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Enzymic peptide bond synthesis and its inhibition

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ENZYMIC PEPTIDE BOND SYNTHESIS AND ITS INHIBITION

by

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INTRODUCTION

The problem of protein synthesis in the living organism is one which has long challenged investigators in the fields of animal and plant metabolism. The solution of this problem has, during recent years, assumed a role of major importance, with the growing realization that the processes operative in normal tissue protein synthesis may be quite similar to those involved in the formation of genes, enzymes, viruses, antibodies and neoplasms.

From the accumulated studies of the physical, chemical, and biological properties of proteins have emerged several rather basic experimental conclusions which must be considered in the elucidation of a mechanism for protein synthesis. Chief amongst these are (1) that proteins consist, in the main, of α-amino acids united by means of peptide linkages; (2) that very precise differences exist not only between the proteins of different species of animals and plants, but between the proteins of the different organs of a single species, indicating that the synthetic process must be highly specific; and (3) that the process of peptide bond synthesis requires energy since the reverse process of hydrolysis proceeds spontaneously and almost to completion under the proper conditions.

As regards the pathway of peptide bond synthesis in vivo, one of the most interesting, and probably the oldest theory of the synthesis is that the process is, in essence, a reversal of proteolysis catalyzed by the same proteolytic enzymes responsible for the hydrolytic reaction. Such a proposal is in agreement with one of the fundamental considerations of catalysis which requires that a substance (catalyst) capable of altering the velocity of a reaction in one direction must exert a similar effect on the velocity of the reverse reaction (1).

Because of the many difficulties inherent in studying the action of proteolytic enzymes in vivo, especially with respect to any potential synthesizing action, complicated as such studies would be by other simultaneous metabolic reactions, recourse must be made to simple, isolated systems wherein the reaction under consideration can be investigated free of confounding factors. Although such simplified systems or "models" may be more or less "non-biological" in nature, the results derived from their study, when tempered by the realization that, at best, they represent but a portion of a complex entity, may be of significance in unravelling the complicated pattern of protein synthesis and proteolytic enzyme action.

(1) Nernst, "Theoretical Chemistry" 4th English ed. p. 617. Macmillan and Co. Ltd., London. 1916.

The demonstrations by Bergmann and associates (2-7) that certain acylated amino acids and peptides under the catalytic influence of various proteolytic enzymes of the catheptic type react with aniline, phenylhydrazine, or amino acid anilides to form peptide bonds, have provided a system which, although limited to some extent by the non-biological nature of some of the substrates, does meet the criteria of isolation and simplicity desirable in a working model. Such a system is a flexible one as well, since alterations of both the acyl group and the amino acid residues are possible and thus the effect of these changes on the ability of the enzyme to catalyze peptide bond synthesis may be followed. A further discussion of the work of Bergmann's group with respect to the specificity and energetic considerations of the reactions has been reserved for the Historical section.

Along with investigations of enzymic peptide bond synthesis *in vitro*, it is of interest to examine the control of these reactions by employing various substances with known or potential inhibitory properties. Knowledge of this type may be of manifold value, since, if the mode of action of the

(2) Bergmann and Fraenkel-Conrat, J. Biol. Chem., 119, 707 (1937).
(3) Bergmann and Fraenkel-Conrat, ibid., 124, 1 (1938).
(4) Bergmann and Behrens, ibid., 124, 7 (1938).
(5) Behrens and Bergmann, ibid., 129, 587 (1939).
(6) Fruton, Irving and Bergmann, ibid., 133, 703 (1940).
(7) Behrens, Doherty and Bergmann, ibid., 136, 61 (1940).

inhibitors on the enzyme is known, it may be of aid in determining the mechanism of the enzyme action as well as the essentiality of certain active centers of the enzyme. In addition it is conceivable that information derived from *in vitro* inhibition studies may be carried over, to some extent, to the control of growth and *in vivo* peptide bond synthesis.

In essence, the investigations reported in this thesis were concerned with the preparation of a variety of acylamino acids and the determination of the conditions influencing their abilities to undergo peptide bond (anilide) formation as catalyzed by the proteolytic enzyme, papain. Many of the corresponding acylamino acid amides were also prepared and a few of these subjected to hydrolytic enzyme studies in a search for possible correlations between the factors governing synthesis and hydrolysis. In addition, studies concerned with the inhibitory effects of a number of compounds on several of the synthetic reactions, as well as attempts to determine the groups in papain essential for its synthesizing properties, will be discussed.

HISTORICAL

Models and Theories of Peptide Bond Synthesis

As might well be expected from the challenging nature of the problem, a rather voluminous literature concerned with attempts to elucidate the probable mechanism of protein synthesis, based on either experimental or hypothetical grounds, or on both, exists. Since the subject has recently been reviewed by Northrop (8), and earlier reviews on more or less specific phases of the problem have been presented by Wasteneys and Borsook (9), Alcock (10) and Bergmann and Fruton (11), only the salient features of some of the previous investigations will be dealt with. Greatest emphasis will be placed on the work related most directly to the investigations reported herein.

Mention has already been made in the introductory remarks that any suggestion pertaining to the mode of biosynthesis of peptide bonds must account for both the specificity and the energetics of the reaction. Observations that the hydrolysis

(8) Northrop, Kunitz and Herriott, "Crystalline Enzymes". 2nd ed. Columbia University Press, New York. 1948.

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

(10) Alcock, Physiol. Rev., 16, 1 (1936).

(11) Bergmann and Fruton, Ann N.Y. Acad. Sci., 45, 409 (1943-44).

of peptide linkages in proteins and simple peptides proceeds spontaneously and practically to completion in the presence of suitable enzymes, as well as mineral acids and bases, indicates that proteolysis involves a decrease in free energy, and thus, the reverse process of peptide bond synthesis is an energy-requiring one.* Some means must therefore be provided whereby this energy can be made available.

The idea that proteins might be synthesized by the action of proteolytic enzymes on amino acids or the higher products of proteolysis is not new. Wasteneys and Borsook (9) have reviewed the earlier work as well as their own investigations, dealing with the attempts to reverse proteolysis in vitro by the action of pepsin or trypsin on peptic digests of proteins. Evidence for peptide bond synthesis included a decrease in amino nitrogen content, the formation of an insoluble protein-like compound called "plastein", and the ability of pepsin to digest plastein under proper pH conditions. The reaction, in the main, seems to be limited to pepsin and trypsin, although Haddock and Thomas (12) have obtained

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* Calculation of the standard free energy of formation of leucylglycine from the component amino acids, has indicated that approximately 3000 calories per mole are necessary for the synthesis of a peptide linkage. (Borsook and Huffman, in Schmidt, "Chemistry of the Amino Acids and Proteins". 2nd ed. p. 865. Charles C. Thomas, Springfield, Ill. 1945).

(12) Haddock and Thomas, J. Biol. Chem., 144, 691 (1942).

plastein by the action of papain on peptic digests of insulin. The former authors have applied the mass action law to the reaction to indicate that with favorable concentrations of reactants and products, the hydrolytic reaction should be readily reversible. Alcock (10) and Bergmann and Fruton (11) have criticized the conclusions drawn by Wasteneys and Borsook on the grounds that peptide bond synthesis was not definitely established, that the optimum pH for synthesis differed markedly from that for hydrolysis, and thus, the reaction was not a simple reversal. Furthermore, the fact that the original proteins from which the peptic digests were derived were not resynthesized indicates the lack of specificity of the reaction and invalidates the application of the mass law treatment to the results. This criticism follows from the fact that the expression of Wasteneys and Borsook (9):

$$\text{protein} + H_2O \rightleftharpoons \text{products}.$$

implies that an equilibrium exists between a specific protein and its split products. If this expression is to be considered valid, the products on undergoing recombination must then yield the same protein from which they were originally derived. Such is definitely not the case. More recently, Northrop (13) has reported that peptic digests of pepsin and trypsin yielded plasteins having neither enzymatic activity nor the general properties of the enzyme protein

(13) Northrop, J. Gen. Physiol. 30, 377 (1947).

from which they were derived. Both Folley (14) and Ecker (15) have determined the molecular weight of plastein and have shown this value to be under 1000.

Studies involving reversal of proteolysis of a somewhat different nature than that of plastein formation have been carried out by Voegtlin and associates (16) who investigated the effects of oxygen tension, sulfhydryl groups and pH on the reversal of proteolysis in tissue autolysates and papain-fibrin digests. They indicated that apparent protein synthesis took place at high oxygen tension, at a pH close to neutrality, in the presence of a relatively high concentration of sulfhydryl groups. The sulfhydryl groups gave rise to the formation of disulfides which, they concluded, were probably essential for the synthesis. Low oxygen tension and low pH favored proteolysis. Bergmann and Fruton (11) have questioned the conclusions regarding pH and sulfhydryl compounds. Such conclusions, they pointed out, were contrary to the observations of Bergmann and Fraenkel-Conrat (2) which indicated that the pH optima and activation requirements of proteolytic enzymes were the same for both the hydrolytic and synthetic reactions, i.e., both required sulfhydryl groups and took place at the same pH. With regard to the specificity of the

(14) Folley, Biochem. J., 26, 99 (1932).

(15) Ecker, J. Gen. Physiol., 30, 399 (1947).

(16) Voegtlin, Maver and Johnson, J. Pharmacol. Exptl. Therap., 48, 241 (1933).

reverse reaction, no experimental proof was forthcoming which might have indicated that the proteins, from which the digests were obtained, were resynthesized on oxygenation. Linderstrøm-Lang and associates (17, 18) reported they were unable to duplicate the results of Voegtlin's group.

The investigations of Bergmann and co-workers (2-7) on the enzymic synthesis of peptide bonds from simple acylamino acids or acylated peptides and aniline, substituted amino acid anilides or phenylhydrazine have already been referred to in the introductory statements. The general equations for these reactions may be written:

(a) For simple acylamino acids and aniline (or phenylhydrazine)

$$R^1CO\text{-}NHCH(R^2)COOH + H_2N\cdot C_6H_5 \longrightarrow R^1CO\text{-}NHCH(R^2)CO\text{-}NH\cdot C_6H_5$$

(b) For acylated peptides and amino acid anilides

$$R^1CO\text{-}NHCH(R^2)CO\text{-}NHCH(R^3)COOH + H_2NCH(R^4)CO\text{-}NH\cdot C_6H_5 \longrightarrow$$

$$R^1CO\text{-}NHCH(R^2)CO\text{-}NHCH(R^3)CO\text{-}NHCH(R^4)CO\text{-}NHC_6H_5$$

It should be pointed out that combinations other than the two given above, e.g. acylamino acids and amino acid anilides, and acylated peptides and aniline, are also possible. In addition, as will be indicated below, the course of reaction (b) does not necessarily follow that indicated in the above equation.

(17) Linderstrøm-Lang and Johansen, Enzymologia, 7, 239 (1939).
(18) Strain and Linderstrøm-Lang, Enzymologia, 7, 241 (1939).

The above reactions, as "models" of peptide bond synthesis, are of interest from several standpoints; in the first place, because of the simple nature of both products and reactants, definite proof of peptide bond synthesis is possible; secondly, because of the insolubility of the anilides, the conditions are greatly in favor of the synthetic reaction and the necessary "driving force" is thus provided; and thirdly, there is evidence that the reaction is specific with respect to both the optical configuration of the acylated amino acids or peptides and the nature of the amino acid residues. In addition, several proteolytic enzymes, including papain, bromelin, pig liver cathepsin and chymotrypsin (19) are capable of catalyzing the synthesis. Recent studies by Fox and Halverson (20) have indicated that the catheptic enzyme, ficin, was also capable of bringing about the synthesis.

With regard to the antipodal specificity of the reaction, it was early demonstrated by Bergmann and Fraenkel-Conrat (2) that the use of acyl derivatives of DL-alanine, DL-leucine, and DL-phenylalanine in the reaction led to the formation of the acyl-L-amino acid anilide only, leaving the acyl-D-amino acid in solution. These results have been confirmed and the method has been used for the resolution of several racemic amino acids. That the specificity is not complete has been

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

(20) Fox and Halverson, Unpublished experiments.

indicated by Behrens and associates (7) who found, on employing an especially active preparation of papain, that acetyl-D-phenylalanylglycine, as well as acetyl-D-phenylalanyl-L-leucine and carbobenzoxy-D-phenylalanylglycine, gave the corresponding anilides when incubated with aniline. However, a comparison of the rates of reaction of both forms showed those of the D-forms to be much lower than those of the corresponding L-isomers. Recent studies by Bennett and Niemann (21), who utilized carbobenzoxy-DL-o-fluorophenylalanine and phenylhydrazine, and by Stevens and Milne (22), who employed several racemic carboallyloxy amino acids and phenylhydrazine as substrates, also indicated that the D-isomers participated in the reaction, but to a lesser extent than the L forms.

Some indications have also been advanced by Bergmann's group that the nature of the amino acid residue in the acylated compound or in the amino acid anilides also influenced the course of the reaction. Behrens, Doherty, and Bergmann (7) reported that neither acetyl-L-phenylalanyl-L-proline nor acetyl-D-phenylalanyl-L-proline reacted with aniline in the presence of papain-cysteine, which is indicative, perhaps, of the need of a hydrogen atom on the peptide nitrogen or, possibly, of the solubility of the anilide. Bergmann and Fraenkel-Conrat (3) pointed out that the reactions between benzoyl-L-

(21) Bennett and Niemann, J. Am. Chem. Soc., 70, 2610 (1948).

(22) Stevens and Milne, Private communication.

leucine with L-leucinanilide or with glycinanilide in the presence of papain are illustrative of the highly developed specificity of enzymic peptide bond synthesis since, in the former case, benzoyl-L-leucyl-L-leucinanilide was formed, whereas in the latter reaction, benzoyl-L-leucinanilide and not benzoyl-L-leucylglycinanilide was obtained. Apparently the latter reaction was not a simple hydrolysis of the anilide to glycine and aniline and later reaction of the free amine with the benzoyl-L-leucine, since, as has been indicated by Behrens and Bergmann (5), glycinanilide was not split by papain under ordinary conditions. These workers reported a similar reaction between acetyl-L-phenylalanylglycine and glycinanilide to ultimately yield acetyl-L-phenylalanylglycinanilide, as well as glycine, aniline, and some unreacted starting materials. In order to account for these products they proposed the following sequence of reactions:

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

Such a scheme is of interest since it is not only illustrative of the simultaneous synthesis and hydrolysis of peptide bonds catalyzed by the same enzyme under the same conditions of pH, activation, etc., but is also indicative of certain

specificity relationships. Thus, while glycinanilide is not hydrolyzed by papain, it reacts with acetyl-L-phenylalanylglycine to give acetyl-L-phenylalanylglycylglycinanilide whose specificity relationship towards the enzyme is such that aniline, and then glycine, can be split off the peptide chain. The free aniline is then available for further reaction to form the final anilide as shown in the above sequence.

In line with the previously mentioned observation that the formation of an insoluble product provided the necessary "driving force" for the synthesis of peptide bonds (anilides) in vitro, Bergmann and Fruton (11) have suggested, that similarly, the energy required for peptide bond synthesis in vivo may be provided, in part, by the removal of insoluble products. They gave as examples of such products the insoluble proteins, collagen and elastin. Northrop (8) has discussed the work of several groups of investigators which indicated that proteins accumulate at surface layers and interfaces as insoluble products, and that the molecules present at the surfaces could act to regulate the formation of more identical molecules. In addition, he suggested that this regulated formation of insoluble products satisfied both the specificity and energy requirements for protein synthesis. While this might well be true with regard to the specificity problem, some question can be raised as to whether the energy requirement is actually met, except for those proteins known

to be insoluble in biological fluids. Since thermodynamic considerations require that the free energy of a specific reaction be the same regardless of the pathways taken in going from reactants to products, then in order to account for the formation of soluble proteins via insoluble intermediates, energy must of necessity be provided for the conversion of the insoluble forms to the soluble forms. This follows from the fact that the net free energy of formation, in solution, of a protein from amino acids or peptides is positive.

An alternative means for providing the necessary energy for peptide synthesis in vivo was advanced by Bergmann and Fruton (11) who postulated that the process is coupled with energy yielding reactions. According to their proposal, the energy liberated by hydrolytic or oxidative reactions could be coupled, by means of common intermediates, to the energy-requiring synthetic reaction. The functions of phosphorylated compounds in carbohydrate metabolism have been indicated by Meyerhof (23) and Kalckar (24) and it is conceivable that the energy derived from these respiratory processes can be linked to the processes of protein synthesis. That such might be the case was indicated in 1940 by Borsook and Dubnoff (25)

(23) Meyerhof, Ann. N.Y. Acad. Sci., 45, 377 (1943-44).

(24) Kalckar, ibid., 45, 395 (1943-44).

(25) Borsook and Dubnoff, J. Biol. Chem. 132, 307 (1940).

who reported the synthesis of hippuric acid from benzoic acid and glycine in the presence of rat liver slices. Inhibition of the respiratory processes by addition of cyanide to the reaction mixture also inhibited the synthetic reaction, indicating that synthesis and respiration were closely bound.

In a recent series of papers dealing with the synthesis of p-aminohippuric acid from p-aminobenzoic acid and glycine in the presence of rat liver slices and liver homogenates, Cohen and McGilvery (26, 27, 28) have indicated that the reaction is closely associated with the citric acid cycle and that energy liberated during oxidation of citric acid cycle components can be transferred through a common intermediate to the synthesizing system. They further indicated that such intermediate was probably adenosine triphosphate and the mechanism of energy transfer may have involved either the formation of p-aminobenzoyl phosphate (acyl phosphate) or of N-phosphoglycine (amide phosphate); the peptide bond could then be formed by the release of phosphoric acid. Further papers dealing with the role of adenosine triphosphate in peptide bond synthesis have recently been published by Bloch (29) and by Speck (30). The former investigator indicated the need for

(26) Cohen and McGilvery, J. Biol. Chem., 166, 261 (1946).
(27) -------- ibid., 169, 119 (1947).
(28) -------- ibid., 171, 121 (1947).
(29) Bloch, J. Biol. Chem., 179, 1245 (1949).
(30) Speck, ibid., 179, 1387, 1405 (1949).

ATP in the synthesis of glutathione in liver homogenates while the latter pointed out that ATP reacts stoichiometrically with gluamate and ammonia to yield glutamine, ADP and phosphate ion.

The question as to whether proteolytic enzymes were instrumental, in part, in bringing about the formation of peptide bonds in any of the above reactions which couple peptide bond synthesis and energy yielding systems is as yet unanswered. If, as has been suggested by Lipmann (31), the acyl phosphates of amino acids, i.e., amino acid phosphates, function instead of amino acids as intermediates in protein synthesis, then in order to uphold the role of proteolytic enzymes in peptide bond synthesis, it would seem that their ability to utilize amino acid phosphates as substrates must be demonstrated. Conversely, the presence of amino acid phosphates in nature must also be demonstrated. The recent work of Neurath and his group (32,33,34) which indicated that trypsin, chymotrypsin and carboxypeptidase possess esterase activity, demonstrated that proteolytic enzymes are not necessarily restricted in their hydrolytic action to peptide groups. Therefore, if proteolytic enzymes were capable of splitting amino acid phosphates, the

(31) Lipmann, Advances in Enzymol., 1, 99 (1941).

(32) Schwert, Neurath, Kaufman and Snoke, J. Biol. Chem., 172, 221 (1948).

(33) Snoke, Schwert, and Neurath, ibid., 175, 7 (1948).

(34) Kaufman, Neurath and Schwert, ibid., 177, 793 (1948).

energy obtained from such hydrolyses should be more than ample to account for that necessary in forming the peptide bond. It is also conceivable that if the energy of hydrolysis of amino acid esters is greater than that required for peptide bond synthesis, then these too may function as possible intermediates.

In addition to amino acid phosphates or N-phosphoamino acids as possible intermediates in protein synthesis, Linderstrøm-Lang (35) has suggested a mechanism involving amino acid aldehydes. Herbst and Shemin (36) have indicated that α-ketacyl amino acids, e.g., pyruvyl alanine, yield dipeptides (alanylalanine) on transamination and might thus function in protein synthesis.

Several theories have recently been advanced to account for the in vivo formation of genes, viruses, enzymes and antibodies by autocatalytic reactions. These have been reviewed by Northrop (8). According to these theories, each molecule of a protein acts as a model or "template" for self-duplication, the amino acids or other intermediates aligning themselves along the template molecule in a predetermined manner. While such a mechanism readily accounts for the specificity of protein formation, the energetics and the process by which the peptide bond is formed between the

(35) Linderstrøm-Lang, Ann. Rev. Biochem., 8, 37 (1939).

(36) Herbst and Shemin, J. Biol. Chem., 147, 541 (1943).

"intermediates" are still left unsolved. It is of interest to speculate that a possible solution to these latter problems lies in invoking high energy intermediates, e.g., amino acid phosphates or N-phosphoamino acids, instead of amino acids, to account for the energetics, and proteolytic enzymes as the agents which ultimately effect closure between these intermediates to form the peptide linkages.

Inhibition of Peptide Bond Synthesis

The value of employing compounds of known or potential enzyme inhibiting properties as tools for determining the mechanism of enzymic reactions as well as for controlling enzymic reactions both *in vitro* and *in vivo* has already been indicated in the introductory remarks. However, before discussing the applicability of inhibitors to the present investigations, it might be appropriate to review briefly some of the general considerations of enzyme action.

It is a generally accepted concept in both the fields of biological and non-biological catalysis that a catalyst exerts its accelerating effects on a reaction by union with the substrate in such a manner as to reduce the energy of activation of the reaction. The earliest indication regarding the probable nature of the complex formed between enzyme

and substrate was advanced by Fischer (37) when he postulated his now classic "lock and key" hypothesis which indicated that there must be a particular "fit" between an enzyme and its substrate before reaction can occur. A quite similar theory was proposed by Ehrlich (38) to explain toxin-antitoxin reactions. This "receptor theory" has been widely promoted, with modifications, in the field of immunology to explain antigen-antibody reactions, c.f. Landsteiner (39). The polyaffinity hypothesis of Bergmann (40), advanced to explain the antipodal specificity of peptidases, may be considered an outgrowth of Fischer's early postulate.

Many of the present concepts concerned with the nature and specificity of enzyme reactions, and founded in part on the effects of inhibitors, assume that enzymes contain "active centers" or essential groups (41) which can combine in a highly specific manner with the substrates. The exact nature of the complexes formed between enzymes and their substrates still remains somewhat obscure. Bayliss (42) has postulated that the enzyme first absorbed the substrate and

(37) Fischer, Ber., 27, 2985 (1894).

(38) Ehrlich, "Studies in Immunity". 2nd ed. John Wiley and Sons, Inc., New York. 1910.

(39) Landsteiner, "The Specificity of Serological Reactions" Rev. ed., Harvard University Press, Cambridge. 1947.

(40) Bergmann, Harvey Lectures, Ser. 31, 37 (1935-36).

(41) Tauber, "The Chemistry and Technology of Enzymes" John Wiley and Sons, Inc., New York. 1949.

(42) Bayliss, "The Nature of Enzyme Action". Longmanns Green and Co., London. 1919.

that the action then took place at the interface. The mathematical treatment applied by Michaelis and Menten (43) suggested the possibility of a chemical union. However, as has been pointed out by Haldane (44), the present knowledge of adsorption phenomena indicates that a sharp line of demarcation between chemical and adsorption reactions does not exist, and at times, differentiation between the two is difficult.

Kalckar (45) has discussed the possible union between enzyme and substrate through groups capable of forming a resonating system. He indicated that stabilization of the complex by resonance might account for the lowering of the energy of activation. Rothen (46) has recently indicated that enzymes may act on substrates at distances greater than 100 A°, his experiments having demonstrated that layers of certain polymer films did not protect bovine albumin from the action of trypsin and pepsin. He concluded that direct contact between enzyme and substrate is not necessary, that enzymatic action may originate through a field of forces resulting from

(43) Michaelis and Menten, Biochem. Z., 49, 333 (1913).

(44) Haldane, "Enzymes" Longmans Green and Co., London. 1930.

(45) Kalckar, in Green, "Currents in Biochemical Research", p. 229. Interscience Publishers, Inc., New York. 1946.

(46) Rothen, J. Biol. Chem., 163, 345 (1946).

the extended resonators suggested by London. Spectroscopic evidence for the formation and breakdown of an intermediary enzyme-substrate complex during the action of catalase on monoethyl hydrogen peroxide has been presented by Stern (47).

In general, the action of inhibitors on enzymatic reactions may be classified under two headings depending on their effects on the velocity of the reactions. The first type, competitive inhibition, involves a direct relationship between inhibitor and substrate such that an increase in the concentration of the substrate tends to reduce the degree of inhibition. Usually there exists a close chemical or structural relationship between the substrate and its inhibitor, the inhibition apparently resulting from competition between the two compounds for the same active centers of the enzyme. Roblin (48) has recently reviewed the subject of competitive inhibition as applied to metabolite antagonists. The second type, non-competitive inhibition, also involves a reduction in the rate of reaction, but variation of the concentration of the substrate has little effect on the degree of inhibition. In addition, there is usually little structural or chemical relationship between inhibitor and substrate.

(47) Stern, J. Biol. Chem., 114, 473 (1936).

(48) Roblin, Chem. Rev., 38, 255 (1946).

Singer (49) has classified enzyme inhibitors into three catagories on the basis of the mechanism of their reactions: (1) compounds which react with the prosthetic groups of enzymes; (2) compounds which are very similar structurally to the natural substrate of the enzyme; and (3) compounds which destroy an essential functional group in the protein component of the enzyme. Examples of the first group are metals, flavins, pyridine nucleotides, etc. The second group has already been described above as competitive inhibitors and the third group contains all those compounds which exhibit reactivity toward a functional group of the protein. Excellent review articles by Herriott (50) and by Olcott and Fraenkel-Conrat (51) have recently appeared on the subject of group reagents for proteins.

It is also possible for an enzymic reaction to be inhibited by reaction of the inhibitor with essential enzyme activators. Such type activators are generally members of a specific class of compounds, e.g. sulfhydryl compounds. They cannot be classed with prosthetic groups since they do not necessarily form a complex with the enzyme nor confer a high degree of specificity on the enzyme system.

(49) Singer Brewers Digest 20, No. 8, 43-6 (85-8T), No. 9, 42-4, 47 (104-6T, 109T) (1945) Original not available for examination: abstracted in C.A. 40, 99^{8} (1946).

(50) Herriott, Advances in Protein Chem., 3, 169 (1947).

