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Enzymic methods of resolving racemates of amino acids and their derivatives

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ABSTRACT. The review is devoted to modern methods of resolving racemates of amino acids and their derivtives by the action of enzymes. Processes of enantioselective enzymic hydrolysis are considered for both *C*- (esters, amides, amino acid nitriles) and *N*-derivatives (acyl- and alkoxycarbonylamino acids) and also for their cyclic derivatives. Examples are given of the catalytic preparation of optically active derivatives from racemic amino acids.

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I. Introduction

Optically active amino acids are used widely in various branches of the national economy, including agriculture (food additives, herbicides), the food industry (sweeteners, aroma-producing agents, products for dietary foods), and medicine (infusion solutions, therapeutic agents). The precipitate growth of the industrial manufacture of optically active amino acids is linked primarily with the discovery of new opportunities for their use. For example, the introduction into manufacture of the artificial sweetening agent aspartame has caused a multiple increase in the volume of manufacture of L-phenylalanine and L-aspartic acid in the last five years (Table 1).[1]

Table 1. Manufacture of L-phenylalanine and L-aspartic acid.[1]

Year	Amino acid	Main area of use	Total cost (millions of dollars)
1979	*L*-Phe	Infusion solutions	7.4
1984	*L*-Phe	Aspartame	~100.0
1979	*L*-Asp	Therapy, aroma-producing agents	1.2
1984	*L*-Asp	Aspartame	~15.0

The improvement of methods of obtaining amino acids using new achievements in biotechnology has enabled a significant reduction in the cost of optically active amino acids which, in its turn, has opened up possibilities for using them in new areas and has significantly extended market salts. The manufacture of lysine is a graphic example of the effect of such technology on the price and volume of manufacture of this amino acid (Table 2).[1]

Table 2. Manufacture of L-lysine.

Year	Manufacturing efficiency (arbitrary units)	Mean cost (dollars pound^{-1})	World production (millions of pounds)	Total cost (millions of dollars)
1956	1	23.7	0.2	5
1969	4	4.45	7.0	31
1980	8	2.10	90.0	189

As a rule chemical synthesis of amino acids leads to a racemic product, the preparation of enantiomers requires an optical resolution step. Existing methods of resolving racemates of amino acids may be divided into three main groups, 1) physicochemical methods, 2) chemical and 3) biotechnological (with the participation of enzymes or cells of microorganisms). Physicochemical methods include mechanical separation, selective crystallisation and chromatographic separation. Chemical separation of a racemic amino acid or a derivative is effected with a chiral compound and one of the diastereomers is removed by crystallisation, chromatography, etc.

The first two methods are suitable for solving analytical problems and for the large scale separation of a limited number of amino acids (for example L-glutamic acid using selective crystallisation), but they are not suitable for the industrial preparation of a large selection of optically active amino acids.

The most widespread methods of obtaining enantiomeric amino acids at the present time are those using enzymes or microbial cells. Work published before 1982 is not considered in the present review, this was included in the review by Shvyadas and Galaev.[2] A high reaction rate and stereospecificity are the features of enzymic reactions. The significant acceleration of the reaction when using enzymes may be demonstrated by the following example. The rate

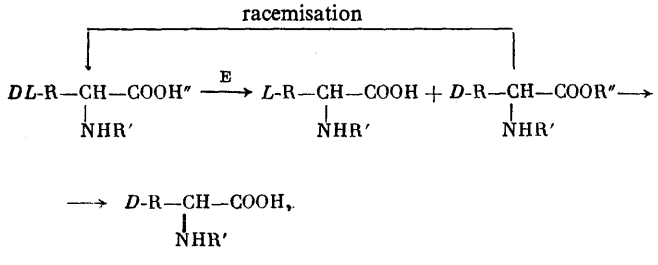
constant for the non-enzymic hydrolysis of urea is 4.15×10^{-5} sec^{-1} at 100 °C while the rate constant for the reaction catalysed by urease is 3×10^{4} sec^{-1} at 20 °C. Another no less important feature of enzymes is their specificity in reaction and in substrate. The specificity may be absolute or almost absolute since some enzymes act on molecules of only one substrate while others act on molecules with marked differences in structure, such as on a whole class of compounds. As a rule enzymes display high reaction specificity but some of them are capable of catalysing 'foreign' reactions. For example the majority of hydrolases catalyse reactions typical of transferases. In addition some groups of enzymes catalyse different types of reaction, i.e. they display a multicatalytic function. In the majority of cases enzymes 'correctly' demonstrate absolute stereospecificity in catalysis when they act on part of a molecule linked with a chiral centre in the substrate or form chiral products from achiral substrates. Racemases and isomerases are exceptions in this respect. Even enzymes possessing wide specificity in relation to substrate structure may display absolute stereospecificity. For example D-amino acid oxidases (EC 1.4.3.3.) catalyse the oxidation of a wide range of primary and secondary D-amino acids while not affecting L-amino acids. L-Amino acid oxidases (EC 1.4.3.2.) act in a similar manner. Stereospecificity is not an obligatory property of enzymes acting on groups distant from a chiral centre in a substrate. Carboxyesterase (EC 3.1.1.1.) catalyses the hydrolysis of the ethyl esters of L- and D-tyrosine at the same rate. On the whole stereospecificity, which is natural for enzymes, is difficult to achieve by chemical means in spite of the fact that asymmetric synthesis has developed significantly in recent years.

By virtue of these factors the enzymic methods of obtaining optically active amino acids have been at the centre of attention of investigators for several decades. They may be divided into two groups, 1) enantioselective enzymic hydrolysis of amino acid derivatives and 2) the enantioselective enzymic derivatisation of amino acids and their derivatives.

II. Enantioselective enzymic hydrolysis of amino acid derivatives

1. Esters of amino acids

The enantioselective enzymic fission of esters as a method of obtaining optically active amino acids was already known at the beginning of the century.[3]



E – enzyme.

The reaction products are the L-amino acid and the ester of the D-isomer which is subjected to non-enzymic hydrolysis of racemisation for subsequent reintroduction into the process for resolving the racemate. Many enzymes of animal and microbial origin are known which possess esterase activity. The best studied of them is α-chymotrypsin, distinguished by high activity and enantioselectivity of action primarily in relation to specific substrate, viz. esters of aromatic *N*-acylamino acids. Its high cost and non-availability in large quantities hinders the application of this enzyme for the separation of racemates in practice. Immobilisation of the enzyme enables the cost of the biocatalyst to be reduced. At the present time the investigation of the chymotryptic hydrolysis of specific substrates is directed mainly towards the development of continuous processes for obtaining optically active amino acids.[4,5] *N*-Acetyl-L-phenylalanine is obtained from its racemic methyl esters in a membrane-reactor (capacity 1.3 kg m^{-2} d^{-1}).[4]

Another way to reduce the cost of biocatalyst is to use cheaper enzymes such as proteases of microbial origin or whole cells. Glaenzer and collaborators[6,7] studied the use of bakers' yeast *Saccharomyces cervisiae* for the enantioselective hydrolysis of raemic *N*-acetylaminoacid esters. It was known previously[4] that the cells of *S. cerevisiae* possess oxido-reductase activity. They catalyse the enantiospecific reduction of ketones, but there are very few data on their hydrolytic activity. It turned out that they are capable of hydrolysing esters of *N*-acetylamino acids with an unbranched chain with a high degree of enantioselectivity (Table 3).

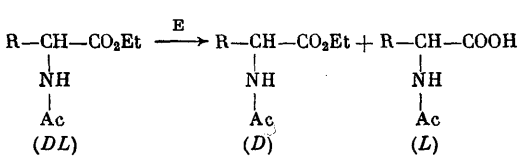


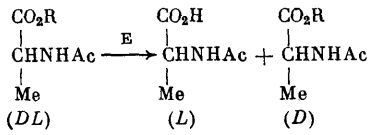
Table 3. Enantioselective anaerobic hydrolysis of *n*-acetylamino acid esters [DL-AcNHCH(R)COOEt] by cells of *Saccharomyces cerevisiae*.

Substrate No.	R	Yield of D-AA ester, %	Enantiomeric excess, %	Substrate No.	R	Yield of D-AA ester, %	Enantiomeric excess, %
1	Me	47	100	4	CH_2CHMe_2	48	92
2	Et	48	96	5	CH_2Ph	38	97
3	iso-Pr	75	13	6	CH_2CO_2Et	49	86
3	iso-Pr	38	80 *	7	$CH_2CH_2CO_2Et$	46	89

* Aerobic hydrolysis.

Substituents in the β-position inhibit the reaction (Table 3, substrate 3), γ-substituents do not show this negative effect (Table 3, substrate 4). Hydrolysis does not occur in the case of substrates with an *N*-acetylamino group in the β-position. When two chemically different ester groups are present (Table 3, substrates 6 and 7) reaction occurs only at the α-ester group, not affecting the ω-group of esters of *N*-acetyl-L-aspartic and -glutamic acids. The hydrolytic action of α-chymotrypsin has similar characteristics.

Changes in the ester component do not as a rule display a significant effect on the course of the hydrolysis of *N*-acetylalanine ester.



R is methyl, ethyl n-propyl, iso-propyl, n-butyl, n-octyl, benzyl, cyclohexyl, t-butyl.

The t-butyl ester is not hydrolysed, the methyl ester is hydrolysed somewhat more slowly than the remaining esters of primary and secondary alcohols, the rates of fission of which are approximately the same and the enantiomeric excess is at least 96%.

Since the esterase actions of *S. cerevisiae* cells and of α-chymotrypsin have much in common it has been suggested that proteinases are the main active principle. To confirm this the hydrolysis of substrate 1 was catalysed by mutant strain ABYS1 of the yeast, which is characterised by a lack of non-specific proteases and carboxypeptidases, and also by the wild strain X-2180-Ia (Table 4).

Table 4. Enzymic hydrolysis of *N*-acetyl-DL-alanine ethyl ester.

