Amino Acid Active Esters. III. Base-Catalyzed Racemization of Peptide Active Esters[1,2]

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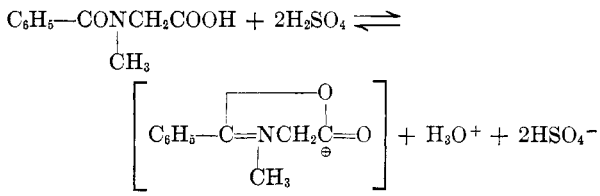
The alkaline hydrolysis of benzyloxycarbonylglycyl-L-phenylalanine *p*-nitrophenyl ester was found to give mainly the racemic benzyloxycarbonylglycylphenylalanine. Since *p*-nitrophenyl esters of this type are useful intermediates in peptide synthesis, we undertook an investigation of the mechanism of this racemization. The related benzyloxycarbonylglycyl-L-N-methylphenylalanine and benzyloxycarbonylglycyl-L-proline *p*-nitrophenyl esters were also prepared. The kinetics of hydrolysis of the above three esters was followed in dioxane–water mixtures by polarimetric and spectrophotometric methods. Benzyloxycarbonylglycyl-L-proline *p*-nitrophenyl ester cyclized to the diketopiperazine which then slowly racemized. The N-methylphenylalanine ester hydrolyzed with only a small amount of racemization while the phenylalanine dipeptide *p*-nitrophenyl ester racemized rapidly and extensively prior to hydrolysis. This racemization is dependent upon pH and takes place with deuterium exchange at the alpha carbon to the ester group. Evidence is presented which supports an oxazolone intermediate in the racemization of benzyloxycarbonylglycyl-L-phenylalanine *p*-nitrophenyl ester.

In the synthesis of peptides, the complete retention of optical activity is an important although rarely achieved goal. While traces of racemized product can often be removed by crystallization it is sometimes necessary to resort to more involved techniques in order to obtain the optically pure product. In unfavorable cases the presence of racemate can prevent the desired product from crystallizing entirely. The success of any multistep synthesis, therefore, depends upon choosing techniques which are known not to cause racemization. The development of methods[4-9] for detecting the extent of racemization has been of inestimable value in devising new peptide syntheses. Many factors have been shown to be intimately connected with racemization. Some of the more important reaction variables which need be considered include the coupling agent, temperature, solvent, and presence of bases such as triethylamine. Despite the importance of this topic to peptide chemistry, relatively little is known with certainty about the mechanisms of racemization and therefore the designing of synthetic routes has been largely empirical.

Racemization is most commonly encountered in amino acid residues when the carboxyl group is activated and the nitrogen is blocked in amide formation. Thus, racemization occurs readily in hydantoins,[10] diketopiperazines,[11] acetylamino acid derivatives,[6] and peptides.

Another class of related compounds which racemize readily are the oxazolones.[12] It is generally believed that oxazolones are intermediates in the racemization of peptides but several cases are reported where loss of optical activity occurs without the possibility of forming such structures.[13,14] Thus, certain acyl derivatives of N-methyltryptophan,[10] N-methylphenylalanine,[11] and proline[11] have been reported to racemize rapidly with ketene or acetic anhydride.

The principal argument against accepting oxazolones as intermediates in these racemizations has been the belief that compounds without an enolizable N—H bond were incapable of yielding such intermediates.[15] Instead, charged oxazolonium ions have been suggested. Evidence that such intermediates are stable under certain conditions has been presented by O'Brien and Niemann.[16] These workers determined the "i factor" for each of a series of acyl amino acid derivatives in concentrated sulfuric acid. Benzoyl sarcosine had an "i factor" of 3.8 indicating that the equilibrium shown below lies far to the right. Oxazolonium ions have also been suggested for the course of the Dakin–West reaction.[17]



(1) Previous paper in this series, M. Goodman and K. C. Stueben, *J. Am. Chem. Soc.*, **84**, 1279 (1962).

(2) Supported by National Science Foundation Grant G 4571.

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(9) N. A. Smart, G. T. Young, and M. W. Williams, *J. Chem. Soc.*, 3902 (1960).

(10) M. Bovarnick and H. T. Clarke, *J. Am. Chem. Soc.*, **60**, 2426 (1938).

(11) An exceptionally fine review of racemization has been compiled by A. Newburger, "Advances in Protein Chemistry," Vol. 4, Academic Press, New York, 1948, p. 339.

(12) H. T. Clarke, "The Chemistry of Penicillin," Princeton Press, 1949, p. 742.

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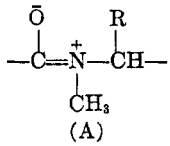
(15) O. H. Borum, *J. Am. Chem. Soc.*, **72**, 1626 (1950).

(16) J. L. O'Brien and C. Niemann, *ibid.*, **79**, 80 (1957).

(17) J. W. Cornforth and D. F. Elliott, *Science*, **112**, 534 (1950).

In addition to the oxazolone theory, the amino acid and peptide derivatives might also racemize by direct proton abstraction followed by enolization. Such racemization would not be expected to be spontaneous but might be induced by the presence of an enolizable acyl amino group attached to the asymmetric carbon. Bovarnik and Clarke tested this hypothesis by examining a series of model compounds in which the enolizable N—H bonds were systematically blocked.[10]

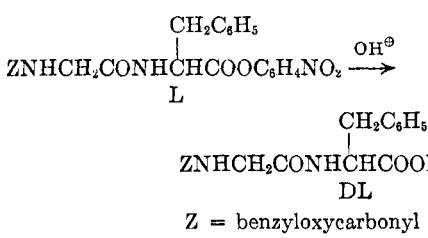
These workers found that the substitution of other groups for hydrogen not only failed to prevent racemization but actually hastened it. Thus, N-acetyl-L-tyrosine anilide possessed a $t_{1/2}$ for racemization of 74 hours while the corresponding L-N-methyltyrosine derivative displayed a $t_{1/2}$ of nine hours. Neuberger[11] rationalized these results by arguing that the presence of an electron donating methyl group would favor the polar form (A) of the amide which, with its positive charge, would then induce ionization of the adjacent C—H bond.



(A)

Several interesting examples of racemization were uncovered in our laboratories during work with dipeptide *p*-nitrophenyl esters.[1,18]

Racemization was found both in the preparation of these derivatives as well as on further manipulation. For example, when benzyloxycarbonylglycyl-L-phenylalanine *p*-nitrophenyl ester was saponified in aqueous dioxane it afforded only the racemic dipeptide. It was of interest to study this reaction and those of certain related peptide derivatives in the hope of shedding further light on the mechanism of racemization.



