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Enzymic Conversion of Racemates into Aminoacid Enantiomers

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The present state of the art and prospects for further development in the employment of enzymes for the resolution of aminoacid racemates or the conversion of racemates of a series of chemical compounds into optically pure aminoacids are considered. A comparative survey of studies involving the use of aminoacylases, proteases, hydantoinases, α-aminocaprolactam hydrolase, and a number of other enzymes for the preparation of L- and D- aminoacids has been carried out. Examples are presented of processes involving the enzymic resolution of racemates on an industrial scale. The kinetic and thermodynamic features of the enzymic reactions employed have been analysed in detail and the limits in the employment of a particular enzyme for the conversion of racemates into optically pure aminoacids are accounted for.
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I. INTRODUCTION

The synthesis of optically pure aminoacids has become an increasingly urgent problem of modern biotechnology in recent years. Its solution by purely chemical means involves many difficulties. The chemical synthesis of optically active aminoacids has been achieved so far only in the manufacture of L-3,4-dihydroxyphenylalanine (L-DOPA) by the enantioselective hydrogenation of an α,β-unsaturated derivative in the presence of rhodium complexes of chiral phosphines[1]. The manufacture of L-DOPA is a rare example of a process in which the cost of an extremely expensive catalyst can be neglected in the synthesis of an expensive final product. The industrial chemical synthesis of the remaining aminoacid enantiomers remains at the present time no more than a theoretical possibility.

The manufacture of L- and D-aminoacids by the physicochemical resolution of racemic mixtures of aminoacids obtained by large-scale chemical synthesis (for the chemical synthesis of aminoacids, see the review of Safonova and Belikov[2]) is also not free from disadvantages. Thus the preparation of an enantiomer by the selective crystallisation of a supersaturated solution of the racemate has been achieved only for glutamic acid[3]. The highly effective enantioselective chromatography, which is being activity developed at the present time, has so far found practical applications only in the preparation of optically active compounds labelled by radioactive isotopes[4]. The available methods for the selective crystallisation of the diastereoisomers of aminoacids and any other optically active compounds require the search for and synthesis of the latter, which limits the practical application of this method.

New possibilities for the synthesis of aminoacids have arisen in connection with the development of genetic engineering methods which have given rise to the highly effective industrial production, in the first place of L-lysine, L-histidine, L-arginine, L-threonine, L-isoleucine, and L-valine[5]. The difficulties involved in the employment of this technology are associated primarily with the isolation of the required compond from the culture liquid containing other metabolic products, including many physiologically active compounds, and also with the establishment and maintenance of the properties of the producing agent.

One of the promising ways of obtaining optically pure aminoacids involves the use of individual enzymes. There are two fundamental possibilities for the enzymic preparation of aminoacid enantiomers. The first involves the synthesis, catalysed by many enzymes (aspartate ammonia-lyase, L-tyrosine phenol-lyase, L-tryptophan indole-lyase, phenylalanine ammonia-lyase, etc.), of L-aminoacids from optically inactive precursors, for example, of L-tryptophan and L-tyrosine from ammonia, pyruvate, indole, or phenol[6]. In the present review we shall not discuss the possibility of the employment of this approach to the synthesis of aminoacids, since it has already been considered in detail[7,8].

The second possibility of employing individual enzymes consists in the enzymic conversion of the simplest racemic aminoacid derivatives into the optically pure L- and D-aminoacids. Such enantioselective enzymic reaction results in the formation of a single unreacted optical isomer and the other optical isomer which has undergone chemical modification. The resulting differences between chemical properties make it possible to separate them readily. The presence of two fairly reactive groups in the aminoacid molecule (the carboxy- and amino-groups), linked to the asymmetric carbon atom, permits a large number of similar enantioselective modifications of the aminoacid molecule by various enzymes.

The first attempts at the employment of living organisms for the separation of aminoacid racemates were based on the ability of animals to assimilar only one optical isomer. When an animal was fed or administered a racemic aminoacid, the D-enantiomer, which had not been assimilated, could be isolated from its urine[9,10]. Despite its evident disadvantages (the permanent loss of half of the starting material, the difficulty of isolating the product from a mixture containing a whole series of all possible organic compounds, and the consequent formation of the aminoacid D-enantiomer which is usually physiologically less active and hence less valuable), this method has still not lost its importance. Thus a patent was taken out in 1976 in Japan for the preparation of D-aminoacids and their esters by treating the racemic aminoacid with the cells of the micro-organisms *Mycoplasma*, *Protaminobacter*, *Acetobacter*, *Pseudomonas*, *Aeromonas*, *Xanthomonas*, and *Bacillus*[11]. As a result of this procedure,

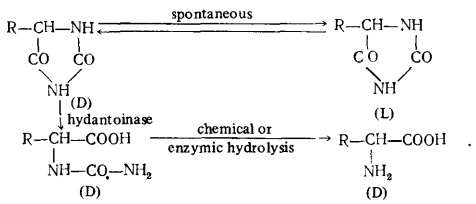
the L-isomer was assimilated by the bacteria, while the D-isomer was isolated from the culture medium after separating the cells by centrifugation.

A much greater success was achieved by employing separate enzyme preparations purified to different extents. In this case only one chemical reaction takes place and it is possible to isolate both aminoacid enantiomers. At the present time all enzymic methods for the resolution of aminoacid racemates can be divided into the following groups, which are listed in order of their increasing practical value and the extent to which they are used: (1) specific methods for the resolution of racemates suitable only for individual aminoacids (lysine, cysteine, and aromatic aminoacids); (2) stereoselective oxidation and decarboxylation of aminoacids; (3) enantioselective synthesis of the amide bond; (4) stereoselective hydrolysis of aminoacid amides and esters; (5) enantioselective hydrolysis of *N*-acylated aminoacids.

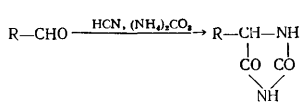
II. ENZYMIC CONVERSION OF CYCLIC AMINOACID DERIVATIVES

1. 5-Substituted Hydantoins

When enzymes are used for the resolution of aminoacid racemates into optical isomers, the problem of the utilisation of the enantiomer which has not reacted arises. It is usually solved by its chemical racemisation and the return of the resulting racemate to the resolution process. However, in the case of hydantoin derivatives of aminoacids the racemisation takes place spontaneously at fairly high rates even under the conditions of enzymic enantioselective hydrolysis. Thus the half-racemisation time of 5-phenylhydantoin at pH 8.5 and 30 °C in 0.25 M solution is 19 min[12]. This made it possible to put forward the following scheme for the enzymic resolution of aminoacid racemates using hydantoinase (dihydropyrimidinase, EC 3.5.2.2) which hydrolyses 5-substituted D-hydantoins stereoselectively:



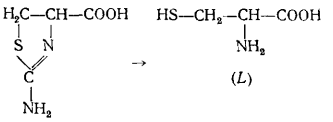
Although the formation of *N*-carbamoylaminoacids from hydantoins in the organism was observed in 1926 [13] and the possibility of the enzymic resolution of racemates was demonstrated as early as 1930, [14] the preparation of optically active aminoacids by this procedure has acquired practical importance only recently. 5-Substituted DL-hydantoins can be synthesised in a single stage from the corresponding aldehydes by the Bucherer reaction[15]:



