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The contribution of substrate structure to enzymic peptide bond synthesis

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THE CONTRIBUTION OF SUBSTRATE STRUCTURE TO ENZYMIC PEPTIDE BOND SYNTHESIS

BY

Milton Winitz

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INTRODUCTION

The elucidation of the process whereby proteins are synthesized in the cell is of fundamental biological importance. Knowledge of the mechanism of protein synthesis is prerequisite to an understanding of not only normal tissue protein synthesis, but also to the mechanism underlying gene, virus, enzyme and antibody formation. The validity of any theory of protein synthesis depends on a clear and precise accounting for both the high degree of specificity and the energy relationships involved in such reaction. Since the peptide bond is the linkage by which the amino acid residues in the protein molecule are held together, the mode of formation of such bonds constitutes an important factor in the clarification of the problem of protein formation as a whole. Despite the expenditure of much effort to the problem, however, the nature of the mechanisms involved in such process still remains quite obscure.

Included among the several postulated mechanisms for protein synthesis is one which suggests that the same proteolytic enzymes responsible for proteolysis are also involved in synthesis (1). Metabolic studies involving

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(1) M. Bergmann, Chem. Rev., 22, 423 (1938).

the use of isotopically labeled amino acids have shown that in the living organism proteins undergo a continual process of degradation and resynthesis (1). Assumption has usually been made that the breakdown process consists of peptide bond cleavage by the proteolytic enzymes. Although the nature of the synthetic reactions leading to peptide bond formation has been the subject of varied speculation, it is conceivable that the reversal of proteolysis is catalyzed by the identical proteases responsible for the hydrolytic reaction. Such a proposal is in agreement with a fundamental concept of catalysis developed by Nernst (2), derived from thermodynamic considerations, that a theoretical catalyst should have the ability to catalyze both the forward and reverse reactions of an equilibrium system. Confirmation of the agreement between enzymatic action and catalytic theory has been made with the carbohydrases and the esterases (3,4). Although the situation is not so well defined

---

(1) R. Schoenheimer, "The Dynamic State of Body Constituents". Harvard Univ. Press, Cambridge, Mass. 1946.

(2) W. Nernst, "Theoretical Chemistry", 4th English Ed. p. 617. Macmillan and Co. Ltd., London. 1916.

(3) G. T. Cori and C. F. Cori, J. Biol. Chem., 131, 397 (1939).

(4) H. Borsook and H. F. Schott, J. Biol. Chem., 92, 535 (1931).

with the proteolytic enzymes, such enzymes have nevertheless been shown to be capable of catalyzing both the synthesis and hydrolysis of peptide bonds (1).

Since a multitude of difficulties are inherent in investigation of *in vivo* protease action with respect to potential synthesizing abilities, recourse must be made to *in vitro* studies wherein the reaction can be investigated free from complicating factors, despite the "non-biological" nature of such studies. The current methods for effecting such studies have been utilization of the formation of insoluble products (peptides of the anilide type (1)) or increase in reactant concentration (2) in order to drive the reaction toward the synthetic end, and use of isotopically labeled compounds to enable the detection and quantitative determination of small amounts of products (3-5).

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

(2) H. Wasteneys and H. Borsook, Physiol. Rev., 10, 110 (1930).

(3) I. D. Frants, Jr., R. B. Loftfield and A. S. Werner, Fed'n. Proc., 8, 199 (1949).

(4) J. Melchior and H. Tarver, Arch. Biochem., 12, 301 (1947).

(5) J. Melchior and H. Tarver, Arch. Biochem., 12, 309 (1947).

It has long since been hypothesized that the synthesis of peptide bonds is due to direct condensation between carboxyl and amino groups in reactions which are the reversal of hydrolysis and accordingly catalyzed by proteolytic enzymes (1). If it is assumed that amino acids are the starting materials for peptide bond formation, then synthesis of these amino acids should be a necessary prerequite to the commencement of protein synthesis. Examination of amino acid and protein synthesis in Torula yeast showed (2) that protein formation was very slow before the accumulation of substantial quantities of amino acids in such cells. The rate of protein synthesis then accelerated while the accumulation of amino acids in the yeast cells ceased.

Determination has been made (3) of the free energy of formation of several peptide bonds composed of various amino acid residues. A postive free energy value of about 1400 to 3700 calories was found in all cases

---

(1) H. Wasteneys and H. Borsook, Physiol. Rev., 10, 110 (1930).

(2) P. Roine, Ann. Acad. Sci. Finnicae, Ser. A. II. Chem. No. 26 (1947). (Original not available for examination; cited in A. I. Virtanen, Ann. Acad. Sci. Finnicae, Ser. A. II, Chem. No. 39 (1950).

(3) H. M. Huffman, J. Phys. Chem., 46, 885 (1942).

investigated. From the free energy value for peptide bond formation from the constituent amino acids, it could be concluded that the equilibrium between a peptide and its split products lies far on the side of hydrolysis. Appreciable synthesis could be expected only if the peptide were effectively removed from the system as it was formed. Recollection has been made by Bergmann and Fruton (1) that various proteases catalyze condensation as well as hydrolysis through action on amino acid derivatives which yield insoluble peptides; they further emphasize that the alleged sharp specificity of the preoteases lends plausibility to the view that their role in protein synthesis is an important one, since operation of some such specificity must be assumed during the formation of the peptide-chain structure of proteins.

The investigations reported herein were carried out with the purpose of studying the effect of residue structure on enzymically catalyzed synthesis of peptide bonds of the anilide type. Particular emphasis was placed on the contribution of the amino acid residue side chains to the quantitative extent to which synthesis might proceed. In addition, attention was given to the

---

(1) M. Bergmann and J. S. Fruton, Ann. N. Y. Acad. Sci., 45, 409 (1944).

different types of reaction that might be effected depending on the structural constitution of the substrates used. Such an investigation, of necessity, had to include catalysis by various enzyme systems, the proteases papain, ficin and chymotrypsin being here used.

HISTORICAL

Proteolytic Enzymes in Protein Synthesis

The role of proteolytic enzymes in the synthesis of peptide bonds by enzymic action on mixtures of protein hydrolyzates was first suggested at the turn of the century (1) by the knowledge that the fat and carbohydrate splitting enzymes are capable of effecting the synthesis, as well as the hydrolysis, of pertinent substrates. Similar impression was drawn from the voluminous data concerning the "resynthesis of protein" through the separation of insoluble products, the so-called "plastein", by the action of pepsin or papain on the partial enzymatic protein hydrolyzate. Thus, it was observed in 1886 by Danilewski (2) that a precipitate is formed if gastric juice is added to concentrated peptone solution. Those who examined the phenomenon during the next 25 years presumed, without any direct evidence, that plastein was a protein probably similar to or the same as that used as the starting material in the hydrolysis

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(1) J. H. Kastle and A. S. Loevenhart, Am. Chem. J., 24, 491 (1900).

(2) Danilewski, cited by V. Henriques and J. K. Gjaldbäk, Z. physiol. Chem., 71, 485 (1911).

(1,2). In 1911, it was observed by Henriques and Gjaldbäk (3-5) that the amino nitrogen decreased during the plastein synthesis. The same finding was made in 1924 (6) with respect to the carobxyl groups. However, as late as 1936, Oppenheimer, in his work, "Die Fermente und ihre Wirkungen", still considered that peptide synthesis had not been proved in the formation of plastein.

If a critical review is made of the literature with regard to plastein synthesis, it will be found that, until the most recent years, the picture of the nature of the reaction remained obscure. Thus, in 1930, Wasteneys and Borsook (7) reviewed much of the earlier literature as well as the greater portion of

---

(1) B. A. E. Taylor, J. Biol. Chem., 3, 87 (1907).

(2) T. B. Robertson, J. Biol. Chem. 3, 94 (1907).

(3) V. Henriques, and J. K. Gjaldbäk, Z. physiol. Chem., 71, 485 (1911).

(4) V. Henriques and J. K. Gjaldbäk, Z. physiol. Chem., 81, 439 (1912).

(5) V. Henriques and J. K. Gjaldbäk, Z. physiol. Chem. 83, (1913).

(6) H. Wasteneys and H. J. Borsook, J. Biol. Chem., 62, 15, 675 (1924).

(7) H. Wasteneys and H. J. Borsook, Physiol. Rev., 10, 110 (1930).

their own investigations dealing with the *in vitro* reversal of proteolysis by the action of pepsin or trypsin on peptic digests of egg albumin. The evidence obtained for peptide bond formation included a decrease in amino nitrogen during the incubation, the formation of an insoluble protein-like precipitate (plastein), the precipitation of the solution of plastein with trichloroacetic acid and the ability of pepsin to digest plastein under properly buffered conditions. The authors explained plastein formation by application of the law of mass action and indicated ready reversibility of the hydrolytic reaction under favorable concentrations of reactants and products. From the assumption that egg albumin hydrolysis and plastein synthesis are the forward and reverse reactions of the overall equilibrium

$$\text{Protein} \xrightleftharpoons{\text{Pepsin}} A + B + C + \ldots M,$$

the equilibrium expression for the reaction was then formulated as

$$K = \frac{(A)\ (B)\ (C)\ \ldots (M)}{\text{Protein}}$$

where K is the equilibrium constant. If this formulation is a valid one, then as the concentration of split products is increased, an appreciable shift of the

proportion of components toward the direction of synthesis should occur.

Criticism of the conclusions drawn by Wasteneys and Borsook has come from many directions. In 1932, the ultracentrifuge determination, by Svedberg, of plastein prepared by Folley (1) from egg albumen, indicated that the molecular weight of the preparation was less than 1000. Hence, it appears that no polypeptide synthesis had occurred. Folley therefore assumed that the decrease of amino nitrogen had been due to the disappearance of the terminal groups of peptides in the hydrolyzate through cyclopeptide formation. As recently as 1947, Ecker (2) arrived at the same result as Svedberg by ultracentrifuge methods. On the other hand, Collier (3) found a very high-molecular fraction, in small amounts, in his plastein preparations.

Perhaps the severest criticism of the conclusions drawn by Wasteneys and Borsook has come from Bergmann and Fruton (4) and Alcock (5). This criticism was based

---

(1) S. J. Folley, Biochem. J. 26, 99 (1932).

(2) P. G. Ecker, J. Gen. Physiol., 30, 399 (1947).

(3) H. B. Collier, Can. J. Res., 18B, 272, 305 (1940).

(4) M. Bergmann and J. S. Fruton, Ann. N. Y. Acad. Sci., 45, 409 (1943-44).

(5) R. S. Alcock, Physiol. Rev., 16, 1 (1936).

on the grounds that no definite demonstration of peptide bond synthesis had been made, that the pH optima for both synthesis and hydrolysis differed markedly, and thereby, the synthetic reaction was not simply a reversal of the hydrolytic one, and that application of the law of mass action to the results was invalidated by the fact that no resynthesis of the original proteins, from which the peptic digest were derived, had occurred. This indicated that the expression of Wasteneys and Borsook (1),

$$\text{Protein} + H_2O \rightleftharpoons \text{Products},$$

implies that an equilibrium exists between a specific protein and its products of hydrolysis. Since recombination of these split products do not yield the same protein from which they were originally derived, the expression is definitely not a valid one.

Still further evidence against the validity of the Wasteneys-Borsook interpretation was presented by Northrop (2). This investigator reported that the plasteins obtained from the peptic digest of pepsin and trypsin had neither the general properties of the

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(1) H. Wasteneys and H. J. Borsook, Physiol. Rev., 10, 110 (1930).

(2) J. H. Northrop, J. Gen. Physiol. 30, 377 (1947).

parent enzyme nor any activity. That a decrease of amino nitrogen in the reaction does occur, however, has been evidenced by even the early investigations, although it has often been claimed, even quite recently (1), that plastein precipitation does not depend on the formation of new peptide bonds.

Virtanen and co-workers (2) assumed, as had Folley (3), on the basis of cryoscopic determinations of molecular weight in formic acid, acetic acid and phenol that the products formed in concentrated protein hydrolyzates with pepsin are small cyclopeptides. Subsequent work by the Virtanen group (4), on the contrary, based on viscosity measurements, cryoscopic determinations in non-polar solvents and diffusion determinations, pointed to large polypeptides with a molecular weight of several thousand. The largest polypeptides synthesized contained only 0.8 to 1.3 per cent α-amino nitrogen of total nitrogen, which corresponded to an average molecular weight of about 7000-12000. These

---

(1) J. A. V. Butler, E. C. Dodds, D. M. P. Philips and J. M. L. Stevens, Biochem. J., 42, 122, (1948).

(2) A. I. Virtanen and H. Kerkkonen, Nature, 161, 888 (1948).

(3) S. J. Folley, Biochem. J. 26, 99 (1932).

(4) A. I. Virtanen, H. Kerkkonen, and T. Laaksonen, Acta Chem. Scand. 2, 933 (1948).

results and additional investigations (1,2) by the Virtanen group has greatly helped to bring some order out of the chaos that has existed in the past and to dispel some of the misconceptions concerning the nature of plastein synthesis. Some of the more salient features of these investigations will be dealt with below.

In general, the Virtanen group carried out the peptic hydrolysis of proteins at pH's varying from 1 to 4 while the plastein synthesis was generally effected at pH 4, the optimum for the reaction. When the peptic hydrolysis was allowed to proceed to the extent that the average size of peptides in the hydrolyzate corresponded to a tetra- or hexapeptide, amino nitrogen was always lowered appreciably in the concentrated hydrolyzate through the action of pepsin. Precipitation of plastein reached a maximum during 24 hours, the decrease of amino nitrogen varying from 10 to 20 per cent. Nevertheless, the plastein precipitate often contained 35 to 40 per cent of the total nitrogen in the initial hydrolyzate due to the co-precipitation of a number of peptides, formed in the original hydrolysis,

---

(1) A. I. Virtanen, H. Kerkkonen, T. Kaaksonen and M. Hakala, Acta Chem. Scand., 3, 520 (1949).

(2) A. I. Virtanen, H. Kerkkonen, M. Hakala and T. Laaksonen, Naturwiss., 37, 139 (1950).

with the synthesized polypeptides. Such plastein precipitates thereby consisted only partially of synthetic products, this being advanced as the reason for the low molecular weights as determined by the ultracentrifuge method.

On the other hand, if peptic hydrolysis was allowed to proceed to the extent where the hydrolyzate contained larger peptides, for example, on the average of a decapeptide, then no decrease in amino nitrogen was noted within 24 hours but, on the contrary, an increase was noted. Despite this, abundant precipitation of plastein was observed. This was interpreted as being due to the fact that the hydrolysis of peptides occurred simultaneously with synthesis. Thus, the contradictory observations of the earlier investigators regarding the decrease of amino nitrogen could be ascribed to the fact that, in plastein synthesis, the variations in the peptide size of hydrolyzates had not been considered.

The introduction of the concept (1-3) that the

---

(1) E. Waldschmidt-Leitz, I. J. Bek and J. Kahn, Naturwiss., 17, 85 (1928-29).

(2) K. Mothes, Naturwiss., 20, 102 (1932).

(3) K. Mothes, Naturwiss., 20, 883 (1932).

direction of intracellular protein reaction is determined by the oxidation-reduction potential, i.e., that it is steered in the direction of hydrolysis by reducing agents and in the direction of synthesis by oxidizing agents, has led to reports by Voegtlin and associates (1-4) concerning the enzymatic synthesis of protein-like products, by means of papain or cathepsin, through the oxidation or aeration of a hydrolyzate mixture. With respect to the intracellular proteinases, these investigators regarded the following conditions as favoring synthetic processes; (a) a relatively high oxygen tension; (b) a relatively high initial concentration of sulfhydryl groups which can give rise to a relatively high concentration of disulfides; (c) a hydrogen ion concentration not far removed from neutrality; (d) a

---

(1) C. Voegtlin, M. E. Maver, and J. M. Johnson, J. Pharmacol., 48, 241 (1932).

(2) M. E. Maver, J. M. Johnson and C. Voegtlin, Natl. Inst. Health Bull. No. 164, 29 (1935).

(3) M. E. Maver and C. Voegtlin, Enzymologia, 6, 219 (1939).

(4) C. Voegtlin and M. E. Maver, U. S. Pub. Health Repts., 47, 711 (1932).

sufficient concentration of suitable protein split-products. Proteolysis, on the other hand, was favored by low oxygen tension and low pH.

The conclusions reached by Voegtlin and co-workers have been demonstrated as invalid by several investigators. Thus, reinvestigation of this work Strain and Linderstrom-Lang (1-3) failed to demonstrate the synthesis of any material that could be precipitated with trichloro-acetic acid. Under the influence of oxidizing agents, no synthesis of peptide bonds was effected but only the union of sulfhydryl containing fragments through the formation of disulfide linkages. Similarly, it was shown by Bergmann and Fruton (4) that the conclusions of the Voegtlin group were contrary to observations (5)

---

(1) H. H. Strain and K. Linderstrom-Lang, Enzymologia, 5, 86 (1938).

(2) H. H. Strain, Enzymologia, 7, 133 (1939).

(3) K. Linderstrom-Lang and G. Johansen, Enzymologia, 7, 239 (1939).

(4) M. Bergmann and J. S. Fruton, Ann. N. Y. Acad. Sci., 45, 409 (1943-44).

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

which indicated that, for both hydrolytic and synthetic reactions, the pH optima and activation requirements of proteolytic enzymes were the same.

In the attempt to effect the enzymatic synthesis of peptide bonds, much credit for decisive progress is due to Max Bergmann and his school. Since 1930, Bergmann studied the specificity of proteolytic enzymes which hydrolyze low-molecular peptides of known structure, and in conjunction with these investigations, the synthesis of peptides by means of proteolytic enzymes was also studied. This investigator concluded (1) that if the peptide synthesized is insoluble to such an extent that even a slight synthesis results in the exceeding of the solubility limit, then considerable peptide synthesis should occur as a result of the continual removal of the reaction product from the system. In other words, the driving force of the reaction was due to the formation and separation of an insoluble product. This may be exemplified by the cysteine-papain catalyzed reaction of benzoylglycine with aniline to yield the insoluble product,

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

benzoylglycinanilide:

$$C_6H_5 \cdot CO\text{-}NH \cdot CH_2 \cdot COOH + NH_2 \cdot C_6H_5 \xrightarrow{papain} C_6H_5 \cdot CO\text{-}NH \cdot CH_2 \cdot CO\text{-}NH \cdot C_6H_5$$

Benzoylglycine + aniline $\xrightarrow{papain}$ Benzoylglycinanilide

The theory of enzymic anilide synthesis and the significance of such experiments in an understanding of biological synthesis of peptide bonds has been discussed by Fox and co-workers (1).

Since the initial enzymic anilide syntheses had been reported by Bergmann and Fraenkel-Conrat (2), many syntheses of a similar nature have been investigated, chiefly by Bergmann and his collaborators. Thus, studies of the effect of variation of blocking groups (3-5),

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

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

(3) S. W. Fox and H. Wax, J. Am. Chem. Soc., 72, 5087 (1950).

(4) D. G. Doherty and E. A. Popenoe, Jr., J. Biol. Chem., 189, 455 (1951).

(5) N. F. Albertson, J. Am. Chem. Soc., 73, 452 (1951).

amino acid residues (1, 2-4), activators (5), buffer concentration (2), amines (6-8), and enzymes (4,5,9) on the degree of reactivity have been conducted. Several of these will be discussed more fully under the appropriate headings.

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(1) S. W. Fox and H. Wax, J. Am. Chem. Soc., 72, 5087 (1950).

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

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

(4) S. W. Fox and M. Winitz, Arch. Biochem., in press.

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

(6) M. Bergmann and H. Fraenkel-Conrat, J. Biol. Chem., 124, 1 (1938).

(7) E. Waldschmidt-Leitz and K. Kühn, Z. physiol. Chem., 285, 23 (1950).

(8) M. Winitz and S. W. Fox, Abstracts, 12th International Congress of Pure and Applied Chemistry, New York, 96 (1951).

(9) M. Bergmann and H. Fraenkel-Conrat, J. Biol. Chem., 124, 321 (1938).

Enzyme Specificity

Enzyme specificity may be classified either as "relative" or as "absolute" specificity. Relative specificity involves enzymic action on various but usually related compounds, these compounds undergoing attack at different rates. Absolute specificity, on the other hand, refers to measurable enzymic action on only one or a group of related substances without appreciable effect on other substances. Relative specificity therefore emphasizes quantitative differences while absolute specificity refers to qualitative ones. Transformation of the former into the latter is feasible in some instances, depending upon the reaction conditions used, while the reverse case is not as general. More pointed references to these types of specificity will be referred to under the topics discussed below.

Antipodal specificity

Although optical antipodes show entirely the same qualitative and quantitative behavior between the D- and the L-form for reactions involving non-asymmetric reactants or catalyzed by non-asymmetric catalysts, the situation is in all known instances fundamentally different when enzymes are the catalysts. An early

report (1) of stereochemical enzyme specificity was made on esterases exposed to the ester of DL-mandelic acid. Interruption of hydrolysis before reaction had gone to completion showed a greater extent of cleavage for the D- than for the L- ester.

Stereochemical or antipodal specificity plays an important role in the field of the proteases. Discussion will be here concerned chiefly with protease specificity in syntheses of the anilide type.

The antipodal specificity of proteolytic enzymes as synthesizing enzymes (2) was revealed when the acyl derivatives of several DL-amino acids were incubated with aniline or phenylhydrazine and cysteine-activated papain. It was observed that, in every instance, enzymic action was confined strictly to the derivative of the natural L-amino acid, the D-amino acid remaining unchanged. This is exemplified as follows:

$$\text{Bz-DL-amino acid} + \text{aniline} \xrightarrow{\text{papain}} \text{Bz-L-amino acid anilide} + \text{Bz-D-amino acid}$$

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(1) H. D. Dakin, J. Physiol. (London), 30, 253 (1904).

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

Thus, no anilide could be obtained from a homogeneous preparation of benzoyl-D-leucine under conditions in which benzoyl-L-leucine was converted to its anilide in almost quantitative yield.

It has been assumed (1) that in the papain catalyzed synthesis of the general type

$$R\cdot CO\text{-}NH\cdot CHR'\cdot CO_2^- + R''NH_2 \rightleftharpoons R\cdot CO\text{-}NH\cdot CHR'\cdot CO\text{-}NHR'' + OH^-$$

that only the N-acyl-L-amino acid amide or hydrazide is formed. However, suggestion has been made (2) that when R' is small, i.e., a methyl group, there may be some loss in enzymatic stereochemical specificity. Further, evidence has been obtained by Bennett and Niemann (3) whose work demonstrates that factors other than configuration about the asymmetric α-carbon atom and possibly the size of the R' group in the N-acylated-α-amino acid could influence the stereochemical course of the above reaction. These investigators observed that in addition to the expected L-isomer, N-carbobenzoxy-o-fluoro-DL-phenylalanine gave significant quantities of the N-acyl-D-amino acid

---

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

(2) M. Bergmann, L. Zervas and J. S. Fruton, J. Biol. Chem., 115, 593 (1936).