(51) Olcott and Fraenkel-Conrat, Chem. Rev., 41, 151 (1947).

Enzymes and enzyme reactions may also be inhibited in other ways. Among these are reaction of the inhibitor with the substrate, inhibition by accumulation of products of the reaction, inactivation by physical agents such as light, sound and pressure (52), inactivation by heating, and inactivation by changes in pH. Certain enzymes are inhibited by naturally occurring "anti-enzymes", e.g., pepsin and trypsin inhibitors (8) and there are many examples in the literature of the inhibition of enzymes by specific immune bodies (53).

Little, if any, information on the inhibition of in vitro enzymic peptide bond synthesis appears in the literature. However, in view of the observations of Bergmann and Fraenkel-Conrat (2) that the enzyme, papain, requires the same conditions of pH, concentration, temperature and activation for the synthesis of anilides as are generally employed in proteolytic experiments, it is then conceivable that the same active centers of papain which are essential for the hydrolytic activities also function in the synthetic processes. It thus becomes plausible to assume that the substances which have been shown to have an inhibitory effect on the proteolytic activity of papain would exhibit a similar effect on

(52) Sumner and Somers, "Chemistry and Methods of Enzymes". Academic Press Inc., New York. 1947.

(53) Sevag, "Immuno-Catalysis". Charles C. Thomas, Baltimore. 1945.

the synthetic activity. On this basis, a survey of the literature dealing with the proteolytic properties of papain, including the effects of activators and inhibitors, was in order.

The literature dealing with the isolation, properties, activation and inhibition of papain is quite extensive and only the essential details will be considered here. The early literature has been reviewed by Mendel and Blood (54), and articles dealing generally with activators and inhibitors of enzymes, with specific reference to papain, have been published by Tauber (55), Hellerman (56), and Bersin (57). The aforementioned review article by Olcott and Fraenkel-Conrat (51), also contains numerous references.

Early workers in the field, notably Vines (58) and Mendel and Blood (54), indicated that papain, the proteolytic enzyme obtained from the latex of the breadfruit plant, Carica papaya, was readily activated by hydrogen cyanide and hydrogen sulfide. Further investigations by Willstätter and his group

(54) Mendel and Blood, J. Biol. Chem., 8, 177 (1910).

(55) Tauber, Ergeb. Enzymforsch., 4, 42 (1935).

(56) Hellerman, Physiol. Rev., 17, 454 (1937).

(57) Bersin, in Nord and Weidenhagen, "Handbuch der Enzymologie" Vol. I, p. 154. Akademische Verlagsgesellschaft, Leipzig. 1940.

(58) Vines, Ann. Botany, 17, 237 (1903).

(59, 60) verified these observations and in addition indicated that while natural papain was capable of hydrolyzing gelatin, it was incapable of hydrolyzing peptone. Hydrogen cyanide activated papain attacked both substrates. They concluded that papain was homogeneous and that hydrogen cyanide played the role of a kinase in extending the specificity range of the enzyme. It was also observed that the pH optima for the digestion of gelatin, peptone ex albumine and fibrin (5, 5 and 7.2 respectively) were very close to their isoelectric points of 4.8, 4.8 and 7.2.

The similarity of plant proteinases, such as papain, to the cathepsins of animal cells with regard to activation by certain substances found in tissues was pointed out by Waldschmidt-Leitz (61). Later studies by Waldschmidt-Leitz and Purr (62) indicated one of these substances to be glutathione. Subsequently, the activation of papain by SH-glutathione and cysteine was demonstrated by Grassman and associates (63).

(59) Willstätter and Grassman, Z. physiol. Chem., 138, 184 (1924).

(60) Willstätter, Grassman and Ambros, ibid., 151, 286, 307 (1926).

(61) Waldschmidt-Leitz, ibid., 188 17 (1930).

(62) Waldschmidt-Leitz and Purr, ibid., 198, 260 (1931).

(63) Grassman, Schoensbeck and Eibeler, ibid., 194, 124 (1931).

In papers published simultaneously by Maschmann and Helmert (64) and by Bersin and Logemann (65), iodoacetic acid was demonstrated to be an inactivator of papain. In addition, the latter group demonstrated that the oxidizing agents, benzoquinone, hydrogen peroxide, iodine and sodium selenite, inhibited the proteolytic action of an activator-free preparation of papain. The fact that hydrogen sulfide, sodium sulfite, and reduced glutathione reactivated papain previously treated with peroxide, iodine and selenite, led Bersin (66) to the conclusions that papain contained sulfhydryl groups essential for its activity and that their subsequent oxidation to disulfide groups resulted in inactivation. The scheme proposed for this reversal was:

$$\text{Papain (Pa-SH (active)} \xrightarrow[\text{HCN, } H_2S\text{, GSH}]{H_2O_2,\ I_2,\ SeO_3^=} \text{Papain (Pa-S-S-Pa) (Inactive)}$$

Quinone and iodoacetate treated papain were not reactivated by the foregoing reducing agents.

Further evidence regarding the presence of sulfhydryl groups in papain was furnished by Hellerman and Perkins (67) who found

(64) Maschmann and Helmert, Z. physiol. Chem., 220, 199 (1933).

(65) Bersin and Logemann, ibid., 220, 209 (1933).

(66) Bersin, ibid., 222, 177 (1933).

(67) Hellerman and Perkins, J. Biol. Chem., 107, 241 (1934).

that iodine, quinone or ferricyanide ion-inactivated papain could be reactivated by a variety of reducing agents such as cysteine, HS-glutathione, thioglycolic acid, hydrogen sulfide, trivalent titanium, and hydrogen cyanide. The action of the activators was presumably the conversion of dithio-papain to sulfhydryl papain. In addition, they reported that papain was extremely sensitive to the action of mercaptide forming reagents, notably cuprous oxide and organo-mercuri compounds of the RHgX type. With the exception of trivalent titanium, all the aforementioned reactivating agents were also effective in reversing the action of the mercaptide-formers. Of especial interest was the action of p-benzoquinone which, as has been previously indicated, was found by Bersin to be irreversible. Hellerman and Perkins have suggested that quinones may inhibit papain irreversibly by the addition of amino or sulfhydryl groups to the olefinic groups. They pointed out, however, that in the case of papain, the addition reaction probably takes place to a limited degree; the main effect of quinone was indicated to be reversible oxidative inactivation.

The effects of many other compounds which presumably inhibit papain and other enzymes by virtue of the reactivity with sulfhydryl groups have been widely studied. These investigations have been summarized in the aforementioned review articles by Hellerman (56), and by Olcott and Fraenkel-Conrat (51).

Investigations carried out by Bergmann and associates (68, 69, 70) concerning the effects of various compounds on the proteolytic activity of papain led them to conclude that papain consists of either two enzymes or of an enzyme system capable of dissociating to give two enzymes. The two enzymes were designated as Papain I and Papain II* (70). Papain I was characterized by its ability to hydrolyze benzoyl isoglutamine and hippurylamide, and by its complete inhibition by phenylhydrazine. Papain II was designated as that component of the enzyme system whichhydrolyzed peptone ex albumine and was activated by phenylhydrazine.** It was further indicated that both enzymes could digest gelatin. Thus, inhibition of Papain I did not prevent hydrolysis of this substrate from proceeding. In addition, it was suggested that Papain I and Papain II mutually inactivated each other through the formation of a reversible complex, "holopapain". Compounds such as hydrogen cyanide, hydrogen sulfide, sulfhydryl compounds and phenylhydrazine were presumably capable

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* Earlier designations for this pair of enzymes were papain polypeptidase and papain proteinase respectively (68), and later, Papain Peptidase I and Papain Peptidase II respectively (69).

** Hydroxylamine inhibited both Papain I and Papain II (69).

(68) Bergmann and Ross, J. Biol. Chem., 111, 659 (1935).

(69) -------- ibid., 114, 717 (1936).

(70) Bergmann, Fruton and Fraenkel-Conrat, ibid., 119, 35 (1937).

of bringing about dissociation of the complex, thereby yielding the two proteolytically active enzymes. In addition, it was hypothesized that Papain I contained an aldehyde group (69) and that the action of phenylhydrazine on the halopapain was to inactivate Papain I, by reaction with the carbonyl group, at the same time that it caused dissociation of the holopapain with the resultant liberation and activation of Papain II.

In view of the suggestion of the foregoing workers concerning the presence of an aldehyde group in papain, Maeda (71) investigated the effects of various carbonyl reagents, including hydroxylamine, sodium bisulfite, phenylhydrazine and dimethylbarbituric acid (specific for aldehyde groups) on the proteolytic action of papain, employing gelatin and hippurylamide as substrates. He found that these reagents inhibited the hydrolysis of gelatin as well as of hippurylamide, although longer contact period between enzyme and inhibitor were required for inactivating papain toward the gelatin than toward the hippurylamide. The results of his investigations led Maeda to the conclusion that papain did possess an aldehyde group. Recently Schales and associates (72) have investigated the effect of carbonyl reagent on the

(71) Maeda, Bull. Chem. Soc. Japan, 12, 319 (1937).

(72) Schales, Suthon, Roux, Lloyd and Schales, Arch. Biochem. 19, 119 (1948).

proteolysis of egg-white by papain as well as by pepsin and trypsin. Moderate inhibition of papain by hydroxylamine, semicarbazide, phenylhydrazine and hydrazine was noted. Dimedon did not inhibit the enzyme and sodium bisulfite had an activating effect. No conclusions were offered regarding the presence or absence of a carbonyl group in papain.

It might here be pointed out that the question as to whether or not papain contains a carbonyl group is still an open one in view of the fact that hydrogen cyanide not only fails to inhibit papain, but indeed, serves as an activator of the compound. In the investigations of Bergmann and Ross, previously mentioned, these authors indicated that hydrogen cyanide was an activator of Papain I, the fraction of the papain system which supposedly contains an aldehyde group. It is not possible to ascertain whether the conditions of activation employed were such that cyanohydrin formation did not occur. It is entirely conceivable that the carbonyl reagents may have either exerted their inhibitory effects by reaction with groups in papain unidentified as yet or by partially denaturing the enzyme.

Reference should be made to the theory proposed by Bersin (73) concerning the probable role of amino groups in the proteolytic action of sulfhydryl enzymes. According to this theory, it is proposed that amino groups of an enzyme

(73) Bersin, Ergeb. Enzymforsch., 4, 68 (1935).

are activated by adjacent sulfhydryl groups. The activated amino groups of the enzyme are then capable of reacting with the peptide linkages of the substrate and this reaction is followed by a dissociation of the peptide bond. This scheme is illustrated below:

1. $-CO-NH- + NH_2\text{-enz} \rightleftharpoons -CO-NH\text{-enz} + NH_2-$

2. $-CO-NH\text{-enz} + H_2O \rightleftharpoons -COOH + NH_2\text{-enz}$

That the above scheme may be invalid for papain is indicated by the recent studies of Balls and Lineweaver (74) on crystalline papain and by the investigations of Greenberg and Winnick (75) on the effect of ketene on papain. Both groups indicated that amino groups are probably unessential for the activity of the enzyme. The latter workers, however, further indicated that phenolic groups of tyrosine may be essential for the proteolytic activity of papain since prolonged action of ketene on the enzyme resulted in inactivation.* In order to avoid a possible reaction of the ketene with sulfhydryl groups, Greenberg and Winnick first oxidized the SH groups with peroxide. This resulted in a reduction of the activity of papain by 55 percent. A consideration of the

* Olcott and Fraenkel-Conrat (51) have cited evidence that ketene reacts more rapidly with amino groups than with phenolic groups. It is thus possible to block the former without affecting the latter.

(74) Balls and Lineweaver, J. Biol. Chem., 130, 669 (1939).

(75) Greenberg and Winnick, ibid., 135, 761 (1940).

fact that prolonged ketene treatment almost completely inactivated peroxide treated papain, resulted in the conclusion that phenolic groups, too, might be essential for papain activity.

Further consideration will be given in the Discussion and Interpretations section to the known and potential papain inhibitors studied in the work reported here, and to their possible modes of action.

EXPERIMENTAL*

Preparation of Compounds Investigated in Enzymic Reactions

Benzoylglycine

This compound was prepared by the conventional Schotten-Baumann reaction utilizing essentially those proportions of reagents recommended by Ingersoll and Babcock (76).

In a typical run, 37.5 gm. (0.5 mole) of glycine (Merck) was dissolved in 500 ml. of 1 N sodium hydroxide. The solution was transferred to a 2 liter, 3-necked, round-bottomed flask equipped with a mechanical stirrer and two dropping funnels and cooled to ca. 10° in an ice bath. Seventy-four gm. (0.525 mole) of benzoyl chloride and 275 ml. (0.55 mole) of 2 N sodium hydroxide were added simultaneously with stirring over a period of about one hour. The ice bath was then removed and the mixture allowed to stir for one hour longer. The solution was then transferred to a beaker and acidified to Congo red with concentrated hydrochloric acid (ca. 60 ml.). The mixture was cooled, the solid filtered off

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* All melting points are uncorrected. All nitrogen analyses, unless otherwise specified, were done by the micro Kjeldahl method.

(76) Ingersoll and Babcock, Organic Syntheses, Coll. 2, 328 (1943).

with suction, and without attempting to dry the material completely, it was boiled with 150 ml. of carbon tetrachloride to remove benzoic acid. The mixture was cooled slightly, filtered, and the extraction procedure with carbon tetrachloride was repeated. The material was filtered, washed with carbon tetrachloride and hexane on the filter, and air dried. It was recrystallized from water-alcohol (3:1). The yield was 75 gm. (84%), with a melting point of 187-190°. Ingersoll and Babcock reported 186-187°.

Benzoyl-DL-valine

This derivative was prepared in 80% yield from DL-valine (Dow) in accordance with the procedure as given for the glycine compound above. Recrystallization from water-alcohol (3:1) gave a product melting at 128-130°. Slimmer (77) has reported the melting point as 132.5°.

Benzoyl-DL-leucine

This compound was prepared in 85% yield from DL-leucine (Dow) utilizing the procedure given for benzoylglycine. In the treatment with hot carbon tetrachloride the material tended to oil somewhat and it was necessary to cool the solution before filtering. Recrystallization from 40% alcohol - 60% water gave a product melting at 136.5-138°.

(77) Slimmer, Ber., 35, 400 (1902).

Fischer (78) reported the melting point as 137-141°.

Benzoyl-L-glutamic acid

The benzoylation procedure which gave the best yields of this compound was essentially the same as that employed for the glycine derivative. One-half mole of L-glutamic acid* was dissolved in 500 ml. of 2 N sodium hydroxide (1 mole) and benzoylated with 74 gm. (0.525 mole) of benzoyl chloride, with the simultaneous addition of 275 ml. (0.55 mole) of 2 N sodium hydroxide. After acylation, the alkaline reaction mixture was acidified in the cold by adding concentrated hydrochloric acid (100 ml.) at a rate such that the temperature did not exceed 10°. Under these conditions, the product oiled out. Upon seeding with a previous preparation, synthesized according to the directions of Fischer (79)**, the reaction mixture yielded a thick creamy mass. This was filtered with suction and pressed out with a rubber dam. The filtrate was reserved for further treatment.

The wet cake was treated with 300 ml. of boiling carbon tetrachloride and once with 300 ml. of hexane. The still wet material was dissolved in 250 ml. of ethyl acetate

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* Obtained from General Mills, Inc. Recrystallized once from hot water.

** Utilization of the method of Fischer in an early preparation, resulted in a yield of 37%.

(78) Fischer, Ber., 33, 2370 (1900).

(79) Fischer, Ber., 32, 2464 (1899).

and the lower aqueous layer (ca 100 ml.) thus obtained combined with the above reserved filtrate. The ethyl acetate layer was first dried rapidly over two portions of anhydrous sodium sulfate and finally over anhydrous calcium sulfate.

The combined aqueous portions were evaporated in vacuo until salts crystallized out; the concentrate was extracted several times with ethyl acetate and the combined extracts, after having been dried over anhydrous sodium sulfate, were added to the main extract which was being dried over anhydrous calcium sulfate.

The ethyl acetate solution was first filtered thru glass wool to remove the drying agent, then thru a #50 Whatman filter paper and finally concentrated in vacuo to 250 ml. Upon adding 100 ml. of carbon tetrachloride to the warm solution, an oil separated out. The mixture was seeded with a previous preparation of benzoyl-L-glutamic acid and placed in the freezer. The material crystallized slowly; in order to hasten the process 200 ml. of hexane was added. The waxy-like solid was filtered, washed with hexane and dried. A white powder (100 gm.) was obtained. Part of the material had passed thru the filter as an oil; after 2 days this had crystallized. It was filtered off, washed and combined with the main batch. The combined solids were then treated twice with 250 ml. portions of carbon tetrachloride, and finally washed with hexane. The yield was 107 gm.

The material was then dissolved in 400 ml. of boiling ethyl acetate, filtered twice through a #50 Whatman filter paper and washed into a 2 l. beaker with an additional 100 ml. of ethyl acetate. The solution was heated to boiling, and carbon tetrachloride (350 ml.) was added to turbidity. On seeding with a previous preparation, crystallization commenced. The mixture was placed in the refrigerator overnight.

The material was filtered, washed with hexane, and dried. Eighty-five gm. of product (68%) melting at 139-140° was obtained.

$[\alpha]_D^{28^\circ} = +18.2 \pm 0.1^\circ$ 5% in 1 N potassium hydroxide

Fischer (79) reported a rotation of -18.7° for the D- isomer.

p-Nitrobenzoylglycine

The procedure for preparation of this compound was quite similar to that given for benzoylglycine with the exception that the solid p-nitrobenzoyl chloride (Eastman white label) was added in an ethereal solution (100 ml. of ether per 0.1 mole of acyl halide). Prior to acidification, the ethereal layer was separated from the aqueous phase and rejected. The solid obtained after acidification with concentrated hydrochloric acid was dried and extracted several times with ether to remove p-nitrobenzoic acid (p-nitrobenzoic acid is insoluble in carbon tetrachloride and hexane). When 0.3 mole (22.5 gm.) of glycine had been used as starting material, the

yield was 55 gm. at this stage. After recrystallization from 75 ml. of ethanol and 500 ml. of water, 45 gm. of material with a melting point of 125-131° was obtained. An additional washing with ether lowered the yield to 44.5 gm. (66%) but raised the melting point to 128-131°. Löb (80) reported a melting point of 129°.

p-Nitrobenzoyl-DL-valine

This derivative was prepared in a manner similar to that described for the glycine compound. During the acylation a deep purple colored solution was obtained; on acidification, however, the color disappeared. The product obtained from 0.3 mole (35.1 gm.) of DL-valine, after washing with ether (three 100 ml. portions), weighed 63 gm. (79%). Recrystallization from 125 ml. of ethanol and 320 ml. of water lowered the yield to 51 gm. (64%). A sample melted at 167-169°. Karrer and Christoffel (81) reported the melting point as 163°.

p-Nitrobenzoyl-DL-leucine

A procedure analogous to that given for the glycine derivative was utilized. A purple solution was obtained during acylation; the color was discharged on acidification.

(80) Löb, Ber., 27, 3093 (1894).

(81) Karrer and Christoffel, Helv. Chim. Acta., 27, 622 (1944).

The product obtained from 0.3 mole (39.3 gm.) of DL-leucine, after washing with ether (200 ml., then 100 ml.), weighed 80.5 gm. (96%). Recrystallization from 400 ml. of ethanol and 300 ml. of water lowered the yield to 70 gm. (83%). The product melted at 230-232° with decomposition. Karrer and Keller (82) reported the melting point as 222-223°.

p-Nitrobenzoyl-L-glutamic acid

Basically the acylation procedure for the preparation of this compound was similar to that described for the other p-nitrobenzoyl derivatives, with the exception that it was necessary to dissolve the L-glutamic acid in 100 ml. of 2 N sodium hydroxide (0.2 mole) per 0.1 mole of amino acid, instead of 100 ml. of 1 N sodium hydroxide (0.1 mole), in order to insure neutralization of both carboxyl groups. During the initial stages of the acylation, a red color, which later disappeared, was evident. On acidification to Congo red with concentrated hydrochloric acid, a gummy solid which rapidly crystallized was obtained. This was filtered off and air dried. (Because the desired product is somewhat soluble in water, the filtrate was concentrated in vacuo until salts separated out; little, if any, acylamino acid seemed to be present in this residue.)

(82) Karrer and Keller, Helv. Chim. Acta., 26, 50 (1943).

The product obtained from 0.2 moles of L-glutamic acid, apparently contained some unreacted amino acid as evidenced by its odor. It was ground in a mortar and extracted with two 150 ml. portions of boiling ether to remove any p-nitrobenzoic acid. The dried residue weighed 43.6 gm. It was further extracted with two portions (250 ml., then 50 ml.) of boiling ethyl acetate and the extracts were combined. The insoluble residue (5 gm.) was rejected.

The product crystallized slowly from the ethyl acetate solution over a 3 day period at room temperature, and finally in the freezer (-12°). The solid was filtered off and dried; the yield was 43.1 gm. (72.5%). A second crop was obtained by adding 300 ml. of hexane to the ethyl acetate filtrate. The oil which settled out crystallized rapidly giving a yield of 3.8 gm. The total yield was 46.9 gm. (79%). The first crop melted at 113.5-116°; the second crop had a melting point of 113-116°. King and associates (83) reported the melting point as 114-116°.

Carbobenzoxy chloride

Carbobenzoxy chloride was prepared from benzyl alcohol and a 20% solution of phosgene according to the directions given by Carter and associates (84). Yields of approximately

(83) King, Speisley and Nimmo-Smith, Nature, 162, 153 (1948).

(84) Carter, Frank and Johnston, Organic Syntheses, 23, 13 (1943).

90% were obtained. The concentration of the acyl halide, which contained some toluene, was about 0.9 gm. per ml. The product was stored at -12° to prevent deterioration.

Carbobenzoxyglycine

The procedure employed was essentially that of Bergmann and Zervas (85); the apparatus used was similar to that described for benzoylglycine. In a typical run 7.5 gm. (0.1 mole) of glycine was dissolved in 50 ml. of 2 N sodium hydroxide (0.1 mole). The solution was cooled in an ice bath and 30 ml. of 4 N sodium hydroxide (0.12 moles, 20% excess) and 19.8 gms. of carbobenzoxy chloride (0.116 moles, 16% excess) were added simultaneously, with stirring, over a period of about 45 minutes. The ice bath was removed, and stirring continued for an hour longer. The solution was extracted once with ether to remove toluene and unreacted carbobenzoxy chloride. The extract was rejected. On acidifying the reaction mixture to Congo red with concentrated hydrochloric acid, an oil which was heavier than water separated out and rapidly crystallized. The material was filtered off and recrystallized from 250 ml. of boiling water. The yield of product, melting at 118-119.5°, was 18.9 gm. (91%). Bergmann and Zervas reported a melting point of 120°.

(85) Bergmann and Zervas, Ber., 65, 1192 (1932).

Carbobenzoxy-DL-valine

One-tenth mole (11.7 gm.) of DL-valine was acylated as described for the glycine derivative. Upon acidification of the reaction mixture, an oil separated out. This was taken up in ethyl acetate by extraction with four 50 ml. portions of the solvent. The extract was dried over anhydrous sodium sulfate and the solvent evaporated off. The thick syrupy residue was placed in the freezer (-12°) overnight. Since the material did not crystallize, it was seeded with a previous preparation (86), placed in a vacuum desiccator, and traces of solvent were removed. After two days, crystallization was complete. The solid was dissolved in 50 ml. of warm benzene. The solution was treated with one gm. of decolorizing carbon (Darco S-51) and filtered. Fifty ml. of hexane was added, the solution was cleared by warming, seeded and set aside to crystallize in the refrigerator. The product was filtered off, dried, and recrystallized from 30 ml. of warm benzene and sufficient hexane to produce a slight turbidity. The yield was 16.3 gm. (65%); the product melted at 76-77.5°.

In a second preparation, ether was substituted for ethyl acetate as the extracting solvent. The syrup, remaining after distilling off the ether, was crystallized from 50 ml. of benzene and 150 ml. of hexane. It yielded 20.5 gm. (82%) of a product melting at 76-78°.

(86) Fox and Fling, Unpublished experiments.

Anal. Calcd. for $C_{13}H_{17}O_4N$: N, 5.58

Found: N, 5.59

Carbobenzoxy-DL-leucine

This compound was previously prepared by Bergmann and Fraenkel-Conrat (2). However, they described it as a syrup. Since there was evidence that the material could be crystallized (86), attempts were made to duplicate this result. Initial trials which utilized the procedure given for carbobenzoxy-DL-valine met with failure although various solvents and solvent pairs were used in attempts to effect crystallization. A portion of the syrupy material which had been kept on a porous plate in a vacuum desiccator yielded a pasty, semi-solid mass after about 3 months, but attempts to recrystallize it failed.

Crystalline carbobenzoxy-DL-leucine was finally obtained in the following manner: Two-tenths of a mole of DL-leucine was treated according to the procedure given for the glycine compound. Acidification of the reaction mixture with concentrated hydrochbric acid yielded an oil which was taken up with ether by extraction with three 75 ml. portions of the solvent. The combined ethereal extracts were washed with 25 ml. of water and re-extracted carefully with 3 portions (75 ml., 50 ml., and 50 ml.) of saturated sodium bicarbonate solution. The combined bicarbonate extracts were then acidified with concentrated hydrochloric acid to Congo red. The

oil which separated was again taken up with ether. The washed ethereal extract was dried over sodium sulfate.

The dry ethereal solution was then concentrated to a syrup by distilling off the solvent in vacuo, a little of the "pasty" preparation previously obtained was added and the material was set into the freezer (-12°). There was some initial evidence of crystallization although no increase was noted overnight. Fifty ml. of benzene was added and the mixture heated to 50°. The syrup did not dissolve. Two hundred ml. of hexane was added and the mixture placed in the freezer. It was stirred periodically with a stirring rod which had a few crystals adhering to it. Crystallization commenced after one day and a white amorphous mass was obtained.

Since it gave evidence of melting at room temperature, the solid was filtered off in a cold room, washed with cold hexane and dried in a vacuum desiccator. Forty gm. of product melting from 44-52° was obtained. This material was dissolved in a minimum amount of warm benzene and filtered. Sufficient hexane was added to the cold filtrate to cause some oiling, and the mixture seeded. The material crystallized slowly, going through a gummy stage, but eventually it yielded a smooth, creamy paste. This was filtered off in the cold, dried by suction and then in a vacuum desiccator. The yield was 35.4 gm. (67%), and the material melted at 45-48°.

