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MECHANISTIC STUDIES OF PEPTIDE OXAZOLONE RACEMIZATION[1,2]

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Abstract—The racemization and ring-opening reactions of the oxazolone from benzyloxycarbonyl-aminoisobutryl-L-phenylalanine with a number of amino acid esters were studied in several commonly used peptide solvents. These studies show that oxazolone racemization is a pseudo first-order reaction when the rate of racemization is much larger than the rate of ring-opening. The ring-opening reaction always proceeds via second-order kinetics. When the rates of racemization and ring-opening are comparable, oxazolone racemization follows second-order kinetics. Since oxazolone racemization probably involves removal of a proton to produce two charged ions, anionic chloride or phosphate ions accelerate the rate of racemization apparently by increasing the ionic strength of the solution. Solvents such as dioxane which can accommodate charge separation by solvation gives rise to much higher extents of oxazolone racemization than solvents such as toluene in which charge separation is not well tolerated.

Perhaps the most important factor controlling the racemization process is the nucleophilicity-basicity ratio of the attacking nucleophile. Of the amino acid esters studied, ethyl glycinate has the most desirable nucleophilicity-basicity ratio. In toluene solution the coupling of this amino acid ester with the oxazolone from benzyloxycarbonylaminoisobutyryl-L-phenylalanine gives the unexpectedly high yield of approximately 75% optically active tripeptide. Under similar conditions methyl aminoisobutyrate, a very hindered nucleophile, gives completely racemized tripeptide.

When a large excess of hydrazine hydrate is allowed to react with the oxazolone from benzyloxycarbonylaminoisobutyryl-L-phenylalanine an optically pure hydrazide is obtained which is identical to that obtained in the traditional manner from the optically active dipeptide ester and hydrazine hydrate. Hydrazine hydrate therefore has an extremely high nucleophilicity-basicity ratio which we attribute to the so-called "alpha effect".

A FEW years ago the first quantitative studies on the racemization and ring-opening of the optically active amino acid oxazolone 2-phenyl-L-4-benzyloxazolone was reported.[5] More recently we described[6] preliminary, kinetic data on the racemization and ring-opening of the peptide oxazolone 2-(1′-benzyloxycarbonylamino-1′-methyl)ethyl-L-4-benzyloxazolone (hereinafter referred to as the L-phenylalanine peptide oxazolone). The conclusions suggested by the kinetic data in these reports were that peptide racemization arises because of the formation of a small steady state concentration of oxazolone which can form under peptide coupling conditions and which racemizes much faster than it can ring-open to give product. The amount of racemization observed depends on the basicity and concentration of the nucleophile present.

[1] For the previous paper in this series see W. J. McGahren and Murray Goodman, *Tetrahedron* **23**, 2017 (1967).

[2] We gratefully acknowledge the support of this research under Grant AMO3868 from the National Institutes of Health.

[3] Submitted to the faculty of the Polytechnic Institute of Brooklyn in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry.

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[5] M. Goodman and L. Levine, *J. Am. Chem. Soc.* **86**, 2918 (1964).

[6] M. Goodman and W. J. McGahren, *J. Am. Chem. Soc.* **87**, 3028 (1965).

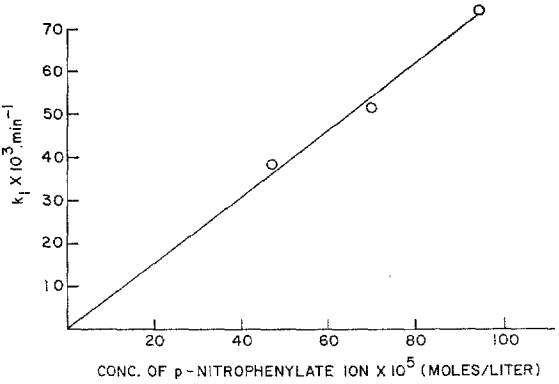


FIG. 1. Second order rate constant for the racemization of L-alanine peptide oxazolone by *p*-nitrophenylate ion in dioxan: $k_{rac} = 78$ liter mole^{-1} min^{-1}.

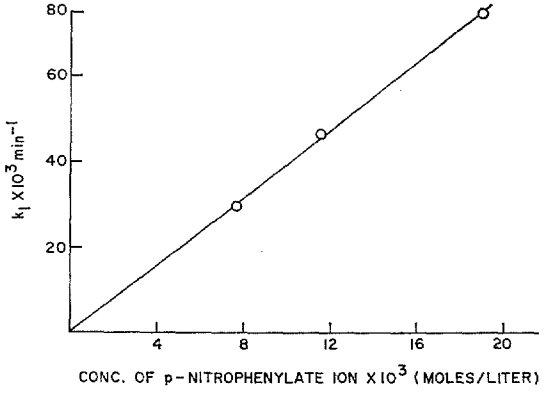


FIG. 2. Second order rate constant for the ring-opening of L-alanine peptide oxazolone by *p*-nitrophenylate ion in dioxan: $k_{ro} = 4{\cdot}0$ liter mole^{-1} min^{-1}

TABLE 1. RACEMIZATION OF L-ALANINE PEPTIDE OXAZOLONE BY *p*-NITROPHENYLATE ION IN DIOXAN

Concentration of racemizing reagent	$k_1 \times 10^2$ / min.$^{-1}$	k_2 / 1 mole^{-1} min^{-1}
$4{\cdot}69 \times 10^{-4}$	$3{\cdot}96 \pm 0{\cdot}09$	78
$7{\cdot}03 \times 10^{-4}$	$5{\cdot}19 \pm 0{\cdot}06$	
$9{\cdot}38 \times 10^{-4}$	$7{\cdot}42 \pm 0{\cdot}10$	

TABLE 2. RING-OPENING OF L-ALANINE PEPTIDE OXAZOLONE BY *p*-NITROPHENYLATE ION IN DIOXAN

Concentration of ring-opening reagent	$k_1 \times 10^2$ / min^{-1}	k_2 / 1 mole^{-1} min^{-1}
$0{\cdot}76 \times 10^{-2}$	$3{\cdot}04 \pm 0{\cdot}04$	4·0
$1{\cdot}15 \times 10^{-2}$	$4{\cdot}70 \pm 0{\cdot}04$	
$1{\cdot}91 \times 10^{-2}$	$7{\cdot}51 \pm 0{\cdot}06$	

Initial kinetic studies on the oxazolone 2-(1′-benzyloxycarbonylamino-1′-methyl)ethyl-L-4-methyloxazolone (hereinafter referred to as L-alanine peptide oxazolone) as given in Tables 1 and 2 are in agreement with these postulations (Figs 1 and 2).

In Tables 1 and 2 the *p*-nitrophenylate ion concentration is obtained from the concentration of tributylamine used in the presence of a large excess of *p*-nitrophenol. Under these conditions the following equilibrium lies far to the right:

$$p\text{-}NO_2C_6H_4OH + N(Bu)_3 \rightleftharpoons p\text{-}NO_2C_6H_4\overset{\ominus}{O}\,\overset{\oplus}{N}H(Bu)_3$$

In all our preliminary experiments virtually no ring-opening occurred during the racemization process. At the completion of racemization essentially no base was consumed. Consequently, oxazolone racemization under these circumstances follows pseudo-first oder kinetics.

When the optically active tripeptide is formed during the course of the racemization process amino acid ester is being consumed and we no longer have a simple kinetic picture. A kinetic scheme was formulated† for those cases where racemization and ring-opening rates are comparable, that is where some amino acid ester is used up before racemization is complete.

$$\begin{array}{ccc} \text{oxazolone}^* + \text{amino acid ester} & \xrightarrow{k_{ro}} & \text{tripeptide}^* \\ \Big\downarrow k_{rac} & & \\ \text{oxazolone} + \text{amino acid ester} & \xrightarrow{k_{ro}} & \text{tripeptide} \end{array}$$

k_{rac} = racemization second order rate constant
k_{ro} = ring-opening second order rate constant

The asterisk indicates the optically active components present. The disappearance of optically active oxazolone is represented by

$$\frac{-dO^*}{dt} = (k_{rac} + k_{ro})[O^*][B] \tag{1}$$

where the concentration of optically active oxazolone or [oxazolone*] = $[O^*]$; concentration of racemic oxazolone or [oxazolone] = $[O]$; and [amino acid ester] = $[B]$. The disappearance of amino acid ester is given by

$$\frac{-dB}{dt} = k_{ro}[O^* + O][B] \tag{2}$$

In the cases where we employ a racemic amino acid ester we assume that the rates of reaction of both antipodes with the enantiomers of the oxazolone are essentially the same. Since $[B] = [O^*] + [O]$

$$\frac{-dB}{dt} = k_{ro}[B]^2 \tag{3}$$

† We wish to take special note of discussions with Prof. Herbert Morawetz of our Institute. Through his efforts we arrived at the kinetic scheme used in this paper.

