The definite physical and chemical differences[4] as well as the antigenicity and characteristic specificity of the various denatured forms of Ea (also ref. 6) studied in this Laboratory show a reproducibility, degree of organization and specificity indicating that they have not reached the final step in the denatured state characterized as a completely random one[15] devoid of any specific structure. The present studies therefore corroborate the concept that various degrees of denaturation exist[16] and suggest that denaturation may take place by more than one pathway to yield different end products.

(15) W. T. Astbury, S. Dickinson and K. Bailey, *Biochem. J.*, **29**, 2351 (1935).

(16) H. P. Lundgren and J. W. Williams, *J. Phys. Chem.*, **43**, 989 (1939); H. P. Lundgren, *J. Biol. Chem.*, **138**, 293 (1941); H. Neurath, J. P. Greenstein, F. W. Putnam and J. O. Erickson, *Chem. Revs.*, **34**, 157 (1944).

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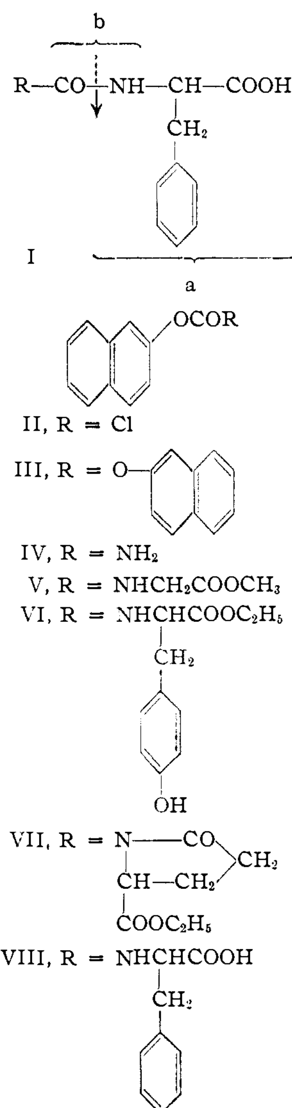
[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF HARVARD UNIVERSITY, DEPARTMENT OF SURGERY OF BETH ISRAEL HOSPITAL AND HARVARD MEDICAL SCHOOL]

Synthesis of Naphthyl Carbonate Derivatives of Amino Acids as Chromogenic Substrates for Carboxypeptidase[1]

BY GEORGE WOLF AND ARNOLD M. SELIGMAN

The synthesis of carbonaphthoxyphenylalanine is described. Since the peptide is colorless, slightly soluble in aqueous solution, moderately stable at *p*H 7.8, unattacked by esterase and lipase (preliminary observation) and hydrolyzed by crystalline carboxypeptidase or by pancreatic tissue which has been subjected to tryptic digestion, it appears to possess properties on which to base a method for the colorimetric demonstration of carboxypeptidase.

The structural requirements of substrates susceptible to enzymatic hydrolysis by carboxypeptidase have been defined.[2] These are a phenylalanine or tyrosine residue with a free carboxyl group (Ia), a peptide link which is hydrolyzed by the enzyme (Ib), and acyl group (I; RCO), which may consist of a peptide chain, a benzoylglycyl group (I; R = $C_6H_5CONHCH_2$–), carbobenzoxy residue (I; R = $C_6H_5CH_2O$–), chloroacetyl group (I; R = $ClCH_2$–) or acetyl group (I; R = CH_3–).



In order to make possible the development of highly colored azo dyes from the hydrolysis product of the substrate for the colorimetric determination or histochemical demonstration of enzymatic activity, analogous compounds with a carbonaphthoxy group (I; R = $C_{10}H_7O$–) were prepared. Following enzymatic hydrolysis, naphthyl carbonate would be expected to lose CO_2 to form β-naphthol which could be converted to an azo dye as in methods developed previously for a variety of enzymes.[3-9]

On the basis of earlier studies[2] with synthetic substrates, it was expected that carbonaphthoxyphenylalanine (VIII) would provide sufficient specificity for demonstrating carboxypeptidase. In addition to the diester, dinaphthyl carbonate

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(2) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).

(3) M. L. Menten, J. Junge and M. H. Green, *J. Biol. Chem.*, **153**, 471 (1944).

(4) L. H. Manheimer and A. M. Seligman, *J. Nat. Cancer Inst.*, **9**, 181 (1948).

(5) M. M. Nachlas and A. M. Seligman, *ibid.*, **9**, 415 (1949).

(6) M. M. Nachlas and A. M. Seligman, *Anat. Record*, **105**, 677 (1949).

(7) M. M. Nachlas and A. M. Seligman, *J. Biol. Chem.*, **181**, 343 (1949).

(8) A. M. Seligman, M. M. Nachlas, L. H. Manheimer, O. M. Friedman and G. Wolf, *Ann. Surg.*, **130**, 333 (1949).