Calf liver dihydropyrimidinase cleaves the ring of dihydropyrimidines with formation of *N*-carbamoyl-β-aminoacids[16] and is identical with the enzyme which converts hydantoin into *N*-carbamoylglycine[17]. It is of interest to note that the opening of the dihydropyrimidine or hydantoin ring is a reversible process and that the reverse reaction can be achieved under acid conditions[16,18]. The bovine liver hydantoinase catalyses the enantioselective hydrolysis of D-hydantoin derivatives of α-aminophenylacetic α-amino-*p*-chlorophenylacetic acids, leucine, alanine, methionine, and valine. The D-derivatives of glutamic and aspartic acids and tryptophan are resistant to the action of the enzyme[12,19]. Hydantoinase has been found in a whole series of microorganisms: *Klebsiella*, *Corynebacterium*, *Agrobacterium*, *Pseudomonas*, and *Bacillus*[20]. The strain *Pseudomonas striata* IFO 12 996 has proved most suitable for practical applications. The hydantoinase is induced by DL-5-methylhydantoin (a hydantoin derivative of alanine). Other hydantoins are inert or actually suppress the biosynthesis of the enzyme[21]. The hydantoinase from *Ps. striata* was purified to a homogeneous state and was obtained in a crystalline form. Its molecular weight is 190 000 and it consists of four identical subunits[22]. Tables 1 and 2 indicate the substrate specificity of the hydantoinase from *Ps. striata*. 5,5-Disubstituted hydantoins are not cleaved by the enzyme. The hydantoinase from *Corynebacterium sepedonicum* and *Aerobacter cloacae* differs somewhat in its substrate specificity from the enzyme obtained from *Ps. striata*[23]. Thus the best substrate for the latter is DL-5-*m*-chlorophenylhydantoin, while for the former the best substrate is DL-5-*p*-chlorophenylhydantoin. A hydantoinase which effectively hydrolyses the hydantoin derivatives of various substituted 5-phenylhydantoins, particularly 5-thienylhydantoin, has been isolated from the micro-organisms *Bacillus sp.* 121-3.[24,25] The strains of the soil micro-organisms T-524, T-639, and especially T-523 effectively convert 4,5-indolylmethylhydantoin into tryptophan, exhibiting not only the hydantoinase but also the N-carbamoylhydrolase activity. The optical purity of the L-tryptophan obtained in this way from the racemic hydantoin reaches 99.1%.[26] L-Glutamic acid can be obtained from DL-5-β-carboxyethylhydantoin with the acid of the micro-organism *Bacillus brevis* ATCC 8185. When it is cultured in a weakly alkaline medium containing 1.0% of DL-5-β-carboxyethylhydantoin, it is possible to convert 90% of the racemate into L-glutamic acid. The enzyme system responsible for this reaction is induced by the addition of the initial substrate to the medium[27]. A reactor capable of processing up to 3 kg of hydantoin per day has been devised on the basis of calf liver hydantoinase incorporated in triacetate fibres. The half-life of the catalyst at pH 8.5 at 30 °C was 20 days[12].

2. 2-Amino-Δ^2-thiazoline-4-carboxylic Acid

L-Cysteine can be obtained from DL-2-amino-Δ^2-thiazoline-4-carboxylic acid using *Pseudomonas* cells[28]:



The reaction yield is close to 100% because the hydrolysis of the thiazoline ring of the L-isomer is accompanied by the racemisation of the D-enantiomer of the substrate in the reaction mixture. The strain *Pseudomonas thiazolinophylum* AI3854 showed the highest activity among the 463 strains of the 37 species tested. The enzyme (or enzyme system) responsible for the conversion is an induced intracellular enzyme closely associated with cell growth. Further degradation of cysteine (or cystine) can be prevented by introducing hydroxylamine or semicarbazide into the reaction medium and inhibiting thereby the pyridoxal 5'-phosphate-dependent cysteine desulphhydrase. The addition of Mn^{2+} (5 mM) and Fe^{2+} (25 mM) ions increases the overall activity of the enzyme

system by a factor of 5–7 and in addition the presence of Mn^{2+} ions at a concentration of 1–10 mM has a stabilising effect on the activity. When intact cells of *P. thiazolinophylum* AI3854 were used, a sigmoid time variation of the accumulation of L-cysteine was observed, but treatment of the cells with ultrasound eliminated this effect and led to a linear relation[29].

Table 1. The substrate specificity of the hydantoinase from *Ps. striata*[20].

D-substrate 

R	Relative rate of hydrolysis*	R	Relative rate of hydrolysis*
H	13	*p*-HO—C_6H_4	16
CH_3	45	*m*-HO—C_6H_4	5
$(CH_3)_2CH$	15	*p*-Cl—C_6H_4	19
CH_3—S—CH_2—CH_2	48	*m*-Cl—C_6H_4	10
$(CH_3)_2CH$—CH_2	48	*p*-CH_3O—C_6H_4	7
C_6H_5	25		

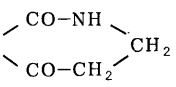
*The rate of cleavage of dihydrouracil  was adopted as 100.

Table 2. Kinetic properties of the hydantoinase from *Ps. striata*[21].

Substrate	K_M^*, mM	pH optimum of activity
Dihydrouracil	1.7	8.8—9.0
DL-5(2-Methylthioethyl)hydantoin	28	8.3—8.5
DL-5-Phenylhydantoin	50	8.0—8.3
Hydantoin	280	8.0—8.3

*K_M = Michaelis constant.

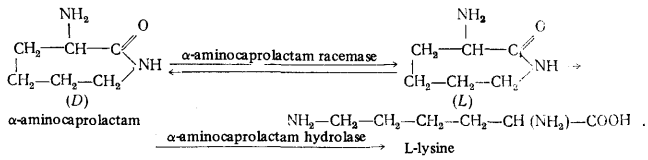
Table 3. The equilibrium constants of the hydrolysis of *N*-acetyl derivatives of aminoacids (25 °C; pH 7.5).[126]

Aminoacid	K_{eq}, hydrolysis*	K_{eq}, synthesis*
Glycine	4.0±1.5	5±1
Alanine	5.4±0.5	5.9±0.7
Aminobutyric acid	5.1±0.6	6±1
Norvaline	11±1	10±1
Norleucine	13±2	12±2

*Path followed by equilibration.

3. α-Aminocaprolactam

A very promising method of preparation of L-lysine from DL-α-aminocaprolactam (DL-ACL), proposed by the Japanese company Torrei Industries[30,31], is based on the use of the L-aminocaprolactam hydrolase–α-aminocaprolactam racemase two-enzyme system:



Caprolactamase has been isolated from various micro-organisms: *Bacillus circulans, Bacillus firmas*[32], *Cryptococcus laurentii, Candida humicola, Trichosporon cutaneum, Achromobacter cycloclastes, Achromobacter obae, Flavobacterium arborescens*, and *Alcaligenes faecalis*[33,34]. The strains of *Cryptococcus laurentii, Candida humicola*, and *Trichosporon cutaneum*, whose properties have in fact been studied in detail, proved to be the most promising[35]. Caprolactamase was purified to a homogeneous state. The enzyme consists of two subunits with a molecular weight of 67 000, a sedimentation constant of 4.4 S, and an isoelectric point $pI = 5.7$.[36,37] Caprolactamase is a metalloenzyme and its activity is fully suppressed in the presence of 7 mM *o*-phenanthroline but can be restored in the presence of Mg^{2+}, Mn^{2+}, or Zn^{2+} ions (but not Co^{2+} and Ni^{2+}) up to 30–70% of the initial value[31,37]. At pH 8.5 and 37 °C the catalytic constant of the enzyme hydrolysis of L-α-aminocaprolactam is 750 s^{-1}.[31] The enzyme hydrolyses L-α-amino-δ-valerolactam three times less effectively and D-α-amino-ε-caprolactam twenty times less effectively than L-α-aminocaprolactam and does not exhibit activity in relation to γ-butyrolactam, δ-valerolactam, ε-caprolactam, cyclic oligomers of ε-caprolactam, α,α-dichloro-ε-caprolactam, and DL-pyrrolidinone carboxylic acid[38]. Caprolactamase has recently been found to show activity in relation to L-lysinamide which is hydrolysed by the enzyme twice less effectively than L-α-aminocaprolactam[36,37]. The Michaelis constant for the hydrolysis of L-α-aminocaprolactam is 2.6 mM and that for the hydrolysis of L-lysinamide is 14 mM.[37] At high substrate concentrations the enzyme is inhibited both by the substrate and by the L-lysine formed. The reaction can be described by the equation[39]

$$\frac{d[S]}{dt} = \frac{k_{cat}[E][S]}{\left(K_M + [S] + \frac{[S]^2}{K_S}\right)\left(1 + \frac{[P]}{K_i}\right)},$$

where E, S, and P are respectively caprolactamase, L-α-aminocaprolactam, and L-lysine, $K_M = 0.023$ M, the substrate inhibition constant $K_S = 1.54$ M, and the product inhibition constant $K_i = 3.23$ M.

