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Factors affecting enzymic peptide bond synthesis

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FACTORS AFFECTING ENZYMIC PEPTIDE BOND SYNTHESIS

By

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INTRODUCTION

The hypothesis that the same enzymes which catalyze the hydrolysis of proteins are also responsible for catalyzing protein synthesis is supported by evidence that proteolytic enzymes catalyze both the hydrolysis and synthesis of the fundamentally important peptide bonds, the fundamental type of bond which links together amino acids in the protein molecule. It is not unreasonable, on this basis, to assume that some of the factors which affect enzymic peptide bond synthesis in simple structures will also affect protein synthesis.

The synthetic ability of proteolytic enzymes has been demonstrated in the formation of peptide bonds from protein split-products and more incisively in the formation of benzamino acid anilides from benzamino acids and aniline as catalyzed by the intracellular enzymes papain, bromelin, cathepsin, and ficin. It has been demonstrated that this anilide synthesis may be utilized in a method for resolution of amino acids. The problem of deciding whether this reaction can be used as a model for protein synthesis has not been solved.

It was the purpose of this investigation to study some of the factors affecting the synthesis of benzamino

acid anilides in order to acquire data which might be basic to the study of the synthesis of peptide bonds in general. This data might also be applied in the development of more effective methods of amino acid resolution utilizing the formation of benzamino acid anilides.

Some Energetic Considerations Related to Peptide Bond Synthesis

The statement of the second law of thermodynamics by Lewis and Randall[1] "Every system which is left to itself will, on the average, change toward a condition of maximum probability" is rigorous and general; however, it does not lend itself to direct application for specific use. More useful for the purpose of biochemistry is the statement[2] (a deduction from the second law of thermodynamics) "All spontaneous processes taking place at constant temperature and pressure are accompanied by a decrease in free energy." Since the free energy is a function of the state of the system only and is not dependent on path; for the reaction: $A + B \longrightarrow C$ the reaction will proceed spontaneously, regardless of the chemical pathway utilized, if the free energy of the

1. Lewis, G. N. and Randall, M. Thermodynamics and the free energy of chemical substances. New York, McGraw-Hill Book Co. 1923. p. 127.
2. Glasstone, S. Thermodynamics for chemists. New York, D. Van Nostrand Co., Inc. 1947. p. 209.

products is greater than the free energy of the reactants.

In addition to this statement of the second law of thermodynamics, there are two other relationships[1] which have application to any consideration of the reaction of amino acids to form peptides. The first is the relationship between the change in free energy for a reaction and the experimentally determined equilibrium constant, K:

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

where for the general reaction

$$lL + mM \longrightarrow nP + qQ$$

K is given by

$$K = \frac{(a_P)^p (a_Q)^q}{(a_M)^m (a_N)^n}$$

For many purposes the activity, a, may be replaced by the concentration without introducing gross errors.

The second relationship of importance expresses the change in free energy of a reaction in terms of heats of formation and the absolute entropies of the substances taking part in the reaction:

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

This equation applies only to isothermal processes. The change in heat content may be measured conveniently by

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1. These thermodynamic relationships are discussed in detail in Glasstone, S. Thermodynamics for chemists.

using either a constant volume or a constant pressure calorimeter. For the constant pressure reaction

$$\Delta H = q$$

where q is the heat absorbed by the system during the reaction. For the constant volume reaction

$$\Delta H = \Delta E - \Delta nRT$$

where ΔE is the quantity of heat absorbed under these conditions and Δn is the number of moles of gas produced in the reaction minus the number consumed. From the heat of combustion of an organic compound and from the heat of formation of the products of combustion from the elements, the heat of formation of the compound can readily be calculated.

The change in absolute entropies as utilized in the equation can conveniently be obtained from the third law of thermodynamics and the proper expression for the heat capacities of the compounds taking part in the reaction. From the third law can be derived:

$$S = \int_0^T \frac{C_p}{T}\, dT - \frac{Q}{T_{tr}}$$

where C_p is the heat capacity under constant pressure restrictions and Q is the heat absorbed during any transition in state at temperature T_{tr} while the substance is warming

from 0° to T°. The change in entropy is then the difference between the sum of the entropies of the products less the sum of the entropies of the reactants.

Using these relationships, the free energy of formation of several peptide bonds composed of different amino acid residues have been determined[1,2]. In all cases the free energy was positive, being about 1400 to 3700 calories. From this value of the free energy of formation of the peptide bond from amino acids, it is obvious that the equilibrium constant for the synthetic reaction will be extremely small, and that only a small amount of the products will be obtained at equilibrium. This fact has also been observed experimentally, for in the hydrolysis of peptides by proteolytic enzymes the reaction proceeds very nearly to completion.

Recently Frantz[3] has been able to evaluate the free energy of peptide bond formation from a study of the equilibrium between glycine and glycylglycine using rat liver peptidase as the catalyst. He used long-lived C_{14}-labeled glycine in order to be able to estimate quantitatively the amount of material present and then separated

1. Huffman, H. M., J. Phys. Chem., 46, 885 (1942).
2. Borsook, H. and Huffman, H. M. In Schmidt, C.L.A. Chemistry of the amino acids and proteins. 2d ed. Springfield, Ill,, Charles C. Thomas. 1945. p. 822.
3. Frantz, I. D. Jr., Loftfield, R. B. and Werner, A. S., Federation Proceedings, 8, part 1, 199 (1949).

the glycine from the glycylglycine in the equilibrium mixture by means of partition chromatography on a starch column. The energy of formation of this particular peptide bond calculated from the equilibrium data was 3300 to 3800 calories. This value is in good agreement with the free energy of the reaction as calculated by Borsook and Dubnoff[1] from the thermal data.

From the considerations mentioned above, it is apparent that the reaction of amino acids to form peptides can be studied only with difficulty, since the yields of peptides are so extremely small. At the present time the following three methods have been used to study peptide bond synthesis in vitro: (A) utilizing derivatives of amino acids which will yield insoluble products[2], so that the concentration is less than that of the equilibrium concentration, (B) increasing the concentration of the reactants in an effort to drive the reaction towards synthesis[3], (C) using labeled compounds so that small amounts of products can be detected and measured quantitatively[4,5,6].

1. Borsook, H. and Dubnoff, J. W., J. Biol. Chem., 132, 307 (1940).
2. Bergmann, M. and Fraenkel-Conrat, H., J. Biol. Chem., 119, 707 (1937).
3. Wasteneys, H. and Borsook, H., Physiol. Rev., 10, 110 (1930).
4. Frantz, I. D. Jr., Loftfield, R. B. and Werner, A. S., Federation Proceedings, 8, part 1, 199 (1949).
5. Melchior, J. and Tarver, H., Arch. Biochem., 12, 301 (1947).
6. Melchior, J. and Tarver, H., Arch. Biochem., 12, 309 (1947).

Some Enzymatic Considerations Related to Peptide Bond Synthesis

Although the properties of enzymes and the types of reactions catalyzed by enzymes were known at least in part since the time of Pasteur, Berzelius[1] in 1825 first pointed out that the properties of the inorganic catalysts were remarkably similar to those active agents which existed in living cells. Kuhne[2] proposed the name enzyme for these active agents in 1867. Nernst[3] showed from thermodynamics that a theoretical catalyst should catalyze both the forward and reverse reactions in an equilibrium system. The agreement between enzymatic action and catalytic theory has been confirmed, especially in connection with the carbohydrases and the esterases. For example, Cori and Cori[4] have shown that the synthesis of glycogen as catalyzed by phosphorylase can also be made to go in the reverse direction with the same enzyme under different conditions. Borsook and Schott[5] found that

1. Berzelius, Lehrbuch der Chemie, Dresden (1825).
2. Kuhne, Virchows Arch., 39, 1130 (1867).
3. Nernst, Theoretical Chemistry, 4th ed., Eng. Trans. London, Macmillan and Co., Ltd. 1916 p. 617.
4. Cori, G. T. and Cori, C. F., J. Biol. Chem., 131, 397 (1939).
5. Borsook, H. and Schott, H. F., J. Biol. Chem., 92, 535 (1931).

fumarase acts as a theoretical catalyst in the succinate-fumarate equilibrium. With respect to the proteolytic enzymes the situation is not as well defined. Bergmann[1] has shown that the proteolytic enzymes can catalyze both the synthesis and the hydrolysis of peptide bonds. (A more complete discussion of this point is found in the Historical section.)

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

HISTORICAL

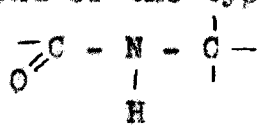
Of the many interesting problems which have been the subject of investigation by the biochemist, there are few which offer a greater challenge and have a more widespread application to biology than the mechanism of protein synthesis in the living organism. The fundamental relationship of this problem to biology is evidenced by the suggestion of Gulick[1] that genes are nucleoproteins that can act as enzymes in the formation of replicas of their own structures. Genes resemble two other nucleoproteins which are also capable of autosynthesis-tobacco mosaic virus and bacteriophage - which seem to act as enzymes in catalyzing duplicates of their own structures from substances present in the host organism[2,3].

In addition to these specific cases, there seems to be little doubt that the biological formation of proteins in general is influenced by proteolytic enzymes. The actual mechanism of protein synthesis and the role of proteolytic enzymes in this synthesis is still largely

1. Gulick, A., Quart. Rev. of Biol., 13, 140 (1938).
2. Stanley, W. M., Ann. Rev. of Biochem., 9, 545 (1940).
3. Northrop, J. H., J. Gen. Physiol., 21, 335 (1938).

an unanswered question. This is due in part to the complexity of the protein molecule and in part to the small equilibrium constant for the reaction of amino acids to form peptides as discussed in the energy considerations related to peptide bond synthesis. Despite the fact that the structural formula of a single well-defined protein molecule cannot be written, there are certain experimental observations which have made possible the formulation of a reasonable theory of protein structure.

It was known before 1900 that proteins, when hydrolyzed with the aid of acids, alkalis or enzymes, yielded amino acids as the principal end products. The suggestion that these amino acids were linked together by a covalent bond of the type



was made almost simultaneously by Hofmeister[1] and Fischer[2]. Fischer termed this linkage the peptide bond. The experimental evidence has been summarized by Vickery and Osborne[3].

1. Hofmeister, F., Ergeb. d. Physiol., 1, 759 (1902).
2. Fischer, E., Ber. 35, 1095 (1902).
3. Vickery, H. B. and Osborne, T. B., Physiol. Rev. 8, 393 (1928).

(1) Hydrolysis of proteins, whether by acids, alkalis or enzymes, results in the liberation of equivalent amounts of amino and carboxyl groups.

(2) Synthetic peptides have been prepared which are susceptible to enzymic digestion under conditions similar to those required for the digestion of proteins.

(3) Proteins contain little amino nitrogen but large amounts are found in the end products of protein hydrolysis.

(4) The peptide bond is found in nature in certain simple substances such as hippuric acid.

(5) Many substances containing the peptide bond give the biuret reaction as well as proteins.

In order to account for the high molecular weight of the proteins as well as the variation in their chemical, physical, and biological properties, it is necessary to assume that each molecule consists of a large number of amino acids linked together by the peptide bond. The kind, relative amount, and order of the amino acid residues in the protein molecule determine to a large extent the properties of the protein. In addition, the spatial configuration of the protein molecule will have an important bearing on certain of its properties. For an excellent review on the various theories on protein

structure and especially the other types of bonds besides the peptide bonds which are found in proteins, the article "Protein Structure" by H. B. Bull should be consulted[1].

A system consisting of amino acids, peptides, and proteins in an aqueous solution represents a combination of an infinite number of equilibrium reactions. In the absence of any catalyst these reactions will proceed at a very slow rate. The role of the proteolytic enzyme is to permit the selective acceleration of a few of these many possible reactions. From the energy considerations mentioned previously, as well as from the experimental observations, it is evident that for the general reaction

$$\text{Protein} \xrightarrow{\text{Enzyme}} \text{Amino acids}$$

the equilibrium is shifted far toward the right. Similarly, the equilibrium for the reaction

$$\text{Peptide} \xrightarrow{\text{Enzyme}} \text{Amino acids}$$

is also far toward hydrolysis. For this reason it is not surprising that the early investigators utilized the hydrolytic reactions in studying the relationships between proteins, peptides and amino acids. Typical of the work

1. Bull, H. B., Adv. in Enzym., 1, 1 (1941).

done on hydrolysis of peptides are the classical investigations of the early workers Fisher and Abderhalden, and more recently, Bergmann[1].

A complete understanding of the mechanism of protein synthesis cannot be obtained from studies of protein hydrolysis alone. Such an understanding requires also that synthetic experiments be undertaken. Although many mechanisms have been proposed for protein synthesis, there is little real experimental evidence existing for any of these proposals.

As discussed above in the introduction, any model of peptide bond synthesis as catalyzed by enzymes must fill two requirements. It must account for the specificity of the proteins as found in nature; and it must furnish some explanation of how the energy is furnished for the synthesis of the peptide bond.

As early as 1901, Sawjalow[2] made the observation that when proteolytic enzymes were added to concentrated solutions of protein split products, precipitates were often observed. Taylor[3] incubated a solution consisting of completely digested protamine sulfate with glycerol

1. Bergmann, M. and Fruton, J. S., Adv. in Enzym., 1, 63 (1941).
2. Sawjalow, W. W., Pfluegers Arch., 75, 171 (1901).
3. Taylor, A. E., J. Biol. Chem., 3, 87 (1907).

extract of liver and found a substance which he believed to be identical with or very similar to the original protamine.

More recently the problem of peptic synthesis has been studied in more complete detail by Wasteneys and Borsook[1]. The greater part of their own work, as well as much of the earlier literature, has been discussed in their review article. Wasteneys and Borsook digested egg albumin with pepsin at pH 1.6, then concentrated this solution and adjusted the pH to 4.0. Upon the addition of fresh pepsin to this concentrate, and after incubation, they were able to separate a precipitate which they called plastein. The evidence for the formation of peptide bonds includes: a decrease in the amount of amino nitrogen during the incubation, the formation of the precipitate, the ability of pepsin to digest this precipitate when placed in the proper buffer solution, the solution of plastein could be precipitated with trichloracetic acid.

According to Wasteneys and Borsook, the formation of plastein can be explained from a consideration of the law of mass action. They assume that the hydrolysis of the egg albumin and the synthesis of the plastein are the forward and reverse steps of the reaction

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

$$\text{Protein} \xrightarrow{\text{Pepsin}} A - B - C - \ldots . M$$

where the equilibrium constant for the reaction is given by

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

where K represents the equilibrium constant. On this basis if one molecule of protein is split to form many moles of split products, and if this simple expression will hold for the complex mixture of protein, enzyme, peptides and amino acids present, then if the concentration of the split products is increased the equilibrium should be appreciably shifted in the direction of synthesis.

A similar experiment has been carried out by Maver, Voegtlin, and Johnson[1,2]. Under suitable conditions of oxygenation a papain digest of fibrin showed the formation of an insoluble material. This work was repeated by Strain and Linderstrom-Lang[3] using fibrin, casein, egg albumin and egg white with papain as the catalyst. These workers reported that in no case could they obtain any material

1. Voegtlin, C., Maver, M. E. and Johnson, J. M., J. Pharmacol., 48, 241 (1932).
2. Maver, M. E. and Voegtlin, C., Enzymologia, 6, 219 (1939).
3. Strain, H. H. and Linderstrom-Lang, K., Comnt. rend. trav. lab. Carlsberg, 23, 11 (1940).

which could be precipitated with trichloracetic acid.