Z = benzyloxycarbonyl

Experimental[19,20]

Materials.—Dioxane was purified by the method described by Fieser.[21] Merck sodium veronal N.F. was used without further purification. Eastman reagent grade *p*-nitrophenol was recrystallized twice from 2% hydrochloric acid, m.p., 112.5–114.5°. Dueterium oxide (min. 99.5% D_2O) was obtained from the Stewart Oxygen Co., San Francisco, California 50% ethanol– 0.1 *M* hydrochloric acid was prepared by diluting 500 ml. of 0.2 *M* hydrochloric acid up to 1 l. with ethanol. Buffer (pH 4.00 ± 0.01) for standardization was obtained from Fisher Scientific Company.

Preparation of Compounds.[22] **Benzyloxycarbonylglycyl-L-phenylalanine *p*-nitrophenyl ester (I)** was prepared according to the previously described method[1,19,23] After repeated recrystallizations from acetonitrile, ethyl acetate–hexane, and ethanol, the pure compound was obtained, m.p. 143–144.5°, $[\alpha]^{25}D$ −6.3 ± 0.9° (*c* 2.1, $CHCl_3$) (lit.[23]).

Anal. Calcd. for $C_{15}H_{23}N_3O_7$: C, 62.88; H, 4.85. Found: C, 63.14; H, 4.92.

This material could be converted to benzyloxycarbonylglycyl-L-phenylalanylglycine ethyl ester in high yield by treatment with glycine ethyl ester. The product of this reaction had $[\alpha]^{25}D$ −12.6° (*c* 2, EtOH) [lit.,[4] $[\alpha]^{25}D$ −13.5 ± 1° (*c* 2, EtOH)] and was shown to contain no racemate when tested according to Anderson's technique.[4]

Benzyloxycarbonylglycyl-L-phenylalanine (II) was prepared by the same procedure described below in detail for the proline analog (III). Purification of the crude product was effected by conversion to the piperazonium salt[24] followed by recrystallization. The free acid was obtained by acidification, m.p. 128.5–130° $[\alpha]^{25}D$ +37.2° (±0.8°) (*c* 2, EtOH), [lit.,[25] m.p. 125–126°, $[\alpha]^{23}D$ +38.5° (*c* 5, EtOH)].

Benzyloxycarbonylglycyl-L-proline (III).—The procedure of Bergmann, *et al.*,[26] was followed with certain modifications. A solution of benzyloxycarbonylglycyl chloride was prepared by allowing 9.2 g. (0.044 mole) of benzyloxycarbonylglycine to react with 10.1 g. (0.048 mole) of phosphorus pentachloride in 200 ml. of anhydrous ether at 0°. When the reaction was over (1 hr.), the ethereal solution was washed with ice water and then dried with anhydrous magnesium sulfate in the cold. This solution was placed in a dropping funnel with a cooling jacket and added dropwise and with stirring to a solution of 5 g. (0.0435 mole) of L-proline in 25 ml. of 2 *N* sodium hydroxide over a 20-min. period. The concurrent dropwise addition of 20 ml. of 2 *N* sodium hydroxide kept the pH between 8 and 10 during the reaction. After an additional 15 min. stirring at ice-bath temperatures, the reaction mixture was acidified and extracted with ethyl acetate three times. Evaporation of the dried extract gave 11 g. of an oil which solidified on triturating with a small amount of ether. Filtration yielded 6.2 g. of crystals, m.p. 120–130°. This material was recrystallized from hot water once and then from ethyl acetate to give 2.0 g. (15%) m.p. 152–154.4°. Further crystallizations were carried out until a constant melting point 155.5–157° and rotation, $[\alpha]^{21}D$ −77.5° (*c* 2, $CHCl_3$) were obtained (lit.,[26] m.p. 156°, no rotation is given).

Benzyloxycarbonylglycyl-L-proline *p*-nitrophenyl ester (IV) was prepared by the same procedure described earlier,[1] m.p. 104–104.5°, $[\alpha]^{21}D$ −102.8° (*c* 2.6, EtOAc).

L-N-Methylphenylalanine hydrochloride (V) was prepared by the method of Fischer.[27] The recrystallized product possessed a rotation of $[\alpha]^{24.5}D$ +49.7° (0.1 *N* NaOH) (calculated on the basis of the weight of free amino acid formed in solution) [lit.,[28] $[\alpha]^{19}D$ +49.06 (0.1 *N* NaOH)].

(18) M. Goodman and K. C. Stueben, *J. Am. Chem. Soc.*, **81**, 3980 (1959).

(19) All melting points are corrected.

(20) Analyses are by Schwarzkopf Laboratories, Woodside, New York. Deuterium analyses were kindly carried out by Prof. D. Denney and Dr. D. Denney, Rutgers University, New Jersey.

(21) L. F. Fieser, "Experiments in Organic Chemistry," 3rd ed., Heath and Co., Boston, 1955, p. 285.

(22) For review of methods of peptide synthesis, see M. Goodman and G. W. Kenner, "Advances in Protein Chemistry," Vol. 12, p. 465, Academic Press, New York, 1957 and T. Wieland, *Angew. Chem.*, **63**, 7 (1951); *ibid.*, **66**, 507 (1954); *ibid.*, **69**, 362 (1957).

(23) This compound has been prepared in partially racemized form by Kenner and his associates, J. A. Farrington, P. J. Hextall, G. W. Kenner, and J. M. Turner, *J. Chem. Soc.*, 1407 (1957).

(24) M. Prigot and C. B. Pollard, *J. Am. Chem. Soc.*, **70**, 2758 (1948).

(25) K. Hoffmann and M. Bergmann, *J. Biol. Chem.*, **134**, 225 (1940).

(26) M. Bergmann, L. Zervas, H. Schleich, and F. Leinert, *Z. Physiol. Chem.*, **212**, 72 (1932).

(27) E. Fischer and W. Lipschitz, *Ber.*, **48**, 360 (1915).

