1953

An acylase system of Lactobacillus arabinosus

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AN ACYLASE SYSTEM OF *LACTOBACILLUS ARABINOSUS*

by

Robert Worth Park

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Biochemistry

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

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Signature was redacted for privacy.

Dean of Graduate College

Iowa State College

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I. INTRODUCTION

A. Delineation of Subject

An acylase is an enzyme which catalyzes the hydrolysis of acylamino acids; that is, speeds up the following reaction:

$$R'\text{-}CO\text{-}NH\text{-}CHR\text{-}COOH + H_2O \longrightarrow R'\text{-}COOH + NH_3^+\text{-}CHR\text{-}COO^-$$

This thesis describes an investigation of the properties and possible physiological importance of an acylase or acylases from the acid producing bacterium, Lactobacillus arabinosus.

B. Objectives

Itschner[1], Drechsler[2], Fox and Warner[3] have shown that

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[1] K. F. Itschner. Bacterial utilization and sequence determination of peptides. Unpublished Ph.D. Thesis. Ames, Iowa, Iowa State College Library. 1951

[2] E. R. Drechsler. Utilization of certain benzoylamino acids by several species of bacteria. Unpublished M.S. Thesis. Ames, Iowa, Iowa State College Library. 1952.

[3] S. W. Fox and C. W. Warner state, on the basis of unpublished experiments, that benzoyl-DL-methionine is partially utilizable for the methionine requirements of L. arabinosus. Ames, Iowa (Private communication) 1953.

Lactobacillus arabinosus has rather marked preferences in its nutritional utilization of benzoylamino acids in lieu of the corresponding amino acids. Thus, L. arabinosus can nutritionally utilize benzoyl-L-leucine and, to some extent, benzoyl-DL-methionine and benzoyl-DL-cystine. However, seven other benzoylamino acids elicit no growth response when present in the growth medium. This metabolic specificity on the part of growing L. arabinosus was thought possibly to be a reflection of the specificity of acylases produced by the organism. An objective of the work described here has been to investigate this possibility; such an objective constitutes an investigation of the correlation between cellular behaviour and enzyme constitution in a specific instance.

A further goal has been to satisfy curiosity concerning enzyme specificity. It has been desired to add to similar work with other organisms and thus increase our knowledge of species specificity and similarities at the molecular level. In particular, it has been sought to expand knowledge of the similarities and dissimilarities of acylase systems from different sources.

Proteins function in nature as enzymes, hormones, and possibly as hereditary agents. As such, they are indispensable directors of life processes. Yet, how proteins are synthesized remains one of the main, largely unsolved,

mysteries of biochemistry. Certain properties of proteases, such as, some degree of specificity and the ability of proteases to catalyze several types of reactions, makes reasonable the working hypothesis that proteases are involved in protein synthesis.

It is believed proper to include among the proteases, the acylase system described in this thesis, and certain experiments will be described that have as their objective the obtaining of indirect evidence as to whether or not the acylase system of Lactobacillus arabinosus takes part in protein synthesis. The approach was to determine if there existed a coinciding pattern of inhibition of both acylase hydrolysis and growth of L. arabinosus by certain compounds. If there existed a coinciding pattern of inhibition, in both instances, it was believed this would constitute evidence, but not proof, that the acylase system was indispensable for the growth and presumably protein synthesis of the organism.

Finally it is appropriate to remark that this thesis is but a small contribution to the large but still quite incomplete picture of the chemical basis of life. The beauty and fascination of this picture, in itself, makes its revelation by the diligence of scientists worthwhile. However, of greater importance are the possible useful applications that may arise from biochemical information. Thus, a more

intimate knowledge of the variation among species of chemical specificity could lead to the rational development of selective biocides and bio-regulators. A more complete knowledge of the reactions leading to cell growth and reproduction may eventually lead to the control of cancer and aging.

II. HISTORICAL

A. Terminology

1. Acylase

An acylase may be defined as an enzyme which catalyzes the hydrolysis of acylamino acids with the liberation of the terminal amino acids according to the following reaction:

$$R'\text{-}CO\text{-}NH\text{-}CHR\text{-}COOH + H_2O \rightarrow R'\text{-}COOH + NH_3^+\text{-}CHR\text{-}COO^-$$

the term acylase shall not apply, however, to enzymes which catalyze the above reaction where R'-CO- is only NH_2-CHR''-, that is, where the only substrates attacked are dipeptides. The group R'-CO-, according to the author's definition, may include, for example, unsubstituted, or halogen substituted aromatic or aliphatic acyl groups; in addition, the R'-CO- may be a substituted amino acid residue, for instance, benzoylglycyl. The above definition of acylase differs somewhat from previous definitions. Mori states that the name, acylase, is generally applicable for the enzyme class, which, in general splits the substituted acid

amides[1]. Krebs, Sykes, and Bartley state that the acylases are enzymes which hydrolyze substituted amides according to the equation:

$$RCO\text{-}NH\text{-}R' \xrightarrow{H_2O} RCOOH + NH_2R'$$

and they add the qualification that acylases are related to peptidases, differing from them in that the acyl residues of the substrate do not contain an amino group[2]. It should be noted that the definition of acylase which we propose differs from previous definitions inasmuch as, according to our definition, an acylase must hydrolyze amides with the liberation of a free amino acid, whereas in previous definitions, the liberated amide component need not be an amino acid. However, our definition does not exclude from the acylases, those amide hydrolyzing enzymes, which liberate both amino acids and also substituted amino acids as the amide components. In addition, our definition of acylase extends to enzymes hydrolyzing the terminal peptide bond of such substrates as acyldipeptides; the older definition limited the acylase reaction to the splitting off of radicals such as acetyl, chloroacetyl, and benzoyl.

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[1]H. Mori, J. Biochemistry (Japan), 29, 225 (1939).

[2]H. A. Krebs, W. O. Sykes, and W. C. Bartley, Biochemical J., 41, 622-30 (1947).

The main justification for the proposed modified definition of acylase is that this definition more accurately describes reactions previously described as acylase-catalyzed. In addition, the definition proposed does not exclude from discussion under this single term of such similar enzyme substrates as the chloroacetylamino acids and the acylglycylamino acids, whereas, the older definition would do so. A new term might be coined for the phenomenon under consideration, but such a term might add to complications and confusion as compared to the slight redefining of a term already in use.

2. Histozyme

This term was first used by Schmiedeberg to describe the enzyme from hog and dog organs which hydrolyzed benzoylglycine (or hippuric acid)[1]. Schmiedeberg believed, without satisfactory experimental proof, that this enzyme played a major role in the metabolic processes of tissue, and he, therefore, named it histozyme, i.e., tissue-enzyme. Since later work demonstrated that a variety of acylamino acids were hydrolyzed by preparations which attacked hippuric acid,

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[1] O. Schmiedeberg, Naunyn-Schmiedeberg's Arch. Exp. Pathol. u Pharmakol., 14, 379-392 (1881).

the term was extended in practice to enzymes acting on acylamino acids in general[1,2].

3. Hippuricase

This term was proposed by Clementi (1923) and has the same meaning as histozyme[3].

4. Carboxypeptidase

A carboxypeptidase is an enzyme which hydrolyzes acyldipeptides with the freeing of the terminal amino acid; the presence of a free terminal carboxyl group is required in substrates susceptible to a carboxypeptidase. Typical substrates include carbobenzoxy- or benzoyldipeptides and tri- and higher-peptides. In addition to acyldipeptides, a carboxypeptidase may hydrolyze simple acylamino acids such as the acetyl- or chloroacetylamino acids. A carboxypeptidase

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[1]T. So, J. Biochemistry (Japan), 12, 107-131 (1930).

[2]F. Leuthardt. Hippuricase (Histozyme). In J. B. Sumner and K. Myrbäck (Editors). The enzymes. Vol. I, part 2. p. 951-955. New York, New York, Academic Press Inc., Publishers. 1951.

[3]A. Clementi, Atti accad. naz. Lincei (5) 32 II, 172 (1923).

is a particular type of acylase. The definition presented is similar to that proposed by Neurath and Schwert[1].

5. Aminoacylase

Smorodinzew introduced the term aminoacylase to describe an enzyme hydrolyzing acylamino acids[2].

B. Limitations of Enzyme Studies

A few comments will be made on the limitations of many acylase studies. The reader may wish to keep these in mind as he reads the next section on the acylases of various species. Many of these studies involved solvent drying of cellular material or extraction with organic solvents at room temperature. Since such conditions often are deleterious to protein[3], it is possible that many of the experiments reported, are based on preparations possessing only a part

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[1] H. Neurath and G. W. Schwert, Chem. Reviews, 46, 126-128 (1950).

[2] I. A. Smorodinzew, Z. Physiol. Chem., 124, 123-139 (1923).

[3] H. L. Fevold. Classification, purification and isolation of proteins. In D. W. Greenberg. Amino acids and proteins. p. 268. Springfield, Illinois, Charles C. Thomas. 1951.

or none of the enzyme activity present in the intact tissue. In addition, procedures for the extraction of cellular material may fail to extract acylases present. If a variety of conditions with regard to pH, reducing agents, and metal ions, are not tried, enzyme activity may be missed. Many of the studies involved the use of crude extracts and only one or a few enzyme concentrations. Limiting the enzyme concentrations used, places a quantitative limitation on the conclusions one can draw as regards absolute activity and relative rates of acylase activity.

Purified enzyme preparations often proved to be quite impure, especially in the earlier work. Commercial enzyme preparations were often used with no mention of the method of preparation or exact source. Where only one or a few substrates were used with enzymes from different sources, it is usually not feasible to compare the specificities of the enzymes, although such comparisons have been attempted. Each enzyme should be tested with a series of substrates in order to gain knowledge of its relative substrate preferences. Work carried out by a single investigator, rather than several, is a more dependable basis for the calculation of relative rates.

In the following review of acylases, discussion is largely limited to the enzymatic hydrolysis of acylamino

acids containing one or two peptide bonds. The enzymatic hydrolysis of acyldipeptides may not always be due to acylase action, unless it has been shown that the split occurred only at the amino group of the terminal amino acid.

C. Acylases Found in Animals and Plants

In the following pages a summary will be given of the acylases found in various plants and animal species. An attempt has been made to review all of the literature that could be found, except that rather brief mention will be made of certain acylases which are well described in review articles.

Mention will be made of experiments dealing with intact organisms which may possibly be indicative of the presence of acylases. No attempt has been made to cover all the literature dealing with experiments of this type. It should be emphasized that one cannot conclude, unambiguously, from the behaviour of the living cell (for instance, as in the nutritional utilization of acyl amino acids by bacteria) that acylases are or are not present. One of the purposes of the experiments described in this thesis has been to see if there is a parallel between the behaviour of viable Lactobacillus arabinosus cells and the acylases derived from these cells.

In the following pages, each of the main headings will embrace the acylases of one or a number of phyla. Under each of these main headings, such as "Invertebrates," the description of the acylases of a particular species will be headed by either the scientific or common name of the species, if available[1].

1. Acylases of invertebrates

a. Cribrina artemisia. An 87% glycerol extract of the mesenteric filament of this sea anemone hydrolyzed chloroacetyl-L-leucine most rapidly at pH 8.15[2].

b. A carniverous species of Platyhelminthes (flattened worms). A glycerin extract of an unstated species of the class, turbellaria, attacked benzoyldiglycine[3].

c. A blood-consuming Platyhelminthes. A glycerin extract of a species of the class, Trematoda (flukes), had no appreciable activity on a solution of benzoyldiglycine[3].

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[1]Use was made of the following book for biological classification: P. D. Strausbaugh and B. R. Weimer, General biology. 3rd ed. New York, John Wiley & Sons, Inc. 1952.

[2]S. Takemura, Science Reports Tohoku Imp. Univ., 4th ser. 12, 531-49 (1938). Chem. Abst., 32, 6273 (1938).

[3]Summary on basis of - E. Pennoit-De Cooman and G. van Grambergen, Verhandl. Kon. Vlaamsche Acad. Wetensch., Letteren, schoone. Kunsten Belgie, Klasse Wetensch, 4, 6, 7-77 (1942); and also - Chem. Abst., 38, 5231 (1944).

d. Moniezia benedeni. No activity on hippurylglycine was noted with a glycerin extract of this parasitic Platyhelminthes[1].

e. Maja squinado. The intestinal juice of this crustacean hydrolyzed chloroacetyltyrosine[2].

f. The fresh-water flea (Daphnia). This plankton crustacean was dried with organic solvents, ground, and extracted with glycerol. At pH 7.2 the glycerol extract attacked chloroacetyl-L-tyrosine[3].

g. Tridacna elongata. Chloroacetyltyrosine is hydrolyzed at pH 6.4 by the intestinal juice from this giant clam which is very common on the coral reefs at the Red Sea town, Chardaque[4].

h. Helix pomatia. An acylase of the gastropod, Helix pomatia, which hydrolyze chloroacetyl-L-tyrosine was obtained by glycerol extraction of the intestinal gland[5].

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[1]Summary on basis of - E. Pennoit-De Cooman and G. van Grambergen, Verhandl. Kon. Vlaamsche Acad. Wetensch., Letteren, schoone. Kunsten Belgie, Klasse Wetensch, 4, 6, 7-77 (1942); and also - Chem. Abst., 38, 5231 (1944).

[2]J. J. Mansour-Bek, Proc. Acad. Sci. Amsterdam 33, 858-70 (1930).

[3]A. D. Hasler, Biol. Bull., 72, 290-298 (1937).

[4]J. J. Mansour-Bek, Fermentforschung, 12, 221-231 (1948).

[5]B. Rosen, Z. Vergleich Physiol., 24, 606 (1937).

2. Acylases of vertebrates

a. Pacific Coast King Salmon. Fruton and Bergmann found no enzyme activity against carbobenzoxy-L-glutamyl-L-tyrosine due to a crystalline, protein-hydrolyzing enzyme from the stomach of this salmon[1]. This finding is in contrast to the observation that carbobenzoxy-L-glutamyl-L-tyrosine is hydrolyzed by enzymes from the stomachs of several species of mammals[2].

b. Ophiocephalus tadiana. The ability of a glycerol extract from the liver of this fresh water fish to hydrolyze benzoylglycine and acetylglycine was investigated by Utjino and Nishiwaki[3]. The experiments were carried out at pH intervals of 1.0 from pH 4 to pH 10. Slight hydrolysis of both substrates with the maximum at about pH 9 was observed. On the basis of the titration figures given, it is doubtful that extracts of the pancreas and intestinal mucosa of this fish hydrolyze benzoylglycine.

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[1] J. S. Fruton and M. Bergmann, J. Biol. Chem., 136, 559-560 (1940).

[2] E. L. Smith. Proteolytic enzymes. In J. B. Sumner and K. Myrback. The enzymes. Vol. I, Part 2. p. 844. New York, New York, Academic Press Inc. 1951.

[3] L. Utjino and I. Nishiwaki, Acta Schol. Med. Univ. Kioto, 29, 1-8 (1951).

c. Naja naja (cobra), Vipera russellii, and B. fasciatus. Venom from each of these snakes has weak acylase activity on chloroacetyl-L-leucine[1].

d. Echis carinata. The acylase activity on chloroacetyl-L-leucine of the venom from this snake is about four times as rapid as the acylase activity of the three preceding species[1].

e. The chicken. Chicken-liver glycerol extract hydrolyzed phenylacetylglycine (9%), phenylpropionylglycine (6%), and possibly benzoylglycine (3%)[2]; no hydrolysis of cinnamoylglycine, furoylglycine, furfuracrylglycine[3], or dibenzoylornithine[4] was detected. Extracts of chicken kidney failed to hydrolyze dibenzoylornithine[4] or furoylglycine[3] and hydrolysis of furfuracrylglycine was questionable[3]. Dibenzoylornithine was not hydrolyzed by glycerol extracts of chicken pancreas, intestinal mucosa, and spleen.

f. The pigeon. According to the Terminology of Krebs, Sykes, and Bartley, the enzyme hydrolyzing acetyl-

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[1]B. N. Ghosly, P. K. Dutt, and D. K. Chowdhury, J. Ind. Chem. Soc., 16, 75-80 (1939).

[2]T. Mori, J. Biochem. (Japan), 29, 225-240 (1939).

[3]S. Utzino, S. Tsunoo, and T. Mori, Ibid., 26, 449-53 (1937).

[4]M. Kaizyu, Tohoku J. Exptl. Med. 36, 255-57 (1939).

sulphamezathine is an acylase[1]. The definition presented on page 5 is not in accord with this. It is not known whether or not the pigeon-liver enzyme which hydrolyzes acetylsulphamezathine will also hydrolyze acylamino acids.

g. The dog. A variety of organs and secretions of this mammal yield acylase preparations splitting acyl derivatives of glycine, α -aminobutyric acid, leucine, tyrosine, and phenylalanine. Preparations from the kidney are usually most active. A qualitative summary of acylase activity found in various hog organs and secretions is presented in Tables 1-4. Most of these experiments were carried out using unconcentrated glycerol or aqueous extracts and, therefore, the conditions probably usually permitted the detection of a limited range of activity.

The data of Table 2 indicate that only one of the two optical forms of benzoyl- -aminobutyric acid is broken down by dog acylase preparations. This is in harmony with the behaviour of mammalian proteases which usually break down only substrates containing amino acid residues of the L configuration. It is of interest to note that the two β -amino compounds tested, DL-benzoyl-β-benzoylaminobutyric acid, and benzoyl-β-alanine were not hydrolyzed.

---

[1]H. A. Krebs, W. O. Sykes, and W. C. Bartley, Biochem. J. 41, 622-30 (1947).

Table 1

Hydrolysis of Glycine Derivatives by Acylase(s) from Dog Organs and Secretions*

Source of enzyme preparation	Substrates			
	Acetyl-glycine	Formyl-glycine	Benzoyl-glycine	α-Bromoiso-capronyl-glycine
Kidney	+[2]	+[2]	2,6,11,14, +17,18,19	
Liver	+[2]	+[2]	2,9,11, +14,18,19	
Pancreatic juice				
Pancreas	+[2]	+[2]	0[2,17], ?[19]	
Intestinal mucosa	+[2]	+[2]	0[13]	+[12]
"Erepsin" from intestinal mucosa			0[15]	
Intestinal juice	+[2]		0[13], ?[9]	0[10]
Skeletal muscle	+[2]	+[2]	+2,11,19,20	
Bone				
Heart	?[2]	0[2]	?[2,19]	
Lung	?[2,16]	?[2], +[14]	?[2], +[14], 0[19]	
Spleen	?[2]		0[2], ?[19]	
Testicle	+[2]	+[2]	?[2,19]	
Blood			0[18]	

*The symbols used and their meaning are as follows: +, hydrolysis observed; 0, no hydrolysis observed; ?, hydrolysis so slight as to be questionable. Superscripts refer to references which are found on page 21.

Substrates								
Bromoiso-capronyl-glycine	Bromoiso-capronyl-diglycine	Benzoyl-diglycine	Benzoyl-DL-leucyl glycine	Glyco-cholic acid	Tauro-cholic acid	Fur-furoyl-glycine	Furyl-propionyl-glycine	Furyla glyc
		?19		O14	+14	O17	O17	?1
		?19		O14, ?11	+11	O17	O17	O1
		+4	+5					
		+19				O11	O17	
+12		O13						
O10	O10	O10						
		O19		+11				
				O14				
		O19						
		O19						
		?19				O17		
		?19						

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Substrates							
Bromoiso-capronyl-diglycine	Benzoyl-diglycine	Benzoyl-DL-leucyl glycine	Glyco-cholic acid	Tauro-cholic acid	Fur-furoyl-glycine	Furyl-propionyl-glycine	Furylacryl-glycine
	?[19]		O[14]	+[14]	O[17]	O[17]	?[17]
	?[19]		O[14], ?[11]	+[11]	O[17]	O[17]	O[17]
	+4	+5					
	+[19]				O[11]	O[17]	
	O[13]						
O[10]	O[10]						
	O[19]		+[11]				
			O[14]				
	O[19]						
	O[19]						
	?[19]				O[17]		
	?[19]						

Table 2

Hydrolysis of Aminobutyric Acid and Alanine Derivatives by Acylase(s) from Dog Organs and Secretions*

Source of Enzyme preparation	Substrate				
	(+) Benzoyl-α-amino-butyric acid	(-) Benzoyl-α-amino-butyric acid	Bz-aminoiso-butyric acid	Bz-β alanine	DL-Benzoyl-β-amino-butyric acid
Kidney	+[11,14]	O[11,14]	O[11,14]	O[11,14]	O[11]
Liver	+[11,14]	O[11,14]	O[11,14]	O[11,14]	O[11]
Skeletal muscle	+[11]	O[11]	O[11]	O[11]	O[11]
Bone	+[14]	O[14]	O[14]	O[14]	

*The symbols used and their meaning are as follows: + , hydrolysis observed; O, no hydrolysis observed; ?, hydrolysis so slight as to be questionable. Superscripts refer to references which are found on page 21.

Table 3

Hydrolysis of Leucine Derivatives by Acylase(s) from Dog Organs and Secretions*

Source of enzyme preparation	Substrate			
	Chloroacetyl-DL-leucine	Acetyl-DL-leucine	Formyl-DL-leucine	Benzoyl-L-leucine
Kidney		+[2]	+[2]	+[11]
Liver		+[2]	+[2]	O[11]
Pancreas		+[2]	+[2]	
Intestinal juice	+[8,10,13]	O[10]		
Muscle		+[2]	+[2]	+[11]
Heart		+[2]	+[2]	
Lung		+[2]	+[2]	
Spleen		+[2]	+[2]	
Testicle		+[2]	+[2]	
Intestinal mucosa		+[2]	+[2]	

*The symbols used and their meaning are as follows: + , hydrolysis observed; O, no hydrolysis observed; ?, hydrolysis so slight as to be questionable. Superscripts refer to references which are found on page 21.

Table 4

Hydrolysis of Phenylalanine and Tyrosine Derivatives by Acylase(s) from Dog Organs and Secretions*

Source of enzyme preparation	Substrate		
	Chloroacetyl-L-tyrosine	Chloroacetyl-DL-phenylalanine	Acetyl-DL-phenylalanine
Kidney			+[2]
Liver			0[2]
Pancreas	+[1]		+[2]
Pancreatic juice			
Intestinal mucosa		+[13]	+[2]
Intestinal juice		0[10,13]	
Muscle			?[2]
Heart			0[2]
Lung			0[2]
Spleen			0[2]
Testicle			0[2]
Leucocytes	?[3]		

*The symbols used and their meaning are as follows: +, hydrolysis observed; 0, no hydrolysis observed; ?, hydrolysis so slight as to be questionable. Superscripts refer to references which are found on page 21.

Substrate				
Acetyl- -phenyl- alanine	Formyl- DL-phenyl- alanine	Benzoyl- DL-phenyl- alanine	Benzoyl- glycyl- DL-phenyl- alanine	Dibenzoyl Tyrosine
+[2]	+[2]	+[2]		
0[2]	?[2]	0[2]		
+[2]	+[2]	+[2]		
			+[2]	
+[2]	+[2]	+[2]		
?[2]	0[2]	+[2]		+[20]
0[2]	0[2]	+[2]		
0[2]	0[2]	+[2]		
0[2]	0[2]	0[2]		
0[2]	?[2]	+[2]		

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lysis so
erences

References for Tables 1-4

1. A. Vescio, Boll. Soc. Ital. Biol. Sper., 18, 287-8 (1943).
2. H. Kimura, J. Biochem. (Japan), 10, 207-223 (1929).
3. R. Willstatter, E. Bamann, and M. Rohdewald, Z. Physiol. Chem. 153, 267-280 (1929).
4. S. Utzino, J. Biochem. (Japan), 9, 465-481 (1928).
5. T. Kawa, J. Biochem. (Japan), 16, 277-297 (1929).
6. V. Baccari, and M. Pontecorvo, Boll. Soc. Ital. Biol. Sper., 16, 329-331 (1941).
7. M. Kawaharada, Tohoku J. Exptl. Med., 47, 92-96 (1944). Abstracted from Chem. Abst. 43, 9089 (1949).
8. M. Matsuo, J. Biochem. (Japan), 33, 147-154 (1941).
9. A. Clementi, Atti Della Accademia Nazionale dei Lincei, (V) 32, ii, 172-174 (1923).
10. M. Matsuo, J. Biochem. (Japan), 33, 59-72 (1941).
11. I. A. Smorodinzew, Z. Physiol. Chem. 124, 123-139 (1923).
12. A. Clementi, Atti Della R. Accademia dei Lincei, Roma (5) 29, ii, 327 (1920).
13. M. Matsuo, J. Biochem. (Japan), 33, 97-109 (1941).
14. I. A. Smorodinzew, J. Russ. Phys. Chem. Soc., 51, 156-82 (1919). Thanks are due to Mr. Sol Shulman who kindly furnished an English summary of this Russian article.
15. O. Cohnheim, Z. Physiol. Chem., 52, 526 (1907).
16. S. Utzino, S. Tsunoo, and T. Mori, J. Biochem. (Japan) 26, 449-453 (1937).
17. Ibid., 439-47.

18. O. Schmiedeberg, Arch. Exp. Path. Pharm., 14, 379-392 (1881).

19. M. Yosioka, Enzymologia, 10, 154-160 (1941).

20. S. Tamura, Acta Schol. Med. Imp. Kioto, 6, 467-470 (1924).

Some evidence concerning the effect of substrate structure on rate of hydrolysis by acylase preparations from dog kidney is given in Table 5.

Table 5

% Hydrolysis[1] of Acylamino Acids by Dog Kidney Acylase Preparation in 20 Hours at pH 7.2

Substrate	% Hydrolysis of L form
Acetylglycine	106
Acetyl-DL-leucine	88
Acetyl-DL-phenylalanine	84
Formylglycine	90
Formyl-DL-leucine	104
Formyl-DL-phenylalanine	56 in 192 hours
Benzoylglycine	23
Benzoyl-DL-phenylalanine	44

[1] H. Kimura, J. Biochem. (Japan), 10, 207-223 (1929).

Smorodinzew[1] found that benzoylglycine was hydrolyzed 23%, while in similar experiments, (+) benzoyl-α-aminobutyric acid was hydrolyzed 13.5% and benzoyl-L-leucine, 17%.

On the basis of Table 5 and the observations of Smorodinzew, the order of decreasing susceptibility to dog kidney acylase is - benzoyl-DL-phenylalanine > benzoylglycine > benzoyl-L-leucine = (+)-benzoyl-α-aminobutyric acid > formyl-DL-phenylalanine; the other substrates of Table 5 are hydrolyzed more rapidly.

It is seen that the formyl derivative of phenylalanine is less susceptible to acylase action than the benzoyl derivative, and the benzoyl derivative less susceptible than the acetyl derivative of phenylalanine. However, the benzoyl derivative of glycine is less susceptible to dog kidney acylase action than the formyl or acetyl derivatives. Hence, for the acyl derivatives of glycine and phenylalanine, one does not observe parallel effects on rate as the nature of the acyl group is varied, at least for the cases cited.

Of the three formyl derivatives, the derivative of phenylalanine is acted upon less rapidly than are the leucine and glycine derivatives. However, the rates of dog kidney

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[1] I. A. Smorodinzew, Z. Physiol. Chem. 124, 123-139 (1923).

acylase action are more nearly equal for the benzoyl derivatives of glycine and phenylalanine than for their formyl derivatives. The data of Table 5 do not point to the approximate independence of the acyl group and the amino acid residue of acylamino acids in influencing substrate susceptibility to the action of dog kidney acylase complex; such an independence has been suggested to hold for bovine pancreatic carboxypeptidase[1].

Table 6 presents data from a paper by Kimura[2] on the acylase activity of preparations from organs of the dog. None of the organs listed is known to excrete proteases into the gastro-intestinal tract. The relative rates of hydrolysis of the substrates listed in Table 6 by preparations from all the tissues listed, except kidney, conform at least in an approximate manner to the following order of decreasing susceptibility to dog acylase complex:- acetyl-DL-leucine > formyl-DL-leucine > acetylglycine. The substrates acetyl-DL-phenylalanine, formylglycine, formyl-DL-phenylalanine, benzoyl-L-leucine, and benzoyl-DL-phenylalanine are all generally less susceptible to acylase action than the three substrates just named; but the extent of hydrolysis is too

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[1]E. L. Smith. Proteolytic enzymes. In J. B. Sumner and K. Myrbäck. The enzymes. Vol. I, part 2. p. 806. New York, N. Y., Academic Press Inc. 1951.

[2]H. Kimura, J. Biochem. (Japan), 10, 207-223 (1929).

Table 6

% Hydrolysis[1] of Acyl Amino Acids by Preparations from Non-Protease-Secreting Dog Organs*

Source of enzyme preparation	Hours incubation	Substrate							
		Acetyl-glycine	Acetyl-leucine	Acetyl-phenyla.	Formyl-glycine	Formyl-leucine	Formyl-phenyla.	Benzoyl-glycine	Benzoyl-phenyla.
Kidney	20	106	44	42	90	52		23	22
	192						28		
Liver	264	12			8			12	0
	288		40	0		12	4		
Skeletal muscle	68		28						
	288	16	48	4	16	28	0	8	8
Heart	288	4	24	0	0	8	0	4	4
Lung	68		12						
	288	12	44	0	4	20	0	4	4
Spleen	68		28						
	288	12							
Testicle	288	12	32	0	8	8	4	4	8

[1] H. Kimura, J. Biochem. (Japan), 10, 207-223 (1929).

* pH of buffer 7.2. Incubation at 37°.

small to establish relative order of breakdown. The possible contradiction to the generalization that the acylase complexes of liver, skeletal muscle, heart, lung, spleen, and the testicle possess similar preferences for the substrates studied is the observation that both acetylglycine and formylglycine are hydrolyzed 16% in 288 hours by skeletal muscle acylase, whereas, the %'s observed are 12 and 4%, respectively, for a preparation from spleen. However, this deviation is not very strong evidence against the generalization stated in view of the possible inaccuracy of the formol titration used and the possibility of microbial contamination during the 288 hour incubation. Perhaps, the strongest argument against the generalization stated is that relative rates on only a few substrates were obtained since the enzyme concentration was not varied so as to obtain appreciable hydrolysis of the less susceptible substrates.

Smorodinzew[1] presents evidence (Table 7), that the relative order of breakdown of three benzoylamino acids by kidney, liver, and skeletal muscle is -benzoylglycine > (+)-benzoyl-α-aminobutyric acid = benzoyl-L-leucine. In addition, the relative rates of both kidney and skeletal muscle acylase complexes are almost the same for these

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[1] I. A. Smorodinzew, Z. Physiol. Chem., 124, 123-139 (1923).

Table 7

Acylase Activity of Dog Tissues As Determined by Smorodinzew[1]

Source of enzyme preparation	% Hydrolysis of		
	Benzoyl-glycine	(+)-Benzoyl-α-amino-butyric acid	Benzoyl-L-leucine
Kidney	23	13.5	17
Liver	2.2	1.3	0
Skeletal muscle	43	17.3	15

[1] I. A. Smorodinzew, Z. Physiol. Chem., 124, 123-139 (1923)

three substrates named. This is limited evidence that the specificities of both tissue-acylases are similar and, furthermore, since the specificity of the skeletal muscle acylase com lex may be similar to the acylases of the 5 other tissues of Table 6, that the acylase complexes of dog kidney, liver, skeletal muscle, heart, lung, spleen, and testicle have in common similar relative rates of substrate hydrolysis. The proof of such a generalization, of course, could be complete only when the acylase complex of each of these types of tissue has been tested on all possible substrates and relative rates obtained. Since this requirement has been fulfilled to a moderate extent only, limited

evidence is on hand to support the generalization stated. However, if one accepts such a generalization, then one can formulate the order of decreasing susceptibility to hydrolysis by dog-tissue acylase complex of ten compounds; this can be done by combination of the conclusions reached in the preceding discussion on the basis of the information of Tables 5, 6, and 7. On this basis the relative order of susceptibility to dog tissue acylase complex is - acetyl-DL-leucine > formyl-DL-leucine > acetylglycine > formylglycine (?) > acetyl-DL-phenylalanine > benzoyl-DL-phenylalanine > benzoylglycine > (+)-benzoyl-α-aminobutyric acid ± benzoyl-L-leucine > formyl-DL-phenylalanine. There is just a moderate amount of evidence for the generalization just stated.

Table 8 presents data from work by Kimura on the acylases of dog intestinal mucosa and pancreas.

According to Kawa, in the presence of calcium ion, the pancreatic juice of the dog hydrolyzes benzoylglycyl-DL-phenylalanine and benzoyl-DL-leucylglycine each at about the same rate[1].

Table 9 presents the data of Matsuo[2] on the acylase activity of dog intestinal juice and dog intestinal mucosa brei.

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[1]T. Kawa, J. Biochem. (Japan), 16, 277-297 (1929).

[2]M. Matsuo, J. Biochem. (Japan), 33, 59-72 (1941).

Table 8

Hydrolysis of Substrates[1] by Acylase Preparations from Dog Pancreas and Dog Intestinal Mucosa*

Substrate	Hours incubation	% Hydrolysis by Preparation of pancreas	% Hydrolysis by Preparation of intestinal mucosa
Acetylglycine	20	12	
	288		44
Acetyl-DL-leucine	20	44	14
	288		44
Acetyl-DL-phenylalanine	20	12	
	288		12
Formylglycine	20	4	
	288	20	12
Formyl-DL-leucine	20	27	6
	288		36
Formyl-DL-phenylalanine	20	35	0
	288		
Benzoyl-glycine	288	8	8
Benzoyl-DL-phenylalanine	288	8	8

* pH of buffer 7.2. Incubation at 37°.

[1] H. Kimura, J. Biochem. (Japan), 10, 207-223 (1929).

Table 9

Acylase Activity[1] of Dog Intestinal Juice and Dog Intestinal Mucosa*

Substrate	% Hydrolysis	
	Intestinal juice	Intestinal mucosa brei
Chloroacetyl-L-phenylalanine	4.4	17.5
Chloroacetyl-L-leucine	19.7	
Benzoyldiglycine	4.4	0
Phthalyldiglycine	1.9	
DL-bromoisocapronyl-diglycine	5.4	
DL-bromoisocapronyl-glycine	4.8	
Benzoylglycine	4.4	

[1] M. Matsuo, J. Biochem. (Japan), 33, 59-72 and 97-109 (1941).

*Added enzyme preparation to pH 7.8 buffer. Seventy-two hour incubation at 37°. % hydrolysis probably must be over 7% to be significant hydrolysis.

Relative rates of substrate hydrolysis by the intestinal mucosa acylase system are somewhat different from those of the tissues listed in Table 6. The most striking difference is the relatively rapid hydrolysis of formyl-DL-phenylalanine as compared to the relative rate with acylases of the seven tissues listed in Table 6. The data on acylase activity of intestinal juice (Table 9) is of limited value because most of the substrates were not significantly hydrolyzed at the one enzyme concentration used. Chloroacetyl-L-leucine was hydrolyzed, but no significant hydrolysis of chloroacetyl-L-phenylalanine or the glycine derivatives was noted. The pH optimum of chloroacetyl-L-leucine hydrolysis is 7.0-8.0[1].

The decreasing order of substrate susceptibility to dog pancreatic acylase complex is acetyl-DL-leucine > formyl-DL-phenylalanine > formyl-DL-leucine > acetyl-DL-phenylalanine > acetylglycine > formylglycine > benzoylglycine ≐ benzoyl-DL-phenylalanine (see Table 8). This relative order of susceptibility is not, however, in general agreement with that for the acylases of the non protease secreting tissues. The ready hydrolyzability of acetyl-DL-leucine as compared to acetyl-DL-phenylalanine by dog pancreas acylase complex is in contrast to what would be expected of bovine

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[1]M. Matsuo, J. Biochem. (Japan), 33, 147-154 (1941).

pancreatic carboxypeptidase. The latter enzyme attacks most rapidly substrates possessing a terminal phenylalanine or tyrosine[1]. However, acetyl-DL-leucine has not been tested with enzyme preparations from bovine pancreas. The observation by Kawa[2] that the rates of hydrolysis of benzoylglycyl-DL-phenylalanine and benzoyl-DL-leucylglycine are about equal is possibly additional evidence for a difference in specificity of bovine and dog pancreatic acylases. The point of attack on the acyldipeptides would have to be established, however, to be sure both activities are of the acylase type.

It seems appropriate to mention that the work reviewed here on dog acylases has a number of defects. For several reasons it is difficult to obtain relative rates of hydrolysis from the data recorded. One reason is the failure to vary enzyme concentrations so as always to obtain significantly positive values of % hydrolysis which are significantly less than complete hydrolysis. Another defect, related to the non-varying enzyme concentration, is the prolonging of enzyme-substrate incubations for long periods, thus increasing the possibility of enzyme inactivation and microbial growth.

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[1]E. L. Smith. Proteolytic enzymes. In J. B. Sumner and K. Myrbäck. The enzymes. Vol. I. Part 2, p. 806. New York, New York, Academic Press Inc. 1951.

[2]T. Kawa, J. Biochem. (Japan), 16, 277-297 (1929).

In spite of the defects cited, an attempt has been made to arrive at some tentative conclusions concerning the effect of structure on relative rates. While such conclusions are not definitely established, they will serve to suggest generalizations which serve as the basis for further investigation. One such generalization reached here concerning dog acylases, is that the acylases from seven non protease secreting tissues (Table 6) have similar relative rates on the leucine, glycine, phenylalanine, and α-aminobutyric acid derivatives studied. Also, the possibility was raised that dog pancreas acylases posses a specificity differing from that of bovine pancreatic acylase(s).

h. The cat. No acylase activity toward furylacrylglycine was detected in kidney, liver, spleen, pancreas, or intestine. Furylpropionylglycine and furfuroylglycine were not split by acylase preparations of the kidney and liver[1].

Yosioka observed hydrolysis of benzoylglycine and benzoyldiglycine by macerations of cat kidney at pH 7.3[2]. He observed slight hydrolysis of benzoylglycine but none of benzoyldiglycine by testicle macerations. Macerations of cat liver, pancreas, muscle, heart, lung, and spleen failed to hydrolyze benzoylglycine or benzoyldiglycine.

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[1]S. Utzino, S. Tsunoo, and T. Mori, J. Biochem. (Japan), 26, 449-53 (1937).

[2]M. Yosioka, Enzymologia, 10, 154-60 (1941).

i. The guinea pig. Clementi reported hydrolysis of benzoylglycine by guinea pig kidney and guinea pig liver acylase preparations[1]. Glycerol extracts of fresh guinea pig liver failed to hydrolyze carbobenzoxyglycyl-D-leucine[2]. Yosioka presents the data (Table 10) on the hydrolysis of benzoylglycine and benzoyldiglycine by guinea pig macerations[3].

Table 10

Hydrolysis[1] of Benzoylglycine and Benzoyldiglycine by Guinea Pig Macerates*

Organ	ML .1 normal NaOH	
	Bz.gly.	Bz.gly.gly
Liver	.12	.10
Pancreas	.09	.09
Muscle	.02	.02
Heart	0	0
Lung	.05	.05
Spleen	0	0
Testicle	.17	0
Kidney	.93	1.90

[1] M. Yosioka, Enzymologia, 10, 154-60 (1941).

*At pH 7.3. Substrate-enzyme incubation 120 hours.

[1] A. Clementi, Atti della Accademia Nazionale dei Lincei, (V) 32, ii, 172-174 (1923).

[2] A. Schmitz and R. Merten, Z. Physiol. Chem., 278, 43-53 (1943).

[3] M. Yosioka, Enzymologia, 10, 154-160 (1941).

It is seen (Table 10) that benzoylglycine and benzoyldiglycine are both markedly attacked by guinea pig kidney enzymes. Ravin and Seligman follow the hydrolysis of carbonaphthoxyphenylalanine using a colorimetric method[1] in order to detect carboxypeptidase activity. They found carboxypeptidase activity in guinea pig duodenal fluid and they found procarboxypeptidase activity in the pancreas. No carboxypeptidase or procarboxypeptidase activity was found in the homogenates of guinea pig liver, kidney, spleen, duodenum, jejunum, stomach, colon, brain, heart, lung, adrenal, thyroid, ovary, testes, and epididymis.

j. The mouse. Yosioka incubated ten glycine and phenylalanine compounds with mouse liver macerations, and determined extents of hydrolysis[2]. All compounds except for chloroacetyl-L-β-phenylalanine and bromoisocapronyldiglycine were used from pH 5 or 6 to pH 9.0. The analytical values for the enzyme-substrate incubations at pH 7.5 are given in Table 11. Of the above compounds, significant hydrolysis of benzoylglycine, benzoyldiglycine, and chloroacetyl-L-β-phenylalanine was observed; also, hydrolysis of α-bromoisocapronyl-DL--phenylalanine was observed after prolonged hydrolysis.

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[1] H. A. Ravin, and A. M. Seligman, J. Biol. Chem., 190, 391-402 (1951).

[2] M. Yosioka, Enzymologia, 10, 154-60 (1941).

Table 11

Acylase Activity[1] of Mouse Liver Macerations at pH 7.5*

Substrate	Hydrolysis as	
	ML .1 N NaOH	mg amino-N
Benzoylglycine	1.40	
Benzoyl-DL-phenylalanine	0	
Benzoyldiglycine	1.45	
Phthalyldiglycine	.01	
Benzoyl-DL-leucylglycine	-.01	
Chloroacetyl-L-α-phenylalanine		.645
Chloroacetyl-DL-β-phenylalanine		.017
α-Bromoisocapronylglycine		.039
α-Bromoisocapronyl-DL-β-phenylalanine		0 (.156 in 120 hours)
α-Bromoisocapronyldiglycine		.465

[1]M. Yosioka, Enzymologia, 10, 154-60 (1941).

*Substrate-enzyme incubations for 24 hours unless otherwise stated.

Yosioka found that the above mentioned hydrolysis of benzoylglycine and benzoyldiglycine was at a pH optimum of about 7.0. He was able to separate benzoylglycine activity from benzoyldiglycine activity by charcoal adsorption at pH 5.8[1]. Yosioka also found that a series of arsenic compounds possessed both activating and inhibitory properties on benzoylglycine hydrolysis by mouse kidney acylase; both in vitro and in vivo experiments were carried out[2].

Rudolph Abderhalden found that mouse ascites tumor cells rapidly hydrolyzed chloroacetyl-L-tyrosine and chloroacetyl-DL-leucine, while the tumor liquid attacked chloroacetyl-DL-leucine slightly but not chloroacetyl-L-tyrosine[3]. $MnSO_4$, $MgSO_4$, $CoCl_2$, and $ZnCl_2$ did not effect the rate of hydrolysis. Fruton, Irving, and Bergmann found that cysteine activates hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine by extracts of mouse carcinoma and sarcoma[4]. A similar activation of carbobenzoxy-D-glutamyl-L-tyrosine hydrolysis by carcinoma extract was observed. Experiments were carried out at pH 4.5-5.2.

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[1] M. Yosioka, Enzymologia, 10, 154-60 (1941).

[2] M. Yosioka, Folia Pharmacologica Japonica, 31, 289-305 (1941): abstracted from Chem. Abst. 35, 5919 (1941).

[3] R. Abderhalden, Fermentforschung, 17, 330-336 (1944).

[4] J. S. Fruton, G. W. Irving, M. Bergmann, J. Biol. Chem. 132, 465-6 (1940).

The ability to hydrolyze chloroacetyl-l-tyrosine by mouse pancreas follows the granule content of the pancreas, which is caused to vary by pilocarpine administration to fasting white mice[1].

k. The rat. Clementi detected acylase activity toward hippuric acid in the kidney of the white rat[2]. Yosioka found that macerations of liver and kidney hydrolyzed hippuric acid and hippurylglycine at pH 7.3, but that these substrates were not hydrolyzed by macerations of pancreas, muscle, heart, lung, spleen, and testicle[3]. Carbonapthoxyphenylalanine is hydrolyzed by rat duodenal fluid and a proenzyme for this substrate is found in rat pancreas tissue[4]. Neither the proenzyme nor enzyme for carbonapthoxyphenylalanine was found in homogenates of liver, kidney, pancreas, spleen, duodenum, jejunum, stomach, colon, brain, heart, lung, adrenal, thyroid, ovary, testes, or epididymis. Jen and Lewis found that dibenzoylcystine is not available for

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[1] P. B. v Weel and C. Engel, Z. Vergleich. Physiol. 26, 67-73 (1938).

[2] A. Clementi, Atti Accad. Naz. Lincei, Mem., Classe Sci. Fis. Mat. e Nat., (V) 32, ii, 172-4 (1923).

[3] M. Yosioka, Enzymologia, 10, 154-160 (1941).

[4] H. A. Ravin and A. M. Seligman, J. Biol. Chem., 190, 391-402 (1951).

the dietary cystine requirements of young white rats[1]; they believed this observation supports the contention that no hydrolysis of the compound occurs in the rat.

1. The rabbit. Jaarsveld and Stokvis reported in 1879 that when hippuric acid was given to rabbits by subcutaneous or venous injections, or by stomach administration, that benzoic acid appeared in the urine[2]. However, in 1883 van de Velde and Stokvis reported that when hippuric acid is administered to rabbit by the jugular vein or subcutaneously, then no benzoic acid is found in the urine provided that the urine is collected by a catheter into 10% HCl[3]. Van de Velde and Stokvis believed these experiments showed that hippuric acid can be rapidly split outside the organism in animal fluids and they concluded that there had not yet been a satisfactory demonstration of the ability of the living organism to split hippuric acid into glycine and benzoic acid. Later workers usually used an antiseptic, such as toluene, to prevent microbial action, which was probably involved here.

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[1] P. C. Jen and H. B. Lewis, Proc. Soc. Exp. Biol. and Med. 39, 301-304 (1938).

[2] G. J. Jaarsveld and B. J. Stokvis, Arch. Exp. Path. Pharm. 10, 288-296 (1879).

[3] V. van de Velde and B. J. Stokvis, Arch. Exp. Path. Pharm. 17, 189-217 (1883).

A qualitative summary of research on rabbit acylases from non protease secreting tissues and from plasma is given in Table 12.

As seen in Table 12, divergent results were obtained by different investigators with benzoyldiglycine and rabbit liver. Utzino failed to detect hydrolysis of benzoyldiglycine using a glycerol press juice of rabbit liver[1]. However, Sugawara finds that this compound is hydrolyzed by rabbit liver cathepsin[2]. That the divergence in results may lie in enzyme inactivation or in incomplete extraction is suggested by the work of Bondi and Frankl who studied the hydrolysis of sodium laurylglycinate[3]. These investigators found that sodium laurylglycinate is hydrolyzed by intact and grated rabbit liver; however, when an enzyme preparation was made by rubbing liver with water, glycerin, and sea sand, and using the supernatant, no hydrolysis of sodium laurylglycinate was noted. In addition, Sugawara found an apparent activation of benzoyldiglycine hydrolysis by cysteine when the enzyme preparation was a glycerin-water maceration of rabbit liver. This apparent activation may have been, in reality, an increase in enzyme stability,

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[1]S. Utzino, J. Biochem. (Japan), 9, 465-481 (1928).

[2]T. Sugawara, Tohoku J. Expt. Med. 48, 223-230 (1945).

[3]S. Bondi and T. Frankl, Biochem. Z. 17, 555-561 (1909).