By selecting micro-organisms capable of growing on media containing only L- or D-α-aminocaprolactam as the only source of nitrogen and the main source of carbon, micro-organisms producing α-amino-ε-caprolactam racemase were selected: *Achromobacter obae, Achromobacter cycloclastes, Alcaligenes faecalis*, and *Flavobacterium arborescens*. The *Achromobacter obae* strain showed the highest activity[30]. The racemase was isolated and partly purified. Its molecular weight was 54 000 and the pH optimum of its activity was 7.5–9.0. The activity of the racemase is fully inhibited by hydroxylamine (10^{-4} M), but is restored following the addition of pyridoxal 5′-phosphate. At higher concentrations (>10^{-4} M) pyridoxal 5′-phosphate is a racemisation inhibitor[40]. Substrate inhibition is characteristic of the racemisation reaction catalysed by lyophilised cells. In addition L-lysine, inevitably present when the hydrolysis of L-α-aminocaprolactam and the racemisation of D-α-aminocaprolactam are carried out in a single reactor, is a powerful non-competitive inhibitor of this reaction. In the real process one must take into account also the inactivation of the racemase in the course of the reaction[39].

The optimum process pH depends on the ratio of the catalytic amounts of racemase and caprolactamase and shifts towards the pH optimum of the enzyme limiting the rate of the entire process. For a periodic reactor, in which the enzyme ratio can be varied during the process, the principal characteristic is the ratio

$$f = \frac{[\text{racemase}]}{[\text{racemase}] + [\text{caprolactamse}]} \quad (0 \leqslant f \leqslant 1) \ .$$

If the concentration is expressed in mole fractions, and k_1 and k_2 are the rate constants for the racemisation and hydrolytic reactions, then the composition of the reaction medium as a function of time can be described by the following system of equations:

$$\frac{d[D\text{-ACL}]}{dt} = fk_1([L\text{-ACL}] - [D\text{-ACL}]);$$

$$\frac{d[L\text{-ACL}]}{dt} = fk_1([D\text{-ACL}] - [L\text{-ACL}]) - (1-f)k_2[L\text{-ACL}];$$

$$[L\text{-ACL}]_0 = [D\text{-ACL}]_0 = 0.5 \ .$$

Initially the optimum condition is that the processes occur at $f = 0$, i.e. when the racemase is absent. Subsequently the optimum condition involves the occurrence of the reaction when [D-ACL]/[L-ACL] = 1 + α, where $\alpha = (k_2/k_1)^{1/2}$. Under these conditions, the optimum enzyme ratio is defined by the expression

$$f = \frac{\alpha(1+\alpha)}{1+(1+\alpha)^2} \ .$$

The optimum composition of the biocatalyst depends only on the ratio α. If the latter remains constant during the reaction, the process can be carried out by the optimum procedure[39].

III. ENANTIOSELECTIVE OXIDATION AND DECARBOXYLATION

Strictly speaking the enantioselective oxidation and decarboxylation of aminoacids does not constitute the resolution of aminoacid racemates because such reactions lead to the loss of half of the starting material. This is the reason why these methods have not found extensive application in the preparation of aminoacid enantiomers from their racemates.

As early as the beginning of this century, it was possible to obtain 60–70% yields of the D-isomers of alanine, leucine, valine[41], norvaline, serine, phenylalanine[42], alloisoleucine, glutamic acid, and histidine[43] by treating the aminoacid racemates with yeast, whose action was due to its decarboxylases. Later this method was employed to obtain the D-isomers (of methionine with the aid of *Proteus vulgaris*[44] and of glutamic acid[45] and lysine[46] with the aid of *E.coli* and *Bacillus cadaveris* respectively). On the other hand, the monoaminodicarboxylic acid decarboxylase, specific exclusively to the D-enantiomers and isolated from *Aspergillus ustus*, makes it possible to obtain their L-enantiomers[47].

The stereoselective oxidation of aminoacids by means of the D-aminoacid oxidase from mammalian kidneys and the L-aminoacid oxidase from snake venom has found a somewhat more extensive application:

$$\text{aminoacid racemate} + O_2 \xrightarrow{\text{oxidase}} \text{unreacted optical isomer} + \alpha\text{-ketoacid} + H_2O_2 + NH_3.$$

The L-enantiomers of alanine, methionine[48], and proline[49] were obtained by treating their racemates with D-aminoacid oxidase.

The possibility of obtaining aminoacid D-enantiomers by treating the racemates with snake venom L-aminoacid oxidase is limited by the specificity of the enzyme. Thus L-threonine, L-proline, L-alanine, L-lysine, and L-ornithine are resistant to the action of the oxidase from *Crotalus adamentus*[50,51]. However, L-alanine is readily oxidised by the enzyme from the venom of *V. libetina*[52] and L-lysine is oxidised by the enzyme from the venom of *Bothrops atrox*[53]. The acylation of the ω-amino-groups of L-lysine, L-ornithine, and L-citrulline also renders them susceptible to oxidation by the enzyme from the venom of *Crotalus adamentus*[50,51]. The reaction products do not inhibit L-aminoacid oxidase, but the α-ketoacids obtained in the oxidation of aromatic aminoacids inhibit D-aminoacid oxidase. The addition of cyanide to the reaction mixture inhibits the catalase usually present as an admixture in preparations of D-aminoacid oxidase. The hydrogen peroxide formed during the reaction under these conditions is no longer decomposed by catalase and is consumed in the oxidation of the α-ketoacid to its nearest lower aliphatic acid homologues, which are much weaker inhibitors of the D-aminoacid oxidase[53].

The ease with which the reaction can be monitored from the evolution of ammonia or the peroxide and also the exceptionally low affinity of oxidases for the uncleaved isomer makes it possible to employ stereoselective oxidation as a method for the analysis of the optical purity of aminoacid stereoisomers. By means of this procedure, it is possible to determine 0.1–0.01% of the admixture of the other isomer[54,55].

The resolution of racemates by stereoselective oxidation and also by stereoselective decarboxylation has not found extensive application, firstly owing to the loss of half of the starting material in the reaction and, secondly, owing to the high cost of snake venom—the source of L-aminoacid oxidase.

IV. ENZYMIC SYNTHESIS OF THE AMIDE BOND

An interesting method for the resolution of aminoacid racemates is the enantioselective synthesis of the amide linkage catalysed by various proteolytic enzymes. It is well known that the hydrolysis of the amide linkage formed by *N*-acylated aminoacid derivatives proceeds until the attainment of equilibrium and under certain conditions the reverse synthetic reaction can be achieved[56–58]. If aniline or phenylhydrazine is used as the amino-group donor for the synthesis of the amide linkage, then the anilides or phenylhydrazides of the *N*-acylated aminoacids, which are sparingly soluble in water and crystallise from the reaction mixture, are formed as a rule. This shifts the equilibrium in the direction of synthesis and makes it possible to carry out the stereoselective synthesis almost quantitatively. Attention was drawn to the enantioselectivity of such synthesis, catalysed by papain, for the first time in a study of the synthesis of acetyl-DL-phenylalanylglycine anilide[58].

A similar method was used to resolve the racemate in benzyloxycarbonyl-DL-glutamic acid[59] and the diastereoisomer of acetyl-DL-phenylalanyl-L-leucine[60]. Later the method was improved[61] and it was possible to resolve by this procedure the racemates of alanine, leucine, isoleucine, norleucine, valine, lysine, serine, threonine, and phenylalanine.

For the synthesis reaction of the anilide to go almost to completion, the *N*-acylaminoacid must be readily soluble and the corresponding anilide must be sparingly soluble. The choice of a suitable *N*-acyl group on the grounds of solubility can entail in its turn difficulties associated with the stereospecificity of the enzyme employed[60]. This question was investigated in greater detail in relation to the papain-catalysed stereoselective synthesis of the phenylhydrazides of *N*-acylated aminoacids[62–64]. The replacement of aniline by phenylhydrazine is dictated by the fact that the phenylhydrazide group can be readily removed by mild oxidation.

The product synthesised was found to have almost fully the *L*-configuration if the acyl group was acetyl or benzoyl. If the acyl group consisted of methoxycarbonyl, ethoxycarbonyl, or benzyloxycarbonyl residues, then a considerable amount of the D-hydrazide, almost comparable to the amount of the L-hydrazide, was formed. This phenomenon was observed regardless of the nature of the aminoacid residue, although the rate of formation of the product is influenced by the nature of both the *N*-acyl group and of the slight change of the aminoacid itself[65-67]. Unfortunately quantitative conclusions as regards this system are hindered by the continuous inactivation of papain by phenylhydrazine[63].