In addition to the fact that this reaction evidently cannot be duplicated by all workers, more serious objections can be raised against the use of this reaction as a model of peptide bond synthesis. Although Northrop[1] found that he could obtain a precipitate from the incubation of the split products of autolyzed pepsin or trypsin, the plastein formed had no enzymatic activity. For this reason he concluded that the enzyme was not resynthesized. If the enzyme, that is, the original protein, cannot be synthesized from its split products, then the equilibrium expression cannot be applied, and the considerations from the law of mass action may not be utilized. In addition, there is evidence that the molecular weight of the plastein is rather small, probably below 1000. This has been demonstrated independently by Ecker, Flosdorf, and Folley[2,3,4]. Another serious objection to the use of this reaction as a model of peptide bond synthesis is the fact that in all cases the identity and quantity of the reacting substances is

1. Northrop, J. H., J. Gen. Physiol., 30, 377 (1947).
2. Ecker, P. G., J. Gen. Physiol., 30, 399 (1947).
3. Flosdorf, E. W., Science, 93, 157 (1941).
4. Folley, S. J., Biochem. J., 26, 99 (1932).

unknown, the steps may be complex and interdependent, and the nature of the product formed has not been clearly defined. In certain cases it has been shown that the type of bond formed has been an intermolecular disulfide linkage between peptides[1,2].

Thus, although there is some experimental evidence that peptide bonds may be synthesized from a concentrated solution of protein split products in the presence of a proteolytic enzyme, the system does not readily lend itself to a study of the factors affecting peptide bond synthesis.

The energy requirements for the synthesis of peptide bonds can also be met by constantly removing the quantity of peptide which is in equilibrium with the reactants. In all probability such an explanation will account for the formation of benzamino acid anilides from benzamino acids and aniline in the presence of papain. Bergmann and Fruton[3] also pointed out the possibility that such a mechanism might account for the

1. Linderstrom-Lang, K. and Johansen, G., Enzymologia, 7, 239 (1939).
2. Geiger, W. B., Patterson, W. I., Mizell, C. R. and Harris, M., Jour. Res. Nat. Bur. Stand., 27, 459 (1941).
3. Bergmann, M. and Fruton, J. S., Ann. N. Y. Acad. Sc., 45, 409 (1944).

synthesis of proteins. Northrop[1] has amplified this suggestion and postulates that the formation of insoluble monolayers of proteins at interfaces[2,3,4] may be one step in a possible mechanism for the synthesis of proteins.[5] No mechanism for the conversion of this insoluble protein to soluble protein in solution in the cytoplasm has been offered. The free energy considerations for the reaction of amino acids to form soluble proteins are still valid, for the conversion of these insoluble proteins is not a spontaneous process but will require energy.

Another proposal that has been suggested for the formation of proteins is that the synthesis of the prptide bond which requires energy is coupled with another reaction which liberates energy. Certain of the carbohydrate metabolic reactions will not take place alone, but will occur if another reaction which liberates

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1. Northrop, J. H., In Northrop, J. H., Kunitz, M., and Herriott, R. M. Crystalline enzymes, 2nd ed. p. 225, Columbia Univ. Press, New York, 1948.
2. Gorter, E., Trans. Faraday. Soc., 33, 1125 (1937).
3. Langmuir, I., Proc. Roy. Soc. London, A., 170, 1 (1939).
4. Langmuir, I., and Schaeffer, V. J., J. Am. Chem. Soc., 60, 1351 (1938).
5. Robertson, T. B., Aust. J. Exp. Biol. and Med. Sc., 3, 97 (1926).

energy takes place at the same time[1,2]. In these coupled reactions, part of the energy liberated from a hydrolysis or oxidation is utilized in a synthetic reaction. Generally such a mechanism involves phosphorylation. Bergmann and Fruton[3] suggest that a similar coupling may take place in protein synthesis, and that the energy required from the peptide bond is furnished from the energy available from carbohydrate metabolism, the energy itself being made available through some energy rich intermediate. No real evidence for any such coupling reaction exists. The nearest approach to this problem has been furnished by Borsook and Dubnoff[4]. They found that rapid synthesis of hippuric acid from benzoic acid and glycine took place in intact liver cells; however, when the cells were minced or when an extract was used, the synthesis was stopped. Here the synthesis of a peptide bond seems to be coupled to respiration, and when respiration ceases, the synthesis of hippuric acid also ceases.

1. Meyerhoff, O., Ann. N. Y. Acad. Sc., 45, 377 (1944).
2. Kalckar, H. M., Ann. N. Y. Acad. Sc., 45, 395 (1944).
3. Bergmann, M. and Fruton, J. S., Ann. N. Y. Acad. Sc., 45, 409 (1944).
4. Borsook, H. and Dubnoff, J. W., J. Biol. Chem., 132, 307 (1940).

Another alternative for the synthesis of peptide bonds is that amino acids as such do not react to give peptides. Bergmann and Grafe[1] have shown that amino acid amides can react with keto acids to give dehydropeptides, which on reduction would yield peptides. At the present time there is no evidence that any such reactions take place in animal or plant tissue. The nearest approach has been the formation of glutamic acid in animal tissues from the keto acid and ammonia[2]. Such a mechanism would account, however, for the observation of Schoenheimer[3] that the labeled C or N supplied from one amino acid is soon found in all other amino acids except lysine. This migration of C and N may also be accounted for by the studies of transamination[4,5] or transmigration of methyl groups[6].

It is obvious that none of the proposals for the mechanism of the synthesis of peptides or proteins in vivo are based on complete evidence. None of the

1. Bergmann, M. and Grafe, K., Z. Physiol. Chem., 187, 187 (1930).
2. Von Euler, H., Adler, E., Gunther, G. and Das, N. B., Z. Physiol. Chem., 254, 61 (1938).
3. Schoenheimer, R. The dynamic state of body constituents. Cambridge. Harvard University Press. 1942.
4. Braunstein, A. E., Enzymologia, 7, 25 (1939).
5. Braunstein, A. R. and Kritzmann, M. G., Enzymologia, 2, 129 (1937).
6. Borsook, H. and Dubnoff, J. W., Ann. Rev. Biochem., 12, 183 (1943).

suggested mechanisms or series of reactions can, according to our present knowledge, completely account for either the energy required or the specificity of the final product. It can also be seen that from the viewpoint of a specific model for the study of the factors affecting the synthesis of peptide bonds, the formation of insoluble anilides from benzamino acids and aniline offers the simplest system for direct experimentation. Bergmann and his co-workers utilized this reaction rather extensively. Table I furnishes a summary of the compounds they prepared.

One experiment listed in Table I is especially interesting. Behrens and Bergmann[1] incubated acetyl-DL-phenylalanylglycine with glycinanilide in the presence of papain and obtained as the products of the reaction glycine, aniline, acetyl-DL-phenylalanylglycinanilide, and the unchanged acetyl-DL-phenylalanylglycine. They were able to isolate also a small amount of acetyl-DL-phenylalanylglycylglycinanilide. From these facts they concluded that the following sequence of reactions had taken place.

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

TABLE I

Summarized enzymatic peptide bond syntheses of Bergmann and collaborators

Substrates		Product	Enzyme	Reference
Carbobenzoxyglycine	Aniline	Carbobenzoxyglycinanilide	Papain	1
Benzoylglycine	Aniline	Benzoylglycinanilide	Papain	1
Benzoylglycinamide	Aniline	Benzoylglycinanilide	Papain	1
Carbobenzoxyglycine	Phenylhydrazine	Carbobenzoxyglycinphenylhydrazide	Papain	1
Benzoylglycine	Phenylhydrazine	Benzoylglycinphenylhydrazide	Papain	1
Benzoyl-DL-leucine	Aniline	Benzoyl-L-leucinanilide	Papain	1
Benzoyl-DL-phenylalanine	Aniline	Benzoyl-L-phenylalanylanilide	Papain	1
Acetyl-L-phenylalanine	Phenylhydrazine	Acetyl-L-phenylalanylphenylhydrazide	Papain	1
Benzoyl-L-alanine	Aniline	Benzoyl-L-alanylanilide	Papain	1
Benzoyl-DL-leucine	Aniline	Benzoyl-L-leucinanilide	Bromelin	1
Carbobenzoxyglycine	Aniline	Carbobenzoxyglycinanilide	Bromelin	1
Benzoyl-DL-leucine	Aniline	Benzoyl-L-leucinanilide	Cathepsin	1

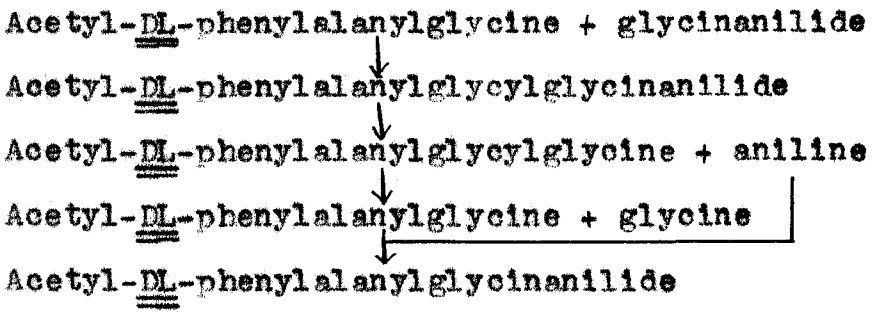
Table I (Continued)

Substrates		Product	Enzyme	Reference
Benzoyl-DL-leucine	Glycinanilide	Benzoyl-L-leucin-anilide	Papain	2
Acetyl dehydrophenyl-alanyl-L-glutamic acid	Aniline	Acetyl dehydro-phenylalanyl-L-glutamic acid monoanilide	Papain	2
Acetyl-L-phenylalanyl-L-glutamic acid	Aniline	Acetyl-L-phenyl-alanyl-L-glutamic acid monoanilide	Papain	2
p-Toluenesulfonylglycine	Aniline	p-Toluenesulfonyl-glycinanilide	Papain	2
Acetyl-DL-phenylalanyl-glycine	Glycinanilide	Acetyl-DL-phenyl-alanylglycine Glycine - Aniline Acetyl-DL-phenyl-alanylglycin-anilide	Papain	3
Carbobenzoxy-L-phenyl-alanylglycine	Glycinanilide	Carbobenzoxy-L-phenylalanyl-glycylglycin-anilide	Papain	3
Benzoyl-L-phenyl-alanylglycine	Glycinanilide	Benzoyl-L-phenyl-alanylglycin-glycinanilide	Papain	3
Carbobenzoxyphenyl-alanylglycine	L-Glutamic acid mono-anilide	Carbobenzoxyphenyl-alanylglycin-L-glutamic acid monoanilide	Papain	3
Carbobenzoxyglycine	L-Glutamic acid mono-anilide	Carbobenzoxyglycin-L-glutamic acid monoanilide	Papain	3

Table I (Continued)

Substrates		Products	Enzyme	Reference
Acetyl-L-phenylalanyl-glycylglycine	Aniline	Acetyl-L-phenylalanyl-glycinanilide	Papain	3
Carbobenzoxy-L-phenyl-alanylglycine	L-Tyrosin-amide	Carbobenzoxy-L-phenyl-alanylglycin-L-tyrosinamide	Papain	3
Benzoyl-L-tyrosine	Glycin-anilide	Benzoyl-L-tyrosyl-glycinanilide	Papain	4
Carbobenzoxy-DL-glutamic acid	Aniline	Carbobenzoxy-L-glutamic acid monoanilide	Papain	5
Acetyl-DL-phenyl-alanylglycine	Aniline	Acetyl-L-phenyl-alanylglycinanilide	Papain	6
Acetyl-L-phenyl-alanyl-L-leucine	Aniline	Acetyl-L-phenyl-alanyl-L-leucin-anilide	Papain	7
Acetyl-D-phenyl-alanyl-L-leucine	Aniline	Acetyl-D-phenyl-alanyl-L-leucin-anilide	Papain	7
Acetyl-aminocinnamoyl-L-leucine	Aniline	Acetyl-aminocinnamoyl-L-leucinanilide	Papain	7
Carbobenzoxy-L-phenyl-alanylglycine	Aniline	Carbobenzoxy-L-phenyl-alanylglycinanilide	Papain	7

1. Bergmann, M. and Fraenkel-Conrat, H., J. Biol. Chem., 119, 707 (1937).
2. Ibid., 124, 1 (1938).
3. Behrens, O. K. and Bergmann, M., J. Biol. Chem., 129, 587 (1939).
4. Bergmann, M. and Fruton, J., J. Biol. Chem., 124, 321 (1938).
5. Fruton, J. S., Irving, G. W. and Bergmann, M., J. Biol. Chem., 133, 703 (1940).
6. Bergmann, M. and Behrens, O. K., J. Biol. Chem., 124, 7 (1938).
7. Behrens, O. K., Doherty, D. G. and Bergmann, M., J. Biol. Chem., 136, 61 (1940).



This series of reactions demonstrates that hydrolytic and synthetic reactions proceed in the same reaction mixture and are catalyzed by the same enzyme preparation under identical experimental conditions. A similar mechanism may be utilized in the reaction between benzoyl-L-leucine and glycinanilide acetate to yield benzoyl-L-leucinanilide[1].

As a model of peptide bond synthesis, the synthesis of benzamino acid anilides as catalyzed by papain appears to be the only available method at the present time which fulfills both the energy and specificity requirements mentioned above. There are many unanswered questions remaining as to the manner in which these two requirements of protein synthesis are fulfilled in a living organism. No isolated model, however perfect, can be expected to exactly parallel protein and peptide bond synthesis *in vivo*.

1. Bergmann, M. and Fraenkel-Conrat, H., *J. Biol. Chem.*, *124*, 1 (1939).

However, in an effort to gain more information as to the mechanism of normal and abnormal growth, more data is first needed on the factors which affect peptide bond synthesis. One of the simplest methods of approach is the study of factors affecting the synthesis of benzamino acid anilides, even though this is an incomplete physiological model.

In all the experiments summarized in Table I, as well as in all enzymatic syntheses of simple peptides published by other workers, the citrate buffer concentration used was 0.2 molar or less. The majority of the experiments used buffers which were less than 0.1 molar. No definite study of the effect of changing the buffer concentration has been published in the literature.

The effect of pH on proteolytic enzymes has long been recognized[1] and schemes have been devised for a system of classification of proteolytic enzymes based on their pH optima. Grassman and Schneider[2] proposed a scheme for the classification of proteolytic enzymes. Recent studies indicate that such a classification system cannot be used for the optimum pH of a proteolytic enzyme depends upon the substrate. Bergmann and Fruton[3] have shown that carbobenzoxy-L-glutamyl-L-tyrosine is hydrolyzed by

1. Sorenson, S.P.L., Biochem. Z., 21, 131 (1909).
2. Grassman, W. and Schneider, F., Ergebnisse der Enzymforschung, 5, 81 (1936).
3. Bergmann, M. and Fruton, J. S., J. Biol. Chem., 127, 627 (1939).

crystalline pepsin to the greatest extent at pH 4.0 while the optimum pH for the hydrolysis of carbobenzoxy-L-glutamyl-L-phenylalanine is at pH 4.5. Harington and Rivers[1] found that the hydrolysis of carbobenzoxy tyrosylcysteine proceeded more rapidly and to a greater extent at pH 4.0 than at 1.8, the usual pH optimum for pepsin activity.

The effect of pH on the hydrolytic activity of papain has been the subject of limited experimentation. Leipert and Hafner[2] reported that papain hydrolyzed 47% of the peptide bonds in casein at pH 5.0. The optimum pH for the initial hydrolysis of casein by papain was found to be 7.0 by Lineweaver and Schwimmer[3], but at this pH only 25% of the peptide bonds were hydrolyzed. These authors also reported that for gelatin the optimum pH was 5.0, that in their studies both the crystallized papain and a commercial preparation showed the same effects with respect to pH. At pH 9.0 and 5.0 the rate of hydrolysis of casein was only 60% of the rate at pH 6.5-7.0. Rocha e Silva[4] studied the effect of pH on the hydrolysis of benzoylarginine amide

1. Harington, C. R. and Rivers, R. V. P., Biochem. J., 38, 417 (1944).
2. Leipert, T. and Hafner, I., Biochem. Z., 229, 427 (1930).
3. Lineweaver, H. and Schwimmer, S., Enzymologia, 10, 81 (1941).
4. Rocha e Silva, M. and Andrade, S. O., J. Biol. Chem., 148, 9 (1943).

and found two distinct pH optima, one at pH 5.0 and the other at 6.8 to 7.0. For egg albumin and hemoglobin the pH optimum for hydrolysis by papain was about 7.0 to 7.5, according to Greenberg and Winnick[1].