(9) A. M. Seligman and M. M. Nachlas, *J. Clin. Invest.*, **29**, 31 (1950).

(III) and the amide (IV), other carbonaphthoxy peptides were readily prepared (V, VI, VII) from the amino acid esters. These esters could not be converted to the acids without simultaneous hydrolysis of the carbonaphthoxy ester linkage to form naphthol. In tissue, however, selective enzymatic hydrolysis by esterase might be expected to occur to form the carbonaphthoxy amino acids.

Preliminary experiments[10] have shown that at *p*H 7.8 and 37°, homogenates of fresh pancreatic tissue (rat), which contain lipase and esterase, in 2 hours hydrolyze slightly the diester (III), but not at all the amide (IV), or the peptides (V–VIII). Following conversion of procarboxypeptidase to carboxypeptidase in these homogenates by the addition of trypsin or homogenate of jejunum, enzymatic hydrolysis was extensive with L- and D,L-carbonaphthoxyphenylalanine (VIII), was slight with the ethyl ester (VII), and was not observed with the amide (IV) or the peptides (V and VI). Crystalline carboxypeptidase[11] hydrolyzed only L- and D,L-carbonaphthoxyphenylalanine (VIII). Crystalline trypsin, pepsin and chymotrypsin failed to hydrolyze these substrates. Enzymatic hydrolysis was indicated by the appearance of naphthol in excess of the amount due to spontaneous hydrolysis at *p*H 7.8 in control experiments. Naphthol was converted to an azo dye and measured colorimetrically. Extensive spontaneous hydrolysis was noted only with the amide (IV), at *p*H 7.8. All substrates were hydrolyzed extensively at higher *p*H. The linkage susceptible to alkaline hydrolysis was presumably the carbonaphthoxy ester link.

Carbonaphthoxyphenylalanine (VIII) was prepared by the addition of carbonaphthoxy chloride[12] (II) and acetone to a solution of the sodium salt of phenylalanine in water in the presence of sodium acetate. Carbonaphthoxyamide (IV) was prepared by the action of ammonia on carbonaphthoxy chloride (II), a method which differs from that used by Gattermann[13] in his preparation of the compound.

Carbonaphthoxyglycine methyl ester (V) was made by the treatment of glycine methyl ester with carbonaphthoxy chloride in pyridine solution. Dinaphthyl carbonate (III) was obtained as a by-product in this reaction; it had been prepared by a different method by Einhorn and Hollandt.[14] Carbonaphthoxy-L-tyrosine ethyl ester (VI) was obtained by the action of carbonaphthoxy chloride on L-tyrosine ethyl ester in pyridine solution. Dinaphthyl carbonate was also obtained from the reaction.

No recognizable product, apart from dinaphthyl carbonate, could be isolated from a reaction of carbonaphthoxy chloride with diethyl glutamate under various conditions, although the acid chloride (II) readily reacted with ethyl 2-pyrrolidone-5-carboxylate, the lactam of diethyl glutamate, to give ethyl N-carbonaphthoxy-2-pyrrolidone-5-carboxylate (VII). An analogous compound, ethyl N-benzoyl-2-pyrrolidone-5-carboxylate, was obtained by Froentjes[15] from benzoyl chloride and ethyl 2-pyrrolidone-5-carboxylate.

(10) H. A. Ravin and A. M. Seligman, *J. Biol. Chem.*, in press.

(11) Obtained from Worthington Biochemical Laboratories, Freehold, New Jersey.

(12) A. Einhorn and L. Rothlauf, *Ann.*, **382**, 256 (1911).

(13) L. Gattermann, *ibid.*, **244**, 29 (1888).

(14) A. Einhorn and F. Hollandt, *ibid.*, **301**, 95 (1898).

Experimental[16]

Carbonaphthoxy Chloride (II).—This compound was prepared essentially by the method of Einhorn and Rothlauf.[12] Through a solution of anhydrous benzene (32 cc.) was passed phosgene until 7 g. of the gas had been taken up. This solution was then poured into a cooled solution of β-naphthol (10 g.) and quinoline (8.9 g.) in anhydrous benzene (25 cc.). The mixture was then warmed on the steam-bath for 10 minutes and filtered. The solvent was removed from the filtrate on the steam-bath and the residue distilled, b.p. 150–152° (9 mm.). The distillate was dissolved in anhydrous ether and crystallized by addition of ligroin, m.p. 66°.