Table 12

Acylase Activity of the Non-Protease-Secreting Tissues and Plasma of Rabbit*

Substrate	Source of acylase preparation									
	Kidney	Liver	Skeletal muscle	Carcinoma	Heart	Lung	Brain	Spleen	Testicle	Plasma	Blood
Benzoylglycine	$+^{17}$, 0^{15}; $?^{12}$	0^{17}, $?^{12}$, $+^{15}$	0^{17}, $+^{15}$		0^{17}, 0^{15}	$0^{15,17}$		0^{15}	$0^{15,17}$		
Benzoyldiglycine	$?^{15}$	$+^{2,11}$, 0^{15}	$0^{15,1}$		0^{15}	0^{15}		0^{15}	0^{15}		
Phthaloyldiglycine		0^{1}	0^{1}								
Chloroacetyl-L-alanine										$+^{3}$	
Laurylglycine	$+^{4}$	$+^{4}$, 0^{7}				0^{4}					0^{4}
Acetylglycine	$+^{17}$	$+^{17}$	$+^{17}$		$+^{17}$	$+^{17}$			$+^{17}$		
Acetyl-DL-leucine	$+^{17}$	$+^{17}$	$+^{17}$		$+^{17}$	$+^{17}$			$+^{17}$		

*The symbols used and their meaning are as follows: + , hydrolysis observed; 0, no hydrolysis observed; ?, hydrolysis so slight as to be questionable. Superscripts refer to references which are found on page 45.

Table 12 (Continued)

Substrate	Source of acylase preparation									
	Kidney	Liver	Skeletal muscle	Carci-noma	Heart	Lung	Brain	Spleen	Tes-ticle	Plasma Blood
Acetyl-DL-phenyl-alanine	+[17]	+[17]	o[17]		o[17]	o[17]			o[17]	
Formyl-glycine	+[17]	+[17]	+[17]		+[17]	+[17]			+[17]	
Formyl-DL-leucine	+[17]	+[17]	+[17]		+[17]	+[17]			+[17]	
Formyl-DL phenyl-alanine	+[17]	+[17]	o[17]		o[17]	o[17]			o[17]	
Lauryl-alaninate	+[4]		o[4]		o[4]	o[4]				
Furfuroyl-glycine	o[7]	o[7]								
Furyl-propionyl-glycine	+[7]	+[7]								

Table 12 (Continued)

Substrate	Source of acylase preparation									
	Kidney	Liver	Skeletal muscle	Carcinoma	Heart	Lung	Brain	Spleen	Testicle	Plasma Blood
Oxymethylfurfuroylglycine	0[7]	0[7]								
Phenylpropionylglycine	+[12]	+[12]								
Phenylfurfuroylglycine	0[13]	0[13]								
Phenylhippuric acid	?[13]	0[13]								
Carbobenzoxyglycyl-D-leucine		0[14]	0[14]			0[14]	0[14]			
Carbobenzoxy-L-glutamyl-L-tyrosine				+[16]						

Table 12 (Continued)

Substrate	Source of acylase preparation									
	Kidney	Liver	Skeletal muscle	Carcinoma	Heart	Lung	Brain	Spleen	Testicle	Plasma Blood
Carbobenzoxy-D-glutamyl-L-tyrosine				+[16]						
Phenyl-α,β-dibromopropionyl-glycine	0[12]	0[12]								
Cinnamoyl-glycine	0[12]	0[12]								
N-pyridin-2-carboxyl-glycine	+[12]	?[12]								
Phenyl-α-bromo-β-oxypropionyl-glycine	0[12]	0[12]								

References to Table 12

(Acylase Activity of Rabbit Tissues and Juices)

1. S. Utzino, J. Biochem. (Japan), 9, 465-481 (1948).
2. T. Sugawara, Tohoku J. Exptl. Med., 48, 171-6 (1944).
3. H. Hanson, Fermentforschung, 14, 189-201 (1934).
4. S. Bondi and T. Frankl, Biochem. Z., 17, 555-561 (1909).
5. F. Itzioka, J. Biochem. (Japan), 24, 139-151 (1936).
6. Meissner and Shepard, "Untersuchungen uber das Enstehen der Hippuric Acid in thierischen Organism." Hannover, 1866. Cited by H. Weiske, Z. fur Biologie, 12, 241-265 (1876).
7. S. Utzino, S. Taunoo, and T. Mori, J. Biochem. (Japan), 26, 449-53 (1937).
8. F. Itzioka, J. Biochem. (Japan), 24, 267-277 (1936).
9. Ibid., 139-151 (1936).
10. F. Itzioka, J. Biochem. (Japan), 26, 75-80 (1937).
11. T. Sugawara, Tohoku J. Exptl. Med., 48, 223-230 (1945).
12. S. Utzino, S. Tsunoo, and T. Mori, J. Biochem. (Japan), 26, 439-47 (1937).
13. Ibid., 477-486 (1937).
14. A. Schmitz and R. Merten, Z. Physiol. Chem., 278, 43-56 (1943).
15. M. Yosioka, Enzymologia, 10, 154-160 (1941).
16. J. S. Fruton, G. W. Irving, and M. Bergmann, J. Biol. Chem., 138, 465-466 (1940).

since the enzyme-substrate incubation was carried out for a long time. Sugawara made the additional interesting observation that injection of chloroform into rabbits decreased the ability of liver enzyme preparations to hydrolyze benzoyldiglycine but did not decrease the ability to hydrolyze gelatin.

The observation that carbobenzoxy-L-glutamyl-L-tyrosine is hydrolyzed by rabbit carcinoma (Table 12), is of comparative interest, since this type of activity is found in some mammalian tissues and digestive juices.

The data of Kimura[1], which are presented in Table 13, give some information on the relative rates of substrate hydrolysis by the acylases of non-protease-secreting tissues from the rabbit.

The data on kidney acylase preparation from rabbit (Table 13), do not yield much information on relative rates since most substrates are completely hydrolyzed; but it is seen that benzoylglycine is hydrolyzed at a slower rate than the other substrates. Of the tissue acylases where comparison is possible, acetyl-DL-leucine was the most rapidly hydrolyzed of the substrates listed (Table 13). Formyl-DL-leucine and formylglycine are the next most susceptible to acylase action,

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[1]H. Kimura, J. Biochem. (Japan), 10, 207-223 (1929).

Table 13

Hydrolysis of Acylamino Acids[1] by Preparations from Non-Protease-Secreting Tissues of Rabbit As % Hydrolysis*

Source of acylase preparation	Hours incubation	Substrate						
		Acetyl-glycine	Acetyl-DL-leucine	Acetyl-DL-phenylala.	Formyl-glycine	Formyl-DL-leucine	Formyl-DL-phenylala.	Benzoyl-glycine
Kidney	20	100	55	56	81	53	52	
	140							21
Liver	20	70	58	16	34	47	7	0
Skeletal muscle	140	7			10	12		
	212		38	0			0	0
Heart	140	16	43	0	13	38	0	0
Lung	140	34	48		19	25		
	212			0			0	0
Testicle	140	32	45	0	15	31	0	0

[1]H. Kimura, J. Biochem. (Japan), 10, 207-223 (1929).

*A buffer of pH 7.2 was used. Incubation at 37°.

but the relative rates vary with the tissue. Formylglycine is the fourth in rate of hydrolysis and acetyl-DL-phenylalanine, formyl-DL-phenylalanine, and benzoylglycine, which are not hydrolyzed except by kidney and liver preparations, seem to follow. Thus, there seem to be significant differences in the relative rates of substrate hydrolysis by the rabbit acylase complexes of the different non-protease-secreting tissues of Table 13. However, the differences are not very great. It may be that similar differences exist in the acylases of the dog, but that the data (Table 6) were not adequate to demonstrate this.

Some information on the hydrolysis of acylglycines is given by the data of Utzino, Taunoo, and Mori[1] (Table 14). Of interest is the observation that phenylpropionylglycine is more readily hydrolyzed (Table 14) by acylase preparations of kidney and liver than is benzoylglycine.

Table 15 indicates, qualitatively, the effect of acylase preparations from pancreas, pancreatic juice, and intestinal mucosa of rabbits on the substrates investigated to date.

Information from a paper by Kimura on hydrolysis of some glycine, leucine, and phenylalanine derivatives by rabbit pancreas and intestinal mucosa preparations is given in Table 16.

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[1]S. Utzino, S. Tsunoo, and T. Mori, J. Biochem. 26, 439-47 (1937).

Table 14

Hydrolysis of Glycine Compounds by Kidney and Liver Acylases According to Utzino, Tsunoo, and Mori[1]

Substrate	Hydrolysis as ml .10 N NaOH	
	Kidney	Liver
Benzoylglycine	.1	.1
Phenylpropionylglycine	.3	.3
Phenyl-α,β-dibromopropionyl-glycine	.364	.046
Cinnamoylglycine	0	.010
N-(Pyridincarboxyl)-glycine	.3	.1
Phenyl-α-bromo-β-oxypropionyl-glycine	.274	.003

[1]S. Utzino, S. Tsunoo, and T. Mori, J. Biochem., 26, 439-47 (1937).

Also, data of Itzioka are presented in Table 17 concerning acylase activity of rabbit pancreas maceration juice.

The observation that rabbit pancreas acylase preparation hydrolyzed acetylglycine more readily than acetyl-DL-phenylalanine (Table 16) is contrary to what one would expect of bovine pancreatic carboxypeptidase[1], although the former

[1]E. L. Smith, Proteolytic enzymes. In J. B. Sumner and K. Myrbäck. The enzymes. Vol. I. Part 2, p. 806. New York, New York, Academic Press Inc. 1951.

Table 15

Acylase Activity of Pancreas, Pancreatic Juice, and Intestinal Mucosa of Rabbit*

Substrate	Pancreas	Pancreatic Juice	Intestinal Mucosa
Benzoylglycine	0[9,15]	0[8]	0[5]
Benzoyldiglycine	0[9,15]	0[8]	0[5]
Phthaloyldiglycine		?[8]	
Benzoyl-DL-leucylglycine	+[9]	0[8]	
Chloroacetylglycylglycine	?[9]	0[8]	+[5]
d-Bromoisocapronylglycylglycine	+[9]	0[8]	
d-DL-bromoisocapronylglycine	0[9]		0[5]
Acetylglycine	+[5]		+[5]
Formylglycine	+[5]		+[5]
Acetyl-DL-leucine	+[5]		+[5]
Formyl-DL-leucine	+[5]		+[5]
Chloroacetyl-L-tyrosine	+[9]		
Chloroacetyl-L-phenylalanine	+[8]	+[9,10]	+[5]
Acetyl-DL-phenylalanine	0[5]		0[5]
Formyl-DL-phenylalanine	0[5]		0[5]

*The symbols used and their meaning are as follows: +, hydrolysis observed; 0, no hydrolysis observed; ?, hydrolysis so slight as to be questionable. Superscripts refer to references which are found on page 51.

References to Table 15

(Acylase Activity of Rabbit Tissues and Juices)

1. S. Utzino, J. Biochem. (Japan), 9, 465-481 (1948).
2. T. Sugawara, Tohoku J. Exptl. Med., 48, 171-6 (1944).
3. H. Hanson, Fermentforschung, 14, 189-201 (1934).
4. S. Bondi and T. Frankl, Biochem. Z., 17, 555-561 (1909).
5. F. Itzioka, J. Biochem. (Japan), 24, 139-151 (1936).
6. Meissner and Shepard, "Untersuchungen uber das Enstehen der Hippuric Acid in theirischen Organism." Hannover, 1866. Cited by H. Weiske, Z. fur Biologie, 12, 241-265 (1876).
7. S. Utzino, S. Taunoo, and T. Mori, J. Biochem. (Japan), 26, 449-53 (1937).
8. F. Itzioka, J. Biochem. (Japan), 24, 267-277 (1936).
9. Ibid., 139-151 (1936).
10. F. Itzioka, J. Biochem. (Japan), 26, 75-80 (1937).
11. T. Sugawara, Tohoku J. Exptl. Med., 48, 223-230 (1945).
12. S. Utzino, S. Tsunoo, and T. Mori, J. Biochem. (Japan), 26, 439-47 (1937).
13. Ibid., 477-486 (1937).
14. A. Schmitz and R. Merten, Z. Physiol. Chem., 278, 43-56 (1943).
15. M. Yosioka, Enzymologia, 10, 154-160 (1941).
16. J. S. Fruton, G. W. Irving, and M. Bergmann, J. Biol. Chem., 138, 465-466 (1940).

substrate has not been tested. However, the observation that chloroacetyl-L-phenylalanine is hydrolyzed more readily than glycine compounds such as chloroacetyldiglycine (Table 17), is in accord with the behaviour of bovine pancreatic carboxypeptidase[1].

The data on the acylase activity of intestine (Table 16), seems to be similar to that for the acylase preparations from the non protease secreting tissues of rabbit (Table 13).

Table 16

% Hydrolysis[1] in 140 Hours of Acylamino Acids by Acylase Systems of Rabbit Pancreas and Intestinal Mucosa*

Substrate	Source of Acylase Preparation	
	Pancreas	Intestinal Mucosa
Acetylglycine	18	60
Acetyl-DL-leucine	13	52
Acetyl-DL-phenylalanine	0	0
Formylglycine	10	15
Formyl-DL-leucine	6	38
Formyl-DL-phenylalanine	0	0
Benzoylglycine	0	0

[1]H. Kimura, J. Biochem. (Japan), 10, 207-223 (1929).

*A buffer of pH 7.2 was used. Incubation at 37°.

[1]E. L. Smith. Proteolytic enzymes. In J. B. Sumner and K. Myrbäck. The enzymes. Vol. I. Part 2, p. 806. New York, New York, Academic Press, Inc. 1951.

Table 17

Acylase Activity[1] of Rabbit Pancreas Maceration Juice

Substrate	ML enzyme sol.	pH	Hours digestion	Hydrolysis as amino-N in 1 ml	Relative enzyme dilution
Benzoylglycine*	2	7.5	72	.01**	.20
Benzoyldiglycine*	2	7.5	72	.02**	.25
Benzoyl-DL-leucyl-glycine	4	7.5	72	.100	.25
d-Bromoisocapronyl-glycine	3	7.5	72	0	.14
d-Bromoisocapronyl-diglycine	3	7.0	24	.057	.14
Chloroacetyl-L-phenylalanine	3	7.5	5	.611	.14
Chloroacetyl-L-tyrosine	4	7.5	24	.145	.14
Chloroacetyl-diglycine	6	7.5	72	.083	.14

[1]T. Itzioka, J. Biochem. (Japan), 24, 139-151 (1936).

*Activated with "Kinase" solution.

**Ml .10 normal NaOH to titrate 4 ml digest solution.

Related to the preceding data are the conclusions of Meissner and Shepard, who stated in 1866, on the basis of indirect evidence, that hippuric acid is decomposed to benzoic acid and glycine in the stomach and intestine of the rabbit[1]. They believed that these components were reunited in the kidney, accounting for the appearance of hippuric acid in the urine.

m. The sheep. Krebs, Sykes, and Bartley found that acetyl-sulphamezathine is hydrolyzed by sheep kidney suspension, kidney cortex, lung, liver, brain, pancreas, spleen, mucosa of jejunum, and blood[2]. These workers also found that acetylsulphanilamide, acetylsulphamezathine, acetylsulfapyridine, acetylsulfathiazole, acetylsulphadiazine, and acetyl-4:6-dimethyloxysulphadiazine were hydrolyzed by sheep kidney. As stated before, the definition of acylase as defined by the writer (page 5), does not encompass the enzyme activity toward these substrates. It is not known if the substrates discussed herein are attacked by acylases as defined on page 5.

n. The cow. Fruton and Bergmann[3] found that ammonium sulfate fractionation of an aqueous extract of beef spleen

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[1]See reference 6 on page 51.

[2]H. A. Krebs, W. O. Sykes, and W. C. Bartley, Biochem. J., 41, 622-630 (1947).

[3]J. S. Fruton and M. Bergmann, J. Biol. Chem., 130, 19-27 (1939).

led to a preparation which hydrolyzed carbobenzoxy-L-glutamyl-L-tyrosine, and carbobenzoxy-L-glutamyl-L-phenylalanine with a certain amount of activation by cysteine at 40°. In addition, carbobenzoxyglycyl-L-tyrosine, carbobenzoxy-L-glutamyl-L-glutamic acid, carbobenzoxy-L-glutamylglycine, carbobenzoxy-L-leucylglycine, carbobenzoxyglycylglycine, and carbobenzoxy-L-isoglutamine are appreciably hydrolyzed only in the presence of cysteine. However, no activation by cysteine of carbobenzoxy-L-glutamyl-L-tyrosine hydrolysis by beef spleen preparation was observed at 25°; the slight activation at 37° may have been due to lessening of enzyme inactivation[1] or due to a second enzyme. The data obtained by Fruton and Bergmann at 37° at one enzyme concentration is presented in Table 18.

The enzyme of beef spleen which hydrolyzes carbobenzoxy-L-glutamyl-L-tyrosine and carbobenzoxy-L-glutamyl-L-phenylalanine, and which is not cysteine-activated, has been named cathepsin I[1] and later cathepsin A[2]. Bergmann has also described it as a pepsinase of spleen because the two substrates just mentioned are hydrolyzed each by porcine

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[1]J. S. Fruton, G. W. Irving, and M. Bergmann, J. Biol. Chem., 138, 249-262 (1941).

[2]H. H. Tallan, M. E. Jones, and J. S. Fruton, J. Biol. Chem., 194, 793-805 (1941).

Table 18

Acylase Activity[1] of Beef Spleen Preparation*

Substrate	pH	Hours incubation	% Hydrolysis with	
			No activator	Cysteine
Carbobenzoxy-L-glutamyl-L-tyrosine	5.5	2	53	61
Carbobenzoxy-L-glutamyl-L-phenylalanine	5.0	4	15	67
Carbobenzoxy-L-glutamyl-D-phenylalanine	5.0	12	1	3
Carbobenzoxy-L-glutamyl-L-glutamic acid	4.7	2	0	55
Carbobenzoxyglycyl-L-tyrosine	4.4	2	5	33
Chloroacetyl-L-tyrosine	7.8	24	0	2
Carbobenzoxyglycyl-L-proline	4.4	12	2	7
Carbobenzoxy-L-leucyl-glycine	4.8	4	2	51
Carbobenzoxyglycyl-glycine	4.7	4	0	35
Carbobenzoxyglycyl-sarcosine	4.5	12	3	7
Carbobenzoxy-L-isoglutamine	5.2	21	7	53

[1] J. S. Fruton and M. Bergmann, J. Biol. Chem., 130, 19-27 (1939).

*Incubation at 37°.

pepsin and cathepsin A at the same rate ratios[1]; however, the activity ratios for the two enzymes were each determined at a different pH. Beef spleen cathepsin A is known to differ from porcine pepsin in that cathepsin A has a pH optimum for carbobenzoxy-L-glutamyl-L-tyrosine at 5.8 (rather than at pH 4) and has a higher specific activity than crystalline porcine pepsin[1].

The cysteine-activated beef spleen enzyme which hydrolyzes carbobenzoxyglycyl-L-phenylalanine has been named beef spleen carboxypeptidase[2], although the former name was cathepsin IV[3]. Cysteine-beef spleen preparation decomposes carbobenzoxyglycyl-L-glutamic acid to carbobenzoxyglycine and L-glutamic acid[4]. No direct evidence was presented that beef spleen carboxypeptidase requires a terminal carboxyl group in substrates hydrolyzed, but this enzyme was described as a carboxypeptidase because a preparation from swine kidney which readily hydrolyzes carbobenzoxyglycyl-L-phenylalanine does not hydrolyze carbobenzoxyglycyl-L-phenylalaninamide[3].

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[1] M. Bergmann, Adv. Enzymol., 2, 49-68 (1942).

[2] H. H. Tallan, M. E. Jones, and J. S. Fruton, J. Biol. Chem., 194, 793-805 (1941).

[3] J. S. Fruton, G. W. Irving, and M. Bergmann, J. Biol. Chem., 141, 763-74 (1941).

[4] J. S. Fruton, G. W. Irving, and M. Bergmann, J. Biol. Chem., 138, 249-262 (1941).

Dialysis caused parallel inactivation of carbobenzoxyglycyl-L-phenylalanine and carbobenzoxyglycyl-L-tyrosine activity, and it is believed the same enzyme hydrolyzes each substrate[1]. Inactivation and activation behaviour of beef spleen preparations indicate that beef spleen carboxypeptidase also hydrolyzes carbobenzoxy-L-glutamyl-L-tyrosine, although the main activity toward this compound in beef spleen preparations before heating is due to cathepsin A[1].

Bergmann tentatively suggests that both pancreatic and spleen carboxypeptidases attack acyldipeptides in which the terminal amino acid must be phenylalanine or tyrosine for action[2]. Their classification seems to imply a similarity in specificity for the two enzymes. In view of the rapid rate at which carbobenzoxyglycylglycine, carbobenzoxy-L-leucylglycine, and carbobenzoxy-L-glutamylglycine were broken down by a cysteine-activated component of beef spleen (Table 18), and in view of the slow rate of breakdown of the first substrate (as compared to carbobenzoxyglycyl-L-phenylalanine hydrolysis) by bovine pancreatic carboxypeptidase[3],

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[1] J. S. Fruton, G. W. Irving, and M. Bergmann, J. Biol. Chem., 141, 763-74 (1941).

[2] M. Bergmann, Adv. Enzymol., 2, 49-68 (1942).

[3] E. L. Smith, Proteolytic enzymes. In J. B. Sumner and K. Myrbäck. The enzymes. Vol. I, Part 2, p. 806. New York, New York, Academic Press Inc. 1951.

it would seem well to regard such a view with extreme caution.

Beef kidney preparations contain about twice as much activity per mg of nitrogen toward carbobenzoxyglycyl-L-leucine in the presence of cysteine as do beef spleen preparations[1]. This cathepsin IV, or beef kidney carboxypeptidase, activity[1,2,3] may also be responsible for part of the hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine since this enzyme activity is partly cysteine activated[1].

Beef kidney contains a cathepsin I, or beef kidney cathepsin A, which hydrolyzes carbobenzoxy-L-glutamyl-L-tyrosine and carbobenzoxy-L-glutamyl-L-phenylalanine in the absence of cysteine[2,3,4]. Bergmann states that beef kidney cathepsin A (then cathepsin I) has a specificity similar to that of beef spleen cathepsin, because both enzymes have the same rate ratios toward the two substrates just mentioned[1,4]. The cathepsin A activity of beef kidney preparations per mg. of nitrogen is about half of the activity

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[1] J. S. Fruton, G. W. Irving, and M. Bergmann, J. Biol. Chem., 141, 762-74 (1941).

[2] J. S. Fruton and M. Bergmann, J. Biol. Chem., 130, 19-27 (1939).

[3] H. H. Tallan, M. E. Jones, and J. S. Fruton, J. Biol. Chem., 194, 793-805 (1952).

[4] M. Bergmann, Adv. Enzymol., 2, 49-68 (1942).

of beef spleen preparations[1]. Glycerol extracts of beef kidney have acylase activity toward benzoylglycine[2,3,4], furfuroylglycine[5], furylpropionylglycine[5], furylacrylglycine[5], phenyl-α,β-dibromopropionylglycine[2], phenylpropionylglycine[2], N-(pyridincarboxyl) glycine, and furfuroyl-DL-alanine[6]; no activity is observed toward phenylhippuric acid[6], cinnamoylglycine[2], phenylfurfuroylglycine[3], oxymethylfurfuroylglycine[5], and furfuroylisoserine[6]. Schaffner and Truelle find that carbobenzoxy-L-glutamyl-L-tyrosine is split by a bovine liver preparation with an optimum at pH 5.6[7]. This enzyme preparation also splits gelatin, but gelatin hydrolysis is inhibited by M/1000 indoacetic acid while carbobenzoxy-L-glutamyl-L-tyrosine splitting is uninfluenced. Jacoby isolated benzoic acid from a four day

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[1] J. S. Fruton, G. W. Irving, and M. Bergmann, J. Biol. Chem., 141, 762-74 (1941).

[2] S. Utzino, S. Tsunoo, and T. Mori, J. Biochem. (Japan), 26, 439-47 (1937).

[3] I. A. Smorodinzew, J. Russ. Phys. Chem. Soc., 81, 156-82 (1919). Thanks are due to Mr. Sol Shulman who kindly furnished an English summary of this Russian article.

[4] I. A. Smorodinzew, Z. Physiol. Chem., 124, 123-134 (1923).

[5] S. Utzino, S. Tsunoo, and T. Mori, J. Biochem. (Japan), 26, 449-53 (1937).

[6] Ibid., 477-482 (1937).

[7] A. Schaffner, and M. Truelle, Biochem. Z., 315, 391-404 (1943).

digest, under toluene, of bovine liver juice and hi uric acid[1]. Abderhalden and Schwab[2] give the data in Table 19 on the enzyme behaviour of liver press juice (which is bovine as far as the writer could tell from the article).

Table 19

Enzyme Activity[1] of Liver Press Juice (Bovine?)*

Substrate	pH	% Hydrolysis
Chloroacetyl-L-alanine	7.8	89
Chloroacetyl-L-tyrosine	8.4	20
Chloroacetyl-o-nitroaniline	7.0	40 (in two hours)

[1]E. Abderhalden, and E. Schwab, Fermentforschung, 14, 43-63 (1933).

*Fifteen hour incubation unless otherwise stated.

Glycerol extracts of bovine liver fail to hydrolyze, significantly, phenylpropionylglycine[3], phenyl-α,β-dibromopropionylglycine[3], cinnamylglycine[3], N-(pyridincar-

[1]M. Jacoby, Z. Physiol. Chem., 30, 168 (1900).

[2]E. Abderhalden, and E. Schwab, Fermentforschung, 14, 43-53 (1933).

[3]S. Utzino, S. Tsunoo, and T. Mori, J. Biochem. (Japan), 26, 439-447 (1937).

boxyl)glycine[1], phenyl-α-bromo-β-oxypro ionylglycine[1], DL-furfuroylisoserine[2], phenylfurfuroylglycine[3], phenylhippuric acid[2], furylacrylglycine[3], furfuroylglycine[3], or furylpropionylglycine[3]. However, α-furfuroyl-DL-alanine is slightly hydrolyzed in 24 hours by a glycerol extract of bovine liver[2].

Benzoyldiglycine and phthaloyldiglycine are not hydrolyzed by glycerin extracts of beef muscle[4].

Enzyme preparations from bovine intestine have been found inactive against N-(pyridincarboxyl)-glycine[1], benzoyldiglycine[5], β-naphthalinsulfoglycylglycine[5], naphthalinsulfoglycyl-L-leucine[6], β-naphthalinsulfoglycyl-DL-phenylalanine[6], β-naphthalinsulfo-DL-leucyl-L-leucine[6], β-naphthalinsulfoglycylglycine[6], and β-naphthalinsulfo-DL-leucylglycine[6]. Abderhalden and von Ehrenwall[7] give

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[1]S. Utzino, S. Tsunoo, and T. Mori, J. Biochem. (Japan), 26, 439-447 (1937).

[2]Ibid., 477-482 (1937).

[3]Ibid., 449-53 (1937).

[4]S. Utzino, J. Biochem. (Japan), 9, 465-81 (1928).

[5]T. Imai, Z. Physiol. Chem., 136, 205-213 (1924).

[5a]M. Metsumoto, Acta Schol. Med. Univ. Imp. Kioto, 10, 229-233 (1928).

[6]S. Otani, Acta Schol. Med. Univ. Imp. Kioto, 17, 163-196 (1934).

[7]E. Abderhalden and E. von Ehrenwall, Fermentforschung, 12, 223 (1930).

data in Table 20 on hydrolysis of acylamino acids by two different "erepsin" solutions, which are presumably purified preparations from bovine intestinal mucosa. The variation

Table 20

Hydrolysis of Acylamino Acids by Two "Erepsin" Preparations (in 24 Hours)[1]

Substrate	% Hydrolysis by "Erepsin" Solution	
	I	II
Chloroacetyl-L-alanine	70	17
Chloroacetyl-DL-leucine	52	42
Chloroacetyl-L-tyrosine	0	0

[1]E. Abderhalden and E. von Ehrenwall, Fermentforschung, 12, 223 (1930).

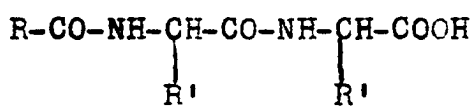
in the activity ratios of the two erepsin preparations toward chloroacetyl-L-alanine and chloroacetyl-DL-leucine may indicate that more than one enzyme is responsible for the hydrolysis of the two substrates.

A proteinase of bovine gastric juice, bovine pepsin, hydrolyzed carbobenzoxy-L-glutamyl-L-tyrosine, carbobenzoxy-L-glutamyl-L-phenylalanine, and carbobenzoxyglycyl-L-tyrosine at pH 4.1[1]. Evidence is at hand to indicate

[1]E. L. Smith, Proteolytic enzymes. In J. B. Sumner and K. Myrbäck. The enzymes. Vol. 1, Part 2. p. 840-845. New York, New York, Academic Press Inc., 1951.

bovine pepsin has a specificity very similar to those of swine, sheep, and chicken pepsins.

A bovine pancreatic carboxypeptidase, which was first crystallized by Anson, hydrolyzes acyldipeptides, acylamino acids, and free or acylated tri- and oligopeptides, with liberation of the carboxyl-terminal amino acid[1,2]. This crystalline enzyme will be referred to as Anson's carboxypeptidase. Anson's carboxypeptidase occurs in the pancreas as a pro-enzyme which is secreted in the pancreatic juice, and is converted to the enzyme by trypsin. Typical substrates for this carboxypeptidase can be represented by the following formula, with the arrow indicating the bond broken:



Compounds containing two peptide bonds are more susceptible to Anson's carboxypeptidase than compounds containing only one peptide bond. The nature of the terminal amino acid

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[1]H. Neurath and G. W. Schwert, Chem. Rev., 46, 69-153 (1950).

[2]E. L. Smith. Proteolytic enzymes. In J. B. Sumner and K. Myrbäck. The enzymes. Vol. 1, Part 2. p. 802-810. New York, New York, Academic Press Inc., 1951.

side chain, R'', has a greater effect on rate of substrate hydrolysis than the nature of the second amino acid side chain, R'. Acyldipeptides containing a terminal phenylalanine, tyrosine, tryptophan, or leucine, are more rapidly split than other substrates. Several chloroacetylamino acids are split at a rather slower rate than the corresponding carbobenzoxyglycylamino acids, but limited data indicate that the acetylamino acids are very slowly hydrolyzed. The rates of hydrolysis of substrates by Anson's carboxypeptidase are given in Table 21, reproduced from the excellent review by Neurath and Schwert[1].

Proteolytic coefficients for several acyldipeptides containing serine, as determined by Harris and Fruton[2], were found to be as follows: carbobenzoxyglycyl-L-serine, .002; carbobenzoxy-L-alanyl-L-serine, .018; carbobenzoxy-L-seryl-L-alanine, .042; carbobenzoxy-L-seryl-L-serine, .003. The authors concluded that the introduction of a terminal serine for the terminal glycine in carbobenzoxyglycylglycine does not change the order of magnitude of the rate constant.

Proteolytic coefficients for the hydrolysis of a number of chloroacetyl-, trichloroacetyl-, and hippurylamino acids

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[1]H. Neurath and G. W. Schwert, Chem. Rev., 46, 89-153 (1950).

[2]J. I. Harris and J. S. Fruton, J. Biol. Chem., 191, 143-151 (1951).

were presented by Ronwin[1] and these are reproduced in Table 21a. In the same paper was a brief presentation of the "dipositive-bond theory" which is a proposed mechanism of enzyme-substrate interaction; this theory was said to explain the failure of N-p-toluenesulfonyl-DL-phenylalanine and N-chloroacetyl-DL-aspartic acid to be hydrolyzed by Anson's carboxypeptidase.

In a recent paper by Steinberg[2], it is suggested that the previously reported release of alanine from ovalbumin by Anson's carboxypeptidase is dependent on the presence of a diisopropylfluorophosphate sensitive contaminant.

The hydrolysis of carbobenzoxyglycyl-L-phenylalanine by Anson's carboxypeptidase is strongly inhibited by certain carboxylic acids[3]. The strongest inhibitor is β-phenylpropionic acid which possesses a side chain identical with that found in the terminal phenylalanine group of substrates most sensitive to Anson's carboxypeptidase action. Certain D-amino acids inhibit the action of Anson's carboxypeptidase, but to a lesser extent than do the corresponding carboxylic acids. Inhibitory power of D-amino acids parallels the

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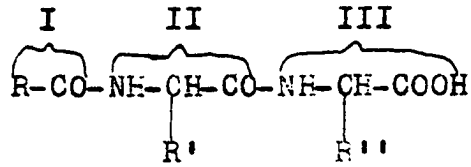
[1]E. Ronwin, J. Am. Chem. Soc., 75, 4026-4030 (1953).

[2]D. Steinberg, J. Am. Chem. Soc., 75, 4875-4876 (1953).

[3]H. Neurath and G. W. Schwert, Chem. Rev., 46, 89-153 (1950).

Table 21a

Substrates for Anson's Carboxypeptidase[1]



	I	II	III	Rate* $C^{25^\circ}_{0.05}$
1	Carbobenzoxy	glycyl	phenylalanine	12.0-14.0
2	Carbobenzoxy	glycyl	tyrosine	6.2
3	Carbobenzoxy	glycyl	tryptophan	4.7
4	Carbobenzoxy	glycyl	leucine	2.6
5	Carbobenzoxy	glycyl	methionine	1.2
6	Carbobenzoxy	glycyl	isoleucine	0.54
7	Carbobenzoxy	glycyl	alanine	0.04
8	Carbobenzoxy	glycyl	aminoisobutyric acid	0.013
9	Carbobenzoxy	glycyl	glycine	0.001
10	Carbobenzoxy	glycyl	thiazolidine-4-carboxylic acid	+

[1] H. Neurath and G. W. Schwert, Chem. Rev., 46, 89-153 (1950).

* $C^{25^\circ}_{0.05}$ denotes the "proteolytic coefficient," calculated from measurements at 25°C and 0.05 M initial substrate concentration, within the pH range of pH 7.3 to pH 7.7. Where the experimental data did not warrant the calculation of proteolytic coefficients, rates are merely denoted by + (moderate or fast) or ± (slow).

Table 21a(Continued)

	I	II	III	Rate* C25° 0.05
11	Benzoyl	glycyl	phenylalanine	+
12	Benzoyl	glycyl	phenylglycine	1.0
13	Benzoyl	glycyl	lysine	+
14	Benzenesulfonyl	glycyl	phenylalanine	+
15	Carbobenzoxy	alanyl	phenylalanine	11.3
16	Carbobenzoxy	alanyl	tyrosine	6.4
17	Carbobenzoxy	methionyl	tyrosine	7.8
18	Carbobenzoxy	tryptophyl	tyrosine	+
19	Carbobenzoxy	tryptophyl	tryptophan	2.3
20	Carbobenzoxy	tryptophyl	alanine	0.12
21	Carbobenzoxy	tryptophyl	glycine	0.007
22	Carbobenzoxy	tryptophyl	proline	0.002
23	Carbobenzoxy	glutamyl	phenylalanine	0.8
24	Carbobenzoxy	glutamyl	tyrosine	0.5
25	Acetyl	dehydro-phenyl-alanyl	phenylalanine	0.003
26	Acetyl	dehydro-phenyl-alanyl	leucine	0.002

Table 21a (Continued)

I	II	III	Rate* $C^{25^\circ}_{0.05}$
27	Carbobenzoxy	phenylalanine	0.0013
28	Carbobenzoxy	tryptophan	0.009
29	Carbobenzoxy	tyrosine	0.001
30	Benzoyl	phenylalanine	±
31	Chloroacetyl	phenylalanine	+
32	Chloroacetyl	tyrosine	1.65
33	Chloroacetyl	tryptophan	+
34	Formyl	phenylalanine	±
35	Acetyl	phenylalanine	±
36	Acetyl	tryptophan	0.045
37	Phenylpyruvyl	phenylalanine	±
38	Phenylpyruvyl	leucine	±

Table 21b

Anson's Carboxypeptidase Action on Chloroacetyl, Trichloroacetyl, and Hippuryl Derivatives of Amino Acids As Determined by Ronwin[1]

Amino acid	Proteolytic coefficient, $C^{25^o}_{0.05\ M}$		
	N-Chloroacetyl derivatives	N-Trichloroacetyl derivatives	Hippuryl derivatives
Phenylalanine	2.22		17.0
Tyrosine	2.00	0.023	9.96
Leucine	0.348	0.016	11.0
Norvaline	.174		
Isoleucine	.087	0.014	8.86
Methionine	.065		2.32
Valine	.030	no act.	1.21
Alanine	.006	no act.	
Aspartic acid	no act.		

[1]E. Ronwin, J. Am. Chem. Soc., 75, 4026-4030 (1953).

susceptibility to hydrolysis of the corresponding acyl-L-amino acids containing at least two peptide bonds. Data on the inhibition of carbobenzoxyglycyl-L-phenylalanine hydrolysis by crystalline Anson's carboxypeptidase are given in Table 22.

Smith and his co-workers have reported that the action of Anson's carboxypeptidase is inhibited by certain "metal poisoners", namely, cyanide, cysteine, phosphate, pyrophosphate, oxalate, and citrate[1]. Largely on the basis of these findings and the observation that magnesium was present in the ash of Anson's carboxypeptidase, it was postulated that protein-bound magnesium played an important part in the catalysis due to Anson's carboxypeptidase[1]. However, Neurath and co-workers[2,3] have reported that the initial rate of carbobenzoxyglycyl-L-phenylalanine and carbobenzoxyglycyl-L-leucine hydrolysis is uninfluenced by the substances said by Smith to be inhibitory. The apparent inhibition by "metal poisons" was ascribed to an increase in enzyme affinity for reaction products[2] due to the added compounds.

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[1]E. L. Smith. Proteolytic enzymes. In J. B. Sumner and K. Myrback. The enzymes. Vol. 1, Part 2. p. 802-810. New York, New York, Academic Press Inc., 1951.

[2]H. Neurath and G. W. Schwert, Chem. Rev., 46, 69-153 (1950).

[3]H. Neurath and O. De Maria, J. Biol. Chem., 186, 653-665 (1950).

Table 22

Inhibition[1] of the Hydrolysis of Carbobenzoxyglycyl-L-phenylalanine by Crystalline Anson's Carboxypeptidase*

Inhibitor	$\frac{C}{C_I}$	K_I
		10^{-3}M
α-Phenylpropionic acid	0.062	
γ-Phenylbutyric acid	1.13	
Phenylacetic acid	0.39	
p-Nitrophenylacetic	2.5	
D-Phenylalanine	6.0	2.0
D-Histidine	2.1	20.0
D-Alanine	1.6	
D-Isoleucine	1.6	
D-Lysine	1.1	

[1]H. Neurath and G. W. Schwert, Chem. Rev., 46, 89-153 (1950).

*C is the proteolytic coefficient in the absence of inhibitor at an initial carbobenzoxyglycyl-L-phenylalanine concentration of .0125; C_i is the proteolytic coefficient in the presence of 0.01 M D-amino acid. K_I is the dissociation constant of the enzyme-inhibitor complex.

For further discussion and references on Anson's bovine pancreatic carboxypeptidase, see the reviews by Neurath and Schwert[1], and by Emil Smith[2]. The discussion just presented is based on these reviews.

Are acylases other than Anson's carboxypeptidase present in bovine pancreas? A carboxypeptidase, which was named protaminase, has been discovered in hog pancreas[3,4]. Protaminase acts on clupein and salmine with liberation of arginine, and this activity has been separated from activity toward chloroacetyl-L-tyrosine. However, clupein was not significantly hydrolyzed by an extract of cow pancreas[3,4] that did hydrolyze chloroacetyl-L-tyrosine. On first thought, these experiments possibly indicate a difference in the acylase complements of bovine and porcine pancreases. However, the apparent species difference may have arisen due to denaturation or extraction differences or due to the different amounts of proteinases present. A conflicting

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[1]H. Neurath and G. W. Schwert, Chem. Rev., 46, 69-153 (1950).

[2]E. L. Smith. Proteolytic enzymes. In J. B. Sumner and K. Myrbäck. The enzymes. Vol. 1, Part 2. p. 802-810. New York, New York, Academic Press Inc., 1951.

[3]E. Waldschmidt-Leitz, F. Ziegler, A. Schäffner, and L. Weil, Z. Physiol. Chem., 197, 219-236 (1931).

[4]E. Waldschmidt-Leitz, and E. Kofranvi, Z. Physiol. Chem., 222, 148-150 (1933).

report is that of Smith, who states that six-times crystallized Anson's carboxypeptidase hydrolyzed salmine[1]; he raises the question of the possible identity of protaminase with carboxypeptidase.

Emil and Rudolf Abderhalden claimed to have separated carboxypeptidase activity, as represented by β-naphthalinsulfoglycyl-L-leucine hydrolysis, from activity toward chloroacetyl-DL-leucine (described as acylase activity) by removing the former activity through formalin treatment[2]. However, the claim that two enzymes were responsible for the activity observed, was later withdrawn[1]. Utzino, Tsunoo, and Mori noted no hydrolysis by glycerol extracts of bovine pancreas of furfuroylglycine, furylpropionylglycine, furylacrylglycine[4], furfuroyl-DL-alanine, furfuroyl-DL-isoserine, and phenylhippuric acid[5]. The Abderhaldens describe the action on haloacylamino acids and acylated peptides of a partially purified preparation from bovine

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[1]E. L. Smith. Proteolytic enzymes. In J. B. Sumner and K. Myrback. The enzymes. Vol. 1, Part 2. p. 827. New York, New York, Academic Press Inc., 1951.

[2]E. Abderhalden and R. Abderhalden, Fermentforschung, 16, 48-61 (1938).

[3]E. Abderhalden and R. Abderhalden, Fermentforschung, 17, 217-223 (1943).

[4]S. Utzino, S. Tsunoo, and T. Mori, J. Biochem. (Japan), 26, 449-453 (1937).

[5]Ibid., 477-482 (1937).

pancreas[1]. They tested the chloroacetyl, bromoacetyl, and iodoacetyl derivatives of tyrosine, leucine, alanine, and glycine; but the leucine and tyrosine derivatives were completely or almost completely hydrolyzed under the conditions of the experiment. The degree of hydrolysis of the alanine and glycine derivatives are given in Table 23.

Table 23

Hydrolysis of Acyl Glycines and Acyl Alanines by Partially Purified Bovine Pancreatic Carboxypeptidase[1]

Compound	% Hydrolysis
Chloroacetyl-DL-alanine	25
Bromoacetyl-DL-alanine	20
Iodoacetyl-DL-alanine	17.5
Chloroacetylglycine	15
Bromoacetylglycine	10
Iodoacetylglycine	0

[1]E. Abderhalden and R. Abderhalden, Fermentforschung, 16, 48-68 (1938).

o. The hog. The earliest report on acylases was in 1881 by Schmiedeberg. He reported that benzoylglycine was

[1]E. Abderhalden and R. Abderhalden, Fermentforschung, 16, 48-61 (1938).

hydrolyzed by an aqueous extract of alcohol-dried and defatted hog kidney and other dog and hog tissues[1]. Schmiedeberg carried out his one day enzyme-substrate incubations in the absence of added antiseptics. He believed the observed hydrolysis was due to an unorganized ferment, and not due to the presence of putrefactive fermentation since no turbidity due to bacterial development appeared in clear histozyme-benzoylglycine solutions after a one day incubation. Later work on acylases was carried out in the presence of antiseptics such as toluene.

Minkowski failed to observe hydrolysis of hippuric acid by a one year old histozyme preparation obtained from Schmiedeberg[2]. Minkowski concluded that Schmiedeberg's observations were not false because of putrefaction. He proceeded to observe that fresh hog kidney brei would split hippuric acid in the presence of 0.2% quinine, 0.5% thymol, and 1.0% phenol. He also succeeded in obtaining an active acylase preparation using Schmiedeberg's procedure. However, Mutch[3] was unable to obtain a preparation from hog kidney

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[1] O. Schmiedeberg, Arch. Exp. Path. Pharmak., 14, 379-392 (1881).

[2] O. Minkowski, Arch. Exp. Path, Pharmak., 17, 445-461 (1883).

[3] N. Mutch, J. Physiol., 44, 176-190 (1912).

by the method of Schmiedeberg, when the precaution was taken of adding an antiseptic. Mutch concluded that probably Schmiedeberg's observations were due largely to bacterial action. Mutch did obtain an active preparation from pig kidney by using the solid remaining after alcohol followed by ether treatment of pig kidney brei. This preparation hydrolyzed both benzoylglycine, and, to a somewhat lesser extent, the dextro-rotary isomer of benzoylalanine. Benzoate inhibited a little, and 0.5% formalin inhibited completely the action of his acylase preparation. Mutch also determined that the equilibrium of hippuric acid hydrolysis was 97 moles of hippuric acid hydrolyzed to 3 moles unhydrolyzed.

More recent work, especially that by Greenstein and co-workers, has demonstrated that a large number of acyl-amino acids are hydrolyzed by protein fractions from hog kidney. Birnbaum, Levintow, Kingsley, and Greenstein have prepared a fraction of hog liver homogenate by acetone and ammonium sulfate fractionation which they designate as acylase I[1]. Fu and Birnbaum report that acylase I hydrolyzes the N-chloroacetyl, straight-chain, aliphatic amino

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[1] S. M. Birnbaum, L. Levintow, R. Kingsley, and J. Greenstein, J. Biol. Chem., 194, 455-470 (1952).

acids at rates whose logarithms are proportional to the inductive constant of the amino nitrogen up to 5 amino acid carbons; an increase in size of the amino acid portion of the chloroacetylated amino acids beyond this, or branching, diminishes the rate[1]. Fones and Lee find the rate of hydrolysis of the monohalogen acetylalanines decreases qualitatively in the order of decreasing electronegativity of the halogen atom[2]. Other acylalanines were used and the results seem to indicate that both electronic and steric factors influence the rate of hydrolysis of acylamino acids by acylase I. The decreasing rates of hydrolysis of some acylalanines by acylase I are trifluoroacetyl > fluoroacetyl > chloroacetyl > propionyl > acetyl > bromoacetyl > formyl > iodoacetyl > benzoyl. The rates of hydrolysis of many acylamino acids by acylase I are given in Table 24.

An interesting observation is that acylase I hydrolyzed certain substrates containing an aromatic ring at about the same rate as the corresponding substrates containing a saturated aliphatic ring[3]. This is in contrast to the

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[1] S. J. Fu and S. M. Birnbaum, J. Am. Chem. Soc., 75, 918-920 (1953).

[2] W. S. Fones and M. Lee, J. Biol. Chem., 201, 847-856 (1953).

[3] S. M. Birnbaum, L. Levintow, R. B. Kingsley, and J. P. Greenstein, J. Biol. Chem., 194, 455-470 (1952).

Table 24

Hydrolysis of N-Acylated Amino Acids by Acylase I from Hog Kidney[1,2,3]

Racemic compound	Rate with* acylase I	Ratio of rates, Acylase I / homogenate
Fluoroacetylalanine	14,700	
Chloroacetylalanine	11,600	34
Propionylalanine	3,100	
Acetylalanine	2,900	
Bromoacetylalanine	2,500	
Iodoacetylalanine	185	
Dichloroacetylalanine	180	
Trichloroacetylalanine	0	
Trifluoroacetylalanine	18,000	
Methylmercaptoacetylalanine	950	
Formylalanine	300	

[1] W. S. Fones and M. Lee, J. Biol. Chem., 201, 847-856 (1953).

[2] S. M. Birnbaum, L. Levintow, M. Kingsley, and J. P. Greenstein, J. Biol. Chem., 194, 455-470 (1952).

[3] K. R. Rao, S. M. Birnbaum, R. B. Kingsley, and J. P. Greenstein, J. Biol. Chem., 198, 507-524 (1952).

*In terms of micromoles of substrate hydrolyzed at 38° per hour per mg of protein N. All digests conducted in phosphate buffer at pH 7.0 and final concentration of about 0.03 M.

