Catalytic Hydrogenolysis.—A solution of 4.77 g. of the distearoyl or 4.43 g. of the dipalmitoyl bis-(glycol)-phosphoric acid phenyl ester (6 mmoles) in a mixture of 95 ml. of chloroform and 20 ml. of 99% ethanol to which had been added 0.91 g. (4 mmoles) of platinic oxide (Adams catalyst) was shaken vigorously in an all-glass hydrogenation vessel in an atmosphere of hydrogen at an initial pressure of 40–50 cm. of water until the absorption of hydrogen ceased. In approximately one hour the theoretical amount of hydrogen (24 mmoles) had been consumed. After replacing the hydrogen with nitrogen and removing the catalyst, the solvents were distilled off under reduced pressure at a bath temperature of 30–35°. The bis-(glycol)-phosphatidic acid was washed with three 50-ml. portions of dilute acetic acid (10%) and was dried *in vacuo* (0.01 mm.) over solid sodium hydroxide. Both phosphatidic acids were free of potassium.

Distearoyl-bis-(glycol)-phosphoric Acid.—For purification the crude distearoyl-(glycol)-phosphoric acid weighing 4.09 g. (95% of theory) was triturated with 35 ml. of 99% ethanol, the mixture was separated by centrifugation and the solid was recrystallized from 140 ml. of boiling 99% ethanol. The bis-(glycol)-phosphatidic acid was recovered in a yield of 75% (3.07 g.) and melted from 92.5–93.5°. At room temperature the distearoyl bis-(glycol)-phosphoric acid is insoluble in ether or acetone, very slightly soluble in methanol, ethanol, ethyl acetate, tetrachloromethane, petroleum ether or benzene, but readily soluble in chloroform. The substance is also readily soluble in warm methanol, ethanol, acetone, ethyl acetate, tetrachloromethane, petroleum ether (b.p. 100–120°) and benzene.

Anal. Calcd. for $C_{40}H_{79}O_8P$ (718.2): C, 66.89; H, 11.09; P, 4.46. Found: C, 67.09; H, 10.96; P, 4.33.

Dipalmitoyl-bis-(glycol)-phosphoric Acid.—For purification the crude dipalmitoyl-bis-(glycol)-phosphoric acid, weighing 3.94 g. (99% of theory), was triturated with 35 ml. of 99% ethanol and the mixture was separated by centrifugation. The precipitate was recrystallized from 140 ml. of warm 99% ethanol; recovery 3.01 g. (76.5%), m.p. 89.0–90.5°. At room temperature the dipalmitoyl bis-(glycol)-phosphoric acid is practically insoluble in methanol, ethanol, acetone, ethyl acetate or petroleum ether, slightly soluble in benzene or tetrachloromethane, and readily soluble in chloroform. It is also readily soluble in hot methanol, ethanol, acetone, ethyl acetate, tetrachloromethane and petroleum ether (b.p. 100–120°).

Anal. Calcd. for $C_{36}H_{71}O_8P$ (662.13): C, 65.30; H, 10.81; P, 4.68. Found: C, 65.46; H, 10.77; P, 4.71.

Acknowledgments.—This work has been supported by a grant from the Nutrition Foundation, Inc., New York, which is gratefully acknowledged. I wish also to express my appreciation to Dr. Norman K. Freeman, Donner Laboratory, University of California, Berkeley, for the infrared absorption spectra and Dr. Robert Gwilliam, University of Toronto, for the X-ray diffraction measurements.