Benzyloxycarbonyl-L-N-methylphenylalanine (VI).—To a solution of 2 g. (0.0093 mole) of L-N-methylphenylalanine hydrochloride in 200 ml. of 0.1 *N* sodium hydroxide maintained at 0° was added 5 g. (0.0294 mole) of benzyloxycarbonyl chloride dropwise with vigorous stirring over a 30-min. period. During this time the pH was maintained between 8–10 by addition of 2 *N* sodium hydroxide. After the addition was complete, stirring was continued for 0.5 hr. longer at 0° and then for 1 hr. at room temperature. The unchanged acid chloride was removed by three extractions with ether. The remaining aqueous solution was acidified and extracted several times with ethyl acetate. After a water wash, the organic layer was dried and the solvent removed under reduced pressure to give an oil which crystallized in a short time. One crystallization from ethyl acetate–petroleum ether afforded 1.4 g. (45%) m.p. 68.5–70.5°. An analytical sample was recrystallized from the same solvent with excellent recovery, m.p. 69.5–70.5°, $[\alpha]^{26}D$ −70.3° (*c* 2.5, EtOAc).

Anal. Calcd. for $C_{18}H_{19}NO_4$: C, 68.99; H, 6.11; N, 4.47; neut. equiv., 313.4. Found: C, 68.92; H, 6.09; N, 4.63; neut. equiv., 314.

Considerably lower yields (25%) were obtained when smaller amounts of water (10 ml.) were used during the carbobenzoxylation. Very probably this is due to the insolubility of the sodium salt of the product which occludes unchanged acid chloride on its surface as it forms. Under these conditions, extremely vigorous stirring in a Waring Blendor or heating to 60° did not improve the yield. The infrared spectrum of this compound is shown in Fig. 1 (in potassium bromide and carbon tetrachloride solution).

L-N-Methylphenylalanine *p*-Nitrophenyl Ester Hydrobromide (VII).—This compound was obtained from the intermediate benzyloxycarbonyl-L-N-methylphenylalanine *p*-nitrophenyl ester (an oil prepared *via* the tris-*p*-nitrophenyl phosphite) by treatment with saturated hydrogen bromide in acetic acid; yield 66% (over-all), m.p. 200–201° dec.

Anal. Calcd. for $C_{16}H_{17}N_2O_4Br$: C, 50.40; H, 4.50; N, 7.35. Found: C, 50.67; H, 4.49; N, 7.22.

The optical rotation of this compound was not determined accurately since the rotational or dimethylformamide changed with time. Approximate values of $[\alpha]^{25}D$ +42.1° (methanol) and +39.4° (DMF) were obtained. The hydrobromide was insoluble in chloroform, acetone, aqueous acid, and water (sl. sol.).

Benzyloxycarbonylglycyl-L-N-methylphenylalanine *p*-Nitrophenyl Ester (VIII).—The acid chloride from 0.21 g. (0.001 mole) of benzyloxycarbonylglycine and 0.23 g. (0.0011 mole) of phosphorus pentachloride in 5 ml. of anhydrous ether was maintained at 0° and 0.38 g. (0.001 mole) of L-N-methylphenylalanine *p*-nitrophenyl ester hydrobromide followed by 5 ml. of cold acetonitrile was added. Then a solution of 0.9 ml. of triethylamine in 10 ml. of acetonitrile was added dropwise and with stirring until a yellow color was just perceptible. Following 10 min. of further stirring the solution was diluted with ethyl acetate, washed with 2 *N* hydrochloric acid, 1% bicarbonate, dried, and evaporated to give 0.49 g. of an oil. A few precipitations from ethanol–petroleum ether and a charcoal treatment gave a product which had an infrared spectrum identical to the crystalline DL-isomer. After drying for 48 hr. at 57° *in vacuo*, the pale brown oil was submitted for analysis.

Anal. Calcd. for $C_{26}H_{27}N_3O_7$: C, 63.53; H, 5.13. Found: C, 63.77; H, 5.47.

The material used for the kinetics described was obtained from another similar preparation except that the compound was dried without the application of heat. It was then stored in a sealed container in the cold.

Benzyloxycarbonylglycyl-L-N-methylphenylalanine Piperazonium Salt (IX).—Using the same procedure previously described for the preparation of benzyloxycarbonylglycyl-L-proline (III), 1.5 g. (0.007 mole) of L-N-methylphenylalanine hydrochloride was converted to the N-blocked dipeptide. However, the product was obtained as an oil (2.4 g.) and efforts to obtain a solid product by crystallization from a number of solvents and the use of silica gel chromatography failed. A successful solution to this problem was realized by converting the crude product to a mixture of the piperazonium salts by the method of Prigot and Pollard.[24]

A solution of piperazine (10%) (anhydrous) in isopropyl alcohol was added to 30 ml. of an ethereal solution of 0.97 g. of the oil obtained above. When precipitation was complete, a tacky solid was obtained. This was triturated with ether and dried to give 0.91 g., m.p. 133–156°. Fractional crystallization of this material from methanol–ether afforded the following crops:

Crops I and II	1.0 g.	m.p. 194–195° dec.
Crops III–V	0.61 g. (53%)	m.p. 139–140°

The first two crops were shown to be the piperazonium salt of benzyloxycarbonyl glycine since a mixed melting point showed no depression with an authentic sample prepared separately, m.p. 195° dec. The last three crops were recombined and crystallized from methanol–ether again to give 0.56 g. (48%), m.p. 139–141°, $[\alpha]^{25}D$ −38.5 ± 0.7° (*c* 2, MeOH). Further crystallization did not change these values.

Anal. Calcd. for $C_{44}H_{54}N_6O_{10}$: C, 63.90; H, 6.58; N, 10.16. Found: C, 63.87; H, 6.35; N, 10.35.

Isolation of Racemic Benzyloxycarbonylglycylphenylalanine (X) from Hydrolysis of the Optically Active Nitrophenyl Ester.—A sample of 3.57 g. (0.0075 mole) of benzyloxycarbonylglycyl-L-phenylalanine *p*-nitrophenyl ester was dissolved in 145 ml. of dioxane and treated with 150 ml. of 0.1 *M* sodium hydroxide (0.015 mole) at room temperature. After 45 min. the clear solution was concentrated half-way under reduced pressure and the pH adjusted to between 8 and 9 with additional base. A small amount of solid was removed by filtration and the filtrate extracted with ethyl acetate three times and acidified. Several more extractions with ethyl acetate followed by evaporation afforded 3.32 g., m.p. 133–142°, $[\alpha]^{18}D$ +3.7° (ethanol) (corrected for the *p*-nitrophenol present (0.044 g.) as determined from optical density at 317 mμ). This was then combined with the same extract from two other separate experiments run under the same conditions and given two recrystallizations from 50% ethanol. The yield was 5.05 g. (66% based on a total of 0.0216 moles of starting ester), m.p. 159–162° of IX (lit.,[28] m.p. 160–162°).