All of these observations were more or less isolated observations which were incidental to other problems. One recent systematic study on the effect of pH on the hydrolysis of proteins and synthetic peptides by papain which has attempted to correlate some of this conflicting data is that of Hoover and Kokes[2]. They attempted to find a differential effect of pH upon the extent of enzymatic hydrolysis. Commercial papain was purified by precipitation from an aqueous solution by the addition of methanol to give a concentration of 70%. They found that the optimum pH for the initial rapid hydrolysis of casein was 7.0, but at this pH only about 25% of the peptide bonds were split. At pH 5.0 the initial rate of hydrolysis was much slower, but 50% of the peptide bonds were hydrolyzed at this lower pH. They found that there was no appreciable difference in the stability of the enzyme preparation over this range of pH values. The same enzyme preparation was used for the hydrolysis of the following three synthetic

1. Greenberg, D. M. and Winnick, T., J. Biol. Chem., 135, 775 (1940).
2. Hoover, S. R. and Kokes, E. L. C., J. Biol. Chem., 167, 199 (1947).

substrates: benzoylargininamide, carbobenzoxy-L-isoglutamine, and hippurylamide. In each case the optimum pH was between 5.0 and 5.5. According to these results, the optimum pH for hydrolysis is partially a matter of definition, since hydrolysis proceeds more rapidly at pH 7.0 but to a greater extent at 5.0. These authors also suggest that papain may consist of at least two active fractions, the activity of the proteinase being highest at pH 7.0 and the activity of the peptidase being about 5.0. This suggestion is still hypothetical for papain has thus far not been resolved into two separate components whose activity and specificity are known.

The effect of pH on the synthesis of peptide bonds has been studied by Bergmann and Fraenkel-Conrat[1] for the reaction of carbobenzoxyglycine and aniline in the presence of papain to yield carbobenzoxyglycinanilide. Under the conditions of the experiment, they found the optimum pH to be about 4.7. This was about the same pH as that for the optimum hydrolysis of hippurylamide[2] and for L-leucinamide[3]. The close agreement between these three optima may be the reason why pH 5.0 citrate buffer has been

1. Bergmann, M. and Fraenkel-Conrat, H., J. Biol. Chem., 119, 707 (1937).
2. Bergmann, M., Zervas, L. and Fruton, J. S., J. Biol. Chem., 111, 225 (1935).
3. Behrens, O. K. and Bergmann, M., J. Biol. Chem., 129, 587 (1939).

used throughout most of the published studies on the synthesis of anilides as catalyzed by proteolytic enzymes.

The effect of concentration of substrates has apparently not been reported. There is some suggestion that Bergmann may have recognized the effect of increased ratios of aniline to benzamino acid, for in most of his experiments four equivalents of aniline were present for each equivalent of the L-form of the amino acid derivative.

With the exception of the studies of the effect of pH which have been previously mentioned, there appear to have been no published systematic studies of reaction conditions which affect enzymic peptide bond synthesis.

EXPERIMENTAL*

Preparation of Materials

General method for the synthesis of benzoylamino acids

The method of preparing the benzoyl derivatives of glycine, alanine, leucine, valine, phenylalanine, methionine and p-methoxyphenylalanine was essentially that of Ingersoll and Babcock[1] as given for benzoylglycine. Since these directions were followed with only slight modifications, the procedure will be given in detail for benzoylglycine and only the modifications, if any, will be listed for the other benzoylamino acids. This procedure was found to be applicable for these amino acids for quantities ranging from 0.1 to 1.0 mole. The procedures of Steiger[2] and Carter and Stevens[3] were also found to give the benzoyl derivatives in good yields.

Benzoylglycine

Thirty grams (0.4 mole) of glycine (Aminoacetic acid,

* All melting points are uncorrected.

1. Ingersoll, S. W. and Babcock, S. H., Organic Syntheses, Coll. 2, 328 (1943).
2. Steiger, R. E., J. Org. Chem., 9, 396 (1944).
3. Carter, H. E. and Stevens, C. M., J. Biol. Chem., 138, 627 (1941).

(Merck) was added to 400 ml. (0.4 mole) of 1.0 M sodium hydroxide in a one liter, three neck, round bottom flask. The flask was fitted with two dropping funnels and an efficient motor stirrer. With vigorous stirring, 56 g. (0.4 mole) of benzoyl chloride and 200 ml. (0.4 mole) of 2 N sodium hydroxide were admitted separately from the dropping funnels at approximately equivalent rates. The solution was kept cold during the addition of the reagents by means of an ice bath which was removed when the addition was complete. The stirring was continued for the next half hour; the reaction mixture had then warmed to room temperature. At this time no odor of benzoyl chloride could be detected. The reaction mixture was transferred to a liter beaker and acidified to about pH 3, as per Congo red indicator paper. After cooling for two hours in the refrigerator, the precipitate was filtered off, all clumps were broken up, and the solid was washed on the filter three times with 100 ml. portions of water. The dried precipitate was boiled for ten minutes with 250 ml. of carbon tetrachloride to remove benzoic acid. The benzoylglycine was allowed to dry in air after filtering from the hot carbon tetrachloride. The product weighed 70 g. For final purification the acid was recrystallized from 500 ml. of 50% ethanol. The long white needles melted at 186-187°. Ingersoll and Babcock[1] reported

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

the m.p. as 187-188°.

Benzoyl-DL-alanine

Benzoyl-DL-alanine was prepared from DL-alanine (Dow, technical grade) and benzoyl chloride using the procedure given above for benzoylglycine. The product obtained from the hot carbon tetrachloride treatment (750 ml. carbon tetrachloride for 0.5 mole of benzoyl-DL-alanine) in one case had a melting point which was the same as that of the material obtained from repeated crystallization. There was no depression when a mixed melting point was taken with a pure sample, m.p. 165-166°. The m.p. is reported in the literature as 165-166° [1]. The yield of several syntheses varied from 87-94%.

Benzoyl-DL-leucine

In the preparation of benzoyl-DL-leucine from DL-leucine (Dow, technical grade) the only modification necessary was due to the tendency of the benzoyl-DL-leucine to oil when precipitated from the alkaline reaction mixture with hydrochloric acid. Purification of the product was facilitated if the oil was crystallized by scratching or seeding before cooling in the refrigerator. Similarly, after the carbon tetrachloride treatment, the suspension was not filtered

1. Fischer, E., Ber., 32, 2451 (1899).

until the mixture had cooled slightly to permit crystallization of the oil which formed upon boiling. The crystals obtained melted at 140-141°. The m.p. is reported in the literature as 137-141° [1]. The yield of several syntheses varied from 83% to 94%.

Benzoyl-DL-valine

Benzoyl-DL-valine was prepared from DL-valine (Dow, Technical grade) with the procedure given above. The melting point was 130-131°. Slimmer[2] reported the m.p. as 132.5°.

Benzoyl-DL-phenylalanine

Benzoyl-DL-phenylalanine was prepared from DL-phenylalanine (Dow, Technical grade) with the procedure given above. The product melted at 187-188°. Fischer reported the m.p. as 187-188° [3]. The yields obtained were 80-97% of theory.

Benzoyl-DL-methionine

The usual procedure was followed in the synthesis of benzoyl-DL-methionine. An almost quanitative yield was obtained of white glistening plates melting at 152° after one

1. Fischer, E., Ber., 33, 2370 (1900).
2. Slimmer, M. D., Ber., 35, 400 (1902).
3. Fischer, E. and Mouneyrat, A., Ber., 33, 2383 (1900).

recrystallization from 60% ethanol. The melting point was reported by Hill and Robson as 151° [1].

O,N-Dibenzoyl-L-tyrosine

O,N-Dibenzoyl-L-tyrosine was prepared from L-tyrosine (Merck) and benzoyl chloride by a slight modification of the procedure for benzoylglycine. Thirty-six grams (0.2 mole) of L-tyrosine were added to 200 ml. (0.2 mole) of 1.0 N sodium hydroxide solution in a three neck, round bottom flask fitted with a stirrer and two dropping funnels. To this solution were added 56 g. (0.4 mole) of benzoyl chloride and 400 ml. (0.4 mole) of 1.0 N sodium hydroxide at equivalent rates. The reaction mixture was kept cold during the course of the addition which required about one hour. After the addition was complete, the mixture was stirred for one-half hour more. The solution was transferred to a beaker and acidified with 6 N hydrochloric acid. A dense white precipitate was formed at once which crystallized upon stirring. The crude product was filtered off, washed with water, and dried in air. The solid was then boiled with 800 ml. carbon tetrachloride, filtered while hot and again dried in air. Final purification was effected by recrystallization from 800 ml. of 60% ethanol. The recrystallized material melted at 210-211°. Schultze reported the m.p. as 211-212° [2].

1. Hill, E. M. and Robson, W., Biochem. J., 30, 248 (1936).
2. Schultze, A., Z. Physiol. Chem., 29, 479 (1900).

Anal: Calcd. for $C_{21}H_{17}O_5N$: neut. equiv., 326

Found: neut. equiv., 324

$[\alpha]_D^{24}$ - 17.5° ± 0.2° (4.0% in pyridine)

DL-Tyrosine

Thirty-six g. (0.2 mole) of L-tyrosine (Huron Mills, technical grade) was refluxed on an oil bath with 120 ml. of technical grade glacial acetic acid and 30 ml. of technical grade 85% acetic anhydride for one hour.[1] The mixture was concentrated under reduced pressure to a heavy syrup. This was refluxed for one hour with 200 ml. of 5 N hydrochloric acid solution.

The solution was again concentrated to a heavy syrup, and treated with 100 ml. of water. This concentration was twice repeated with the addition of water, 5 g. of Darco G-60 was added, and the solution heated to boiling and filtered. The pH was brought to 5.0 ± 0.1 with sodium hydroxide and the solution was allowed to stand overnight in the refrigerator. The dried precipitate weighed 31.5 g. (85% recovery) The color of the product was yellow, but the material was satisfactory as an intermediate in further syntheses. The product showed no rotation on the polarimeter.

1. Fling, Preparation of amino acids and derivatives and their effect on the growth of Lactobacillus arabinosus. Unpublished Ph. D. thesis. Ames, Iowa. Iowa State College Library. 1946.

O,N-Dibenzoyl-DL-tyrosine

The DL-tyrosine obtained from the racemization procedure was benzoylated in the same manner as the L-tyrosine, using a 3:2:1 molar ratio of sodium hydroxide, benzoyl chloride and DL-tyrosine respectively. Forty-eight grams of DL-tyrosine when benzoylated, gave a 71% yield of material melting at 226-228° after one recrystallization from 50% ethanol.

Anal: Calcd. for $C_{21}H_{17}O_5N$: N, 3.60%

Found: N, 3.61%

DL-Tyrosine ethyl ester hydrochloride

This compound was prepared according to the directions of Rohmann[1]. A suspension of 30 g. of L-tyrosine (Merck) in 1000 ml. of absolute ethanol was treated with dry hydrogen chloride for four hours. The solution obtained was refluxed for two and one-half hours and left to stand overnight. The solution was evaporated to dryness on a water bath under reduced pressure. The dried residue was dissolved in 100 ml. of ethanol, and the DL-tyrosine ethyl ester hydrochloride was precipitated with 800 ml. of dry ether. The oil obtained was crystallized by scratching and cooling.

1. Rohmann, F., Ber., 30, 1979 (1897).

The slightly gummy, yellow crystals were dissolved in 100 ml. of ethanol, heated for 5 minutes with 5 g. of Norite-A and filtered through a thin layer of Super-Cel filter aid. The ester was precipitated from this solution by adding 2 liters of dry ether. The oil which formed was readily crystallized with a seed of the crude material. After drying overnight under vacuum, the material weighed 18 g. (45%), m.p. 155°. The nitrogen analysis of this compound gave consistently low results, in spite of repeated crystallization. The analyses for chlorine agreed with the calculated value. The nitrogen analysis of the compounds prepared from this intermediate gave values which agreed with the calculated values.

Anan: Calcd. for $C_{11}H_{16}O_3NCl$: Cl, 14.4%

Found: Cl, 14.3%

N-Benzoyl-DL-Tyrosine ethyl ester hydrochloride

The procedure of Fox[1] for N-benzoyl-L-diiodotyrosine ethyl ester was followed. Twenty-five g. (0.1 mole) of tyrosine ethyl ester was dissolved in 100 ml. of water, and treated with 200 ml. (0.4 mole) of 2 N sodium carbonate solution, using a large separatory flask. After shaking vigorously, the mixture was extracted with two 100 ml. portions of ethyl acetate. The ethyl acetate fraction was placed in a separatory flask and was treated with 12 ml.

1. Fox, S. W., J. Am. Chem. Soc., 68, 194 (1946).

(0.1 mole) of benzoyl chloride and 50 ml. (0.1 mole) of 2 N sodium carbonate. These reagents were added in four approximately equal portions. The mixture was shaken vigorously for one-half hour. No more carbon dioxide was evolved after the first ten or fifteen minutes. The ethyl acetate layer was separated, washed twice with 100 ml. portions of water, dried with 15 g. of Drierite, and evaporated to dryness in an evaporating dish. The oily residue crystallized on cooling in the refrigerator. The material obtained from recrystallizing from a 1:9 mixture of ethyl acetate and benzene weighed 27 g. (82%), m.p. 119-120°. A small portion was again recrystallized from ethyl acetate-benzene for analysis, m.p. 119-121°.

Anal: Calcd. for $C_{18}H_{19}O_4N$: N, 4.5%

Found: N, 4.5%

N-Benzoyl-DL-Tyrosine

The procedure of Fox[1] for N-benzoyl-L-diiodotyrosine was followed. Twenty g. (0.065 mole) of N-benzoyl-DL-tyrosine ethyl ester hydrochloride was added to 50 ml. of 5 N sodium hydroxide solution and the mixture was heated under reflux for twenty minutes on the water bath. After five minutes all the solid material present had gone into solution. The solution was diluted to 150 ml. with water and then acidified with 6 N hydrochloric acid to approximately pH 3 with Congo red indicator paper. A white

1. Fox, S. W., J. Am. Chem. Soc., 68, 194 (1946).

oily precipitate formed at once which solidified on cooling. This solid was washed with 50 ml. of water and dried in air. After recrystallizing from hot 30% ethanol and drying in a vacuum, 16 g. (33%) of N-benzoyl-DL-tyrosine was obtained, m.p. 195-196°. Fischer reported the melting point as 195-197° [1].

Anal: Calcd. for $C_{16}H_{14}O_3N$: N, 4.9%

Found: N, 4.9%

L-Tyrosine ethyl ester hydrochloride

L-Tyrosine ethyl ester hydrochloride was prepared from L-tyrosine (Merck) using the same method as given for the DL-tyrosine ethyl ester hydrochloride. The ester hydrochloride was obtained in 83% yield, m.p. 163-165°. Rohmann reported a melting point of 166° [2].

N-Benzoyl-L-Tyrosine ethyl ester hydrochloride

N-Benzoyl-L-tyrosine ethyl ester hydrochloride was prepared from the ester hydrochloride and benzoyl chloride in the same manner as described for the N-benzoyl-L-tyrosine ethyl ester hydrochloride. The small white crystals obtained from recrystallization from ethyl acetate-benzene

1. Fischer, E., Ber., 32, 3638 (1900).
2. Rohmann, F., Ber., 30, 1879 (1900).

1:9, melted at 112-114°.

N-Benzoyl-L-tyrosine

N-Benzoyl-L-tyrosine was prepared in the same manner as the N-benzoyl-DL-tyrosine. The oil obtained from the hydrolysis could be crystallized by rubbing under hexane and chilling. Upon recrystallization from ethyl acetate-benzene, small white rosettes were obtained which melted at 154°. After recrystallization from ethyl acetate-benzene, 1:9, the melting point was 165-166°. Fischer[1] reported the m.p. as 165-166°.

