinuclidine·HCl.

The trebly-labeled experiment was performed with 1.1 equivalents of N-methylmorpholine to give racemate which had identical isotopic ($^3\text{H}/^{14}\text{C}$) compositions in the D enantiomer and L starting material, a result consistent only with an oxazolone mediated racemization.

Active Esters

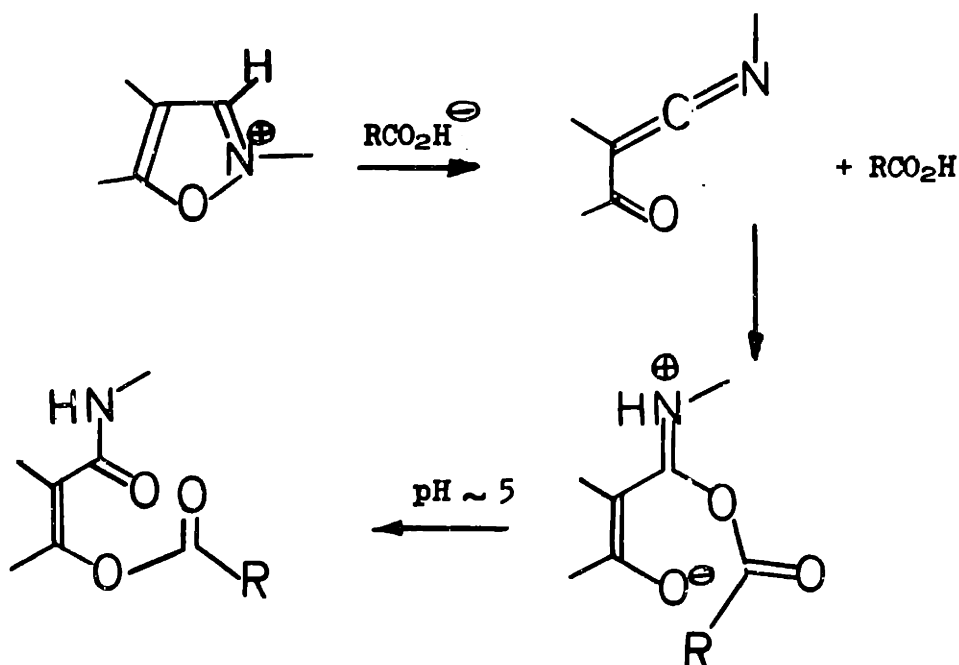
Peptide synthesis by fragment condensation has generally been limited to the acyl azide coupling method. The low yields and side reactions that are often encountered with this procedure make alternative methods of coupling desirable. The search for stable crystalline (purifiable) active esters which could provide these alternatives began in the early 1950's and, although moderate successes have been scored recently, represents a dismal chapter in peptide history. The brief popularity of new methods, uninspired application of dehydrating agents, and general desperation characterize the decade 1950-1960.* The esters developed in this period shared a peculiar trait - although they are excellent acylating agents, their preparation requires secondary activating agents. This requirement leads



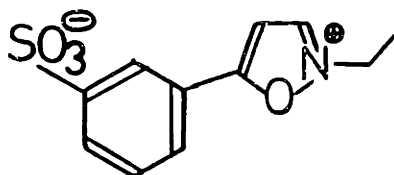
* A review of methods is given by M. Bodanszky and M. Ondetti in "Peptide Synthesis," Interscience, New York, 1966, Chapter 5.

to disastrous results when the acids involved are peptide acids. The dehydrating agents not only produce the active esters, but oxazolones as well. Since the formation of the latter always involve an intramolecular reaction leading to a five-membered ring, while the esterification was a bimolecular reaction, various degrees of racemization were consistently observed during the preparation peptide active esters. Further, their preparation in optically pure form did not guarantee freedom from racemization in the subsequent aminolysis.

It required the genius of Woodward to save active ester technology from an early death. Isoxazolium salts, reacting with carboxylates provided both the energy for activation and the incipient alcoholate in the same molecule. Esterification was "promoted" to

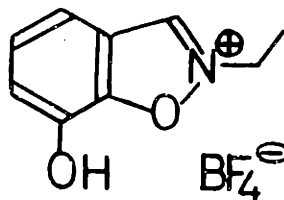
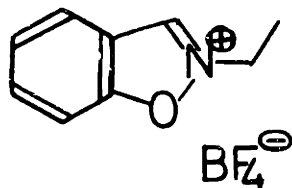


a unimolecular reaction on the dehydrating agent! Woodward's reagent K^{29} rapidly became the badly needed supplement to the acyl azide



WRK

method of peptide coupling. Although the recently published results of racemization assay may cause WRK to lose some of its current popularity, it will long be remembered as the prototype of newer and apparently more successful benzisoxazolium salts³⁰ (the Kemp-Chien Reagent).⁸ These two esters, then were chosen for the mechanistic



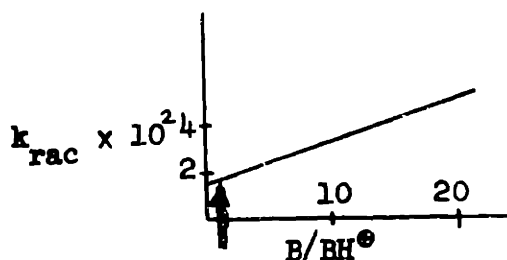
Kemp-Chien
reagent

(29) R. B. Woodward, R. A. Olofson and H. Mayer, J. Am. Chem. Soc., **83**, 1010 (1961).

(30) D. S. Kemp and R. B. Woodward, Tetrahedron, **21**, 3019 (1965).

study from among the bestiary of active esters.

The O esters of *N*-ethylsalicylamide had previously been found to give unacceptable levels of racemization (Ref. 4) but possessed other desirable characteristics. They were typical phenyl esters in their racemization behavior when treated with tertiary bases, thus the possibility existed that the specific base-catalyzed oxazolone formation could be suppressed by the addition the corresponding amine salts. The ester of benzoyl-L-leucine exhibited an inordinately large intercept for k_{rac} vs. B/BH^{\oplus} indicating that

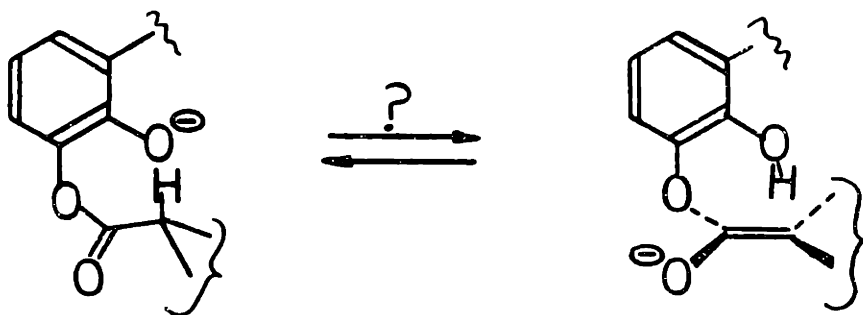


general base-catalyzed reactions, possibly enolization, contributed significantly to the racemization process. The ester derived from trebly-labeled material was exposed to conditions (indicated in the graph above) under which ca. 80% of the racemization rate could be attributed to the general base-catalyzed processes. Subsequent reaction with ethyl glycinate followed by separation of the Young peptides gave a sample of racemate, the D activity of which had the same $^3H/^{14}C$ ratio as the L starting material. Again, only an oxa-

zolone mechanism would give this result, consequently, the intercept of the graph above must represent general base-catalyzed oxazolone formation:



The ester derived from the Kemp-Chien reagent was selected for the mechanistic study because it met all of the criteria. Its catechol structure raised the unique possibility of racemization via an intramolecular process as depicted below:



The trebly-labeled ester was exposed to strongly racemizing, weakly racemizing and optimal coupling conditions, all with the same result. No kinetic isotope effect was observed for the racemization process, a result which can only be accommodated by an oxazolone mechanism.

In conclusion, all the methods tested gave the same answer to the mechanistic problem - the oxazolone was the culprit in racemization. More specifically, three of the four cases indicated a general base-catalyzed oxazolone formation. The fourth case (the Kemp-Chien ester) is examined in some detail in Chapter III, but the reader is urged to examine Chapter II as well.

EXPERIMENTAL

I. Reagents

All amino acids were purchased from Calbiochem Corp. and bore the "A grade" label. Solvents, as well as other reagents, were of "reagent grade" quality unless otherwise noted and were used without further purification. Dimethyl sulfoxide and dimethyl formamide were dried over molecular sieves (Linde, 4A) at least a week before use. Radiotracer labelled compounds were obtained from New England Nuclear Corp.

II. Apparatus

Melting points were determined in capillary tubes with a Thomas-Hoover apparatus and are reported corrected. Infrared spectra were obtained with a Perkin-Elmer Infracord Model 137 and calibrated to the 1603 cm^{-1} band of polystyrene. Varian Associates' Models A-60 and T-60 spectrometers were used to obtain NMR spectra with TMS as the external standard. Radioactivity assays were determined with a Packard Instruments Model 3000 Tri-Carb Liquid Scintillation Counter, using internal ^{14}C and ^3H standards. Polarimetry was performed with Perkin-Elmer 181 or 141 and Zeiss 0.005° (photoelectric) polarimeters.

III. Methods

1. Analytical Samples.

Microanalyses were performed by the Scandanavian Microanalytical Laboratories of Copenhagen. Samples were recrystallized to constant melting point, treated with activated charcoal and recrystallized three additional times. For the ultimate recrystallization only glassware that had been cleaned with $H_2SO_4-Na_2Cr_2O_7$ solution and oven-dried in a dust-free atmosphere was employed. The ultimate solution was filtered hot through a fine porosity sintered glass funnel and sacrificially crystallized. The crystals were powdered with a glazed porcelain mortar and pestle, dried in vacuo at 56° for 1-2 hours and sealed in sample tubes before mailing.

2. Radioactivity Measurements.

Suitably purified samples were counted as dilute (10-30 mg in 15 ml scintillation liquid) solutions in potassium-free glass vials. The scintillation liquid was prepared by dissolving 150 mg "Dimethyl POPOP", 3.5 g "POP" and 50 g naphthalene in 500 ml dioxane. Activities are reported as disintegrations per minute per millimole as calculated by the following equation:

$$\frac{\text{dpm}}{\text{mmole}} = \frac{(\text{counts per minute-background}) \cdot (\text{MW})}{(\text{sample wt}) \cdot (\% \text{ efficiency})}$$

The efficiency was determined by counting appropriate internal standards at optimum voltage (gain) settings for each isotope. Calibration of the apparatus for dual channel counting (double labeling experiments) was accomplished by the following procedure: Internal standards of ^3H and ^{14}C were separately counted in the channel optimized for ^{14}C . The efficiency as a function of the lower discrimination setting for this channel was determined for both ^3H and ^{14}C . A similar set of curves were obtained for each isotope in the ^3H optimized channel where the efficiency as a function of the upper discriminator for this channel was determined. Calculation of disintegrations due to ^3H and ^{14}C at any discriminator setting were made with the aid of the charts.

Resolution of Acetyl-DL-leucine

The procedure used by DeWitt¹ to resolve α -phenyl ethyl amine was adapted. To a suspension of 11.2 g acetyl-DL-leucine in 15 ml H_2O was added 7.9 g (+) α -phenyl ethyl amine.² Another 55 ml H_2O was added and the mixture heated to boiling then allowed to cool

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- (1) H. E. DeWitt and A. W. Ingersoll, J. Am. Chem. Soc., 73, 5782 (1951).
 - (2) A. Ault, Org. Syn., 49, 93 (1969).

(insulated) slowly to room temperature. The solids were collected (ca 8 g) and recrystallized from 45 ml hot water to give magnificent spars, 4.5 g mp. 195-198°. The polarimetric data suggest that the literature values are in error since that salt was presumably contaminated with the more levorotatory acetyl-L-leucine (-) amine salt.

<u>mp</u>	<u>$[\alpha]_D^{24}$ (c=4, MeOH)</u>	<u>Source</u>
1) 195-198	- 7.2	Resolution above
2) 193-196	- 7.2	Salt of Ac-L-Leu-OH with (+) amine
3) 185-190	- 8.8	Literature ¹ resolution of α -phenyl ethyl amine with Ac-L-Leu-OH
4) 178-183	-13.2	Salt of Ac-L-Leu-OH with (-) amine

The combined mother liquors were evaporated to dryness and the resulting solids were recrystallized from minimum H₂O to give another 700 mg spars, mp 192-195°, raising the total yield to 55%.

Preparation of L-Leucine

A solution of 1.1 g (+) amine salt of acetyl-L-leucine in 7 ml 3N NCl was refluxed for 3 hours then evaporated to dryness in vacuo. The residue was combined with 2 ml H₂O and the evaporation repeated.

The resulting solids were dissolved in a minimum volume of H₂O (ca 5 ml) and the pH adjusted to 5.5 by the dropwise addition of 4N LiOH solution. After the addition of ca 40 ml absolute ethanol the suspension was chilled overnight to give shiny plates, 360 mg (73%). $[\alpha]_D^{23} + 16.0$ (c=2, 5N HCl). Lit.³ +16.0.

Benzoyl Chloride, 7-¹⁴C

Benzoic acid [7-¹⁴C (100 μc) was carefully transferred in ether solution to a solution containing 13 g (0.108 mol) benzoic acid. The solution was evaporated at reduced pressure and a small sample removed for isotopic analysis. The remaining white solid was combined with 13 ml (0.18 mol) thionyl chloride and heated with stirring under reflux for 1.5 hours. The reflux condenser was replaced by a short-run distilling head and the liquid was distilled at atmospheric pressure until the bath temperature reached 120°. After cooling the residue was distilled at aspirator pressure and the water-white fraction boiling at 66-68° was collected and used without further purification. 14.2 g (93%).

(3) Ref. 4, p. 2075.

Attempted Deuteration of Acetylleucine

The procedure described by Greenstein and Winitz⁴ to racemize L-leucine was adapted.

Solutions of DOAc were prepared by heating equimolar amounts of D₂O and acetic-anhydride until the phases coalesced, then used as quickly as possible.

To a hot solution of DOAc (1 mole) was added 6.55 g L-leucine (0.05 mol) and when solution was obtained, 13 ml acetic anhydride. The resulting (exothermic) reaction mixture was gently boiled for 10 min, allowed to stand at ambient temperature for one hr, then evaporated at reduced pressure. The residue was triturated with 4.5 ml D₂O (omission of this step results in production of substantial amounts of intractable resin at the expense of crystalline product), and the evaporation was repeated (in vacuo) giving a white crystalline solid. The solid was submitted to two repetitions of the deuteration procedure and the ultimate solid was recrystallized from acetone to give 5.1 g large transparent tablets mp 156-163°. Nmr analysis indicated 80% deuteration of the N-acetyl function and 90% deuteration of the α position. Similar results were obtained using acetyl-L-leucine in the place of L-leucine.

(4) J. P. Greenstein and M. Winitz, Chemistry of the Amino Acids, John Wiley and Sons, Inc., New York, N.Y., 1961, p. 834.

Benzoyl-L-Leucine⁵

A solution of 13.1 g L-leucine (0.1 mol) in 50 ml 2N NaOH was stirred vigorously and chilled by means of an ice bath. When the temperature reached 4^o, dropwise and simultaneous addition of 11.7 ml benzoyl chloride (14.1 g, 0.1 mol) and 60 ml 2N NaOH was commenced in such a manner that the reaction mixture remained strongly basic. The reaction was stirred at 0-5^o for 30 min after the last addition then extracted once with 50 ml ether. The aqueous phase was acidified to pH 1 by the addition of 3N HCl, extracted with three 100 ml portions of ether, and the combined ether phases were dried (MgSO₄) and evaporated. The water-white oil was taken up in 80 ml dry ether and warmed as pet ether was added to the cloud point, then seeded and allowed to cool slowly to room temperature to afford large transparent prisms in two crops (12.5 and 8.2 g; 88%), mp 106-107^o. The remaining mother liquor was treated with excess cyclohexylamine and the resulting flocculent precipitate was recrystallized from methanol-ether to give 2.4 g of the salt, mp 145-146^o to raise the total yield to 95%. [7-¹⁴C]-Benzoyl-L-leucine and benzoyl-L[4,5-³H]leucine were prepared by identical procedures.

(5) M. W. Williams and G. T. Young, J. Chem. Soc., 881 (1963).

Benzoyl Leucine Oxazolone^e

(2-phenyl-4-isobutyl oxazol-5-one)

Benzoyl-L-leucine, 7.05 g (30 mmol) was dissolved in a mixture of 10 ml dioxane and 40 ml acetic anhydride and heated under reflux for 20 minutes. After cooling to room temperature, the solvent was removed at reduced pressure and the residue was evaporated with three 15 ml portions of toluene to remove the excess acetic anhydride. The oily residue was taken up in ca 40 ml warm hexane and chilled in the freezer to give 5.6 g white needles, mp 55-56° in two crops. (86%)

Deuteration of Benzoyl Leucine Oxazolone

(2-phenyl-4-deutero-4-isobutyl oxazol-5-one)

A mixture of 13 g D₂O (0.65 m), and acetic anhydride, 80 ml (0.8 m) was heated together until the phases coalesced, then the exothermic reaction mixture was allowed to cool to room temperature and used within a few minutes. In 25 ml of this mixture (0.4 m exchangeable DOAc) was dissolved 5.45 g (25 mol) azlactone and the solution heated under reflux for 20 minutes, cooled then evaporated in vacuo. The procedure was repeated with another 25 ml deuteration solution and the residue crystallized from hexane to give 4.0 g white needles, mp 55-57°. No signal could be detected for the α -hydrogen region in the nmr

(6) H. Clarke, Ed., "The Chemistry of Penicillin," Princeton Univ. Press, Princeton, N.J., 1949.

spectrum of this substance.

Acid Hydrolysis of the Deuterated Oxazolone

To a hot solution of 6.9 g azlactone in 25 ml DOAc was added 10 ml D₂O. The exothermic mixture was allowed to cool until solids began to separate then evaporated to dryness in vacuo. The residue was recrystallized from ethyl acetate-pet ether to give 6.8 g (91%) in two crops, mp 137-139°. The readily exchangeable (nitrogen and oxygen-bound) deuterium was removed by evaporating the solids with two 50 ml portions of methanol before resolution. C.A.T. nmr analysis using internal triphenyl silane standard gave < 3% α -Hydrogen after 49 scans of the region 3 to 6.5 ppm.

Cinchonine Salts of Benzoyl Leucine

To a suspension of 294 mg (1 mmol), finely powdered cinchonine in 100 ml boiling ethyl acetate was added 235 mg (1 mmol) benzoyl-L-leucine. The solution was boiled down to 50 ml, at which time all the solids were in solution, and allowed to stand for 24 hours to give 500 mg shining cubes mp 190.5-193° $[\alpha]_D^{23} + 122.6$ (c=3.65; MeOH), insoluble in ethyl acetate.

Similar treatment of benzoyl-D-leucine gave no solids until the solution was evaporated, triturated with ethyl acetate-pet ether and chilled for 2 weeks; 270 mg small transparent blades mp 163-168, readily soluble in ethyl acetate.

