TABLE II

BARBITURIC ACID DERIVATIVES

No.	Compound	M.p., °C.	Solvent for recrystallization	Formula	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Nitrogen, % Calcd.	Nitrogen, % Found[a]
1	5-(2-Acetoxypropyl)-5-(1-methylbutyl)-	159–160	Water	$C_{14}H_{22}N_2O_5$	56.36	56.26	7.43	7.12		
2	5-Benzyl-5-ethyl-2-thio-	193–194	Alcohol	$C_{13}H_{14}N_2O_2S$	59.53	59.72	5.38	5.33		
3	5-*n*-Butyl-2-isopropyl-thio-	257–258	Alcohol	$C_{11}H_{18}N_2O_2S$	54.51	54.92	7.48	7.83	11.56	11.3
4	5-*n*-Butyl-5-isopropyl-2-thio-	152–153	Alcohol	$C_{11}H_{18}N_2O_2S$	54.51	55.05	7.48	7.03	11.56	11.3
5	5-Ethyl-2-amylthio-[b]	268–270	Alcohol	$C_{11}H_{18}N_2O_2S$	54.51	54.54	7.48	7.40	11.56	11.7
6	5-Ethyl-2-benzylthio-	258–260	Alcohol	$C_{13}H_{14}N_2O_2S$	59.53	59.93	5.38	5.78		
7	5-(2-Hydroxypropyl)-5-(1-methylbutyl)-	215–216[c]	Aq. alcohol	$C_{12}H_{20}N_2O_4$	56.24	56.68	7.87	7.70	10.94	10.8

[a] Kjeldahl nitrogen. [b] The structure of the amyl group was not determined. [c] Melting points as high as this were rarely obtained. Nevertheless, crude products with melting points as low as 165–185° gave better than 90% yields of pure acetate (compound 1) when treated with acetic anhydride.

Reaction of 5-Ethyl-5-(1-methylbutyl)-2-thiobarbituric Acid with Aluminum Chloride.—Six grams of anhydrous aluminum chloride was added to a solution of 2.00 g. of the barbituric acid in 30 ml. of hot toluene and heated under reflux for 18 hours. The mixture was poured into 300 ml. of water and the phases were separated. The aqueous phase was extracted with a minimal amount of toluene to remove black oily droplets, adjusted to *p*H 1 and extracted continuously with ether for 24 hours. Evaporation of the ether gave 1.45 g. of a light brown crystalline solid, m.p. 175–193°. The product was recrystallized from alcohol and aqueous alcohol; after two recrystallizations it melted at 190–192°. By reworking the filtrates an 80% yield was obtained. Further recrystallization of 5-ethyl-2-thiobarbituric acid raised the melting point to 195–196° but it still retained a very light brown color; Wheeler and Jamieson[9] and Einhorn[10] report the melting point as 190–192°.

NEW YORK, N. Y.

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF IOWA STATE COLLEGE]

Enzymic Synthesis of Peptide Bonds. V. Instances of Protease-Controlled Specificity in the Synthesis of Acylamino Acid Anilides and Acylpeptide Anilides[1-3]

BY FRANK JANSSEN, MILTON WINITZ AND SIDNEY W. FOX[4]

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Instances of protease-controlled specificity in the synthesis of different substituted peptides from the same substrates, benzoylphenylalanine and glycinanilide, are presented. Similar enzyme-controlled specificities, when benzoyltryptophan was the acid component or alaninanilide was the aminoid reactant, were observed. The compounds obtained from the ficin-catalyzed reactions were predominantly transamidation products whereas those from the chymotrypsin-catalyzed reactions were coupling products. Benzoylphenylalanine and glycinanilide yielded in the presence of papain a mixture of benzoylphenylalaninanilide and benzoylphenyllalanylglycylglycinanilide. Theoretical implications of these instances of specificity are considered.