Benzoyl-p-methoxy-L-phenylalanine

This compound was prepared according to the usual procedure from p-methoxy-L-phenylalanine and benzoyl chloride. The method of Behr and Clarke[2] was used to synthesize the p-methoxy-L-phenylalanine, which melted at 264-265°[3]. The rotation observed for the methoxy-L-phenylalanine was $[\alpha]_D^{23} = -5.45° \pm 0.73°$ for a 2.21% solution in 1 N hydrochloric acid. The benzoyl derivative melted at 135-137° and the rotation observed was $[\alpha]_D^{24} = -3.78° \pm 0.98°$

1. Fischer, E., Ber., 32, 3638 (1900.
2. Behr, L. D. and Clarke, H. T., J. Am. Chem. Soc., 54, 1630 (1932).
3. This compound was prepared by S. W. Fox.

for a 2.76% solution in ethanol. Behr and Clarke[1] reported the melting point of the methoxy-L-phenylalanine as 264-265°, that of the benzoyl derivative as 134-136°. The values for the specific rotation of these compounds cannot be compared with those previously reported, since Behr and Clarke used the green line of mercury for their determinations.

General method for preparing the carbobenzoxyamino acids

The procedure of Bergmann and Zervas[2] for preparing the carbobenzoxy derivatives of glycine, alanine and phenylalanine was followed for preparing these compounds as well as the carbobenzoxy derivatives of leucine and valine.

Carbobenzoxy-DL-alanine

A solution of 44.5 g. (0.5 mole) of DL-alanine in 250 ml. of 2 N sodium hydroxide was placed in a three neck, round bottom flask fitted with an efficient motor stirrer and cooled in an ice bath. This solution was treated with 250 ml. of 2 N sodium hydroxide and 85 g. (0.5 mole) of carbobenzoxy chloride. The reagents were added in approximately equivalent rates through separatory funnels. After

1. Behr, L. D. and Clarke, H. T., J. Am. Chem. Soc., 54, 1630 (1932).
2. Bergmann, M. and Zervas, L., Ber., 65, 1192 (1932).

the addition had been completed, the ice bath was removed and the solution stirred for one-half hour more. The solution was acidified with 6 N hydrochloric acid to about pH 3 with Congo red indicator paper. The oil which separated crystallized upon stirring and cooling. After standing for two hours in the refrigerator, the precipitate was filtered off, washed twice with 100 ml. portions of water and dried in the air. The air dry solid was washed twice with 300 ml. portions of hexane. The carbobenzoxy-DL-alanine obtained weighed 92 g. (82%) and melted at 110-112°. Bergmann and Zervas found the melting point to be 114-115° [1].

Anal: Calcd. for $C_{11}H_{13}O_4N$: N, 6.70%

Found: N, 6.73%

Carbobenzoxy-DL-phenylalanine

Carbobenzoxy-DL-phenylalanine was prepared from 83 g. (0.5 mole) of DL-phenylalanine and 85 g. (0.5 mole) of carbobenzoxy chloride according to the procedure given above for carbobenzoxy-DL-alanine. After washing the product obtained with hexane, 142 g. (94%) of carbobenzoxy-DL-phenylalanine was obtained, melting at 89-90°. Bergmann and Zervas[2] reported a melting point of 103°. An analytical sample was recrystallized from carbon tetrachloride, a sample melting at 101-102°.

1. Bergmann, M. and Zervas, L., Ber., 65, 1192 (1932).
2. Ibid.

Anal: Calcd. for $C_{17}H_{17}O_4N$: N, 4.68%

Found: N, 4.59%

Carbobenzoxy-DL-valine[1]

Carbobenzoxy-DL-valine was prepared from 58.5 g. of DL-valine and 85 g. (0.5 mole) of carbobenzoxy chloride according to the usual procedure. Upon acidification an oil was obtained which at first resisted efforts at crystallization. After washing the oil with three 500 ml. portions of water, decanting each time, and finally rubbing under several 300 ml. portions of hexane, 113 g. (90%) of crystals were obtained which melted at 74-76°.

Anal: Calcd. for $C_{13}H_{17}O_4N$: N, 5.55%

Found: N, 5.56%

Carbobenzoxy-DL-leucine[2]

This compound was prepared from 66 g. of DL-leucine and 85 g. (0.5 mole) of carbobenzoxy chloride in the usual manner. This preparation also resulted in an oil which was crystallized using the same treatment which was successful in obtaining crystalline carbobenzoxy-DL-leucine. The weight of the product was 125 g. (94%) after washing, m.p. 46-49°.

Anal: Calcd. for $C_{14}H_{19}O_4N$: N, 5.27%

Found: N, 5.24%

1. Previously prepared and characterized by Fling, M., Wax, H. and Fox, S. W., Unpublished experiments.
2. Ibid.

Chloracetylaniline

Following the procedure of Abderhalden[1] with slight modifications, 47 g. (0.5 mole) of aniline in 250 ml. of dry benzene was treated slowly with 28 g. (0.25 mole) of chloracetyl chloride. The reaction was carried out in a three neck, round bottom flask fitted with a motor stirrer and cooled in an ice bath. After all the chloracetyl chloride had been added, the stirring was continued for one-half hour more. At this time the mixture was very heavy with crystals. The mixture was cooled in the refrigerator, the dark, red-brown crystals were filtered off and washed with three 100 ml. portions of benzene and two 50 ml. portions of water. After drying in the vacuum desiccator, 61 g. of light tan crystals were obtained, melting at 130-134°. Abderhalden[2] reported a melting point of 135°.

Glycinanilide

The procedure of Hill and Kelsey[3] was modified slightly. One thousand ml. of 95% ethanol was saturated with ammonia by rapidly bubbling the gas through the ethanol

1. Abderhalden, E. and Brockmann, H., Fermentforschung, 10, 164 (1928).
2. Ibid.
3. Hill, A. J. and Kelsey, E. B., J. Am. Chem. Soc., 42, 1709 (1920).

while cooling the mixture in an ice bath. When saturated the ethanol contained 135 g. (8.0 moles) of ammonia. To this solution was added 61 g. (0.4 mole) of chloracetylaniline; the mixture was placed in a glass lined pressure bomb and allowed to stand at room temperature for five days. At the end of this period, the solution was evaporated down to dryness under reduced pressure. This crude material was purified by two different methods.

For one portion the procedure of Fox and Halverson[1] was used. Twenty g. of the dried residue was treated with 34 g. of picric acid dissolved in 2000 ml. of hot water. A yellow flocculent precipitate formed at once. This precipitate was filtered off after standing twenty-four hours in the cold. After drying in air, the yellow crystals were washed with 300 ml. of benzene, then recrystallized from boiling water. After drying, the picrate melted at 190-191°. Abderhalden[2] reports a melting point of 186°. Forty grams of this picrate was dissolved in 2000 ml. of chloroform and then treated with 1225 ml. of water and 17 ml. of 10 N sodium hydroxide. The chloroform layer was separated and extracted with 750 ml. of water and 5 ml. of 10 N sodium hydroxide. After separating, the chloroform layer was dried with 20 g. of Drierite and then evaporated

1. Fox, S. W. and Halverson, J. S., Unpublished experiments.
2. Abderhalden, E. and Brockmann, H., Fermentforschung, 10, 164 (1928).

to dryness under reduced pressure. The residue left was gummy and could not be crystallized by rubbing under hexane. The gum was redissolved in 1000 ml. of chloroform and was again extracted with 500 ml. of 1 N sodium hydroxide. After drying over 20 g. of Drierite, the chloroform solution was evaporated to dryness. Almost colorless small crystals were obtained, melting at 60-62°. Hill and Kelsey report the dihydrate of glycinanilide as melting at 61-62° [1].

The second method of purification of glycinanilide utilized the differences in solubility between the glycinanilide and the secondary base in water. The dried residue was suspended in 3 parts of hot water and the insoluble secondary base filtered off. The filtrate was decolorized with Norite-A and then cooled in an ice bath. The cold solution was saturated with ammonia gas. The crystals which formed were filtered off and dried under reduced pressure for three days.

Anal: Calcd. for $C_9H_{10}O_3N$ .2 H_2O 15.05%

Found: N, 15.15%

Benzoyl-DL-leucinamide

The procedure of Max[2] was slightly modified. Six g. (0.0255 mole) of benzoyl-DL-leucine was covered with 14 ml. of acetyl chloride in a stoppered flask and chilled

1. Hill, A. J. and Kelsey, E. B., J. Am. Chem. Soc., 42, 1704 (1920).
2. Max, J., Ann., 369, 276 (1902).

in a freezer for about 10 minutes. The chilled mixture was treated with 5.5 g. (0.0265 mole) of phosphorus pentachloride in one portion. Upon shaking the mixture, all of the solid material went into solution. After 10 minutes the reaction mixture was cooled with cold running water and 100 ml. of dry hexane was added. There was formed at once a dense white precipitate which was filtered off and washed with two 150 ml. portions of dry hexane. The acid chloride was utilized at once without purification or identification. The crude chloride was added to 150 ml. of ammonia-saturated anhydrous ether which had been prepared by bubbling ammonia into 150 ml. of ether cooled in an ice bath. Additional ammonia was bubbled into the mixture after the addition of the chloride for about 25 minutes. The solid material present was filtered off and dried in the air. The dry material was washed first with 50 ml. of water, then with 50 ml. of 10% sodium carbonate solution and then with two more 25 ml. portions of water. The product weighed 3 g. after recrystallization from 1500 ml. of water. M.p. 169-170°. The reported m.p. was 168°.

Benzoyl-DL-valinamide

This compound was prepared by the same procedure as for benzoyl-DL-leucinamide. Six g. (0.024 mole) of

benzoyl-DL-valine gave 2.1 g. of benzoyl-DL-valinamide, m.p. 220-221°. Fox[1] reports the m.p. as 217-219°.

Enzymatic hydrolysis of amides

For these experiments 0.002 mole of each of the benz-amino acid amides were incubated with 10 mg. of papain and 10 mg. of cysteine hydrochloride in 10 ml. of 1.0 M citrate buffer, pH 5.0. The incubations were carried out in tightly stoppered tubes at 40° C. for three days. At the end of this time, any solid material present was filtered off and the filtrates analyzed for ammonia by a modification of the Folin method[2]. This modification utilizes the fact that ammonia is cold distilled more rapidly from a solution made alkaline with sodium borate, than from any other sodium salt[3]. In addition, the use of sodium carbonate might also be disadvantageous if acidic buffers were used, since the liberation of carbon dioxide would interfere with the subsequent titration.

Ammonia and moisture free air was bubbled through a solution of 2 ml. of the filtrate from the amide hydrolysis mixtures described above, and 5 ml. of a saturated solution of sodium borate. The effluent air was then bubbled

1. Fox, S. W., Unpublished experiments.
2. Hawk, P. B., Oser, B. L. and Summerson, W. H., Practical Physiological Chemistry. Philadelphia, Blakiston Co. 1947. p. 828.
3. Conway, E. J., Micro-diffusion analysis and volumetric error. London, C. Lockwood & Son Ltd. 1939.

through 10 ml. of 1% boric acid solution. This cold distillation of ammonia was continued for three hours at a rate of about three bubbles of air per second. The boric acid solution was then titrated with 0.01 M standard acid using modified methyl red indicator[1]. From these titrations the amount of ammonia could be determined and the extent of hydrolysis of the amides calculated. This procedure, when checked with known amounts of ammonium sulfate, gave 95% - 3% recovery.

Method of treating papain

The procedure of Grassmann[2] as modified by Bergmann and Fraenkel-Conrat[3] was used. Twenty-five g. of a commercial papain preparation was suspended in 1000 ml. of water and stirred for twenty minutes. The insoluble material was filtered off and discarded. Hydrogen sulfide was bubbled through the filtrate for four hours. To this solution was added 1500 ml. of absolute methanol. A precipitate formed at once. After standing overnight in the refrigerator, the precipitate was filtered off, resuspended in 1000 ml. of water and the hydrogen sulfide treatment repeated. The precipitated enzyme recovered from this

1. 83 mg. methylene blue and 125 mg. methyl red in 100 ml. 95% ethanol.
2. Grassmann, W., Biochem. Z., 279, 131 (1935).
3. Bergmann, M. and Fraenkel-Conrat, H., J. Biol. Chem., 119, 707 (1939).

second treatment was washed first with two 250 ml. portions of methanol, then with 250 ml. of absolute methanol, and finally with 250 ml. of dry ether. The precipitate was dried under reduced pressure over phosphorus pentoxide, and was stored at 5° in a desiccator.

General method for the synthesis of benzamino acid anilides

All experiments were carried out with the same general procedure for adding the components of the incubation mixture and for obtaining and purifying the products of the reactions. Typically, 3 ml. of 3.0 M citric acid solution and 0.23 g. of redistilled aniline were added to 6.0 ml. of a 1 N sodium hydroxide solution containing the stated amount of benzoylated amino acid. To this was added 2.0 ml. of an enzyme solution prepared by dissolving 2.00 g. of papain[1] in 80 ml. of a 1.0 M citrate buffer solution (pH 5.0), filtering through pyrex wool, and treating the filtrate with 2.00 g. of cysteine hydrochloride (Merck). The solution was brought to 45 2 ml. by use of the appropriate buffer solution. The pH was adjusted to the indicated value with 10 N sodium hydroxide solution with a glass electrode.

1. Unless stated specifically, all enzymatic reactions used commercial papain. These studies utilized preparations obtained from Merck, Difco, and Nutritional Biochemicals. Comparable results were obtained from all of the preparations used when checked against the ability of the enzyme to catalyze the synthesis of benzoylglycinanilide and benzoyl-L-alaninanilide.

The volume was brought to 50 ml. with 1.0 M citrate buffer of the proper pH, the incubation mixture was placed in 22 x 150 mm. test tubes, stoppered tightly, and incubated for 72 hours at 40°C. The tubes were shaken by hand at approximately hourly intervals throughout the working day. The products were filtered, washed first with a small amount of water, then with two 10 ml. portions of 1 N sodium hydroxide solution and then copiously with water. The precipitates were allowed to dry in air a minimum of twelve hours, and were then weighed. In each case the melting point of the products obtained through this washing technique were in close agreement with the melting points of analytically pure samples. For the preparation of large amounts of the anilides, the modifications which were made in this general procedure are specifically stated. Similarly, in studying the effect of various factors on the synthesis of benzamino acid anilides, all modifications are stated in the specific procedure.

Procedure for the synthesis of carbobenzoxyamino acid anilides

Two-tenths of a mole of the carbobenzoxyamino acid was weighed out into a liter Erlenmeyer flask and covered with 650 ml. of 1.0 M citrate buffer, pH 5.0. To this mixture was added 55.5 g. (0.6 mole) of redistilled aniline, and 300 ml. of a filtered 1.0 M citrate buffer solution

containing 2.0 g. of papain and 1.5 g. of cysteine hydrochloride. The volume was made up to 1000 ml., after adjusting the pH to 5.0 with 10 N sodium hydroxide. The flasks were tightly stoppered and incubated at 40°. After incubating for the period of time indicated in Table II, the solid material present was filtered off, the precipitate washed first with two 400 ml. portions of water to remove water soluble impurities, then with two 300 ml. portions of 3 N hydrochloric acid to remove any excess aniline. The solid residue was then treated with two 100 ml. portions of 1 N sodium hydroxide to dissolve any residual carbobenzoxyamino acid and was finally washed copiously with water.

Table II

Yields of carbobenzoxyamino acid anilides

Carbobenzoxyamino acid	Weight, g.	Time of incubation, days	Weight of anilide, g.	Yield percent
Carbobenzoxy-DL-valine	50.2	7	14.4	44
Carbobenzoxy-DL-leucine	53.0	8	12.7	37
Carbobenzoxy-DL-alanine	44.6	3	28.0	94
Carbobenzoxy-DL-phenylalanine	60.0	3	36.2	97

The anilides were recrystallized from 50% ethanol. The melting points, rotations, and nitrogen analyses for the anilides are reported in Table III.

