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The Participation of the Amide Group in the Solvolysis of Phosphoric Acid Esters. II. Phosphotriesters in Neutral Ethanolic and Aqueous Media*

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The syntheses of phosphotriesters of *N*-acylethanolamine, and *N*-acetylserinamide are recorded. Treatment of these triesters in ethanolic potassium acetate at 78° results in the quantitative formation of a Δ^2-oxazoline or dihydrooxazine and a phosphodiester ion. The solvolysis of these triesters proceeds equally well in aqueous media (*p*H 4–7). The finding that, under both sets of conditions, the rate of expulsion of the phosphodiester fragment is independent of potassium acetate concentration or *p*H supports the hypothesis that the facile solvolysis is due to the effective anchimeric assistance of the *un-ionized amide (peptide) function*. In ethanol, formation of diphenylphosphate ion from the serine derivative IVb occurs 1200 times faster than from the simple phosphotriester ethyl diphenylphosphate.

While many of the functional groups located in the amino acid side chains of proteins have been implicated in the catalytic action of enzymes, little direct evidence has been adduced so far to support the possible role of the peptide bond in these processes.

The amide group has long been known to be able to participate in displacement reactions, by nucleophilic attack of either the carbonyl oxygen or the amide nitrogen atom. Much of the early literature has been reviewed by Winstein and Boschan (1950) and more recently by Cohen and Witkop (1961) and Bruice (1962). As a result of attempts to devise model systems for the catalytic center of esteratic and proteolytic enzymes, particular attention has been focused upon intramolecular reactions of amide groups and carboxylic acid derivatives. Bernhard *et al.* (1962) have investigated the unusually rapid alkaline hydrolysis of the β-benzyl esters of carbobenzoxyaspartylserinamide and related compounds. The mechanism of these reactions appears to involve intramolecular participation of the amide anion, leading to the transient formation of succinimide derivatives. Similar cyclic intermediates had earlier been reported to be formed during the alkaline solvolysis of carbobenzoxy-L-aspartamine methyl ester and carbobenzoxy-L-glutamine methyl ester (Sondheimer and Holley, 1954, 1957). In an extension of these studies, Adler *et al.* (1963) have described the conversion of poly-β-benzyl-L-aspartate to polysuccinimide by catalytic amounts of sodium methoxide. On the other hand, poly-γ-benzyl-L-glutamate gave rise to the sodium salt of D,L-2-pyrrolidone-5-carboxylic acid in the presence of stoichiometric sodium methoxide. The solvolysis of the amide bond may itself be accelerated by a neighboring amide function, both under acidic (Cohen and Lipowitz, 1961) and alkaline (Morawetz and Otaki, 1962) conditions.

The study of the influence of the amide (peptide) function in the solvolysis of esters of phosphoric acid is of interest in connection with catalytic mechanisms. Furthermore, information pertinent to the chemistry of phosphoproteins may also be derived. In a previous communication (Zioudrou and Schmir, 1963), the facile solvolysis of certain phosphotriesters in alkaline media was shown to occur via intramolecular participation of a neighboring amide group (in its anionic form). The present report describes the results of a study of the solvolysis in neutral ethanolic and aqueous media of phosphotriesters derived from serine and ethanolamine. Efficient assistance to the solvolytic process has been found to be provided by the *un-ionized amide group*.

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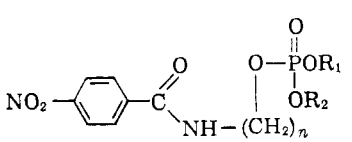
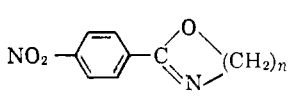
RESULTS

Earlier studies (Zioudrou and Schmir, 1963) have demonstrated the ready transformation of phosphotriesters of type I to heterocyclic compounds II and phosphodiesters, in the presence of alkoxide ion at room temperature. It has now been observed that



I



II

Ia $R_1 = R_2 = C_6H_5$ $n = 2$ IIa $n = 2$
Ib $R_1 = R_2 = C_6H_5CH_2$ $n = 2$ IIb $n = 3$
Ic $R_1 = R_2 = C_6H_5$ $n = 3$
Id $R_1 = C_6H_5$ $n = 2$
R_2 = 2-*p*-nitrobenzamidoethyl

treatment of such phosphotriesters with ethanolic potassium acetate at 78° results in the formation of the same products. High yields (80–95%) of Δ^2-oxazoline IIa and of the dihydroöxazine IIb have been isolated from triesters Ia and Ic, respectively; in both cases, diphenyl hydrogen phosphate was also obtained in

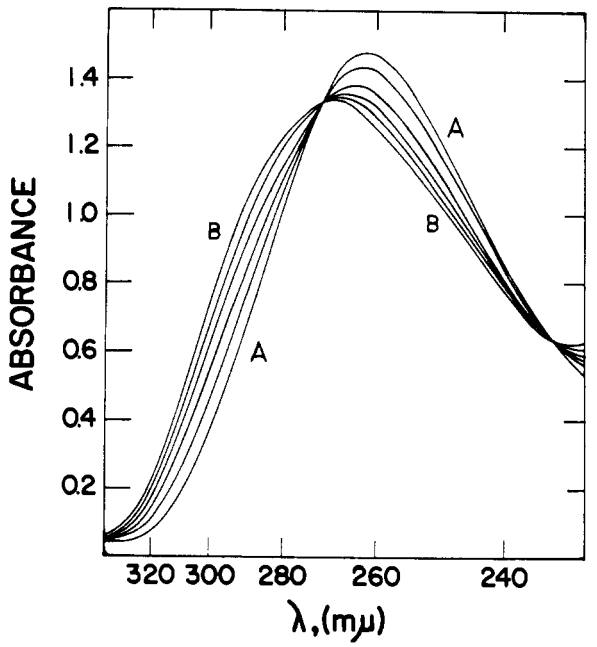
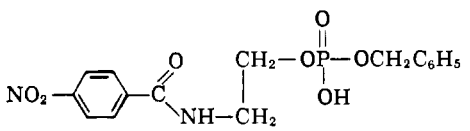


FIG. 1.—Spectral changes of triester Ia in ethanolic potassium acetate at 78°. Ia at 10^{-4} M; potassium acetate at 10^{-3} M. (A) Spectrum at zero time; (B) after 4 hours.

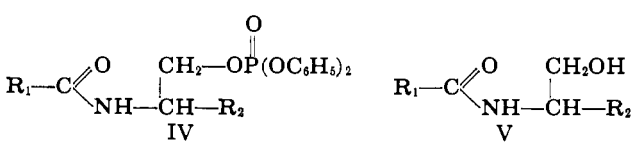
yields of 70–87%. Correspondingly, Ib afforded the oxazoline IIa and dibenzyl hydrogen phosphate, accompanied by a by-product, for which structure III is proposed. Elementary analysis, ultraviolet spectrum,



III

and neutralization equivalent are in agreement with this assignment.

