ON PROTEOLYTIC ENZYMES

V. ON THE SPECIFICITY OF DIPEPTIDASE*

By MAX BERGMANN, LEONIDAS ZERVAS,† JOSEPH S. FRUTON, F. SCHNEIDER, AND H. SCHLEICH

(From the Laboratories of The Rockefeller Institute for Medical Research, New York, and the Kaiser Wilhelm Institute for Leather Research, Dresden)

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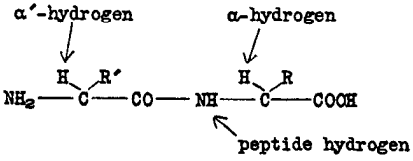
In recent years it has become possible to synthesize peptides of any desired amino acids and thus to obtain a wide selection of substrates for the investigation of the specificity of the proteolytic enzymes. It was found that dipeptidase splits all dipeptides corresponding to this general structure.



Thus it is essential for dipeptidase action that the substrate shall contain the peptide linkage, a free carboxyl, and an amino group. The carboxyl must be situated on the carbon atom next to the peptide nitrogen; the amino group must be on the carbon next to the peptide carbonyl. These results are in agreement with the conclusions drawn by Grassmann for the dipeptides of the simple amino acids (2). The peptide hydrogen is also essential (*cf.* Levene and Simms (3), Abderhalden *et al.* (4), and Bergmann *et al.* (5)). Finally, all dipeptides of natural amino acids have α- and α'-hydrogen in a definite spatial arrangement. If one of these 2 hydrogen atoms is missing, dipeptidase action is either completely or partly inhibited.

* For Paper IV of this series see (1).

† Fellow of the Rockefeller Foundation.

In the experiments here reported further information regarding these criteria of specificity has been obtained.

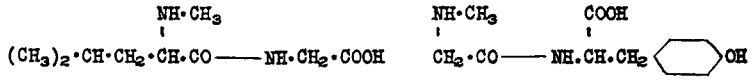
The essential character of the *amino group* had been drawn from the finding that N-acylated dipeptides were not split by dipeptidase. Upon acylation the basic character of the amino group is removed but is retained when an alkyl group is introduced instead of the acyl residue, as in sarcosylglycine which was first studied by Levene and his coworkers (6). We have examined the splitting of N-methyl-*dl*-leucylglycine (I), studied by Abderhalden and his coworkers (4, 7), because the methyl-free leucylglycine is very easily split and is therefore employed as a sensitive test substrate

TABLE I

Splitting with Glycerol Extract of Intestinal Mucosa

Substrate	Time	Hydrolysis
	hrs.	*per cent*
dl-Leucylglycine	1	70
	2½	86
dl-Leucylglycylglycine	1	78
	2½	109
Chloroacetyl-*l*-tyrosine	2½	2
N-Methyl-*dl*-leucylglycine	2	4
	3½	2
Sarcosyl-*l*-tyrosine	2	1
	3½	2

for dipeptidase. In agreement with Abderhalden's results, we could find no splitting with erepsin under the usual experimental conditions (Table I). A similar finding was obtained in the case of sarcosyl-*l*-tyrosine (II). Dipeptidase from intestinal mucosa requires, therefore, the complete NH_2 group in the substrate and the basicity of the nitrogen is not in itself sufficient.

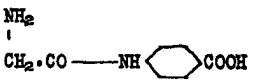


I

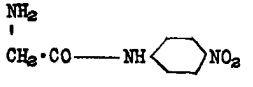
II

The necessity of the *free carboxyl* in the peptide for dipeptidase action has been the subject of much research. Grassmann and his

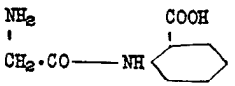
coworkers (2) have demonstrated the essential character of the carboxyl group for the action of yeast dipeptidase. We have assumed the same for dipeptidase from intestinal mucosa and have never found in our experiments any evidence to the contrary. However, Balls and Köhler (8) have reported from the laboratory of Waldschmidt-Leitz that dipeptidase from intestinal mucosa splits dipeptides of aromatic amines. According to these investigators the enzyme is thus able to split glycyl-*p*-aminobenzoic acid (III) and glycyl-*p*-nitraniline (IV) but not glycyl-*o*-aminobenzoic



III



IV



V

acid (V). None of these dipeptides bears a carboxyl group on the carbon atom attached to the peptide nitrogen. Peptide (IV) has no free carboxyl group at all.

Waldschmidt-Leitz and Balls (9) explain these results by attributing to the carboxyl group of glycyl-*o*-aminobenzoic acid an inhibiting effect on the enzyme. In the hydrolyzable *p* compound the carboxyl group is further removed, therefore the inhibition cannot occur. There can be no doubt that such inhibition by the carboxyl group is difficult to understand in an enzyme which, like dipeptidase, is capable of splitting compounds which have the peptide linkage and the carboxyl group in close spatial proximity. These results fit an enzyme which, like aminopeptidase, is inhibited by a free carboxyl in the vicinity of the peptide linkage of the substrate. It has been possible for us to demonstrate that aminopeptidase is indeed the active principle in this case.