Benzyloxycarbonylglycyl-DL-phenylalanine *p*-nitrophenyl ester (XI) was prepared from the DL-acid (IX) isolated from hydrolysis of the L-ester above *via* reaction with tris(*p*-nitrophenyl phosphite); yield 63%, m.p. 135.5–136.5°.

Anal. Calcd. for $C_{25}H_{23}N_3O_7$: C, 62.88; H, 4.85. Found: C, 62.96; H, 4.85.

Hydrolysis in Deuterium Oxide.[20] Isolation of Unhydrolyzed Ester.—The hydrolysis of the optically active ester (I) was repeated in 64% dioxane–36% deuterium oxide in the same manner described in the kinetic procedure below except that the solution was 0.3 molar in buffer (pH 8) and the latter had been prepared with 6 *N* deuterosulfuric acid rather than with hydrochloric acid. No 8 *M* base was added, however, and the pH was allowed to drift down slightly. After 25 min., the solution was acidified with deuterosulfuric acid and then rinsed into a flask with dioxane. The solvent was replaced by ethyl acetate and the solution then washed repeatedly with 2 *N* hydrochloric acid. After removing the solvent *in vacuo*, the remaining solid was crystallized three times from ethanol to give 0.15 g. of benzyloxycarbonylglycyl-DL-phenylalanine *p*-nitrophenyl ester, m.p. 135.5–136.5°, $[\alpha]^{25}D$ 0° (*c* 2.5, pyridine). This material showed no depression in melting point on admixture with an authentic sample of XI. A 4% solution of this solid in chloroform

(28) J. R. Vaughan and R. L. Osato, *J. Am. Chem. Soc.*, **74**, 676 (1952).

was placed in a 1-mm. cell and examined in the infrared from 2850 cm.$^{-1}$ to 1800 cm.$^{-1}$. Comparison of this spectrum with that of a solution of the undeuterated ester showed the presence of a weak band at 2195 cm.$^{-1}$ for C—D. In addition, the unhydrolyzed ester isolated above was shown to contain approximately one deuterium atom per molecule by combustion analysis.[20]

Hydrolysis of Benzyloxycarbonyl-L-phenylalanine *p*-Nitrophenyl Ester.—A sample of 0.84 g. (0.002 mole) of this ester[29] was dissolved in 50 ml. of dioxane and treated with 40 ml. of 0.1 *M* sodium hydroxide. After 45 min. the solution was concentrated, adjusted to pH 9 with additional base and extracted with ethyl acetate. The aqueous layer was acidified and extracted again with ethyl acetate. After drying and evaporating the organic extract, crude benzyloxycarbonyl-L-phenylalanine was obtained which had $[\alpha]^{25}D$ +5.7° (*c* 5.5, HOAc) (after correcting for the *p*-nitrophenol present) [lit.,[30] $[\alpha]^{23}D$ +5.2° (*c* 5.2, HOAc)].

Racemization in Pyridine. Racemization of Benzyloxycarbonylglycyl-L-phenylalanine *p*-Nitrophenyl Ester in Pyridine.—A solution (10 ml.) of 0.1917 g. of the blocked dipeptide in anhydrous pyridine[30] was prepared and stored under nitrogen at room temperature. Samples were withdrawn over a period of 4 days and the observed optical rotation measured in a 2-dm. tube using the D line of sodium (Table I).

Other *p*-Nitrophenyl Esters in Pyridine.—In contrast to the above behavior a 4.4% solution of benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester in pyridine had $[\alpha]^{25}D$ −26.9° and showed no change even after 48 hr. Benzyloxycarbonylglycyl-L-proline *p*-nitrophenyl ester had $[\alpha]^{25}D$ −126° in pyridine (1% solution) and showed no change after 24 hr. However, benzyloxycarbonyl-L-N-methylphenylalanine *p*-nitrophenyl ester underwent some changes. The values shown in Table I are for a 1.4% solution in a 2-dm. tube using the D line of sodium.

Isolation of Partially Racemized Benzyloxycarbonylglycyl Phenylalanine *p*-Nitrophenyl Ester (X).—On allowing a sample of benzyloxycarbonylglycyl-L-phenylalanine to react with tris-*p*-nitrophenyl phosphite in pyridine for 3 hr., a product having m.p. 136–137°, $[\alpha]^{23.5}D$ +1.86° (*c* 2.2, $CHCl_3$) was obtained. When redissolved in pyridine and allowed to stand overnight, a quantitative recovery of material having m.p. 134.5–136°, $[\alpha]^{23.5}D$ +0.81° (*c* 4, $CHCl_3$) was obtained.

Formation of Racemic Benzyloxycarbonylglycyl-L-N-methylphenylalanine *p*-Nitrophenyl Ester (XII).—Acidification of an aqueous solution of 0.6 g. (0.726 mmole) of benzyloxycarbonylglycyl-L-N-methylphenylalanine piperazonium salt liberated the free acid which was extracted into ethyl acetate. Drying and evaporation of the organic layer gave the dipeptide derivative as a sirup. This reacted with 0.4 g. (0.9 mmole) of tris-*p*-nitrophenyl phosphite in the usual way to yield 0.78 g. of oil. On crystallization of this material from ethanol–ether–petroleum ether, 0.4 g. (56%) crystals, m.p. 95.5–99.5°, was obtained. Further crystallization gave m.p. 99.5–100.5°. A 2% solution of this material had no appreciable rotation in pyridine or ethyl acetate.

Anal. Calcd. for $C_{26}H_{25}N_3O_7$: C, 63.53; H, 5.13. Found: C, 64.02; H, 5.13. Further crystallization did not improve this analysis.

Hydrolysis of 90 mg. of this material in 0.55 ml. dioxane and 0.9 ml. 12 *N* hydrochloric acid in a sealed tube at 56° for 14 hr. gave a solution which was again devoid of any optical activity.

Preparation of Buffer Solutions.—Concentrated buffer solutions were prepared by dissolving the appropriate amounts of sodium veronal and hydrochloric acid in a mixture of dioxane and water.

The amount of hydrochloric acid to be added to provide a given pH was calculated from available data[31] in purely aqueous media. Thus, to prepare "pH 8" buffer, a mixture of 19.38 g. (0.0938 mole) of sodium veronal, 10.82 ml. of 3.446 *N* hydrochloric acid (0.0374 mole), and 137.5 ml. of dioxane (thermostated at 25°) were placed in a 250-ml. volumetric flask and made up to the mark with de-ionized water at 25.0°. This concentrated buffer solution was never used without further dilution with dioxane. A mixture of four parts of concentrated buffer with one part of dioxane gave a solution 0.3 *M* in buffer containing 64% dioxane by this volume. This solution's pH reading was 9.75 on the meter but will be referred to as "pH 8." The use of 1/3 the quantities of sodium veronal and acid results in 0.1 *M* pH buffer.