Table III

Properties of Carbobenzoxyamino acid anilides

Compound	Melting point	N calcd. %	N found %	$[\alpha]_D^{24}$
Carbobenzoxy-L-leucinanilide	139-141°	8.22	8.11	
Carbobenzoxy-L-valinanilide	182-184°	8.52	8.47	
Carbobenzoxy-L-phenylalanin-anilide	158-159°	7.50	7.38	- 46.9° - .7° [1]
Carbobenzoxy-L-alaninanilide	164-165°	9.40	9.27	- 12.0° - .6° [2]

1. c = 3.7% in chloroform
2. c = 4.1% in chloroform

RESULTS

Effect of pH on Enzymatic Peptide Bond Synthesis

Since it is known that enzyme activity is affected by the pH of the reaction media, and in particular that the synthesis of carbobenzoxyglycinanilide as catalyzed by papain is affected by the pH of the buffer solution used, it was desirable to ascertain whether this effect was general for other amino acids. For this reason the benzoyl derivatives of glycine, DL-alanine, DL-leucine, DL-phenylalanine, p-methoxy-L-phenylalanine, L-tyrosine, and the O,N-dibenzoyl derivative of L-tyrosine were incubated with aniline in the presence of papain in citrate buffers ranging from pH 3.0 to 7.5. The identity of the products was established in each case by melting points and mixed melting points with samples whose identity had been established by analysis.

The data presented in Fig. 1 (p. 56) is typical of many of the results obtained. In this figure, the percent yield of benzoylglycinanilide obtained is plotted against the pH of the incubation solution. In Curve 1 is depicted the yield of anilide obtained from 0.005 mole of benzoylglycine and 0.005 mole of aniline when incubated with 50

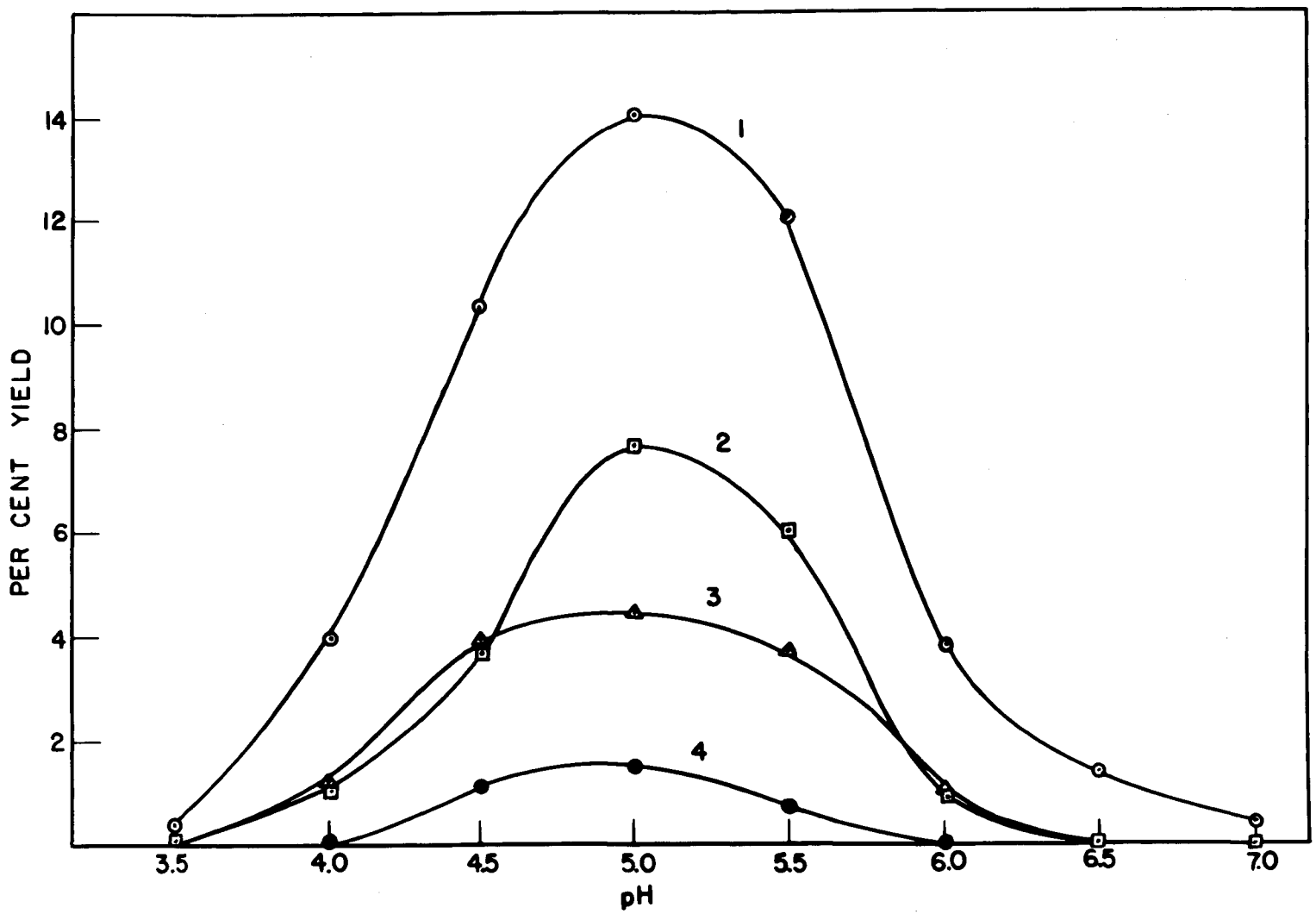


Fig. 1. Effect of pH and buffer concentration in yield of benzoylglycinanilide.

mg. of papain and 50 mg. of cysteine hydrochloride in 1.0 M buffers of the indicated pH values. The total volume of the incubation solution was 50 ml. Similarly, Curve 2 presents the data from an experiment in which all conditions were the same except that 0.1 M citrate buffer was used rather than 1.0 M buffer. Curves 3 and 4 represent the data obtained from similar experiments using 1.0 and 0.1 M citrate buffers respectively, but with only half the amount of benzoylglycine and half the amount of aniline used in the first two series. Under these conditions, the pH optima for the enzymatic synthesis of benzoylglycinanilide was virtually independent of the buffer concentration and the concentration of the reactants.

The data for the effect of pH on the yield of benzoyl-L-alaninanilide is presented in Fig. 2 (p. 58). Curve 1 presents the yields of benzoyl-L-alaninanilide obtained when 0.005 mole of benzoyl-DL-alanine and 0.0025 mole of aniline were incubated with papain in 1.0 M citrate buffers of the indicated pH. Curve 2 represents a similar series using 0.1 M buffer. As was the case for benzoylglycinanilide, the pH optimum for the synthesis of benzoyl-L-alaninanilide was independent of buffer concentration for the experimental conditions used.

A different type of curve was obtained for the effect of pH on the yield of benzoyl-L-leucinanilide. In Fig. 3

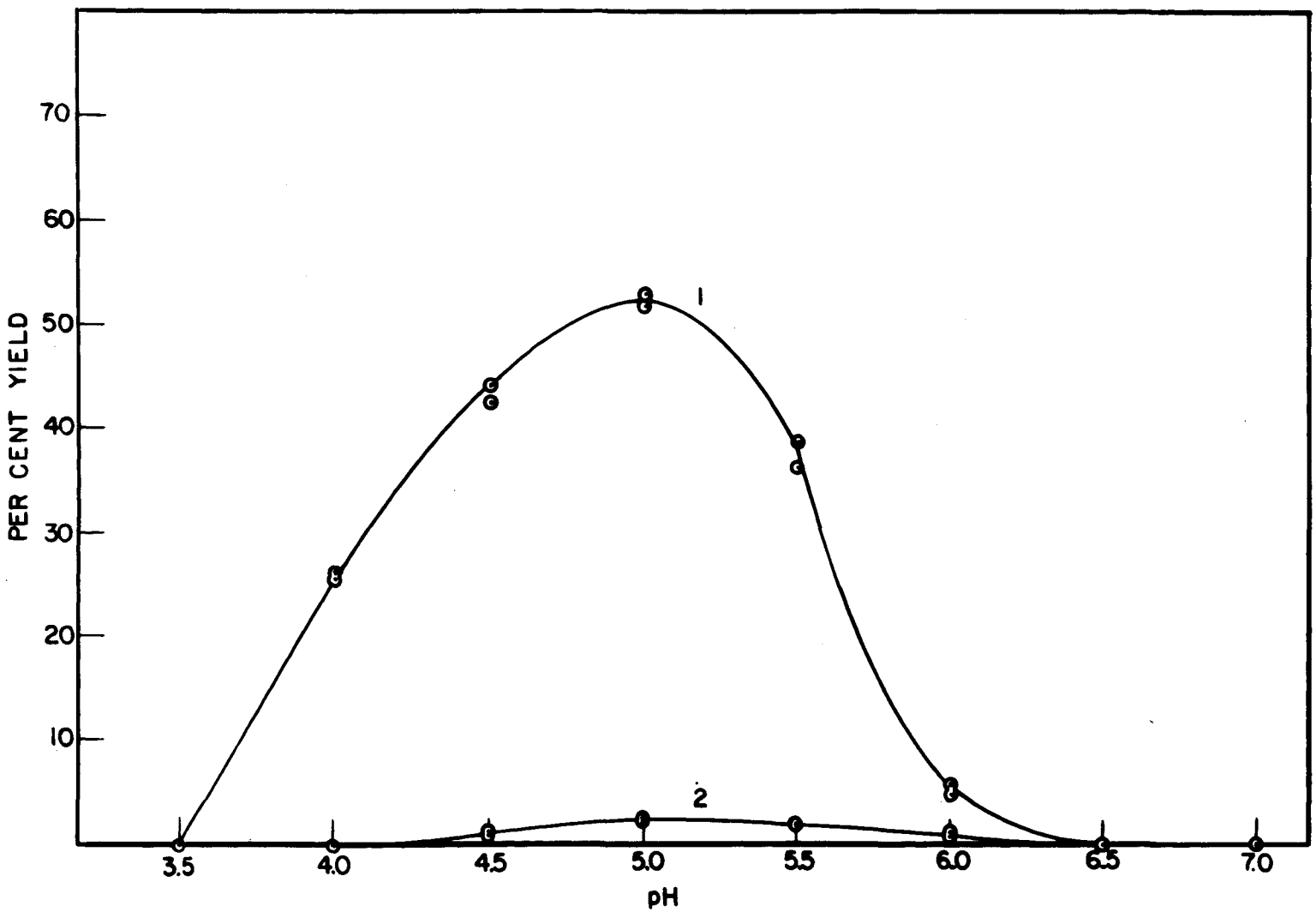


Fig. 2. Effect of pH and buffer concentration on yield of benzoyl-L-alaninanilide.

(p. 60) are plotted the yields of benzoyl-L-alaninanilide (Curve 1) and benzoyl-L-leucinanilide (Curve 2). In both series the conditions were those that have been described in the Experimental section for the general method of preparing the benzamino acid anilides. The experiment using benzoylalanine was repeated as a confirmation of the first series using benzoylalanine (Fig. 2), since different lots of the commercial papain were used in Fig. 2 and Fig. 3. The results using these two different lots of enzyme were comparable within the limits of experimental error. The optimum pH for the synthesis of benzoyl-L-leucinanilide was about pH 6.2, while the optimum pH for the synthesis of benzoylglycinanilide and benzoyl-L-alaninanilide was about pH 5.0.

In Fig. 4 (p. 61) is represented the effect of pH on the yield of benzoyl-L-phenylalaninanilide (Curve 1) and benzoyl-p-methoxy-L-phenylalaninanilide (Curve 2). In each case the optimum pH for the papain catalyzed synthesis was about pH 6; the yield of anilide becoming less as the incubation solution became more acidic or more alkaline.

For N-benzoyl-L-tyrosine anilide and for O,N-dibenzoyl-L-tyrosinanilide the pH optimum for synthesis was about pH 6 or slightly higher. The effect of pH on the yield of these anilides is represented by Fig. 5 (p.63). Curve 1

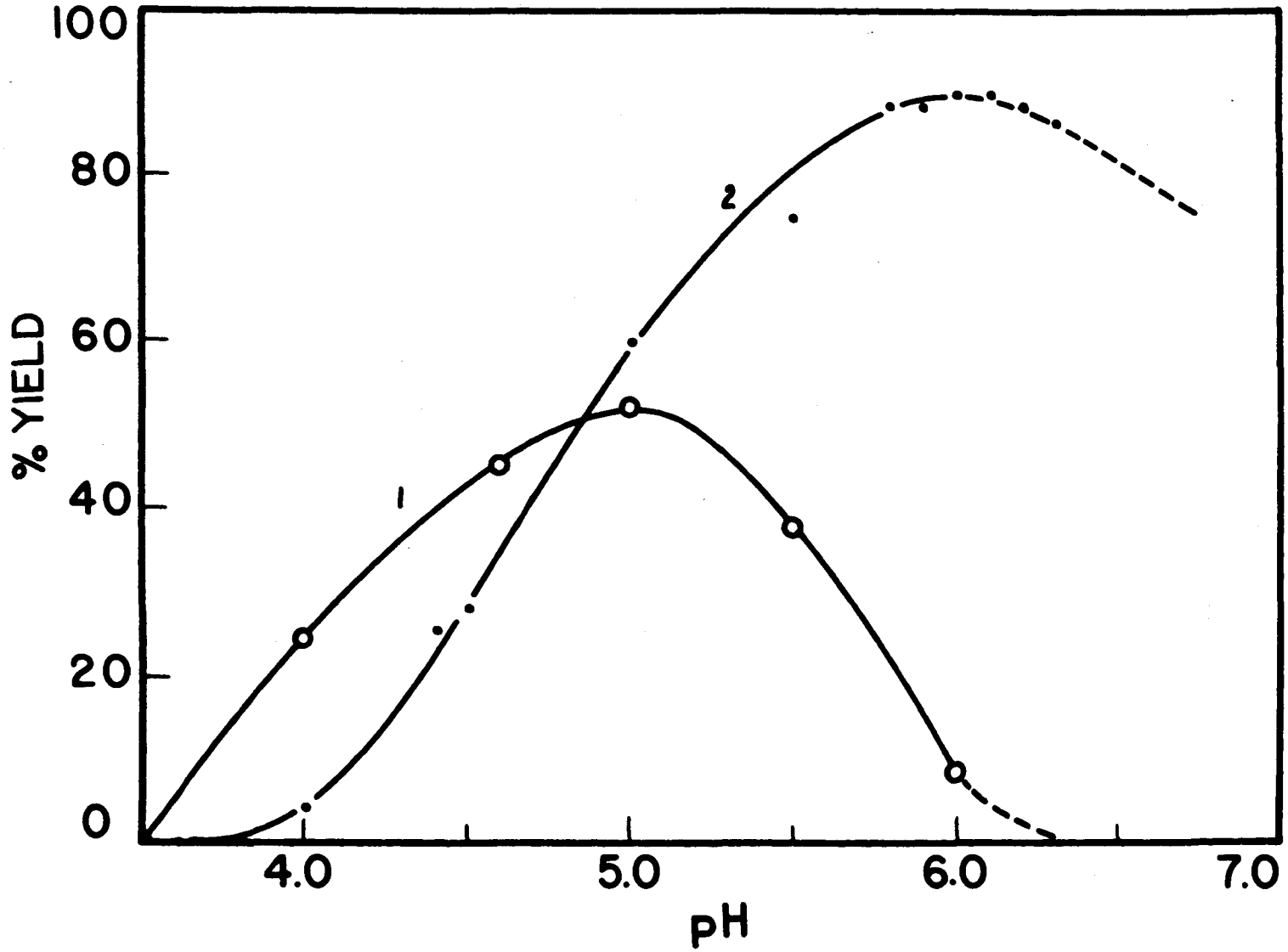


Fig. 3. Effect of pH on yield of benzoyl-L-alaninanilide and benzoyl-L-leucinanilide.

