large quantity of water and concentrating it *in vacuo* in an atmosphere of carbon dioxide, the hydroxytryptophan deposited gradually. The fine precipitate, which has a decomposition point of 248–249°, was filtered off and dried for analysis.

Anal. Calcd. for $C_{11}H_{12}O_3N_2$: C, 59.97; H, 5.49; N, 12.73. Found: C, 59.91; H, 5.54; N, 12.66.

The ninhydrin and other tests gave the same results as in the case of the hydrochloride salt.

Acknowledgment.—The authors wish to express their hearty thanks to Dr. David E. Green in reading the manuscript and to Dr. Bernard J. Katchman for his help in preparing the manuscript.

Summary

The synthesis of α-hydroxytryptophan is described.

OSAKA, JAPAN RECEIVED APRIL 3, 1950

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF IOWA STATE COLLEGE]

Enzymic Synthesis of Peptide Bonds. III. The Relative Effects of Some Amino Acids and their Acyl Substituents[1]

BY SIDNEY W. FOX AND HARRY WAX[2]

The comparative extents of reaction of the benzoyl derivatives of monoaminomonocarboxylic acids with aniline in the presence of papain–cysteine have been shown to be an expression of "preferential" rates of reaction.[3,4] The anilide model was introduced by Bergmann and Fraenkel–Conrat[5]; the evaluation of many conditions which influence the reaction[3,6] has made feasible a simple gravimetric method for systematic and precise comparisons of the reactivity of various substrates. Prior to application of the method to substrates involving two or more amino acid residues, it was of interest to gain some insight into how the relative reactivities of various amino acid residues may be affected by the nature of acyl substituents. In the course of such studies new information on the influence of other factors, such as *p*H, has been uncovered.

The effects for four benzoylamino acids showed reactivity in the descending order of leucine, alanine, glycine and valine.[3] Three of these amino acids, leucine, valine and glycine plus one other, glutamic acid, were employed in the present investigation. The four blocking groups studied were: benzoyl, *p*-nitrobenzoyl, carbobenzoxy, and carboallyloxy. Nearly all of the compounds were previously known; few of the anilides which resulted from enzymic reaction, however, had been previously described. Since establishment of identity of these materials is prerequisite to employment of the gravimetric method used in these studies, the constants of the anilides were compiled either from accumulated quantities of product in enzyme studies or from special preparations. The constants for the various anilides are given in Table I. Of the sixteen acylamino acids submitted to reaction with aniline in the presence of papain–cysteine, only carboallyloxy-L-glutamic acid failed to deposit a precipitate, even though this was tested over the wide *p*H range and the two buffer concentrations employed for the other acylamino acid reactants.[7]

The typical dependency upon *p*H of the reactions studied is shown in Figs. 1 and 2, which also illustrate the marked activating effect of citrate buffer as previously noted with the benzoylamino acids alone, under somewhat different conditions.[6] The moderately atypical behavior of the carbobenzoxy derivatives is shown in Fig. 3.

A summary of the optimal *p*H ranges found is given in Table II. These results emphasize, in conjunction with the optima of around 6 found for aromatic amino acids,[6] the effect of substrate upon the *p*H optimum of an enzyme. The importance of substrate to *p*H optimum has long

(1) Journal Paper No. J-1735 of the Iowa Agricultural Experiment Station, Ames, Iowa, Project 1111. This project is supported in large part by the National Cancer Institute of the National Institutes of Health, Public Health Service.

(2) Much of the work presented in this paper is from the Ph.D. thesis of Harry Wax, Iowa State College, 1949.

(3) Fox, Minard, Wax, Pettinga and Strifert, *Fedn. Proc.*, **8**, 198 (1949); Fox, Pettinga, Halverson and Wax, *Arch. Biochem.*, **25**, 21 (1950).