Balls and Köhler employed for their observations a mixture of ereptic enzymes which contained, besides dipeptidase, much aminopeptidase. They did not perform experiments with homogeneous dipeptidase. We have repeated their experiments with yeast dipeptidase and have found that it is entirely inactive. On the other hand, it was possible to split glycyl-*p*-nitraniline as well as glycyl-*p*-aminobenzoic acid with aminopeptidase from intestinal mucosa (Tables II and III). It is therefore evident that if erepsin

TABLE II

Hydrolysis of Glycyl-p-Nitraniline

Substrate	Glycerol extract of intestinal mucosa		Aminopeptidase		Yeast dipeptidase	
	Time	Hydrolysis	Time	Hydrolysis	Time	Hydrolysis
	hrs.	*per cent*	*hrs.*	*per cent*	*hrs.*	*per cent*
dl-Leucylglycine	1	100	1¼	1	1	58
					3	100
dl-Leucylglycylglycine	1	80	1¼	32	1	1
			3	77		
Glycyl-*p*-nitraniline*	2	9	3	6	2½	1
	5	24	7	7	5	0
	20	40	20	11	20	0

* The amination of chloroacetyl-*p*-nitraniline (8) was carried out with ammoniacal methyl alcohol for 48 hours. After evaporating down the solution, the residue was dissolved in a measured volume of N HCl, filtered, and the peptide precipitated from the filtrate with the calculated quantity of N NaOH. Yellow needles, difficultly soluble in water, with a melting point of 167° (corrected), were obtained. The material was titrated in 0.3 cc. aliquots. During hydrolysis the color changed from light yellow (peptide) to deep yellow (nitraniline).

TABLE III

Hydrolysis of Glycyl-p-Aminobenzoic Acid

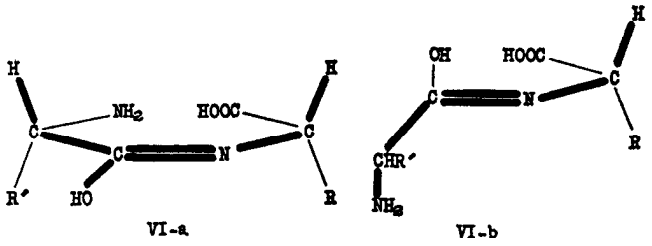
Substrate	Glycerol extract of intestinal mucosa*		Aminopeptidase		Yeast dipeptidase†	
	Time	Hydrolysis	Time	Hydrolysis	Time	Hydrolysis
	hrs.	*per cent*	*hrs.*	*per cent*	*hrs.*	*per cent*
dl-Leucylglycine	1	77	1¼	1	2	83
dl-Leucylglycylglycine	1	80	1¼	32	2	7
	2	102	3	77	7	38
Glycyl-*p*-aminobenzoic acid	2	19	3	7	3	0
	6	45	6	10	7	0
	15	69	20	20		

* 0.3 cc. of extract was employed.

† 7 mg. of dry preparation were employed.

hydrolyzes these two dipeptides, as in the experiments of Balls and Köhler, the hydrolytic action is to be attributed to the aminopeptidase. Since the only experimental support for a difference in specificity for dipeptidase from yeast and intestinal mucosa has disappeared, it may now be stated without reservation that dipeptidase splits only those dipeptides which bear a free carboxyl group on the same carbon as the peptide nitrogen.

For the *stereochemical aspect of dipeptidase action* the peptide hydrogen, as well as the α- and α'-hydrogens, is of fundamental significance. It was pointed out above that the peptide hydrogen is essential for enzymatic hydrolysis by dipeptidase. We assume that this hydrogen atom becomes rearranged under the influence of the enzyme in such a way that the amide form of the dipeptide is changed to the imide form, —CO—NH— → —C(OH) = N—. The imide form makes possible two different spatial arrangements of the carbon-nitrogen double bond.

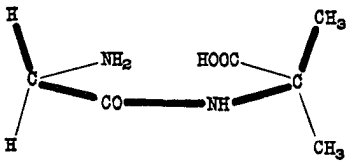


In isomer (VI-a) the amino and carboxyl groups lie in the closest possible proximity, whereas in (VI-b) they are distant from each other. We are of the opinion that during dipeptidase action dipeptides exist in the configuration (VI-a) so that there results a hexagon, whose corners are formed by the carboxyl group, α-carbon, peptide nitrogen, carbon of the peptide bond, α'-carbon, and amino group. The simultaneous combination of the carboxyl and the amino group with the enzyme restricts the free rotation within the peptide and confers upon the hexagon a rigid structure. The six corners of the hexagon must lie nearly in a single plane and we shall fall into no great error if we draw the hexagon as a plane and

refer to it as such in what follows. However, the 2 α-hydrogen atoms and the side chains R and R′ are not situated in the plane of the hexagon but in a way similar to that of the substituents in a partially hydrogenated benzene ring.[1] The spatial configuration of the naturally occurring optically active amino acids is such that their α-hydrogen atoms lie in *cis* position in the dipeptide hexagon, the same being the case for the side chains R and R′. On the one side of the hexagon plane there are therefore the two voluminous side chains R and R′ which do not allow the enzyme to approach the hexagon plane from this side, thus preventing combination with the reactive groups of the dipeptide from this side. On the other side of the hexagon plane there are only the 2 α-hydrogen atoms which have only a small atomic volume. We are therefore of the opinion that the enzyme approaches that side of the hexagon upon which the 2 α-hydrogen atoms are situated and that this condition determines the stereochemical selectivity of dipeptidase.