Reaction time, h	*S. cerevisiae*		Wild strain X-2180-1 A		Mutant strain ABYSI	
	Enantiomeric excess, %	Yield of D-ester, %	Enantiomeric excess,%	Yield of D-ester, %	Enantiomeric excess, %	Yield of D-ester,%
24	92	not determined	89	52	1	95
48	100	47	100	43	2	81

In many cases the use of enzymes and microbial cells for the fission of racemic esters is limited by the low water solubility of the substrates. The use of two-phase systems of 'water–immiscible organic solvent' is not always possible. For example, on adding chloroform or ethyl acetate to the aqueous system containing the enzyme and substrate, the hydrolysis of esters of *N*-acetylamino acids (alanine, phenylalanine, phenylglycine) is not observed.[8] This is evidently linked with a shift in the direction of ester synthesis in the 'hydrolysis–synthesis' equilibrium of the given system. To solve this problem Fadnavis and collaborators[8] have proposed carrying out the enzymic hydrolysis of esters in reverse micelles. The authors considered that cells of microorganisms, possessing enzymic activity, may in principle be considered as a system containing an enzyme immobilised by inclusion in or addition to the cell wall. To confirm this hypothesis the hydrolysis of esters of *N*-acetylamino acids has been carried out with cells of *S. cerevisiae* crosslinked with glutaraldehyde in an aqueous medium and in reverse micelles [organic phase was chloroform–isooctane (1:9 by volume), the detergent being bis(2-ethylhexyl)sulphosuccinate, sodium salt] (Table 5).

Table 5. Hydrolysis of *N*-acetyl-DL-amino acid methyl esters of bakers' yeast in aqueous medium and in reverse micelles.

No	Substrate*	Product**	Reaction time, h		Yield, %		Enantiomeric excess, %	
			water	reverse micelles	water	reverse micelles	water	reverse micelles
1	Phg	1*a*	72	48	64	60	96	98
		1b	72	48	60	46	>99	>99
2	Phe	2*a*	48	24	57	53	98	98
		2b	48	24	56	48	99	99
3	Ala	3*a*	60	32	72	65	97	96
		3b	60	32	52	40	>99	>99

* *N*-Ac-DL-AA methyl ester.
** a, *N*-Ac-L-AA, b, *N*-Ac-D-AA.

Table 6. Hydrolysis of *N*-Z-amino acid esters [Z-NH-CH(R)CO_2R'] by lipase.

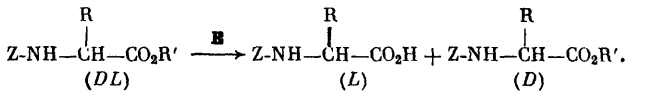
No.	R	$R'=CH_2CH_2Cl$						$R'=CH_2CF_3$	
		A. niger		*P. fluorescens*		*C. cylindracea*		PP	
		A	B	A	B	A	B	A	B
1	Methyl	36	89	46	16	38	7	39	21
2	Ethyl	40	96	49	52	36	7	40	97
3	n-Propyl	27	86	43	58	–	–	39	98
4	n-Butyl	32	85	52	60	42	30	38	92
5	n-Pentyl	44	95	25	80	47	66	40	87
6	n-Hexyl	31	94	47	87	–	–	33	61
7	iso-Propyl	14	92	8	53	–	–	Not a substrate	
8	$Ch_2=CHCH_2$	34	86	40	52	31	27	38	93
9	Benzyl	32	94	12	57	35	63	40	>99
10		37	94	40	70	35	43	44	89

Note. A is the extent of conversion, %; B the excess of L-enantiomer, %.

No enzymic hydrolysis of the D-enantiomer was observed, spontaneous hydrolysis in the reverse micelles was less than 1.5% in 72 h (less than in aqueous medium). The advantages of using reverse micelles are evident in the case of substrates with low solubility, such as in the hydrolysis of 5 g substrate 1 (Table 5) in 200 ml in 72 h. The enantiomeric excess of unreacted D ester was 30% in aqueous medium and 75% in the reverse micelle medium.

The search for enzymes capable of hydrolysing esters of racemic amino acids led to the discovery of the esterase activity of lipases.[9,10] It is known from the literature that lipases are capable of the fission

of esters of racemic alcohols and carboxylic acids, they are also readily available, inexpensive and stable. Investigation of the hydrolysis of the substrate *N*-Z-α-amino-DL-heptanoic acid ester with lipase from six microorganisms showed that the best results were obtained with the lipase from *Aspergillus niger* and satisfactory results with the lipase from *P. fluorescens* and *C. cylindracea*. Using these three enzymes and a porcine pancreas lipase (PPL) the ester hydrolysis has been effected for a series of natural and synthetic acids (Table 6).



In the general case the enantioselectivity of lipase action decreases in the series PPL ≈ *A. niger* lipase > *P. fluorescencs* lipase > *C. cylindracea* lipase. Three types of unnatural amino acids: 1) with unbranched hydrocarbon chain (Table 6, substrates 2–6), 2) with an unsaturated bond (Table 6, No. 8) and 3) with a heteroaromatic ring (Table 6, No. 10) in the side chain R were separated with high values of the enantiomeric excess (85–95%) by lipases from *A. niger* and from pancreas (except for substrate 6) although the reactions were not optimised. The good result was particularly important in the case of substrate 10 (Table 6) since other enzymic processes (such as the use of acylase I) do not lead to resolution of the racemate of this heteroaromatic amino acid.

It is known that transferring from esters of amino acids with an *N*-acylamino group to esters of amino acids with a free amino group leads to a reduction in the enantioselectivity of enzymic hydrolysis.[11] However in many cases the selectivity is sufficiently large and it is possible to separate enantiomeric amino acids with a free amino group by enzymic enantioselective hydrolysis of their esters. One of the most widely used biocatalysts for this purpose is α-chymotrypsin. Investigations of recent years in this direction may be separated into two main groups. The first group contains work on the development of efficient continuous processes using immobilised α-chymotrypsin.[12,13] The cost of the process may be reduced by racemising the D-isomer and subsequently reintroducing it into the separation process.[14] Such processes may be effected in two-phase 'water–immiscible organic solvent' systems.[15,16]

The second group comprises work devoted to the chymotryptic fission of racemic amino acids containing substituents in the side chain R[17] or at the α-carbon atom.[18] The methyl (or ethyl) esters of *ortho*-(*meta*-, *para*-)fluoro-DL-phenylalanine may be separated into enantiomers in high yield and enantiomeric purity.[17]

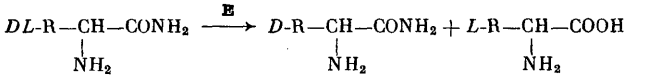
It is known that the introduction of an α-substituent significantly reduces the rate of enzymic hydrolysis. For example, on introducing an α-methyl group into the *N*-acetyltyrosinamide molecule the hydrolysis rate is reduced 10^5 times which is seemingly linked with the incorrect orientation of the bond being hydrolysed.[19] The effect is less significant for amino acid esters. However there is a need for optically active α-aminoacids. They are used in investigations of the link between structure and activity in peptide hormones, conseqently the enzymic separation of their racemic derivatives is of significant interest. The resolution of the methyl esters of α-methyl-DL-amino acids (tryptophan, phenylalanine and *para*-fluorophenylalanine) has been effected (37 °C, pH 5.0, time 30–40 h).[18]

The high enantioselectivity of enzyme action enables the preparation of L-isomers in good yield (33–36%) and optical purity (96% for α-methyl-L-tryptophan). Contamination by the isomer was attributed to spontaneous non-enzymic hydrolysis since no hydrolysis of D-esters by chymotrypsin was observed.

The use of enzymes (or enzyme complexes) from microorganisms or whole cells is an alternative to the use of expensive enzymes of animal origin for esters of amino acids with a free amino group as for esters of *N*-acylamino acids. The fission of the methyl esters of DL-phenylalanine and DL-tyrosine and the benzyl ester of DL-alanine has been effected on a preparative scale (25 g).[20] The scope for ester hydrolysis by the alkaline protease from *Bacillus licheniformis*, the main component of which is subtilisin A (Carlsberg subtilisin), was investigated. The enzyme is stable and active at room and higher temperatures (the half-live for inactivation at 60 °C is about 5 h) and over a wide pH range (6–12). The enantioselective hydrolysis of a racemate leads to the preparation of the product in high yield (L-phenylalanine 48%, D-phenylalanine methyl ester 42.5%) and enantiomeric purity (L-enantiomer 90%, D-enantiomer 100%). Subtilisin 8350, obtained by replacing six amino acids in the protein chain of subtilisin BPN′ from *Bacillus amiloliquefaciens*, has been used for the separation of the esters of several amino acids with a free amino group and also of *N*-acylamino acids.[21] The modified enzyme proved to be 50 times more stable than the initial enzyme in water and 100 times more stable in anhydrous dimethylformamide. Practically 50% conversion was achieved in the resolution of racemic esters. The yields of product and unreacted substrate were more than 40% and the enantiomeric excess was more than 91% (the initial enzyme possessed low selctivity).

2. Amino acid amides

In the general case the enantioselective enzymic hydrolysis of amino acid amides proceeds by the scheme.



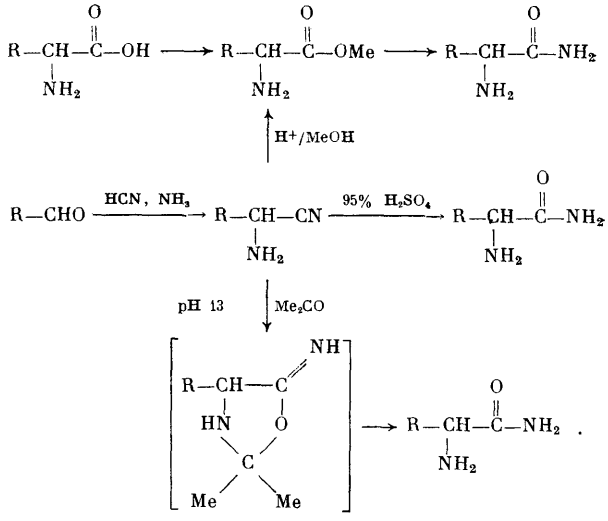
Enzymes possessing amidase activity are encountered in many microorganisms. Both purified enzymes and their complexes[22] and whole cells[23–25] may be used for the resolution of racemates. The possibility of using the proteolytic complex pronase from *Streptomyces griseus*, containing serine proteases and aminopeptidases, for the separation into enantiomers of the racemic amides of leucine, phenylalanine and tryptophan has been studied.[22] The hydrolysis of amino acid amides catalysed by pronase is characterised by high values of the rate, enantioselectivity and degree of conversion of the L substrate. DL-Leucine amide may also be separated into antipodes in 50% yield by whole cells of *Enterobacter cloacae*, *Pseudomonoas sp.* etc.[23] The L-aminopeptidase from *Pseudomonas putida* enabled separation of Met and Phg amides into optical antipodes.[24] Enzymes are encountered possessing an enantioselective action in relation to a D substrate.[26–28] DL-Valine amide may be separated into antipodes using either cells of *Pseudomonas rasea*, which hydrolyse L-amino acid amides (yield of L-valine was 50%, 40 °C, time 5 h),[25] or cells of *Rhodococcus erythropolis*, which hydrolyse D-amino acid amides (yield of D-valine 38–47%, 40 °C, time 2 h).[27] The D-amidase from a strain of *Ochrobactrum anthropi*, isolated from soil, has been partially purified and characterised.[28] Data on substrate specificity are given in Table 7. In addition the rates of hydrolysis for L-substrates (with the exception of norvaline and norleucine) have been determined and were less than 0.1% of the hydrolysis rate of the D-enantiomer.