Anal. Calcd. for $C_{14}H_{19}O_4N$: N, 5.28

Found: N, 5.17, 5.14.

Carbobenzoxy-L-glutamic acid

The method of Bergmann and Zervas (85) was employed with slight modifications. In a typical run 59 gm. (0.4 moles) of recrystallized L-glutamic acid was suspended in 650 ml. of water in a 2 l., 3-necked, round bottom flask equipped with a mechanical stirrer and dropping funnel. Fifty gm. (1.24 moles) of magnesium oxide was added and the suspension layered with 50 ml. of ether. The mixture was cooled in an ice bath and 75 gm. (0.44 mole) of carbobenzoxy chloride was added over a 45 minute period. The ice bath was removed and the stirring continued for 3 hours. The thick suspension was then filtered with suction; the cake of magnesium oxide was pressed out with a rubber dam and rejected. The filtrate was extracted once with 200 ml. of ether to remove toluene and unreacted acyl halide and the extract rejected. On acidifying to Congo red with concentrated hydrochloric acid (100 ml.), an oil separated out. This was taken up in ethyl acetate by extracting with three 250 ml. portions of the solvent. The combined extracts were dried over sodium sulfate, and concentrated to ca. 200 ml. *in vacuo*. The material was transferred to a beaker, heated to boiling and about 300 ml. of carbon tetrachloride added. The oil which separated out crystallized rapidly on cooling (seeding was employed after the initial preparation of this material had been achieved). The white, creamy mass was filtered, washed with carbon

tetrachloride, dried under an infra-red lamp and ground to a fine powder. The yield was 90 gm. (80%). The product melted at 116-119°. Bergmann and Zervas reported a melting point of 120°. A sample which had been crystallized twice from ethyl acetate-carbon tetrachloride melted from 119-120.5°.

$$[\alpha]_D^{26} = -8.9^\circ \pm 0.2^\circ \qquad 10\% \text{ in ethanol}$$

Carboallyloxyglycine

This compound has been previously described by Stevens and Milne (22) as a non-crystalline material. The following method was employed in attempts to isolate a crystalline product: Fifteen gm. (0.2 mole) of glycine, dissolved in 100 ml. of 2 N sodium hydroxide (0.2 mole), were treated in the cold with 50 ml. of 4 N sodium hydroxide (0.2 mole) and 25 gm. (0.2 mole) of allyl chloroformate* (carboallyloxy chloride) in 50 ml. of ether. The acylating procedure was similar to that described for carbobenzoxyglycine. The alkaline reaction mixture was extracted once with 50 ml. of ether and acidified to Congo red with concentrated hydrochloric acid. The oil which separated was taken up in ether, the ether re-extracted with saturated sodium bicarbonate solution, and the bicarbonate solution, after acidification, was re-extracted with ether.

* A generous sample of this compound was furnished by the Columbia Chemical Division, Pittsburg Plate Glass Co., Pittsburg, Pa. It was redistilled and stored at -12°.

After drying the solution over anhydrous sodium sulfate and anhydrous calcium sulfate, the ether was evaporated off *in vacuo* and the last traces removed in a vacuum desiccator over sulfuric acid. The light colored syrup which remained was treated with 25 ml. of benzene (in which it was not completely soluble) and 100 ml. of hexane. On cooling at -12°, the syrup gradually yielded a waxy-like solid. This was filtered off in the cold and stored in the refrigerator. The yield of material was 9.0 gm. (28%). It melted over a range of about 15-35°. No attempt was made to recrystallize it.

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

(The low nitrogen value may be due to the presence of solvent impurities or condensation of moisture on the cold material while weighing.)

Carboallyloxy-DL-valine

This compound was prepared in a manner similar to that described for the glycine derivative. The syrup obtained was crystallized from ethyl acetate-hexane. The yield of product from 0.3 mole of DL-valine was 35 gm. (58%). The compound melted at 49.5-52°. Stevens and Milne (22) obtained the material as a liquid.

Anal. Calcd. for $C_9H_{15}O_4N$: N, 6.96.
Found: N, 7.11, 7.08.

Carboallyloxy-DL-leucine

This compound was prepared as described for the glycine derivative. The syrup obtained was crystallized from ethyl-acetate-hexane instead of benzene-hexane. The yield from 0.3 mole of DL-leucine was 44.3 gm. (69%). The compound melted at 40-42°. Stevens and Milne (22) reported 41-43°.

Anal. Calcd. for $C_{10}H_{17}O_4N$: N, 6.51

Found: N, 6.56, 6.52.

Carboallyloxy-L-glutamic acid

The acylating procedure used for preparing this derivative was similar to that described for the preparation of carbobenzoxy-L-glutamic acid. Failure to obtain either an oil or a solid on acidifying the filtered reaction mixture necessitated the addition of sodium chloride to salt-out the desired product. This was taken up in ether, and the ether was extracted with saturated sodium bicarbonate solution. After acidification of the bicarbonate extract and the addition of sodium chloride, the oil obtained was again taken up in ether. The ethereal extract was thoroughly dried (essential) over anhydrous sodium sulfate and anhydrous calcium sulfate, and the solvent removed in vacuo. Final traces of ether were removed by placing the material in a vacuum desiccator over sulfuric acid. After standing about 3 weeks, some crystal formation was noted around the edges and on the surface of the

syrupy residue. Further recrystallization was induced by stirring. Finally, the mass was rubbed up with cold hexane and yielded a white, crystalline mass. This was dried in a vacuum desiccator. The yield of material from 0.1 mole of L-glutamic acid was 18 gm. (78%). The compound melted at 55-58°.

$[\alpha]_D^{28} = -0.94^\circ \pm 0.1^\circ$ 5% in 1 N potassium hydroxide

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

Found: N, 6.04.

Benzoylglycinamide

The method of Fischer (87) was employed. Twenty grams of benzoylglycine gave 9.8 gm. (49%) of the amide which melted at 183.5-184.5°. Fischer reported 183°.

Benzoyl-DL-leucinamide

This compound was prepared according to the directions of Max (88). The material obtained from 5.9 gm. of benzoyl-DL-leucine weighed 1.5 gm. (25%) and melted at 168-169°. Max reported 171°.

An attempt to prepare the amide by ammonolysis of benzoyl-DL-leucine ethyl ester with ammoniacal absolute alcohol

(87) Fischer, Ber., 38, 613 (1905).

(88) Max, Ann., 369, 276 (1909).

at room temperature was unsuccessful; the original material was obtained.

Preparation of Intermediates and Compounds Not Investigated in Enzymic Reactions*

DL-Glutamic acid

Commercial L-glutamic acid (General Mills, Inc.) was racemized according to the procedure of Arnow and Opsahl (89). In a typical run, 20 gm. of the amino acid was heated for 4 hours in an oven at 190-195°. The brown melt was cooled slightly and 30 ml. of 20% hydrochloric acid solution was carefully added. The solution was filtered through a coarse sintered glass funnel and refluxed for 4 hours. It was then transferred to a beaker, cooled, treated with 20 ml. of concentrated hydrochloric acid and seeded with a small quantity of partially racemized glutamic acid. Crystallization was allowed to proceed in the refrigerator. The solid was filtered off, washed with alcohol and ether and air dried.

The crude glutamic acid hydrochloride (16.7 gm.) was dissolved in 21 ml. of boiling water, treated with 2 gm. of charcoal (Darco G-60) and filtered hot. Sufficient 2.5 N

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* Many of the compounds, especially the acylamino acid amides described in this section were prepared in anticipation of carrying out studies similar to those reported for the parent acids. Since these studies were not performed, the synthesis of the compounds are reported as a matter of possible interest and information to others.

(89) Arnow and Opsahl, J. Biol. Chem., 134, 649 (1940).

sodium hydroxide (45 ml.) was added to bring the solution to pH 4.5. The product, which crystallized out in the cold, was filtered and washed with alcohol and ether. The yield was 8.7 gm. (43%). A solution of 0.5 gm. of the material in 25 ml. of 5 N hydrochloric acid showed no rotation.

In a subsequent experiment, in which 200 gm. of L-glutamic acid was employed, the crude hydrochloride (175 gm.) was recrystallized from 325 ml. of 20% hydrochloric acid solution instead of converting it to the free acid. The yield was 124 gm. (62%). Treatment of the hydrochloric acid filtrate with sodium hydroxide yielded a small quantity (11 gm.) of the free acid.

Carbobenzoxy-DL-glutamic acid

This compound was prepared in 85% yield using the same procedure as described for the L-isomer. It exhibited a lesser tendency to oil and did not seem to dissolve in ethyl acetate as readily as the L-compound. A sample melted at 118-121°. Fruton and associates (90) reported 119°.

Glycine ethyl ester hydrochloride

The method of Harries and Weiss (91) was used. Twenty-five gm. of glycine suspended in 80 ml. of absolute alcohol,

(90) Fruton, Irving and Bergmann, J. Biol. Chem., 133, 703 (1940).

(91) Harries and Weiss, Ann., 327, 365 (1903).

was heated, at reflux, with dry hydrogen chloride. The product which separated weighed 42.2 gm (90%) and melted at 141-143°. Harries and Weiss reported 144°.

DL-Leucine ethyl ester hydrochloride

This compound was prepared in a manner similar to the above. The alcohol soluble product was precipitated with an excess of anhydrous ether. The yield from 30 gm. DL-leucine was 31 gm. (69%). The product melted at 108-110.5°. Röhmann (92) reported 112°.

Benzoyl-DL-valine ethyl ester

A modification of the method of Fox (93) for the benzoylation of diiodo-L-tyrosine ethyl ester hydrochloride was followed. A solution of 7.25 gm. (0.04 mole) of DL-valine ethyl ester hydrochloride in 19 ml. of water, contained in a 1 liter, 3-necked flask equipped with a mechanical stirrer and dropping funnel, was treated with 90 ml. of 2 N sodium carbonate (0.09 mole) and 200 ml. of chloroform. The mixture was cooled in an ice bath and a solution of 6.5 gm. of benzoyl chloride (0.046 moles, 15% excess) in some dry chloroform was added with stirring over a 30 minute period. The ice bath was removed and the stirring continued for an additional 30 minutes. The

(92) Röhmann, Ber., 30, 1980 (1897).

(93) Fox, J. Am. Chem. Soc., 68, 194 (1946).

chloroform was separated off and the aqueous layer was extracted twice with chloroform (25 ml.) and twice with ether (25 ml.). All extracts were combined and dried over sodium sulfate. After evaporating off the solvent in vacuo, a yellow syrup was obtained. This was dissolved in 25 ml. of benzene and treated with 275 ml. of hexane. A seed, obtained by pretreating a small quantity of the benzene solution, was introduced and the mixture set aside to crystallize in the cold. Filtration gave 8.2 gm. (82%) of material with a melting point of 65-68°.

Anal. Calcd. for $C_{14}H_{19}O_3N$: N, 5.62.
Found: N, 5.40, 5.43.

Benzoyl-DL-leucine ethyl ester

This compound was prepared in the same manner as the valine derivative. From 7.8 gm. (0.04 mole) of DL-leucine ethyl ester hydrochloride, 8.5 gm. (82%) of product with a melting point of 76-77.5° was obtained. Bouveault and Locquin (94) reported a melting point of 79° ; Max (88) reported 73-75°.

p-Nitrobenzoylglycine ethyl ester

This compound was prepared in a manner analogous to that given for benzoyl-DL-valine ethyl ester. The reaction mixture

(94) Bouveault and Locquin, Bull. Soc. Chim. France., [3] 35, 968 (1906).

was extracted with chloroform only. The syrup remaining after evaporation of the solvent was crystallized from benzene-hexane. The solid obtained was recrystallized from the same solvent pair. The yield from 5 gm. of glycine ethyl ester hydrochloride was 8 gm. (88%) of material melting at 140-142°. Curtius (95) reported the melting point as 142°.

Carbobenzoxyglycine ethyl ester

The general procedure employed for benzoylglycine ethyl ester was followed (carbobenzoxy chloride was substituted for benzoylchloride). The oily residue, obtained after concentrating the chloroform-ether extract of the reaction mixture, was crystallized at -12° from benzene-hexane. The product derived from 5.6 gm. (0.04 mole) of glycine ethyl ester hydrochloride weighed 4.6 gm. (49%); a sample melted at 33-34°. The melting point was determined by placing a crystal of the material on the bulb of a thermometer and allowing it to warm over a hot plate.

Anal. Calcd. for $C_{12}H_{15}O_4N$: N, 5.90.

Found: N, 5.91, 5.89.

Carbobenzoxy-DL-valine ethyl ester

The material obtained by treating 5.5 gm. (0.03 mole) of DL-valine ethyl ester hydrochloride, as described for benzoyl-

(95) Curtius, J. prakt. Chem., 94 (N.S.), 120 (1916).

glycine ethyl ester, yielded an oil which was soluble in benzene-hexane and crystallized on long standing at -12°. Recrystallization from benzene-hexane gave 2.7 gm. (32%) of product with a melting point of 32-33°. The melting point was determined as for the glycine derivative above.

Anal. Calcd. for $C_{15}H_{21}O_4N$: N, 5.02.

Found: N, 5.07.

Carbobenzoxy-DL-leucine ethyl ester

This derivative was prepared by treating 5.9 gm. (0.03 mole) of DL-leucine ethyl ester hydrochloride with 5.9 gm. (.034 mole) of carbobenzoxy chloride using the general procedure described for benzoylglycine ethyl ester. The residue, obtained after evaporation of the extraction solvent, was crystallized from benzene-hexane. The solid was filtered off in the cold; a second crop was obtained from the filtrate. A combined yield of 6.9 gm. (78%) was obtained. A sample melted at 18.5-19°. The melting point was determined as described for carbobenzoxyglycine ethyl ester .

Anal. Calcd. for $C_{16}H_{23}O_4N$: N, 4.78.

Found: N, 4.85, 4.73.

Carboallyloxy-DL-valine ethyl ester

Treatment of 5.4 gm. (0.03 mole) of DL-valine ethyl ester hydrochloride with 4.2 gm. (0.034 mole) of allylchloroformate, according to the general acylating procedure, yielded an oil

which showed no tendency to crystallize from benzene-hexane at -12°. Removal of the solvents yielded an oil which solidified at -12°. This was treated with cold hexane and filtered in the cold. Additional material crystallized out from the filtrate. The combined yields weighed 2.5 gm. (36%) and melted at 9-11°. The melting point was determined by placing a crystal of the material on the bulb of a thermometer previously cooled to 0° and allowing it to warm at room temperature. The compound was stored at -12°.

Anal. Calcd. for $C_{11}H_{19}O_4N$: N, 6.11.

Found: N, 5.89.

Carbobenzoxyglycinamide

Carbobenzoxyglycylchloride was synthesized according to the method of Bergmann and Zervas (85). Addition of the acid chloride, obtained from 4.2 gm. (0.02 mole) of carbobenzoxyglycine, to 50 ml. of anhydrous ether, previously saturated with ammonia, gave a white precipitate. Ammonia was passed through the mixture for 15 minutes. After cooling overnight, the solid was filtered off. This material was extracted with 40 ml. of boiling ethyl acetate and the residue remaining after the extraction was extracted continuously for 7 hours, with ethyl acetate, using a Butt extractor. The residue of ammonium chloride in the extraction thimble was rejected. The ethyl acetate extracts were combined, heated to boiling

and filtered hot. The filtrate was evaporated to a low volume. Upon cooling, crystallization took place. The material was filtered off. An additional crop was obtained by adding hexane to the filtrate. The combined yield was 2.4 gm. (58%) with a melting point of 133-136°.

Anal. Calcd. for $C_{10}H_{12}O_3N_2$: N, 13.4.

Found: N, 13.3, 13.3.

A second preparation of the amide was obtained by treating 1.8 gm. (0.0075 mole) of carbobenzoxy glycine ethyl ester, in 20 ml. of absolute alcohol, with a stream of ammonia for 45 minutes. After 4 days at room temperature, crystals appeared. The alcohol was removed in vacuo and the solid residue recrystallized from 15 ml. of boiling water. Filtration gave 1.1 gm. (71%) of material melting at 136-137.5°. A mixed melting point, run with the above preparation, gave 135-136.5°.

Carboallyloxyglycinamide

An attempt was made to prepare carboallyoxyglycine ethyl ester by treating 5.6 gm. (0.04 mole) of glycine ethyl ester hydrochloride with 5.6 gm. (0.046 mole) of allylchloroformate in 25 ml. of carbon tetrachloride. The general procedure described for benzoylglycine ethyl ester was used. A non-crystallizable oil (7.3 gm.) was obtained.

The oil was dissolved in 50 ml. of absolute alcohol and dry ammonia gas was passed into the solution for 15 minutes.

The flask was stoppered and after 6 weeks (this period is probably too long) at room temperature the alcohol was distilled off *in vacuo*. The solid residue was dissolved in a small quantity of ethyl acetate, and on the addition of hexane, the material crystallized. The yield was 5.5 gm. or 87%, based on the .04 mole of glycine ethyl ester hydrochloride used in the preparation of the intermediate acylamino acid ester; the melting point was 107-107.5°.

Anal. Calcd. for $C_6H_{10}O_3N_2$: N, 17.7.

Found: N, 17.2.

Carboallyloxy-DL-leucinamide

Attempts to prepare the corresponding ester from 5.9 gm. (0.03 mole) of DL-leucine ethyl ester hydrochloride, according to the general acylating procedure, yielded a non-crystallizable oil (7.1 gm.).

The oil was dissolved in 50 ml. absolute alcohol and treated with gaseous ammonia as described above. The syrupy residue which remained after evaporation of the alcohol was dissolved in 25 ml. of hot ethyl acetate. One hundred ml. of hexane was added and after 4 hours at -12°, crystallization commenced. After filtering, washing with hexane, and drying, 1.5 gm. (23%) of product with a melting point of 83-85° was obtained.

Anal. Calcd. for $C_{10}H_{18}O_3N_2$: N, 13.1.

Found: N, 13.1.

Enzyme Studies

Conditions for enzymic synthesis of anilides

Acylamino acid solutions. Half molar solutions of the various acylamino acids were prepared shortly before use by dissolving the necessary quantity of the compound in an equivalent amount of 5.8 N sodium hydroxide and diluting with water. Each milliliter of these solutions contained 0.0005 mole of the compound.

Papain preparations and solutions. Various commercial preparations of the enzyme were used; however, most of the work reported was carried out using two lots (#1953 and #2924) obtained from Nutritional Biochemicals Corporation; these had almost equal anilide synthesizing abilities. The enzyme solution generally employed in the investigations contained, the soluble portion of 16 mgm. of papain and 6.4 mgm. of cysteine hydrochloride (Merck) as the activator per half ml. These solutions were prepared by suspending the requisite amount of the enzyme in water, adding the necessary quantity of cysteine hydrochloride, stirring occasionally over a half-hour period to break up the lumps, and centrifuging down the insoluble matter. About 10% of the papain was insoluble under these conditions. Fresh solutions were prepared shortly before use.

Papain, free of natural activators, was prepared, with some modifications, according to the method of Grassman (96). One hundred gm. of commercial papain was suspended in 2 l. of water and the suspension cooled in an ice bath while a slow stream of hydrogen sulfide was bubbled through for 5 hours. The solids were centrifuged down and rejected; the enzyme was precipitated by adding sufficient methanol (4.6 l.) to the supernatant to give a 70% methanol solution. The precipitated enzyme was allowed to settle in the cold, most of the alcoholic solution syphoned off and the remainder removed by centrifugation. The enzyme residue was redissolved in 2 l. of water and the treatment with hydrogen sulfide and methanol repeated. The residue which remained was washed once with methanol and once with ether, transferred to a clay plate and dried *in vacuo*. The hard brown solid thus obtained was ground to a fine powder in a mortar. It weighed 31 gm. A whiter product was obtained if the enzyme was washed twice with methanol and twice with ether previous to drying. Transfer to a clay plate was also unnecessary since the drying could be carried out more conveniently in the centrifuge bottles.

Aniline. Commercial aniline was redistilled at atmospheric pressure and the almost colorless fraction was collected.

Citric acid. A U.S.P. grade of citric acid (Pfizer) was used in preparing the citrate buffers.

(96) Grassman, *Biochem. Z.*, *279*, 131 (1935).

Preparation of buffer solutions. The enzymic syntheses were carried out in 1 M and 0.1 M citrate solutions at different pH values ranging from about pH 3.0 to pH 6.5 in steps of 0.5 pH unit. It was necessary, in order to avoid the tedious process of adjusting the pH of each reaction individually, to prepare buffers of specific pH values and concentrations, such that, after addition of all reactants, the final pH and buffer concentrations would be reasonably close to the desired value. The following preliminary studies were therefore carried out:

Benzoylglycine was used as representative of the acylated monoaminomonocarboxylic acids and carbobenzoxy-L-glutamic acid was used as representative of the acylated glutamic acids. Half molar solutions of these compounds were prepared as described. A solution of papain and cysteine was prepared as described; it was diluted with an equal volume of water. Solutions of 3 M, 1 M and 0.3 M citric acid and of 18 N and 6 N sodium hydroxide were also prepared.

One series of 10 ml. beakers containing 1.67 ml. of 3 M citric acid and one series of 10 ml. beakers containing 1.67 ml. of 0.3 M citric acid were set up for each of the two acylamino acids. To each beaker was then added 1 ml. (0.0005 mole) of the acylamino acid solution, 0.1 ml. (0.0011 mole) of redistilled aniline, and 1 ml. of the diluted papain-

cysteine solution* (extract of 16 mgm. papain - 6.4 mgm. cysteine). The contents of each beaker were then titrated to the desired pH values (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 or 6.5) with 18 N sodium hydroxide, for those solutions containing 3 M citric acid, and with 6 N sodium hydroxide, for those solutions containing 0.3 M citric acid; a microburette was used for the titrations. Since the pH values of the solutions containing 0.3 M citric acid were above pH 4 before titration, it was necessary to add a measured volume of the 1 M citric acid in order to attain the lower pH values. All volumes were adjusted to 5 ml., to give final citrate concentrations of either 1.0 or 0.1 M, except for those cases where the 1 M citric acid had been added.

New series of beakers containing 1.67 ml. of either 3 M or 0.3 M citric acid were then set up. To each beaker was added the requisite amount of 18 N or 6 N sodium hydroxide or 1 M citric acid as indicated by the titrations above; the volumes were adjusted to 2.9 ml. (This was a convenient volume since addition of 1 ml. of the acylamino acid solution,

* The proportions of acylamino acid, aniline, papain and cysteine used were quite similar to those employed by Fruton and associates (90), for the enzymic resolution of carbobenzoxy-DL-glutamic acid. Preliminary studies with carbobenzoxy-L-glutamic acid wherein similar concentrations of the acylamino acids and aniline were used, but the papain and cysteine concentrations were varied, indicated that these proportions were suitable for this substrate. The concentration of acylamino acids specified was without regard to optical configuration. Thus, for the L-acylamino acids the ratio of acid to aniline was about 2:1. For the DL-acylamino acids, based on the L-form only, it was approximately 4:1.

0.1 ml. of aniline and 1 ml. of the diluted papain-cysteine solution gave a final volume of 5.0 ml.) The pH of each of these solutions was then determined; these values were those required for the stock buffer solutions, before addition of the reactants. In order to check the method, the specified amounts of substrates and enzyme solutions were added to each of the beakers, and the pH values of the resultant mixtures determined; these were within a few tenths of a pH unit of the desired value.

Finally large quantities (200 ml.) of stock solutions of the various buffers were prepared by adding to 115 ml. quantities of 3 M or 0.3 M citric acid the calculated amounts of 18 N or 6 N sodium hydroxide or of 1 M citric acid necessary to give the desired pH values. Final adjustments were made with the use of a pH meter and the solutions were diluted to 200 ml. With the exception of those buffers requiring added citric acid (actually these were not buffers but citric acid solutions stronger than 0.173 M), the citrate concentrations of these solutions were 1.73 M or 0.173 M. Dilution of 2.9 ml. of these buffer solutions to 5 ml. with the reactants, thus gave a final citrate concentration of 1 M or 0.1 M respectively, except for those cases where additional citric acid had been required. It was found that all of the 1.73 M series of buffer solutions, with but one exception, served equally well for both types of acylamino acids. However, it was necessary to prepare separate series of 0.173 M

buffer solutions for the acylated monoaminomonocarboxylic acids and for the acylated glutamic acids. The data, pertinent to the preparation of the three series of buffer solutions, are summarized in Table 1.

Effects of pH and buffer concentrations on anilide syntheses. The following procedure was adopted for carrying out the studies on the effects of varying pH and buffer concentrations on the enzymic syntheses of the various acylamino acids. The reactions were carried out in 16 mm. X 50 mm. shell vials. To duplicate series of eight shell vials were added 2.9 ml. of the appropriate stock buffers of both concentration series. (Table 1) One-tenth ml. of redistilled aniline, 1 ml. of the acylated amino acid solution and 1 ml. of a papain-cysteine solution (prepared as described but diluted with an equal volume of water), were added in the order given.

The contents were stirred well and the pH values determined. (It was necessary, in the cases of all the acyl glutamic acids in the 0.1 M buffers at pH 6.0 and 6.5, to add a few drops of 2 N sodium hydroxide in order to attain these values). The vials were then stoppered with paraffined corks and incubated for 72 hours at 40° ± 1° in a thermostatically controlled water bath. The contents were shaken by hand every 12 hours.

At the end of the reaction period, the pH values were again determined (in almost all cases they had not varied more

Table 1

pH and Concentration Relationships of Citrate Buffer Solutions for Anilide Synthesis Studies

A. 1.73 M buffer solutions for both acylated monoaminomonocarboxylic acids and acylated glutamic acids.

pH		Citrate Concentration, M	
Before adding reactants	After adding[1] reactants	Before adding reactants	After adding reactants
2.6	3.3 ± 0.1	1.725	1.0
3.1	3.8 ± 0.1	1.725	1.0
3.7	4.3 ± 0.1	1.725	1.0
4.3	4.7 ± 0.1	1.725	1.0
5.0	5.3 ± 0.1	1.725	1.0
5.5	5.7 ± 0.15	1.725	1.0
6.1	6.2 ± 0.15	1.725	1.0
6.5[2]	6.6 ± 0.15	1.725	1.0
6.0[3]	6.5 ± 0.1	1.725	1.0

[1]The actual pH obtained after addition of the reactants varied somewhat with the nature of the acylamino acid. The variations are denoted by the ranges.

[2]For acylated monoaminomonocarboxylic acids only.

[3]For acylated glutamic acids only.