Resolution of Benzoyl-DL-Leucine

To a suspension of 588 mg, (2mmol), finely powdered cinchonine in 200 ml boiling ethyl acetate was added 470 mg, (2 mmol), benzoyl-DL-leucine and the solution boiled down to 100 ml and seeded with the L salt. After 20 hours at room temperature the cubes were collected and washed with ethyl acetate to give 450 mg (85%), mp 189-191

$[\alpha]_D^{23}$ M 119.4 (c=4.2 MeOH).

To a rapidly stirred solution of 3N HCl (10 ml) was added 1.17 g of the salt followed quickly by 30 ml ethyl acetate. The stirring was continued until the initial suspension cleared to give two simple phases, the layers were separated, and the aqueous phase was extracted with another 25 ml ethyl acetate. The combined organic phases were washed with 3N HCl and brine solutions, then dried and evaporated. The water-white oil was crystallized from ether-pet ether to give 420 mg transparent prisms mp 105-106.5° (81%).

The resolution was performed on a 30 mmol scale for the preparation of [7-¹⁴C]benzoyl-L-leucine, α -deuterated. (73% based on the racemic acid).

Isotopic Purification⁷ of Labeled Benzoyl-L-Leucines

The resolved [7-¹⁴C]benzoyl-L-[α -²H]leucine, 3 g and benzoyl-L-[4,5-³H]leucine, were dissolved in 17.5 ml ethyl acetate containing

(7) D. S. Kemp, S. W. Wang, G. Busby, III and G. Hugel, J. Am. Chem. Soc., 92, 1043 (1970)

0.65 g unlabeled benzoyl-DL-leucine. The solution was seeded with racemic acid and the precipitated solids (0.73 g) collected after 3-1/2 hr at 3°. The mother liquor was used to dissolve another 242 mg unlabeled racemate and the seeding repeated to yield 235 mg solids. This process was repeated with another 242 mg racemate and the ultimate mother liquor was evaporated. The residue was treated with ethyl acetate-pet ether to give 3.5 g large prisms, mp 105-107°, $[\alpha]_D^{25} -6.5$, (c=2, EtOH). Specific activity 8.70×10^5 dpm/mmol, $^3\text{H}/^{14}\text{C} = 6.7$. Another sample of trebly-labeled acid was prepared by the same overall procedure (deuteration, resolution and isotopic purification) and the Young tests for the various coupling procedures described below were performed on both samples. Note that the α -deuterated (^{14}C -labeled) acid is not diluted in specific activity by the washing procedure above, but the α -hydrogen (^3H -labeled) acid is diluted.

Consider the following calculation:

The α -deuterated acid, ^{14}C activity 8,315 dpm/mg was combined with ^3H labeled acid and washed by the described procedure. The ultimate L-acid had specific activity (total) 7,950 dpm/mg, with the $^3\text{H}/^{14}\text{C}$ ratio = 2.16. Thus one mg of the trebly-labeled acid has $\frac{7,950}{2.16} = 3,679$ dpm ^{14}C which corresponds to $\frac{3,679}{8,315} = 0.442$ mg α -deuterated substances. The remaining 0.695 mg is α -protium species and is responsible for $(7,950 - 3,679) = 4,271$ dpm ^3H , consequently the activity of

α -protium species in $\frac{5,437 \text{ dpm}}{0.696 \text{ mg}} = 7,820 \text{ dpm/mg}$. The ratio of OH to OD is thus 2.3 in the starting material.

A Test of the Accuracy of Dual Channel Counting

Samples of ^3H labeled benzoyl-L-leucine were counted at optimum voltage and discriminator settings and the average value for the specific activity was determined to be $2.57 \times 10^6 \text{ dpm/mmol}$ against internal ^3H standard. The ^{14}C labeled substance was determined to have specific activity $1.97 \times 10^5 \text{ dpm/mmol}$ against internal ^{14}C standard.

The calibration curves determined that the efficiency for ^3H and ^{14}C were $< 0.1\%$ and 58.5% respectively, for channel A when the voltage (gain) was 11.5 and the discriminator settings were 225 to 1000. For channel B at voltage 50 and discriminator 50 to 375 the corresponding figures were: ^3H 27%, ^{14}C 10.6%.

A sample consisting of 12.70 μg ^{14}C labeled acid and 15.75 mg ^3H labeled acid was counted simultaneously in channels A and B at the discriminator settings described above.

A

found 6190 counts/min

→ $^{14}\text{C} = 10,600 \text{ disint/min}$

(6,190/58.5%)

or specific activity = $1.96 \times 10^5 \text{ dpm/mmol}$

$\left(\frac{(10,600) \cdot \text{MW mg/mmol}}{12.70 \text{ mg}} \right)$ compare $1.97 \times 10^5 \text{ dpm/mmol}$

B

found 47,300 counts/min

- 1,120 ¹⁴C contribution

(10.6% x 10,600)

46,180 ³H cpm

→ 171,000 dpm ³H

(46,180 / 27%)

specific activity = 2.55×10^8 dpm/mmol

compare 2.57×10^8 dpm/mmol.

All samples were counted at two discriminator settings in each channel, and the figures reported represent the average values. (The numbers were always within 2% of each other.)

Ethyl Benzoyl-L-Leucylglycinate

The procedure described by Young⁵ was used. Benzyloxycarbonyl-L-leucine was coupled with ethyl glycinate via the carbodiimide method in ca 40% yield. The dipeptide (ethyl benzyloxy carbonyl-L-leucylglycinate), 10.7 g, was dissolved in acetic acid (25 ml) containing 1.1 g 5% palladium on charcoal catalyst and hydrogenolyzed for 3 hours under a continuous stream of hydrogen at atmospheric pressure (a description of the apparatus employed may be found in Ref. 4, page 1233).

The catalyst was removed by filtration (celite) and the solvent removed at reduced pressure. The syrupy residue was diluted with 30 ml water, overlaid with 75 ml ether and stirred vigorously as 5.7 g NaHCO_3 was cautiously added in small portions. The stirring was continued as a solution of 4.3 ml benzoyl chloride in 60 ml ethyl acetate was added dropwise and simultaneously with a slurry of 5.5 g NaHCO_3 in 30 ml water. Fifteen minutes after the addition was complete 5 ml pyridine was added and a few minutes later the layers were separated. The organic phase was washed successively with 3N HCl, 1% citric acid and sat. bicarbonate solutions, then dried (MgSO_4) and evaporated. The solid residue was twice recrystallized from ethyl acetate-pet ether to yield 7.8 g (80%) ethyl benzoyl-L-leucylglycinate, mp 155-157°, $[\alpha]_D^{25}$ - 32.9 (c=3, EtOH). Lit.⁵ - 34.0.

Ethyl Benzoyl-DL-leucylglycinate

A solution of 2-phenyl-4-isobutyl-oxazol-5-one (benzoylleucine oxazolone) 10 mmol (2.17 g), in 10 ml dry DMF was combined with a warm solution of 30 mmol glycine ethyl ester hydrochloride and 6 ml triethylamine in 20 ml DMF. The resulting suspension was stirred at ca 70° for 1/2 hr, then poured into 100 ml ice-water and extracted into three 50 ml portions of ethyl acetate. The combined organic phases were washed successively with 3N HCl, 2N NaOH and brine, then evaporated. The white crystalline residue was recrystallized from aqueous

ethanol to give 2.8 g (88%) brilliant spars mp 145-147^o, Lit.⁵
145-147^o.

Proof of the Separability of the Young Peptides⁷

Racemic Young peptide, (prepared via the oxazolone) 78.9/mg
sp. act. 1846 d/mg, ¹⁴C/³H 1.32, and unlabeled L peptide, 369.8 mg
were dissolved in 15 ml hot ethyl acetate, the solution was cooled
to room temperature then seeded with a microdrop of a slurry of freshly
precipitated racemate. After 4 hours at 3^o the solids were collected
and recrystallized to mp 144.5-145.5 sp. act. 973 dpm/mg ¹⁴C/³H
1.34 (96% pure) calculated activity: 1013 dpm/mg.

The remaining racemate, 17.2 mg, was combined with 115.3 mg
unlabeled racemate and 577.6 mg unlabeled L peptide and recovered as
described above; mp 144-145.5, sp. act. 113 dpm/mg ¹⁴C/³H 1.35 calc.
act. 121 dpm/mg, (94% pure).

The remaining racemate, 58.8 mg, and 248 mg unlabeled L peptide
were combined in 10 ml solvent and recovered as before to give mp
144.5-146, sp. act. 114 dpm/mg, ¹⁴C/³H 1.31.

Racemization of Methyl Benzoyl-L-Leucinate by Methoxide:

Polarimetric Analysis

A solution of 0.2 M base was prepared by cautiously dissolving
potassium tert-butoxide, 570 mg (98.5% pure), 5 mmol, in 25 ml dry
methanol. When the solution had cooled to room temp. 10 ml was com-

bined with 274 mg methyl benzoyl-L-leucinate, 1.5 mmol, (instant solution in a total volume of 11 ml). After the time lapses indicated below, 1.0 ml aliquots were quenched by addition of 0.5 ml aliquots of 20% acetic acid in methanol (v/v) and sealed until analyzed polarimetrically at the sodium D-line (1 dm microcell).

t(min)	α_D^{23}
1	-0.481
1.3	0.478
30	0.456
70	0.435
115	0.395
220	0.325

Racemization During Saponification
of Methyl Benzoyl-L-Leucinate

Isotopically purified methyl benzoyl-L-leucinate, 220 mg (0.88 mmol), sp. act. 1.675×10^6 dpm/mmol, was dissolved in a minimum volume of acetone (1-2 ml) and stirred rapidly at room temp. as 2.0 ml 1 N NaOH was added all at once. After 90 min the solution was partitioned between 30 ml each ethyl acetate and 1 N HCl, the aqueous phase was saturated with salt, the layers were separated and the organic phase was washed with brine, then dried and concentrated. A 1 ml

aliquot of the product in 25 ml ethyl acetate was combined with 149 mg benzoyl-L-leucine and the solids recrystallized from ether-pet ether to mp 106-107°, sp. act. 403 dpm/mg, indicating a total yield of acid: 201 mg (97%). The solution of 193 mg remaining acid was combined with 317.3 mg unlabeled racemic acid and 2.053 g unlabeled L acid, the volume concentrated to 10 ml, chilled and seeded with racemate. The solids were collected 3 hrs later and recrystallized from ethyl acetate-pet ether to mp 138-140, 150 mg. This substance was combined with 1.0224 g unlabeled L acid in 5 ml ethyl acetate and recovered as before to give racemate, mp 138-140°. This substance, 92.4 mg, was taken through another dilution with 628.8 mg unlabeled L acid to give an ultimate racemate mp 138-140°, sp. act. 6.4 dmp/mg. The L acid was recovered from the mother liquors and recrystallized, mp 105-107°, sp. act. 3.7 dpm/mg (calc 2.96 dpm/mg). The activity of the D enantiomer is 9.1 dpm/mg indicating a yield of D acid 0.2 mg or 0.21% racemate.

Racemization and Hydrolysis of Methyl benzoyl-L-leucinate by Methoxide: Isotopic Dilution Analysis

A solution of 375 mg methyl benzoyl-L-leucinate, freed of labeled D enantiomer, sp. act. 1.675×10^5 dpm/mmol in 10 ml of 0.2 M potassium tert-butoxide in methanol was allowed to stand in a desiccator for 3 hrs. The reaction was quenched by the addition of 553 mg (2.36 mmol), benzoyl-DL-leucine, the solvent was evaporated and the residue was partitioned between ether and sat. bicarbonate solution. The organic phase

was washed with sat. bicarbonate and brine, combined with the back-extracts of the aqueous phases and evaporated. The combined aqueous phases were acidified (3 N HCl) then extracted into ethyl acetate, dried, concentrated and the solid recrystallized to mp 138-140^o, sp. act. 94 dpm/mg or 19% hydrolysis. The evaporate from the neutral phase was dissolved in 10 ml methanol and treated with 3 ml 1 N NaOH with occasional addition of water to keep a one phase system. The mixture was evaporated 2 hrs later, the glassy residue was partitioned between ethyl acetate and water, the aqueous phase was acidified and extracted into ethyl acetate. The organic phase was washed with brine then dried and concentrated. An aliquot (1 ml) of the product in 25 ml ethyl acetate was combined with unlabeled L acid, 125 mg, and recrystallized to mp 106-107^o, sp. act. 63 dpm/mg indicating 244.8 mg L acid remaining in the 24 ml. The 24 ml was combined with 469.5 mg unlabeled racemic acid and 2.026 g unlabeled L acid, the volume adjusted to 12 ml, the chilled solution seeded with racemate. The solid was collected after 2 hrs and recrystallized from ethyl acetate-pet ether, mp 138-140^o (400 mg).

Sp. act.	65.7 dpm/mg
Calc. for L	60.2 dpm/mg
∴ D activity =	71.2

The remaining racemic acid, 178 mg, was combined with unlabeled L acid, 1.255 g, and recovered and recrystallized as before to mp 138-140°.

Sp. act.	37.1 dpm/mg
found for L	4.08 dpm/mg (Calc. 3.98)
∴ D activity =	71.1 dpm/mg indicating a yield of D acid 21.6 mg or 16.3% racemi- zation

Material balance:

70 mg hydrolysis product
255 mg L acid
<u>22.5 mg D acid</u>
347.5 mg (1.47 mm) or 98.5%

Racemization of Methyl Benzoyl-L-leucinate by

Methoxide: Isotopic Dilution Analysis via the Azide

The reaction solution was prepared by dissolving 1.0 g (4 mmol) methyl [7-¹⁴C]benzoyl-L-leucinate, sp. act. 1.675×10^8 dpm/mmol, in a solution of 25 ml methanol containing 5 mmol (570 mg of 98%) potassium tert-butoxide. Aliquots of 8 ml were quenched with excess acetic acid at 5 min, 60 min and 210 min after combination of reagents. The quenched solutions were evaporated, the residues in ethyl acetate were

washed with sat. bicarbonate and water then evaporated. The resulting solids were quantitatively taken up in hot ethanol, treated with 0.5 ml hydrazine hydrate and allowed to stand at room temp. for 12 hrs. The solvent was removed at reduced pressure, the residue was taken up in 20 ml water and 0.5 ml each conc. HCl and glacial acetic acid, then overlaid with ether and diazotized in a cold room (+3°) using an ice-acetone bath at -10°, with sat. sodium nitrite solution. The ethereal phases were worked up in the usual fashion* and each treated with excess ethyl glycinate at 3° for 22 hrs. The residues from the coupling workup were taken through the isotopic dilution sequence with unlabeled L and DL Young peptides to give the following results.

	% rac	k_{rac} (min ⁻¹)	% act. due to D enantiomer
1. (5 min)	0.5	1×10^{-3}	75
2. (60 min)	7.1	1.2×10^{-3}	83
3. (210 min)	17.0	0.9×10^{-3}	89

The Isotope Effect for Racemization of Methyl-benzoyl-L-leucinate by Methoxide

The trebly-labeled methyl benzoyl-L-leucinate, specific activity: 7,500 dpm/mg; $^3\text{H}/^{14}\text{C} = 2.16$, $\alpha\text{H}/\alpha\text{D} = 2.3$, 3 μmoles , were dissolved in

* Detailed experimental conditions are described elsewhere in this section.

20 ml of a 0.2 M solution of potassium tert-butoxide in methanol and allowed to stand in a desiccator for 3 hrs at room temp. The reaction was quenched by the addition of 0.5 ml acetic acid and the solution was evaporated at reduced pressure. The residue was partitioned between ethyl acetate and sat. sodium bicarbonate, the layers were separated and the organic phase was washed with sat. bicarbonate and brine, then dried ($MgSO_4$) and divided into 2 equal portions A and B.

1. Determination via the azide coupling and Young test

The neutral fraction A was evaporated and the residue was dissolved in ethanol and treated with hydrazine; the hydrazide was converted to the azide by the usual technique (cold room) then coupled with ethyl glycinate. When solids appeared during these conversions care was taken to quantitatively dissolve them to avoid any racemate-enantiomer fractionations. The residue from the work up of the coupling reactions was combined with unlabeled racemic Young peptide, 128.4 mg, and the corresponding L enantiomer 397.4 mg and both substances recovered by the usual techniques.

	3H	^{14}C	$^3H/^{14}C$
Activity DL	1,035	373	
Activity L	1,226	613	2.00
Activity D	844	131	6.46

The remaining racemate, 64.4 mg, was combined with unlabeled L 318.2 mg and both substances recovered

	$^3\text{H}/^{14}\text{C}$		
Activity DL	408	75.8	
Activity L	110	57.2	1.92
Activity D	706	94.4	7.49

Calculations

Activity of undiluted product is 5,840 dpm/mg.

1. Yield of L peptide

found activity 1,226 ^3H + 613 ^{14}C

$$= 1,839 \text{ dpm/mg}$$

total L diluent added = 461.6 mg

$$\begin{aligned} \text{Yield of } \underline{\text{L}} \text{ peptide} &= \frac{461.6 \text{ mg (1839 dpm/mg)}}{(5,840 - 1839) \text{ dpm/mg}} \\ &= 212 \text{ mg.} \end{aligned}$$

Since all the ^3H -labeled molecules bear an αH and all ^{14}C -labeled molecules bear an αD , the $^3\text{H}/^{14}\text{C}$ ratio of the starting material corresponds to an $\alpha\text{H}/\alpha\text{D}$ ratio. The $^3\text{H}/^{14}\text{C}$ ratio of the products then will reflect the $\alpha\text{H}/\alpha\text{D}$ origin of the products:

$$\frac{({}^3\text{H}/{}^{14}\text{C})_{\text{SM}}}{(\alpha\text{H}/\alpha\text{D})_{\text{SM}}} = \frac{({}^3\text{H}/{}^{14}\text{C})_{\text{P}}}{(\frac{\alpha\text{H-derived}}{\alpha\text{D-derived}})_{\text{P}}}$$

For the L peptide $({}^3\text{H}/{}^{14}\text{C})_{\text{P}} = 2.0$ the starting material had

${}^3\text{H}/{}^{14}\text{C} = 2.16$; $\alpha\text{H}/\alpha\text{D} = 2.3$ ∴ the L peptide has $\frac{\alpha\text{H-derived}}{\alpha\text{D-derived}}$ ratio 2.12

or 1.44 mg $\alpha\text{-H}$ derived and 0.68 mg $\alpha\text{-D}$ derived.

2. Yield of D peptide

10.18 mg (calculated as above)

$${}^3\text{H}/{}^{14}\text{C} \text{ D peptide} = 7.5 \therefore \frac{\alpha\text{H-derived}}{\alpha\text{D-derived}} = 8.0$$

or 9.04 mg $\alpha\text{-H}$ derived and 1.14 mg $\alpha\text{-D}$ derived

3. % racemization

$$\alpha\text{-H species} = \frac{2(9.04)}{14.4 + 9.04} = 11.8\%$$

$$\alpha\text{-D species} = \frac{2(1.14)}{6.8 + 1.14} = 3.3\%$$

4. Isotope effect

Assuming that no methoxide is consumed, and the reaction is

$$\text{P-first order, } k_{\text{rac}}^{\text{H}} = \frac{+\ln H_0/H}{t}$$

$$\text{and } \frac{k_{\text{rac}}^{\text{H}}}{k_{\text{rac}}^{\text{D}}} = \frac{\ln H_0/H}{\ln D_0/D} = 3.75$$

2. Determination of saponification

The remaining neutral fraction B was evaporated at reduced pressure and the residue was saponified in acetone 1 N NaOH as previously described. The acidic fraction was isolated and combined with unlabeled benzoyl-L-leucine, 1.527 g and the corresponding racemate, 231 mg, and both substances were recovered and purified by recrystallization.