Table 24 (Continued)

Racemic compound	Rate with* acylase I	Ratio of rates, Acylase I / homogenate
Hydroxyacetylalanine	30	
p-Toluenesulfonylalanine	0	
Methanesulfonylalanine	0	
Benzoylalanine	25	
p-Fluorobenzoylalanine	24	
p-Nitrobenzoylalanine	21	
p-Chlorobenzoylalanine	4	
p-Bromobenzoylalanine	3	
p-Iodobenzoylalanine	3	
p-Tolylalanine	2	
p-Anisoylalanine	0	
m-Anisoylalanine	0	
m-Nitrobenzoylalanine	5	
2,4-Dichlorobenzoylalanine	0	
Chloroacetylaminobutyric acid	33,600	34
Acetylvaline	1,660	
Chloroacetylvaline	4,970	35
Chloroacetylnorvaline	40,500	33
Acetylleucine	5,400	

Table 24 (Continued)

Racemic compound	Rate with acylase I	Ratio of rates, Acylase I / homogenate
Chloroacetylleucine	16,500	27
Acetylnorleucine	14,400	
Chloroacetylnorleucine	30,400	36
Acetylisoleucine	376	
Acetylalloisoleucine	250	
Chloroacetylaminoheptylic acid	28,200	
Chloroacetylserine	11,600	
Chloroacetylthreonine	720	19
Chloroacetylallothreonine	2,580	
Dichloroacetyldiaminopropionic acid	455	
Dichloroacetylornithine	304	
Dichloroacetyllysine	140	
N-Acetyl-S-benzylcysteine	100	
Acetylmethionine	24,200	
Chloroacetylmethionine	100,000	34
Acetylethionine	15,400	
Acetylaspartic acid	5	
Chloroacetylaspartic acid	4	0.1
Acetylglutamic acid	3,080	
Chloroacetylglutamic acid	12,700	27

Table 24 (Continued)

Racemic compound	Rate with acylase I	Ratio of rates, Acylase I / homogenate
Chloroacetylasparagine	129	16
Carbobenzoxyglutamic acid	28	
Acetylphenylalanine	138	
Chloroacetylphenylalanine	460	15
Chloroacetyltyrosine	330	3
Acetyltryptophan	5	
Chloroacetyltryptophan	12	4
Acetylproline	0.7	
Chloroacetylproline	6	
Chloroacetylaminocaprylic acid	7,700	
Chloroacetylphenylserine	00	
Chloroacetylaminocyclohexylpropionic acid	350	
Chloroacetylaminocyclohexylbutyric acid	132	
Chloroacetylaminocyclohexylacetic acid	4,600	
Chloroacetylaminophenylacetic acid	4,500	
Chloroacetylglycine	2,640	48
Acetylhistidine	150	
Acetylarginine	410	
Chloroacetylisoleucine	1,010	28
Chloroacetylalloisoleucine	950	33

behaviour of Anson's carboxypeptidase which breaks down substrates containing aromatic rings at a much faster rate than the corresponding aliphatic compounds. Thus, kidney acylase I hydrolyzes chloroacetylphenylalanine at close to the same rate as chloroacetylaminocyclohexylpropionic acid (Table 24). On the other hand, the rates of hydrolysis of these substrates by Anson's carboxypeptidase were, chloroacetylphenylalanine 2750, and chloroacetylcyclohexylpropionic acid 16[1].

In general, the acyl-D-amino acids are very slowly hydrolyzed by acylase I as compared to the corresponding acyl-L-amino acids[2]. However, the ratio of the rates of hydrolysis of the trifluoroacetyl-D and L-alanines is 1:90, which is in decided contrast to the 1:20,000 found for the corresponding chloroacetyl derivatives.

Table 24 includes certain ratios of the rate on a substrate by acylase I to the rate by the crude hog kidney homogenate. Most of these ratios are about 30, and except for a few compounds which will be discussed shortly, it is believed that acylase I action is due to a single enzyme

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[1] S. M. Birnbaum, L. Levintow, R. B. Kingsley, and J. P. Greenstein, J. Biol. Chem., 194, 456-470 (1952).

[2] W. S. Fones and M. Lee, J. Biol. Chem., 201, 847-856 (1953).

component[1]. Certain observations of possible relation to the mechanism of hog kidney acylase action will be mentioned. Mazza and Pannain observed approximately equal hydrolysis of acetylglycine and benzoylglycine and their methyl esters by a pig kidney glycerol extract, and concluded that the aminoacylase of hog kidney differed from carboxypeptidase[2]. Utzino, Tsunoo, Fujita and Mori report that phenylhip uric acid is not split by hog kidney acylase and conclude that a peptide bond hydrogen is necessary for acylase action[3]. Ferrous sulfate at .09 molarity and potassium cyanide inhibit hydrolysis of benzoylglycine[4]. However, it was reported that N-chloroacetyl-DL-alanine amide and N-chloroacetyl-DL-leucine amide are resistant to hog kidney acylase action indicating that a free carboxyl group is necessary for acylase action[5].

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[1]K. R. Rao, S. M. Birnbaum, R. B. Kingsley, and J. P. Greenstein, J. Biol. Chem., 198, 507-523 (1952).

[2]F. P. Mazza and L. Pannain, Atti Della Reale Accademia Nazionale dei Lincei, 6th Series, Rendiconti, Classe di Scienze Fisiche, Metamaliche e Naturali, 19, 97-102 (1934).

[3]S. Utzino, S. Tsunoo, and T. Mori, J. Biochem. (Japan), 26, 449-453 (1937).

[4]T. Mori, J. Biochem. (Japan), 29, 225-240 (1939).

[5]S. J. Fu and S. M. Birnbaum, J. Am. Chem. Soc., 75, 918-920 (1953).

The ability of hog kidney acylase to hydrolyze asymmetrically acylated racemic amino acids has been used as the basis for the resolution of racemic amino acids by Greenstein and other workers[1,2,3,4]. Racemates split by hog kidney acylase in resolution procedures, and not mentioned previously, are the γ-lactone of chloroacetyl-DL-homoserine, N-acetyl-S-benzyl-DL-homocysteine[5], α,γ-diaminobutyric acid[6], DL-N-chloroacetyl-α-amino-ε-hydroxy-n-caproic acid, DL-N-chloroacetyl-α-amino-δ-hydroxy-n-valeric acid[7] and chloroacetyl-DL-isovaline[8]. Also, Utzino and Yoneya studied

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[1]For a list of 11 references see - S. M. Birnbaum, L. Levintow, R. B. Kingsley, and J. P. Greenstein, J. Biol. Chem., 194, 455-470 (1952).

[2]S. M. Birnbaum and J. P. Greenstein, Arch. Biochem. Biophy., 39, 108-118 (1952).

[3]A. Meister, L. Levintow, R. B. Kingsley, and J. P. Greenstein, J. Biol. Chem., 192, 535- 541 (1951).

[4]S. Utzino and S. Nishio, Acta Schol. Med. Univ. Kioto, 29, 134-137 (1951).

[5]S. M. Birnbaum and J. P. Greenstein, Arch. Biochem. Biophy., 42, 212-218 (1953).

[6]S. J. Fu, K. R. Rao, S. M. Birnbaum, and J. P. Greenstein, J. Biol. Chem., 199, 207-215 (1952).

[7]L. Berlinguet and R. Gaudry, J. Biol. Chem., 198, 765-769 (1952).

[8]C. G. Baker, S. J. Fu, S. M. Birnbaum, H. A. Sober, and J. P. Greenstein, J. Am. Chem. Soc., 74, 4701-4702 (1952).

the action of kidney hog enzyme on some acyllysines and acylamino-n-caproic acids and found that only α-chloroacetyl-ε-benzoyl-DL-lysine was split so as to be resolvable[1].

Akijuki was able to separate and distinguish three types of acylase activity in hog kidney[2]. One type of activity, which was named glycinehistozyme, hydrolyzed benzoylglycine, chloroacetylglycine, and chloroacetylasparagine, but not benzoylasparagine, or benzoyltyrosine. Akijuki's glycinehistozyme may be closely similar to the acylase I preparation[3] of Birnbaum, Levintow, Kingsley, and Greenstein.

A second enzyme preparation from hog kidney, which was designated by Akijuki as asparaginhistozyme, hydrolyzed benzoylasparagine, chloroacetylglycine, and chloroacetyltyrosine, but not benzoylglycine or benzoyltyrosine[4]. Somewhat similar results were observed by Nawa[5]. Of interest in this connection, are the variations in concentration of

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[1]S. Utzino and T. Yoneya, Chem. Ber., 85, 860-862 (1952).

[2]H. Akijuki, J. Biochem. (Japan), 25, 43-59 (1937).

[3]S. M. Birnbaum, L. Levintow, R. B. Kingsley, and J. P. Greenstein, J. Biol. Chem., 194, 455-470 (1952).

[4]H. Akizuki, J. Biochem. (Japan), 25, 43-59 (1937).

[5]K. Nawa, J. Biochem. (Japan), 28, 237-249 (1938).

activity for the various substrates in preparing acylase I from hog kidney homogenate (Table 24). The majority of the activities, including that toward chloroacetylglycine, undergo an increase in specific activity of 27 to 35. However, the activities toward the chloroacetyl derivatives of asparagine, phenylalanine, and threonine undergo a concentration of 16, 15, and 19, respectively (see Table 24). While the difference in activity ratios for these two groups of compounds was not ascribed to the presence of two different enzymes, by Rao, Birnbaum, Kingsley, and Greenstein[1], the combined observations of Akijuki and Nawa, and the data of Rao et al., would seem to be at least fair evidence for the existence of an acylasparagine-hydrolyzing enzyme, as distinguished from an acylglycine hydrolyzing enzyme. It seems likely that neither Akijuki nor Rao et al. were able to obtain preparations containing only the acylase active toward acylasparagines; conclusive evidence for such an enzyme must await a more complete separation of activities. It should be mentioned that Fones and Lee[2] raise the possibility of the enzyme inhomogeneity of

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[1] K. R. Rao, S. M. Birnbaum, R. B. Kingsley, and J. P. Greenstein, J. Biol. Chem., 198, 507-524 (1952).

[2] W. S. Fones and M. Lee, J. Biol. Chem., 201, 847-856 (1953).

acylase I because of some lack of parallel in the effect on rates of hydrolysis by acyl substituents for the acylalanines and acylphenylalanines.

The third acylase fraction reported by Akizuki was active toward benzoyltyrosine and chloroacetyltyrosine, but not toward benzoylglycine, benzoylasparagine, chloroacetylglycine, or chloroacetylasparagine[1]. This fraction was called tyrosinehistozyme. Rao, Birnbaum, Kingsley, and Greenstein[2] note that the activity toward chloroacetyl derivatives of tryptophan and tyrosine is only three to four times as great for acylase I as in the homogenate (Table 24), while the concentration is in the range 15 to 35 for the other chloroacetylamino acids listed by them, with the exception of chloroacetylaspartic acid. These workers raise the possibility that chloroacetyltyrosine and chloroacetyltryptophan are hydrolyzed by an acylase distinct from an enzyme hydrolyzing the other substrates of acylase I, with the exception of the acylaspartic acids. Their data seem to be a partial confirmation of Akiyui's

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[1]H. Akizuki, J. Biochem. (Japan), 25, 43-59 (1937).

[2]K. R. Rao, S. M. Birnbaum, R. B. Kingsley, and J. P. Greenstein, J. Biol. Chem., 198, 507-524 (1952).

earlier statement that there exists a distinct tyrosine-histozyme in hog kidney. Nawa, also, describes a tyrosin-benzoacylase[1].

Along with acylase I, a second fraction was isolated from hog kidney and designated acylase II[2]. The activity per mg. of N toward acylaspartic acids in acylase II represented a several fold concentration of activity over that present in the homogenate, whereas activity toward other acylamino acids was diminished. In contrast, acylase I possessed little activity toward acylaspartic acids (Table 24). The hydrolysis rates on several compounds by acylase II, which was also called aspartic acid acylase, is given in Table 25.

Certain of the earlier workers give data on the relative rates of hydrolysis of benzoylglycine and acetylglycine or chloroacetylglycine by porcine kidney acylase preparations, which differs from that presented by Fodor, Price, and Greenstein. Thus, Mazza and Pannain find that acetylglycine is hydrolyzed 18.8% in one hour, and benzoylglycine, 12.3% in one hour by a glycerin extract of pig kidney at the same

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[1]K. Nawa, J. Biochem., 28, 237-249 (1938).

[2]S. M. Birnbaum, L. Levintow, R. B. Kingsley, and J. P. Greenstein, J. Biol. Chem., 194, 455-470 (1952).

Table 25

Acylase II[1] Activity*

Compound	Rate of hydrolysis with	
	Homogenate	Aspartic acid acylase
Acetyl-DL-aspartic acid	11	27
Chloroacetyl-DL-aspartic acid	32	142
Chloroacetyl-DL-glutamic acid	480	6
Acetyl-DL-methionine	615	9
Chloroacetyl-DL-alanine	440	5
Chloroacetyl-DL-leucine	630	7
Chloroacetyl-DL-serine	455	3

[1]S. M. Birnbaum, L. Levintow, R. B. Kingsley, and J. P. Greenstein, J. Biol. Chem., 194, 455-470 (1952).

*The values are measured in terms of micromoles of substrate hydrolyzed at 38° per mg. of N.

enzyme concentration[1]. Mori reports that hippuric acid is hydrolyzed 16%, and chloroacetylglycine, 10% by hog kidney autolysate[2]. According to Nawa, hippuric acid is hydrolyzed 26%, and acetylglycine, 63%, by a treated glycerol extract of hog kidney, which he called glycinebenzoacylase. In contrast, Fodor, Price, and Greenstein report rates of 0.6 for benzoylglycine, 50 for acetylglycine, and 133 for chloroacetylglycine[3]. Nawa calls a fraction from hog kidney, glycinephenacetoacylase, since it is quite active on phenylacetylglycine as compared to benzoylglycine; another fraction hydrolyzes benzoylglycine but phenylacetylglycine to only a small extent[4]. Mori states that heating the maceration juice of hog kidney at 70°, largely destroys acylase activity toward furylacrylglycine, while the hippuric acid splitting enzyme is resistant[1]. Also, Baccari and Pontecorvo report that the acylase activity of hog kidney toward benzoylglycine is only partially extractable by 50-100%

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[1]F. P. Mazza and L. Pannain, Atti della Reale Accademia Nazionale dei Lincei, 6th Series, Rendiconti, Classe di Scienze Fisiche, Matematiche e Naturali, 19, 97-102 (1934).

[2]H. Mori, J. Biochem., 29, 225-240 (1939).

[3]P. J. Fodor, V. E. Price, and J. P. Greenstein, J. Biol. Chem., 182, 467-470 (1950).

[4]K. Nawa, J. Biochem. (Japan), 28, 237-249 (1938).

glycerol[1]. From the preceding observations, it seems likely that acylglycines are hydrolyzed by more than one acylase of hog kidney, or that decided experimental defects existed in some of the work reported. It is quite possible that the earlier workers were using poor analytical methods and often conditions conducive to microbial spoilage of enzyme-substrate digests; however, it seems unlikely that these flaws would lead to three reports differing from that of Fodor and all. One of the glycineacylases is probably the main acylase component of acylase I. An acylase having pronounced activity toward benzoylglycine, may be largely absent from the hog kidney homogenate prepared by Greenstein et al.[2] due to incomplete extraction from the kidney tissue or because of denaturation. It seems likely that this second glycineacylase and possibly other glycineacylases exist in hog kidney.

Tamura detected no hydrolysis of the DL and meso forms of dibenzoyldiaminosuccinic acid by a glycerol extract of hog kidney which hydrolyzed hippuric acid[3]. Other work on

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[1] V. Baccari and M. Pontecorvo, Bolletino della Societa Italiano di Biologia Sperimentale, 16, 329-331 (1941). Chem. Abst., 40, 6511 (1946). The writer depended largely on Chem. Abst. to review this Italian article.

[2] P. J. Fodor, V. E. Price, and J. P. Greenstein, J. Biol. Chem., 182, 467-470 (1950).

[3] T. Tamura, J. Biochem., 37, 335-349 (1938).

the hydrolysis of acylamino acids by hog kidney preparations include that by Schmorodinzew[1,2]; Utzino, Tsunoo, and Mori[3]; Clementi[4]; Matsui[5]; Kawa[6]; and Tamura[7].

In a study of the enzyme components of hog liver, Fodor and Greenstein, were able to separate activities toward chloroacetyl-L-alanine and glycyl-L-alanine[8]. However, in 1952, Rao, Birnbaum, Kingsley, and Greenstein suggested that glycyl-L-alanine and other glycylamino acids are hydrolyzed, not only by dipeptidases, but also by the acylase hydrolyzing chloroacetyl-L-alanine[9]. Attempts to

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[1]I. A. Smorodinzew, Z. Physiol. Chem., 124, 123-139 (1923).

[2]I. A. Smorodinzew, J. Russ. Phys. Chem. Soc., 51, 156-182 (1919). Thanks are due to Sol Shulman for an English summary of this Russian article.

[3]S. Utzino, S. Tsunoo, and T. Mori, J. Biochem. (Japan), 26, 449-53 and 477-482 (1937).

[4]A. Clementi, Atti della Accademia Nazionale dei Lincei, (5), 32, ii, 172-174 (1923).

[5]K. Matsui, J. Biochem. (Japan), 33, 183-199 (1941).

[6]T. Kawa, Acta Schol. Med. Univ. Imp. Kioto, 11, 121-(1924).

[7]S. Tamura, Acta Schol. Med. Univ. Imp. Kioto, 6, 467-470 (1924).

[8]P. J. Fodor and J. P. Greenstein, J. Biol. Chem., 181, 549-558 (1949).

[9]K. R. Rao, S. M. Birnbaum, R. B. Kingsley, and J. P. Greenstein, J. Biol. Chem., 198, 507-524 (1952).

separate the activity of acylase I toward chloroacetyl- and glycylamino acids were unsuccessful. Co (II) has a variable effect on the hydrolysis of chloroacetyl- and glycylamino acids by acylase I, but the extent of activation or inhibition was about the same for the corresponding glycyl- and chloroacetylamino acids. However, the action of acylase II is not effected by Co (II). Acylase I hydrolyzed the chloroacetylamino acids 3 to 30 times as rapidly as the corresponding glycylamino acids, as a rule. This behaviour on the part of hog kidney acylase differs a great deal, quantitatively, from the behaviour of Anson's carboxypeptidase, which hydrolyzes dipeptides at a much slower rate than the corresponding acyldipeptides[1].

Swine kidney contains a carboxypeptidase, which is cysteine-activated, and which hydrolyzes carbobenzoxyglycyl-L-phenylalanine[2,3,4]. The pH optimum for the hydrolysis of this substrate is 5.4[2]. This enzyme was first named

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[1]E. L. Smith, Proteolytic enzymes. In J. B. Sumner and K. Myrback. The enzymes. Vol. I, Part 2. p. 807. New York, New York, Academic Press Inc. 1951.

[2]M. Bergmann and J. S. Fruton, J. Biol. Chem., 145, 247-252 (1942).

[3]J. S. Fruton, G. W. Irving, and M. Bergmann, J. Biol. Chem., 141, 763-774 (1941).

[4]A. A. Plentl and I. H. Page, J. Biol. Chem., 155, 363-378 (1944).

cathepsin IV[1], but more recently Tallan, Jones, and Fruton, have suggested the name swine kidney carboxypeptidase[2]. Swine kidney carboxypeptidase is said to hydrolyze carbobenzoxyglycyl-L-tyrosine about 1.4 times as rapidly as carbobenzoxyglycyl-L-phenylalanine[3]. However, the enzyme preparations used have not been described as homogeneous. Dialysis largely inactivates activity toward the two substrates mentioned[4]. As mentioned before, similar enzymes are in bovine kidney and bovine spleen preparations, but at only a specific activity of 1/5 and 1/11, respectively, of the activity in hog kidney[5]. Also, about 3/4 of the hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine by hog kidney preparation is cysteine-activated[5]. Interaction of oxygen and cysteine forms an inhibitor of carbobenzoxyglycyl-L-phenylalanine hydrolysis[6].

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[1] M. Bergmann, Adv. Enzymol., 2, 49-68 (1942).

[2] H. H. Tallan, M. E. Jones, and J. S. Fruton, J. Biol. Chem., 194, 793-805 (1952).

[3] M. Bergmann and J. S. Fruton, J. Biol. Chem., 145, 247-252 (1942).

[4] A. A. Plentl and I. H. Page, J. Biol. Chem., 155, 363-378 (1944).

[5] J. S. Fruton, G. W. Irving, and M. Bergmann, J. Biol. Chem., 141, 763-774 (1941).

[6] G. W. Irving, J. S. Fruton, and M. Bergmann, J. Biol. Chem., 144, 161-68 (1942).

Also present in swine kidney preparations is an enzyme described by Bergmann[1] as a pepsinase, or cathepsin I, and which is now named swine kidney cathepsin A[2]. Swine kidney cathepsin A hydrolyzes carbobenzoxy-L-glutamyl-L-tyrosine and carbobenzoxy-L-glutamyl-L-phenylalanine at a rate ratio of 2 to 1[3,4]. This enzyme acts in the absence of cysteine and is inactivated at 50°. Beef kidney and beef spleen contain similar enzymes[3]. Carbobenzoxy-L-glutamic acid acts as an inhibitor of carbobenzoxy-L-glutamyl-L-tyrosine hydrolysis by swine kidney preparations[5].

Fodor, Price, and Greenstein presented the data (Table 26) concerning the number of bonds split in acyldipeptides by a hog kidney aqueous extract[6]. It is seen in Table 26 that substrates containing a terminal D-amino acid are not split by the hog kidney preparation. Replacement with a

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[1]M. Bergmann, Adv. Enzymol., 2, 49-68 (1942).

[2]H. H. Tallan, M. E. Jones, and J. S. Fruton, J. Biol. Chem., 194, 793-805 (1952).

[3]J. S. Fruton, G. W. Irving, and M. Bergmann, J. Biol. Chem., 141, 763-774 (1941).

[4]P. C. Zamecnik and M. L. Stephenson, J. Biol. Chem., 169, 349-357 (1947).

[5]I. D. Frantz and M. L. Stephenson, J. Biol. Chem., 146, 459 (1947).

[6]P. J. Fodor, V. E. Price, and J. P. Greenstein, J. Biol. Chem., 180, 193-208 (1949).

Table 26

Bonds Split in Acyldipeptides by Hog Kidney Aqueous Extract[1]

Substrate	Bonds available	Bonds hydrolyzed
Chloroacetylglycyl-L-alanine	2	2
Chloroacetylglycyl-D-alanine	2	0
Chloroacetylglycyl-DL-phenylalanine	4	2
Chloroacetyl-DL-phenylalanylglycine	4	2
Chloroacetylsarcosyl-DL-alanine	4	0
Chloroacetyl-DL-alanylglycine	4	2
Chloroacetylglycylglycine	2	1
Chloroacetylglycyl-DL-leucine	4	1
Chloroacetyl-DL-leucylglycine	4	2
Chloroacetyl-N-methyl-DL-alanine	2	0

[1] P. J. Fodor, V. E. Price, and J. P. Greenstein, J. Biol. Chem., 180, 193-208 (1949).

methyl group of the hydrogen of the second peptide bond from the carboxyl end of acyl dipeptides prevents enzymatic hydrolysis.

The rates of breakdown of chloroacetyl-L-alanine and chloroacetylglycyl-L-alanine by hog kidney aqueous extract was 1161 and 25, respectively, as micromoles per hour per

mg of enzyme nitrogen[1]. The point of attack on the acyldipeptides is not known[2].

Fodor and Greenstein succeeded in fractionating the activities of hog kidney toward chloroacetyl-DL-alanine and chloroacetyl-DL-alanylglycine[2]. The pH optimum for chloroacetyl-DL-alanylglycine and chloroacetylglycyl-DL-alanine breakdown was found to be 8.0. In the introduction of the paper under discussion, the authors stated that a question under consideration is whether the acylamino acids and acyldipeptides are hydrolyzed by the same enzyme or different enzymes[2]. But they stated no definite conclusion on this point, as a result of their experiments. It would have been desirable to determine the activities of their enzyme fractions on chloroacetylalanine, and chloroacetylglycylalanine, as well as chloroacetyl-DL-alanylglycine, since the rate of substrate breakdown by an enzyme could be decisively effected by the nature of the terminal amino acid.

Otani observed hydrolysis by swine kidney glycerin extracts of β-naphthalinsulfoglycylglycine to β-naphthalin-

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[1] P. J. Fodor, V. E. Price, and J. P. Greenstein, J. Biol. Chem., 180, 193-208 (1949).

[2] P. J. Fodor, and J. P. Greenstein, J. Biol. Chem., 181, 549-558 (1949).

sulfoglycine and glycine[1]. Also split were p-toluolsulfoglycylglycine, β-naphthalinsulfoglycyl-L-leucine, β-naphthalinsulfoglycyl-DL-phanylalanine, but not β-naphthalinsulfoglycine, p-toluolsulfoglycine, β-naphthalinsulfo-DL-leucylglycine, or β-naphthalinsulfo-DL-leucyl-L-leucine. He was able to separate the enzymes acting on benzoylglycine and β-naphthalinsulfoglycylglycine by adsorption on kieselguhr at pH 3.3. By adsorptive techniques, Kazama was able to separate enzymatic activities toward β-naphthalinsulfodiglycine, benzoyldiglycine, and benzoylglycine or a glycerol extract of hog kidney brei[2]. Mayeda also separated benzoylglycine activity from benzoyldiglycine activity; he observed that the benzoyldiglycine hydrolyzing enzyme was not a dipeptidase[4].

Additional evidence is in the literature on the presence in hog kidney of separate enzymes, one group of enzymes having its primary action on acylated amino acids having one CO-NH bond, and other enzymes having their primary action on acyldipeptides. Fodor, Price, and Greenstein observe that

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[1]S. Otani, Acta Schol. Med. Univ. Imp. Kioto, 17, 163-196 (1934).

[2]T. Kazama, Acta Schol. Med. Univ. Imp. Kioto, 23, 154-159 (1939).

[3]H. Mayeda, Acta Schol. Med. Univ. Imp. Kioto, 18, 199-204 (1936).

the rates of hydrolysis by hog kidney extract on chloroacetylglycine and chloroacetylglycylglycine are 133 and 6, respectively[1]. Matsui observed that chloroacetylglycine is hydrolyzed 71%, and chloroacetylglycylglycine is hydrolyzed 91% by the globulin fraction from a glycerin extract of hog kidney, under the same conditions[2]. In contrast, a heat-d sucrose solution extract broke down chloroacetylglycine 86%, and chloroacetylglycylglycine 18%; and for an autolysate of hog kidney the figures were 100% and 0%, respectively[2]. The sucrose extract failed to hydrolyze hippurylglycine, hippurylasparagine, or hippuryltyrosine, but did hydrolyze chloroacetylasparagine and chloroacetyltyrosine[2]; similar results were obtained by Mori[3]. Utzino and Nakayama observed that on heating hog kidney maceration juice at 70° for 15 minutes, benzoyldiglycineacylase activity disappeared while appreciable benzoylglycineacylase activity remained[4].

Further observations on hydrolysis of acyldipeptides by hog kidney enzymes will be mentioned. Yosioka observed

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[1]P. J. Fodor, V. E. Price, and J. P. Greenstein, J. Biol. Chem., 178, 503-509 (1949).

[2]K. Matsui, J. Biochem. (Japan), 33, 183-199 (1941).

[3]H. Mori, J. Biochem. (Japan), 29, 225-240 (1939).

[4]S. Utzino and M. Nakayama, Enzymologia, 8, 280-288 (1928).

appreciable enzyme activity on benzoyldiglycine and benzoylglycine by macerations of hog kidney at pH 7.3[1]. Bondi and Eissler isolated lauric acid and glycine from a 7 day digest under toluene of laurylalanylglycine with hog kidney brei[2]. Nishihira called a purified preparation, "dipeptidopeptidase", because it split out terminal dipeptides from benzoyltriglycine, tetraglycine, benzoyltetraglycine, and benzoyldiglycylasparagine[3]. Dipeptidopeptidase did not attack benzoyldiglycine or benzoylglycine. In fact, the writer has not found a mention in the literature of an acyldipeptide containing two CO-NH bonds being enzymatically hydrolyzed with the primary attack being on the CO-NH bond second from the carboxyl end. However, the point of enzymatic attack on acyldipeptides has often not been determined.

A qualitative summary of acylase activity in preparations from certain swine components, other than kidney, is given in Table 27.

Kimura found that the pH optimum for hog liver acylase action on acetylglycine, acetyl-DL-leucine, and acetyl-DL-phenylalanine was about 7.2[4]. Acetylglycine and acetyl-DL-

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[1]M. Yosioka, Enzymologia, 10, 154-160 (1941).

[2]S. Bondi and F. Eissler, Biochem. Z., 23, 510-513 (1910).

[3]M. Nishihira, Enzymologia, 9, 356-363 (1941).

[4]H. Kimura, J. Biochem. (Japan), 10, 225-250 (1929).

Table 27

Acylase Activity in Some Porcine Components*

Substrate	Source of enzyme preparation				
	Liver	Spleen	Skeletal muscle	Intestinal mucosa	Leucocytes
Benzoylglycine	0[4], ?[1], +[2,6]	+[2]		0[2]	
Acetylglycine	+[1,4]				
Formylglycine	+[1]				
Chloroacetylglycine	+[4]				
Phenylacetylglycine	+[6]				
Phenylpropionylglycine	+[2]	0[2]		0[2]	
Phenylfurfuroylglycine	0[3]				
Phenyl-α,β-dibromo-propionylglycine	0[2]	0[2]			
N-(pyridylcarboxyl)-glycine	?[2]	?[2]			
Phenyl-α-bromo-β-oxypropionylglycine	+[2]	0[2]			
Cinnamoylglycine	+[2]	?[2]		0[2]	
Furfuroylglycine	+[7]	+[7]		0[7]	
Furylpropionylglycine	+[7]	+[7]		0[7]	

*The symbols used and their meaning are as follows: +, hydrolysis observed; 0, no hydrolysis observed; ?, hydrolysis so slight as to be questionable. Superscripts refer to references which are found on page 105.

Table 27 (Continued)

Substrate	Source of enzyme preparation				
	Liver	Spleen	Skeletal muscle	Intestinal mucosa	Leucocytes
Furylacrylglycine	$+^{7}$	$+^{7}$		0^{7}	
Phenylhippuric acid	0^{3}	0^{3}			
Furfuroylisoserine		0^{3}			
Benzoylalanine	$+^{6}$		$+^{6}$		
Furfuroyl-DL-alanine		$+^{3}$			
Acetyl-DL-leucine	$+^{1}$				
Formyl-DL-leucine	$+^{1}$				
Benzoyl-L-leucine	$+^{16}$				
Furfuroylisoserine		0^{3}			
Benzoylasparagine	0^{4}		0^{4}		
Acetylasparagine	$+^{4}$				
Chloroacetylasparagine	$+^{4}$				
Benzoylaspartic acid	0^{4}		0^{4}		
Benzoylglutamic acid	0^{6}		0^{6}		
Ornithuric acid	0^{6}		0^{6}		
Acetyl-DL-phenylalanine	$+^{1}$				
Formyl-DL-phenylalanine	0^{1}				
Benzoyl-DL-phenylalanine	$+^{16}$				
Monobenzoyltyrosine	$+^{16,0^{6}}$		0^{6}		

Table 27 (Continued)

Substrate	Source of enzyme preparation				
	Liver	Spleen	Skeletal muscle	Intestinal mucosa	Leucocytes
Dibenzoyltyrosine	0^{6}		$+^{6}$		
Chloroacetyl-L-tyrosine	0^{4}, $+^{10}$			$+^{14}$	$+^{5}$
Benzoylglycylglycine	$^{9,10,12}_{0^{4,11}}$ $+^{10}$			$+^{14}$	
Phthaloyldiglycine	$^{+10}_{0^{9,11}}$			0^{13}	
Carboethoxyglycyl-DL-leucine	$+^{10}$				
Chloroacetylalanine				$+^{14}$	
Chloroacetylleucine				$+^{14,15}$	
Chloroacetyl-L-phenylalanine				$?^{15}$	
Acetyl-DL-phenylalanyl-DL-alanine				$+^{8}$	
Benzoylglycylasparagine	0^{4}				
Benzoylglycyltyrosine	0^{4}				

References to Table 27

1. H. Kimura, J. Biochem. (Japan), 10, 225-50 (1929).
2. S. Utzino, S. Tsunoo, and T. Mori, J. Biochem. (Japan), 26, 439-47 (1937).
3. Ibid., 477-482 (1937).
4. K. Matsui, J. Biochem. (Japan), 33, 183-199 (1941).
5. R. Willstatter, E. Bamann, and M. Rohdewald, Z. Physiol. Chem., 153, 267-280 (1929).
6. T. So, J. Biochem. (Japan), 12, 107-131 (1930).
7. S. Utzino, S. Tsunoo, and T. Mori, J. Biochem. (Japan), 26, 449-53 (1937).
8. E. Waldschmidt-Leitz, W. Grassmann, and A. Schaffer, Ber. Deut. Chem. Ges., 60, 359-364 (1927).
9. T. Sugawara, Tohoku J. Expt. Med., 48, 171-176 (1944).
10. E. Waldschmidt-Leitz, A. Schäffner, J. J. Bek, and E. Blum, Z. Physiol. Chem., 188, 17-47 (1930).
11. S. Utzino, J. Biochem. (Japan), 9, 465-481 (1928).
12. S. Utzino and F. Sakai, J. Biochem. (Japan), 33, 457-465 (1941).
13. S. Utzino, J. Biochem. (Japan), 9, 453-463 (1928).
14. A. K. Balls and F. Köhler, Ber. Deut. Chem. Ges., 64, 383-387 (1931).
15. M. Hino, Tohoku J. Expt. Med., 47, 344-347 (1944).
16. T. Kawa, Acta Schol. Med. Univ. Imp. Kioto, 11,121-129 (1928).

leucine were broken down at about equal rates while acetyl-DL-phenylalanine was just slightly hydrolyzed at the one enzyme concentration used. The corresponding formyl derivatives and benzoylglycine and benzoyl-DL-phenylalanine were less readily hydrolyzed.

Utzino and Sakai described the action of two purified fractions from swine liver, "cathepsin", and "ereptase", on benzoyldiglycine[1]. "Cathepsin" broke down benzoyldiglycine to benzoylglycine with a greater rate at pH 4.5 than 7.2. "Erepsin" hydrolyzed benzoyldiglycine and benzoylglycine at pH 7.2 in the absence of cysteine, but did not attack benzoyldiglycine appreciably at pH 4.5 in the presence of cysteine. "Cathepsin" did not hydrolyze benzoylglycine appreciably at pH 4.5. A similar report was given earlier by Waldschmidt-Leitz, Schäffner, and Bek, who stated that benzoyldiglycine is hydrolyzed with H_2S activation at a pH optimum of about 4-4.5 by a glycerol extract of dried hog liver[2]. Also hydrolyzed by the hog liver preparation were chloroacetyl-L-tyrosine, carbethoxyglycyl-DL-leucine, and phthalylglycylglycine; the hydrolysis of these compounds

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[1] S. Utzino and F. Sakai, J. Biochem. (Japan), 33, 457-465 (1941).

[2] E. Waldschmidt-Leitz, A. Schäff J. J. Bek, and E. Blum, Z. Physiol. Chem., 188, 1747 (1930).

was described as being due to a carboxypeptidase. Hydrolysis of these three compounds was activated by an enzyme preparation from spleen. It is conceivable that the liver enzyme mentioned here, which hydrolyzed benzoyldiglycine with H_2S activation at a somewhat acid pH optimum[1], is similar to the carboxypeptidases of hog kidney, bovine kidney, and bovine spleen, which attacks carbobenzoxyglycyl-L-phenylalanine and carbobenzoxyglycyl-L-tyrosine with cysteine activation at a similar pH optimum.

Some fractionation of acylase activity from hog intestinal mucosa was achieved by Balls and Kohler as Table 28 shows.

Table 28

Fractionation[1] of Glycerol Extract of Hog Intestinal Mucosa

Substrate	pH	Hydrolysis* as n/10 NaOH by Solution		
		B	C	D
Chloroacetylalanine	7.8	.11	.67	.64
Chloroacetylleucine	7.8	.40	.86	.32
Chloroacetyltyrosine	7.4	.07	.05	---

[1]A. K. Balls and F. Kohler, Ber. Deut. Chem. Ges., 64 383-387 (1931).

*At same enzyme concentration. 20 hour incubation.

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[1]E. Waldschmidt-Leitz, A. Schäffner, J. J. Bek, and E. Blum, Z. Physiol. Chem., 188, 17-47 (1930).

Fractions B and C hydrolyze chloroacetylleucine more rapidly than chloroacetylalanine, whereas, the converse is true for solution D. As seems to be generally true for preparations from mammalian tissues, other than the pancreas (after activation), chloroacetyltyrosine is not appreciably broken down (Table 28).

Present in hog gastric mucosa is a proenzyme, pepsinogen, which is activated by acid to pepsin[1]. Pepsin is one of the proteinases of gastric juice and acts optimally on proteins at a pH of about 2. Crystalline pepsin, in addition to being an endoprotease, is an acylase as shown by its ability to split off the terminal amino acid of certain peptides or peptide derivatives containing tyrosine and phenylalanine, linked to other or the same amino acids. Thus carbobenzoxy-L-tyrosine is hydrolyzed to carbobenzoxy-L-glutamic acid and tyrosine[2]. The rate of hydrolysis by pepsin is greatest for those synthetic substrates in which two aromatic amino acids (phenylalanine or tyrosine) are linked to each other; the two amino acids can be different or the same[3]. It has

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[1]E. L. Smith. Proteolytic enzymes. In J. B. Sumner and Karl Myrback, eds. The enzymes. Vol. 1, Part 2. p. 840-845. New York, New York, Academic Press Inc. 1951.

[2]J. S. Fruton and M. Bergmann, J. Biol. Chem., 127, 627-641 (1939).

[3]L. E. Baker, J. Biol. Chem., 193, 809-819 (1951).

previously been stated that the pH optimum for the hydrolysis of certain substrates including carbobenzoxy-L-glutamyl-L-tyrosine is about 4[1], but more recently Baker[2] has shown that the pH optimum depends on conditions for this compound; the optimum is about pH 2 at low substrate and enzyme concentrations. Data from a review on proteolytic enzymes by Emil Smith[1] and from several original articles in the literature are presented in Table 29 to indicate the substrate preferences of porcine pepsin.

As is seen on examining Table 29, apparently a free carboxyl group, such as in a glutamyl residue or carboxyl-terminal amino acid, promotes hydrolysis by porcine pepsin. Baker[2] observed no hydrolysis of carbobenzoxy-L-phenylalanyl-L-phenylalanamide (Table 29) after prolonged hydrolysis. However, this compound is quite insoluble, and Baker states, ".....conclusive proof for the necessity of a carboxyl group in this class of substrate must await the availability of a more soluble substrate in which the carboxyl group is blocked." It would seem best to say that pepsin is not a carboxypeptidase since a carboxyl-terminal amino acid is not an absolute

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[1]E. L. Smith. Proteolytic enzymes. In J. B. Sumner and Karl Myrback, eds. The enzymes. Vol. 1, Part 2, p. 840-845. New York, New York, Academic Press Inc. 1951.

[2]L. E. Baker, J. Biol. Chem., 193, 809-819 (1951).

Table 29

Substrate Preferences of Crystalline Porcine Pepsin

Substrate	pH	Hours incubation	Substrate M	mg Protein N per ml	Lit. Ref.	% Hydrolysis
N-Acetyl-L-tyrosyl-L-tyrosine	2.0	4	.001	0.12	1	34
Acetyl-L-phenylalanyl-L-phenylalanine	2.0	3.25	.0005	0.05	1	68
N-Carbobenzoxy-L-tyrosyl-L-phenylalanine	2.0	5	.0005	0.10	1	29
N-Acetyl-D-tyrosyl-L-tyrosine	2.0	48	.004	0.12	1	0
Acetyl-D-phenylalanyl-L-tyrosine	2.0	48	.004	0.12	1	0
Acetyl-D-phenylalanyl-L-diiodotyrosine	2.0	48	.0005	0.12	1	0
N-Acetyldehydrotyrosyl-L-tyrosine	2.0	24	.004	0.12	1	0
Acetyldehydrophenylalanyl-L-tyrosine	2.0	24	.004	0.12	1	0
N-Carbobenzoxy-O-acetyl-L-tyrosyl-L-phenylalanine	2.0	24	.002	0.12	1	0
Carbobenzoxy-L-phenylalanyl-L-phenylalanine amide	2.0	144	.002	0.1	1	0

Table 29 (Continued)

Substrate	pH	Hours incubation	Substrate M	mg Protein N per ml	Lit. Ref.	% Hydrolysis
Carbobenzoxy-L-glutamyl-L-tyrosine	2.0	72	.002	0.06	1	16
Carbobenzoxy-L-glutamyl-L-tyrosine	4.0	24	.05	1.4	2	53
Carbobenzoxy-L-glutamyl-L-phenylalanine	4.0	24	.05	1.4	2	28
Carbobenzoxy-L-glutaminyl-L-phenylalanine	4.0	24	.05	1.4	2	17
Carbobenzoxyglycyl-L-tyrosine	4.0	24	.05	1.4	2	10
Carbobenzoxy-L-glutamyl-L-tyrosylglycine	4.0	24	.05	1.4	2	39
Carbobenzoxyglycyl-L-glutamyl-L-tyrosine	4.0	24	.05	1.4	2	48
Glycyl-L-glutamyl-L-tyrosine	4.0	24	.05	1.4	2	32
L-Glutamyl-L-tyrosine	4.0	24	.05	1.4	2	1

Table 29 (Continued)

Substrate	pH	Hours incubation	Substrate M	mg Protein N per ml	Lit. Ref.	% Hydrolysis
Carbobenzoxytyrosylcysteine*	4.0	48	.02	1.46**	3	39
Carbobenzoxytyrosylcystine*	4.0	48	.02	1.46**	3	7
Carbobenzoxycysteyltyrosine*	4.0	48	.02	1.46**	3	53
Carbobenzoxycystyltyrosine*	4.0	48	.02	1.46**	3	21
Carbobenzoxy-S-benzylcysteyltyrosine*	4.0	48	.02	1.46**	3	26
Tyrosylcysteine*	4.0	48	.01	1.46**	3	22
Tyrosylcystine*	4.0	48	.01	1.46**	3	25
Cysteyltyrosine*	4.0	48	.01	1.46**	3	31
Cystyltyrosine*	4.0	48	.01	1.46**	3	5

*Compounds are the L forms.

**The enzyme concentration was described as being 1% pepsin; this was converted to protein nitrogen by using the nitrogen content of pepsin, 14.6%, as given by J. K. Northrop, M. Kunitz, and R. M. Herriott. Crystalline enzymes. 2nd ed., rev. p. 74. N. Y., Columbia University Press. 1948.

Table 29 (Continued)

Substrate	pH	Hours incubation	Substrate M	mg Protein N per ml	Lit. Ref.	% Hydrolysis
Carbobenzoxy-L-glutamyl-L-tyrosineamide	4.0	68	.05	1.4	4	25
Carbobenzoxy-L-glutaminyl-L-tyrosine-amide	4.0	68	.05	1.4	4	5
Chloroacetyl-L-tyrosine	4.0	24	.05	1.4	4	0
Carbobenzoxy-L-glutamyl-L-diiodo-tyrosine	4.0	24	.05	1.6	4	0
Carbobenzoxy-L-glutamyl-L-glutamic acid	4.0	96	.05	1.6	4	5
Carbobenzoxy-L-glutamylglycine	4.0	96	.05	1.6	4	5
Carbobenzoxy-L-tyrosyl-L-tyrosine	4.0	96	.05	1.6	4	29
Carbobenzoxyglycyl-L-tyrosine	4.0	96	.05	1.6	4	43
Carbobenzoxy-L-methionyl-L-tyrosine	4.0	24	.05	1.2	5	35
L-methionyl-L-tyrosine	4.0	24	.05	1.2	5	25

References to Table 29

1. L. E. Baker, J. Biol. Chem., 193, 809-819 (1951).

2. E. L. Smith. Proteolytic enzymes. In J. B. Sumner and K. Myrbäck, eds. The enzymes. Vol. I, Part 2. p. 844. New York, New York, Academic Press Inc., 1951.

3. C. R. Harrington and R. V. Pitt Rivers, Biochem. J., 38, 417 (1944).

4. J. S. Fruton and M. Bergmann, J. Biol. Chem., 127, 627-641 (1939).

5. C. A. Dekker, S. P. Taylor, and J. S. Fruton, J. Biol. Chem., 180, 155-173 (1949).

requirement for a substrate break-down, but that pepsin is probably similar to carboxypeptidase since pepsin hydrolysis is facilitated by a carboxyl group rather near to the bond broken.

For a discussion of the kinetics of pepsin-catalyzed hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine and its ethyl ester see the paper by Casey and Laidler[1].

Enzymes from the gastric juice of cows, sheep, and chicken possess substrate specificities very similar to those of porcine pepsin for a group of substrates (those of reference 2 of Table 29) and hence are described as

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[1]E. J. Casey and K. J. Laidler, J. Am. Chem. Soc., 72, 2159-2164 (1950).

pepsins[1]. Porcine pepsin differs immunologically from chicken pepsin but not from bovine pepsin[2].

In 1886 Nenchi reported the hydrolysis of hippuric acid by minced pancreas (probably swine pancreas) in the presence of a 0.5% phenol solution[3]. Also a commercial enzyme preparation from the pancreas of an unstated species, Grubler "trypsin", split hippuric acid[3]. In 1903, Emil Fisher and Peter Bergell described certain enzymatic properties of another commercial pancreatic enzyme preparation, pancreatin, from the firm of Rhenania-Aachen[4]. Pancreatin acted on peptone from silk fibroin, causing the crystallization from solution within 15 minutes of tyrosine; no glycine or alanine were detectable after incubation for a day. Also hydrolyzed were β-naphthalinsulfoglycyl-L-tyrosine, carbethoxyglycyl-DL-leucine, and carbethoxyglycyltyrosine; tyrosine and leucine, respectively, were isolated from enzymatic digests of the first two compounds mentioned. Not hydrolyzed were β-naphthalinsulfoglycyl-DL-leucine,

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[1]E. L. Smith, Proteolytic enzymes. In J. B. Sumner and K. Myrbäck, ed. The enzymes. Vol. I, Part 2. p. 844. New York, New York, Academic Press Inc. 1951.

[2]R. M. Herriott, C. R. Bartz, and J. H. Northrop, J. Gen. Physiol. 20, 797 (1937).

[3]M. Nenchi, Arch. Exp. Path., 20, 367-384 (1886).

[4]Emil Fisher and Peter Bergell, Ber. Deut. Chem. Ges. 36, 2592-2608 (1903).

β-naphthalinsulfo-L-alanylglycine, and glycyl-L-alanine; also no hydrolysis of hippuric acid was detected. Probably pH control was poor in these experiments of Fisher and Bergell. In 1910, Bondi and Eissler reported slight hydrolysis of butyrylalanylglycine and no hydrolysis of laurylalanylglycine by Kohlbaum "trypsin"[1].