Integrating this equation leads to the following:

$$\frac{1}{[B]} = k_{ro}t + \frac{1}{B_o} \tag{4}$$

where B_o = initial concentration of amino acid ester. Rewriting this equation gives:

$$[B] = \frac{B_o}{1 + k_{ro}B_o t} \tag{5}$$

Substituting this in Eq. (1) yields the equation:

$$\frac{-dO^*}{O^*} = (k_{ro} + k_{rac})\left(\frac{B_o}{1 + k_{ro}B_o t}\right) dt \tag{6}$$

$$= \left(\frac{k_{ro} + k_{rac}}{k_{ro}}\right) \frac{d(1 + k_{ro}B_o t)}{1 + k_{ro}B_o t} \tag{7}$$

Integrating this equation gives us the following expression:

$$\ln \frac{O_o^*}{O^*} = \frac{k_{ro} + k_{rac}}{k_{ro}} . \ln (1 + k_{ro}B_o t) \tag{8}$$

where O_o^* = initial concentration of optically active oxazolone. Hence, from the slope of a plot of log O_o^*/O^* *versus* log $(1 + B_o k_{ro} t)$ one can obtain the value of $(k_{ro} + k_{rac})/k_{ro}$ and since k_{ro} is measured independently the value of k_{rac} can be derived. Figs. 3 and 4 illustrate cases where this method was used.

An alternate approach can be developed from Eq. (1). The appearance of optically active tripeptide follows the equation

$$\frac{dP^*}{dt} = k_{ro}[O^*][B] \tag{9}$$

where $[P^*]$ is the concentration of optically active tripeptide. From these two equations we can write

$$\frac{\Delta O^*}{\Delta P^*} = \frac{k_{ro} + k_{rac}}{k_{ro}} \tag{10}$$

Therefore, the ratio of the disappearance of optically active oxazolone to the formation of optically active peptide is a constant from which the racemization rate constant can be obtained.

The term $\Delta O^*/\Delta P^*$ is best calculated as the normalized expression

$$\frac{O_o^* - O_t^*}{O_o^*} \Big/ \frac{P_t^*}{P^*_{\text{theoretical}}} \tag{11}$$

where O_o^* = initial rotation of optically active oxazolone

O_t^* = rotation of optically active oxazolone at time t

P_t^* = rotation of optically active tripeptide at time t

$P^*_{\text{theoretical}}$ = rotation of optically active tripeptide which would be observed if no racemization occurred.

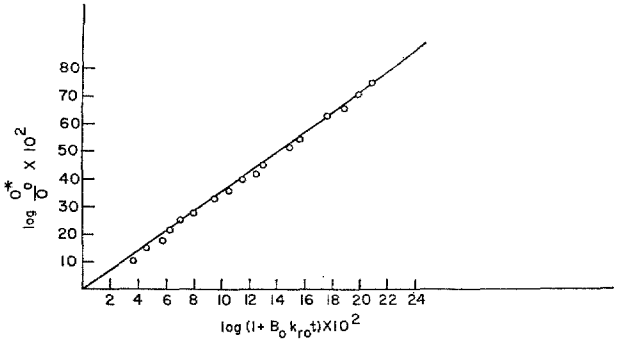


FIG. 3. Second order rate constant for the racemization of L-phenylalanine peptide oxazolone by ethyl glycinate in dioxan: $k_{rac} = 4{\cdot}5$ liter mole^{-1} min^{-1}.

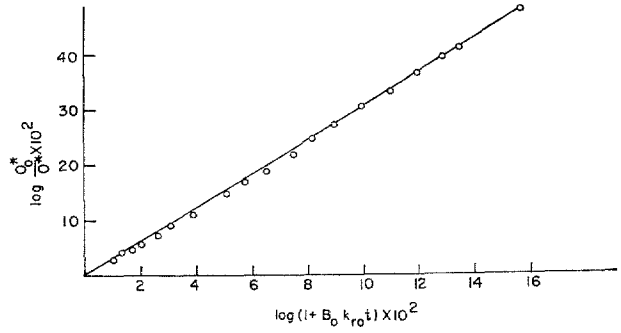


FIG. 4. Second order rate constant for the racemization of L-phenylalanine peptide oxazolone by methyl DL-alaninate in chloroform: $k_{rac} = 1{\cdot}0$ liter mole^{-1} min^{-1}.

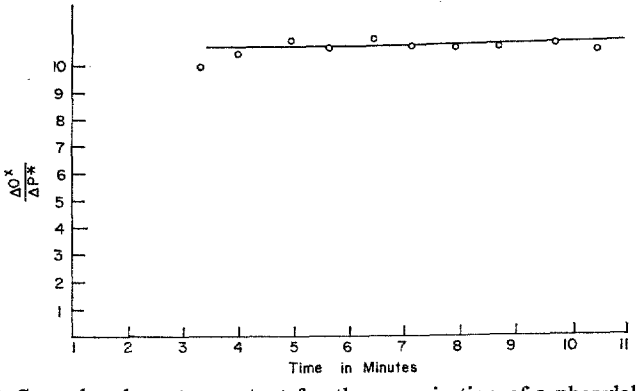


FIG. 5. Second order rate constant for the racemization of L-phenylalanine peptide oxazolone by methyl DL-alaninate in ethyl acteate: $k_{rac} = 6{\cdot}8$ liter mole^{-1} min^{-1}.

A plot of $\Delta O^*/\Delta P^*$ *versus* time gives a constant value. Fig. 5 illustrates a case where this method was used.

The ring-opening rate constant k_{ro} was determined separately by IR spectroscopy or calculated from a half-life value as measured by TLC. In order to interpret the kinetic data, a method was devised to correct for the appearance of optically active tripeptide. This was accomplished by running identical reactions for various lengths of time such as 5, 10 and 20 min. At the appropriate time, sufficient n-tributylamine

was added instantly to racemize all remaining optically active oxazolone. A curve of the optical rotation contribution of tripeptide *versus* time was obtained in this manner so that each individual, observed, polarimetric reading could be corrected to reflect

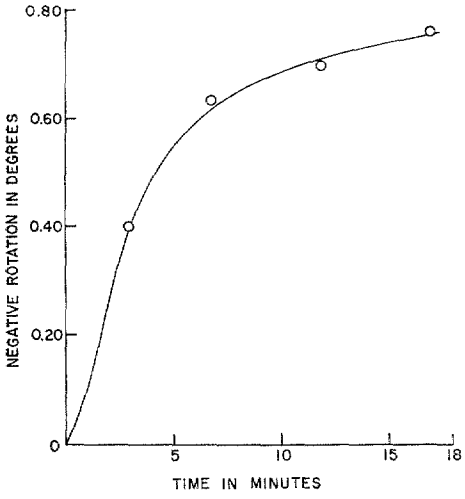


FIG. 6. Correction curve for optically active tripeptide in the racemization of L-phenylalanine peptide oxazolone by ethyl glycinate in chloroform.

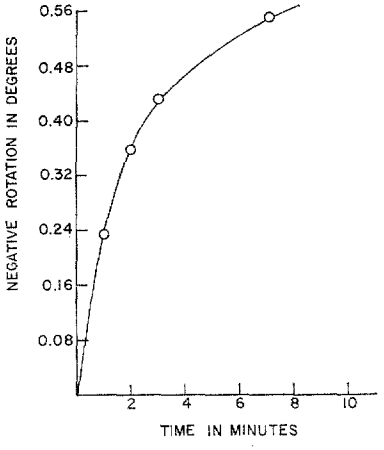


FIG. 7. Correction curve for optically active tripeptide in the racemization of L-phenylalanine peptide oxazolone by ethyl glycinate in toluene.

true oxazolone optical rotation at any given time. Representative correction curves are shown in Figs 6 and 7. With these data the kinetic equation

$$\ln \frac{O_0^*}{O} = \frac{k_{ro} + k_{rac}}{k_{ro}} \cdot \ln (1 + k_{ro} B_0 t) \text{ is readily solved.}$$

We also established the per cent optical purity of the tripeptide product which formed by comparing its rotation with that for the same material prepared via a route that avoids racemization.

When the reactions of L-phenylalanine peptide oxazolone and a racemic amino acid ester such as methyl-DL-alaninate were studied it was necessary separately to prepare Z-Aib-L-Phe-L-Ala-OCH_3[7] and Z-Aib-L-Phe-D-Ala-OCH_3. Equal weights of these isomers were mechanically mixed to get the required species Z-Aib-L-Phe-DL-Ala-OCH_3.