Exactly the same procedure is followed to make the "pH 7" buffers used except that a greater amount of acid is required (0.081 mole).

Instruments and Apparatus.—Polarimeter readings were obtained in jacketed 2- or 4-dm. tubes maintained at 25° by means of circulating water from a thermostated bath. The polarimeter was a Rudolph Research Model 70 capable of being read to 0.01° and was used in conjunction with a sodium lamp. Spectral measurements were carried out with a Beckman Model DU spectrophotometer using 1-cm. matched cells. Full ultraviolet spectra were obtained with a Cary Model 11 recording spectrophotometer. Measurements of pH were obtained with a Cambridge Research Model pH meter equipped with an external shielded glass electrode and a calomel electrode. Before use, the electrodes were soaked for 2–3 hr. in the same dioxane–water medium employed in the kinetic run. They were then rinsed with distilled water, dried, and standardized rapidly in pH 4.00 buffer at 25°. Immediately after the standardization, the electrodes were rinsed again, dried, and then placed in the reaction mixture.

The reaction vessel used in some experiments (method B below) was a glass container of about 35-ml. capacity surrounded by a jacket through which water from a constant temperature bath circulated. The top of this container was open and was large enough to allow the electrodes and microburet to pass through.

Kinetic Procedure.—The large difference between the optical rotation of the proline and N-methylphenylalanine peptides on one hand and the phenylalanine peptide on the other necessitated the use of two different methods for following the reactions. The first two compounds possessed large specific rotations and could therefore be used in relatively small concentrations and still give sizeable observed rotations. This permitted the incorporation of sufficient buffer to maintain the pH within reasonable limits. These reactions were followed continuously (method A below). Because of low specific rotation, the analogous phenylalanine compound was used in high concentration. The concentration of buffer theoretically required to maintain constancy of pH in this case was far above its solubility and a system was devised which continuously renewed the buffer (method B below). All solvents and solutions were thermostated at 25° for at least 15 min. before use.

Method A. 1. Polarimetry.—About 40–60 mg. of the compound to be studied was weighed into a 25-ml. volumetric flask and dissolved in 5.0 ml. of dioxane. To initiate the run, concentrated buffer of the desired pH was added from a rapid delivery pipet up to the mark (about 5 sec. were required), shaken to ensure homogeneity and then quickly poured into the thermostated (24.95 ± 0.05°) 4-dm. polarimeter tube. Zero time was taken as the beginning of the buffer addition. The solution, now 64% dioxane and 0.3 *M* in buffer maintains the pH constant

(29) M. Goodman and K. C. Stueben, *J. Org. Chem.*, **24**, 112 (1959).

(30) The pyridine was refluxed and distilled from barium oxide and calcium hydride in succession; shown to have less than 0.002% of water by Karl Fischer titration.

(31) H. T. S. Britton, "Hydrogen Ions," 4th ed., p. 360, Chapman and Hall, Ltd., London, 1955.

to about 0.05 unit. Readings were begun immediately, their frequency depending on the rate of change of rotation. Usually two readings per minute were taken for the first 30–60 min. The infinity reading, taken at some time greater than ten half-lives, was then determined from a series of ten readings.

2. **Determination of *p*-Nitrophenol.**—The solutions were prepared in exactly the same way as described above in the polarimetric work and were thermostated in the same bath. At stated times, 1.0-ml. samples were removed and quenched in 25.0 ml. of 50% ethanol–0.1 *M* hydrochloric acid. The optical density at 350 mμ was then determined on a spectrophotometer. The quenched solutions from the proline runs were very stable and gave almost identical readings even after several hours but those from the N-methylphenylalanine reaction mixture were found to increase by about 35% in optical density after standing 2 hr. The spectral ieadings for the latter case, therefore, were determined rmmediately after quenching.

Method B.—Approximately 0.5 g. of the phenylalanine peptide was weighed into a 25-ml. flask and dissolved in 5.0 ml. of dioxane. To initiate a run, concentrated buffer of the desired pH was added from a rapid delivery pipet up to the mark. This solution (0.1 *M* in buffer and containing 64% dioxane) was shaken briefly to ensure homogeneity and then poured into the thermostated reaction vessel (25.0 ± 0.1°). The pH of the solution was measured immediately and maintained constants to ±0.03 unit by addition of small increments of 8 *M* sodium hydroxide from a microburet. Efficient stirring was achieved by use of a magnetic stirrer.

At stated time intervals, 2.0-ml. samples were removed and added to 0.1 ml. of 4 *M* hydrochloric acid to stop the reaction. The optical rotations of the colorless solutions were then determined in a 2-dm. tube at 25°. The rotation of any given solution was stable for at least 2 hr. at room temperature but as a precaution these quenched solutions were stored at −10° until used. To follow the liberation of *p*-nitrophenol, aliquots of 0.25 ml. were removed, quenched in 50% ethanol–0.1 *M* hydrochloric acid and made up to 50.0 ml. with this same solvent. The optical density at 350 mμ was then determined. These solutions were also stored at −10° until used. They showed no significant change after 4 hr. of storage.

Specific Rotations of Hydrolysis Products.—Optical rotations of the following compounds were determined in the same solvents in which the kinetic measurements were made:

Z-gly-L-proOH in 64% dioxane, pH 7 or 8 buffer
$[\alpha]^{25}D$ −65.0°(*c* 0.5)
Z-gly-L-*N*-methylpheOH piperazonium salt in 64% dioxane pH 7 or 8 buffer
$[\alpha]^{25}D$ −38.7 ± 0.3° (*c* 0.6)
Z-gly-L-pheOH in 64% dioxane, acid medium
$[\alpha]^{25}D$ +32.6° ± 0.3° (*c* 1.5)