Table 1 (Continued)

B. "0.173" M buffer solutions for acylated monoaminomonocarboxylic acids only.

pH		Citrate Concentration, M	
Before adding reactants	After adding[1] reactants	Before adding reactants	After adding reactants
1.5	3.1 ± 0.1	0.69 [2]	0.40
1.7	3.6 ± 0.1	0.38 [2]	0.22
1.9	4.1 ± 0.1	0.225[2]	0.13
3.1	4.7 ± 0.15	0.173	0.10
4.1	5.1 ± 0.15	0.173	0.10
5.2	5.6 ± 0.15	0.173	0.10
5.9	6.1 ± 0.15	0.173	0.10
6.6	6.65± 0.1	0.173	0.10

[1]The pH obtained after addition of the reactants varied somewhat with the nature of the acylamino acid. The variations are denoted by the ranges.

[2]These are citric acid solutions, i.e., no sodium hydroxide was added.

Table 1 (Continued)

C. "0.173" M buffer solutions for acylated glutamic acids only.

pH		Citrate Concentration, M	
Before adding reactants	After adding[1] reactants	Before adding reactants	After adding reactants
1.5	3.1 ± 0.1	0.69 [2]	0.40
1.7	3.6 ± 0.1	0.38 [2]	0.22
2.0	4.1 ± 0.1	0.173[2]	0.10
3.6	4.6 ± 0.1	0.173	0.10
4.9	5.1 ± 0.1	0.173	0.10
6.5	5.6 ± 0.1	0.173	0.10
11.4	6.0[3]	0.173	0.10
11.4	6.5[3]	0.173	0.10

[1]The pH obtained after addition of the reactants varied somewhat with the nature of the acylglutamic acid. The variations are denoted by the ranges.

[2]These are citric acid solutions, i.e., no sodium hydroxide was added.

[3]In order to attain this pH it was necessary to add a few drops of 2 N sodium hydroxide to the reaction mixture.

than 0.2 pH unit) and the contents of the vials were transferred to 25 mm. X 150 mm. test tubes. In the case of the acylated monoaminomonocarboxylic acids, 15-20 ml. of 1 N sodium hydroxide was used in the transferring procedure and, in addition, sufficient 6 N sodium hydroxide was added to those tubes containing the 1 M buffer solutions of pH values below 3.5 to give an alkaline reaction to phenolphthalein. The treatment with sodium hydroxide insured the removal of any unreacted acylamino acid, many of which were insoluble in the reaction mixture, and were therefore potential contaminants of the anilides. For the acylated glutamic acids, 15-20 ml. of 1 N hydrochloric acid was used in the transferring procedure; here the danger of contamination of the anilide was not too great since the acylated glutamic acids investigated were all soluble under the reaction conditions. (Sodium hydroxide, naturally, cannot be used because the anilides contain a free carboxyl group.) The insoluble anilides were then filtered with suction, washed with several portions of water and dried in the air. Careful washing of the acylated glutamic acid anilides was essential since they tended to be gelatinous in nature and absorbed significant amounts of citrate. The compounds were then weighed to the

nearest milligram* and the melting points determined on a melting point block. All of the studies were repeated.

Purification of the acylamino acid anilides. The accumulated yields of each of the anilides were pooled in order to obtain sufficient material for characterization and determination of melting points and rotation. In certain instances where only small quantities of the materials were available, more was synthesized enzymically using proportionally larger quantities of the reactants. The reactions were carried out at the optimum pH for the substrate determined from the above type studies.

All the acylated monoaminomonocarboxylic acid anilides were washed with 1 N sodium hydroxide, then with water and dried previous to recrystallization. All were recrystallized from dioxane-water by first dissolving in the minimum quantity of hot dioxane, treating the solution with a small quantity of decolorizing carbon (Darco G-60) and adding water to the hot filtrate to incipient crystallization. The recrystallization procedure was repeated with the omission of the carbon. The acylated glutamic acid anilides were recrystallized twice from

* As has been indicated in the experimental section, the carboallyloxyglycine was somewhat impure. On this basis the yields of carboallyloxyglycinanilide should have been corrected for the impurities in the starting compound. However, the percentage of impurities, based on the deviation of the nitrogen content from theoretical, were within the limits of accuracy of the method used in conducting the anilide syntheses studies. No corrections were therefore applied.

dioxane-hexane in a manner similar to that employed above. Hexane was used instead of water since the latter gave gelatinous instead of crystalline precipitates. The melting points for all the anilides, the optical rotations and the analytical data for those not previously prepared or characterized are summarized below.

Benzoylglycinanilide. Melting point: first recrystallization, 213-215°; second recrystallization, 213-215°. Bergmann and Fraenkel-Conrat (2) reported 212.5°.

Benzoyl-L-valinanilide. Melting point: first recrystallization, 215-217°; second recrystallization, 215-217°. Fox and associates (97) reported a melting point of 218-220° on an analyzed sample, prepared with the utilization of somewhat different conditions.

Benzoyl-L-leucinanilide. Melting point: first recrystallization, 214-215°; second recrystallization, 213.5-215°. Bergmann and Fraenkel-Conrat (2) reported a melting point of 213°.

Benzoyl-L-glutamic acid anilide. Melting point: first recrystallization, 169.5-171°; second recrystallization, 169-171°.

Anal. Calcd. for $C_{18}H_{18}O_4N_2$: N, 8.58

Found: N, 8.34. (micro Dumas)

(97) Fox, Minard, Wax, Pettinga and Strifert, Federation Proc., 8, (Part 1), 198 (1949).

$[\alpha]_D^{28} = +1.25^\circ \pm 0.25^\circ$ 2% in 95% ethanol

$[\alpha]_D^{28} = +4.55^\circ \pm 0.3^\circ$ 2% in 0.5 N potassium hydroxide

p-Nitrobenzoylglycinanilide. Melting point: first recrystallization, 213.5-215.5°; second recrystallization, 213.5-215.5°.

Anal. Calcd. for $C_{15}H_{13}O_4N_3$: N, 14.1.

Found: N, 14.1. (micro Dumas)

p-Nitrobenzoyl-L-valinanilide. Melting point: first recrystallization, 214-215.5°; second recrystallization, 215-216°.

Anal. Calcd. for $C_{18}H_{19}O_4N_3$: N, 12.3.

Found: N, 12.0 (micro Dumas)

$[\alpha]_D^{28} = -15.4^\circ \pm 0.3^\circ$ 2% in dioxane

p-Nitrobenzoyl-L-leucinanilide. Melting point: first recrystallization, 188-190°; second recrystallization, 188-189.5°.

Anal. Calcd. for $C_{19}H_{21}O_4N_3$: N, 11.8.

Found: N, 12.0 (micro Dumas)

$[\alpha]_D^{28} = +14.1^\circ \pm 0.5^\circ$ 2% in 95% ethanol

p-Nitrobenzoyl-L-glutamic acid anilide. Melting point: first recrystallization, 190.5-192°; second recrystallization, 191-192°.

Anal. Calcd. for $C_{18}H_{17}O_6N_3$: N, 11.3.

Found: N, 11.2. (micro Dumas)

$[\alpha]_D^{28} = + 10.5^\circ \pm 0.3^\circ$ 2% in 0.5 N potassium hydroxide

Carbobenzoxyglycinanilide. Melting point: first recrystallization, 144-144.5°; second recrystallization, 144-144.5°. Bergmann and Fraenkel-Conrat (2) reported 144°.

Carbobenzoxy-L-valinanilide. Melting point: first recrystallization, 182-183.5°; second recrystallization, 182-183.5°.

Anal. Calcd. for $C_{19}H_{22}O_3N_2$: N, 8.58.

Found: N, 8.59. (micro Dumas)

$[\alpha]_D^{28} = - 33.0^\circ \pm 0.5^\circ$ 2% in chloroform

Carbobenzoxy-L-leucinanilide. Melting point: first recrystallization, 138-141°; second recrystallization, 138-141°. Bergmann and Fraenkel-Conrat (2) prepared this compound but reported no melting point.

Anal. Calcd. for $C_{20}H_{24}O_3N_2$: N, 8.24.

Found: N, 8.46 (micro Dumas)

$[\alpha]_D^{28} = - 47.6^\circ \pm 0.3^\circ$ 2% in chloroform

Carbobenzoxy-L-glutamic acid anilide. Melting point: first recrystallization, 195-196°; second recrystallization, 195-196°. Behrens and Bergmann (5) reported 193-195°.

Carboallyloxyglycinanilide. Melting point: first recrystallization, 134-136°; second recrystallization, 134-136°.

Anal. Calcd. for $C_{12}H_{14}O_3N_2$: N, 12.0.

Found: N, 11.7, 11.6.

Carboallyloxy-L-valinanilide. Melting point: first recrystallization, 168-168.5°; second recrystallization, 168-169°.

Anal. Calcd. for $C_{15}H_{20}O_3N_2$: N, 10.1.

Found: N, 10.0.

$[\alpha]_D^{28} = -49.0° \pm 0.5°$ 2% in chloroform

Carboallyloxy-L-leucinanilide. Melting point: first recrystallization, 160-161.5°; second recrystallization, 160.5 - 162°.

Anal. Calcd. for $C_{16}H_{22}O_3N_2$: N, 9.65.

Found: N, 9.48, 9.43.

$[\alpha]_D^{28} = -64.0° \pm 0.3°$ 2% in chloroform

Enzymic hydrolysis of acylamino acid amides

Acylamino acid amide solutions. A 0.01 M alcoholic solution of benzoylglycinamide was prepared by dissolving 62.4 mgm. of the amide in 35 ml. of 95% ethanol. Each 2 ml. of this solution contained 0.00002 moles of the amide.

Similarly, 0.02 M alcoholic solutions of benzoyl-DL-valinamide* and benzoyl-DL-leucinamide were prepared by dissolving 110 mgm. and 163.2 mgm. respectively of the two compounds in 25 ml. of 95% ethanol. Each 2 ml. of these solutions contained 0.00002 moles of the L-form of the amide.

Papain-cysteine solution. Solutions of papain and cysteine hydrochloride in both 1 M pH 5.0 and 0.1 M pH 5.0 citrate buffer solutions were prepared by suspending 40 mgm. of the commercial enzyme and dissolving 40 mgm. of the activator in 10 ml. of the buffer solutions. After the solutions had stood one-half hour with intermittent stirring, the solids were centrifuged down and rejected. Each 0.25 ml. of the supernatant solutions contained 1 mgm. cysteine hydrochloride and the soluble portion of 1 mgm. papain.

Ammonium sulfate standards. A solution of ammonium sulfate containing 0.3 mgm. nitrogen per 0.75 ml. was prepared by dissolving 188.5 mgm. of dry, analytical grade ammonium sulfate in 100 ml. of 1 M pH 5.0 citrate buffer. The solution was stored in the cold. Dilution of this solution with an equal volume of the citrate buffer previous to use , yielded a solution containing 0.15 mgm. nitrogen per 0.75 ml. Further dilution of this second solution with an equal volume of the buffer gave a solution containing 0.075 mgm. nitrogen per 0.75 ml.

* The benzoyl-DL-valinamide was prepared by Dr. S. W. Fox.

Preparation of modified Nessler's reagent. Nessler's solution was prepared according to the formula of Koch and McMeekin (98). Immediately prior to use, one volume of the reagent was mixed with one-half volume of a freshly prepared 2.5% solution of potassium persulfate and with one-half volume of a freshly prepared 1% solution of potassium gluconate.* Only small quantities of the modified reagent were prepared at one time since, according to Gentzkow (99), who described its preparation and use, the solution is not stable for longer than 15 minutes.

Preparation of the standard curve. Two ml. portions of 95% ethanol were pipetted into 22 mm. X 175 mm. test tubes, which had been previously calibrated to contain 25.0 ml., and after the alcohol had been removed by evaporation at 100°, 0.75 ml. portions of the above prepared ammonium sulfate solutions were pipetted in. Duplicates were used. (This procedure furnished a control on the alcohol, since the amides were dispensed from alcoholic solutions.) To these tubes were then added 0.25 ml. quantities of the papain-cysteine solution and after stoppering with paraffined rubber

* A purified preparation of potassium gluconate was kindly furnished by Dr. Ethelda Norberg.

(98) Hawk, Oser and Summerson, "Practical Physiological Chemistry", 12th ed., p. 1230, The Blakiston Co., Philadelphia. 1947.

(99) Gentzkow, J. Biol. Chem., 143, 531 (1942).

stoppers, they were allowed to stand at 40° for a period equal to that of the hydrolytic experiments.

The contents of the tubes were then diluted to about 20 ml. with distilled water, 4 ml. of the modified Nessler's reagent was added and the volumes were made up to the mark (25.0 ml.). After 5 minutes had been allowed for the color to develop, the percents transmission, at 480 mμ, were determined on a Coleman spectrophotometer, Model 11. The values were plotted on semi-log paper against the nitrogen concentrations. An individual curve was determined for each run.

Conditions for hydrolysis of the acylamino acid amides. Two ml. aliquots of each of the above prepared alcoholic solutions were pipetted into 23 mm. X 175 mm. test tubes which had previously been calibrated to contain 25.0 ml. To each tube was then added 2 small glass beads, and the alcohol was evaporated off at 100° leaving the dried residues of the amides.

To half of the tubes of each series was added 0.75 ml. of 1 M pH 5.0 citrate buffer; to the remaining tubes of each series was added 0.75 ml. of 0.1 M pH 5.0 citrate buffer. Several of the tubes from each series were then set aside as controls and to the remainder was added 0.25 ml. of the papain-cysteine solution in the appropriate concentration of buffer. All tubes, including the controls, were then incubated at 40° for a period of about 2 days with occasional shaking, since the amides were incompletely soluble in the reaction mixtures.

Also carried along at 40° were portions of the papain-cysteine solutions.

At the end of the reaction period, the contents of all the tubes were diluted to about 20 ml. To the control tubes were added, immediately before Nesslerization, 0.25 ml. portions of the proper papain-cysteine solution. These tubes served not only to control possible non-enzymic hydrolysis of the amide by the citrate buffer during the incubation period, but also to control hydrolysis by the alkaline Nessler's reagent. All tubes were Nesslerized, as described for the standards, and after 5 minutes, but not longer than 15 minutes, the contents were filtered through Pyrex glass wool into cuvettes, and the percents transmission determined. The amounts of ammonia liberated were determined from the plotted standard curve.

Inhibition of peptide bond synthesis

Preparation of test solutions. One-tenth molar solutions of the test substances (potential inhibitors) were prepared in either aqueous or alcoholic solutions. In the cases of those substances which were soluble in water the proper quantity of material (0.0025 mole) was dissolved in 12.5 ml. of 0.2 M pH 5.0 citrate buffer and after readjustment of the pH to 5.0 (usually with 2 N sodium hydroxide) the solution was diluted to 25 ml. with water. The resultant solutions were thus 0.1 M in the test substances. Portions of these

solutions were then diluted with an equal volume of 0.1 M pH 5.0 citrate buffer to give 0.01 M solutions of the test compounds. Substances so treated included betaine hydrochloride, 2,3,5-triphenyl tetrazolium chloride,* hydroxylamine hydrochloride, cholestenone-6-sulfonic acid,** ethyl carbamate, copper sulfate, 2-methyl-1,4-naphthoquinone bisulfite,*** maleic acid,**** furoic acid, carboallyloxy-L-glutamic acid, sodium bisulfite, fumaric acid,***** dichloroacetyl-DL-valine,****** phenylhydrazine hydrochloride, formaldehyde, barbituric acid, and potassium ferricyanide.

Aqueous solutions of iodoacetic acid in concentrations of 0.001 M and 0.0001 M and of basic phenylmercuric nitrate ******* in concentrations of 0.01 M and 0.001 M were similarly prepared. Sodium nitrite solutions in concentrations of 0.11 M and 0.011 M were prepared but the citrate buffer was omitted until immediately before use.

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* Prepared by Mrs. E. H. Atkinson.

** Prepared by Mr. W. McGukin.

*** Kindly furnished by Abbott Laboratories, North Chicago, Ill.

**** Prepared by Dr. F. N. Minard.

***** Kindly furnished by Dr. J. S. Pruzansky.

****** Prepared according to the method of Woolett and Coulter, J. Am. Chem. Soc., 56, 1922 (1934).

Non-acidic, water insoluble compounds were dissolved in 95% ethanol so as to give 0.1 M and 0.01 M solutions. Substances so treated included 2,5-diphenyl-3-(p-iodophenyl)-tetrazolium chloride,* p-iodoaniline,* o-hydroxyphenyl acetic acid,** p-benzoquinone, coumarin and phenacyl bromide. Indoleacetic acid was dissolved in absolute alcohol and an equivalent amount of 6 N sodium hydroxide was added; 0.1 M and 0.01 M solutions were prepared. The 2-hydroxy-5-methyl-chloropropiophenone*** was treated in warm absolute alcohol with an equivalent amount of 2.5 M sodium acetate (100) to convert it to the unsaturated 2-hydroxy - 5-methylacrylophenone; 0.1 M and 0.01 M solutions were prepared. Because of its insolubility in most solvents chrysene (Eastman recrystallized) was added as a solid in 2 mgm. quantities.

Procedures for following effects of test substances on enzymic anilide synthesis. Preliminary tests were carried out to determine the order of addition of the various reactants to the solutions of the test substances. Results

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* Prepared by Mrs. E. H. Atkinson.

** Kindly furnished by Dr. J. Levine, Federal Security Agency, Washington. This compound was acidic but decomposed on being neutralized with sodium hydroxide. It was therefore used without neutralization since it had little effect on the final pH of the reaction mixture.

*** Kindly furnished by Dr. W. P. Geiger. New York University College of Medicine. New York.

(100) Geiger, W. P., Private communication.

indicated that pronounced differences in the degree of inhibition of the reaction did not exist whether the papain-cysteine solution was added to the inhibitor solutions previous to the addition of the substrates or was added to the reaction mixture last. Therefore, in most of the studies the enzyme-activator solution was added last.

Preliminary studies also indicated that the extents of inhibition varied inversely with the length of the reaction period and that compounds which inhibited the initial stages of the reactions quite markedly, showed little or no inhibitory effects if measurements were made after a 3 day period. Consequently, it was necessary to set a time limit on the reaction. With the utilization of the procedure previously given for the determination of the effects of pH and buffer concentrations on the anilide syntheses, time-yield studies were carried out for several of the reactions at their pH optima in 1 M citrate buffer, over a 3 day period. Based on these results, the reaction time limit was arbitrarily set as that time in which the yield of the anilide was 50% of the 3 day value. For the two compounds, carbobenzoxy-L-glutamic acid and benzoyl-DL-leucine, which were the only ones used as substrates in the inhibitor studies, this time was about 2 hours.

Because of the rather short durations of the reactions, it was necessary to provide a means of keeping the reaction

in check until all the reactants had been added to all the reaction vessels. It was also necessary to provide a means for "quenching" the reaction after the 2 hour period. It was found that if the reaction mixtures were kept at 10° or below, essentially no synthesis took place within a 2 hour period. Therefore cooling of the reaction mixtures was adopted as the method for keeping the reaction in check and for quenching the reaction.

The following general procedure was adopted for carrying out the inhibition studies: One-half ml. aliquots of the previously prepared test solutions were pipetted into 16 mm. X 50 mm. shell vials.* In those cases where the test substances had been dissolved in alcohol, the solvent was removed *in vacuo* over sulfuric acid and replaced with one-half ml. of 0.1 M pH 5.0 citrate buffer. Two and nine-tenths ml. of the appropriate 0.73 M citrate buffer (pH 4.4 for carbobenzoxy-L-glutamic acid and pH 5.25 for benzoyl-DL-leucine) were then added, and after the solutions were stirred, 0.1 ml. of aniline and 1.0 ml. of the appropriate acylamino acid solution (0.5 M) were added. The vials were then cooled in an ice bath, until the temperature of the contents had fallen below 10°, and one-half ml. of a cold solution of papain-cysteine (prepared as previously described) was added. All the reaction mixtures

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* It was necessary to pipet the basic phenylmercuric nitrate (0.01 M) from a hot solution since it was insoluble in the cold; chrysene was added as a solid (2 mgm.) and one-half ml. of a 0.1 M pH 5.0 citrate buffer was added.

were stirred well with a glass rod and the vials were incubated at 40°±1° for two hours.* (The vials were stoppered with paraffined corks after the contents had warmed up to 40°.) The vials were shaken several times during the reaction period.

At the end of 2 hours the vials were cooled in an ice bath, and the contents were transferred to 25 mm. X 150 mm. test tubes with the aid of 15-20 ml. of 1 N sodium hydroxide when benzoyl-DL-leucine was the substrate or 15-20 ml. of 1 N hydrochloric acid when carbobenzoxy-L-glutamic acid was the substrate. The residual anilides were filtered, washed, dried, and weighed to the nearest milligram.

In a few cases the anilides were contaminated with insoluble test substances and it was necessary to either wash out the contaminants with a selective solvent or to dissolve the anilides in solvents in which the contaminants were insoluble. It should be pointed out, that had the test substances been completely insoluble in both the reaction mixture and the wash solvents (1 N sodium hydroxide and water for benzoyl-L-leucinanilide and 1 N hydrochloric acid and water for carbobenzoxy-L-glutamic acid anilide) the errors introduced, had they not been removed, would have been quite large in some instances and negligible in others, depending on the

* The pH values of several of the reaction mixtures were determined. Within the limit of error of the pH meter, these were the same regardless of the nature of test substance, i.e. pH 4.95 when carbobenzoxy-L-glutamic acid was the substrate and pH 5.75 when benzoyl-DL-leucine was the substrate.

molecular weight of the test substance and on the yields of the anilides. Thus, for example, the usual control yield of benzoyl-L-leucinanilide was about 40 mgm. The contaminating test substances of this anilide were in the main the reduced forms of 1,3,5 -triphenyltetrazolium chloride and of 1,5-diphenyl-3-(p-iodophenyl)-tetrazolium chloride (formazans), p-iodoaniline, 2-hydroxy-5-methylacrylophenone, p-benzoquinone, phenacyl bromide and cholestenone-6-sulfonic acid.

Based on the fact that there was present at the highest concentrations of test substances, 0.00005 mole of the materials, the maximum amounts of contamination expected from the above named compounds would have been about 16 mgm., 23 mgm., 11 mgm., 7 mgm., 5 mgm., 10 mgm. and 23 mgm, respectively. In other words the contaminants, had they been completely insoluble, could have introduced errors ranging from about 12 to 58 percent of the weight of the controls. Hexane was used in attempts to remove all the above named contaminants except the cholestenone-6-sulfonic acid. The anilides, contained in 25 mm. X 150 mm. test tubes, were treated with about 25 ml. of the solvent, filtered and washed with more solvent until the washings ran clear. A total of about 50 ml. of the solvent was employed. A control sample was similarly treated to correct for possible solubility of the anilide in the solvent. Cholestenone-6-sulfonic acid was removed by washing the anilide with about 25 ml. of 1 N hydrochloric acid and after filtering,

the residue was further washed with 50 ml. of water. A control was similarly treated. The final weights of the anilides, after washing out the contaminants were then corrected for losses due to the solubility of the anilide in the wash solvents.

The usual control yield of carbobenzoxy-L-glutamic acid anilide was about 90 mgm. The contaminants, in the main, appeared to be a reduced form of 1,5-diphenyl-3-(p-iodophenyl)-tetrazolium chloride (this was not simply a formazan as it was insoluble in hexane), 2-hydroxy-5-methylacrylophenone, p-benzoquinone, cholestenone-6-sulfonic acid and coumarin. The possible amounts of these contaminants, assuming complete insolubility in the reaction mixture and washings were about 23 mgm., 7 mgm., 5 mgm., 23 mgm., and 7 mgm. respectively, or about 5 to 25% of the control yield. The tetrazolium compound was corrected for by dissolving the anilide in 1 N sodium hydroxide, and after centrifuging the colloidal suspension, the amount of the insoluble residue was estimated (2-3 mgm.) and the original weight was corrected. The anilides contaminated with 2-hydroxy-5-methylacrylophenone and p-benzoquinone were washed with hexane, as described for benzoyl-L-leucinanilide. It was obvious from the fact that the anilides contaminated with cholestenone-6-sulfonic acid and coumarin, weighed more than the controls that little or no inhibition had taken place and no attempts were made to correct for these contaminations. Because of the variations existing between the

weights of replicate samples, and the uncertainties regarding the complete removal of contaminants, it was arbitrarily established that yields of anilides deviating by 15% or less from the control yields should not be considered as indicative of inhibition.

In a few of the studies, it was found necessary or desirable to add the papain solutions, without added cysteine, to the test substance solutions previous to the addition of the other components of the reaction mixture. In these cases one-half ml. of the test solution and one-half ml. of a papain solution, which contained the soluble portion of 16 mgm. papain, were mixed. After the mixture had stood for the desired one-half hour, 2.9 ml. of the buffer, in which was dissolved 6.4 mgm. of cysteine hydrochloride, was added. One ml. of the acylamino acid solution was then added, and after the mixture had been cooled in an ice bath to below 10°, 0.1 ml. of redistilled aniline was added. The remainder of the treatment was as described above. Melting point checks were run on samples chosen at random.

RESULTS

Peptide Bond Synthesis and Hydrolysis

Effects of varying pH, buffer concentration and acylamino acid on peptide bond synthesis

The need for determining the effects of variation of pH on the synthesis of the various acylamino acid anilides investigated, was suggested by results (not reported herein) obtained in connection with preliminary studies on the synthesis and inhibition of the synthesis of carbobenzoxy-L-glutamic acid anilide. The conditions employed in these initial studies followed those given by Fruton, Irving and Bergmann (90), who utilized 0.02 M citrate buffer at an apparent pH of 5.0. It was noted that addition to the reaction mixture of certain substances, e.g., hydantoinpropionic acid, DL- and L-glutamic acids, etc., whose potential inhibitory properties were being tested, not only failed to inhibit, but actually stimulated the reaction. This was evidenced by higher yields ofcarbobenzoxy-L-glutamic acid anilide. The addition of an equivalent amount of propionic acid also resulted in stimulation. It thus became evident that acidic substances were stimulating the reaction and that the synthetic conditions given in the literature were possibly not

the optimum ones.* Although Bergmann and Fraenkel-Conrat (2) have indicated that the enzymic synthesis of carbobenzoxy-glycinanilide was quite pH sensitive, with an optimum at about pH 4.6, no other instances of similar studies were cited by the authors. In fact, it appears that the conditions employed throughout most of the investigations of the Bergmann group were usually the same except with respect to the nature of the acylated substrate. It thereby became evident that the determination of the pH optimum for the synthesis of carbobenzoxy-L-glutamic acid anilide was fundamental to further studies.