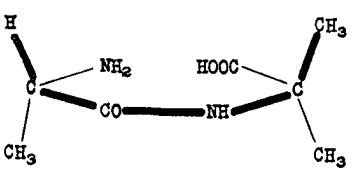
This view leads to the problem as to whether the 2 α-hydrogen atoms enter into active combination with dipeptidase or merely favor the action of the enzyme because of their small volume. The second possibility seemed the more probable one.

In order to decide this question we have investigated the following dipeptides with dipeptidase.



Glycyl aminoisobutyric acid

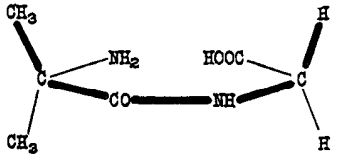
VII



l-Alanyl aminoisobutyric acid

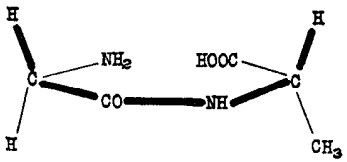
VIII

[1] In analogy to the Haworth formula of pyranosides, in formulas (VI-a) to (XVI) the heavy lines represent valences in front of the plane of the paper while the light lines represent valences behind the plane of the paper.



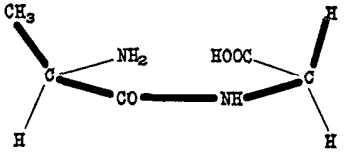
Aminoisobutyryl glycine

IX



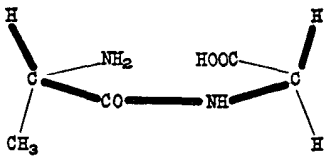
Glycyl-l-alanine

X*



d-Alanyl glycine

XI



l-Alanyl glycine

XII

Abderhalden and Zeisset (10) found no splitting for dipeptide (VII) with erepsin but did observe hydrolysis in the case of (VIII) and of (IX), without stating which component of the erepsin was the effective agent in the splitting. We found hydrolysis by erepsin from intestinal mucosa for all three dipeptides. We found further that an active and homogeneous preparation of aminopeptidase from erepsin was entirely inactive toward these three dipeptides. Finally, we noted an appreciable splitting of all three dipeptides with a dry preparation of yeast dipeptidase. It was smallest in the case of (IX), where the aminoisobutyric acid bears the amino group (Tables IV to VI).

These experiments show that dipeptidase can also act on dipeptides of amino acids which have no α-hydrogen but in its place a methyl group. The substitution of methyl for hydrogen on the side of the hexagon approached by the enzyme does not prevent the splitting by dipeptidase completely, but merely slows it down.

* Since all natural amino acids have the same spatial configuration and belong to the so called *l* family, we designate them as *l* compounds and their antipodes as *d* compounds regardless of their optical behavior.

This is particularly clear when peptides (VII) and (IX) are investigated under the same conditions as glycyl-*l*-alanine (X) and *l*-alanylglycine (XII). The two last named peptides differ from (VII) and (IX) only in the fact that in place of the methyl groups on the upper side of the hexagon there are hydrogen atoms.

TABLE IV

Hydrolysis of Peptides of α-Aminoisobutyric Acid

Substrate	Glycerol extract of intestinal mucosa			Aminopeptidase		Yeast dipeptidase*	
	Time	Hydrolysis A	Hydrolysis B	Time	Hydrolysis	Time	Hydrolysis
	hrs.	*per cent*	*per cent*	*hrs.*	*per cent*	*hrs.*	*per cent*
dl-Leucylglycine	2	95	90	2½	3		
Glycylglycine						1	71
dl-Leucylglycylglycine	2	90	92	2½	96		
Chloroacetyl-*l*-tyrosine	1	1					
Glycylaminoisobutyric acid	5	5		3	3	3	4
	8		21	6	3	6	8
	8½	13				10	12
Glycyl-*l*-alanine	2		60				
	4		79				
l-Alanylaminoisobutyric acid	3¼	62		3	0	1	16
	5	77		5½	0	3	35
	6¾	89				10	45
Aminoisobutyrylglycine	3	22	24	3	0	3	25
	5	38		5	0	6	31
	6		51			10	38
	7	43					
l-Alanylglycine	3		65				
	6		91				

* 4 mg. (0.4 peptidase unit) of dry preparation were employed per 1 cc. of reaction mixture.

At this point our experimental results and interpretation conflict with a specificity law whose general validity was held to be quite firmly established. It was stated that dipeptides containing only one antipode of a natural amino acid are not split by dipeptidase (11). Our proposed theory of the mechanism of dipeptidase

action requires, however, that certain exceptions to this rule should be observed. For example, *l*-leucyl-*d*-alanine (XIII) is composed of the natural *l*-leucine and the antipode of the natural

TABLE V

Inhibition of Dipeptidase Action by Alanine. Glycerol Extract of Intestinal Mucosa Was Employed

0.1 mM of *dl*-alanine was added to each reaction mixture.