Activity of the L enantiomer:

^3H	^{14}C	$^3\text{H}/^{14}\text{C}$	yield L _ 270.5 mg:
748	380	1.97	$\alpha\text{H} = 183 \text{ mg}; \alpha\text{D} = 87.5 \text{ mg}$

The racemate, 147 mg, was combined with 1.005 mg unlabeled L acid and the racemate recovered, then combined (100 mg) with 700 mg unlabeled L and both substances were recovered and purified as usual.

			$^3\text{H}/^{14}\text{C}$	
Activity of DL	357.5	46		
Activity of L	7.8	3.94	1.98	
Activity of D	707.2	88.06	8.03	yield D = 12.9 mg;
				11.5 mg αH -derived
				1.4 mg αD -derived

% racemization $\alpha\text{H} = 11.8\%$

$\alpha\text{D} = 3.15\%$

Isotope effect = 3.94

The Isotope Effect for Racemization During Coupling
via the 3-Acyloxy-N-Ethylsalicylamides

1. Optimal Conditions

The trebly-labeled ester 1 mmol, sp. act. 8.70×10^5 dpm/mmol, $^3\text{H}/^{14}\text{C}$: 6.3, was dissolved in 6 ml dry DMF and chilled for one hr at 3° , then 0.11 ml (1.1 eq) ethyl glycinate was added, and the reaction mixture was allowed to stand at 3° overnight. The solution was quenched with dil. HCl and extracted with several portions of ethyl acetate. The combined organic phases were washed successively with dil. NaOH, sat. bicarbonate and brine solutions, then dried and concentrated to 25 ml. A 1 ml aliquot of the solution was combined with 91 mg unlabeled Young L peptide and the L peptide, mp 155° , recovered and recrystallized from ethyl acetate-pet ether. Activity 297 dpm/mg; $^3\text{H}/^{14}\text{C}$: 6.26. The remaining solution of product was combined with unlabeled racemate, 206 mg, and unlabeled L peptide, 751 mg, in a total volume of 30 ml ethyl acetate, the solution was seeded with a micro-drop of a freshly precipitated slurry of racemate and chilled for 6 hr to give solids. After recrystallization from ethyl acetate-pet ether the mp was $145-147^\circ$, specific activity 339 dpm/mg; $^3\text{H}/^{14}\text{C}$: 6.2.

The remaining racemate, 95 mg, was combined with 478.4 mg unlabeled L peptide and the racemate recovered and purified as before, mp $144-$

146°, sp. act. 37.4 dpm/mg; $^3\text{H}/^{14}\text{C}$: 6.5. The remaining racemate, 37.4 mg, was combined in 7 ml ethyl acetate with 192.8 mg unlabeled L peptide, and both substances recovered as before.

Activity of racemate: 117.1 dpm/14.3 mg; $^3\text{H}/^{14}\text{C}$ 6.4
Activity of L enantiomer: 5.3 dpm/mg; $^3\text{H}/^{14}\text{C}$ 6.4

Calculations

1. Yield of L peptide in aliquot

$$(X \text{ mg}) [2.720 \text{ dpm/mg}] = (X + 91 \text{ mg}) [297 \text{ dpm/mg}]$$

$$X = 11.5 \text{ mg, or } 268 \text{ mg remains in the } 24 \text{ ml.}$$

$$\text{Total yield} = 279.5/320 = 87.5\%$$

2. Activity of the D enantiomer

a. After first dilution L act. = 665 dpm/mg

$$\text{D activity} = 2 \times \text{DL activity} - \text{L activity}$$

$$= 2(339) - 665$$

$$= 13 \text{ dpm/mg.}$$

b. After second dilution L act. = 60 dpm/mg

$$\text{D activity} = 2(37.4) - 60$$

$$= 14.8$$

c. After third dilution L act. = 5.3 dpm/mg

$$\text{D activity} = 2(8.2) = 5.3$$

$$= 11.1 \text{ dpm/mg.} \quad 65\% \text{ due to D.}$$

3. Yield of D peptide

$$X \text{ mg } [2720 = (x + 103 \text{ mg}) [11.1]$$

$$X = 0.425 \text{ mg } \underline{D} \text{ peptide}$$

$$\text{and mg DL} = 0.85$$

4. % racemization

$$\% \text{ rac.} = \frac{\text{mg DL}}{\text{mg L} + \text{mg D}} = \frac{0.85}{268} = 0.32\%$$

The constant $^3\text{H}/^{14}\text{C}$ ratio indicates the isotope effect = 1.

2. Slightly Racemizing Conditions

A solution of 2 mmol of the active ester in 10 ml dry DMF was treated with 2 mmol glycine ethyl ester hydrochloride and 2 mmol triethylamine then stirred at room temp. for 1 day. The reaction mixture was worked up as before and the Young test applied to give:

L peptide 96% yield; $^3\text{H}/^{14}\text{C}$ 6.4; 1.0 % rac., D peptide $^3\text{H}/^{14}\text{C}$ 6.5

40% of the final measured activity was due to D enantiomer.

3. Strongly Racemizing Conditions

A solution of 2 mmol active ester in 10 ml DMF was treated with 230 mg (2.0 mmol) tetramethylguanidine. After 1 hr at room temp. 2 mmol glycine ethyl ester hydrochloride was added and the resulting solution was stirred at room temp. for 15 hr. The usual workup and Young test gave:

L peptide $^3\text{H}/^{14}\text{C}$ 6.3; 32% rac, D peptide $^3\text{H}/^{14}\text{C}$ 6.5

88% of the final measured activity was due to the D enantiomer

The Isotope Effect for Racemization During Coupling
with O-Esters of N-Ethylsalicylamide

A solution prepared by dissolving 1 mmol of the trebly-labeled active ester, sp. act. 1.87×10^6 dpm/mmol; $^3\text{H}/^{14}\text{C}$ 2.2, 2 mmol triethylammonium fluoroborate and 1 mmol triethylamine in 5 ml dry DMF was allowed to stand for 10 min at room temp., after which time 1 mmol ethyl glycinate was added. The reaction mixture was quenched after 24 hr at room temp. by the addition of 20 ml dil. HCl, and the aqueous phase was extracted with several portions of ethyl acetate. The combined organic phases were washed with N NaOH, sat. bicarbonate and brine solutions, then dried and concentrated to 25 ml and the Young test applied.

85% yield; L peptide $^3\text{H}/^{14}\text{C}$ 2.2

19% rac; D peptide $^3\text{H}/^{14}\text{C}$ 2.2

Racemization Tests for Couplings via Acyl Azides

The general procedure described by Young⁵ was employed with some major modifications. The ^{14}C -labeled hydrazide, 2.17 g, (8.5 mmol) was suspended in 10 ml water at 0° and stirred rapidly as 2 ml each conc. HCl and glacial acetic acid were added.

All subsequent operations were performed in a cold room maintained at $+3^{\circ}$, using pre-equilibrated reagents and glassware.

The clear, acidic hydrazide solution was placed in an ice-acetone bath (ca. -10°), overlayers with 15 ml ether and stirred rapidly as a solution of 1.2 g sodium nitrite in a minimum volume of water was added dropwise over the course of 8 min. After another 5 min stirring the layers were separated, the aqueous phase was extracted with three 25 ml portions of ether and the combined organic phases were thoroughly mixed (volume \sim 90 ml).

One third of the acyl azide solution was dried briefly over $MgSO_4$, then divided and one portion was treated with 0.18 ml ethyl glycinate (1) while the other was treated with ca. 100 mg triethylamine followed 10 min later by 0.18 ml ethyl glycinate (2).

The remaining ethereal azide solution was extracted cautiously with four 15 ml portions of sat. bicarbonate solution (after which the pH of the aqueous phase is \sim 8), dried briefly over $MgSO_4$ then divided into four equal portions (3-6).

3 was treated with 100 mg triethylamine followed 10 min later by 0.18 ml ethyl glycinate.

4 was treated with 100 mg glacial acetic acid and, after thorough mixing, 0.18 ml ethyl glycinate.

5 was treated with 0.36 ml ethyl glycinate.

6 was treated with 0.18 ml ethyl glycinate and, after 1 hr, was brought into a room at 24° and allowed to stand for 20 hr.

Solutions 1-5 were allowed to stand at 3° for 20 hr, during which time crystals of product appeared in all the solutions. The workup was conducted at room temp; the ether was evaporated and all resulting solids were carefully dissolved in ethyl acetate, washed with acid, bicarbonate and brine solutions, dried then concentrated to 25 ml. The Young test results are tabulated below.

Conditions	% Yield	% rac.	% of final measured activity due to the D enantiomer
1	62	0.1 ± 0.03	30
2	66	0.1	30
3	70	2.3	92
4	67	0.27	55
5	73	0.36	80
6	62	0.29	70

Racemization Tests for Couplings via
the Mixed Anhydride

The general procedure described by Anderson⁸ was used with minor modifications. Tritium labeled benzoyl-L-leucine, sp. act. 2.5×10^6 dpm/mmol, 1 mmol was dissolved in 5 ml dry tetrahydrofuran and allowed to equilibrate for 20 min with a bath at -14° (the bath temperature was obtained by preparing a slurry of 50 g each ice, water and sodium nitrate in a small Dewar flask). The base indicated below was added followed by 0.14 ml iso-butylchloroformate. One minute after the addition of the chloroformate, 0.11 ml ethyl glycinate was added, the bath was removed 1-2 min later, and the stirred reaction mixture was allowed 1 hr at ambient temperature. The suspension was partitioned between ethyl acetate and dilute HCl, the aqueous phase was saturated with salt, the layers were separated and the organic phase was washed with sat. bicarbonate and brine solutions, dried ($MgSO_4$) then concentrated to 25 ml and the Young test applied as usual.

	N-methylmorpholine		% of final measured activity due to D enantiomer
	% yield	% rac	
1 eq.(0.11 ml)	60	0.4	20
2 eq.(0.22 ml)	60	15	85
Triethylamine			
1 eq.(0.14 ml)	25	1.5	45
2 eq.(0.28 ml)	70	39	95

(8) G. W. Anderson, J. E. Zimmerman and F. M. Callahan, J. Am. Chem. Soc., 89, 5012 (1967).

The Isotope Effect for Racemization
During Couplings via the Mixed Anhydride

The trebly-labeled benzoylleucine, 1 mmol, sp. act. 1.9×10^6 dpm/mmol, $^3\text{H}/^{14}\text{C}$ 2.2, was converted to the mixed anhydride with 1 eq. iso-butylchloroformate at -14° using 1.1 eq. N-methyl morpholine. After a one-minute activation time, 1.1 eq. ethyl glycinate was added and the reaction mixture treated as previously described. The Young test gave a 66% yield of the L peptide $^3\text{H}/^{14}\text{C}$ 2.2, 2.4% rac., D peptide $^3\text{H}/^{14}\text{C}$ 2.1. Of the final measured activity, 60% was due to the D enantiomer.

The Isotope Effect for Racemization
During Coupling via the Acyl Azide

A. Racemizing Conditions

The acyl hydrazide, 1.3 mmol, sp. act. 1.90×10^6 d/mmol, $^3\text{H}/^{14}\text{C}$ 2.2 was diazotized in the cold room as previously described. The resulting ethereal azide solution was extracted exhaustively with sat. bicarbonate, dried and the total volume (35 ml) was treated with 100 mg triethylamine followed 10 min later by 0.18 ml ethyl glycinate. After 20 hr at 3° the reaction mixture was worked up as before to give the following results:

Yield 66%; L peptide $^3\text{H}/^{14}\text{C}$ 2.2

Rac. 1.8%; D peptide $^3\text{H}/^{14}\text{C}$ 2.4

Of the final measured activity $> 90\%$ was due to the D enantiomer.

B. Optimal Conditions

An independently prepared sample of the acyl hydrazide with sp. act. 8.7×10^5 dpm/mmol, $^3\text{H}/^{14}\text{C}$ 6.5 was diazotized according to the usual procedure. The resulting ethereal azide solution was extracted with a single portion of sat. bicarbonate solution, dried then treated with ethyl glycinate.

Yield 65%; L peptide $^3\text{H}/^{14}\text{C}$ 6.4

Rac. 0.16%; D peptide $^3\text{H}/^{14}\text{C}$ 6.5

65% of the final measured activity was due to the D enantiomer.

Polarimetric Study of the Racemization
of the O-Acyl-N-Ethylsalicylamide Ester*

All runs were performed in a water-jacketed polarimeter microcell (1 dm) held at 25° by means of a constant temperature circulating bath. Standard solutions of the active ester, triethylamine and triethylammonium fluoborate were combined, and the resulting solutions were made up to constant ionic strength (0.2 M) by addition of tetraethylammonium fluoborate. The rotation at the sodium D line was observed as a function of time; plots of \ln vs. time gave good straight lines over three half-lives.

* The method described by Kemp and Chien (J. Am. Chem. Soc., 89, 2745 (1967)).

Active ester concentration 2% (20 mg/ml).

Triethyl amine concentration 0.2 M.

Concentrations(M)		$k_{\text{obs}} \times 10^2$
$\text{Et}_3\text{N} \cdot \text{HBF}_4$	$\text{Et}_4\text{N} \cdot \text{BF}_4$	
0.2	0	1.4
0.1	0.1	2.2
0.025	0.175	3.6
0.012	0.188	4.7
0.01	0.19	5.3

A plot of $k_{\text{obs}} \times 10^2$ vs. $\text{Et}_3\text{N}/\text{Et}_3\text{N} \cdot \text{HBF}_4$ resulted in a straight line with slope 0.19 and intercept 1.6 (least squares analysis). In one run, an isotopic dilution was performed after three half-lives to determine the total remaining active ester concentration ($\underline{\text{I}}$ + $\underline{\text{II}}$). Only 9% of the initial radioactivity was recovered.

CHAPTER II

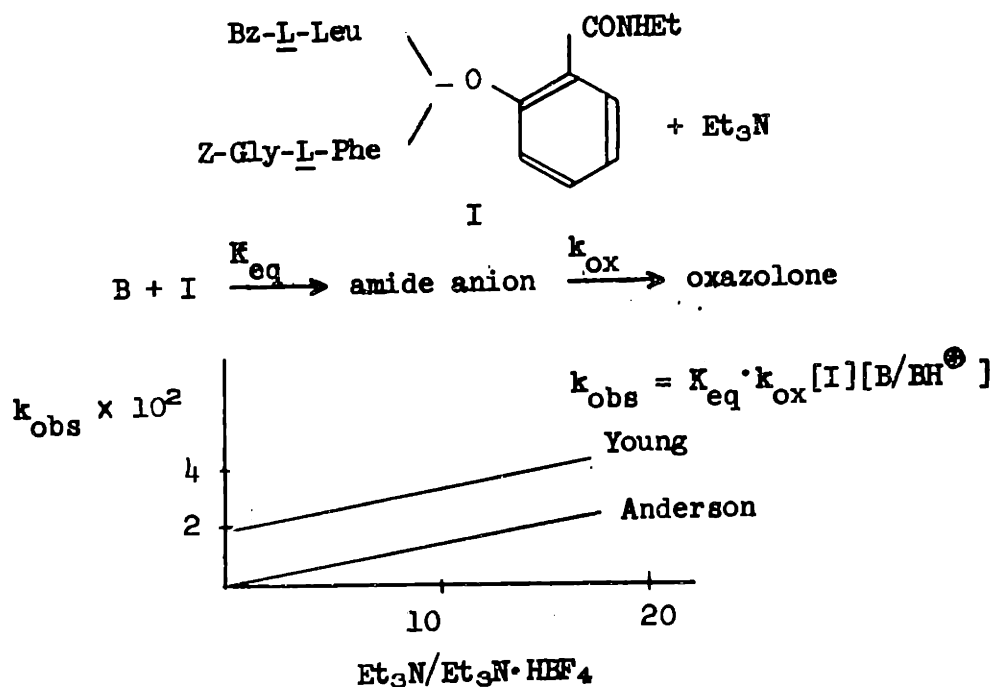
How generalizable is all this?

G. M. Whitesides

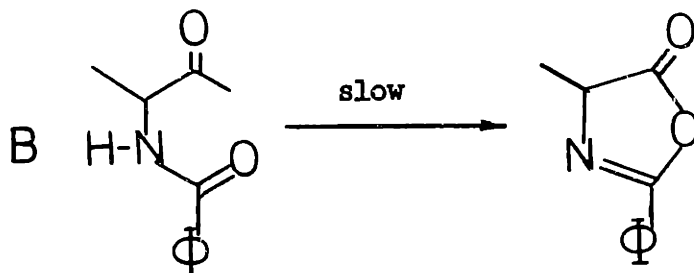
The mechanistic conclusions reached in the previous chapter are not necessarily valid for other peptide systems. The uncertainty arises because the benzamide of the Young peptide may not be a reliable model for a normal peptide bond. The effect of the phenyl group on the racemization behavior of oxazolones has already been described; the same phenyl group may have other consequences as well.

Comparison of the racemization rates of the Young (Bz-L-Leu) and Anderson (Z-Gly-L-Phe) O-esters of N-ethylsalicylamide I, bears out these suspicions (Fig. 1). The Young ester racemizes at considerably faster rate than the Anderson ester. Since the slopes,

Fig. 1



which represent $K_{eq} \cdot k_{ox}$ for the two esters, are parallel,* the increased racemization rate of the Young case can be traced to the larger intercept. The evidence of the preceding chapter has shown that this intercept represents the rate of general base-catalyzed oxazolone formation. The effect of the benzamide grouping, then, is an overall

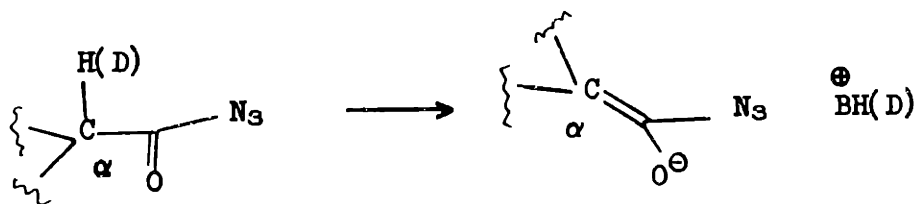


increased rate of racemization due to this secondary oxazolone mechanism. The important consequence for the mechanistic study is that this effect creates a two-fold racemization rate difference in the two models at the high N/NH^{\oplus} ratios expected under normal peptide coupling conditions.

With this rate difference in mind the azide results of the previous chapter were bothersome since the isotope effect was distinguishable from unity. Assuming a primary isotope effect of three for

* The increased stability of a benzamide anion vs. an alkylamide anion would be reflected in a larger K_{eq} but smaller k_{ox} for the Young case when compared to the Anderson case. The parallel slopes are, consequently, not surprising.

the process:



the oxazolone pathways need be only 6 times faster than the hydrogen enolization rate to give an observed isotope effect of 1.1. The actually observed value (1.1), suggested that both mechanisms might clearly be seen in a system with a slower overall rate of oxazolone-catalyzed racemization.

In summary, the predictive value of the results from the Young model was questionable. The Anderson model, in which a genuine peptide bond would be involved in oxazolone formation, represented a case from which generalizations to other peptide systems could be made with more certainty. The present chapter describes the attempts to measure the isotope effect for racemization using the Anderson model.