At the same time, observations by Pettinga (101) indicated the utility of higher concentrations of buffers. Initial studies conducted at several pH values in both 1.0 M and 0.1 M citrate buffers indicated that not only was the pH optimum for the synthesis of carbobenzoxy-L-glutamic acid anilide lower than that implied by the work of Fruton and associates (90), but that somewhat higher yields of the anilide were obtained at the higher buffer concentrations.

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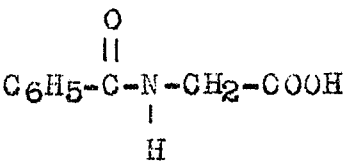
* Iodoacetic acid, however, completely inhibited the reaction. In view of the demonstrated inhibitory properties of this compound toward papain, this result was not unexpected.

(101) Pettinga, "Factors Affecting Enzymic Peptide Bond Synthesis". Unpublished Ph.D. Thesis. Ames, Iowa. Iowa State College Library. 1949.

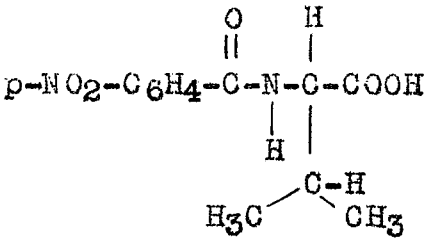
The use of the acylamino acids studied was suggested by the fact that the literature reported practically no investigations in which attempts had been made to correlate the effects of varying both the nature of the amino acid and the nature of the acyl group on the ability of the compounds to react, under enzymic influence, with aniline. The desirability of such type studies was further indicated by the work of Fox and associates (97), who observed that the two structurally similar acylamino acids, benzoyl-DL-valine and benzoyl-DL-leucine, gave markedly different yields of the corresponding anilides under similar reaction conditions.* Based on these observations, which indicated that slight structural differences in the amino acid residue gave rise to pronounced differences in reactivity, it was decided that further investigations, in which not only the effects of varying the amino acid but also the effects of varying the acyl groups, were in order. Consequently, the benzoyl, p-nitrobenzoyl, carbobenzoxy and carboallyloxy derivatives of the four amino acids, glycine, DL-valine, DL-leucine and L-glutamic acid were prepared and their abilities to undergo enzymic peptide bond (anilide) syntheses were studied at varying pH values and at two buffer concentrations. A typical representative

* Unpublished experiments by Fox, Pettinga and Halverson have indicated that these differences were not due to differences in the solubility of the anilides, but rather to differences in the rate of formation of the anilides.

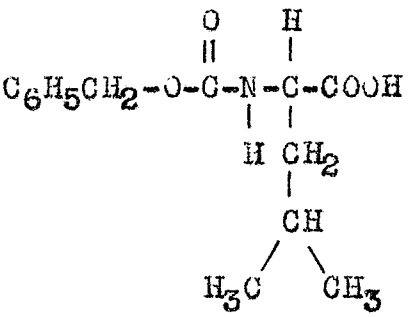
of each of these compounds is illustrated below:



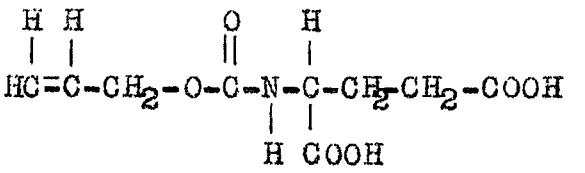
Benzoylglycine



p-Nitrobenzoylvaline



Carbobenzoxyleucine



Carboallyloxyglutamic acid

The choice of the particular compounds studied was dictated partially by the availability of the starting material and partially by the interrelationships existing between either the amino acid residues or the acyl groups. Thus, glycine was selected because it does not exist in optically active forms; DL-valine and DL-leucine were selected because of the marked differences in the reactivity of their benzoyl derivatives noted by Fox and coworkers, and L-glutamic acid was chosen as representative of the monoaminodicarboxylic acids.

The results on the synthesis of the various anilides, obtained by varying both pH and buffer concentrations, are indicated graphically in Figures 1 through 4. The ordinates

represent percent yield of the anilides based, in the cases of the optically active forms, on the theoretically possible yield of the L form; the abscissas represent the initial pH values of the reaction mixtures. All curves shown are for reactions carried out for 72 hours at 40° ± 1°. The proportions of the reactants employed were: 0.0005 mole of acylamino acid (regardless of configuration), 0.0011 mole of aniline, a centrifuged solution of 16 mgm. commercial papain, and 6.4 mgm. of cysteine hydrochloride all in 5.0 ml. of 1.0 M or 0.1 M citrate buffer.* The optimum pH ranges and the percentage yields in these ranges for the various acylamino acids are indicated in Table 2.

An examination of both the tabulated data and the curves indicates the following facts and relationships:

1. Without exception, the pH optima for those systems which gave a measurable yield of the anilide were lower when 0.1 M citrate buffer was employed than when the 1.0 M buffer was used.
2. With but one exception, i.e., carbobenzoxy-L-leucinanilide, the yields of the anilides, when

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* As has been indicated in the experimental section, the citrate concentrations for some of the citrate buffers were of necessity somewhat higher than 0.1 M for reaction mixtures having pH values of about 4 or lower. These slightly higher concentrations probably had little effect on the reaction since either low or no yields at all were generally obtained at these lower pH values in the dilute buffers.

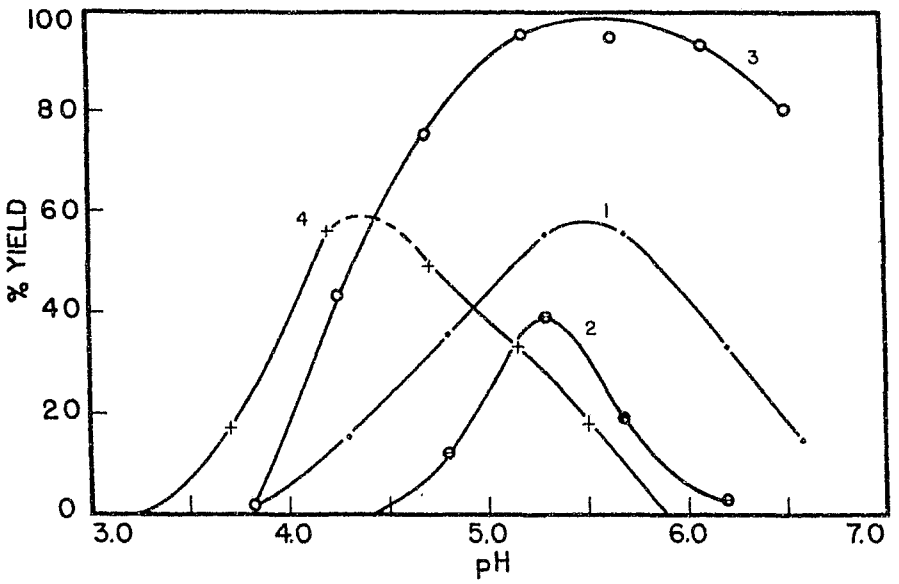

Fig. 1a

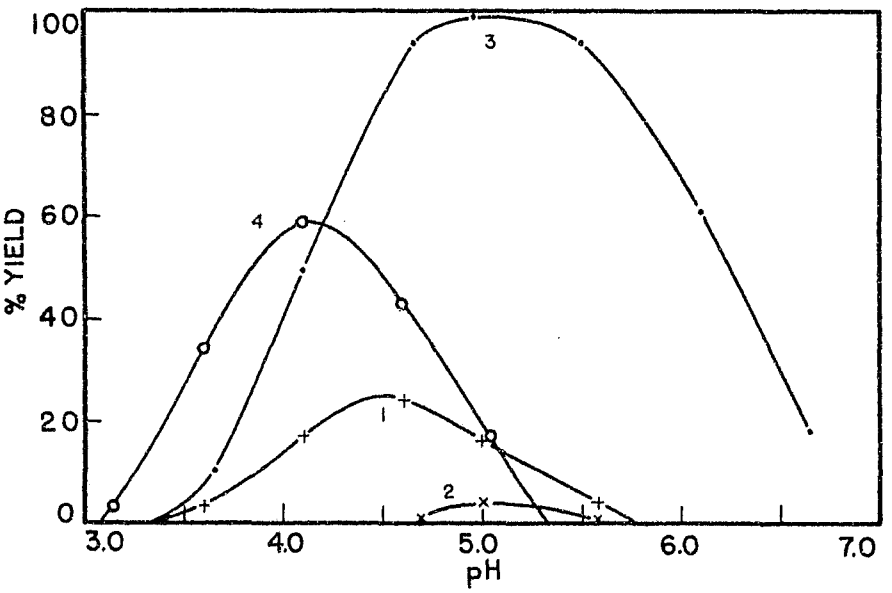

Fig. 1b

Fig. 1. Effects of pH and Citrate Buffer Concentrations on Yields of Benzoylamino Acid Anilides

Buffer concentrations were: Fig. 1a, 1.0 M; Fig. 1b, 0.1 M. Yields were based on theoretical amounts of L-forms. Curves 1,2,3 and 4 are for the glycine, L-valine, L-leucine and L-glutamic acid derivatives respectively.

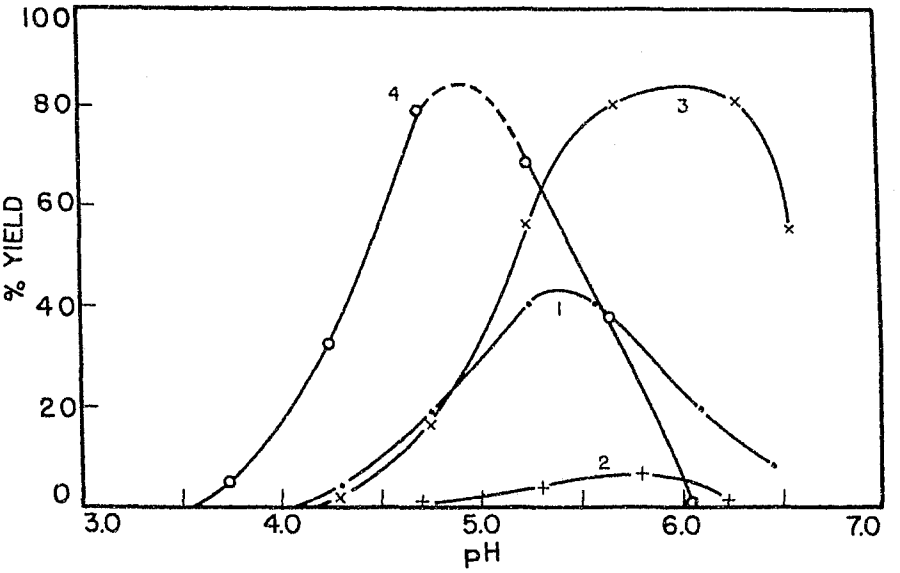

Fig. 2a

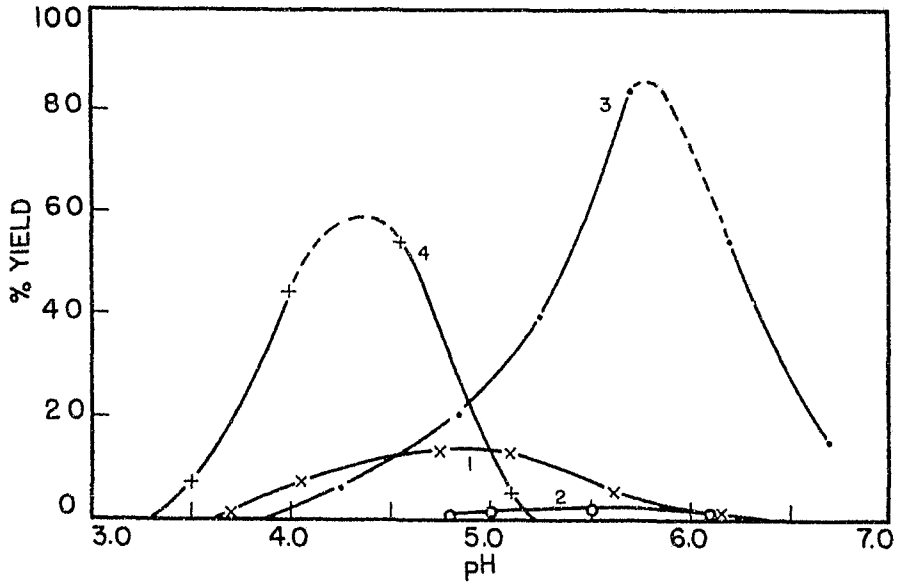

Fig. 2b

Fig. 2. Effects of pH and Citrate Buffer Concentration on Yields of p-Nitrobenzoylamino Acid Anilides

Buffer concentrations were: Fig. 2a, 1.0 M; Fig. 2b, 0.1 M. Yields were based on theoretical amounts of L-forms. Curves 1,2,3 and 4 are for the glycine, L-valine, L-leucine, and L-glutamic acid derivatives respectively.

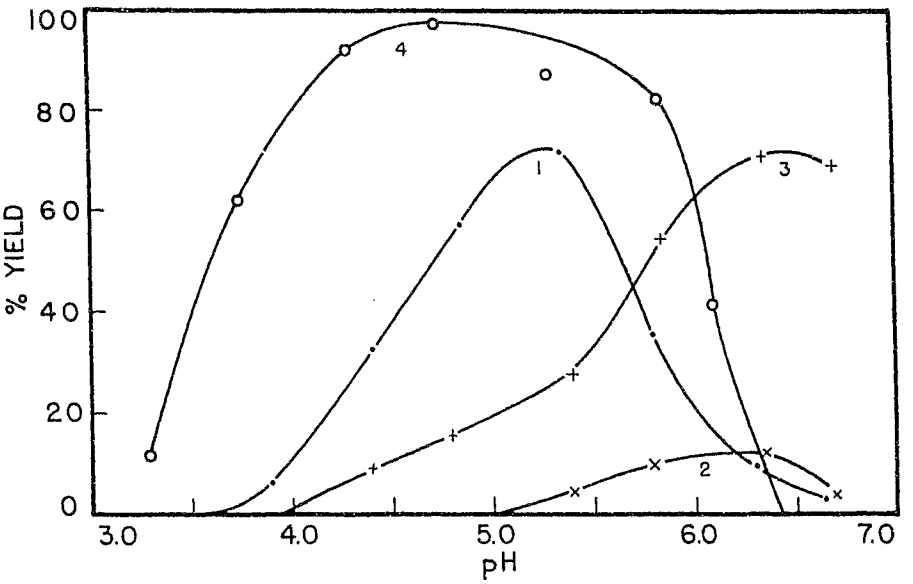

Fig. 3a

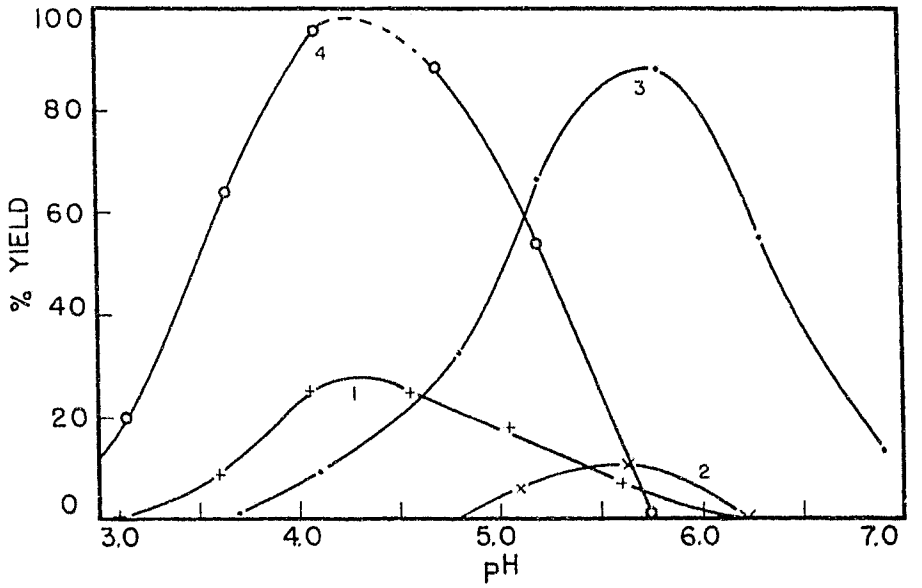

Fig. 3b

Fig. 3. Effects of pH and Citrate Buffer Concentrations on Yields of Carbobenzoxyamino Acid Anilides

Buffer concentrations were: Fig. 3a, 1.0 M; Fig. 3b, 0.1 M. Yields were based on theoretical amounts of L-forms. Curves 1,2,3 and 4 are for the glycine, L-valine, L-leucine, and L-glutamic acid derivatives respectively.

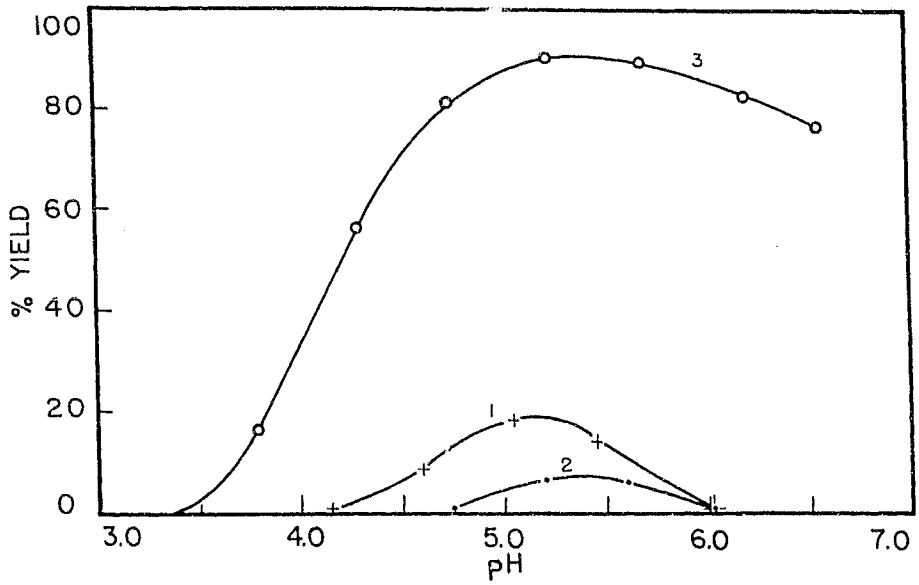

Fig. 4a

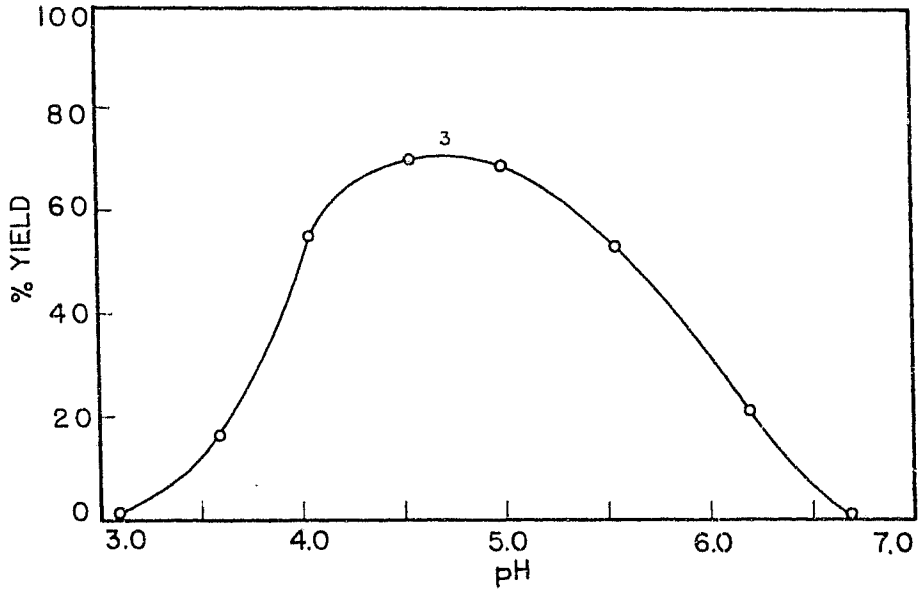

Fig. 4b

Fig. 4. Effects of pH and Citrate Buffer Concentrations on Yields of Carboallyloxyamino Acid Anilides

Buffer concentrations were: Fig. 4a, 1.0 M; Fig. 4b, 0.1 M. Yields were based on theoretical amounts of L-forms. Curves 1,2,3 and 4 are for the glycine, L-valine, L-leucine, and L-glutamic acid derivatives respectively. Absence of a curve for any of the compounds indicates that measurable yields were not obtained.

Table 2

Optimum pH Ranges and Approximate Yields of Anilides for Various Acylamino Acids[3]

Acylamino Acid	Optimum pH Range[1]		Percent Yields of Anilides[2]	
	1 M Buffer	0.1 M Buffer	1 M Buffer	0.1 M Buffer
Benzoyl glycine	5.4-5.6	4.5-4.7	60	25
Benzoyl-DL-valine	5.2-5.4	4.9-5.1	40	4
Benzoyl-DL-leucine	5.5-5.8	5.0-5.2	100	100
Benzoyl-L-glutamic acid	4.3-4.5	4.1-4.3	60	60
p-Nitrobenzoyl glycine	5.3-5.6	4.8-5.0	45	15
p-Nitrobenzoyl-DL-valine	5.7-5.9	5.4-5.6	7	2
p-Nitrobenzoyl-DL-leucine	5.9-6.1	5.7-5.9	85	85
p-Nitrobenzoyl-L-glutamic acid	4.7-5.0	4.3-4.5	85	60
Carbobenzoxyglycine	5.1-5.4	4.2-4.4	70	30
Carbobenzoxy-DL-valine	6.2-6.4	5.5-5.7	15	10
Carbobenzoxy-DL-leucine	6.3-6.5	5.6-5.8	70	90
Carbobenzoxy-L-glutamic acid	4.6-4.9	4.1-4.3	95	95
Carboallyloxyglycine	5.1-5.3	-----	20	--
Carboallyloxy-DL-valine	5.3-5.5	-----	8	--
Carboallyloxy-DL-leucine	5.3-5.6	4.6-4.8	95	70
Carboallyloxy-L-glutamic acid	-----	-----	--	--

[1] See text for conditions.

[2] Given to the nearest 5% except where the yields were below 10%.

Table 2 (Continued)

[3]The studies were not carried out concurrently. The data for the curves were obtained by studying the acylamino acids in pairs. Thus the acylglycine and the corresponding acyl-DL-valine compounds were studied together; similarly the acyl-DL-leucine and corresponding acyl-L-glutamic acid derivatives were investigated at the same time. The above results, taken from Figures 1 to 4, are the mean values of duplicate runs. The yields are the mean values obtained at the optimum pH ranges.

1.0 M citrate was employed, were equal to or greater than the yields in the 0.1 M buffers*; in two instances, carboallyloxyglycinanilide and carboallyloxy-L-valinanilide, measurable yields were obtained only when the 1.0 M buffers were used.

3. In the case of both the 1.0 M and 0.1 M citrate buffers, the pH optima for all the acylglutamic acids which gave measurable yields of anilide were lower than the corresponding acyl derivatives of the monoaminomonocarboxylic acids.

4. With few exceptions the pH optima of the acyl derivatives of glycine, DL-valine and DL-leucine showed the following relationships to each other: acylglycine $\leqq$ acyl-DL-valine $\leqq$ acyl-DL leucine.

5. Although changing the acyl group of any one amino acid residue resulted in a different pH optimum for that residue, the order of change was not the same for all the amino acids observed. For example, the order of pH optima for the acylglutamic acids in 1.0 M citrate buffer was benzoyl < carbobenzoxy < p-nitrobenzoyl; for the corresponding acyl-DL-

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* It was noted that the carbobenzoxy-DL-leucine tended to oil out readily from the 1.0 M buffer solutions. This may account for fact that a lower yield of the anilide was obtained in the higher concentration buffer.

leucine derivatives, the order was benzoyl < p-nitrobenzoyl < carbobenzoxy.

6. In regards to the yields of the anilides of the acylmonoaminomonocarboxylic acids, the order, in both concentrations of the buffers, was almost consistently acyl-DL-leucine > acylglycine > acyl-DL-valine. With the exception of carboallyloxy-L-glutamic acid which failed to yield an anilide, the acyl derivatives of glutamic acid gave yields of anilides comparable to those of the corresponding leucine derivatives.

Enzymic hydrolysis of peptide bonds

In order to determine whether variations in the nature of the amino acid residue and the acylating group would affect the hydrolytic abilities of papain in the same manner as they affected the synthetic abilities, the preparations of amides, corresponding to the acylamino acids studied, were undertaken. Investigations comparable to those made on the acylamino acids, i.e. effects of varying both pH and buffer concentrations, have not as yet been carried out and only results of a preliminary nature have been obtained.

Since benzoyl-DL-leucine and benzoyl-DL-valine had been shown to have pronounced differences in reactivities as substrates in the anilide syntheses, studies were carried out

to determine whether similar differences existed in the reactivities of their amides as hydrolytic substrates for papain. Also investigated as a third member of the series was benzoylglycinamide. The procedures for carrying on these studies have been presented in the experimental section. The proportions of reactants were: benzamino acid amide, 0.00002 moles, based on the L-form when DL-compounds were used, a centrifuged solution of 1.0 mgm. of commercial papain and 1.0 mgm. of cysteine hydrochloride all in 1.0 ml. of 1 M or 0.1 M pH 5 citrate buffer. The results are summarized in Table 3.

Although the results are somewhat incomplete, they tend to indicate that the order of hydrolysis was benzoylglycinamide > benzoyl-DL-leucinamide > benzoyl-DL-valinamide in the 1.0 M buffer, and benzoyl-DL-leucinamide = benzoyl glycinamide > benzoyl-DL-valinamide in the 0.1 M buffer. These results agree, to some extent, with those observed for the anilide syntheses, since both the glycine and leucine derivatives were more reactive than the valine compounds. However, the order of these reactivities appear to differ in going from the 1.0 M buffer to the 0.1 M buffer. In addition, benzoyl-DL-leucinamide was hydrolyzed to a

Table 3

Effects of Varying Substrate and Buffer Concentrations on the Hydrolysis of Several Benzamino Acid Amides

Substrate	Percent Hydrolysis			
	Run No. 2[1]		Run No. 3[2]	
	1 M Buffer	0.1 M Buffer	1 M Buffer	0.1 M Buffer
Benzoylglycinamide	31	22	36	21
Benzoyl-DL-valinamide	4[3]	-	4[3]	4[3]
Benzoyl-DL-leucinamide	12[3]	-	13[3]	23[3]

[1]65 hours at 40°.