Substrate	Time	Hydrolysis	
		No alanine	Alanine
	hrs.	*per cent*	*per cent*
Glycylglycine	1	30	18
	2	59	34
l-Alanylaminoisobutyric acid	2	36	24
	4	67	35
Aminoisobutyrylglycine	2	18	14
	4	29	16

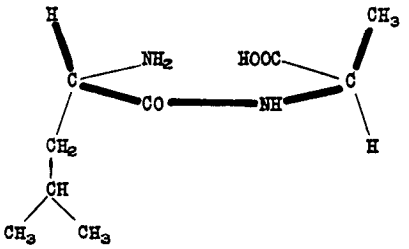
TABLE VI

Poisoning of Enzymatic Action of Extract of Intestinal Mucosa with HCN

3 mg. of HCN were added to each reaction mixture.

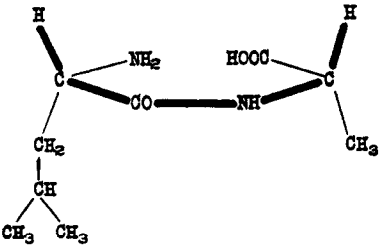
Substrate	Time	Hydrolysis	
		Without HCN	With HCN
	hrs.	*per cent*	*per cent*
Glycylglycine	4	79	7
	6		7
l-Alanylaminoisobutyric acid	3		0
	5	77	0
Aminobutyrylglycine	3	22	0
	5	38	0

alanine. Its hexagon shows on its upper face precisely the same arrangement as in the two peptides (VII) and (VIII) of aminoisobutyric acid.



l-Leucyl-d-alanine

XIII



l-Leucyl-l-alanine

XIV

Since this is, however, the side of the hexagon upon which, according to our view, dipeptidase combines with the substrate, the enzyme should be able to combine with *l*-leucyl-*d*-alanine and thus split it. Similarly, *d*-alanylglycine (XI) should be hydrolyzed. Experiments with yeast dipeptidase have confirmed this expectation and shown further that *l*-leucyl-*d*-alanine is split more rapidly than the sluggish glycylglycine, but more slowly than *l*-leucylglycine or *l*-leucyl-*l*-alanine (XIV). Hydrolysis was also obtained in the case of *l*-leucyl-*d*-alanine with erepsin from intestinal mucosa, but not with aminopeptidase from the same source (Table VII).

It is of interest that aminoisobutyrylglycine is split more rapidly than *d*-alanylglycine. Thus the substitution of α'-hydrogen by methyl on the side of the hexagon not approached by the enzyme in this case accelerates the splitting.

As a component of dipeptides subjected to dipeptidase action, *d*-alanine does not obey the hitherto accepted specificity rule. The rule must therefore be restricted as follows: If a dipeptide contains the antipode of a natural amino acid, $NH_2 \cdot CHR \cdot COOH$, and R is CH_3 or larger, then steric hindrance of dipeptidase action results, which in the case of R = C_4H_9 or bigger side chains[2] leads to complete inhibition of hydrolysis in the usual experimental period of time.

[2] The precise behavior of *d*-aminobutyric acid and the corresponding aminovaleric acid remains to be investigated. It was previously found (12) that the dipeptide, glycyldehydrophenylalanine, is not split by dipeptidase. In view of the results reported in this paper this finding cannot be explained by the absence of α-hydrogen.

The following method for the determination of the configuration of primary amino acids was recently reported (13): combination with a natural amino acid to a dipeptide which is subjected to the action of dipeptidase. The validity of this method is not affected by the above remarks since it is employed only for amino acids which are much more complex than alanine.

TABLE VII

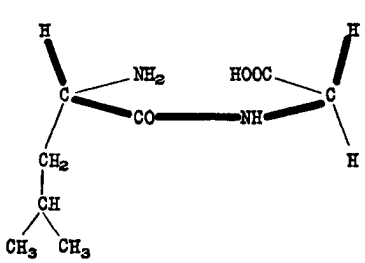
Hydrolysis of Peptides of d-Alanine

Substrate	Glycerol extract of intestinal mucosa		Aminopeptidase		Yeast dipeptidase*	
	Time	Hydrolysis	Time	Hydrolysis	Time	Hydrolysis
	hrs.	*per cent*	*hrs.*	*per cent*	*hrs.*	*per cent*
dl-Leucylglycine	1	70	2	0	2	74
	2½	86	4	0	4	90
Glycylglycine	1	39			2	36
	2½	74			4	80
					6	90
dl-Leucylglycylglycine	1	78	2	27	2	4
	2½	109	4	35	4	7
Chloroacetyl-*l*-tyrosine	2½	2				
Gelatin	2½	2				
l-Leucyl-*d*-alanine	1	26	2	0	2	25
	3	54	4	3	4	51
	5	70			6	69
l-Leucyl-*l*-alanine	1½	100				
d-Alanylglycine	1½	5			4	11
	4	13			8	28
	8	23				
l-Alanylglycine	1½	42				
	3	63				

* 4 mg. of dry preparation were employed.