Treatment of triester IVa under the same condition led to the isolation of diphenyl hydrogen phosphate and of the *acyclic alcohol* Va. It is probable (see Discussion) that the expected cyclic product, 2-



IV V

IVa R_1 = $C_6H_5CH_2OCONHCH_2$, R_2 = H
IVb R_1 = CH_3, R_2 = $CONH_2$
Va R_1 = $C_6H_5CH_2OCONHCH_2$, R_2 = H
Vb R_1 = CH_3, R_2 = $CONH_2$

carbobenzoxyaminomethyl-Δ^2-oxazoline, suffered hydrolysis in the course of the isolation procedure.

Ultraviolet spectroscopy furnished a convenient method of determining the rates of cyclization of triesters I in ethanolic potassium acetate solution. The spectral transformation attendant upon the conversion of Ia to oxazoline IIa and diphenyl hydrogen phosphate is shown in Figure 1. The rate of increase of absorbance at 310 mμ was employed to calculate first-order rate constants for the cyclization of triesters Ia, Ic, and Id in the presence of varying concentrations of potassium acetate (Table I). The cyclization of Ia in ethanolic solution proceeds equally well *in the absence of potassium acetate*. The rate constant reported in Table I was obtained from the initial 40% of the reaction, since the oxazoline was found to be unstable to prolonged reaction periods. Disappearance of the oxazoline may be a consequence either of the ready hydrolysis of the oxazolinium ion (Fry, 1949; Porter *et al.*, 1960; Martin and Parcell, 1961) because of the adventitious presence of traces of water or of conversion of the oxazolinium ion to *N*-(2-ethoxyethyl)-*p*-nitrobenzamide. Ring-opening by nucleophilic attack upon position 5 of the oxazolinium ion is well known (cf. Fry, 1950). In the case of Ib, the rate constant derived from the rate of change of the absorbance at 310 mμ consists of the sum of the first-order rate constants for two parallel first-order reactions: (1) cyclization to oxazoline; (2) formation of the diester III as a result of attack on the triester by solvent. The rate constants for these competitive reactions are in the ratio of 2.1:1.

TABLE I
RATE OF CYCLIZATION IN ETHANOL[a]

Compound[b]	[CH_3COOK] × 10^3 (M)	t (°C)	$k \times 10^3$ (min^{-1})
Ia[c]	0.0	78	8.6
	1	78	9.9
	2	78	9.8
	5	78	10.1
	10	78	9.4
	1[d]	78	9.3
	2	56	1.24
	2	30	0.06
Ib	2	78	0.72[e]
Ic	2	78	2.2
Id	2	78	10.5
VI	2	56	46
	2	30	3.1

[a] Rate measured by increase in absorbance at 310 mμ. [b] All compounds except Id at about 2×10^{-4} M; Id at 1.3×10^{-4} M. [c] $\Delta H‡$ 21.8 kcal/mole; $\Delta F‡$ (30°) 25.95 kcal/mole; $\Delta S‡$ (30°) − 13.7 eu. [d] Containing 9×10^{-3} M LiCl. [e] Rate constant for disappearance of triester is 1.06×10^{-3} min^{-1}.

The solvolysis in ethanolic potassium acetate of triesters IVa and IVb seemed of particular interest since, in the one instance (IVa), the *p*-nitrobenzoyl function of triesters I is replaced by the peptidelike carbobenzoxyglycyl group, while compound IVb may be considered the prototype of phosphotriesters derived from serine residues incorporated in peptide linkage. With both IVa and IVb, the only observable spectral changes during the solvolysis resulted from the formation of diphenyl phosphate ion. The marked increase

TABLE II
SOLVOLYSIS OF TRIESTERS IVA AND IVB, AND OF ETHYL DIPHENYLPHOSPHATE IN ETHANOL[a,b]

Compound[c]	[CH_3COOK] × 10^3 (M)	[LiCl] × 10^3 (M)	$k \times 10^3$ (min^{-1})
IVa	5	—	12.9
	100	—	13.4
	—	5	12.1
IVb	10	—	43.0
	—	100	45.0
Ethyl diphenyl phosphate	5	—	0.039[d]

[a] At 78°. [b] Rate measured by increase of absorbance at 270 or 272 mμ. [c] Triesters at $1–1.4 \times 10^{-3}$ M. [d] Rate constant for displacement of diphenylphosphate ion is 0.037×10^{-3} min^{-1}.

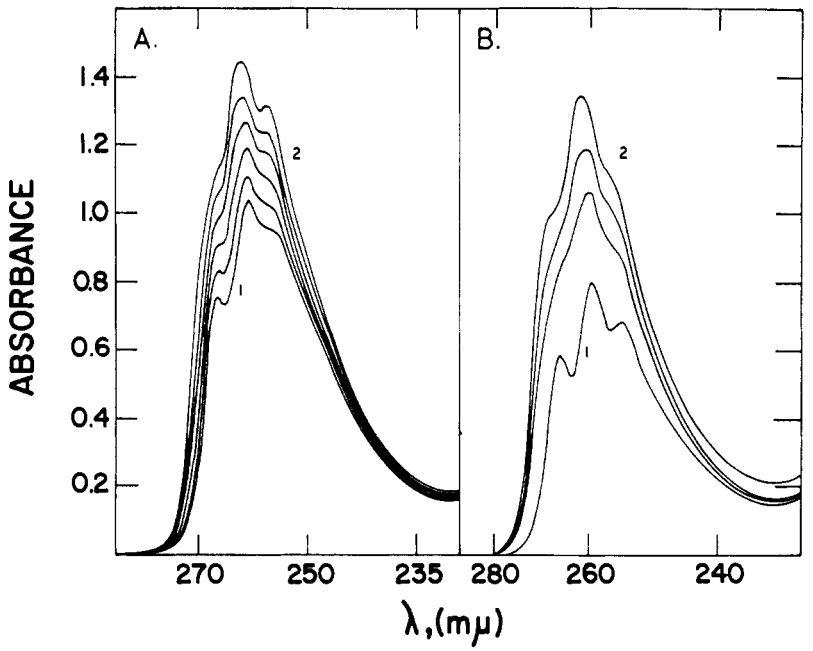


FIG. 2.—(A) Solvolysis of IVa (1.5×10^{-3} M) in 25% ethanol-water at pH 7.4 (78°). (1) Spectrum at zero time; (2) after 6 hours. (B) Solvolysis of IVb (1.36×10^{-3} M) in 25% ethanol-water at pH 4.2 (78°). (1) Spectrum at zero time; (2) after 2 hours.

in absorbance at 270 or 272 mμ provided a simple and rapid means of studying the kinetics of the appearance of diphenyl phosphate ion from these triesters. The results of these experiments are summarized in Table II.