Results and Discussion

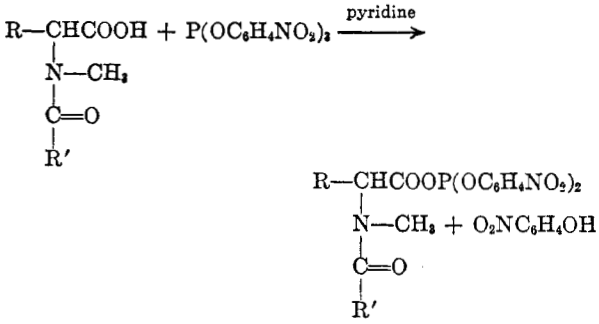
In order to study the influence of structure upon the racemization of dipeptide active esters we synthesized Z-gly-L-N-methylphenylalanine, Z-gly-L-proline, and Z-glyphenylalanine *p*-nitrophenyl esters, where Z-gly represents benzyloxycarbonylglycyl. Initially, it had been planned to prepare all of these materials by the same route, namely, the coupling of benzyloxycarbonylglycine with the desired amino acid *p*-nitrophenyl ester. Although this procedure was successful with both of the phenylalanine derivatives it gave only a 22% yield of benzyloxycarbonylglycyl-L-proline *p*-nitrophenyl ester. The difficulties encountered in this latter preparation have already been discussed in an earlier paper.[1] The alternate synthesis from the dipeptides and tris(*p*-nitrophenyl) phosphite[18] was therefore used. Benzyloxycarbonylglycyl-L-proline gave an 84% yield of the *p*-nitrophenyl ester, identical in all respects to the product obtained by the first method. However, considerable racemization was encountered with both of the phenylalanine peptides. Benzyloxycarbonylglycyl-L-phenylalanine afforded a product which had lost about 60% of its optical activity. At least part of the racemization encountered here is due to the action of pyridine on the product. This was subsequently shown by preparing a solution of the optically active *p*-nitrophenyl ester (I) in scrupulously dry pyridine and observing the loss of rotation with time. Under these conditions several days were required for the optical rotation to decrease to 50% of its initial value (Table I). This reaction was found to be quite sensitive to the presence of moisture, and the use of pyridine containing as little as 0.1% water resulted in a marked increase[32] in the rate of racemization over that found above. Very possibly, the hydroxyl ion produced from the pyridine–water equilibrium is responsible for this increase in rate since aqueous base has been shown to bring about rapid racemization of the ester itself.

TABLE I
RACEMIZATION OF *p*-NITROPHENYL ESTERS IN PYRIDINE

p-Nitrophenyl ester	Time (days)	Observed rotation
Benzyloxycarbonylglycyl-L-phenylalanine	0	−1.41°
	2	−0.98°
	9	−0.22°
	12	−0.10°
	15	−0.08°
Benzyloxycarbonyl-N-methyl-L-phenylalanine	0	−2.76°
	0.2	−2.75°
	2	−2.27°
	4	−1.86°

An attempt to effect the conversion of benzyloxycarbonylglycyl-L-N-methylphenylalanine to its *p*-nitrophenyl ester by use of the phosphite technique in dry pyridine resulted instead in the formation of a 56% yield of the *racemic* ester. This result was completely unexpected because the closely related proline dipeptide (IV) was prepared in high yield and optical purity by the same route. Since a sample of the optically active benzyloxycarbonylglycyl-N-methylphenylalanine ester (VIII) was not appreciably racemized on standing several hours in dry pyridine most of the loss of optical activity occurred during the preparation rather than after it. The mixed phosphite–carboxyl anhydride intermediate may be responsible for much of the racemization observed here and in the unmethylated case.

(32) Although 0.1% water corresponds to one equivalent, hydrolysis is not a contributing factor here as evidenced by the fact that a quantitative recovery of partially racemized ester was obtained after standing twelve hours in this same solvent.



This intermediate is a great deal more reactive than the *p*-nitrophenyl ester and would therefore have a greater tendency to racemize. Direct abstraction of hydrogen from the active carbon does not appear to be an attractive mechanism since proline would also be expected to racemize essentially as readily as the N-methylphenylalanine peptides if this were the case. The difference between the behavior of the proline peptide ester on the one hand and the phenylalanine and N-methylphenylalanine derivatives on the other can be explained on the basis of molecular structure. The oxygen forming the peptide linkage between glycine and proline in Z-gly-pro-$OC_6H_4NO_2$ is prevented from attacking the carbonyl containing the *p*-nitrophenyl ester because it is held out of the plane of that carbonyl by the rigid five-membered ring. In the case of the phenylalanine peptides (Z-gly-N-methyl-L-phe-$OC_6H_4NO_2$ and Z-gly-L-phe-$OC_6H_4NO_2$) the peptide oxygen can attack the ester carbonyl.

The product of these displacements, an oxazolone or oxazolonium ion, would be expected to racemize very quickly. It is clear from these results that a peptide containing a sufficiently activated C-terminal amino acid can still racemize under preparative conditions despite the absence of enolizable N—H amide bonds.

Attempts to convert benzyloxycarbonyl-L-valyl-L-tyrosine to its *p*-nitrophenyl ester by means of tris(*p*-nitrophenyl) phosphite in pyridine have also resulted in racemization according to a recent report.[33] These same workers, however, claim that benzyloxycarbonyl-S-benzyl-L-cysteinyl-L-tyrosine yields an optically pure product (m.p. 108–115°) by this route, somewhat surprising because of the close similarity in structure of these two compounds. The optical purity of the *p*-nitrophenyl ester was established by saponification and isolation of the dipeptide acid. Unfortunately the yield of the dipeptide was not disclosed. In view of the present finding that racemization can occur during hydrolysis, this point is of great importance since inactive product from two racemizations might be removed by recrystallization. It is of interest to point out that the method[33] which gave the product with the highest melting point and optical rotation involved coupling benzyloxycarbonyl-S-benzyl-L-cysteine with L-tyrosine *p*-nitrophenyl ester. Unfortunately, saponification to the dipeptide acid was again used to judge the optical purity of the active ester.

As a result of our findings it was necessary to prepare the optically active benzyloxycarbonylglycyl-N-methylphenylalanine *p*-nitrophenyl ester (VIII) by coupling benzyloxycarbonylglycine with the amino acid *p*-nitrophenyl ester. The desired peptide ester was obtained as an oil with correct analysis and gave an infrared spectrum superposable with the crystalline DL-compound (XII) (m.p. 99.5–100.5).

The observation that the N-methyl dipeptide ester (VIII) was not crystalline was in accord with the unusual properties of its precursors. Substitution of one N—H bond with an *N*-methyl drastically affects the solubility, crystallinity, and in one case, the absorption in the infrared of these compounds. These observations are discussed at the end of this paper in more detail.