TORONTO, CANADA

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF IOWA STATE COLLEGE]

Enzymic Synthesis of Peptide Bonds. VI. The Influence of Residue Type on Papain-catalyzed Reactions of Some Benzoylamino Acids with Some Amino Acid Anilides[1,2]

BY SIDNEY W. FOX, MILTON WINITZ AND CORNELIUS W. PETTINGA

RECEIVED MARCH 30, 1953

Each of thirteen benzoylamino acids has been submitted to reaction with glycinanilide in the presence of papain. Only one, benzoylglycine, participated in a synthesis leading to a larger peptide. The failure of benzoylaminoisobutyric acid to react was explainable on the basis of steric hindrance. Benzoyltryptophan has been shown earlier to react in another *p*H range. Benzoylglutamic acid and benzoyltyrosine fail to react at *p*H's in which the corresponding reactions with aniline had previously been shown to proceed rapidly. All eight other reactions proved to be transacylations yielding glycine-free products. On reacting with each of four amino acid anilides, benzoylglycine yielded benzoylglycylamino acid anilide. When benzoylalanine was employed instead of benzoylglycine there resulted two syntheses and two transacylations. The acylamino acid and the amino acid anilide thus each contribute to selectivity in synthesis. The specificities observed when the carboxoid or aminoid component is systematically varied contrasts, at the two amino acid residue level, with the broad preferences observed in reactions of benzoylamino acid with aniline. From these results it is apparent how a single protease participating in peptide bond synthesis may favor unique synthetic reactions, and reject or divert others. To emphasize that the substrate contributes to this specificity to a degree comparable to the influence of the enzyme, these phenomena are referred to as *zymosequential specificity*. These observations suggest the possibility that, in protein synthesis, each peptide intermediate becomes part of the protease to give, in effect, a new enzyme at each step.

In any consideration of mechanisms of protein synthesis, adequate explanation of replication of highly specific protein structures must be paramount. One mechanism, among those which have been postulated, suggests that the enzymes which are known to catalyze proteolysis, also mediate protein synthesis.[3] Any pathway which invokes the agency of proteases is more worthy of consideration if it also explains the variability of this replication from one tissue to another and from one species to another. Experimentally valid instances of the type of model peptide bond synthesis which correspond to the latter, have been described.[4] In these comparisons the enzyme preparation was the sole initial variable.

This paper presents evidence for influences which may be considered as a basis for uniquely limited replication of protein molecules.

Experimental Procedure

Reactants.—These have been described in previous papers.[4-8]

Enzyme Experiments.—The essential features have been described previously. The enzymes were from the same

(1) Journal Paper No. J-2281 of the Iowa Agricultural Experiment Station, Ames, Iowa, Project 1111. This work was supported by the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

(2) Presented at the Twelfth International Congress of Pure and Applied Chemistry, New York City, September 12, 1951. The essential results are described in the Ph.D. theses of Cornelius W. Pettinga, 1949, and Milton Winitz, 1951.

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

(4) F. Janssen, M. Winitz and S. W. Fox, THIS JOURNAL, **75**, 704 (1953).

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

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

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

(8) S. W. Fox and H. Wax, THIS JOURNAL, **72**, 5087 (1950).

Table I

Reactivity of Benzoylamino Acids with Glycinanilide at Initial *p*H of 5.3 as Catalyzed by Papain and Ficin

Bz-amino acid	M.p. of crude product after washing, °C.	Mixed m.p. with anilide from bz-amino acid and aniline and purified crude product, °C.	Product	Yield, % Papain	Yield, % Ficin
Bz-glycine	246–248	.....[a]	Bz-glycylglycinanilide	63	23
Bz-DL-alanine	174–176	174–176	Bz-alaninanilide	26	20
Bz-DL-valine	218–220	219–220.5	Bz-valinanilide	1	Trace
Bz-DL-leucine	212–213	212–213	Bz-leucinanilide	29	21
Bz-DL-methionine	160–162	161–162	Bz-methioninanilide	28	20
Bz-DL-isoleucine	213–215	217–219	Bz-isoleucinanilide	1	0
Bz-L-glutamic acid	.....	.....	.....	0	0
Bz-DL-tryptophan	.....	.....	.....	0	0

[a] No reference substance in this case. *Anal.* See Table IV.

commercial lots reported in the preceding paper.[4] It was necessary to weigh the alaninanilide and valinanilide hydrogen citrates by difference because of their hygroscopicity.

Products.—Anilides not recorded in earlier papers in this series are described in Table IV. Each of these was purified to constant m.p. by recrystallization from 50% aqueous ethanol.