The required starting material, radiotracer-labeled Z-Gly-L-(α -deuterated)Phe, was expected to present no synthetic difficulties since the α -deuteration of amino acids via the benzoyl oxazolones was now a well-trodden laboratory route. The deuteration was accomplished without event, acid hydrolysis served to remove the benzoyl

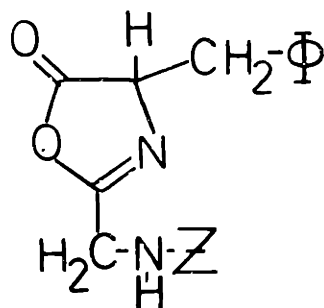
group, and the racemic, α -deuterated phenylalanine was coupled with ^{14}C -labeled Z-Gly via the Kemp-Chien reagent. Resolution of the resulting racemic dipeptide acid with (-) α -phenyl ethylamine gave a sample of the required Z-[1- ^{14}C]-Gly-L-[2- ^2H]Phe that was > 99% optically pure. This material was combined with Z-Gly-L-[3- ^3H]Phe and the mixture was freed of any radiolabeled D enantiomer by the techniques described in the previous chapter.

When the trebly-labeled material was coupled with ethyl glycinate via either the Kemp-Chien reagent or the mixed anhydride method, and the racemization assay applied to the resulting peptide, small but measureable kinetic isotope effects for racemization were observed!

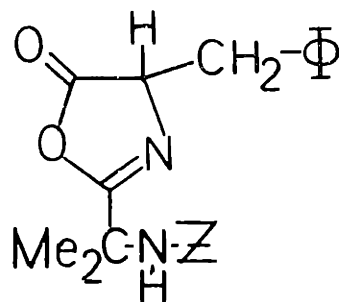
	% αH	racemization αD	kH/kD
Mixed anhydride	0.013	0.009	1.5
Kemp-Chien Reagent, (23 $^\circ$)	0.06	0.04	1.5
Kemp-Chien Reagent, (3 $^\circ$)	0.014	0.013	1.1

These results suggest a racemization mechanism in which the breakage of the $\text{C}_{\alpha}\text{-H}$ bond is rate-determining. By the arguments of the preceding chapter, enolization alone could account for this phenomenon. Unfortunately, the arguments for the Young case may not obtain in the present case.

It will be recalled that the advantage of the Young model lay in the assurance that the oxazolone derived from benzoyl-L-leucine would racemize at such a rate that any peptide arising from it would be racemic, i.e., $k_{\text{rac}} \gg k_{\text{coup}}$ (Scheme I). This feature is not necessarily shared by the oxazolone derived from Z-Gly-L-Phe (II).



II



III

Goodman's¹ studies with optically active oxazolones, in which the peptide oxazolone (III) was found to give only partially racemized

(1) See page 25.

coupling product (i.e., $k_{\text{rac}} \approx k_{\text{coup}}$) with ethyl glycinate, suggest that oxazolone II may also give partially racemic peptide. If the racemization rate for the oxazolone is in balanced competition with the coupling rate, substitution of the α -H in III by α -deuterium should upset this balance, and a kinetic isotope effect for the racemization of the oxazolone would be seen.²

The consequences for the present mechanistic study would be at the very least, annoying. If both oxazolone and enolization pathways are able to show kinetic isotope effects for racemization, the mechanistic problem cannot be solved by a method that is designed to measure the kinetic isotope effect. The initial objectives were to:

1. Determine the relative rates for racemization and aminolysis of the Anderson oxazolone by ethyl glycinate.
2. Develop experimental conditions under which the kinetic isotope effect allows a distinction between the two racemization mechanisms.

In order to attain the first objective, the Anderson oxazolone was required in an optically pure form. The literature records num-

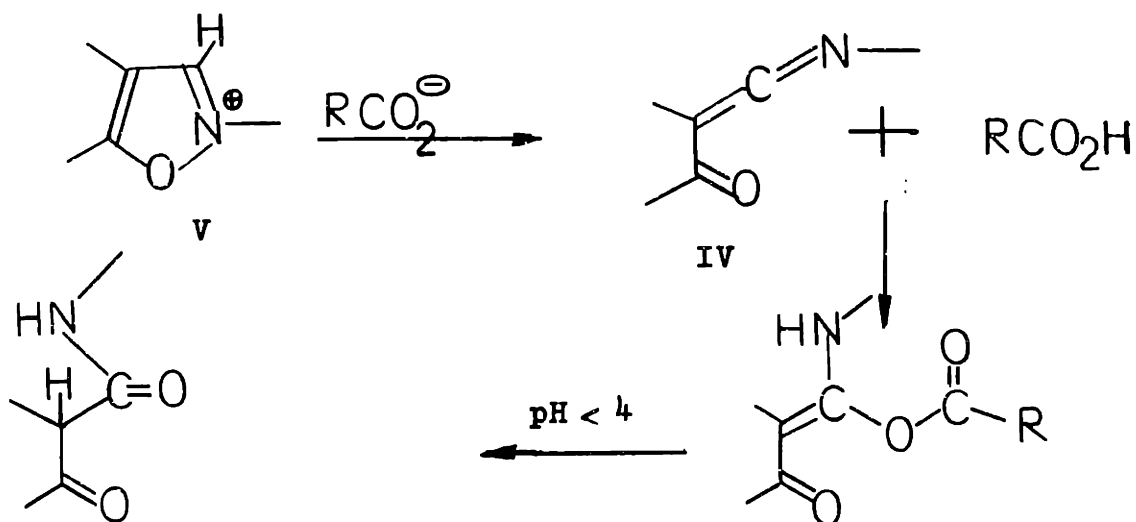
(2) Another reaction in which the rate-determining step is followed by a kinetic isotope effect has recently been reported: T. J. Katz and S. A. Cerefice, J. Am. Chem. Soc., 91, 6519 (1969).

erous attempts to prepare this substance,³ but, even though its derivatives have shown some optical activity, quantitative assessment of the optical purity of the oxazolone itself has been impractical due to its existence as an unstable heavy syrup. In our hands, assessing the minimum optical purity of the Anderson oxazolone would be an easy matter. Goodman⁴ reports that the reaction of oxazolone III with excess hydrazine in cold methanolic tetrahydrofuran yields a hydrazide that is optically pure. The hydrazide that would result from similar treatment of the Anderson oxazolone is a substance whose optical purity could be assessed with great accuracy due to the isotopic dilution assay described for this compound by Kemp.⁵

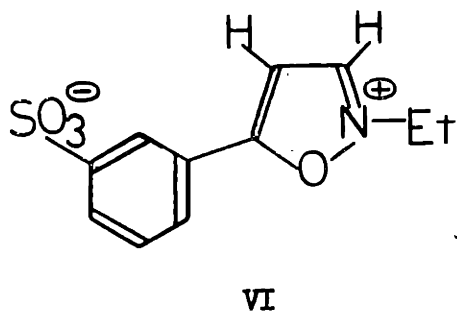
None less a method of generating the oxazolone under mild conditions was required. The published attempts, which suffer their greatest problems in the purification of this exceedingly soluble substance, appeared unpromising. A much more attractive method was suggested by the observations of Woodward's students^{3a,6} regarding the

-
- (3) (a) D. S. Kemp, Ph.D. Thesis, Harvard, 1964.
(b) E. Schnabel, Ann., 688, 238 (1965).
(c) W. J. McGahren and M. Goodman, Tet., 23, 2017 (1967).
- (4) M. Goodman and C. Glaser in "Peptides: Chemistry and Biology," B. Weinstein and S. Lande, Eds., M. Dekker, New York, 1970, p. 307.
- (5) D. S. Kemp, S. W. Wang, G. Busby and G. Hugel, J. Am. Chem. Soc., 92, 1052 (1970).
- (6) D. Woodman, Ph.D. Thesis, Harvard, 196

behavior of keto-ketimines IV derived from isoxazolium salts V by the action of tertiary bases. These intermediates are extremely mild



dehydrating agents and under carefully defined conditions are able to convert peptide acids to oxazolones. Woodman's⁶ investigations suggested that the keto-ketimine derived from Woodward's reagent K VI would be ideal for our purposes, since the side products would be water soluble and could be removed by simple extraction.



This method, in fact, proved to be satisfactory. The Anderson oxazolone could be prepared from Z-Gly-L-Phe with greater than 95% retention of optical integrity, albeit in low yield. The trebly-labeled acid was converted to the corresponding oxazolone which, in ethereal solution, was (1) sampled for optical purity with the hydrazine quench and (2) treated with excess ethyl glycinate. Application of the isotopic dilution assay to the hydrazide and peptide products gave the following results.

	% rac		isotope effect (k_{rac}^H/k_{rac}^D)
	α -H	α -D	
(1) Z-Gly-Phe-NHNH ₂	7	3	
(2) Z-Gly-Phe-GlyOEt	54(64)*	24(34)	3.7(3.3)

Assuming that the coupling and racemization reactions are both bimolecular, the fraction racemate, β , can be expressed as

$$\beta = \frac{\% \text{ rac}}{100} = \frac{k_{rac}}{k_{coup} + k_{rac}} ; k_{rac}/k_{coup} = \beta/(1-\beta)$$

* See the details in the experimental section for the explanation of the numbers in parentheses.

Since secondary isotope effects are expected to be negligible,

$$k_{\text{rac}}^{\text{H}}/k_{\text{rac}}^{\text{D}} = \frac{\beta^{\text{H}}/1-\beta^{\text{H}}}{\beta^{\text{D}}/1-\beta^{\text{D}}} = \text{isotope effect for racemization.}$$

In another series of experiments, the oxazolone was allowed to react with ethyl glycinate in the presence of triethylamine in ether (3) and dimethylformamide (4) and directly with ethyl glycinate in dimethylformamide (5):

	% rac		isotope effect $k_{\text{rac}}^{\text{H}}/k_{\text{rac}}^{\text{D}}$
	α -H	α -D	
(3) Et ₃ N-GlyOEt(Et ₂ O)	87(100)	87(100)	1
(4) Et ₃ N-GlyOEt(DMF)	87(100)	87(100)	1
(5) GlyOEt (DMF)	73(87)	34(42)	3.8(7)

These results are in complete accord with the studies of Goodman⁵ and establish an insurmountable obstacle in the path of our original goal, i.e., the study of racemization mechanism under optimal peptide conditions. Only in the presence of tertiary amines could the enolization and azlactone mechanisms be distinguished; milder conditions would show isotope effects for both mechanisms and therefore could allow no distinction. In this light, the results of the mixed anhydride and catechol ester couplings are not easily rationalized, since, under

neutral conditions, both mechanisms should show isotope effects for racemization. A possible explanation may lie in the fact that the carboxylate and phenolate by-products increase the rate of the racemization of the azlactone.

Although the mechanistic study was thwarted by the optical lifetime of the Anderson oxazolone, the acyl azide method was still of interest for the reasons discussed earlier in this chapter. The acyl azide was generated by the usual method and, using ether as the coupling medium (with the hope that the specific base-catalyzed formation of oxazolone would be suppressed in this solvent), our suspicions were confirmed. Experiment (2) is ambiguous since the oxazolone shows

Ether with	% rac		$k_{\text{rac}}^{\text{H}}/k_{\text{rac}}^{\text{D}}$
	$\alpha\text{-H}$	$\alpha\text{-D}$	
(1) $\text{Et}_3\text{N-GlyOEt}$	1.6	0.5	2.8
(2) GlyOEt	0.03	0.011	2.9

an isotope effect under these conditions, but the racemization in experiment (1) cannot be oxazolone-mediated. The latter experiment, then, represents the first proof of enolization for any peptide-activated species capable of oxazolone formation.

The experiments were repeated in dimethylformamide as the reaction medium with even more dramatic results. Again, experiment (3) is

ambiguous, but experiment (4), in which massive amounts of racemization are observed for the first time with acylazides, must represent a competition between the oxazolone and enolization mechanisms. Assuming

DMF with	% rac		$k_{\text{rac}}^{\text{H}}/k_{\text{rac}}^{\text{D}}$
	$\alpha\text{-H}$	$\alpha\text{-D}$	
(3) GlyOEt	0.48	0.22	2.3
(4) Et ₃ N-GlyOEt	50.3	42.4	1.3

an isotope effect of 3 for the enolization process, it can be calculated that the rate of enolization in (4) is 1/3 as fast as the rate of oxazolone formation, whereas in experiment (1) the enolization rate is 10 times the oxazolone rate. Herein may lie the explanation of the solvent effects previously noted for racemization. By changing the solvent from DMF to ether, the specific base-catalyzed oxazolone formation may be reduced 30-fold. Unfortunately, the more polar solvents will be required for the coupling of large peptide fragments. Fortunately, other means of reducing specific base-catalyzed racemization are available and are discussed in the next chapter.

The enolization demonstrated for the acyl azide presents a new facet of the racemization curse and warns against highly activating groups in peptide synthetic schemes that do not encounter the oxazolone problem.

EXPERIMENTAL

Determination of the Amount of Tritium Label
in the α Position of L-[2,3³H]phenylalanine

Tritium-labeled benzoyl-L-phenylalanine¹ (prepared from L-phenylalanine "2,3³H"* by the same procedure as used for benzoyl-L-leucine), 800 mg, sp. act. 13,000 dpm/mg, was dissolved in 35 ml of a mixture of dioxane-acetic anhydride, 1:7 (v/v), heated under reflux for 20 min, then evaporated at reduced pressure. The resulting oil was refluxed in 20 ml of acetic acid-acetic anhydride solution, 3:1 (v/v), then evaporated. This latter procedure was repeated three additional times with fresh acid-anhydride solution, then a portion of the residue was removed, while the remaining oil was taken through another cycle. The azlactones thus obtained were hydrolyzed by addition to a boiling mixture of 3 ml acetic acid in 10 ml water, the crude solids were collected and recrystallized twice from acetone to mp 185-187°. Both samples of the racemic benzoylphenylalanine had the same activity; 12,870 dpm/mg, indicating a loss of 130 dpm/mg or a total loss of 0.047 μ Ci tritium. An aliquot of the combined distillates from the evaporations were counted indicating a total of 0.025 μ Ci tritium recovered.

(1) E. Fischer and A. Mouneyrat, Ber., 33, 2383 (1900).

* Purchased from New England Nuclear Co., Boston, Mass.

Since some of the label was sure to be lost during the evaporations, the results demonstrate that only 1% of the tritium label is in the α position in the phenylalanine used for the subsequent studies.

2-Phenyl-4-benzyl-oxazol-5-one
(Benzoylphenylalanine Azlactone)

Racemic benzoylphenylalanine,¹ 30 g, (0.11 mol), was dissolved in a mixture of 30 ml dioxane and 200 ml acetic anhydride and the solution was heated at 100° for 30 min. After standing an additional hour at room temp. the solution was evaporated at reduced pressure and the residue was evaporated with two 50 ml portions of toluene to remove excess acetic anhydride. The residue was taken up in hot hexane, filtered and chilled to give 24 g fine needles (86%), mp 70-71°, Lit.² 70-72°.

Benzoyl-DL-phenylalanine, α -Deuterated

A solution of acetic acid-O-D was prepared by refluxing 92 g acetic anhydride (0.9 mol) with 15.5 g deuterium oxide (0.775 mol) for 30 min. After cooling to room temp. the solution (100 ml) was divided into 4 portions each containing ca 0.35 moles exchangeable deuterium.

(2) E. Mohr and F. Stroschein, Ber., 42, 2521 (1909).

The recrystallized azlactone, 20 g (0.08 moles), was combined with a portion of the deuteration solution heated under reflux for 15 min, then cooled and evaporated in vacuo. The residue was taken through two additional cycles with fresh deuteration solution and the ultimate golden residue was heated with the final portion of the deuteration mixture. The refluxing solution was cautiously treated with 5 ml deuterium oxide and after 10 min another 5 ml heavy water was added, followed by vigorous refluxing for 30 min. The solvent was evaporated in vacuo and the resulting crystalline solid was washed free of color with cold dry ether to give 18 g (84%) racemic α -deuterated benzoylphenylalanine, mp 184-186°.

Acid Hydrolysis of α -Deuterated Benzoylphenylalanine

Eighteen grams of α -deuterated benzoylphenylalanine was dissolved in $5\frac{1}{2}$ liters of hot 3 N HCl and the solution kept at 100° for 14 hrs. Concentration at reduced pressure to a volume of 2 liters followed by chilling to 0° gave a precipitate of benzoic acid, mp 120-121°, which was filtered off and the filtrate was taken to dryness at reduced pressure. Excess HCl was removed by two further evaporations with 250 ml portions of water and the resulting yellow powder was taken up in 100 ml each ether and water. The aqueous phase was washed twice with 100 ml portions of ether then treated with 18 g freshly distilled aniline to bring the pH to 4.5-5. The curdy precipitate was dissolved by heating

as 80 ml absolute ethanol was added, the hot solution filtered, and the filtrate was diluted with 200 ml hot absolute ethanol. The large plates of α -deuterated racemic phenylalanine were collected after overnight chilling and washed with ethanol and ether, 5.8 g. A second crop of 1.7 was obtained from the mother liquors: total yield 7.5 g, 68%. A small sample was converted to the N-trifluoroacetyl derivative³ and the mass spectrum determined. The latter indicated 94% α -deuteration when compared with the spectrum of a sample of unlabeled N-trifluoroacetyl phenylalanine.*

(-) α Phenylethylammonium Salt of Benzyloxycarbonyl
glycyl-L-phenylalanine

The salt was prepared by combining equimolar amounts of the acid and base in absolute ethanol. An analytical sample from ethanol had mp 181-183°, with $[\alpha]_D^{23} + 18.6^\circ$ (c=0.5, MeOH). C, 67.79; H, 6.56; N, 8.72. $C_{27}H_{31}N_3O_5$ requires C, 67.91; H, 6.54; N, 8.80.

To a solution of 121 mg (1 mM) (-) α -phenylethylamine in 8 ml hot ethanol was added 357 mg ZGly-DL-Phe, (1 μ mol), asymmetrically

(3) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley and Sons, Inc., New York, N.Y., 1961, p. 914.

* Technical assistance from Mr. James Althaus (M.I.T.) is gratefully acknowledged.

labeled (D enantiomer only) with sp. act. 605 dpm/mg. The solution was chilled, seeded with the L(-) salt and kept in the freezer overnight with occasional swirling. The solid was collected and washed with water and ether to give 200 mg, mp 179-181^o, activity 83.2 dpm/mg, indicating a composition of 91% L- and 9% D salts. The remaining 175 mg salt was recrystallized from ethanol (4 ml) to give 105 mg mp 180.5-182.5^o, with specific activity 22.5 d/mg indicating 98% L and 2% D salts.

Resolution of Benzyloxycarbonyl[1-¹⁴C]glycyl-L-
[2-D]phenylalanine

A solution prepared by dissolving 9.7 g (27.2 mmol) of the doubly-labeled racemic acid in 250 ml hot ethanol containing 3.3 g (27.2 mmol), (-) α -phenylethylamine was seeded with the L(-) salt and chilled with occasional swirling for 20 hrs. The precipitate, 5.5 g, mp 175-180^o, was recrystallized from ethanol to give 4.6 g (70%), mp 180-181.5^o, $[\alpha]_D^{23} + 18.5^{\circ}$ (C=0.5, MeOH). The salt was added to a rapidly stirred suspension of 100 ml each ethyl acetate and 3 N HCl, the layers were separated and the aqueous phase extracted twice with 50 ml portions of ethyl acetate. The combined organic phases were washed with water and brine, then dried and evaporated. The residue was taken up in 50 ml hot ethyl acetate, seeded with racemate and chilled at 3^o for 2 hrs to give 430 mg, mp 140-145^o. Concentration of the filtrate to

15 ml and chilling gave, after recrystallization 2.53 g, mp 130-131.5^o,
[α]_D²³ + 38.6 (c=2.5, ethanol) Lit. +39.2, sp. act. 20,750 dpm/mg;
3.37 μ Ci/mmol.