The solvolysis of IVa and IVb was also studied in aqueous buffered solutions at 78°. The spectral consequences of the formation of diphenyl phosphate ion are illustrated in Figure 2. First-order rate constants for the solvolytic processes in the pH range 3.4–7.4 were obtained from the absorbance increases at 270 mμ, and are listed in Table III. In the case of IVb, the solvolytic products consisting of *N*-acetyl-DL-serinamide Vb and diphenyl hydrogen phosphate were isolated in a preparative experiment.

When the cyclization of Ia was studied in aqueous solution, the change in absorbance at 310 mμ was found to consist of a rapid increase followed by a slower decrease (Figure 3). It was assumed that the solvolytic process involved cyclization of Ia to oxazoline IIa, followed by hydrolysis of IIa to a mixture of *N*- and *O*-*p*-nitrobenzoyl ethanolamine (Martin and Parcell, 1961). Rate constants k_1 (for cyclization) and k_2 (for oxazoline hydrolysis) were calculated from the absorbance data at four different pH values (Table IV). These values of k_1 and k_2 were employed to compute the predicted variation of absorbance as a function of time and the theoretical curves were compared to the absorbance data (Figure 3). The validity of the derived values of k_2 was further supported by the determination of the rates of hydrolysis of synthetic oxazoline in the same pH range (Table V). Since k_2 is strongly pH-dependent, while k_1 is essentially independent of pH, the time t_{max} and the extent β_{max} of maximum accumulation of the oxazoline intermediate will also vary with pH. Values for t_{max} and β_{max} for each pH were calculated from the equations that follow (Frost and Pearson, 1961b), where $\kappa = k_2/k_1$ and are listed in Table IV.

$$k_1 t_{max} = \frac{1}{\kappa - 1} \ln \kappa \qquad \beta_{max} = \kappa^{\kappa/(1-\kappa)}$$

TABLE III
SOLVOLYSIS OF TRIESTERS IVA AND IVB, AND OF ETHYL DIPHENYLPHOSPHATE IN AQUEOUS SOLUTION[a,b]

Compound[c]	pH[d]	$k \times 10^3$ (min^{-1})
IVa	7.4	23.6
	6.4	25.2
	5.0	23.4
	4.2	24.0
	3.4	22.6
IVb[e]	7.4	56.5
	6.4	50.5
	4.2	48.5
	6.4[f]	6.2
	6.4[g]	0.32
Ethyl diphenylphosphate	7.4	0.32[h]
	5.0	0.33

[a] At 78° in 25% ethanol-water. [b] Rate measured by increase in absorbance at 270 mμ. [c] Triesters at 1.3–1.6 × 10^{-3} M. [d] pH determined at 78°. For composition of buffers, see Experimental. [e] $\Delta H^{\ddagger}$ 21.65 kcal./mole; $\Delta F^{\ddagger}$ (30°) 24.9 kcal/mole; $\Delta S^{\ddagger}$ (30°) −10.8 eu. [f] At 56°. [g] At 30°. [h] Rate constant for displacement of diphenylphosphate ion is 0.31 × 10^{-3} min^{-1}.

TABLE IV
SOLVOLYSIS OF IA IN AQUEOUS SOLUTION[a,b,c]

pH[d]	k_1[e] × 10^3 (min^{-1})	k_2[f] × 10^3 (min^{-1})	t_{max} (min[g])	Oxazoline$_{max}$ (%[h])
7.4	14.7	1.2	187	80.0
6.4	14.6	1.85	162	76.2
5.6	14.3	3.0	139	66.0
5.0	14.4	8.8	88	46.2

[a] At 78° in 25% ethanol-water. [b] Ia at 1.5–2 × 10^{-4} M. [c] Rates measured by change in absorbance at 310 mμ. [d] pH determined at 78°. [e] Rate constant for cyclization to oxazoline. [f] Rate constant for hydrolysis of oxazoline. [g] Time of maximum accumulation of oxazoline. [h] Fraction of Ia present as oxazoline at time of maximum accumulation.

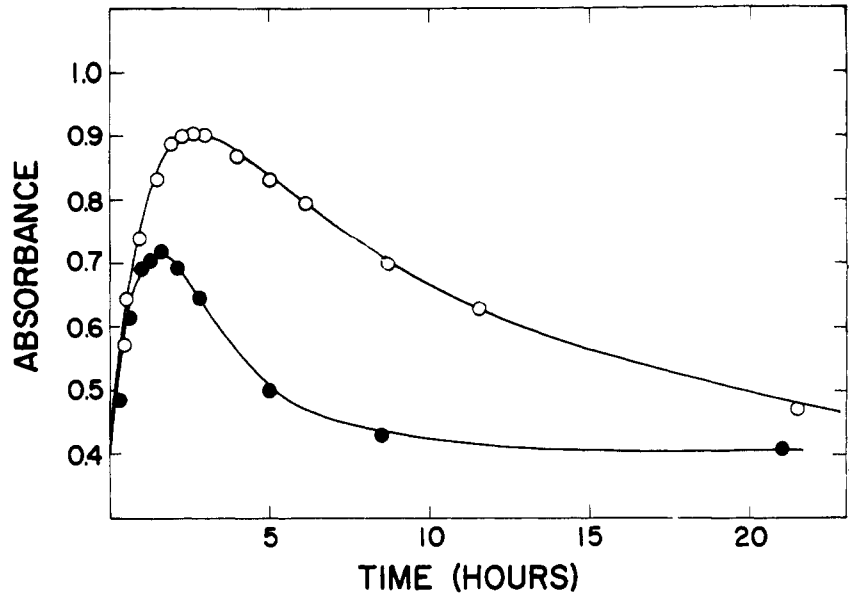


FIG. 3.—Solvolysis of Ia (1.65×10^{-4} M) in 25% ethanol-water at 78°; absorbance measured at 310 mμ. ○, *p*H 6.4; ●, *p*H 5.C. Solid lines are theoretical curves computed as described in Experimental.

To provide a quantitative basis for the evaluation of the reactivity of triesters I and IV, the rates of solvolysis of the simple phosphotriester ethyl diphenylphosphate were determined in ethanolic (Table II) and in aqueous (Table III) solution. Under these conditions, ethyl diphenylphosphate yields largely diphenylphosphate ion (95%), but also about 5% of free phenol. Presumably, the former product results from solvent attack with carbon-oxygen cleavage (see the neutral hydrolysis of trimethyl phosphate; Blumenthal and Herbert, 1945; Barnard, *et al.*, 1955) and the latter results from nucleophilic displacement on phosphorus.