(3) E. L. Bennett and C. Niemann, J. Am. Chem. Soc., 70, 2610 (1948).

phenylhydrazide. These same workers later showed (1) that stereochemical specificity in the papain-catalyzed synthesis of phenylhydrazides of acylated phenylalanines is, in part, determined by the nature of the acyl group present in the acylated phenylalanines. Thus, for an acyl-DL-phenylalanine, $R \cdot CO-NH \cdot CH(CH_2C_6H_5) \cdot COOH$, the papain-catalyzed synthesis of the phenylhydrazide proceeds with almost complete stereochemical specificity for the L-antipode when R equals CH_3- or C_6H_5-. However, when R equals CH_3O-, C_2H_5O- or $C_6H_5CH_2O-$, this stereochemical specificity is lost to a striking degree and the rate of formation of the phenylhydrazide of the D-antipode is of a magnitude almost as great as that of the L-antipode.

Milne and Stevens (2) not only showed lack of absolute stereochemical specificity in a different series of compounds but also demonstrated enzymatic synthesis when the starting material was a pure derivative of the D-configuration. Both the D-and DL-

---

(1) E. L. Bennett and C. Niemann, J. Am. Chem. Soc., 72, 1798 (1950).

(2) H. B. Milne and C. M. Stevens, J. Am. Chem. Soc., 72, 1742 (1950).

forms of N-carboallyloxyleucine, when incubated with phenylhydrazine in the presence of activated papain, gave appreciable quanities of phenylhydrazides of the D-antipode.

The effect of certain combinations of amino acid side chains and acyl groups on the retention or loss of stereochemical specificity has also been studied (1). Although carbobenzoxy-DL-alanine, in the presence of activated papain and phenylhydrazine, formed only the carbobenzoxy-L-alanylphenylhydrazide, carbobenzoxy-DL-phenylalanine yielded both the phenylhydrazides of the D- and the L- antipodes. Similar studies (2) have been carried out on the enzyme-catalyzed hydrolysis of the corresponding amides and esters.

Bergmann and associates (3) investigated the enzymatic combination of acetyl-DL-phenylalanylglycine with aniline. Here the amino acid residue which contains the assymmetric carbon atom, i.e., phenylalanine, is

---

(1) E. L. Bennett and C. Niemann, J. Am. Chem. Soc., 70, 2610 (1948).

(2) S. Kaufman, H. Neurath and G. Schwert, J. Biol. Chem., 177, 793 (1949).

(3) M. Bergmann and O. K. Behrens, J. Biol. Chem., 124, 7 (1938).

not directly involved in the coupling reaction since the free carboxyl group belongs to the glycine which contains no assymmetric center. Nevertheless, only the acetyl-L-phenylalanylglycine was transformed into its anilide by activated papain. Repetition with a more highly active papain preparation showed that the D-isomer also reacted to give the anilide. However, the rate of formation of the D-antipode was shown to be much slower than that of the L-antipode.

Enzyme specificity in relation to substrate structure

The enzymic preference for particular substrates is a more subtle type of specificity than the stereochemical specificity exhibited by proteolytic enzymes. This specificity embodies the particular sensitivity of proteinases to the presence and special nature of the side chains in the substrate. Thus, in the hydrolysis of synthetic substrates, papain is able to distinguish between glycyl and leucyl while chymotrypsin is able to distinguish between leucyl and phenylalanyl (1). This is illustrated by the hydrolysis of two peptides, by papain, which differ only in the central amino acid

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

residue, i.e., a glycyl in one instance and a leucyl in the other (1).

Bz-leucylleucylglycine $\xrightarrow{\text{Papain}}$ Bz-leucine + leucylglycine

Bz-leucylglycylglycine $\xrightarrow{\text{Papain}}$ Bz-leucylglycine + glycine

In both cases, the peptide bond between the leucine and glycine residues was resistant to enzymic cleavage whereas the bonds between the two leucine residues, in the former case, and the two glycine residues, in the latter, were susceptible to hydrolytic action. These same investigations showed that the enzymic action of papain is not limited to peptide linkages between particular amino acids but is capable of cleaving varied peptide bonds irrespective of the presence of neutral, acidic, or basic amino acids on the carbonyl or imino side of the linkage split. However, if the substrate subjected to enzymic attack contains several peptide linkages, these linkages are attacked at widely different velocities. Haphazard cleavage is not effected at all linkages but preference is shown for only certain ones. Thus, "the specificity of a proteinase is characterized by the rates at which the peptide bonds of a number of substrates are hydrolyzed

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(1) M. Bergmann, L. Zervas and J. S. Fruton, J. Biol. Chem., 115, 593 (1936).

in the presence of the enzyme" (1).

Proteinase cleavage of a peptide linkage is affected by more distant groups as well as the two amino acid residues which directly participate in the peptide bonds (2). Demonstration of this effect was clearly made by the decreased cleavage of the tyrosyl-glycine linkage by chymotrypsin on passing from carbobenzoxy-tyrosylglycineamide to carbobenzoxytyrosylglycylglycineamide.

Since the enzymatic synthesis of peptide bonds is performed by the same proteases that cleave these bonds, and since these syntheses and hydrolyses represent component processes of equilibrium reactions, it is to be expected that similar residue specificities should be observed for enzymatic syntheses as were observed for hydrolyses. Thus, in a synthesis of the general type

$$R\cdot CO\text{-}NH\cdot CHR'\cdot COOH + NH_2\cdot R'' \overset{\text{enzyme}}{\rightleftharpoons} R\cdot CO\text{-}NH\cdot CHR'\cdot CO\text{-}NH\cdot R''$$

the nature of R, R' and R" should play a role similar to that in hydrolysis with regard to the rate at which the reaction proceeds and the nature of reaction effected.

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(1) M. Bergmann and J. S. Fruton, Advances in Enzymol., 1, 89 (1941).

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

The effect of residue side chains (R') was demonstrated by Fox and co-workers (1) in the papain-catalyzed anilide synthesis of some benzoylamino acid anilides of the monoaminomomocarboxylic acid category. Different degrees of reactivity were noted for the glycine, alanine, leucine and valine residues upon their incorporation into anilides. The behavior varied, under identical experimental conditions, from virtual inactivity for valine to semi-complete reactivity for leucine. In view of these results, the connotation of "specificity" was here considered inappropriate, the term "preference" being used in its stead. Distinction was made between the preference used in this connotation, which was defined on a rate basis, and the type of preference noted in the hydrolytic experiments wherein enzymic action was confined to one of two or more peptide bonds within the same substrate molecule (2).

The investigations of Fox and Wax (3), in which the

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

(2) M. Bergmann, L. Zervas and J. S. Fruton, J. Biol. Chem., 115, 600 (1936).

(3) S. W. Fox and H. Wax, J. Am. Chem. Soc., 72, 5087 (1950).

effects of various blocking groups were studied, gave confirmation to the "preference" concept advocated above. The previously observed decreasing preferences for leucine, glycine and valine (1) were here supported for the p-nitrobenzoyl, carboallyloxy, and benzoyl blocking groups, with a shift in order between glycine and leucine only when carbobenzoxy was the substituent. In addition, the use of various blocking groups (R) gave some indication of their relative contribution to the quantitative extent to which the enzyme-catalyzed reaction proceeded over a given time interval.

The effect of a variety of substituted anilines (R'') was reported by Waldschmidt-Leitz and Kühn (2) in papain-catalyzed anilide syntheses using hippuric acid. The results demonstrated that the extent to which a synthesis proceeded was governed, in several instances, by whether an ortho-, meta- or para-substituted aniline was used. Thus, o-toluidine showed a far lesser ability to react than the meta- or para-derivatives, reaction of o-anisidine was much slower than p-phenetidine, and

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

(2) E. Waldschmidt-Leitz and K. Kühn, Z-physiol. Chem., 285, 23 (1950).

o-aminobenzoic acid, in distinction to the para-derivative, exhibited no coupling. No special position of the ortho-derivatives were noted with the aminophenols and the phenylenediamines. Cognizance should be taken of the fact, however, that the ortho-derivative of the latter reacted with only one, while the para- and meta-derivatives reacted with two hippuric acid residues. It should also be mentioned that the negative outcome of synthetic experiments with N-methyl aniline was ascribed to the absence of the free amino group necessary for reactivity.

Enzyme specificity and the "cosubstrate" hypothesis.

The difficulties with which interpretation of studies on specificity are beset are illustrated by the following example (1,2). Neither acetyl-L-phenylalanylglycine nor glycyl-L-leucine undergo cleavage in the presence of cysteine-papain. However, if both substrates are placed in contact with the enzyme, a tetrapeptide, acetyl-L-phenylalanylglycylglycylleucine, is synthesized. The specificity requirements of the enzyme are now met by this substrate and the terminal leucine and glycine

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(1) M. Bergmann and J. S. Fruton, Advances in Enzymol., 1, 63 (1941).

(2) M. Bergmann, Advances in Enzymol., 2, 49 (1942).

residues are split off in the order given. Thus, with the aid of the acetyldipeptide as "cosubstrate", hydrolysis of glycylleucine by cysteine-papain was made feasible.

A mechanism similar to that given above was investigated by Behrens and Bergmann (1) in a synthesis of the anilide type. These workers reported that reaction between acetyl-L-phenylalanylglycine and glycinanilide led to the ultimate formation of acetyl-L-phenylalanylglycinanilide, as well as to glycine, aniline, and some unreacted starting materials. The reaction apparently did not proceed via a simple cleavage of glycinanilide followed by coupling of the liberated aniline with the acetyldipeptide, since glycinanilide was not split by cysteine-papain under the conditions investigated. In order to account for the products obtained, the following sequence of reactions was proposed:

acetyl-L-phenylalanylglycine + glycinanilide
↓
acetyl-L-phenylalanylglycylglycinanilide
↓
acetyl-L-phenylalanylglycylglycine + aniline
↓
acetyl-L-phenylalanylglycine + glycine
↓
acetyl-L-phenylalanylglycinanilide

(1) O. K. Behrens and M. Bergmann, J. Biol. Chem., 129, 587 (1939).

This scheme demonstrates not only the simultaneous occurrence of hydrolytic and synthetic action of the same enzyme under the same conditions, but also attempts to explain the specificity relationships of the enzyme and the substrate. Thus with the formation of acetyl-L-phenylalanylglycylglycinanilide, a compound is formed which meets the specificity requirements of the enzyme. This relationship is such that first aniline, and then glycine, can undergo a sequential cleavage from the peptide chain. A subsequent synthesis of the final anilide product is then effected from the liberated aniline and the acylated dipeptide.

Evidence has also been advanced by the Bergmann group that the nature of the amino acid residue in the amino acid anilides played a significant role in the determination of the course of the reaction. It was reported by Bergmann and Fraenkel-Conrat (1) that the papain-catalyzed reactions of benzoylleucine with leucinanilide and glycinanilide, respectively, were illustrative of the highly developed specificity of enzymic peptide bond synthesis, since the benzoyldipeptide anilide resulted in the former case, whereas the benzoylamino acid anilide was formed in the latter.

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(1) M. Bergmann and H. Fraenkel-Conrat, J. Biol. Chem., 124, 1 (1938).

The mechanism of this latter reaction was recently interpreted (1) as occurring via a cosubstrate sequence.

Enzyme Induced Replacement Reactions

Transamidation or transpeptidation reactions have been defined (2) as the protease catalyzed "replacement of one participant in a peptide bond by another, closely related, molecular species". Such reactions, of the general nature,

$$R\cdot CO\text{-}NH\cdot CHR'\cdot CO\text{-}NH_2 + NH_2\cdot R'' \xrightarrow{\text{protease}} R\text{-}CO\text{-}NH\cdot CHR'\cdot CO\text{-}NH\cdot R'' + NH_3$$

were first observed by Bergmann and Fraenkel-Conrat (3) in 1937. These investigators showed that by the action of papain, hippuric anilide was formed more rapidly from aniline and hippuryl amide than from aniline and hippuric acid. The driving force of the reaction was the formation and separation of the insoluble anilide.

Since the free energy change in reactions which involve the exchange of the amide group of naturally occurring or synthetic amides with ammonia or other amines is probably small, the exchange may proceed without

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(1) C. W. Pettinga, Unpublished Ph.D. Thesis. Ames, Iowa, Iowa State College Library. 1949.

(2) R. B. Johnston, M. J. Mycek and J. S. Fruton, J. Biol. Chem., 185, 629 (1950).

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

the removal of an insoluble reaction product. Thus, it was recently shown by Fruton (1) that ammonia may replace aniline in the same system. Isotopically labeled ammonia, in the presence of papain, was incorporated in the amides of hippuric acid and carbobenzoxy-L-methionine by replacement reactions. A considerably greater extent of isotope incorporation was found than could be expected by direct coupling from the products of hydrolysis (acylamino acid and ammonia). Conclusion was therefore made that the enzyme catalyzed transamidation reaction occurred via a hypothetical transient intermediate in which the carbon atom of the amide bond was linked to two $-NH_2^s$ groups.

$$R\cdot CO\text{-}NH_2 + N^{15}H_3 \rightleftharpoons \left[R\cdot C(OH)(NH_2)(N^{15}H_2) \right] \rightleftharpoons R\cdot CO\text{-}N^{15}H_2 + NH_3$$

$R = C_6H_5\cdot CO\text{-}NH\cdot CH_2\text{-}$

A later report by the Fruton group (2) demonstrated that such transamidation reactions were favored by a shift in pH from 5 to 8. Cysteine-activated papain was

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(1) J. S. Fruton, Yale J. Biol. Med., 22, 263 (1950).

(2) R. B. Johnston, M. J. Mycek and J. S. Fruton, J. Biol. Chem., 185, 629 (1950).

also found capable of inducing the replacement of the amide NH_2 group of a variety of acylamino acid amides by the -NHOH group of hydroxylamine. As with the isotope experiments, the extent of transamidation underwent marked increase with a shift in pH from 5 to 7.5. Similar results were obtained with beef spleen cathepsin.

The presence of enzymes which catalyze the exchange of hydroxylamine with the amide group of glutamine and asparagine in cell-free extracts and resting cells of Proteus vulgaris has recently been reported by Waelsch and co-workers (1-4). The products of the enzymatic exchange were α-glutamo- and β-aspartohydroxamic acids. The ability of these enzymes to utilize the amide bond energy for further synthetic processes, e.g., peptide linkages, was thereby demonstrated. The cell free enzyme extracts not only catalyzed the replacement of the amide group by hydroxylamine but split synthetic glutamo- and aspartohydroxamic acids and showed glutaminase and

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(1) H. Waelsch, E. Borek and N. Grossowicz, Abstracts, American Chemical Society, 116th meeting, Atlantic City, 54C (1949).

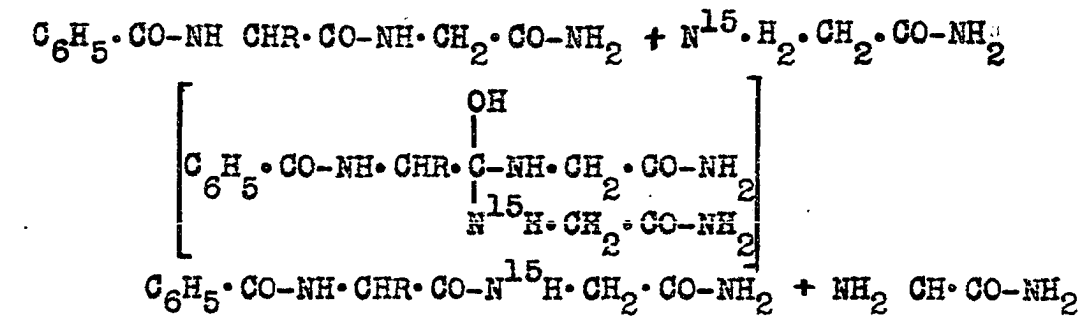
(2) H. Waelsch, E. Borek, N. Grossowicz and M. Schow, Fed'n. Proc., 9, 154 (1950).

(3) N. Grosowicz, E. Wainfan, E. Borek and H. Waelsch, J. Biol. Chem., 187, 111 (1950).

(4) E. Borek, N. Grossowiczzand H. Waelsch, Fed'n. Proc., 9, 154 (1950).

asparaginase activities. Stumpf and Loomis (1) also have reported the enzymatic synthesis of α-glutamohydroxamic acid from glutamine and hydroxylamine. Here, the source of the enzyme preparation was pumpkin seedlings.

Later work by the Fruton group (2) extended transamidation reactions (replacement reactions involving amide bonds) to include replacement of one of the components of a bond linking two amino acid residues (transpeptidation reactions). The reaction observed was formulated as follows, the postulatulated intermediate being enclosed in brackets:



$$C_6H_5\cdot CO\text{-}NH\ CHR\cdot CO\text{-}NH\cdot CH_2\cdot CO\text{-}NH_2 + N^{15}H_2\cdot CH_2\cdot CO\text{-}NH_2$$

$$\left[C_6H_5\cdot CO\text{-}NH\cdot CHR\cdot \underset{\displaystyle N^{15}H\cdot CH_2\cdot CO\text{-}NH_2}{\overset{\displaystyle OH}{\overset{|}{C}}}\text{-}NH\cdot CH_2\cdot CO\text{-}NH_2\right]$$

$$C_6H_5\cdot CO\text{-}NH\cdot CHR\cdot CO\text{-}N^{15}H\cdot CH_2\cdot CO\text{-}NH_2 + NH_2\ CH\cdot CO\text{-}NH_2$$

R = $-CH_2\ C_6H_4OH$

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(1) P. K. Stumpf and W. D. Loomis, Arch. Biochem. 25, 451 (1950).

(2) R. B. Johnston, M. J. Mycek and J. S. Fruton, J. Biol. Chem., 187, 205 (1950).

Thus, crystalline pancreatic chymotrypsin was shown to catalyze the replacement of the glycinamide moiety of benzoyl-L-tyrosylglycinamide by isotopic glycinamide containing N^{15} in the glycine N. Transamidation reactions were also effected with cathepsin C, pancreatic trypsin and ficin.

The enzyme catalyzed incorporation of C^{14} labeled glycine into glutathione via peptide bond formation was demonstrated by Johnston and Bloch (1). This uptake of C^{14}- glycine was shown to be due to an exchange reaction (transpeptidation).

When the possible metabolic significance of replacement reactions of the foregoing type was considered, the hypothesis was advanced (2) that the energy for the formation of the peptide bonds of proteins might be funneled through a small number of amides or peptides such as glutamine or glutathione. Recent investigations of Speck (3) and Johnston and Bloch (1) suggested the intervention of adenosinetriphosphate

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(1) R. B. Johnston and K. Bloch, J. Biol. Chem., 188, 221 (1951).

(2) J. S. Fruton, Yale J. Biol. Med., 22, 263 (1950).

(3) J. F. Speck, J. Biol. Chem., 179, 1405 (1949).

in the biosynthesis of glutamine and glutathione from the component free amino acids. This work has provided support to the suggestion of Lipmann (1) that adenosinetriphosphate participates in a transphosphorylation reaction with free amino acids (e.g., L-glutamic acid) to form a phosphoric acid anhydride (e.g., γ-L-glutamyl phosphate) which then reacts with ammonia or a peptide, with the formation of glutamine or a γ-glutamyl peptide such as glutathione. These products might then participate in replacement reactions catalyzed by intracellular proteases (2).

On the basis of the foregoing investigations, a speculative scheme regarding the metabolic pathways leading to the formation of the peptide bonds of proteins has been proposed (3), as follows:

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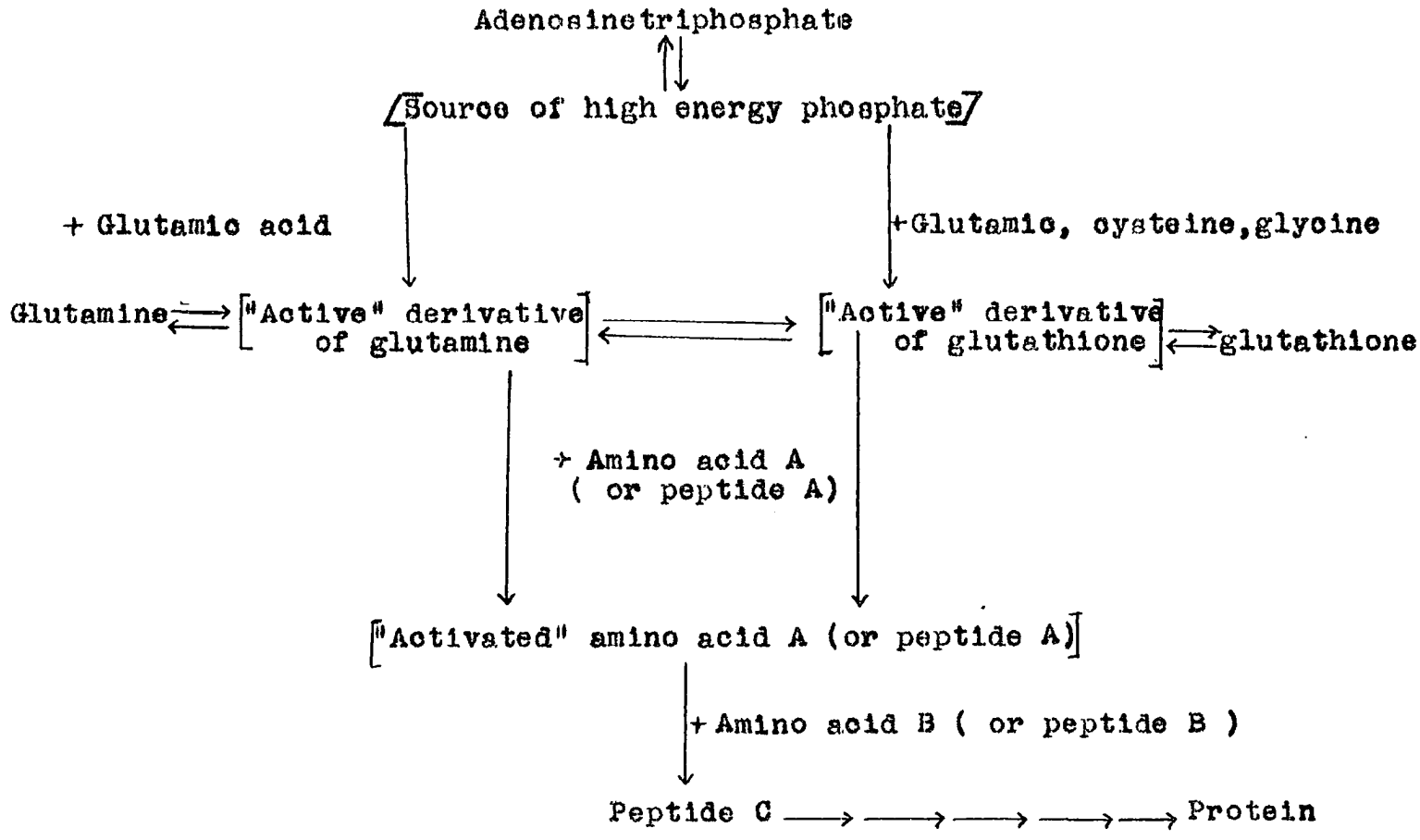
(1) F. Lipmann, Fed'n. Proc., 8, 597 (1949).

(2) J. S. Fruton, Yale J. Biol. Med., 22, 263 (1950).

(3) J. S. Fruton, R. B. Johnston and Melvin Fried, J. Biol. Chem., 190, 39 (1951).