Among the few studies devoted to the enzymic separation of amino acid amides some[29–33] occupy a special place. Regarding the industrial enzymic separation of amino acids to be promising and fully competitive with asymmetric synthesis and chemical separation, the authors have carefuly developed all the stages of obtaining optically active amino acids from racemic amides, beginning with the preparation of the starting materials. It is known that amides may be obtained from amino acids through the corresponding esters. It is probably more expedient to use as starting materials the α-aminonitriles which are intermediates in the synthesis of amino acids from aldehydes by the Strecker reaction. The conversion of α-aminonitriles into the corresponding amides may be effected by various routes. A promising method[29] has been proposed which is a one-stage conversion of an aminonitrile into the amide through an intermediate oxazolidine in alkaline medium in the presence of catalytic quantities of acetone (yield of product was over 90%).

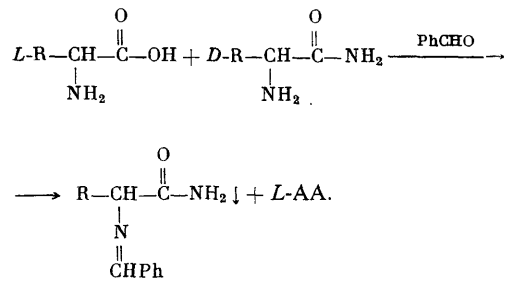
Table 7. Amidase activity of the enzyme from *Ochrobactrum anthropi.*

Substrate D-AA amide	V_{rel}*, %	Substrate D-AA amide	V_{rel}*, %	Substrate D-AA amide	V_{rel}*, %
Phe	100	Norleu	40	Phg	15
Tyr	97	Ala	33	Pro	9.7
Trp	96	Met	28	Lys	2.5
Leu	46	Norval	15	His	1,5

* V_{rel} is hydrolysis rate.



Many microorganisms are known which produce aminopeptidases (such as *Aspergillus* etc.). However, their use for separating amino acid amides, in the authors' opinion, is limited by low activity, narrow substrate specificity and undesirable side effects. A search carried out showed that the enzyme preparation of aminopeptides from *Pseudomonas putida* possessed the best characteristics. The high (almost 100%) enantioselectivity was combined with a very broad substrate specificity. The relative activity varied from 20% (L-phenylalanine amide) to 100% (D-phenylglycine amide), substrate concentration may be as high as 20% without affecting the activity of the enzyme. A considerable virtue is that the reaction may be conducted over a wide pH range. Traditional methods such as ion exchange chromatography or extraction are used for the separation of the L-amino acid and the D-amino acid amide. In those cases when the D-amino acid is required the authors suggest using one of the other methods developed by them. The first consists of the use of biocatalysts possessing no enantioselective action. Such a biocatalyst was found which was an enzyme with amidase activity towards L and D substrates from cell preparations of *Rhodococcus erythropolis.* The yields of the D-amino acids obtained by this method were very high in comparison with the usual chemical hydrolysis. Another method is based on the formation of a Schiff's base, from benzaldehyde and the D-amino acid amide, which is practically insoluble in water (and therefore readily isolable).



Acid hydrolysis at temperatures below 100 °C of the compound obtained give the D-amino acid without racemisation. The D- and L-amino acids obtained in this way possess high optical purity (99% and more).

In those cases when the D-enantiomer is not widely used it is expedient to subject the D-substrate to racemisation for subsequent reintroduction into the separation process. For this purpose a racemisation process was developed for the Schiff's base, i.e. for an *N*-(or un)substituted benzilidene-amino acid amide, in aqueous organic medium in the presence of base.[31] The racemate of the Schiff's base obtained was hydrolysed to the DL-amino acid amide which was subjected to enantioselective hydrolysis by an enzyme from *Pseudomonas putida.* The method[29–32] developed has enabled separation of racemates of phenylglycine, *p*-hydroxyphenylglycine, valine, phenylalanine, tryptophan, methionine, lysine, thienylglycine and their derivatives. The structural elements for the amino acids required for the development of the enzymic activity from *Pseudomonas putida* are an unsubstituted amino group and an α-hydrogen atom. Since α-alkylated amino acids are of significant scientific and commercial interest a new biocatalyst has been developed from *Mucobacterium neoarum* which possesses amidase activity towards α-alkylated amino acids.[33] The enantiomers of α-methylvaline, -leucine, -phenylalanine, -4-methoxyphenylalanine and α-ethylphenylalanine have been separated in high yield and enantiomeric purity (for example, the yield of α-methyl-L-leucine was 49.5% and of α-methyl-D-leucine amide 48.7%).

3. Amino acid nitriles

It is known that many strains of microorganisms such as *Corynebacterium sp., Nocardia rhodochrous, Pseudomonoas sp., Pencillium sp., Escherichia coli* and several others are capable of catalysing the hydrolysis of nitriles to the corresponding amides or acids and because of the variety of microorganisms the substrate spectrum is extremely broad. This comprises aliphatic and aromatic nitriles, α-hydroxy and α-aminonitriles.[34,35]

Many strains have been investigated[36] and among them was *Brevibacterium sp.* R312 which hydrolyses practically every water soluble nitrile to the corresponding acid but the reaction does not occur enantioselectively. The selection of strain R312 was effected in a medium containing fluoroacetamide, which is toxic for it, since hydrolysis to fluoroacetic acid occurs by the action of the non-specific amidase. Screening enabled selection of several mutants not carrying out this reaction and one of them A_4 was chosen for subsequent investigations. The spectrum of nitrilase activity of A_4 and R312 proved to be absolutely identical which confirmed the hypothesis that the enzyme system causing the hydrolysis of nitriles comprises two enzymes, a nitrilase and an amidase. Investigation of the amidase activity of whole cells and cell extracts showed that R312 hydrolyses practically all amides investigated with the exception of acrylamide, methacrylamide, L- and D-asparagine and D-glutamine, though the latter is hydrolysed by cell extracts. The amidase activity of A_4 is appreciably less. It hydrolyses only formamide, urea, nicotinamide, L-glutamine and amides of α-amino acids, although on hydrolysis of amides of DL-amino acids the yield did not exceed 50% and the hydrolysis product was the L-amino acid.

Additional investigations confirmed the enantioselectivity of the action of the enzyme. D-Aminopropionamide is not a substrate but the D-enantiomer is converted quantitatively into L-alanine. The use of strain A_4 enabled separation of the racemate of α-amino-γ-methylthiobutyronitrile quantitatively in 2–3 h. The hydrolysis products are L-methionine and D-methionine amide, the latter may be subjected to racemisation or hydrolysis to the D-amino acid by strain R312. Similar processes enabled the preparation of L-alanine, L-α-aminobutyric acid, L-phenylalanine, L-valine and L-leucine.

In the process of separating racemic aminonitriles with a strain of *Pseudomonas MY-1*[37] it was evident that two enzymes were participating, a DL-aminonitrilase and an enantioselective amidase. Separation of the enantiomers of DL-phenylglycine nitrile to the L-amino acid and the D-amino acid amide occurs quantitatively in 0.5 h at 15 °C. Optically active L-Phe may be obtained from the whole mass of racemic derivative as a result of a combined process in which hydrolysis of the DL-aminonitrile occurs to the DL-amino acid amide under the action of cells of *Acinetobacter calcoaceticus* with subsequent hydrolysis of the amino acid amide and racemisation of the D-phenylalanine amide by cells of *Arthrobacter* ATCC 31652.[38]

There are however data indicating that the participation of enantioselective amidases in the hydrolysis process is not the sole route for the resolution of racemic nitriles. It has been shown[39] that optically active amino acids and their amides (such as L-leucine amide) may be obtained from racemic nitriles by the enantioselective action of a nitrilase (strain 311).

4. Acylamino acids

The enantioselective hydrolysis of *N*-acylamino acids by acylase is the most widespread method for the large scale separation of enantiomers of amino acids.

$$\underset{\substack{|\\ \text{NHCOR}'\\ (DL)}}{\text{R—CH—COOH}} \xrightarrow{\text{E}} \underset{\substack{|\\ \text{NH}_2\\ (L)}}{\text{R—CH—COOH}} + \underset{\substack{|\\ \text{NHCOR}'\\ (D)}}{\text{R—CH—COOH}}.$$

The industrial development of this method, including the process of racemising the D-enantiomer for subsequent reintroduction into the separation process, was effected in the fifties and sixties by Tanabe Seijaku Co. Ltd.

At the end of the sixties a fermentor was built containing acylase immobilised by ionic binding to DEAE-Sephadex for the continuous production of amino acids. This was the first industrial application of immobilised enzymes. Since 1969 several types of fermentors have been developed for obtaining L-methionine, L-valine, L-phenylalanine and other amino acids from their racemic *N*-acyl derivatives.[40,41]

Enzymes possessing acylase activity are isolated from animal, plant and microbial sources. The most studied acylase of animal origin is the enzyme from porcine kidney, viz. acylase I, which is specific for aliphatic amino acids.