It was due to the work and thought of Waldschmidt-Leitz and his collaborators during the 1920's that the term and concept of carboxypolypeptidase (later carboxypeptidase) arose. In 1925, Waldschmidt-Leitz and Harteneck removed by adsorption a leucylglycine hydrolyzing activity from the glycerin extract of hog pancreas[2]. The remaining material hydrolyzed gelatin and was called "trypsin". "Trypsin" later proved to be enzymatically impure. In 1928, Waldschmidt-Leitz, Schaffner, Schlatter, and Klein reported that "trypsin" hydrolyzed β-naphthalinsulfonylglycyltyrosine and certain tri and higher peptides containing tyrosine in the terminal position, but not glycyltyrosine[3]. They concluded that a free amino group is not necessary for the action of "trypsin", that attack took place on the carboxyl group, and that

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[1]S. Bondi and F. Eissler, Biochem. Z., 23, 510-513 (1910).

[2]E. Waldschmidt-Leitz and A. Harteneck, Z. Physiol. Chem., 147, 286-308 (1925).

[3]E. Waldschmidt-Leitz, A. Schaffner, H. Schlatter, and W. Klein, Ber. Deut. Chem., Ges., 61, 299-306 (1928).

"trypsin" attacks peptides with tyrosine at the carboxyl end. Waldschmidt-Leitz and Klein observed that acylation of certain dipeptides renders them susceptible to "trypsin" hydrolysis[1]. They report the hydrolysis by "trypsin-kinase" of carbethoxyglycyl-leucine, acetylglycylglycine, acetylphenylalanylalanine, benzoyldiglycine, and phthalyl-diglycine, but detected no hydrolysis of benzoylpentaglycine, or of glycylleucinamide. The "kinase" was from porcine intestinal mucosa and served as an activator. A further step in the development of the theory of protease specificity was the conclusion by Waldschmidt-Leitz and Klein that a free carboxyl group is needed for the attack of "trypsin"[2]. Observations concerning the action of "trypsin" are reproduced in Table 30.

In 1929, Waldschmidt-Leitz and Purr introduce the term, carboxy-polypeptidase, to describe the polypeptide splitting component of pancreas-"trypsin"[3]. The term was used to distinguish this pancreas peptidase from the amino-polypeptidase of the intestine. The carboxy-polypeptidase

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[1] E. Waldschmidt-Leitz and W. Klein, Ber. Deut. Chem. Ges., 61, 640-645 (1928).

[2] E. Waldschmidt-Leitz and W. Klein, Ber. Deut. Chem. Ges., 61, 2092-2096 (1928).

[3] E. Waldschmidt-Leitz and A. Purr, Ber. Deut. Chem. Ges., 62, 2217-2226 (1929).

Table 30

Enzymatic Behaviour toward Amino Acid Derivatives by Preparation from Hog Pancreas as Reported by Waldschmidt-Leitz and Klein in 1928[1].

Substrate	% Hydrolysis by "Trypsin"	% Hydrolysis by "Trypsin-Kinase"
Benzoyl-DL-alanyl-decarboxyleucine	0	0
DL-bromoisocaproonyldiglycine	0	0
DL-bromoisocaproonylglycyl-L-tyrosine	65	75
Chloroacetyl-DL-phenylalanine	31	45
Chloroacetyl-L-tyrosine	18	38
Carbethoxyglycyl-L-tyrosine	32	66
Benzoylglycyl-L-tyrosine	77	88
β-Naphthallinsulfonyl-L-tyrosine	0	0
L-phenylalanyl-L-arginine	25	69

[1] E. Waldschmidt-Leitz and W. Klein, Ber. Deut. Chem. Ges., 61, 2092-2096 (1928).

described in this paper had been separated from the gelatin splitting component of the so-called "trypsin". Additional properties, other than the peptidase properties of the old "trypsin" which had been mentioned, are that carboxy-polypeptidase split chloroacetylleucine and clupein but not leucylglycylglycine. The pH optimum for chloroacetyltyrosine splitting was found to be pH 7.4.

About this time, Kawa failed to detect hydrolysis of benzoylglycylglycine by a commercial preparation, Grubler "trypsin," which did hydrolyze benzoyl-DL-leucylglycine[1].

Some additional observations on the enzymatic behaviour of swine pancreas preparations (species not always clearly stated) are given in Table 31.

Saito and Saito[2] reported that 10^{-2} molar cyanide inhibits the hydrolysis of chloroacetyltyrosine by a carboxypeptidase preparation. They also studied the effect of various metal salts on the enzyme action and their findings include the observation of some activation by $CoSO_4$, $NiSO_4$, $FeSO_4$, and several iron complex salts.

Can the acylase activity of hog pancreas be explained in terms of a carboxypeptidase identical in specificity with that of the carboxypeptidase from cow pancreas which has

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[1] T. Kawa, J. Biochem. (Japan), 10, 277- (1929).

[2] T. Saito and K. Saito, J. Biochem. (Japan), 40, 261-263 and 265-271 (1953).

Table 31

Some Observations on Acylase Activity of Porcine Pancreas Preparations

Substrate	Reference	Action* of enzyme preparation
β-Naphthalinsulfoglycylglycine	1	0
β-Naphthalinsulfoglycyl-L-leucine	1	+
β-Naphthalinsulfoglycyl-DL-phenylalanine	1	+
β-Naphthalinsulfo-DL-leucylglycine	1	0
β-Naphthalinsulfo-DL-leucyl-L-leucine	1	0
Chloroacetyl-m-aminobenzoic acid	2	+
Chloroacetyl-o-aminobenzoic acid	2	0
Chloroacetyl-p-aminobenzoic acid	2	0
Chloroacetyl-L-alanine	3	0
DL-leucylglycyl-L-tyrosine	3	0
Furfuroylglycine	4	+
Furylpropionylglycine	4	+
Furylacrylglycine	4	0
Chloroacetyl-L-tryptophan	5	+

*The symbols used and their meaning are as follows: +, hydrolysis observed; 0, no hydrolysis observed; ?, hydrolysis so slight as to be questionable. Superscripts refer to references which are found on page 123.

Table 31 (Continued)

Substrate	Reference	Action* of enzyme preparation
Leucylglycyl-L-tyrosine	5	0
L-Alanylglycyl-L-tyrosine	5	0
Leucylglycylleucine	5	+
Pyruvoyl-DL-phenylalanine	6	+
Carbobenzoxy-L-tyrosyl-L-tyrosine	7	+
Carbobenzoxyglutaminyl-L-tyrosine	7	+
L-Glutaminyl-L-tyrosine	7	0
α-Furfuroyl-DL-alanine	8	+
Furfuroylisoserine	8	?
Phenylhippuric acid	8	0
Hippuric acid	9	0
Phenylpropionylglycine	9	0
Phenyl-α, β-dibromopropionylglycine	9	0
Cinnamoylglycine	9	0
N-(Pyridylcarboxyl)-glycine	9	+
Phenyl-α-bromo-β-oxypropionylglycine	9	0
Chloroacetyl-L-prolyl-L-phenylalanine	10	+
DL-α-Bromopropionyl-L-prolyl-L-phenylalanine	10	+
DL-α-Bromoisocapronyl-L-prolyl-L-alanine	10	+

Table 31 (Continued)

Substrate	Reference	Action* of enzyme preparation
Phthalylglycylglycine	11	+
DL-α-Bromoisocapronylglycine	12	+
DL-α-Bromoisocapronyl-DL-leucine	12	+
DL-α-Bromoisocapronyl-DL-tyrosine	12	+
β-Naphthalinsulfoglycyl-L-tyrosine	12	+
Carbobenzoxyglycylglycine	13	+
Glycylglutamic acid anhydride	14	+
Chloroacetyl-o-aminobenzoic acid	15	+
Chloroacetyl-p-aminobenzoic acid	15	+
Chloroacetyl-m-aminobenzoic acid	15	+
α-Bromoisocapronyl-o-aminobenzoic acid	14	+
α-Bromoisocapronyl-p-aminobenzoic acid	15	+
α-Bromoisocapronyl-m-aminobenzoic acid	15	+
α-Naphthalinsulfoglycylglycine	16	0
Chloroacetylglycylglycine	16	0
Benzoylglycine	16	?
Benzoyldiglycine	16	+

References to Table 31

1. S. Otani, Acta Schol. Med. Univ. Imp. Kioto, 17, 163-196 (1934).

2. E. Waldschmidt-Leitz and A. K. Balls, Ber. Deut. Chem. Ges., 64B, 45-48 (1931).

3. E. Abderhalden and E. Schwab, Fermentforschung, 12, 559-571 (1931).

4. S. Utzino, S. Tsunoo, and T. Mori, J. Biochem. (Japan), 26, 449-453 (1937).

5. E. Abderhalden and E. Schwab, Fermentforschung, 12, 432-461 (1931).

6. M. Bergmann and H. Schleich, Z. Physiol. Chem., 207, 235-240 (1932).

7. M. Bergmann, L. Zervas, L. Salzmann, and H. Schleich, Z. Physiol. Chem., 224, 17-26 (1934).

8. S. Utzino, S. Tsunoo, and T. Mori, J. Biochem. (Japan), 26, 447-482 (1937).

9. Ibid., 439-447 (1937).

10. E. Abderhalden and R. Merkel, Fermentforschung, 15, 1-23 (1938).

11. E. Abderhalden and G. Effkemann, Fermentforschung, 14, 27-42 (1933).

12. E. Abderhalden and H. Hanson, Fermentforschung, 16, 37-47 (1938).

13. E. Abderhalden, R. Abderhalden, H. Weidle, E. Baertich, and W. Morneweg, Fermentforschung, 16, 118-120 (1938).

14. M. Itibakasa, J. Biochem. (Japan), 32, 355-369 (1940).

15. T. Utsunomiya, J. Biochem. (Japan), 35, 103-118 (1942).

16. T. Kazama, Acta Schol. Med. Univ. Imp. Kioto, 23, 169-175 (1939).

been crystallized by Anson (see pages 64-70)? Emil Smith states that since it has been demonstrated that Anson's carboxypeptidase has a wide range of specificity, there is little basis for assuming the existence of more than one pancreatic carboxypeptidase[1]. Many of the observations previously mentioned support such a contention. However, certain data in the literature seem to the writer to raise the possibility that other carboxypeptidases are present in hog pancreas. Some of this evidence will now be considered. Also, material on other types of acylases will be considered.

Pertinent to the question under discussion are the proteolytic coefficients for the hydrolysis of chloroacetyl-L-tyrosine and carbobenzoxyglycylglycine by Anson's carboxypeptidase; these are--chloroacetyl-L-tyrosine, 1.65[2], and for carbobenzoxyglycylglycine, 0.0024[3]--that is, the rate ratios are about 690 to one, respectively. Benzoylglycylglycine and phthaloylglycylglycine, compounds often used with porcine pancreas preparations, would be expected to be attacked by

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[1]Emil L. Smith. Proteolytic enzymes. In J. S. Sumner and Karl Myrback, eds. The enzymes. Vol. 1. p. 805. New York, New York, Academic Press Inc. 1951.

[2]H. Neurath and G. W. Schwert, Chem. Rev. 46, 129 (1950).

[3]E. L. Smith. Proteolytic enzymes. In J. S. Sumner and Karl Myrback, eds. The enzymes. Vol. 1, Part 2, p. 806. New York, New York, Academic Press Inc. 1951.

Anson's carboxypeptidase at a rate similar to that for carbobenzoxyglycylglycine[1]. In the discussion that follows, mention will be made of data that seem to indicate that hog pancreas preparations attack benzoylglycylglycine and similar compounds at a relatively more rapid rate than would be expected for Anson's carboxypeptidase.

Data from a paper by Abderhalden and Effkemann[2] are presented in Table 32.

Table 32

Enzyme Activity[1] of Preparation from Hog Pancreas ("Trypsin-kinase")

Substrate	Amino Nitrogen Liberated
Chloroacetyl-L-tyrosine	1.15
Chloroacetyl-DL-leucine	.40
Phthalylglycylglycine	.54

[1]E. Abderhalden and G. Effkemann, Fermentforschung, 14 27-42 (1933).

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[1]See the preceding two references (page 124) for discussions on the relation between substrate structure and break-down by Anson's carboxypeptidase.

[2]E. Abderhalden and G. Effkemann, Fermentforschung, 14, 27-42 (1933).

The rate of breakdown by the hog pancreas preparation of phthaloylglycylglycine (Table 32) is much more rapid, as compared to the rate on chloroacetyl-L-tyrosine and chloroacetyl-DL-leucine, than would be expected for Anson's carboxypeptidase; but, unfortunately, the proteolytic coefficients are not available for all of these specific compounds. Also, there is a possibility that the "kinase" from intestinal mucosa, which was used as an activator in Abderhalden and Effkemann's experiment, may have been responsible for the breakdown of phthalyldiglycine. Very similar data were presented by Waldschmidt-Leitz and Purr[1].

Other observations of the hydrolysis of acylglycines by hog pancreas preparations have been recorded. As pointed out before, acylglycines would be expected to be very slowly hydrolyzed by Anson's carboxypeptidase. It has been mentioned (page 10) that Waldschmidt-Leitz and Klein observed the hydrolysis of acetylglycylglycine, benzoyldiglycine, and phthaloyldiglycine, by "trypsin-kinase". Also, Kawa (page 119) observed the hydrolysis of benzoyl-DL-leucylglycine by a commercial pancreatic preparation. Yosioka observed breakdown of benzoylglycylglycine at pH 7.3 by a maceration

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[1]E. Waldschmidt-Leitz and A. Purr, Ber. Deut. Chem. Ges., 62B, 2211-2226 (1930).

of swine kidney in a 120 hour digestion[1]. Kawaharada also observed benzoyldiglycine hydrolysis[2]. In an experiment by Kazama, benzoyldiglycine, and to a slight extent benzoylglycine, were hydrolyzed by glycerol extract of hog pancreas, but no splitting of chloroacetyldiglycine or β-naphthalinsulfoglycylglycine occurred[3]. Sugawara recorded that on heating a swine pancreas maceration at 75° for 30 minutes, activity toward chloroacetylglycylglycine, phthaloyldiglycine, and benzoyldiglycine is destroyed, but that activity toward chloroacetyl-L-leucine and chloroacetyl-L-phenylalanine still remains[4]. However, activity toward the latter two substrates was higher in both the unheated and heated solutions.

Kazama claims to have distinguished by acetone fractionation of hog kidney glycerin extract, enzyme activity toward chloroacetyl-L-phenylalanine, β-naphthalinsulfoglycyl-L-leucine, and benzoylglycyl-L-leucine[5]. The activity toward these compounds of three fractions from hog pancreas is

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[1] M. Yosioka, Enzymologia, 10, 154-160 (1941).

[2] M. Kawaharada, Tohoku J. Exptl. Med., 47, 85-91 (1944).

[3] T. Kazama, Acta Schol. Med. Univ. Imp. Kioto., 23, 169-175 (1939).

[4] T. Sugawara, Tohoku J. Exptl. Med., 48, 185-189 (1944).

[5] T. Kazama, Acta Schol. Med. Univ. Imp. Kioto., 23, 160-168 (1939).

presented in Table 33. The data (Table 33) seem to the writer to be fair but not conclusive evidence that separate enzymes hydrolyze each of the three substrates listed.

Table 33

Enzyme Activity at pH 7.65 of Three Fractions from Hog Pancreas According to Kazama[1]

Substrate	% Hydrolysis by fraction		
	3	4	5
Chloroacetyl-L-phenylalanine	83	31	0
Benzoylglycyl-L-leucine	68	53	30
β-Naphthalinsulfoglycyl-L-leucine	100	13	0

[1]T. Kazama, Acta Schol. Med. Univ. Imp. Kioto., 23, 160-168 (1939).

Matsui studied the enzyme properties of several different preparations from hog pancreas, and concluded that separate enzymes caused the hydrolysis of chloroacetylglycylglycine and benzoylglycylglycine[1]. He believed that the pancreas enzyme which hydrolyzed benzoylglycylglycine, also hydrolyzed benzoylglycyltyrosine, and benzoylglycylasparagine. He believed this was so since hippuryltyrosine was so rapidly

[1]K. Matsui, J. Biochem. (Japan), 33, 183-199 (1941).

hydrolyzed and since the activities on hippuryl derivatives of tyrosine, asparagine, and glycine appeared together. Table 34 presents some data from the paper by Matsui[1].

By examining Table 34, it can be seen that fraction 1 from hog pancreas hydrolyzed benzoylglycylglycine, but chloroacetylglycylglycine to only a slight extent. Fraction 5 hydrolyzed chloroacetylglycylglycine, but not benzoylglycylglycine. Matsui seemed to have had good evidence for supposing that separate enzymes hydrolyzed these two substrates. Only in fraction 3 of Table 34 is there chloroacetylglycine-acylase activity, and, therefore, the data of Matsui probably indicate the presence of an enzyme which attacks chloroacetylglycine, but which has no appreciable activity toward chloroacetylglycylglycine or benzoylglycine; if this enzyme acts on benzoylglycylglycine, it is at least the second enzyme which does so, since activity toward this substrate by fraction 3 is less than toward chloroacetylglycine.

The second enzyme fraction of Table 34 hydrolyzes benzoylglycylglycine 21%, and chloroacetyltyrosine 63%. Therefore, it appears that either an enzyme or enzymes differing from Anson's carboxypeptidase are present, or that an acylase corresponding to Anson's carboxypeptidase and also another

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[1] K. Matsui, J. Biochem. (Japan), 33, 183-199 (1941).

Table 34

Enzymatic Activity of Some Preparations from Hog Pancreas According to Matsui[1]

Substrate	% Hydrolysis by fractions from hog pancreas				
	1. Autolysate	2. Autolysate with H_2S	3. Heated sucrose extract	4. Purified autolysate	$MgSO_4$ extract
Acetylglycine	0	0			
Acetylasparagine	0	0			
Acetyltyrosine	13	3			
Chloroacetylglycine	0	0	92	0	0
Chloroacetylasparagine	0	0		0	
Chloroacetyltyrosine	90	63		0	
Benzoylglycylglycine	85	21	63	0	0
Benzoylglycylasparagine	98	90		0	
Benzoylglycyltyrosine	90	71		44	
Chloroacetylglycylglycine	5		0		24
Chloroacetylglycylasparagine	56				
Hippuric acid	0		0		
Glycylglycine	64	1		0	
Glycylasparagine	61	4			
Glycyltyrosine	58	6			

[1] K. Matsui, J. Biochem. (Japan), 33, 183-199 (1941).

acylase (or acylases) are present in the enzyme preparations from hog pancreas.

Matsui states that the hippurylamino acids which he studied are rapidly hydrolyzed to 100% (based on one bond), and that the percentage does not rise after this[1]. Dipeptidases were present but not benzoylglycineacylase activity. Therefore, he concludes that hydrolysis occurs between the hippuryl residue and the end amino acid.

According to several investigators, there is a carboxypeptidase in pig pancreas, protaminase, which splits off basic amino acids from the protamines. In 1925 Waldschmidt-Leitz and Harteneck reported that clupeinsulfate and histonesulfate are hydrolyzed by "trypsin" from hog pancreas[2]. Waldschmidt-Leitz, Ziegler, Schäffner, and Weil recorded in 1931 that a glycerol extract of hog pancreas attacks clupein and salmine with the liberation of free arginine; they named the responsible enzyme protaminase[3,4]. No significant amount of protaminase activity was detected in a preparation from

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[1]K. Matsui, J. Biochem. (Japan), 33, 183-199 (1941).

[2]E. Waldschmidt-Leitz and A. Harteneck, Z. Physiol. Chem., 149, 203-220 (1925).

[3]E. Waldschmidt-Leitz, F. Ziegler, A. Schäffner, and L. Weil, Z. Physiol. Chem., 197, 219-236 (1931).

[4]Also see E. Waldschmidt-Leitz and E. Kofranvi, Z. Physiol. Chem., 225, 148-150 (1933).

cow pancreas. Esterified clupein and salmine were not significantly attacked by protaminase, indicating that protaminase was a carboxypeptidase. Chloroacetyltyrosine activity was separated from enzyme activity toward clupein sulfate. The pH optimum for clupein sulfate hydrolysis was 8.0. Weil also states that protaminase, freed of carboxypeptidase activity toward chloroacetyltyrosine, hydrolyzed partially hydrolyzed clupein sulfate, but does not attack the methyl ester of clupein[1]. Waldschmidt-Leitz and Kofranyi described the use of protaminase and other enzymes to determine partially the structure of clupein[2]. Lebreton and Mocoroa reported the hydrolysis of scombrine at pH 7.4 by pancreatic juice; "kinase" increased the hydrolysis rate[3]. Portis and Altman found that a crude protaminase preparation hydrolyzed salmine with liberation of about one-half of the arginine bound in the molecule[4]. Arginase and urease were used to determine specifically liberated arginine. Trypsin and chymotrypsin hydrolyzed salmine without liberation of arginine.

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[1]L. Weil, J. Biol. Chem., 105, 291-299 (1934).

[2]E. Waldschmidt-Leitz and E. Kofranyi, Z. Physiol. Chem., 236, 181-191 (1935).

[3]M. E. Lebreton and F. Mocoroa, Compt. Rend., 92, 1492-1494 (1931).

[4]R. A. Portis and K. I. Altman, J. Biol. Chem., 169, 203-209 (1947).

Emil Smith reported that Anson's carboxypeptidase hydrolyzes salmine and stated that this observations raises the question of the possible identity of protaminase with carboxypeptidase[1]. A possible answer to the question may be found in the work of Calvery on the proteolysis of crystalline egg albumin[2]. Following exhaustive pepsin hydrolysis of the egg albumin, a protaminase preparation freed of proteinase and chloroacetyltyrosine activity hydrolyzed the treated egg albumin with the liberation of an additional 6% of the total amino nitrogen; this corresponded to the amino nitrogen of the basic amino acids in egg albumin. Anson's carboxypeptidase hydrolysis following the pepsin hydrolysis would liberate an additional 30% of the total amino nitrogen. However, the same end point was reached (in relation to amino nitrogen freed) by Anson's carboxypeptidase hydrolysis whether it followed pepsin, or pepsin plus protaminase exhaustive hydrolysis[2]. Possibly both Anson's carboxypeptidase and protaminase split off terminal basic amino acids from peptides, but only Anson's carboxypeptidase may be capable of hydrolyzing off other terminal amino acids from peptides as well.

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[1]E. L. Smith. Proteolytic enzymes. In J. B. Sumner and K. Myrbäck, eds. The enzymes. Vol. 1, Part 2. p. 828. New York, New York, Academic Press Inc. 1951.

[2]H. O. Calvery, J. Biol. Chem., 102, 73-89 (1933).

An unusual obeservation was made by Schmitz and Merten who reported hydrolysis of carbobenzoxyglycyl-D-leucine by an extract of hog pancreas[1]. The pH optimum was 7.8. The hydrolysis was activated by Co(II), but not by Mg(II). Manganese, zinc, and ferrous ions inhibited breakdown. Glycyl-D-leucine and D-leucylglycine were split by the hog kidney extract used. Schmitz and Merten thought possible conclusions, as a result of their experiments, were that D-peptidases were present in the extract or that Bergmann's polyaffinity theory was wrong.

The studies of Kimura[2] may indicate the presence in hog pancreatic tissue of acylases similar to those found in other mammalian tissues, and which differ from Anson's carboxypeptidase. The formol titration values for enzyme-substrate incubations at pH 7.2 are presented in Table 35.

It is seen (Table 35) that acetylleucine is more rapidly broken down than acetylglycine or acetylphenylalanine by the hog pancreas preparation in the 20 hour period. This would not be expected of Anson's carboxypeptidase. The increase in rate on acetyl-DL-phenylalanine in the 20-44 hour period (Table 35) is, at first appearance, anomalous. The increase

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[1] A. Schmitz and R. Merten, Z. Physiol. Chem., 278, 43-56 (1943).

Table 35

Hydrolysis at pH 7.2 of Acylamino Acids by Glycerol Extract of Hog Pancreas[1]

Substrate	Hydrolysis as ccm .10 KOH in	
	20 hours	44 hours
Acetylglycine	.30	.80
Acetyl-DL-leucine	.50	.55
Acetyl-DL-phenylalanine	.10	2.5
Formylglycine	0	.15
Formyl-DL-leucine	.20	.40
Formyl-DL-phenylalanine	.55	.55
Benzoylglycine	---	.05

[1]H. Kimura, J. Biochem. (Japan), 10, 225-250 (1929).

in rate may have been due to microbial contamination or conversion of a proenzyme to an enzyme; quite possibly an enzyme similar to Anson's carboxypeptidase was formed. Of the formyl derivatives (Table 35), formylphenylalanine is more rapidly hydrolyzed and it seems difficult to interpret this.

According to Abderhalden and Schwab[1], separate enzymes hydrolyze chloroacetyl-L-tyrosine and chloroacetyl-L-alanine;

[1]E. Abderhalden and E. Schwab, Fermentforschung, 12, 559-571 (1931).

but the evidence presented does not seem satisfactory to the writer. Silver nitrate is reported to inhibit chloroacetyl-L-tyrosine splitting by "trypsin-kinase", but to a slight extent phthaloyldiglycine splitting, and chloroacetyl-DL-leucine splitting completely[1]. Also, DL-leucylglycine inhibited the action of "trypsin" on chloroacetyl-L-tyrosine[1]. "Trypsin" from hog pancreas was reported to hydrolyze the DL-α-bromoisocapronyl derivatives of glycine, DL-leucine, and L-tyrosine while an impure carboxypeptidase preparation from cow pancreas failed to do so[2].

Certain of the more interesting points concerning the acylases of hog pancreas will be recapitulated. Quite probably hog pancreas contains the proenzyme of a carboxypeptidase which is similar in specificity to the bovine enzyme, Anson's carboxypeptidase, which preferentially splits off terminal phenylalanine, tyrosine, tryptophan, and leucine, from acylated dipeptides, polypeptides, and from haloacylated amino acids. Evidence exists for a second carboxypeptidase, protaminase, which splits off basic amino acids from the protamines and protamine derived peptides. It is rather likely that a separate acylase exists which hydrolyzes acylated dipeptides

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[1]E. Abderhalden and G. Effkemann, Fermentforschung, 14, 27-42 (1933).

[2]E. Abderhalden and H. Hanson, Fermentforschung, 16, 37-47 (1938).

containing terminal glycine, for instance, benzoyldiglycine and phthaloyldiglycine. The activity on these compounds could be due to the first enzyme mentioned in this paragraph; but since whether or not activity on these terminal-glycine compounds is observed, varies with the method of enzyme preparation and investigator, it is probable that a separate enzyme, differing from Anson's carboxypeptidase, is responsible for the hydrolysis of benzoyldiglycine and phthaloyldiglycine. In addition, a separate enzyme may exist which attacks chloroacetylglycylglycine.

As had been pointed out, Otani believes that three separate enzymes are present in hog pancreas which attack chloroacetyl-L-phenylalanine, benzoylglycyl-L-leucine, and naphthalinsulfoglycyl-L-leucine, respectively. Probably the first activity is due to an enzyme like Anson's carboxypeptidase. The acylamino acid portions of naphthalinsulfoglycyl-L-leucine and chloroacetylglycylglycine are rather similar in certain respects; it might be worthy of investigation to see if the same enzyme attacks these two substrates.

Some investigators observed hydrolysis of benzoylglycine by hog pancreas material and some did not. It is possible, if pancreas acylases are similar to those of other mammalian tissues, that this substrate is attacked by an enzyme differing from those previously mentioned in the summary.

Acetyl and formyl derivatives of leucine, glycine, and phenylalanine are attacked by hog pancreas acylases which probably include acylases differing from Anson's carboxypeptidase, but of unknown relation to the other acylases of swine pancreas which are discussed here.

Evidence has been presented that acylases differing from Anson's carboxypeptidase are present in hog pancreas. Probably the proenzyme of hog pancreas, corresponding to the proenzyme of Anson's carboxypeptidase, becomes functional only after secretion when it serves as a digestive enzyme. In view of the wide occurrence in mammalian tissues of acylases, it would not be surprising that hog pancreas would contain similar enzymes, which function as tissue enzymes, and not only as digestive enzymes as does Anson's carboxypeptidase.

p. The horse. Acylase activity has been detected in horse kidney toward benzoylglycine[1,2,3], (+)benzoyl-α-aminobutyric acid, benzoyl-L-leucine, glycocholic acid, and

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[1] V. Baccari and M. Pontecorvo, Bollettino della Societa Italiano di Biologia Sperimentale, 16, 329-331 (1941).

[2] I. A. Smorodinzew, Z. Physiol. Chem., 124, 123-131 (1923).

[3] I. A. Smorodinzew, J. Russ. Phys. Chem. Soc., 51, 156-182 (1919). Thanks are due to Mr. Sol Shulman who kindly furnished an English summary of this Russian article.

taurocholic acid, but activity has been found lacking toward (-)-benzoyl-α-amino-n-butyric acid, benzoyl-β-alanine, benzoyl-DL-β-aminobutyric acid, benzoyl-α-aminoisobutyric acid[1,2]. Abderhalden and Schwab[3] gave additional information (Table 36) on acylase activity of extracts from horse tissues.

Table 36

Acylase Activity[1] of Preparations from Horse Tissues at pH 8.0

Source of Enzyme Preparation	Hours	Substrate		
		Chloroacetyl-L-alanine	Chloroacetyl-L-tyrosine	Sarcosyl-L-tyrosine
Kidney	2	28	5	30
	13		57	
Suprarenal capsule	10	14	10	
Lung	15	33	0	42

[1]E. Abderhalden and E. Schwab, Fermentforschung, 14, 43-53 (1933).

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[1]I. A. Smorodinzew, Z. Physiol. Chem., 124, 123-131 (1923).

[2]I. A. Smorodinzew, J. Russ. Phys. Chem. Soc., 51, 156-182 (1919). Thanks are due to Mr. Sol Shulman who kindly furnished an English summary of this Russian article.

[3]H. Hanson, Fermentforschung, 14, 189-201 (1934).

The dried plasma of the horse hydrolyzed chloroacetyl-L-alanine, but had no appreciable activity on chloroacetyl-L-tyrosine[1]. The leucocytes of the horse have questionable activity toward chloroacetyl-L-tyrosine[2]. The high activity toward chloroacetyl-L-alanine of these preparations from the horse may indicate the presence of an enzyme like that found in acylase I of hog kidney.

q. Man. According to the work of Emil and Rudolph Abderhalden[3,4] human tissues usually contain acylases, but activity toward chloroacetyl-L-leucine is more general than chloroacetyl-L-tyrosine activity. Some data on the acylase activity of human organs, from two papers, is presented in Table 37; the studies included an investigation of the effect of age on acylase activity.

The effect of human gland extracts on chloroacetyl-L-leucine and β-naphthalinsulfoglycyl-L-leucine was also compared. These data are presented in Table 38.

Using glycerol extracts of human intestine and pancreas, Blum and Yarmoshkevich have found that much acylase activity

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[1]H. Hanson, Fermentforschung, 14, 189-201 (1934).

[2]R. Willstätter, E. Bamann, and M. Rohdewald, Z. Physiol. Chem., 153, 267-280 (1929).

[3]E. Abderhalden and R. Abderhalden, Fermentforschung, 17, 217-223 (1943).

[4]R. Abderhalden, Z. Alterforsch., 4, 124-137 (1943).

Table 37

Acylase Activity of Tissues and Fluids of Homo Sapiens[1,2]

Source of enzyme preparation	% Splitting by single preparation or as range by several preparations for substrate	
	Chloroacetyl-L-leucine	Chloroacetyl-L-phenylalanine
Kidney of fetus	70-90	
Kidney of adult	90-100	85-90
Kidney of aged	75-100	
Liver of fetus		35-52
Liver of adult	85-100	42-52
Liver of aged		32-37
Lung of fetus	25	22
Lung of adult	35	15
Lung of aged	32	10
Brain of fetus	17	16
Brain of adult	50	20
Brain of aged	52	25
Skeletal muscle of fetus	50	0
Skeletal muscle of adult	55	0

[1]E. Abderhalden and R. Abderhalden, Fermentforschung, 17, 217-223 (1943).

[2]R. Abderhalden, Z. Alterforsch., 4, 124-137 (1943).

Table 37 (Continued)

Source of enzyme preparation	% Splitting by single preparation or as range by several preparations for substrate	
	Chloroacetyl-L-leucine	Chloroacetyl-L-phenylalanine
Skeletal muscle of aged	55	0
Heart muscle of fetus	30	8
Heart muscle of adult	38	5
Heart muscle of aged	38	0
Testicle of adult	68	0
Testicle of aged	70	0
Uterus	5	0
Thyroid gland	100	0
Spleen	90	42
Thymus	45	-
Placenta	10	12
Cerebrospinal fluid	0	0
"Ovarialcystenflussigkeit"	0	0
Serum	0	0

Table 38

Acylase Activity on Chloroacetyl-L-Leucine and β-Naphthalinsulfoglycyl-L-Leucine by Extracts of Human Tissues[1]

Source of enzyme preparation	% Hydrolysis of	
	Chloroacetyl-L-leucine	β-Naphthalinsulfoglycyl-L-leucine
Thyroid gland	100	0
Skeletal muscle	55	25
Kidney	88	8
Pancreas	100	35

[1]E. Abderhalden and R. Abderhalden, Fermentforschung, 17, 217-223 (1943).

toward chloroacetyl-L-tyrosine appears by the 5th or 6th month of embryonic development[1]. Rudolf Abderhalden detected no hydrolysis of chloroacetyl-L-tyrosine or β-naphthalinsulfoglycyl-L-leucine by cerebrialspinal fluid[2]. Where carbonaphthoxyphenylalanine was the test substrate, procarboxypeptidase was found in the pancreas of, and carboxypeptidase in the duodenal fluid of man; no carboxypeptidase or procarboxypeptidase activity was present in the

[1]E. Blum and A. I. Yarmoshkevich, Bull. Biol. Med. Exptl. U.R.S.S., 1, 113-114 (1936).

[2]R. Abderhalden, Fermentforschung, 17, 173-177 (1943).

homogenates of the liver, kidney, spleen, duodenum, jejunum, stomach, colon, brain, heart, lung, adrenal gland, thyroid gland, ovary, testes, epididymis, serum, or urine[1]. Hayakawa studied the acylase activity of human gastric mucosa of patients afflicted with cancer or ulcers of this tissue; he found significant activity toward chloroacetyl-L-leucine, chloroacetyl-L-phenylalanine, and chloroacetyldiglycine in the stated order of magnitude[2]. No significant activity by the gastric mucosa was detected toward benzoyldiglycine or benzoylglycine. Acylase activity was higher in the gastric mucosa of ulcer patients than in that of cancer patients.

Extracts of human breast carcinoma breakdown carbobenzoxy-L-glutamyl-L-tyrosine and carbobenzoxy-D-glutamyl-L-tyrosine in the presence of cysteine at about pH 5[3]. The same investigators found that human bone sarcoma extract hydrolyzed carbobenzoxy-L-glutamyl-L-tyrosine, but not carbobenzoxy-D-glutamyl-L-tyrosine.

Secretin, which is a hormone having a molecular weight of 3000, was said to stimulate the secretion of carboxypep-

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[1]H. A. Ravin and A. M. Seligman, J. Biol. Chem., 190, 391-402 (1951).

[2]M. Hayakawa, Tohoku J. Exptl. Med., 53, 243-249 (1951).

[3]J. S. Fruton, G. W. Irving, and M. Bergmann, J. Biol. Chem., 132, 465-466 (1940).

tidase (chloroacetyl-L-tyrosine splitting) and trypsin by the human pancreas[1].

3. Acylases of algae

a. Spirogyra. Hippuric acid does not serve as a nutrient for Spirogyra cells nor is it markedly inhibitory[2].

4. Acylases of bacteria (Phylum schizomycophyta)

Van Tieghem stated in 1864, that it is a known fact that hippuric acid found in the urine of herbivacious animals is broken down by fermentation to benzoic acid[3]. He further states, that in his experiments carried out with ammonium hippurate in a nutrient solution, the splitting of hippuric acid to benzoic acid and glycine was always accompanied by the presence of "la torulacee de uree". Van Tieghem believed that this splitting proceeded parallel with the life and development of an organized vegetable ferment. In 1876, Hoppe-Seyler mentions the splitting of hippuric acid to

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[1]G. Agren, J. Physiol., 94, 553-559 (1939).

[2]T. Bokorny, Arch. Ges. Physiol. 172, 466-496 (1918).

[3]Van Tieghem, Compt. Rend. 58, 210-214 (1864).

glycine and benzoic acid, and the splitting of taurocholic acid to taurine and cholic acid[1] in the presence of dead lower organisms. No experimental details were given.

Several other papers possibly related to the acylases of bacteria will be briefly mentioned. According to Carbone and Rusconi, benzoylglycine is hydrolyzed to varying degrees by cultures of certain micrococci, bacilli, and bacterium[2]. Stapp describes the utilization of hippuric acid as a nutrient for the nitrogen and carbon source of seven bacilli[3]. Bacterium erythrogenes and Septosporium bifurcum Fre. used hippuric acid for growth[4].

a. Some species of water vibrio. Autolysates from 6 of 10 species of water vibrio hydrolyzed hippuric acid[5]. No activity toward benzoylglycine was detected in the remaining 4 species. Activity disappeared when the cultures were grown for three days instead of one day. The enzyme-substrate experiments were carried out at pH 7.0-7.6.

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[1]F. Hoppe-Seyler, Pflugers Arch. 12, 1-17 (1876).

[2]D. Carbone and A. Rusconi, Bollettino della Societa Medico-Chirureica di Pavia, V, 15, 382-389 (1910).

[3]C. Stapp, Centralb. Bakt. Parasitenk., II Abt., 51, 1-71 (1920).

[4]S. Bierems, Centralb. Bakt. Parasitenk., II Abt., 23, 109-110 (1909).

[5]K. Oshima, Japan J. Med. Sci. II, Biochem., 5, 101-122 (1944).

b. Staphylococcus aureus. Imaizumi studied the acylases of an extract of autolyzed Staphylococcus aureus[1]. Some of his results are presented in Table 39. The observed activities (Table 39) seemed to group themselves into three groups based on their behaviour when heated at different temperatures[1]. These groups were: stable at 70° but inactivated at 80°, benzoylglycine; some loss of activity at 60° and great or complete loss of activity at 70°, benzoyldiglycine, acetylglycine, and DL-bromoisocapronylglycine; only partial loss of activity at 80° but complete loss at 100°, chloroacetyl-L-phenylalanine.

Kameda and Toyoura report the yields of benzoic acid from the incubation of one gram portions of hippuric acid, benzoyl-DL-α-aminobutyric acid, and benzoyl-DL-phenylalanine in the presence of Staphylococcus aureus (Terashima); these yields were, respectively, as mg of benzoic acid: 525, 51 and 0[2]. It was reported that centrifugates from bacterial digests hydrolyzed chloroacetylasparagine and benzoylasparagine to a moderate extent, but the hydrolysis was ascribed to amidase action since no benzoic acid was isolated from

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[1] M. Imaizume, J. Biochem. (Japan), 27, 199-211 (1938).

[2] Y. Kameda and E. Toyoura, J. Pharm. Soc. Japan, 72, 400-402.

Table 39

Acylase Activity of Extract of Autolyzed *Staphylococcus aureus*[1]

Substrate	pH	ccm 0.1 n NaOH after 24 hours	pH optimum
Benzoylglycine	7.5	1.15	7.0-8.0
Benzoyldiglycine	8.0	1.42	
Phthaloylglycine	7.0	0	
Phthaloyldiglycine	8.0	0	
Acetylglycine	8.0	0.96	7.0-8.0
Acetyldiglycine	8.0	0	
Formylglycine	7.5	0	
Formyl-L-tyrosine	7.5	0	
Acetyl-L-glutamic acid	7.5	0	
Benzoyl-L-glutamic acid	7.0	0	
Chloroacetyl-L-phenylalanine	7.0	1.35	
Chloroacetyl-L-tyrosine	7.0	0.3	7.0
DL-α-Bromoisocapronylglycine	7.0	0.65	7.0
DL-α-Bromopropionylglycine	7.0	0.42	

[1]M. Imaizume, J. Biochem. (Japan), 27, 199-211 (1938).

the digest of the benzoyl compound[1]. Pulverized cells of Staphylococcus aureus failed to break down dibenzoylornithine or benzoyldiglycine while they did break down benzoylglycine[2]. Enzyme preparations from the dried cells or the culture filtrate of Staphylococcus aureus failed to attack chloroacetyl-L-phenylalanine or benzoyldiglycine[3].

The effect of some acylamino acids as inhibitors of the growth of Staphylococcus (Terashima strain) has been described[4,5]. The most effective antibacterial agents are lauryl-DL-phenylalanine, and o-laurylaminobenzoic acid, followed by lauryl-DL-valine, lauryl-DL-α-amino-n-butyric acid, lauryl-DL-alanine[4,5]. Laurination of the amino acids was more effective in producing antibacterial compounds than was caprinylation. It seems possible that antibacterial

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[1]S. Utzino and M. Imaizumi, Z. Physiol. Chem., 253, 51-64 (1938).

[2]S. Tomota and H. Saitoo, Tohoku J. Exptl. Med., 39, 211-214 (1940).

[3]M. Imaizumi, J. Biochem. (Japan), 27, 45-64 (1938).

[4]Y. Kameda and E. Toyoura, J. Pharm. Soc. Japan, 67, 3 (1947).

[5]Y. Kameda, E. Toyoura, S. Ohshima, M. Tsuji, and C. Iriye, J. Pharm. Soc. Japan, 68, 143-144 (1948). Both reference 2 and 3 are reported on the basis of Chem. Abst., 44, 1564-1565 (1950).

action was greater for those compounds most resistant to acylase action.

c. Staphylococcus pyrogenes citreus Nr. 1. A glycerol extract of this organism hydrolyzed benzoylglycine over the pH range 5.0 to 8.0 with an optimum of about pH 7.6[1].

d. A strain of Staphylococcus from bubo. The yields of benzoic acid after exposing one gram quantities of substrates to this microorganism were as follows, where the yields are in mg of benzoic acid: hippuric acid, 266; benzoyl-DL-α-aminobutyric acid, 45; benzoyl-DL-phenylalanine, 0[2].

e. Soil bacteria KT 1, KT 3, KT 4, KT 9, KT 13, KT 17, KT 2, KT 6, and KT 7. These bacteria which were isolated from soil, possessed the ability to metabolize hippuric acid and/or phenylacetylglycine[3]. Some were found to break down hippuric acid permitting the isolation of benzoic acid[4]. KT I metabolized hippuric acid, benzoyl-DL-leucine, DL-α-

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[1]T. Abe, Tohoku J. Exptl. Med., 49, 27-32 (1947).

[2]Y. Kameda and E. Toyoura, J. Pharm. Soc. Japan, 72, 400-402 (1952).

[3]Y. Kameda and E. Toyoura, J. Pharm. Soc. Japan, 67, 172- (1947). Chem. Abst., 44, 1565 (1950).

[4]Ibid., 213.

benzamidobutyric acid, benzoyl-DL-valine, and benzoyl-DL-alanine, and also metabolized were the corresponding nonyl or hendecyl derivatives[1]. Kameda and Toyoura reported yields (Table 40) of benzoic acid on allowing three acylamino acids to be exposed to the non-pathogenic bacteria, KT I and KT 3 in the presence of toluene.

Table 40

Yields of Benzoic Acid per One Gram of Substrate on Exposure to Microorganisms in the Presence of Toluene[1]

Microorganism	mg Benzoic acid from		
	Hippuric acid	Benzoyl-DL-α-aminobutyric acid	Benzoyl-DL-phenylalanine
KT I	414	133	121
KT 3	415	103	110

[1]Y. Kameda and E. Toyoura, J. Pharm. Soc. Japan, 72, 400-402 (1952).

f. Streptococcus faecalis, Leuconostoc mesenteroides, Leuconostoc citrovorum, Lactobacillus arabinosus, Lactobacillus brevis, Lactobacillus casei, and Lactobacillus pentoacetacus. A systematic study of the nutritional

[1]Y. Kameda and E. Toyoura, J. Pharm. Soc. Japan, 67, 1,2 (1947).

utilization of benzoylamino acids by 4 acid-producing organisms for their amino acid requirements has been carried out by Itschner[1] and Drechsler[2]. A summary of their work is given in Table 41 which is reproduced from a thesis by Drechsler[2].

As shown in Table 40, Lactobacillus arabinosus, ATCC 8014, uses benzoyl-DL-leucine for its leucine requirements and also to a slight extent, dibenzoyl-L-cystine for its cystine requirements; the other seven benzoylamino acids tested were not utilized. According to Fox and Warner[3], under certain conditions, L. arabinosus requires methionine, and then benzoyl-DL-methionine partially meets its methionine requirements. Hegsted has determined the availability to L. arabinosus of certain acylamino acids and acyldipeptides in place of the corresponding amino acid[4]. These results are presented in Table 42.

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[1]K. F. Itschner, Bacterial utilization and sequence determination of peptides. Unpublished Ph.D. thesis. Ames, Iowa. Iowa State College Library. 1951.

[2]E. R. Drechsler, Utilization of certain benzoylamino acids by several species of bacteria. Unpublished M.S. thesis. Ames, Iowa. Iowa State College Library. 1952.

[3]S. W. Fox and C. W. Warner, Ames, Iowa. (Private communication.) (1953).

[4]D. M. Hegsted, J. Biol. Chem., 157, 141-146 (1945).

Table 41

Utilization of Benzoylamino Acids by Four Species of Bacteria[1]

Compound	Action by			
	L. arab. ATCC No. 8014	L. brevis ATCC No. 8287	Strep. faec. ATCC No. 8043	Leuc. mesen. ATCC No. 8042
Benzoyl-DL-alanine	---*	---	NU&	---
α-Benzoyl-L-arginine	NU	U	NU	NU
Benzoyl-L-aspartic acid	---	NU	---	NU
Dibenzoyl-L-cystine	S#	S	---	U
Benzoyl-L-glutamic acid	NU	NU	NU	NU
Benzoylglycine	---	U	---	U
α-Benzoyl-L-histidine	---	NU	NU	NU

[1]E. R. Drechsler, Utilization of certain benzoylamino acids by several species of bacteria. Unpublished M.S. thesis. Ames, Iowa. Iowa State College Library, 1952.

*Blank spaces indicate parent amino acid is non-essential.

&Indicates "not utilized."

**Indicates "utilized."

#Indicates "slight" or questionable utilization.

Table 41 (Continued)

Compound	Action by			
	L. arab. ATCC No. 8014	L. brevis ATCC No. 8287	Strep. faec. ATCC No. 8043	Leuc. mesen. ATCC No. 8042
Benzoyl-DL-leucine	U	NU	NU	NU
α-Benzoyl-L-lysine	---	NU	NU	NU
Benzoyl-DL-isoleucine	NU	NU	NU	NU
Benzoyl-DL-methionine	---	NU	U	NU
Benzoyl-DL-phenylalanine	NU	NU	---	NU
Benzoyl-L-proline	---	NU	---	NU
N-Benzoyl-DL-serine	---	NU	---	NU
N-Benzoyl-DL-threonine	NU	NU	NU	NU
Benzoyl-DL-tryptophan	NU	NU	NU	NU
N-Benzoyl-L-tyrosine	---	NU	---	NU
Benzoyl-DL-valine	NU	NU	NU	NU

Table 42

% Nutritional Utilization of Amino Acid Derivatives by Lactobacillus arabinosus[1]

Compound	% Utilization based on amino acid content
Acetyl-DL-leucine	30-60
Acetyl-DL-isoleucine	0
Acetyl-L-valine	0
Benzoyl-L-valine	0
Benzoyl-L-valyl-L-valine	0
Benzoyl-L-valyl-D-valine	0
Benzoyl-D-valyl-D-valine	0
Benzoyl-D-valyl-L-valine	0

[1]D. M. Hegsted, J. Biol. Chem., 157, 141-146 (1945).