The biological synthesis of peptide bonds through the agency of proteases has been suggested[5] and deserves as rigorous an evaluation as it is possible to obtain. In the consideration of any hypothesis of the biosynthesis of peptide bonds, the discernible problems which must be solved include those of the energetics and of the specificity of synthesis. The thermodynamic feasibility of the mechanism involving reversal of hydrolysis has been frequently discussed; a hypothetical means for elimination of the restriction of an exponentially unfavorable limiting equilibrium has been presented.[6]

Study of the information available has thrown open to question the concept that the specificity of the proteases is sharp enough to mediate the formation of sufficiently unique end-products.[7-9] Not only must the enzyme or other system (templet?[9,10]) select from a variety of biologically available junior peptide and amino acid fragments, but it would seem that proteosynthetic agents from different sources must necessarily exhibit some differences in their abilities to catalyze reactions from the same substrate(s). This latter type of specificity, of the many kinds that may be considered, is the principal subject of this paper.

Evidence for enzyme-controlled specificity in peptide bond synthesis has been offered.[11] Chymotrypsin was found to catalyze the coupling of benzoyltyrosine and glycinanilide whereas no reaction was recorded with papain-cysteine. The experimental details available indicate that each

(1) Paper IV. S. W. Fox and M. Winitz, *Arch. Biochem. Biophys.*, **35**, 419 (1952).

(2) Journal Paper No. J-2140 of the Iowa Agricultural Experiment Station, Ames, Iowa, Project 1111. This project has been supported by the National Cancer Institute of the National Institutes of Health, Public Health Service, and by the Rockefeller Foundation.

(3) Presented in part at the Twelfth International Congress of Pure and Applied Chemistry, New York City, September 12, 1951. Some of the work is described in the Ph.D. thesis of Milton Winitz, 1951, and in the M.S. thesis of Frank Janssen, 1952.

(4) Author to whom inquiries should be addressed.

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

(6) S. W. Fox, *Proc. Natl. Acad. Sci.*, **37**, 291 (1951).

(7) S. W. Fox, C. W. Pettinga, J. S. Halverson and H. Wax, *Arch. Biochem.*, **25**, 21 (1950).

(8) P. C. Caldwell and C. Hinshelwood, *J. Chem. Soc.*, 3156 (1950).

(9) F. Haurowitz, "Chemistry and Biology of Proteins," Academic Press, Inc., New York, N. Y., 1950, p. 348.

(10) A. Claude, *Adv. Prot. Chem.*, **5**, 423 (1949).

(11) J. S. Fruton, *Cold Spring Harbor Symp. Quant. Biol.*, **6**, 55 (1938).

enzyme was employed at the *p*H ordinarily used with that catalyst; non-reactivity may have thus merely reflected insufficient testing of *p*H, as has been pointed out for a similar example,[12] in which small changes of *p*H produced profound differences in reactivity. Since the original studies on specificity, not only *p*H,[12,13] but buffer concentration[12,14] has been demonstrated to be critical. It may be noted that the effect of buffer concentration, for example, produces what may be interpreted as qualitatively different results in some cases. No carboallyloxylglycinanilide or carboallyloxy-L-valinanilide formed in 0.1 *M* citrate buffer whereas substantial yields were obtained in 1.0 *M* citrate.[14] Other conditions such as volume of reaction mixture (Fig. 2 of an earlier paper[12]) have been shown to be critical in determining whether reaction could be observed or not.

Similar considerations are germane for some reports of demonstration of protease-controlled hydrolytic specificity,[15,16] in which it is not clear whether the results observed were due to *p*H, enzyme, or other conditions. In anilide synthesis, both the nature of the reaction and the law of the single variable require that any experimentally valid example of protease-controlled specificity will include two reactions with two enzymes acting on the same substrates, and with all other initial conditions as identical as possible. Both reactions, furthermore, should yield products of different constitution.

The effect of *p*H on the reaction of benzoyltyrosine and glycinanilide with papain–cysteine in one case and chymotrypsin in the other is shown in Table I. These results confirm the essential comparison reported in the literature and indicate that non-reactivity of the substrates in presence of papain is not overcome simply by employment of a high *p*H for this enzyme.