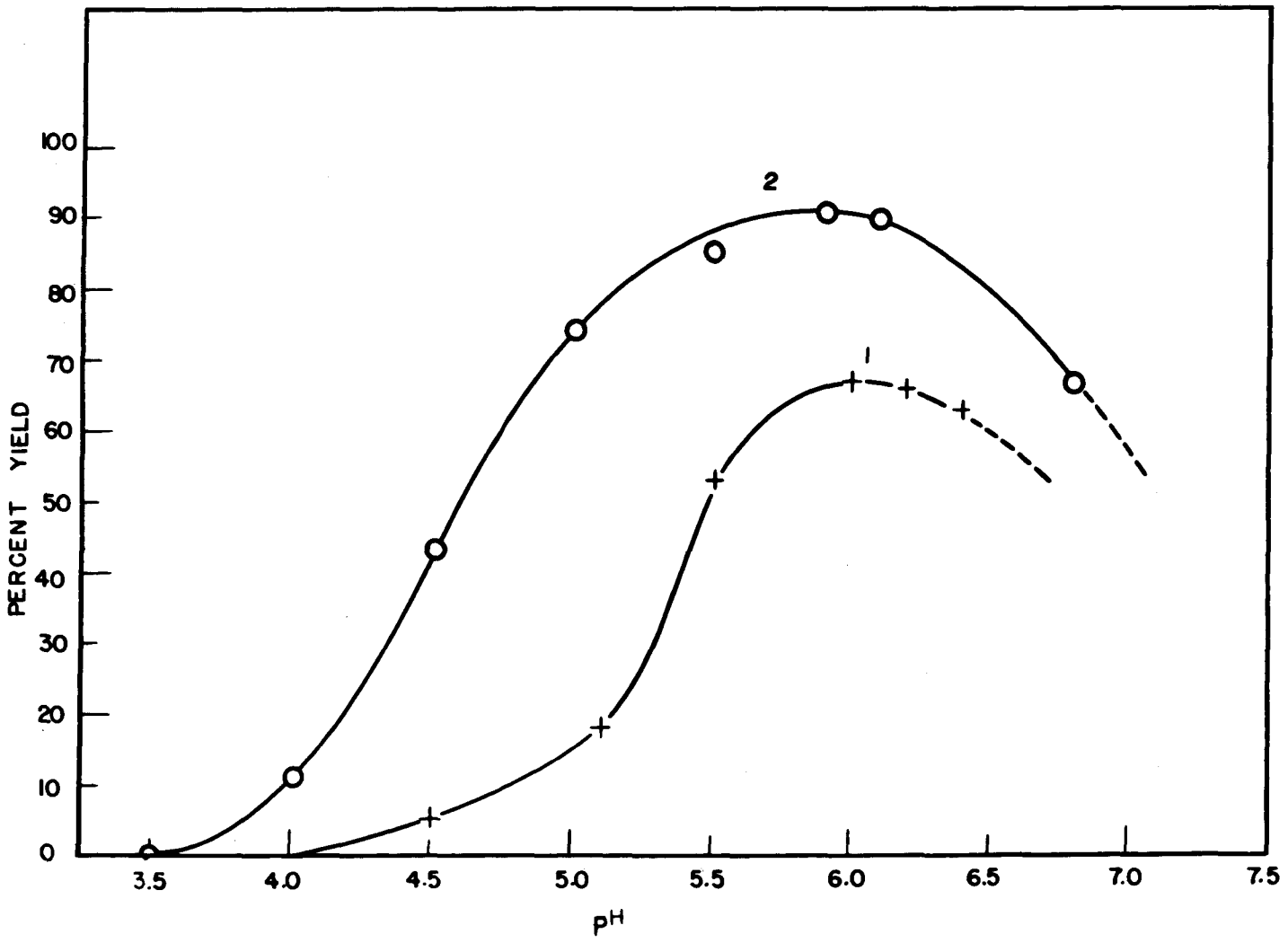


Fig. 4. Effect of pH on yield of benzoyl-L-phenylalaninanilide and benzoyl-p-methoxy-L-phenylalaninanilide.

represents the data for the monobenzoyl derivative and Curve 2, the dibenzoyl derivative.

Effect of Buffer Concentration

Initially these pH studies were carried out using citrate buffer concentrations of 0.1 M. It was noticed that when more concentrated buffer was employed, such as 1.0 M, the reaction frequently proceeded more rapidly. Thus, for a given reaction time, the over-all yield was increased. In the case of benzoylglycinanilide, the use of more concentrated buffer increased the yield of anilide about three-fold for a three day incubation period (Fig. 1, p. 56). One of the most striking examples of the effect of increased buffer concentration is presented in Fig. 2 (p. 58); in this experiment the conditions were such that 1.0 M buffer caused greater than a twenty-fold increase in yield of benzoyl-L-alaninanilide at the optimum pH. Because of this effect of buffer concentration, the enzymatic studies in the remainder of this thesis were carried out using 1.0 M buffer.

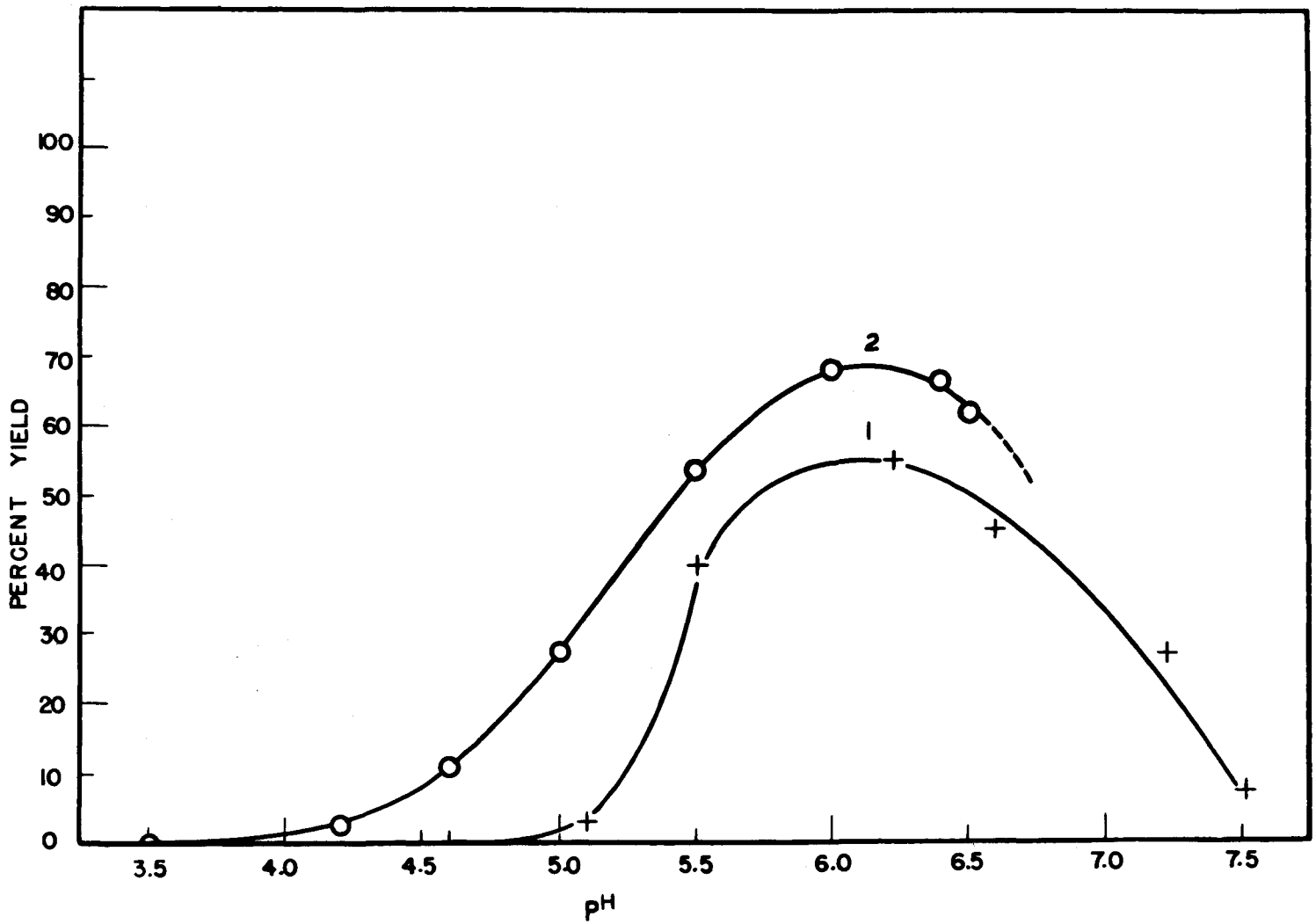


Fig. 5. Effect of pH on yield of N-benzoyl-L-tyrosinanilide and O,N-dibenzoyl-L-tyrosinanilide.

Effect of Concentration of Reactants

A quantitative evaluation of the effect of concentration of all the reactants is brought out in Fig. 6 (p. 65). With the amounts of all the components held constant, the total volume of buffer was varied for both benzoylglycine and benzoylalanine. The incubation time was three days at 40°. In each case the yield was increased with increasing concentration of the reactants. In the experiments where the total volume was 40 and 20 ml., a large part of the benzoylamino acids did not go into solution when the reaction mixture was prepared. The products obtained from these two-phase reactions were identical to those obtained from the reactions where all the reactants were in solution initially.

Effects of Other Experimental Conditions

In Table IV (p.66) are presented the effects of variations of a number of conditions. The effects of alteration of any one condition in most of this work seemed to depend upon the combination of other conditions employed. In the experiment reported, controlled variations revealed that 6 days' incubation gave slightly

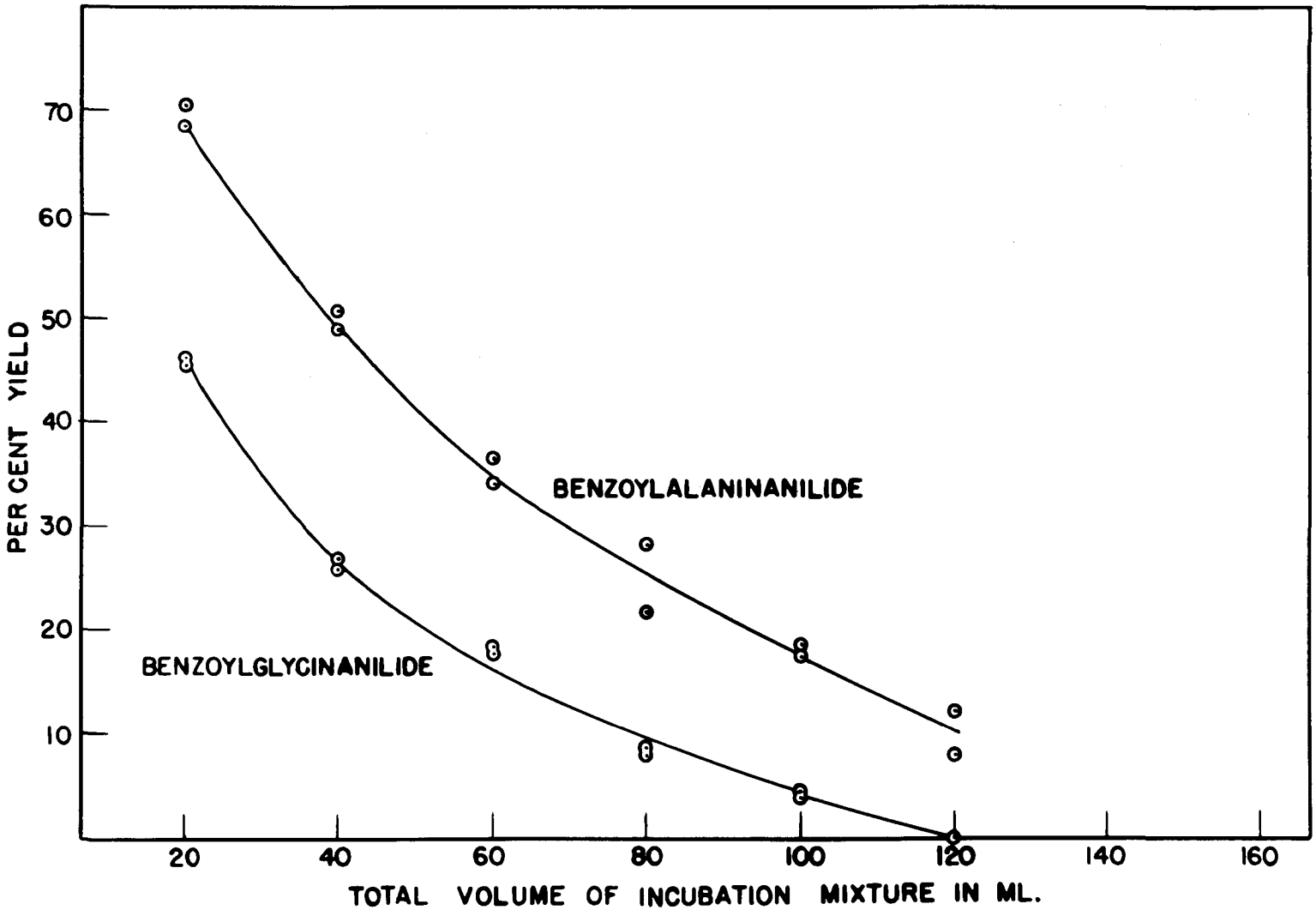


Fig. 6. Effect of total volume at incubation mixture on yields of benzoylglycinanilide and benzoyl-L-alaninanilide.

Table IV

Effects of conditions on yield of benzoyl-L-alaninanilide

Buffer Concentration, Molar	mM Aniline	Aniline/acid Molar Ratio	Enzyme	Time, Days	Yield, Percent
0.10	25	1:1	Commercial	3	2.4, 2.7
0.10	25	1:1	Commercial	6	3.0, 3.6
1.00	25	1:1	Commercial	3	44, 46
1.00	50	2:1	Commercial	3	70, 71
1.00	100	4:1	Commercial	3	84, 85
1.00	25	1:1	Commercial	6	51, 52
1.00	25	1:1	Hydrogen sulfide-treated	3	48, 49
1.00	100	4:1	Hydrogen sulfide-treated	6	85, 86

larger yields than three days' incubation, the commercial sample employed (Difco papain, control no. 384352) catalyzed almost as well as an hydrogen sulfide-treated enzyme, much greater increases in yield resulted from more concentrated buffer, and from using a high ratio of aniline to benzamino acid.

A similar experiment was set up for benzoylphenylalanine which was, in contrast to benzoylalanine, relatively insoluble in the buffer mixture. The results in Table V (p. 68) can be seen to be qualitatively similar to those for the alanine derivative.

Effect of the Type of Substitution of the Amino Acid

Another factor of importance in some anilide syntheses was the type of substitution of the amino acid. Various derivatives of phenylalanine have been investigated; the results are presented in Table VI (p. 70). Under the experimental conditions used, it would appear that there existed a sharp specificity in reactivity between N-benzoyltyrosine and O,N-dibenzoyltyrosine. Under otherwise identical experimental conditions, the dibenzoyltyrosine reacted rapidly but the monobenzoyltyrosine was

Table V

Effects of conditions on yields of benzoyl-L-phenylalanin-anilide

Buffer Concentration, Molar	m M Aniline	Aniline/acid Molar Ratio	Enzyme	Time, Days	Yield, Percent
0.10	25	1:1	Commercial	3	12, 14
0.10	25	1:1	Commercial	6	17, 18
1.00	25	1:1	Commercial	3	40, 42
1.00	50	2:1	Commercial	3	52, 53
1.00	100	4:1	Commercial	3	75, 76
1.00	25	1:1	Commercial	6	46, 48
1.00	25	1:1	Hydrogen sulfide-treated	3	44, 45
1.00	100	4:1	Hydrogen sulfide-treated	6	86, 88

unreactive. The comparative results showed that the failure of the DL compound to react was, in this case, not due to combination of the D and L antipodes, since O,N-dibenzoyl-DL-tyrosine yielded O,N-dibenzoyl-L-tyrosinanilide, as well as O,N-dibenzoyl-L-tyrosine. It was first suspected that the presence of the free hydroxyl group in the monobenzoyltyrosine was responsible in some manner for its unreactivity, since both the dibenzoyl derivative and benzoylphenylalanine gave anilides in good yield. Accordingly, benzoyl-p-methoxy-L-phenylalanine was incubated with aniline in the presence of papain. Again the corresponding anilide was obtained (Table V). Subsequently the effect of pH on the formation of all of these benzoylated amino acids was studied, as has been discussed previously. As a matter of curiosity, the monobenzoyltyrosine was also included in this study. It was found that at pH 5.0 the yield of anilide was virtually zero, but that at higher pH values the anilide was obtained in good yield (Fig. 5).