Also Krehl and Fruton observed a 6% utilization of acetyl-L-leucine by L. arabinosus, ATCC 8014, while carbobenzoxyglycyl-L-leucine was utilized 68% in 72 hours on the basis of its L-leucine content[1]. Acetyldehydroleucine was not available at all[1]. Spies and Chambers stated that L. arabinosus 17-5 made use of formyl-L-methionine to the

[1]W. A. Krehl and J. D. Fruton, J. Biol. Chem., 173, 479-485 (1948).

extent of about 5% while the D-isomer was utilized .6%[1]. Utilization data of Eades is given in Table 43. Eades considered it likely that the chloroacetyl and acetylamino acids were hydrolyzed by the organism prior to utilization of the amino acids[2].

In the work just mentioned, four derivatives of leucine have been reported as being utilized by Lactobacillus arabinosus, namely, the benzoyl, acetyl, chloroacetyl, and carbobenzoxyglycyl derivatives. Some utilization of formylmethionine, benzoylmethionine, and benzoylcystine has been reported. In contrast, other amino acid derivatives tested are not utilized.

Malin, Camien, and Dunn (Table 44) published information on the nutritional availability of benzoylglycine, benzoylglycylglycine, and carbobenzoxyglycyl-L-tyrosine for the glycine requirements of several acid producing organisms. The benzoylglycyl- and benzenesulfonylglycyl- derivatives of glycine displayed little or no availability for the glycine requirements of the organisms listed in Table 44.

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[1]J. R. Spies and D. C. Chamber, J. Biol. Chem., 183, 709-712 (1950).

[2]C. H. Eades, J. Biol. Chem., 187, 147-152 (1950).

Table 43

Nutritional Utilization by Acid-Producing Bacteria of Amino Acid Derivatives[1]

Derivative	% Utilization on the basis of amino acid content by		
	L. arabinosus ATCC 8014	L. casei ATCC 7469	Leuconostoc mesenteroides ATCC 8042
Acetyl-DL-leucine	50	0	0
Chloroacetyl-DL-leucine	50	0	0
Acetyl-DL-phenylalanine	0	0	0
Chloroacetyl-DL-phenylalanine	0	0	0
Acetyldehydroleucine	0	0	0
Acetyldehydrophenylalanine	0	0	0
Acetyl-DL-tryptophan	0	50	0
Chloroacetyl-DL-tryptophan	0	50	0
Acetyldehydrotryptophan	0	0	0
Acetyl-DL-valine	0	0	0
Acetyldehydrovaline	0	0	0

[1] C. H. Eades, J. Biol. Chem., 187, 147-152 (1950).

Table 44

Availability to Some Acid-Producing Bacteria for the Glycine Requirements of Several Amino Acid Derivatives[1]

Organism	% Utilization of		
	Benzoyl-glycine	Benzoyl-diglycine	Carbobenzoxyglycyl-L-tyrosine
Streptococcus faecalis R, ATCC 8043	80	0	-
Lactobacillus brevis, ATCC 8287	94		5
Leuconostoc citrovorum, ATCC 8081	40	-	5
Leuconostoc mesenteroides P 60	105	5	-
Lactobacillus pentoaceticus ATCC 367	141	5	5

[1]R. B. Malin, M. N. Camien, and M. S. Dunn, Arch. Biochem., Biophys., 32, 106-112 (1951).

Spies and Chambers reported that formyl-L-methionine is more stimulatory to the growth of Streptococcus faecalis than is L-methionine[1]. Formyl-L-valine is 2-5% available for the valine requirements of Streptococcus faecalis 9790[2].

[1]J. R. Spies and D. C. Chambers, J. Biol. Chem., 183, 709-712 (1950).

[2]J. L. Stokes, M. Gunness, I. M. Durjer, and M. C. Caswell, J. Biol. Chem., 160, 35-49 (1945).

In addition, the compounds listed in Table 45 have been found unavailable for the amino acid requirements of Streptococcus faecalis ATCC # 9790. As can be seen in Table 41, only benzoyl-DL-methionine, of the 11 benzoyl-amino acids tested by Itschner and Drechsler, was utilized

Table 45

Certain Compounds Not Available for the Amino Acid Requirements of Streptococcus Faecalis, ATCC 9790[1,2]

Substrate	Reference Number
Benzoyl-DL-lysine	1
ϵ-Benzoylaminocaproic acid	1
ϵ-Benzoylamino-α-bromocaproic acid	1
N-Benzoyl-DL-threonine	1
N-Benzoyl-O-methyl-DL-threonine	1
Benzoyl-DL- tryptophan	1
Carbobenzoxyglycyl-L-leucine	2
Acetyl-L-leucine	2
Acetyldehydroleucine	2

[1]J. L. Stokes, M. Gunness, I. M. Durjer, and M. C. Caswell, J. Biol. Chem., 160, 35-49 (1945).

[2]W. A. Krehl and J. D. Fruton, J. Biol. Chem., 173, 479-485 (1948).

by Streptococcus faecalis, ATCC 8043. However, Malin, Camien, and Dunn (Table 44) tested benzoylglycine and found it was available for the glycine requirements of the same strain of Streptococcus faecalis. Also, carbobenzoxyglycyl-L-leucine and acetyl-L-leucine are not available to Strep. faecalis, ATCC 9790 (Table 45).

Lactobacillus brevis, ATCC 8287, does utilize benzoylglycine (Tables 41 and 44), α-benzoyl-L-arginine (Table 41), and, to a slight extent, dibenzoylcystine, but not 14 other benzoylamino acids (Table 41).

Berger, Johnson, and Peterson carried out enzyme experiments using preparations from Leuconostoc mesenteroides, strain Pd-60, and presented the data of Table 46.[1]

Leuconostoc mesenteroides multiplies on dibenzoyl-L-cystine and benzoylglycine[2] when the corresponding amino acid (which is essential) is omitted from its growth medium, but 15 other benzoylamino acids are not nutritionally available (Table 41). Benzoyldiglycine is not available for the glycine requirements[3] of Leuconostoc mesenteroides

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[1]J. Berger, M. J. Johnson, and W. H. Peterson, J. Biol. Chem., 124, 395-408 (1938).

[2]V. Nurmikko and A. I. Virtanen, Acta Chemica Scandinavica, 5, 97-101 (1951). Also see Tables 41 and 44.

[3]V. Nurmikko and A. I. Virtanen, Acta Chemica Scandinavica, 5, 97-101 (1951). Also see Table 44.

Table 46

Acylase System of Leuconostoc mesenteroides[1]

Substrate	pH	% Hydrolysis
Chloroacetyl-L-tyrosine	6.6	1
Benzoylglycine	6.27	81
Benzoyldiglycine	6.22	33
Benzoyltriglycine	6.32	14
Chloroacetylglycine	6.09	40
Chloroacetyldiglycine	6.51	14

[1]J. Berger, M. J. Johnson, and W. H. Peterson, J. Biol. Chem., 124, 395-408 (1938).

and several acyl derivatives of leucine, phenylalanine, and valine are not nutritionally available in lieu of the corresponding amino acid (Table 43).

Lactobacillus casei, ATCC 7469, can make use of acetyl-DL-tryptophan and chloroacetyl-DL-tryptophan for its tryptophan requirements; but several acyl derivatives of leucine, phenylalanine, and valine are not nutritionally available in lieu of the corresponding amino acid (Table 43).

The striking aspect of the observations described in this section is the fairly sharp nutritional preferences of the acid-producing bacteria for acylamino acids. For each

bacterium, only a few benzoylamino acids are available, while the majority are not. However, benzoylglycine seems to be generally nutritionally available to the acid-producing bacteria.

g. Escherichia coli. According to Kameda and Toyoura, benzoic acid cannot be isolated when hippuric acid or benzoyl-DL-phenylalanine are incubated in the presence of E. coli[1]. Fruton and co-workers reported on utilization of amino acid derivatives by a series of E. Coli mutants which required a specific amino acid for growth. The compounds tested included in their structure the amino acid which is essential to the strain of E. coli that was used. It was found that acetyl-L-leucine and carbobenzoxyglycyl-L-leucine are, at most, a few per cent available for the leucine requirements of a leucineless mutant[2,3]. Carbobenzoxyglycyl-L-phenylalanine and carbobenzoxyglycyl-L-tyrosine were used to a slight extent for the phenylalanine and tyrosine requirements of two Escherichia coli mutants[4,5].

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[1]Y. Kameda and E. Toyoura, J. Pharm. Soc. Japan, 72, 400-402 (1952). The above summary is based on an English summary.

[2]S. Simmonds, E. L. Tatum, and J. Fruton, J. Biol. Chem., 170, 483 (1947).

[3]S. Simmonds, J. I. Harris, and J. S. Fruton, J. Biol. Chem., 188, 251-262 (1950).

[4]J. S. Fruton and S. Simmonds, Cold Spring Harbor Symposia Quantitative Biology, 14, 55-64 (1950).

[5]J. S. Fruton, J. Biol. Chem., 169, 91-101 (1947).

Acetyl-L-proline was not nutritionally available, and carbobenzoxy-L-prolyl-L-proline, carbobenzoxyglycyl-L-proline, and acetyl-L-phenylalanyl-L-proline were less than 1% available for the proline requirements of a proline requiring strain of E. coli[1].

Tomota and Saitoo detected no significant hydrolysis of benzoylglycine, benzoyldiglycine or dibenzoylornithine by dried and pulverized bacillus coli communis[2]. Imaizumi reported the following titration data, as ccm 0.1 n NaOH, for the substrates listed: chloroacetyl-L-phenylalanine, .35; acetylglycine, 1.08; and formylglycine, .115[3] where an extract of autolyzed bacteria was used. The organism was described as Bac. coli. Not hydrolyzed were benzoylglycine, benzoyldiglycine, phthaloylglycine, phthaloyldiglycine, acetyldiglycine, formyl-L-tyrosine, acetyl-L-glutamic acid, benzoyl-L-glutamic acid, chloroacetyl-L-tyrosine, DL-bromoisocapronylglycine, and DL-bromopropionylglycine[3]. The pH optimum for acetyl- and formyl-glycine splitting was at pH 7.0-8.0.

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[1]S. Simmonds and J. S. Fruton, J. Biol. Chem., 174, 705-715 (1948).

[2]S. Tomota and H. Saitoo, Tohoku J. Exptl. Med., 39, 211-214 (1940).

[3]M. Imaizumi, J. Biochem. (Japan), 27, 199-211 (1938).

h. Proteus OX 19. Benzoylglycine and benzoyl-DL-phenylalanine were not broken down by exposure to this Proteus[1].

i. Bacillus typhi, Bacillus dysentheriae, Bacillus enterditis Gartneri, Bacillus paratyphi A and B, and "Sarcina". Imaizumi studied the acylase activity of extracts of non-gelatin-liquifying bacteria of the species listed in the heading[2]. Chloroacetyl-L-phenylalanine was broken down by all of these extracts, except for that from Bacillus paratyphi B. Benzoyldiglycine was not attacked at all.

Tomota and Saitoo detected no hydrolysis of benzoylglycine or benzoyldiglycine by extracts of Salmonella enteritidis Gartneri, Nr 171.

j. Bacillus prodigiosus, Bacillus proteus, Bacillus subtilis, Bacillus pyocaneus. Table 47 presents information from a paper by Imaizumi[3] on the specificity of acylase preparations from the above bacilli.

Somewhat at variance with the preceding data of Imaizumi is the report by Tomota and Saitoo who found no

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[1]Y. Kameda and E. Toyoura, J. Pharm. Soc. Japan, 72, 400-402 (1952). The above summary is on basis of an English summary of this otherwise Japanese article.

[2]M. Imaizumi, J. Biochem. (Japan), 27, 65-79 (1938).

[3]Ibid., 199-211.

Table 47

Action of Acylase Preparations from Bacilli[1]

Substrate	Hydrolysis at 24 hours as ccm 0.1 n NaOH by preparation from			
	Bacillus prodigiosus	Bacillus proteus	Bacillus subtilis	Bacillus pyocaneus
Benzoylglycine	0.67	0	0	.45
Benzoyldiglycine	0.98	0	0	
Phthaloylglycine	0	0	0	
Phthaloyldiglycine	0	0	0	
Acetylglycine	0	0.65	0	
Acetyldiglycine	0	0	0	
Formylglycine	0	0	0	
Formyl-L-tyrosine	0	0	0	
Acetyl-L-glutamic acid	0	0	0	
Benzoyl-L-glutamic acid	0	0	0	
Chloroacetyl-L-phenylalanine	0.46	1.05	1.20	.38
Chloroacetyl-L-tyrosine	0	0.55	0	
DL-bromoisocapronylglycine	0	0	0	
DL-bromopropionylglycine	0	0	0	

[1] M. Imaizumi, J. Biochem. (Japan), 27, 45-64 and 199-211 (1938).

activity on benzoylglycine by a suspension of dried and pulverized Bacillus prodigiosus, and of other bacteria[1]. Some of their data is given in Table 48.

Table 48

Acylase Activity on Benzoylglycine, Benzoyldiglycine, and Dibenzoylornithine by Several Species of Bacteria[1]

Bacterial species	Enzyme activity as ml .1 n NaOH		
	Benzoylglycine	Benzoyl-diglycine	Dibenzoyl-ornithine
Bacillus subtilis	1.81	0	-.13
Bacillus proteus	.16	0	0
Bacillus prodigiosus	-.04	0	-.23
Bacillus pyocaneus	.06	0	-.10

[1]S. Tomota and H. Saitoo, Tohoku J. Exptl. Med., 39, 211-214 (1940).

Chloroacetylasparagine and benzoyl-L-asparagine were broken down by extracts of Bacillus proteus, Bacillus subtilis, Bacillus prodigiosus, Bacillus pyocyaneus, but since no benzoic acid was isolated from the digests with benzoyl-

[1]S. Tomota and H. Saitoo, Tohoku J. Exptl. Med., 39, 211-214 (1940).

L-asparagine, it was thought probable that only amidase action was involved[1].

k. Mycobacterium phlei. Table 49 lists the quantities of acids isolated from digests of Mycobacterium phlei with acylamino acids.

Table 49

Hydrolysis of Acylamino Acids in the Presence of Mycobacterium phlei[1]

Substrate	Mg of acid liberated from 1/200 mole of N-acylated amino acids on exposing them to M. phlei
Hippuric acid	236
Benzoyl-DL-α-aminobutyric acid	145
Benzoyl-DL-phenylalanine	5
Dibenzoyl-L-cystine	21 (from 1/400 mol)
Phenylacetylglycine	129
Laurylglycine	952
Lauryl-DL-phenylalanine	295

[1]Y. Kameda and E. Toyoura, J. Pharm. Soc. Japan, 72, 402-403 (1952).

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[1]S. Utzino and M. Imaizumi, Z. Physiol. Chem., 253, 51-64 (1938).

The ready hydrolysis of lauryl-DL-phenylalanine as compared to the slight hydrolysis of benzoyl-DL-phenylalanine (Table 49) is rather striking.

5. Acylases of true fungi (Phylum eumycophyta)

Kossowicz described the utilization of hippuric acid as a nitrogen source by a number of molds[1,2,3,4]. Bieremns found that a mold similar to Cordicyps militaris Linne used hippuric acid in growth[4].

a. Saccharomyces cerevisiae. Euler and Josephson reported that an enzyme preparation from yeast failed to split benzoyldiglycine[5]. By adding acetone to yeast autolysate at -15 to -20°, a precipitate was obtained which failed to decompose benzoyldiglycine or β-naphthalinsulfoglycyltyrosine at pH 7.0[6]. Grassmann and Dyckerhoff reported that benzoylglycine, carbethoxyglycyl-DL-leucine, acetylphenylalanylalanine, and β-naphthalinsulfoglycyl-L-tyrosine

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[1] A. Kossowicz, Z. Garungephysiol., I, 60-62, 121-123, and 314-315 (1912).

[2] A. Kossowicz, Z. Garungephysiol., II, 81-83 (1913).

[3] A. Kossowicz, Biochem. Zeit., 67, 391-399 (1914).

[4] S. Bieremns, Centr. Bakteriol. Parasatenk., 2nd part, 23, 109-110 (1909).

[5] H. von Euler and K. Josephson, Ber. Deut. Chem. Ges., 60, 1341-1349 (1927).

[6] W. Grassmann and H. Dyckerhoff, Z. Physiol. Chem., 175, 18-37 (1928).

were not hydrolyzed by a dipeptidase preparation from yeast[1]. Also, a "proteinase" preparation failed to split benzoyldiglycine or β-naphthalinsulfoglycyltyrosine[1]. However, Waldschmidt-Leitz did obtain a preparation from yeast which was active against acetyl-DL-phenylalanyl-DL-alanine[2]. Utzino found that phthaloylglycylglycine is split by yeast press juice[3]

b. Penicillium notatum 176. Ito studied the specificity of the acylase complex in a H_2O-glycerin extract from the mycelium maceration of Penicillium notatum 176[4]. The titration values for a 24 hour incubation at pH 6.0 and pH 7.5 are presented in Table 50. The pH optimum for benzoylglycine hydrolysis was found to be about pH 7. No significant hydrolysis of benzoyldiglycine was detected over the pH range 4.0 to 9.0[4].

c. Aspergillus niger. H. Otani discovered that an extract of ground Aspergillus niger would break down β-naphthalinsulfoglycylglycine with the formation of

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[1]W. Grassmann and H. Dyckerhoff, Ber. Deut. Chem. Ges., 61, 656-670 (1928).

[2]E. Waldschmidt-Leitz, W. Grassmann, and A. Schaffner, Ber. Deut. Chem. Ges., 60, 359-364 (1927).

[3]S. Utzino, J. Biochem. (Japan), 9, 453-463 (1928).

[4]Y. Ito, J. Biochem. (Japan), 37, 237-247 (1950).

Table 50

Specificity[1] of an Acylase System of *Penicillium notatum* 176

Substrate	Hydrolysis in 24 hours as as ml .10 n NaOH at	
	pH 6.0	pH 7.5
Benzoylglycine	.25	.37
Benzoyl-DL-methionine	0	0
Benzoyl-DL-phenylalanine	0	0
Benzoyldiglycine	.07	.09
Acetylglycine	.28	.17
Acetylglutamic acid	.50	.17
Acetyl-DL-methionine	0	0
Chloroacetylleucine	.95	0.62
Chloroacetylphenylalanine	.22	0.71

[1]Y. Ito, J. Biochem. (Japan), 37, 237-247 (1950).

β-naphthalinsulfoglycine[1]. The pH optimum was 7.1-7.3. The action of the enzyme was inhibited by DL-phenylalanine, L-leucine, and glycine; the compounds are listed in the order of decreasing inhibitory power.

[1]H. Otani, Acta Schol. Med. Univ. Imp. Kioto, 17, 269-287 (1935).

β-Naphthalinsulfoglycine was only slightly inhibitory. Of these compounds, only β-naphthalinsulfoglycine inhibited the hydrolysis of β-naphthalinsulfoglycylglycine by a preparation from hog liver. Also the mold enzyme was shown to differ from the hog liver enzyme by differences in behaviour toward heat and pH.

An enzyme preparation from Aspergillus niger attacked p-toluolsulfoglycylglycine and benzolsulfoglycylglycine, but not β-naphthalinsulfoglycine[1]. In experiments carried out in a pH 7.1 buffer, and using a similar enzyme preparation, hydrolysis of benzoylglycine benzoyldiglycine, and benzoyl-DL-leucylglycine, but not benzoylglycyl-DL-phenylalanine or benzoyl-DL-phenylalanine was observed[2]. Other workers have reported the hydrolysis by Aspergillus niger enzymes of hippuric acid[3,4,5,6], benzoyl alanine[7], acetylglycine[7]

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[1]H. Ōtani, Acta Schol. Med. Univ. Imp. Kioto., 17, 269-287 (1935).

[2]Ibid., 330-333.

[3]A. W. Dox and R. E. Neidig, Z. Physiol. Chem., 85, 68-71 (1913).

[4]A. W. Dox, J. Biol. Chem., 6, 461-467 (1909).

[5]J. Nikitinsky, Jährbucher fur Wissenschaftliche Botanik, 40, 1-93 (1904).

[6]K. Shibata, Beit. Chem. Physiol. Pathologie, 5, 384-394 (1904).

[7]A. W. Dox and W. E. Ruth, Biochem. Bull., 3, 23-25 (1913).

and furfuroylglycine[1]. Benzoylalanine and acetylglycine were hydrolyzed 23% and 87%, respectively, under the same conditions[2]. The analytical values for the hydrolysis in 24 hours of benzoylglycine, benzoyldiglycine, and benzoyl-DL-leucylglycine as mg amino nitrogen were .058, .070, and .435, respectively[6].

d. Aspergillus oryzae. A commercial enzyme mixture obtained from this mold is called Takadiastase. Takadiastase and other preparations from Aspergillus oryzae have been reported to hydrolyze benzoylglycine[4,5,6], phenylacetylglycine[7], benzoyl-DL-alanine[8] and glycocholic acid[9].

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[1] A. W. Dox and R. E. Neidig, Biochem. Bull., 2, 407-409 (1913).

[2] A. W. Dox and W. E. Ruth, Biochem. Bull., 3, 23-25 (1913).

[3] H. Otani, Acta Schol. Med. Univ. Imp. Kioto, 17, 330-333 (1935).

[4] A. W. Dox and R. E. Neidig, Z. Physiol. Chem., 85, 68-71 (1913).

[5] C. Neuberg and K. Linhardt, Biochem. Z., 147, 372-376 (1924).

[6] A. W. Blagoweschenski and K. A. Nikolaeff, Biochem. Z., 276, 368-375 (1935).

[7] C. Neuberg and J. Noguchi, Biochem. Z., 147, 370-377 (1924).

[8] C. Hoppert, Biochem. Z., 149, 510-512 (1924).

[9] W. Grassmann and K. Pado Basu, Z. Physiol. Chem., 198, 247-250 (1931).

The benzoylglycine activity of Takadiastase is not readily soluble in water and the efficiency of various extractive solutions has been studied[1]. Phosphate buffer of pH 8.0 was the best extractive agent of those studied.

Ellis and Walker studied the effect of mono substitution on the rate of hydrolysis of hi puric acid by Takadiastase[2]. The substituting groups were chloro, bromo, iodo, nitro, and methyl. It was found that meta substitution, especially by nitro or bromo groups, accelerated hydrolysis and that ortho substitution inhibited hydrolysis. Para substitution had negliable effect. Ortho-iodohi uric acid inhibited acylase action, and Ellis and Walker believed that this indicated that the ortho group was inhibiting the breakdown of the enzyme-substrate complex.

e. Penicillium NRRL 1978 B_2 and Pencillium 176 Yabuta. Kameda and Toyoura obtained the yields of benzoic acid given in Table 51 on incubating three benzoylamino acids in the presence of these two species of Penicillium.

f. Aspergillus clavatus, Aspergillus fumigatus, Penicillium expansum, Penicillium roqueforti, Penicillium

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[1] O. A. Blagoweschenski and K. A. Nekolaeff, Biochem. Z., 276, 368-375 (1935).

[2] S. Ellis and B. S. Walker, J. Biol. Chem., 142, 291-297 (1942).

Table 51

Amount of Benzoic Acid Liberated by Exposing One Gram Portions of Benzoylamino Acids to Penicillium NRRL 1978 B_2 and Penicillium 176 Yabuta[1]

Microorganism	Yield of benzoic acid as mg from		
	Hippuric acid	Benzoyl-DL-α-amino butyric acid	Benzoyl-DL-phenylalanine
Penicillium NRRL 1976 B_2	83	54	79
Penicillium 176 Yabuta	455	134	123

[1]Y. Kameda and E. Toyoura, J. Pharm. Soc. Japan, 72, 400-402 (1952).

camemberti, Penicillium chrysogenum, Penicillium brevicaule, Penicillium glaucum, Citromyces glaber, Rhizopus tonkinensis, Mucor circinellordes, Cladosporium herbarum, Fusarium oxysporium, Monascus purpureus, Butrytis strophanoderis. A summary of some work on the acylase activity of mold preparations is given in Table 52.

Dox and Ruth compared the breakdown of benzoylalanine and acetylglycine by the press juice from a number of molds[1] (Table 52). With one exception, acetylglycine was broken

[1]A. W. Dox and W. E. Ruth, Biochem. Bull., 3, 23-25 (1913).

Table 52

Acylase Activity of Mold Enzyme Preparations*

Microorganism	Acylase activity on			
	Hippuric acid	Acetyl-glycine	Benzoyl-alanine	Furfuroyl-glycine
Aspergillus clavatus	+[1]	+[3]	+[3]	+[4]
Aspergillus fumigatus	+[1,5]	+[3]	+[3]	+[4]
Penicillium expansum	+[1]	+[3]	+[3]	+[4]
Penicillium roqueforti	+[1]	+[3]	+[3]	+[4]
Penicillium camemberti	+[1,2]			+[4]
Penicillium chrysogenum	+[2]			
Penicillium brevicaule	+[2]			
Cladosporium herbarum		0[3]	+[3]	
Fusarium oxysporium		+[3]	+[3]	+[4]

*The symbols used and their meaning are as follows: +, hydrolysis observed; 0, no hydrolysis observed; ?, hydrolysis so slight as to be questionable. Superscripts refer to references which are found on page 176.

References to Table 52

1. A. W. Dox and R. E. Neidig, Z. Physiol. Chem., 85, 68-71 (1913).

2. A. W. Dox, J. Biol. Chem., 6, 461-467 (1909).

3. A. W. Dox and W. E. Ruth, Biochem. Bull., 3, 23-25 (1913).

4. A. W. Dox and R. E. Neidig, Biochem. Bull., 2, 407-409 (1913).

down 1½ to 6 times as rapidly by the mold extracts listed in Table 52 under reference 3. The exception was for the extract of Cladosporium herbarum which hydrolyzed benzoyl-alanine but not acetylglycine (Table 52).

Quantitative information on the breakdown of several acylamino acids by mold extracts is presented in Table 53. It is interesting to note in Table 53 that the two substrates containing terminal phenylalanine, benzoylglycyl-DL-phenyl-alanine, and benzoyl-DL-phenylalanine, were resistant to all of the mold extracts listed. Also, benzoyl-DL-leucyl-glycine was always more rapidly hydrolyzed than was benzoyl-glycylglycine by the mold extracts listed in Table 53.

g. Citromyces glaber, and Citromyces pfefferianus. Hippuric acid supported limited growth of these molds by partially replacing NH_4NO_3 as a nitrogen source[1].

[1] F. A. McDermott, Mycol. Centralbl., 3, 159-160 (1913).

Table 53

Mold Enzyme Action on Amino Acid Derivatives as Determined by Ōtani[1]

Microorganism	Amino nitrogen released by 24 hour enzyme action on				
	Benzoyl-DL-phenylalanine	Benzoyl-glycine	Benzoyl-diglycine	Benzoyl-glycyl-DL-phenylalanine	Benzoyl-DL-leucyl-glycine
Mucor circinellordes	0	.337	.005	0	.481
Rhizopus tonkinensis	0	.319	.058	0	.522
Citromyces glaber	0	.232	.029	0	.215
Penicillium glaucum	0	.087	.046	0	.261
Cladosporium herbarum	0	.824	.203	0	.406
Botrytis strephanoderis	0	.075	.019	0	.597
Monascus purpureus	0	0 (in 96 hrs. .058)	.012	0	.435

[1] H. Ōtani, *Acta Schol. Med. Univ. Imp. Kioto*, 17, 330-333 (1935).

h. Aspergillus parasiticus. An aqueous extract of a mat of Aspergillus parasiticus hydrolyzes chloroacetyl-L-tyrosine with pH optimum of 7.1 in phosphate buffer[1].

i. Mortierella renispora PRL 26, Gliocladium roseum PRL 79, Gliocladium roseum PRL 86, Trichoderma viride PRL 92, Fusarium sp. PRL 232, Chaetomium sp. PRL 319, Alternaria tenuis PRL 369, and Streptomyces sp. PRL 376. McConnell, Spencer, and Trew[2] reported on the hydrolysis of some carbobenzoxy-α-L-glutamylamino acids by the culture medium from the above molds. Some results from their paper is presented in Table 54. They also determined the activation energies for the enzymatic hydrolysis of carbobenzoxy-α-L-glutamyl-L-glutamic acid and carbobenzoxy-α-L-glutamyl-DL-alanine.

6. Acylases of vascular plants (Phylum tracheophyta)

a. Oats and barley. Water cultures of oats and barley make limited use of hippuric acid as a nitrogen source[3].

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[1] M. J. Johnson, Z. Physiol. Chem., 224, 163-175 (1934).

[2] W. B. McConnell, E. V. Spencer, and J. A. Trew, Can. J. Chem., 31, 697-704 (1953).

[3] M. A. Thompson, Chem. Zentral., 1901, II, 556 (1901).

Table 54

Peptidase Activity[1] in Extracellular Fungal Proteases

Substrate	Approx. pH	Percent hydrolysis							
		Enzyme*							
		PRL 26	PRL 79	PRL 86	PRL 92	PRL 316	PRL 319	PRL 369	PRL 376
α-L-Glutamyl-L-glutamic acid	3.5	0	0	0	0	0	0	0	0
N-cbzo-α-L-Glutamyl-L-glutamic acid	5.5	9.6	76	97	45	3.0	40	51	1.0
N-cbzo-α-L-Glutamyl-DL-alanine	7.5	16	23	40	9.6	18	0	0	7.6
N-cbzo-α-L-Glutamyl-glycine	11.0	33	18	2.1	10	28	28	24	10

[1]W. B. McConnell, E. V. Spencer, and J. A. Trew, Can. J. Chem., 31, 697-704 (1953).

*The enzyme preparations used were lyophilized culture filtrates. A more complete name of the mold corresponding to each number is given in the heading of this section.

b. Carica papaya. The specificity of a protease fraction, papain peptidase I, which is derived from the latex of this tree, has been investigated by Bergmann, Zervas, and Fruton[1]. Papain peptidase I has both endopeptidase action and acylase activity. Some of the compounds hydrolyzed and the bonds split are shown below:

Benzoylglycine|amide

Carbobenzoxyglycyl|glycine

Carbobenzoxyglycyl|glycylglycine

Carbobenzoxyglycyl|glycylglycylglycine

Carbobenzoxy-L-glutamyl-d-|-glycine

Carbobenzoxy-L-glutamylglycyl|glycine

Benzoylglycyl|2nd carbobenzoxy-L-lysyl|1st glycine

Carbobenzoxy-L-leucylglycyl|glycine

It was concluded from the above information that pepsin peptidase I splitting was directed toward the acylamino group of acylated peptides; of leucine, glutamic acid, lysine, or glycine carbonyl, the glycine carbonyl group was preferentially split off.

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[1]M. Bergmann, L. Zervas, and J. S. Fruton, J. Biol. Chem., 115, 593-611 (1936).

c. Tomato plant. Wood and Fontaine described the preparation of some halogenated phenoxyacetyl derivatives of L, DL, and D-amino acids[1]. These compounds were tested for their ability to produce formative changes in tomato plants. All of the acylated L- and DL-amino acids produced formative changes in tomato plants, whereas, the three 2,4-dichlorophenoxyacetyl-D-amino acids produced no formative changes in plants although they did produce parthenocarpic fruit and caused fruit to set. It has been assumed that the free halogenated phenoxyacetic acids are responsible for plant growth regulation by derivatives of these compounds. Wood and Fontaine suggested that the tomato plant was unable to split the amide linkage of 2,4-dichlorophenoxyacetyl-D-amino acids. The compounds which were tested were as follows:

N-(2,4-Dichlorophenoxyacetyl)-L-alanine

N-(2,4-Dichlorophenoxyacetyl)-DL-alanine

N-(2,4-Dichlorophenoxyacetyl)-L-aspartic acid

N-(2,4-Dichlorophenoxyacetyl)-D-aspartic acid

N-(2,4-Dichlorophenoxyacetyl)-DL-aspartic acid

N-(2,4-Dichlorophenoxyacetyl)-L-glutamic acid

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[1] J. W. Wood and T. D. Fontaine, J. Org. Chem., 17, 891-896 (1952).

N-(2,4-Dichlorophenoxyacetyl)-DL-glutamic acid

N-(2,4-Dichlorophenoxyacetyl)-glycine

N-(2,4-Dichlorophenoxyacetyl)-DL-histidine, methyl ester, HCl

N-(2,4-Dichlorophenoxyacetyl)-DL-isoleucine

N-(2,4-Dichlorophenoxyacetyl)-L-leucine

N-(2,4-Dichlorophenoxyacetyl)-DL-leucine

N-(2,4-Dichlorophenoxyacetyl)-L-methionine

N-(2,4-Dichlorophenoxyacetyl)-D-methionine

N-(2,4-Dichlorophenoxyacetyl)-DL-methionine

N-(2,4-Dichlorophenoxyacetyl)-L-phenylalanine

N-(2,4-Dichlorophenoxyacetyl)-D-phenylalanine

N-(2,4-Dichlorophenoxyacetyl)-DL-phenylalanine

N-(2,4-Dichlorophenoxyacetyl)-L-proline

N-(2,4-Dichlorophenoxyacetyl)-DL-proline

N-(2,4-Dichlorophenoxyacetyl)-DL-serine

N-(2,4-Dichlorophenoxyacetyl)-DL-threonine

N-(2,4-Dichlorophenoxyacetyl)-DL-tryptophan

N-(2,4-Dichlorophenoxyacetyl)-valine

N,N'-bis-(2,4-Dichlorophenoxyacetyl)-L-lysine

N,N'-bis-(2,4-Dichlorophenoxyacetyl)-DL-lysine

N,O-bis-(2,4-Dichlorophenoxyacetyl)-DL-tyrosine methyl ester

N-(4-Chlorophenoxyacetyl)-glycine

N-(2,4,5-Trichlorophenoxyacetyl)-glycine

N-(2,4,5-Trichlorophenoxyacetyl)-DL-valine

N,N'-bis-(2,4-Dichlorophenoxyacetyl)-L-cystine

It is possible that the physiological effects observed with the above acylated L amino acids are due to release of halogenated phenoxyacetic acids by acylases present in the tomato plant.

III. EXPERIMENTAL PROCEDURES

A. Preparation of Compounds

1. Compounds previously reported but synthesized by new or modified procedures

a. Copper 1-aminocyclobutane carboxylate. Five grams (.0357 mole) of 5 cyclobutane spirohydantoin (page 193) were refluxed for 53 hours in a solution of 5 1/2 gm. (ca. .135 mole) NaOH and 75 ml H_2O. At the end of this time, some ammonia was still being given off. Dem'yanov and Tel'nov in carrying out a similar preparation, reported that ammonia evolution had ceased at the end of 14 hours[1]. The reaction mixture was neutralized and evaporated *in vacuo* on a water bath. The evaporation was completed by blowing a stream of air on the mixture while heating on a water bath. The solid remaining after evaporation was extracted with hot ethanol and the ethanol extract discarded. The residue was extracted with two 75 ml portions of hot water. To the combined aqueous extracts were added, during heating of the solution, 4 grams of cupric acetate dihydrate.

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[1] N. Y. Dem'yanov and S. M. Tel'nov, Bull. Acad. Sci. U.R.S.S., Classe Sci. Math. Nat., Sér. Chem., 1937, 529-538 (1937). The translation of their Russian article was generously carried out by Dr. Kenneth Shaw.

After standing for 4 days, the purple copper salt which formed in the above solution was recovered by decanting off the mother liquor and an easily suspended light blue solid. The copper salt was used without drying and weighing in the preparation of 1-aminocyclobutane carboxylic acid.

The above procedure is a simplification of that reported by Dem'yanov and Tel'nov[1]. Dem'yanov and Tel'nov used cupric oxide (rather than cupric acetate) and this necessitated a tedious extraction with hot water and a subsequent evaporation of the aqueous extract which contained the copper-1-aminocyclobutane carboxylate free of cupric oxide. The use of cupric acetate in the preparation reported here avoided the steps just mentioned.

b. 1-Aminocyclobutane carboxylic acid. The copper 1-aminocyclobutane carboxylate, the preparation of which was just described, was suspended in 150 ml of hot water, the solution was slightly acidified with 1 ml of concentrated acetic acid, and the copper salt was decomposed with H_2S. Most of the copper sulfide was filtered off, the greenish solution was treated with activated charcoal, and

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[1]N. Y. Dem'yanov and S. M. Tel'nov, Bull. Acad. Sci. U.R.S.S., Classe Sci. Math. Nat., Ser. Chem., 1937, 529-538 (1937). The translation of this Russian article was generously carried out by Dr. Kenneth Shaw.

refiltered, and the solution evaporated to a low volume and refiltered. The filtrate was evaporated to incipient crystallization (volume about 10 ml), 5 ml of ethanol was added, the mixture was cooled in an ice bath, and .9 gm of solid was filtered off. The filtrate on further evaporation yielded another .45 gm of solid. Each of the above solids was washed with a little ethanol following the filtration. Total yield was 31% of theory based on 5-cyclobutane spirohydantoin.

Analysis: Calc. for $C_5H_9NO_2$:
N, 12.16%
Found: N, 12.32%

The procedure described is essentially that of Dem'yanov and Tel'nov[1], but the method of preparing the copper salt of 1-aminocyclobutane carboxylic acid was somewhat modified as indicated on page 184.

c. Benzoyl-L-leucine. To 6.57 gm. (.050 mole) of Mann, methionine-free, L-leucine was added 50 ml. of one normal NaOH. The resulting solution was cooled to below 5° and mechanically stirred while, simultaneously, 22.5 ml (.055) mole) of 2 normal NaOH and 4.77 ml. (7.39 gm. or

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[1]N. Y. Dem'yanov and S. M. Tel'nov, Bull. Acad. Sci. U.R.S.S. Classe Sci. Math. Nat., Ser. Chem., 1937, 529-538 (1937). The translation of this Russian article was generously carried out by Dr. Kenneth Shaw.

.0525 moles) of benzoyl chloride were added over a period of 90 minutes. The cooling bath was removed and the mixture mechanically stirred for an additional 45 minutes. The mixture was then acidified to Congo Red paper with 4.5 ml of concentrated HCl and the mixture was allowed to stand in the refrigerator. After standing two days the precipitate was stil a semisolid.

Several attempts were made to cause the above material to crystallize without success. The unsuccessful procedures included the following:

1. The mother liquor was removed and the oily residue was stirred under Skelly D.
2. The above mixture of product in Skelly D was frozen with a dry ice-acetone bath.
3. The mixture from 2 (above) was evaporated and dried <u>in vacuo</u>.
4. The product was then dissolved in methanol and this solution was slowly evaporated in a stream of air.
5. A small portion of the product from 4 (above) was heated <u>in vacuo</u> up to 95° bath temperature in an attemot to distil off contaminating benzoic acid, the resulting solid was frozen in a dry ice-

acetone bath, and then an unsuccessful attempt was made to crystallize the solid from 50% ethanol.

6. The bulk of the material from 4 (above) was extracted with hot carbon tetrachloride.

The preparation was concluded by the following operations which finally led to a solid product. The bulk of the preparation was dissolved in acetone and filtered free of solid. The acetone solution was evaporated in vacuo and the residue was heated in vacuo at a bath temperature of 97° causing some crystals to form on the side-arm of the Claisen head. The residue was largely dissolved in about 20 ml. of dry ether, a moderate amount of a white solid was filtered off and discarded, and the ether solution was treated with a little Norit and calcium sulfate, and filtered again. The ether solution was warmed and Skelly A was added to the solution until it became turbid; the mixture was placed in the refrigerator. A process of freezing the mixture in a dry ice-acetone mixture and allowing to thaw while scratching the sides of the flask with a glass stirring rod was repeated twice, and the mixture was placed in the refrigerator; on examining the mixture about 30 minutes later, clusters of crystals seemed to be forming. After two days the solid product was washed twice by grinding up with 40 ml. portions of Skelly A and

removing the wash solvent by filtration. The grayish product was dried over calcium chloride in vacuo. The weight of this material was 6.0 gm and it melted at 58-63°. Fisher obtained a compound by crystallization from the same solvent mixture which he described as containing 1 mole of ether for every 2 moles of benzoyl-L-leucine and which had an indistinct melting point at 60°[1]. The dried material obtained here failed to show clearly defined crystalline properties, but macroscopic crystals were clearly visible at the initiation of crystallization. The bulk of the ether addition compound was crystallized from boiling water in two portions. The melting points of the two fractions were, respectively, 104.5-105.5, and 104-105.5°, and the weights of the fractions were 0.15 gm. and 2.4 gm. Combined yield was 21% of theory. Fisher reported a melting point of 105-107° for benzoyl-L-leucine prepared by resolution of benzoyl-DL-leucine[2].

d. L-leucine methyl ester. The following procedure is an application of the general method of Hillmann[3] for

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[1]E. Fischer, Untersuchungen uber aminosauren, polypeptide, and proteine, p. 123. Berlin, Verlag von Julius Springer, 1906.

[2]Ibid., 126.

[3]G. Hillmann, Z. Naturforsch., 1, 682-683 (1946).

the preparation of amino acid esters from amino acid ester hydrochlorides.

Gaseous ammonia was bubbled into $CHCl_3$ at room temperature to give a solution which was 1.417 normal in NH_3 according to titration with HCl to a methyl red end-point. Leucine methyl ester hydrochloride (4.9 gm., 0.25 mole) was suspended in 10 ml of dried $CHCl_3$ and the mixture was cooled. The NH_3-$CHCl_3$ solution was cooled in an ice bath. During a period of five minutes, 17.9 ml. (.025 mole of NH_3) of the $CHCl_3$-NH_3 solution was added to the suspension of the ester hydrochloride. The flask containing the reaction mixture was shaken and cooled in an ice bath during this addition. As the NH_3-$CHCl_3$ solution was being added, an opalescence appeared in the reaction mixture. Following the addition of the NH_3-$CHCl_3$ solution, the reaction mixture was shaken in the ice bath for an additional 15 minutes, and then allowed to stand in the ice bath for 25 minutes. Some activated charcoal (Norite) and $CaSO_4$ was added, and the reaction mixture was filtered through a fluted filter paper, removing all but a little charcoal. The filtrate was evaporated <u>in vacuo</u> at 0°, to an oily residue. The oily residue was dissolved in ether and used directly in the preparation of carbobenzoxyglycyl-<u>L</u>-leucine.

2. New compounds

a. N-phenylacetyl-1-aminocyclobutane carboxylic acid. Seven ml. of 2 N NaOH, .230 gm. (.002 mole) of 1-aminocyclobutane carboxylic acid, and .523 ml. of phenylacetylchloride (.004 mole) were shaken up until most of the turbidity disappeared. The white solid obtained on acidification with hydrochloric acid was extracted with toluene and crystallized from 50% ethanol. Yields of 47 ml. and 194 mg. or yields of 10 and 42, respectively, were obtained in two runs. The melting point was 194-194.5° and this remained constant on repeated recrystallization.

Anal. Calc'd for $C_{13}H_{15}O_3N$: N, 6.025%;
Neut. equiv., 233.
Found: N (micro-Kjeldahl) 5.97, 6.13%;
Neut. equiv., 233.

b. N-Benzoyl-1-aminocyclobutane carboxylic acid. To 0.2302 gm. (.002 mole) of 1-aminocyclobutane carboxylic acid in 8 ml. of 1 normal NaOH was added .29 ml. (.0025 mole) of benzoylchloride. The mixture was shaken until most of the odor of benzoylchloride had disappeared and then acidified with hydrochloric acid. The mixture was put in the refrigerator overnight, cooled in an ice bath, and then the mother liquor was drawn off. After drying

in vacuo the precipitate was extracted with hot carbon tetrachloride. This substance was crystallized from 50% ethanol giving 69 mg. of white crystals melting at 201.5-202.5°. A second crystallization from 50% ethanol gave 48 mg. (11% of theory) of crystals melting at 202.5°.

Anal. Calc'd for $C_{12}H_{13}O_3N$: N, 6.41;
Neut, equiv., 219
Found: N (micro-Kjeldahl) 6.34, 6.31, 6.46;
Neut. equiv., 210, 224.

3. Known compounds synthesized as described in the literature

The compounds listed in Table 55 were synthesized as described in the literature without more than minor modifications of procedure.

B. Compounds Received as Gifts or Purchased from Commercial Sources

The compounds listed in Table 56 were synthesized or isolated by others.

Table 55

Known Compounds Synthesized as Described in the Literature

Compound	Reference
Diethyl cyclobutane-1,1-dicarboxylate	A. W. Dox and L. Yoder, J. Am. Chem. Soc., 43, 677-684 (1921).
Cyclobutane-1,1-dicarbonamide	Ibid.
Cyclobutane-1,1-dicarbonbromamide	C. K. Ingold, S. Sako, and J. T. Thorpe, J. Chem. Soc., 121, 1177-1198 (1922).
5-Cyclobutane spriohydantoin	Ibid.
Carbobenzoxyglycine	H. E. Carter, R. L. Frank, and H. W. Johnson. In L. I. Smith, ed. Organic syntheses. Vol. 23. New York, John Wiley & Sons, Inc. 1943.
Carbobenzoxyglycylchloride[a]	M. Bergmann and L. Zervas, Ber. Deut. Chem. Ges., 65, 1192-1201 (1932).
Carbobenzoxyglycyl-L-leucine	M. A. Stahmann, J. S. Fruton, and M. Bergmann, J. Biol. Chem., 164, 753-760 (1946).

[a]Unless thorough cooling with an ice bath was carried out during this preparation, little yield was obtained. The petroleum ether used in washing should be chilled before use. In distilling off ether, an ice bath was used as a heating bath.

Table 55 (Continued)

Compound	Reference
L-Leucine methyl ester hydrochloride[b]	C. S. Smith and A. E. Brown, J. Am. Chem. Soc., 63, 2605 (1941), and M. Fling, Preparation of amino acids and derivatives. Unpublished Ph.D. Thesis. Ames, Iowa. Iowa State College Library. 1946.
Trimethylene bromide	S. O. Kamm and C. S. Marvel. In Organic syntheses. Col. vol. I. 2nd ed. N. Y., John Wiley & Sons, Inc. 1941.
Chloroacetyl-DL-leucine[c]	E. Fisher and O. Warburg, Ann. Chem., Justus Liebigs, 340, 157 (1905).

[b]The method of Smith and Brown for the preparation of the D-isomer was used. The thesis by Fling describes the L-isomer. Mann, methionine free L-leucine was used in this preparation, but difficulty in obtaining a pure product was experienced unless the commercial leucine was first recrystallized from 60 (vol) 10 ethanol.

[c]In a preparation involving a .5 mole of DL-leucine, 1.0 N NaOH and chloroacetylchloride were added simultaneously over a period of 90 minutes to a solution of sodium DL-leucinate. During the crystallization from water of the product obtained by acidification of the reaction mixture, purification was aided by mechanical separation of yellow material which separated, for the most part, before lighter crystalline material. Final crystallization was from acetone.

Table 56

Compounds Synthesized or Isolated by Others

Substance	Source and/or Description
Benzoyl-DL-alanine	These compounds were available from previous work in this laboratory. For literature references, origin, and descriptions, see - E. R. Drechsler. Utilization of certain benzoylamino acids by several species of bacteria. Unpublished Ph.D. thesis. p. 12-16. Ames, Iowa, Iowa State College Library, 1952.
Benzoyl-L-glutamic acid	
Benzoyl-DL-methionine	
Benzoyl-DL-phenylalanine	
N-Benzoyl-DL-threonine	
Benzoyl-DL-valine	
α-Benzoyl-L-arginine	
Benzoyl-L-aspartic acid	
α-Benzoyl-L-histidine	
Benzoyl-DL-leucine	This compound was obtained due to the courtesy of Dr. Milton Winitz. For preparation see Milton Winitz, The Contribution of Substrate Structure to Enzymatic Peptide Bond Synthesis. Unpublished Ph.D. Thesis. p. 43-45. Ames, Iowa, Iowa State College Library, 1952.