(4) The terminology of preferential hydrolysis has been recently emphasized, for a carboxypeptidase, by Kabrowska, Kazenko and Laskowski, *Science*, **110**, 95 (1949), and preferential results have been found for the previously allegedly highly specific leucine aminopeptidase by Smith and Polglase, *J. Biol. Chem.*, **180**, 1209 (1949). The fact that the term, "preference," is more apt than "specificity" is thus applicable to proteases other than papain. That specificity should not be interpreted literally has been recognized for some time, *e. g.*, Tauber has stated, "A specificity that is less than 100 per cent. is not rare in enzymology," in "Chemistry and Technology of Enzymes," 1949, p. 129, *cf.* also, *e. g.*, Stahmann, Fruton and Bergmann, *J. Biol. Chem.*, **164**, 753 (1946). In the reported conflict between the Bergmann concept of specificity of pepsin and the results of hydrolytic experiments of Bull, *Rec. Chem. Prog.*, **10**, 195 (1949), if the situation is recognized to involve preference rather than specificity, the conflict does not necessarily exist. It may also be noted that even the concept of antipodal specificity requires refinement beyond the original first approximation, as in the result of experiments with the enzymic synthesis of phenylhydrazides; Bennett and Niemann, THIS JOURNAL, **72**, 1798 (1950); Milne and Stevens, *ibid.*, **72**, 1742 (1950).

(5) Bergmann and Fraenkel-Conrat, *J. Biol. Chem.*, **119**, 707 (1937).

(6) Fox and Pettinga, *Arch. Biochem.*, **25**, 13 (1950).

(7) Two explanations of this non-reactivity include (1) the probability that the expected anilide is soluble (*cf.* ref. 3 for a critical discussion of the contribution of solubility), and (2) that the particular structure of carboallyloxyglutamic acid is inhibitory to enzyme–substrate interaction. Addition of the acid in other anilide syntheses gave no inhibition, and the solubility explanation seems, therefore, to be more acceptable at present, although yet other steric explanations are not excluded.

TABLE I

ACYLAMINO ACID ANILIDES PREPARED

Acylamino acid anilide	M. p.[a] found, °C.	M. p. reported, °C.	Nitrogen, % Calcd.	Found	$[\alpha]^{28}D$
Benzoylglycinanilide	213-215	212.5[b]			
Benzoyl-L-valinanilide	215-217	218-220[c]			
Benzoyl-L-leucinanilide	213.5-215	213[b]			
Benzoyl-L-glutamic acid anilide	169-171		8.58	8.34[d]	+4.61 ± 0.3°[e]
p-Nitrobenzoylglycinanilide	213.5-215.5		14.1	14.1[d]	
p-Nitrobenzoyl-L-valinanilide	215-216		12.3	12.0[d]	−15.4 ± 0.3°[f]
p-Nitrobenzoyl-L-leucinanilide	188-190		11.8[d]	12.0[d]	+14.1 ± 0.5°[g]
p-Nitrobenzoyl-L-glutamic acid anilide	191-192		11.3	11.2[d]	+10.5 ± 0.3°[h]
Carbobenzoxyglycinanilide	144-144.5	144[b]			
Carbobenzoxy-L-valinanilide	182-183.5		8.58	8.59[d]	−33 ± 0.5°[i]
Carbobenzoxy-L-leucinanilide	138-141[j]		8.24	8.46[d]	−47.6 ± 0.3°[i]
Carbobenzoxy-L-glutamic acid anilide	195-196	193-195[k]			
Carboallyloxyglycinanilide	134-136		12.0	12.2[l]	
Carboallyloxy-L-valinanilide	168-169		10.1	10.0[l]	−48.0 ± 0.5°[i]
Carboallyloxy-L-leucinanilide	160.5-162		9.65	9.46[l]	−64.0 ± 0.3°[i]

[a] All m. p.'s are uncorrected. [b] Ref. 5. [c] Ref. 3; the 3° discrepancy represents the m. p. correction. [d] By micro Dumas. [e] 2% in 0.5 *N* potassium hydroxide; in 95% ethanol, $[\alpha]^{28}D$ was 1.25° ± 0.25°. [f] 2% in dioxane. [g] 2% in 95% ethanol. [h] 2% in 0.5 *N* potassium hydroxide solution. [i] 2% in chloroform. [j] Compound reported in ref. 5, but no m. p. given. [k] Behrens and Bergmann, *J. Biol. Chem.*, 129, 587 (1939). [l] By micro-Kjeldahl.

been recognized,[8,9] but the catholic spectrum of substrate preferences exhibited by papain permitted a particularly incisive demonstration of this relationship.