Reactivities Found Within the Monoamino-monocarboxylic Acid Series

In contrast to the appreciable yield of anilides obtained from the benzoyl derivatives of glycine,

Table VI

Reaction of aromatic amino acid derivatives with aniline at pH 5 under the influence of papain

Acyl Component	Yield of Anilide Percent
N-Benzoyl-DL-phenylalanine	12.2, 12.3
N-Benzoyl-DL-tyrosine	0, 0
N-Benzoyl-L-tyrosine	0, 0
O,N-Dibenzoyl-L-tyrosine	6.9, 6.3
N-Benzoyl-p-methoxy-L-phenylalanine	44.5, 44.7

alanine, and leucine, Minard[1] found that benzoyl-DL-valine, when incubated with aniline in the presence of papain, yielded only small amounts of the anilide. Repetition of this work showed that the yield of benzoyl-L-valinanilide, under the usual experimental conditions, varied from 4 to 7% and no appreciable difference in yield resulted when hydrogen sulfide-treated enzyme was used rather than a commercial preparation. It was suggested that the reason for the relative non-reactivity of benzoyl-DL-valine might possibly be due to the relatively greater solubility of benzoyl-L-valinanilide in comparison to the anilides of benzoylalanine and benzoylleucine. For this reason an attempt was made to determine the solubilities of benzoylvalinanilide, benzoylalaninanilide and benzoylglycinanilide in citrate buffer solution. Two different methods were tried; the first involved the determination of the amount of aniline present in the acid hydrolysate of a saturated solution of the anilide in citrate buffer, while the second involved Kjeldahl nitrogen determinations on chloroform extracts of a saturated anilide solution in citrate buffer. Neither method gave satisfactory results, since

1. Minard, F. N., Synthesis of some valine derivatives as potential antibacterial agents. Unpublished Ph. D. Thesis. Ames, Iowa. Iowa State College Library. 1949.

none of the solubility values could be duplicated on repetition. The values obtained did show that in all probability no great differences in solubility existed, and that in each case the solubility of the anilide was in the order of decigrams per liter of citrate buffer.

From these unacceptable results, it was concluded that another experimental approach might show that a reason other than the solubility of the anilide might be the determining factor in the relatively low percent yield of benzoyl-L-valinanilide obtained under the experimental conditions employed. If it could be demonstrated that these benzoylamino acid anilides were formed at appreciably different rates, and that equilibrium had not been reached in the customary three day incubation period; then any differences in yield might be due primarily to differences in rate of reaction and not to differences in solubility of the anilides.

The importance of rates of reaction is illustrated in Fig. 7 (p. 73) which demonstrates that these syntheses have not reached equilibrium by the third day. It is obvious that the rate of reaction of benzoylvaline was the slowest of the group studied; the slope of the curve remains almost constant for the three day period.

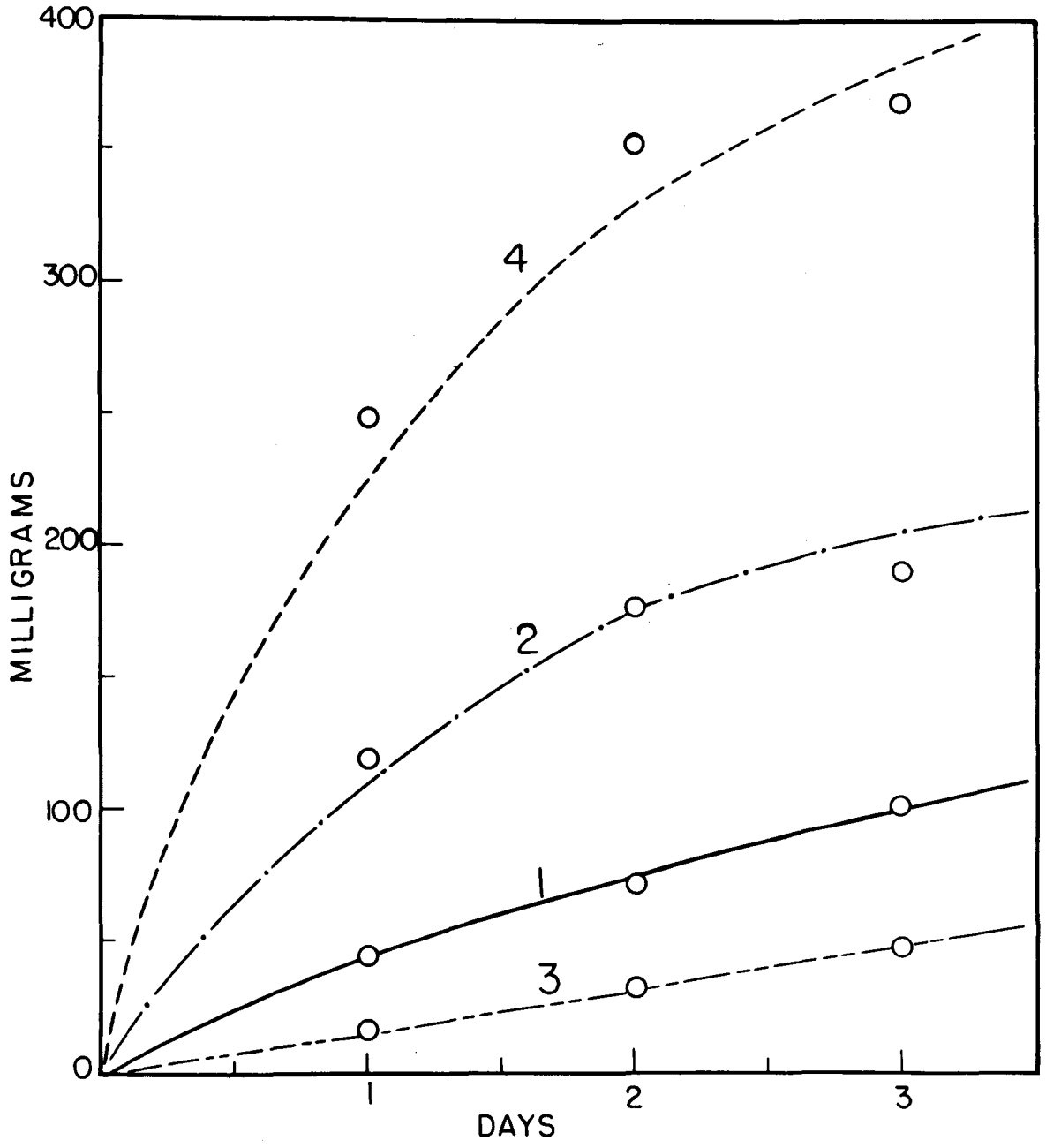


Fig. 7. Rate of synthesis of benzoylamino acid anilides.
Glycine (Curve 1), Alanine (Curve 2), Valine (Curve 3), Leucine (Curve 4).

Another conceivable manner in which the properties of the solid anilide could be determinant of the extent of the reaction is that in which the solution becomes supersaturated with the anilide. In such a case, the formation of the anilide would be decreased if the equilibrium conditions were reached. In order to check on this, the rate experiments of Fig. 7 were repeated with 200 mg. of anilide initially added to enough tubes so that daily filtration of the same tubes was unnecessary. In the event of supersaturation, precipitation would be most facilitated when in contact with added solid acting as seed material. In no case, within the limits of experimental error, was any enhancement of yield observed during the three day period.

Another approach to the problem of the relative unreactivity of benzoyl-DL-valine in the anilide synthesis was attempted using hydrolytic reactions rather than synthetic reactions. Benzoylvalinamide, benzoylleucinamide, and benzoylglycinamide were incubated with papain in pH 5.0, 1.0 M citrate buffer and the extent of hydrolysis determined as has been described in the Experimental section. The benzoylvalinamide was hydrolyzed to a considerably lesser extent than was

either the benzoylleucinamide or the benzoylglycinamide. The results are tabulated in Table VII below.

Table VII

Extent of hydrolysis of benzoylated amino acid amides

Amino acid	Percent hydrolysis
Glycine	59
Valine	3.7
Leucine	32

Although benzoylvaline was relatively unreactive under the usual experimental conditions employed, it was desirable to determine whether a large quantity of the anilide could be prepared under especially favorable conditions for synthesis. The study of the factors affecting anilide synthesis had shown that a high ratio of aniline to benzamino acid and high reactant concentrations increased the yield of anilides. Fox and Halverson[1] had shown that the pH optimum for the synthesis of benzoylvalinanilide was between 5.0 and 5.5. The rate study discussed previously had shown

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

that the rate of synthesis of the anilide was slow and that at the end of the third day no diminution in the rate of synthesis could be observed.

Accordingly, 22.1 g. (0.1 mole) of benzoyl-DL-valine and 20 g. (0.215 mole) of redistilled aniline were incubated in 500 ml. of pH 5.0, 1.0 M citrate buffer with 3.2 g. of papain and 1.28 g. of cysteine hydrochloride. After three days, 7.1 g. of anilide were filtered off and at the end of an additional seven days another 5.5 g. were obtained. The melting point of the crude product was 218-220°. This crude material contained a small amount of chloroform insoluble material; in order to obtain a reading in the polarimeter, the anilide was dissolved in 200 ml. of chloroform and treated with 4 g. of Norite-A while hot. The anilide was recovered from the filtrate by evaporation of the solvent. The melting point of this material was 219-220°; the specific rotation was $[\alpha]_D^{24} = -78.2^\circ \pm 0.9^\circ$ (c = 4% in chloroform). Fox and Halverson[1] found the melting point to be 220-221°, and the rotation to be $[\alpha]_D^{23} = -80.6^\circ \pm 0.9^\circ$.

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

Enzymatic Reactions of Benzoylated Amino Acids With Glycinanilide

A series of benzoylated amino acids were incubated with glycinanilide in place of aniline. In each case 0.001 mole of the benzoylated amino acid and 0.150 g. (0.001 mole) of glycinanilide were suspended in 10 ml. of 1.0 M, pH 5.0 citrate buffer which contained 10 mg. of commercial papain and 10 mg. of cysteine hydrochloride. The reaction mixtures, in tightly stoppered test tubes, were incubated for three days at 40°. After incubation, the solid material was filtered off, washed with 50 ml. of 1 N hydrochloric acid to remove any unreacted glycinanilide, then with 100 ml. of 1 N sodium hydroxide, and finally with two 50 ml. portions of water. The residues were weighed after drying in air for twelve hours. Melting points were taken after recrystallization from 50% ethanol. The results are reported in Table VIII (p. 78). 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 anilides whose identity had been established. For all the benzoylated amino acids except glycine, the product was the corresponding benzoylamino acid anilide. In the case of glycine, the

product was identified by nitrogen analysis as benzoylglycylglycinanilide. Since Behrens and Bergmann[1] have

Table VIII

Reactivity of benzoylated amino acids with glycinanilide

Amino acid	Weight of benzoylated amino acids used, g.	Weight of product, g.	Melting point	Product	Percent yield, g.
Glycine	0.179	0.212	222-223°	Benzoylglycylglycinanilide	23
Valine	0.221	0.050	218-220°	Benzoyl-L-valinanilide	8
Leucine	0.235	0.111	212-213°	Benzoyl-L-leucinanilide	46
Alanine	0.193	0.137	174-176°	Benzoyl-L-alaninanilide	34
Phenylalanine	0.269	0.034	215-216°	Benzoyl-L-phenylalaninanilide	20

shown that glycinanilide is not hydrolyzed in the presence of papain, these reactions must follow the same mechanism which they have shown for the reaction of acetylphenylalanylglycine and glycinanilide. On the basis of this

assumption, the following series of reactions must have occurred (benzoyl-DL-leucine will be used as a specific example). The benzoyl-DL-leucine reacted with glycinanilide to yield benzoyl-L-leucylglycinanilide. This compound was hydrolyzed to give aniline and benzoyl-L-leucylglycine, which in turn was hydrolyzed to give benzoyl-L-leucine and glycine. The free aniline liberated from the previous hydrolysis then reacted with benzoyl-L-leucine to give the anilide. For some reason the benzoylglycylglycinanilide which was formed when benzoylglycine was incubated with glycinanilide was resistant to hydrolysis by papain under the experimental conditions used.

DISCUSSION AND CONCLUSIONS

The results depicted in Fig. 1 through Fig. 5 indicate the effect of pH on the synthesis of benzoylamino acid anilides. The variations in pH optima with variations in substrate are of interest in connection with the findings of Northrop[1], who observed that the pH optimum for the digestion of proteins by trypsin or pepsin varied with different proteins. He showed that when it is assumed that pepsin reacted with the positive protein ion and trypsin with the negative ion, the pH optimum could be fairly well predicted from the titration curves of the proteins. Kunitz and Northrop[2] demonstrated that trypsin exists in an active and an inactive form which are in equilibrium with each other, and that the equilibrium is shifted towards the inactive form as the pH becomes higher than 8.0. As a result, the pH optimum of trypsin is dependent both on the amount of the active form of the enzyme present and on the extent of the ionization of the protein. The rate of digestion is proportional to the product of the concentration of the negative protein ions times the concentration of the

1. Northrop, J. H., J. Gen. Physiol., 5, 263 (1922).
2. Kunitz, M. and Northrop, J. H., J. Gen. Physiol., 17, 591 (1934).

active form of the enzyme. There is a point at which this product becomes a maximum and the position of this maximum, the optimum pH, will depend upon the type of protein used.

Willstatter, Grassmann and Ambros[1] have shown that the effect of pH on the action of papain and similar enzymes can be accounted for on the assumption that papain attacks the undissociated protein molecule. No data is available at the present time to show that papain exists in an active and an inactive form similar to those of trypsin. Related data available shows that papain is irreversibly inactivated only at the more extreme ranges of hydrogen ion concentration, that is, below pH 2 and above pH 13[2].

On the basis of the assumption that papain acts on the undissociated protein molecule, the difference in the pH optimum for papain acting on benzoylated amino acids to form anilides might be supposed to be based on differences in isoelectric points of the substrates. However, the pH optima for the two structurally similar benzoyl amino acids, benzoylleucine and benzoylalanine, are less closely related than are the pH optima for such

1. Willstatter, R., Grassmann, W. and Ambros, O., Z. Physiol. Chem., 151, 286 and 307 (1926).
2. Lineweaver, H. and Schwimmer, S., Enzymologia, 10, 81 (1941).

relatively dissimilar compounds as benzoylleucine and benzoyltyrosine. It is of interest in this connection that the pH optimum for the synthesis of benzoylglutamic acid anilide, as catalyzed by papain, is about pH 4.5[1]. It would appear that the pH optima for the two acidic compounds, benzoylglutamic acid and N-benzoyltyrosine, would be fairly close, if the relationship between isoelectric point and maximum reactivity were assumed to be valid for these simple substrates.

Fruton and Bergmann[2] have observed that the pH optimum for crystalline pepsin may vary with simple substrates which are structurally very closely related. Under otherwise identical experimental conditions, the pH optimum for the action of crystalline pepsin on carbobenzoxy-L-glutamyl-L-tyrosine was 4.0, while the pH optimum for the same crystalline pepsin was 4.5 for carbobenzoxy-L-glutamyl-L-phenylalanine.

It has been suggested that the differences in pH optimum for the synthesis of these anilides might be due to the presence of more than one enzyme in the commercial preparations of papain that were employed. At

1. Wax, H. and Fox, S. W., Unpublished experiments.
2. Fruton, J. S. and Bergmann, M., J. Biol. Chem., 127, 627 (1939).

least two different crystalline enzymes have been reported to have been isolated from papain preparations[1,2]. The importance of using pure enzyme preparations cannot be discounted in any enzymatic studies. The use of crystalline preparations was contraindicated in this work by a consideration of the quantities of enzyme needed for these studies and by the consideration that crystallinity is not indicative of homogeneity in many cases. For example, if the presence of more than one pH optimum for crude papain is indicative of the presence of more than one enzyme, then similarly, more than one enzyme must have been present in the crystalline pepsin used in the studies just mentioned. In the event that pure crystalline enzymes become available from commercial papain in appreciable quantity, the effect of pH on the synthesis of these anilides using these pure preparations should be studied.