Acylases from animals belonging to four classes have been investigated.[42] These are fish (silver carp), amphibians (frog), birds (pigeon) and mammals (mouse, rat, cat, dog, cow, pig) in addition to human acylase. Freshly prepared kidney homogenates served as the enzyme source. Substrates used are the *N*-acetyl derivatives of DL-amino acids (norvaline, norleucine, valine, leucine, glutamic acid, phenylalanine and tryptophan) and *N*-chloroacetyl-DL-phenylalanine and *N*-formyl-DL-methionine. The character of the substrate specificity proved to be similar for the majority of animals. The maximum rate was displayed in the hydrolysis of *N*-formyl-methionine. The specific activity of the enzymes varied more than 16 times (from 4.2 for frog to 65.5 μmole min^{-1} mg $protein^{-1}$ for cow) so assessment of substrate specificity was carried out in relative units (for *N*-formylmethionine V_{max} = 100%). The rate of hydrolysis for the unnatural amino acid *N*-acetylnorleucine was 30–49%. Comparison of the hydrolysis rates of amino acids with branched and unbranched side chains (leucine, valine and norleucine, norvaline) showed that the latter were more suitable substrates (the difference in rate was 1.2–4.0 times). The *N*-acetyl derivatives of aromatic and dicarboxylic amino acids were poor substrates ($0.5\% \leqslant V_{rel} \leqslant 10\%$). As might have been expected replacement of the acetyl group by chloroacetyl leads to a 2–3-fold increase in rate. The substrate specificities of porcine acylase (PA) and human acylase (HA) differed somewhat. The maximum hydrolysis rate was observed for *N*-acetylnorleucine (V_{rel} = 208% for PA, 124% for HA), *N*-acetylnorvaline was in the second place (181% for PA, 110% for HA) and *N*-formylmethionine in third place (100%). The remaining substrates behaved in a similar manner with the other acylases. The data obtained are in good agreement with the previously proposed structure for the active centre as a narrow hydrophobic cleft of length five methylene units,[43] and suggest that the original and main role of the enzyme is the hydrolysis of *N*-formylmethionine.

Enzymes possessing acylase activity are encountered in many microorganisms.[2] They are inexpensive, available in large quantities and are used widely for the resolution of racemates of acylamino acids.[44–53] Up to the present time development has been conducted

on the application of free enzymes and microbial cells,[44–47] however the advantage of using immobilised enzymes was demonstrated indisputably even in the late sixties (the efficiency of using an immobilised enzyme for three months was more than 10 times greater than for the free enzyme).[54] The various methods of immobilising aminoacylases have been discussed.[55] The substrate specificity of microbial acylases is appreciably broader than that of acylase I. Substrates were not only the aliphatic amino acids but also derivatives of aromatic and dicarboxylic acids.[2] An investigation has been carried out of specificity in relation to the acyl group of phenylalanine for fungal acylase (for which aromatic amino acids are good substrates) and for acylase I.[56] The relative rate was studied at the pH optimum determined for each acyl group for fungal acylase (pH 6–7.5) and at pH 7 for acylase I. The hydrolysis rate fell in the series formyl > chloroacetyl > acetyl > propanoyl > butanoyl > pentanoyl > hexanoyl > ethyloxycarbonyl ⩾ benzyloxycarbonyl (Z) [t-butyloxycarbonyl (Boc) ≈ 0] on catalysis with fungal acylase and in the series butanoyl > chloroacetyl > propanoyl > pentanoyl > acetyl > hexanoyl > formyl > ethoxycarbonyl ⩾ benzyloxycarbonyl (t-butyloxycarbonyl ≈ 0) on catalysis with acylase I. Derivatives of the urethane type were significantly less reactive than the usual acyl derivatives, neither t-butyloxy nor benzyloxycarbonyl derivatives were subject to hydrolysis in practice. The correlation between the chain length of the hydrocarbon fragment of the acyl group [$H-(CH_2)_n-C(O)$, n = 0 to 5] and the relative rate of hydrolysis is shown in Fig. 1. The hydrolysis of N^{α}-formyl-N^{ε}-t-butyloxycarbonyl-DL-ornithine was effected by acylase (yield of L-amino acid was 48.5%, of D-amino acid derivative 44%) which confirms the suitability of the formyl group for practical separations.

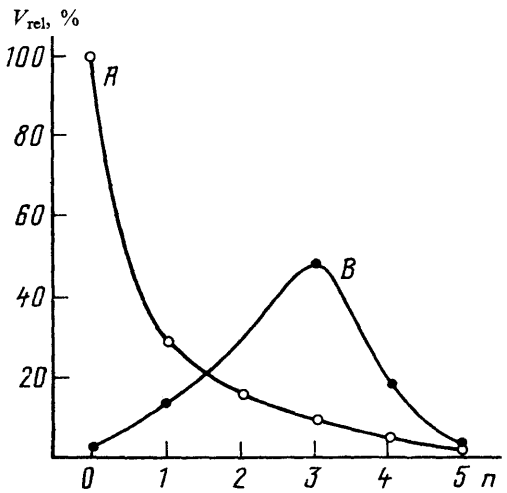


Figure 1. Dependence of the relative hydrolysis rate of *N*-acylphenylalanine by *A*, fungal acylase and *B*, acylase I on the number of methylene units (*n*) in the acyl group.

The kinetics of the enantioselective hydrolysis of *N*-acetyl-DL-amino acids (methionine, leucine, alanine, valine, serine, tryptophan, phenylalanine and phenylglycine) by acylase from *Aspergillus oryzae*, free and immobilised on DEAE-Filtradex R50, have been investigated.[57,58]

With the aim of broadening the application of acylase for obtaining optically active amino acids a search for new enzymes possessing acylase activity has been carried out[59,60] as has a clarification of such activity for enzymes of other classes.[61,62] The acylase from *Comamonas testosteroni* specific for acyl-L-proline was discovered in this way.[59] The high enantioselectivity enables resolution of *N*-acetyl-DL-proline into enantiomers. Replacement of the acetyl group by chloroacetyl accelerates the rate of hydrolysis of the *N*-derivatives of L-proline 3.6-fold. No reaction took place in the case of the benzoyloxycarbonyl and t-butyloxycarbonyl derivatives, the activity in relation to the derivative of other amino acids was one to two orders of magnitude less than for *N*-acetyl-L-proline. An acylase from *Bacillus thermoglucosidius* has been investigated.[60] The enzyme is specific for aliphatic amino acids (V_{rel} = 100% for *N*-acetyl-L-methionine), the hydrolysis rate for aromatic amino acids was approximately one order of magnitude less.

The majority of *N*-acetyl derivatives of acidic and basic L-amino acid and the *N*-acetyl-D-amino acids investigated (methionine, leucine, valine) were inert to the action of this enzyme (V_{rel} < 1%). Replacement of the acetyl group by chloroacetyl leads to an increase in rate V_{rel} of approximately 10-fold. The benzoyloxycarbonyl and t-butyloxycarbonyl derivatives were inert. The enzyme investigated has much in common with acylase I and with the acylase from *Asp. oryzae*. They all contain Zn^{2+}, are activated by Co^{2+} ions, have pH optima 8.0–8.5 and have similar amino acid compositions. However the activity of the acylase from *Bacillus thermoglucosidius* was approximately two orders of magnitude greater with the *N*-chloroacetyl derivative of DL-alanine and DL-norleucine. In addition the enzyme is distinguished by a high stability under extreme conditions. On heating to 70 °C for 10 min its activity was fully preserved, to 80 °C 90% was retained. It was stable to organic solvents (ethanol up to 50%, isopropanol up to 30%). The activity (80%) was retained in the presence of 5% sodium dodecyl sulphate and 85% in 8 M urea at 37 °C for 30 min. These special features of the acylase from *B. thermoglucosidius* suggest it may have promise in the preparation of optically active amino acids.

The same authors detected acylase activity towards acylamino acids in the purified dipeptidase from *B. stearothermophilus* which was 3–30% of its activity in the hydrolysis of dipeptides.[61] However this enzyme displayed higher specificity towards aromatic amino acids (V_{rel} = 100% for *N*-acetyl-L-methionine, 110% for *N*-acetyl-L-tyrosine and 160% for *N*-acetyl-L-phenylalanine). As in the previous case replacement of the acetyl group by chloroacetyl leads to an increase in V_{rel} of at least 10-fold. The ability of polymixin acylase to hydrolyse *N*-acyl derivatives of amino acids, although with low enantioselectivity has been shown.[62] Of all the acyl groups investigated containing from 2 to 16 carbon atoms the decanoyl (C_{10}) derivatives were most readily subject to hydrolysis. It must also be mentioned that when preparing the enzyme under specific conditions it is able to remove a Z protecting group (although with a low rate).

D-Amino acids are being used more widely in recent years. Communications on the presence of D-acylase activity in certain microorganisms is of special interest in this respect. D-Aminoacylases from microorganisms of *Pseudomonas*[63,64] and *Streptomyces*[65,66] species were known previously. Low enantioselectivity was a disadvantage of these enzymes which limited their use for the manufacture of D-amino acids. An investigation has been carried out[67] of 800 strains of microorganisms isolated from soil and

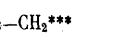
one strain *Alcaligenes dentrificans* DA 181 was found to be a source of D-aminoacylase, possessing high activity and enantioselectivity towards *N*-acyl-D-amino acids. The enzyme, expressed under optimum conditions, possesses an activity of 250 μmol h^{-1} ml^{-1} (D-acylase from *Pseudomonas Sp.* has 10 μmol h^{-1} ml^{-1},[64] and from *Streptomyces olivaceus* 84 μmol h^{-1} ml^{-1} [65]). The maximum specific activity, 108 600 μmol h^{-1} ml^{-1}, was displayed in the hydrolysis of *N*-acetyl-D-methionine (*Pseudomonas sp.* 6.8 μmol h^{-1} ml^{-1} and *Str. olivaceus* 571 μmol h^{-1} ml^{-1}). The enzyme catalyses the hydrolysis of L substrates to an insignificant extent (Table 8).

Table 8. Relative hydrolysis rate of D- and L-substrates by the aminoacylase from *A. dentrificans*.

Substrate	V_{rel}, %		Substrate	V_{rel}, %	
	D-enantiomer	L-enantiomer		D-enantiomer	L-enantiomer
N-Ac-Met	100	0.1	N-Ac-Trp	33	0.6
N-Ac-Phe	81	0.3	N-Ac-Asp	17	0.0
N-ClAc-Val	66	0.0	N-Ac-aloIleu	12	*
N-Ac-Leu	60	0.4	N-Ac-Val	6	0.0
N-Ac-Ala	25	0.8	N-Ac-Phg	5	*

* Not determined.

Table 9. Separation of amino acid racemates by aminoacylase.

R	Acyl group	Enzyme*	Co^{2+}	Yield, %		Enantiomeric purity, %	
				L	D	L	D
Et	Ac	AI	–	40	32	>99.5	>99.5
Et	ClAc	AI	–	40	41	>99.5	>99.5
Pr	Ac	AA	+	33	32	>99.5	>99.5
$CH_2=CHCH_2-$	ClAc	AI	–	41	33	>99.5	>99.5
trans-Me–CH=CH–CH_2	Ac	AA	+	33	38	99	93
cis-Me–CH=CH–CH_2	Ac	AI	–	44	47	>99.5	>99.5
	ClAc	AI	+	37	42	99	84
	Ac	AA	+	50	50	95	98
	Ac	AA	+	45	41	99	**
	ClAc	AI	+	51	31	93	**
	ClAc	AI	+	43	46	91	80
	ClAc	AI	+	30	63	97	48

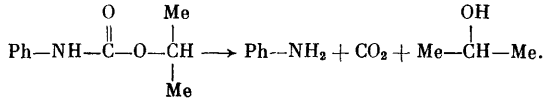
*AI is acylase I, AA is acylase from *Aspergillus sp.*
** The free amino acid was not isolated.
*** *N*-Acyl derivatives of α-methyl-AA.