Adenosinetriphosphate
Source of high energy phosphate
+ Glutamic acid
+Glutamic, cysteine,glycine
Glutamine
"Active" derivative of glutamine
"Active" derivative of glutathione
glutathione
+ Amino acid A (or peptide A)
"Activated" amino acid A (or peptide A)
+ Amino acid B (or peptide B)
Peptide C
Protein

In the foregoing scheme, the reactions designated by means of solid arrows are considered to be reversible enzyme-catalyzed reactions, and the various hypothetical intermediates are enclosed in brackets. In this scheme, adenosinetriphosphate, in causing the formation of such key funneling agents of "peptide bond energy" would thus link the process of peptide synthesis to the exergonic reactions in the breakdown of carbohydrates and fats. The selective utilization of this energy for the synthesis of naturally occurring peptides and the elongation of peptide chains in the formation of proteins could then be effected via the catalysis of trans-peptidation reactions by the intracellular proteases.

Recent investigations by the Fruton group (1) dealt with the action of proteinases such as chymotrypsin and papain in transamidation reactions leading to the elongation of peptide chains. Crystalline chymotrypsin was found to catalyze a transamidation reaction in which the amide NH_2 group of benzoyl-L-tyrosinamide was replaced by isotopic glycinamide, to form benzoyl-L-tyrosylglycinamide labeled with N^{15} in the glycine N. Papain-catalyzed transamidation reactions in which the

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(1) J. S. Fruton, R. B. Johnston and Melvin Fried, J. Biol. Chem., 190, 39 (1951).

amide NH_2 groups of several carbobenzoxyamino acid amides were replaced by amino acid amides or by peptides. D-Phenylalaninamide was also shown capable of serving as a replacement agent in such transamidation reactions.

Further illustration of the elongation of a peptide chain by means of a protease-catalyzed replacement reaction was demonstrated by Brenner and co-workers (1). The formation of L-methionyl-L-methionine and of L-methionyl-L-methionyl-L-methionine by chymotrypsin was effected. Report (2) has been made that chymotrypsin also catalyzes the hydrolysis of ester analogues of sensitive amides (e.g., benzoyl-L-tyrosine ethyl ester).

In a recent publication by Hanes and co-workers (3), it was concluded that in the presence of added amino acids and of certain enzymes present in sheep kidney extract, glutathione can undergo a transpeptidation reaction in which cysteinylglycine and a γ-glutamyl dipeptide are formed. This conclusion was reached with

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(1) M. Brenner, H. R. Muller and R. W. Pfister, Helv. chim. acta, 33, 568 (1950).

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

(3) C. S. Hanes, F. J. R. Hird and F. A. Isherwood, Nature, 166, 288 (1950).

the finding that, after incubation of sheep kidney extracts with glutathione and one of several amino acids (leucine, phenylalanine, valine), the presence of new ninhydrin-reactive substances could be demonstrated by means of paper chromatography. These substances migrated at rates different from those of any of the products of hydrolysis of glutathione or of the other components introduced at the start. Although no authentic samples were available for direct comparison, the new spots were provisionally attributed to γ-glutamyl peptides of the added amino acids (e.g., γ-glutamylphenylalanine).

Waley and Watson (1) reported the rearrangement of the amino acid residues of lysyltyrosyllysine and lysyltyrosylleucine by the action of a mixture of trypsin and chymotrypsin. Among products was included a dipeptide, lysyllysine, in which the sequence of the amino acid residues differed from that of the original peptides. It was assumed that a transpeptidation reaction occurred via the "carboxyl transfer" mechanism of Hanes (2) and not via the unreported "amine transfer" mechanism.

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(1) S. G. Waley and J. Watson, Nature, 167, 360 (1951).

(2) C. S. Hanes, F. J. R. Hird and F. A. Isherwood, Nature, 166, 288 (1950).

EXPERIMENTAL

Preparation of Compounds Investigated

Benzoylamino acids

All the benzoyl derivatives of the amino acids used in these studies, with the exception of N-benzoyltyrosine, were prepared by the conventional Schotten-Baumann procedure. The proportions of reagents, as recommended by Ingersoll and Babcock (1), were used in all cases.

In a typical benzoylation, 0.1 mole of the amino acid was dissolved in 150 ml. of 1 N sodium hydroxide in a 250 ml. round-bottomed flask. The addition of 0.1 mole of benzoyl chloride, by means of a medicine dropper, was effected over a period of one hour with intermittent, vigorous shaking. The solution was kept cold by means of an ice bath which was removed after the addition of benzoyl chloride had been completed. After the solution had been allowed to stand for one-half hour, during which time it had warmed to room temperature, no odor of benzoylchloride was detectable. The reaction mixture was then placed in an ice bath and acidified with 6 N hydrochloric acid to approximately pH 3, with Congo red as indicator. After being cooled for about two hours at 4°, the precipitate was filtered off, all clumps were

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(1) A. W. Ingersoll and S. H. Babcock, Org. Syntheses, Coll. 2, 328 (1943).

broken, and the solid was washed on filter paper three times with 75 ml. portions of ice water. The precipitate was dried in air overnight and treated with 150 ml. of boiling carbon tetrachloride to remove any contaminating benzoic acid. Final purification was effected by recrystallization from ethanol-water.

All of the benzoylated amino acids used in these studies had previously been described in the literature. These compounds are listed in Table I, together with the observed melting points, the literature values and references. Identification of the benzoylamino acids was made by melting points and the mixed melting points were determined in those cases in which pure samples were available.

Since a considerable discrepancy existed between the melting points of N-benzoyl-S-benzyl-DL-penicillamine observed in these studies and in another laboratory (1), micro-Kjeldahl analyses were run and showed agreement for this compound to within less than 3% relative error. The higher and sharper melting point found in this laboratory suggests a purer preparation than that described in the

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(1) H. M. Crooks in T. H. Clark, "The Chemistry of Penicillin", p. 462. Princeton University Press, Princeton, New Jersey. 1949.

Table I

Melting Points of Benzamino Acids

Amino Acid	M.p. of Benzoyl Amino Acid in this Study, C° Uncorrected	Literature Value
Glycine	187½-189	187-188 (1)
Alanine	165-166	165-166 (2)
Valine	131-132	132½ (3)
Leucine	141-142	137-141 (3)
Isoleucine	136-137	115-118 (4)
Norvaline	152½	152½ (5)
Norleucine	134	134 (3)
Aminoiso-butyric Acid	200-201	198 (6)

(1) A. W. Ingersoll and S. H. Babcock, Org. Syntheses, Coll. 2, 328 (1943).

(2) E. Fischer, Ber. 32, 2454 (1899).

(3) E. Fischer, Ber., 33, 2370 (1900).

(4) C. Mourew and A. Valeur, Compt. rend., 141, 115 (1905).

(5) M. D. Slimmer, Ber., 35, 400 (1902).

(6) E. Mohr and T. Geis, Ber., 41, 798 (1908).

Table I (Continued)

Amino Acid	M.p. of Benzoyl Amino Acid in this Study, C° Uncorrected	Literature Value
α-Amino-n-butyric Acid	145-146	145-146 (1)
α-Aminoheptylic Acid	133	135 (2)
α-Aminocaprylic Acid	127½-128	128 (2)
α-Aminopelargonic Acid	127-128	128 (2)
S-Benzylpenicellamine	163½-164	156-159 (3)
Phenylalanine	187-188	187-188 (1)

(1) E. Fischer and A. Mouneyrat, Ber., 33, 2383 (1900).

(2) N. F. Albertson, J. Am. Chem. Soc., 68, 451 (1946).

(3) H. M. Crooks in T. H. Clarke, "The Chemistry of Penicillin", p. 462. Princeton University Press Princeton, New Jersey. 1949.

Table I (Continued)

Amino Acid	M.p. of Benzoyl Amino Acid in this Study, C° Uncorrected	Literature Value
Glutamic	139-140	139-140 (1)
Tyrosine (N-Benzoyl)	165-166	165-166 (2)
Tyrosine (O,N-Dibenzoyl)	226-228	226-228 (3)
Methionine	151	152 (4)

(1) H. Wax, Unpublished Ph.D. Thesis. Ames, Iowa, Iowa State College Library. 1949.

(2) E. Fischer, Ber., 32, 3638, (1900).

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

(4) E. M. Hill and W. Robson, Biochem. J., 30, 248 (1936).

earlier literature.

Benzoyldipeptides

Almost all of the benzoyldipeptides used in these studies were prepared by Mr. Louis Carpino and all were of the DL-configuration. These are listed in Table II together with their melting points. Where success was achieved in the separation of diastereomers, if such diastereomers exist, each is listed separately. In these instances the diastereomeric racemates were arbitrarily designated as the A and B forms.

N-Benzoyl-L-tyrosine

Eleven grams of N-benzoyl-L-tyrosine ethyl ester was added to 30 ml. of 5 N sodium hydroxide solution. The mixture was refluxed over a water bath for twenty minutes. After five minutes had elapsed, all the solid material had gone into solution. The solution was diluted to 150 ml. with distilled water and then acidified with 6N hydrochloric acid to approximately pH 3 with Congo red paper as indicator. An oil was obtained which underwent crystallization to a white precipitate after it had been rubbed under hexane and chilled. Upon recrystallization from ethyl acetate-benzene, the observed melting point was 164½-165½°. Fischer (1) reported the melting point as 165-166°.

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(1) E. Fischer, Ber., 32, 3638 (1900).

Table II

Physical Constants of Benzoyldipeptides

Benzoyldipeptide	M.p. in C.° (uncorr.)	Lit. value and ref.	% N, calcd.	% N, found
Bz-glycylglycine	207-208	208(1)		
Bz-glycylalanine	194-197	202(2)		
Bz-glycylleucine	151-153		9.58	9.43
Bz-glycylvaline	134-136	135-136(3)		
Bz-glycylphenylalanine	175	172(4)		
Bz-alanylglycine	154-156	161(5)		
Bz-leucylglycine	161-164	167(6)		
Bz-valyglycine	176-179		10.06	10.18
Bz-valylvaline (A-Form)	220-224		8.74	9.12
Bz-valylvaline (B-Form)	230-232	231-232(7)		
Bz-leucylvaline (A-Form)	218-220		8.37	8.22
Bz-leucylvaline (B-Form)	195-198		8.37	8.19

(1) E. Fischer, Ber., 38, 608 (1905).
(2) T. Curtius and E. Lambotte, J. prakt. Chem., 70, 114 (1904).
(3) E. Abderhalden, E. Rindtorff and A. Schmitz, Fermentforschung, 10, 213
(4) T. Curtius and E. Muller, J. prakt. Chem. N. F. 70, 223 (1904).
(5) S. Goldschmidt and C. Steigerwald, Ber., 58, 1352 (1925).
(6) E. Fischer and A. Brunner, Ann., 340, 148 (1905).
(7) J. W. Hinman, E. L. Caron and H. N. Christensen, J. Am. Chem. Soc., 72, 1620 (1950).

Phthalylglycine

The method of Drechsel (1) was followed. Two hundred grams of phthalic anhydride (General Chemical Co.) was heated in 1 l. Erlenmeyer flask, over an oil bath, until completely melted. One hundred grams of glycine (Merck, N. F.) was then added and the heating was continued until complete solution had taken place, as was testified by a dark yellow but clear solution. At this point, the reaction mixture was allowed to come to room temperature. The solid which was obtained on cooling was dissolved in 4 l. of hot distilled water, Darco was added to decolorize the yellow solution, and the hot solution was filtered over suction. The supernatant, which contained some crystalline solid, was then reheated to dissolve all solid material present. Recrystallization was effected at 4°. The white, crystalline precipitate was filtered over suction, washed with two 500 ml. portions of ice water, and dried in air. The yield of product obtained was 275 g. The melting point was 192-193° as compared to 191-192° obtained by Drechsel (1)

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(1) E. Drechsel, J. prakt. chem., 27, 418 (1883).

Phthalylglycyl chloride by using phosphorus pentachloride

The procedure which was used was a modification of Gabriel's method (1) as developed by Sheehan and Frank (2). A suspension of 10.4 g. (0.05 moles) of phosphorus pentachloride in 100 ml. of benzene, contained in a 250 ml. Erlenmeyer flask and protected from moisture with a calcium chloride drying tube, was treated with 10.3 g. (0.05 moles) of phthalylglycine. The reaction mixture was heated in a 60° water bath. The mixture was shaken at frequent intervals until the turbidity had completely disappeared and a clear solution was obtained. After two hours, the reaction mixture was removed from the water bath. The solution was concentrated under reduced pressure and the resulting dry residue was dissolved in a minimal amount of benzene at 40°. The phthalylglycyl chloride was precipitated by the addition of Skelly D. The yield of shimmering, white crystals was 9.2 g. The melting point of 83-85° was the same as that observed by Sheehan and Frank (2).

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(1) S. Gabriel, Ber., 40, 2648 (1907).

(2) J. C. Sheehan and V. S. Frank, J. Am. Chem. Soc., 71, 1855 (1949).

Phthalylglycyl chloride by using thionyl chloride

The method of Grassmann and Schulte-Uebbing (1) was used with a slight modification. To 8.2 ml. of thionyl chloride, contained in a 25 ml. round-bottomed flask, was added 4.1 g. of phthalylglycine. The reaction mixture was refluxed over a steam bath until the solution of phthalylglycine was complete and the evolution of hydrogen chloride had ceased. The excess thionyl chloride was distilled off under reduced pressure and the last traces of thionyl chloride were eliminated by two evaporations after the addition, in each case, of 10 ml. of chloroform. The chloride remained behind as a white, crystalline residue. The yield of product was 3.7 g. and the melting point was 83-85°.

Phthalylglycinanilide from phthalylglycyl chloride

The method used was essentially the same as that reported by Scheiber (2). To a solution of 3.7 g. of phthalylglycyl chloride in 40 ml. of benzene was added 3.7 ml. of crude aniline, in small portions, over a period of 20 minutes, with intermittent shaking by hand.

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(1) W. Grassmann and E. Schulte-Uebbing, Chem. Ber., 83, 244 (1950).

(2) J. Scheiber, Ber. 46, 1103 (1913).

The reaction mixture was meanwhile kept cold by means of an ice bath. The precipitate obtained was filtered over suction. The filtrate was evaporated to about 5 ml. under reduced pressure and more precipitate was obtained by the addition of petroleum ether. The combined precipitates were recrystallized from 50% ethanol-water. The white crystals, obtained in 4.4 g. yield, were filtered over suction and dried in the vacuum desiccator. The melting point was 230-231°. Scheiber (1) reported a melting point of 231-232°.

Phthalylglycinanilide from phthalylglycine

A previously undescribed method for the preparation of this compound was suggested by the method reported by Shriner and Fuson (2) for the preparation of anilide derivatives of fatty acids. To 51 g. of phthalylglycine, contained in a 200 ml. round-bottomed flask, was added 102 ml. of thionyl chloride. The mixture was refluxed on a water bath and solution was observed to be complete in about fifteen minutes. After the solution was allowed

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(1) J. Scheiber, Ber. 46, 1103 (1913).

(2) R. L. Shriner and R. C. Fuson, "The Systematic Identification of Organic Compounds", 2nd Ed., p. 132. John Wiley and Sons, Inc., New York. 1940.

to reflux for fifteen minutes more, it was cooled to room temperature. The cool solution was placed in a dropping funnel and was then added, over a period of twenty minutes, to a solution of 102 ml. of crude aniline in 1 l. benzene, with agitation by means of a motor stirrer. The reaction was carried out in an ice bath. Stirring was allowed to continue for a total of one hour and the reaction mixture, which by this time contained a dense, gelatinous precipitate, was allowed to warm to room temperature. The precipitate was filtered over suction and washed with cold, distilled water to remove any aniline hydrochloride formed in the reaction. Further precipitate was obtained by the addition of petroleum ether to the benzene filtrate, which had previously been concentrated to a small volume under reduced pressure. The combined precipitates were then recrystallized from 50% ethanol-water to give small, needle-like, white crystals. The yield of phthalylglycinanilide was 49 g. and the melting point was 230-231°.

Glycinanilide hydrochloride

The procedure used was that of Sheehan and Frank (1). A suspension of 1.29 g. of phthalylglycinanilide in 50 ml. of 95% ethanol was refluxed for one hour over a steam bath with an equivalent amount of hydrazine hydrate

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(1) J. C. Sheehan and V. S. Frank, J. Am. Chem. Soc., 71, 1855 (1949).

(0.224 ml.). The reaction mixture was then concentrated to dryness under reduced pressure. The dry residue was warmed to 50° with 25 ml. of approximately 2 N hydrochloric acid for about five minutes and was subsequently allowed to cool to room temperature over a period of one hour. The insoluble residue of phthalylhydrazide was removed by filtration. Evaporation of the solvent under reduced pressure followed by recrystallization of the residue from ethanol gave 0.84 g. of glycinanilide hydrochloride which melted at 191-195°. Sheehan and Frank (1) reported a melting point of 192-195°.

Glycinanilide from the hydrochloride

A solution of 0.84 g. of glycinanilide hydrochloride in 50 ml. of distilled water, in an ice bath, was saturated with ammonia gas. After further cooling in the refrigerator overnight, a white precipitate had formed. The precipitate was filtered over suction and recrystallized from hot, distilled water. The yield of the crystalline dihydrate of glycinanilide obtained was 0.65 g. The

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(1) J. C. Sheehan and V. S. Frank, J. Am. Chem. Soc. 71, 1855 (1949).

melting point of 60½-61½° was in close agreement with the 62° previously reported in the literature (1).

Chloroacetylaniline

This compound was prepared by the procedure of Abderhalden and Brockmann (2). To a solution of 94 g. (1.0 mole) of crude aniline in 500 ml. of dry benzene, contained in a 1 l., three-necked, round-bottomed flask, 56 g. (0.5 mole) of chloroacetyl chloride was added over a period of twenty minutes. The reaction mixture was cooled in an ice bath and agitation was effected by means of a motor stirrer. After the addition of chloroacetyl chloride was complete, the stirring was further continued for one hour. The mixture was cooled in the refrigerator overnight. The dense precipitate of reddish-brown crystals was filtered over suction and washed with three 250 ml. portions of benzene to remove any surplus aniline. The precipitate was then washed with three 100 ml. portions of cold, distilled water to remove the aniline hydrochloride which had been formed in the reaction. After the precipitate was dried under the infra-red lamp for eight hours, an 80 g. yield of light tan crystals was obtained.

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(1) J. V. Dubsky and C. Granacher, Ber., 50, 1703 (1917).

(2) E. Abderhalden and H. Brockmann, Fermentforschung, 10, 164 (1928).

The melting point of 132-134° was in good agreement with the 135° reported in the literature (1).

Glycinanilide from chloroacetylaniline

The method used was a modification of the procedure of Hill and Kelsey (2). Ammonia gas was rapidly bubbled through 1500 ml. of 95% ethanol until the ethanol was saturated, the process being effected in an ice bath. Upon saturation, the ethanol contained 210 g. of ammonia. After the addition of 90 g. of chloroacetylaniline to this solution, the reaction mixture was placed in the 40° incubator for four days. After this period, the insoluble material was filtered off and the filtrate was then concentrated to dryness under reduced pressure. The crude material was purified by the procedure of Fox and Halverson (3).

Forty g. of the crude material obtained above was dissolved in a solution of 68 g. of picric acid in 3 l. of hot, distilled water. Formation of a yellow precipitate was immediate. After it had been cooled overnight at 4°, the yellow precipitate was suction

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(1) E. Abderhalden and H. Brockmann, Fermentforschung, 10, 164 (1928).

(2) A. J. Hill and E. B. Kelsey, J. Am. Chem. Soc., 42 1709 (1920).

(3) S. W. Fox and J. S. Halverson, Unpublished experiments.

filtered, dried in air and washed with 600 ml. of benzene. After recrystallization from water, the dried picrate showed a melting point of 188-190°. Alderhalden (1) and Brockmann reported the melting point at 186°. Sixty g. of this picrate was dissolved in 3 l. of chloroform and this solution was then treated with 1800 ml. of water containing 26 ml. of 10 N sodium hydroxide. Separation of the chloroform layer was followed by treatment of this chlroform fraction with 1 l. of water containing 8 ml. of 10 N sodium hydroxide. After its separation from the aqueous layer, the chloroform layer was dried with 30 g. of Drierite and was then concentrated to dryness under reduced pressure. The gummy residue was redissolved in a minimal amount of chloroform and precipitation was effected with hexane. The tiny, colorless crystals obtained were dried in air. This was followed by recrystallization from hot water. The large, colorless, needle-like cyrstals which formed melted at 60 1/2 to 61°.

An improved method was also used in the preparation of glycinanilide from chloroacetylaniline. Eighty grams

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(1) E. Abderhalden and H. Brockmann, Fermentforschung, 10, 164 (1928).

of chloroacetylaniline was dissolved in 1 l. of 95 per cent ethanol. To this solution was added 1.5 l of concentrated aqueous ammonia. The reaction mixture was placed in the 40° water bath for three days. After this period, the solution was evaporated to about one-third of its original volume. The concentrate was treated with 200 ml. of concentrated aqueous ammonia and placed in the refrigerator overnight. The crystalline precipitate of secondary base (m.p. 148-150°) was filtered off over suction. A copious precipitate of the hydrate of glycinanilide was then obtained by seeding the filtrate with a crystal of glycinanilide hydrate. The white, crystalline product was obtained in 64 g. yield and melted at 63-64° without further purification.

Chloroacetyl-D-phenylalanine

The procedure used for the preparation of the D-antipode was essentially the same as that used by Leuchs and Suzuki (1) for chloroacetyl-DL-phenylalanine. The solution of 3.40 g. of D-phenylalanine in 21 ml. of

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(1) H. Leuchs and W. Suzuki, Ber., 37, 3306 (1904).

1 N sodium hydroxide was effected, the reaction mixture being cooled in an ice bath. To this solution was added 2.35 g. (1.55 ml.) of chloroacetyl chloride, via a medicine dropper, over a period of one hour with intermittent shaking and cooling. The solution was then acidified to pH 2 with concentrated hydrochloric acid and the somewhat gummy residue was extracted with three 75 ml. portions of ether. The ether extracts were combined and the solution was concentrated to a brown syrup under reduced pressure. This syrup completely crystallized when placed under Skellysolve "D" at 4°. This material, when recrystallized from 65 ml. hot water, melted at 115-118°. Further recrystallization brought the melting point up to 125-126°. A melting point of 126° was reported (1) for the L-antipode.

Glycyl-D-phenylalanine

The same procedure reported by Fischer and Schoeller (2) for the L-antipode was used, with slight modifications. A suspension of 2.3 g. of chloroacetyl-D-phenylalanine in 70 ml. of concentrated, aqueous ammonia, contained in

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(1) E. Fischer and W. Schoeller, Ann. 357, 1, 1907.

(2) S. Simmonds, E. L. Tatum and J. S. Fruton, J. Biol. Chem., 169, 91 (1947).

a 200 ml. round-bottomed flask, was heated in a pressure bomb for three hours at 100°. The clear, slightly brownish solution which resulted was concentrated to dryness under water pump pressure and a temperature of 50°. The crystalline residue was dissolved in 75 ml. of boiling, distilled water and precipitation was effected by the addition of 1 l. of absolute ethanol. The resulting flocculent, white crystals were filtered over suction and dried under the infra-red lamp. The rotation observed was $[\alpha]_D^{22} = 41.8^\circ \pm 1.0^\circ$ for a 1.5 % solution in distilled water. This value is in agreement with the literature value of $[\alpha]_D^{21} = 41.7^\circ$ (2% in water) for this compound (1).