Results

Reactions of Benzoylamino Acids with Glycinanilide.—The results are summarized in Tables I and II. It may be seen in Table I that at *p*H 5.3, two bz-amino acids fail to react, five undergo transacylation, whereas only bz-glycine participates in a synthetic reaction. The anilides obtained by transacylation displayed the m.p.'s of the known bz-L-amino acid anilides. These m.p.'s were not depressed by mixture with anilides obtained from reaction of the bz-amino acid with aniline. The constants for the bz-glycylglycinanilide are presented in Table IV.

Table II

Reactivity of Benzoylamino Acids with Glycinanilide at Various Initial *p*H's in Papain-catalyzed Reactions

Bz-amino acid	*p*H	M.p., °C.	Product	Yield, %
Bz-DL-leucine	5.0	210–212	Bz-leucinanilide	3
	5.5			34
	5.9			45
Bz-DL-isoleucine	5.0			0
	5.5	208.5–211	Bz-isoleucinanilide	1
	5.9			1
Bz-DL-valine	5.0			0
	5.5	212–214	Bz-valinanilide	1
	5.9			5
Bz-DL-norvaline	5.0	174–176	Bz-norvalinanilide	5
Bz-DL-norleucine	5.0	176–178	Bz-norleucinanilide	4
Bz-DL-α-amino-*n*-butyric acid	5.0	163–165	Bz-α-aminobutyric acid anilide	21
Bz-DL-α-aminoisobutyric acid	5.0	.....	.....	0
N-Bz-L-tyrosine	5.0	.....	.....	0
	6.1	.....	.....	0
	7.3	.....	.....	0

In Table II are recorded some effects of variation in *p*H on three of the reactions of Table I, as well as the effects of five other substrates in the same experiment. Of a total of thirteen reactants tested with glycinanilide[9] the results demonstrate the selective type of reaction observed with bz-glycine, and indicate rejection of all of the other tested reactants for synthetic reactions. Under the conditions, eight of the carboxoid reactants undergo transacylation, to provide one type of rejection. Three others, bz-glutamic acid, bz-tryptophan and N-bz-tyrosine fail to react entirely under conditions in which reactions are observed for other residues.

Reactions of Bz-glycine and of Bz-alanine with Amino Acid Anilides.—When the bz-amino acid was held constant and the amino acid anilide varied, the results presented in Table III were obtained. For these four monoaminomonocarboxylic acid anilides, no qualitative difference was ob

Table III

Reactivity of Bz-glycine and Bz-alanine with Amino Acid Anilides as Catalyzed by Papain at *p*H 5.6

Reaction	Yield, %
Bz-glycine + Glycinanilide → Bz-glycylglycinanilide[a]	63
Bz-glycine + Alaninanilide → Bz-glycylalaninanilide	69
Bz-glycine + Leucinanilide → Bz-glycylleucinanilide	51
Bz-glycine + Valinanilide → Bz-glycylvalinanilide	56
Bz-alanine + Glycinanilide → Bz-alaninanilide[a]	26
Bz-alanine + Alaninanilide → Bz-alaninanilide	46
Bz-alanine + Leucinanilide → Bz-alanylleucinanilide	43
Bz-alanine + Valinanilide → Bz-alanylvalinanilide	50

[a] These reactions were carried out at *p*H 5.3.

Table IV

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

Anilide	M.p.,[a] °C.	Nitrogen, % Calcd.	Nitrogen, % Found
Bz-glycylglycinanilide	246–248[b]	13.5	13.4
Bz-glycylalaninanilide	235.5–236.5	12.9	12.9
Bz-glycylvalinanilide	251–252	11.9	11.8
Bz-glycylleucinanilide	197–199	11.4	11.3
Bz-alanylvalinanilide	271.5–272	11.4	11.2
Bz-alanylleucinanilide	243–243.5	11.0	10.9

[a] Melting points are uncorrected. [b] A m.p. of 238–240° was reported by T. Curtius and R. Wüstenfeld, *J. prakt. Chem., N. S.*, **70**, 80 (1904).