DISCUSSION

Mechanism of the Cyclization Reaction.—The course of the solvolysis of triesters of type I in ethanolic potassium acetate or in aqueous buffered media is depicted in Figure 4A for triester Ia. It is proposed that the cyclization reaction is a consequence of intramolecular nucleophilic displacement by the *un-ionized amide group* upon the alkyl carbon of the triester. The fate of the intermediate cyclic product IIa varies with the solvolytic medium: in ethanolic potassium acetate solution IIa accumulates, while in the absence of the salt IIa undergoes further slow conversion to acyclic products. In aqueous solution hydrolysis of IIa ensues. Completely analogous mechanisms may be written for triesters Ib, Ic, and Id.

TABLE V
HYDROLYSIS OF 2-*p*-NITROPHENYL-Δ^2-OXAZOLINE[a,b,c]

*p*H[d]	$k \times 10^3$ (min^{-1})
7.7	1.1
7.4	1.2
6.4	2.0
5.6	3.0
5.0	8.8
4.4	25.1

[a] At 78° in 25% ethanol-water. [b] Oxazoline at $1.4–1.5 \times 10^{-4}$ M. [c] Rate measured by decrease in absorbance at 310 mμ. [d] *p*H determined at 78°. For composition of buffers, see Experimental.

The following experimental evidence supports the postulated mechanism: (1) The oxazoline IIa and the oxazine IIb have been isolated in high yield from triesters Ia, Ib, and Ic in ethanolic potassium acetate. (2) The rate of cyclization of Ia in ethanol is independent of the concentration of added potassium acetate in the range $1–10 \times 10^{-3}$ M salt and proceeds almost equally as fast in the complete absence of potassium acetate (Table I). The slight diminution in rate in the latter case may be due to the low ionic strength of the medium. (3) The intermediacy of the oxazoline IIa in the aqueous solvolysis of Ia has been detected spectroscopically. The rate of disappearance of the intermediate was found to be identical to that of synthetic IIa at four different *p*H values. The rate constant k_1 for cyclization is independent of *p*H in the range 5.0–7.4, in agreement with the proposed participation of the neutral amide function.

In a classic paper, Winstein and Boschan (1950) have discussed a number of instances of assistance to solvolysis provided by the un-ionized amide group. More recently, the neutral solvolyses of 2-benzamidoethyl bromide and of 2-benzamidoethyl *p*-toluenesulfonate have been explained on the basis of an analogous mechanism (Heine, 1957; Scott *et al.*, 1957). The highly specific cleavage of methionine-containing polypeptides induced by cyanogen bromide or other alkylating agents probably occurs via participation of the neutral amide species (Witkop, 1961). Synthetic application of the principle of neutral amide displacement has been described by Peter *et al.* (1963), in the preparation of *N*-methyl amino acids. Scott *et al.* (1957) have demonstrated that more complex displacing groups, such as the ureido and urethano function, may also act via a neutral mechanism.

It appears therefore that phosphotriesters of type I may undergo intramolecular nucleophilic displacement either via the amide anion (Zioudrou and Schmir, 1963) or via the un-ionized amide function. This functional duality of the amide group has been noted previously (Heine, 1957; Scott *et al.*, 1957; Stirling, 1960; Bruice, 1962).

Several additional features of the cyclization process may be noted on inspection of the data of Table I. The rate of formation of the five-membered oxazoline

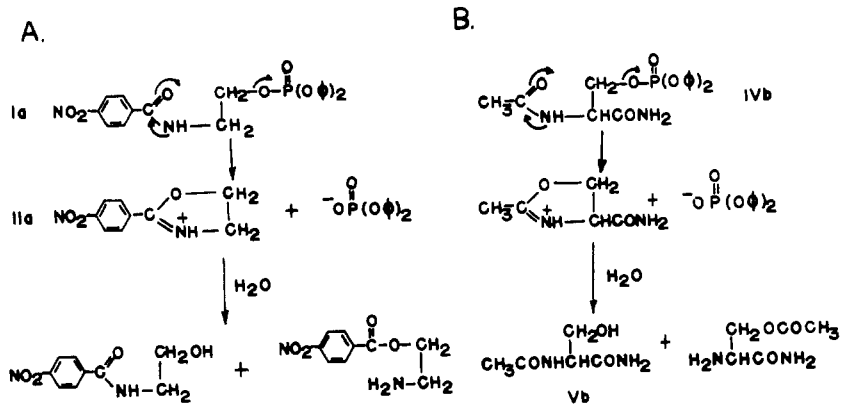


FIG. 4.—Mechanism of solvolyses of phosphotriesters I and IV.

from Ia is only about five times faster than the rate of cyclization to the six-membered oxazine (from Ic). The influence of ring size on the rates of nucleophilic ring closures is extremely variable: in bromide-ion displacement by the neutral urethano function, the five-membered ring is formed 20 times faster than the six-membered one (Scott *et al.*, 1957); Bruice and Benkovic (1963) have pointed out that the ratios of the rate constants for the formation of the five-membered ring to that for the formation of the six-membered ring have been found to be 2.3 for attack on the ester carbonyl group by the trialkylamino function, 230 for ring closure of succinate and glutarate monoesters, and 800 for cyclization of ω-aminoalkyl bromides.

The nature of the leaving group plays a marked role in determining the rate of the cyclization process. Thus, diphenyl phosphate ion is expelled about thirteen times faster than dibenzyl phosphate ion (compare Ia and Ib). Measurement of the rate of cyclization of 2-*p*-nitrobenzamidoethyl *p*-toluenesulfonate (VI) showed that the leaving tendency of the *p*-toluenesulfonate ion exceeds that of the diphenylphosphate ion by a factor of 30–40 (Table I).

As mentioned above, the cyclization of Ib is accompanied by simultaneous solvent debenzylation to III, which is stable to further reaction. Other benzyl esters of phosphoric acid (dibenzyl hydrogen phosphate, tetrabenzyl pyrophosphate) have been shown to undergo carbon-oxygen cleavage under neutral conditions (Kumamoto and Westheimer, 1955; Dudek and Westheimer, 1959).