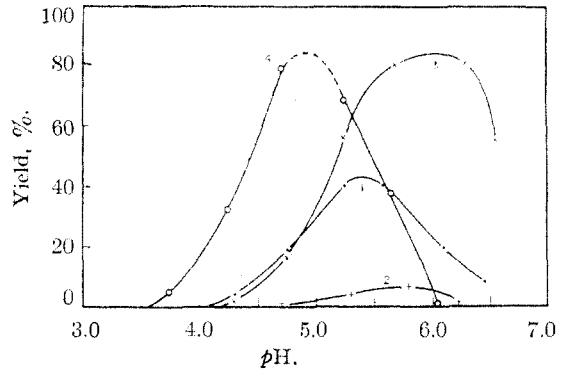

Fig. 1.—*p*H–activity curves for *p*-nitrobenzoylamino acids in 1.0 *M* citrate. In all of the graphs, 1, 2, 3 and 4 represent glycine, valine, leucine and glutamic acid, respectively.

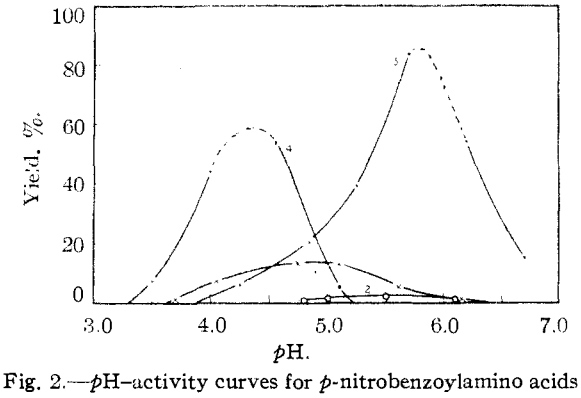

Fig. 2.—*p*H–activity curves for *p*-nitrobenzoylamino acids in 0.1 *M* citrate.

(8) Willstätter and Grassmann, *Z. physiol. Chem.*, **138**, 184 (1924).
(9) Willstätter, Grassmann and Ambros, *ibid.*, **151**, 286, 307 (1926).

TABLE II

OPTIMUM *p*H RANGES FOR SYNTHESIS OF ANILIDES OF VARIOUS ACYLAMINO ACIDS

Acylamino acid	Optimum buffer *p*H range 1 *M*	0.1 *M*	Yields of anilides, % 1 *M* Buffer	0.1 *M* Buffer
Benzoylglycine	5.4-5.6	4.5-4.7	60	25
Benzoyl-DL-valine	5.2-5.4	4.9-5.1	40	4
Benzoyl-DL-leucine	5.5-5.8	5.0-5.2	100	100
Benzoyl-L-glutamic acid	4.3-4.5	4.1-4.3	60	60
p-Nitrobenzoylglycine	5.3-5.6	4.8-5.0	45	15
p-Nitrobenzoyl-DL-valine	5.7-5.9	5.4-5.6	7	2
p-Nitrobenzoyl-DL-leucine	5.9-6.1	5.7-5.9	85	85
p-Nitrobenzoyl-L-glutamic acid	4.7-5.0	4.3-4.5	85	60
Carbobenzoxyglycine	5.1-5.4	4.2-4.4	70	30
Carbobenzoxy-DL-valine	6.2-6.4	5.5-5.7	15	10
Carbobenzoxy-DL-leucine	6.3-6.5	5.6-5.8	70	90
Carbobenzoxy-L-glutamic acid	4.6-4.9	4.1-4.3	95	95
Carboallyloxyglycine	5.1-5.3	.....	20	0
Carboallyloxy-DL-valine	5.3-5.5	.....	8	0
Carboallyloxy-DL-leucine	5.3-5.6	4.6-4.8	95	70