[2]51 hours at 40°.

[3]Based on assumption that only the L-form was hydrolyzed.

greater extent in the 0.1 M buffer than in the 1.0 M buffer.*

Such results definitely indicate the need for studying the hydrolytic reactions at varying pH values as well as at different buffer concentrations. It may well be the case, as has been previously observed in the synthetic studies, that the pH optima are higher in 1.0 M citrate than in 0.1 M citrate, and therefore, carrying out the reaction at the single pH of 5.0, as was done in the above case, does not give as complete a picture of the reactivities of the substrates.

Inhibition of Peptide Bond Synthesis

A detailed discussion has already been given in the Experimental section regarding the conditions chosen for carrying out the inhibition studies. It was noted that a 2 hour reaction period was chosen for the two substrates employed viz. carbobenzoxy-L-glutamic acid and benzoyl-DL-leucine, because preliminary studies had indicated that many compounds which had seemingly inhibited synthesis in the early stages of the reaction appeared to be without

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* Similar results indicating that benzoyl-DL-leucinamide was hydrolyzed to a greater extent in the 0.1 M buffer were obtained in a previous run. However, these results have not been cited because of poor temperature control during the course of the reaction.

effect if measurements were made after a 3 day period. When viewed from the kinetic standpoint, such results were not unexpected. Figure 5 illustrates to what extent the two synthetic reactions varied with an increase in time as measured by the yields of the anilides. In essence, both reactions were completed within a 24 to 32 hour period. If then, an inhibited reaction, i.e., a retarded reaction, was allowed to continue for 72 hours, it is not improbable that a theoretical yield of the product could be obtained because of this longer reaction period. Further examination of Figure 5 indicates that both reactions went to about 50% completion in 2 hours. This time interval was that selected as the standard reaction period.

The concentrations of test substances (potential inhibitors) employed were, in the majority of cases, 0.01 M and 0.001 M. The former concentrations were then only slightly greater than the concentration of cysteine hydrochloride which was always 0.008 M (6.4 mgm./5 ml.). Thus, even if the test substance were capable of reacting quantitatively with the activator, a sufficient amount still remained to react, in part at least, with the enzyme. The highest concentration of basic phenylmercuric nitrate used (0.001 M) was limited by the solubility of the compound. The concentrations of iodoacetic acid employed, 0.0001 M and 0.00001 M, were based on preliminary observations which indicated the high potency of this compound as an inhibitor.

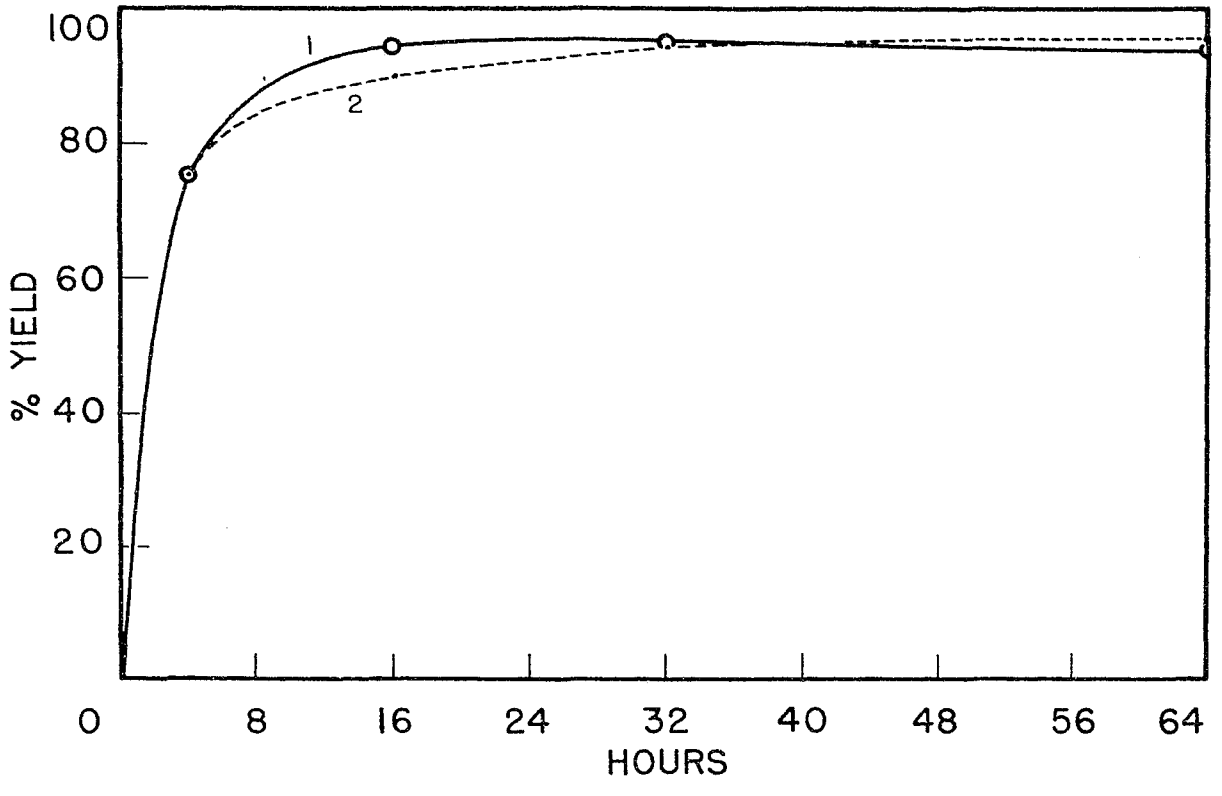

Fig. 5. Rates of synthesis of carbobenzoxy-L-glutamic acid anilide (curve 1) and of benzoyl-L-leucinanilide (curve 2)

As has been noted in the Experimental section, several procedures were utilized in studies of the effects of the test compounds on peptide bond synthesis as catalyzed by papain. Table 4 is a summary of the results obtained when the reactants were added in the following order: inhibitor solution, buffer solution, aniline, acylamino acid solution and papain-cysteine solution. In this case then, the papain was not pretreated with the inhibitor, but was pretreated with the activator (cysteine).

In evaluating the results given in Table 4, and in subsequent tables of a similar nature, it was necessary to set some arbitrary limit to the values that could be considered as indicative of inhibition (or activation) and also to the values that could be considered as falling within the range of experimental error. As was indicated in the Experimental section this value was set at ±15%, i.e. 15% greater or or lesser yield of the anilides compared with the controls were considered as indicative of neither inhibition nor activation. On this basis, the following statements may be deduced from Table 4:

1. The percents of inhibition of the synthetic reactions studied were, within the limits of experimental error, independent of the nature of the substrates when iodoacetic acid, copper sulfate, p-benzoquinone, sodium bisulfite and hydroxylamine were used as inhibitors. The order of these effects, with the

Table 4

Effects of Test Substances on Enzymic Anilide Syntheses; Papain Not Pretreated by Inhibitors

Test Substance	Concentration[1] of Test Substance, M	Percent Inhibition[2] Carbobenzoxy-L-glutamic Acid Anilide[3]	Benzoyl-L-leucinanilide[4]
Iodoacetic acid	0.0001	100	100
Iodoacetic acid	0.00001	100	92
Dichloroacetyl-DL-valine	0.01	0	8
Phenacyl bromide	0.01[8]	98	67
Phenacyl bromide	0.001	56	0
p-Iodoaniline	0.01	0	24
p-Iodoaniline	0.001	0	20
Basic phenylmercuric nitrate	0.001	31	66
	0.0001	0	11
Copper sulfate[5]	0.01	100	100
Copper sulfate	0.001	38	41
Maleic acid	0.01	90	65
Maleic acid	0.001	30	14
Fumaric acid	0.01	73	12
2-Hydroxy-5-methylacrylophenone	0.01[8]	20	43
	0.001	17	9
p-Benzoquinone[6]	0.01[8]	18	22
p-Benzoquinone	0.001	13	10
2-Methyl-1,4-naphthoquinone bisulfite	0.01	100	91
	0.001	51	22
Sodium bisulfite	0.01	100	100
Sodium bisulfite	0.001	80	66
Hydroxylamine	0.01	26	36
Hydroxylamine	0.001	0	8
Phenylhydrazine	0.01[8]	-10	12

Table 4 (Continued)

Test Substance	Concentration[1] of Test Substance, M	Percent Inhibition[2] Carbobenzoxy-L-glutamic Acid Anilide[3]	Percent Inhibition[2] Benzoyl-L-leucinanilide[4]
2,3,5-Triphenyltetrazolium chloride[7]	0.01	52	16
	0.001	0	8
2,5-Diphenyl-3-(p-iodophenyl)-tetrazolium chloride[7]	0.01	31	0
	0.001	9	7
Chrysene	Saturated	0	0
Cholestenone-6-SO_3H	0.01[8]	0	0
Ethyl carbamate	0.01	10	0
Betaine	0.01	10	0
Coumarin	0.01[8]	0	0
Indoleacetic acid	0.01[8]	10	12
Furoic acid	0.01	0	7
o-Hydroxyphenylacetic acid	0.01	12	13
Carboallyloxy-L-glutamic acid	0.01	0	0

[1]The amounts of test substances added were such as to give these final concentration had they been completely soluble.

[2]Percent inhibitions were calculated with respect to the control yields. The time of the synthetic reaction was 2 hours.

[3]The pH values of the reaction mixtures were 4.95 ± 0.05.

[4]The pH values of the reaction mixtures were 5.75 ± 0.05.

[5]Some reaction between the copper sulfate and the cysteine was observed.

[6]Some reaction between the p-benzoquinone and the aniline was observed.

[7]Apparent reactions between the tetrazolium salts and the acylamino acids, especially carbobenzoxy-L-glutamic acid were observed.

[8]Test substance not completely soluble at these concentrations.

exclusion of iodoacetic acid which was by far the most effective inhibitor studied, was sodium bisulfite = copper sulfate > hydroxylamine > p-benzoquinone, for 0.01 M concentrations of test substances; for 0.001 M concentrations of test substances, the order was sodium bisulfite > copper sulfate > hydroxylamine $\cong$ p-benzoquinone.

2. The percents of inhibition of the synthetic reactions studied appeared, in certain instances, to be dependent upon the nature of the substrates. Thus, in concentrations of 0.01 M, phenacyl bromide, maleic acid, fumaric acid, 2,3,5-triphenyltetrazolium chloride and 2,5-diphenyl-3-(p-iodophenyl)-tetrazolium chloride inhibited the synthesis of carbobenzoxy-L-glutamic acid anilide more effectively than that of benzoyl-L-leucinanilide. In concentrations of 0.001 M, both phenacyl bromide and maleic acid still effectively inhibited the first above mentioned synthesis, but had little or no effect on the second. Although 2-methyl-1,4-naphthoquinone bisulfite inhibited both reactions equally as well at concentrations of 0.01 M, it apparently inhibited the synthesis of carbobenzoxy-L-glutamic acid anilide more strongly at a concentration of 0.001 M.

3. In contrast to the above observations 0.001 M basic phenylmercuric chloride, and 0.01 M 2-hydroxy-5-

methyl-acrylophenone and p-iodoaniline inhibited the synthesis of benzoyl-L-leucinanilide to a greater extent than that of carbobenzoxy-L-glutamic acid anilide. A possible explanation for the observation that p-iodoaniline was as effective an inhibitor of the synthesis of benzoyl-L-leucinanide at both "0.01 M" and "0.001 M" concentrations may depend on the fact that the compound was insoluble in both these cases.

It was noted (Table 4) that p-benzoquinone apparently reacted with the aniline, a factor which might have greatly diminished its inhibitory power. Therefore, studies were conducted to determine whether the addition of enzyme to the inhibitor solution 30 minutes prior to the addition of the other components would lead to significantly different results. At the same time several additional compounds were investigated, with the inclusion of two, sodium bisulfite and hydroxylamine, which had been studied previously (see Table 4). The procedure was modified to allow the papain solution, without added cysteine, to react with the inhibitor solution for 30 minutes. The buffer solution containing the proper amount of cysteine, the acylamino acid solution and the aniline were then added in the order given. The results are summarized in Table 5.

Table 5

Effects of Test Substances on Enzymic Anilide Syntheses
Papain Pretreated by Inhibitor

Test Substance	Concentration[1] of Test Substance, M	Percent Inhibition[2] Carbobenzoxy-L-glutamic Acid Anilide[3]	Benzoyl-L-Leucinanilide[4]
p-Benzoquinone	0.01	20	73
p-Benzoquinone	0.001	5	39
Sodium bisulfite	0.01	100	96
Sodium bisulfite	0.001	64	40
Hydroxylamine	0.01	61	85
Hydroxylamine	0.001	6	16
Barbituric acid	0.01	17	22
Barbituric acid	0.001	7	12
Nitrous acid[5]	0.01	100	100
Nitrous acid	0.001	34	42
Formaldehyde	0.01	42	46
Formaldehyde	0.001	0	0
Potassium ferricyanide	0.01	100	100
Potassium ferricyanide	0.001	12	23

[1]These were the final concentrations after all reactants had been added. Actually the enzyme was pretreated for 30 minutes with concentrations of 0.05 M and 0.005 M.

[2]Percent inhibitions were calculated with respect to the control yields. The time of the synthetic reaction was 2 hours.

[3]The pH values of the mixtures were 4.95 ± 0.05.

[4]The pH values of the mixtures were 5.75 ± 0.05.

[5]Apparent diazotization of aniline and coupling was observed.

A comparison of Tables 4 and 5 tends to indicate that, with utilization of the modified procedure, p-benzoquinone was a more effective papain inhibitor only in the case where benzoyl-DL-leucine was employed as the substrate. Hydroxylamine, however, inhibited both reactions to a greater degree than was previously noted. Although 0.01 M sodium bisulfite was a complete inhibitor under both sets of conditions, it was apparently slightly less effective by the latter method in 0.001 M concentration.

Both nitrous acid and potassium ferricyanide (Table 5) inhibited the two reactions completely at concentrations of 0.01 M. Although nitrous acid was moderately effective at the lower concentration, potassium ferricyanide showed marked decrease in inhibitory powers. Formaldehyde inhibited both reactions equally at the higher concentration but was without effect at the lower.

Reference has previously been made to the investigations of several workers which indicated that the inhibition of papain by iodoacetic acid was not reversed by the addition of sulfhydryl activators. These claims were seemingly verified by the results of Table 4 which demonstrated that essentially complete inhibition of both synthetic reactions was realized with 0.0001 M and 0.00001 M iodoacetic acid, despite the fact that the cysteine concentration was 0.008 M. It was of interest, however, to determine whether iodoacetic acid had

completely inactivated papain or whether the inhibition was merely a retardation of the reaction. Consequently, in certain instances the reactions, carried out in accordance with the procedure followed for the compounds of Table 4, were allowed to proceed for a 72 hour period. The results are shown in Table 6. The 2 hour results have been taken from Table 4.

Table 6

Effect of Reaction Times on Inhibition of Anilide Syntheses by Iodoacetic Acid

Concentration of Iodoacetate, M	Percent Inhibition[1] Carbobenzoxy-L-glutamic Acid Anilide[2]		Benzoyl-L-leucinanilide[3]	
	2 hours	72 hours	2 hours	72 hours
0.0001	100	35	100	98
0.00001	100	24	92	79

[1]Percents inhibition were calculated with respect to the control yields.

[2]The pH values of the mixtures were 4.95 ± 0.05.

[3]The pH values of the mixtures were 5.75 ± 0.05.

The results presented in Table 6 indicate greater differences in the degrees of the inhibition of papain, with time, at both concentrations of iodoacetic acid when carbobenzoxy-L-glutamic acid was the substrate than when benzoyl-DL-leucine was employed as the substrate. These results will be interpreted in the section to follow.

Table 7

Essentiality of Cysteine as Activator in Anilide Syntheses

Papain Preparation	Cysteine Added	Percent Yield of Anilide[1]			
		Carbobenzoxy-L-glutamic acid Anilide[2]		Benzoyl-L-leucinanilide[3]	
		2 hours	24 hours	2 hours	24 hours
Commercial	No	12	38	5	6
Commercial	Yes	52	94	52	94
H_2S-treated	No	7	25	4	5
H_2S-treated	Yes	67	92	75	94

[1]Based on theoretically possible yields.

[2]The pH values of the mixtures were 4.95 ± 0.05.

[3]The pH values of the mixtures were 5.75 ± 0.05.

Although Bergmann and Fraenkel-Conrat (2) have indicated the essentiality of cysteine or other activators for the synthetic activities of papain, it was of interest to recheck these observations and also to ascertain whether the effects of omitting the activator would be independent of the nature of the substrate. Studies were therefore carried out with two papain preparations, a commercial preparation and a hydrogen sulfide-treated preparation. Portions of the two preparation solutions were preactivated with cysteine hydrochloride; no activator was added to the remaining portions. Carbobenzoxy-L-glutamic acid and benzoyl-DL-leucine were used as the substrates. The results are summarized in Table 7.

An examination of the results of Table 7 reveals that while cysteine is necessary for the complete activation of papain, it is apparently not required to the same extent for both substrates. Thus, the synthesis of carbobenzoxy-L-glutamic acid proceeded to 25% of completion within 24 hours in the presence of the "activator-free" enzyme. The synthesis of benzoyl-L-leucine, on the other hand, took place to a very limited degree.

DISCUSSION AND INTERPRETATIONS

Peptide Bond Synthesis and Hydrolysis

The importance of precise pH control in enzymic reactions was early emphasized by Sörensen (102) who showed that the anomalous results obtained in studies of the reactions in different acids could be reconciled if the hydrogen ion concentration were considered. Indeed, in any investigation concerned with the properties of an enzyme, a determination of the effects of the variation of pH on the activity of the enzyme is of primary importance. While most studies have appeared to indicate that enzymes exhibit their maximum activity at a definite pH, i.e., an optimum pH, further evidence has also indicated that the nature of the substrate may influence the pH optimum. In 1922, Northrop (103) reported that the pH optimum for the digestion of several proteins by pepsin or trypsin varied with the different proteins and could be predicted from the titration curves of the proteins if it were assumed that pepsin reacted with the positive protein ion and trypsin with the negative protein ion.

(102) Sörensen, Biochem. Z., 21, 131 (1909).

(103) Northrop, J. Gen. Physiol., 5, 263 (1922).

Mention has already been made concerning the observations of Willstätter and associates (59,60) which indicated that the optimum pH values for the digestion of gelatin, peptone, and fibrin by papain were close to the isoelectric points of these substrates, namely, 5, 5, and 7.2, respectively. Lineweaver and Schwimmer (104) in studies with crystalline papain, found that similar relationships existed for the crystalline enzyme. Thus the pH optimum when gelatin was used as the substrate was 5.2, whereas for denatured casein, denatured egg albumin and denatured hemoglobin, the optimum values were approximately 7. In a study concerned with the effects of pH on the activity of purified trypsin, Kunitz and Northrop (105) observed that the activity of the enzyme dropped off rather rapidly as the pH became greater than 8.0, despite the fact that the substrate (casein) was present in a digestible form at the higher alkalinities. They concluded that trypsin exists in both an active and inactive form which are in equilibrium with each other and that the equilibrium is shifted in the direction of the inactive form at pH values greater than 8.0.

It thus becomes evident from the above cited investigations that the optimum pH of an enzymic reaction may be dependent on the existence of both the enzyme and the

(104) Lineweaver and Schwimmer, Enzymologia, 10, 81 (1941-42).

(105) Kunitz and Northrop, J. Gen. Physiol., 17, 591 (1934).

substrate in definite forms. Wilson (106) has discussed the effects of pH on enzymes and substrates and has indicated that both may exist in "active" forms, the amount of such forms present at any one time being functions of the pH. The optimum pH is then that value at which the product of the concentrations of the active forms of the enzyme and the substrate is a maximum. It is quite conceivable that similar reasoning may be applied in an attempt to explain, in part, the differences in the pH optima noted for the various acylamino acids (see Table 2 and Figures 1 through 4).

In regard to the pH optima for the acyl derivatives of L-glutamic acid, it was noted that in all cases in both 1.0 M and 0.1 M, the optimum values were consistently lower than those of the corresponding derivatives of the monoaminomonocarboxylic acids. Such results suggest that the differences may, in part, be attributed to the second carboxyl group of glutamic acid, and that the synthetic reactions take place most readily when this group exists in an undissociated form. The sharp decreases noted in the yields of the anilides at the lower pH values may be due to a decrease in the activity of papain at the higher acidities. Indeed, such conclusion is borne out by the investigations of Lineweaver and Schwimmer (104) which indicated that crystalline papain was rapidly

(106) Wilson, in Elvehjem and Wilson, "Respiratory Enzymes", p. 203. Burgess Publishing Company, Minneapolis. 1939.

inactivated below pH 3 and above pH 12. The maximum stability was reported to be in the range of pH 5 to 6.

The differences noted between the pH optima of the acyl-L-glutamic acids, although slight, may be due to small variations in the strengths of these compounds as acids as a result of alteration of the acyl group. On this basis, it might be concluded that the acid strengths of the acyl-L-glutamic acids stand in the order benzoyl > carbobenzoxy > p-nitrobenzoyl. However, the fact that this order is neither borne out by the optima for the acylglutamic acids in 0.1 M buffer, nor does it hold, in general, for the corresponding acyl derivatives of the monoaminomonocarboxylic acids, makes such a conclusion an unlikely one.

It is conceivable that structural differences, other than those which affect acidity of the substrates, play an important role in determining differences in pH optima. An illustration of such a phenomenon has been cited by Pettinga (101), in the case of the synthesis of N-benzoyl-L-tyrosinanilide. Because of the acidic phenolic group, it was expected that the substrate, N-benzoyl-DL-tyrosine would show an optimum pH close to that of benzoyl-L-glutamic acid for the synthesis of its anilide. In contrast, however, the optimum value obtained was above pH 5.5.

In reference to the pH optima of the acylmonoaminomonocarboxylic acids, the higher values observed for these compounds as contrasted with the acylglutamic acids may, in part,

be dependent on the possibility that relatively high concentrations of the undissociated forms of these acids exist at the higher pH values. If the undissociated forms of the acylamino acids are then the "active" forms and, as has been indicated, papain is most active in the pH range of 5 to 6, it might then be expected that the pH optima for the acylmonoaminomonocarboxylic acid would be somewhat higher than those of the acylated dicarboxylic (glutamic) acids. A possible verification of the proposal that the undissociated forms of the acylamino acids are also the active forms may be found in the work of Bergmann and Fraenkel-Conrat (2). Studies by these workers on the enzymic synthesis of benzoylglycine anilide from benzoylglycine and benzoylglycinamide indicated that the latter compound reacted more readily with aniline than did the former. Since the amide probably existed in a completely undissociated form at the pH of the reaction (4.77), the differences in rate may well have been due to the greater concentration of undissociated amide as contrasted with that of undissociated acid. It should also be stated, however, that Bergmann and Fraenkel-Conrat suggested that the difference in the two rates gave evidence that the transformation of the amide into the anilide did not proceed through the intermediate stage of benzoylglycine, i.e. hydrolysis, but that the amino group in the molecule of benzoylglycinamide was directly replaced by the aniline residue.

The results of Table 2 also indicate that, with but the single exceptional case of carbobenzoxy-DL-leucine, the yields of all the anilides obtained from the various acylamino acids were, for those reactions carried out in the 1.0 M citrate buffer, equal to or greater than the yields obtained when the 0.1 M buffers were used. While such results may also be due to differences in the ionic strengths, preliminary studies* carried out with carbobenzoxy-L-glutamic acid as the substrate, indicated than when 3 M, 1 M and 0.1 M acetate buffers were used, the yields of the anilide varied inversely with the buffer strength. On this basis it is conceivable that the buffers may, in some manner, either exert a catalytic effect on the reaction or act as activators or inhibitors of papain. Instances have been cited in the literature where variation in both buffer strength and buffer type have led to variations in reaction rates. Hammett (107) has discussed the decomposition of nitramide in the presence of various concentrations of acetate and benzoate ions and has indicated that the rate of breakdown was greater in the presence of higher concentrations of the buffers; the rate was consistently greater in acetate than in the corresponding concentrations of benzoate. Furthermore, it was emphasized that the differences

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* Not presented in this thesis.

(107) Hammett, "Physical Organic Chemistry", p. 216. McGraw-Hill Book Co. Inc., New York. 1940.

noted in reaction rates at the various concentrations were independent of ionic strength. This was evidenced by the fact that the addition of sodium chloride to the reaction mixture had no effect. Hammett has cited this type of catalysis as "base catalysis".

Observations similar to some of those cited above and reported herein are not without precedence in enzymology. Howell and Sumner (108), in a study of crystalline urease, found that the activity of the enzyme was not only dependent upon pH, but upon the concentration of the substrate (urea), the buffer type and buffer concentration as well. In their investigations on the effects of citrate, acetate, and phosphate buffers on urease activity, they found that the pH optima for urease, when the substrate concentration was 2.5%, were 6.4, 6.5 and 6.9 for acetate, citrate and phosphate respectively. The order of activity of the enzyme in the different buffers was citrate > phosphate > acetate. When 0.1% solutions of urea were employed, the pH optima were 6.7 for acetate, 6.7 for citrate and 7.6 for phosphate. The order of activity of the enzyme in the different buffers was unchanged. When pH and urea concentration were held constant a decrease in buffer concentration resulted in an increase in urease activity until a point was reached beyond which further dilution had little effect or resulted in decreased activity.

(108) Howell and Sumner, J. Biol. Chem., 104, 619 (1934).

From the above discussion it becomes apparent that both the concentration and the nature of a buffer may have a marked effect on the activity of an enzyme system. It becomes further apparent, from the results reported herein, that citrate ion may have functioned to supplement the catalytic activity of papain in the anilide syntheses.