In the three cases studied (alaninate, phenylglycinate and phenylalaninate) this latter procedure was unnecessary since the experimentally determined specific rotation of the L-DL-tripeptide species agreed with the value calculated by adding the specific rotations of the separate isomers.

Tables 3 through 6 represent a summary of the kinetic data obtained on allowing L-phenylalanine peptide oxazolone to react with various amino acid esters. Equal concentrations of reactants were employed except in those ring-opening reactions where it was convenient to use higher concentrations of nucleophile.

Ethyl acetate is a very popular solvent with peptide chemists but because it absorbs at 1825 cm^{-1} IR spectroscopy could not be used to obtain ring-opening rate

[7] Aib denotes α-aminoisobutyryl residue. Other abbreviations follow standard conventions.

TABLE 3. RACEMIZATION AND RING-OPENING OF L-PHENYLALANINE PEPTIDE OXAZOLONE BY ETHYL GLYCINATE

Solvent	k_{rac} / 1 mole^{-1} min^{-1}	k_{ro} / 1 mole^{-1} min^{-1}	% Retention of optical activity
Chloroform	2·1	5·0	66
Toluene	9·3[a]	13·3[a]	78
Dioxan	4·5	1·75	14
Ethyl acetate	3·2	3·6[b]	38

[a] Reactions in toluene were too fast for good kinetic studies.
[b] Rate constant calculated from an estimated half-time value obtained by TLC.

TABLE 4. RACEMIZATION AND RING-OPENING OF L-PHENYLALANINE PEPTIDE OXAZOLONE BY METHYL DL-ALANINATE

Solvent	k_{rac}	k_{ro} / 1 mole^{-1} min^{-1}	% Retention of optical activity
Chloroform	1·0 l. mole^{-1} min^{-1}	0·5	32
Toluene	2·2 l. mole^{-1} min^{-1}	2·4	52
Dioxan	$10·34 \pm 0·3 \times 10^{-2}$ min^{-1}	0·14	0
Ethyl acetate	6·8 1 mole^{-1} min^{-1}	0·75[a]	11

[a] Rate constant calculated from estimated half-time value obtained using TLC.

TABLE 5. RACEMIZATION AND RING-OPENING OF L-PHENYLALANINE PEPTIDE OXAZOLONE BY METHYL DL-PHENYLGLYCINATE

Solvent	k_{rac} / 1 mole^{-1} min^{-1}	k_{ro} / 1 mole^{-1} min^{-1}	% Retention of optical activity
Chloroform	0·18	0·4	—
Toluene	0·7	2·1	74
Chloroform plus equimolar $NEt_3 \cdot HCl$[a]	0·35	0·25	—

[a] The same concentration, namely 0·03045M, of oxazolone, methyl DL-phenylglycine and $NEt_3 \cdot HCl$ used.

TABLE 6. RACEMIZATION AND RING-OPENING OF L-PHENYLALANINE PEPTIDE OXAZOLONE BY METHYL α-AMINOISOBUTYRATE

Solvent	$k_{rac} \times 10^2$ / min^{-1}	$t_{1/2rac}$ / min	k_{ro} / 1 mole^{-1} min^{-1}	% Retention of optical activity
Chloroform	$3·30 \pm 0·08$	21·0	0·0024	0
Toluene	$3·03 \pm 0·10$	22·8	0·006	0
Dioxan	$8·52 \pm 0·04$	8·1	0·008	0
Ethyl acetate	$8·21 \pm 0·05$	8·4	—	0

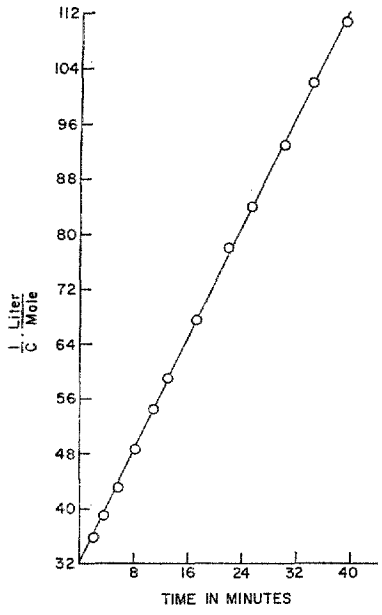


FIG. 8. Second order rate constant for the ring-opening of L-phenylalanine peptide oxazolone by methyl DL-phenylglycinate in toluene: $k_{RO} = 2{\cdot}1$ liter mole^{-1} min^{-1}.

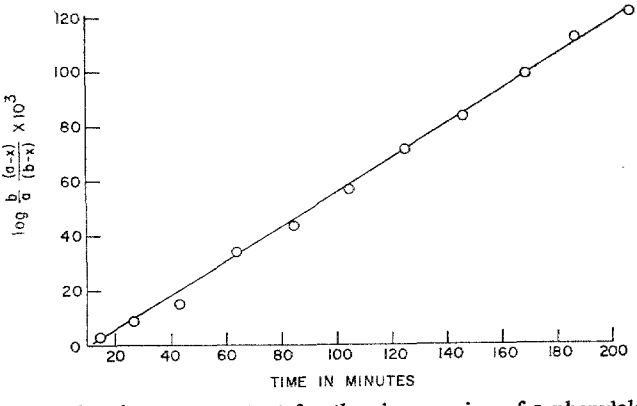


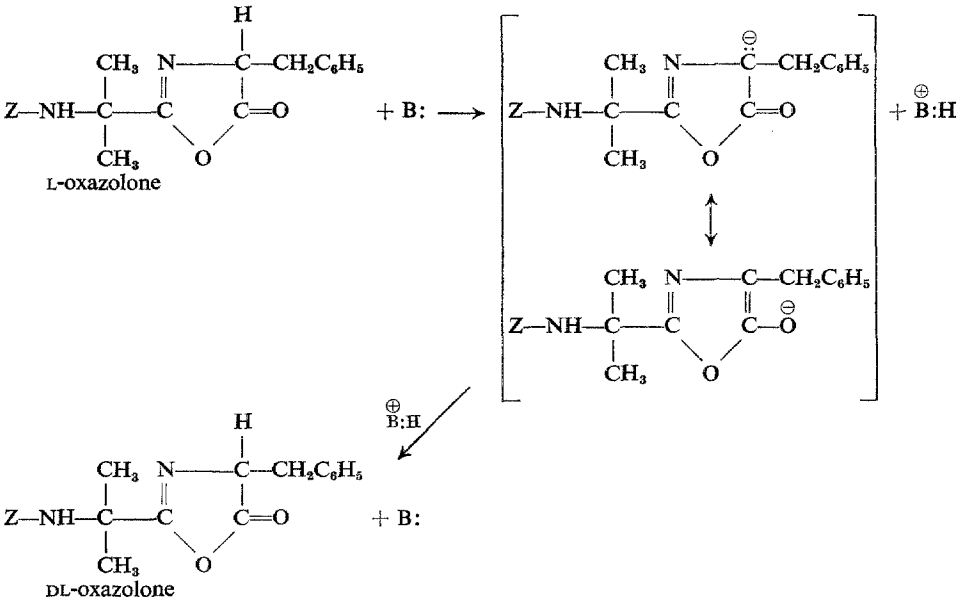
FIG. 9. Second order rate constant for the ring-opening of L-phenylalanine peptide oxazolone by methyl aminoisobutyrate in chloroform: $k_{RO} = 2{\cdot}4 \times 10^{-3}$ liter mole^{-1} min^{-1}.

constants in this solvent. Instead, we estimated half-times of ring-opening reactions from TLC and from these values rate constants were calculated. The reactions of the oxazolone with ethyl glycinate in toluene were too fast for good kinetic studies so that the rate constants obtained are only approximate. Figures 8 and 9 are typical of the plots obtained for the second order ring-opening reactions.

The importance of solvent in the racemization process

Tables 3 through 6 clearly indicate the effect of solvent on the optical activity retention in a peptide coupling reaction. Of the four solvents studied toluene and

chloroform are the least likely to allow racemization to occur. The oxygenated solvents, particularly dioxan, appear to facilitate the racemization process. The following scheme results in irreversible loss of optical activity. Solvents which can best



tolerate the separation of charges should lead to greater racemization. The ability of solvents to favor this reaction should increase with increasing value of their dielectric constants.[8]

We would expect that use of dioxan solvent would tend to prevent racemization. Dioxan itself, however, can act as a Lewis base and assist in the removal of the proton from the oxazolone. In pure dry dioxane L-phenylalanine peptide oxazolone loses approximately 25% of its optical activity in 24 hr, whereas in dry chloroform over the same period there is no loss of optical activity. This experiment is indicative that dioxan can participate in the equilibrium leading to racemization. The effect is evidently enhanced by the addition of a nucleophile, perhaps because dioxane can accommodate the separation of charges by solvation.