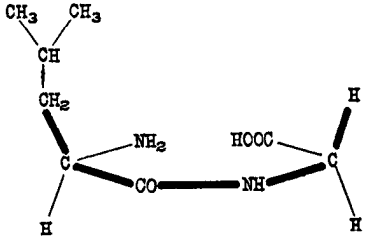
Since a large amount of consistent information regarding the specificity of dipeptidase is now at hand, it is worth while to discuss the cause for the stereochemical selectivity of the enzyme. The splitting of the peptides, glycylglycine, glycylaminoisobutyric acid, and aminoisobutyrylglycine, shows definitely that the enzyme requires no asymmetry of the amino acids or peptides. The fact

that stereochemical selectivity occurs in the case of peptides containing asymmetric amino acids will be elucidated with *l*-leucylglycine as an example. When the dipeptidase combines with *l*-leucylglycine on the upper side of the hexagon (*cf.* (XV)), it finds the carboxyl group, the peptide hydrogen, and the amino group arranged in a clockwise order. The reactive groups of the enzyme which combine with carboxyl, peptide hydrogen, and amino group must be arranged in the enzyme in such a manner that they fall exactly on the above groups in the peptide.



l-Leucylglycine

XV



d-Leucylglycine

XVI

If we now subject *d*-leucylglycine (XVI) to dipeptidase action, we have again on the upper side of the hexagon the carboxyl group, peptide hydrogen, and the amino group arranged in clockwise order. However, the enzyme cannot combine with these groups because its approach is prevented by the voluminous C_4H_9 group. Were the enzyme to act on the lower face of the hexagon, no splitting would result because the carboxyl group, peptide hydrogen, and amino group are arranged in a counter-clockwise order so that the reactive groups of the enzyme cannot combine with all the necessary reactive groups of the substrate.

If the enzyme would combine with only two reactive groups of the substrate, for example the amino group and the peptide hydrogen, it would not matter upon which side of the hexagon plane it reacts with the substrate and *d*-leucylglycine would be split exactly as its antipode. The last cause for optical selectivity of dipeptidase lies, therefore, in the fact that the enzyme combines with more than two points of the substrate. It seems probable that this theory of polyaffinity applies to other enzymes with asymmetrical specificity as well. We are investigating this question for car-

boxypeptidase and aminopeptidase and are using the steric hindrance of their action towards peptides of *d*-alanine as a convenient proof.

It may appear too early to propose such a detailed picture of the mechanism of dipeptidase action, especially in view of the lack of any knowledge regarding its chemical nature. However, the fact that the proposed theory could predict specificity phenomena (splitting of peptides of *d*-alanine) indicates that it possesses a high degree of probability.

EXPERIMENTAL

Synthesis of Peptides of α-Aminoisobutyric Acid. Glycyl-α-Aminoisobutyric Acid

Carbobenzoxyglycyl-α-Aminoisobutyric Acid Ethyl Ester—From 6.5 gm. of α-aminoisobutyric acid ethyl ester hydrochloride a solution of the free ester in ethyl acetate was prepared in the usual manner. To this solution there were added with strong cooling 4 gm. of carbobenzoxyglycyl chloride (14) in several portions. After standing 1 hour at room temperature the α-aminoisobutyric acid ester hydrochloride (2.3 gm.) which had separated out was filtered off by suction, the filtrate washed with dilute HCl, water, and bicarbonate, dried over Na_2SO_4, and evaporated under diminished pressure. Needles with a melting point of 84° (corrected) were obtained. Yield, 4 gm.

For analysis the substance was recrystallized from ethyl acetate-petroleum ether.

$C_{16}H_{22}O_5N_2$(322.2). Calculated, N 8.70; found, N 8.61

Carbobenzoxyglycyl-α-Aminoisobutyric Acid—4 gm. of the above ester were shaken for a short time with 14 cc. of N NaOH (1.1 moles) and 20 cc. of absolute alcohol, whereupon a clear solution was obtained. After 45 minutes the solution was acidified with dilute HCl, the free acid crystallizing out. On concentrating the filtrate under diminished pressure, a second crop of crystals was obtained. Total yield, 3.5 gm.; m.p., 154° (corrected). The needles crystallized in clusters.

For analysis the material was recrystallized from water, whereupon the melting point rose to 155.5° (corrected).

$C_{14}H_{18}O_5N_2$(294.2). Calculated, N 9.52; found, N 9.63

Free Dipeptide—1.4 gm. of the carbobenzoxypeptide were dissolved in absolute methyl alcohol, a small amount of water and 1 cc. of glacial acetic acid added, and the solution hydrogenated in the presence of palladium, Mohr, in the usual manner. The hydrogenation was completed in about 30 minutes. On evaporation under diminished pressure crystals (prisms) were obtained which were transferred to the filter by means of absolute alcohol. Yield, 0.7 gm.

For analysis the material was recrystallized from water-alcohol.