Benzoylglycyl-D-phenylalanine

This compound was prepared from glycyl-D-phenylalanine by the same general procedure of Ingersoll and Babcock (2) as previously described for the benzoylamino acids. The benzoyl derivative melted at 164-167 1/2°.

Amino Acid Anilides

The citrate salts of the leucine-, alanin- and valinanilides used in these studies were prepared by Mr. Armand McMillan. All are of the DL-configuration. The analytical values obtained for these compounds are shown in Table III.

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(1) S. Simmonds, E. L. Tatum and J. S. Fruton, J. Biol. Chem., 169, 91 (1947).

(2) A. W. Ingersoll and S. H. Babcock, Org. Syntheses, Coll. 2, 328 (1943).

Table III

Physical Constants of Amino Acid Anilide Citrates

Compound *	M.p.	N Calcd.	N Found
Alaninanilide citrate	87°	7.37	7.7
Valinanilide citrate	87°	7.29	7.3
Leucinanilide citrate	157-158°	7.04	7.0, 7.1

* All compounds are of the DL configuration

Enzyme Studies

Enzymic reactions of benzoylamino acids with aniline

In all experiments involving the enzymic coupling of benzoylamino acids with aniline, the same general procedure was used for addition of components, incubation of the reaction mixture and isolation and purification of the reaction products.

Benzoylamino acids.

The benzoyl derivatives of glycine, alanine, valine, leucine, aminoisobutyric acid, norvaline, norleucine, ∝-amino-n-butyric acid, ∝-aminoheptylic acid, ∝-aminopelargonic acid, ∝-aminocaprylic acid and S-benzylpenicillamine were investigated. All were DL-isomers. A weight of derivative corresponding to 1.0 mM of benzoyl-DL-amino acid (0.50 mM in the case of benzoylglycine) was used in each instance.

Enzyme preparations and solutions.

All enzymatic reactions used commercial grades of either papain or ficin. The work reported here involved ficin No. 40349 obtained from Merck and Co. and papain No. 3781 obtained from Nutritional Biochemicals Corporation. The enzymes were used without further purification in the preparation of enzyme solutions. The enzyme solutions were prepared by dissolving 1.6 g. of the suitable enzyme and 0.64 g. of cysteine hydrochloride (Merck) with 200 ml. of citrate buffer (pH 5.0, 1M). The mixture was stirred occasionally over a half-hour period to break up the lumps and then filtered through glass wool. A volume of 2.0 ml. of solution thereby contained the soluble portion of 16 mg. of enzyme and 6.4 mg. of cysteine hydrochloride.

Fresh enzyme solutions were prepared shortly before use.

Aniline solution.

Commercial aniline was redistilled under reduced pressure and the almost colorless fraction collected. Aniline solutions in citrate buffer (pH 5.0, 1 M) were prepared to the extent that each 8.0 ml. of solution contained 0.046 ml. of aniline.

Citrate buffer solution.

In the preparation of the citrate buffer solution, a U.S.P. grade of citric acid (Pfizer) was used.

Typical enzymic run.

In a typical experiment, 1.0 mM of benzoyl-DL-amino acid or 0.50 mM of benzoylglycine was treated in a 15 ml. test tube with 0.046 ml. (0.50 mM) of redistilled aniline diluted to 8.0 ml. with citrate buffer (pH 5.0, 1.0 M). To each tube was added 2.0 ml. of enzyme solution. The tubes were stoppered and incubated at 40° for 72 hours with shaking by hand at twelve intervals. The products were filtered over suction and each was washed with two 5 ml. portions of 1 N sodium hydroxide solution and two 5 ml. portions of water, allowed to dry in air overnight and weighed.

It should be emphasized that conditions for almost

quantitative yields of even benzoylvalinanilide are known (1,2); for the present studies, standarized conditions permitting convenient comparisons in a sensitive range were however chosen. Some idea of the validity of reporting yields under standard conditions, instead of with rate curves, may be obtained from Fig. 2 of an earlier paper (3). These rate studies have been here extended to include the papain- and ficin-catalyzed anilide syntheses of the benzoyl derivatives of isoleucine, ∝-aminoheptylic acid and ∝-aminocaprylic acid as well as the previously studied alanine. The yields of anilide obtained after one, two and four days were determined for each substrate.

In order to observe the effect of pH on the relative reactivities of amino acid residues, benzoylvaline and benzoylisoleucine were compared over a pH range of from 3.9 to 5.9. All other conditions, such as reaction

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

(2) D. G. Doherty and E. A. Popenoe, Jr., J. Biol. Chem., 189, 455 (1951).

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

time, aniline concentration and buffer concentration were the same as those previously described. The enzyme used in this case was papain.

For reference purposes, workable quantities of anilides were obtained from larger papain-catalyzed preparations and purified to constant melting point by recrystallization from aqueous ethanol.

Enzyme Dilution Studies

Benzoylamino acids.

Investigation was made of the benzoyl derivatives of glycine, α-aminocaprylic acid, leucine, alanine, valine and isoleucine. All were the DL-isomers.

Enzyme solutions.

The same commercial grades of both papain and ficin were here used as were used in the preceding studies. The solutions were prepared by dissolving 0.80 g. of enzyme in 250 ml. of citrate buffer for one-half hour. This was followed by filtration through glass wool. Aliquots of the filtrate were diluted with citrate buffer (pH 5.0, 1M) such that four different solutions resulted, each 5.0 ml. of which contained 8 mg., 6 mg., 4 mg. or 2 mg. of enzyme respectively.

Aniline and cysteine hydrochloride solution.

A solution in citrate buffer (pH 5.0, 1 M) was prepared such that each 5.0 ml. of solution contained 0.50 mM of aniline and 13 mg. of cysteine hydrochloride.

Typical enzymic run.

To 5.0 ml. of the aniline solution described immediately above was added 1.0 mM of benzoyl-DL-amino acid (0.50 mM in the case of glycine). Five ml. of enzyme solution was added to each tube. Each benzoylamino acid thereby underwent reaction in the presence of 8, 6, 4 and 2 mg. of enzyme respectively. The pH in every case was 5.0. Reaction and treatment of products was effected under the usual conditions.

Enzymic Reactions of Glycine Containing Benzoyldipeptides With Aniline.

Benzoyldipeptides.

The benzoyl derivatives of glycylglycine, glycylvaline, glycylleucine, glycylalanine, glycylphenylalanine, valylglycine, alanylglycine and leucylglycine were investigated. Where the benzoyldipeptide had an asymmetric center, the DL-isomer was used. The only optically active benzoyldipeptide investigated was benzoylglycyl-D-phenylalanine. A weight of benzoyldipeptide corresponding to 0.50 mM was used in each case.

Enzyme preparations and solutions.

Enzyme solutions of both ficin and papain were prepared as previously described under "Enzymic reactions

of benzoylamino acids with aniline".

Aniline solution.

A solution of redistilled aniline in citrate buffer (pH 5.2, 1.0 M) was prepared wherein each 3.0 ml. of solution contained 0.023 ml. of aniline.

Typical enzymic run.

In a typical experiment, 0.50 mM of benzoyldipeptide was treated in 3 dram vials (19x65mm) with 0.023 ml. (0.25 mM) of aniline diluted to 3.0 ml. with citrate buffer (pH 5.0, 1.M). To each tube was added 2.0 ml. of enzyme solution, i.e., the soluble portion of 16 mg. of enzyme and 6.4 mg. of cysteine hydrochloride. The tubes were stoppered and incubated at 40° for 72 hours with shaking by hand at twelve intervals. The products were filtered over suction and each was washed with 5 ml. portions of 1 N sodium hydroxide and two 5 ml. portions of water, dried in air overnight and weighed.

Enzymic Reactions of Non-glycine Containing Benzoyldipeptides With Aniline

Benzoyldipeptides.

The benzoyldipeptides investigated were the A and B forms of benzoyl-DL-valyl-DL-valine and benzoyl-DL-leucyl-DL-valine.

Typical enzymic run.

Both forms of benzoyl-DL-valyl-DL-valine were reacted under the same conditions as described under "Enzymic reactions of glycine containing benzoyldipeptides

with aniline". With the benzoylleucylvalines, however, the procedure was varied in that a higher aniline concentration and a higher pH was used, i.e., 1.0 mM of aniline and a pH of 5.7 respectively.

Enzymic Reactions of Benzoylamino Acids With Glycinanilide

Benzoylamino acids.

The benzoyl derivatives investigated included those of glycine, alanine, valine, leucine, methionine, norvaline, norleucine, α-amino-n-butyric acid, tyrosine (mono- and dibenzoylated), isoleucine, tryptophan, phenylalanine, aminoisobutyric acid and glutamic acid. All benzoyl derivatives were the DL-isomers except those of glutamic acid and tyrosine (monobenzoylated) which were of the L-configuration.

Glycinanilide solution.

A solution of glycinanilide in citrate buffer (pH 5.0, 1M) was prepared wherein each 3.0 ml. of solution contained 0.50 mM of the dihydrate of glycinanilide.

Typical enzymic run.

To 0.50 mM of the benzoylamino acid in a 3 dram vial (19x65 mm) was added 3.0 ml. of the glycinanilide solution described immediately above. The remainder of the procedure is the same as that described under "Enzymic reactions of glycine containing benzoyldipeptides with aniline". In addition there were made several other runs

in which several of the benzoylamino acids were reacted with glycinanilide at various pH's. These reactions were catalyzed by both ficin and papain.

Enzymic Reaction of Benzoylamino Acids With Amino Acid Anilide Citrates

Benzoylamino acids.

The benzoylamino acids used in this study included the derivatives of glycine, alanine, valine, leucine, α-amino-n-butyric acid, norvaline, isoluecine and aminoisobutyric a acid. All were the DL-antipodes.

Amino acid anilide citrates.

These included the citrates of leucinanilide, valinanilide and alaninanilide, all of the DL-configuration. Since both valinanilide and alaninanilide were found to be very hygroscopic and therefore had to be weighed quite rapidly, solutions of these were made in 1 M citrate buffer wherein every 3.0 ml. contained 1.0 mM of the respective amino acid anilide citrate. No such hygroscopic properties were observed with leucinanilide citrate, however.

Typical enzymic run.

The treatment of 1.0 mM of benzoylamino with 3.0 ml. of a valinanilide or alaninanilide solution was effected in 3 dram vials (19x65mm). For those cases in which leucinanilide was the amine used, 3.0 ml.

of 1M citrate buffer was added to 1.0 mM of benzoylamino acid and 1.0 mM of leucinanilide citrate. The pH of the reaction mixtures was 5.6. The remainder of the procedure was similar to that given under "Enzymic reactions of glycine containing benzoyldipeptides with aniline". The only modification involved the washing of the reaction products with two 5 ml. portions of 1 N hydrochloric acid to remove any unreacted and undissolved amino acid anilide citrate.

Enzyme Specificity Studies

Benzoylamino acids.

The acylamino acids used included benzoyl-L-tyrosine, benzoyl-DL-tryptophan, benzoyl-DL-methionine and benzoyl-DL-phenylalanine. The weight of each benzoyl derivative used was equivalent to 0.50 mM based on the L-antipode.

Enzyme preparations.

Both papain (Merck No. 3781) and crystalline chymotrypsin (Worthington Biochemical Laboratories No. C, 21) were used.

Typical enzymic run.

Typically, 1.0 mM of the benzoyl-DL-amino acid and 0.50 mM of glycinanilide dihydrate were weighed out into 3 dram vials. To each vial was added 5.0 ml.

of citrate buffer (pH 7.1, 1 M). Into one series of vials, which included all the benzoyl-DL-amino acids listed above, were placed 16 mg. of crude papain and 6.4 mg. of cysteine hydrochloride. To another series was added 5.0 mg. of crystalline chymotrypsin. The tubes were incubated in the 40° water bath for periods ranging from 32 to 48 hours. The reaction products obtained were treated in the usual manner.

In another experiment, the reactivities of papain and chymotrypsin were compared, in the reaction of N-benzoyl-L-tyrosine with glycinanilide and aniline, over a pH range. The concentrations of substrate and enzyme were the same as given above. The pH range studied extended from 5.0 to 7.5 in 1 M citrate buffer. Reaction time was 48 hours and reaction products were treated by the usual procedure.

RESULTS

Enzymic Reactions of Benzoylamino Acids With Aniline

The necessity for determining the effects of variation of substrate structure on the relative extents of synthesis of several benzoylamino acid anilides was suggested by the results of Fox and co-workers (1,2) with the valine and leucine derivatives. These investigators observed a low rate of formation of benzoylvalinanilide as contrasted to a high rate of formation of the closely homologous benzoylleucinanilide. Investigation was therefore invited to the problem of the variation of amino acid residue structure on anilide yield. This structural variation took the form of changing the length and degree of branching of the hydrocarbon side chain of the amino acid residue.

In the resolution of this problem, which, for the sake of simplicity was confined to the monoaminomonocarboxylic acid series, several unnaturally occurring

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

(2) S. W. Fox and H. Wax, J. Am. Chem. Soc., 72, 5087 (1950).

amino acids were included. This appeared necessary since there was an insufficient number of naturally occurring amino acids in the monoaminomonocarboxylic acid series to give as much information as desired. Such an investigation also demanded the study of more than one enzyme system, since the problem involved might be one of enzyme-substrate interaction rather than the effect of substrate structure alone. Each of the related proteases, papain and ficin, was therefore used in these investigations.

A study of the benzoyl derivatives of thirteen systematically varied amino acids was made. These were incubated with aniline in the presence of activated papain or activated ficin in citrate buffer at pH 5.0. The pH of 5.0 was chosen because it was close to optimal for the formation of benzoylvalinanilide (1). The anilides obtained and their physical constants are shown in Table IV. One discrepancy between melting points appears for benzoylisoleucine. Samples supplied by Dr. Jesse P. Greenstein demonstrated that the sample here used was benzoylisoleucine, whereas the material in the earlier literature (2) is benzoylalloisoleucine (3).

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

(2) C. Mourew and A. Valeur, Compt. rend., 141, 115(1905).

(3) K. F. Itschner and S. W. Fox, Unpublished experiments.

Table IV

Physical Constants of Pure Benzoylamino Acid Anilides

Benzoylamino Acid	M.p. of Anilide C.°, Uncorr.	Lit. Value and Ref.	N Calcd.	N,* Found Micro Kjeldahl	$[\alpha]_{24}^{D}$ C 1% in chloroform, Error ± 4°
Bz-glycine	212 1/2	212 1/2(1)			
Bz-alanine	175-176	175-176(1)			
Bz-valine	220-221	220-221(2) 210-211(3) 217-218(4)			
Bz-leucine	213	213			
Bz-isoleucine	220-220 1/2	218-218 1/2(4)	9.03	8.94	-49°
Bz-norvaline	182 1/2-183 1/2	184-185(1)	9.46	9.61	-81°
Bz-norleucine	181-182	182-183(4)	9.03	9.00	-66°
Bz-Aminoisobutyric acid					
Bz-α-amino-n-butyric acid	169 1/2-170 1/2	170-171(4)	9.92	10.1	-90°
Bz-α-aminoheptylic acid	184-185		8.69	8.91	-81°
Bz-α-aminocaprylic acid	175-176		8.28	8.30	-69°
Bz-α-aminopelargonic acid	158-158 1/2		7.95	7.96	-70°
Bz-S-benzylpenicillamine					

Table IV (Cont'd.)

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

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

(3) D. G. Doherty and E. A. Popenoe, Jr., J. Biol. Chem., 189, 455 (1951).

(4) N. F. Albertson, J. Am. Chem. Soc., 73, 452 (1951).

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* Nitrogen analyses were kindly furnished by Mr. Armand McMillan.

The results are reported in Table V. The melting points obtained were in such good agreement with the melting points of the corresponding benzoylamino acid anilides that mixed melting points were taken with pure samples of those anilides whose identity had been established. For all the benzoylated amino acids which were investigated, the product was the corresponding benzoylamino acid anilide.

Table V

Reactivity of Benzoylated Amino Acids with Aniline in Papain- and Ficin-Catalyzed Reactions.

Amino Acid	Wt. of benzoylated DL-amino acids used, g.	Weight of product		Melting point	Product **
		Papain	Ficin		
α-Aminoheptylic acid	0.249	0.1065	0.0358	182-184°	Bz-α-aminoheptylic acid anilide
α-Amino-n-butyric acid	0.207	0.0872	0.0651	166-168°	Bz-α-amino-n-butyric acid anilide
Norleucine	0.235	0.0822	0.0478	181-182°	Bz-norleucinanilide
α-Aminocaprylic acid	0.263	0.0846	0.0375	170-172°	Bz-α-aminocaprylic acid anilide
Leucine	0.235	0.0742	0.0407	208-210°	Bz-leucinanilide
α-Aminopelargonic acid	0.277	0.0680	0.0274	153-155°	Bz-α-aminopelargonic acid anilide
Norvaline	0.221	0.0559	0.0274	177-179°	Bz-norvalinanilide

Table V (Cont'd.)

Reactivity of Benzoylated Amino Acids with Aniline in Papain- and Ficin-Catalyzed Reactions.

Amino Acid	Wt. of benzoylated DL-amino acids used, g.	Weight of product		Melting point	Product **
		Papain	Ficin		
Alanine	0.193	0.0443	0.0317	174-176°	Bz-norvalinanilide
Glycine	0.0895	0.0153	0.0063	211-212°	Bz-glycinanilide
Valine	0.221	0.0079	0.0012	217-219°	Bz-valinanilide
Isoleucine	0.235	0.0043	trace	214-216°	Bz-isoleucinanilide
α-Aminoisobutyric acid	0.207	None	None	-	-
S-Benzoylpenicillamine	0.343	None	None	-	-

* Melting point of crude product.

** All products are of the L-configuration.

In Table VI, a ranking of the substrate preferences with both papain and ficin is given, as well as the % of anilide yield in each case. Although emphasis was herein placed on the contribution of substrate structure to the enzyme-substrate interaction, the results with ficin-cysteine and papain-cysteine are seen to be qualitatively different. The differences are expressed in the order of substrate activities, or of "preferences". These results are in agreement with earlier observations that papain/ficin yield ratios were different for glycine, valine and leucine when the yield of benzoylalaninanilide was the same (1). Particularly noteworthy are the marked differences in ranking for the alanine, α-amino-caprylic acid and α-aminoheptylic acid derivatives.

Information of the effect of pH on the order of substrate activities seemed of importance in view of the fact that the relative degree of enzyme-substrate interaction might differ for different substrates at different pH's. Such pH studies have been reported in the literature (2) for the papain-catalyzed anilide

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(1) S. W. Fox and J. S. Halverson, Unpublished experiments.

(2) S. W. Fox, C. W. Pettinga, Arch. Biochem., 25, 13 (1950).

Table VI

Ranking of Substrate* Preferences
with Papain and Ficin

Ranking with Papain	% Yield	Ranking with Ficin	% Yield
1. ∝-Aminoheptylic acid	64	1. ∝-Amino-n-butyric acid	46
2. ∝-Amino-n-butyric acid	61	2. Norleucine	31
3. Norleucine	53	3. Leucine	26
4. ∝-Aminocaprylic acid	50	4. Alanine	24
5. Leucine	48	5. ∝-Aminoheptylic acid	22
6. ∝-Aminopelargonic acid	39	6. ∝-Aminocaprylic acid	22
7. Norvaline	38	7. Norvaline	19
8. Alanine	33	8. ∝-Aminopelargonic acid	16
9. Glycine	12	9. Glycine	5
10. Valine	5	10. Valine	1
11. Isoleucine	3	11. Isoleucine	trace
12. ∝-Aminoisobutyric acid	0	12. ∝-Aminoisobutyric acid	0
13. S-Benzylpenicillamine	0	13. S-Benzylpenicillamine	0

* In each case the benzoyl-DL-amino acid was employed as substrate.

syntheses involving the benzoyl derivatives of glycine, alanine and valine. Since both benzoylvaline and benzoylisoleucine showed low reactivity, it was therefore of interest to compare these substrates, under the same conditions, over a pH range to ascertain whether the order of preference could be inverted. When these substrates were compared over the pH range of 3.9 to 5.9, the order of preference remained the same for both the ficin- and the papain-catalyzed reactions, with benzoylvaline as the preferred substrate. This is illustrated in Table VII.

Table VII

Effect of pH on Yields of Anilide from Benzoylvaline and Benzoylisoleucine

pH	Per Cent Anilide Yield	
	Benzoylvaline	Benzoylisoleucine
3.9	None	None
4.3	None	None
4.7	0.7	None
5.0	4.5	1.3
5.6	24.6	11.3
5.8	27.1	15.8
5.9	27.5	16.6

Since some question might be raised concerning the validity of reporting yields under standard conditions, instead of with rate curves, rate studies were carried out in the ficin- and papain-catalyzed synthesis of anilides using the isoleucine, ∝-aminoheptylic acid, ∝-aminocaprylic acid and alanine derivatives. The yield of anilide, in each instance, was determined after a one, two and four day period. In all cases investigated, the ranking of substrate preferences remained the same as those shown in Table VI for both papain and ficin. These results are further supported by similar rate studies, reported in the literature (1), for the benzoyl derivatives of valine, leucine, alanine and glycine.

Enzyme Dilution Studies

A factor of importance in the anilide syntheses was the concentration of enzyme used in the studies. Enzyme dilution studies were carried out in the attempt to elucidate whether enzyme preferences change with changing protease concentration. Particular emphasis

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

was afforded such studies in view of observations (1) that the papain/ficin yield ratios were different for glycine, valine and leucine when the benzoylalaninanilide yield was the same (1). Investigation of the reactivity of the foregoing benzoylamino acids, in addition to the α-aminocaprylic acid and isoleucine derivatives, was made with different concentrations of both ficin and papain.

Enzyme solutions were prepared from a standard enzyme solution by dilution wherein the four concentrations obtained were 1.0, 0.75, 0.50 and 0.25 times as concentrated as the standard enzyme solution. Such solutions were made for both papain and ficin. The conditions of reaction are given in the Experimental Section. The yields of anilide obtained with the various enzyme concentrations are presented in Table VIII.

In order to obtain weighted comparisons, the yields of two anilides were plotted against a series of concentrations of each of the proteases. The relationships are plotted in Figures 1 and 2. The ordinates represent the percent yield of anilide based on the theoretically

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(1) S. W. Fox and J. S. Halverson, Unpublished experiments.