When we first encountered the difficulty of measuring racemization rates of the L-phenylalanine peptide oxazolone by ethyl glycinate because of the formation of optically active tripeptide, we decided to obtain an estimate of the rate constant by using a base of comparable pK_a with ethyl glycinate which could not couple with the oxazolone. Ethyl glycinate has a pK_a of 7·73 while 2-cyanoethyldiethylamine has a pK_a value of 7·65 in water.[9] This tertiary amine was prepared by the method of Stevenson and Williamson[10] and the racemization rate constants of the L-phenylalanine peptide oxazolone with this amine were measured in dioxan and chloroform

[8] *Handbook of Chemistry* (Edited by N. A. Lange; 10th Edition) p. 1222. *McGraw-Hill*, New York (1961).

[9] E. J. Cohn and J. T. Edsall, *Proteins, Amino Acids and Peptides as ions and dipolar ions* p. 99. Reinhold, New York (1943).

[10] G. W. Williamson and D. Williamson, *J. Am. Chem. Soc.* **80**, 5943 (1958).

in solutions which were equimolar in amine and oxazolone (0·03045M) and the values found were $k_1 = 9{\cdot}59 \pm 0{\cdot}22 \times 10^{-3}$ min^{-1} and $1{\cdot}298 \pm 0{\cdot}025 \times 10^{-2}$ min^{-1}, respectively. As the pK_a values indicate, ethyl glycinate and the tertiary cyanoamine have approximately the same ability in water to accept or donate a proton. In the organic solvents dioxan and chloroform, however, the cyanoamine is ten times and six times, respectively, less effective than ethyl glycinate in its capacity to racemize

TABLE 7. RACEMIZATION OF L-PHENYLALANINE PEPTIDE OXAZOLONE BY TRIETHYLAMINE HYDROCHLORIDE IN CHLOROFORM

Conc of triethylamine hydrochloride × 10M	$k_1 \times 10^4$ / min^{-1}	$t_{1/2}$ / min
1·51	6·97 ± 0·32	993
2·66	10·92 ± 0·24	634
4·12	14·70 ± 0·17	470

L-phenylalanine peptide oxazolone as judged from half-times of reactions. These results support the contention that solvent is intimately involved in the racemization process.

Nucleophilicity-Basicity ratio

In order to simplify our discussion we shall refer to the ability of any amino acid ester to racemize the L-phenylalanine peptide oxazolone as representative of its basicity and the ability to ring-open or couple with the oxazolone as indicative of its nucleophilicity.

Tables 3 through 6 show that the nature of amino acid ester is directly related to racemization occurring during the coupling process. Of the amino acid esters studied ethyl glycinate has the best per cent retention of optical activity (i.e., the most favorable nucleophilicity-basicity ratio). With methyl alaninate the retention is not as good. Methyl aminoisobutyrate exhibits an extraordinary unfavorable nucleophilicity to basicity ratio as might be expected from steric considerations. Young's model systems[11–13] show decided advantages from the point of view of racemization detection. However, since he employed ethyl glycinate in all his model systems he may have encountered an abnormally high optical purity in his products compared to cases where other amino acids serve as the attacking agents. Conversely, we believe that methyl aminosiobutyrate provides the most stringent test for a coupling method.

The "Chloride ion effect"

Young[14] has studied an effect which he attributes to the basicity of chloride ion in organic solvents. We studied the effect of triethylamine hydrochloride on L-phenylalanine peptide oxazolone in chloroform and found that by itself this hydrochloride shows very little ability to racemize the oxazolone (Table 7).

In the presence of an amino acid ester such as methyl DL-phenylglycinate, triethylamine hydrochloride accelerates the rate of racemization somewhat and apparently

[11] N. A. Smart, G. T. Young and M. W. Williams, *J. Chem. Soc.* 3902 (1960).
[12] M. W. Williams and G. T. Young, *J. Chem. Soc.* 881 (1963).
[13] A. L. Heard and G. T. Young, *J. Chem. Soc.* 5807 (1963).
[14] M. W. Williams and G. T. Young, *J. Chem. Soc.* 3701 (1964).

retards the rate of ring-opening (Table 5). It does not appear that the basicity of the chloride ion as such is involved. The data are surprising in view of Young's contention[14] that chloride ion and glycine are bases of equivalent strength in chloroform. We also studied the racemization of L-phenylalanine peptide oxazolone in dioxan containing aqueous buffered solutions in the ratio of 3:2. Under the conditions employed in Table 8 virtually no ring-opening occurs as measured by UV spectroscopy.

TABLE 8. RACEMIZATION OF L-PHENYLALANINE PEPTIDE OXAZOLONE IN THE SYSTEM DIOXAN:BUFFER SOLUTION::3:2[15]

Conditions	k_1 / min^{-1}	$t_{1/2}$ / min
Pure solvent mixture dioxan:water (3:2)	$12{\cdot}2 \pm 0{\cdot}8 \times 10^{-4}$	567
pH 7·2 buffer solution	$11{\cdot}6 \pm 0{\cdot}24 \times 10^{-2}$	5·96
pH 7·0 buffer solution	$9{\cdot}3 \pm 0{\cdot}38 \times 10^{-2}$	7·41
pH 6·5 buffer solution	$5{\cdot}1 \pm 0{\cdot}47 \times 10^{-2}$	13·6
pH 4·0 buffer solution	$0{\cdot}35 \pm 0{\cdot}02 \times 10^{-2}$	198
Water $1{\cdot}25 \times 10^{-3}$M in hydroxide ion	$2{\cdot}3 \pm 0{\cdot}04 \times 10^{-3}$	298

In view of the extremely low concentration of OH ion present in the "pH 7·2" buffered system, it appears that the large acceleration of the racemization process in this solution may be due to the increased ionic strength of the system. This is borne out by the fact that a relatively higher concentration of OH ion by itself merely doubles the rate of racemization. In addition, the "pH 4·0" buffered system only doubles the rate of oxazolone racemization when compared to the solvent system dioxan–water (3:2). This observation indicates that the effect does not arise from general base catalysis of oxazolone enolization by buffered anions. We believe that the presence of a base is necessary for racemization and its effectiveness is increased as the ionic strength of the system is increased. Further experiments are under way to test this hypothesis.

To conclude our discussion of over-all kinetic data, we must mention the extraordinary capacity of hydroxide ion and tributylamine, respectively, to racemize L-phenylalanine peptide oxazolone in methanol solution (Table 10 and 11). This type of study was not pursued extensively because the data obtained did not strictly obey first-order kinetics even though no effective ring-opening occurred during racemization. Tables 9 and 10 do show that the use of polar solvents such as methanol in peptide coupling reactions may be hazardous.

Reactions of L-*phenylalanine peptide oxazolone and* L-*alanine peptide oxazolone with hydrazine hydrate*

As we have already stated ethyl glycinate has a very favorable nucleophilicity-basicity ratio. When this ester is allowed to react with L-phenylalanine peptide oxazolone in toluene the resultant tripeptide is only 22% racemized. When a huge excess of the basic compound hydrazine hydrate was allowed to react with this same oxazolone no racemization was detected.

[15] *Handbook of Chemistry and Physics* (44th Edition) p. 1717. Chemical Rubber Publishing, Ohio (1963).