The use of aminoacylases for the separation of enantiomers enables rarely encountered and unnatural optically active amino acids to be obtained. The racemic acyl derivatives of such amino acids and also of α-methylamino acids are separated into enantiomers by acylase I and by the acylase from *Aspergillus sp.* in preparative quantities (Table 9).[68]

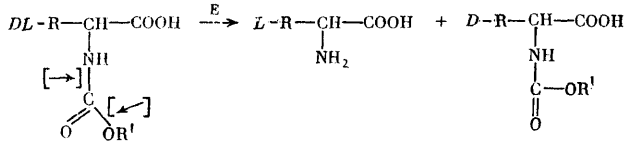
The values for the enantiomeric purity given in Table 9 characterise the significant enantiospecificity of the action of the enzyme (the amino acids isolated were not recrystallised). In the case of *N*-acyl-α-methyl-AA, the activity in relation to which was low (V_{rel} < 1%, for *N*-acetylmethionine V_{rel} = 100%), hydrolysis was effected with acylase I enclosed in a membrane which made it possible to use large quantities of protein many times in small reaction volumes. It was not expedient to use the acylase from *Asp. oryzae* since its specific activity was two orders of magnitude lower than that of acylase I.

5. Carbamates of amino acids

Many pesticides contain carbamate groups, such as NH(CO)OMe. It was shown previously that breakdown of *N*-phenylcarbamates occurs in soil under the action of microorganisms.[69]



With the development of an efficient method for the chemical synthesis of carbamates of amino acids[70] the possibility arose of obtaining optically active amino acids by enzymic breakdown of these derivatives. With this aim Kula and collaborators attempted to effect the hydrolysis of carbamates of several amino acids with an esterase from porcine liver, a hydantoinase from *Pseudomonas fluorescens* DSM 84, the urease from black beans, an acylase from porcine kidney and an acetylcholinesterase from porcine and human erythrocytes.[71,72] Enantioselective enzymic hydrolysis of the carbamates of amino acids occurs according to the following scheme.



Enantioselective removal of the *N*-alkoxycarbonyl group was observed only in the case of acylase and acetylcholinesterase. Of the three *N*-substituted DL-amino acids investigated, the alkoxycarbonyl derivatives of alanine, valine and *N,N'*-α,ε-disubstituted DL-lysine, acylase hydrolysed the carbamates of alanine and valine with both methoxy- and with ethoxycarbonyl (EOC) substituents. In the case of the alkoxycarbonyl derivative of lysine complete hydrolysis occurred but only with a methoxy carbonyl (MOC) group. Acetylcholinesterase effected the decomposition of the *N*-MOC derivatives of all three amino acids but in the case of lysine hydrolysis occurred regioselectivetly at the α substituent. The *N*-EOC derivatives were not hydrolysed. The pH optimum for the action of acetylcholinesterase was 7.5–8 and for acylase 7.5. The Michaelis constant (K_M) for the hydrolysis of *N*-MOC-DD-alanine was 68 mM for acetylcholinesterase and 8.2 mM for acylase. The activity of each of the enzymes in the hydrolysis of valine and lysine

derivatives was approxitimately the same but was one order of magnitude less in the hydrolysis of α,ε-bis(MOC)-DL-Lys. The activity of acetylcholinesterase was 15–20 times less than the activity of acylase but the size of the latter in the hydrolysis of *N*-MOC-DL-Val(Ala) was less than 1% activity of acylase in the hydrolysis of *N*-acetylmethionine (values of K_M are of the same order) (Table 10).

Table 10. Hydrolysis of amino acid carbamates by acylase from porcine kidney (temperature 37 °C).

Carbamate of DL-AA	α, μmole min^{-1} mg^{-1}	K_M, mM	Time, h	L-AA detected, %	
				A	B
N-MOC-Ala	0.63	8.2	78	47.5	43.0
N-EOC-Ala	0.56	8.2	70	45.5	44.0
N-MOC-Val	0.45	9.1	70	45.5	44.5
N-EOC-Val	0.40	9.1	76	42.5	34.5

Note. α is specific activity; A before chromatographic separation; B after chromatographic separation.

When considering the mechanism of the enzymic hydrolysis reaction of carbamates of amino acids the authors suggested two possible routes for the reaction.[72] The first was fission of the ester bond of the carbamate. The products of this reaction would be an alcohol and a substituted carbamic acid. Derivatives of carbamic acids are unstable and the intermediate obtained is spontaneously decomposed into an amino acid and carbon dioxide. Enzymic attack at the carbonyl atom with fission of the amide bond is a more probable route. The products of this reaction are the amino acid and a monoester of carbonic acid which decomposes to an alcohol and CO_2. In either case two volatile products are formed which may readily be removed from the reaction. This enables the reaction to proceed to completion and simplifies the isolation of the amino acid.

However the low values of the carbamates activity of acylase and acetylcholinesterase exclude the possibility of using these enzymes for the large scale resolution of amino acid racemates. In this situation there are two next steps, 1) a search for new sources of enzymes possessing high activity and 2) the selection of mutant strains. Kula and Sambale chose the first route.[73,74] They investigated 42 strains of microorganisms isolated from soil, which had previously been subjected to treatment with pesticides containing carbamate structures, and 36 strains from the German Microorganism Collection (from *Agrobacterium rodiobacter, Agrobacterium rhizogenes, Arthrobacter globiformis, Bacillus coagulans, Corynebacterium sepedonicum, Enterobacter cloacae, Mucobacterium spegmatis, Pseudomonas fluorescens, Pseudomonas putida*). Seven strains from the German Collection displayed low activity towards the alanine derivative and four towards the lysine derivative. The greatest activity was shown by the strain of *Pseudomonas fluorescens* DSM 50124 at 2.3 nmol min^{-1} mg^{-1} for *N*-MOC-Ala and 1.6 nmol min^{-1} mg^{-1} for *N*-MOC-Val.

The strains of microorganisms isolated from soil were cultured in media enriched with one of the substrates *N*-MOC-Ala, *N*-MOC-Val or *N,N'*-α,ε-bis(MOC)-Lys and the presence of carbamatase activity was then determined. It turned out that such activity was possessed by significantly fewer strains cultured in media enriched with the valine derivative than in the case of the alanine and lysine derivatives. This may be linked with the bacteriostatic properties of valine.[75] The majority of strains possessing carbamatase activity effected the hydrolysis of all three substrates but the activity towards the lysine derivative was the least. It is interesting to note that strains cultured on a medium enriched with *N*-MOC-DL-Ala possessed higher activity towards valine and lysine derivatives than those cultured on media enriched with *N*-MOC-DL-Val or *N,N'*-α,ε-bis(MOC)-DL-Lys. Six samples were isolated for further investigation (Table 11). The D-enantiomers of the substrates investigated were stable to the action of the enzyme. The hydrolysis of racemic substrates led to a product containing contamination by the D-isomer only in the case of alanine, but this is apparently linked with the presence of an alanine racemase.

Table 11. Hydrolysis of *N*-methoxycarbonyl-DL-amino acids by various strains of microorganisms isolated from soil.

Strain	α, nmole min^{-1} mg^{-1}			Hydrolysis of *N*-Moc-DL-Val				
	N-MOC-*DL*-Ala	*N*-MOC-*DL*-Val	*N,N'*-α,ε-bis(MOC)-DL-Lys	[protein], mg ml^{-1}	[substrate] mM	Time, h	[product], mM	Yield, %
A3 Xanthomanas maltophilia	166	126	96	0.2	180	65.5	18.5	10.6
A4 Methylobacterium extorques	318	81	45	1.2	180	65.0	29.5	16.9
L8 (not determined)	19.5	4.1	8.9	1.5	180	63.5	9.2	5.4
L10 Pseudomonas putida	59.0	8.8	0.0	0.5	180	67.0	46.0	26.3
L12 not determined	8.3	6.1	0.0	4.0	180	64.0	74.7	42.7
L18 Alcaligenes sp.	20.9	51.8	0.0	1.4	350	64.5	162.5	46.5

In the majority of cases the hydrolysis of *N,N'*-α,ε-bis(MOC)-Lys led to *N*-ε-MOC-LYs, *N*-α-MOC-Lys was not detected in the reaction products. Four strains gave unsubstituted lysine, two others a mixture of lysine and *N*-ε-MOC-Lys.

In the course of further investigations a strain was found possessing DL-carbamatase activity (121 nmol min^{-1} mg^{-1} for the alanine derivative, 170 nmol min^{-1} mg^{-1} for valine and 6.0 nmol min^{-1} mg^{-1} for lysine).[74] Two enzymes were isolated by purification, an L-carbamatase (MW 160000) and a D-carbamatase (MW 90000). Their substrate specificities towards the amino acid side chain and the alcohol component of the carbamate group were investigated (Table 12). The L-carbamatase displayed the greatest activity towards *N*-MOC-L-Phe while the best substrate for the D-carbamatase proved to be *N*-MOC-D-Ala. An increase in the number of carbon atoms in the alcohol component of the carbamate group causes a change in activity, which is extremely marked in the case of the L-carbamatase and insignificant in the case of D-carbamatase.

The stereoselective hydrolysis of *N,N'*-α,ε-bis(MOC)-DL-Lys occurs only at the α-amino group. Such a regioselective enzyme might play an important role in peptide synthesis for removing *N*-α-protecting groups.

Table 12. Specific activity of purified enzymes.

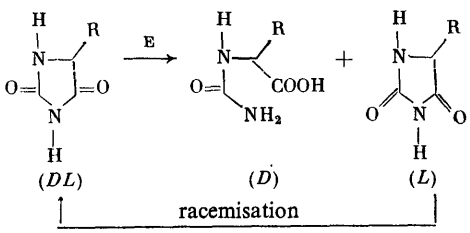
DL-Amino acid carbamate	L-Carbamatase activity, μmole min $^{-1}$ mg^{-1}	D-Carbamatase activity, μmole min $^{-1}$ mg^{-1}
N-(MOC)-*DL*-Val	12.1	4.5
N-(EOC)-*DL*-Val	1.1	2.1
N-(MOC)-*DL*-Ala	25.6	51.3
N-(EOC)-*DL*-Ala	8.9	26.7
N-(MOC)-*DL*-Phe	42.6	0.1
N-(MOC)-*DL*-Leu	3.3	1.0
N,N'-αε-bis(MOC)-DL-Lys	1.0	0.5

In conclusion it must be recorded that the specific activity of the strains found in the primary selection were inadequate for the development of an enzymatic process. However the fact that many microorganisms belonging to different species possess carbamatase activity raises hopes that further search and investigations will lead to the obtaining of enzymes suitable for the industrial resolution of amino acid carbamates.