(9) One other bz-amino acid than those tabulated has been tested, *i.e.*, bz-phenylalanine. The special behavior of this substrate at higher *p*H's has been recorded.[4] All of the non-reacting substrates in Tables I and II, bz-glycine, and many of those undergoing transacylation were tested by two or more authors or other investigators with the same results (four investigators confirmed the synthetic reaction of bz-glycine). With bz-phenylalanine, however, only one of these authors has recorded at *p*H 5.0 with glycinanilide a reaction which was not found in experiments of others. Inasmuch as the discrepancy has not been satisfactorily explained, this reaction has been omitted from the tables.

served with bz-glycine. Bz-alanine, however, behaved differently than did bz-glycine, and the reactions in which it participated involved transacylation or coupling, depending upon the exact amino acid anilide. This last reflects in a systematically varied series an isolated instance observed by Bergmann and Fraenkel-Conrat.[10] Bz-leucine was found to give with leucinanilide bz-leucylleucinanilide, whereas with glycinanilide the product was bz-leucinanilide. The behavior of bz-leucine was like that of bz-alanine and unlike that of bz-glycine (Table III).

Discussion

Of the total of thirteen reactants tested with glycinanilide, Tables I and II illustrate the selective type of reaction with bz-glycine, and indicate rejection of the other possible synthetic reactions. The non-reactivity of bz-aminoisobutyric acid is ascribable to steric hindrance within the substrate[5] and is without significance here. Non-reactivity of the bz-tyrosine–glycinanilide pair has been mentioned earlier[4]; the significant features in the present work are that the reaction with glycinanilide is rejected under conditions in which reaction with aniline is known to proceed rapidly.[6] On the other hand, the non-reactivity of valine and of isoleucine under the action of ficin (Table I) is very likely due to the fact that these residues show threshold reactivity in the comparative studies employed here.

It becomes important to ask if the transacylations are explainable on the basis that all except one of the bz-aminoacylglycinanilides, bz-glycylglycinanilide in this case, are not sufficiently insoluble to separate.[7] This explanation is eliminated by virtue of the fact that many of these same sequences can be synthesized enzymically from the corresponding benzoyldipeptide and aniline.[2] In the cases of rejected reaction without transacylation, *i.e.*, cases in which no apparent reaction occurred, such a technical explanation is also unacceptable; bz-tyrosine fails to react with glycinanilide over a wide *p*H range in papain- and ficin-catalyzed reactions, but the product of a coupling reaction is quite insoluble enough to separate if another enzyme, chymotrypsin, is employed.[4]

As is indicated in Table III, the product of the reaction of bz-glycine with each of the four amino acid anilides tested is, in every instance, the bz-dipeptide anilide. No selectivity is observed in this series. When, however, bz-alanine is allowed to react with the same four amino acid anilides, selectivity is observed. The alaninanilide reactions differ among themselves and from the glycinanilide reactions with the same carboxoid components. A difference between alaninanilide and glycinanilide in a reaction with bz-phenylalanine at another *p*H has also been observed.[4]

The possibility that replacement reactions of bz-amino acids with glycinanilide follow the co-substrate mechanism of Behrens and Bergmann[11] has been considered. Such a mechanism would by analogy require the sequence

bz-leucine + glycinanilide ⟶ bz-leucylglycinanilide
bz-leucylglycinanilide ⟶ bz-leucylglycine + aniline
bz-leucylglycine ⟶ bz-leucine + glycine
bz-leucine + aniline ⟶ bz-leucinanilide

This mechanism is improbable for the following reasons. Bz-leucylglycine and aniline yield bz-leucylglycinanilide.[2] Secondly, the *p*H optimum for bz-valine + glycinanilide (> 5.5) is above that for bz-valine + aniline (5.3).[7,8] Finally, complete lack of reactivity of bz-tyrosine with glycinanilide over *p*H 5.0–7.5 suggests a mechanism other than direct coupling with aniline, inasmuch as the latter reacts rapidly at *p*H 6.0.[4,6] A more likely mechanism is that of direct transacylation (see refs. 12 and 13 for general treatment and related terminologies). In any event the cosubstrate mechanism appears to be inapplicable here, and its pertinence in other similar reactions should be tested against this enlarged outlook.