TABLE 9. RACEMIZATION OF L-PHENYLALANINE PEPTIDE OXAZOLONE IN METHANOL SOLVENT

A—abs MeOH
B—MeOH containing approximately 0·5% water and the stated concentration of hydroxide ion

Conditions	k_1 / min^{-1}	$t_{1/2}$ / min
A	$1·1 \pm 0·03 \times 10^{-3}$	640
B, $4·7 \times 10^{-4}$M in OH^-	$5·4 \pm 0·6 \times 10^{-2}$	13
B, $4·5 \times 10^{-4}$M in OH^-	$3·3 \pm 0·5 \times 10^{-2}$	20·7
B, $4·3 \times 10^{-4}$M in OH^-	$1·0 \pm 0·04 \times 10^{-2}$	69·2

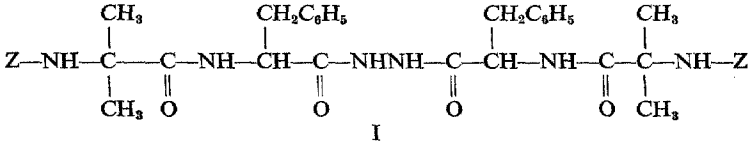
TABLE 10. RACEMIZATION OF L-PHENYLALANINE PEPTIDE OXAZOLONE BY TRIBUTYLAMINE IN ABSOLUTE METHANOL

Conc of tributylamine / M	$k \times 10$ / min^{-1}	$t_{1/2}$ / min
$6·27 \times 10^{-4}$	$3·0 \pm 0·046$	2·3
$5·90 \times 10^{-4}$	$2·0 \pm 0·029$	3·4
$5·54 \times 10^{-4}$	$0·24 \pm 0·003$	29·4

Edwards[16] has discussed the factors determining the reactivity of nucleophilic reagents. The enhanced nucleophilicity of hydrazine hydrate is largely due to what he terms the "alpha effect" or the presence of an unshared electron-pair on the atom adjacent to the nucleophilic atom.

Nowak and Siemion[17] observed that when the oxazolone from acetyl-L-leucine is allowed to react with hydrazine hydrate products exhibiting optical activity are formed.

We obtained proof of the observation that the oxazolone-hydrazine hydrate coupling reaction occurs with complete retention of optical activity by allowing hydrazine hydrate to react with the dipeptide ester Z–Aib–L–Phe–OCH_3. The product which we isolated was identical with that from the reaction of a huge excess of hydrazine hydrate with the oxazolone. Unfortunately, we were unable to obtain a crystalline hydrazide by the conventional method or by the reaction of peptide oxazolone with hydrazine hydrate. Since the conventional method of hydrazide preparation is one of the standard steps in the azide coupling route and is known to avoid racemization we can conclude that our evidence clearly shows that hydrazine hydrate does not racemize the peptide oxazolone. The reaction between L-phenylalanine peptide oxazolone and dilute equimolar hydrazine hydrate solution yields in addition to the above mentioned hydrazide a compound which we believe to be the dihydrazide I.



I

[16] J. O. Edwards and R. C. Pearson, *J. Am. Chem. Soc.* **84**, 16 (1962).
[17] K. Nowak and I. Z. Siemion, *Rocz Chem.* **35**, 153 (1961).

One might expect that the bifunctional nucleophile hydroxylamine would also react with peptide oxazolones with retention of optical activity. In some exploratory work we found that on the addition of L-phenylalanine peptide oxazolone to an equivalent amount of hydroxylamine in methanol solution the optical rotation falls rapidly to a steady sizeable reading which parallels what we observed in the case of hydrazine hydrate. However, over a period of 14 to 16 hr the optical rotation value drops slowly almost to zero. We believe that the reaction of the oxazolone with hydroxylamine occurs with retention of optical activity but that the resultant hydroxamic acid racemizes slowly in the solution of relatively high ionic strength. Further work is now in progress in our laboratory to examine these reactions.

CONCLUSIONS

We have prepared a model optically active peptide oxazolone and used it to compare racemization and coupling rates with various amino acid esters in several solvents. Racemization of oxazolones may follow pseudo-first-order or second-order kinetics depending on the attacking amino acid ester and the nature of the solvent. The racemization rate can be accelerated by the presence of substances such as chlorides or phosphates which increase the ionic strength of the solution and facilitate the charge separation necessary for racemization.

The coupling or ring-opening reaction is always a second-order reaction, the rate of which is governed by the nucleophilicity of the amino compound in the solvent in question. Our findings are in keeping with the notion that peptide coupling, where the possibility of oxazolone formation exists, is best carried out in non-polar solvents in the absence of salts such as hydrochlorides.

As with many model systems we must recognize the limitations before extrapolating the results obtained to other systems. We have not investigated the ease of oxazolone formation. We know, for example, that in the presence of DCCi the dipeptide acid Z–Aib–L–Phe–OH forms oxazolone almost instantly. From Khorana's work[18] we know that certain carboxylic acids can give acylureas with DCCi. Schuessler and Zahn[19] isolated symmetrical acylamino acid anhydrides as intermediates in DCCi peptide coupling.

As an example, benzyloxycarbonylglycine with DCCi gave a symmetrical anhydride which was isolated and characterized and subsequently allowed to couple with DL-phenylalanine methyl ester to get Z–Gly–DL–Phe–OCH_3. In a very recent paper DeTar *et al.*[20] have made a quantitative study of the stoichiometries of the reactions of peptide acids of the general structure Z–AA–OH and acyl–AA–OH with DCCi in various solvents.

It is important to emphasize that there remains a need to study the ease of formation of oxazolones under peptide coupling conditions.

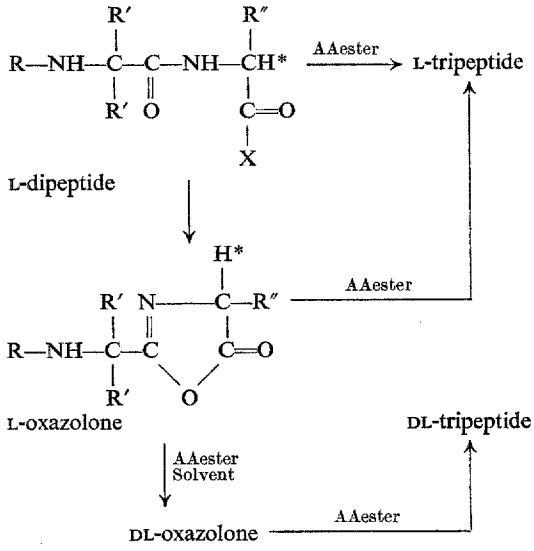
Our model system has the advantage that it represents the most unfavorable situation possible in any peptide coupling, namely, that in which all the acyl peptide is present as oxazolone.

Our studies also show that each amino acid must be examined in our model system

[18] M. Smith, J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.* **80,** 6204 (1958).
[19] H. Schuessler and H. Zahn, *Chem. Ber.* **95,** 1076 (1962).
[20] DeL. F. DeTar, R. Silverstein and F. F. Rogers, Jr., *J. Am. Chem. Soc.* **88,** 1024 (1966).

in a variety of solvents. Situations must also be examined where the nucleophilies are dipeptides and tripeptides.

Our work indicates that if an activated acyl dipeptide is allowed to couple with an amino nucleophile a number of possible routes to tripeptide product are available as shown below.



where * denotes asymmetric carbon atom and X denotes an activated group.

We have demonstrated the unexpected route where optically active oxazolone yields optically active peptide product. The nature of the solvent and the nucleophilicity-basicity ratio of the attacking nucleophile determine to what extent this route is followed. Finally, it should be pointed out that stable, optically active oxazolones provide the physical organic chemist with a very powerful tool to investigate the basicity and nucleophilicity of amine compounds in organic solvents.

EXPERIMENTAL

Instruments and apparatus. The kinetics of racemization were studied on a Model 80 Rudolph polarimeter equipped with a Model 200A Oscillating Polarizer. Monochromatic light was obtained by a prism monochromator equipped with an independent Xenon light source (Hanovia 901B). Center-fill, 2 dm polarimeter tubes with a bore of 3 mm in diameter were used (Polarimeter tube Type 14, Catalogue of O.C. Rudolph and Sons, Caldwell, New Jersey).

The temp of the tube compartment was kept constant at 25 ± 0·2° by a circulating pump connected to a constant temp bath. The voltage applied to the photoelectric cell was controlled by a Keithley voltage supply Model 240.

The oxazolones which we studied do not lend themselves particularly well to uv spectroscopy because in the critical 241 mμ area only a shoulder was observed [uv spectrum (dioxan): of L-phenylalanine peptide oxazolone, 257 mμ (ε 514), 241 mμ (ε 461); uv spectrum (dioxan): of Z–Aib–L–Phe–OH, 257 mμ (ε 440), 241 mμ (ε 257)]. However, some studies were carried out on oxazolone ring-opening in buffered dioxan–water solns using a Perkin–Elmer 350 recording spectrophotometer.

Ring-opening kinetics were studied on a Perkin–Elmer Model 21 IR spectrophotometer using matched 0·5 mm NaCl cells. Our studies were based on the disappearance of the 1825 cm^{-1} absorption of the oxazolones. In Chf and dioxan measurements were readily made. In toluene, however, because of some solvent absorption in this region, a high Gain Control was necessary. Time was recorded on a "Precision Time-it" device calibrated in minutes and hundreds of a minute and sold by Precision Scientific Co. of Chicago, Illinois.