Isotropically Pure Benzyloxycarbonylglycyl-L-
phenylalanine, Trebly Labeled⁴

The resolved acid, benzyloxycarbonyl[1,¹⁴C]glycyl-L-[2-D]phenyl-
alanine, 2.31 g, sp. act. 3.37 μ Ci/mmol, was combined with 4.82 g
benzyloxycarbonylglycyl-L-[3-³H]phenylalanine, sp. act. 1.55 μ Ci/mm,
and 1.426 g of the corresponding unlabeled racemic acid in 250 ml hot
acetonitrile. The solution was chilled to 3^o and stirred rapidly as
a drop of freshly precipitated slurry of racemate was added. After
100 min at 3^o the suspension was filtered and 1.53 g racemate dissolved
in the filtrate. The solution was seeded as described and two hrs
later the racemate was collected, and the process repeated with a
further 1.426 g racemate. The ultimate filtrate was concentrated at
reduced pressure and the solids recrystallized twice from ethyl acetate
to give the trebly labeled acid, mp 129-131^o, 5.4 g, sp. act. 9,360
dpm/mg ³H/¹⁴C = 0.895, α H/ α D = 3.2.

(4) D. S. Kemp, S. W. Wang, G. Busby, III, and G. Hugel, J. Am. Chem. Soc., 92, 1052 (1970).

In order to define limits of isotopic purity for this substance, the recovered racemate from the first dilution was recrystallized (acetonitrile) to mp 161-163° and 160 mg combined with 800 mg unlabeled L acid in 28 ml acetonitrile. Recovery and purification of both substances by the usual procedures gave a sample of L acid, mp 129-131°, sp. act. ³H 494; ¹⁴C 624 dpm/mg.

The racemate, 112 mg, was combined with 566 mg L acid and the racemate recovered and recrystallized, mp 160-162°

	³ H	¹⁴ C
activity DL	30.3	137
(Calc) L	45	57
activity of D	15.6	217

Consequently, the resolved substance contained 0.66% racemate while the initial tritiated substance contained 0.046% racemate. Assuming a 90% recovery of racemate at each dilution and a purity of 99% for the final L acid, the substance can be calculated to have less than 0.00001% of its tritium activity and less than 0.0001% of its ¹⁴C activity in the D enantiomer.

Anderson Test Using the Mixed Anhydride Procedure

The trebly labeled acid, 393 mg (1.1 mmol) was added to a solution of N-methylmorpholine, 111 mg (1.1 mmol), in 5 ml dry tetrahydrofuran.

The acid was dissolved at room temperature and the resulting solution allowed to equilibrate with a bath at -14° (sodium nitrate and ice-water in a small Dewar flask) for 15 min. The solution was stirred rapidly as 0.15 ml (1.13 mmol) isobutylchloroformate was added, followed 30 sec later by 0.11 ml ethyl glycinate, (1.1 mmol). After 1 min the bath was removed and the reaction mixture was allowed to stir at ambient temperature for 15 min. The reaction was quenched by the addition of 40 ml ethyl acetate and 10 ml 1 N HCl, the layers were separated and the organic phase was washed with saturated bicarbonate and salt solutions, then dried and evaporated. The residue was combined with 453 mg unlabeled L enantiomer and 95.3 mg unlabeled racemate in 30 ml ethanol, cooled to 15° and seeded with a freshly precipitated slurry of racemate. Frequent swirling during the first 10 min followed by occasional swirling gave a precipitate of racemate, 86.3 mg, mp $132-133^{\circ}$ within 1 hr. The racemate was collected and subjected to the dilution sequence below while the L enantiomer was recovered from the filtrate, recrystallized and its activity determined.

Dilution Scheme for Mixed Anhydride Product

Recovered racemate	Added L	Added DL	Activity L		mp(DL)	Yield of L 377 mg
			³ H	¹⁴ C dpm/mg		
1. Reaction product	453 mg	95.3	1,520	1,760	132-133°	αH = 285 mg αD = 92 mg
2. 86.3 mg	766.2				131-133°	
3. 69.8	696.7				130.5-132°	
4. 57.9	474.5				131.5-133°	
			0.23	0.26		
		(calc)	0.25	0.29		
activity of racemate			0.97	0.84		
activity of D			1.71	1.42		
yield of D (mg)			0.017 mg	α Hydrogen derived		
			0.004 mg	α Deuterium derived		
racemization: αH			0.0125%	αD 0.0087%		isotope effect = 1.45

Anderson Tests with 3-Benzylloxycarbonylglycyl-L-phenylalanyloxy-2-hydroxy-N-ethylbenzamide

- A. A solution of the trebly-labeled active ester⁴ 519 mg, (1.0 mmol) in 5 ml dry dimethylformamide was treated with 110 mg ethyl glycinate (1.1 mmol) and allowed to stand 4 hrs at 23°.
- B. As in A but at 0-3° for 20 hrs.

After the allotted times the reaction solutions were partitioned between 50 ml each 1 N HCl and ethyl acetate, the organic phases were washed with water, twice with 1 N NaOH, water, then evaporated at reduced pressure. The residues were combined with unlabeled racemic and L Anderson tripeptides and the dilution sequence performed as previously described.

	Yield	% rac		Isotope effect*
		αH	αD	
A	85%	0.06	0.04	1.3
B	90%	0.014	0.013	1.1

* For small (< 1%) racemization levels the isotope effect is merely

$$\frac{({}^3\text{H}/{}^{14}\text{C})_{\text{sm}}}{({}^3\text{H}/{}^{14}\text{C})_{\text{prod}}}$$

Preparation of Optically Active 2-(Carbobenzoxamidomethyl)-
4-Benzyl-oxazol-5-one (The Anderson Azlactone)

A solution of 1 mmol triethylamine and 1.2 mmol finely powdered N-ethyl-5-phenylisoxazolium-3'-sulfonate (Woodward's Reagent K) in 10 ml acetonitrile was stirred at ice-bath temperature for 20 min, then at room temp. until almost all the solid dissolved in the pale yellow solution (ca 15 min).

All subsequent operations were performed in a cold room at +3⁰ with pre-equilibrated reagents and glassware.

The reaction was returned to the ice-bath and a supersaturated solution of benzyloxycarbonylglycyl-L-phenylalanine, 0.95 mmol, in cold acetonitrile was added all at once and stirring continued for 15 min. The reaction solution was partitioned between ether (50 ml) and water (30 ml), the layers were separated and the organic phase was washed twice with water, once with sat. bicarbonate then dried over MgSO₄. The resulting solution was used immediately after preparation. (Yield: ca 15%).

GENERAL PROPERTIES:

I. Infrared Spectrum

The infrared spectrum of the freshly prepared oxazolone solution was essentially superimposable on that of the oxazolone described by Kemp.*

II. Optical Activity

A portion of the oxazolone solution (5 ml) was placed in a 1 dm polarimeter cell giving $\alpha_D^{23} -0.256^\circ$. Treatment of the polarimetry solution with 50 mg triethylamine gave a solution with $\alpha_D^{23} 0.001^\circ$ within 1 min after mixing.

III. Optical Purity⁴

A portion of the ethereal solution prepared from trebly-labeled starting material (20 ml) was combined at 3^o with a quenching solution⁵ composed of 1 ml hydrazine hydrate in 5 ml each dry methanol and tetrahydrofuran and allowed to stand for 1 hr at 3^o. After evaporation the oily residue was taken up in 25 ml absolute ethanol and 1.01 g unlabeled L-hydrazide was added. After heating to effect complete solution, 125 ml cold absolute ethanol was added followed by a solution of 302 mg unlabeled racemic hydrazide in 20 ml hot ethanol. When the

(5) M. Goodman and C. Glaser in "Peptides: Chemistry and Biology," B. Weinstein and S. Lande, Eds., M. Dekker, New York, 1970, p. 307.

* D. S. Kemp, Ph.D. Thesis, Harvard, 1964.

solution had equilibrated to room temperature, a small drop of an ethanolic slurry of freshly precipitated racemate was added and the mixture rapidly stirred at ambient temperature for 12 hrs. The solid was collected and recrystallized from ethanol to give racemate A, 300 mg, mp 167-170°, while the filtrate was concentrated and the resulting solids recrystallized from ethanol to give the L-enantiomer mp 140-142° after shrinking at 105°, sp. act. ³H 98.7 dpm/mg; ¹⁴C 107.5 dpm/mg. Yield of α-Hydrogen derived 20.9 mg; and 6.44 mg α-Deuterium derived L hydrazides.

The racemate A, 214 mg was combined with 721 mg unlabeled L-hydrazide in the manner described above. The resulting solid was sacrificially recrystallized to mp 167-170°, sp. act. ³H 21 dpm/mg, ¹⁴C 14.4 dpm/mg. Since the calculated activity of the L-enantiomer is ³H 12.75 dpm/mg and ¹⁴C 13.9 dpm/mg, the activity of the D-enantiomer is 29.2 dpm/mg ³H and 14.8 dpm/mg ¹⁴C. Yield: α-Hydrogen derived D-enantiomer 0.794 mg or 7.3% racemization; α-Deuterium derived D-enantiomer 0.113 mg or 3.5% racemization.

In another determination of optical purity according to the sequence described above but using a larger volume of quenching solution, the racemate derived from the α-Hydrogen species was less than 2%.

The Isotope Effect for Racemization During
Peptide Coupling via the Oxazolone

A. Ether

The freshly prepared oxazoline solution (estimated concentration 0.06 mmol/30 ml) was treated at 3° with 1 mmol ethyl glycinate (15-fold excess) and allowed to stand in the cold for 12 hrs. The residue after evaporation was taken up in ethyl acetate, washed with diluted HCl, water, sat. bicarbonate then dried and evaporated. The residue was quantitatively dissolved in 5 ml absolute ethanol and placed in a 1 dm polarimeter cell giving $\alpha_D^{23} +0.025^\circ$ indicating ca 10 mg excess L Anderson tripeptide. The polarimetry solution was taken through the Anderson test to give 54% racemized αH species and 24% racemized αD species.

B. Dimethylformamide

The freshly prepared ethereal oxazolone solution, 50 ml, (ca 0.003 M in oxazolone) was concentrated at 0° to one-tenth of its original volume and divided into three equal portions, one of which was combined with each of the following solutions at 2°.

- a. 5 ml DMF containing 100 mg freshly distilled ethyl glycinate.
- b. 5 ml DMF containing 60 mg triethylamine.
- c. 5 ml ether containing 60 mg triethylamine.

After 15 min at 3^o, 100 mg ethyl glycinate was added to solutions b and c and all three solutions were allowed to stand in the cold for 12 hrs. The reaction mixtures were partitioned between 50 ml ethyl acetate and 20 ml 1 N HCl, and the organic phases were washed with water and sat. NaHCO₃, dried and evaporated. The residues were combined with unlabeled L and racemic Anderson tripeptides and taken through the usual dilutions.

	% _{rac}		Isotope Effect
	α H	α D	
A(DMF)	73(87)*	34(42)	3.8(7)
B(DMF-Et ₂ N)	87(100)	87(100)	1
C(Ether-Et ₃ N)	87(100)	87(100)	1

* The 13% excess L peptide must be due to the W.R.K. ester that survives the aqueous extractions. The figures in parentheses are adjusted to represent the results expected for the pure L-oxazolone.

Preparation of Trebly-Labeled Benzyloxycarbonylglycyl-
L-phenylalanylhydrazide

A solution of the trebly-labeled acid, 1.11 g (3.1 mmol), was dissolved in a minimum volume of methanol at room temp. and titrated to a persistent yellow end point with cold ethereal diazomethane. The solvent was evaporated and the residue was taken up in 5 ml ethanol, 1 ml hydrazine hydrate added, and the solution heated at 60° for 30 min. After standing an additional 3 hrs at room temp. the solution was seeded and the solid was recrystallized from 30 ml ethanol to give the hydrazide, mp 142-144° after softening at 105°, 1.07 g (93%) Lit.* mp 140-142°, Sh 107°. Unlabeled and racemic hydrazides of ZGly-Phe were prepared by this procedure in comparable yields.

Preparation of Trebly-Labeled Benzyloxycarbonylglycyl-
L-phenylalanylazide

All procedures were performed in a cold room at +3° with pre-equilibrated reagents and glassware.

The trebly-labeled hydrazide, 970 mg (2.6 mmol), was suspended in 6 ml water and treated with 1.5 ml each conc. HCl and glacial acetic

* Excessive heating during recrystallization gives less satisfactory results. D. S. Kemp, private communication.

acid. The resulting smooth cream was placed in an ice-acetone bath at -10° , overlayers with 15 ml ether and stirred rapidly as a solution of 500 mg sodium nitrite in a minimum volume of water was added dropwise. After an additional 10 min stirring at -10° the layers were separated and the aqueous phase extracted with two 25 ml portions of ether. The combined ethereal phases were washed cautiously but persistently with 4 x 15 ml portions of sat. bicarbonate solution (to bring the pH of the last wash up to ~ 8) then dried over anhydrous magnesium sulfate, and used immediately afterward. Yield of acylazide about 60% (vidi infra).

The Isotope Effects for Racemization

via the Acyl Azides

Ether

1. A portion of the cold ethereal azide solution, 60 ml (containing ca 1.2 mmol acyl azide) was treated with 0.3 ml (3 mmol) ethyl glycinate and the immediately precipitated oil was allowed to stand for 24 hrs at 3° (crystals appear).

2. A portion of the cold ethereal solution, 30 ml (containing ca 0.6 mm acyl azide) was treated with 100 mg (1.0 mmol) triethylamine and after 15 min, ethyl glycinate, 0.15 ml (1.5 mmol), was added to the clear solution. The precipitated oil was allowed to stand as in 1.

Dimethylformamide

The freshly prepared ethereal azide solution (containing ca 2 mmol acyl azide) was combined with 10 ml dry DMF and the ether was stripped off at 0°. The resulting solution was divided into two portions (6 ml each).

3. One portion was treated with 150 mg (1.5 mmol) triethylamine (immediate yellowing) and, after 15 min, ethyl glycinate, 0.16 ml, was added.

4. The other portion was treated directly with 0.16 ml ethyl glycinate. Both solutions were allowed to stand at 3° for 24 hrs.

Workup (Room Temp.)

Ether

The solvent was evaporated at reduced pressure and the resulting solids were completely dissolved in warm ethyl acetate, washed with 1 N HCl, water, sat. bicarbonate, dried over magnesium sulfate and evaporated.

Dimethyl Formamide

The reaction solutions were partitioned between 20 ml each ethyl acetate and water and the organic phases worked up as above.

	α_H	$\%_{\text{rac}}$	α_D	Isotope Effect
1. Ether	0.03		0.011	2.6
2. Ether-Et ₃ N	1.6		0.54	2.9
3. DMF-Et ₃ N	50.3		43	1.3
4. DMF	0.48		0.22	2.1

CHAPTER III

Mindig szeretek egy kis extrát csinálni.

J. Rebek, Sr.

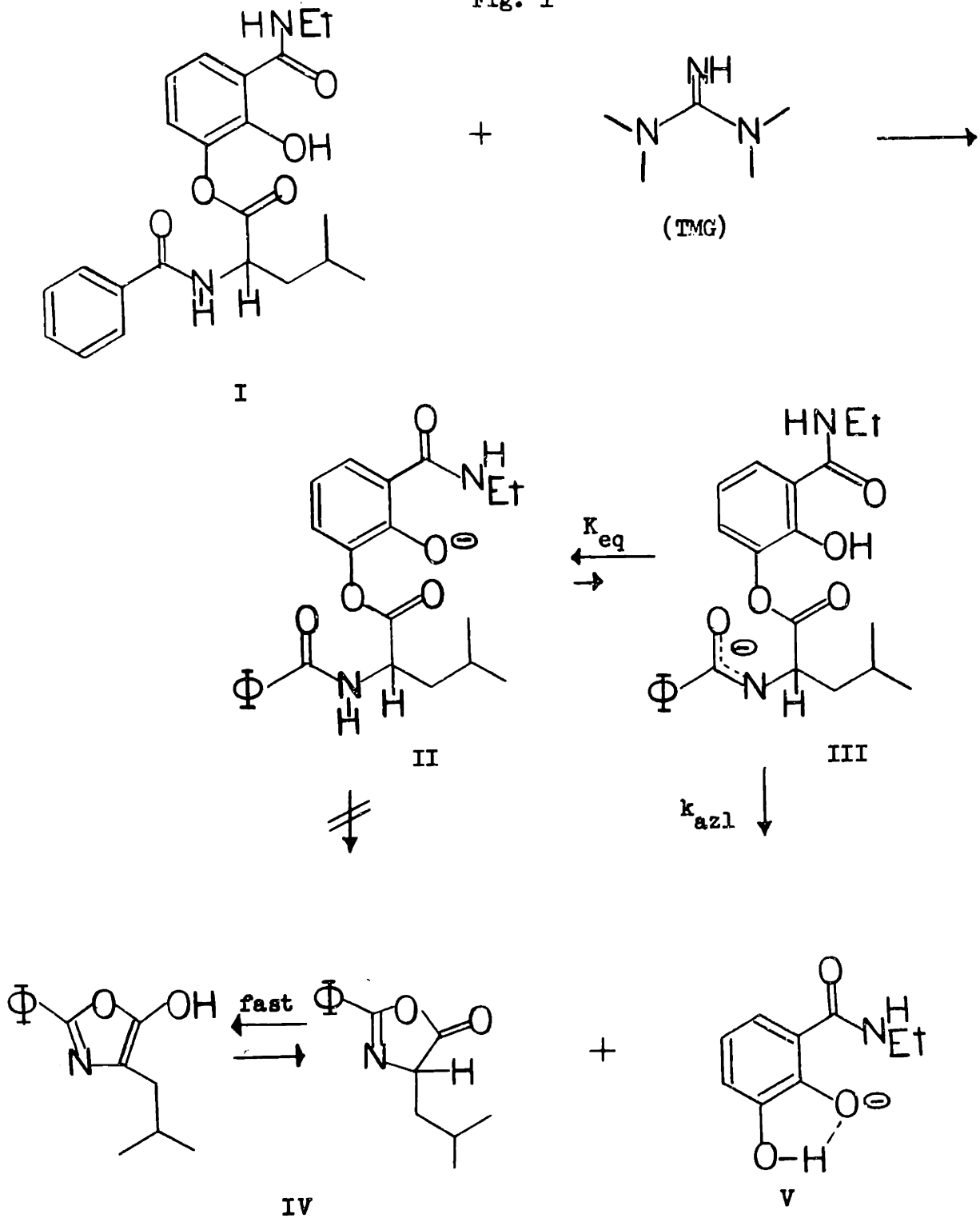
The isoxazole-derived catechol monoesters occupy a unique position in peptide chemistry; they represent the only peptide reagents that owe their existence to rational design on two levels. The synthetic design of these reagents has been described in Chapter 1. The present chapter is devoted to their design as a natural consequence of the acid-base chemistry of racemization processes.