Carbonaphthoxyphenylalanine (VIII).—Phenylalanine (1 g.) and sodium acetate (0.65 g.) were dissolved in 2 *N* sodium hydroxide (3.03 cc.) and water (50 cc.). The solution was cooled and its *p*H adjusted to about 8. To this solution was added with vigorous stirring powdered carbonaphthoxy chloride (1.2 g.) and acetone (30 cc.). Stirring was continued for 10 minutes, until most of the acid chloride had disappeared. The *p*H of the solution was then adjusted to about 7 and the acetone evaporated on the steam-bath. The aqueous residue was then cooled in ice, acidified with dilute hydrochloric acid and extracted with several portions of ether. The collected ether extracts were re-extracted with several portions of dilute sodium bicarbonate solution. Any solid dinaphthyl carbonate, which is insoluble in both ether and bicarbonate, and appeared at this stage, was removed by filtration. The collected aqueous extracts were then acidified with dilute hydrochloric acid and extracted with ether. The ether solution was washed with water, dried and concentrated. Carbonaphthoxyphenylalanine was precipitated by the addition of ligroin and recrystallized from ethyl acetate by addition of ligroin. It was obtained as colorless prisms, 1.1 g. (55%), very soluble in acetone and alcohol and slightly soluble in water (0.5 mg. per cc.). Incubation in phosphate buffer, *p*H 7.8, for 2 hours resulted in 20% hydrolysis and at *p*H 7.0 in 8% hydrolysis.[10]

Carbonaphthoxy-DL-phenylalanine was prepared from DL-phenylalanine, m.p. (with decomposition) 160°.

Anal. Calcd. for $C_{20}H_{17}O_4N$: C, 71.64; H, 5.11. Found: C, 71.42; H, 5.04. Carbonaphthoxy-L-phenylalanine prepared from L-phenylalanine[17]: m.p. (with decomposition) 116°. *Anal.* Found: C, 71.71; H, 5.28.

Carbonaphthoxyamide (IV).—Carbonaphthoxy chloride (1 g.) was added to a saturated aqueous solution of ammonia (50 cc.) with shaking. The precipitate was collected by filtration and recrystallized from alcohol, 0.8 g. (89%), m.p. (with decomposition) 198° (Gattermann[13] gives a m.p. 187°).

Anal. Calcd. for $C_{11}H_9O_2N$: C, 70.57; H, 4.84. Found: C, 70.70; H, 4.70.

Carbonaphthoxyglycine Methyl Ester (V).—Carbonaphthoxy chloride (1.15 g.) was added to a solution of glycine methyl ester (0.5 g.) in anhydrous pyridine (5 cc.). The solution was warmed on the steam-bath for 30 minutes, treated with dilute hydrochloric acid and extracted with ether. The ethereal extract was washed with sodium carbonate solution and water, dried and concentrated. The residue was dissolved in hot alcohol. Upon cooling, dinaphthyl carbonate crystallized and was collected by filtration. Carbonaphthoxyglycine methyl ester crystallized upon concentration of the filtrate. It was recrystallized from 80% alcohol, 0.72 g. (51%), m.p. 99°.

Anal. Calcd. for $C_{14}H_{13}O_4N$: C, 64.89; N, 5.06. Found: C, 64.94; H, 5.00.

Dinaphthyl Carbonate[14] (III).—Dinaphthyl carbonate obtained as a by-product in the above reaction, was recrystallized from alcohol, m.p. 170°.

(15) W. Froentjes, *Rec. trav. chim.*, **62**, 97 (1943).

(16) Analyses by Mrs. Shirley Golden. Melting points uncorrected.

(17) Obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

Anal. Calcd. for $C_{21}H_{14}O_3$: C, 80.26; H, 4.50. Found: C, 80.16; H, 4.78.

Carbonaphthoxy-L-tyrosine Ethyl Ester (VI).—Carbonaphthoxy chloride (0.5 g.) was added to a solution of L-tyrosine ethyl ester (0.5 g.) in anhydrous pyridine (10 cc.). The solution was kept at room temperature for 2 hours, treated with dilute hydrochloric acid and extracted with ether. The ether extract was washed with sodium carbonate solution, water, dried and evaporated. The residue was dissolved in hot alcohol. Upon cooling dinaphthyl carbonate crystallized and was collected by filtration. Carbonaphthoxy-L-tyrosine ethyl ester could be obtained on concentration of the filtrate. It was recrystallized from ethyl acetate by the addition of ligroin and then from ether by the addition of ligroin, 0.39 g. (41%), m.p. (with decomposition) 137°.

Anal. Calcd. for $C_{22}H_{21}O_5N$: C, 69.66; H, 5.57. Found: C, 69.86; H, 5.86.