The accumulated observations indicate, under

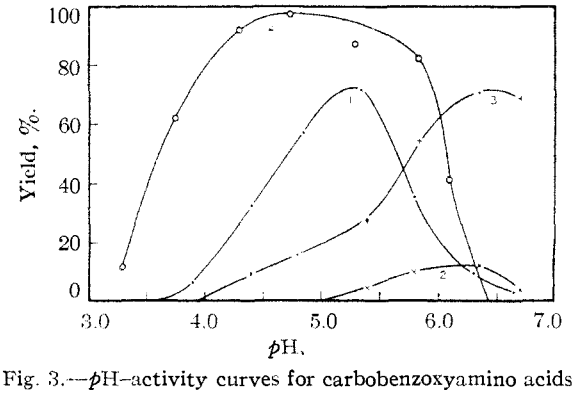

Fig. 3.—*p*H–activity curves for carbobenzoxyamino acids in 1.0 *M* citrate.

similar conditions, a *p*H optimum of approximately 4 for benzoylglutamic acid, approximately 5 for the benzoyl derivatives of glycine, alanine[3] and valine, and of about 6 for the benzoyl derivatives of leucine, phenylalanine[6] and tyrosine.[6] The optimum for glutamic acid as contrasted to the monoaminomonocarboxylic acids might be explained on the basis of a greater reactivity of the residues when in the more undissociated forms; this explanation does not, however, appear to be reconcilable with the *p*H optimum of about 6 for tyrosine.

The finding that the optimum for leucine is more categorical with those of the benzenoid amino acids than with the others of the monoaminomonocarboxylic acid series suggests that leucine, alone of the latter group, possesses some property in common with the aromatic amino acids. It is clear from Table II that the *p*H optima need also to be qualified by designated buffer concentrations. Probably other conditions are also of importance in this connection.

While the results tabulated in Table II provide data which permit some comparisons of yields, they were compiled from eight separate experiments. Variations in yield between experiments, as observed throughout much work of this sort, made desirable also simultaneous comparisons for a more precisely controlled evaluation. A typical result is presented in Table III.

TABLE III

COMPARATIVE YIELDS OF ACYLAMINO ACID ANILIDES IN A SINGLE EXPERIMENT

Acylamino acid	Yield based on L-reactant, %
Benzoylglycine	22
Benzoyl-DL-valine	5.8
Benzoyl-DL-leucine	39
p-Nitrobenzoylglycine	15
p-Nitrobenzoyl-DL-valine	0.8
p-Nitrobenzoyl-DL-leucine	18
Carbobenzoxyglycine	41
Carbobenzoxy-DL-valine	0
Carbobenzoxy-DL-leucine	13
Carboallyloxy-DL-valine	2
Carboallyloxy-DL-leucine	81

Almost all of the data support earlier conclusions on preferences involving glycine, valine, and leucine[3] and indicate that the blocking group quantitatively modifies the effects observed. The preference of leucine over valine has been observed for all four blocking groups, and also in the hydrolyses of benzamino acid amides. In the comparisons between leucine and valine, the amino acid residue which participated in the formation of the peptide bond was the primary determinant of the extent or rate of reaction. Glycine, in one comparison, however, did not occupy a position intermediate between valine and leucine. The fact that the carbobenzoxy amino acids exhibited a different order of preferences than the other acylamino acids is a point of special interest in that it suggests that various peptide blocking groups may also modify the preferences observed for the amino acid residues participating in formation of the peptide bond. Pertinent systematic experiments with acylated peptides are under way.

Experimental Details

New Acylamino Acids.—Nearly all of the acylamino acids were previously known.[10] Methods for obtaining crystalline carbobenzoxy-DL-valine and crystalline carbobenzoxy-DL-leucine have recently been described.[11] The carboallyloxy derivatives were prepared essentially by the method of Stevens and Watanabe.[12] The carboallyloxyglycine was obtained as a sirup which was partially crystallized by addition of ether, and chilling to −12°. The material melted 15–35°.

Anal. Calcd. for $C_6H_9O_4N$: N, 8.80. Found: N, 8.29.

The carboallyloxy-DL-valine was successfully crystallized from ethyl acetate–hexane; m. p. 49.5–52°.

Anal. Calcd. for $C_9H_{15}O_4N$: N, 6.96. Found: N, 7.10.