The ester I, derived from benzoyl-L-leucine, is completely deprotonated by tetramethylguanidine (TMG) in polar aprotic solvents (pK_a phenol ~ 8 ,¹ pK_a TMGH[⊕] ~ 13). The resulting anion II must be in equilibrium with the isomeric anion III, with a pH-independent equilibrium constant defined by the relative acidities of the phenol and amide, which in water may be estimated to be 10^{-6} III/II. While II is not expected to collapse readily into an azlactone by virtue of its poor leaving group, the isomeric III is the direct precursor to azlactones in specific base-catalyzed processes² and should be unstable to azlactone formation. The fragile grasp that azlactones hold on optical integrity in basic media insures that k_{azl} is the slowest step in the racemization of such a system. The rate of racemization is thus given by (1) for the reactions of Fig. 1.

(1) S. W. Chien, Ph.D. Thesis, M.I.T., 1967.

(2) D. S. Kemp and S. W. Chien, J. Am. Chem. Soc., 89, 2745 (1967).

Fig. 1



$$\frac{-d[\alpha]}{dt} = k_{azl} [III] = K_{eq} \cdot k_{azl} [II] = K_{eq} k_{azl} [TMG] \quad (1)$$

when $[TMG] \leq [I]$

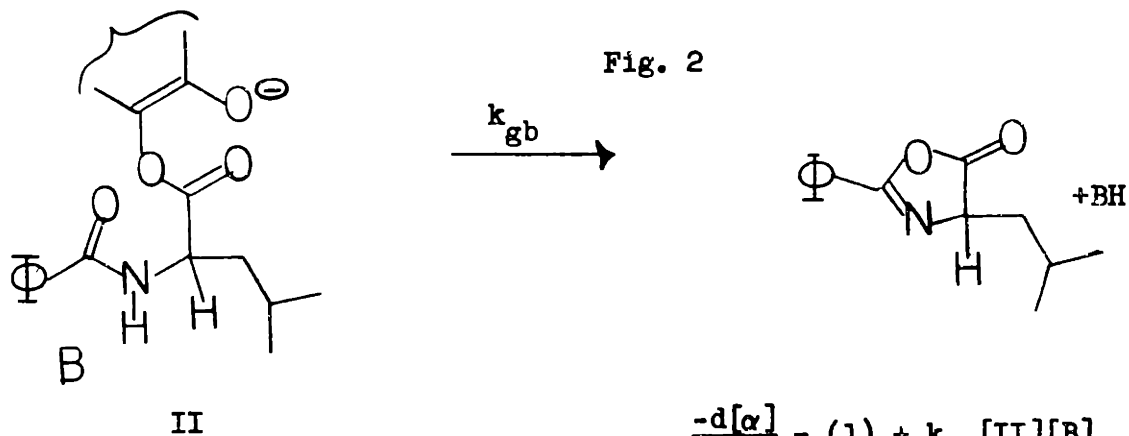
The introduction of excess base has some surprising consequences in this system. If II can be racemized by general base-catalyzed processes (Fig. 2), an increase in racemization rate should be observed, (2) or (3) and this system becomes excruciatingly uninteresting when compared to other active esters. (The internal racemization to give VI is independent of excess base and offers the only dubious distinction)

The rational design factor rises from the assumption that general base-catalyzed reactions are not major contributors to the racemization process, leaving the system internally buffered against the specific base-catalyzed process! The increase in pH (required to dissolve amino acid nucleophiles in aprotic media during peptide coupling) will not affect the racemization rate of I until the pK_a of the dianion IV is reached (Fig. 3). The racemization rate should begin to increase only around pH 18 according to Eq. (4).

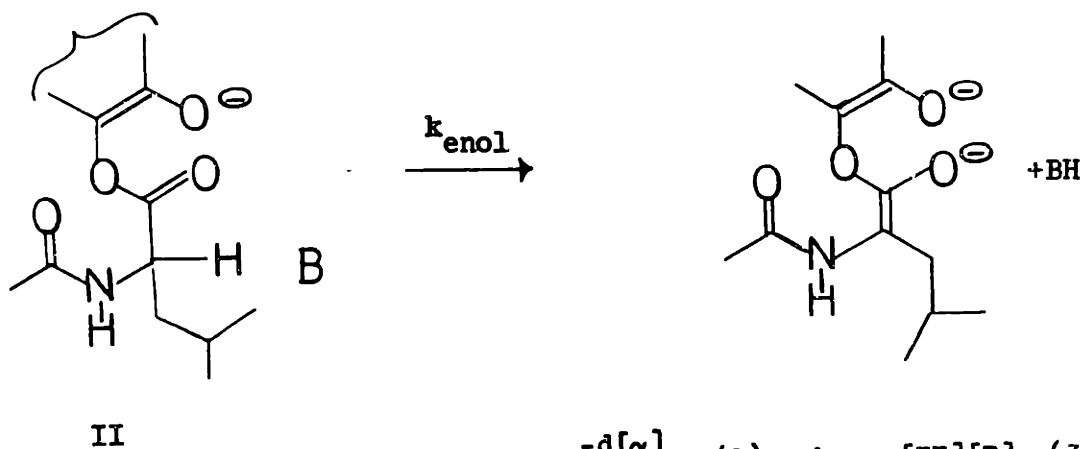
The species dominating this system as a function of pH is approximated in Fig. 4.

Conventional active esters must show increasing racemization rates in the pH range 10-16 due to increased concentration of the amide anions

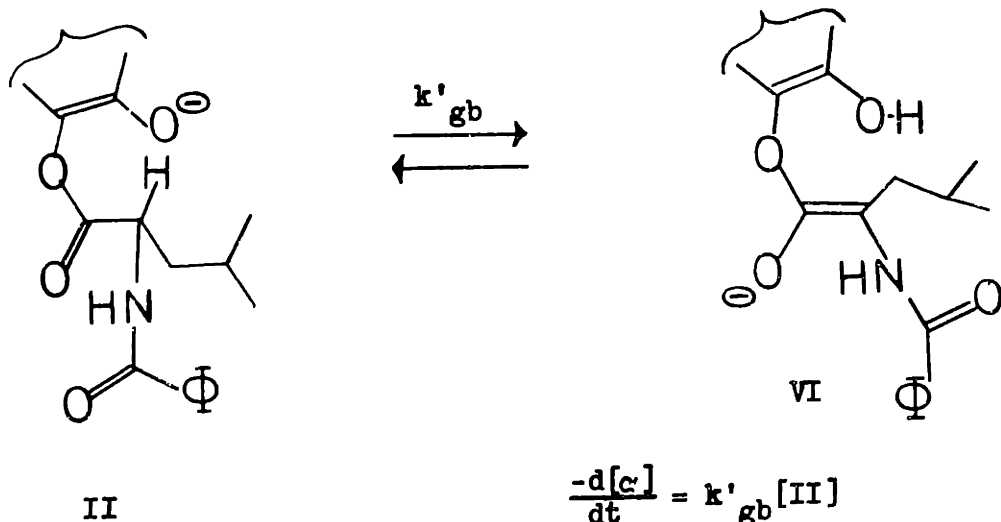
Fig. 2



$$\frac{-d[\alpha]}{dt} = (1) + k_{gb}[\text{II}][\text{B}] \quad (2)$$



$$\frac{-d[\alpha]}{dt} = (1) + k_{enol}[\text{II}][\text{B}] \quad (3)$$



$$\frac{-d[\alpha]}{dt} = k'_{gb}[\text{II}] \quad (4)$$

Fig. 3

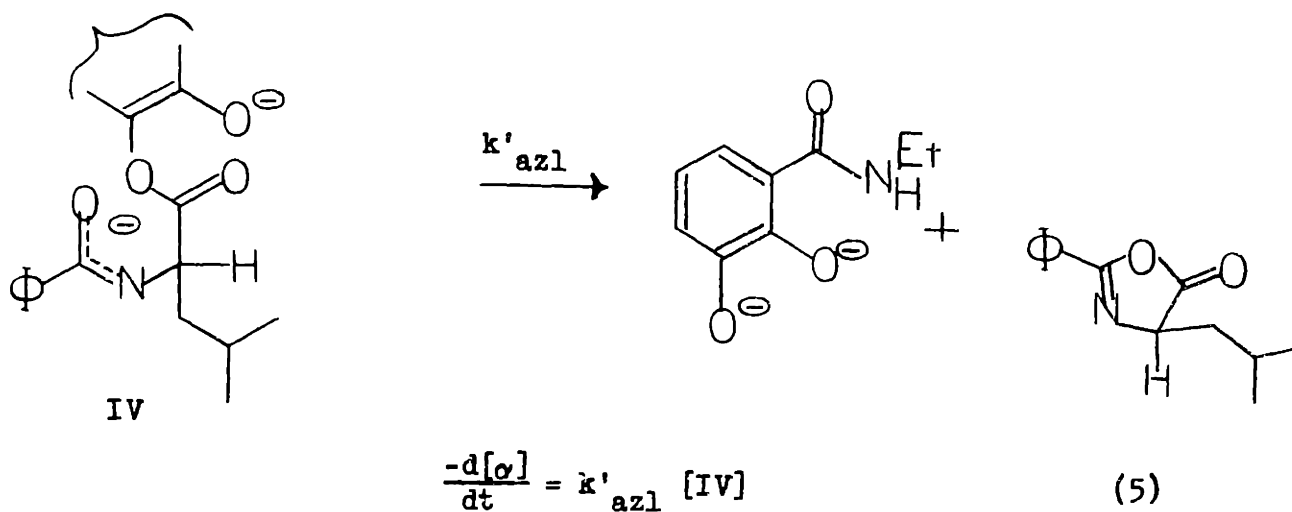
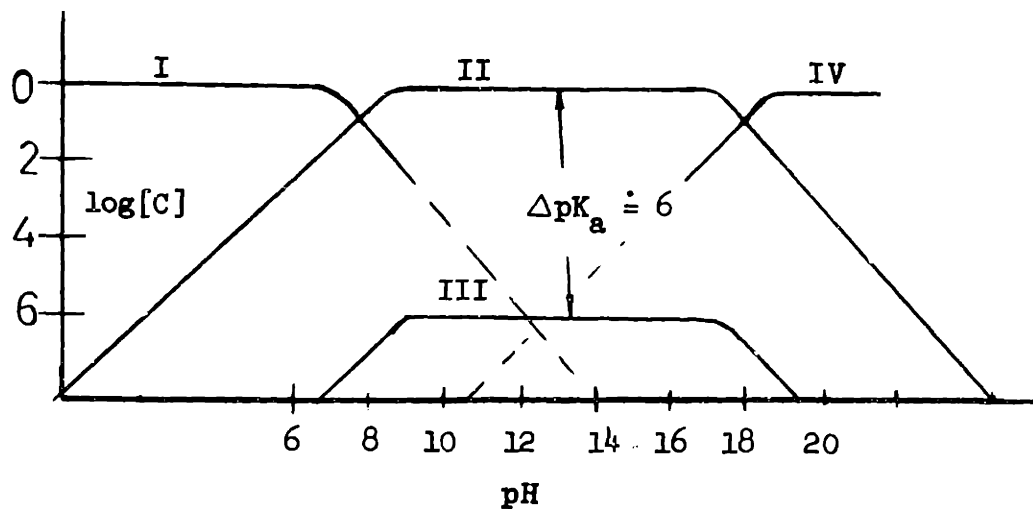


Fig. 4

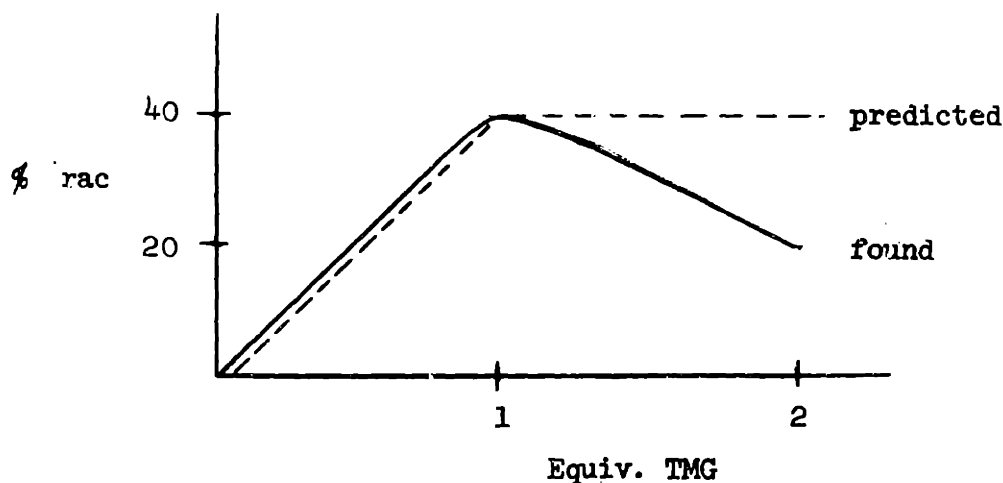


For the sake of familiarity, the figures are those expected in aqueous medium (pH). The actual values for DMF or DMSO can be approximated by shifting the graph about 4 units to the right on the scale above (i.e., pK_I in DMF ~ 12). C. Ritchie and G. Megerle, *J. Am. Chem. Soc.*, **89**, 1447 (1967).

responsible for specific base-catalyzed azlactone formation. Experimental verification of the unique behavior of the catechol esters in this pH range was obtained by Edith Chien.³

The isoxazole-derived ester of benzoyl-L-leucine (I) was treated with 0.2 eg. TMG for 1 hr to initiate the racemization process. Ethyl glycinate was then added and the resulting mixture of Young peptides was analyzed for racemic content using the isotopic dilution assay. The results of these analyses for coupling reactions in various aprotic solvents is given in Fig. 5.

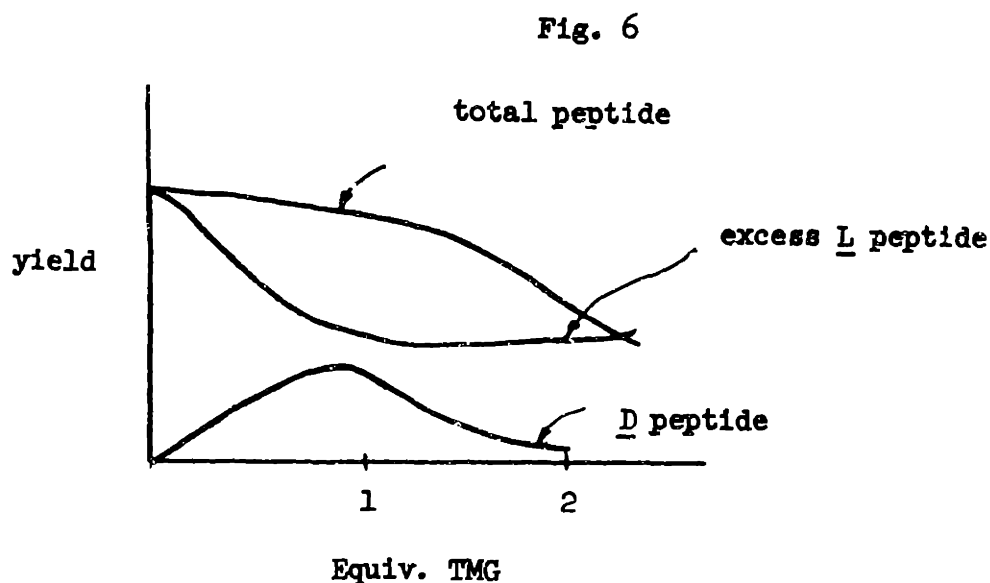
Fig. 5



(3) D. S. Kemp and Edith Chien, unpublished observations.

In accord with theory, the rate of racemization for less than 1 eq. TMG is directly proportional to the base concentration and the addition of excess base does not increase racemic content. The downward curve in the region 1 to 2 eq. TMG suggested that some process was reducing the yield of racemic product (or specifically D product) at a somewhat faster rate than the destruction of L product. Closer examination of Chien's data was in complete agreement with this notion.

Fig. 6.



The yield of excess L* peptide remained constant when more than 1 equivalent of TMG was used, but the yield of D peptide (and con-

* It is important to remember that the quantities actually measured in this isotopic assay are total yields of L peptide and D peptide, excess L refers to the difference in these two quantities.

sequently total peptide) steadily decreased as more base was present.

These facts offer a new argument for azlactone-catalyzed racemization, an argument that is independent of the azlactone proof by the isotope effect of Chapter 1. There can be no reasonable process that destroys D active ester or D peptide faster than it destroys excess L active ester or excess L peptide. Consequently this apparently selective destruction of D peptide must be merely a result of the destruction of potentially D peptide.

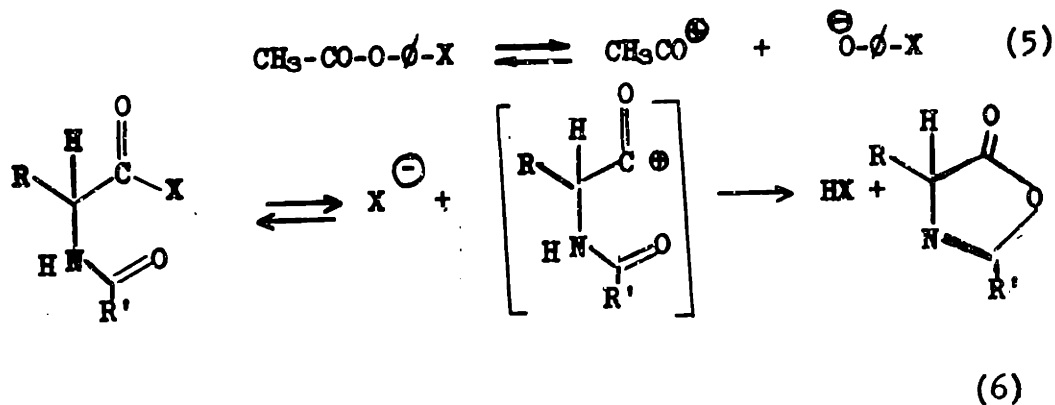
This potential D peptide must be of a different structural type than active ester, i.e., a true intermediate such as an azlactone. (The internal enolization product (VI), cannot be such an intermediate: if its destruction were faster than C-protonation, a decrease in yield should result whenever any TMG is present.)

The intriguing notion of suppressing racemization by selective destruction of potentially racemic product offers a new solution to the racemization problem and merits further investigation in this "age of desperation" in peptide synthesis. The present example, although a synthetically unattractive case, provides a system from which this notion might be expanded if the forces that cause this effect could be measured with some precision. Our goals, then, were to

- (a) Provide experimental proof for both the sufficiency and necessity of the presumed azlactone intermediate, hopefully by developing techniques that could also be used in other cases.