The experiments reported are not offered as proof *per se* that proteases participate in protein synthesis. Evidence which at least suggests such participation has been obtained from other studies such as the inhibition of bacterial growth by D-amino acids[14] in a pattern analogous to inhibitions of protease–substrate interactions supporting the Polyaffinity Hypothesis.[15] In an extension of the bacterial experiments mentioned,[16] Yaw and Kakavas have found D-valine to be more inhibitory to *B. abortus* in broth than was D-leucine. This work has however amplified the earlier inhibition experiments and the interpretation of their relationship to protein synthesis. Whether or not proteases are involved in protein synthesis, the present results illuminate protease–substrate chemistry. The systematic experiments also provide a basis for understanding the selectivity necessary for protein replication, in the event that proteases are directly active in this process.

In the light of present knowledge, it seems unlikely that proteases are sufficient in number for each to participate in a single step in the stepwise synthesis[17–19] of large peptide or protein molecules. With, however, this much emphasis upon the contribution of the substrate to the reaction[20] the substrate appears to play more than the role usually assigned to it, and there is suggested a principle of action which permits more serious consideration of the intermediacy of only one or a small number of

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

(11) O. K. Behrens and M. Bergmann, *ibid.*, **129**, 587 (1939).

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

(13) M. Bergmann, *Chem. Revs.*, **22**, 430 (1938).

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

(15) M. Bergmann, L. Zervas, J. S. Fruton, F. Schneider and H. Schleich, *ibid.*, **109**, 325 (1935).

(16) K. E. Yaw and J. C. Kakavas, *J. Bact.*, **63**, 263 (1952).

(17) C. B. Anfinsen and J. B. Steinberg, *J. Biol. Chem.*, **189**, 739 (1951).

(18) P. N. Campbell and T. S. Work, *Biochem. J.*, **52**, 217 (1952).

(19) For evidence suggesting non-stepwise synthesis, see M. V. Simpson and S. F. Velick, *Federation Proc.*, **12**, 268 (1953).

(20) Not only in synthetic reactions, but also in hydrolytic reactions, when studies are carried out on large substrates, evidence is at hand that the reactivity of any one linkage is governed by the nature of the other residues, T. L. Hurst and S. W. Fox, 123rd meeting of the American Chemical Society, Los Angeles, Abstracts, 28c. *Cf.* also J. S. Fruton, M. Bergmann and W. P. Anslow, Jr., *J. Biol. Chem.*, **127**, 627 (1939), for a discussion of this factor as an influence on rates of proteolytic reaction.

proteases for protein synthesis. This interpretation emphasizes that it is the joint action of enzyme and substrate that contributes to the specificity of the reaction. This joint action may lead to a self-regulated order of residues. Such regulation, controlled by the simultaneous influence of enzyme and substrate, is here referred to as *zymosequential specificity*.

This concept is related to one proposed by Bergmann to explain a postulated periodicity in protein structure.[13,21] Bergmann stated that the mutual interdependence of enzyme and substrate determined which one of four types of reaction (hydrolysis, synthetic coupling, replacement of two types) occurred. It is now clear that protein structures are not generally periodic[22,23] and it becomes necessary to explain a highly exact ordering of individual residues. The experiments reported here, particularly the pronounced selectivity of coupling of bz-amino acid with glycinanilide, indicate that the particular reactants limit the nature of the possible syntheses. The introduction of a glycine residue into the aminoid component (Tables I and II), furthermore, is seen to alter the synthetic capabilities of the system from one of a broad gradation of preference[7] to a more highly selective situation. Extrapolation to larger molecular intermediates should thus lead to increasingly selective reaction, in the presence of any one protease. The initial reactions in a stepwise synthesis would thus predetermine in the fashion indicated by the results presented here, the identity of the later reactions. This would constitute connotations of zymosequential specificity. In conformity with the observation that different proteases can catalyze different interactions, each protease should selectively interact with its available substrates in its own characteristic way.[4]

The following influences leading to selectivity in reaction can be summarized: a single instance of synthesis from a series of possibilities (Tables I and II), non-reactivity of some components under conditions in which others react and in which they react in some cases when only one amino acid residue is present, selectivity imposed each by the aminoid and carboxoid reactants (Table III and ref. 4) and a replacement of preferential action by specificity in reactions involving two amino acid residues instead of one.