Table VIII

Benzoylamino Acid Anilide Yields at Different Enzyme Concentrations

Amino Acid Derivative	Anilide Yield, % Papain	Ficin	Enzyme conc. used Conc. Std. Enzyme Soln.
Glycine	4.3	1.3	1.0
	2.9	1.0	0.75
	0.9	0.2	0.50
	-	-	0.25
α-Aminocaprylic Acid	55.8	11.2	1.0
	41.0	10.1	0.75
	18.0	8.1	0.50
	9.9	5.1	0.25
Leucine	19.2	11.2	1.0
	15.6	9.6	0.75
	11.0	7.1	0.50
	6.2	3.8	0.25
Alanine	18.9	15.7	1.0
	16.2	13.2	0.75
	11.4	9.6	0.50
	3.5	3.7	0.25
Valine	2.0	trace	1.0
	0.5	-	0.75
	trace	-	0.50
	-	-	0.25
Isoleucine	0.5	-	1.0
	-	-	0.75
	-	-	0.50
	-	-	0.25

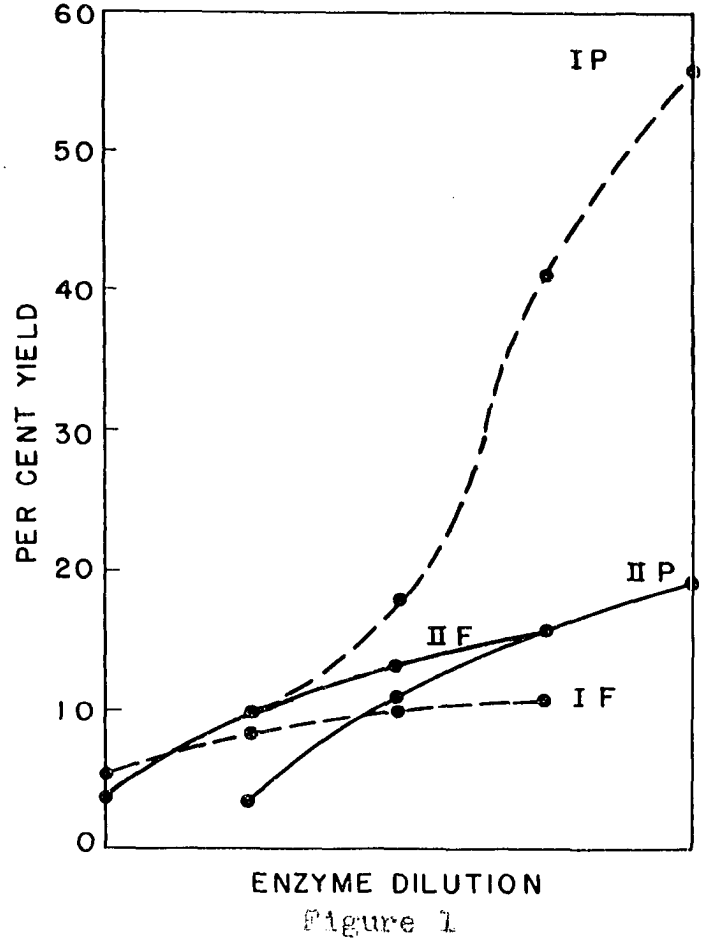

Figure 1

Figure 1: Enzyme Dilution Studies

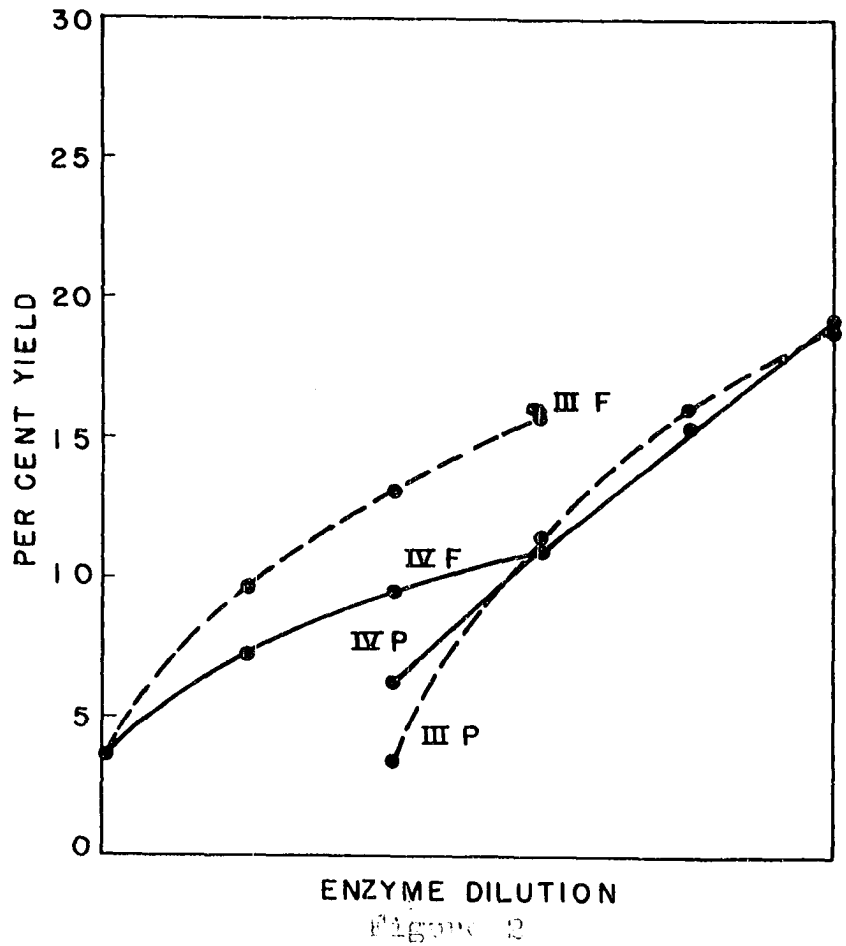

Figure 2

Figure 2: Enzyme Dilution Studies

possible yield of L-form. The abscissas represent the relative concentration of enzyme solution based on the amount of dilution of a standard enzyme preparation. Where the benzoylalaninanilide curves crossed, in Figure 1, the corresponding enzyme concentrations gave yield of the α-aminocaprylic acid analog which were several fold different. A similar situation is found in Figure 2 where, at the intersection of the benzoyl-leucinanilide curves, the corresponding enzyme concentrations gave a noticeable difference in benzoylalaninanilide yields. These relationships are presented in Tables IX and X.

Table IX

Yield of Benzoyl-α-aminocaprylic Acid Anilide at Enzyme Concentrations Giving Same Yield of Benzoylalaninanilide

Enzyme	Yield of Benzoyl-alaninanilide, %	Yield of Benzoyl-α-amino-Caprylic Acid Anilide, %
Papain	16	41
Ficin	16	11

Table X

Yield of Benzoylalaninanilide at Enzyme Concentrations Giving Same Yield of Benzoylleucinanilide

Enzyme	Yield of Benzoyl-leucinanilide, %	Yield of Benzoyl-alaninanilide, %
Papain	10 1/2	11
Ficin	10 1/2	16

Examination of Figure 1 shows that for the lower concentrations of ficin, the yields obtained of benzoyl-alaninanilide and benzoyl-α-aminocaprylic acid anilide approach the same value. However, with increasing protease concentration, the per cent yield of benzoylalaninanilide increases to a greater extent than does the α-aminocaprylic acid analog. A similar situation for the ficin-catalyzed reactions is found in Fig. 2. Here, although the yields

of the alanine and leucine derivatives are the same at the lowest enzyme concentration, the yield of the alanine derivative increases to a proportionately greater extent with increasing ficin concentration than does the yield of the leucine analog. These results demonstrate that the substrate preferences of enzymes may change with changing enzyme concentration.

Enzymic Reactions of Glycine-Containing Benzoyldipeptides With Aniline

Although several reports have appeared in the literature (1-3) concerning the enzymatic syntheses of anilides involving glycine-containing acylated peptides, no systematic studies have appeared with respect to the effect of the glycine residue on the type of reaction and the degree of reaction effected. Such studies were here undertaken in an effort to gain some insight regarding the effect of residue structure, and its relative position in the peptide chain, on enzyme specificity.

All of the benzoyldipeptides used in these studies

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(1) O. K. Behrens, and M. Bergmann, J. Biol. Chem., 129, 587 (1939).

(2) O. K. Behrens, D. G. Doherty and M. Bergmann, J. Biol. Chem., 136, 61 (1940).

(3) M. Bergmann and O. K. Behrens, J. Biol. Chem., 124, 7, (1938).

contained a glycine residue either adjacent to the acyl group or on the free carboxyl end. These acylated dipeptides were reacted with aniline in citrate buffer in activated papain- or activated ficin-catalyzed reactions. Most of the benzoyldipeptides investigated were of the DL-configuration. The only optically active acyldipeptide studied was benzoylglycyl-D-phenylalanine.

The results of the reactions investigated are summarized in Table XI. The melting points obtained with the latter five compounds were in such good agreement with the melting point of benzoylglycinanilide that mixed melting points were taken with a pure sample of benzoylglycinanilide whose identity has been established. The product was found to be benzoylglycinanilide in all of these instances. With the former three compounds, however, the unreported benzoyldipeptide anilides were obtained. The physical constants of these new anilides are given in Table XII.

Table XI

Reactivity of Benzoyl-DL-Dipeptides with Aniline in Papain- and Ficin-Catlayzed Reactions

Dipeptide	Wt. of benzoylated DL-dipeptides used, g.	Weight of Product Papain	Ficin	Melting* Point	Product
Leucylglycine	0.1455	0.0823	0.0908	197-199	Benzoylleucylglycinanilide
Valylglycine	0.1385	0.0771	0.0838	210-211	Benzoylvalylglycinanilide
Alanylglycine	0.1245	0.0153	0.0076	160-161⅓	Benzoylalanylglycinanilide
Glycylglycine	0.1175	0.0185	0.0109	210-212	Benzoylglycinanilide
Glycylleucine	0.1455	0.0323	0.0285	210-212	Benzoylglycinanilide
Glycylalanine	0.1245	0.0326	0.0148	210-212	Benzoylglycinanilide
Glycylvaline	0.1385	0.0176	0.0154	210-212	Benzoylglycinanilide
Glycylphenylalanine	0.1630	0.0224	0.0264	210-212	Benzoylglycinanilide

*Melting point of crude product.

Table XII

Physical Constants of New Anilides from Papain- and Ficin-Catalyzed Reactions of Benzoyldipeptides with Aniline

Anilide*	Melting point, C.° (uncorr.)	N, Calcd.	N, Found	$[\alpha]_{28}^{D}$ C** 0.5% Error = ± 1.0°
Benzoylalanyl-glycinanilide	161⅓-162⅔°	12.92 %	12.80 %	-
Benzoylleucyl-glycinanilide	200-201°	11.44 %	11.23 %	23.3 °
Benzoylvalyl-glycinanilide	210-211⅓°	11.90 %	11.69 %	28.9°

* Stereochemical identities of anilides are not known.

** Solvent was a 1:1 mixture of chloroform and 95% ethanol.

The types of reaction observed may be placed into two categories:

1. Coupling reaction

$$C_6H_5 \cdot CO\text{-}NH \cdot CHR' \cdot CO\text{-}NH \cdot CHR'' \cdot COOH + NH_2 \cdot C_6H_5 \longrightarrow$$
$$C_6H_5 \cdot CO \cdot NH \cdot CHR' \cdot CO\text{-}NH \cdot CHR'' \cdot CO\text{-}NH\text{-}C_6H_5$$

2. Transamidation reaction

$$C_6H_5 \cdot CO\text{-}NH \cdot CHR' \cdot CO\text{-}NH \cdot CHR'' \cdot COOH + NH_2 \cdot C_6H_5 \longrightarrow$$
$$C_6H_5 \cdot CO\text{-}NH \cdot CHR' \cdot CO\text{-}NH \cdot C_6H_5 \ (+ \ NH_2 \cdot CHR'' \cdot COOH)$$

In a typical coupling reaction, the benzoyldipeptide coupled with the aniline, in the presence of either ficin or papain, to yield the insoluble benzoyldipeptide anilide as the reaction product. This may be illustrated by the reaction of benzoylleucylglycine with aniline, benzoylleucylglycinanilide being the product obtained. A typical transamidation reaction may be exemplified by the reaction of benzoylglycylleucine with aniline, the product in this case being benzoylglycinanilide. Presumably, free leucine remains behind in solution. The reactions investigated, together with the per cent yields, are summarized in Table XIII.

Table XIII

Reactivity of Benzoyldipeptides[1] with Aniline as Catalyzed by Papain and Ficin at pH 5.2

Benzoyldipeptide	Product Obtained	% Yield[2] Papain	Ficin
Coupling Reactions			
Benzoylleucylglycine	Benzoylleucylglycinanilide	90	99
Benzoylalanylglycine	Benzoylalanylglycinanilide	19	9
Benzoylvalylglycine	Benzoylvalylglycinanilide	87	95
Transamidation Reactions			
Benzoylglycylglycine	Benzoylglycinanilide	30	17
Benzotlglycylleucine	Benzoylglycinanilide	51	45
Benzoylglycylalanine	Benzoylglycinanilide	51	23
Benzoylglycylvaline	Benzoylglycinanilide	28	24
Benzoylglycylphenylalanine	Benzoylglycinanilide	35	42
Benzoylglycine	Benzoylglycinanilide	49	14

[1]Benzoyldipeptides used are DL-isomers.
[2]Yields are based on quantity of aniline used.

Study of Table XIII reveals that in all instances where the glycine residue was on the free carboxyl end, except in the case of benzoylglycylglycine, a coupling reaction was observed. These results are compatible with those found in the literature for the papain-catalyzed reaction of acetyl-DL-phenylalanylglycine (1) and carbobenzoxy-L-phenylalanylglycine (2) with aniline, both of which showed coupling reactions. Agreement is also found with the case of benzoylglycylglycine, which showed a transamidation reaction, in the report (3) that the papain-catlayzed reaction of the acylated tripeptide, acetyl-L-phenylalanylglycylglycine, with aniline gave the acyldipeptide anilide, acetyl-L-phenylalanylglycinanilide, as the reaction product.

Further study of Table XIII reveals that in all instances investigated where the glycine residue was adjacent to the acyl substituents, a transamidation

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(1) M. Bergmann and O. K. Behrens, J. Biol. Chem., 124, 7 (1938).

(2) O. K. Behrens, D. G. Doherty and M. Bergmann, J. Biol. Chem. 136, 61 (1940).

(3) O. K. Behrens and M. Bergmann, J. Biol. Chem., 129, 587 (1939).

reaction was effected. The reaction product was benzoylglycinanilide in every case. In this respect, it is interesting to note that benzoyglycinanilide is also the reaction product of the enzyme-catalyzed condensation of benzoylglycine with aniline. The only report in the literature which mentions a similar type of transamidation reaction to give benzoylglycinanilide as product is the reaction of benzoylglycinamide with aniline in the presence of cysteine-activated papain (1).

In view of the stereochemical specificity requirements of proteases (1,2) in syntheses of the anilide type, it was of interest to ascertain whether such requirements held for the reactions of the transamidation type described above. The optically active benzoylated dipeptide, benzoylglycyl-D-phenylalanine, was therefore reacted with aniline in the presence of activated papain and activated ficin. Yields of benzoylglycinanilide corresponding to 23% and 31% were obtained for the papain- and ficin-catalyzed reactions, respectively.

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(1) O. K. Behrens and M. Bergmann, J. Biol. Chem., 129, 587 (1939).

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

Enzymic Reactions of Non-glycine Containing Benzoyldipeptides With Aniline

Reactions of several benzoyldipeptides containing no glycine were also investigated. Study of such benzoyldipeptides was more complicated than those previously described since non-glycine containing acyldipeptides contain two asymmetric centers and may therefore exist as four isomers or two racemic diastereomeric pairs. These are the L-D, D-L and the L-L, D-D racemates (1). The peptides were made by amination of the α-bromo intermediates and advantage could sometimes be taken of the differences in the solubility properties of the diastereomers to effect their separation. The problem remained, however, with regard to whether the diastereomeric pairs were of the D-L, L-D form, the L-L, D-D form, or mixtures. Such identification has previously been made via total synthesis of the peptide using optically active starting materials and unequivocal techniques (2) and via the different rates of halogen cleavage, by alkali, of the isomeric racemic pairs of the α-haloacylamino acids (3).

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(1) E. Fischer and A. H. Koelker, Ann., 354, 39 (1907).

(2) J. W. Hinman, E. L. Caron and H. N. Christensen, J. Am. Chem. Soc., 72, 1620 (1950).

(3) E. Abderhalden and E. Schwab, Fermentforschung, 10, 179 (1928).

Since solubility differences during synthesis resulted in the separation of the diastereomeric pairs of the benzoylvalylvalines and the benzoylleucylvalines, these benzoyldipeptides were further investigated in syntheses of the anilide type. The D-L, L-D form of benzoylvalylvaline had been prepared and described by Hinman and co-workers (1). The stereochemical configuration of the diastereomeric racemates of the benzoylvalylvalines could thereby be established. A comparable description appeared in the literature for the benzoylleucylvalines (2).

Reactions of the two diastereomeric forms of benzoylvalylvaline with aniline, as catalyzed by papain and ficin, were first studied. A product was obtained only with the L-L, D-D form, the D-L, L-D form showing no reaction, as follows:

1. Benzoyl | D-valyl-L-valine / L-valyl-D-valine | + aniline ⟶ no reaction

2. Benzoyl | L-valyl-L-valine / D-valyl-D-valine | + aniline ⇄ Benzoyl-L-valyl-L-valinanilide.

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(1) J. W. Hinman, E. L. Caron and H. N. Christensen, J. Am. Chem. Soc., 72, 1620 (1950).

(2) E. Abderhalden and E. Schwab, Fermentforschung, 10, 179 (1928).

Positive identification of the stereochemical configuration of the product was made in collaborative studies with Mr. Kenneth F. Itschner. Hydrolysis of the product with 6 N hydrochloric acid at 100° was allowed to proceed for twenty-four hours. Microbiological assay of the acid hydrolyzate showed the presence of two-L-valine residues, within the limits of experimental error. This established the identity of the product as benzoyl-L-valyl-L-valinanilide, which was obtained in 20 per cent yield.

Similar studies were carried out with the benzoylleucylvalines. The results obtained are shown in the following:

1. Benzoyl [D-leucyl-L-valine / L-leucyl-D-valine] + aniline ⟶ no reaction

2. Benzoyl [L-leucyl-L-valine / D-leucyl-D-valine] + aniline ⟶ Benzoyl-L-leucyl-L-valinanilide.

This time, acid hydrolysis of the product followed by microbiological assay showed the presence of one L-leucine and one L-valine residue. The stereochemical configuration of the reaction product was therefore again the L-L form, i.e., benzoyl-L-leucyl-L-valinanilide (10 per cent yield). Determination of the stereochemical configuration of the reaction product also established the

stereochemical identity of the benzoyldipeptide reactant as the D-D, L-L form. This, in turn, identified the benzoyldipeptide where no reaction was observed as the D-L, L-D form.

The results obtained above were not unexpected in view of the stereochemical specificity properties of the enzymes used. Also, they suggested a new and less tedious method for the stereochemical identification of dipeptide diastereomers. A proposed method, together with its theoretical aspects, will be elaborated more fully in the Discussion and Conclusions section.

The physical constants of the new anilides are given in Table XIV.

Enzymic Reactions of Benzoylamino Acids With Glycinanilide

In 1938, Bergmann and co-workers (1) succeeded in the synthesis of a peptide bond between two amino acid residues when the papain-catalyzed reaction of benzoylleucine with leucinanilide yielded the benzoyldipeptide anilide, benzoylleucylleucinanilide. Later reports in the literature demonstrated that the

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(1) M. Bergmann and H. Fraenkel-Conrat, J. Biol. Chem., 124, 1 (1938).

Table XIV

Physical Constants of New Anilides from Papain- and Ficin-Catalyzed Reactions of Benzoyldipeptides with Aniline

Anilide*	Melting point, C.°**	N, Calcd.	N, Found
Bz-L-valyl-L-valinanilide	286-287	10.63 %	10.48 %
Bz-L-leucyl-L-valinanilide	238½-239½	10.27 %	10.11 %

* Stereochemical configuration identified via microbiolgoical assay after acid hydrolysis.

** All melting points are uncorrected.

protease, chymotrypsin (1), as well as papain (2), is capable of inducing the formation of a peptide bond between two amino acid residues. Since recent studies (3) have indicated that ficin was capable of catalyzing reactions of the anilide type with benzoylamino acids and aniline, the problem as to whether it was also capable of catalyzing the synthesis of a peptide bond between two amino acid residues invited investigation. Therefore, both the papain-cysteine and ficin-cysteine catalyzed syntheses of peptide bonds with a variety of benzoylamino acids and glycinanilide were studied.

A series of benzoylamino acids were reacted with glycinanilide in citrate buffer in papain-cysteine or ficin-cysteine catalyzed reactions. All of the benzoylamino acids investigated were of the DL-configuration with the exception of the tyrosine (monobenzoylated) and glutamic acid derivatives, which were of the L-configuration. The types of reactions observed fell

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(1) M. Bergmann and J. Fruton, J. Biol. Chem., 124, 1 (1938).

(2) O. K. Behrens and M. Bergmann, J. Biol. Chem., 129, 587 (1939).

(3) S. W. Fox and J. S. Halverson, Unpublished experiments.

into two categories;

1. Coupling reactions

$$C_6H_5 \cdot CO \cdot NH \cdot CHR \cdot COOH + NH_2 \cdot CH_2 \cdot CO \cdot NH \cdot C_6H_5 \xrightarrow{\text{enzyme}}$$

$$C_6H_5 \cdot CO\text{-}NH \cdot CHR \cdot CO\text{-}NH \cdot CH_2 \cdot CO\text{-}NH \cdot C_6H_5$$

e.g. Benzoylglycine + glycinanilide ⟶

Benzoylglycylglycinanilide

2. Transamidation reactions

$$C_6H_5 \cdot CO \cdot NH \cdot CHR \cdot COOH + NH_2 \cdot CH_2 \cdot CO\text{-}NH \cdot C_6H_5 \longrightarrow$$

$$C_6H_5 \cdot CO\text{-}NH \cdot CHR \cdot CO \cdot NH \cdot C_6H_5$$

$$(+ \quad NH_2 \cdot CH_2 \cdot COOH)$$

e.g. Benzoylalanine + glycinanilide ⟶

Benzoylalaninanilide (+ glycine)

A typical coupling reaction is shown where the benzoylamino acid, benzoylglycine, when reacted with the amino acid anilide, glycinanilide, in the presence of the activated enzyme, yields as product the insoluble benzoyldipeptide anilide, benzoylglycylglycinanilide. On the other hand, when benzoylglycine is replaced by benzoylalanine, all other conditions remaining the same, a transamidation reaction takes place in which the benzoylamino acid anilide, benzoylalaninanilide, rather than the benzoyldipeptide anilide is formed. Presumably, free glycine remains

behind in solution.

Eighteen different benzoylamino acids were reacted with glycinanilide in reactions catalyzed by cysteine-papain or cysteine-ficin. These results are summarized in Tables XV and XVI. With the exception of the product obtained from the reaction which involved benzoylglycine, all products possessed melting points which were in good agreement with the melting points of the corresponding benzoyl-L-amino acid anilides. Mixed melting points, when taken with pure samples of known anilides, showed positive identification of these products as the benzoyl-L-amino acid anilides. With benzoylglycine, however, the product was the benzoyldipeptide anilide, benzoylglycylglycinanilide.

From examination of Table XVII it is apparent that at pH 5.3, only one instance of a coupling reaction was noted, that instance being when benzoylglycine was reacted with glycinanilide. In all other cases where a benzoylamino acid was reacted with glycinanilide, and reaction occurred, it was a transamidation reaction. Thus, reaction of benzoylmethionine with glycinanilide gave benzoylmethioninanilide, reaction of benzoylleucine with glycinanilide gave benzoylleucinanilide, and so on.