Despite a number of evident advantages, namely the simplicity of the enantioselective synthesis of the anilide or phenylhydrazide and the exceptionally convenient separation of the unreacted D-isomer, this method has not found extensive applications for the resolution of aminoacid racemates into optical isomers and its possible comparative use has been pointed out in only isolated studies[68]. This is associated, firstly, with the difficulty of choosing a suitable acyl derivative of the aminoacid satisfying all the essential requirements, secondly, with the necessity for a careful selection and constant monitoring of the conditions in the resolution process, and, thirdly, with the fact that the starting materials and the reaction products are not aminoacids themselves but fairly complex derivatives of the latter. This necessitates additional synthetic stages followed by the hydrolysis of these compounds. Since the anilides of the acylated aminoacids are fairly stable, refluxing for many hours in 6 M HCl must be used for the preparation of the free aminoacid[59]. This is why, even if the L-compound does not contain initially admixtures of the other isomers, such treatment can lead to partial racemisation[66,69] and in the case of certain aminoacids, for example, methionine[70] and tryptophan[71], to chemical degradation.

Despite the fact that the stereoselective synthesis catalysed by various proteolytic enzymes is hardly used for the resolution of aminoacid racemates into optical isomers, reactions of this type are being vigorously investigated. They have already found applications in the stereoselective synthesis of various *N*-acylated oligopeptides. Apart from papain, the synthesis of the amide linkage can be catalysed by α-chymotrypsin[72], nagarase, pepsin, and thermolysin[73]. The employment of proteolytic enzymes with different specificities makes it possible to incorporate various aminoacids in the oligopeptides synthesised. The kinetics of both the direct synthesis of the amide linkage, for example, the papain-catalysed synthesis of hippurylanilide from aniline and hyppuric acid[74] as well as the formation of the dipeptide from *N*-benzoyl-L-tyrosine and glycineanilide catalysed by α-chymotrypsin[75], and the "synthesis with transfer", where instead of the *N*-acylated aminoacid one uses its more reactive derivative, usually the ester, as the carboxyl donor for the formation of the amide linkage, have been studied in detail. Proteolytic enzymes such as trypsin[76] and α-chymotrypsin[77] are catalysts of reactions of this kind. Since the hydrolysis of amide and ester substrates of these enzymes proceeds via the formation of the acyl-enzyme, it follows that, in accordance with the principle of microreversibility, the synthesis should also proceed via these intermediate states. The use of aminoacid esters as donors of the carboxy-group facilitates the acylation stage compared with the acylation of the free aminoacid, which accelerates the entire process, because the deacylation stage is not rate-limiting[78], and makes it possible to use aminoacid esters with a free amino-group as donors of the carboxy-group[79]. Furthermore, for a specific ratio of the kinetic parameters of the hydrolytic and synthetic reactions, it is possible to achieve yields exceeds the thermodynamic values in "synthesis with transfer"[80,81].

The study of the equilibrium constants of the hydrolytic-synthetic reactions of the amide or peptide linkage[82-85] permitted the conclusion that the free energy of the synthesis, calculated in relation to the unionised forms of the starting materials and reaction products, is as a rule negative, i.e. the equilibrium is displaced towards synthesis. However, when the reactions are carried out in aqueous solutions, the vast majority of one or both starting materials exist in the ionised form (since the p*K* of the carboxy-groups of such compounds are in the range 2–3.5 and the p*K* of the amino-groups are in the range 6–9.5) and the synthesis of the amide and peptide linkages is no longer such a thermodynamically favourable process[56,57].

Various methods whereby the reaction equilibrium can be displaced towards the required product have been vigorously developed recently[86-88]. Such methods are based on the alteration of the ionisation constants of the reactants and the decrease of the concentration of water in the system, the employment of two-phase "water–water-immiscible organic solvent" systems, or the creation of conditions for a kinetically controlled equilibrium as a result of the immobilisation of the enzyme on a charged carrier. Admittedly this entails an appreciable decrease of the rate of synthesis and additional components, hindering the isolation of the required product, are introduced into the system.

Summarising, one may say that, although the stereoselective method of synthesis of amides, hydrazides, or peptides under the influence of proteolytic enzymes has not found applications as a procedure for the resolution of aminoacid racemates into optical isomers, the study of such reactions has made it possible to understand many features of the thermodynamics of the important reaction involving the synthesis of the peptide linkage and provided synthetic chemists with a convenient method whereby one can synthesise various oligopeptides stereoselectively under mild conditions and with a high yield[89-91].

V. ENANTIOSELECTIVE HYDROLYSIS OF AMINOACID ESTERS AND AMIDES

The stereoselective hydrolysis of aminoacid esters was first used for the resolution of racemates into optical isomers in 1906,[92] i.e. it was contemporary with the classical diastereoisomeric crystallisation methods. A common disadvantage of the method described was the long time involved and their laborious nature, since the employment of low-purity enzyme preparations required much time for the hydrolytic reaction and led to contamination by products of side reactions.

Despite these difficulties, tryptophan[93-97], phenylalanine[98-100], norleucine[100], methionine[94], valine[98,99], and a number of benzene ring-substituted phenylalanine[98,99,101,102] racemates have been resolved by the stereoselective hydrolysis of their esters under the influence of pancreatic enzymes.

The studies discussed above were extremely empirical. The isolation of proteolytic pancreatic enzymes in a pure form, the discovery and isolation of numerous proteases from various micro-organisms, and the development of a convenient method whereby the kinetics of reactions of this kind can be followed (pH-stat measurements) renewed the interest in the stereoselective hydrolysis of esters as a method for the resolution of aminoacid racemates into optical isomers.

1. Characteristics of the Action of α-Chymotrypsin on Aminoacid Esters

Despite the fact that α-chymotrypsin is one of the most thoroughly investigated enzymes[103], the mechanism of the hydrolysis by the latter of aminoacid esters has not been

ultimately elucidated. As for the esters of *N*-acylated aminoacids, the formation of an intermediate acyl-enzyme has been postulated in all the investigations for esters with a free amino-group, but the question which stage, acylation or deacylation, is rate-limiting still remains open[104–109].

The hypothesis that in the hydrolysis of aminoacid esters by α-chymotrypsin the reactive form of the Michaelis complex is a complex of the enzyme with the substrate protonated in the amino-group can now be regarded as having been convincingly demonstrated[104,105,109–112]. It is supported by the higher reactivity in hydrolytic reactions of the forms of aminoacid esters protonated in the amino-group[113] and also by the possibility of the formation of a hydrogen bond between the NH_3^+ group and the carbonyl group of Ser[214,112]. The formation of a hydrogen bond in the Michaelis complex would ensure a more favourable binding of the substrate and, in accordance with the "better binding–better catalysis" principle[103], might occur in the catalytic stage of the chymotrypsin catalysis. The results of the crystallographic study of α-chymotrypsin indicate the presence in the active centre of the enzyme, alongside the hydrophobic section, of the aspartic acid residue Asp[194], participating in the formation of a salt bridge, which is necessary for the maintenance of the catalytically active tertiary structure[114,115] and is capable of electrostatic interaction with the protonated amino-group of the substrate. The mechanism of the enzymic (chymotrypsin) hydrolysis of aminoacid esters with a free amino-group, proposed on the basis of these considerations, describes satisfactorily the pH dependence of all the kinetic parameters[109].

A characteristic property of α-chymotrypsin is its high stereospecificity with respect to the L-isomers of aminoacid esters with a free amino-group, which makes it possible to use this reaction for the separation of aminoacid racemates. It is of interest to examine factors determining the high stereospecificity of α-chymotrypsin. The idea that the stereospecificity is determined by the structure of the substrate and is not an invariant property of the enzyme has been confirmed in the study of the hydrolysis of the *p*-nitrophenyl esters of *N*-acetylated aminoacids[116,117]. As the side chains of the substrate becomes more hydrophobic, the deacylation of the L-acyl-enzymes is accelerated, while that of the corresponding D-acyl-enzymes is retarded.