Table 56 (Continued)

Substance	Source and/or Description
Benzoyl-DL-leucinamide	Prepared by Dr. Harry Wax. For description, see Harry Wax. Enzymatic Peptide Bond Synthesis and its Inhibition. Unpublished Ph.D. Thesis. Ames, Iowa, Iowa State College Library. 1949.
D-leucine	This compound was prepared by Dr. Marguerite Fling. For description of preparation see Marguerite Fling. Preparation of amino acids and derivatives and their effect on the growth of Lactobacillus arabinosus. Unpublished Ph.D. Thesis. p. 15-17. Ames, Iowa. Iowa State College Library, 1946.
D-valine	This compound was also prepared by Dr. Marguerite Fling. For description of preparation see the above thesis by Fling, p. 24-27.
D-phenylalanine	This compound was prepared by Mr. John Mosser. For description of preparation see John D. Mosser. Resolution of DL-phenylalanine by the quarternary base of methyl cinchonine. Unpublished M.S. Thesis. Ames, Iowa, Iowa State College Library. The sample of D-phenylalanine was described as follows on the label: $[\alpha]_D^{30^\circ} = 32.2 \pm .7^\circ$, M.P. 262-264° C.

Table 56 (Continued)

Substance	Source and/or Description
Potassium salt of Penicillin G (crystalline)	Commercial Solvents lot number 50061401-P, potency 1595 units per mg.
Aureomycin hydrochloride	This was received as a gift from Dr. D. Catron. This sample was Commercial Solvents aureomycin hydrochloride from lot number 691 MH 176.
Bacitracin	Commercial Solvents lot number B 5 0221-A. Potency 55 units/mg. This was received as a gift from Mr. J. F. Griffith.
Gramicidin	This was Sharp and Dohme gramicidin. It was received from Dr. S. W. Fox

C. General Bacteriological Procedure

1. Bacterial cultures

The bacteria used were originally obtained from the American Type Culture Collection (ATCC). Those used in this work were Streptococcus faecalis R, ATCC No. 8043; Lactobacillus brevis, ATCC No. 8287; and Leuconostoc mesenteroides, P-60, ATCC No. 8042. Also used was a culture received in this laboratory with the designation, Lactobacillus arabinosus 17-5, ATCC No. 8014 and which has been maintained in this laboratory for over 5 years. There is no reason to believe that this culture differs from the original strain, but it is possible that alteration could have occurred.

Bacterial cultures were maintained in the refrigerator in agar stabs made up as follows[1]:

Glucose	10 gm.
Bacto yeast extract	10 gm.
Peptone	8 gm.

[1] M. J. Horn, D. B. Jones, and A. E. Blum. U. S. Dept. Agr. Misc. Pub., 696 (1950).

Agar	15 gm.
H_2O	1,000 ml.

About once a month transfers were made with a loop into two tubes of agar which were incubated one or more days at 37° in an air incubator; after incubation, the two cultures were transferred to the refrigerator to replace the previous cultures. One culture was used for general inoculation purposes and the other was used for the next month's transfer.

2. Preparation of inocula

Unless otherwise stated, cells for inoculation were prepared by inoculating 10 ml. of peptone medium from agar slants maintained in the refrigerator and incubating for 24 hours at 37°. Then the cells were centrifuged down and the supernatant decanted, the cells were resuspended in 10 ml. of .9% saline solution, recentrifuged, and the wash saline was discarded. Finally the cells were resuspended in 10 ml. of .9% saline solution and this suspension was used as the inoculum. The inoculum was generally used within a day of preparation and was kept in the refrigerator when not in use. The medium[1] for the growth of the inoculum cells had the following composition:

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[1] M. J. Horn, D. B. Jones, and A. E. Blum, U. S. Dept. Agr. Misc. Pub., 696 (1950).

Glucose		10 gm.
Peptone		8 gm.
Yeast extract		1 gm.
Sodium acetate		1 gm.
Salt Solution A	See page 202 for composition	5 ml.
Salt Solution B		5 ml.
Distilled water		1,000 ml.

3. Media

The research reported in this thesis involved assays for 9 amino acids. The media to assay the majority of these amino acids were modifications of the synthetic medium described by Horn, Jones, and Blum[1]. Modifications included the omission of the amino acid being assayed and certain other modifications first used by Dr. Thomas Hurst. Composition of the complete medium upon which other media used in this work were based was as described in Table 57.

L-leucine, L-histidine, L-arginine, L-threonine, and L-valine were assayed by use of Streptococcus faecalis and a medium of the composition given on pages 201-203 except

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[1]M. J. Horn, D. B. Jones, and A. E. Blum, U. S. Dept. Agr. Misc. Pub., 696 (1950).

Table 57

Composition of Complete Synthetic Medium[a] (double strength)

Component	Concentration/liter	Comment
Glucose	40 gm.	
Sodium acetate	15 gm.	
DL-Alanine	400 mg.	
L-Arginine hydrochloride	400 mg.	
DL-Aspartic acid	800 mg.	
L-Cystine	400 mg.	
L-Glutamic acid	419 mg.	
Glycine	400 mg.	
L-Histidine·HCl·H_2O	400 mg.	
DL-Isoleucine	400 mg.	
DL-Leucine	400 mg.	
DL-Lysine·HCl or 2 HCl*	300 mg.	
DL-Methionine	400 mg.	
DL-Norleucine	400 mg.	

[a] M. J. Horn, D. B. Jones, and A. E. Blum, U. S. Dept. Agr. Misc. Pub., 696 (1950).

* Dorothy De Fontaine (Private Communication, Iowa State College, 1953) recommends Mann L-lysine dihydrochloride over the Mann monohydrochloride in phenylalanine assays. Use of a sample of the monohydrochloride may have been responsible for some high blanks observed in phenylalanine assays.

Table 57 (Continued)

Component	Concentration/liter	Comment
DL-Phenylalanine	400 mg.	
L-Proline	400 mg.	
DL-Serine	400 mg.	
DL-Threonine	400 mg.	
DL-Tryptophan	800 mg.	
L-Tyrosine	400 mg.	
DL-Valine	400 mg.	
K_2HPO_4	1 gm.	Added as 10 ml. aqueous solution (Salt solution A)
KH_2PO_4	1 gm.	
$MnSO_4 \cdot 4H_2O$	20 mg.	Added as 10 ml. aqueous solution (Salt solution B)
$MgSO_4 \cdot 7H_2O$	400 mg.	
NaCl	20 mg.	
$FeSO_4 \cdot 7H_2O$	20 mg.	
Adenine	10 mg.	10 ml. of solution. Added as Hydrochloric acid and heat may be necessary to dissolve these compounds.
Guanine	10 mg.	
Uracil	10 mg.	

Table 57 (Continued)

Component	Concentration/liter	Comment
Thiamin hydrochloride	2 mg.	Added as a solution in 10 ml. of 50% ethanol
Pyridoxamine hydrochloride or pyridoxine hydrochloride	.40 mg.	
Calcium pantothenate	.40 mg.	
Riboflavin	.40 mg.	
Nicotinic acid	.80 mg.	
p-Aminobenzoic acid	.40 mg.	
Biotin	.01 mg.	
Folic acid	.002 mg.	
Distilled water	800 ml.	

Directions: Dissolve cystine and tyrosine in a little dilute HCl before adding to the main solution. Before adding the vitamin solution, dissolve the other ingredients. Adjust pH with NaOH to 6.8. Dilute to 1,000 ml. and store under toluene in the refrigerator.

[a]A modification of the medium given in Horn, Jones, and Blum., U. S. Dept. Agr. Misc. Pub., 696, 1950. These modifications were suggested by Dr. Thomas Hurst.

that the amino acid being assayed was omitted; pyridoxamine hydrochloride was the form of vitamin B_6 used. However, in some of the experiments reported herein, L-leucine was assayed for by use of Lactobacillus arabinosus and the complete medium of pages 201-203 less leucine; pyridoxine hydrochloride was the form of vitamin B_6 used in this instance. L-phenylalanine analysis was carried out with Lactobacillus arabinosus and the complete medium (pages 201-203) containing pyridoxamine hydrochloride less phenylalanine, but with the sugar content modified to 10 gm. of arabinose and 70 gm. of glucose per liter. Lueconostoc mesenteroides was the assay organism for L-aspartic acid; the complete medium containing pyridoxamine hydrochloride (pages 201-203) was modified for this assay with regard to the glutamic acid, phosphate, and sodium acetate concentrations so that it contained 209 mg. of L-glutamic acid, 4 gm. of K_2HPO_4, 4 gm. of KH_2PO_4, and 40 gm. of sodium acetate per liter. The medium for L-glutamic acid assay was also a modification of the complete medium containing pyridoxamine hydrochloride (page 201-203), but with major modifications of containing just 200 mg. of DL-aspartic acid, 200 mg. of L-asparagine, 50 mg. of L-glutamine, and 40 gm., of sodium acetate per liter; Lactobacillus arabinosus was the assay organism for L-glutamic acid.

Lactobacillus brevis was used to assay L-methionine. The medium used included an H_2O_2-treated peptone solution which was kindly donated by Dorothy De Fontaine and which was made as follows:

reparation of H_2O_2-Treated Peptone

Fifty grams of Difco Bacto Peptone was dissolved in 250 ml. of water and 250 ml. of 1 N HCl was added. Five ml. of 30% H_2O_2 were added to the solution and the mixture was allowed to stand overnight at room temperature. The solution was then steamed 45 minutes in the autoclave and autoclaved 15 minutes at 15 p.s.i. The solution was stirred while hot, cooled, and neutralized with NaOH. The solution was steamed one hour and diluted to one liter.

The medium used for methionine analysis is a modification of that used by Kuiken, Lyman, and Hale[1], for the assay of tryptophan and has been devised and used in this laboratory by De Fontaine, Warner, and Hurst. It was made as follows: The following were added together and dissolved.

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[1]K. A. Kuiken, C. M. Lyman, and F. Hale, J. Biol. Chem., 171, 551-560 (1947).

200 ml. of H_2O_2-treated peptone

200 mg. of L-cystine and 200 mg. of L-tyrosine dissolved in HCl

200 mg. of DL-tryptophan

20 gm. of succinic acid

40 gm. of glucose

6 gm. of sodium acetate

The salts, purines, and vitamins listed for the complete medium (pages 201-203) with pyridoxine hydrochloride

The pH of the above solution was adjusted to 6.8 with NaOH and the volume was made to one liter.

The concentrations of the above media were all twice that for the fully diluted medium. In the work reported in this thesis the double strength media described were diluted with equal volumes of solutions or water before the assays or growing of cells were carried out.

4. General assay procedures

The principle of microbiological assay is to compare the growth response of a microorganism in the presence of an unknown amount of material with the growth response in the presence of graded known amounts of the material being

analyzed for. Except for a few experiments, in the work described here, the growth response was determined by titrating with dilute NaOH the acid liberated during growth.

The solutions to be assayed were 2.5 ml. portions contained in 18 x 150 mm. Pyrex culture tubes or test tubes. To each of these solutions was added 2.50 ml. portions of a medium lacking the amino acid being assayed. The medium was also added to 2.50 ml. portions of solutions containing known amounts of the amino acid being assayed and to controls. Unless otherwise stated, the known graded doses of amino acids were contained in solutions of the same buffer composition as the unknowns. However, in the enzyme experiments covering a pH range from pH 4.5-7.5 most of the known amino acid solutions were in buffers of pH 6.0, but in addition, cultures were grown at one concentration of amino acid with buffers of pH 4.5 and 7.5.

The tubes for the standard curve contained each or some of the following amounts of L-amino acids as micromoles per tube: .381, .305, .229, .191, .1526, .1143, .0764, .0572, .0381, .0191, and 0.

After the addition of medium to the solutions to be analyzed, the racks of tubes were covered with cloths and autoclaved for 15 minutes at 15 pounds per square inch,

the tubes were allowed to cool, and each tube (except for a pair of controls) inoculated with one drop (about .05 ml.) of a saline suspension of the assay organism. The tubes were then incubated for 72 hours at 37° in an air incubator. The extent of growth was determined by adding 5 drops of .5% phenolphthalein 50% ethanol solution to each tube and titrating to an end point with dilute NaOH. If the number of tubes was too great for titration within a few hours of the termination of incubation, the remaining tubes were kept at 2-3°. The amounts of amino acids in the unknowns were determined from the titration values and a plot of ml. of base versus amount of amino acid (standard curve). Duplicates or triplicates were usually run.

In the preparation of the known amino acid solutions, the L-leucine, L-valine, L-phenylalanine, and L-methionine were U.S.P. reference standards except that methionine-free Mann L-leucine recrystallized from 50% ethanol was used in the enzyme experiments involving all but Enzyme Preparation II and the Mann L-leucine was used in the utilization experiments.

D. Enzyme Studies

1. Enzyme preparations

a. In solution with acetate, phosphate, and metal salts. The synthetic medium used in growing L. arabinosus was made by mixing 1.5 liters of double strength leucineless synthetic medium containing pyridoxine hydrochloride and 1.5 liters of distilled water containing 30 mg. of L-leucine. The resulting solution was then autoclaved for 20 minutes at 15 p.s.i. This medium was inoculated with 30 ml. of saline suspension of L. arabinosus prepared from a 24 hour, 30 ml. peptone culture. The peptone had been inoculated directly from an agar stab culture. The 3 liter culture was incubated 24 hours at 37° and then removed to a cold room maintained at 2-3°.

The culture was allowed to stand in the cold room 1 hour, and the cells were then removed from the medium by centrifuging with an air-driven Sharples super centrifuge at 45,000 revolutions per minute. The centrifugation proper took 15 minutes. The cells collected in the centrifuge cylinder were suspended in 300 ml. of buffer-metal ion solution of the following composition:

Buffer-metal Ion Solution

About 800 ml. H_2O

0.50 gm. K_2HPO_4 (.00287 mole)

0.50 gm. KH_2PO_4 (.00367 mole)

0.010 gm. $MnSO_4 \cdot 4H_2O$ (.000044 mole)

0.20 gm. $MgSO_4 \cdot 7H_2O$ (.000812 mole)

0.01 gm. NaCl plus salt from pH adjustment (.00017 mole or more)

0.01 gm. $FeSO_4 \cdot 7H_2O$ (.00035 mole)

7.5 gm. sodium acetate (.0916 mole)

Solution was adjusted to pH 6.0 with HCl (and NaOH). Distilled water was added to give 1 liter of solution. Redetermination of pH after standing gave 6.29.

The cells were centrifuged for 30 minutes from the suspending solution in an International Centrifuge. The supernatant liquid was decanted and the cells washed again using 30 ml. of buffer-metal ion solution. The washed cells were then resuspended in buffer-metal ion solution and made to a volume of 22.5 ml. with the same solution. Following the growth of cells, preparation of this cell suspension was carried out in a room maintained at 2-3°.

The test tube containing the cell suspension was placed in an ice bath and transported to the Bacteriology Department. Here 21 ml. of the cell suspension was treated in the chamber of the Magnetostriction Oscillator, Model S-102[1], with sonic vibrations for 30 minutes at ca. 9 kilocycles per minute. The chamber of the instrument was cooled with water near 0° during the treatment. The voltage was set at 95. Following treatment, the mixture was transferred to a centrifuge tube in an ice bath, and transported back to the cold room for centrifugation.

The treated cells were centrifuged at the full speed of the International centrifuge for 35 minutes to give a cream-colored residue and a blackish, rather opaque, supernatant. The supernatant was pipetted off, and recentrifuged for 65 minutes to give a translucent supernatant and a blackish residue. The supernatant was pipetted off and its volume found to be 15.8 ml. One-half ml. of this supernatant was withdrawn for a plate count, and then 5.1 ml. of the buffer-metal ion solution were added to the remaining supernatant, giving an enzyme preparation such that 100 ml. of the L. arabinosus culture were

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[1]The Head of the Bacteriology Department at Iowa State College, Dr. C. Werkman, kindly gave permission to use this instrument.

required to produce 1 ml. of the enzyme preparation. This enzyme concentration will be referred to as a relative concentration of 100 X. Buffer-metal ion solution was added to portions of this 100 X solution to give enzyme solutions of 20 X, 10 X, and 2 X. These solutions were kept in the refrigerator (about 6^{o}) except during use.

A Kjeldahl nitrogen determination indicated that the cell suspension contained 4.14 mg. N per ml. The 100 X enzyme solution contained .408 mg. N/ml. Therefore, the sonic treatment released about (.408/4.14) x 100%, or about 13%, of the cellular nitrogen into soluble form. A plate count of the 100 X enzyme solution showed no viable cells in 1 ml. of a 10^{5} dilution, whereas, a plate count of 10^{9} dilution of the cell suspension, from which the enzyme preparation was prepared, gave 7.6×10^{2} cells per ml. These data lead to the estimate that there were more than 7.6×10^{6} as many viable cells per ml. in the cell suspension as there were in the 100 X enzyme preparation.

The enzyme preparation just described will be referred to as Enzyme Preparation I. The buffer-metal ion solution has the same composition in concentrations of salts as does the synthetic medium used in the growth of L. arabinosus. It was believed that such a solution would make more likely the detection of acylase activity, since utilization of benzoyl-L-leucine occurred under similar conditions.

b. Enzyme preparation II (lyophilized). The inoculum was prepared as follows: Material from an L. arabinosus agar stab culture was streaked out on an agar plate and the plate allowed to incubate at 37° for 3 days. Small, oval or round, smooth white colonies were observed. One of these colonies was inoculat-d into peptone broth and this culture incubated for 24 hours. This peptone culture served as the inoculum for a fresh agar stab culture. A transfer was made from this stab culture into 10 ml. of peptone medium which was incubated at 37° for 24 hours. Three ml. portions of this latter culture were transferred into each of two 150 ml. portions of peptone medium. These 150 ml. cultures were in turn incubated for 24 hours at 37°. The cells from these cultures were washed with .9% NaCl solution and resuspended in 2, 150 ml. portions of .9% sterile NaCl solution. These cell suspensions served as the inocula of the large portions of synthetic medium.

The synthetic medium used for the growth of Lactobacillus arabinosus cells in quantity had the same composition as Enzyme Preparation I. However, the components other than glucose and vitamins for 3 ten-liter portions of fully diluted medium were autoclaved in 3 8.95 liter portions contained in 12 liter round flasks, after pH adjustment, for 25 minutes at 15 p.s.i. Twenty-three

minutes were required to bring the steam pressure up to 15 lb. and 25 minutes were required to bring the pressure down to atmospheric pressure following autoclaving. Three one liter portions of glucose solutions, each containing 200 gm. of glucose, were also autoclaved for 25 minutes at 15 p.s.i. These solutions were allowed to stand in an incubator at 37° for one day and then three 50 ml. portions of Seitz-filtered (bacteria removed) vitamin solution and liter portions of glucose solution were added to the flask containing the other components of the medium giving 3, 10 liter portions of synthetic medium. The 3 flasks containing this medium were allowed to stand in the incubator an additional 24 hours before inoculation.

Three, 100 ml. suspensions of L. arabinosus cells were used to inoculate the above 10 liter portions of synthetic medium. The resulting cultures were incubated at 37° for 24 hours. At the end of the 24 hour incubation, the cells were removed from the medium by centrifugation in an air-driven Scharples super centrifuge (Type T-304-24 BRY) at ca. 45,000 revolutions per minute. This centrifugation required 30 minutes. The centrifugation and subsequent operations, unless otherwise stated, were carried out in a room maintained at 2-3°. The cells collected on the centrifuge cylinder were suspended in 3.00 liters of

acetate buffer. This buffer had an ionic strength of .10 and a pH of ca. 5.5. It was made by diluting .10 mole of sodium acetate and .0159 mole of acetic acid to 1.00 liter. The cells were centrifuged from the acetate buffer with an International centrifuge at full speed for 15 minutes. Two of the 250 ml. centrifuge tubes containing the cell suspension were broken during the centrifugation, but most of the cellular material was recovered. Following decantation of the supernatant acetate buffer from the cells, the cells were resuspended in acetate buffer and the volume was made up to 300 ml. This suspension, subsequently, will be referred to as the "cell suspension." Two hundred and seventy-six ml. of this cell suspension were then centrifuged as in the prior centrifugation (but the centrifuge tubes did not break). The cells on being centrifuged down formed 4-6 layers alternating between a lower thin brown or brownich black layer and an upper thicker grayish white layer. The supernatants were decanted from each of the two 250 ml. centrifuge bottles used, leaving a cell paste at the bottom of the tubes. The tubes containing the cells were placed in an ice bath and transported from the cold room to the Bacteriology Department for sonic treatment. The cells were suspended with acetate buffer in the treatment chamber of the Raytheon magnetostriction oscillator (Sonic

Generator) and made to a volume of ca. 30 ml. with acetate buffer. The cells were treated for 3 hours at a rheostat setting of 90 which gave a voltage reading of 140 volts. The treatment chamber was cooled during this time with water at 7-3°.

Following the sonic treatment, the suspension was washed into a container kept in an ice bath and the suspension was transported back to the cold room. Here the volume of the treated cells was made to 92 ml. with acetate buffer. Eighty-six ml. of this suspension of "treated cells" were centrifuged for 15 minutes at ca. 13,000 r.p.m. by use of a Cervall centrifuge. The supernatant resulting was decanted to give a "cellular residue." The supernatant was again centrifuged and the supernatant pipetted to give off a small black residue and made to 80 ml. with acetate buffer. This solution will be referred to as the "crude enzyme solution."

Seventy-five ml. of the crude enzyme solution were placed in a 250 ml. three necked flask and the mixture was cooled with the aid of an ice bath and slow stirring to 1°. Then 38.8 ml. of 45.4 wt. % ethanol-acetic acid buffer were added; the added solution had the same composition in sodium acetate and acetic acid as did the aqueous acetate buffer (page 215). The ethanol-acetate solution was introduced

below the surface of the crude enzyme solution through a capillary over a period of one hour with continuous mechanical stirring. The solution temperature ranged from 4°, when some heating occurred during the start of ethanol addition, to -7° at the termination of the addition; salt and ice were added to the cooling bath during the addition in order to lower the temperature. The ethanol concentration of the enzyme solution was ca. 14.6 wt. % (by calculation) at this point.

The ethanol concentration of the enzyme solution was further increased by the addition of 250 ml. of 92.5 wt. % ethanol-acetate over a period of ca. 2 hours with continuous stirring; the ethanol-acetate was .100 molar in sodium acetate and .0159 molar in acetic acid. The temperature was lowered from -5 to -13° during this addition. An opaque mixture resulted. The final ethanol concentration was ca. 65 wt. %.

The ethanol-enzyme mixture was centrifuged in two batches at ca. 13,000 r.p.m. for 5 minutes using a Cervall centrifuge (model 85). Four of the eight holes in the head of the centrifuge were filled with dry ice just prior to centrifugation and the other four holes used for the 50 ml. tubes containing the enzyme-alcohol mixture. The temperature of the mixture was ca. -13° just prior to centrifugation,

and 2° at the termination of this operation. The supernatant ethanol was decanted from the precipitates thus collected. The solid obtained by alcohol precipitation was transferred with the aid of stirring and 25 ml. of cold water to a lyophilization flask. The material was allowed to stand, with occasional stirring, in order to promote dissolution of the solid phase. Most of the solid finally dissolved, giving a gray opaque solution. This aqueous solution was frozen in a shell on the sides of the flask with a ethanol-acetone-dry ice bath and allowed to be lyophilized in a room at ordinary temperatures for 7 hours. The flask containing the produce as a light gray shell, was removed from the apparatus, and was slapped and shook, causing the enzyme preparation to drop into a tared weighing bottle. This material was a fine, light gray powder weighing 745 mg. Nitrogen content was 11.8% according to micro-Kjeldahl determinations. This material will be referred to as Enzyme Preparation II.

Viable cell counts were made of the fractions obtained in the preceding experiment by making dilutions in physiological saline and plating out on agar plates. The following viable cell counts were obtained where the values are cells per ml. corrected to the dilution of the culture from which the fraction was obtained: cell suspension,

4.3×10^8; treated cells, 3.4×10^8; cellular residue, 3.1×10^8; supernatant, 3.7×10^2.

c. Enzyme Preparation III A and III B (lyophilized). Two lyophilized enzyme fractions were obtained from L. arabinosus in a manner very similar to that used in the obtaining of Enzyme Preparation II. However, sonic treatment of the cells was for only 30 minutes. The cells were grown in 12 liters of synthetic medium. Enzyme Preparation III A was gotten by lyophilizing the precipitate formed on adding ethanol to a concentration of about 33 wt. %; this fraction weighed 75.3 mg. Enzyme Preparation III B was a lyophilized preparation which was the material precipitating between 33 and 65 wt. % ethanol concentration; this fraction weighed 37.2 mg. Before centrifuging the above fractions, the temperature was -15°.

2. Enzyme-substrate incubations

Stock substrate solutions were .0003048 molar in the L-component. In making them up, NaOH equivalent to the substrate was added. A few drops of toluene were added to the solutions and they were stored in a refrigerator when not in use.

In order to study the effect of pH on acylase activity, buffer solutions were made up in a manner similar to that described by Michaelis[1]. To a solution .100 molar both in sodium veronal and sodium acetate was added ca. 0.10 normal HCl until the desired pH, as read on a Beckmann, Industrial Model M, pH meter, was reached. The solution was made to one liter with water for every 333.3 ml. of the sodium veronal-sodium acetate solution. The result was a solution which had an ionic strength of .0667 regardless of the pH. Buffers varying from pH 4.5 to pH 7.5 were made up to study the effect of pH on enzyme activity. The buffer solutions were stored at room temperature (with a few drops of toluene) because crystallization would gradually occur in the more acid buffers if stored in the cold. The buffer solutions would frequently become moldy a few weeks after being made up and were then discarded.

As pointed out before, Enzyme Preparation I was a solution of enzyme in a buffer metal ion solution of the composition given on page 210. Enzyme Preparation II A and II B were tested for activity by dissolving in a veronal-acetate buffer of pH 6.0 made like the buffers just described, but having an ionic strength of .04.

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[1]L. Michaelis, Biochem. Z. 234, 139 (1931).

The solutions for the enzyme-substrate incubations were usually made up in 16 x 150 mm. Pyrex test tubes or culture tubes some time before the incubation was to be carried out. At the same time, controls and tubes by enzyme action were prepared. After components, other than enzyme solution had been added to the tubes, a drop of toluene was added to each tube. The tubes were stoppered and stored at 2-3° until a short time before use. Then the tubes, which were held in a wire rack, were placed in an air incubator maintained at 37° and allowed to prewarm for at least one-half hour before enzyme addition.

Except as will be mentioned, solutions of Enzyme Preparation II were prepared by weighing out the solid and dissolving it in cold water. Enzyme solutions were kept in the refrigerator (about 6°) except when in use. Solutions of Enzyme Preparation II and III, unless otherwise stated, were made up just prior to use. Just before enzyme additions, substrate-buffer solutions were removed from the incubator, and the corks removed. The enzyme solution was added. The final volume of solution in each tube following enzyme addition was 2.5. In the pH studies with Enzyme Preparation II this volume was made up as follows:

1.25 ml.	.0003048 Molar Substrate veronal-acetate
0.75 ml.	of buffer solution having ionic strength .0667

$\frac{0.50 \text{ ml.}}{2.50 \text{ ml.}}$ of enzyme in water

Resulting solutions had an ionic strength of .02.

In experiments involving Enzyme Preparation III A and III B enzyme substrate incubations were started by adding 1.25 ml. of the enzyme solution to 1.25 ml. portions of substrate. In experiments to test the acylase activity of Enzyme Preparation IIIA and IIIB, 1.25 ml. portions of enzyme in veronal-acetate buffer of ionic strength .04 were added to 1.25 ml. portions of substrate solution.

A series of compounds were tested for the ability to inhibit the hydrolysis of benzoyl-L-leucine and carbobenzoxy-glycyl-L-leucine by Enzyme Preparation II. In these experiments, the enzyme was dissolved in the veronal-acetate buffer of ionic strength .0667 and pH 6.0 which had been cooled in the refrigerator. To a mixture of .50 ml. inhibitor solution and 1.25 ml. substrate solution was added .75 ml. portions of enzyme-buffer solution. The solutions of penicillin, aureomycin, and bacitracin were made up just a few minutes before the enzyme-substrate incubations. D-leucine and D-phenylalanine were weighed out and added to each test tube as a solid because they were not soluble enough to give .5 molar solutions. It was also necessary to autoclave these two compounds with the substrate

solutions (1.25 ml.) plus .50 ml. H_2O to effect complete solution.

After enzyme addition the tubes were restoppered and placed back in the 37° incubator. The tubes were incubated, the rack of tubes removed from the incubator, the corks removed, and the tubes autoclaved 2 minutes at 15 p.s.i. in order to stop enzyme action. However, in experiments with Enzyme Preparation I, medium was first added and then the tubes autoclaved for 15 minutes at 15 p.s.i. Unless otherwise stated, 4 hours elapsed between the addition of enzyme solutions, and the start of autoclaving. Tubes for controls and the standards were treated in a like manner, except for the addition of enzyme solutions.

In the pH versus enzyme-activity experiments, the pH's of tubes replicating the enzyme-substrate solutions were determined at 0 time, that is just after adding enzyme solution, and after 4 hour's incubation. The pH used in graphing pH versus % hydrolysis was the average of these two readings. In the pH versus enzyme-activity experiments, a Beckmann, Industrial Model M, pH meter was used in making the readings and the pH meter was standardized against a pH 7.0 buffer. In other than the pH versus % hydrolysis experiments, one pH reading was made of a replicate tube within about one hour after the addition of enzyme.

3. Assays for liberated amino acids

a. General. The extent of enzymatic hydrolysis was determined by assay for liberated amino acid as described under "General Assay Procedures" on pages 206-209.

b. Effect of substrates, substrate products, buffers, enzyme preparations and potential inhibitors on accuracy of assays. In order for the assays to be accurate, substances other than the amino acid being assayed for must not interfere with the assay or the effect of these substances on the assay must be corrected for. In each determination enzyme blanks and substrates blanks were run; where enzyme or substrate were equivalent to more than 0 micromoles of the amino acid being assayed for, the corresponding correction was made in the data for enzymatic hydrolysis of substrate.

The possibility existed that the buffers used in the enzyme experiments would effect the amino acid assays. In all cases, the graded doses of amino acids were in buffers corresponding to those used in the enzyme-substrate experiments, except that in the pH versus % hydrolysis experiments, a complete standard curve was run only in the pH 6.0 veronal-acetate buffer. An assay was run at one amino acid level in pH 4.5 and 7.5 veronal-acetate buffers. Also,

tubes containing no amino acid and containing pH 4.5, 6.0, and 7.5 veronal-acetate buffers were assayed in these experiments. If the buffers had an appreciable effect on the assay titration values, then a correction was made, but usually no correction was made.

Mixtures corresponding to 10% and 40% hydrolysis of the substrates studied were assayed to determine if the products of enzymatic hydrolysis or the substrates would affect the assay for amino acid. The tubes corresponding to 10% hydrolysis of substrate contained 2.5 ml. of pH 6.0 veronal-acetate buffer of ionic strength .02 which contained .343 micromole of substrate plus .0381 micromole of amino acid plus .0381 micromole of benzoic acid or carbobenzoxy-glycine. The tubes corresponding to 40% hydrolysis of substrate contained .2285 micromole of substrate plus .1522 micromole of amino acid and .1522 micromole of benzoic acid or carbobenzoxyglycine.

The compounds tested for acylase inhibitory activity were tested to see if they affected the assay for leucine. This was done by adding these compounds in the amounts used in the enzyme experiments to a solution containing 30-40 micrograms of L-leucine and veronal-acetate buffer to give 2.5 ml. of pH 6.0, ionic strength .02 veronal-acetate buffer. These solutions were assayed for leucine and

where the titration values differed from that for 30 micrograms of L-leucine in the veronal-acetate buffer, the difference was applied as a correction in the calculation of the % of enzymatic hydrolysis.

E. Utilization Experiments

Carbobenzoxyglycyl-L-leucine and several benzoylamino acids were tested for their ability to meet the amino acid requirements of certain acid-producing bacilli. These experiments were on the whole repetition of experiments carried out by other investigators. However, utilization experiments with benzoyl-L-leucine had not been reported although experiments with benzoyl-DL-leucine have been reported.

In general, utilization experiments were carried out by the methods reported in the section on "General Assay Methods" on pages 206-208. The test compounds were contained in water (before the addition of medium). Incubation was for 72 hours at 37°. Results are reported as % utilization; % utilization is equal to amino acid found in the assay divided by amino acid content of the substrate all times 100.

F. Growth Inhibition Experiments

Experiments were carried out to measure the effect of isocaproate on the growth of Lactobacillus arabinosus in synthetic medium containing either leucine or benzoyl-L-leucine. Controls were run in which no isocaproic acid was added. In preparing tubes for the experiment, 2.5 ml. portions of leucineless medium containing pyridoxamine were added to 16 x 150 mm Pyrex culture tubes which had been calibrated for measurement of optical density. In addition, 1.25 ml. portions of either L-leucine or benzoyl-L-leucine solution were added where the stated portions each contained 0.381 micromole of the compound in solution. The tubes were plugged with cotton and autoclaved for 15 minutes at 15 p.s.i. Meanwhile, a 0.04 molar solution of sodium caproate had been filtered into a sterile test tube using a bacterial filter. To the tubes containing medium plus either L-leucine or benzoyl-L-leucine were added aseptically 1.25 ml. portions of either sterile water or the sodium caproate solution which had been filtered free of bacteria. The tubes were then each inoculated with 0.10 ml. portions of a saline suspension of Lactobacillus arabinosus which was prepared from a 14 hour peptone culture. The cultures in synthetic medium were incubated for 13 hours at 37°.

Percent transmittance was measured using a Coleman Universal Spectrophotometer at 610 millimicrons wave length. In the experiment described, triplicate tubes were run.

IV. EXPERIMENTAL RESULTS

A. Utilization Experiments

Experimental results on the utilization of benzoyl-L-leucine and carbobenzoxyglycyl-L-leucine by Lactobacillus arabinosus and Streptococcus faecalis 8043 during a 72 hour period are presented in Tables 58-61.

Table 58

Utilization of Benzoyl-L-leucine by Lactobacillus arabinosus for Its Leucine Requirements*

Amount Bz-L-leucine as micromoles per tube	Ml. .100 N NaOH to titrate	% Utilization**
.381	6.38,6.37	97
.305	5.94,5.97	98
.228	5.30,5.40	99
.152	4.24,4.24	85
.114	4.04,4.03	97
.076	3.48,3.69	107
.038	2.56,2.62	92
0	1.51,1.51	--
		Average 96%

*72 hour incubation at 37°.

**% utilization equals (L-leucine found in assay L-leucine content of benzoyl-L-leucine) x 100.

Table 59

Utilization of Carbobenzoxyglycyl-L-leucine by Lactobacillus arabinosus for Its Leucine Requirements*

Amount Cbzo-gly-leu as micromoles per tube	Ml. .100 N NaOH to titrate	% Utilization**
.381	6.42,6.34	96
.305	6.00,5.94	97
.228	5.56,5.41	102
.152	4.35,4.30	90
.114	4.08,4.06	100
.076	3.51,3.57	103
.038	2.60,2.67	110
0	1.51,1.51	---
		Average 100%

*72 hour incubation at 37°.

**% Utilization equals (l-leucine found in assay/L-leucine content of carbobenzoxyglycyl-L-leucine) x 100.

Table 60

Utilization of Benzoyl-L-leucine by Streptococcus faecalis 8043 for its Leucine Requirements*

Amount Benzoyl-L-leucine as micromoles per tube	Ml. .053 N NaOH to titrate	% Utilization**
.762	1.58,1.49	0
.381	1.60,0.62	0
.305	1.62,1.62	0
.228	1.65,1.66	0
.152	1.56,1.55	0
.076	1.58,1.59	0
0	1.60,1.58	-
		Average 0%

*72 hour incubation at 37°.

**% utilization equals (L-leucine found in assay/L-leucine content of benzoyl-L-leucine) x 100.

Table 61

Utilization of Carbobenzoxyglycyl-L-leucine by *Streptococcus faecalis* 8043 for its Leucine Requirements*

Amount Cbzo-gly-leu as micromoles per tube	Ml. 100 N NaOH to titrate	% Utilization**
.762	1.96,1.95	2.5
.381	1.95,(over),1.58	2.0
.305	1.74,1.69	2.0
.228	1.73,1.77	3
.152	1.70,1.69	3
.076	1.74,1.66	6
0	1.60,1.58	-
		Average 3%

*72 hour incubation at 37°.

**% Utilization equals (L-leucine found in assay/L-leucine content of carbobenzoxyglycyl-L-leucine) x 100.

B. Effect of Enzyme Substrates and Substrate roducts on Amino Acid Assays

Table 62

Recovery of Amino Acids with Synthetic Mixtures Corresponding to 10% and/or 40% Hydrolysis of Substrate*

Mixtures corresponding to hydrolysis of substrate	Assay Organism	Recovery as % of .381 micromoles where added solution corresponds to % hydrolysis of	
		10%	40%
Benzoyl-L-leucine	Strep. faecalis	10,9.5	40,46
Carbobenzoxyglycyl-L-leucine	Strep. faecalis	12.6,11.4	38½,44
Benzoyl-DL-phenylalanine	L. arabinosus	11.2,10.9	31,36
Benzoyl-DL-methionine	L. brevis	11,11½	40,39
Benzoyl-DL-valine	Strep. faecalis	10½,11½	44,44
Benzoyl-L-glutamic acid	L. arabinosus	8.3,12.5	
Benzoyl-L-aspartic acid	Leuc. mesent.	12.0,10.2	
α-Benzoyl-L-histidine	Strep. faecalis	about 17	
α-Benzoyl-L-arginine	Strep. faecalis	9.6,10.7	
Benzoyl-DL-threonine	Strep. faecalis	9.5,9.9	

*Solution corresponding to 10% hydrolysis contained .343 micromole substrate, .0381 micromole L-amino acid plus .0381 micromole benzoic acid or carbobenzoxyglycine. The solutions corresponding to 40% hydrolysis of substrate contained .2285 micromole of substrate, .1522 micromole of amino acid and .1522 micromole of benzoic acid or carbobenzoxyglycine. These compounds were added with veronal-acetate buffer. After the addition of medium, total volume of solution in each tube was 5 ml.

C. Hydrolysis of Various Acylamino Acids by Enzyme Preparations from Lactobacillus arabinosus

1. Acylase activity of Enzyme Preparation I

Table 63

Acylase Activity of Enzyme Preparation I*

Mg. enzyme N per ml.	Hydrolysis of			
	Benzoyl-L-leucine		Carbobenzoxyglycyl-L-leucine	
	pH	% hydrolysis	pH	% hydrolysis
.041	6.31	97,90	6.34	97,97
.0204	6.33	70,63	6.32	94,84
.0041	6.31	29,28	6.38	35,35

*% Hydrolysis of .0001524 molar substrate in 4 hours at 37°. The enzyme-substrate solution had one-half the molar concentration of salts as did the buffer metal ion solution described on page 210.

2. The acylase activity of Enzyme Preparation II

a. Acylase activity on various acylamino acids over a pH range.

Table 64

Hydrolysis of Benzoyl-L-leucine by Enzyme Preparation II*

pH of enzyme-substrate solution at zero time	pH of enzyme-substrate solution after 4 hr. incubation	% Enzymatic hydrolysis
4.50	4.49	3.4, 2.0
5.02	5.00	20, 28
5.57	5.52	62, 54½
6.09	6.03	67, 54
6.40	6.42	54, 50
[illegible]	6.92	36, 39½
7.38	7.48	30½, 30

*For conditions see Figure 1.

Figure 1. Hydrolysis of benzoyl-L-leucine by Enzyme Preparation II. Conditions: .0001524 molar substrate, enzyme concentration 9.24 micrograms/ml., 4 hr., enzyme-substrate incubations at 37°, and veronal-acetate buffers of ionic strength .02.

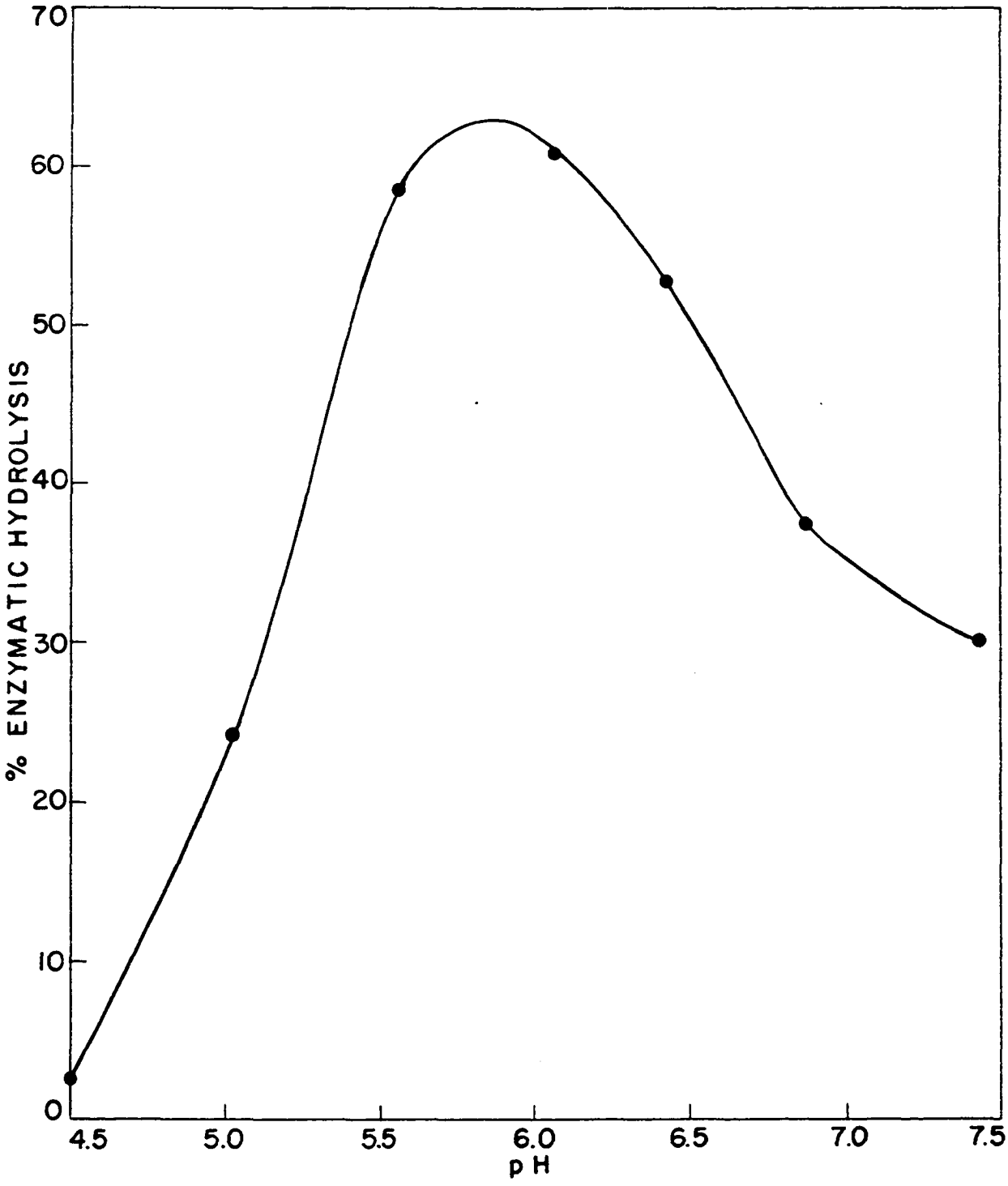
70
60
50
40
30
20
10
0
% ENZYMATIC HYDROLYSIS
4.5
5.0
5.5
6.0
6.5
7.0
7.5
pH

Table 65

Hydrolysis of Carbobenzoxyglycyl-L-leucine by Enzyme Preparation II*

pH of enzyme-substrate solution		
at zero time	after 4 hr. incubation	% Enzymatic hydrolysis
4.50	4.50	2.4,2.0
5.00	5.00	24.6,28.2
5.50	5.52	50,46
6.02	6.07	45,45
6.38	6.40	39,38
6.87	6.92	16.6,25.4
7.42	7.42	8.6,10.6

*For conditions see Figure 2.

Figure 2. Hydrolysis of carbobenzoxyglycyl-L-leucine by Enzyme Preparation II. Conditions: .0001524 molar substrate, enzyme concentration 3.85 micrograms/ml., 4 hr. enzyme-substrate incubations at 37°, and veronal-acetate buffers of ionic strength .02. In an experiment run simultaneously and with the same enzyme solution, benzoyl-L-leucine was hydrolyzed 28%.

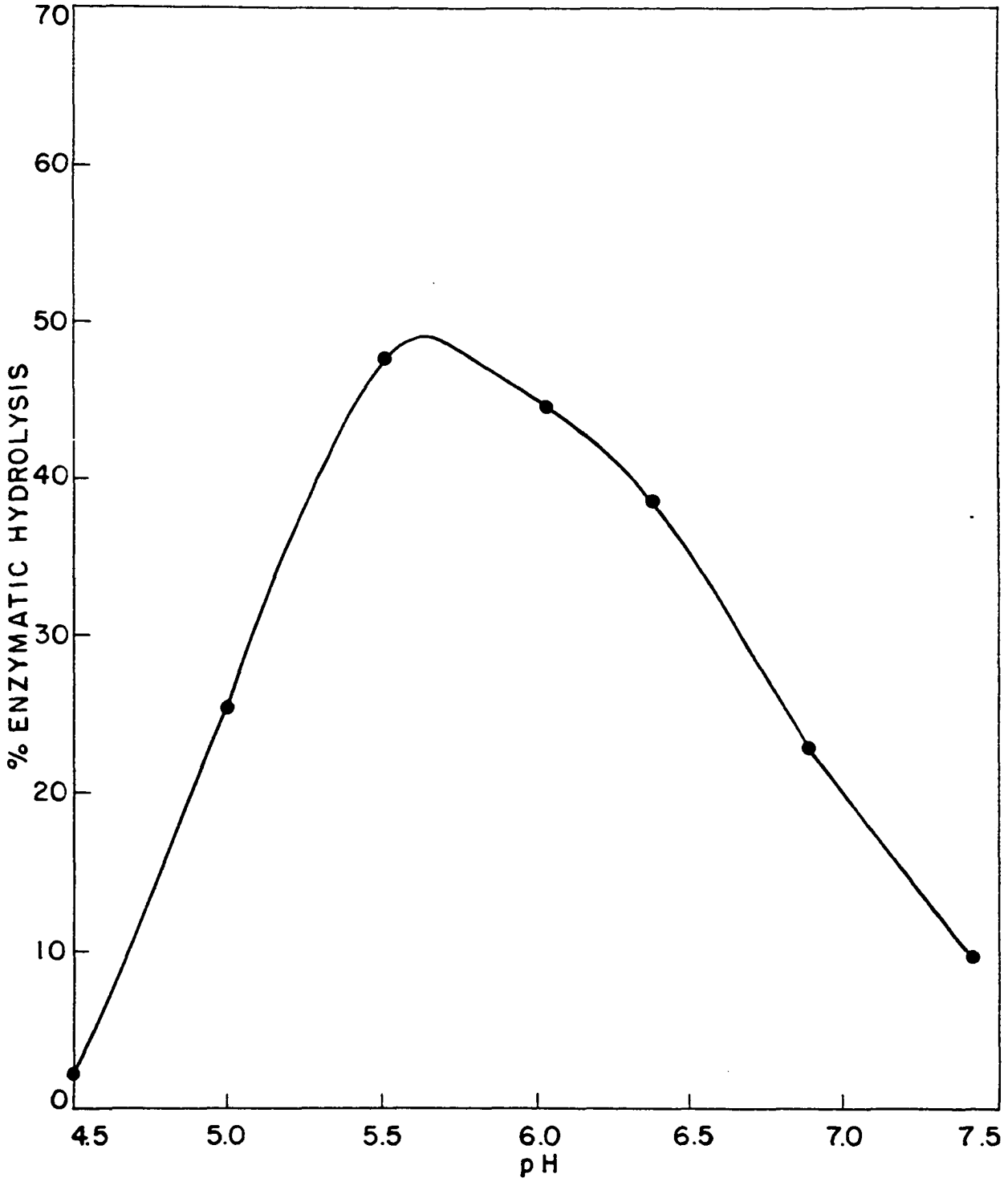
70
60
50
40
30
20
10
0
% ENZYMATIC HYDROLYSIS
4.5
5.0
5.5
6.0
6.5
7.0
7.5
pH

Table 66

Hydrolysis of Benzoyl-DL-methionine by Enzyme Preparation II at 30.8 Micrograms of Enzyme per Ml.*

pH of enzyme-substrate solution		
at zero time	after 4 hr. incubation	% Enzymatic hydrolysis
4.50	4.52	1.1,1.0
5.02	4.96	1.1,1.0
5.53	5.58	2.9,3.1
6.08	6.18	7.5,8.1
6.39	6.40	10,8.5
6.90	6.90	8.3,9.2
7.40	7.39	4.7,4.8

*For conditions see Figure 3.