Kinetic procedure

Polarimetry. All kinetics were followed at 589 mμ on solns at 25 ± 0·2° in capped, center-fill, 2 dm tubes which could be adequately filled by 2 ml of soln. Since most of our studies involved L-phenylalanine peptide oxazolone we shall describe the technique used by outlining specific experiments carried out.

Exactly 22·3 mg L-phenylalanine peptide oxazolone were weighed into a 2-ml volumetric flask and the stoppered flask, together with the polarimeter tube and a 2-ml volumetric pipet, were thermostated at 25° for 15 min. An adequate volume of nucleophile at the required concentration in a given solvent was suspended in the 25° constant temp bath. In order to start the reaction 2 ml of the nucleophile soln were pipetted into the volumetric containing the oxazolone. In all cases the oxazolone dissolved instantly, the timer was started and the soln volume adjusted to the fiducial mark by addition of one or 2 drops of nucleophile soln. The reaction soln was quickly transferred to the polarimeter tube and readings were commenced. With practice the first reading could be obtained in from 1·5 to 2·0 min. Subsequent readings were recorded every 0·5, 1, 2, 4 or 10 mins depending on the reaction rate. Generally speaking, results were considered satisfactory if they were reproducible in two different runs using separately prepared lots of the free amino acid ester. The rotation at zero time was found in each case by extrapolating three or four initial readings to zero time.

Certain difficulties were encountered with kinetic studies in the solvents toluene and AcOEt. The tripeptides formed from L-phenylalanine peptide oxazolone and the various amino acid esters are fairly insoluble in these solvents so that usually after 50% to 60% completion of racemization reaction a ppt appeared in the polarimeter tube which prevented further readings being taken.

UV spectrophotometry. As already mentioned not too much use was made of this technique. However, in our studies of the racemization of L-phenylalanine peptide oxazolone in dioxan–water buffered solns it was advantageous to check the amount of ring-opening which had occurred during racemization. This was done by uv measurements at 241 mμ on solns in matched quartz cells with 1 mm spacers.

IR spectrophotometry. The kinetics of the second-order, ring-opening reactions of L-phenylalanine peptide oxazolone by amino acid esters were easily measured by following the disappearance of the strong CO absorption band at 1825 cm^{-1}. Specifically, the procedure was as follows.

An exact amount, 55·75 mg L-phenylalanine peptide oxazolone was weighed into a 5-ml volumetric flask and the stoppered flask was suspended in a 25° constant temp bath. At the same time an adequate volume (10 or 25 ml) of a suitable concentration of amino acid ester in a given solvent was placed in the constant temp bath.

With the nucleophile soln in two matched 0·5 mm NaCl cells the Perkin–Elmer 21 IR spectrophotometer was calibrated for quantitative response in the area 5·5 μ. The soln was removed from the sample cell and the cell cleaned with solvent which was blown off by a dry N jet. By means of a 5-ml volumetric pipet the amino acid ester soln was transferred to the oxazolone reaction flask, the electric timer was started and the volume adjusted to the fiducial mark. The stoppered reaction flask was re-suspended in the constant temp bath and a 0·25 ml sample was transferred, using a 1 ml syringe, to the sample cell and scanning was carried out between 3·5 and 6·5 μ. The time at which the recorder pen reached the max of the 1825 cm^{-1} peak was noted. The sample cell was cleaned out and dried and a fresh aliquot was taken from the reaction flask and scanned. In this manner 12 to 14 readings were readily obtained during the course of the reaction. The absorbance at zero time was determined by extrapolating the initial 3 or 4 readings to zero time. The precipitation of tripeptides in toluene soln did not interfere with IR readings.

Thin-layer chromatography (TLC). Thin-layers (10 × 20 mm.) of Silica Gel H were prepared,[21] activated for 20 min in an oven at 110–120° and stored in a desiccator. We used TLC extensively as a criterion of purity in synthetic work. The developing solvents used ranged from Chf to Chf with from 1% to 10% MeOH added for materials such as blocked amino acids and peptides. For more polar materials with free amino or carboxyl groups the system benzene:dioxan:acetic acid::50:50:x was used. The value of x varied from 1 to 10 depending on the polarity and mol wt of the substance to be chromatographed. In our most commonly used system and the one used in all kinetic studies the value of x was 2.

Samples were applied using an ultra micro pipet about 0·5 inches from one end of the thin-layer. For determination of purity 10 to 25 λ of 10 mg per ml solns were applied. In our kinetic work 5

[21] T. M. Lees and P. J. DeMuria, *J. Chromatog.* **8**, 108 (1962).

samples were applied to each plate using a 10 μl Hamilton syringe. On one side of the thin-layer, 4λ of a solution of L-phenylalanine peptide oxazolone exactly one-half the concentration of the initial reaction concentration of oxazolone was applied. On the other side 4λ of exactly half the total expected concentration of tripeptide in the completed reaction was applied. At time intervals of 2 min 4λ aliquots from the thermostatted reaction mixture were applied to the remaining three positions on each plate. The plates were developed as mentioned using benzene:dioxan:acetic acid:: 50:50:2. In all cases whether for purity determination or for kinetic purposes peptides and amino acid derivatives were detected as described by Mazur.[22] After removal from the development tank each plate was left in the air for 10 to 15 min to allow solvents and AcOH to evaporate. The plate was then placed in a sealed container which contained a 50 ml beaker with 2 or 3 drops of freshly added t-butylhypochlorite and left there for 10 to 15 mins. On removal from the container the plate was left in an air stream in the hood for 15 min and then sprayed with an aqueous soln which was 1% in soluble starch and 1% in KI. Under these conditions compounds containing the group —NH— show up at once as purple spots. Oxazolones and compounds containing the group =N— show up after 10 to 15 min.

The half-time of the coupling reaction was estimated by locating the reaction sample whose tripeptide spot density most closely matched that of the control tripeptide spot. The oxazolone appeared as either a single spot or sometimes as two spots depending on the humidity to which the plate was exposed. This spot or spots, by matching with the control material, could be used to confirm the findings from the tripeptide spots. By making a repeat study and taking a large number of samples in the approximate half-time area it was possible to estimate half-time values to within a few minutes. This technique was used only for reactions carried out in AcOEt for reasons already mentioned.

Correction curves for optically active tripeptides. In the reaction of L-phenylalanine peptide oxazolone with some amino acid esters in certain solvents the nucleophilicity-basicity ratio of the nucleophile was such that the tripeptide formed was only partially racemized. In these cases it was necessary to find a way to measure the contribution of the tripeptide to optical rotation at any given time so that the observed rotation could be corrected to reflect the true oxazolone optical rotation at that time. It was known that an excess of tributylamine instantly racemizes all remaining oxazolone optical activity without any such effect on the tripeptide. A number of reactions were set up as for normal kinetic measurements by weighing exactly 22·3 mg L-phenylalanine peptide oxazolone into 2-ml volumetric flasks and adding the concentration of amino acid ester in a given solvent so that the concentration of the oxazolone and nucleophile in the reaction flask were the same initially. The 2-ml flasks were stoppered and suspended in the constant temp bath for time intervals of approximately 5, 10, 15 and 20 min, respectively. The volumetric flask to be used for the 5 min reading was removed from the bath after about 4½ min and 0·1 ml tributylamine added, the stopper replaced and the flask was shaken. At the instant of shaking the exact time interval was recorded using a stopwatch and later at a convenient opportunity the optical rotation was measured. The other reaction flasks were handled in a similar fashion at the appropriate time intervals. Each of these readings had to be corrected for the presence of an excess of 0·1 ml tributylamine. This correction was made by setting up the reaction using twice the amount of oxazolone and nucleophile solution in a 4-ml volumetric flask and allowing the reaction to proceed to completion of racemization (usually overnight). A reading of the optical rotation was obtained on 2 ml of this soln. The remaining 2 ml were diluted with 0·1 ml tributylamine and again a reading of the optical rotation was made. The ratio of the two readings provided a correction factor for the presence of the excess 0·1 ml tributylamine on the time study readings.

In the case of solvents toluene and AcOEt still another complication arose. On addition of 0·1 ml tributylamine and shaking the volumetric flask the tripeptide began to precipitate very shortly thereafter so that it was impossible to get rotation readings. To circumvent this difficulty the tripeptide correction values in these two solvents were obtained a little differently. An exact amount, 22·3 mg, L-phenylalanine peptide oxazolone was weighed into 4-ml volumetric flasks and 2 ml of the amino acid ester in toluene or AcOEt soln were added using a volumetric pipet. After addition of 0·1 ml tributylamine at a given time and shaking, 2 ml Chf were added. The addition of Chf prevented precipitation of the tripeptide or if this had already occurred the precipitated material was re-dissolved. Of course, it was necessary to correct for the dilution and mixed solvent effect. This correction was made by obtaining a rotation reading on a dilute soln of the Z–Aib–L–Phe–DL–amino acid ester in

[22] R. H. Mazur, *J. Org. Chem.* **28**, 2498 (1963).

question in either AcOEt or toluene. Then 2 ml of this soln were diluted with 2 ml Chf and 0·1 ml tributylamine and another rotation reading was obtained. The ratio of the two readings provided the necessary correction factor.