In view of these facts, and inasmuch as proteases have the unique ability among enzymes of functioning also as substrates,[24] the possibility of a converse relationship may be considered. Can substrates function as part of the protease complex? If so, at each step the new protease–substrate complex may become in effect a new enzyme different from that operating at any other step in synthesis. Evidence that may confirm or deny the applicability of such a mechanism to protease–substrate action should clarify concepts related to zymosequential specificity.

Acknowledgments.—The assistance of Jacquetta S. Halverson, Dorothy De Fontaine and Armand J. McMillan is appreciated.

(21) M. Bergmann, *J. Mt. Sinai Hosp.*, **6**, 181 (1939).

(22) R. J. Block in C. L. A. Schmidt, "Chemistry of the Amino Acids and Proteins," Charles C Thomas, Springfield, Ill., 1944, p. 1093.

(23) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 481 (1951).

(24) J. H. Northrop, M. Kunitz and R. M. Herriott, "Crystalline Enzymes," Columbia University Press, New York, N. Y., 1948.

AMES, IOWA

[CONTRIBUTION FROM THE DEPARTMENTS OF MICROBIOLOGY AND NEUROLOGY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY, AND THE NEUROLOGICAL INSTITUTE, PRESBYTERIAN HOSPITAL, NEW YORK]

Immunochemical Studies on Blood Groups. XIII. The Action of Enzyme from Snail (Busycon) Liver on Blood Group A and O(H) Substances (Hog)[1a]

BY CALDERON HOWE[1b] AND ELVIN A. KABAT

RECEIVED JUNE 8, 1953

Enzymes from snail liver were shown to split methylpentose from blood group A and O(H) substances and to increase their cross-reactivity with horse anti-SXIV. Blood group A activity was reduced, but O(H) activity was unaffected. N-Acetylhexosamine was liberated from A substances but not from O(H) substances, suggesting structural differences between these two antigens.

The degradation of chitin by extracts of snail hepatopancreas (*Helix pomatia*, sp.) was first described by Karrer and his associates,[1c] who showed that N-acetylglucosamine was liberated by the hydrolytic action of an enzyme which they named chitinase. Freudenberg and Eichel[2] subsequently showed that similar enzyme preparations reduced the specific blood group activity of polysaccharides obtained from human urine, with the liberation of N-acetylglucosamine and a 25% increase in reducing power. Neuberger and Pitt Rivers[3] and Zechmeister and Tóth[4] have since demonstrated that extracts of whole digestive tract and liver from *Helix pomatia* exhibited β-glucosaminidase activity. More recently, Utusi, *et al.*,[5] and Yosizawa[6] have reported the effects of

(1) (a) This investigation was carried out in part under grants from the National Institutes of Health, Public Health Service (RG34), and the William J. Matheson Commission. (b) Senior Fellow in Virus Diseases of the National Research Council, 1949–1951; Fellow of the National Foundation for Infantile Paralysis, 1951–1952; Markle Scholar in Medical Science, 1952. (c) P. Karrer and A. Hofmann, *Helv. Chim. Acta*, **12**, 616 (1929); P. Karrer and G. von François, *ibid.*, **12**, 986 (1929).

(2) K. Freudenberg and H. Eichel, *Ann. Chem.*, **518**, 97 (1935).

(3) A. Neuberger and R. V. Pitt Rivers, *Biochem. J.*, **33**, 1580 (1939).

(4) L. Zechmeister and G. Tóth, *Naturwissenschaften*, **27**, 367 (1939).

(5) M. Utusi, K. Huzi, S. Matumoto and T. Nagaoka, *Tohoku J. Exp. Med.*, **50**, 175 (1949).

(6) Z. Yosizawa, *ibid.*, **55**, 35 (1951).