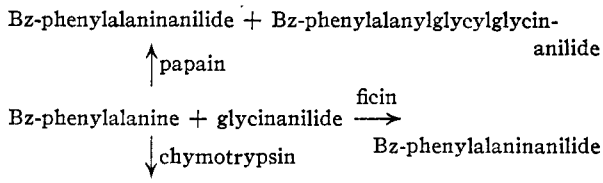
TABLE I

VARIATION IN YIELDS OF BENZOYLTYROSYLGLYCINANILIDE WITH PAPAIN, CHYMOTRYPSIN AND DIFFERING *p*H

Substrates, N-Bz-L-tyrosine + glycinanilide

Enzyme	Initial *p*H	Yield, %
Papain	5.0	0
	7.0	0
	7.5	0
Chymotrypsin	7.4	16
	7.7	18
	7.9	22

The *p*H-activity data for the reaction of benzoylphenylalanine and glycinanilide in the presence of ficin, papain and chymotrypsin, without regard to nature of the product, demonstrated that the ranges for the three enzymes overlap to a considerable degree. Each of these enzymes yielded products in a range which was represented by initial *p*H's of 6.5–7.5. Table II presents results typical of experiments in which benzoylphenylalanine and glycinanilide were allowed to react in the presence of each of the three enzymes under otherwise initially identical conditions. It may be seen that the initial crude products were specific and that they differed to the extent that they reacted in a modified biuret test to give a unique color in each case. The initial ficin and chymotrypsin products were almost pure and represented transamidation and coupling reactions, respectively. Papain yielded a mixture consisting of benzoylphenylalaninanilide and predominantly of benzoylphenylalanylglycylglycinanilide (Fig. 1 and Table VI). For the formation of the latter, one mechanism compatible with the facts would involve formation of glycylglycinanilide from two molecules of glycinanilide followed by coupling with benzoylphenylalanine. The reaction yielding a glycylglycinanilide residue is analogous to the reaction of methionine ester[17] and of phenylalanine ester.[18] No evidence is at hand, however, to support or reject the suggested condensation mechanism in the present case.



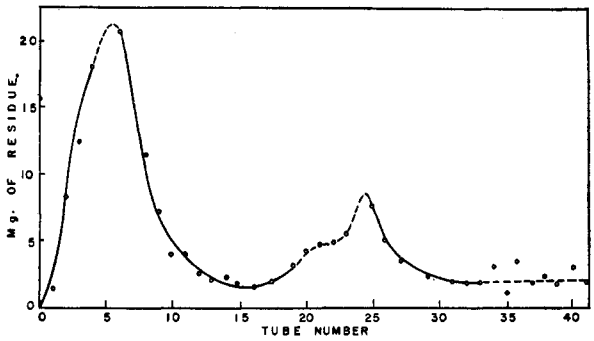


Fig. 1.—Graph of counter-current distribution of product from papain-catalyzed reaction of Table I. The main component gave the constants reported for Bz-phenylalanylglycylglycinanilide in Table VI. The second component showed m.p. and mixed m.p. of Bz-phenylalaninanilide. The solvent system was prepared by agitation of 400 ml. of chloroform, 1600 ml. of cyclohexane, 1600 ml. of 95% ethanol and 400 ml. of water, and used by the method of L. C. Craig and O. Post, *Anal. Chem.*, 21, 500 (1949).

Thus is illustrated not only a qualitative specificity between chymotrypsin and ficin but also a considerable difference in the nature of the reaction as catalyzed by the closely related ficin and papain. The larger *p*H drop of the ficin reaction, a phenomenon repeatedly observed, appears to be of no moment when viewed against the nature of the products obtained over the *p*H range involved in these experiments. In other studies involving initial and final *p*H's, respectively, of 6.6 → 5.9, 6.4 → 6.0 and 6.1 → 5.9, the product was virtually

(12) S. W. Fox and C. W. Pettinga, *Arch. Biochem.*, **25**, 13 (1950).

(13) (a) N. F. Albertson, THIS JOURNAL, **73**, 452 (1951); W. H. Schuller and C. Niemann, *ibid.*, **74**, 4630 (1952).

(14) S. W. Fox and H. Wax, *ibid.*, **72**, 5087 (1950).