$C_5H_{12}O_3N_2$.	Calculated.	C 44.98, H 7.55, N 17.49
160.1	Found.	" 45.26, " 7.46, " 17.48

3.360 mg. required 2.05 cc. of 0.01 N KOH in 90 per cent alcohol, thymolphthalein being used as indicator. Calculated, 2.10 cc.

l-Alanyl-α-Aminoisobutyric Acid

Carbobenzoxy-l-Alanyl-α-Aminoisobutyric Acid—From 5 gm. of α-aminoisobutyric acid ethyl ester hydrochloride a solution of the free ester in ethyl acetate was prepared in the usual manner. Into this solution there was filtered with cooling an absolute ethyl acetate solution of carbobenzoxy-*l*-alanyl chloride which was prepared (5) from 4.5 gm. of carbobenzoxy-*d*-alanine. After standing 3 hours the ester hydrochloride which had separated out was filtered off by suction, the filtrate washed with dilute HCl, water, and bicarbonate, dried over Na_2SO_4, and evaporated under reduced pressure. A syrup (3.8 gm.) resulted which could not be crystallized. This oil was dissolved in 20 cc. of absolute alcohol and shaken with 13 cc. of N NaOH for 1½ hours after cooling at the start. After acidifying with dilute HCl to Congo red acidity, most of the alcohol was removed by evaporation under reduced pressure. The aqueous residue was extracted with ethyl acetate; the ethyl acetate solution washed several times with water, dried over Na_2SO_4, and evaporated under reduced pressure. The crystalline residue was recrystallized from a small quantity of ethyl acetate. Yield, 2.2 gm. of lancet-shaped crystals.

For analysis the material was recrystallized from ethyl acetate. M.p., 165° (corrected).

$C_{15}H_{22}O_5N_2$(308.2). Calculated, N 9.09; found, N 9.29

Free Dipeptide—1.7 gm. of the carbobenzoxypeptide were dissolved in absolute methanol, and after addition of a small quantity of water and 7 cc. of glacial acetic acid hydrogenated with palladium, Mohr, in the usual manner. The hydrogenation was completed in about 30 minutes. The filtered solution was evaporated under reduced pressure, giving in quantitative yield long prisms which were transferred to the filter by means of absolute alcohol.

For analysis the material was recrystallized from water and alcohol.

$C_7H_{14}O_3N_2$.	Calculated.	C 48.28, H 8.10, N 16.09
174.1	Found.	" 48.61, " 8.15, " 16.17

4.125 mg. required 2.34 cc. of 0.01 N KOH in 90 per cent alcohol, thymolphthalein being used as indicator. Calculated, 2.37 cc. $[\alpha]_D^{20} = +34.52°$ (2 per cent in H_2O).

α-Aminoisobutyrylglycine

Carbobenzoxy-α-Aminoisobutyric Acid—From 14 gm. of α-aminoisobutyric acid ethyl ester hydrochloride a solution of the free ester in ether was prepared in the usual manner. To this solution there were added with cooling 7 gm. of benzylcarbonyl chloride in several portions. After standing 4 hours at room temperature, the ester hydrochloride which had separated out (6.2 gm.) was filtered off by suction and the filtrate shaken with a little pyridine to decompose the unchanged chloride. The solution was washed with dilute HCl, water, and bicarbonate, dried over Na_2SO_4, and evaporated under diminished pressure. The resulting syrup (9.5 gm.) was dissolved in 25 cc. of absolute alcohol and shaken with 19 cc. of 2 N NaOH (1.1 moles) for 1½ hours with initial cooling. After being acidified with dilute HCl to Congo red acidity, most of the alcohol was removed by evaporation under reduced pressure. The aqueous residue was extracted with ether and the ethereal layer shaken with bicarbonate. The bicarbonate layer

was acidified with dilute HCl and extracted several times with ether. The ethereal solution was then washed with water, dried over Na_2SO_4, and evaporated under diminished pressure, yielding a syrup which crystallized on standing and could be transferred to the filter by means of petroleum ether. Yield, 6.5 gm. of hexagonal plates.

For analysis the substance was recrystallized twice from ether-petroleum ether. M.p., 78° (corrected).

$C_{12}H_{15}O_4N$(237.1). Calculated, N 5.91; found, N 6.00

Acid Chloride—To a solution of 3 gm. of carbobenzoxy-α-aminoisobutyric acid in 20 cc. of absolute ether 3 gm. of PCl_5 were added with strong cooling. After being shaken for 15 minutes, the filtered solution was evaporated under anhydrous conditions at 0° under diminished pressure, yielding a syrup which was washed several times with petroleum ether, and employed for the next reaction.

α-Aminoisobutyrylglycine—To a solution of about 4.5 gm. of free glycinebenzyl ester (15) in dry ethyl acetate was added a dry ethyl acetate solution of carbobenzoxy-α-aminoisobutyryl chloride (from 3 gm. of acid) described above. The filtrate of glycylbenzyl ester hydrochloride, which had separated out, was washed with dilute HCl, water, and bicarbonate, dried over Na_2SO_4, and evaporated under diminished pressure. The resulting syrup was dissolved in absolute methanol; a few drops of water and 1 cc. of glacial acetic acid were added and hydrogenated with palladium, Mohr, in the usual manner. The hydrogenation was complete after 1½ hours. After the palladium was filtered off, the solution was evaporated under diminished pressure and after repeated evaporation with absolute alcohol yielded plates which were transferred to the filter by means of absolute alcohol. Yield, 0.5 gm.

For analysis the material was recrystallized from water-alcohol.