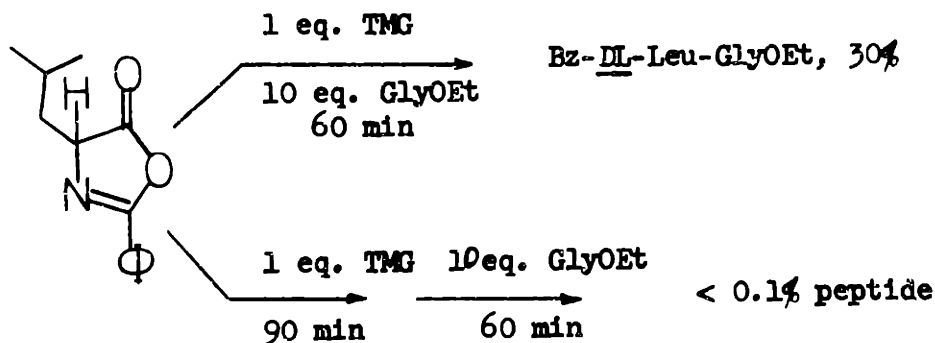
- (b) Confirm the unique acid-base chemistry of these catechol esters.
- (c) Define the stability of these esters in terms of the equilibrium between the ester and azlactone, since this value must reflect the inherent "racemizability" of the active ester.

Of special relevance to this last objective is the study by Jencks⁴ in which the equilibrium was measured for reaction (5). The extraordinary sensitivity of this equilibrium to the nature of X may have important consequences in the analogous process for peptide reagents (6).



The evidence in Chapter 1 has demonstrated the existence of azlactones in the racemization of catechol monoesters. The large amounts of racemization observed in the present case suggested that the azlactone concentration might be high enough to permit actual isolation of this intermediate. Experiments designed to do so were unsuccessful (a failure that could subsequently be attributed to the

short lifetime of the azlactone in this system). Isotopic dilution proved to be an excellent method of determining small concentrations of azlactones, and with this technique the destruction of the azlactone in basic media was easily assessed. Only 3% of the initial radioactivity due to the Young azlactone survived a 90-min treatment with an equiv. TMG in DMSO. Although the final products of azlactone destruction were not determined,* it was necessary to establish that the altered azlactone was not capable of producing racemic Young peptide upon reaction with ethyl glycinate. Again, isotopic dilution readily confirmed this:



* Self-acylation leading to polymeric material is the most probable path. The disappearance of azlactone absorption in the I.R. when this azlactone is treated with tertiary amines has been noted: M. W. Williams and G. T. Young, J. Chem. Soc., 3701 (1964).

(4) M. Gerstein and W. P. Jencks, J. Am. Chem. Soc., 86, 4655 (1964).

To prove the sufficiency of the azlactone intermediate for explaining the downward trend in Chien's racemization curve, the loss of potentially excess L peptide must proceed at a constant rate in the region of excess base. Polarimetric assessment of the racemization of the anion II (the only reasonable source of excess L peptide and optical activity) showed the necessary insensitivity of racemization rate to excess base. (Table 1). The constant, first-order rate of

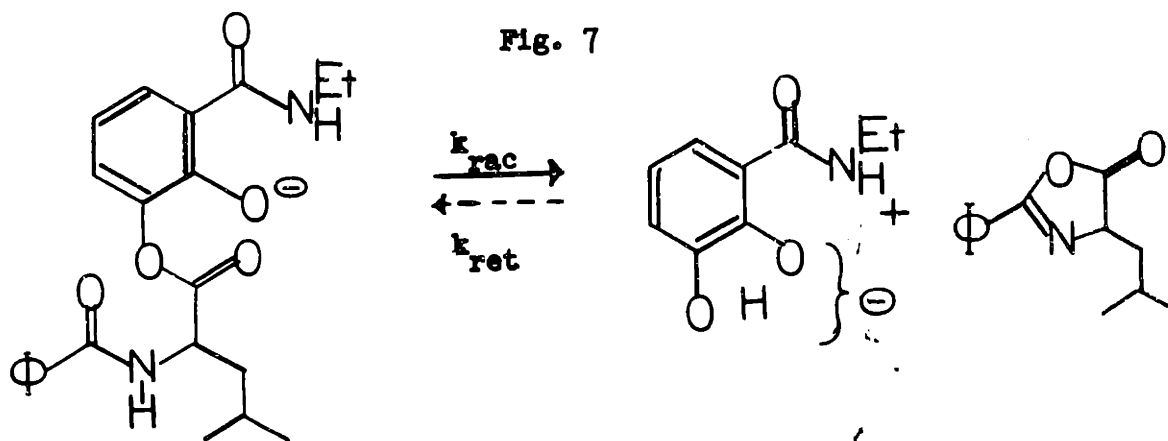
TABLE 1

Base Catalyzed Racemization of I; DMSO, 23°

<u>Unit conc.(M)</u>		<u>$k_{rac} \times 10^3$</u>	<u>Optical half-life</u>
Ester	TMG		(min)
0.058	0.058	8.4/min	82
0.039	0.058	8.2	85
0.2	0.2	7.8	90
0.4	0.4	8.2	85

racemization also confirms the plateau in the pH profile of Fig. 4, consequently the general-base catalyzed mechanisms of Fig. 2 cannot be significant racemization-contributing processes. The internal enolization process, while consistent with the polarimetric results, has already been discredited.

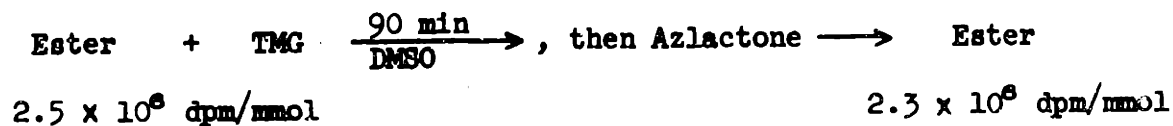
The demonstrated instability of the azlactone to TMG placed unfortunate obstacles in the path of reaching the third goal, i.e., the stability measure of the active ester anion in terms of azlactone partitioning. Fig. 7. Ideally the K_{eq} should be determined by



measuring both the forward and backward reaction velocities, but the known instability of the catechol moiety in base,¹ as well as the destruction of the azlactone made such a direct approach impractical. In fact, no available evidence pointed to the existence of the return reaction. The feasibility of the return reaction was established by an isotopic dilution experiment, but one which differed from those previously described. In the earlier experiments the instantaneous concentrations of radioactive substances were determined by appropriate

quenches. In the experiments which follow, the design provided for partitioning of radiolabeled species before the system was quenched. In a sense the two types of experiments are differential and integral respectively. The following experiment is of the latter type:

The active ester, with known specific activity (labeled in the amino acid moiety) was allowed to equilibrate with TMG (1 eq) in DMSO for 90 min. Unlabeled azlactone was then added in large excess and, after a few minutes equilibration time, the active ester was recovered by an appropriate procedure. The specific activity of the recovered ester was only 90% of that of the starting material, demonstrating that a process was generating active ester from unlabeled azlactone.



This demonstrates the feasibility of the return reaction but does not require its existence under reaction conditions.

Further labeling experiments of the integrative type were designed to trace the components, active ester and azlactone through their meanderings in this complex system with more certainty. Such experiments were feasible only if the azlactone and active ester carried different radiolabels, according to the scheme in Fig. 8. The ^{14}C labeled L-ester, TMG, and ^3H labeled azlactone were allowed to equili-

(1) Product Distribution^a after 90 Min in DMSO

L-Ester	Azlactone
58% ¹⁴ C	14% ¹⁴ C
13% ³ H	18% ³ H

(2)

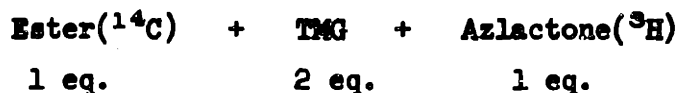
	¹⁴ C	¹⁴ C
L-Ester		D-Ester ^b
55% ¹⁴ C		18% ¹⁴ C
11% ³ H		11% ³ H

(a) The figures represent the radioactivity recovered; 58% of the original ¹⁴C activity was retained in the L-active ester while 13% of the original ³H activity of the azlactone was converted to L-active ester.

(b) These figures were determined by diluting with racemic ester and subtracting the known isotopic content due to the L-enantiomer.

The label distribution in the D-ester from experiment (2) is not easily rationalized. If all D-ester comes from the reaction of azlactone with phenolate, more ³H activity should appear in this product than ¹⁴C activity. The observed ratio ³H/¹⁴C < 1 suggests the direct leakage of ¹⁴C activity from L-ester to D-ester, a result expected for an enolization mechanism. The internal racemization of Fig. 2 becomes attractive but the evidence of Chapter 1 argues against such a process. A possible explanation is that the azlactone composition

at this point in Exp. (2) is also $^3\text{H}/^{14}\text{C} < 1$, although it was not determined. A slight excess of TMG causing faster destruction of the ^3H azlactone could be the cause. Excess base (Exp. (3)) indeed caused



(3) Product Distribution after 90 min in DMSO

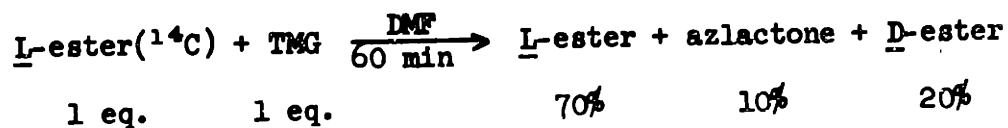
<u>L</u> -Ester	Azlactone
57% ^{14}C	6% ^{14}C
6% ^3H	5% ^3H

(4) Product Distribution after 24 hrs in DMSO

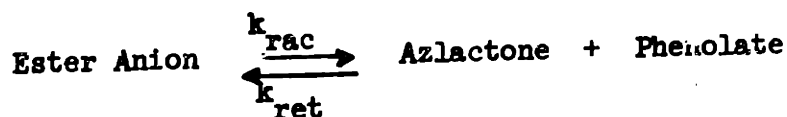
<u>DL</u> -Ester	<u>DL</u> -Benzoyl leucine	Azlactone
22% ^{14}C	29% ^{14}C	< 0.1% ^{14}C
7% ^3H	3.5% ^3H	< 0.1% ^3H

faster destruction of the azlactone, but, in complete accord with the polarimetry results, showed little effect on the rate of loss of L(^{14}C) active ester. These integrative experiments complete the proof of the azlactone intermediate.

Our last objective, the definition of the equilibrium constant, was realized using DMF as the reaction medium. The nature of the equilibrium, in which the azlactone is present only in low concentrations, coupled with the increased stability of the azlactone in this solvent, greatly aided the determination of the return reaction velocity. The instantaneous concentrations of azlactone, D ester and L ester were determined by an isotopic dilution experiment using a single radiotracer.



The complete recovery of radioactivity demonstrates that material balance is maintained. Since the concentration of the D ester is greater than that of the azlactone from which it originates, the system must be at equilibrium (the initial concentrations of both D-ester and azlactone are zero). The equilibrium constant for the reaction



is thus:

$$\frac{[\text{Azlactone}][\text{Phenolate}]}{[\text{Ester Anion}]} = 0.011 \text{ M} = K_{\text{eq}} = k_{\text{rac}}/k_{\text{ret}}$$

The forward reaction velocity can be calculated since the system is at its optical halflife at 60 min.

$$\%_{\text{rac}} = \frac{2 \times [\text{D ester}] + [\text{Azlactone}]}{[\text{Material Balance}]} = 50\%$$

$$k_{\text{rac}} = -0.011/\text{min}$$

The return reaction rate constant is consequently $\sim 1 \text{ M}^{-1} \text{ min}^{-1}$.

The only other activated species for which comparable data exist are the *p*-nitrophenyl esters. Young⁵ reports that the PNP ester-oxazolone ratio is about 2:1 (benzoyl leucine ester with *N*-methylpiperidine as the base). Goodman's data also indicate a 2:1 ratio of ester to oxazolone at equilibrium* for the PNP ester of benzoyl phenylalanine using tributylamine.

(5) M. N. Williams and G. T. Young, J. Chem. Soc., 3701 (1964).

(6) M. Goodman and L. Levine, J. Am. Chem. Soc., 86, 2918 (1964).

* Goodman reports the ester-oxazolone ratio is 17:1 when ten-fold excesses of base and phenol are present.

For mixed anhydrides the equilibrium must lie far on the side of the oxazolone, since successful use of this method is critically dependent on very short activation times (30 sec to 2 min), i.e., before equilibrium can be established. The same conclusions may be drawn for carbodiimide-derived activated species, where the equilibrium is driven to the oxazolone by the essentially irreversible nature of the dehydration. Oxazolone-ester-equilibrium constants for a number of activated species would be very desirable for the rational design of peptide reagents. A study of the enigmatic acyl azides may provide valuable information regarding the inherent "racemizability" of activated species.

The specific destruction of potentially racemic peptide products formally represents a new means of reducing racemization. The azlactones, replete with functional groupings, should be easily trapped by many reagents compatible with peptide synthesis. A few possibilities are listed below.

1. The behavior of azlactones as dipolarophiles⁷ suggests that they might be specifically destroyed by singlet oxygen.

2. The reaction of hydrazoic acid with azlactones to give tetrazole acetic acids⁸ is an especially attractive possibility; the side products could easily be separated by extraction.

3. The high kinetic α -acidity of azlactones could be used to advantage; reaction with some diazonium salts should give readily separable products.

(7) R. Huisgen, H. Gotthardt and H. Bayer, Angew. Chem., 76, 185 (1964).

(8) H. Behringer and W. Grimme, Ber., 92, 2967 (1960).

EXPERIMENTAL

The preparation of benzoylleucine derivatives is described elsewhere in this thesis.

Racemization of 3-(Benzoyl-L-Leucyloxy)-2-Hydroxy

N-Ethylbenzamide by Tetramethylguanidine

All runs were made in a 1 dm water-jacketed polarimeter microcell held at 25° by means of a constant temperature circulating bath. The rotation at the D line of sodium was observed as a function of time. Aliquots of standard solutions of the active ester and TMG in DMSO were combined, made up to volume, then placed in the polarimeter cell. Plots of ln α vs. time gave good straight lines over three half-lives. Intercepts of ln α at zero time were directly proportional to initial concentrations of the active ester, indicating that Beer's law was followed over the range of concentrations used (0.06 to 0.2 M).

The Reaction of Oxazolone with Tetramethylguanidine

A. (Control)

A solution prepared by dissolving 88.4 mg oxazolone (0.4 mmol), sp. act. 2.56×10^6 dpm/mmol (³H), and 55 mg TMG (0.4 mmol), in 2.0 ml DMSO was thoroughly mixed and a 1.0 ml aliquot immediately added to a freshly prepared solution of 1 mmol each glycine ethyl ester

hydrochloride and TMG in 3 g DMSO. After 1 hr 181.7 mg racemic Young peptide was added and the reaction solution was worked up in the usual fashion to give peptide, mp 144-146°, sp. act. 2.36×10^5 dpm/mmol, indicating a 29% yield from the oxazolone.

B.

A solution of oxazolone and TMG in the proportions and solvent above was allowed to stand at room temp. 95 min, then a 0.5 ml aliquot was added to a solution of unlabeled oxazolone, 310 mg, and acetic acid, 0.5 ml, in 20 ml ethyl acetate. After washing with sat. bicarbonate, dil. HCl and brine, the dried organic phase was evaporated and the residue triturated with warm pet. ether. The supernatant was drawn off, filtered and chilled. Recrystallization from a minimum volume of pet. ether gave oxazolone, mp 55-57° sp. act. 24.5 dpm/mg indicating the presence of only 3% of the original oxazolone radioactivity.

C.

Of the remaining solution in B, a 0.5 ml aliquot was treated with a solution of ethyl glycinate (prepared as in A) and allowed to stand for 1 hr. Racemic Young peptide, 182.4 mg, was added and recovered after workup, sp. act. 11.3 dpm/mg, or 8.2×10^{-4} mmol labeled product.

Demonstration of the Incorporation of Oxazolone
into Active Ester

A solution of 1 mmol L-active ester, sp. act. 2.55×10^6 dpm/mmol (^{14}C) in 5 ml DMSO was treated with 1 mmol TMG and, after brief flushing with nitrogen, the intensely yellow reaction solution was allowed to stand at 23° for 1 hr. The mixture was used to dissolve 3.15 mmol unlabeled oxazolone and immediately diluted with 50 ml ether. The clear supernatant was decanted from the resulting orange oil and the washing procedure repeated two additional times. The combined ethereal phases were washed with water then dried (MgSO_4) and evaporated. Trituration with carbontetrachloride* gave 80 mg active ester, mp $147-149^\circ$, sp. act. 2.30×10^6 dpm/mmol. The orange oil was treated with 1 ml acetic acid, dissolved in ethyl acetate, washed with dilute acid and sat. bicarbonate solution then dried. Evaporation gave the ester mp $148-150^\circ$, sp. act. 2.31×10^6 dpm/mmol.

The Exchange Reaction Between Active Ester
and Oxazolone Mediated by Tetramethylguanidine

A.

A thoroughly mixed solution of benzoyl-L-leucyl active ester, 0.4 mmol, sp. act. 1.93×10^6 dpm/mmol (^{14}C) and 0.36 mmol TMG in ca

* The solubility of the oxazolone in this solvent is greater than 150 mg/ml.

1 ml DMSO was treated with benzoylleucine oxazolone, 0.43 mmol, sp. act. 2.58×10^6 dpm/mmol (^3H) and the volume adjusted to 2.0 ml. After 90 min at room temp. aliquots were quenched with unlabeled oxazolone and L-active ester solutions; the usual workups recovered the diluents having the following properties:

	mg diluent	activity/mg	$^3\text{H}/^{14}\text{C}$	mmoles (origin)	
				(ester)	(oxazolone)
L-ester	398	340 dpm	0.29	0.058	0.013
Oxazolone	310	231 dpm	1.74	0.014	0.0185

B.

A solution prepared as in A was allowed to stand 90 min at room temp. after which 0.5 ml aliquots were extracted and quenched for L- and DL-active esters.

	mg diluent	activity/mg	$^3\text{H}/^{14}\text{C}$	mmoles (origin)	
				(oxazolone)	(ester)
L-ester	254	480 dpm	0.266	0.0113	0.055
DL-ester	203	794 dpm	0.421		
D(calc.)	101.5	580 dpm	0.88	0.0111	0.0164

The Reaction of Active Ester with Excess

Tetramethylguanidine in the Presence of Oxazolone

A solution prepared by dissolving 0.4 mmol L-active ester, 1.93×10^6 dpm/mmol (^{14}C), 0.4 mm oxazolone, 2.5×10^6 dpm/mmol (^3H) and TMG, 0.87 mmol, in a total volume of 2 ml DMSO was allowed to stand 90 min at room temp. Aliquots of 0.5 ml each were quenched and diluted for L-active ester and oxazolone.

	mmol			mmoles (origin)	
	diluent	activity/mg	$^3\text{H}/^{14}\text{C}$	(ester)	(oxazolone)
L-active ester	2.063	148.4 dpm	0.129	0.0575	0.00575
Oxazolone	2.063	53.7	1.19	0.00571	0.00526

After an additional 24 hrs at room temp., a 0.5 ml aliquot was quenched and diluted for racemic benzoylleucine and racemic active ester, and the remaining solution diluted for oxazolone.

				mmoles (origin)	
	mg diluent	activity/mg	$^3\text{H}/^{14}\text{C}$	(ester)	(oxazolone)
DL-acid	552.6	117 dpm	0.152	0.0296	0.0035
DL-ester	301	192	0.391	0.022	0.0072
Oxazolone	250	< 1		< 0.0001 (i.e. < 0.1%)	

The Partitioning of the Active Ester in DMF-TMG

The reaction solution was prepared by mixing 139.4 mg L-active ester (0.35 mmol), sp. act. 1.95×10^6 dpm/mmol, and 40 mg TMG (0.34 mmol), in 2.0 ml DMF at room temp. After 1 hr 0.5 ml aliquots were quenched with solutions of L-active ester, oxazolone, and DL-active ester and the solutions worked up as usual to recover the diluents. The results are given below.

	mg diluent	activity/mmol	mmol	%
L-ester	396.3	1.15×10^5	0.0626	71.5
Oxazolone	291.5	1.27×10^4	0.00875	10
D (calc.)	213	2.45×10^5		
			<u>0.0178</u>	20
		total activity \Rightarrow	0.0892 mm	
		theoretical	0.0875 mm	

$$\% \text{ racemization} = \frac{2(\text{D-active ester}) + \text{oxazolone}}{\text{total}} = 50\%$$

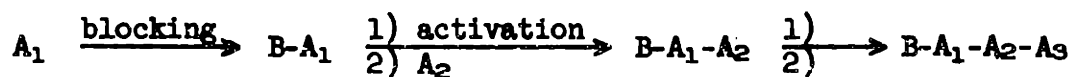
CHAPTER IV

Throughout this study reference has been made to the definition relating racemization to the two parameters; racemization rate and coupling rate. Some factors that affect the former have been explored in previous chapters. The present chapter defines the variables that affect the latter.

$$\text{fraction rac} = \frac{v_{\text{rac}}}{v_{\text{coupling}} + v_{\text{rac}}}$$

Aside from its obvious importance in the racemization problem, kinetic data bears other practical rewards. In any synthesis that offers several paths connecting starting materials to product the most successful strategy places low yield (slow) steps early in the synthetic sequence. Peptide synthesis—always a multi-step procedure—is especially sensitive to variations in synthetic strategy, but has historically been limited either to low-efficiency linear sequences, or low yield fragment condensations via the azide procedure. The isoxazole-derived esters, of which the Kemp-Chien reagent appears to be the most useful, introduce alternative strategies for peptide synthesis.