Ethyl N-Carbonaphthoxy-2-pyrrolidone-5-carboxylate (VII).—To a solution of ethyl 2-pyrrolidone-5-carboxylate[18] (0.8 g.) in anhydrous pyridine (5 cc.) was added carbonaphthoxy chloride (1 g.). The mixture was warmed on the steam-bath for 30 minutes, treated with dilute hydrochloric acid and extracted with ether. The ether extract was washed with dilute sodium carbonate solution, water, dried and evaporated. The residue was dissolved in hot alcohol. Upon cooling, dinaphthyl carbonate crystallized. It was collected by filtration. After evaporation of some alcohol, the pyrrolidone carboxylate crystallized from the filtrate. It appeared in long, colorless needles, 0.7 g. (44%) m.p. 119°, on recrystallization from alcohol.

Anal. Calcd. for $C_{18}H_{17}O_5N$: C, 66.12; H, 5.23. Found: C, 66.28; H, 5.24.

(18) E. Fischer and R. Boehner, *Ber.*, **44**, 1332 (1911).

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Synthesis of Ketomethyldihydronaphthoic Acid Derivatives as Possible Chromogenic Substrates[1]

BY GEORGE WOLF AND ARNOLD M. SELIGMAN

The syntheses of 1-keto-methyl-1,2-dihydro-2-naphthoyl-DL-phenylalanine (XIV) and of methyl 1-keto-2-methyl-1,2-dihydro-2-naphthoate (I) are described. Hydrolysis of the peptide or ester link is followed by decarboxylation and aromatization to 2-methyl-1-naphthol which can be converted to highly colored azo dyes. However, these substrates are not hydrolyzed by carboxypeptidase or esterase and lipase, respectively.

In a previous publication[2] reasons for expecting carbonaphthoxyphenylalanine to be a suitable chromogenic substrate for carboxypeptidase[3] were given. In that case enzymatic hydrolysis of the peptide linkage resulted in the formation of the unstable naphthyl carbonate, with subsequent loss of CO_2 and formation of naphthol. Because of the possibility of hydrolysis of the carbonaphthoxy ester linkage at alkaline *p*H, another acyl derivative of phenylalanine was sought which, upon enzymatic hydrolysis, would produce a carboxylic acid unstable enough to decompose spontaneously and immediately to a naphthol, which then could be converted to an azo dye by coupling with an appropriate diazonium salt. It was expected that these requirements might be fulfilled by 1-keto-2-methyl-1,2-dihydro-2-naphthoylphenylalanine (XIV). Following hydrolysis to the β-keto acid, 1-keto-2-methyl-1,2-dihydro-2-naphthoic acid, decarboxylation to the unstable 1-keto-2-methyl-1,2-dihydro naphthalene (II) would result in rearrangement to 2-methyl-1-naphthol (III). The aromatization of the intermediate ketone (II) would be an additional driving force in the decarboxylation of the β-keto acid formed from XIV. That this conversion does indeed take place was readily demonstrated by the hydrolysis of the β-keto ester (I) in dilute alkali, which led to an instantaneous conversion to the naphthol (III).

In preliminary experiments[4] with the peptide (XIV), no enzymatic hydrolysis by the carboxypeptidase in tryptically activated pancreatic homogenate was obtained at *p*H 7.8 and 37° in 2 hours. The β-keto ester (I) was not hydrolyzed by esterase[5] or lipase[5] at *p*H 7.8 at 37° in 2 hours with rat liver and pancreas.

The ketomethyldihydronaphthoylphenylalanine (XIV) was prepared from methyl 1-keto-2-methyl-1,2,3,4-tetrahydronaphthoate (VI), readily obtainable from 1-tetralone by the method of Bachmann and Thomas.[6] The ester (VI) was hydrolyzed to ketomethyltetrahydronaphthoic acid (VII) by cold, dilute alkali within 24 hours in good yield. This reaction is remarkable in that generally a tertiary ester group as in VI is more resistant to hydrolysis. The acid (VII) is stable at room temperature for periods of several days. It was converted into ketomethyltetrahydronaphthoyl chloride (VIII), which reacted readily with ammonia to give the amide (IX), and with glycine ester and phenylalanine ester to afford (after saponification) the respective ketomethyltetrahydronaphthoyl amino acid derivatives (X and XI). Upon treatment with N-bromosuccinimide, the acid chloride (VIII) gave presumably the bromo-acid chloride (XII) which, at once and without isolation, was made to react with phenylalanine ethyl ester in the presence of an excess of pyridine to form ketomethyldihydronaphthoylphenylalanine ethyl ester. The ester was readily

(1) This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service, (in part) by a grant from the American Cancer Society (Massachusetts Division), and (in part) by an institutional grant to Harvard University from the American Cancer Society.

(2) G. Wolf and A. M. Seligman, THIS JOURNAL, **73**, 2080 (1951).

(3) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).

(4) H. A. Ravin and A. M. Seligman, *J. Biol. Chem.*, in press.

(5) M. M. Nachlas and A. M. Seligman, *ibid.*, **181**, 343 (1949).

(6) W. E. Bachmann and D. G. Thomas, THIS JOURNAL, **63**, 598 (1941).