A 78% yield of carboallyloxy-L-glutamic acid was obtained by three weeks of desiccation of the original sirup, followed by rubbing with hexane after the initial crystals appeared; m. p. 55–58°; $[\alpha]^{28}D$ 0.94 ± 0.1° (5% in 1 *N* potassium hydroxide).

Anal. Calcd. for $C_9H_{13}O_6N$: N, 6.05. Found: N, 6.04.

Enzymic Synthesis of Acylamino Acid Anilides.—The procedures given in the earlier publications[3,6] were followed in essence. Principal differences were the employment of a higher aniline:acylamino acid ratio and a higher concentration of papain. For the studies of Table II and Figs. 1–3, incl., solutions of the acylamino acids were made up to 0.5 molar in the monosodium salts. To each of duplicate series of 16 × 50 mm. vials was added 2.9 ml. of citrate buffer which would give in the final solution 1.0 or 0.10 *M* citrate concentration of appropriate *p*H values in the range of 3.0–6.5. To each of these was added 0.1 ml. of redistilled aniline, 1.0 ml. of acylamino acid solution, and 1.0 ml. of a papain–cysteine solution. This last was prepared from 1.12 g. of papain (Nutritional Biochemicals lot 1953 or lot 2924, found to be approximately equivalent in activity), 0.45 g. of cysteine hydrochloride, and 70 ml. of water.

The contents were stirred well and the *p*H values determined. It was necessary, in the case of all the acylglutamic acids, in the 0.1 *M* buffers at *p*H 6.0 and 6.5 to add a few drops of 2 *N* sodium hydroxide in order to approximate the recorded values. The vials were then stoppered with paraffined corks and incubated for 72 hours at 40 ± 1° in a thermostatically controlled water-bath. The contents were shaken by hand every 12 hours.

At the end of 72 hours, the *p*H values were again determined (in almost all cases they had not varied more than 0.2 *p*H) and the contents of the vials were transferred to 25 × 150 mm. test-tubes. In the case of the acylated monoaminomonocarboxylic acid anilides, 15–20 ml. of 1 *N* sodium hydroxide was used in the transfer and, in addition, sufficient 6 *N* sodium hydroxide solution was added to those tubes containing the 1 *M* buffer solutions of *p*H values below 5.5 to give a reaction alkaline to phenolphthalein. For the acylglutamic acids, 15–20 ml. of 1 *N* hydrochloric acid was used in the transfer. The anilides were then filtered with suction, washed with several portions of water and dried in the air. Careful washing of the

(10) For numerous improvements in methods of synthesis, the Ph.D. thesis of Wax, Iowa State College, 1949, may be consulted.

(11) Fox, Fling, Wax and Pettinga, THIS JOURNAL, **72**, 1862 (1950).

(12) Stevens and Watanabe, *ibid.*, **72**, 725 (1950).

acylglutamic acid anilides was essential since they tended to be gelatinous in nature and absorbed significant amounts of citrate. The above washing procedure served to remove acylamino acid impurities and to give products of correct intrinsic and mixed m. p.'s. The compounds were then weighed to the nearest milligram[13] and the melting points checked on a melting point block. All of the studies were repeated.

The proportions of reagents employed for the experiments in Table III were the same as in earlier runs as previously described except that the molar ratio of aniline to acyl-L-amino acid was 2:1 instead of 1:1 and the quantities of acylglycines were equimolar to that of the L-acids. The citrate buffer concentration was 1.0 *M* throughout. The total reaction volume of 10 ml. was made up in tubes of 12 × 100 mm. size. The results are averages of duplicates.

Purification of Anilides.—The accumulated yields of each of the anilides from many runs were pooled in order to obtain sufficient material for characterization and determination of melting points and rotation. In certain instances in which only small quantities of the materials were available, more was synthesized enzymatically by use of proportionally larger quantities of the reactants.

All of the acylated monoaminomonocarboxylic acid anilides were washed with 1 *N* sodium hydroxide (except the glutamic acid derivatives which were washed with water alone), then with water and dried prior to recrystallization. All were purified by dissolving in the minimum quantity of hot dioxane, treating the solution with a small quantity of decolorizing carbon (Darco G-60) and adding water to the hot filtrate to incipient crystallization. The recrystallization procedure was repeated with the omission of the carbon. The acylglutamic acid anilides were recrystallized twice from dioxane–hexane to constant m. p.