Since some of the benzoylamino acids described in Tables XV and XVII gave only slight or no yields of anilide at pH 5.3, variation of pH was attempted with

Table XV

Reactivity of Benzoylamino Acids with Glycinanilide at pH 5.3 in Papain- and Ficin-Catalyzed Reactions

Amino Acid	Wt. of benzoylated DL-amino acid used, g.	Weight of Product Papain	Ficin	Melting* Point C.°	Product
Glycine	0.0895	0.0981	0.0358	246-248	Benzoylglycylglycinanilide
Alanine	0.193	0.0342	0.0268	174-176	Benzoylalaninanilide
Valine	0.221	0.0015	trace	218-220	Benzoylvalinanilide
Leucine	0.235	0.0456	0.0317	212-213	Benzoylleucinanilide
Methionine	0.253	0.0456	0.0329	160-162	Benzoylmethioninanilide
Isoleucine	0.235	0.0014	None	213-215	Benzoylisoleucinanilide
Phenylalanine	0.269	None	None	-	-
Glutamic Acid**	0.126	None	None	-	-
Tryptophan	0.309	None	None	-	-

* Melting point of crude product
** Benzoyl-L-glutamic acid was used.

Table XVI

Reactivity of Benzoylamino Acids with Glycinanilide at Various pH's in Papain-Catalyzed Reactions

Amino Acid	Wt. of benzoylated DL-amino acids used, g.	pH	Weight of Product	% Yield	Melting Point, C.°	Product
Leucine	0.235	5.0	0.0046	3	210-212	Benzoylleucinanilide
		5.5	0.0530	34		
		5.9	0.0685	45		
Isoleucine	0.235	5.0	None	-	-	-
		5.5	0.0024	1	208½-211	Benzoylisoleucin-anilide
		5.9	0.0022	1		
Tryptophan	0.309	5.0	None	-	-	-
		5.5	0.0038	2	196-196½	Benzoyltryptophan-anilide
		5.9	0.0061	3		
		6.6	0.0525	28		
Valine	0.221	5.0	None	-		
		5.5	0.0010	1	212-214	Benzoylvalinanilide
		5.9	0.0072	5		
Glutamic Acid**	0.126	3.6	None	-	-	-
		4.1	None	-	-	-
		5.0	None	-	-	-
		5.3	None	-	-	-

Table XVI (Cont'd.)

Reactivity of Benzoylamino Acids with Glycinanilide at Various pH's in Papain-Catlayzed Reactions

Amino Acid	Wt. of benzoylated DL-amino acids used, g.	pH	Weight of Product	% Yield	Melting Point, C.°	Product
Phenylalanine	0.269	5.0	None	-	-	-
Methionine	0.253	5.0	0.0127	8	159½-162½	Benzoylmethionin-anilide
Norvaline	0.221	5.0	0.0068	5	174-176	Benzoylnorvalin-anilide
Norleucine	0.235	5.0	0.0068	4	176-178	Benzoylnorleucin-anilide
α-Amino-n-butyric acid	0.207	5.0	0.0348	21	163-165	Benzoyl- -amino-n-butyric acid anilide
Aminoiso-butyric acid	0.207	5.0	None	-	-	-
Tyrosine (N-Bz)**		5.0-7.5	None	-	-	-
Glycine	0.0895	5.0	0.0690	44	247-248	Benzoylglycylglycin-anilide

* Melting point of crude product
** Benzoyl-L-amino acid was used.

Table XVII

Reactivity of Benzoyl Derivatives of Amino Acids With Glycinanilide as Catalyzed by Papain of Ficin at pH 5.3

Benzoyl Derivative of DL Acid	Benzoyl-Anilide Formed	Per Cent Yield Papain	Ficin
Glycine	Bz-glycylglycinanilide	63	23
Alanine	Bz-alaninanilide	26	20
Valine	Bz-valinanilide	1	trace
Leucine	Bz-leucinanilide	29	21
Methionine	Bz-methioninanilide	28	20
Isoleucine	Bz-isoleucinanilide	1	None
Phenylalanine	-	None	None
Glutamic Acid	-	None	None
Tryptophan	-	None	None

several benzoylamino acids in an effort to find conditions more conducive to anilide formation. These results, summarized in Table XVI, further indicate that benzoylglycine was the only benzoylamino acid to give a coupling type reaction with glycinanilide. The results suggest, too, that the pH optima for all reactions studied is, in most if not in all cases, above pH 5.0.

It should be noted (Table XVI) that no reaction was observable between N-benzoyltyrosine and glycinanilide over the pH range of 5.0-7.5. This is of interest in the light of recent experiments (1) which demonstrated that, at pH 6.0, high yields of N-benzoyltyrosinanilide were obtained from the cysteine-papain catalyzed reaction of N-benzoyltyrosine with aniline. Observations of an analogous nature with crystalline chymotrypsin (2), previously noted in the literature, were offered as evidence for enzyme specificity. This will be discussed later, in fuller detail, under the appropriate sections.

For reference purposes, workable quantities of anilides were obtained from larger papain-catalyzed preparations and purified to constant melting point by recrystallization

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

(2) M. Bergmann and J. Fruton, J. Biol. Chem., 124, 321 (1938).

from aqueous ethanol. The melting points for the pure benzoyl-L-amino acid anilides obtained, together with the literature values and literature references, are given in Tables IV and XVIII.

Table XVIII

Melting Points of Anilides from Papain- and Ficin-Catalyzed Reactions of Benzoylamino Acids with Glycinanilide

Anilide	Melting point, C.°	Lit. value and ref.
Benzoylglycylglycinanilide	246½-247½	238-240 (1)
Benzoyltryptophananilide	199-200	199-200 (2)
Benzoylmethioninanilide	162-162½	159 (3)

(1) T. Curtius and R. Wustenfeld, J. prakt. Chem., 70, 80 (1904).
(2) S. W. Fox and J.S. Halverson, Unpublished results.
(3) C. A. Dekker and J. S. Fruton, J. Biol. Chem., 173, 471 (1948).

Enzymic Reaction of Benzoylamino Acids With Acid Anilides

Previously published results (1) have shown that the nature of the amino acid anilide employed in the anilide syntheses was a principal determinant in the type of products obtained. Statement was made to the effect that the different behavior of the amino acid anilides employed "illustrates the highly developed specificity of enzymatic peptide syntheses". Studies of this nature appeared to afford a means of further clarifying the concept of "preferences" in protease-substrate interaction. Such studies have therefore been here extended to include two amino acid anilides, valinanilide and alaninanilide, which have heretofore been unreported, in addition to the previously reported leucinanilide and glycinanilide (1).

In these investigations, the benzoyl derivatives of glycine and alanine were studied most extensively, with the derivatives of leucine, valine, aminoisobutyric acid, norvaline and ∝-amino-n-butyric acid studied to a somewhat lesser extent. Reaction of these acylamino acids with amino acid anilides, as catalyzed by papain-cysteine, gave both coupling and transamidation reactions.

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(1) M. Bergmann and H. Fraenkel-Conrat, J. Biol. Chem., 124 1, (1938).

The results of the reactions investigated are summarized in Table XIX. Where transamidation reactions occurred, the benzoylamino acid anilide which was obtained as the product was, in every instance, a previously described compound. The identity of the products was, in these instances, established by means of mixed melting points with pure samples. Where coupling reactions occurred, however, the benzoyldipeptide anilides which were obtained were in all instances undescribed. These products were recrystallized from ethanol-water to constant melting point. The physical constants of these new anilides are given in Table XX.

In Table XXI, the coupling and transamidation reactions are listed, as well as the per cent of anilide yield in each case. It can be seen that in all reactions which involved benzoylglycine, a coupling reaction was observed. That the type of reaction, coupling or transamidation, can not be attributed to the acylamino acid alone is shown by the fact that benzoylalanine undergoes both types of reactions, depending upon the amino acid anilide used. A similar argument can be used to show that the type of reaction effected can not be entirely attributed to the specific amino acid anilide used in the reaction. These factors will be discussed in fuller detail in the Discussion and Conclusion section.

Table XIX

Reactivity of Benzoyl Derivatives of Amino Acids With Amino Acid Anilides as Catalyzed by Papain at pH 5.6

Amino Acid	Wt. of benzoylated DL-amino acids, used, g.	Amino Acid Anilide Citrate	Wt. amino acid anilide citrate used, g.	Wt. of Product	Melting Point, C°	Product
Glycine	0.0895	Alanin-anilide	0.374	0.112	235½-236½	Bz-glycylalanin-anilide
Glycine	0.0895	Valin-anilide	0.402	0.0976	251-252	Bz-glycylvalin-anilide
∝-Amino-n-butyric acid	0.207	Valin-anilide	0.402	0.0757	260-262	Bz-∝-amino-n-butyric acid anilide
Norvaline	0.221	Valin-anilide	0.402	0.0876	241-243	Bz-norvalylvalin-anilide
Glycine	0.0895	Leucin-anilide	0.416	0.0930	197-199	Bz-glycylleucin-anilide
Alanine	0.193	Leucin-anilide	0.416	0.0815	237-239	Bz-alanylleucin-anilide
Alanine	0.193	Valin-anilide	0.402	0.0920	263-265	Bz-alanylvalin-anilide
Leucine	0.235	Alanin-anilide	0.374	0.0883	213-214	Bz-leucinanilide

Table XIX

Reactivity of Benzoyl Derivatives of Amino Acids With Amino Acid Anilides as Catalyzed by Papain at pH 5.6

Amino Acid	Wt. of benzoylated DL-amino acids, used, g.	Amino Acid Anilide Citrate	Wt. amino acid anilide citrate used, g.	Wt. of Product	Melting Point, C°	Product
Valine	0.221	Alanin-anilide	0.374	0.0065	206-209	Bz-valinanilide
α-Amino-n-butyric acid	0.207	Alanin-anilide	0.374	0.1031	167-169	Bz-α-amino-n-butyric acid anilide
Alanine	0.193	Alanin-anilide	0.374	0.0628	174-176	Bz-alaninanilide
Norvaline	0.221	Alanin-anilide	0.374	0.1224	178-179	Bz-norvalinanilide
Aminoiso-butyric acid	0.207	Alanin-anilide	0.374	None	-	-
Isoleucine	0.235	Alanin-anilide	0.374	None	-	-
Aminoiso-butyric acid	0.207	Valin-anilide	0.402	None	-	-
Isoleucine	0.235	Valin-anilide	0.402	None	-	-
Valine	0.221	Valin-anilide	0.402	None	-	-

Table XX

Physical Constants of New Anilides from Papain-Catlayzed Reactions of Benzoylamino Acids with Amino Acid Anilides

Anilide	Melting* Point, C.	N, Calcd.	N, Found	$[\alpha]_{28}^{D}$ C=0.5%** error ±1.0°
Bz-glycylalaninanilide	237-238	12.92 %	12.94 %	-23.1°
Bz-glycylvalinanilide	255½-256	11.90 %	11.82 %	-38.1°
Bz-alanylvalinanilide	271½-272	11.44 %	11.19 %	-54.5°
Bz-α-amino-n-butyryl-valinanilide	264-264½	11.02 %	10.89 %	-58.2°
Bz-norvalylvalinanilide	249½-251	10.63 %	10.46 %	-59.5°
Bz-glycylleucinanilide	204-204½	11.44 %	11.27 %	- 8.1°
Bz-alanylleucinanilide	243-243½	11.02 %	10.88 %	

* Melting points are uncorrected.

** Solvent was a 1:1 mixture of chloroform and 95% ethanol.

Table XXI

Reactivity of Benzoyl Derivatives of Amino Acids With Amino Acid Anilides as Catlayzed by Papain at pH 5.6

Benzoyl Derivative of	Amino Acid Anilide	Product	% Yield
Coupling			
Glycine	Alaninanilide	Bz-glycylalaninanilide	69
Glycine	Valinanilide	Bz-glycylvalinanilide	56
Alanine	Valinanilide	Bz-alanylvalinanilide	50
α-Amino-n-butyric acid	Valinanilide	Bz-α-amino-n-butyryl-valinanilide	40
Norvaline	Valinanilide	Bz-norvalylvalinanilide	44
Glycine	Leucinanilide	Bz-glycylleucinanilide	51
Alanine	Leucinanilide	Bz-alanylleucinanilide	43
Transamidation			
Leucine	Alaninanilide	Bz-leucinanilide	57
Alanine	Alaninanilide	Bz-alaninanilide	46
Valine	Alaninanilide	Bz-valinanilide	4
α-Amino-n-butyric acid	Alaninanilide	Bz-α-amino-n-butyric acid anilide	73
Norvaline	Alaninanilide	Bz-norvalinanilide	83
No Reaction			
Aminoisobutyric acid	Alaninanilide	--	None
Isoleucine	Alaninanilide	--	None
Aminoisobutyric acid	Valinanilide	--	None
Isoleucine	Valinanilide	--	None
Valine	Valinanilide	--	None

Enzyme Specificity

In the enzymic investigations previously described, the two closely related proteases, ficin and papain, were used. With regard to the type of reaction effected, these enzymes gave the same qualitative results in all instances studied. In the hope that two different enzymes might be found that would, under the same set of conditions, give different reaction products, attention was directed to the study of another protease which possessed widely different specificity properties than either ficin or papain. The proteolytic enzyme, chymotrypsin, was chosen.

Bergmann and Fruton (1) were the first to report the use of chymotrypsin in syntheses of the anilide type. These investigators found that at the pH optimum for chymotrypsin, benzoyltyrosine will react with glycinanilide in a coupling type reaction to yield the benzoyldipeptide anilide, benzoyltyrosylglycinanilide. In the presence of papain, however, no reaction was observed.

1. Benzoyltyrosine + glycinanilide $\xrightarrow{\text{chymotrypsin}}$ Benzoyltyrosylglycinanilide

2. Benzoyltyrosine + glycinanilide $\xrightarrow{\text{papain}}$ no reaction

This set of reactions was presented by the authors as

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(1) M. Bergmann and J. S. Fruton, J. Biol. Chem., 124, 321 (1938).

evidence for enzyme specificity. These reactions were repeated in this work over the pH range of 7.3 to 7.9 for the former and 5.0 to 7.5 for the latter reaction. Table XXII shows that the same qualitative results were obtained as those previously reported (1).

In view of the report (1) that papain could induce the synthesis of benzoyltyrosinanilide from benzoyltyrosine and aniline, attempts were made to effect a similar synthesis in a chymotrypsin-catalyzed reaction over the pH range of 6.1 to 7.5. The reactions are illustrated in the following;

1. Benzoyltyrosine + aniline $\xrightarrow{\text{chymotrypsin}}$ No reaction

2. Benzoyltyrosine + aniline $\xrightarrow{\text{papain}}$ Benzoyltyrosinanilide (1)

In this case again, only one enzyme showed reactivity while the other failed to catlayze any reaction. However, in this instance, it was the papain-catalyzed reaction that gave a product whereas no reaction was obtained where chymotrypsin was used.

A demonstration of specificity would be more meaningful if, under the same set of conditions, two different enzymes would catalyze reactions that gave products, but different products. In an effort to establish such a

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

Table XXII

Effect of pH on Yeilds of Anilide from the Chymotrypsin- and Papain-Catalyzed Reaction of Benzoyl-L-tyrosine* with Glycinanilide **

Enzyme Used	Product	pH	Yield of Product, g.	% Yield	Melting Point, C.°
Papain	-	5.0	None	-	-
		5.5	None	-	-
		6.1	None	-	-
		6.7	None	-	-
		7.0	None	-	-
		7.5	None	-	-
Chymotrypsin	Bz-L-tyrosyl-glycin-anilide	7.4	0.0326	15.6	224-225
		7.7	0.0381	18.3	224-225
		7.9	0.0465	22.3	224-225

* The amount of benzoyl-L-tyrosine used was 0.1425 g.

** The amount of glycinanilide used was 0.093 g.

case of specificity, several benzoylamino acids, as well as benzoyl-DL-phenylalanine, were reacted with both aniline and glycinanilide in papain-, ficin- and chymotrypsin-catalyzed reactions. The results are summarized in Table XXIII.

Examination of Table XXIII reveals that in no instance was an insoluble product obtained in the chymotrypsin-catalyzed reactions which involved the benzoyl derivatives of tryptophan, methionine and glycylphenylalanine. The comparable papain- and ficin-catalyzed reactions, however, gave products with melting points which were in good agreement with those of known anilides. After the product had been recrystallized from ethanol-water, mixed melting points with pure samples established the identity of these products.

It is significant that at least one benzoyl derivative reacted to give an insoluble product, under the same conditions, in the chymotrypsin-, papain- and ficin-catalyzed reactions. The chymotrypsin-catalyzed reaction may be exemplified in the following:

Bz-phenylalanine + glycinanilide $\xrightarrow{\text{chymotrypsin}}$ Bz-phenylalanylglycinanilide

The anilide obtained was recrystallized from ethanol-water to constant melting point. The analytical data obtained

Table XXIII

Reactivity of Benzoyl Derivatives with Aniline and Glycinanilide in Papain-, Ficin- and Chymotrypsin-Catalyzed Reactions

Benzoyl-derivative of	Wt. of benzoyl derivative used, g.	Amine Used	Wt. of amine used, g.	pH	Enzyme used	Wt. of Product g.	Melting Point, C.°	Main Product
Phenylalanine*	0.269	Glycinanilide	0.090	7.1	Chymotrypsin	0.0036	235-238 1/2	Bz-phenyl glycine
Tryptophan*	0.309	Glycinanilide	0.090	7.1	Chymotrypsin	None		—
Methionine*	0.235	Glycinanilide	0.090	7.1	Chymotrypsin	None		—
Phenylalanine*	0.269	Glycinanilide	0.090	7.1	Papain	0.0030	213-216	Unidenti
Tryptophan*	0.309	Glycinanilide	0.090	7.1	Papain	0.0561	192-195	Bz-tryptop
Methionine*	0.253	Glycinanilide	0.090	7.1	Papain	0.0880	156-158	Bz-methi
Glycylphenylalanine*	0.163	Aniline	0.098	6.7	Papain	0.305	210-212	Bz-glycin
Glycylphenylalanine**	0.163	Aniline	0.098	6.7	Ficin	0.0163	209-211	Bz-glycin

Table XXIII

Reactivity of Benzoyl Derivatives with Aniline and Glycinanilide in Papain-, Ficin- and Chymotrypsin-Catalyzed Reactions

Wt. of benzoyl derivative used, g.	Amine Used	Wt. of amine used, g.	pH	Enzyme used	Wt. of Product g.	Melting Point, C.°	Main Product
0.269	Glycinanilide	0.090	7.1	Chymotrypsin	0.0036	235-2381/2	Bz-phenylalanyl-glycinanilide
0.309	Glycinanilide	0.090	7.1	Chymotrypsin	None		--
0.235	Glycinanilide	0.090	7.1	Chymotrypsin	None		--
0.269	Glycinanilide	0.090	7.1	Papain	0.0030	213-216	Unidentified
0.309	Glycinanilide	0.090	7.1	Papain	0.0561	192-195	Bz-tryptophanilide
0.253	Glycinanilide	0.090	7.1	Papain	0.0880	156-158	Bz-methionanilide
anine* 0.163	Aniline	0.098	6.7	Papain	0.305	210-212	Bz-glycinanilide
0.163	Aniline	0.098	6.7	Ficin	0.0163	209-211	Bz-glycinanilide

Table XXIII

Reactivity of Benzoyl Derivatives with Aniline and Glycinanilide in Papain-, Ficin- and Chymotrypsin-Catalyzed Reactions

Benzoyl derivative of	Wt. of benzoyl derivative used, g.	Amine Used	Wt. of amine used, g.	pH	Enzyme Used	Wt. of Product g.	Melting Point, C.°	Main Produc
Glycylphenal-alamine**	0.163	Aniline	0.098	6.7	Chymotrypsin	None	-	-
Phenylal-anine**	0.269	Glycin-anilide	0.186	6.8	Papain	0.0104	213-216	Uniden
Phenyl-alanine**	0.269	Glycin-anilide	0.186	6.8	Ficin	0.0131	196-201	Uniden
Phenyl-alanine**	0.269	Glycin-anilide	0.186	6.8	Chymotrypsin	0.1108	239-241	Bz-phe glyci
Glycylphenyl-alanine**	0.1630	Glycin-anilide	0.186	6.6	Papain	0.0100	246-248	Bz-gly anili
Glycylphenyl-alanine**	0.1630	Glycin-anilide	0.186	6.6	Ficin	0.0255	246-248	Bz-gly anili
Glycylphenylalanine**	0.1630	Glycin-anilide	0.186	6.6	Chymotrypsin	None		-

* Buffer was 1M citrate.
** Buffer was 0.5M citrate-0.5M phosphate.

Table XXIII

Reactivity of Benzoyl Derivatives with Aniline and Glycinanilide in Papain-, Ficin- and Chymotrypsin-Catalyzed Reactions

Wt. of benzoyl derivative used, g.	Amine Used	Wt. of amine used, g.	pH	Enzyme Used	Wt. of Product g.	Melting Point, C.°	Main Product
0.163	Aniline	0.098	6.7	Chymotrypsin	None	-	-
0.269	Glycinanilide	0.186	6.8	Papain	0.0104	213-216	Unidentified
0.269	Glycinanilide	0.186	6.8	Ficin	0.0131	196-201	Unidentified
0.269	Glycinanilide	0.186	6.8	Chymotrypsin	0.1108	239-241	Bz-phenylalanylglycinanilide
0.1630	Glycinanilide	0.186	6.6	Papain	0.0100	246-248	Bz-glycylglycinanilide
0.1630	Glycinanilide	0.186	6.6	Ficin	0.0255	246-248	Bz-glycylglycinanilide
anine** 0.1630	Glycinanilide	0.186	6.6	Chymotrypsin	None		-

* Buffer was 1M citrate.
** Buffer was 0.5M citrate-0.5M phosphate.

by micro Kjeldahl determination was in agreement with that for benzoylphenylalanylglycinanilide.

The reactions observed with the ficin- and papain-catalyzed reactions appear more complex than that noted with chymotrypsin. In the former instances, the product obtained melted below the melting points of the expected products, benzoylphenylalaninanilide (219-220°) or benzoylphenylalanylglycinanilide (244-245°). Although the melting point of the product was raised considerably after several recrystallizations from ethanol-water no substance which gave a constant melting point was obtained. A possible explanation of these results may lie in the assumption of mixed products, i.e., benzoylphenylalanin-anilide and benzoylphenylalanylglycinanilide. These mixed products could be due to the simultaneous occurrence of transamidation and coupling reactions when papain and ficin serve as the catalysts.

The physical constants of the pure anilides obtained in these specificity studies, after recrystallization from 50 per cent ethanol-water to constant melting points, are presented in Tables XVIII and XXIV.

Table XXIV

Physical Constants of Anilides Obtained in the Enzyme Specificity Studies

Anilide	Melting Point, C°	Lit. value and reference	N, Found	N, Calcd.
Bz-L-tyrosyl-glycinanilide	225½-226	226 (1)		
Bz-phenylalanyl-glcyinanilide	244-245 (uncorr.)	-	10.30 %	10.47 %

(1) M. Bergmann and J. S. Fruton, J. Biol. Chem., 124, 321 (1938).

DISCUSSION AND CONCLUSIONS

Enzymic Reactions of Benzoylamino Acids With Aniline

The results depicted in Table VI indicate that in syntheses of the anilide type, the ranking of substrate preferences with papain and ficin is not the same in all instances. In particular, marked differences in the order of substrate preferences appear for the alanine, α-aminocaprylic acid and α-aminoheptylic acid residues. Such differences would tend to place greater emphasis on protease-substrate interaction rather than on the effect of substrate structure alone. The contribution of residue structure in determining the degree of enzyme preference becomes clear, however, when the branched-chain isomers are compared with their normal analogs, as is shown in Table XXV.