Consider the scheme below, in which amino acids are added one at a time to the C-terminus of an N-blocked peptide.



At the present time, the Kemp-Chien reagent is the only coupling method which can be used efficiently in this scheme, since it can both activate peptide acids and couple with amino acid anions without significant racemization. This scheme, which requires only one blocking group,* represents the shortest path between amino acids and peptides.

In order to gain information regarding optimal conditions for peptide synthesis via the Kemp-Chien reagent and thus refine the coupling technique, a kinetic study of aminolysis for these esters was devised.

The peptide literature, unfortunately, is not a source for such kinetic data; probably because of the awesome number of possible combinations (there exist over 1600 dipeptides). A reasonable approach is implicit in the work of Fless and Boissannas¹ who report half times for the reactions of certain 2,4,5-trichlorophenyl (TCP) esters. The

* The efficiency of this scheme is dependent on the use of amino acid anions as nucleophiles. The Kemp-Chien reagent appears unique in its ability to maintain optical integrity under the conditions required to generate amino acid anions in aprotic medium (see Chap. III).

(1) J. Fless and R. Boissannas, Helv. Chem. Acta, 46, 1609 (1963).

Z-L-Phe ester was treated with most of the common amino acid methyl esters (1) and also benzyl amine. Then the Z-TCP esters of most



of the common amino acids were treated with benzyl amine (2).



From the published data we have calculated rate constants for the more interesting reactions, the coupling of two amino acids (3),* (Table 1).

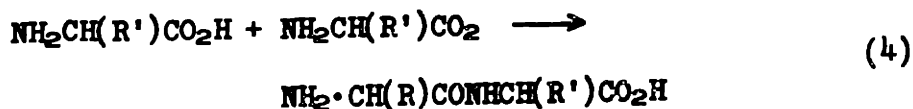


* Assuming that steric effects of side chains are additive in the transition state, k_{coup} for reactions (1), may be normalized to the k_{coup} for reaction with benzylamine. The k_{coup} for reaction (2), when multiplied by the normalization factors, give k_{coup} for reactions (3).

TABLE 1*

	<u>R</u>	<u>R'</u>	<u>$k_2 M^{-1} \text{min}^{-1}$, calc.</u>
1	H(Gly)	Gly	3.0
2	Me(Ala)	Ala	1.1
3	Ala	<u>i</u> pr(Val)	1.3
4	Val	Ala	0.045
5	Val	Val	0.05
6	Val	Gly	0.07

Although these rate constants need only be valid for the TCP esters at specified conditions (dioxane; 25°), the relative rates should reflect the process (4).

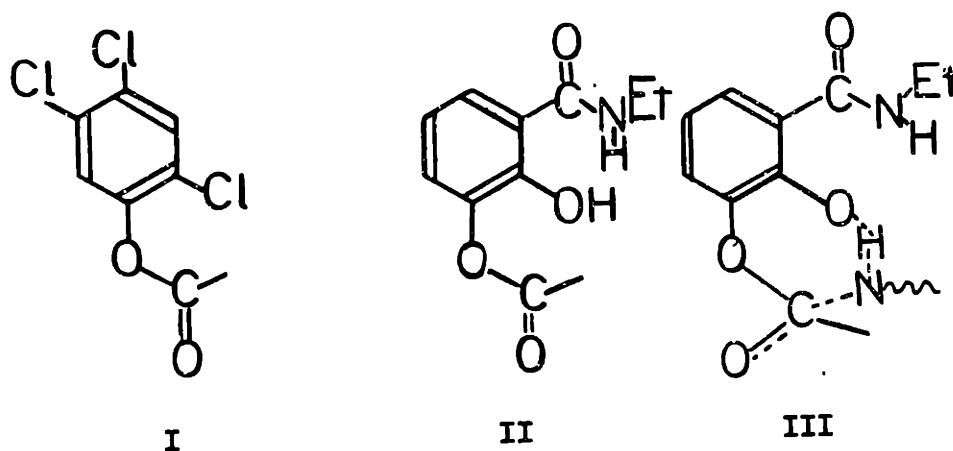


The most striking aspect is the sensitivity of the coupling rate to the side chain of the active ester component (3 vs. 5) when com-

* Although the original publication does not specify the configuration (L, DL or D) of the amino esters used in reaction (1), our own results vidi infra allow us to deduce that the amino esters used were of the L configuration.

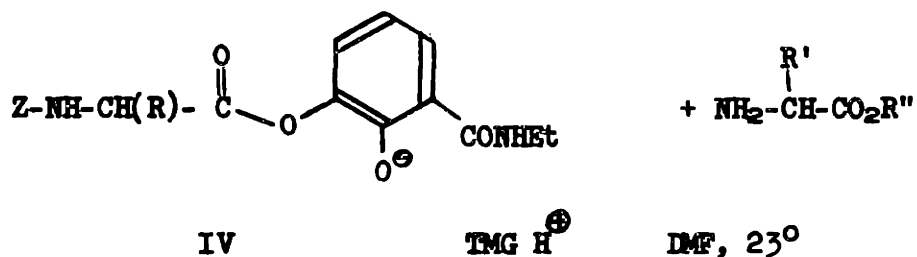
pared to the insensitivity of the rate to the side chain of the nucleophilic component (2 vs. 3), (4 vs. 5 vs. 6). The two extremes (1 and 5) probably set the range of rates (10^2) that can be expected for coupling with TCP esters I, but not necessarily for other esters.

The esters derived from the Kemp-Chien reagent are a priori expected to have greater sensitivity (greater rate ranges) due to the more crowded environment near the aminolysis center and the restrictions imposed by hydrogen-bridged structures III, possibly important in the rate determining step.



The coupling of amino acids via the Kemp-Chien reagent (Table II; see also Table III) suggests that this method is only slightly more sensitive to steric factors than the TCP ester, and, inasmuch

TABLE II*



	<u>Ester component</u>	<u>Amine component</u>	<u>k₂, M⁻¹min⁻¹</u>
1	Gly	GlyOEt	10 ^(±10%) (3)
2	<u>L</u> -Ala	<u>L</u> -AlaOEt	1(1.1)
3	<u>L</u> -Ala	<u>D</u> -AlaOEt	0.6
4	<u>L</u> -Ala	<u>L</u> -ValOEt	0.5(1.3)
5	<u>L</u> -Val	<u>L</u> -AlaOEt	0.06(0.045)
6	<u>L</u> -Val	<u>L</u> -ValOBz	0.03(0.05)
7	<u>L</u> -Val	GlyOMe	0.15(≈ 0.07)

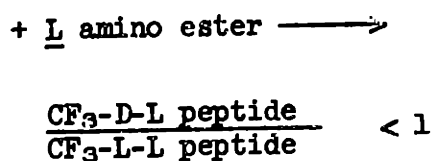
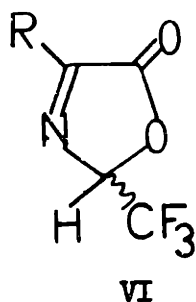
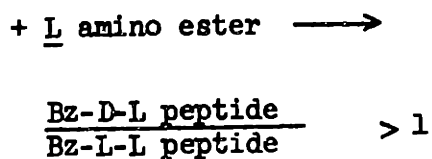
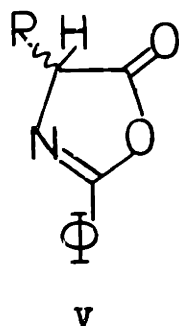
as the conditions can be compared, the two systems are of similar reactivity.

* Kinetics by isotopic dilution for peptide product; see experimental section for details.

The most interesting feature of Table II is the rate difference in reactions 2 and 3, which must reflect the preference for L + L vs. L + D couplings (Nature knows best). Although there is no reason to expect the two reactions to have the same rates, this feature of active ester couplings has not been previously described.*

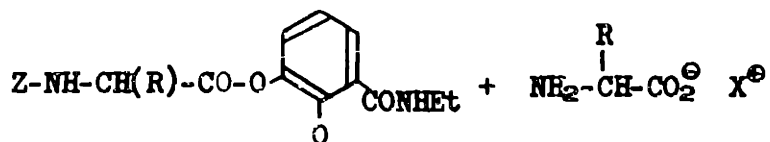
The reaction of esters IV with the strongly basic amino acid anions was next examined (Table III). The amino acid anions appear

* For coupling of oxazolones, (V), the opposite preference seems to hold, but the pseudo-oxazolones (VI) behave as active esters.



F. Weygand, W. Steglich and X. Baracio de la Lama, Tetrahedron (1966), Suppl. 8, part I, p. 9.

TABLE III



DMSO, 23°

or

DMSO-DMF at 0-3°

	<u>Ester component</u>	<u>X</u>	<u>Amine component</u>	<u>k₂M⁻¹min⁻¹</u>
8	Gly	TMA*	Gly	36
9	Gly	TMA	Gly	15 at 0-3°
10	Gly- <u>L</u> -Phe	TMA	Gly	36
11	Gly- <u>L</u> -Phe	TMA	Gly	15 at 0-3°
12	Gly	TMGH	<u>L</u> -Pro	0.08
13	Gly	TMGH	Sarcosine [†]	0.8
14	Gly	TMGH	<u>L</u> -Phe	2.6
15	<u>L</u> -Val	TMA	<u>L</u> -Val	0.2
16	<u>L</u> -Val	TMA	<u>D</u> -Val	0.06

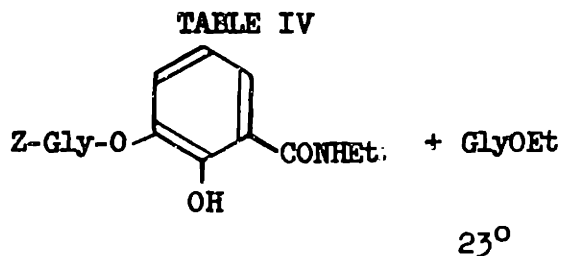
to react about 4 times faster than their alkyl ester counterparts, in those cases where comparisons may be made (1 vs. 8; 6 vs. 15).

* Me₄N

† Sarcosine = N-methyl Glycine

The effect of temperature appears to be small (10 vs. 11 and 8 vs. 9).

Solvent effects for couplings involving neutral active esters and amine components are given in Table IV. The dramatic decrease



	<u>Solvent</u>	<u>k₂M⁻¹mir.⁻¹</u>
17	DMF	0.2
18	DMSO	0.22
19	Dioxane	0.15
20	Pyridine	0.45
21	DMF + 1 eq. Et ₄ N [⊕] BF ₄ [⊖]	0.15

(50-fold; 1 vs. 17) in aminolysis rate when TMG is not present in the reaction medium may be of much importance to the racemization question. It would seem that the small temperature dependence on aminolysis, coupled with this large base dependence argues that peptide synthesis should be conducted at low temperatures and in the presence of TMG, unless the racemization rate shows dependencies that parallel

those of the coupling rate. Available evidence³ indicates that the racemization rate is, in fact, more sensitive to temperature than the coupling rate. (By analogy with the reactions 10 and 11 the coupling



Fraction rac: $23^{\circ}; 0.001 = k_{\text{rac}}^{\text{RT}} / k_{\text{coup}}^{\text{RT}}$; $k_{\text{rac}}^{\text{RT}} / k_{\text{rac}}^{\text{O}} = 20.$
 $3^{\circ}; 0.0001 = k_{\text{rac}}^{\text{O}} / 0.5 k_{\text{coup}}^{\text{RT}}$

rate should suffer only a two-fold decrease when conducted at 0° rather than 23°.)

Recently obtained results⁴ suggest that the racemization rate is, unfortunately, sensitive to the presence of TMG in much the same manner that the coupling rate is sensitive for the system above.

$$\frac{\text{fraction rac (neutral)}}{\text{fraction rac (TMG)}} \sim 1$$

(3) D. S. Kemp, S. W. Chien, G. Busby and G. Hugel, J. Am. Chem. Soc., 92, 1049 (1970).

(4) D. S. Kemp and C. Banquer, unpublished.

Chapter III, in which a specific-base-catalyzed oxazolone formation is shown to be the mechanism of racemization of the Kemp-Chien esters, suggests another possible method of racemate reduction. Because the slow step in this process is unimolecular, whereas the coupling is bimolecular, the fraction racemate should be an inverse function of concentration. Studies in these Laboratories⁴ have, in fact, confirmed this surmise.

EXPERIMENTAL

Methods

All coupling kinetics were followed by isotopic dilutions measuring yields of peptide product as a function time. Second order rate constants were obtained from at least four points which followed the reactions for three half-lives. Water (or ice-water) baths were used to maintain constant temperature.

Materials

Peptides for use as diluents were generally prepared via the Kemp-Chien reagent, using procedures described by Chien.¹ Isolated yields of peptides having physical properties in excellent agreement with literature values were generally between 70-80%.

Amino acid alkyl esters were liberated from the corresponding hydrochlorides with tetramethylguanidine (TMG) and used directly in those cases where a second equivalent of TMG was present (Table II). The neutral couplings (Table IV), ethyl glycinate that had been freshly distilled was used exclusively.

Tetramethylguanidinium salts of the amino acids were generated in situ. Tetraalkylammonium (TMA) salts were prepared by dissolving the amino acid in the appropriate volume of 10% (standardized) aqueous

(1) S. W. Chien, Ph.D. Thesis, M.I.T., 1967.

tetramethylammonium hydroxide followed by azeotropically distilling the water with benzene, using a Dean-Stark apparatus. Valine salts thus prepared were highly crystalline, alanine salts were amorphous powders, but the glycine salt was a wax. Standard solutions of these salts were prepared for the kinetic studies.

Active esters were prepared by the method described by Kemp.³ Physical data for new benzyloxycarbonyl amino acid 3-esters of 2,3-dihydroxy-N-ethylbenzamide are reported below.

<u>Ester of Z-</u>	<u>M.P.</u>	<u>Rotation, 23°</u> (C = 2, EtOAc)	<u>Analysis</u>	<u>Calc.</u>
L-Serine	116.5-117.5°	[α] ₅₄₆ = 49.0° [α] ₅₇₈ = -42.3°	C 59.63 H 5.47 N 6.99	59.70 5.51 6.96
L-Methionine	106-107.5°	[α] ₅₄₆ = -42.0° [α] ₅₇₈ = 36.3°	C 59.21 H 5.94 N 6.23 S 7.54	59.19 5.87 6.27 7.18
L-Phenylalanine	89.5-91.5°	[α] _D = -47.0°	C 67.44 H 5.66 N 5.98	67.52 5.67 6.06
L-Valine	77-79°	[α] _D = -48.5	C 63.69 H 6.32 N 6.83	63.76 6.32 6.76

Techniques

A. The couplings (Table II) were performed as follows. Equimolar amounts of the amino acid alkyl ester hydrochloride and TMG were combined in a small volume of DMF (ca. 10 ml). The precipitated TMG·HCl was filtered off (by suction filtration through a coarse porosity sintered-glass funnel in such a manner that the filtrate was directly collected in a 25 ml volumetric flask) and washed with a few ml DMF. The filtrate was combined with another equivalent TMG and the resulting solution was equilibrated with a water bath at $23 \pm 1^\circ$. The solutions were rapidly stirred (micro magnet) and standard solutions of radiolabeled-active esters (1 equiv.), were injected via calibrated (1 ml) syringe at the beginning of marked time, and the reaction mixture was quickly made up to volume. After the appropriate time lapses, aliquots of the reaction solution were removed and quenched with dil. HCl over-layered with ethyl acetate. Standard solutions of unlabeled peptide product were added via pipette and the peptide product was reisolated by suitable extraction procedures. Recrystallization to constant melting point was followed by analysis for isotopic activity. The yields were calculated according to the following expression:

$$\frac{\text{mmoles product}}{\text{in aliquot}} = n = \frac{(\text{mmoles diluent})(\text{sp. act. of product})}{(\text{sp. act. S.M.} - \text{sp. act. product})}$$

$$\% \text{ yield} = n / \text{theoretical yield for the aliquot.}$$

plots of $\frac{1}{\text{init. conc.}} \left(\frac{1}{(1 - \% \text{ yield})} - 1 \right)$ vs. time were made and the values of the slopes are reported as k_2 .

B. Reactions involving TMG salts as nucleophiles (Table III) were performed with equimolar amounts of ester and amine components and the usual technique described in A.

C. The reactions for which TMA salts were required - Table III, 8-11(glycine) and 15 and 16(valine) - were conducted under pseudo first order conditions. Ten to forty-fold excesses of the amine component were employed then the reaction mixture was treated as in A. A first order rate constant, k_{obs} , was determined from four points at each of four anion concentrations. The plot of $k_{\text{obs}}/[\text{anion}]$ vs. $[\text{anion}]$ gave a (horizontal) line which was extrapolated to zero anion concentration. The intercept found is reported as k_2 . (Sixteen points were used to define each rate constant for reactions 8-11, eight points each for 15 and 16.)

Reaction 14 was also conducted under pseudo first order conditions using the anion solution derived from L-phenylalanine with two equivalents of TMG (eight points).

D. Neutral couplings (Table IV) were run with equimolar amounts of active ester and ethyl glycinate.

BIOGRAPHICAL NOTE

The author was born in Beregszasz, Hungary, on April 11, 1944. He immigrated to the United States in 1949, and attained American citizenship through the naturalization of his parents. He graduated from the University of Kansas (1966) where he was introduced to chemical research by Professor Albert W. Burgstahler. Since 1966 the author has continued his research under the supervision of Professor Daniel S. Kemp as a National Science Foundation Predoctoral Fellow. The author has accepted a position as Assistant Professor of Chemistry at the University of California, Los Angeles.

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