(15) M. Bergmann and J. S. Fruton, *J. Biol. Chem.*, **118**, 405 (1937).

(16) M. Bergmann and J. S. Fruton, *Adv. Enzymol.*, **1**, 63 (1941).

(17) M. Brenner, H. R. Mueller and R. S. Pfister, *Helv. Chim. Acta* **33**, 568 (1950).

(18) H. Tauber, THIS JOURNAL, **74**, 847 (1952).

TABLE II

REACTIONS OF BENZOYLPHENYLALANINE WITH GLYCINANILIDE AS CATALYZED BY THREE PROTEASES

Protease	Initial *p*H	Final *p*H	Yield, mg.	M.p. of crude product, °C.	Modified biuret test of crude product	M.p. of recrystallized product, °C.	Product
Papain	6.4[a]	6.3	8.9	236–243 with premelting	Violet	240–245[b] with premelting	Mixture[c]
Ficin	6.4[a]	5.8	19	215–218	Green	221–222[d]	Bz-phenylalaninanilide
Chymotrypsin	6.4[a]	6.2	192	241.5–244	Blue	244–245	Bz-phenylalanylglycinanilide

[a] Buffer was 1.0 *M* citrate. [b] Mixed m.p. with Bz-phenylalanylglycinanilide 224–232°. [c] See Fig. 1. [d] Mixed m.p. with pure product from Bz-phenylalanine and aniline 221–222°.

TABLE III

REACTIONS OF BENZOYLPHENYLALANINE WITH ALANINANILIDE AS CATALYZED BY THREE PROTEASES

Protease	Initial *p*H	Final *p*H	Yield, mg.	M.p. of crude product, °C.	Mixed m.p. with pure product, °C.	Product
Papain	6.4[a]	5.7	72	217–219.5	221–222	Bz-phenylalaninanilide
Ficin	6.4[a]	5.6	10	215–219	220–222	Bz-phenylalaninanilide
Chymotrypsin	6.4[a]	5.7	12	242–244.5	.....	Bz-phenylalanylalaninanilide

[a] Buffer 1.0 *M* citrate.

TABLE IV

REACTIONS OF BENZOYLTRYPTOPHAN WITH GLYCINANILIDE AS CATALYZED BY THREE PROTEASES

Protease	Initial *p*H	Final *p*H	Yield, mg.	M.p. of crude product, °C.	M.p. of recrystd. product, °C.	Mixed m.p., °C.	Product
Papain	7.4	6.8	87	197–202	195–197	197–199	Bz-tryptophananilide
Ficin	7.4	6.8	29	199–203	196–198	199–202	Bz-tryptophananilide
Chymotrypsin	7.4	7.4	12	198–199 with premelting at 125–140	200–201[a]	180–190	Bz-tryptophylglycinanilide

[a] M.p. after second recrystn.: 200–201°; buffer, mixed citrate (0.5 *M*)–phosphate (0.5) *M*.

pure benzoylphenylalaninanilide in each case. No explanation for the larger *p*H decrease for this reaction is at hand. The answer may be in the nature of the other reactions which occur in solution but are not expressed in precipitates. In an experiment initially at *p*H 7.2 the final *p*H was 6.5; at this higher level the benzoylphenylalaninanilide was again the predominant product, but there was also isolated a component melting with decomposition at 270°.

In the reaction of benzoylphenylalanine and alaninanilide as portrayed in Table III, the *p*H drop in the reaction was virtually identical for all systems. Again there may be observed a specificity of reaction as determined by the enzyme used. With this aminoid reactant ficin and papain each yielded the same precipitated product, benzoylphenylalaninanilide, but chymotrypsin promoted coupling. The influence of substrate may be seen when one compares the action of papain on glycinanilide and on alaninanilide in their respective reactions with benzoylphenylalanine.

In Table IV may be seen the results of replacing the benzoylphenylalanine by benzoyltryptophan. This latter component did not react at an initial *p*H of 5.0. The identity of each purified product as in Tables II and III, was determined by analysis, aided by mixed melting points. The benzoyltryptophananilide and benzoyltryptophylglycinanilide possessed closely similar melting points, yet it may be seen that the mixed melting point of these two anilides showed a depression of approximately 20°.