$C_6H_{12}O_3N_2$.	Calculated.	C 44.98, H 7.55, N 7.49
160.1	Found.	" 45.28, " 7.44, " 7.38

3.805 mg. required 2.30 cc. of 0.01 N KOH in 90 per cent alcohol, thymolphthalein being used as indicator. Calculated, 2.38 cc.

l-Leucyl-d-Alanine

Carbobenzoxy-l-Leucylhydrazide—To a solution of 8.4 gm. of *l*-leucine methyl ester hydrochloride in 50 cc. of H_2O there were added 90 cc. of $CHCl_3$ and then with strong cooling and shaking three 1 gm. portions of MgO and three 4 gm. portions of benzylcarbonyl chloride were added. 15 minutes after the last addition the excess chloride was decomposed with pyridine and the reaction mixture acidified with 5 N HCl. The $CHCl_3$ layer was washed twice with water, then with bicarbonate and dilute HCl, dried over Na_2SO_4, and evaporated under diminished pressure. A syrup resulted which was dried by repeated evaporation with methyl alcohol and dissolved in 50 cc. of absolute alcohol. 3.2 gm. of hydrazine hydrate were added and the reaction mixture was left at room temperature for 24 hours. A slight precipitate was filtered off; the alcohol evaporated off under diminished pressure and completely removed by two evaporations with ether. Needles; yield, 12.5 gm.; m.p., 121°. For analysis the substance was recrystallized from ethyl acetate.

$C_{14}H_{21}O_3N_3$.	Calculated.	C 60.21, H 7.88, N 14.69
279	Found.	" 59.99, " 7.76, " 14.54

Carbobenzoxy-l-Leucyl-d-Alanine Methyl Ester—8 gm. of carbobenzoxyleucylhydrazide were dissolved in 100 cc. of H_2O, 10 cc. of acetic acid, and 5 cc. of concentrated HCl. With strong cooling (3°) and shaking, 3.5 gm. of $NaNO_2$ were added slowly and the oil which separated out was extracted with ether. The ether solution was washed twice with cold H_2O and bicarbonate, filtered through a dry filter, and dried over Na_2SO_4. The dry ether solution of the azide was then mixed with a dry ether solution of *d*-alanine methyl ester from 3.2 gm. of *d*-alanine[3] and the reaction mixture was allowed to stand overnight at room temperature. Some of the substance crystallized out in long needles. These were filtered off, and the filtrate washed with dilute HCl, bicarbonate, and water,

[3] The *d*-alanine methyl ester was prepared from *d*-alanine hydrochloride which had been obtained by resolution of benzoyl-*dl*-alanine with brucine according to Fischer (16). The rotation of the *d*-alanine hydrochloride was $[\alpha]_D^{24} = -10.2°$ (10 per cent in H_2O).

passed through a dry filter, and concentrated under diminished pressure. In this manner more of the substance was obtained. To the filtrate petroleum ether was added, yielding another crop. Yield, 3 gm.; m.p., 129–130°.

For analysis the substance was recrystallized from ethyl acetate and petroleum ether.

$C_{18}H_{26}N_2O_5$.	Calculated.	C 61.71, H 7.45, N 8.00
350	Found.	" 61.63, " 7.46, " 8.00

This compound was also prepared by coupling carbobenzoxy-*l*-leucyl chloride with *d*-alanine methyl ester but the yield was even lower.

Free Dipeptide—A solution of 1.3 gm. of the ester in 7 cc. of acetone and 3.9 cc. of N NaOH (1.1 moles) was allowed to stand 40 minutes at room temperature with occasional shaking. The solution was filtered and to the filtrate 4.2 cc. of N HCl were added. The acetone was removed under diminished pressure, whereupon an oil appeared which could not be crystallized. It was extracted with ethyl acetate; the ethyl acetate solution dried over Na_2SO_4 and evaporated under diminished pressure. The resulting syrup was dissolved in absolute methyl alcohol and hydrogenated with palladium as catalyst in the presence of acetic acid. The hydrogenation was completed in 30 minutes. The filtered solution was evaporated under reduced pressure and this procedure repeated twice after addition of absolute alcohol to remove all water and acetic acid. It was taken up in absolute alcohol and crystallized on standing in the ice box. Yield, 0.4 gm.

For analysis the substance was recrystallized from absolute alcohol.

$C_9H_{18}O_3N_2$.	C 53.47, H 8.91, N 13.86
202	" 53.35, " 9.05, " 13.63

5.35 mg. required 2.69 cc. of 0.01 N KOH in 90 per cent alcohol, thymolphthalein being used as indicator. Calculated, 2.65 cc. $[\alpha]_D^{22} = +76.0°$ (2.5 per cent in H_2O).

l-Leucyl-l-Alanine

Carbobenzoxy-l-Leucyl-l-Alanine Methyl Ester—This was prepared in the same manner as in the case of *l*-leucyl-*d*-alanine. M.p., 92–93°.

$C_{18}H_{26}N_2O_5$.	Calculated.	C 61.71, H 7.45, N 8.00
350	Found.	" 61.64, " 7.37, " 8.17

Free Dipeptide—This was prepared in the same manner as in the case of *l*-leucyl-*d*-alanine.

6.97 mg. required 3.45 cc. of 0.01 N KOH in 90 per cent alcohol, thymolphthalein being used as indicator. Calculated, 3.39 cc. $[\alpha]_D^{24} = +22.9°$ (5 per cent in methyl alcohol).[4]

d-Alanylglycine

Carbobenzoxy-d-Alanine—This was prepared in the same manner as the *l* form (14). M.p., 84°.