In view of the fact that three of the acylglutamic acids gave rather high yields of their respective anilides in both buffer concentrations, the failure of carboallyloxy-L-glutamic to react was somewhat unexpected. Several likely explanations for this failure may be advanced: (1) the configuration of the carboallyloxy-L-glutamic acid is such that it is either incapable of reacting with the enzyme or, if the initial reaction is between papain and aniline, with the enzyme-aniline complex; (2) carboallyloxy-L-glutamic acid is an inhibitor of papain; and (3) carboallyloxy-L-glutamic acid anilide is soluble in the reaction mixture. While definite evidence for or against the first possibility has not been obtained, a comparison of the yields of the three other acyl-L-glutamic acid anilides with those of the corresponding L-leucine derivatives tends to indicate that the glutamic acid compounds react as readily as those containing leucine. In view of the high yields of carboallyloxy-L-leucinanilide, it might then be expected that carboallyloxy-L-glutamic acid would exhibit a similar degree of reactivity. In addition,

if the failure of carboallyloxy-L-glutamic acid to react is due to "improper" configuration, then this configuration must be markedly different from those of the other acyl-glutamic acids.

In regards to carboallyloxy-L-glutamic acid acting as an inhibitor, experiments were performed (Table 4) to test the effects of this compound on the enzymic synthesis of carbobenzoxy-L-glutamic acid anilide and benzoyl-L-leucin-anilide. No inhibition was noted at a level of 0.01 M.

The third possibility i.e. that the anilide is soluble in the reaction mixture, might be checked by synthesizing carboallyloxy-L-glutamic acid anilide chemically and comparing its solubility with those of some enzymically formed anilides e.g. carboallyloxy-L-leucinanilide.

Previous reference has been made in the Results section to the observations of Fox and coworkers (97) that, under the same reaction conditions, benzoyl-DL-leucine consistently gave a markedly higher yield of benzoyl-L-leucine anilide than did benzoyl-DL-valine of its corresponding anilide. Although the concentrations of reactants employed in the present investigation differed somewhat from those used by the above investigators, the results obtained were comparable. In addition, it should be pointed out that the same relations held for all the acyl derivatives of DL-leucine and DL-valine irrespective of buffer concentration.

Comparison of the reactivities of the acyl derivatives of glycine, with those of DL-leucine and DL-valine, showed the following relationship, acyl-DL-leucine > acylglycine > acyl-DL-valine, held with few exceptions. Such results would tend to indicate that a specifity or "preference" of papain for the various monoaminomonocarboxylic acids, which is highly sensitive to configurational changes of the amino acid residue, may exist.

While the results obtained from the benzamino acid amide hydrolysis studies (Table 3) verified the activity relationship, acyl-DL-leucine > acyl-DL-valine, which was shown in the corresponding anilide synthesis studies, the glycine compound was no longer intermediate but exhibited greater reactivity than either the amide of DL-leucine or of DL-valine. It should be emphasized that the reactions investigated were carried out at the same pH of 5.0 and it is highly conceivable that this value is not optimum for all the compounds studied.

In support of the above assumption that the pH optima of the various acylamino acid amides may differ, the observations of Hoover and Kokes (109), and Fruton and Bergmann (110) may be cited. The former group, utilizing papain, reported that the pH optima for the three synthetic substrates, benzoyl-

(109) Hoover and Kokes, J. Biol. Chem., 167, 199 (1947).

(110) Fruton and Bergmann, ibid., 127, 627 (1939).

argininamide, carbobenzoxy-L-isoglutamine, and benzoylglycinamide were 5.5 for the former compound and 5.0 for the two latter compounds. Similar type results were obtained by Bergmann and Fruton in studies on the specificity of crystalline pepsin. When carbobenzoxy-L-glutamyl-L-tyrosine and carbobenzoxy-L-glutamyl-L-phenylalanine were utilized as substrates, the pH optima for the two compounds, 4.0 and 4.5 respectively, not only differed between themselves but were far removed from the generally accepted optimum pH range of 1.8 to 2 for pepsin.

Inhibition of Peptide Bond Synthesis

Selection and properties of the test substances

Preliminary to discussing the results obtained from the inhibitor studies, it might be well to consider the reasons for having selected these compounds and to conjecture briefly as to their nature and probable modes of action.

The compounds which were tested as potential inhibitors of peptide bond synthesis were selected, in the main, because of previous indications in the literature that they, or their analogs, were capable of inhibiting the proteolytic activity of papain. It was therefore of interest to determine whether similar inhibitory effects would be demonstrated against the synthetic reactions. A few of the compounds were studied

because of evidence in the literature that they were either *in vitro* or *in vivo* inhibitors of enzymes related to papain, e.g., cathepsins, sulfhydryl containing enzymes, etc. In some instances, there was little or no previous evidence that the compounds were inhibitory.

An individual treatment of each of the compounds studied is given in detail below.

Iodoacetic acid. Dickens (111) has suggested that iodoacetic acid probably functions through alkylation of sulfhydryl groups as is illustrated below:

$$R\text{-}SH + I\text{-}CH_2\text{-}COOH \longrightarrow R\text{-}S\text{-}CH_2\text{-}COOH + HI.$$

He further reported that bromo and chloroacetic acids reacted similarly, but at slower rates. The ratios of reactivity of the various halogen compounds was I:Br:Cl = 15:9:0.15. Bersin and Logemann (65), and Greenberg and Winnick (75) have indicated that the action of iodoacetic acid is not reversed by cysteine, SH glutathione and cyanide. Maschmann (112) reported that papain, inactivated by iodoacetate, was reactivated after ethanol precipitation; Greenberg and Winnick could not duplicate these results. Michaelis and Schubert (113) have shown that iodoacetate can react with aromatic and

(111) Dickens, Biochem. J., 27, 1141 (1933).

(112) Maschmann, Biochem. Z., 279, 225 (1935).

(113) Michaelis and Schubert, J. Biol. Chem., 106, 331 (1934).

aliphatic amine groups as follows:

$$R\text{-}NH_2 + I\text{-}CH_2\text{-}COOH \longrightarrow R\text{-}N(CH_2\ COOH)_2 + HI.$$

This reaction is slower than the one involving SH groups.

Dichloroacetyl-DL-valine. The reasons for the investigation of this compound were two-fold. In the first place, the compound was investigated as a possible analog of iodoacetic acid. Secondly, it was studied as a possible competitive inhibitor of the acylamino acids.

Phenacyl bromide. The action of phenacyl bromide (ω-bromoacetophenone) and related lachrymators and vesicants on sulfhydryl enzymes has been reported by Mackworth (114). Its reaction with SH groups is, in all likelihood, similar to that of iodoacetate, viz.:

$$R\text{-}SH + BrCH_2\text{-}\overset{\overset{O}{\|}}{C}\text{-}C_6H_5 \longrightarrow R\text{-}S\text{-}CH_2\text{-}\overset{\overset{O}{\|}}{C}\text{-}C_6H_5 + HBr$$

Iodoaniline. The use of iodoaniline was suggested by the investigation of Kocholaty and Krejci (115) who reported that Clostridium histolyticum proteinase was activated by SH compounds and inhibited, to some extent, by iodoacetate, iodine, etc. Iodoaniline was not found to be a very effective inhibitor, but since its effect on the proteinase was equal to that of maleic acid, which in turn is a fairly potent inhibitor of papain, it was plausible to assume that it

(114) Mackworth, Biochem. J., 42, 82 (1948).

(115) Kocholaty and Krejci, Arch. Biochem., 18, 1 (1948).

(iodoaniline) might show stronger inhibitory properties against papain. It was also conceivable that iodoaniline might react, competitively with aniline in the anilide syntheses, although this was rather unlikely in view of the concentration ratios employed.

Phenylmercuric nitrate (basic). Hellerman and Perkins (67) demonstrated that papain was inhibited by mercuri-organo compounds, e.g., C_6H_5HgOH, C_6H_5HgCl, etc. They postulated the formation of mercaptides, as given in the reaction below:

$$En\text{-}SH + X\text{-}HgC_6H_5 \longrightarrow En\text{-}S\text{-}HgC_6H_5 + HX$$ (where En = enzyme)

The inactivation of papain was reversed by cysteine, hydrogen sulfide and hydrogen cyanide. Cook and associates (116,117) inhibited the activities of several respiratory enzymes with phenylmercuric nitrate; the effects were reversed by cysteine and homocysteine.

Copper sulfate. The inhibitory action of cupric ion on papain was studied by Hellerman and Perkins (67) who suggested that it probably catalyzed the oxidation of SH groups to disulfide linkages. Bernheim and Bernheim (118) indicated that cupric ion reacts readily with cysteine, probably by

(116) Cook, Kreke, McDevitt and Bartlett, J. Biol. Chem., 162, 43 (1946).

(117) Cook, Perisutti and Walsh, ibid., 162, 51 (1946).

(118) Bernheim and Bernheim, Cold Springs Harbor Symposium Quant. Biol., 7, 174 (1943).

catalyzing the oxidation of SH groups to both disulfide linkages and higher oxidation states of sulfur.

Potassium ferricyanide. The effects of potassium ferricyanide on papain were also investigated by Hellerman and Perkins (67). They reported that the inactivation of the enzyme was probably due to the oxidation of sulfhydryl groups to disulfide linkages; cysteine, added in sufficient quantity to the enzyme solution, protected papain from the inactivating effects of the oxidant.

Maleic acid. The inhibitory effect of maleic acid on papain has been demonstrated by Morgan and Friedmann (119), Ganapathy and Sastri (120), and Greenberg and Winnick (75). Morgan and Friedmann (121) have suggested that an addition of sulfhydryl groups to the double bond takes place, viz.:

$$HOOC\text{-}CH{=}CH\text{-}COOH + RSH \longrightarrow \underset{\displaystyle COOH}{\overset{\displaystyle CH_2}{\diagup}}\!\!-\underset{\displaystyle COOH}{\overset{\displaystyle CH}{|}}\!\!-S\text{-}R + H_2O$$

Greenberg and Winnick believed the action was oxidation of SH groups by maleic acid with a corresponding reduction of the maleic acid to succinic acid. They further indicated that the reaction was reversed by cysteine and cyanide.

Fumaric acid. This compound was selected in order to determine whether the trans-isomer of maleic acid would inhibit

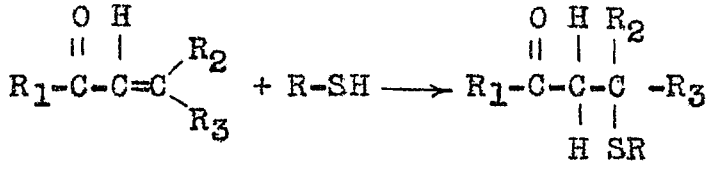
(119) Morgan and Friedmann, Biochem. J., 32, 862 (1938).

(120) Ganapathy and Sastri, ibid., 33, 1175 (1939).

(121) Morgan and Friedmann, ibid., 32, 733 (1938).

papain. No evidence was found in the literature to indicate that fumaric acid reacted with sulfhydryl groups or was inhibitory to SH enzymes. Hopkins and associates (122) found that fumaric acid did not inhibit succinic dehydrogenase (an SH enzyme) whereas maleic acid did. They further found that fumaric acid protected succinic dehydrogenase from oxidation by oxidized glutathione.

2-Hydroxy-5-methylacrylophenone. Geiger (123), in an extensive study of thirty-five α,β -unsaturated ketones, indicated that almost all of the compounds had marked antibacterial and antifungal properties, as well as the ability to inhibit SH-enzymes. Of these compounds, 2-hydroxyacrylophenone and its derivatives were among the most active. Previous studies by Geiger and Conn (124) indicated similar properties for the antibiotics clavacin and penicillic acid, which possess the general structure of α,β -unsaturated ketones. They suggested that the following reaction took place between the unsaturated ketones and sulfhydryl compounds:



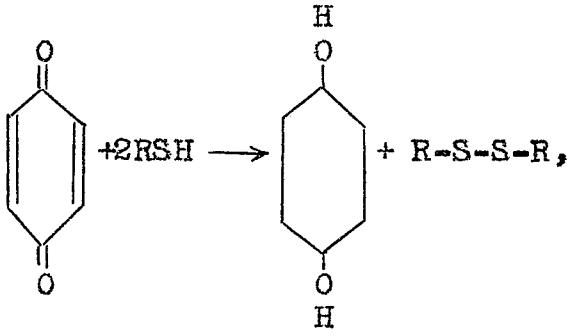
where R_1 was an aryl group and R_2 or R_3 (or both) was hydrogen.

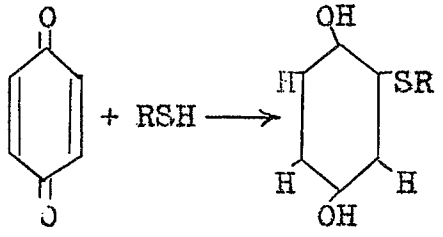
(122) Hopkins, Morgan and Lutwak-Mann, Biochem. J., 32, 1829 (1938).

(123) Geiger, Arch. Biochem., 16, 423 (1948).

(124) Geiger and Conn, J. Am. Chem. Soc., 67, 112 (1945).

p-Benzoquinone. The inhibitory effect of p-benzoquinone on papain was demonstrated by Bersin and Logemann (65) and by Hoffmann-Ostenhof and Biach (125). The former group reported that the inactivation was irreversible. Hellermann and Perkins (67) suggested that quinone reacted with sulfhydryl enzymes in two ways: (1) oxidation; and (2) addition of SH groups to the double bonds. They further indicated that the inhibition of papain by p-benzoquinone was reversed by cysteine, etc., and concluded that it was the oxidation reaction that predominated. Snell and Weissberger (126) postulated that the following reactions took place:

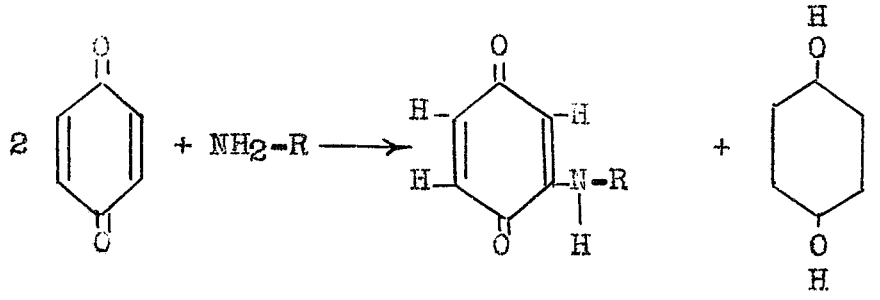
a. 

b. 

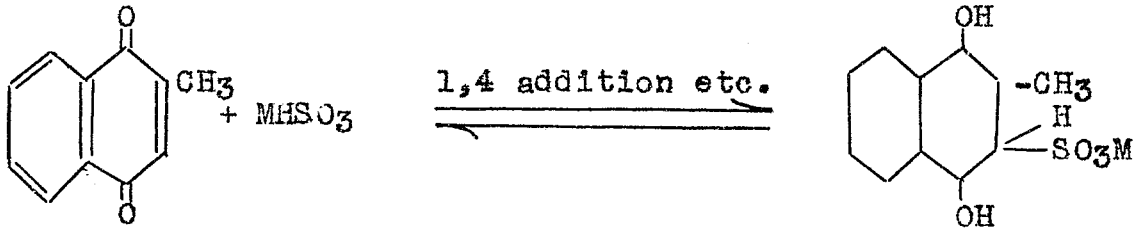
(125) Hoffmann-Ostenhof and Biach, Experientia, 2, 405 (1946).

(126) Snell and Weissberger, J. Am. Chem. Soc., 61, 450 (1939).

Hoffmann-Ostenhof (127) has suggested a possible reaction with amines, viz:



2-Methyl-1,4-naphthoquinone bisulfite. The reaction of bisulfites with 2-methyl-1,4-napthoquinone (synthetic Vitamin K) has been formulated by Moore (128) as follows:



The compound is water soluble and capable of yielding the free quinone on reversal. The ability of the quinone to react with sulfhydryl compounds, such as cysteine, has been demonstrated by Fieser (129); thiol ethers were formed. Potter and DuBois (130) have reported that Vitamin K inhibited succinic dehydrogenase (an SH enzyme); however, it was only one-tenth as effective as p-benzoquinone. The inhibition of choline

(127) Hoffmann-Ostenhof, Experientia, 3, 176 (1947).

(128) Moore, J. Am. Chem. Soc., 63, 2049 (1941).

(129) Fieser, Ann. Internal Med., 15, 648 (1941).

(130) Potter and DuBois, J. Gen. Physiol., 26, 391 (1942-43).

acetylase by methyl naphthoquinone as well as by the sulfonates of substituted 1,2 and 1,4-naphthoquinones has been demonstrated by Nachmansohn and Berman (131).

Sodium bisulfite. This compound was studied both as a control for the above derivative of Vitamin K and to ascertain whether it would inhibit papain by reacting with carbonyl groups, the presence of which has been suggested by several investigators. Maeda (71) reported bisulfite inhibited papain; Bersin and Logemann (65) and Schales and associates (72) found that it activated the enzyme. Winnick and coworkers (132) observed that asclepain, an enzyme similar to papain, was slightly activated by sodium bisulfite. Clarke (133) has formulated the following reaction between bisulfite and disulfide linkages:

$$R\text{-}S\text{-}S\text{-}R + HSO_3^- \longrightarrow R\text{-}S\text{-}H + R\text{-}S\text{-}SO_3^-$$

This would tend to indicate that bisulfite should have an activating effect on papain.

Hydroxylamine. The investigations of Bergmann and Ross (69), Maeda (71), and Schales and associates (72) on hydroxylamine as a reagent for the carbonyl groups in papain, have already been mentioned in the Historical section. The three above groups all reported the inhibition of papain, either partial or complete.

(131) Nachmansohn and Berman, J. Biol. Chem., 165, 551 (1946).
(132) Winnick, Davis and Greenberg, J. Gen. Physiol., 23, 275 (1939-40).
(133) Clarke, J. Biol. Chem., 97, 235 (1932).

Phenylhydrazine. The same investigators mentioned in connection with hydroxylamine studied this compound. Bergmann and Ross indicated that only Papain I was inhibited (see Historical). Maeda reported that after 3 days contact of papain with phenylhydrazine, the enzyme no longer hydrolyzed hippurylamide or gelatin. Schales reported only partial inhibition of papain when egg albumin was used as the substrate. The reaction may be between phenylhydrazine and the carbonyl groups of papain, although Hellerman (56) has further pointed out that phenylhydrazine can also act as an oxidizing or a reducing agent under proper conditions.

The work of Bergmann and Fraenkel-Conrat (2) has indicated that phenylhydrazine can be used instead of aniline as a substrate for peptide bond synthesis catalyzed by papain. However, phenylhydrazine would react only if cysteine were used as the enzyme activator; papain which had been activated by cyanide was inhibited by phenylhydrazine. Their observations regarding the failure of phenylhydrazine to inhibit papain-cysteine but its ability to inhibit papain-cyanide led the above workers to the conclusion that the Papain I portion of holopapain (see page 28) was responsible for the synthetic activities. It should be noted that phenylhydrazine had no inhibitory effects on Papain II, either in the presence or absence of activators.

Barbituric acid. This compound was tested in view of the report of Maeda (71) that diethylbarbituric acid, which was claimed to be specific for aldehyde groups, inhibited papain. Conrad and Reinbach (134) have shown that barbituric acid reacts readily with aldehydes.

Nitrous acid. Deamination of amino groups with nitrous acid has been studied by a number of investigators (51) in investigations concerning the essentiality of amino groups for the action of specific enzymes. Philpot and Small (135) have indicated that, in 1 N concentrations, the action (deamination) of nitrous acid on pepsin at 0°, was complete in one-half hour. They further indicated that nitrous acid reacts with the tyrosine groups, although the reaction rate is much slower in this case. The reaction with tyrosine (phenolic) groups was postulated as:

$$R\text{-}C_6H_4OH + HNO_2 \longrightarrow R\text{-}C_6H_3(NO)OH + H_2O.$$

Formaldehyde. Olcott and Fraenkel-Conrat (51) have reviewed the literature concerning formaldehyde as a group reagent. They have indicated that in neutral solutions, the reaction of formaldehyde is predominantly with amino groups. The probable reaction is:

$$R\text{-}NH_2 + H_2C{=}O \longrightarrow R\text{-}N{=}CH_2 + H_2O$$

The reaction can be reversed by both dialysis and dilution.

(134) Conrad and Reinbach, Ber., 34, 1339 (1901).

(135) Philpot and Small, Biochem. J., 32, 542 (1938).

In alkaline solution (pH 11) guanidyl, indole, and amide groups react almost as readily as amino groups. Fraenkel-Conrat and Olcott (136) have indicated that formaldehyde introduces methylene bridges between amino groups and labile hydrogen positions of phenolic and imidazole rings.

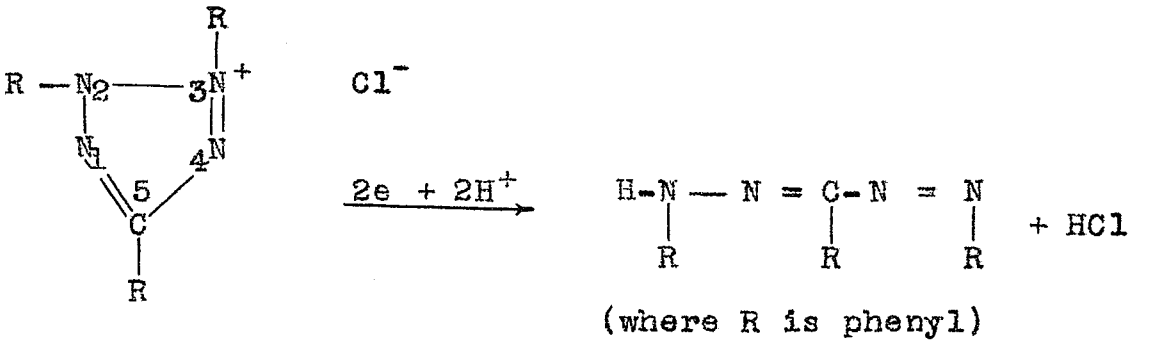
2,3,5-Triphenyltetrazolium chloride. This compound was employed as a potential inhibitor in view of reports that it was readily reduced by vital tissues. Atkinson (137) has recently reviewed this work. It was conceivable that the compound might act on the sulfhydryl groups of papain, although Kuhn and Jerohel (138) have indicated that no reaction took place with cysteine or SH glutathione below a pH of 9.0. Mattson and associates (139) have indicated that triphenyltetrazolium chloride is reduced to an insoluble red triphenylformazan by the following reaction:

(136) Fraenkel-Conrat and Olcott, J. Biol. Chem., 174, 827 (1948).

(137) Atkinson, "Syntheses, Properties and Applications of Some Tetrazolium Compounds" Unpublished M.S. Thesis. Ames, Iowa, Iowa State College Library. (1949).

(138) Kuhn and Jerchel, Ber., 74, 949 (1941).

(139) Mattson, Jensen and Dutcher, Science, 106, 294 (1947).



(where R is phenyl)

2,5-Diphenyl-3-(p-iodophenyl)-tetrazolium chloride. This compound was tested as an analog of 2,3,5-triphenyltetrazolium chloride.

Chrysene. The observation by Rondoni and Beltrami (140) that benzpyrene inhibited animal cathepsins in vitro and the reference by Rondoni (141) to the unpublished work of Gaetani, which indicated that papain was inhibited by the carcinogens, methylcholanthrene and benzpyrene, led to the testing of the related hydrocarbon, chrysene, as a potential papain inhibitor. In addition, the report by Rusch and Kline (142) that phospholipid oxidation was catalyzed by such compounds as cysteine and glutathione, and inhibited by anthraquinone, benzanthraquinone as well as by the hydrocarbons phenanthrene, anthracene, 1,2-benzanthracene, etc., suggested that perhaps SH groups were involved in the activation and inhibition

(140) Rondoni and Beltrami, Enzymologia, 3, 251 (1937).

(141) Rondoni, ibid., 12, 128 (1947).

(142) Rusch and Klein, Cancer Research, 1, 465 (1941). Original not available for examination. Abstracted in C.A., 35, 8080^5 (1941).

processes. Feigenbaum (143), however, has recently indicated that 3,4-benzpyrene, methylcholanthrene, phenanthrene, etc., did not inhibit papain.

Cholestenone-6-sulfonic acid. Rusch and Klein (see above) also reported that estrogenic hormones and related substances, such as dehydroandrosterone, calciferol, cholesterol and desoxycholic acid, inhibited the oxidation of phospholipides. Feigenbaum (143), however, found that cholesterol and ergosterol had no inhibitory effects on papain.

Because of the relationships existing between cholestenone-6-sulfonic acid and some of the above named compounds, it was tested as a potential inhibitor of peptide bond synthesis.

Ethyl carbamate. Huggins and associates (144) recently observed that ethyl carbamate inhibited prostatic cancer. It was therefore of interest to determine whether the inhibitory effects were due to a possible reaction of the compound with proteosynthetic enzymes.

Betaine. No reports concerned with the action of betaine on papain or related type enzymes have appeared in the literature. Since, however, it has been indicated (145) that

(143) Feigenbaum, Exptl. Med. Surg., 2, 304 (1947).
(144) Huggins, Yü, and Jones, Science, 106, 147 (1947).
(145) Bodansky, J. Biol. Chem., 165, 605 (1946).

glycine and its derivatives, e.g. dimethylglycine, inhibited phosphatase and that glycine inhibited urease (146), it was of interest to examine betaine (trimethylglycine) for possible inhibitory properties.

Coumarin. Thimann and Bonner (147) recently indicated that both coumarin and the antibiotic, protoanemonin, inhibited the growth of oat coleoptiles and pea stems. The fact that such effects were similar to those produced by iodoacetate, arsenite and organo-mercurials led them to suggest that the action was probably on SH enzymes. Consequently, coumarin was tested for SH reactivity on papain.

Indoleacetic acid. The testing of indoleacetic acid as a possible papain inhibitor was prompted by reports of Scudi and Jelinek (148), and Smith (149). The former group indicated that amorphous penicillin inhibited the sulfhydryl enzyme, urease, whereas crystalline penicillin had no effects. The latter workers observed that amorphous penicillin inhibited the germination of radish seeds and found that this effect was due, in the main, to the presence of indoleacetic acid. It was therefore conceivable that indoleacetic acid

(146) Kato, Biochem. Z. 136, 498 (1923).

(147) Thimann and Bonner, Science, 109, 444 (1949).

(148) Scudi and Jelinek, ibid., 100, 312 (1944).

(149) Smith, ibid., 104, 411 (1946).

was the factor responsible for the inhibition of urease and the use of this compound as a potential SH reagent was indicated.

Furoic acid. Smith (see above) also reported the presence of furoic acid in amorphous penicillin. It too was tested as a possible SH inhibitor.

o-Hydroxyphenylacetic acid. The isolation of o-hydroxyphenylacetic acid from amorphous penicillin by Fischbach and associates (150), when correlated with the above cited work of Scudi and Jelinek, suggested that this compound be tested as a possible SH inhibitor.