In Table XXV, the yield and consequently the enzyme preference in the normal or straight chain series is almost consistently greater than that in the branched-chain series. This suggests that steric hindrance is the underlying factor for the previously observed differences in reactivity between benzoylvaline and benzoylleucine (1). Thus, a substantial yield of

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

Table XXV

Yields of Benzoylamino Acid Anilides as a Function of Location of Substituent Methyl Group

Straight Chain Acid	% Yield	Branched-Chain Acid	% Yield
$CH_3 \cdot CH(NHBz) \cdot COOH$ Bz-alanine	38	$CH_3 \cdot C(NHBz) \cdot COOH$ CH_3 Bz-aminoisobutyric acid	0
$CH_3 \cdot CH_2 \cdot CH(NHBz) \cdot COOH$ Bz-α-amino-n-butyric acid	69	$CH_3 \cdot CH \cdot CH(NHBz) \cdot COOH$ CH_3 Bz-valine	7
$CH_3 \cdot CH_2 \cdot CH_2 \cdot CH(NHBz) \cdot COOH$ Bz-norvaline	46	$CH_3 \cdot CH \cdot CH \cdot CH(NHBz) \cdot COOH$ CH_3 Bz-isoleucine	4
$CH_3 \cdot CH_2 \cdot CH_2 \cdot CH(NHBz) \cdot COOH$ Bz-norvaline	45	$CH_3 \cdot CH \cdot CH_2 \cdot CH(NHBz) \cdot COOH$ CH_3 Bz-leucine	53

benzoylalaninanilide is obtained under the same conditions in which the corresponding α-methyl derivative fails to form. That a methyl substituent in the β-position interferes, at least partially, with the enzyme-substrate interaction is evidenced by the lower yields of anilide with benzoylvaline than with its normal analog, benzoyl-α-amino-n-butyric acid. A similar situation exists between benzoylisoleucine and benzoylnorvaline. When the methyl group occupies the γ-position, as with leucine, it is, according to this picture, too remote from the α-carbon atom to cause interference.

In the comparison between norvaline and its γ-methyl homolog (leucine), a greater extent of reaction is found for leucine. A tentative explanation for this difference is that when the methyl is sufficiently removed from the site of interaction, it is not inhibitory, but contributes instead to the attraction between R-group and the enzyme (1).

As a first approximation, it may be stated that the lesser reactivity generally shown in the branched-chain series is due to the greater degree of steric hindrance resulting from the presence of a methyl side

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(1) E. Smith, Fed'n. Proc., 8, 581 (1949).

chain closely adjacent to the α-carbon atom. It follows from this first approximation that a greater degree of hindrance should result by replacing the methyl substituent with one of increased size. The results obtained with all available benzoylamino acids with β-substituents are presented in Table XXVI.

Table XXVI.

Comparison of Yields from Benzoylamino Acids of Varying Size of β-Substituents

Amino Acid Residue	% Yield
$CH_3 \cdot CH(H) \cdot CH(NH_2) \cdot COOH$ -amino-n-butyric acid	69
$CH_3 \cdot CH(CH_3) \cdot CH(NH_2) \cdot COOH$ valine	7
$CH_3 \cdot CH(CH_2CH_3) \cdot CH(NH_2) \cdot COOH$ isoleucine	4
$CH_3 \cdot C(CH_3)(S-CH_2-C_6H_5) \cdot CH(NH_2) \cdot COOH$ S-benzylpenicillamine	0

The results shown in Table XXVI substantiate the hypothesis. Structural considerations reveal that although the hydrogen on the β-carbon of α-amino-n-butyric acid does not inhibit, the methyl (valine) or the ethyl group (isoleucine) causes the reaction to proceed to a lesser extent. It should be here pointed out that isoleucine might logically be considered a β-methylvaleric acid as well as a β-ethylbutyric acid. As the former, its yield might be no more limited than that of the valine analog. However, one could assume that benzoylisoleucine would occur in both configurations, so that actually a smaller yield of the product than of the valine analog is reasonable. In any event, the reaction is highly hindered.

Inspection of the S-benzylpenicillamine substrate reveals that the hindrance here is great enough to account for its complete lack of activity. Since a sulfur atom appears in this substrate, the results might be questioned on the basis of possible inhibition due to this sulfur atom. This would seem to be ruled out, however, by the fact that benzoylmethionine has been found to react to an appreciable extent in work in this laboratory.

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(1) D. G. Doherty and E. A. Popenoe, Jr., J. Biol. Chem., 73, 452 (1951).

Similar activity has been reported elsewhere (1). With this exception, all of the correlations reported herein involve amino acids with hydrocarbon side-chains.

From the observations made above, the following generalization can be formulated, namely; that included among the factors which govern enzyme preference for acylamino acids are the size of the residue side chain and the distance of this side chain from the α-carbon atom. The smaller the side chain and the further it is removed, up to a limiting distance, from the α-carbon atom, the greater is the reactivity.

The relative order of reactivity of the branched acids tested are qualitatively the same with the closely related ficin as with papain. It is here worthy of repetition that when the dominant effect of the branch is eliminated, the relationships among substrates are qualitatively different for the two enzymes, as Table VI shows. The effects of the branch are qualitatively similar to those observed by Cason and Wolfhagen (2)

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(1) D. G. Doherty and E. A. Popenoe, Jr., J. Biol. Chem., 73, 452 (1941).

(2) J. Cason and H. J. Wolfhagen, J. Org. Chem., 14, 155 (1949).

with sodium hydroxide solution as the hydrolytic agent for fatty acid esters. Pancreatic cholesterol esterase has recently been shown (1) to give results of a similar nature.

Albertson (2) indicated the correlation of structure and yield with papain for the normal acids, through benzoylnorleucine. The present work (Table V) furnishes data which extend the series through benzoylaminopelargonic acid, and which point out peak reactivity for benzoylaminoheptylic acid.

Studies by the Bergmann group (3), on the action of dipeptidase on α-aminoisobutyric acid dipeptides, revealed a very low reactivity for such peptides. In that instance, the relative inactivity was ascribed to the absence of a hydrogen atom in the α-position. According to the steric hindrance picture developed here, the presence of the α-methyl rather than the absence of the α-hydrogen is responsible. The subtle but significant distinction is that lack of activity is correlative not

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(1) L. Sewell, J. W. Cassidy and C. R. Treadwell, Fed'n. Proc., 10, 256 (1951).

(2) N. F. Albertson, J. Am. Chem. Soc., 73, 452 (1951).

(3) M. Bergmann, C. Zervas, J. S. Fruton, F. Schneider and H. Schleich, J. Biol. Chem., 109, 325 (1935).

to inability to meet rather specific structural requirements, but rather to the presence of an interfering group within the substrate.

Invocation of the principle of steric hindrance in enzyme-substrate interation is not new; its application, which has been frequent (1-9), has however been largely limited to the effects of D-residues acting on enzymes equipped to catalyze reactions with L-forms. The present experiments emphasize steric hindrances operating in L-substrates subjected to the action of enzymes active upon L-substrates, a relationship which involves a more natural type of enzyme-substrate interaction.

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(1) M. Bergmann, C. Zervas, J. S. Fruton, F. Schneider and H. Schleich, J. Biol. Chem., 109, 325 (1935).

(2) E. Smith, Fed'n. Proc., 8, 581 (1949).

(3) M. Bergmann, L. Zervas and J. S. Fruton; J. Biol. Chem. 115, 593 (1936).

(4) E. Abderhalden and W. Zeisset, Fermentforschung, 10, 120 (1929).

(5) M. Fling and S. W. Fox, J. Biol. Chem. 160, 329 (1945).

(6) Y. Kobayashi, M. Fling and S. W. Fox, J. Biol. Chem., 174, 391 (1948).

(7) R. Merten, Biochem. Z., 318, 185 (1947).

(8) E. Elkins-Kaufman and H. Neurath, J. Biol. Chem., 178, 645 (1949).

(9) H. T. Huang and C. Niemann, J. Am. Chem. Soc., 73, 1555 (1951).

Another consequence of the above observations is the conclusion that the papain-substrate fit must be a close one. This is best explained by Smith's modification (1) of the Polyaffinity theory (2) in that fuller reaction in the anilide series is obtained with residues with long side-chains; these reactions are more rapid than those involving alanine and much more rapid than those involving glycine. The concept of closeness of fit is also in accord with other related observations (3,4).

Enzyme Dilution Studies

With regard to the enzymic synthesis of anilides, statement has been made to the effect that "the process involves a compromise in solubilities as the N-acyl-DL-acid must be soluble in the buffer mixture, yet the N-acyl-L-amino acid anilide must be sparingly soluble if the reaction is to proceed to completion. The nature of the acyl group controls the solubility of the N-acyl-DL-amino acid and the corresponding anilide, and, as might be

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(1) E. Smith, Fed'n. Proc., 8, 581 (1949).

(2) M. Bergmann, C. Zervas, J. S. Fruton, F. Schneider and H. Schleich, J. Biol. Chem., 109, 325 (1935).

(3) M. Bergmann, L. Zervas and J. S. Fruton, J. Biol. Chem, 115, 593 (1936).

(4) H. Neurath and G. W. Schwert, Chem. Rev., 46, 145 (1950).

expected, the more soluble amino acids require more insoluble acyl groups" (1). Thus, what appear to be assumptions relating anilide solubility and yield have been made; they lack the proper support of experimental data on solubilities, however.

The above assumptions are in contradiction with the interpretation of Fox and co-workers (2) that although anilide type syntheses depend upon slight solubility, the anilide yields are not primarily a function of the solubility properties of the products under the usual conditions of incomplete reaction. This conclusion could be based on some thermodynamic relationships. First, the relationship between the free energy change for a reaction and the experimentally determined equilibrium constant, K, may be expressed as follows;

$$\Delta F = -RT \ln K.$$

Secondly, for isothermal processes, the change in the free energy of a reaction may be expressed in terms of heats of formation and the absolute entropies of the substances taking part in the reaction, as in the following:

$$\Delta F = \Delta H - T\Delta S.$$

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(1) D. G. Doherty and E. A. Popenoe, Jr., J. Biol. Chem., 189, 455 (1951).

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

From the above expressions, the energy requirement of 1400 to 3700 calories (see p.4) for the synthesis of a peptide bond could be met by constantly removing the quantity of peptide which is in equilibrium with the reactants. Such an explanation could also account for the formation of benzamino acid anilides from benzamino acids and aniline in the presence of papain.

According to the interpretations of the Fox group (1), the occurrence of an anilide synthesis may be considered as a two step reaction:

1. Acylamino acid + aniline $\longrightarrow$ acylamino acid anilide (soln.)
2. Acylamino acid anilide (soln.) $\rightarrow$ acylamino acid anilide (ppt.)

From rate studies on the enzyme-induced formation of benzoylvalinanilide, it was shown that the quantitative extent of the reaction is independent of the equilibrium solubility of the anilide, with the first step as the rate limiting step. Since this is the enzyme-controlled step, the extent of the overall synthesis must be enzyme-controlled. Thus, for those anilide syntheses that did occur to a measurable extent, " the quantitative differences

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

in the extents have been shown to depend primarily on the kinetics of the enzyme-controlled step."

The rate and solubility studies of Waldschmidt-Leitz and Kühn (1) gave strong support to the interpretations of Fox and co-workers. These investigators showed that the slight solubility or insolubility of the synthetic reaction products was not the only factor involved in the occurrence of or the rate of a synthesis. Thus, comparison of the aniline derivative of hippuric acid with the somewhat more soluble phenylenediamine derivatives and the even more soluble aminophenol derivatives showed differences in reaction rates which could be directly traced to solubility differences. However, such explanation was inapplicable in a number of other instances. Consideration of aniline, m-toluidine or p-phenetidine on the one hand, and benzylamine or cyclohexylamine on the other, showed that although all the corresponding hippuric acid derivatives were insoluble, the former compounds coupled easily in

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(1) E. Waldschmidt-Leitz and K. Kühn, Z. physiol. Chem., 285, 23 (1950).

syntheses of the anilide type while the latter possessed no coupling action whatever.

The results reported herein give further strength to the concept that under the usual conditions of incomplete reaction, the extent to which an anilide synthesis proceeds is not primarily dependent upon the solubility properties of the reaction product. Thus, examination of Figure 1, wherein anilide yield is plotted against protease concentration, shows that at the point of like yield of benzoylalaninanilide for both papain and ficin, an almost fourfold difference in the yield of the α-aminocaprylic acid derivative exists for the two enzymes. If anilide yield was primarily a function of the solubility properties, the yields of the latter derivative should here be equal for both proteases. Since conditions in both syntheses were identical, with the exception of the variable due to the different enzymes involved, then the differences in reactivity must be attributed to something other than mere insolubility alone. Emphasis is thereby shifted to differences in interaction between the substrate and each of the proteases involved. Further study and recognition of such relationships may be expected to yield at least further standardization of syntheses of the type described.

Enzymic Reactions of Glycine Containing Benzoyldipeptides With Aniline

Although no systematic studies of glycine containing acylated peptides have been previously reported in enzymic peptide syntheses, such studies have nevertheless been effected on the hydrolytic end. Extensive investigation was made by Bergmann, Zervas and Fruton (1) with the papain peptidase I-catalyzed cleavage of a number of such peptides. Conclusions reached by these investigators showed that the acylamino group of acylated peptides directed the hydrolytic action of papain peptidase I to the peptide linkage immediately adjacent to the acylamino group. If an acylated polypeptide contained linkages with leucine-, glutamic acid-, or lysine-carbonyl, in addition to peptide bonds with glycine-carbonyl, cleavage of the peptide bond involving the glycine-carbonyl was effected. Furthermore, in all instances investigated, the directive influence of the glycine-carbonyl was stronger than that of the acylamino group. Thus, if no glycine-carbonyl was adjacent to the acylamino group, but was nonetheless present as part of another peptide bond in another portion of the polypeptide molecule, then the point of cleavage

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(1) M. Bergmann, L. Zervas and J. S. Fruton, J. Biol. Chem., 115, 593 (1936).

was shifted away from its proximity to the acylamino group. A distinct preference for glycine residues in the enzymic hydrolysis was thereby shown.

Similar preferences for glycine residues in protease-catalyzed syntheses were observed in the investigations reported herein. Several of the benzoyldipeptides studied contained linkages with leucine-, valine-, alanine- or glycine-carbonyl, in addition to a peptide bond involving glycine on the carboxyl end, i.e., benzoylleucylglycine, benzoylvalylglycine, benzoylalanylglycine, and benzoylglycylglycine. With the former three cases, wherein no glycine-carbonyl was adjacent to the acylamino group but where glycine did exist as the terminal residue on the carboxyl end, an enzymically induced coupling reaction resulted with aniline (Table XIII). With benzoylglycylglycine, however, where a glycine residue is adjacent to the benzoylamino group, the directing influence of the glycine-carbonyl was stronger than that of the carboxyl-glycine and an enzymically catalyzed transamidation reaction with aniline resulted. Similar transamidation reactions with other benzoylglycylamino acids (Table XIII) emphasized the significance of the directing influence of the glycine residue. These results with glycine-containing benzoyldipeptides demonstrated that the type of reaction which occurred, transamidation or

coupling, was determined at least in part by the structure of the residues involved, as well as by the relative positions of these residues within the peptide chain.

It could be argued that the reactions described above as "transamidation" reactions are, in essence, merely two-step reactions which involve hydrolysis succeeded by synthesis, as in the following;

1.

$$\text{Benzoylglycylamino acid} \xrightarrow[\text{cleavage}]{\text{enzymic}} \text{Benzoylglycine} + \text{amino acid}$$

2.

$$\text{Benzoylglycine} + \text{aniline} \xrightarrow[\text{synthesis}]{\text{enzymic}} \text{Benzoylglycinanilide}$$

"Transamidation", however, is defined in this instance as the direct replacement of the terminal residue by the aniline residue without the formation of the intermediate stage of hippuric acid (1-4). Such reaction was first reported (1) for the papain-catalyzed formation of anilides from hippurylamide where the rate

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

(2) J. S. Fruton, Yale J. Biol. and Med., 22, 263 (1950).

(3) R. B. Johnston, M. J. Mycek and J. S. Fruton, J. Biol. Chem., 185, 629 (1950).

(4) R. B. Johnston, M. J. Mycek and J. S. Fruton, J. Biol. Chem., 187, 205 (1950).

of anilide formation exceeded that from hippuric acid. This divergence between the two rates gave evidence that the transformation of the amide into the anilide did not proceed through the intermediate stage of benzoylglycine, i.e., hydrolysis, but that the amino group in the molecule of benzoylglycinamide was directly replaced by the aniline residue.

That at least some of the reactions reported herein are transamidation reactions, as defined above, is shown by inspection of Table XIII. It should be here noted that the enzyme-catalyzed reaction of either a benzoylglycylamino acid or benzoylglycine with aniline gave benzoylglycinanilide as the reaction product. However, comparison of the yields of benzoylglycinanilide obtained by the "transamidation" reactions, which involved the reaction of the benzoyldipeptides with aniline, and the coupling reaction, which involved the reaction of benzoylglycine with aniline, show that for the ficin-catalyzed reactions at least, the transamidation reaction yields are in all instances greater than the yield of benzoylglycinanilide obtained by reaction of benzoylglycine with aniline. Thus, both benzoylglycylalanine and benzoylglycylvaline gave a 24 per cent yield of benzoylglycinanilide as opposed to a 14 per cent yield when benzoylglycine was used.

Of even greater significance is the three-fold greater yield obtained with benzoylglycylleucine and benzoylglycylphenylalanine than with benzoylglycine.

Similar consideration of the yields from the papain-catalyzed reactions give little or no indication of the occurrence of transamidation. Comparison of the benzoylglycinanilide yields obtained from the corresponding benzoylglycylamino acids for both proteases shows greater yields for the papain-catalyzed reactions in nearly all cases. However, the yield of benzoylglycinanilide obtained with papain for the coupling reaction, i.e., benzoylglycine plus aniline, was more than three times as great as that obtained with ficin and in most cases close to or greater than the yields obtained in the "transamidation" reactions.

From the above results with papain, no conclusive statement can be made with respect to the occurrence of transamidation reactions. That the reaction products were obtained via transamidation reactions or through the coupling of aniline with a hippuric acid intermediate or even by means of a combination of the foregoing processes, are all possibilities. Conclusive evidence has, however, been obtained for transamidation in the ficin-catalyzed reactions. It has not been ascertained to what extent, if any, the reaction product is synthesized via

the hippuric acid intermediate, however.

In order to investigate the influence of stereochemical configuration on enzymic activity in reactions of the transamidation type, benzoylglycyl-D-phenylalanine and the corresponding acyl-DL-peptide were compared. After three days of incubation, the acyl-D-peptide yielded an appreciable quantity of hippurylanilide for both the ficin- and papain-catalyzed reactions. Transamidation reaction was again indicated, at least for the ficin-catalyzed reaction which showed a 31 per cent yield as contrasted to a 14 per cent yield from the coupling reaction involving hippuric acid. The acyl-DL-peptide gave a 42 per cent yield of product over the same time interval, indicating that the L-form reacted to a more appreciable extent than the D-form.

The above results demonstrate that absolute antipodal specificity (1,2) does not obtain for the

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

(2) M. Bergmann and O. K. Behrens, J. Biol. Chem., 124, 7 (1938).

transamidation reaction investigated. It is rather a case of relative antipodal specificity (1-3), in which both the D- and L- forms react, but at a slower rate for the former. A comparable case has been reported (4) in the enzymic cleavage of benzoylglycyl-L-leucylglycine and its D-isomer, the latter of which, although cleaved to give an appreciable yield of hippurylanilide, nevertheless reacted to a lesser extent than did the former. These findings were attributed to steric hindrance or to a difference in the affinity of the enzyme for the D- and L- forms of the peptide.

Reports in the literature may be interpreted to indicate that transamidation could conceivably exist as a common event in *in vivo* protein synthesis. Thus, a mutant strain of *E. coli* has been reported (5) which, although it required proline for growth,

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(1) O. K. Behrens, D. G. Doherty and M. Bergmann, J. Biol. Chem., 136, 61 (1940).

(2) E. L. Bennett and C. Niemann, J. Am. Chem. Soc., 72, 1798 (1950).

(3) H. B. Milne and C. M. Stevens, J. Am. Chem. Soc., 72, 1742 (1950).

(4) M. Bergmann, L. Zervas, J. S. Fruton, J. Biol. Chem., 111, 225 (1935).

(5) S. Simmonds and J. S. Fruton, J. Biol. Chem., 174, 705 (1948).

showed even more growth when proline peptides were used in its stead. Analogous results were obtained for a microorganism, called Strain SF, which showed a greater growth rate in a leucylglycine containing medium than in a medium containing both leucine and glycine as the source of nitrogen (1,2). In studies involving a variety of glycine containing peptides (3), greater growth stimulation was demonstrated for five lactobacilli than could be accounted for on the basis of glycine content alone. The reports (4,5) that partial hydrolyzates of various proteins have more active growth promoting properties than their constituent amino acids, are also worthy of consideration.

Enzymic Reactions of Non-glycine Containing Benzoyldipeptides With Aniline

In syntheses of the anilide type, the study of acylated peptides containing at least two asymmetric

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(1) S. Simmonds and J. S. Fruton, Science, 109, 561 (1949).

(2) S. Simmonds and J. S. Fruton, Science, 111, 329 (1950).

(3) R. B. Malin, M. N. Camien and M. S. Dunn, Arch. Biochem. Biophys., 32, 106 (1951).

(4) H. Sprince and D. W. Woolley, J. Am. Chem. Soc., 67, 1734 (1945).

(5) M. Klungsor, R. J. Sirny and C. A. Elvehjem, J. Biol. Chem., 189, 557 (1951).

centers has been cultivated relatively little. In this regard, the optically active acyldipeptides, acetyl-L-phenylalanyl-L-leucine and acetyl-L-phenylalanyl-L-glutamic acid as well as their optically active D-L diastereomeric forms have been studied (1). The active isomers were separately treated with aniline in the presence of cysteine-papain. Those isomers containing the D-residues were found to couple with aniline under the influence of the enzyme, although to a lesser extent than the L-L forms. The asymmetric course of the reactions was ascribed to the asymmetry of the phenylalanine residue. It should be noted, however, that this residue was not directly involved in the coupling reaction since it was the free carboxyl of the terminal residue (L-leucine or L-glutamic acid) that had undergone the anilide formation.

No comparable reports have previously appeared in the literature in which the diastereomeric racemates had been studied. The possibility of two diastereomeric forms, the D-L, L-D and the L-L, D-D forms for acyl-dipeptides containing two asymmetric centers has been discussed in the Results section. From the considerations discussed above, the D-L antipode of the former diastereomer could undergo a coupling reaction in the

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(1) O. K. Behrens, D. G. Doherty and M. Bergmann, J. Biol. Chem., 136, 61 (1940).

anilide syntheses. Due to the antipodal specificity properties of the proteases involved (1), however, similar coupling reaction would be far less probable for the L-D isomer, although not entirely overruled (2-5). Consideration of the L-L, D-D diastereomeric pair would lead one to conclude that the L-L form should be capable of undergoing coupling reaction in anilide type syntheses at an even greater rate than the D-L form. The D-D antipode should undergo no reaction, however, because of the stereochemical inhibition exhibited by the two asymmetric centers.