According to the view which has become firmly established at the present time[103], the driving force of the chymotrypsin catalysis is the hydrophobic interaction between the side chain (R^2) of the substrate having the general formula $R^2–CH(NHR^1)–COR^3$ and the groups located in the hydrophobic region of the active centre of the enzyme. The "better binding–better catalysis" rule is then observed. However, it is applicable only to the variation of R^2. It has been shown[118] that, when the *N*-acyl group in ester derivatives of L-aniline is altered, the "better binding–less effective reaction" relation is observed. In order to interpret this effect, the hypothesis of the "unproductive binding" of the substrate molecule by the enzyme was put forward. The binding of the molecules of the L- and D-substrates can be illustrated schematically on the basis of the model involving the three-point binding of the substrate to the active centre of α-chymotrypsin (Fig. 1). Evidently in the case of the L-substrate the "correct" binding is at the same time also productive. Therefore the enhancement of the hydrophobic properties of the aminoacid side chain (the group R^2) leads to an increase of the efficiency of the catalysis (Fig. 1*a*). The unproductive binding can be explained in this instance by the fact that the *N*-acyl group R^1, which is fairly hydrophobic, can itself occupy a site in the hydrophobic section of the active centre and the carbonyl group attached to the bond undergoing dissociation in the substrate will not be correctly oriented in relation to the general nucleophile of the active centre (Fig. 1*b*).

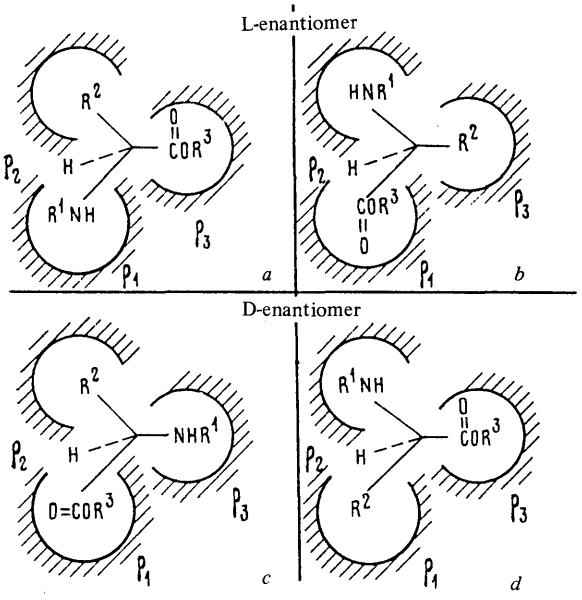


Figure 1. Different versions of the three-point binding of the substrate molecule to the active centre of α-chymotrypsin; ρ_1 = hydrogen bond acceptor; ρ_2 = hydrophobic region of the active centre; ρ_3 = generalised nucleophile of the active centre.

The hydrolysis of D-aminoacid derivatives takes place in exactly the opposite manner: the "correct" binding of the substrate, i.e. binding such that the group R^2 is in the hydrophobic section of the active centre, leads to an unproductive enzyme–substrate complex (Fig. 1*c*), since the formation of the productive complex is in this case sterically hindered and requires that the *N*-acylamide group occupy the site provided in the active centre for the hydrogen centre[119]. On the other hand, the "incorrect" orientation of the *N*-acylamide group in the hydrophobic region ρ_2 is in fact productive (Fig. 1*d*). It is clear from the foregoing that the enhancement of the hydrophobic properties of the aminoacid group R^2 entails an increase of the stereospecificity of the enzyme, while the enhancement of the hydrophobic properties of the *N*-acyl group R^1 should actually reduce it. This conclusion has been confirmed experimentally[120,121].

For the resolution of aminoacid racemates, it is important to know how the stereospecificity of the action of α-chymotrypsin changes on passing to substrates with a free amino-group. It was found that the character of the influence of the hydrophobic properties of the group R^2 on the stereospecificity of the chymotrypsin hydrolysis of the methyl esters of aminoacids is the same for substrates with free and acetylated amino-groups, but the ratio of the second-order rate constants for the enzyme hydrolysis of L- and D-substrates is approximately 10 times greater for acetylated aminoacids than for aminoacids with a free amino-group. The

D-substrate having a free amino-group is hydrolysed by α-chymotrypsin faster than its acylated analogue[112].

2. The Problem of the Spontaneous Hydrolysis of α-Aminoacid Esters

In the stereoselective hydrolysis of aminoacid esters by proteolytic enzymes the spontaneous (non-enzymic) hydrolysis of the aminoacid esters is one of the concomitant processes interfering with the effective resolution of their racemic mixture. It is therefore necessary to seek optimum conditions which correspond both to relatively low rates of spontaneous hydrolysis and to an adequate activity of the biocatalyst employed. The most sensitive parameters of such optimisation are the pH of the solution and the reaction temperature. It is therefore very urgently necessary to compare the pH profile of the catalytic activity of the enzyme and the pH dependence of the rate of spontaneous hydrolysis.

Until recently, the hydrolysis of aminoacid esters has been studied only over a narrow range of alkaline pH (9–12), which did not allow the estimation of the rate of the process in neutral and weakly acid media[122–125]. The following kinetic mechanism has been proposed on the basis of a detailed study of the pH dependence of the hydrolysis of the ethyl ester of DL-tryptophan in the pH range 4.5–10.5:[113]

$$\left.\begin{array}{l}\xleftarrow{k_5[OH^-]}\\ \xleftarrow{k_3[H_2O]}\end{array}\right| -EH^+ \overset{K_E}{\rightleftharpoons} E- \left|\begin{array}{l}\xrightarrow{k_1[H^+]}\ ,\\ \xrightarrow{k_2[H_2O]}\ ,\\ \xrightarrow{k_4[OH^-]}\ ,\end{array}\right. \qquad (1)$$

together with the corresponding equation for the rate of hydrolysis:

$$V = k_1[E][H^+] + k_2[E][H_2O] + k_3[EH^+][H_2O] + k_4[E][OH^-] + k_5[EH^+][OH^-] = k_{abs}[E]_{tot},$$

where EH^+ and E are the form of the aminoacid ester protonated in the amino-group and the non-protonated form and k_1, k_2, k_3, k_4, and k_5 are the rate constants for the acid, neutral, and alkaline hydrolysis. The possibility of the acid hydrolysis of the protonated form of the substrate, which is unlikely in view of the electrostatic repulsion of the interacting species, is not reflected in mechanism (1). Analysis of the pH dependence of k_{obs} obtained and the literature data for the hydrolysis of *N*-acylated aminoacid esters made it possible to find or at least to estimate all the kinetic parameters of mechanism (1):

$$k_1 \cong 10^{-5} \text{ litre mol}^{-1}\text{s}^{-1},\ k_2 \cong 10^{-7} \text{ litre mol}^{-1}\text{s}^{-1},\ k_3 = 2.7 \times 10^{-7} \text{ litre mol}^{-1}\text{s}^{-1},$$
$$k_4 = 1.2 \text{ litre mol}^{-1}\text{s}^{-1},\ k_5 = 79 \text{ litre mol}^{-1}\text{s}^{-1}.$$

The pH dependence of the hydrolysis of the ethyl ester of DL-tryptophan permits certain practically important solutions. Thus, in order to diminish the contribution of the spontaneous hydrolysis as a side process in the enzymic resolution of aminoacid racemates as a result of the stereoselective hydrolysis of their esters, it is necessary to employ proteolytic enzymes with a pH optimum of the catalytic activity in the region of acid and neutral pH (neutral and acid proteases). The possibility of employing alkaline proteases is to a large extent limited, because in the region of the alkaline pH the rate of spontaneous hydrolysis is so high that it is difficult to avoid the contamination of the final product even when high enzyme concentrations are used and the process is carried out over a short time interval.

In the study of the possibility of employing a particular proteolytic enzyme for the resolution of aminoacid racemates by the enantioselective hydrolysis of aminoacid esters, it is important to know the characteristics of the reaction at substrate concentrations close to saturation, i.e. under the conditions of the preparative resolution of aminoacid racemates. It has been shown[126,127] that the full kinetic curves for the enantioselective hydrolysis of the ethyl ester of DL-tryptophan, catalysed by α-chymotrypsin, are described by the mechanism

$$E\cdot D\text{-}S \overset{K_i'}{\rightleftharpoons} D\text{-}S + E + L\text{-}S \overset{K_S}{\rightleftharpoons} E\cdot L\text{-}S \xrightarrow{k_2} EA \xrightarrow{k_3} E + P,$$
$$P \quad (K_i \text{ with } E\cdot P), \qquad P'$$

where *E* is α-chymotrypsin, L-S and D-S are the ethyl esters of L- and D-tryptophan respectively, P is L-tryptophan, P' is ethanol, $K_i' = (1.8 \pm 0.3) \times 10^{-3}$ mol litre^{-1} is the constant of the competitive inhibition by the ethyl ester of D-tryptophan, $K_i = (25 \pm 5) \times 10^{-3}$ mol litre^{-1} is the constant of the competitive inhibition by L-tryptophan, $k_{cat} = k_2k_3/(k_2 + k_3) = 100 \pm 10$ s^{-1} is the catalytic constant, and $K_M = K_Sk_3/(k_2 + k_3) = (2.4 \pm 0.1) \times 10^{-3}$ M is the Michaelis constant (all the constants were determined at 37 °C and pH 6.3).