The Solvolysis of IVa and IVb.—The favored mechanism for the solvolysis of triesters IVa and IVb is shown in Figure 4B, in complete analogy to the mechanism of Figure 4A. While the existence of the intermediate oxazoline was not directly demonstrated, the following evidence has been adduced in support of the proposed pathway: (1) The rates of formation of diphenylphosphate ion (presumably reflecting the rates of cyclization) from IVa and IVb in ethanol (Table II) or aqueous solutions (Table III) differ from the corresponding rates of cyclization of Ia by a factor of less than 3. (2) The ΔH^{++} for cyclization of Ia in ethanol is 21.8 kcal/mole while ΔH^{++} for solvolysis of IVb in ethanol-water is 21.6 kcal/mole. (3) The alcohol Va has been isolated from the solvolysis of IVa in ethanolic potassium acetate. Formation of diphenyl hydrogen phosphate via nucleophilic displacement by ethanol would have been expected to produce the ether *N*-2-ethoxyethyl-*p*-nitrobenzamide. *N*-Acetyl-DL-serinamide Vb was isolated from IVb in aqueous solution. (4) The rate of solvolysis of IVa in ethanol is independent of potassium acetate concentration. Replacement of potassium acetate by lithium chloride has no effect. Both triesters release diphenyl phosphate ion in aqueous solution at a rate independent of *p*H in the range 3.4–7.4. (5) The formation of diphenylphosphate ion from IVb in ethanol is 1200 times faster than the corresponding reaction of the related simple triester ethyl diphenyl phosphate (Table II). The relative rates in buffered solution are about 160:1. The solvolysis of ethyl diphenylphosphate probably involves nucleophilic attack of solvent upon the alkyl carbon atom. The large rate differences are strongly suggestive of the operation of another mechanism in the solvolysis of IVa and IVb. It may be noted that the triesterified phosphoserine derivative IVb is the most reactive phosphotriester of this study. Its half-life in aqueous solution (*p*H 6.4) is 36 hours at 30° and 14 minutes at 78°.

An alternative mechanism for the formation of diphenylphosphate ion from IVb which could not be excluded a priori consists of a base-catalyzed β-elimination, resulting in the production of α-acetamidoacrylamide. Acrylic acid derivatives exhibit considerable light absorption in the region of 240 mμ (Riley *et al.*, 1957; Greenstein and Winitz, 1961) with molar extinction coefficients of the order of 4000–5000. No significant increase in absorbance in the 230–240 mμ range was observed during the solvolysis of IVb from *p*H 4.2 to 7.4 (Figure 2). To eliminate the possibility that accumulation of the acrylamide was not seen because of its rapid subsequent hydrolysis (see Bergmann and Grafe, 1930; Patchornik and Sokolovsky, 1962), the stability of α-acetaminoacrylic acid (*N*-acetyldehydroalanine) was determined under the solvolytic conditions of this study (Table VII-Experimental). While strongly *p*H dependent, the hydrolytic behavior of this compound is such that the acrylamide should accumulate, were it formed.[1] Finally, hydrolysis of the acrylamide would be expected to yield acetamide and pyruvic acid amide (see Riley *et al.*, 1957), and not the alcohol Vb which was in fact isolated. It is therefore considered extremely unlikely that the solvolysis of IVb proceeded via a β-elimination reaction.

The ambient nature of the amide function suggested the possibility that nucleophilic displacement on phosphorus by the nitrogen atom of triesters I and IV might occur, with release of free phenol. Since

[1] It is realized that the stability of *N*-acetyldehydroalanine may be affected by the presence of the ionized carboxyl group. It is unlikely, however, that the corresponding amide would become so reactive that it would go undetected, especially at *p*H 7.4, where the acid has a half-life of 47 days at 78°.

TABLE VI
ISOLATION OF PRODUCTS

Compound	Reaction Time[a] (hours)	Products (% yield)		
			Diphenyl Hydrogen Phosphate	Free Phenol
Ia	14	IIa, 81	70	0.8
Ic	40	IIb, 80	87	1.2
IVa	12	Va, 93	70	0.6

[a] In 0.2 M ethanolic potassium acetate at 78°.

measurement of phenol upon completion of solvolysis revealed the presence of only traces of free phenol (Table VI), this alternative intramolecular process does not appear to take place in the systems described in this communication.

EXPERIMENTAL

All melting points are uncorrected. Microanalyses were performed by Dr. S. M. Nagy (Massachusetts Institute of Technology). Ultraviolet spectra were determined on a Perkin-Elmer Model 350 recording spectrophotometer.

The following triesters were prepared according to the general method described in a previous paper (Zioudrou and Schmir, 1963) by phosphorylation of *N*-(2-hydroxyethyl)- or *N*-(3-hydroxypropyl)-*p*-nitrobenzamide with the appropriate phosphorochloridate in pyridine at low temperature: *N*-*p*-nitrobenzoyl-*O*-diphenylphosphoryl ethanolamine (Ia), *N*-*p*-nitrobenzoyl-*O*-dibenzylphosphoryl ethanolamine (Ib), *N*-*p*-nitrobenzoyl-*O*-diphenylphosphoryl-3-amino-propanol-1 (Ic), and di-(2-*p*-nitrobenzamidoethyl) phenyl phosphate (Id). 2-*p*-Nitrobenzamidoethyl *p* toluenesulfonate (VI), 2-*p*-nitrophenyl-Δ^2-oxazoline (IIa), 2-*p*-nitrophenyl-5,6-dihydro-1,3-oxazine (IIb), ethyl diphenyl phosphate, dibenzyl hydrogen phosphate and diphenyl hydrogen phosphate were materials used in the previous study also.

N-(*N*-Carbobenzoxyglycyl)-ethanolamine (Va)

This substance was prepared by the general method of Woodward *et al.* (1961) (procedure A) and by the *p*-nitrophenyl ester method (see Iselin *et al.*, 1957) (procedure B).

Procedure A.—*N*-Carbobenzoxyglycine (4.2 g, 20 mmoles) (Bergmann and Zervas, 1932) was dissolved in 50 ml of anhydrous acetonitrile in the presence of 3 ml (20 mmoles) of anhydrous triethylamine. To the solution was added 5.04 g (20 mmole) of *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Pilot Chemicals), and the mixture was stirred in the cold for 30 minutes and an equal length of time at room temperature, during which time all reactants went into solution. After addition of a solution of 1.22 g (20 mmoles) of ethanolamine (Matheson, Coleman and Bell) in 10 ml of acetonitrile, the reaction mixture was stirred for 18 hours. The solvent was removed *in vacuo* and the residue was triturated with 150 ml of cold water and chilled. The crystalline product (3.5 g) was collected by filtration, washed with cold water, and air-dried. The aqueous filtrate was extracted with 3 × 50 ml of ethyl acetate, the organic phase was dried over $MgSO_4$, and the solvent was removed *in vacuo*, yielding a crystalline residue (0.7 g). The combined products (4.2 g, 84%, mp 111–113°) were recrystallized from chloroform, the mp increasing to 113–115°. (Reported 113–114°, Ben-Ishai, 1956).