(13) As has been indicated, the carboallyloxyglycine was somewhat impure. The percentage of impurities based on the deviation of the nitrogen content from theoretical, however, was approximately within the limits of accuracy of the method used in conducting the anilide synthesis studies. No corrections in yield of anilide because of impurities in the reacting acid were therefore applied.

in a manner similar to that employed above. Hexane was used instead of water for these derivatives since the latter gave gels in the presence of water.

Acknowledgments.—The analytical services of Mr. Armand McMillan are gratefully acknowledged. Miss Janet Wilkerson performed a number of experiments of which Table III represents a typical set of results.

Summary

The yields and *p*H optima, in 1.0 *M* and 0.1 *M* citrate buffer, for the papain-catalyzed formation of anilides from the benzoyl, *p*-nitrobenzoyl, carbobenzoxy and carboallyloxy derivatives of glycine, valine, leucine, and glutamic acid have been tabulated. The previously observed decreasing preferences for leucine, glycine and valine have been confirmed in this work for a total of four types of blocking groups, with a shift in order between glycine and leucine when carbobenzoxy, only, was the substituent. The more rapid and complete reaction in 1.0 than in 0.1 *M* citrate buffer has been confirmed for virtually all of these acylamino acids. Of the entire series of sixteen acylamino acids only carboallyloxyglutamic acid failed to yield an anilide.

The *p*H optima were lower in 0.1 than in 1.0 *M* citrate buffer. Accumulated observations indicate incompletely explained differences in *p*H optimum for different substrates.

The constants of the resultant new acylamino acid anilides are recorded.

AMES, IOWA RECEIVED JANUARY 18, 1950

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE OHIO STATE UNIVERSITY]

Chemical Interactions of Amino Compounds and Sugars. V.[1] Comparative Studies with D-Xylose and 2-Furaldehyde[2]

BY TZI-LIEH TAN,[3] M. L. WOLFROM AND A. W. LANGER, JR.[3]

It has been demonstrated[1,4] that the "browning" of sugar solutions, when heated in the presence or absence of amino acids at elevated temperatures, was accompanied by the formation of a small amount of furan bodies. These furan compounds, which decompose readily in the presence of mineral acids,[4b,5] amino acids[6] and other chemicals[5,6] to form color, have been considered as possible intermediates in the coloration or "browning" of various food products containing reducing sugars and amino acids. In the preceding paper[1] of this series, evidence was presented in favor of this supposition. To continue our studies on the role of furan compounds in the "browning" reaction, we have investigated and compared the reactions of glycine with a sugar, D-xylose, and with a furan compound, 2-furaldehyde. This paper describes the results of our study of coloration rates and some properties of the colored products formed in both systems.

(1) Previous communication in this series: M. L. Wolfrom, R. D. Schuetz and L. F. Cavalieri, THIS JOURNAL, **71**, 3518 (1949).

(2) This paper reports research undertaken in coöperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number 300 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of the Army.

(3) Research Associate (T.-L. Tan) and Research Fellow (A. W. L.) of The Ohio State University Research Foundation (Project 366).

(4) (a) B. L. Scallet with J. H. Gardner, THIS JOURNAL, **67**, 1934 (1945); (b) B. Singh, G. R. Dean and S. M. Cantor, *ibid.*, **70**, 517 (1948); (c) C. D. Hurd, C. D. Kelso and E. Rondesvedt, Report to the Quartermaster Food and Container Institute for the Armed Forces, July to September, 1946; (d) R. G. Rice, *Abstracts Papers Am. Chem. Soc.*, **112**, 3A (1947); (e) S. Akabori, *Ber.*, **66**, 143 (1933); *Proc. Imp. Acad. (Tokyo)*, **3**, 362 (1927).

(5) J. J. Blanksma and G. Egmond, *Rec. trav. chim.*, **65**, 309 (1946).

(6) R. G. Rice, Z. I. Kertesz and E. H. Stotz, THIS JOURNAL, **69**, 1798 (1947).