In all of the comparisons recorded in Tables II–IV the critical factor of *p*H range was common to all of the enzyme systems studied within each experiment; the differences were those of identity of enzyme preparation employed. These studies, accordingly, embrace valid instances of protease-controlled specificity. Without such an example at hand, the concept of a mechanism of synthesis of protein by reversal of proteolysis would be considerably less tenable than it is. The manner in which identity of involved amino acid residues may modify such protease-controlled specificity has been studied and will be the subject of a later communication. Such modification and extension of the experiments recorded here should also permit more adequate evaluation of the degree to which the anilide type of model may explain the forces underlying biological specificities.

Experimental

Reactants.—The benzoylamino acids are well known. Glycinanilide[19] has also been described. Since alaninanilide was known only as an oil and as the picrate,[19] the base obtained from the haloacyl intermediate was converted to a solid hydrogen citrate salt in order to contain the same anionic component as the buffer used in the anilide syntheses. For purposes of future reference other amino acid anilide citrates prepared in this work are also described here.

α-Bromopropionylaniline[20] (3.6 g.) was dissolved in 125 ml. of ethanolic ammonia (saturated in an ice-bath) and heated in a pressure bottle in a boiling water-bath for 20 hours. The contents of seven such bottles were concentrated to an oily residue which was then dissolved in 3 *N* hydrochloric acid and shaken in a funnel with chloroform and sufficient sodium hydroxide solution to neutralize the acid. After washing with water and drying, the chloroform extract was concentrated. Seventeen grams of an oil was collected at 145–155° at 1 mm. To 10 g. of oil was added 11 g. of citric acid and 30 ml. of 60% ethanol. The

(19) E. Abderhalden and H. Brockmann, *Fermentforschung*, **10**, 159 (1929).

(20) A. Tigerstadt, *Chem. Ber.*, **25**, 2919 (1892).

TABLE V

CONSTANTS OF AMINO ACID ANILIDE HYDROGEN CITRATES

Amino acid anilide hydrogen citrate	M.p., °C. uncor.	Nitrogen, % Calcd.	Found
Alanine	87, foaming 128°	7.87	7.7
Valine	87, foaming 118°	7.29	7.3
Leucine	157–158, foaming 162°	7.04	7.0, 7.1

solution was concentrated under reduced pressure, the residual gum treated with 300 ml. of boiling ethyl acetate, and this liquid discarded. The residue was dried in a desiccator and stirred under hexane until solid; yield 22 g. For analysis, a sample was dried to constant weight in a pistol.

The hydrogen citrates of valinanilide and of leucinanilide were prepared similarly; their constants are presented in Table V. The leucinanilide hydrogen citrate obtained by recrystallization from amyl acetate was definitely microcrystalline, being composed of prisms. The other two citrates appeared as jagged chunks microscopically; their crystallinity was doubtful.

Products.—The constants of the new anilides produced are given in Table VI. Benzoylphenylalaninanilide has been described.[5] All melting points were determined on a Fisher–Johns block.

Enzyme Experiments.—1.00 mmole of the benzoyl-DL-amino acid and 1.00 mmole of the glycinanilide or alaninanilide hydrogen citrate were weighed into screw-cap vials. A 5.0-ml. volume of the appropriate buffer was then pipetted into each vial. After standing with occasional shaking, the *p*H was readjusted to the desired value. Then were added 16.0 mg. of enzyme (Nutritional Biochemicals papain lot no. 3781, or Merck ficin, or 5.0 mg. of Armour lot no. 10705 crystallized chymotrypsin–magnesium sulfate) plus 13.0 mg. of L-cysteine hydrochloride with each of the first two enzymes. Incubation proceeded at 40 ± 1° for 3 days (5 days for Table IV).