Procedure B.—*N-Carbobenzoxyglycine p-Nitrophenyl Ester.* To a solution of 10.45 g (0.05 mole) of carbobenzoxyglycine and 6.95 g (0.05 mole) of *p*-nitrophenol in a mixture of 75 ml of methylene chloride and 12 ml of tetrahydrofuran was added 10.3 g (0.05 mole) of *N,N′*-dicyclohexylcarbodiimide. The reaction mixture was stirred at room temperature for 4 hours, following which the precipitated *N,N′*-dicyclohexylurea was removed by filtration. After removal of the solvent *in vacuo*, the solid residue was crystallized from benzene, yielding 9.8 g (59% of theory) of carbobenzoxyglycine *p*-nitrophenyl ester, mp 125–128° (reported mp 128°; Farrington *et al.*, 1957). Addition of cyclohexane to the mother liquor afforded another 2.85 g of product. Total yield, 77%.

A solution of 15 g (0.046 mole) of carbobenzoxyglycine *p*-nitrophenyl ester and of 6.1 g (0.10 mole) of ethanolamine in 65 ml of tetrahydrofuran was kept at room temperature for 3 hours. The solvent was removed *in vacuo* and the crystalline residue was dissolved in 350 ml of water. The yellow solution was stirred for 1 hour with the mixed ion-exchange resin Rexyn IRG-501 (Fisher Scientific Co.) to remove ethanolamine and *p*-nitrophenol. Upon concentration of the nearly colorless supernatant solution to a small volume, and cooling, colorless crystals deposited: 5.47 g (yield 47%), mp 114–115°, melting point undepressed on mixture with the product of method A.

N-Acetyl-DL-serinamide

N-Acetyl-DL-serinamide was prepared from LD-serine methyl ester hydrochloride by the procedure of Rothstein (1949), but without distillation of the intermediate *N,O*-diacetyl-DL-serine methyl ester, and melted at 138–140° (reported 138–139°, Rothstein, 1949).

N-(*N*-Carbobenzoxyglycyl)-*O*-diphenylphosphoryl Ethanolamine (IVa)

To a solution of 2.52 g (10 mmole) of Va in 12 ml of anhydrous pyridine kept at −15° was added in one portion 3 ml of diphenyl phosphorochloridate. The reaction mixture was kept for 1 hour in the cold and then allowed to reach room temperature during 1.5 hours. It was poured into 150 ml of an ice-water mixture and kept for an hour at 5°, during which time the triester precipitated in the form of a heavy gum. The turbid supernatant liquid was decanted and the gum was triturated with cold water to remove traces of pyridine. Finally, it was dissolved in 25 ml of cold ethanol, and crystallized by the addition of ice-cold water to turbidity. After 6 hours in the ice chest, the crystals (3.8 g, 78%) were collected, dried over P_2O_5 *in vacuo*, and melted at 63–64°. Recrystallization from ethanol-water or methylene chloride–petroleum ether did not change the mp.

Anal. Calcd. for $C_{24}H_{25}N_2O_7P$ (484.46), C, 59.40; H, 5.00; N, 5.70; P, 6.40. Found: C, 59.10; H, 5.32; N, 6.00; P, 6.40. λ_{max} at 267, 261, and 257 mμ; ϵ at 261 mμ 870 (ethanol).

N-Acetyl-*O*-diphenylphosphoryl-DL-serinamide (IVb)

N-Acetyl-DL-serinamide (910 mg, 6.2 mmoles) was dissolved in a mixture of 20 ml of anhydrous pyridine and of 3 ml of anhydrous *N,N*-dimethylacetamide. The solution was cooled at −15° and 1.8 ml of diphenylphosphorochloridate was added; it was then kept for 1 hour in the cold and for 2 hours at room temperature. The yellow solution was concentrated *in vacuo* at 15° and triturated with 100 ml of ice-water. The crystalline material (180 mg) which deposited after 1 hour in the cold was collected and dried; mp 47–49°.

After recrystallization from methanol-water in the cold it melted at 49–50°. It was identified as triphenyl phosphate[2] by mixed mp with authentic material and comparison of infrared spectra in chloroform.

Anal. Calcd. $C_{18}H_{15}O_4P$ (326.7): C, 66.3; H, 4.54; P, 9.52. Found: C, 67.3; H, 5.03; P, 9.6. ϵ at 261 mμ 1200 (ethanol).

The clear filtrate (100 ml) was extracted with 5 × 20 ml of chloroform followed by extraction with 6 × 20 ml of ethyl acetate. The extracts were combined and dried over $MgSO_4$, and the solvents were removed *in vacuo*. The thick sirupy residue was dissolved in 10 ml of cold methanol, traces of insoluble material were removed by filtration, and ice-cold water was added to turbidity. Crystallization was induced by scratching and chilling overnight in the ice chest. The crystals (1.2 g, 52%) were collected, washed with cold water, and dried over P_2O_5 *in vacuo;* mp 122–125°. After two recrystallizations from methanol-water the product melted at 132–133°.

Anal. Calcd. for $C_{17}H_{19}N_2O_6P$ (378.32): C, 54.01; H, 5.07; N, 7.40; P, 8.20. Found: C, 53.95; H, 4.98; N, 7.10; P, 8.12. ϵ at 261 mμ 710 (ethanol).

Isolation and Characterization of Products

Phosphotriesters Ia, Ic, and IVa in Ethanolic Potassium Acetate Solution.—Solutions of the triesters (1–2 mmoles) in 50 ml of 0.2 M ethanolic potassium acetate were heated for appropriate periods of time (Table VI) by immersion in a glycerol bath maintained at 78°, with exclusion of atmospheric moisture. At the end of the reaction, the solvent was removed *in vacuo*, the residue was triturated with 10–15 ml of water and chilled for 15 minutes, and the crystalline oxazoline IIa or oxazine IIb was collected by filtration and dried. The products were recrystallized from hot ethanol and characterized by comparison of mp, mixed mp, and ultraviolet and infrared spectra to those of the authentic materials.

In the case of triester IVa, the suspension resulting from addition of 20 ml of water to the residue was extracted with 5 × 20 ml of ethyl acetate. The extracts were dried over $MgSO_4$, the solvent was removed *in vacuo*, and carbobenzoxyglycyl ethanolamine (Va) was obtained by crystallization from hot chloroform with a few drops of petroleum ether. The mp and infrared spectrum of the product were identical to those of authentic material.

Prior to the isolation of diphenyl hydrogen phosphate the aqueous filtrates were brought to a volume of 20–30 ml, and free phenol formed during the reaction was estimated colorimetrically using an aliquot of 0.1–0.2 ml, according to the method of Folin and Ciocalteu (1927). The isolation of diphenyl hydrogen phosphate was achieved by acidification of the aqueous solution with concentrated HCl in the cold. Yields of all products are detailed in Table VI.