Determination of per cent retention of optical activity. The calculation of the per cent retention of optical activity following the reaction of L-phenylalanine peptide oxazolone with a given nucleophile in a particular solvent is best illustrated by a specific example. Let us consider the reaction of this oxazolone with ethyl glycinate in Chf.

TABLE 11. PHYSICAL DATA ON HYDROCHLORIDES OF AMINO ACID ESTERS

Ester hydrochloride	M.P.	Lit. M.P.[24]	$[\alpha]_D^{25}$
H–L–Phe–OCH_3	158·5–159·5°	159°	+18·9(c = 4·5, MeOH) +40·0(c = 2·0, EtOH)
H–D–Phe–OCH_3	157–158°	158°	−36·4(c − 1·8, EtOH)
H–DL–Phe–OCH_3	156–158°	158°	—
H–L–Ala–OCH_3	Impure solid	—	+5·9(c = 2·7, EtOH)
H–D–Ala–OCH_3	Impure Solid	—	—
H–DL–Ala–OCH_3	156–158·5°	158–158·5°	—
H–D–ϕgly–OCH_3	199–200°	—	+133·1(c = 1·0, MeOH)
H–L–ϕgly–OCH_3[25]	197–199°	—	+131·0(c = 1·0, MeOH)
H–DL–ϕgly–OCH_3	200–202°	—	—
H–Gly–OEt	143–145°	144°	—
H–Aib–OCH_3	173–175°	—	—

The specific rotation of Z–Aib–L–Phe–Gly–OEt was measured in Chf. A reaction was set up wherein 22·3 mg oxazolone were dissolved in 2 ml 0·03045M ethyl glycinate in Chf and this reaction was allowed to proceed to completion (overnight). To verify completeness of reaction an identical reaction, set up for the same period, was treated with 0·1 ml tributylamine. When the reading obtained on this latter sample was corrected for the presence of 0·1 ml tributylamine it had to agree with the former within ±2%. From these final readings a calculation was made for the specific rotation of the tripeptide present. The ratio of this calculated specific rotation with the measured specific rotation of Z–Aib–L–Phe–OEt gave the per cent retention figure.

Treatment of kinetic data. Integrated forms of standard first- and second-order rate expressions as given by Frost and Pearson[23] were employed where suitable. All other cases were handled as previously described.

The following general formula was used for calculation of the half-life of a second-order reaction

$$t_{1/2} = \frac{2^{n-1} - 1}{a^{n-1}k_n(n-1)}$$

where n = order of reaction, a = concn of reactant of interest and k = rate constant.

Concentrations of oxazolones in solutions. In all kinetic experiments involving L-phenylalanine peptide oxazolone the solns were 0·03045M in this compound initially. In the case of those experiments carried out on L-alanine peptide oxazolone solns were 0·03843M in this material at the start of each reaction.

Fischer esterification of amino acids. In general, 10 g of the amino acid were suspended in 100 ml MeOH or EtOH. Dry HCl gas was passed into the suspension until all the material was in soln. Generally, the soln became quite warm. The solvent was removed under reduced press and the crude product was recrystallized from alcohol–ether. Yields were always 75% or above. The optically active methyl alaninate hydrochlorides did not crystallize in our hands and were obtained as solids by prolonged subjection to high vacuum. Pertinent physical data on the ester hydrochlorides which we prepared are given in Table 11. The literature m.p. values are taken from Greenstein and Winitz.[24]

[23] A. F. Frost and R. G. Pearson, *Kinetics and Mechanism.* Wiley, New York (1958).

[24] J. P. Greenstein and M. W. Winitz, *Chemistry of the Amino Acids*, pp. 929–932. Wiley, New York (1961).

[25] Abbreviation ϕgly represents the phenylglycyl residue.

Preparation of free amino acid esters for kinetic studies

Ethyl glycinate. The procedure of Fischer[26] was used. To a slurry of 12·5 g (90 mmole) ethyl glycinate hydrochloride in 12 ml water, 25 ml ether were added and the flask was cooled in an ice-salt bath. Then 10 ml 33% NaOHaq were added followed by enough K_2CO_3 so that the aqueous layer was saturated. After shaking well and allowing to settle with cooling the ether was removed by decantation. The remaining slurry was twice extracted with 15 ml ether. The ether extracts were combined and dried over $MgSO_4$ for 1 hr. The salt was removed by filtration and the filtrate was transferred in aliquots to a micro-distillation set-up with a small built-in Vigreaux column. The ether was removed under slightly reduced press and the fraction of the remaining liquid which distilled at 30° under 1 mm press was collected (1–2 ml) and stored under N in the freezer. It was always used on the day prepared [lit.[26] b.p. 43–44° under 11 mm press].

Methyl DL-*alaninate.* To a soln of 10 g (71 mmole) of the hydrochloride salt of DL-alanine methyl ester in 10% K_2CO_3aq, solid K_2CO_3 was added until the water soln was saturated. The suspension was extracted 5 times with 10 ml ether. The ether extracts were combined and handled as described for ethyl glycinate. The fraction boiling at 106–108° under atm press was collected (1–2 ml), stored under N in the freezer and used within 3 days.

Methyl DL-*phenylglycinate.* This free ester was obtained in the manner of the alanine ester. The fraction boiling at 96–98° under 0·1 mm press was collected and stored under N in the refrigerator. This ester solidifies in a room at 20°.

Methyl DL-*phenylalaninate.* This material was prepared as described for alanine ester. The fraction boiling at 80° under 0·5 mm press was collected and stored under N in the freezer and used within 3 days.

Methyl α-aminoisobutyrate. Again this ester was prepared as described in the preparation of alanine methyl ester. The fraction boiling at 125–130° under atm conditions was collected and stored under N in the freezer [lit.[27] b.p. 130–138° under atm conditions].

Purification of solvents for kinetic studies

Dioxan. Reagent grade dioxan was refluxed for 48 hr with Na, distilled and stored in a dark closet for not more than 3 weeks.

Chloroform. The procedure recommended by Vogel[28] was followed. A liter of reagent grade Chf was shaken 6 times with 500 ml water, then dried over $CaCl_2$ for 24 hr and distilled. The fraction boiling at 61° was collected and stored in the dark for not more than 3 weeks.

Ethyl acetate. Vogel's procedure[28] was used. One liter of reagent grade AcOEt, 100 ml Ac_2O and a few drops of conc H_2SO_4 were refluxed for 4 hr and fractionated. The distillate was shaken with 25 g K_2CO_3 and re-distilled. The fraction boiling at 77° was stored in a dark closet.

Toluene. This solvent was purified according to Fieser.[29] A liter of toluene was stirred for 1 hr at 30° with 80 ml conc H_2SO_4. The toluene was decanted and the process repeated. The toluene was then distilled and the fraction boiling at 111° was collected and stored in the dark.

Preparation of buffered solutions. Buffer solns were made up according to the Handbook[15] and diluted with dioxan to get the systems mentioned in Table 9.

Preparation of Compounds

1. *2-Cyanoethyldiethylamine.* A soln of 20 ml (300 mmole) cyanoethylene and 10 ml (100 mmole) Et_2NH was refluxed for 3 hr. The excess of cyanoethylene was distilled off and the remaining liquid was distilled under 0·15 mm press at 36°. Dry HCl gas was passed into the distillate to give a faintly yellowish solid which was recrystallized from EtOH ether to give 8·5 g pure white crystals, m.p. 127–129° [lit.[10] m.p. 125–128°]. The free base was regenerated by treating 8·5 g of the hydrochloride with 10 ml sat K_2CO_3aq and extracting 3 times with 10 ml ether. The ether extracts were combined, dried over $MgSO_4$. The salt was removed by filtration and the ether was removed under reduced press. The fraction of the remaining liquid which distilled at 38° under 0·5 mm press was collected (4–5 ml).