Figure 3. Hydrolysis of benzoyl-DL-methionine by Enzyme Preparation II. Conditions: .0001524 molar in L-substrate, enzyme concentration 30.8 micrograms/ml., 4 hr. enzyme-substrate incubations at 37°, and veronal-acetate buffers of ionic strength .02. Hydrolysis of benzoyl-L-leucine in a simultaneous experiment at 9.24 micrograms enzyme per ml. was 38%.

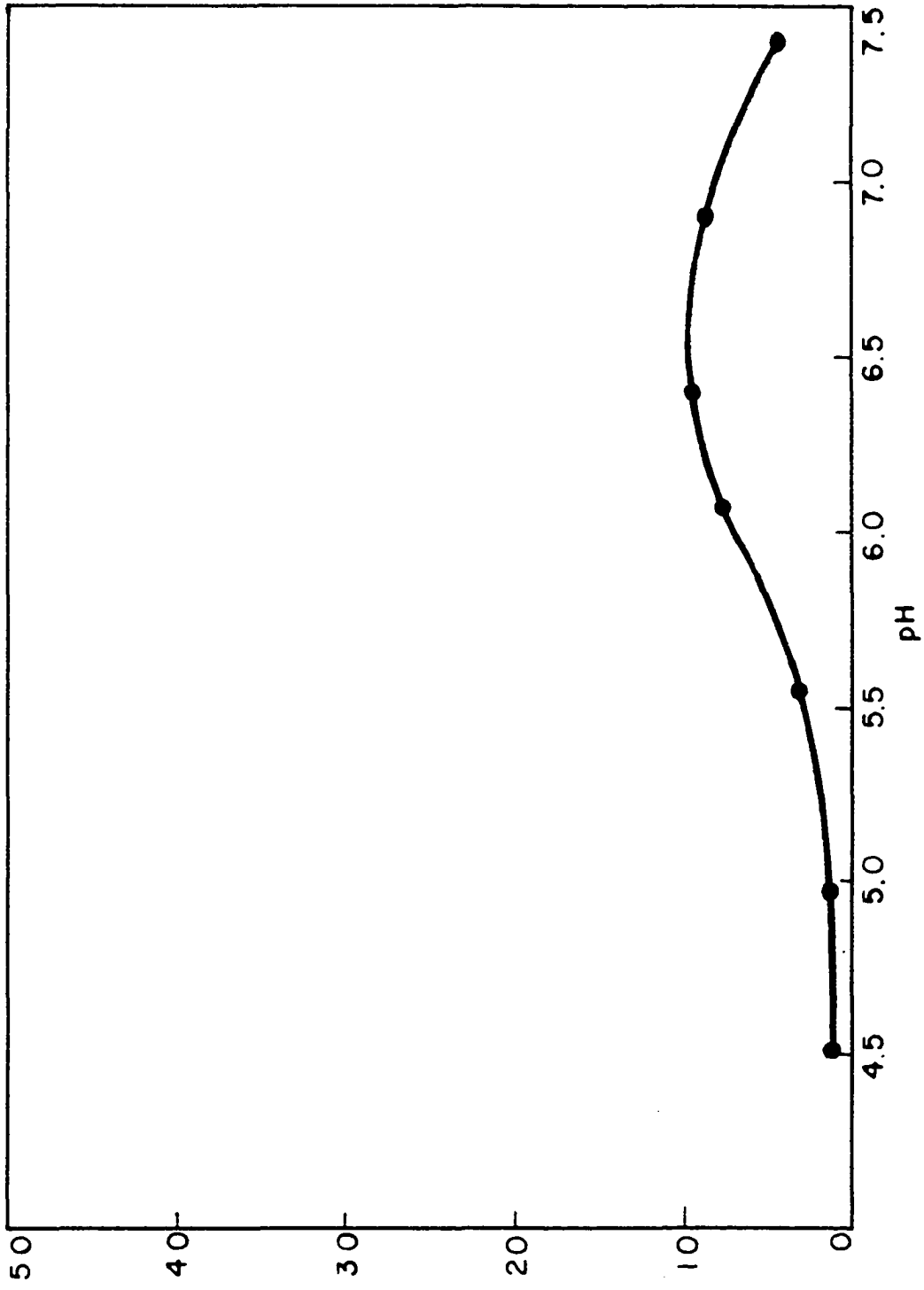
7.5
7.0
6.5
6.0
5.5
5.0
4.5
pH
0
10
20
30
40
50
% ENZYMATIC HYDROLYSIS

Table 67

Hydrolysis of Benzoyl-DL-methionine by Enzyme Preparation II at 185 Micrograms of Enzyme per Ml.*

pH of enzyme-substrate solution		
at zero time	after 4 hr. incubation	% Enzymatic hydrolysis
4.62	4.58	.7,1,.7
5.12	5.07	4,3½,4
5.62	5.65	9.2,9.1,9.2
6.13	6.09	20,19,19
6.51	6.47	39,31,31
6.82	6.73	45,49,44
6.98	6.95	38,45½,46
7.48	7.47	28½,26½,28½

*For conditions see Figure 4.

Figure 4. Hydrolysis of benzoyl-DL-methionine by Enzyme Preparation II. Conditions: .0001524 molar in L-substrate, enzyme concentration 185 micrograms/ml., 4 hr. enzyme-substrate incubations at 37°, and veronal-acetate buffers of ionic strength .02. The enzyme solution was kept in an ice bath during the addition of enzyme to the substrate. Hydrolysis of benzoyl-L-leucine in a simultaneous experiment at 9.24 micrograms enzyme per ml. was 51%.

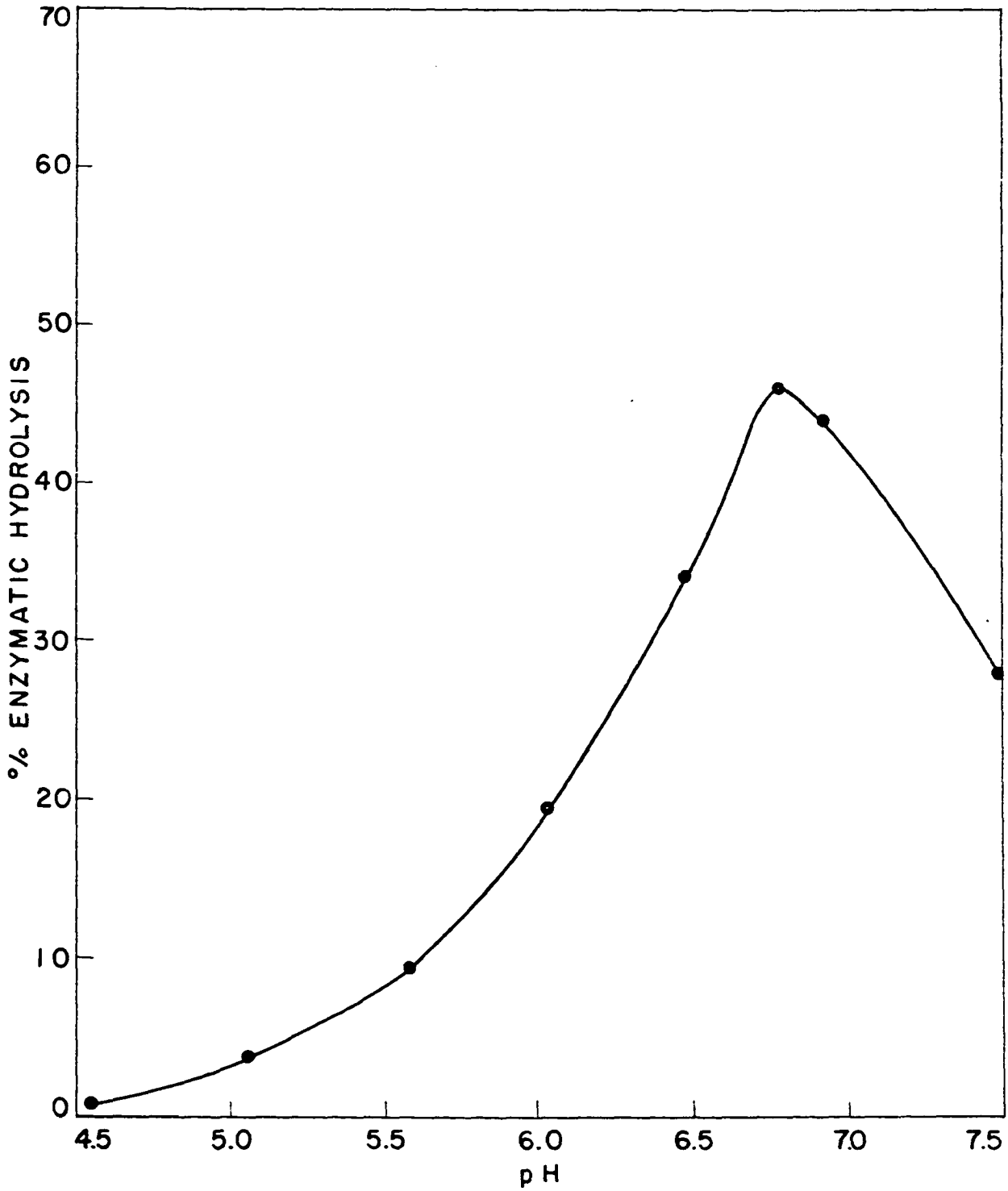
70
60
50
40
30
20
10
0
% ENZYMATIC HYDROLYSIS
4.5
5.0
5.5
6.0
6.5
7.0
7.5
pH

Table 68

Hydrolysis of Benzoyl-DL-valine by Enzyme Preparation II at 185 Micrograms of Enzyme per Ml.*

pH of enzyme-substrate solution		
at zero time	after 4 hr. incubation	% Enzymatic hydrolysis
4.52	4.46	0,0
5.01	5.00	0,0
5.51	5.52	1.8,0
6.00	6.00	1.1,10.6
6.30	6.32	11.8,14.8
6.88	6.82	20.8,22.8
7.40	7.32	19.4,11.8

*For cond'tions see Figure 5.

Figure 5. Hydrolysis of benzoyl-DL-valine by Enzyme Preparation II. Conditions: .0001524 molar in L-substrate, enzyme concentration 185 micrograms/ml., 4 hr. enzyme-substrate incubations at 37°, and veronal-acetate buffers of ionic strength .02. Hydrolysis of benzoyl-L-leucine in a simultaneous experiment at 9.24 micrograms enzyme per ml. was 35%.

50
40
30
20
10
0
% ENZYMATIC HYDROLYSIS
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
pH

Table 69

Hydrolysis of Benzoyl-DL-valine by Enzyme Preparation II at Micrograms Enzyme per Ml.*

pH of enzyme-substrate solution		% Enzymatic hydrolysis
at zero time	after 4 hr. incubation	
4.54	4.58	0,0,0
5.08	5.08	2.5,2.5,2.0
5.51	5.55	4.5,2,6.5
6.03	6.05	8,10.5,15.5
6.37	6.38	23,23,24½
6.68	6.72	36½,33,34
6.89	6.85	46,42,41½
7.39	7.40	48,41

*For conditions see Figure 6.

Figure 6. Hydrolysis of Benzoyl-DL-valine by Enzyme Preparation II. Conditions: substrate .0001524 molar in L-form, enzyme concentration 464 micrograms/ml., 4 hr. enzyme-substrate incubations at 37°, and veronal-acetate buffers of ionic strength .02. Hydrolysis of benzoyl-L-leucine in a simultaneous experiment with 9.24 micrograms of enzyme per ml. was 49%.

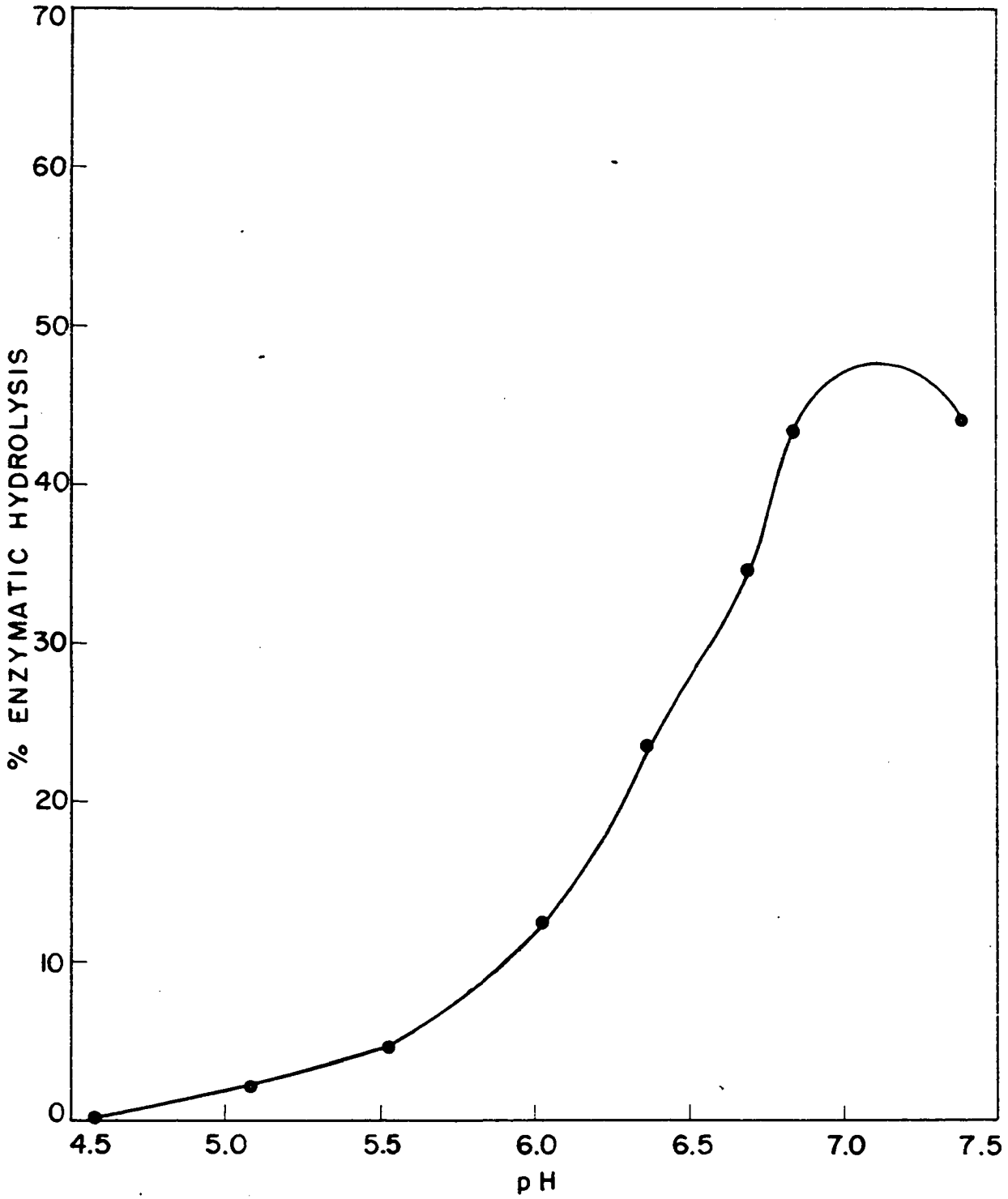
70
60
50
40
30
20
10
0
% ENZYMATIC HYDROLYSIS
4.5
5.0
5.5
6.0
6.5
7.0
7.5
pH

Table 70

Hydrolysis of Benzoyl-DL-phenylalanine by Enzyme Preparation II*

pH of enzyme-substrate solution		% Enzymatic hydrolysis
at zero time	after 4 hr. incubation	
4.51	4.53	.2,.3
5.02	5.00	.8,.6
5.58	5.53	1.7,1.7
6.12	6.06	3.0,3.3
6.40	6.40	6.6,5.3
6.92	6.90	6.6,3.9
7.42	7.42	2.7,1.6

*For conditions see Figure 7.

Figure 7. Hydrolysis of benzoyl-DL-phenylalanine by Enzyme Preparation II. Conditions: substrate .0001524 molar in L-form, enzyme concentration 92.4 micrograms/ml., 4 hr. enzyme-substrate incubations at 37°, and veronal-acetate buffers of ionic strength .02. Hydrolysis of benzoyl-L-leucine in a simultaneous experiment at 9.24 micrograms enzyme per ml. was 56%.

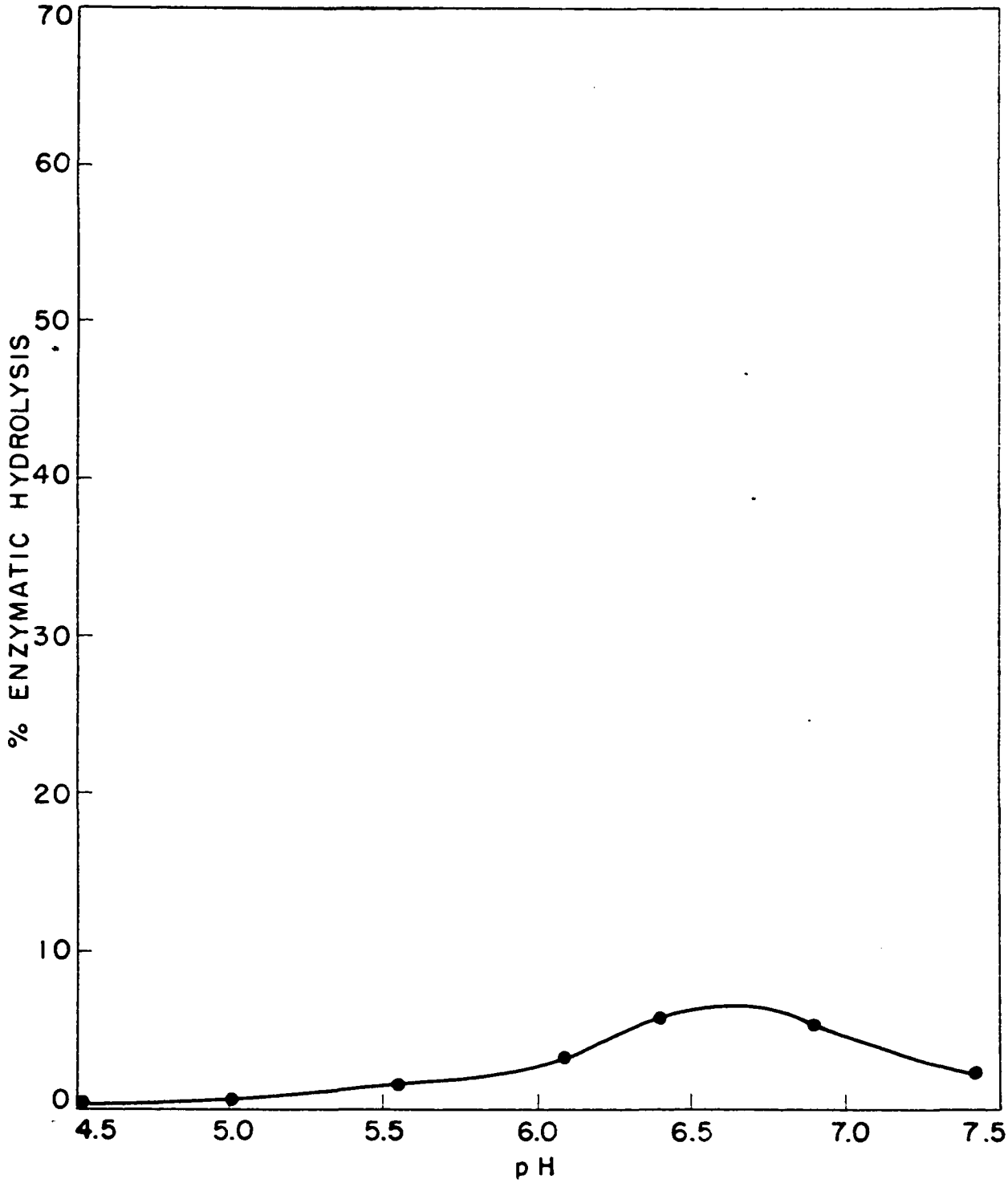
70
60
50
40
30
20
10
0
% ENZYMATIC HYDROLYSIS
4.5
5.0
5.5
6.0
6.5
7.0
7.5
pH

Table 71

Hydrolysis of Benzoyl-L-glutamic Acid by Enzyme Preparation II*

pH of enzyme-substrate solution		% Enzymatic hydrolysis
at zero time	after 4 hr. incubation	
4.65	4.68	2,1½,1½
5.16	5.15	0,0,0
5.70	5.66	0,0,1½
6.21	6.22	0,0,6.5
6.50	6.50	1,1½,-
6.93	6.96	0,-,-
7.43	7.42	0,8,19

*The -'s indicate that the titration values for these tubes were about equal to the titration values for the uninoculated tubes. Conditions: .0001524 molar substrate, enzyme concentration 742$_0$micrograms/ml., 4 hr. enzyme-substrate incubations at 37°, and veronal-acetate buffers of ionic strength .02. Ten per cent hydrolysis corresponded to .3 ml. increase in .10 normal base required to titrate cultures. Hydrolysis of benzoyl-L-leucine in simultaneous experiment at 9.24 micrograms enzyme per ml. was 39%.

Table 72

Hydrolysis of Benzoyl-L-aspartic Acid by Enzyme Preparation II*

pH of enzyme-substrate solution		% Enzymatic hydrolysis
at zero time	after 4 hr. incubation	
4.52	4.59	0,0
5.08	5.04	0,0
5.57	5.58	1.4,1.0
6.12	6.11	0,0
6.40	6.42	0,0
6.90	6.97	0,0
7.40	7.46	0,0

*Conditions: .000152^4 molar substrate, enzyme concentration 185 micrograms/ml., 4 hr. enzyme-substrate incubations at 37°, and veronal-acetate buffers of ionic strength .02. Three percent hydrolysis corresponded to .3 Ml. increase in .050 normal base required to titrate assay cultures. Hydrolysis of benzoyl-L-leucine in a simultaneous experiment at 18.5 micrograms of enzyme per ml. was 100%.

Table 73

Hydrolysis of α-Benzoyl-L-arginine by Enzyme Preparation II*

pH of enzyme-substrate solution		% Enzymatic hydrolysis
at zero time	after 4 hr. incubation	
4.58	4.60	0,0
5.07	5.08	0,0
5.62	5.70	0.5,0.6
6.21	6.20	0.6,0.2
6.42	6.55	0,0
6.94	6.97	0,0
7.48	7.50	0,0

*Conditions: .0001524 molar substrate, enzyme concentration 185 micrograms/ml., 4 hr. enzyme-substrate incubations at 37°, and veronal-acetate buffers of ionic strength .02. Two per cent hydrolysis corresponded to .3 ml. increase in .050 normal base required to titrate assay cultures. Hydrolysis of benzoyl-L-leucine in a simultaneous experiment at 18.5 micrograms of enzyme per ml. was 95%.

Table 74

Hydrolysis of α-Benzoyl-L-histidine by Enzyme Preparation II*

pH of enzyme-substrate solution		% Enzymatic hydrolysis
at zero time	after 4 hr. incubation	
4.59	4.59	0,0
5.05	5.08	0,9
5.65	5.62	0,-
6.19	6.19	0.3,1.5
6.41	6.42	1.0,-
6.96	6.98	1.0,-
7.36	7.42	0.5,0

*The -'s indicate values that were discarded because the titration value was about equal to the uninoculated control. Conditions: .0001524 molar substrate, enzyme concentration 185 micrograms/ml., 4 hr. enzyme-substrate incubations at 37°, and veronal-acetate buffers of ionic strength .02. Two per cent hydrolysis corresponded to .3 ml. increase in .050 normal base required to titrate assay cultures. Hydrolysis of benzoyl-L-leucine in a simultaneous experiment at 18.5 micrograms of enzyme per ml. was 92%.

Table 75

Hydrolysis of N-Benzoyl-DL-threonine by Enzyme Preparation II*

pH of enzyme-substrate solution		% Enzymatic hydrolysis
at zero time	after 4 hr. incubation	
4.57	4.49	0,0
5.02	4.98	0,0
5.50	5.49	0,0
6.02	5.95	0,0
6.30	6.30	0,0
6.92	6.80	0,0
7.40	7.35	0,0

*Conditions: .0001524 molar substrate (of L-form), enzyme concentration 185 micrograms/ml., 4 hr. enzyme-substrate incubations at 37°, and veronal-acetate buffers of ionic strength .02. Hydrolysis of 3.7% corresponded to increase of .3 ml. in .050 normal base required to titrate assay cultures. Hydrolysis of benzoyl-L-leucine in a simultaneous experiment at 9.24 micrograms of enzyme per ml. was 38%.

Table 76

Hydrolysis of Benzoyl-DL-leucinamide and Chloroacetyl-DL-leucine by Enzyme Preparation II in a Simultaneous Experiment*

Substrate	Mg. enzyme per ml.	% Enzymatic hydrolysis
Chloroacetyl-DL-leucine	9.24	59,73,55
Chloroacetyl-DL-leucine	4.62	24,32,31
Benzoyl-DL-leucinamide	9.24	1.1,0
Benzoyl-L-leucine	9.24	48,42,58

*Conditions: .0001524 molar substrate (of L-form), pH about 6.0, 4 hr. enzyme-substrate incubations at 37°, and veronal-acetate buffer was of ionic strength .02.

b. Relative yields from hydrolysis of various acylamino acids by Enzyme Preparation II and pH optima. The data of the preceding section seem to indicate that carbobenzoxy-glycyl-L-leucine, benzoyl-L-leucine, benzoyl-DL-methionine, benzoyl-DL-valine, and benzoyl-DL-phenylalanine are hydrolyzed by an enzyme preparation from Lactobacillus arabinosus. In order to express simply, and in an approximate manner, the ability of this acylase preparation to hydrolyze the substrates studied, the data of section a. are used to calculate relative yields where

$$\text{Relative Yield} = \frac{(\text{Weight of enzyme to hydrolyze Bz-leu})}{(\text{Weight of enzyme to hydrolyze substrate})} \times \frac{(\% \text{ Hydrolysis of substrate})}{(\% \text{ Hydrolysis of Bz-leu at pH 6.0})} \times 100.$$

No significant hydrolysis of benzoyl-L-glutamic acid, benzoyl-L-aspartic acid, -benzoyl-L-arginine, -benzoyl-L-histidine, or benzoyl-DL-threonine was noted. Calculated relative yields of hydrolyses are presented in Table 77.

In addition, an experiment was carried out in which the extent of hydrolysis of several substrates at one enzyme concentration, when the enzyme additions were within

Table 77

Calculated Relative Yield for Hydrolysis by *Lactobacillus arabinosus* Acylase Preparation (Enzyme Preparation II) and pH Optima*

Substrate	pH optimum	Relative yield per unit weight of acylase at pH			Enzyme concentration as mg./ml.
		5.0	6.0	7.0	
Benzoyl-L-leucine	5.85	39	100	80	9.24
Benzoyl-DL-leucinamide			Less than 3		9.24
Carbobenzoxyglycyl-L-leucine	5.65	91	163	71	3.85
Chloroacetyl-DL-leucine			135		9.24
Benzoyl-DL-methionine	6.75	0.34	1.8	4.1	185
Benzoyl-DL-phenylalanine	6.6	0.12	0.52	0.82	92.4
Benzoyl-DL-valine	7.1	0.07	0.45	1.9	464

*In veronal-acetate buffer of ionic strength .02. Substrates were .000152 molar in L-component. Enzyme-substrate incubations were for 4 hours at 37°. Relative rates are calculated from data of section a. and expression on page 262.

Table 77 (Continued)

Substrate	pH optimum	Relative yield per unit weight of acylase at pH			Enzyme concentration as mg./ml.
		5.0	6.0	7.0	
Benzoyl-L-glutamic acid		Less than 0.32**			742
Benzoyl-L-aspartic acid		Less than 0.30**			185
α-Benzoyl-L-arginine		Less than 0.21**			185
α-Benzoyl-L-histidine		Less than 0.22**			185
N-Benzoyl-DL-threonine		Less than 0.49**			185

**That is less than relative rates corresponding to an increase over blanks of 0.30 ml. of base used in titration of assay cultures.

a few minutes, was determined. The results are presented in Table 78. It can be seen that the relative yield of breakdown per unit weight of enzyme is apparently higher at lower enzyme concentrations for the substrates benzoyl-DL-methionine, benzoyl-DL-valine and benzoyl-DL-phenylalanine. However, this increase in relative yield may not be

Table 78

Hydrolysis of Substrates at One Enzyme Concentration in a Single Experiment*

Substrate	Measured pH	% Hydrolysis
Benzoyl-L-leucine	5.81	43,60,73,56
Chloroacetyl-DL-leucine	5.73	53,46,42,46
Benzoyl-DL-methionine	5.80	5.7,5.7,5.2,5.2
Benzoyl-DL-valine	5.73	1.0,1.3,2.9,0
Benzoyl-DL-phenylalanine	5.82	.8,1.0,1.0

*The enzyme concentration was 11.1 micrograms of Enzyme Preparation II per ml. of enzyme-substrate solution. The buffer was of veronal-acetate of ionic strength .02, substrate concentration was .000152 molar in the L-form, the enzyme-substrate incubation was for 4 hours at 37°.

much greater than the increase in the usual relative variation in assay results at the lower levels of released amino acid.

3. The acylase activity of Enzyme Preparation III A and III B

a. Hydrolysis of benzoyl-L-leucine and carbobenzoxyglycyl-L-leucine.

Table 79

Acylase Activity of Enzyme Preparation III A and III B*

Fraction	Mg/ml of enzyme	% Hydrolysis	
		Benzoyl-L-leucine	Carbobenzoxyglycyl-L-leucine
A	.0094	20,18	35,38
B	.0232	42,34	88,72
B	.00464	8,9	19,19

*In veronal-acetate buffer of pH ca. 6.0 and of ionic strength .04. Four hour incubation at 37°.

b. Recovery of acylase activity in preparation of alcohol precipitated and lyophilized preparations. Table 80 presents data on the hydrolysis of benzoyl-L-leucine by 3 enzyme fractions. The enzyme concentrations in the enzyme-substrate solution was derived from the cells grown in 5 ml. of synthetic medium. As can be seen from Table 80, recovery of activity toward benzoyl-L-leucine apparently exceeded 100%. This may have been due to proenzyme activation.

Table 80

Recovery of Enzyme Activity*

Enzyme solution	% hydrolysis of benzoyl-L-leucine
Unfractionated solution	67,58
Enzyme Preparation III A (.047 mg. enzyme/ml.)	91,64
Enzyme Preparation III B (.0232 mg. enzyme/ml.)	35,34

*The buffer compositions of the three preparations were made to correspond in terms of veronal and acetate. Substrate was .0001524 molar. Incubation was for 4 hours at 37°.

4. Acylase activity of cell suspensions

The ability of a cell suspension to hydrolyze benzoyl-L-leucine is described in Table 81.

Table 81

Comparison of Acylase Activity of Cell Suspension and Almost Cell-Free Preparation*

Fraction	mL of medium to prepare enzyme present in 1 ml of enzyme-substrate solution	Maximum viable cells per ml.	% Hydrolysis of benzoyl-L-leucine**
Cell suspension	10	4.3×10^9	66,66
Cell suspension	5	2.15×10^9	26,26
Lyophilized enzyme preparation (enzyme preparation II)	0.25 (7.2 mg/ml.)	92	42,49

*The lyophilized enzyme preparation and the cell suspension from which it was derived are described on pages 213-219. The results of the viable cell counts from which the above cell concentrations are calculated are given on pages 218-219.

**Conditions: 0.000152 molar benzoyl-L-leucine, in acetate buffer of ionic strength .05 and pH 5.4, 4-1/3 hour incubation at 37°.

D. The Effect of Various Compounds on Hydrolysis by Acylase

The ability of various compounds to inhibit the hydrolysis of benzoyl-L-leucine and carbobenzoxyglycyl-L-leucine by Enzyme Preparation II was investigated. The results are presented in Table 82 and are expressed as % inhibition. Of the compounds tested, only isocaproic acid and D-phenylalanine caused significant inhibition of the hydrolysis of benzoyl-L-leucine and carbobenzoxyglycyl-L-leucine by an enzyme preparation from Lactobacillus arabinosus (Enzyme Preparation II).

E. The Effect of Isocaproate on the Growth of Lactobacillus arabinosus

As seen in Table 84, isocaproate inhibited the growth of L. arabinosus when the laucine was supplied as benzoyl-L-leucine; no inhibition was observed when L-leucine as such was supplied.

Table 82

Effect of Added Compounds on Hydrolysis of Benzoyl-L-leucine by Enzyme Preparation II*

Compound	Concentration	pH	% Inhibition**
Isocaproic acid	.010 molar	6.28	91,93
Isocaproic acid	.001 molar	6.12	78,87
Isocaproic acid	.0001 molar	6.12	56,53
D-Leucine	0.1 molar	6.10	0,0
D-Valine	0.1 molar	6.18	0,0
D-Phenylalanine	0.1 molar	6.00	75,78
1-Aminocyclobutane carboxylic acid	.01 molar	6.01	1,3
5-Cyclobutane spiro-hydantoin	.01 molar	6.07	0,0
Penicillin G potassium salt	1 mcg./ml.	5.82	23,16
Bacitracin	200 mcg./ml.	6.01	0,0
Aureomycin hydrochloride	50 mcg./ml.	6.02	5,20

*Substrate .000152 molar and in veronal-acetate buffer of ionic strength .02. Enzyme-substrate incubations were for 4 hours at 37°. The hydrolysis of the controls (no added compound) was from 55 to 86% and the control for isocaproic acid and D-phenylalanine was hydrolyzed 55%.

**Per cent inhibition is equal to % decrease from control in hydrolysis of substrate divided by % hydrolysis of control all times 100. Assay titration values were corrected by amount of base equivalent to deviation from known-amount-of-leucine control caused by added compound. The 0's indicate that enzymatic hydrolysis in the presence of the added compound was equal to or greater than enzymatic hydrolysis in the absence of the compound.

Table 83

Effect of Added Compounds on Hydrolysis of Carbobenzoxy-glycyl-L-leucine by Enzyme Preparation II*

Compound	Concentration	pH	% Inhibition**
Isocaproic acid	.010 molar	6.29	97,94
Isocaproic acid	.001 molar	6.07	89,92
Isocaproic acid	.0001 molar	6.33	70,65
D-Leucine	0.1 molar	6.12	0,0
D-Valine	0.1 molar	6.08	0,0
D-Phenylalanine	0.1 molar	6.12	97,90
1-Aminocyclobutane carboxylic acid	.01 molar	6.16	28,0
5-Cyclobutane spiro-hydantoin	.01 molar	6.32	0,0
Penicillin G potassium salt	1 mcg./ml.	5.82	23,16
Bacitracin	200 mcg./ml.	6.01	0,0
Aureomycin hydrochloride	50 mcg./ml.	6.02	5,20

*Substrate was .000152 molar and in veronal-acetate buffer of ionic strength .02. Enzyme-substrate incubations were for 4 hours at 37°. The hydrolysis of the controls (no added compound) was from 50 to 73% and the control for isocaproic acid and D-phenylalanine was hydrolyzed 61%.

**Per cent inhibition is equal to % decrease from control in hydrolysis of substrate divided by % hydrolysis of control all times 100. Assay titration values were corrected by amount of base equivalent to deviation from known-amount-of-leucine control caused by added compound. The 0's indicate that enzymatic hydrolysis in the presence of the added compound was equal to or greater than enzymatic hydrolysis in the absence of the compound.

Table 84

Result of Isocaproate Addition on Growth of Lactobacillus arabinosus in Presence of L-leucine or Benzoyl-L-leucine*

Leucine source added to leucineless medium	Concentration of isocaproate	% Transmittance after 13-14 hours**
.381 micromole benzoyl-L-leucine	none	25,30,34
.381 micromole benzoyl-L-leucine	0.01 molar	63,58,65
.381 micromole-L-leucine	none	22,23,24
.381 micromole L-leucine	0.01 molar	22,24,23

*Total volume in each tube was 5.0 ml. and incubation was at 37°.

**The tubes were removed after 13 hours incubation and about 50 minutes were required to complete the readings. The values (% transmittance) in the first column were taken before those in the second column and those in the second column, before those in the third column.

V. DISCUSSION

A. Characteristics of the Acylase System of *Lactobacillus arabinosus* and Comparison with Other Acylases

1. Substrate preferences of the acylase system of *Lactobacillus arabinosus*

Of nine benzoylamino acids tested, only benzoyl-L-leucine was rapidly hydrolyzed by an enzyme preparation from *Lactobacillus arabinosus* (page 263). Hydrolyzed at a decidedly slower rate were benzoyl-DL-methionine, benzoyl-DL-phenylalanine, and benzoyl-DL-valine. In addition carbobenzoxyglycyl-L-leucine and chloroacetyl-L-leucine were hydrolyzed at somewhat faster rates than was benzoyl-L-leucine. Thus, the data available seem to point to the presence in *L. arabinosus* of a rather specific acylase system which attacks most rapidly substrates which contain a terminal L-leucine residue, with the release of free leucine. The tentative name, *Lactobacillus arabinosus*, Acylase, is suggested for this enzyme system. While the present data seem to point to a strong preference by *Lactobacillus arabinosus* Acylase for leucine terminal substrates, the validity of this generalization for substrates containing two or more CO-NH bonds is not well established,

since, of such substrates, only carbobenzoxyglycyl-L-leucine has been tested.

Lactobacillus arabinosus Acylase is not greatly enhanced in its activity by the presence of a second CO-NH bond in its molecule, since the hydrolysis of carbobenzoxyglycyl-L-leucine is only about twice as rapid as is the hydrolysis of benzoyl-L-leucine. The substitution of a chloroacetyl for a benzoyl group in benzoyl-L-leucine, results in only a small increase in the relative rate of hydrolysis by Lactobacillus arabinosus Acylase.

2. Comparison of the substrate preferences of Lactobacillus arabinosus acylase with the acylases from other sources

Certain of the descriptions of acylases given in the Historical of this thesis will now be summarized in a comparison of the substrate preferences of Lactobacillus arabinosus Acylase with the preferences of certain acylases from other sources.

Anson's carboxypeptidase, which is from bovine pancreas (pages 64-72), rapidly hydrolyzes acyldipeptides containing a terminal phenylalanine, tyrosine, tryptophan, leucine, and methionine; the proteolytic coefficients for the carbobenzoxyglycylamino acids containing these amino acids range

from 14 to 1.2 (page 174). Substrate preferences of Lactobacillus arabinosus Acylase (page 263) are decidedly sharper with regard to the influence of the terminal acid. Hydrolysis by Anson's carboxypeptidase is more decidedly facilitated by the presence of a second CO-NH bond in a substrate than is true for Lactobacillus arabinosus Acylase; the proteolytic coefficients for the hydrolysis of carbobenzoxyglycyl-L-tyrosine and chloroacetyl-L-tyrosine by Anson's carboxypeptidase are 6.2 and 1.65, respectively, but the relative rates by Lactobacillus arabinosus Acylase on carbobenzoxyglycyl-L-leucine and chloroacetyl-DL-leucine are 163 and 120, respectively, at pH 6.0. The electronegativity of the acyl group in acylamino acids seems to affect only slightly the rate of hydrolysis by Lactobacillus arabinosus. Thus, the rates of breakdown of benzoyl-L-leucine and chloroacetyl-DL-leucine are 100 and 135 at pH 6.0. In contrast, Fones and Lee report that the relative rates on the chloroacetyl- and acetyl- derivatives of DL-phenylalanine are 3,151 and 15 in veronal buffer for Anson's carboxypeptidase[1].

The present knowledge concerning the acylase complement present in Lactobacillus arabinosus presents a rather simple

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[1] W. S. Fones and M. Lee, J. Biol. Chem., 201, 847-856 (1953).

picture, with the central object being the rather sharp preference of Lactobacillus arabinosus Acylase for substrates containing carboxy-terminal leucine. In sharp contrast is the picture for the acylase complement of hog kidney. The complexity of this picture and the limited specificity will be painted by summarizing the discussion (pages 76-101) concerning the acylases of hog kidney. The various acylases discovered in hog kidney will now be listed and described and comparisons made:

1. The main component of Acylase I which hydrolyzes a variety of acyl aliphatic amino acids at a rapid rate. The rate of substrate hydrolysis by this enzyme increases up to 5 amino acid carbons for the N-chloroacetyl, straight-chain, aliphatic amino acids; the increase in size of the amino acid portion of the chloroacetylated amino acids beyond 5 carbons, or branching, diminishes the rate. Chloroacetylamino acids are hydrolyzed several times as rapidly as are acetyl derivatives. The rates on chloroacetyl-DL-alanine and benzoyl-DL-alanine were found to be 11,609 and 25, respectively (pages 79 and 80) in contrast with almost equal rates on the chloroacetyl and benzoyl derivatives of leucine by L. arabinosus Acylase. The main component of acylase I possesses much less specific substrate preferences with regard to the terminal amino acid than does Lactobacillus

arabinosus Acylase. However, the action of the main enzyme acylase of acylase I is more selectively influenced by the nature of the acyl group in acylamino acids serving as substrates than is true for Lactobacillus arabinosus acylase. Limited evidence indicates the primary action of acylase I is on substrates containing 1 CO-NH bond, but on the other hand, the presence or absence of the second CO-NH group does not have a striking effect on the rate of hydrolysis by Lactobacillus arabinosus Acylase.

2. An enzyme acting more rapidly than benzoyldiglycine than does the enzyme described under 1. Possibly other enzymes acting on acylglycine are present in hog kidney.

3. An enzyme hydrolyzing acyltyrosines. Possibly this enzyme or other separate enzymes hydrolyze acyltryptophans. Evidence is very scanty that acyltryptophans are hydrolyzed by an enzyme differing from the main component of acylase I.

4. An enzyme hydrolyzing acylasparagines. Possibly this enzyme or other enzymes hydrolyze acylphenylalanines and threonines.

5. An aspartic acid acylase.

6. An enzyme hydrolyzing some acyldipeptides including chloroacetyl-DL-alanylglycine. This enzyme differs from the

main component of acylase I and has a pH optimum of 8.0 for the substrate mentioned.

7. A carboxypeptidase which acts on carbobenzoxyglycyl-L-tyrosine and carbobenzoxyglycyl-L-phenylalanine with cysteine activation. The pH optimum for the hydrolysis of the latter compound is about 5.3.

8. An enzyme hydrolyzing carbobenzoxy-L-glutamyl-L-tyrosine and carbobenzoxy-L-glutamyl-L-phenylalanine without cysteine activation.

9 and 10. Separate enzymes which hydrolyze benzoyldiglycine and which have acylase action on β-naphthalinsulfoglycine exist. These activities differ from that for benzoylglycine. Whether or not the first two activities are identical with some of the preceding enzymes is not known.

Some evidence exists for the widespread occurrence in mammalian tissues of acylases similar to some of those found in hog kidney and bovine pancreas. There is a little evidence that some differences exist in the substrate preferences of the acylases from different mammalian species. For a detailed presentation of evidence on this subject see the Historical (pages 16-145).

Possibly related to the specificity of constituent acylases are the utilization by acid-producing bacteria of certain acylamino acids for their amino acid requirements.

Benzoylglycine is utilized by Lactobacillus brevis 8287, Leuconostoc mesenteroides 8042, Streptococcus faecalis 8043, Leuconostoc mesenteroides P 60, Leuconostoc citrovorum 8081, and Lactobacillus pentoaceticus 367. An enzyme preparation from Leuconostoc mesenteroides hydrolyzes benzoylglycine at about twice the rate as chloroacetylglycine. With the exception of the general hydrolysis of acylated glycine, it seems rather likely that marked species differences and marked specificities exist in the substrate specificity of the acylases in a group of acid-producing bacteria. This is supported by the observation that each acid-producing bacterium investigated can make detectable nutritional utilization of just one or a few acylamino acids of a goodly number tested. Also, the acylamino acid utilized varies with the bacterial species. The evidence is limited, however, in that only a few acylated dipeptides were used. Also, direct enzymatic evidence is usually lacking.

The specific utilization pattern of acylamino acids other than acylglycines will now be summarized. Streptococcus faecalis makes use of benzoyl-DL-methionine, formyl-L-methionine, and, to a slight extent, formyl-L-valine; the experiments involved acyl derivatives of 11 amino acids. Leuconostoc mesenteroides utilized only dibenzoyl-L-cystine of the acyl derivatives of 16 amino acids.

Lactobacillus brevis 8287 utilizes α-benzoyl-L-arginine and to a slight extent dibenzoyl-L-cystine of 16 benzoylamino acids tested. Acetyl-DL-tryptophan and chloroacetyl-DL-tryptophan are available to Lactobacillus casei 7469 for its tryptophan requirements; but several acyl derivatives of leucine, phenylalanine, and valine are not nutritionally available in lieu of the corresponding amino acid. As reported in this thesis, the acylase system of Lactobacillus arabinosus seems to have its greatest preference for substrates possessing a terminal L-leucine residue. For a detailed discussion of past work relating to the acylases of the acid-producing bacteria see pages 152-162.

The work summarized in the Historical of this thesis (pages 6-183 supports the contention that enzymes of the acylase type are widely and generally distributed throughout the plant and animal kingdoms. Evidence is fairly limited concerning the substrate preferences of these acylases. However, based on present knowledge, the acylases of certain acid-producing bacteria are rather unique in the sharp substrate preferences displayed and in the decided specificity differences among the acylases of different species.

3. Comparison of acylase inhibition patterns

The data on page 276 indicate that the breakdown by Lactobacillus arabinosus acylase of benzoyl-L-leucine and carbobenzoxyglycyl-L-leucine is inhibited at high concentrations of D-phenylalanine and at low concentrations of isocaproate. D-Leucine and D-valine failed to significantly inhibit acylase action at molar concentrations 657 times that of the substrates. 1-Aminocyclobutane carboxylic acid, 5-cyclobutane spirohydantoin, penicillin G, bacitracin, and aureomycin did not inhibit acylase action. The strong inhibition of Anson's carboxypeptidase action by β-phenylpropionic acid parallels the inhibition by isocaproic acid of Lactobacillus arabinosus acylase. This is so since both inhibitors possess structures corresponding in part to the structures of substrates quite susceptible to hydrolysis by the respective enzymes. The inhibition by D-amino acids, however, presents no parallel in one regard. Both Lactobacillus arabinosus acylase and Anson's carboxypeptidase are inhibited by D-phenylalanine. This inhibition does not present a parallel in the case of Lactobacillus arabinosus acylase between the structure of inhibiting D-amino acid and structure of terminal L-amino acid in susceptible substrates--in terms of inhibiting power and susceptibility

to hydrolysis, respectively. If such a parallel existed, then hydrolysis of benzoyl-L-leucine by Lactobacillus arabinosus acylase would be more strongly inhibited by D-leucine than by D-phenylalanine and such is not the case. Also, the inhibition of Anson's carboxypeptidase by D-phenylalanine is decidedly more pronounced than is the inhibition of Lactobacillus arabinosus acylase (see pages 72 and 276).