Carboallyloxy-L-glutamic acid. As has already been indicated, the failure of this compound to react with aniline under the catalytic influence of papain suggested that it be tested for possible papain inhibiting properties.

Comparison of inhibitors

In a discussion of the abilities or non-abilities of the compounds studied in the work reported herein to inhibit peptide bond synthesis, as catalyzed by papain, it is necessary to consider the possible reactions they may have undergone and attempt to evaluate the effects of such reactions on the overall synthetic process.

(150) Fischbach, Eble, and Levine, Science, 106, 373 (1947).

Mention has already been made in the Historical section that a substance may inhibit an enzymic reaction, non-competitively, by reaction with certain "essential groups" of the enzyme. While it has not been experimentally established that the type of inhibitions encountered in the present studies were non-competitive, it is reasonable to assume, in view of the structural dissimilarities between inhibitors and substrates, that such was probably true in the majority of cases. It was further indicated in the aforementioned section, that an enzymic reaction could be inhibited by destruction of essential activators of the enzyme. Conversely, of course, the presence of a sufficient concentration of activators may well diminish the effects of potential inhibitors. In view of the data presented in the Results section (Table 7), which substantiated the previous observation by Bergmann and Fraenkel-Conrat (2) that cysteine was essential for the proteosynthetic activities of papain, it becomes evident that in an evaluation of the various compounds studied, due consideration must be given to the fact that cysteine was present in the reaction mixture.

The possible reactions of the compounds tested with either or both of the substrates (acylamino acid and aniline) are also factors which must be weighed. Indeed it is quite conceivable that a reaction can be inhibited by removal or destruction of the substrate; on the other hand if a substrate, capable of reacting with an inhibitor, is

present in sufficient concentration to completely inactivate the inhibitor without itself undergoing marked diminution in concentration, it might then be expected that no inhibition would be noted.

From the foregoing it is apparent that in considering the various means by which the substances studied might have acted as inhibitors or have had their inhibitory powers diminished, the relative concentrations of the components of the reaction system are quite important. An additional factor, that of the relative reactivities of the substrates and the test substances with the enzyme must also be considered. From the standpoint of kinetics, if both substrate and inhibitor were added to an enzyme-containing solution at the same time, the degree of inhibition would then be dependent upon the relative reactivities of the substrate and the inhibitor with essential groups or active centers of the enzyme. Thus if the substrate reacted at a much greater rate than the inhibitor, little or no inhibition would be expected. If the reactivities were comparable, an intermediate degree of inhibition might result. If the inhibitor had the greater reactivity, then almost complete inhibition would be predicted.* All of the above mentioned factors, i.e. reaction with the papain, reaction with the cysteine,

* The foregoing assumes that the enzyme is the limiting factor.

reaction with the substrates and relative reactivities of inhibitors and substrates with the enzyme, must then be considered in an evaluation of the results obtained in the present study.

Reference to the results tabulated in Table 4, reveals that dichloroacetyl-DL-valine, ethyl carbamate, betaine, indoleacetic acid, furoic acid, o-hydroxy-phenylacetic acid and carboallyloxy-L-glutamic acid failed to inhibit either of the two synthetic reactions. In view of the lack of experimental evidence for possible modes of actions of these compounds, their failures to inhibit can probably best be explained with the assumptions that they were either incapable of reacting with any of the other components of the reaction system or that if any reactions did result they did not take place to extents which would markedly affect the synthetic processes.

The failure of either chrysene or cholestenone-6-sulfonic acid to inhibit anilide formation is not surprising in light of conflicting evidence in the literature (see above individual discussions of these compounds) regarding the inhibitory properties of related compounds. It is conceivable that, under the conditions employed herein, these compounds did not react to a sufficient degree with any of the components of the reaction mixture. The failure of coumarin to inhibit the reactions does not necessarily invalidate the suggestion

of Thimann and Bonner (147) that this compound reacted with SH enzymes. In the first place coumarin may be a rather weak SH reagent and incapable of reacting markedly or rapidly with the SH groups of cysteine or papain. Secondly, under the in vivo conditions employed by Thimann and Bonner, a much more sensitive SH enzyme system or systems, without the protective effects of relatively large concentrations of sulfhydryl activators, may have been inhibited.

In view of the observation by Hoffmann-Ostenhof and Biach (125) that p-benzoquinone in quite low concentrations (0.0001 M) inhibited the ability of papain or papain-HCN to digest gelatin, the failure of this compound to markedly inhibit the synthetic reactions was somewhat unexpected. However, in the previous discussion devoted to p-benzoquinone, it was pointed out that the compound can either add or oxidize SH groups, and that the latter effect is readily reversed by cysteine. It is thus conceivable that the cysteine functioned rather effectively in diminishing the inhibitory action of p-benzoquinone both by reversing any oxidation of the SH groups of papain and by adding to the double bonds. If such reactions did take place, they apparently were far from quantitative, since complete reaction of the cysteine with the quinone would have reduced the concentration of the

Mackworth (114) has also indicated the desirability of such preactivation of papain by cysteine, in studies concerned with the inhibitory effects of sulfhydryl reagents.

In contrast with the results obtained with p-benzoquinone, those obtained with 2-methyl-1,4-naphthoquinone bisulfite indicate that this compound, at a concentration of 0.01 M, was quite an effective inhibitor of both synthetic reactions. Whether the strong inhibitory effects can be attributed to the structure of the compound per se or to the fact that it is capable of reversibly dissociating to yield the free quinone and bisulfite (see under the discussion of this compound), and that the latter compound actually inhibits the reaction, is not known. In reference to sodium bisulfite, the results of Table 4 which indicate strong inhibitory powers at concentrations of 0.01 M and 0.001 M, emphasize the need for studying the free quinone in order to determine whether it possesses any inhibitory powers.

As has been mentioned in the discussion devoted to the mode of action of sodium bisulfite, its ability to reduce disulfide linkages to sulfhydryl groups implied that the compound should function as an activator. It is therefore necessary to consider other possible reactions. In light of the results obtained (Tables 4 and 5) consideration must be given to the suggestion of Bergmann and Ross (69) and the conclusion of Maeda (71) that papain contains an aldehyde

group. The latter worker reported that sodium bisulfite inhibited the hydrolysis of gelatin by papain, after the enzyme had first been pretreated for 24 hours with the carbonyl reagent.

However, in view of the observations of Sizer and Tytell (151), that the activity of urease (a sulfhydryl enzyme) was markedly influenced by the oxidation-reduction potential of the reaction mixture, it is of interest to speculate as to the applicability of their findings to the present case. According to these workers urease exhibited its maximum activity in the presence of 0.005 M cysteine or at about E_h+5. In the presence of strong oxidizing agents, e.g. potassium permanganate or potassium ferricyanide, or of strong reducing agents, e.g. sodium sulfide or sodium hydrosulfite, the activity of the enzyme dropped sharply. Plots of the enzyme activity against the oxidation-reduction potential of the reaction mixture gave curves similar to the typical pH-activity curves for enzymes. Such results led the authors to suggest that the control of oxidation-reduction potentials as well as of pH may be important in some enzyme reactions. Since Sizer and Tytell have implied that differences in oxidation-reduction potentials may similarly affect papain, it is plausible that the sodium bisulfite may have so altered the

(151) Sizer and Tytell, J. Biol. Chem., 138, 631 (1941).

potential of the reaction mixture as to actually result in an appreciable deactivation of papain.

Of further interest is the work of Fruton and Bergmann (152) which indicated that papain, previously activated with cysteine, had greater enzymatic activity toward benzoylargininamide and carbobenzoxyisoglutamine than cyanide treated papain. Although the authors interpreted these results as an alteration of specificity, and considered the activators as coenzymes, it seems likely that the differences may have actually been due to changes in the oxidation-reduction potentials of the reaction mixtures. The work of Sizer and Tytell and of Fruton and Bergmann suggests the determination of the effects of altering redox potentials on the activity of papain. It is also of interest to speculate as to whether the optimum redox potential would be dependent upon the nature of the substrate.

With reference to the presence of aldehyde groups in papain the results of studies conducted with the other carbonyl reagents, phenylhydrazine (Table 4), hydroxylamine (Table 4 and 5) and barbituric acid (Table 5), need to be examined. The fact that phenylhydrazine did not inhibit the anilide syntheses was not unexpected in view of the aforementioned observations of Bergmann and Fraenkel-Conrat (2)

(152) Fruton and Bergmann, J. Biol. Chem., 133, 153 (1940).

that phenylhydrazine can be utilized instead of aniline for the enzymic syntheses of phenylhydrazides and that phenylhydrazine inhibited only Papain I-HCN and not Papain I-cysteine.

The results obtained with hydroxylamine, especially those in Table 5, tend to indicate that this carbonyl reactor, at a concentration of 0.01 M, was a rather effective papain inhibitor. Conceivably the inhibition may have been due to a reaction with carbonyl groups in papain, which the cysteine was incapable of preventing although oxidation-reduction effects as considered for bisulfite may have been involved. The observations that the synthetic reactions were inhibited to a greater degree when the papain was pretreated with hydroxylamine (compare results of Tables 4 and 5) may indicate that the compound had, during the pretreatment period, reacted to such extent with the enzyme as to appreciably reduce the concentration of unaltered enzyme and thus the rate of the synthetic reactions.

Although barbituric acid inhibited the synthetic reactions to some extent, (Table 5) it was not a very potent inhibitor. This may have been due to a slow rate of reaction between the inhibitor and an essential group, perhaps an aldehyde group, of the enzyme. Maeda (71) found that papain treated for 40 hours with diethylbarbituric acid inhibited the hydrolysis of hippurylamide completely, but had very little effect on the hydrolysis of gelatin.

The results obtained with the tetrazolium salts (Table 4) are of interest, because it is believed that in part the inhibitory effects may have been due to the reaction of these compounds with the acylamino acid substrates, especially carbobenzoxy-L-glutamic acid. This was evidenced by the formation of an oily deposit on addition of the acylamino acid solution to the solutions containing the tetrazolium compounds. While the nature of these reaction products has not been established, it is plausible that they are polar compounds (salts) formed by the interaction of the positive nitrogen atom of the tetrazolium compound and the carboxyl groups of the acylamino acids. It should be noted, however that the extents of inhibition of the synthesis of carbobenzoxy-L-glutamic acid anilide by 0.01 M 2,3,5-triphenyltetrazolium chloride and by 0.01 M 2,5-diphenyl-3-(p-iodophenyl)-tetrazolium chloride, of 52 percent and 31 percent respectively, were greater than can be accounted for on the basis of a quantitative reaction between these compounds and the acylamino acid, which was present in a concentration of 0.1 M. Presumably, then, the tetrazolium compounds may have reacted with other components of the reaction mixture. The fact that red colored compounds characteristic of the reduced forms (formazans) of the tetrazolium salts were obtained, seemed to indicate that reactions with the cysteine may have taken place. Although such reactions would be contrary to the observations of Kuhn and Jerchel (138) which indicated that

cysteine did not reduce triphenyltetrazolium chloride, it is conceivable, that under the conditions employed in the present investigation, reduction may have taken place to a limited extent.

No explanation can be advanced for the observation that the synthesis of carbobenzoxy-L-glutamic acid was inhibited to a greater extent by both tetrazolium compounds than was the synthesis of benzoyl-L-leucinanilide, unless these differences are bound in some way to the apparent differences in the reactivities of the two acylamino acids with the tetrazolium compounds. Perhaps the differences in pH of the two reaction mixtures were also contributing factors. Aldons (153) in a recent study of the effect of pH on iodoacetic acid toxicity to yeast cells, signified that pH also plays an important role in enzyme-inhibitor reactions since the inhibitor was shown to be more effective below pH 5. Such results suggest that there may also be an optimum pH for the inhibition of an enzyme by an inhibitor. Possibly changes in the oxidation-reduction potentials of the reaction mixtures by the tetrazolium compounds may have contributed, in part, to the differences.

Consideration of the results obtained (Table 4) in studies concerned with 2-hydroxy-5-methylacrylophenone, p-iodoaniline, and basic phenylmercuric nitrate indicates that

(153) Aldons, J. Biol. Chem., 176, 83 (1948).

these compounds apparently inhibited the synthesis of benzoyl-L-leucinanilide to a greater extent than that of carbobenzoxy-L-glutamic acid anilide. On the other hand phenacyl bromide, maleic acid, and fumaric acid were more potent inhibitors of the formation of the second named compound. These variations may have been due to the difference existing between the pH of the two reaction mixtures, i.e. 4.95 for the glutamic acid derivative and 5.75 for the leucine compound, a factor which might have altered the reactivities of the inhibitors with papain or cysteine.*

A rather interesting mechanism to account for the variation in the degrees of inhibition of an enzymic reaction with the nature of the substrate has recently been advanced by Singer (154) who observed that the percent of inhibition of wheat germ lipase by a fixed concentration of p-chloromercuribenzoate (a sulfhydryl reagent) varied with the structure of the substrate ester. Since the extent of inhibition varied directly with the molecular size of the substrate, Singer suggested that sulfhydryl reagents may possibly interfere sterically with the approach of the substrates to

* The same explanation, and others to follow, may be applicable to the previously noted observation that p-benzoquinone (Table 5) inhibited the synthesis of the leucine compound to a greater degree than that of the glutamic acid compound.

(154) Singer, J. Biol. Chem., 174, 11 (1948).

the active surface of the enzyme. It seems likely that a similar mechanism might be invoked to interpret the results obtained with the above mentioned compounds. However, Singer's mechanism requires some modification since it implies that the order of the degrees of inhibition of reactions involving different substrates would remain unaltered despite changes in the nature of the inhibitor. It is thus necessary to assume that different inhibitors in reacting with a specific group of the enzyme may so orient themselves with respect to the active surface of the enzyme that they may tend to hinder the approach of one substrate more readily than another; the order of these effects may vary with the nature of the inhibitor. It seems desirable to verify this assumption by employing more acylamino acid substrates than in the work reported here.

The observations that fumaric acid markedly inhibited the synthesis of carbobenzoxy-L-glutamic acid anilide with but little effect on that of benzoyl-L-leucinanilide, whereas maleic acid (0.01 M) inhibited both reactions appreciably were of interest. The work of Hopkins and associates (122), which indicated that fumaric acid did not inhibit succinic dehydrogenase, whereas maleic acid did (presumably by reaction with SH groups) may be cited as evidence that fumaric acid may have functioned differently in the inhibition of the anilide synthesis than did maleic acid. These workers further indicated that fumaric acid as well as malonic acid and

succinic acid protected the dehydrogenase from oxidation by oxidized glutathione (GSSG); they emphasized the fact that these were dicarboxylic acids.

Potter and DuBois (130) also noted that malonic acid protected succinic dehydrogenase from the action of various sulfhydryl reactors. They postulated that the two carboxyl groups of malonic acid, by uniting with groups adjacent to the sulfhydryl groups of the enzyme, e.g. -N-H, formed a protective bridge over the SH groups, and thus prevented the approach of the sulfhydryl reagents. It should be pointed out since malonic acid is a known competitive inhibitor of succinic acid, that the groups to which it had become attached were probably those which make up the active centers of the enzyme. Conceivably then fumaric acid is capable of reacting with these same groups, although, it apparently does not function as a competitive inhibitor of succinic dehydrogenase.

In view of the observations of the aforementioned investigators, it seems likely that fumaric acid may have similarly united, not with the sulfhydryl groups of papain, but with other groups essential for the formation of the enzyme-substrate complex. Because of the similarity in the structure of fumaric acid and glutamic acid i.e. two carboxyl groups, it is plausible that fumaric acid functioned as a competitive inhibitor of the synthesis of carbobenzoxy-L-

glutamic acid anilide. Such proposal, of course, requires testing by means of kinetic studies, studies of reversal ratios, variation in the nature of the acylglutamic acid and perhaps variation in the dicarboxylic acid used as the inhibitor. The proposal would also seem to indicate that derivatives of monoaminomonocarboxylic acids would require different active centers, unless these derivatives were capable of displacing fumaric acid from the active groups to which it was attached.

The pronounced inhibitory effects noted with 0.01 M copper sulfate (Table 4) and 0.01 M potassium ferricyanide were probably due to the oxidation of sulfhydryl groups of both the papain and cysteine. The fact that these effects were noticeably less at concentrations of 0.001 M may be illustrative of the protective action of the cysteine on the enzyme. It is likely, that alterations in the oxidation-reduction potentials of the reaction mixtures were also involved. In reference to the inhibitory action of nitrous acid (Table 5), the results cannot be taken as evidence that amino groups are essential for the synthetic activity of papain, since reaction with the cysteine, undoubtedly, took place as well. Similar reasoning may be applicable to the case of formaldehyde (Table 5). Such results indicate the desirability of treating papain with various group reagents and separating

the treated enzyme from the reagents, previous to the addition of the normal components of the reaction mixture.*

The extremely potent inhibitory power of iodoacetic acid is emphasized by the results of Table 4. It is, however, difficult to explain the fact that the haloacid at a concentration of 0.00001 M inhibited the reactions completely, despite the presence of a relatively high (0.008 M) concentration of cysteine. Since both enzyme and activator were added simultaneously to the inhibitor containing solution, the results indicate that the reaction of iodoacetic acid with the SH groups of papain may have proceeded at a tremendously greater rate than with that of cysteine.

In contrast to the results of Table 4, those presented in Table 6 indicated that the extent of inhibition of papain by iodoacetic acid varied with the nature of the substrate. The difference in the pH of the two reaction mixtures might have accounted for the variations, although the previously cited work of Aldons (153) would suggest that the synthesis of carbobenzoxy-L-glutamic acid anilide should have been the more highly suppressed. It is plausible that the amounts of iodoacetic acid used were insufficient to react completely with all the papain, and that a small enough quantity of free enzyme remained to catalyze the synthetic reactions albeit at a slower rate. However, under these conditions,

* Studies of this nature are in progress in these laboratories.

both syntheses should have proceeded to approximately the same extent since Figure 5 has shown marked similarity in the rates of the two reactions. Conceivably, the rates of the two reactions may differ from each other at lower levels of enzyme. It is also likely that the differences may be due to variable steric effects produced by the iodoacetic acid, such that carbobenzoxy-L-glutamic acid is capable of complexing with the altered enzyme more readily than benzoyl-L-leucine.

The data of Table 7, give evidence that differences in the relative reactivities of the substrates, dependent on the state of activation of the enzyme may exist. Apparently both synthetic reactions proceeded at equal rates in the presence of cysteine, however pronounced differences were noted after 24 hours in the extents of the reactions in the absence of cysteine. Such results indicated that cysteine may not be required to the same extent as a papain-activator for both reactions. Possibly the differences in the pH of the two reaction mixtures may have been involved.

The results of the foregoing investigations have confirmed the value of employing simple synthetic substrates for studying both the synthetic and hydrolytic activities of the enzyme papain. The studies concerned with the inhibition of peptide bond synthesis suggest extension of the work to additional acylamino acids. In order to eliminate the possible confounding effects of variations in the pH, which may have accounted for the differences in the extents of inhibition with

change in substrate, the reactions could either be carried out at a uniform pH, or substrates having approximately the same pH optima might be used. In addition, it would be of interest to utilize the corresponding acylamino acid amides as hydrolytic substrates in conjunction with the inhibitors, in order to correlate the effects of the inhibitors on the abilities of papain to synthesize and hydrolyze peptide bonds.

SUMMARY

The syntheses of a number of acylamino acids have been described and the effects of altering experimental conditions on the papain-catalyzed syntheses of the corresponding acylamino acid anilides were studied. The factors investigated included: variation of the nature of the acyl group (benzoyl, p-nitrobenzoyl, carbobenzoxy and carboallyloxy), variation in the nature of the amino acid residue (glycine, DL-valine, DL-leucine, and L-glutamic acid), variation of the pH (from ca. 3.0 - 6.5) and variation of the citrate buffer concentration (1.0 M and 0.1 M). The extents of hydrolysis of the amides of benzoylglycine, benzoyl-DL-valine and benzoyl-DL-leucine by papain at pH 5.0 in 1.0 M and 0.1 M citrate buffers were also determined.

The efficacies of various substances as inhibitors of the papain catalyzed syntheses of carbobenzoxy-L-glutamic acid anilide and benzoyl-L-leucine anilide at the pH optima of the syntheses, and in 1.0 M citrate buffers have been tested. Various group reagents were employed in attempts to determine the essentiality of certain groups of the enzyme for its synthetic ability.

1. The pH optima for the syntheses of all the acyl-L-glutamic acid anilides were lower, in both 1.0 M and 0.1 M

citrate buffers than the optima for the anilides of the corresponding acyl derivatives of glycine, L-valine and L-leucine. The former values were in the range of pH 4.1 to 5.0.

2. With but few exceptions the pH optima for the acyl derivatives of the monoaminomonocarboxylic acids increased in the order: acylglycine, acyl-DL-valine and acyl-DL-leucine. These values fell in the range of pH 4.2 to 6.5.

3. The pH optima for the syntheses of all the acylamino acid anilides were from 0.2 to 0.9 pH units lower in the 0.1 M buffers than the corresponding values in the 1.0 M buffers.

4. The yields of the anilides when 1.0 M buffers were employed were, in almost all instances, greater than the yields of the same compounds in 0.1 M buffers at the pH optima of the reactions.

5. Carboallyloxy-L-glutamic acid was the only acylamino acid studied which failed to yield an anilide in either 1.0 M or 0.1 M buffer solutions. Carboallyloxyglycine and carboallyloxy-DL-valine did not give measurable yields of anilides in 0.1 M citrate buffers.

6. Although variations in the nature of acyl group attached to any one amino acid residue resulted in changes in the pH optima of the synthetic reactions, the orders of the changes were not the same for all the amino acids studied.

7. The yields of the anilides obtained by altering the nature of the acyl group attached to any one amino acid

residue also varied with these changes. However the effects were not too pronounced and, in the main, there was a greater dependency on the nature of the amino acid than on the nature of the acyl group; no definite order in the yields was noted with variation of the acyl groups.

8. The orders of the yields of the anilides of the acylated monoaminomonocarboxylic acids in both 1.0 M and 0.1 M buffers were in all cases acyl-L-leucine > acylglycine > acyl-L-valine. The acyl derivatives of glutamic acid which gave insoluble products, showed yields of their respective anilides comparable to those of the corresponding leucine derivatives. Such results indicated that variation in the nature of the amino acid residue altered the reactivities of the substrates in a definite manner and to a greater extent than did changes in the acyl groups. The results supported the view that an enzymic preference for the amino acid residues existed.

9. At pH 5.0 in both 1.0 M and 0.1 M citrate buffers, benzoyl-DL-leucinamide was hydrolyzed to a greater extent by papain than was benzoyl-DL-valinamide. These results, when correlated with those of the synthetic experiments indicated a preference by papain for leucine residues relative to valine residues.

10. The extents of inhibition of the papain-catalyzed syntheses of carbobenzoxy-L-glutamic acid anilide and

benzoyl-L-leucinanilide by a number of substances varied with either the nature of the acylamino acid substrate or the pH and the time of the synthetic reaction.

11. The degrees of inhibition were apparently independent of either the nature of the substrate or the pH when copper sulfate, sodium bisulfite, hydroxylamine, nitrous acid, formaldehyde and potassium ferricyanide were used as inhibitors. p-Benzoquinone inhibited both reactions weakly and to the same extent when the enzyme was not pretreated with this compound. Pretreatment of papain with the quinone resulted in a greater degree of inhibition of the synthesis of the acylleucinanilide, but did not markedly change the extent of formation of the glutamic acid derivative.

12. The extents of inhibition of each of the two synthetic reactions was dependent on either the nature of the substrate or the pH in the cases of phenacyl bromide, maleic acid, fumaric acid, 2-methyl-1,4-naphthoquinone bisulfite, 2,3,5-triphenyltetrazolium chloride, and 2,5-diphenyl-3-(p-iodophenyl)-tetrazolium chloride. These compounds inhibited the synthesis of carbobenzoxy-L-glutamic acid to a greater degree than that of benzoyl-L-leucinanilide. Converse results were obtained with p-iodoaniline, basic phenylmercuric nitrate and 2-hydroxy-5-methylacrylophenone. Possible reasons for these differences have been discussed.

13. The papain-catalyzed syntheses were inhibited by several sulfhydryl reagents (iodoacetic acid, phenacyl bromide,

basic phenylmercuric nitrate, maleic acid and oxidizing agents). Such results thus indicated that sulfhydryl reagents inhibit the synthetic activities of papain as well as the proteolytic abilities as previously shown.

14. Studies conducted with carbonyl reagents gave results, which when compared with previous observations showed that these reagents were capable of inhibiting both the synthesis and hydrolysis of peptide bonds.

15. During the course of the investigation the following compounds previously not reported in the literature were prepared: carbobenzoxy-DL-valine,* m.p.76-78°; carbobenzoxy-DL-leucine,* m.p. 45-48°; carboallyloxy-DL-valine, m.p. 49.5-52°; carboallyloxy-L-glutamic acid, m.p. 55-58°; benzoyl-DL-valine ethyl ester, m.p. 65-68°; carbobenzoxyglycine ethyl ester, m.p. 33-34°; carbobenzoxy-DL-valine ethyl ester, m.p. 32-33°; carbobenzoxy-DL-leucine ethyl ester, m.p. 18.5-19°; carboallyloxy-DL-valine ethyl ester, m.p. 9-11°; carbobenzoxyglycinamide, m.p. 136-137.5°; carboallyloxyglycinamide, m.p. 107-107.5°; carboallyloxy-DL-leucinamide, m.p. 83-85°; benzoyl-L-glutamic acid anilide, m.p. 169-171°; p-nitrobenzoyl-glycinanilide, m.p. 213.5-215.5°; p-nitrobenzoyl-L-valinanilide, m.p. 215-216°; p-nitrobenzoyl-L-leucinanilide, m.p. 188-190°; p-nitrobenzoyl-L-glutamic acid anilide, m.p. 191-192°; carbobenzoxy-L-valinanilide, m.p. 182-183.5°; carbobenzoxy-

* Previously prepared in these laboratories by Fox and Fling (86).

L-leucinanilide,* m.p. 138-141°; carboallyloxyglycinanilide, m.p. 134-136°; carboallyloxy-L-valinanilide, m.p. 168-169°; and carboallyloxy-L-leucinanilide, m.p. 160.5-162°.

* Previously prepared by Bergmann and Fraenkel-Conrat (2) but melting point not reported.

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