TABLE VI

CONSTANTS OF BENZOYLAMINO ACID ANILIDES

Anilide	M.p., °C.	Nitrogen, % Calcd.	Found	$[\alpha]^{25}D$
Bz-phenylalaninanilide	220–222	8.15	7.9, 7.9	$+24.8 \pm 1.0^{a}$
Bz-phenylalanylglycinanilide	244–245	10.47	10.3	$+ 6.5 \pm 1.0^{b}$
Bz-phenylalanylglycylglycinanilide[f]	247–249[h]	12.22	11.8, 11.8	$+34.9 \pm 1.5^{c}$
Bz-phenylalanylalaninanilide	243–244	10.15	9.8, 9.8	-17.4 ± 1.0^{d}
Bz-tryptophananilide	199–200	10.96	11.0, 11.1	$+43.4 \pm 1.0^{e}$
Bz-tryptophylglycinanilide	200–201			$+22.6 \pm 1.5^{e}$
Monohydrate[g]	125–140	12.22	11.8, 11.8	

Solvent for rotations consisted of 1 part of 95% ethanol to 1 part of chloroform, by volume. [a] $c = 0.50$, [b] $c = 2.47$, [c] $c = 0.33$, [d] $c = 5.68$, [e] $c = 0.76$. [f] Calcd.: phenylalanine, 32.1; glycine, 24.9. Found: phenylalanine, 34.5; glycine, 24.7, by microbiological assay with *Lactobacillus brevis* and medium of S. W. Fox, T. L. Hurst and K. F. Itschner, THIS JOURNAL, **73**, 3573 (1951). [g] H_2O lost at 125° *in vacuo* for 2 hr. Calcd.: H_2O, 3.9. Found: H_2O, 3.5. [h] A m.p. of 236–240° was reported for material which was believed to be isomerically impure; O. K. Behrens and M. Bergmann, *J. Biol. Chem.*, 129, 597 (1939).

Crude anilides were recrystallized from aqueous ethanol to constant values. The modified biuret test (Table II) consisted of a standard biuret run in aqueous ethanol (1:2) as a solvent.

Acknowledgments.—The help of Mr. A. J. McMillan in synthesizing amino acid anilides and in analyzing products for nitrogen content is appreciated. Jacquetta Strifert Halverson first prepared the benzoyltryptophananilide. Appreciation is expressed to Dr. Randolph T. Major of Merck and Co., Inc., for the ficin and to Dr. Otto K. Behrens of Eli Lilly and Co. for his generosity in revealing unpublished notebook records of his own experiments on anilide synthesis.

AMES, IOWA

[CONTRIBUTION FROM THE STATE UNIVERSITY OF NEW YORK COLLEGE OF FORESTRY]

Preliminary Separations of Maple Hydrol Lignin

BY MÄRTA GRANATH AND CONRAD SCHUERCH

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A method has been developed for the separation of a dimer-rich fraction from hydrol lignin. Material higher in molecular weight than dimers is largely removed by solvent precipitation and monomers are then separated by countercurrent distribution. The extent of separation has been established by molecular weight determinations, distribution coefficients of monomers, paper chromatography and the isolation of the most important monomer.

The monomeric units isolated from various degradations have been important evidence of the aromatic structure of lignin. As yet there is little experimental evidence to indicate how these units are combined in the original polymer, for little progress has been made in the isolation of larger molecular fragments. Gustavsson and co-workers[1] have studied dimer-rich fractions obtained by the chromatography of spruce and birch ethanol lignin, and Pearl and Dickey[2] have separated and identified several crystalline dimeric compounds from the alkaline oxidation of lignosulfonic acids. Because of the complexity of lignin degradations, it seems profitable to study the dimeric fractions of several isolated lignins for comparison.

The present investigation concerns the separation of a dimer-rich fraction from hydrol lignin.[3] Hydrol lignin has the advantage of being more

(1) C. Gustafsson, K. Sarkanen, S. Kahila and E. Niskasaari, *Paper and Timber*, **33**, 74 (1951).

(2) I. A. Pearl and E. E. Dickey, THIS JOURNAL, **74**, 614 (1952).

(3) C. P. Brewer, L. M. Cooke and H. Hibbert, *ibid.*, **70**, 57 (1948).