6. Cyclic derivatives of amino acids

a) 5-Monosubstituted hydantoins

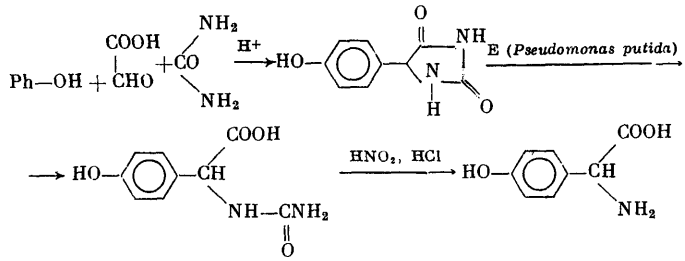
Enzymes possessing hydantoinase activity are mainly encountered in the microorganisms *Hansemula sp.*,[76] *Pseudomonas*,[77–79] *Agrobacterium*,[80] *Arthrobacter*,[81,82] *Corynebacterium*,[83] *Bacillus*,[84] etc. They catalyse the fission of hydantoins into *N*-carbamoyl-α-amino acids.



Hydantoins are intermediate compounds in the synthesis of α-amino acids from aldehydes, KCN and urea or ammonium carbonate.[85] They have an important advantage in comparison with other derivatives of amino acids. They racemise spontaneously under the conditions of the enzymic reaction and make it possible to convert practically 100% of a racemate into one of the enantiomers.

Removal of the *N*-carbamoyl group is possible chemically with an equimolar quantity of sodium nitrite in acidic medium. Complete retention of configuration was observed and the degree of conversion was close to 100%.[86]

This method enabled development of one of the most economic processes for the large scale manufacture of such amino acids as D-*p*-hydroxyphenylglycine used in the preparation of the semi-synthetic penicillin amoxicillin and the cephalosporins cephadroxil and cefatriazine.[86] Development of the process included the design of a simple and economical method for the chemical synthesis of the substrate DL-5-(*p*-hydroxyphenyl)hydantoin from phenol, glyoxylic acid and urea in an acidic medium.



The manufacture of D-phenylglycine and D-valine, which are used to the synthesis of antibiotics and pesticides, was developed in a similar manner.

The catalytic conversion of DL-5-(*p*-hydroxyphenyl)hydantoin also occurs by the action of cells of *Pseudomonas sp.* AJ-11220 however in this case the product is not the *N*-carbamoyl-D-amino acid but D-*p*-hydroxyphenylglycine.[87,88] Other similar processes have been studied.[89,90]

Yokozeki and collaborators have investigated strain AJ-11220, have optimised the conditions of expression of cells and of carrying out the enzymic hydrolysis (pH optimum 8.0, optimum T = 43 °C) and determined the substrate specificity of the enzyme (Table 13).[87]

Table 13. Substrate specificity of the enzyme from *Pseudomonas sp.* AJ-11220.

Hydrolysis produce	V_{rel}, %	Hydrolysis produce	V_{rel}, %
D-cyanoethylglycine	100	*D*-His	33
D-Met	80	*D*-Trp	21
Gly	80	*D*-Ile	19
D-Gln	72	*D*-Lys	18
D-Phg	62	*D*-Val	15
D-*p*-hydroxy-Phg	61	*D*-Glu	12
DL-Ala	58	D-3, 4-methylenedioxy-Phe	11
D-Phe	41		

All the products with the exception of glycine and alanine have the D configuration. In the case of alanine the authors linked the formation of a racemic product with the presence of alanine racemase in the cells, which was confirmed by subsequent experiments.

The possibility of obtaining D-lysine from its hydantoin precursor has been demonstrated for the first time.[87] It must also be noted that dihydrouracil is hydrolysed to a very insignificant extent by

AJ-11220 cells although it is the best substrate for the enzyme from *Pseudomonas putida*.[86] All these data suggest that the enzyme participates in the catalytic process with a different substrate specificity. To investigate this a partial purification was carried out and the substrate specificity of the fractions isolated was determined.[88] Cells of *Pseudomonas sp.* AJ-11220, suspended in 0.02 M tris HCl buffer (pH 7.5) containing either 10 mM $CoCl_2$ and $CaCl_2$ (variant I), or 10 mM dithiothreitol (variant II), were subjected to the action of an ultrasonic oscillator with subsequent fractionation on a column of DEAE-Toyopearl 650 M previously equilibrated with the appropriate buffer. Isolation by the first variant led to a 27-fold purification of the enzyme, viz. D-hydantoin hydrolyase (D-HH), with an overall activity yield of 63% (Table 14). No L-hydantoin hydrolase was detected. The hydrolase of *N*-carbamoyl-AA (CH) found in the unpurified extract was also not detected after fractionation on DEAE-Toyopearl.

Table 14. Partial purification of D-hydantoin hydrolase.

Fraction	Total quantity of protein, mg	Formation			
		D-enantiomer		L-enantiomer	
		A*, nmole min^{-1}	α, nmole min^{-1} mg^{-1}	A*, nmole min^{-1}	α, nmole min^{-1} mg^{-1}
Unpurified extract	540	36 000	67	0	0
DEAE-Toyopearl	13	22 620	1795	0	0

*A is total activity.

Purification according to variant II led to the observation that D-hydantoinase activity, found in the unpurified extract, was not detected after fractionation on DEAE-Toyopearl. The results of purifying the D and L hydrolases of *N*-carbamoyl-AA are shown in Table 15.

Table 15. Partial purification of *N*-carbamoyl-L- and D-amino acid hydrolases.

Fraction	Total quantity of protein, mg	Formation			
		D-enantiomer		L-enantiomer	
		A, nmole min^{-1}	α, nmole min^{-1} mg^{-1}	A, nmole min^{-1}	α, nmole min^{-1} mg^{-1}
Unpurified extract	475	7200	15	2290	5
DEAE-Toyopearl	10	2600	261	0	0

The data obtained enable certain conclusions to be drawn as to the mechanism of the asymmetric hydrolysis of DL-hydantoins of amino acids by *Pseudomonas sp.* AJ-11220. Only the D-isomer of the hydantoins is converted into the corresponding *N*-carbamoyl-D-amino acid by D-HH (pH optimum 8.0, optimum T 55 °C). *N*-Carbamoyl-D-amino acids are hydrolysed to the D-amino acid by D-CH (pH optimum 7.0, optimum T 50 °C). The L-hydantoins of amino acids racemise spontaneously and the D-isomers obtained are introduced into the hydrolysis process. When using preparations of D-HH and D-CH, obtained by fractionation on DEAE-Toyopearl, in the hydrolysis of DL-5-(*p*-hydroxyphenyl)hydantoin (pH 8.0) the product D-(*p*hydroxyl)phenylglycine, was obtained quantitatively. The substrate specificity of the three partially purified enzymes was also determined (Table 16).

Table 16. Substrate specificity of enzymes (D-hydantoin hydrolases and hydrolases of *N*-carbamoyl-D- and L-amino acids).

Substrate, hydantoin derivative of AA	V_{rel}, % (D-HH)	Substrate, *N*-carbamoyl-D- or L-AA	V_{rel}, %	
			D-CH	L-CH
D-Met	100	Met	100	47
DL-Met	54	Phg	94	–
L-Met	30	*p*-hydroxy-Phg	88	–
D-Phe	57	DOPA	62	0
D-Val	40	Leu	61	98
D-Phg	34	Ala	56	44*
D-*p*-hydroxy-Phg	33	Asn	48	2
DL-*p*-hydroxy-Phg	23	Phe	32	10
L-Lys	28	Glu	32	3
D-Tyr	19	Trp	31	0
DL-DOPA	18	Tyr	30	9
β-Ala	9	Val	18	100
DL-Leu	8	Gly	8	0
D-Trp	6	β-Ala	8	–
Gly	5	–	–	–
L-Glu	5	–	–	–
L-Asp	5	–	–	–
L-Ala	2			

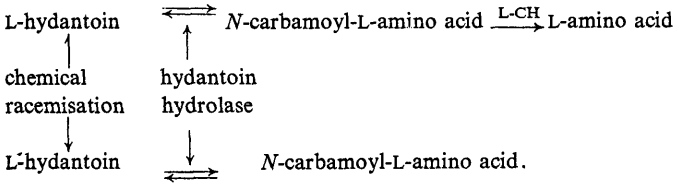
* Product was DL-alanine (due to the presence of alanine racemase in the unpurified extract).

Table 17. Substrate specificity of hydantoin hydrolase enzyme from strain AJ-3940.

Substrate, hydantoin derivates of AA	V_{rel}, %
L-Trp	100
5-Hydroxy-L-Trp	12
L-Phe	108
L-Tyr	71
L-3,4-DOPA	105
DL-3,4-Methylenedioxy-Phe	112
DL-3,4-Dimethoxy-Phe	4
L-Met	2
Gly,L-Ala,L-Ile,L-Asp,L-Glu	0

The strain *Flavobacterium sp.*-3912 was investigated in a similar manner. This enzyme hydrolyses DL-5-indolylmethylhydantoin to L-tryptophan in fairly high yield. The mutant strain AJ-3940, in which the routes decomposing tryptophan are blocked (yield of L-tryptophan was almost 100%), was also investigated.[91–93] The pH optimum for strain AJ-3940 was 8.5 and optimum T about 50 °C. Data on the substrate specificity are shown in Table 17.

Hydantoin hydrolyase activity towards both L and D substrates was observed at all stages of purifying the enzyme from strain AJ-3912 (ratio of specific activities L/D = 2.6 to 2.7). The *N*-carbamoyl-AA hydrolase activity was also detected at all stages of the purification but only towards the L substrate. Since no *N*-carbamoyl-AA racemase was detected in the strain, the following mechanism is proposed for the enzymic conversion of hydantoins of DL-amino acids into L-amino acids.[93]



The pH optimum for the hydrolysis of the hydantoin derivative was 9.7, the reverse reaction proceeds at a pH close to neutral values (7–8). The pH optimum for the action of L-CH is 8.0 and the optimum temperature for the purified enzymes is 40 °C. Data on the substrate specificity of L-CH are shown in Table 18.