On the basis of the above theoretical considerations and the findings reported herein, a new method is proposed for the determination of the stereochemical configuration of diastereomeric dipeptides. This method may be outlined as follows:

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

(2) E. L. Bennett and C. Niemann, J. Am. Chem. Soc., 72 1798 (1950).

(3) H. B. Milne and C. M. Stevens, J. Am. Chem. Soc., 72, 1742 (1950).

(4) E. L. Bennett and C. Niemann, J. Am. Chem. Soc., 70, 2610 (1948).

(5) E. L. Bennett and C. Niemann, J. Am. Chem. Soc., 72, 1800 (1950).

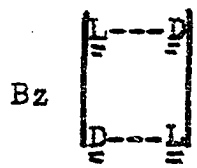
1. Acylation of dipeptide

Form 1: $\text{dipeptide}_1 + \text{Bz-Cl} \longrightarrow \text{Bz-dipeptide}_1$

Form 2: $\text{dipeptide}_2 + \text{Bz-Cl} \longrightarrow \text{Bz-dipeptide}_2$

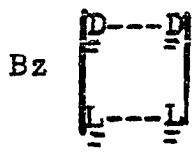
2. Enzymic resolution

Bz-dipeptide_1



+ aniline ⟶ no reaction or Bz-D---L-anilide

Bz-dipeptide_2



+ aniline ⟶ Bz-L---L-anilide

3. Microbiological Utilization of Anilide Hydrolyzate

Bz-D---L-anilide ⟶ 50% utilization

Bz-L---L-anilide ⟶ 100% utilization

After suitable separation of the diastereomeric forms of the dipeptide on the basis of physical properties, i.e., solubility, etc., each form should be acylated with a suitable acylating reagent by the usual Schotten-Baumann procedure. The benzoyl derivative is recommended in this respect since the enzymic reactions in which it is involved (1,2) exhibit a greater degree of

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(1) E. L. Bennett and C. Niemann, J. Am. Chem. Soc., 72, 1798 (1950).

(2) E. L. Bennett and C. Niemann, J. Am. Chem. Soc., 70, 2610 (1948).

absolute stereochemical specificity than do those which involve the carbobenzoxy, carbomethoxy, carboethoxy (1,2) or carboallyloxy (3) derivatives.

In the enzymic resolution, such factors as pH, buffer concentration and substrate concentration are of extreme importance (4). It is here recommended that the enzymic reactions be effected over the pH range of 4.5 to 6.0, at intervals of about 0.5 of a pH unit, for papain-catalyzed reactions involving monoaminomonocarboxylic acids as the terminal residue. This range should be somewhat lower for peptide derivatives involving glutamic acid (5) as the terminal residue and somewhat higher where tyrosine or phenylalanine terminal residues are involved (6). High concentrations of substrate, buffer and enzyme should be used for optimal results.

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(1) E. L. Bennett and C. Niemann, J. Am. Chem. Soc., 72, 1798 (1950).

(2) E. L. Bennett and C. Niemann, ibid., 70, 2610 (1948).

(3) H. B. Milne and C. M. Stevens, J. Am. Chem. Soc., 72, 1742 (1950).

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

(5) S. W. Fox and H. Wax, J. Am. Chem. Soc., 72, 5087 (1950).

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

The above proposed method offers many advantages over the classical methods, which involve total synthesis using optically active amino acids, for the determination of configuration of diastereomeric dipeptides. Such advantages are expressed in terms of time and expense.

Enzymic Reactions of Benzoylamino Acids With Glycinanilide

The results of Bergmann and Fraenkel-Conrat (1) have shown that the structural nature of the amino acid anilide employed in the anilide syntheses determined the type of reaction product obtained. This interpretation was based on papain-catlayzed syntheses with benzoyleucine which, with leucinanilide, gave the benzoyldipeptide anilide as the reaction product. When glycinanilide was used in lieu of leucinanilide, however, the reaction product was the acylamino acid derivative, benzoylleucinanilide.

Pettinga (2) repeated and extended the above investigations with glycinanilide to include the benzoyl derivatives of alanine, glycine and valine as well as that of leucine. Suggestion was made by this investigator that the reactions of benzoylamino acids with glycinanilide

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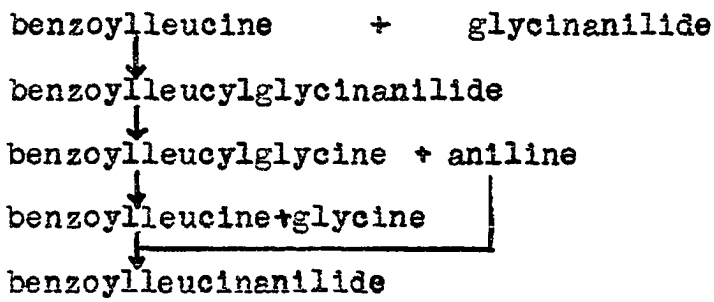
(1) M. Bergmann and H. Fraenkel-Conrat, J. Biol. Chem., 124, 1 (1938).

(2) C. W. Pettinga, Unpublished Ph.D. Thesis. Ames, Iowa. Iowa State College Library. 1949.

followed the cosubstrate mechanism of Behrens and Bergmann (1). On the basis of this assumption, the following series of reactions, illustrated by benzoylleucine, were postulated to have occurred:



Thus, the reaction sequence was assumed to proceed along the following lines. First, benzoylleucine and glycinanilide combine to form benzoylleucylglycinanilide. This synthesis is followed by two successive hydrolytic steps in which first aniline and subsequently glycine are produced. Finally, the liberated aniline is coupled with benzoylleucine with the formation of benzoylleucinanilide. This sequence of reactions gave a plausible explanation for the fact that glycinanilide, which itself is not hydrolyzed by cysteine-papain (1), was readily split in the presence of benzoylleucine. However, no

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(1) O. K. Behrens and M. Bergmann, J. Biol. Chem., 129, 587 (1939).

adequate explanation could be given for the resistance to cleavage of the benzoylglycylglycinanilide formed from the incubation mixture of benzoylglycine and glycinanilide.

Although the above postulated mechanism was reasonable on the basis of the cosubstrate hypothesis, it has nevertheless been demonstrated as invalid by the investigations presented herein. According to the above mechanism, the papain-catalyzed reaction of benzoylleucylglycine and aniline should proceed along the following lines. First, cleavage of the benzoylleucylglycine should be effected with the formation of benzoylleucine and glycine. Second, a coupling reaction should occur between benzoylleucine and aniline to give benzoylleucinanilide. These steps are identical with the final two steps of the mechanism postulated in the preceding paragraph. That such is not the case was evidenced by the fact that the product obtained from the incubation of benzoylleucylglycine with aniline, in these investigations, was benzoylleucylglycinanilide. This product was obtained in high yield and apparently uncontaminated with any benzoylleucinanilide. These results not only invalidate the above postualted mechanism but also cast some doubt upon the validity of the original cosubstrate mechanism of Behrens and Bergmann (1).

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(1) O. K. Behrens and M. Bergmann, J. Biol. Chem., 129, 587 (1939).

For reactions of the general type (Table X and XI),

Benzoylamino acid + glycinanilide → benzoylamino acid anilide (+ glycine)

a transamidation mechanism is here suggested. Evidence for such a mechanism may be obtained from several lines. First, possibility of the intermediate formation of a benzoylaminoacylglycinanilide via a cosubstrate mechanism has been discarded by the foregoing considerations. Second, resistance of glycinanilide to the hydrolytic action of cysteine-papain (1) eliminates the possibility of a coupling reaction between the benzoylamino acid and free aniline in solution. Third, results indicate that the pH optima for the transamidation reactions (Table XVI) probably differ from those of the coupling reactions (benzoylamino acid with aniline), e.g., although not determined for peak activity, the pH optimum for the reaction of benzoylvaline with glycinanilide appears to be above pH 5.5 (Table XI), whereas the pH optimum for the formation of benzoylvalinanilide by a coupling reaction is close to 5.0 (2). Finally, complete lack

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(1) O. K. Behrens and M. Bergmann, J. Biol. Chem., 129, 587 (1939).

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

of reactivity for N-benzoyl-tyrosine with glycinanilide over the pH range of 5.0 to 7.5 (Table XVI) suggests a mechanism other than direct coupling with aniline, which shows great reactivity at pH 6.0 (1).

A "transamidation" reaction may be pictured as a process of group transfer, without intervening hydrolysis, in which either the carboxyl- or the amino- moiety of an existing peptide would be transferred to linkage with the appropriate group of another amino acid (or peptide) molecule (2). By this route, formation of a new peptide bond could be effected at the expense of an existing one, according to either of the following schemes:

Amine transfer

$$R' \cdot CO\text{-}NH \cdot R'' + R''' \cdot COOH \longrightarrow R''' \cdot CO\text{-}NH \cdot R'' + R' \cdot COOH$$

Carboxyl transfer

$$R' \cdot CO\text{-}NH \cdot R'' + NH_2 \cdot R''' \longrightarrow R' \cdot CO\text{-}NH \cdot R''' + NH_2 \cdot R''$$

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

(2) C. S. Hanes, F. J. R. Hird and F. A. Isherwood, Nature, 166, 288 (1950).

Transamidation reactions of the latter type (carboxyl transfer) have been reported by various investigators (1-4). Carboxyl transfer was also involved in the transamidation reactions with benzoyldipeptides mentioned in a previous section. However, although the alternative mode of transamidation (amine transfer) is the scheme involved in the glycinanilide experiments described here, no previous demonstration of this scheme has been reported in the literature (4).

Enzymic Reaction of Benzoylamino Acids With Amino Acid Anilides

In view of the results obtained in these investigations (Tables XV, XVI, XVII and XXI), it is of interest to compare the reactions exhibited by both benzoylglycine and benzoylalanine with the variety of

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

(2) R. B. Johnston, M. J. Mycek and J. S. Fruton, J. Biol. Chem., 185, 629 (1950).

(3) C. S. Hanes, F. J. R. Hird and F. A. Isherwood, Nature, 166, 288 (1950).

(4) S. G. Waley and J. Watson, Nature, 167, 360 (1951).

amino acid anilides used.

1. Reactions of benzoylglycine

Bz-glycine + glycinanilide → Bz-glycylglycinanilide

Bz-glycine + alaninanilide → Bz-glycylalaninanilide

Bz-glycine + leucinanilide → Bz-glycylleucinanilide

Bz-glycine + valinanilide → Bz-glycylvalinanilide

2. Reactions of benzoylalanine

Bz-alanine + glycinanilide → Bz-alaninanilide

Bz-alanine + alaninanilide → Bz-alaninanilide

Bz-alanine + leucinanilide → Bz-alanylleucinanailide

Bz-alanine + valinanilide → Bz-alanylvalinanilide

It will be noted that in all cases where benzoylglycine was studied with an amino acid anilide, an enzymatically induced coupling reaction resulted, the product in all cases being the benzoyldipeptide anilide. Thus, benzoylglycine with glycinanilide gave benzoylglycylglycinanilide, benzoylglycine with alaninanilide gave benzoylglycylalaninanilide, and so on. When benzoylalanine was used in lieu of benzoylglycine, however, reaction with both glycinanilide and alaninanilide gave transamidation reactions while reaction with leucinanilide and valinanilide gave coupling reactions.

Differences in the type of reactivity with glycinanilide and alaninanilide, under the same set

of conditions, have therefore been exhibited by benzoylglycine and benzoylalanine. The only variables introduced were inherent in the compositional differences of the amino acid residues of the acylamino acids employed. Examination of the structural make-up of these residues reveals that the side chain of the alanine residue contains a methyl group whereas the glycine residue has a hydrogen in its stead. If the differences in the properties shown by these residues were, at least in part, a function of the size of the hydrocarbon side chain, then all residues with larger side chains than that of the alanine residue should exhibit the same type of reaction (transamidation) with glycinanilide and alaninanilide as was shown by benzoylalanine. Thus, substitution of the CH_3- group of the alanine residue by $CH_3 \cdot CH_2$- (α-amino-n-butyric acid), $CH_3 \cdot CH(CH_3)$- (valine), $CH_3 \cdot CH_2 \cdot CH_2$-(norvaline) and $CH_3 \cdot CH(CH_3) \cdot CH_2$- (leucine) lead to no change in the type of reaction which resulted (Tables XVI, XVII and XXI). From the foregoing discussion, it may be concluded that the type of reaction which occurs, transamidation or coupling, depends in part upon the structure of the amino acid residue of the amino acid anilide.

Consideration of the reactions of benzoylalanine shows that the type of reaction effected is also influenced by the structure of the amino acid anilide. Differences in type of reaction of benzoylalanine with glycinanilide or alaninanilide (transamidation), as opposed to leucinanilide or valinanilide (coupling), were observed. Here, however, the differences cannot be attributed to the acylamino acid but must lie within the amino acid anilide employed. It may be significant that the size of the side chains of the residues involved in the transamidation reactions, i.e., H-(glycinanilide and CH_3-(alaninanilide) is less than that of the residues involved in the coupling reactions, i.e., $CH_3 \cdot CH(CH_3)$-(valinanilide) $CH_3 \cdot CH(CH_3) \cdot CH_2$-(leucinanilide). The effect of other structural considerations, such as the degree of branching, cannot however be excluded. Certainly, further study is here necessitated before any definite conclusions can be drawn.

The several interpretations that can be extrapolated from the foregoing discussion are here summarized. First, that certain benzoylamino acids have a greater propensity than others to undergo coupling reactions under the same conditions. This ability,

possessed to the most considerable extent by benzoylglycine in these studies, is determined in part by the structural features of the amino acid residue under consideration. Second, that the degree of coupling reaction is conditioned in part by the nature of the amino acid anilide involved. Since glycinanilide and alaninanilide are more prone to undergo transamidation as opposed to leucinanilide and valinanilide, which possess the coupling ability to a greater extent, the possibility of steric factors is here invoked. Third, that for a given enzyme system (papain in this case), the type of reaction which results depends upon an interaction of the coupling propensities of the amino acid residues of the reactant benzoylamino acids and amino acid anilides. As a fourth factor, the fact that different enzymes may exert different degrees of influence on the type of reaction which occurs should here be mentioned. A more lengthy discussion of the effect of different enzymes on reaction type will be treated under the heading of "Enzyme Specificity" below.

As a point of interest, it should be here noted that the same steric effects were observed in transamidation reactions involving reaction of benzoylamino acids with alaninanilide as were observed

in the coupling reactions of benzoylamino acids with aniline, described earlier. The relationships observed are shown in Table XXVII. Since full description of these relationships was given earlier for the coupling reactions, and since the same principles hold, no extended discussion will be given here.

Enzyme Specificity

Numerous claims of enzyme specificity in the cleavage of peptide substrates have been cited in the literature (1,2). Such studies emphasize that the preferential hydrolytic action of proteases on particular peptide bonds is conditioned by the nature and the relative positions of the residues involved in such linkage. These same preferences for residue order and kind should also hold in the reversal of the hydrolytic processes, i.e., synthesis of peptide bonds. However, no adequate demonstration of enzyme specificity in this "reversal of hydrolysis" has heretofore been made.

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(1) M. Bergmann, L. Zervas, J. S. Fruton, J. Biol. Chem., 124, 321 (1938).

(2) M. Bergmann, L. Zervas, J. S. Fruton, J. Biol. Chem., 111, 225 (1935).

Table XXVII

Reactivity of Benzoylamino Acids With Alaninanilide as Catalyzed by Papain

Branch Chain	% Yield	Straight Chain	% Yield
$CH_3 \cdot C(NHBz) \cdot COOH$ CH_3 Bz-aminoisobutyric acid	0	$CH_3 \cdot CH(NHBz) \cdot COOH$ Bz-alanine	46
$CH_3 \cdot CH \cdot CH(NHBz) \cdot COOH$ CH_3 Bz-valine	4	$CH_3 \cdot CH_2 \cdot CH(NHBz) \cdot COOH$ Bz-amino-n-butyric acid	73
$CH_3 \cdot CH \cdot CH(NHBz) \cdot COOH$ C_2H_5 Bz-isoleucine	0	$CH_3 \cdot CH_2 \cdot CH(NHBz) \cdot COOH$ Bz-amino-n-butyric acid	73
$CH_3 \cdot CH \cdot CH_2 \cdot CH(NHBz) \cdot COOH$ CH_3 Bz-leucine	57	$CH_3 \cdot CH_2 \cdot CH_2 \cdot CH(NHBz) \cdot COOH$ Bz-norvaline	85

From the data presented in the Results section, it may be seen that no completely acceptable demonstration of enzyme specificity has here been made. Although the chymotrypsin-, ficin- and papain-catalyzed reactions of benzoylphenylalanine with glycinanilide all yielded products, the products in the latter two cases were unidentified. If, as was postulated in the Results section, these unidentified products were mixtures of anilides, then a valid instance of enzyme specificity could be here invoked. Such invocation could be based on the occurrence of a coupling reaction, in the presence of chymotrypsin, to give the benzoyldipeptide anilide whereas in the presence of ficin or papain, the benzoylamino acid anilide may form as well via some other mechanism.

Previous claim has been made regarding demonstration of protease specificity in synthetic reactions (1). Such claim was based upon the formation of benzoyltyrosylglycinanilide from benzoyltyrosine and glycinanilide in a chymotrypsin-catalyzed reaction. However, no reactivity with the same substrates was found for papain (2).

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(1) M. Bergmann and J. S. Fruton, J. Biol. Chem., 124, 321 (1938).

(2) J.S. Fruton, Cold Spring Harbor Symposia Quant. Biol., 6, 55 (1938).

The foregoing demonstration of specificity may be criticized in several respects. First, each of the proteases were employed at the traditional pH's. Lack of reactivity could here be attributed to insufficient testing of pH. The validity of such criticism is particularly justifiable in view of the recent findings (1) of the importance of pH control in syntheses of the anilide type. Secondly, the fact that the syntheses were not run at the same pH introduces a second variable into a system already containing one variable due to protease differences. Pointed statement could not therefore be justifiably made that the observed "specificity" was due to enzyme effects or pH effects alone. Finally, the inability of papain to induce the formation of a reaction product prohibits the formulation of any definite statement concerning its specificity. The first two criticisms do not apply to the specificity studies reported here since the only variable was due to the proteases involved.

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

SUMMARY

The effects of variation of substrate structure on the relative extents of the papain- and ficin-catalyzed syntheses of a number of benzoylated amino acid anilides have been studied. Among the naturally and unnaturally occurring amino acids investigated were the benzoyl derivatives of glycine, alanine, valine, leucine, ∝-amino-n-butyric acid, norvaline, norleucine, ∝-aminoheptylic acid, ∝-aminocaprylic acid, ∝-amino pelargonic acid, S-benzoylpenicilliamine, isoleucine and aminoisobutyric acid. It has been shown that the previously observed greater reactivity of benzoylleucine than of benzoylvaline in the papain-catalyzed synthesis of anilides is in accord with steric hindrance by the branching methyl in the valine substrate. This explanation invokes the principle of steric hindrance in enzyme-substrate interaction involving L-type substrates. The theoretical inferences of this sort of result have been indicated.

The relative rankings of the thirteen benzamino acids in the above reactions were shown to differ when ficin was the catalyst, from the results obtained with papain. The effect of the position of the methyl substituent, however, again dominated in the contrast between leucine and valine.

The relationships of yield to dilution of enzyme have been found to vary both with each of the enzymes and with the substrate.

The papain- and ficin-catalyzed syntheses of peptide bonds with a variety of benzoylated amino acids and glycinanilide in citrate buffer have been studied. Eighteen different benzoyl derivatives of amino acids were used. Coupling and transamidation reactions of the following types were observed:

1. Coupling

Benzoylamino acid + glycinanilide ⟶

Benzoyl-α-aminoacylglycinanilide

2. Transamidation

Benzoylamino acid + glycinanilide ⟶

Benzoylamino acid anilide.

The type of reaction which occurred seemed to depend, at least in part, upon the nature of the benzoylated amino acid employed. In some instances, the variation of yield of anilide with pH was also studied. Evidence was presented that the transamidation reactions proceeded via the unreported amine transfer mechanism. Evidence was also presented against invocation of a cosubstrate mechanism where transamidation was observed.

The above studies were extended to include papain-catalyzed reactions which involved alaninanilide, leucinanilide and valinanilide. Again, coupling and

transamidation reactions were observed. These results showed that the type of reaction which occurred depends not only upon the nature of the benzoylamino acid, but also upon the nature of the amino acid anilide employed. The transamidation reactions observed with alaninanilide as the substrate followed the steric hindrance concept, invoked above, for the coupling reactions of benzoylamino acids and aniline.

The papain- and ficin-catalyzed reaction of several glycine-containing benzoyldipeptides with aniline was also studied; of these, some gave coupling reactions and some gave transamidation reactions under the conditions investigated.

1. Coupling

Benzoyl-α-aminoacylglycine + aniline $\longrightarrow$

Benzoyl-α-aminoacylglycinanilide

2. Transamidation

Benzoylglycylamino acid + aniline $\longrightarrow$

Benzoylglycinanilide (+ amino acid)

The type of reaction which occurred, transamidation or coupling, depended upon the directive influence of the glycine residue, i.e., its relative position in the peptide chain. The transamidation reactions were shown to proceed via the carboxyl transfer mechanism.

Studies with the racemic diastereomeric pairs of

non-glycine containing benzoyldipeptides revealed that in the enzyme-induced coupling with aniline, only the L-L isomer of the D-D, L-L pair showed reactivity to give the anilide as product. The D-L, L-D racemate showed no reactivity under the conditions studied. As a result of the foregoing observations a new and simplified method was proposed for the identification of diastereomeric dipeptides. This method involved enzyme syntheses in conjunction with microbiological assay of the acid hydrolyzate of the product from the enzymic syntheses.

Enzyme specificity studies were effected with several benzoyl derivatives in the ficin-, chymotrypsin-, and papain-catlayzed anilide syntheses. The results were discussed in the light of contemporary beliefs of enzyme specificity in protease-catalyzed syntheses.

The newly obtained anilides have been characterized.

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APPENDIX

The melting points of the crude products obtained in the anilide syntheses, as well as their mixed melting points with pure samples, when available, are given in Table XXVIII.

Table XXVIII

Melting Points and Mixed Melting Points of Crude Products

Product	M.p. of crude product, C°.	M.p. of pure anilide, C°.	Mixed m.p., C°.
Bz-dipeptide aniline			
Bz-glycinanilide	210-212	212½	211-212
Bz-amino acid glcyinanilide			
Bz-glycylglycinanilide	246-248		
Bz-alaninanilide	174-176	175-176	174-176
Bz-valinanilide	218-220	220-221	219-220½
Bz-leucinanilide	212-213	213	213
Bz-methioninanilide	160-162	162-162½	161-162
Bz-isoluecinanilide	213-215	220-220½	217-219
Bz-tryptophananilide	196-196½	199-200	196½-198
Bz-norvalinanilide	174-176	182½-183½	178-181
Bz-norleucinanilide	176-178	181-182	179-181½
Bz-α-amino-n-butyric acid anilide	163-165	169½-170½	167-168½
Bz-amino acid alaninanilide			
Bz-leucinanilide	213-214	213	213
Bz-valinanilide	206-209	-	-
Bz-α-amino-n-butyric acid anilide	167-169	169½-170½	169½-170½
Bz-alaninanilide	174-176	175-176	174-176
Bz-norvalinanilide	178-179	182½-183½	179-181