The *p*-nitrophenyl ester of benzyloxycarbonyl-L-N-methylphenylalanine could not be obtained in crystalline form and therefore was not analyzed but converted directly to the hydrobromide by treatment with hydrogen bromide. This salt was insoluble in many of the customary solvents.

Finally, a dipeptide acid, benzyloxycarbonylglycyl-L-N-methylphenylalanine was also prepared as a standard to gauge the extent of racemization in the hydrolysis of the corresponding ester. As such, this compound was also difficult to crystallize. However, its piperazonium salt was easily crystallizable and therefore the peptide was isolated in this form.

Kinetics.—Unfortunately, Z-gly-L-phenylalanine *p*-nitrophenyl ester (I) possessed a very low specific rotation in aqueous media necessitating the use of high concentrations both of I and the buffer in order to get appreciable rotations and still maintain reasonable constancy of pH. Both benzyloxycarbonylglycyl-L-proline *p*-nitrophenyl ester and the benzyloxycarbonylglycyl-L-N-methylphenylalanine analog had high specific rotations in dioxane–water mixtures[34] and so the hydrolysis of these compounds could be followed continuously in the polarimeter.

Reaction of Benzyloxycarbonylglycyl-L-proline *p*-Nitrophenyl Ester in Base.—The hydrolysis of benzyloxycarbonylglycyl-L-proline *p*-nitrophenyl ester[1] was studied in 64% dioxane at pH's 7 and 8 as described above. As can be seen from the summary of the kinetic data in Table II, the liberation of *p*-nitrophenol took place considerably faster than the change in optical rotation. Both reactions followed pseudo-first-order kinetics and proceeded more rapidly in going from pH 7 to pH 8. It was obvious here that a fast reaction

(33) B. Iselin and R. Schwyzer, *Helv. Chim. Acta*, **43**, 1760 (1960).

(34) While the "pH" values of these dioxane–water solutions are not directly comparable with the pH in pure water they are nevertheless useful for internal comparison.

TABLE II
SUMMARY OF KINETIC DATA

Peptide Ester	"pH"	Pseudo-first-order rate constants liberation of *p*-nitrophenol, k min.$^{-1}$	Pseudo-first-order rate constants for racemization k min.$^{-1}$
Benzyloxycarbonyl-glycyl-L-proline *p*-nitrophenyl ester	7	1.9×10^{-2}	2.4×10^{-3}
	8	1.9×10^{-1}	8.7×10^{-3}
Benzyloxycarbonylglycyl-*N*-methyl-L-phenylalanine *p*-nitrophenyl ester	7	1.0×10^{-3}	1.3×10^{-3}
	8	6.6×10^{-3}	7.4×10^{-3}
Benzyloxycarbonylglycyl-L-phenylalanine *p*-nitrophenyl ester	7	1.9×10^{-3}	2.9×10^{-2}
	8	2.6×10^{-2}	2.1×10^{-1}

was taking place first to give an optically active intermediate which was then undergoing further reaction, perhaps racemization since the final rotation of the solution was very close to zero. At first it was suspected that the intermediate was an oxazolonium ion. Although the structure of the proline compound is such that the ester carbonyl is near the attacking oxygen this reaction would not be favored for the stereochemical reasons mentioned earlier. In addition, it was difficult to see why the ring formation should be pH dependent. By stopping the reaction after the liberation of *p*-nitrophenol was complete and isolating the neutral fraction, it was possible to obtain the pure intermediate, the benzyloxycarbonylglycyl-L-proline diketopiperazine. The proof of structure and the general chemistry of this reaction were discussed in a previous paper.[1] Since the racemization of diketopiperazines is a well known phenomenon, no attempt was made to examine this reaction further.

Hydrolysis of Benzyloxycarbonylglycyl-L-N-methylphenylalanine *p*-Nitrophenyl Ester.—The second compound studied was benzyloxycarbonylglycyl-L-N-methylphenylalanine *p*-nitrophenyl ester. As a consequence of the fact that this substance could not be obtained in crystalline form it was impossible to guarantee its optical purity. Indeed, because of the ease with which this compound had racemized during preparation by the phosphite method, no attempt was made to remove all traces of solvent by heating and therefore the material used for the kinetic study was only about 95% pure as indicated by spectrophotometric analysis. However, if racemization occurred extensively, as expected from its previous behavior this would be of little consequence. As the reaction progressed, it became apparent that only a limited amount of racemization, *if any*, was taking place. At both "pH" 7 and 8 only 83% of the expected optical rotation was present at the end of the hydrolysis. While these results indicate some possible racemization, the uncertain optical purity of the starting material demands caution. It is of interest that the rate of reaction is approximately the same whether it be followed by the change in optical rotation or by the liberation of *p*-nitrophenol (Table II). Although the rate of change of optical activity conformed to a pseudo-first-order plot, the liberation of *p*-nitrophenol did so only after the reaction was well underway. This behavior may be indicative of some competing reaction.

Racemization–Hydrolysis of Benzyloxycarbonylglycyl-L-phenylalanine *p*-Nitrophenyl Ester.—In contrast to the above behavior, the unmethylated compound, benzyloxycarbonylglycyl-L-phenylalanine *p*-nitrophenyl ester, gave an almost completely racemic product when hydrolyzed under essentially identical conditions. As can be seen from Table II the rate of racemization increases with pH and takes place a great deal faster than the hydrolysis. It was possible, therefore, to isolate ester which was identical to the DL-isomer (XI) prepared previously. The reaction and isolation were repeated in the presence of deuterium oxide. In analogy with the racemization of a number of amino acids and their derivatives[35-38] exchange of the asymmetric C—H for C—D with its characteristic change in infrared spectrum[39-41] was expected. Examination of this racemized ester in chloroform solution by infrared techniques showed the presence of a weak band at 2195 cm.$^{-1}$ confirming the presence of a C—D bond. In addition, deuterium analysis indicated greater than 0.8 deuterium atoms per ester molecule.[20] Benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester, however, hydrolyzed in a normal fashion with no sign of racemization.