$C_{11}H_{13}O_4N$.	Calculated.	C 59.16, H 5.87, N 6.28
223	Found.	" 59.14, " 5.74, " 6.56

$[\alpha]_D = +13.9°$ (8.5 per cent in glacial acetic acid).

Carbobenzoxy-d-Alanylglycine Ethyl Ester—A dry ether solution of carbobenzoxy-*d*-alanyl chloride prepared from 3.3 gm. of carbobenzoxy-*d*-alanine in the same manner as the *l* form (5) was mixed with a dry ether solution of glycine ethyl ester prepared from 4.9 gm. of the hydrochloride. The reaction mixture was allowed to stand 30 minutes, whereupon some glycine ethyl ester hydrochloride separated out. This was filtered off and the filtrate washed with dilute HCl, bicarbonate, and water. The solution was then dried over Na_2SO_4 and evaporated down under diminished pressure, whereupon the substance crystallized out in needles. Yield, 3.5 gm.; m.p., 98°. For analysis the substance was recrystallized from ethyl acetate-petroleum ether.

$C_{15}H_{20}O_5N_2$.	Calculated.	C 58.44, H 6.49, N 9.09
308	Found.	" 58.39, " 6.56, " 8.98

Carbobenzoxy-d-Alanylglycine—To a solution of 2.5 gm. of ethyl ester in 8 cc. of acetone 10 cc. of N NaOH were added. After standing for 30 minutes with occasional shaking, the acetone was evaporated off, yielding an oil which crystallized on standing in the ice box. Yield, 1.7 gm.; m.p., 103–104°. For analysis the substance was recrystallized from ethyl acetate.

[4] Fischer (17) gives $[\alpha]_D^{20} = +23.5°$.

$C_{13}H_{16}O_5N_2$.	Calculated.	C 55.71, H 5.71, N 10.00
280	Found.	" 55.61, " 5.67, " 10.01

Free Dipeptide—0.9 gm. of carbobenzoxy compound was dissolved in absolute methyl alcohol and hydrogenated in the usual manner after addition of a small amount of water and acetic acid. On evaporation under diminished pressure the peptide was obtained in a yield of 0.4 gm. For analysis it was recrystallized from H_2O-alcohol.

$C_5H_{10}O_3N_2$.	Calculated.	C 41.08, H 6.90, N 19.18
146	Found.	" 41.01, " 6.95, " 19.23

7.30 mg. required 4.94 cc. of 0.01 N KOH in 90 per cent alcohol, thymolphthalein being used as indicator. Calculated, 5.00 cc. $[\alpha]_D^{23} = -50.7°$ (4.3 per cent in H_2O).[5]

l-Alanylglycine

Carbobenzoxy-l-Alanylglycine Ethyl Ester—This was prepared in the same manner as in the case of *d*-alanylglycine. M.p., 100°.

$C_{15}H_{20}O_5N_2$.	Calculated.	C 58.44, H 6.49, N 9.09
308	Found.	" 58.41, " 6.44, " 9.16

Carbobenzoxy-l-Alanylglycine—This was prepared in the same manner as in the case of *d*-alanylglycine. M.p., 104°.

$C_{13}H_{16}O_5N_2$.	Calculated.	C 55.71, H 5.71, N 10.00
280	Found.	" 55.64, " 5.75, " 10.21

Free Dipeptide—This was prepared by hydrogenation of the carbobenzoxy compound as in the case of *d*-alanylglycine.

7.17 mg. required 4.75 cc. of 0.01 N KOH in 90 per cent alcohol, thymolphthalein being used as indicator. Calculated, 4.91 cc.

Glycyl-l-Alanine—This was prepared by the method of Fischer (19).

N-Methyl-dl-Leucylglycine—This was prepared by the method of Fischer (20).

Sarcosyl-l-Tyrosine—This was prepared by the method of Abderhalden, Schwab, and Valdecasas (4).

[5] Fischer (18) found for *l*-alanylglycine $[\alpha]_D^{18} = +50.3°$.

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Enzymatic Studies

In the experiments reported above the total volume of each reaction mixture was 1 cc. in which were contained 0.05 mM of substrate[6] in 0.5 cc. of H_2O previously neutralized with 0.2 N NH_4OH, 0.1 cc. of M/3 phosphate buffer of the required pH (usually 7.4), and 0.4 cc. of the enzyme solution. The temperature in all cases was 40°.

The extent of hydrolysis was determined by titration of the carboxyl groups in 90 per cent alcohol with 0.01 N KOH, thymolphthalein being used as indicator (Grassmann and Heyde (21)). 100 per cent splitting represents an increase of 1 cc. in the titration of 0.2 cc. of the reaction mixture.

The aminopeptidase employed in these experiments was prepared from a glycerol extract of intestinal mucosa by adsorption of the dipeptidase with $Fe(OH)_3$ according to Waldschmidt-Leitz, Balls, and Graser (22).

The dry preparation of yeast dipeptidase was prepared according to Grassmann and Klenk (23).

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[6] In the case of *dl*-peptides 0.1 mM was employed and splitting calculated only for the natural form.

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