N-p-Nitrobenzoyl-O-dibenzylphosphoryl Ethanolamine (Ib) in Ethanolic Potassium Acetate.—A solution of 1.9 g (4 mmoles) of phosphotriester Ib in 100 ml of 0.2 M ethanolic potassium acetate was kept at 78° for 75 hours with protection from atmospheric moisture. The solvent was removed *in vacuo* and the residue was triturated with 25 ml of cold water and chilled. Crystalline material (308 mg, 40%) was collected by filtration, dried, and melted at 172–177°. Recrystallization from hot acetone raised the mp to 177–178°; mixed mp with authentic 2-*p*-nitrophenyl-Δ^2-oxazoline IIa showed no depression.

[2] The unexpected formation of triphenyl phosphate in approximately 10% yield (based on phosphorochloridate) is under investigation.

The aqueous filtrate was extracted with 3 × 15 ml of diethyl ether; evaporation of the ethereal phase to dryness afforded 35 mg of an oil with a strongly aromatic odor, presumably ethyl benzyl ether. Traces of ether dissolved in the aqueous layer were removed *in vacuo* and the aqueous solution was acidified in the cold with concentrated HCl to Congo reaction. Addition of a few ml of acetone to the resulting emulsion yielded a homogeneous solution which was chilled overnight. The crystalline deposit (620 mg, 41%) was collected by filtration, washed thoroughly with water, dried *in vacuo* over P_2O_5, and melted at 130–133°. After two recrystallizations from hot acetone, the product (*p*-nitrobenzamidoethyl benzyl hydrogen phosphate, III) melted at 135–137°.

Anal. Calcd. for $C_{16}H_{17}N_2O_7P$ (380.3): C, 50.5; H, 4.24; N, 7.36; P, 8.16. Found: C, 50.52; H, 4.52; N, 7.76; P, 8.20. Neutralization equivalent, 389.

The filtrate obtained after isolation of the diester III was further acidified with concentrated HCl and kept in the ice chest. A crystalline material (470 mg) sintering at 77° and melting at 110°, was collected and dried. Fractional crystallization from acetone afforded another 80 mg of diester III, mp 135–137°. The mother liquor was evaporated to dryness and the residue was crystallized by dissolving it in 1.5 ml of acetone and adding 6 N HCl in the cold. After 2 days in the ice chest, 300 mg of crystalline dibenzyl hydrogen phosphate was obtained (mp 78–80°; no depression on admixture with authentic material). An additional 50 mg of dibenzyl hydrogen phosphate, mp 78–80°, was obtained from mother liquors. Total yield of dibenzyl hydrogen phosphate, 350 mg (31.5%); total yield of *p*-nitrobenzamidoethyl benzyl hydrogen phosphate III, 700 mg (46%).

*N-Acetyl-O-diphenylphosphoryl-*DL*-serinamide (IVb) in Acetate Buffer.*—A solution of 80 mg (0.21 mmole) of IVb in 25 ml of ethanol and 75 ml of 0.01 M acetate buffer (*p*H 3.7) was heated for 2 hours 45 minutes at 78° under a reflux condenser. The solvent was removed *in vacuo* at 30° and the residue was dried overnight over P_2O_5 *in vacuo*. On addition of 5 ml of ice-cold 3 N HCl and chilling for 2 hours, 33 mg (63%) of diphenyl hydrogen phosphate was collected (mp 68–70° after drying *in vacuo* over P_2O_5; mixed mp with authentic material showed no depression). The filtrate was neutralized by addition of 45 ml of 0.34 M ethanolic sodium ethoxide and evaporated to dryness. The residue was extracted with 30 ml of methanol, insoluble sodium chloride removed by filtration and the solvent evaporated *in vacuo*. After addition of 50 ml of water and treatment with the mixed ion-exchange resin Rexyn IRG-501, the solution was evaporated to dryness *in vacuo* and the residue was dried thoroughly over P_2O_5. Addition of 0.5 ml of ethanol and 10 ml of ether followed by chilling yielded 16 mg (53%) of *N*-acetyl-DL-serinamide (Vb), mp 137–139°. The mixed mp with authentic material was undepressed and infrared spectra (KBr disk) were identical.

Kinetic Measurements

Ethanolic Potassium Acetate.—The stock solution for each run was distributed among ten to twelve 5-ml ampoules. The sealed ampoules were immersed in a glycerol bath maintained at constant temperature by means either of refluxing absolute ethanol (for 78°) or of refluxing acetone (for 56°). At specified times, ampoules were removed from the bath, the reaction was quenched by immersion of the ampoule in cold water, and the absorbance was measured using a Beckman Model DU spectrophotometer.

The cyclization of triesters Ia, Ib, Ic, Id, and of VI was followed by the increase in absorbance at 310 mμ resulting from the formation of oxazoline IIa or oxazine IIb. The solvolysis of triesters IVa and IVb and of ethyl diphenyl phosphate was followed by the increase in absorbance at 270 or 272 mμ due to the appearance of diphenyl phosphate ion. The progress of each reaction was measured for at least 7 half-lives. The values of the final optical densities as well as complete spectral profiles were compared to those of the expected products at the same concentrations. The first-order rate constants were calculated using the expression $k = (2.303/t) \log (D_t - D_f/D_i - D)$, where D_f = optical density at infinite time, D_i = initial optical density, and D = optical density at time t. Since immersion of the ampoules in the bath was followed by a temperature drop of several degrees, D_i was taken as the absorbance value at the time at which constant temperature was regained (usually 10–15 minutes after immersion). In the one run where potassium acetate was omitted (Table I), D_f could not be measured directly and was assumed to be equal to the value found in the presence of potassium acetate.

In the case of triester Ib, the first-order rate constant obtained as above represents the sum of the rate constants for the two competitive processes, cyclization and diester formation. The competing reactions of equations (1) and (2) (where A = Ib, B = IIa and C = III) lead to the integrated rate expression (3), where A_t =

$$A \xrightarrow{k_1} B \tag{1}$$

$$A \xrightarrow{k_2} C \tag{2}$$

$$\ln \frac{A_t}{A_t - \frac{Bk}{k_1}} = kt \tag{3}$$

A + B + C and $k = k_1 + k_2$. (see Frost and Pearson, 1961a). Conversion of equation (3) to equation (9)

$$D_i = A_t\epsilon_A \tag{4}$$

$$D_f = B_f\epsilon_B + C_f\epsilon_C \tag{5}$$

$$D = A\epsilon_A + B\epsilon_B + C\epsilon_C \tag{6}$$

$$\frac{A_t}{B_f} = \frac{k}{k_1} \tag{7}$$

$$A_t = B_f + C_f \tag{8}$$

$$k = \frac{2.303}{t} \log \frac{D_i - D_f}{D_i - D} \tag{9}$$

$$\frac{k}{k_1} = \frac{D_i(\epsilon_B - \epsilon_C)}{D_f\epsilon_A - D_i\epsilon_C} \tag{10}$$

via equations (4–8) (where D_t, D_f, and D are as defined, B_f and C_f are concentrations of B and C at infinite time, and ϵ_A, ϵ_B, and ϵ_C are molar extinction coefficients for A, B, and C, respectively) allows the determination of k. The individual rate constants k_1 and k_2 were evaluated by means of equation (10), using $\epsilon_A = \epsilon_C = 1500$, $\epsilon_B = 5000$.