With the foregoing evidence at hand, several possible mechanisms can be considered for the racemization during hydrolysis. The first of these involves the intermediate formation of an oxazolone (XIII) as shown in the following scheme:

(35) H. R. V. Arnstein and R. Bently, *Nucleonics*, (6) **6**, 11 (1950).
(36) H. Erlenmeyer, *et al.*, *Helv. Chim. Acta*, **20**, 367 (1937).
(37) See Bibliography of Research on Deuterium and Tritium Compds. 1945–1952. Natl. Bur. Stds. (U.S.) *ca.* **562**, (1956). Also First Supplement (1953–1954) for further references. For an exception to this generality see:
(38) H. Erlenmeyer, H. Schenkel, and A. Epprecht, *Nature*, **138**, 547 [*Helv. Chim. Acta*, **19**, 1053 (1936)].
(39) For infrared techniques to estimate deuterium content see: N. R. Trenner, R. W. Walker, B. Arison, and R. P. Buks, *Anal. Chem.*, **21**, 285 (1949).
(40) N. R. Trenner, R. W. Walker, B. Arison, and C. Trumbauer, *ibid.*, **23**, 487 (1951). An exception to this generality has been published.
(41) N. R. Trenner, B. Arison, and R. W. Walker, *Appl. Spectros.*, **7**, 166 (1953).

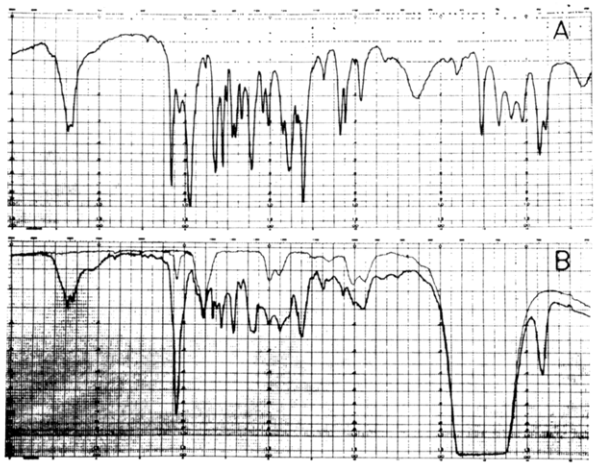
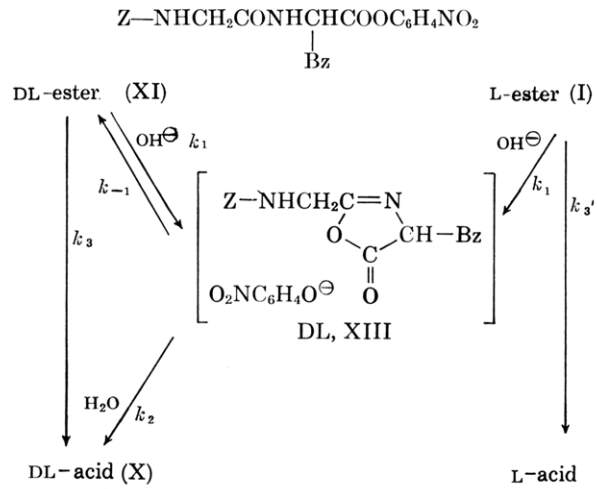


Fig. 1.—Infrared spectra of benzyloxycarbonyl-L-N-methylphenylalanine in a potassium bromide pellet (A) and in carbon tetrachloride solution (3%) (B) (see text for discussion).

Suggested Racemization and Hydrolysis Mechanism for Benzyloxycarbonylglycyl-L-phenylalanine *p*-Nitrophenyl Ester

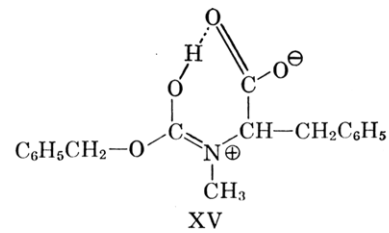


Once formed, the oxazolone would probably racemize almost instantly. It could then react either with hydroxide ion to give the racemic acid (X) or with *p*-nitrophenoxide ion to reform the ester (racemized). Although the nucleophilicities of these anions are such as to favor attack by the hydroxide ion, the internal return of *p*-nitrophenoxide ion in structure XIII might cause k_{-1} to be competitive with k_2. If the equilibrium $\underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}}$ were rapidly established it would have little effect on the kinetics of liberation of *p*-nitrophenol (k_3,k_3') except at the very beginning of the reaction, and indeed, the liberation of *p*-nitrophenol does not conform to a first-order plot during the very early stages of the reaction. Assuming that the oxazolone racemized instantly, the rate of racemization will be equal to the rate of formation of XIII and in addition this would conform to the first-order plot actually observed. Further support for the oxazolone theory comes from the observation that the N-methyl dipeptide ester does not racemize extensively. This indicates that the peptide bond is intimately involved in racemization. In media of higher dielectric constant than employed here or with more active leaving groups oxazolonium ion formation may be encouraged for the N-methyl peptide with its attendant racemization.

Taken in their entirety both the kinetic and synthetic results make a strong case for the intermediacy of oxazolones in racemizations. However, the possibility that certain other mechanisms are also operating to some small extent cannot be eliminated completely.

Infrared of N-Methyl Compound.—Benzyloxycarbonyl-L-N-methylphenylalanine, the first intermediate needed in the synthesis of VII possessed a sodium salt which was only sparingly soluble in water and an infrared spectrum which was entirely different from the unmethylated compound. The preparation of the N-methyl compound from the amino acid and benzyloxycarbonyl chloride proceeded in a much lower yield than usual unless a large volume of water and a considerable excess of the acid chloride were used.

Based on experience with similar compounds, a somewhat broad band from both the carboxyl and benzyloxycarbonyl groups was expected in the 1705–1725-cm.$^{-1}$ range of the infrared spectrum of VI. Instead, however, this substance showed two strong peaks at 1758 and 1635 cm.$^{-1}$ and a much weaker absorption at 1705 cm.$^{-1}$ (see Fig. 1). Essentially the same results were obtained in both Nujol and potassium bromide. On the other hand, a solution spectrum (see Fig. 1) taken in carbon tetrachloride had only one broad band at the expected 1710-cm.$^{-1}$ region. The absorption at 1705 cm.$^{-1}$ commonly referred to as belonging to the carboxyl carbonyl is in reality that of the hydrogen-bonded dimer. It is expected that if this carbonyl is freed somehow it will absorb at a higher frequency. Keeping this in mind, an explanation for the behavior of this compound can be formulated. An intramolecularly hydrogen bonded form (XV) would contain not only the



XV

free carbonyl of the acid but also the hydrogen bonded benzyloxycarbonyl group. Such a large shift in the frequency of the acceptor oxygen is not usual, however, unless resonance stabilization is involved as shown.[42]

(42) R. N. Jones and Camille Sandorfy, "Chemical Applications of Spectroscopy, Techniques of Organic Chemistry," Vol. 9, A. Weissberger, ed., Interscience, New York, 1956, p. 521ff.