[26] E. Fischer, *Ber. Dtsch. Chem. Ges.* **34,** 433 (1901).
[27] M. T. Leplawy, D. S. Jones, G. W. Kenner and R. C. Sheppard, *Tetrahedron* **11,** 39 (1960).
[28] A. I. Vogel, *Practical Organic Chemistry* (3rd Edition) p. 176. Wiley, New York (1962).
[29] L. F. Fieser, *Experiments in Organic Chemistry* (3rd Edition) p. 292. D. C. Heath, Boston (1957).

2. *Benzyloxycarbonylaminoisobutyryl-L-phenylalanyl-DL-alanine methyl ester.* An exact amount, 100 mg, of each of the compounds Z–Aib–L–Phe–L–Ala–OCH_3 and Z–Aib–L–Phe–D–Ala–OCH_3 was weighed and transferred to a small vial together with 3 stainless steel balls. The vial was securely stoppered and shaken in a vibrator for 30 min. The specific rotation of the resultant mixture was was given by $[\alpha]_D^{25}$ −50·8 (c = 1·0, Chf). The theoretical value obtained by calculation from the specific rotations of the two isomers is $[\alpha]_D^{25}$ −50·4 in Chf.

3. *Benzyloxycarbonylaminoisobutyric-DL-phenylalanyl-DL-alanine methyl ester.* This material was prepared by the azide procedure described in the previous paper[1] in 35% yield, m.p. 187–189°. In this reaction the tripeptide precipitated from the AcOEt soln as it formed and was recovered by filtration. (Found: C, 64·24; H, 6·85; N, 9·00. Calc. for $C_{25}H_{31}N_3O_6$: C, 63·96; H, 6·61: N, 8·95% This tripeptide was also isolated from AcOEt solns used in the study of the ring-opening of L-phenylalanine peptide oxazolone by methyl DL-alaninate, m.p. 180–183°. The IR spectra and TLC R_fs of the synthesized and isolated materials were identical.

4. *Benzyloxycarbonylaminoisobutyryl-L-phenylalanyl-DL-phenylglycine methyl ester.* A mechanical mixture was made of the appropriate isomers as described in No. 2, $[\alpha]_D^{25}$ −39·2° (c = 1·0, Chf). The theoretical value obtained by calculation from the specific rotations of the two isomers is $[\alpha]_D^{25}$ −39·3 in Chf.

5. *Benzyloxycarbonylaminoisobutyryl-DL-phenylalanyl-DL-phenylglycine methyl ester.* This material was prepared by the azide procedure described[1] in 30% yield, m.p. 208–210°. (Found: C, 67·43; H, 6·30; N, 8·01. Calc. for $C_{30}H_{33}N_3O_6$: C, 67·79; H, 6·21; N, 7·91%.) A small amount of material isolated from AcOEt solns which were used in the kinetic study of the ring-opening of L-phenylalanine peptide oxazolone by methyl DL-phenylglycinate had m.p. 202–204°. The IR spectra and TLC R_fs on both the synthesized and isolated materials were identical.

6. *Benzyloxycarbonylaminoisobutyryl-DL-phenylalanyl-DL-phenylalanine methyl ester.* This material was prepared by the azide procedure described[1] in 55% yield, m.p. 167–169°. (Found: C, 68·36; H, 6·50; N, 7·78. Calc. for $C_{31}H_{35}N_3O_6$: C, 68·25; H, 6·42; N, 7·70%.) The material isolated from dioxan solns used in the ring-opening studies of L-phenylalanine peptide oxazolone by methyl DL-phenylalaninate[6] had an IR spectrum which was identical with the synthesized material.

7. *Benzyloxycarbonylaminoisobutyryl-DL-phenylalanylglycine ethyl ester.* Product isolated from dioxan solns which were used in the kinetic study of the ring-opening of L-phenylalanine peptide oxazolone by ethyl glycinate had an m.p. 162–163°. Synthesized tripeptide[1] had m.p. 161·5–162·5°. IR spectra and TLC R_fs of synthesized and isolated products were identical.

8. *Benzyloxycarbonylaminoisobutyryl-DL-phenylalanylaminoisobutyric methyl ester.* The tripeptide isolated from toluene solns used in the kinetic study of ring opening of L-phenylalanine peptide oxazolone by methyl aminoisobutyrate had m.p. 170–171·5°. Synthesized material[1] had m.p. 169·5–170·5°. IR spectra and TLC R_f values of synthesized and isolated materials were identical.

9. *Reactions of L-phenylalanine peptide oxazolone with hydrazine hydrate solution.* A dilute soln, approximately 0·03M of 85% hydrazine hydrate in Chf:MeOH::4:1 soln was prepared and 100 mg (0·28 mmole) L-phenylalanine peptide oxazolone[1] were dissolved in 10 ml of this soln. The solvent was removed under reduced press to get a white solid which was taken up in CH_2Cl_2 soln and treated with Norite. After removal of the Norite by filtration the soln was concentrated to small volume and diluted with ether and left standing overnight in the refrigerator. A white solid weighing 35 mg was recovered, m.p. 97–100° with a trace persisting beyond 120°, $[\alpha]_D^{25}$ −57·3° (c = 0·5, Chf). (Found: C, 63·84; H, 6·24; N, 11·75. Calc. for $C_{21}H_{26}N_4O_4$: C, 63·31; H, 6·53; N, 14·07%.) The analyst reported a grey residue of 0·08 mg from a sample of 4·411 mg. If we consider that dihydrazide had been formed the calculated values would be: Calc. for $C_{42}H_{48}N_6O_8$: C, 64·24; H, 6·45; N, 11·29%.) The analytical results indicate that some dihydrazide did form and this high value of the specific rotation bolsters this notion. A Rast mol wt determination gave a value of 471. The calculated value for the hydrazide is 398 and for the dihydrazide 784. Hence, it appears that 20% or more of the product was dihydrazide.

In another reaction 150 mg (0·4 mmole) L-phenylalanine peptide oxazolone in 10 ml MeOH was added to a large excess (1·5 ml) of 85% hydrazine hydrate soln. Removal of the solvent gave a white solid whose physical characteristics were exactly those of Z–Aib–L–Phe–$NHNH_2$ prepared in the traditional manner[1] $[\alpha]_D^{25}$ −33·8° (c = 1·0, Chf). Found: C, 63·24; H, 6·61; N, 13·89. Calc. for $C_{21}H_{26}N_4O_4$: C, 63·31; H, 6·53; N, 14·07%.) A Rast mol wt determination gave a value of 383 as

opposed to the theoretical value 398. The IR spectra of both materials obtained from the reactions of L-phenylalanine peptide oxazolone and hydrazine hydrate were identical.

10. *Reactions of L-alanine peptide oxazolone with hydrazine hydrate solution.* A dilute soln, approximately 0·03M, of 85% hydrazine hydrate in Chf:MeOH::4:1 soln was made up and 203 mg (0·7 mmole) L-alanine peptide oxazolone[1] were added to 20 ml of this soln. The solvent was removed without delay under reduced press and the solid obtained was allowed to precipitate from CH_2Cl_2–ether soln to get 150 mg, m.p. 140–141°, $[\alpha]_D^{25}$ −15·4° (c = 1·1, MeOH). (Found: C, 58·60; H, 6·71; N, 13·54. Calc. for $C_{15}H_{22}N_4O_4$: C, 55·90; H, 6·83; N, 17·39%.) The analyst reported a residue of 0·04 mg from a sample of 4·523 mg. If we assume that dihydrazide had formed the analytical calculation would be: (Calc. for $C_{30}H_{40}N_6O_8$: C, 58·49; H, 6·53; N, 13·54%.) From the elemental analyses and the high specific rotation value it appears that some dihydrazide did form. A Rast mol. wt. determination gave a value of 469. The calculated mol. wt of the hydrazide is 322 and of the dihydrazide 612. Hence, it appears that the product obtained was at least 50% dihydrazide.

A soln of 340 mg (1·1 mmole) L-alanine peptide oxazolone in 25 ml MeOH was added to 1·5 ml 85% hydrazine hydrate soln. The product was recovered by evaporation of solvent and was a brittle white solid at 20° or below which became tacky if allowed to reach higher temps, $[\alpha]_D^{25}$ −7·2° (c = 1·5, Chf), −6·9° (c = 0·5 MeOH).

11. *Benzyloxycarbonylaminoisobutyryl-L-alanine hydrazide.* To a soln of 180 mg (0·56 mmole) Z–Aib–L–Ala–OCH_3 in 2 ml hot MeOH, 0·25 ml 85% hydrazine hydrate were added. Removal of solvent under reduced press yielded a material, hard and brittle at 20° or below that became tacky at 25°. Repeated attempts to obtain crystalline material failed. $[\alpha]_D^{25}$ −7·3° (c = 1·0, MeOH).

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