Summarising the discussion of α-chymotrypsin as a potential catalyst of the enantioselective hydrolysis of aromatic acid esters, one may make the following conclusions: firstly, α-chymotrypsin is highly stereospecific. Secondly, the pH optimum of the catalytic efficiency of the action of α-chymotrypsin on aminoacid esters with a free amino group is in the pH range 6.5–7.0, where the non-enzymic hydrolysis of the substrate proceeds to only an insignificant extent; the rates of the enzymic hydrolysis of the L-isomer of the substrate, of the enzymic hydrolysis of the D-isomer, and of the spontaneous hydrolysis of the racemate at the pH optimum of the α-chymotrypsin activity are approximately 10 000 : 1 : : 0.0001, which leads to a very insignificant contamination of the final product by the other isomer. Thirdly, the optimum pH range from the standpoint of the relative contributions of the enzymic and non-enzymic hydrolytic reactions is in fact also most convenient for the isolation of the reaction product from the reaction mixture, because in this particular pH range aminoacids are least soluble and the substrate hydrolysed is fairly soluble. Fourthly, L-tryptophan and ethanol, formed in the enzymic reaction, have an insignificant influence on the process kinetics, which makes it possible to achieve high degrees of conversion of the substrate.

Thus α-chymotrypsin is an exceptionally convenient catalyst for the resolution of aromatic aminoacid racemates by the hydrolysis of their esters. However, a significant disadvantage of α-chymotrypsin is its animal origin and hence its relatively high cost. A possible way of overcoming this disadvantage may be the use of immobilised preparations of the enzyme with a fairly high stability, which would make possible their repeated employment in the process. The immobilisation of α-chymotrypsin has been thoroughly investigated[6] and there is no doubt that active preparations of the immobilised enzyme with a high stability and effective mechanical properties necessary for practical applications can be obtained. Another solution of the problem is to seek proteolytic enzymes of microbial origin with properties close to those of α-chymotrypsin.

3. The Stereospecificity of Proteolytic Enzymes

Bacterial proteases have been studied only recently from the standpoint of their suitability for the resolution of aminoacid racemates[126–129]. Unfortunately, all the enzymes or

enzyme complexes of bacterial origin investigated are appreciably inferior to their activities and, what is most important, in their enantioselectivity to α-chymotrypsin. The proteases from *Bacillus subtilis* and the extracellular protease from *Actinomyces* are the most suitable for the stereoselective hydrolysis of aminoacid esters. The catalytic constants for the hydrolysis of the ethyl ester L-tryptophan at the pH optimum are 6.5 ± 0.5 and 3.2 ± 0.4 s^{-1} for these enzymes and their enantioselectivities, i.e. the ratios of the catalytic constants for the enzyme hydrolysis of the L- and D-isomers of the ethyl ester of tryptophan, are 200 and 150 respectively. For α-chymotrypsin, these quantities are 100 s^{-1} and 10 000.[126,129] Furthermore, the pH optimum of the catalytic activity of these substrates corresponds to a weakly alkaline region where the solubility of the substrate is low and the contribution of the non-enzymic hydrolysis increases.

The relatively low stereospecificity of the microbial proteolytic enzymes can be associated with the fact that contacts with the aminoacid residues, remote from the bond undergoing cleavage in the hydrolysis of oligopeptide and polypeptide substrates, are exceptionally important for such an enzyme in contrast to, say, α-chymotrypsin[130]. Thus the replacement of an acetylaminoacid amide by an oligopeptide, fully covering the active centre of the enzyme, entails an increase of the efficiency of the action of chymotrypsin by a factor of 4000,[131] of trypsin by a factor of 300,[132] and for the microbial enzymes elastase and the protease from *Streptomyces griseus*, the corresponding increase in the efficiency of hydrolysis is by a factor of 10^7.[130,133–135] Such increase of the efficiency of hydrolysis is apparently due to the stronger binding of the oligopeptide substrates compared with the acetyl aminoacid amide and hence to their more "accurate" orientation, which should lead to an increase of the stereospecificity of the action of these enzymes. However, it is entirely likely that, among the large number of microbial proteases, there is an enzyme corresponding to the highest requirements which must be met by the catalyst of the stereoselective hydrolysis of aminoacid esters for the resolution of the racemate into optical isomers, while the addition of metal ions will make it possible to increase the stereospecificity of bacterial proteases[128,136,137].

The racemates of acetyl-leucine[138], methionine, and α-aminophenylacetic acid[139] have been resolved into optical isomers by the enantioselective hydrolysis of their amides by proteolytic enzymes. However, it is important to note that the stereoselective hydrolysis of aminoacid amides has not found extensive applications owing to the low rates of hydrolysis of the amide substrates compared with the ester derivatives of aminoacids.

VI. THE USE OF ACYLAMIDOHYDROLASES FOR THE RESOLUTION OF RACEMATES

The stereoselective hydrolysis of *N*-acylated aminoacids has come to be most widely used nowadays among all the methods for the resolution of racemates. The application of this method was initiated in 1922, when it was observed that preparations of animal tissues catalyse the hydrolysis of *N*-benzoyl-L-aminoacids only[140]. Subsequently the possibility of the hydrolysis of not only benzoyl derivatives but also of other *N*-acylaminoacids was demonstrated[141–144]. Extensive practical application of the stereoselective hydrolysis of acylated aminoacids became possible only after a series of studies by Greenstein and co-workers who were able to resolve the racemates of virtually all natural and a number of synthetic aminoacids by employing a pig kidney homogenate or partly purified preparations of aminoacid acylase[145].

The enzyme catalysing the hydrolysis of *N*-acylated aminoacids has come to be called aminoacid acylase (EC 3.5.1.14). Aminoacid acylases belong to the group of enzymes hydrolysing non-peptide bonds (EC 3.5..). However, the similarity of the properties of the C–N bond in acylated aminoacids and peptides and also the necessity for a free carboxy-group for the manifestation of the acylase activity indicate that the acylase is related to a number of enzymes hydrolysing the peptide linkage (EC 3.4..), for example, carboxylpeptidases (EC 3.4.2), which split off the *C*-terminal aminoacid from peptides and are capable of hydrolysing *N*-acylated aminoacids with an aromatic side chain. Furthermore, aminoacid acylases exhibit a dipeptidase activity[146], which relates them to the group of dipeptidases (EC 3.4.3.). Such similarity of the substrate specificities of acylases and other enzymes sometimes leads to confusion. Thus in certain studies it has been suggested that the determination of the acylase activity in the liver be used for the diagnosis of virial hepatitis, although in fact this involves the monitoring of the hydrolysis of *N*-butyryl-γ-L-glutamyl-β-naphthylamide and the determination of the β-naphthylamine liberated in the reaction[147,148]. The activity determined is in fact due to an amidase (EC 3.5.1.4) and not an acylase.

Apart from acylase I, at least a further two enzymes capable of hydrolysing *N*-acylated aminoacids have been isolated from animal tissues. The first of these, catalysing the hydrolysis of acyl derivatives of aspartic acid, was called pig kidney acylase II (EC 3.5.1.15). The second enzyme, which hydrolyses *N*-acylated aromatic aminoacids (tyrosine, phenylalanine, tryptophan, and β-phenylserine) has been called acylase III.[149,150] Both these enzymes have been hardly investigated and the existence of acylase (III) has been altogether questioned and its activity has been attributed to carboxypeptidase. Only in recent years studies have been published confirming the presence of a special enzyme hydrolysing the *N*-acyl derivatives of aromatic aminoacids[151,152]. There are indications in the literature also of the presence of a special enzyme responsible for the hydrolysis of *N*-formylmethionine[153].