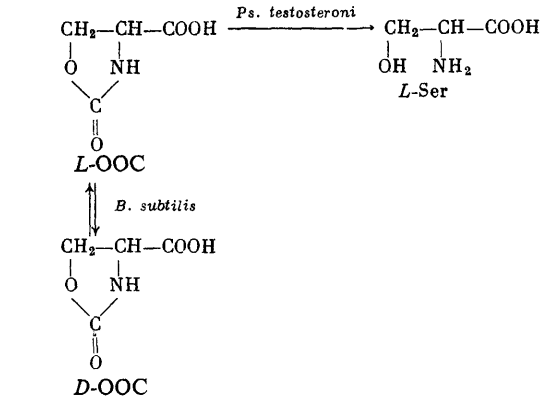
Table 18. Substrate specificity of *N*-carbamoyl-L-amino acid hydrolase.

Substrate, *N*-carbamoyl-AA	V_{rel}, %
DL-3,4-Methlenedioxy-Phe	100
L-Phe	82
L-Tyr	59
L-Trp	55
DL-3,4-Dimethoxy-Phe	24
L-Met	24
Gly	5
L-Ile	2

Another strain, *Arthrobacter sp.* DSM 3747, has been investigated.[94] This strain is able to catalyse the hydrolysis of DL-5-indolylmethylhydantoin to L-tryptophan, which showed that the conversion proceeded according to a scheme similar to that proposed previously,[93] but the racemisation of 5-indolymethylhydantoin in the presence and absence of cells of *Arthrobacter sp.* occurs in a different way (Fig. 2). This formed the basis for proposing that a third enzyme participates in the process, a hydantoin racemase, which was isolated during subsequent work.[95]

b) DL-2-Oxooxazolidine-4-carboxylic acid

A method is known for the quantitative preparation of L-cysteine from DL-2-aminothiazolin-4-carboxylic acid (ATC), an intermediate in the chemical analysis of DL-cysteine, by a combined enzymic process of hydrolysis of L-ATC and racemisation of D-ATC by cells of *Pseudomonas thiazolinophilum* AJ-3854.[96,97] The process was applied industrially and subsequently attempts were made to develop a similar process to obtain L-serine from DL-2-oxooxazolidine-4-carboxylic acid (OOC), which is an intermediate in the synthesis of DL-serine.[98] It is necessary to effect two reactions for the complete conversion of the racemic derivative into the optically active amino acid, viz. the hydrolysis of L-OOC to L-serine and the racemisation of D-OOC. In all 849 strains were investigated 12 of which proved to be capable of catalysing the first reaction and 8 the second. The strain of *Pseudomonas testosteroni* AJ-2770 was the best source of hydrolysis activity and *Bacillus subtilis* AJ-1996 of racemisation. By the combined process L-Ser was obtained from D-OOC in 30% yield with cells of *Ps. testosteroni* and *B. subtilis*. When using L-OOC as substrate the reaction proceeded in a similar manner. This enabled the conclusion to be made that racemisation is not the rate-limiting stage. L-Ser was not obtained from D-OOC if cells of one of the strains was not present. The combined process occurs according to the scheme:



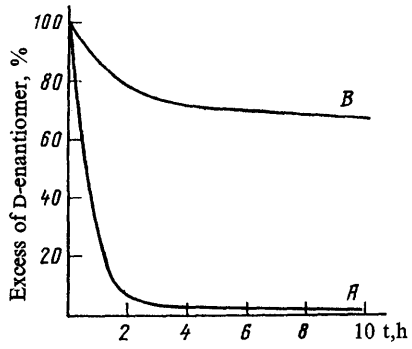


Figure 2. Racemisation of D-indolylmethylhydantoin *A*, in the presence and *B*, in the absence of cells of *Arthrobacter sp.*

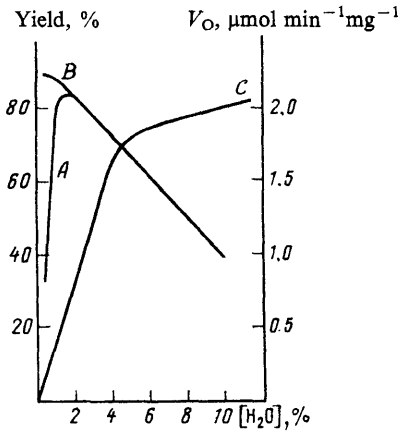


Figure 3. Effect of water content on the yield of *N*-acetyl-L-tryptophan ethyl ester (*A*, reaction time 24 h; *B*, at low H_2O concentrations the reaction time was increased to 15 d) and *C*, on the reaction rate. *N*-Ac-L-Trp 0.05 g (0.02 mmole), α-chymotrypsin 0.01 g, 0.1 M phosphate buffer pH 6.8, 30 °C.

III. Enantioselective enzymic derivatisation of amino acids and their derivatives

Reactions catalysed by enzymes are, as a rule, reversible. However by creating particular conditions (for example varying reagent concentration, removing one of the products from the reaction) it is possible to secure a shift in the equilibrium in the direction required. In practice this is achieved by carrying out the reaction in media containing significant quantities of organic solvent, such as in aqueous organic media (including alcohol–water), in two-phase systems (water–immiscible organic solvents), in systems of reverse micelles and in two-phase aqueous polymeric systems. The enzymic synthesis of aminoacid derivatives is in the development stage. The greatest number of studies in this area is devoted to the synthesis of α-amino acid esters.

1. Synthesis of amino acid esters

One of the first reports on the ability of enzymes to catalyse the synthesis of amino acid esters was made in 1965.[99] The rate of the enzymic reaction and the yield of product depends on many factors, among which are the decisive role played by the state of the enzyme, the properties of the carrier (including the specific surface) when using immobilised catalysts, the composition and pH of the aqueous phase, the concentration of amino acid and alcohol, the composition of the organic phase, the rate of the volumes of the organic and aqueous phases, etc. The synthesis of *N*-acetyl-L-tyrosine ethyl ester using immobilised α-chymotrypsin in a two-phase system has been optimised with respect to these parameters (yield of product was 85–90%).[100] *N*-Acetyl-L-tryptophan ethyl ester was obtained in 86–95% yield under similar conditions (two-phase water–chloroform system, time 2–8 h),[101] which enabled the process to be used for separating racemic *N*-acetyltryptophan (time 48 h, yield of L-enantiomer 45%).[102] The esterification of acyl derivatives of aromatic amino acids in two-phase systems and in aqueous organic media has been investigated.[103,104] The ratio of aqueous and organic phase proved to have a significant effect on the extent of conversion. On changing this ratio from 1:10 to 1:80 the yield was increased from ~38 to ~92%. In a water–chloroform system at constant V_{aq}:V_{org} ratio (1:40) the effect of ethanol concentration on the yield of product was fairly weak (Table 19). The yield of product in ethanol was satisfactorily high. The reaction system was a suspension of hydrated α-chymotrypsin which may easily be removed by filtration. Another important virtue of the one-phase system was considered to be the significantly less noticeable inhibition by the substrate concentrations (Table 20).

Table 19. Effect of ethanol concentration on yield of product.

[EtoH] in $CHCl_3$, M	Yield of *N*-Ac-L-Trp ethyl ester, %
1	83 *
3	86 *
5	77 **
8.6 (50 об.%)	79 **
17.1	78 **

* Two-phase system.
** One-phase system.

Table 20. Effect of substrate concentration.

N-Ac-L-Trp	Reaction time, h	Yield of *N*-Ac-L-Trp ethyl ester, %	
		EtOH	$CHCl_3$ (1 M EtOH)
0.05	24	81	78
0.10	24	86	78
0.15	24	75	78
0.20	24	70	78
0.20	48	85	78
0.20	72	83	78
0.20	96	85	4.4
0.25	96	58	3.0

The dependence of the ester yield on the water content of the system when carrying out esterification for 24 h has a maximum in the region of 2–4% H_2O (Fig. 3, curve *A*), however the shape of the curve is changed on increasing the synthesis time (at these and lower H_2O concentrations) to 15 d (Fig. 3, curve *B*). This is seemingly linked with the significant reduction in reaction rate (Fig. 3, curve *C*) due to the reduction in the extent of hydration of α-chymotrypsin. It is possible that the negative effect may be reduced by using the modified enzyme, hexylchymotrypsin, for the synthesis of esters.[105] The size of V_{max} for such an enzyme is increased.

Another possible method of increasing the reaction rate at low H_2O concentrations is immobilisation of the enzyme, such as the adsorption of α-chymotrypsin into chitin.[106] When carrying out

esterification in ethanol containing 3.4% water the yield of *N*-acetyl-L-tryptophan ethyl ester was 28% for the free enzyme (reaction time 24 h) and 85% for α-chymotrypsin adsorbed onto chitin (reaction time 12 h). The use of other polysaccharides, such as microcrystalline cellulose and aminoethylcellulose, led to a lower yield (20 and 23% respectively after 24 h) than for the free enzyme. It is known that α-chymotrypsin in solutions containing hydrophilic solvents at high concentration loses its catalytic activity to a significant extent due to partial unfolding of the polypeptide chain. The enzyme bound to chitin probably retains the native conformation at H_2O concentrations below 5%. It is possible that a significant portion of the water in such solutions is also adsorbed by the chitin and contributes to the stabilisation of the native conformation.

Investigation of the esterification at various temperatures showed that the yield was practically unchanged in the temperature range 10–35 °C and at higher temperatures it fell sharply.[104] Under the conditions described above esterification was effected for aromatic *N*-acetyl-L-amino acids (tryptophan, tyrosine and phenylalanine), the analogous derivatives of L-alanine and L-leucine did not react. The yields of the ethyl esters of *N*-acetyl-D-tryptophan and -phenylalanine were extremely insignificant. This indicates the retention of substrate specificity and enantioselectivity under the given conditions. However the specificity towards the alcoholic component of the reaction was other than in the case of hydrolysis. Methanol and 2-methylpropan-2-ol (t-butanol) did not react, the other primary and even secondary alochols investigated gave a product in high yield (Table 21).

Table 21. Effect of structure of the alcohol component on the synthesis of *N*-acetyl-L-tryptophan esters.