The rate data obtained solvolysis of ethyl diphenyl phosphate may be similarly treated to yield the over-all constant k (from equation 9). The mole fraction of phenol present at infinite time allows computation of k/k_1 (equation 7) and hence of the individual rate constants for the competing reactions.

Buffered Aqueous Solutions.—Sodium chloroacetate buffers were used below *p*H 4, sodium acetate buffers in the *p*H range 4.0–5.8, and sodium phosphate buffers from *p*H 6.3 to 7.9. The total concentration of chloroacetate, acetate, or phosphate ion in the reaction mixture was 0.01 M, and the solvent was 25% (v/v) ethanol-water. Buffer *p*H was measured with a Radiometer TTT1a *p*H meter equipped with a Metrohm high-temperature glass electrode, and the solution under measurement was placed in a water-jacketed microtitration cell maintained at 78°. It was noted that *p*H values observed at 78° differed by less than 0.1 *p*H unit from those measured at 25°.

The procedure employed for the kinetic runs was the same as that described for the experiments in ethanolic solution. The solvolysis of triesters IVa, IVb, and ethyl diphenyl phosphate was followed at 270 mμ and first-order rate constants were computed as for the experiments in ethanol. Ultraviolet spectra determined at the end of the reactions were compared to those of the expected products.

The hydrolysis of IIa was followed by the decrease in absorbance at 310 mμ.

In the case of Ia, the consecutive increase and decrease of absorbance at 310 mμ were interpreted according to equation (11), where A = Ia, B = IIa, and C = hydrolysis products (mixture of *N*- and *O*-*p*-nitrobenzoyl ethanolamine).

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C \tag{11}$$

The rate constants k_1 and k_2 were evaluated from the absorbance data at 310 mμ by means of the usual equations for consecutive first-order reactions (see Frost and Pearson, 1961b). The absorbance D at time t is given by equation (12) or its equivalent (13), where ϵ_A, ϵ_B, and ϵ_C are as defined, $\tau = k_1t_1$, $\kappa = k_2/k_1$,

$$D = A\epsilon_A + B\epsilon_B + C\epsilon_C \tag{12}$$

$$\frac{D}{A_t} = (\epsilon_A - \epsilon_C)e^{-\tau} + \frac{(\epsilon_B - \epsilon_C)}{\kappa - 1}(e^{-\tau} - e^{\kappa\tau}) + \epsilon_C \tag{13}$$

$$\tau_{max} = k_1t'_{max} = \frac{1}{\kappa - 1} \ln \frac{(\epsilon_B - \epsilon_C)\kappa}{(\kappa - 1)(\epsilon_A - \epsilon_C) + (\epsilon_B - \epsilon_C)} \tag{14}$$

$$k_1t'_{max} = \frac{1}{\kappa - 1} \ln \kappa = k_1t_{max} \tag{15}$$

and $A_t = A + B + C$. The time t'_{max} at which *maximal absorbance* will be observed may be found by setting $dD/d\tau = 0$, yielding expression (14). In the event that $\epsilon_A = \epsilon_C$, equation (14) becomes (15), where t_{max} is the time at *which maximum accumulation of the intermediate B* has occurred. Thus, $t'_{max} = t_{max}$ *only when* $\epsilon_A = \epsilon_C$. In the present study, $\epsilon_A = \epsilon_C = 2500$ and $\epsilon_B = 6500$, so that the simplified equation (15) (or its equivalent 16) could be used.

$$t'_{max}(k_1 - k_2) = \ln \frac{k_1}{k_2} \tag{16}$$

In a given run, k_2 was estimated from the first-order decay of the absorbance at 310 mμ in the latter stages of the reaction. A value of k_1 satisfying the equality (16) was then found, and these (or further refined) values of k_1 and k_2 were employed to calculate the variation of D as a function of t (equation 13). Comparison of the computed curves to the experimental data is shown in Figure 3.

Stability of N-Acetyldehydroalanine in Buffered Solution

N-Acetyldehydroalanine was prepared by the method of Bergmann and Grafe (1930) and melted at 198–200° (reported 198–200°); ϵ_{max} at 240 mμ 4300 (25% ethanol-water).

The hydrolysis of *N*-acetyldehydroalanine in buffered solution (25% ethanol-water) at 78° was followed by the decrease in absorbance at 240 mμ, using the sealed-

TABLE VII
HYDROLYSIS OF N-ACETYLDEHYDROALANINE[a,b,c]

pH[d]	$k \times 10^4$ (min^{-1})
7.4	0.1
6.4	0.5
5.6	2.1
5.0	5.8
4.2	31.6

[a] At 78° in 25% ethanol-water. [b] Compound at 2.7×10^{-4} M. [c] Rate measured by decrease of absorbance at 240 mμ. [d] pH determined at 78°. For composition of buffers, see Experimental.

ampoule technique described. Results are given in Table VII.

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Equilibrium Constants for the Synthesis of Hydroxamic Acids*

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Hydroxamic acid formation from hydroxylamine and unactivated carboxylic acids proceeds readily in aqueous solution at elevated temperatures at pH 4–6 or at 25° in dilute acid. The equilibrium constants for the formation of acetohydroxamic, hexanohydroxamic, octanohydroxamic, and *N*-acetyl-L-tyrosine hydroxamic acids have been determined at 25°. At pH 7 the apparent equilibrium constants for the formation of simple hydroxamic acids are near 1, while that for acetyl-L-tyrosine hydroxamic acid is 0.042.

The determination of activated acyl groups by their conversion to hydroxamic acids has been an extremely useful analytical tool because of the specificity of the reaction for different types of acyl groups and the ease with which the ferric complex of hydroxamic acids can be determined spectrophotometrically. The requirements for the formation of a hydroxamic acid from an acyl compound are, first, that the rate of the reaction be appreciable and, second, that the equilibrium of the reaction be favorable under the particular experimental conditions employed. It is sometimes assumed that hydroxylamine will react only with activated "energy-rich" acyl compounds, and that either the rate or the equilibrium constants are unfavorable for reactions of hydroxylamine with "low-energy" acyl compounds, including carboxylate ions.

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