The biological role of acylases has not been ultimately elucidated. It has been suggested that they are involved in the catabolism of the *N*-acetylaminoacids formed in the hydrolysis of *N*-acetylated proteins[154,155].

1. The Pig Kidney Acylase

The pig kidney aminoacid acylase I has been isolated in a homogeneous state and its physical and chemical properties have been investigated[156–162]. It is a two-subunit enzyme with a molecular weight of 86 000, consisting of 772 aminoacid residues. Each enzyme subunit contains two SH groups, one of which is significantly more active with respect to various SH reagents. The modification of two SH groups per molecule of the enzyme leads to its complete inactivation. Each subunit contains two S–S bonds. The reduction of two S–S bonds per enzyme molecule also results in complete enzyme inactivation[158]. The acylase is likewise fully inactivated by the photochemical oxidation or chemical modification of four histidine residues[160] and two tryptophan residues[159] per enzyme molecule.

It has been recently reported that acylase I is a zinc-dependent metalloenzyme[161] containing one zinc atom per subunit.

Consideration of the substrate specificity of the pig kidney aminoacid acylase makes it possible to isolate two factors: (a) the specificity of the enzyme with respect to the type of aminoacid residue and (b) its specificity with respect to the nature of the acyl group.

Acylase I exhibits a fairly broad specificity and hydrolyses at a high rate the derivatives of the majority of aliphatic aminoacids[161,163]. The catalytic constant of the hydrolysis of acetyl-L-methionine at pH 7.0 and 37 °C is 330 s^{-1} (determined for an electrophoretically homogeneous enzyme)[157]. The Michaelis constants for the majority of acetyl derivatives of L-aminoacids amount to several millimoles. An increase of the chain length in the unbranched aminoacid residue entails an increase of the efficiency of the hydrolysis. If the results are expressed within the framework of the "structure–reactivity" correlation

$$\lg \frac{V_{max}}{K_M} = \alpha\pi + \text{const} \quad ,$$

where V_{max} is the maximum velocity of the enzymic reaction, K_M the Michaelis constant, π the Hansch hydrophobic constant for the substituent which is varied in the given series (π characterises the free energy of the transfer of the hydrocarbon residue from water to a standard hydrophobic phase—1-octanol, amounting to 0.51 for the CH_2 group in the unbranched substituent), and α the proportionality coefficient, it is found that the data obtained are described by a linear relation with a proportionality coefficient $\alpha \cong 0.7$. On passing from a linear aminoacid residue to a branched residue with the same number of carbon atoms (norvaline–valine, norleucine–leucine), the efficiency of the hydrolysis falls by a factor of 5–10. Thus the correlation obtained between the kinetic efficiency and the hydrophobic properties of the substrate shows that the simple "extraction" model of the utilisation of the substrate binding energy in the course of the enzymic reaction is valid for the pig kidney acylase (a detailed explanation of the model of the utilisation of the binding energy in the enzymic reaction has been given, for example, by Berezin and Martinek[103]). The hydrophobic section of the active centre of the acylase is a narrow "slit" whose length corresponds to at least four methylene units[163]. The results obtained agree with data for the inhibition of the acylase by free aminoacids[164] and with the finding that aromatic aminoacid derivatives are hydrolysed 2–3 times more slowly than methionine derivatives. The presence of electron-donating substituents in the aromatic ring of the aminoacid side chain results in a further decrease of the rate of hydrolysis[165]. Acyl derivatives of proline and hydroxyproline are resistant to the action of acylase I.[149] As regards the specificity of acylase I to the nature of the acyl component of the substrate, the enzyme hydrolyses different acyl-aminoacid derivatives. Among derivatives of unsubstituted aliphatic acids, acetylaminoacids are the best substrates[166]. The formyl and propionyl derivatives are hydrolysed less effectively than the acetyl derivatives. The appearance of electron-accepting substituents in the acyl component increases the rate of hydrolysis. The reactivity of the substrates increases in the sequence iodoacetyl < bromoacetyl < chloroacetyl < fluoroacetyl derivatives[149]. The benzoyl derivatives of aminoacids are poor substrates. The presence of electron-accepting substituents in the *para*-position in the benzoyl derivatives of aminoacids accelerates and the presence of electron-donating substituents retards the hydrolysis catalysed by acylase I.[165]

There are data showing that acylase I is capable of catalysing the hydrolysis of dipeptides[146,164,167], although the rate of hydrolysis is lower by 2–3 orders of magnitude compared with the chloroacetyl derivatives of aminoacids. It is of interest to note that the enzyme preparation investigated cleaved dipeptides with the L–L configuration but did not cleave those with the D–D and D–L configurations[146]. It was concluded that, for the manifestation of the catalytic activity of acylase I, it is essential that not only the C-terminal but also the *N*-terminal aminoacids should be in the L-configuration. At the same time, it is known that the acylase hydrolyses equally effectively both diastereoisomers of DL-α-chloropropionyl-L-alanine[167].

However, one should note that commercial preparations of acylase (I) without additional purification were used in the above investigations[146,167]. Presumably the observed activity in relation to dipeptides, which is lower by at least two orders of magnitude than the activity of acylase I in relation to *N*-acetyl-L-methionine, is a consequence of an admixture of another enzyme—a dipeptidase. Kördel and Schneider[164] used an electrophoretically homogeneous preparation of acylase (I), but in this case too the criterion of homogeneity employed can hardly guarantee the absence of less than 1% of an impurity protein. Thus the question of the dipeptidase activity of acylase (I) must be regarded as open.

It has been shown that the Michaelis constants for non-specific substrates (acetyl derivatives of aromatic aminoacids and derivatives of aliphatic aminoacids with a bulky acyl component) are approximately equal to the constants for the competitive inhibition of the hydrolysis of specific substrates in the presence of non-specific substrates[165]. If the three-stage mechanism of the enzymic reaction is valid for acylase I, then in this case such equality would imply that the rates of hydrolysis of non-specific substrates of acylase I are limited by the enzyme acylation stage[165]. However, the validity of the three-stage mechanism of the acylase-catalysed hydrolysis has not so far been in any way demonstrated.

At acid pH it has been possible to observe kinetically and with the aid of sedimentation equilibria the dissociation of acylase I into inactive subunits[168]. The dissociation constant is 2.0×10^{-7} M at pH 7.2 and 1.1×10^{-3} M at pH 5.8. The dissociation into inactive monomers under the influence of the substrate explains the substrate inhibition of the activity of acylase I at acid pH and the fact that there is a "flash" on the product accumulation curve under these conditions[168,169].

The pH optimum of the catalytic activity is in the pH range 7.0–7.5.[164] The Michaelis constant for the hydrolysis of acetyl-L-methionine catalysed by acylase I is independent of pH and the pH dependence of the maximum rate is bell-shaped and can be described in terms of the ionisation of two ionogenic groups having $pK_a = 6.7$ and $pK_b = 8.9$.[170]

One should note that, in the presence of high concentrations of the product of the hydrolytic reaction catalysed by acylase I, namely the acetate ion and the aminoacid, the hydrolysis of *N*-acetylaminoacids proceeds only as far as the attainment of equilibrium in the system and does not go to completion[171–173]. However, only qualitative data were available until recently for the kinetics of the synthesis of *N*-acetyl derivatives of aminoacids catalysed by the pig kidney acylase I.