Alcohol	Yield after 24 h, %	Alcohol	Yield after 24 h, %
Methanol	0	Butan-2-ol	83
Ethanol	81	Butan-2-ol	65
Propan-1-ol	83	2-Methylpropan-1-ol	80
Propan-2-ol	78	2-Methylpropan-2-ol	0

Table 22. Initial esterification rate (V_0) and yield of acetylamino acid ester.

Solvent	*N*-Ac-L-Trp-OEt		*N*-Ac-L-Tyr-OEt	
	$V_0 \cdot 10^2$, μmole $min^{-1} g^{-1}$	Yield % (24 h)	$V_0 \cdot 10^2$, μmole $min^{-1} g^{-1}$	Yield %(24 h)
Acetone	6.1	71	18	74
Nitromethane	4,3	60	31	89
Acetonitrile	2.3	56	13	80
Propylene carbonate	1.1	45	6.8	66
Tetrahydrofuran	0.88	30	4.5	44
1,4-Dioxan	≪0.5	12	6.5	46
Ethanol	12	81	15	82

Attempts were also made to conduct the synthesis in other hydrophilic solvents containing 1.7 M ethanol (Table 22).[107] Esterificaiton of *N*-acetyl-L-tryptophan did not take place in dimethylformamide, dimethylacetamide and dimethylsulphoxide.

An investigation was also conducted on the effect of aqueous phase composition, buffer concentration and pH (Table 23) which showed that the yield was extremely insignificant (5%) in pure H_2O. When using phosphate buffer the optimum concentration was 0.1 M. At higher concentrations the activity of the enzyme was reduced. This may be linked with a reduction in the quantity of water available for the stabilisation of the enzyme due to the increase in the quantity bound to phosphate. It is interesting that other buffers at the same molar concentration at other pH (5.0 and 9.0) gave results (70 and 74%) similar to that obtained when using phosphate buffer (pH 6.8). The minimal effect of pH may be explained by the inorganic salts playing the role of adsorbents of the enzyme after mixing the solution of α-chymotrypsin with a large excess of organic solvent.

Table 23. Effect of buffer on the synthesis of *N*-acetyl-L-tyrosine ethyl ester.

Buffer	pH	*M*	$V_0 \cdot 10^2$, μmole $min^{-1} g^{-1}$	Yield, % (24, h)
Phosphate	6.8	0.05	4.1	78
	6.8	0.1	4.9	78
	6.8	0.2	2.4	49
	6.8	0.5	2.3	24
Acetate	5.0	0.1	4.4	70
Borate	9.0	0.1	4.7	74
H_2O	–	–	*	5

* Not determined.

Table 24. Esterification of *N*-t-butyloxycarbonyl-L-amino acids using papain (37 °C, 8 h).

Substrate, *N*-Boc-AA	Volume ratio buffer: CH_2Cl_2: alcohol	Yield, %	Substrate, *N*-Boc-AA	Volume ratio buffer: CH_2Cl_2: alcohol	Yield, %
Leu	3 : 1 : 1	75	Tyr	3 : 5 : 1	90
Phe	3 : 1 : 1	76	Ser	3 : 5 : 1	75
Trp	3 : 1 : 1	78	Thr	3 : 5 : 1	40
Met	3 : 1 : 1	76	Glu	3 : 50 : 1.5	90
Cys(Bzl)	3 : 1 : 1	74	Glu *	3 : 50 : 1.5	78
Val	3 : 0.5 : 1	27	Gln	1.5 : 50 : 1.5	85
Ile	3 : 0.5 : 1	22	Asp	3 : 50 : 1.5	83
Gly	3 : 5 : 1	82	Asp *	3 : 50 : 1.5	70
Ala	3 : 5 : 1	88	Asn	1.5 : 50 : 1.5	82

* Benzyl alcohol was used as the alcohol component.

Papain is another enzyme widely used for obtaining optically active esters.[108–113] Attempts were undertaken to separate enantiomers of *N*-t-butyloxycarbonylamino acids by the enzymic synthesis of esters. Methylene chloride was used as the organic phase and 1 M citrate–phosphate buffer (pH 4.2) as the aqueous phase. By varying the ratios of buffer : methylene chloride : alcohol, esters were obtained for the majority of amino acids in high yield (Table 24). Yields were not high in the case of sterically hindered valine, isoleucine, threonine) and basic (lysine, arginine, histidine) amino acids.

As is seen from Table 24 the optimum ratio of organic and aqueous phases $\beta = V_{org}/V_{aq}$ varied from $\beta = 17$ for hydrophilic amino acids (aspartic and glutamic acids) to $\beta = 0.3$ for the hydrophobic (phenylalanine, leucine, tryptophan and methionine). Esterification of the dicarboxylic acids occurred at the α-carboxyl group to the monoester. The possibility of using enzymic synthesis for the separation of amino acid racemates was demonstrated by the synthesis of *N*-t-butyloxycarbonyl-L-aspartic acid from the racemic *N*-protected amino acid and benzyl alcohol. No D-ester was detected in the product and it was shown that the D-enantiomer was not esterified by papain. In subsequent investigations the possibility was shown of obtaining esters from the analogous *N*-derivatives of alanine and aliphatic alcohols $C_nH_{2n+1}OH$ (n = 2, 4, 6) and diols $HO(CH_2)_{2m}OH$ ($1 \leqslant m \leqslant 5$).[109] Immobilisation of papain itself on XAD-7 (neutral cross-linked polymethylacrylate) or Sepharose enabled the reaction to be carried out for n = 8, 10, 12, 16 and m = 6 as well. When using the immobilised enzyme the amount of alcohol was reduced 5-fold and the enantioselectivity of papain action towards α-amino acids was retained. The process may be used for resolving racemic *N*-t-butyloxycarbonylamino acids (alanine and aspartic acid).[110] As in the cases of α-chymotrypsin the enzymic synthesis using papain may be conducted in an aqueous alcoholic medium.[111–113] Esterification of *N*-benzyloxycarbonylamino acids catalysed by papain was carried out in an alcohol–water system with a minimum quantity of water (4–5 eq. H_2O/eq. AA). Ethyl, methyl, n-propyl and n-butyl alcohols were used as the alcohol component of the reaction (Table 25).[112]

Table 25. Synthesis of *N*-protected amino acid esters.

N-Protected-DL-AA	Product, *N*-Z-L-AA-OR		*N*-Protected-DL-AA	Product, *N*-Z-L-AA-OR	
	R	Yield, %		R	Yield, %
Ala	Et	48.5	Ser	Et	26.5
Ala	Me	42.5	Met	Et	47.5
Ala	n-Pr	37.5	Asp	Et	31.5
Ala	N-Bu	40.0	Gln	Et	44.5
Ala *	Et	36.5	Trp	Et	46.0
Ala **	Et	17.5	Phe	Et	43.5
NorVal	Et	49.0	Tyr	Et	47.5
NorLeu	Et	48.5	4-F-Phe	Et	41.0
Val	Et	19.0	4-NO_2-Phe	Et	37.5
Leu	Et	45.0	3,4-Cl_2-Phe	Et	45.5

Note. Reaction conditions in example 1: 50 °C, 10.7 g Z-DL-Ala in 80 ml abs. EtOH containing 2 ml citrate buffer (pH 4.5), time 24 h. Yield of Z-D-Ala was 42.5% (enantiomeric excess > 95%).

* *N*-Boc-L-Ala-OEt.

** *N*-Fmoc-L-Ala-OEt.

Immobilised papain was used in another study for esterification.[113] Of eight carriers investigated the best proved to be Sephadex G-50 (yields of esters of *N*-t-butyl- and *N*-benzyloxycarbonylamino acids were 40 to 93%). Investigation of the effect of volume, concentration and pH of the aqueous phase showed that the maximum yield was achieved at a water content of about 5%. The dependence of yield on pH had a sharp maximum at pH 4.2 and on increasing the concentration of citrate buffer from 0 to 3 M the yield was increased from 0 to 93%.

Some studies have appeared recently demonstrating the possibility of carrying out enzymic reactions in anhydrous organic solvents.[114–116] It has been shown[117] that α-chymotrypsin precipitated from n-propanol is capable of catalysing the synthesis of *N*-acetyltryptophan ethyl ester in cyclohexane with a high enantioselectivity which may be adjusted by varying the precipitation conditions. On precipitating in the presence of *N*-acetyl-D-tryptophan the ratio of the initial rates of the enzymic reactions $V_0^L/V_0^D = 70$ (V_0^L = 0.5 μmol mg^{-1} h^{-1}, V_0^D = 7.1 mmol mg^{-1} h^{-1}). When precipitating in the presence of *N*-Ac-L-Trp the enantioselectivity was strongly increased (V_0^L = 2.7 μmol mg^{-1} h^{-1}, no synthesis of D-ester was observed after 120 h).

2. Synthesis of amino acid amides

It was found that the D-enantioselective aminopeptidase from *Ochrobactrum anthropi*, which is able to hydrolyse the amides of D-amino acids and the *p*-nitroanilides and esters of amino acids and certain peptides, catalyses the aminolysis of D-amino acid esters under certain conditions.[118,119] D-Alanine-3-pentylamide was synthesised quantitatively in 1 h from D-alanine methyl ester and 3-aminopentane in various organic solvents saturated with water (Table 26).

Table 26. Enzymic aminolysis of alanine methyl ester by 3-aminopentane.

Substrate	Solvent	Yield, %
D-Ala-OMe	n-Butyl acetate	98
DL-Ala-OMe	n-Butyl acetate	48
L-Ala-OMe	n-Butyl acetate	0
D-Ala-OMe	Benzene	98
D-Ala-OMe	1,1,1-Trichloroethane	91
D-Ala-OMe	Toluene	67

When using a DL-substrate the product contained no L-amino acid amide. The enzyme possesses a wide specificity towards the acceptor of the acyl group. Products were syntheised in high yield from D-alanine and n-butylamine, benzylamine and neopentylamine in a similar manner. At the same time the specificity of the enzyme towards the acyl group proved to be appreciably worse. Only esters of D-alanine, glycine and D-α-aminobutyric acid gave a product in high yield (98, 80 and 40% respectively). The methyl esters of 19 other D-α-amino acids, β-alanine and various carboxylic acids were not substrates in this enzymic reaction. In water the aminolysis of

D-alanine methyl ester occurs in 78% yield after 7.5 min with subsequent complete hydrolysis to D-alanine in 30 min.

The information presented in the review on the enzymic methods of resolving racemic amino acids and their derivatives demonstrates the intense development of the sphere of science and manufacturing activity. The widespread use of enantioselective enzymic processes together with processes for the enzymic synthesis of amino acids from achiral substrates enables the need for optically active amino acids to be satisfied to a significant extent.

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