The hydrolysis of β-naphthalinsulfoglycylglycine by an enzyme preparation from Aspergillus niger (page 170) was inhibited by DL-phenylalanine, L-leucine, and glycine. It would be of interest to know which of the phenylalanine isomers was responsible for the observed inhibition.

4. pH and acylase activity

As indicated on page 263, the pH optima for the hydrolysis of benzoyl-L-leucine and carbobenzoxyglycyl-L-leucine by Lactobacillus arabinosus acylase are 5.85 and 5.65, respectively; while the pH optima for the hydrolysis of benzoyl-DL-methionine, benzoyl-DL-valine, and benzoyl-DL-phenylalanine lie in the range 6.6 to 7.1. The optional pH for the action of Anson's carboxypeptidase is about

pH 7.3[1]. The breakdown of carbobenzoxyglycyl-D-leucine by a hog pancreas extract occurred more rapidly at pH 6.0 (page 134). The maximum rate of hydrolysis of carbobenzoxyglycyl-L-phenylalanine by a cysteine-activated enzyme from swine kidney is 5.4 (page 95). The pH optimum for the hydrolysis of chloroacetyl-DL-alanylglycine and chloroacetylglycyl-DL-alanine by a swine kidney preparation was found to be pH 8.0 (page 98). Hog liver preparations were found to hydrolyze substrates at the indicated pH optimum: benzoyldiglycine, 4-4.5 (page 106); acetylglycine, acetyl-DL-leucine, and acetyl-DL-phenylalanine, 7.2 (page 106). Carbobenzoxy-L-glutamyl-L-phenylalanine was optimally hydrolyzed by porcine epsin at pH 2 to 4.0 depending on substrate and enzyme concentration (page 108-109).

Staphylococcus aureus extracts hydrolyzed several acylamino acids at optima ranging between 7.0 and 8.0 (page 148). Hydrolysis of some acylglycines by extracts of Bacillus pyrogenes citreus Nr. 1, and Bacillus coli communis occurred between pH 7 and 8.0 (pages 150 and 163). A pH optimum of 7.1 was found for the hydrolysis of β-naphthalinsulfoglycylglycine by an extract of Aspergillus niger (page 169-170), and for the hydrolysis of chloroacetyl-L-tyrosine by an extract of Parasiticus aspergillus (page 178).

Thus the present evidence indicates that, with the exception of the mammalian pepsins, acylases usually act optimally at pH's in the range 4.0-8.0.

5. Enzymes present in the acylase system of *Lactobacillus arabinosus*

Several experimental observations make it seem probable that one enzyme hydrolyzes benzoyl-L-leucine and carbobenzoxyglycyl-L-leucine. One such observation is the approximate constancy of the activity ratio's on the two substrates for four different enzyme preparations of differing activity. Another observation supporting the contention is the similar pattern for the inhibition of hydrolysis of the two substrates. Thus isocaproic acid and D-phenylalanine both inhibited the hydrolysis of the two substrates. It seems improbable that the observations would be as they are if benzoyl-L-leucine and carbobenzoxyglycyl-L-leucine were each hydrolyzed by a separate enzyme from *Lactobacillus arabinosus*.

No good evidence has been presented concerning whether or not additional enzymes are responsible for the observed hydrolysis of benzoyl-DL-methionine, benzoyl-DL-valine, and benzoyl-DL-phenylalanine. The pH optima for the

hydrolysis of these substrates differ from those for the hydrolysis of benzoyl-L-leucine and carbobenzoxyglycyl-L-leucine but this does not demonstrate that more than one enzyme is acting on the group of substrates in view of the varying pH optima for the hydrolysis of pepsin substrates.

B. Relationships between Behaviour of Acylases and Cellular Behaviour

1. The relation between Lactobacillus arabinosus acylase hydrolysis and utilization of acylamino acids by Lactobacillus arabinosus

An objective of this dissertation has been to see if there existed a parallel between utilization of acylamino acids by Lactobacillus arabinosus and the relative rates of enzymatic hydrolysis of acylamino acids by enzyme preparations derived from the organism. The benzoyl-, chloroacetyl-, and carbobenzoxyglycyl- derivatives of leucine were both appreciably utilized and enzymatically hydrolyzed. Benzoyl-DL-methionine was hydrolyzed at a moderate rate by Lactobacillus arabinosus acylase and this compound was also to some extent available for the methionine requirements of Lactobacillus arabinosus. α-Benzoyl-L-arginine, α-benzoyl-L-histidine, benzoyl-L-glutamic acid, and N-benzoyl-DL-

threonine were not nutritionally utilized nor were they hydrolyzed by Lactobacillus arabinosus acylase. Benzoyl-DL-phenylalanine and benzoyl-DL-valine were hydrolyzed at a low relative rate by Lactobacillus arabinosus acylase, but these compounds were not detectably utilized by L. arabinosus. Thus, there is a close parallel between nutritional utilization of the acylamino acids and their breakdown by enzyme preparations derived from the cell with the possible exception of benzoyl-DL-phenylalanine and benzoyl-DL-valine. However, the relative rate of hydrolysis of benzoyl-DL-phenylalanine and benzoyl-DL-valine are so low as to not be in decided quantitative disagreement with the utilization experiments. Eades[1], Itschner[2], and Drechsler[3] have suggested that the utilization of acylamino acids involves the prior hydrolysis by the organism of these compounds. The evidence presented in this thesis supports such a suggestion, at least for Lactobacillus arabinosus[4].

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[1]C. H. Eades, J. Biol. Chem., 187, 147-152 (1950).

[2]K. F. Itschner. Bacterial utilization and sequence determination of peptides. Unpublished Ph.D. Thesis. Ames, Iowa, Iowa State College Library. 1951.

[3]E. R. Drechsler. Utilization of certain benzoylamino acids by several species of bacteria. Unpublished M.S. Thesis. Ames, Iowa, Iowa State College Library. 1952.

[4]For a brief discussion of the validity of enzyme studies in explaining the behaviour of living cells, see - F. Schlenk. Bacterial enzymes and the theory of action. In C. H. Werkman and P. W. Wilson, eds. Bacterial physiology. p. 270-271. New York, New York, Academic Press Inc. 1951.

Evidence has been presented that some of the enzyme preparations described in this thesis were contaminated with viable cells. In the making of comparisons between cell-free enzyme behaviour and the behaviour of Lactobacillus arabinosus cells, the objection might be raised that results observed with the enzyme preparations could be due to contaminating cells. However, the contamination with cells was slight compared with the number of cells from which the preparations were derived. Also, the ability of suspended Lactobacillus arabinosus cells to hydrolyze benzoyl-L-leucine was relatively moderate (Table 81, page 268). It can be seen from the information of Table 81 that the cells which contaminate the lyophilized enzyme preparation (Enzyme Preparation II) could be responsible for only an insignificant part of the hydrolysis of benzoyl-L-leucine by this preparation. It seems probable, therefore, that the observed results of the enzyme experiments are due essentially to cell-free enzyme material.

2. Cell environment and acylase activity

In essence the work reported in this thesis has involved the extraction of enzymes from Lactobacillus arabinosus cells and the study of the action of the enzyme solution on

solutions of certain compounds in an attempt to analyze and explain certain metabolic behaviour of the cell. However, it is well to keep in mind that the cell environment may differ from that of the enzyme studies. Acylase molecules of the cells growing in a medium are at least partially confined to little "islands" in the nutrient sea and are not uniformly dispersed throughout the whole body of liquid as in the enzyme studies. Furtherfore, the possibility exists that acylase molecules may be localized, perhaps in an orderly fashion, in sub-cellular particles. These possibilities are raised because other enzymes have been shown to be so localized. Thus enzymes carrying out reactions of the citric acid cycle and fatty acid oxidation have been shown to be localized in the mitrochrondia of liver cells[1,2]. It has been suggested that the four heme proteins of the cytochrome system are fixed in space so that the hemes are juxtaposed[1]. Enzymes may be held in an insoluble state by lipid material; this is suggested, for instance, by the

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[1]A. L. Lehninger. The organized respiratory activity of isolated rat-liver mitochondria. In J. T. Edsall, ed. Enzymes and enzyme systems. p. 1-14. Cambridge, Massachusetts, Harvard University Press. 1951.

[2]D. E. Green. The cyclophorase system. In J. T. Edsall, ed. Enzymes and enzyme systems. p. 15-46. Cambridge, Massachusetts, Harvard University Press. 1951.

observation[1] that treatment with butanol of hog kidney particulate fraction solubilizes an aminopeptidase. Also, Lactobacillus arabinosus acylase may be excreted into the medium during the utilization experiments.

The pH of the cellular environment is not identical with that of the external medium according to Mitchell[2].

In the specific phase of metabolism under consideration in this thesis, viz., the metabolism of acylamino acids, one finds that there is a close parallel between cellular behaviour and enzyme behaviour. While it seems reasonable to deduce that, therefore, the acylase action explains the cell behaviour, these observations do not, unfortunately, make clear to the author many deductions concerning the cell environment and the physical state of the enzyme in the cell. In the utilization experiments, acylase action probably takes place between pH 4.5 and 8.0 judging from the pH versus % hydrolysis curves (pages 235-255); but this may not correspond to intracellular pH since the experiments reported here have not excluded the excretion of acylases

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[1]D. S. Robinson, S. M. Birnbaum, and J. P. Greenstein, J. Biol. Chem., 202, 1-26 (1953).

[2]P. Mitchell. Physical factors affecting growth and death. In C. H. Werkman and P. W. Wilson, eds. Bacterial physiology. p. 140-141. New York, New York, Academic Press, Inc. 1951.

into the medium; nor has localization of the acylases on the external-cell surface been excluded.

The observed close parallel between the utilization experiments and the enzyme studies, besides being due to the participation of acylases in metabolism, may arise from several circumstances. First, the conditions of the enzyme experiments may approximate the environment of the acylases (within or without the cell) during the utilization experiments. Or, secondly, the enzyme environment during the utilization experiments may differ with regard to solutes or physical condition of the acylases; but this may not result in enzyme behaviour differing greatly from that observed in the experiments with essentially cell-free enzymes. This discussion of intracellular environment and acylase activity does not lead to many conclusions and is intended primarily to point to the questions left unanswered.

3. Role of Lactobacillus arabinosus acylase in cellular metabolism

What is the role, if any of acylases in the metabolic machinery of Lactobacillus arabinosus? The possibilities that they serve in the hydrolysis and/or synthesis of protein and/or peptides will be considered. The author is not aware of other possible roles.

a. As digestive enzyme. The observation that the acylase system of Lactobacillus arabinosus is capable of splitting carbobenzoxyglycyl-L-leucine makes it seem probable that it functions to split at least some proteins and peptides containing carboxy-terminal leucine which Lactobacillus arabinosus encounters under natural conditions. It is difficult to say, on the basis of present evidence, whether or not the specificity displayed by the acylase system toward the benzoylamino acids carries over to most substrates containing two or more peptide bonds; but such a circumstance, i.e. where carboxy-terminal leucine is most readily split out, would not be surprising. If such were the case, the specificity of the acylase system would be of disadvantage to Lactobacillus arabinosus in the process of making available a variety of free amino acids for assimilation

b. In protein synthesis. One of the central unsolved mysteries of biochemistry is the manner in which protein is synthesized. For a number of reasons which will be discussed, a not unreasonable working hypothesis is that proteases, for instance, acylases, participate in protein synthesis. An attempt has been made in the work reported in this thesis to test this hypothesis by means of inhibitor experiments. Before discussing the inhibitor experiments,

mention will be made of observations relating to protein synthesis, and then possible pathways of protein synthesis will be discussed.

Any theory of protein synthesis must explain the presence in proteins of rather definite sequences of amino acid residues linked together by CO-NH bonds. Evidence that proteins contain a definite amino acid sequence is contained in the reports of Sanger, Thompson, and Tuppy[1,2] on the complete amino acid sequence in the insulin molecule. However, McFadden and Smith reported that several preparations of γ-globulins contained a number of N-terminal amino acids in other than molar proportions[3]; these and other observations may indicate the frequent occurrence in nature of mixtures of closely related protein molecules[4].

The equilibrium degrees of hydrolysis of 0.1 molar alanylglycine and benzoylglycine are 99.99 and 99.95%, respectively, according to Borsook and Dubnoff[5]. These

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[1]F. Sanger and H. Tuppy, Biochem. J., 49, 463-481 and 482-490 (1951).

[2]F. Sanger and E. O. P. Thompson, Biochem. J., 53, 353-366 and 366-374 (1953).

[3]M. L. McFadden and E. L. Smith, J. Am. Chem. Soc., 75, 2784-2785 (1953).

[4]S. W. Fox made similar statements in the following article: S. W. Fox, The American Naturalist, 87, 253-256 (1953).

[5]H. Borsook and J. W. Dubnoff, J. Biol. Chem., 132, 307-324 (1940).

authors point out that this is evidence that *in vivo* CO-NH bond synthesis cannot be simply the reverse of hydrolysis; synthesis must be coupled with an energy yielding reaction. This conclusion is supported by several *in vivo* observations.

Neglein reported that the uptake of L-histidine by rat sarcoma slices is appreciable in the presence of glucose, but that no uptake occurs in the absence of glucose and oxygen[1]. Glucose doubles the uptake of L-histidine under aerobic conditions. Siekevitz and Zamecnik stated that the uptake of labeled alanine by rat liver microsomes is increased 2 to 3 times by the aerobic oxidation of α-ketoglutarate or succinate in the presence of magnesium, inorganic phosphate, and adenylic acid[2]. Glucose and ammonium sulfate were observed to stimulate the uptake of methionine by *Escherichia coli*; this uptake was inhibited by 2,4-dinitrophenol[3]. Glucose kept down the plasma amino acid level of hepatectomized dogs[4]. Shimura observed the stimulation of protein formation in the excised silk gland

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[1] E. Negelein, Biochem. Z., 323, 214-234 (1952).

[2] Siekevitz and Zamecnik, Fed. Proc. 10, 246 (1951).

[3] Melchior, Klioze, and Klotz, J. Biochem., 189, 411-420 (1951).

[4] E. V. Flock, M. A. Block, F. C. Mann, J. H. Grindlay, and J. L. Bollman, J. Biol. Chem., 198, 427-437 (1952).

of the silk worm by a variety of substances; these substances included α-ketoglutaric acid; pyruvic acid; the vitamins B_1, B_2, and B_6; ascorbic acid; pantothenic acid; and adenosine triphosphate[1]. The incorporation of threonine-1,2-C^{14} and valine-2-C^{14} into rat liver particles were stimulated 5-fold by the combination of adenosine triphosphate, magnesium chloride, citrate, and an amino acid mixture according to the report of Kit and Greenberg[2]. Hotchkiss reported during a symposium that there is an inverse relationship in the uptake by respiring staphylococci of phosphate and amino acids during glucose oxidation[3]. Pantothenic acid, pyridoxine, and pteroylglutamic acid have been observed to favor antibody formation in rats[4].

Not only is protein synthesis probably linked to energy yielding reactions, but it often involves a state of dynamic equilibrium involving rapid synthesis and breakdown of

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[1]K. Shimura, F. Koide, H. Itabashi, and H. Fukai, Symposia on Enzyme Chem. (Japan) 7, 51-3 (1952). Kensuke Shimura kindly sent an English translation of the Japanese text.

[2]S. Kit and D. M. Greenberg, J. Biol. Chem., 194, 377-381 (1952).

[3]R. D. Hotchkiss. Discussion. In W. D. McElroy and B. Glass, eds. Phosphorus metabolism. Vol. 1. B. 639-640. Baltimore, Maryland, The John Hopkins Press. 1951.

[4]A. E. Axelrod, Metabolism, II, 1-8 (1953).

protein; the net rate of synthesis is the difference between the two processes. Evidence for the rapid turnover of protein includes that of Pietro and Rittenberg on the rate of protein synthesis in humans[1]. Simpson suggested that the incorporation and release of amino acids from protein might be interrelated; he bases this suggestion on the observations that anaerobiosis, presence of cyanide, and presence of dinitrophenol (conditions which block off the energy supply) inhibit the release of labeled methionine and leucine from the proteins of rat liver slices[2].

One might wonder if peptides are intermediates in protein synthesis. The presence of peptides in viable cells has been demonstrated in several instances. For instance, Salander and Patton have described a "liver peptide" from chick liver which is increased in concentration by feeding of vitamin B_{12}[3]. This peptide appeared to yield aspartic acid, glutamic acid, glycine, cysteine and phosphorus on hydrolysis. Peptides are present in human plasma[4] and in

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[1]A. S. Pietro and D. Rittenberg, J. Biol. Chem., 201, 457-473 (1953).

[2]M. V. Simpson, J. Biol. Chem., 201, 143-154 (1953).

[3]R. C. Salander and A. R. Patton, J. Nutrition, 47, 469-476 (1952).

[4]K. Schmid, J. Am. Chem. Soc., 75, 60-68 (1953).

the juice of corn leaves[1]. A large peptide has been found in the livers of the cow, guinea pig, hog, horse, lamb, and rat[2]. Additional peptides are present in the liver of the guinea pig. The tripeptide, glutathione, is a common constituent of tissue[3]. Steinberg and Afinsen observed non-uniform labeling of ovalbumin by aspartic acid, glutamic acid, and alanine in synthesis by hen oviducts in vitro[4]. They believed that their findings pointed to a stepwise synthetic process probably involving the formation of peptides as intermediates.

Incorporation of labeled amino acids has been observed to be most rapid for the microsomal fraction of the cell[5]. However, the presence of mitrochondria and the substrates and cofactors of oxidative phosphorylation are required for incorporation[5].

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[1]Dorothy De Fontaine. Quantitative chemical distribution of amino nitrogen from leaves of two strains of corn. Unpublished M.S. Thesis. Ames, Iowa, Iowa State College Library. 1952.

[2]H. Borsook, C. L. Deasy, A. J. Haagen-Smit, G. Keighley, and P. H. Lowry, J. Biol. Chem., 179, 705-719 (1949).

[3]F. G. Hopkins, Biochem. J., 15, 286-305 (1921), and R. A. Gortner, Outlines of biochemistry. p. 499. New York, New York, John Wiley and Sons, Inc., 1949.

[4]D. Steinberg and C. B. Anfinsen, J. Biol. Chem., 199, 25-42 (1952).

[5]G. H. Hogeboom, W. C. Schneider, and Mary J. Striebich, Cancer Research, 13, 617-632 (1953).

Ribonucleic acid of living cells has been observed to be closely associated with the protein synthesizing loci[1] and in fact 50% of cell pentose nucleic acid has been found to be associated with a microsomal fraction[2]. Possibly related to the pathway of nucleic acid formation (and protein synthesis?) is the observation that adenine-4,6-^{14}C is most rapidly incorporated into the pentose nucleic acid of mouse liver nuclear fraction and that the least rapid incorporation is in the microsomal fraction[3]. Hypotheses involving the participation of nucleic acid in protein synthesis will be discussed later.

A number of biochemical syntheses of simple molecules containing the CO-NH bond have been investigated. Whether or not these syntheses involve mechanisms used by nature in the synthesis of the more complex protein molecules has not been established, but the investigation of such possibilities seems worthwhile. Perhaps, the most protein-like of the simple molecules under discussion, is glutathionine, γ-glutamylcysteinylglycine. This tripeptide is synthesized by pigeon liver extracts from the constituent amino acids

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[1]A. E. Mirsky, Scientific American, 188, 47-57 (1953).

[2]G. H. Hogeboom, W. C. Schneider, and Mary J. Striebich, Cancer Research, 13, 617-632 (1953).

[3]E. L. Bennett, J. Biochem., 11, 487-496 (1953).

in the presence of magnesium and potassium ions, and adenosine triphosphate[1,2]. A co-factor, such as coenzyme A, is not required. The synthesis takes place in two steps; the first step is the formation of γ-L-glutamyl-L-cysteine from L-glutamic acid and L-cysteine; and the second step involves the condensation of L-glutamyl-L-cysteine and glycine. Enzymes hydrolyzing glutathionine are not required for its synthesis. Glutathione serves as a prosthetic group[3] of glyceraldehyde-3-phosphate dehydrogenase[2].

Further evidence that peptides may be formed as intermediates in protein synthesis is afforded by experiments in which peptides appear to accumulate when penicillin is added to bacterial cultures. Hotchkiss found that when a strain of Staphylococcus aureus was incubated with a solution containing glucose, amino acids, and penicillin, peptides would accumulate in the solution, but no cellular protein was formed; in the absence of penicillin, amino acids would be incorporated into cellular protein in amounts about equivalent to that incorporated into peptides in the presence of penicillin[4].

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[1]J. E. Snoke, S. Yanari, and K. Bloch, J. Biol. Chem., 201, 573-586 (1953).

[2]J. E. Snoke, J. Am. Chem. Soc., 75, 4872-4873 (1953).

[3]I. Krimsky and E. Racker, J. Biol. Chem., 198, 721-729 (1952).

[4]R. D. Hotchkiss, J. Exp. Med., 91, 351-364 (1950).

Gale and co-workers reported that Staphylococcus aureus in glucose solution linked together several amino acids, including cysteine, with glutamic acid with the accumulation of the resultant peptides in the external medium[1,2]. The presence of these amino acids inhibited the transfer of glutamic acid against a concentration gradient into the cells. Glutamic acid transfer was also inhibited by penicillin. However, valine, leucine, and isoleucine accelerated the accumulation of free glutamic acid in the bacterial cell. Compounds having in common a uridine-5-pyrophosphate-amino sugar group were reported to accumulate in Staphylococcus aureus H cells when penicillin was added to a growing culture[3,4,5]. In addition, two of the three compounds isolated contained amino acid residues. The significance of these observations is not clear.

Simmonds and Fruton made observations rather similar to those of Hotchkiss. They reported that a gram negative

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[1]E. F. Gale, Ames, Iowa. Lecture. May 22, 1951.

[2]E. F. Gale and M. B. Van Halteren, Biochem. J., 50, 34-43 (1951).

[3]J. T. Park, J. Biol. Chem., 194, 877-884 (1952).

[4]Ibid., 885-895.

[5]Ibid., 897-904.

bacillus would grow in the presence of salts plus either glycine and leucine, or L-leucylglycine[1]. The organism was slightly inhibited in the presence of 500 units/ml. of pencillin and L-leucylglycine; but 5 units of pencillin caused pronounced inhibition when glycine and leucine but no L-leucylglycine were present. They offered the hypothesis that assimilation of glycine by the organism involved its prior incorporation into a peptide and that the bacteriostatic effect of pencillin was due to inhibition of peptide synthesis.

Interesting reports have appeared within the last few years on the biological synthesis of CO-NH bonds in compounds differing somewhat from the peptides resulting from the hydrolysis of proteins. One such report is that by Maas, who reported that pantothenate is synthesized from β-alanine and pantoate by an extract of Escherichia coli cells in the presence of adenosine triphosphate as an energy source and Mg^{++} or Mn^{+} and K^{+} or NH_4^{+} as activators[2]. Coenzyme A dependence was excluded. Hippuric acid was synthesized from benzoic acid plus glycine by extracts of an acetone powder of rat liver in the presence of adenosine

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[1]S. Simmonds and J. S. Fruton, Science, 111, 329-331 (1950).

[2]W. K. Maas, J. Biol. Chem., 198, 23-32 (1952).

triphosphate; coenzyme A was required and cysteine, Mg and K were also present[1]. Benzoylphosphate could not substitude for the presence of benzoic acid plus adenosine triphosphate. Schachter and Taggart have demonstrated the enzymatic formation of hippurate from S-benzoyl coenzyme A and glycine in the presence of a fraction from hog kidney[2]. Kielley and Schneider reported that mitrochrondria possessed practically all of the para aminohippuric acid synthesizing ability in rat liver homogenates[3]. Para-aminobenzoic acid was observed to conjugate with δ-benzoyl-L-ornithine due to the catalysis of washed chicken kidney residues in the presence of adenosine triphosphate, magnesium sulfate, and cytochrome C[4]. In the presence of rather high concentrations of cyanide, dried cell preparations of Clostridium kluyveri and acetylphosphate caused a relatively non-specific acetylation of amino acids and proteins[5].

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[1]H. Chantrenne, J. Biol. Chem., 189, 227-233 (1951).

[2]D. Schachter and J. V. Taggart, J. Biol. Chem., 203, 925-934 (1953).

[3]R. K. Kielley and W. C. Schneider, J. Biol. Chem., 185, 869-880 (1950).

[4]R. W. McGilvery, and P. P. Cohen, J. Biol. Chem., 183, 179-189 (1950).

[5]E. R. Stadtman, J. Katz, and H. A. Barker, J. Biol. Chem., 195, 779-785 (1952).

Lipmann discussed the acetylation of sulfonamide by pigeon liver extract in the presence of adenosine triphosphate and similar reactions[1]. He suggested that a phosphate bond transfer is operative in the process of incorporation of amino acids into protein.

We have discussed some observations possibly pertinent to protein synthesis. What then are some hypotheses concerning the mode of protein synthesis which are reconcilable with observation? Several workers believe that protein synthesis involves the participation of nucleic acid and a template mechanism which accounts for the specific pattern of protein. Haurowitz suggested that the template conferring specificity in protein synthesis is an extended protein layer and that the physical process leading to a specific sequence of amino acids is analogous to crystallization[2,3] Peptide bond formation is said to be effected by non-specific enzymes and nucleic acid is said to function to maintain the protein template layer in an extended layer. The enzymes involved may be of the cathepsin or papain

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[1]F. Lipmann, Federation Proc., 8, 597-602 (1949).

[2]F. Haurowitz, Biol. Reviews, 27, 247-280 (1953).

[3]F. Haurowitz. Chemistry and biology of proteins. p. 325-355. New York, Academic Press Inc. 1950.

type, but are said not to confer specificity since, according to Haurowitz, proteolytic enzymes isolated from organs are non-specific. Similar proposals were made by Caldwell and Hinshelwood[1].

Dounce suggested a template hypothesis involving the reaction of amino acids with diphosphonucleic acids and a subsequent peeling off involving the change of phosphoamide linkages to carboxylamide linkages[2]. An argument against these template hypothesis is based on the observation, already mentioned, that peptides accumulate in the presence of penicillin, which would seem to indicate a stepwise mechanism of protein synthesis; whereas, a template hypothesis more suggestive of a mechanism in which any one degree of peptide bond formation would not be differentiated from another by an inhibitor. It is possible, of course, that the peptide formation observed involved a process not connected with protein synthesis. Also, it is difficult to reconcile unequal labeling of ovalbumin with the formation of a protein on a single template; however, exchange reactions following protein formation could account for the unequal

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[1]P. C. Caldwell and C. Hinshelwood, J. Chem. Soc., 3156-3159 (1950).

[2]A. L. Dounce, Enzymologia, 15, 251-258 (1952).

labeling observed. The possible role of nucleic acid as the hereditary transmittor of specific patterns of life is, however, an argument for its participation in the formation of protein which is so important in biological specificity[1].

A second possible hypothesis concerning the mode of protein synthesis is that proteases are to a large extent responsible for protein synthesis. An argument for such a hypothesis is stated by Fruton:

> Perhaps the strongest reason for assuming, as a working hypothesis, the view that proteolytic enzymes play an important role in protein synthesis is the fact that they are the only known biocatalysts which by virtue of their sharp specificity could direct, precisely and reproducibly, the couple sequence of successive peptide synthesis required for the formation of a protein[2].

However, the evidence for a sharp specificity on the part of proteases is far from incontrovertible. The description of present knowledge on acylase specificity in the Historical of this thesis would seem to indicate that both specific and rather unspecific acylase are present in nature. However, Janssen, Winitz, and Fox have offered evidence that different products may be formed by different proteases in the synthesis of acylamino acid anilides and acyldipeptide

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[1] A. L. Dounce, Enzymologia, 15, 251-258 (1952).

[2] J. S. Fruton, In D. E. Green. Currents in biochemical research. p. 133. New York, New York, Interscience Publishers, Inc. 1946.

anilides starting with the same reactants[1]. It has been suggested by S. W. Fox[2] that while the substrate specificity of the protease involved in a single step of protein synthesis may be limited, the cumulative effect of a series of protease mediated reactions might give a product with a limited number of amino acid sequences. As has been mentioned, Haurowitz, in contrast to Fruton's statement, does not believe that proteases possess sufficient specificity to account for the specificity of protein synthesis[3,4].

While there is disagreement as to the ability of proteases to impart the specificity of protein structure, the fact that they are the only well known group of enzymes involved in reactions of the peptide bond which possess at least some specificity of action, would make their investigation in connection with protein synthesis of possible value.

As pointed out, protein synthesis is more than a simple reversal of peptide bond hydrolysis. While proteases have

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[1]F. Janssen, M. Winitz, and S. Fox, J. Am. Chem. Soc., 75, 704-707 (1953).

[2]S. W. Fox, Ames, Iowa. Comment on protease specificity. Private communication. 1953.

[3]F. Haurowitz, Biol. Reviews, 27, 247-280 (1953).

[4]F. Haurowitz. Chemistry and biology of proteins. p. 326-355. New York, Academic Press Inc. 1950.

been primarily studied as hydrolytic enzymes, they seem to possess a versatility of reaction which increases the possibility that they can catalyze reactions involved in protein synthesis. This versatility of reaction is displayed in various exchange reactions, other than hydrolysis, on the carbonyl group of peptide or similar bonds. For instance, proteases catalyze transpeptidation reactions[1] as in the replacement of the glycinamide moiety of benzoyl-L-tyrosylglycinamide by isotopic glycinamide in the presence of chymotrypsin[2]. Or transamidation can occur as in the replacement of the $-NH_2$ group of glycyl-L-phenylalaninamide by the -NHOH group of hydroxyamine in the presence of cathepsin C[2]. Certain transpeptidation reactions may lead to the formation of high molecular weight peptides from peptides of lower molecular weight according to Virtanen[3,4,5]. Protease directed exchange

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[1] C. S. Hanes, G. E. Connell, and G. H. Dixon. Transpeptidation and transamidation reactions. In W. D. McElroy and B. Glass, eds. Phosphorus metabolism. Vol. II. p. 95-108. Baltimore, John Hopkins Press. 1952.

[2] R. B. Johnston, M. J. Mycek, and J. S. Fruton, J. Biol. Chem., 187, 205-211 (1950).

[3] A. I. Virtanen, H. K. Kerkkonen, T. Laaksonen, and M. Hakala, Acta Chem. Scand., 3, 520-524 (1949).

[4] A. I. Virtanen, Ann. Acad. Scient. Fennicae, Ser. A. II, 39, 3-25 (1950).

[5] A. I. Virtanen, H. Kerkkonen, M. Hakala, and T. Laaksonen, Naturwissenschaften, 6, 139-140 (1950).

reactions include the replacement of an alkoxy group with an amino acid ester; for instance, chymotrypsin catalyzes the formation of dipeptides and tripeptides from methionine isopropyl ester[1]. A discussion of transpeptidation and how transpeptidation and glutathione formation could account for protein synthesis is presented in a thesis by Winitz[2]. A detailed consideration of transpeptidations involving γ-glutamyl peptides and α-amino-acyl peptides is presented in a paper by Hanes, Hird and Isherwood[3].

The versatility of reaction displayed by proteases could conceivably function in protein synthesis by:

1. a reshuffling of amino acid sequences, or transpeptidation, without net synthesis of peptide bonds.

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[1]Brenner, Müller, and Pfister, Helv. Chimica Acta 33, 568-591 (1950).

[2]Milton Winitz. The contribution of substrate structure to enzymatic peptide bond synthesis. Unpublished Ph.D. Thesis. p. 7-42. Ames, Iowa, Iowa State College Library, 1951.

[3]C. S. Hanes, F. J. R. Hird, and F. A. Isherwood, Biochem. J. 51, 25-35 (1952).

2. coupling of larger peptides, at which stage peptide bond formation may not involve a large negative free energy[1,2,3].
3. or by a selective hydrolysis of peptides formed from non-specific reactions, leaving only a few peptides available for further conversion to protein.

In addition, the participation of proteases in protein synthesis might involve transfer reactions involving high energy bonds. Such bonds might be involved in high energy phosphate groups or in CO-S bonds such as those that occur in coenzyme A. Such reactions would reconcile protease-mediated protein synthesis with the requirement for an energy input. Such a hypothesis seems possible because of the versatility of proteases in catalyzing exchange reactions and because of the intrinsic ability of amino acids to undergo non-enzymatic reactions of the type conceivably involved. An example of such non-enzymatic reactions was

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[1] A. I. Virtanen, H. K. Kerkkonen, T. Laaksonen, and M. Hakala, Acta Chem. Scand., 3, 520-524 (1949).

[2] A. I. Virtanen, Ann. Acad. Scient. Fennicae, Ser. A. II, 39, 3-25 (1950).

[3] A. I. Virtanen, H. Kerkkonen, M. Hakala, and T. Laaksonen, Naturwissenschaften, 6, 139-140 (1950).

that observed by Koshland where acetylphosphate was observed to react with some amino acids at pH 7.3 and 39°[1]. Also, Chantrenne found that dibenzoylphosphate reacted with glycine at pH 7.4 and 37°[2].

A possible mechanism or peptide bond synthesis might be as follows:

(1) R-COOH + acyl-S-Co A $\xrightarrow{\text{protease I}}$ R-CO-S-Co A + acyl-OH

(2) R-CO-S-Co A + R' -NH_2 $\xrightarrow{\text{protease II}}$ R-CO-NH-R' + HS-Co A

where the carboxyl and amino reactants are amino acids and/or peptides and Co A stands for coenzyme A exclusive of the sulfhydryl group. Similar reactions involving high energy phosphate bonds such as acylphosphate groups might be involved. Living organisms, as has been pointed out, possess several prerequisites for carrying out protein synthesis via protease directed reactions involving energy transfer reactions; these include available energy in the form of high energy phosphate bonds, as in adenosine triphosphate, and high energy CO-S bonds, as in acetyl

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[1]D. E. Koshland, Jr., J. Am. Chem. Soc., 73, 4103-4108 (1951).

[2]H. Chantrenne, Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim., 26, 297-314 (1948).

coenzyme A; proteases possessing the ability to carry out a variety of exchange reactions and possessing certain amount of specificity with regard to amino acid residues in substrates; and finally, amino acids having the intrinsic ability to undergo reactions of the type possibly involved. Whether nature actually carries out protein synthesis by use of proteases depends on the specific nature of specificity possessed by her enzyme complement.

Interesting reviews on protein synthesis have been presented Borsook[1,2].

Now to be discussed are experiments undertaken to gain indirect evidence as to whether or not the acylase system described in this thesis was involved in protein synthesis. Fox, Fling, and Bollenback have reported that D-leucine inhibited the growth of Lactobacillus arabinosus in a medium containing no peptide nor protein[3]. Kobayashi, Fling, and Fox suggested that D-amino acids hinder bacterial

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[1]H. Borsook, Physiol. Rev., 30, 206-219 (1950).

[2]H. Borsook. The biosynthesis of proteins and peptides, including isotopic tracer studies. In L. Zechmeister, ed. Progress in the chemistry of organic natural products. Vol. IX. p. 292-353. Wien, Austria, Springer Verlag. 1952.

[3]S. W. Fox, M. Fling, and G. N. Bollenback, J. Biol. Chem., 155, 465-468 (1944).

growth by interfering with proteolytic enzymes in protein synthesis[1]. The observation that D-amino acids inhibited hydrolysis of substrates by Anson's carboxypeptidase made it seem possible that the acylase system of Lactobacillus arabinosus would also be inhibited by D-amino acids. It was believed if a parallel pattern of inhibition of both cell growth and inhibition of hydrolysis by the acylase system of L. arabinosus was observed, that this would constitute evidence, but not proof, that the acylase system was essential to the life of the organism and probably took part in protein synthesis.

As has been mentioned, experiments were carried out to test the effect of D-leucine, D-valine, D-phenylalanine, penicillin, bacitracin, aureomycin, isocaproic acid, 1-aminocyclobutane carboxylic acid, and 5-cyclobutane spirohydantoin on the rate of hydrolysis of benzoyl-L-leucine, and carbobenzoxyglycyl-L-leucine by Lactobacillus arabinosus acylase. Of the compounds tested, only isocaproic acid and to some extent D-phenylalanine caused inhibition of substrate hydrolysis. No significant inhibition of hydrolysis was caused by .10 molar D-leucine which was

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[1] Y. Kobayashi, M. Fling, and S. W. Fox, J. Biol. Chem., 174, 391-398 (1948).

657 times the molar concentration of the substrates and at a concentration which caused decided inhibition of Lactobacillus arabinosus growth, according to Fling[1].

One can conclude from the above results that inhibition of the growth of Lactobacillus arabinosus by D-leucine, D-valine, penicillin, bacitracin, and aureomycin does not involve inhibition of the acylase in L. arabinosus which hydrolyzes benzoyl-L-leucine and carbobenzoxyglycyl-L-leucine. The lack of acylase inhibition by D-leucine did not disprove that inhibition of bacterial growth by D-leucine involves inhibition of some protease since a protease other than the acylase studied might be inhibited.

The observation that isocaproic acid inhibited Lactobacillus arabinosus acylase action suggested a further experiment. The ability of isocaproate to inhibit the growth of L. arabinosus in synthetic medium containing L-leucine was determined and no inhibition was observed. In relation to acylase participation in protein synthesis, this result could be interpretated in at least three ways, viz.: the isocaproate was reaching the Lactobacillus arabinosus acylase, but no inhibition of growth was observed

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[1]M. Fling. Preparation of amino acids and derivatives and their effect on the growth of Lactobacillus arabinosus. Unpublished Ph.D. Thesis. p. 60. Ames, Iowa, Iowa State College Library. 1946.

since the acylase was not essential for the protein synthesis of the organism; the isocaproate was not reaching the acylase in effective quantities because of its being rapidly catabolized or because of its failure to penetrate the cell and at the same time the acylase is essential to the protein synthesis of the organism; or, finally, the isocaproate may have failed to reach the acylase and the acylase is not essential for the protein synthesis of Lactobacillus arabinosus[1].

The experiment described above was enlarged to determine if the isocaproate was reaching the Lactobacillus arabinosus acylase which hydrolyzes benzoyl-L-leucine; the objective of this enlargement was to further limit the conclusions which might be drawn from the previously mentioned experiment. The extension made was to determine if isocaproate would inhibit the growth of L. arabinosus in synthetic medium when leucine was furnished as benzoyl-L-leucine. The observed results were that 0.01 molar isocaproate decidedly inhibited the growth of Lactobacillus arabinosus when no leucine but only benzoyl-L-leucine was present to meet the leucine needs of the bacterium.

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[1]The author appreciates the helpful comments of Dr. Fritz Schlenk on these inhibition experiments. Iowa State College. 1953.

The preceding results would be explained by the following statements. The inhibition of Lactobacillus arabinosus growth by isocaproate when benzoyl-L-leucine was the leucine source was due to inhibition of the acylase active on this latter compound and therefore the isocaproate was reaching the acylase; that some other reaction was not being inhibited was made likely by the failure to observe inhibition when leucine was added as free leucine. Since isocaproate was reaching the Lactobacillus arabinosus acylase and no inhibition of growth was observed when free leucine was present, it seems rather likely that the Lactobacillus arabinosus acylase which acts on benzoyl-L-leucine and carbobenzoxyglycyl-L-leucine does not participate in synthesis of protein from free amino acids.

There are at least two possible arguments, however, against the conclusion that an L. arabinosus acylase does not participate in protein synthesis. It is conceivable that the acylase functions both as a synthesizing and hydrolyzing enzyme and that only a small quantity of the acylase is needed for the synthetic function. The acylase might carry out its synthesizing function at a locus which the isocaproate cannot reach, whereas, the remainder of the acylase can be reached and inhibited by the isocaproate. A further possibility is that isocaproate reached all of the acylase

but that the acylase participates in a peptide-bond-forming reaction which was not sufficiently rate limiting to slow down growth when the reaction was inhibited by isocaproate. However, this latter possibility does not seem likely because of the considerable amount of energy needed for peptide bond formation.

In spite of the preceding arguments it is believed that the experiments described make it rather doubtful that the carboxypeptidase described in this thesis participates at any stage in the synthesis of protein from free amino acids.

C. Comment on Experimental Methods Used in Enzyme Studies

The use of microbiological assay for the detection of amino acids liberated during enzymatic hydrolysis of acylamino acids possesses certain advantages and disadvantages. An advantage was the ability of the method to detect liberated amino acids at concentrations used in growth of the bacterium, that is at physiological concentrations. Thus microbiological assay detected amino acids corresponding to 2 to 10% hydrolysis of .000152 molar substrates. The sensitive ninhydrine colorimetric method as described by Troll and Cannan[1] will only detect concentrations about ten times this level. The specificity of the method

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[1] W. Troll and R. K. Cannan, J. Biol. Chem., 200, 803-811 (1953).

enabled leucine to be determined in the presence of other amino acids which were present in molar concentrations several hundred fold the concentration of leucine and/or substrate.

A disadvantage of the analytical method used in these enzyme studies include its limitation to substrates which do not appreciably influence the assay. Thus it was not seen how the method could be applied to following the hydrolysis of benzoylglycine by acylase. Experiments to detect the hydrolysis of benzoyl-DL-alanine were not satisfactory. Another drawback to the method was the work involved in preparing materials and in carrying out assays for a variety of amino acids. Less specific methods, if applicable, often involve less labor. In addition, microbiological assay has the customary variation in results of the order of 10% and other methods are usually more precise.

It probably would have been more desirable to dissolve the solid enzyme preparations in saline or other salt solutions rather than in distilled water as was usually done in these studies; the acylase preparations seemed to be rather unstable when dissolved in water and kept at ca. 6°. However, incomplete observations suggest that the

acylase activity may undergo activation in acetate buffer of ionic strength 0.1 and pH 5.5.

The use of veronal-acetate buffers of constant ionic strength permitted the study of the affect of the single variable, pH, on the rates of substrate hydrolysis due to Lactobacillus arabinosus acylase. However, veronal is relatively insoluble, and the use of a more soluble buffer combination would permit the use of higher buffer concentrations and the storage of the buffer solutions in the cold so as to minimize spoilage.

VI. SUMMARY AND CONCLUSIONS

1. The ability of a crude lyophilized enzyme preparation from Lactobacillus arabinosus to hydrolyze 9 benzoylamino acids and carbobenzoxyglycyl-L-leucine over the pH range 4.5 to 7.5 was measured by assaying microbiologically for the liberated amino acids. The breakdown of chloroacetyl-DL-leucine at pH 6.0 was also determined.

2. In an experiment carried out at one enzyme concentration at a pH of 6.0 with substrates .152 millemolar in the L-components, the observed extents of hydrolysis (of L-forms) were as follows: benzoyl-L-leucine, 58%; chloroacetyl-DL-leucine, 47%; benzoyl-DL-methionine, 5.5%; benzoyl-DL-valine, 1.3%; and benzoyl-DL-phenylalanine, 1%. Under the preceding conditions, carbobenzoxyglycyl-L-leucine is somewhat (about 60%) more susceptible to hydrolysis than is benzoyl-L-leucine. The pH optima for the benzoylamino acids just mentioned and for carbobenzoxyglycyl-L-leucine, ranged from 5.65 to 7.1.

3. No significant hydrolysis was observed of benzoyl-L-glutamic acid, benzoyl-L-aspartic acid, -benzoyl-L-histidine, -benzoyl-L-arginine, or N-benzoyl-DL-threonine when the conditions were such that hydrolysis over a relative rate of 0.49 would be detected and when the rate on benzoyl-L-leucine was set equal to 100.

4. Benzoyl-L-leucine was almost completely utilizable for the leucine requirements of Lactobacillus arabinosus. Carbobenzoxyglycyl-L-leucine was 100% utilizable in terms of its leucine content and this extent of utilization was somewhat more than previously reported for the 72 hour growth period used.

5. The activity ratios on benzoyl-L-leucine and carbobenzoxyglycyl-L-leucine for three different enzyme preparations from Lactobacillus arabinosus which possessed considerably different activity per unit of protein were about the same.

6. Benzoyl-DL-leucinamide was not detectably hydrolyzed by an enzyme preparation from L. arabinosus under conditions which caused 46% hydrolysis of benzoyl-L-leucine.

7. The hydrolysis of benzoyl-L-leucine and carbobenzoxyglycyl-L-leucine by the above mentioned enzyme preparation was inhibited by .0001 molar isocaproic acid and .1 molar D-phenylalanine. No significant inhibition was noted with .1 molar D-leucine, 0.1 molar D-valine, .01 molar 1-aminocyclobutane carboxylic acid, .01 molar 5-cyclobutane spirohydantoin, 1 mcg./ml. of penicillin G, 200 mcg./ml. of bacitracin, or 50 mcg./ml. of aureomycin.

8. Isocaproate inhibited the growth of Lactobacillus arabinosus in synthetic medium containing benzoyl-L-leucine

as the leucine source; no inhibition was noted when L-leucine was the sole leucine source.

9. The preparation of the new compounds, N-phenylacetyl-l-aminocyclobutane carboxylic acid, and N-benzoyl-l-amino-cyclobutane carboxylic acid is described.

10. Modified procedures are described for the preparation of the previously described compounds, benzoyl-L-leucine and copper l-aminocyclobutane carboxylate.

11. There was a close parallel between the rates of acylase action and the, for the most part, previously reported nutritional utilizations of acylamino acids by Lactobacillus arabinosus. It is believed that these observations constitute good evidence that the utilization phenomena are due to the action of an acylase or acylases produced by Lactobacillus arabinosus.

12. In view of the observations given under 5 (page 319), and considering the rather specific common inhibition pattern for enzyme activity on benzoyl-L-leucine and carbobenzoxyglycyl-L-leucine (statement 7, page 319), it seems likely that only one enzyme from L. arabinosus is responsible for the observed hydrolysis of benzoyl-L-leucine and carbobenzoxyglycyl-L-leucine.

13. In view of the statements of 6 (page 319) and 12 (page 320), it seems likely that the enzyme which hydrolyzes benzoyl-L-leucine and carbobenzoxyglycyl-L-leucine is a carboxypeptidase.

14. Since no inhibition of acylase action by D-leucine was observed, the previously reported inhibition of Lactobacillus arabinosus growth by this compound was probably not due to inhibition of the L. arabinosus acylase (or carboxypeptidase) active on benzoyl-L-leucine and carbobenzoxyglycyl-L-leucine.

15. It is believed that the observations concerning the effect of isocaproate on acylase action and growth of Lactobacillus arabinosus (statements 7 and 8, page 319) make it appear rather unlikely that the carboxypeptidase described in this thesis participates in protein synthesis.

VII. ACKNOWLEDGMENTS

The author wishes to express his sincere thanks for the guidance and inspiration provided by Dr. S. W. Fox. The furnishing of chemicals and advice for microbiological assays by Dr. Thomas Hurst, Dorothy De Fontaine, and Mrs. Carol Warner is appreciated. Thanks are due to Dr. C. H. Werkman, Head of the Bacteriology Department of Iowa State College, for permitting use of a magnetostriction oscillator. And finally the author expresses his appreciation to his wife, Marilyn, for typing the preliminary draft of this thesis.