At the present time, at least three possible explanations for this variation in pH optimum can be submitted. The first of these has already been mentioned; more than one enzyme may be present in the crude preparations used and for each pH optimum a corresponding enzyme is present. Another possibility is that more than one active center

1. Balls, A. K. and Lineweaver, H., J. Biol. Chem., 130, 669 (1939).
2. Jansen, E. F. and Balls, A. K., J. Biol. Chem., 137, 459 (1941).

is present in a single enzyme molecule, each active center having definite specificities and a definite pH optimum. The third possibility is that one enzyme having one active center is present, and that the differences in pH optima are due to properties of the substrate, such as the isoelectric point. The present evidence is too scanty to permit discounting completely any of these possibilities. What evidence does exist on the basis of the anilide syntheses would tend to show that the third possibility is more remote than the first two.

This difference in observed pH optima may well be general for all proteolytic enzymes, and may be of particular significance in relation to specificity requirements for proteolytic enzymes which have been established. The majority of the data now available on the structural requirements of proteolytic enzymes has been obtained at only one hydrogen ion concentration. A re-evaluation of these structural requirements for proteolytic enzymes may be necessary from at least a quantitative point of view. It has already been pointed out that at pH 5.0, even under the favorable experimental conditions used (1.0 M citrate buffer, etc.), relatively high specificity was observed for mono- and di-benzoyltyrosine. Yet, at

higher pH values both compounds were highly reactive and formed anilides in good yield. The specificity of papain would appear to depend in large measure on the experimental conditions employed.

Such variations in reactivity for these amino acid derivatives may possibly explain the observations of Hoover and Kokes[1] on the effect of pH on the extent and rate of hydrolysis of casein. They found casein to be hydrolyzed at a more rapid initial rate at pH 7.0; at this pH, peptide bonds involving such amino acids as tyrosine, phenylalanine, and leucine might be hydrolyzed. The hydrolysis of casein was observed to be more complete at pH 5.0; at this pH, peptide bonds involving such amino acids as alanine, glycine, and valine might be broken. The possibility is not remote that studies on both the rate of synthesis and hydrolysis of peptide bonds in substrates of known structure at various pH levels might reveal information valuable in elucidating the actual mechanism of enzymatic protein hydrolysis.

The reaction of a benzoylated amino acid with aniline in the presence of papain in citrate buffer solution to form an insoluble anilide is very likely a complex of reactions. The reason for increased yield of

1. Hoover, S. and Kokes, E. L. C., J. Biol. Chem., 167, 199 (1947).

anilide in a given period of time when increased buffer concentrations are used (Fig. 1 and Fig. 2) may be due to one or more of the steps in the reaction being acid catalyzed. Since the yield of anilide is not only dependent upon pH, but also upon buffer concentration, or the concentration of the undissociated molecules of the acid, one of the reactions in the complex series must be catalyzed by the undissociated acid molecules as well as by hydrogen ions. Hellerman[1] has stressed the importance of buffer concentration as long ago as 1937. Little emphasis has been placed on this point both before and after that time. To quote Hellerman:

> In addition to those changes in ionic species which are brought about by pH control (e.g., by the use of buffers) the activity of enzymes in general may be altered through various "salt effects". The most general kind of salt effect would result from a change in the thermodynamic environment resulting from the presence in reaction mixtures of added ions or molecules (buffer or other salts, amino acids, proteins, etc.). The magnitude of general salt effects may, perhaps, be gauged by studies of the variation of enzyme activity with the ionic strength of the medium. There is needed systematic work on this point.

The importance of the effect of pH and buffer concentration in the development of methods of resolution of amino acids is brought out in Fig. 2. The use of 1.0 M

1. Hellerman, L., Physiol. Rev., 17, 454 (1937).
* Referring to enzymes and protein substrates.

buffer and careful control of pH was also useful in improving the yield of benzoyl-L-methioninanilide. Dekker and Fruton[1] have reported a method for the resolution of DL-methionine which involves the enzymatic synthesis of benzoyl-L-methioninanilide. When their published directions were followed exactly, only a negligible yield of anilide was obtained in 24 hours. When the 0.1 M citrate buffer they reported using was replaced with 1.0 M buffer and the pH adjusted to 5.0, the yield of anilide obtained in 24 hours was virtually quantitative. This not only enables a greater yield of the L-form to be obtained, but also would facilitate the separation of the D-form, since it would be less contaminated with unreacted L-methionine.

The effect of the concentration of reactants on yield (Fig. 6) can be explained on the basis of kinetic theory. For any order of reaction except zero order, the speed of the reaction is proportional to the concentration of the reacting substances. At the present time there is insufficient knowledge of the actual mechanism of the anilide synthesis to be able to relate the speed of the over-all reaction to the concentration of any one reactant. However, kinetic studies might lead to a better understanding of the mechanism of these anilide syntheses, and thus furnish a possible basis for

1. Dekker, C. A. and Fruton, J. S., J. Biol. Chem., 173, 471 (1948).

understanding the mechanism of peptide bond synthesis in living organisms.

The effects of a number of factors on the synthesis of benzoylalaninanilide and benzoylphenylalaninanilide have been given in Tables IV and V, respectively. The effect of increasing the concentration of aniline in the incubation mixture results in an increased yield of anilide, which is in accordance with the theoretical considerations mentioned above. The fact that six days' incubation resulted in slightly greater yields than did three days' incubation indicates that equilibrium is not reached in a three day period, although after three days the reaction is proceeding at a much diminished rate. The slight differences in yield between using a commercial enzyme preparation and a hydrogen sulfide-treated papain demonstrates that for synthetic purposes, for methods of resolution involving the anilides, and for studies on the effects of various experimental conditions on anilide synthesis, the commercially available papain is as suitable as the hydrogen sulfide-treated preparations. The importance of buffer concentration is again emphasized.

The differences which have been found for the variations in rate of formation of the benzoylamino acid

anilides are of interest from the point of view of enzyme specificity. The term "specificity" which has been applied in comparisons between, for example, basic and acidic residues in substrates, is inappropriate here, since all of the benzoylated amino acids studied showed at least some reactivity. In accordance with these facts the term "preference" is employed instead. Preference, in this connotation, has been found to be on a kinetic basis, and is used in place of the more ambiguous phrase, partial specificity.

In considering these preferences it is helpful to consider the synthesis as occurring in two steps:

(a) Acylamino acid + aniline = acylamino acid anilide (solution)

(b) Acylamino acid anilide (solution) = acylaminoacid anilide (ppt.)

One critical determinant of whether reaction (a) will proceed to give an appreciable yield of anilide (others are enzyme-substrate interaction requirements, etc.) is whether or not the solubility of the particular anilide is less than that necessary to satisfy the equation:

$$K_a = \frac{(\text{anilide})}{(\text{acylamino acid})\ (\text{aniline})}$$

When it is less, the reaction proceeds towards the right; the anilide is synthesized. It is conceivable that in

the synthesis of an anilide of limited yield, such as benzoyl-L-valinanilide, reaction (a) may proceed until enough acid and aniline are used up so that the solubility of the anilide is no longer less than the concentration required to maintain K_a. If this were a valid reason for the relative non-reactivity of benzoyl-valine, the corresponding curve of Fig. 7 would become horizontal before the third day. Since it does not do so, the quantitative extent of the individual reaction for such a relatively short time period is independent of the equilibrium solubility of the anilide.

As mentioned in the Results section, another conceivable way in which a property of the solid anilide would be determinant is that in which the solution becomes supersaturated with the anilide and step (b) is rate-limiting. In order to check on this, the experiments on added anilide were carried out. When added anilide was added as seed material to facilitate crystallization of the anilide, no enhancement of synthesis was noted. For this reason, step (b) is not rate-limiting and step (a) must be the rate-limiting one of the two. It is necessary to bear in mind that step (a) is very likely a complex series of reactions and that either one of these steps or a number of the steps may be rate-limiting. Since the enzyme-controlled part of

the over-all reaction is (a), the extent of the reaction in a given period of time must be enzyme-controlled.

Such biological significance as these studies have needs to be considered against the known dynamic nature of protein synthesis, and the fact that in a biological system possessing lower concentrations of buffer, substrates, and enzyme, the approach to equilibrium would theoretically be far slower. In the absence of data for special enzymes for protein synthesis, and in the presence of data that the same enzymes can catalyze both the synthesis and hydrolysis of peptide bonds under essentially similar conditions, the proteolytic enzymes deserve serious consideration as primary catalytic agents of protein synthesis. This point is emphasized by the fact that the same preference for the leucine residue over the valine residue is shown in the hydrolysis of the benzamino acid amides as was shown for the synthesis of the benzamino acid anilides. The data for the rate of hydrolysis of the amides has not been obtained. For a complete comparison between hydrolysis and synthesis, such rate studies would be necessary.

These studies on the reactivity of valine and leucine derivatives are also of interest in relation to the

observations of Roche and Mourgue[1]. These workers observed a substantial liberation of leucine from casein during hydrolysis with papain, with little recovery of valine. They suggested that the leucine residues were located at readily accessible portions of the peptide chain, whereas valine would lie, according to their explanation, well within the peptide chain. Such an explanation will not, of course, explain the preferences observed in the synthesis of anilides or for the hydrolysis of the amides. For anilide synthesis the critical factor seems to be the kinetically determined enzyme preference rather than the position in the substrates.

With respect to the reactions where glycinanilide was employed in place of aniline, the significance of the results cannot be evaluated at the present time. The reason why benzoylglycylglycinanilide is resistant to hydrolysis under the conditions employed, while the other benzoylamino acid derivatives of glycinanilide are susceptible to hydrolysis is, as yet, not known. The need of repeating this experiment using other amino acid anilides in place of glycinanilide is obvious. In all probability the effect of experimental conditions upon these reactions, as well as kinetic studies, will reveal

1. Roche, J. and Mourgue, M., Compt. rend., 218, 86 (1944).

information which will be of value in understanding the mechanism of peptide bond synthesis as well as enzyme preferences.

The utility of the anilide synthesis has been demonstrated in published methods for the resolution of amino acids[1,2,3]. The effects of various experimental conditions on the yield of acylamino acid anilides that have been discussed can be applied directly to the improvement of the existing methods and can be utilized in the development of additional methods of resolution.

The validity of anilide syntheses as models of the biological synthesis of peptide bonds becomes worthy of more serious consideration since quantitative differences in anilide syntheses, in a given period of time, are now found to depend primarily upon enzymatic behavior rather than on anilide properties. The essential difference between anilide syntheses and the biological synthesis of peptide bonds may well be in the manner in which energy is supplied. Recently, peptide bond syntheses were reported in which high energy phosphate compounds, such as adenosine triphosphate, were utilized in the formation of glutamine

1. Fruton, J. S., Irving, G. W. and Bergmann, M., J. Biol. Chem., 133, 703 (1940).
2. Behrens, O. K., Doherty, D. G. and Bergmann, M., J. Biol. Chem., 136, 61 (1940).
3. Dekker, C. A. and Fruton, J. S., J. Biol. Chem., 173, 471 (1948).

and glutathione[1,2]. Similar methods of supplying energy may well be involved in many peptide bond syntheses in living organisms as well as in experiments done in glassware. No matter how the energy is supplied for peptide bond synthesis does not obviate the possibility that proteolytic enzymes may be the primary catalysts employed in biological synthesis of peptide bonds.

1. Bloch, K., J. Biol. Chem., 179, 1245 (1949).
2. Speck, J. F., J. Biol. Chem., 179, 1387 (1949).

SUMMARY

The effects of various experimental conditions upon the papain-catalyzed syntheses of benzoylamino acid anilides have been studied. The factors have included pH, substitution in the side-chain of phenylalanine, citrate buffer concentration, benzoylamino acid: aniline ratio, volume of buffer employed for the reaction, and time of reaction. Rates of formation were studied for the anilides of the benzoyl derivatives of glycine, alanine, valine, and leucine. The extent of hydrolysis of the amides of benzoylglycine, benzoylvaline, and benzoylleucine were determined.

1. Significant differences were found in the effect of pH on the papain catalyzed syntheses of benzoylamino acid anilides. The optimum pH for the syntheses of the anilides of benzoylglycine and benzoylalanine was 5.0 - 0.5. The optimum pH for the syntheses of the anilides of the benzoyl derivatives of leucine, phenylalanine, tyrosine, and p-methoxyphenylalanine was between 6.0 and 6.5.

2. At pH 5.0, the pH customarily used for papain, the differences in reactivity between benzoylphenylalanine and N-benzoyltyrosine virtually amounted to specificity. No demonstrable yield of N-benzoyltyrosinanilide was

obtained at this pH, but good yields of benzoylphenylalaninanilide resulted. When citrate buffers at higher pH values such as 6.0 and 6.5 were used, good yields of benzoyltyrosinanilide as well as benzoylphenylalaninanilide were obtained. These results demonstrated that specificity requirements of proteolytic enzymes are determined to an appreciable extent by the hydrogen ion concentration.

3. The use of 1.0 M citrate buffer in place of the customarily used 0.1 M citrate buffer resulted in increased yields of benzoylamino acid anilides. The increase in yield was typically three- or four-fold, for the experimental conditions used in these studies.

4. The benzoylamino acid anilides were obtained in increased yield when the ratio of aniline to benzoylamino acid was increased.

5. The volume of buffer employed in the reaction affects the yields of anilides obtained. When the concentration of all the reactants was increased by diminishing the volume of citrate buffer while holding the amounts of benzoylamino acid, aniline, papain, and cysteine hydrochloride constant, the amount of anilide obtained in a three day period was increased.

6. For the benzoylamino acids studied, commercial papain catalyzed almost as well as a treated papain preparation. For methods of resolution of amino acids, enzymic syntheses, and for the studies reported in this thesis, the use of commercial papain is apparently as suitable as the treated preparations of papain.

7. Six days' incubation resulted in only slightly greater yields of benzoylalaninanilide and benzoylphenylalaninanilide than did three days' incubation. The rate studies on the formation of the anilides of the benzoyl derivatives of glycine, alanine, valine, and leucine also indicated that equilibrium was not reached in three days.

8. It was shown from the rate studies on the formation of the anilides of the benzoyl derivatives of glycine, alanine, valine and leucine that the rate of reaction increased in the order: valine, glycine, alanine, and leucine. The "seeded" rate studies indicated that variations in yield were not due to supersaturation effects. On the basis of these rate studies it was concluded that the variations in yields of anilides obtained were due to differences in rate of reaction rather than to physical properties of the anilides.

9. Benzoylleucinamide was hydrolyzed to a significantly greater extent than was benzoylvalinamide when these amides were incubated in citrate buffer in the presence of papain-cysteine. This greater reactivity of the leucine derivative in contrast to the valine derivative for this hydrolysis reaction indicated that in both hydrolysis as well as synthesis an enzymic preference existed for leucine derivatives.

10. The significance of these anilide syntheses as models of peptide bond synthesis was discussed.

11. Data pertinent to the development of methods of resolution of amino acids such as alanine, valine, leucine, methionine, phenylalanine, and tyrosine were presented.

12. In the course of this work, the following compounds not previously reported in the literature were prepared: DL-tyrosine ethyl ester hydrochloride, m.p. 155°; O,N-dibenzoyl-DL-tyrosine, m.p. 226-228°, O,N-dibenzoyl-L-tyrosinanilide, m.p. 243-244°; N-benzoyl-L-tyrosinanilide, m.p. 208-208.5°; benzoyl-L-alaninanilide, m.p. 175-176°; benzoyl-p-methoxy-L-phenylalaninanilide, m.p. 223-224°; carbobenzoxy-L-alaninanilide, m.p. 164-165°; carbobenzoxy-L-phenylalaninanilide, m.p. 158-159°; and benzoylglycylglycinanilide, m.p. 223-225°.

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