2. Kinetic and Thermodynamic Characteristics of the Action of Acylase

The application to the study of acylases of a recently developed method for the quantitative determination of aminoacids, based on the spectrophotometric measurement of the product of the interaction of the aminoacid with *o*-phthalaldehyde in the presence of mercaptoethanol[177], permitted a detailed investigation of the kinetics of the hydrolysis-synthesis of acetyl-L-methionine catalysed by the pig kidney acylase I and made it possible to put forward a kinetic mechanism of the action of the enzyme and to determine the

kinetic parameters of the reaction[126,170,178]:

$$E + S \overset{K_M}{\rightleftharpoons} ES \underset{V_{synth}}{\overset{V_{hydr}}{\rightleftharpoons}} EPP' \leftarrow \left[\begin{array}{l} \xrightarrow{K'_M} EP + P' \xleftarrow{K_i} \\ \xrightarrow{\quad} EP' + P \longleftarrow \end{array} \right] \rightarrow E + P + P' \quad (2)$$

where E is the acylase, S is acetyl-L-methionine, P is sodium acetate, P' is L-methionine, $K_M = (25 \pm 3) \times 10^{-3}$ M and $K'_M = (140 \pm 20) \times 10^{-3}$ M are the Michaelis constants for the hydrolytic synthetic reactions respectively, $K_i = (43 \pm 5) \times 10^{-3}$ M is the acetate ion binding constant, and $V_{hydr} = (17 \pm 2) \times 10^{-5}$ mol litre^{-1} s^{-1} and $V_{synth} = (2.1 \pm 0.3) \times 10^{-5}$ mol litre^{-1} s^{-1} are the maximum rates of hydrolysis and synthesis respectively at an acylase concentration of 1 mg ml^{-1}. The kinetic parameters are linked by the Haldane relation

$$K_{eq} = \frac{K'_M K_i V_{hydr}}{K_M V_{synth}} ,$$

where $K_{eq} = 3.6 \pm 0.4$ is the equilibrium constant of the hydrolytic-synthetic reaction of acetyl-L-methionine. The empirical Eqn.(3) [179] for the description of the action of a membrane reactor with acylase I under conditions where the reaction equilibrium begins to have an effect in essence confirms the kinetic mechanism (2):

$$v = \frac{V_{max}}{1 + (S_L + S_{0D})/K_1} \cdot \frac{S_L - (S_{0L} - S_L)^2/K_{eq}}{K_M[1 + (S_{0L} - S_L)/K_2] + S_L} , \quad (3)$$

where the S_L and S_D are the concentrations of acetyl-L-methionine and acetyl-D-methionine respectively (the symbols with subscript 0 represent the initial concentrations), $K_1 = 1.45 \pm 0.08$ M and $K_2 = (3 \pm 1) \times 10^{-3}$ M are the inhibition constants, $V_{max} = 3.37 \times 10^{-5}$ mol litre^{-1} s^{-1} is the maximum velocity at an acylase (I) concentration of 1 mg ml^{-1}, K_M $(0.8 \pm 0.5) \times 10^{-3}$ M is the Michaelis constant, and $K_{eq} = 2.75$ is the equilibrium constant.

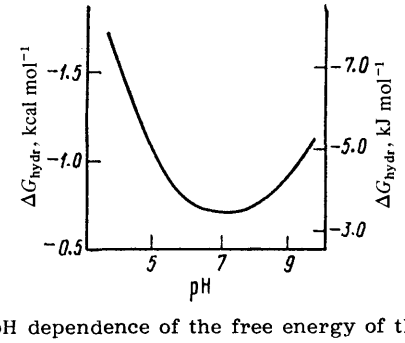


Figure 2. pH dependence of the free energy of the hydrolysis of acetyl-L-methionine.

Since the equilibrium constants of the hydrolytic-synthetic reactions of *N*-acetylaminoacids depend little on the nature of the aminoacid (Table 3), the proposed kinetic-thermodynamic approach is suitable for the description of the interaction of acylases with substrates having different structures.

There is no doubt that the experimental equilibrium constants of the hydrolytic-synthetic reactions of *N*-acetyl derivatives of aminoacids are not thermodynamic constants in the strict sense of the word, because the concentrations of the reactants in the reaction mixture are much higher than in dilute solutions for which the activity coefficient is unity.

The lack of literature data for the activity coefficients of compounds of this kind, due to the exceptional difficulty and ambiguity of the measurements of these quantities, resulted in a general employment at the present time of equilibrium constants calculated from the concentrations of the reactants as estimates of the true thermodynamic equilibrium constants[56,57,78,82]. Since the equilibrium constant K_0 calculated in terms of the unionised forms of the reactants is independent of the pH and the entire contribution to the pH dependence of K_{eq} is due to the ionisation of specific ionogenic groups, it follows that, taking into account all the equilibria in system (4), it is possible to calculate the dependence of K_{eq} and hence of ΔG_{hydr} on the pH (Fig.2).[170,178]

Fig.2 shows that the equilibrium in this reaction is displaced towards synthesis precisely at the pH values where the majority of both the native acylases of animal and microbial origin and the immobilised preparations of these enzymes are active. This conclusion has a definite practical importance because the reversibility of the reaction in the stereoselective hydrolysis of the racemic acetylaminoacid reduces the productivity of the process:

$$\begin{array}{l} CH_3{-}CO{-}NH{-}CHR{-}COOH \overset{K_3}{\rightleftharpoons} CH_3{-}CO{-}NH{-}CHR{-}COO^- , \\ \quad\downarrow\uparrow K_0 \\ CH_3{-}COOH \overset{K_4}{\rightleftharpoons} CH_3{-}COO^- , \\ \quad + \\ NH_2{-}CHR{-}COOH \rightleftharpoons NH_2{-}CHR{-}COO^- , \\ \quad\downarrow\uparrow K_5 \qquad\qquad\qquad\quad \downarrow\uparrow K_5 \\ NH_3^+{-}CHR{-}COOH \overset{K_1}{\rightleftharpoons} NH_3^+{-}CHR{-}COO^- ; \end{array}$$

$$\Delta G = -RT \ln K_{eq} ;$$

$$K_{eq} = K_0 \frac{(1 + K_4/[H^+])(1 + K_1/K_2 + K_1/[H^+] + [H^+]/K_5)}{1 + K_3/[H^+]} ; \quad (4)$$

pK_1 2.3; pK_2 9.2; pK_3 3.8; pK_4 4.75; pK_5 7.4 .

The search for new microbial acylases or the creation of immobilised enzyme preparations with a pH optimum of their catalytic activity in the pH range where the reversibility of the hydrolytic reaction does not have an effect even at high acetate ion concentrations would make it possible to eliminate this kind of deficiency. A possible method for the creation of immobilised acylase preparations with a modified pH optimum of their activity involves their incorporation in the structure of polyelectrolyte complexes.[180]

It is also of interest to note that the free energy of the hydrolysis of acetyl-L-methionine, calculated relative to the unionised forms of the starting materials and reaction products, is 7.46 kcal mol^{-1} (31.0 kJ mol^{-1}), i.e. the synthesis is a thermodynamically favourable process. The virtual failure to observe the synthesis of the acetamide linkage under the usual conditions is due to the fact that both the starting materials and the reaction products are present in the reaction mixture in the form of ions and the concentration of the unionised forms is negligible. The ΔG_0 obtained for acetyl-L-methionine exceeds somewhat the free energy of hydrolysis calculated relative to the unionised forms, usually obtained for the peptide bond[82], and shows that the synthesis of the acetamide linkage is thermodynamically even more favourable than the synthesis of the peptide linkage. Analogous results have been obtained also in the study of the reversible hydrolysis of *N*-phenylacetyl derivatives of aminoacids catalysed by penicillin amidase from *E. coli*[181,182].

The pig kidney acylase I exhibits a high stereospecificity. Among the large number of acetylaminoacids, only the D-isomer of acetylmethionine is appreciably hydrolysed by this enzyme; the ratio of the rates of hydrolysis of acetyl-L-methionine and acetyl-D-methionine, taken separately, is 10 000 : 1.[52] In the presence of L-methionine, the action of acylase I and acetyl-D-methionine is appreciably inhibited

and in the hydrolysis of the racemic acetyl-DL-methionine the ratio of the rates of hydrolysis of the L- and D-isomers is close to 40 000 : 1. A similar if not slightly greater ratio of the rates of hydrolysis of the optical isomers obtains for the acyl derivatives of other aminoacids. However, it has been observed that, when the acyl group is trifluoroacetyl, then a marked decrease of the stereospecificity of acylase I is observed for certain aminoacids[183].

The high stereospecificity of acylase I in the hydrolysis of acetyl and chloroacetyl derivatives of various aminoacids made it possible to resolve the racemates of many natural and synthetic aminoacids into optical isomers. On the other hand, it is necessary to note that acylase I is unsuitable for the resolution of the racemates of aspartic acid, proline, hydroxyproline, and aromatic aminoacids.

3. Microbial and Plant Acylases

Acylase activity (mainly in the hydrolysis of acetylmethionine) has been detected in tobacco leaves[184], in potato tubers, in many fruits (it is particularly high per unity weight in the radish and the pumpkin), in virtually all gramineous and leguminous plants (with the exception of polished rice whose treatment may lead to the breakdown of the enzyme system), and in the peel of mandarins, oranges, and figs[185,186]. An appreciable activity has been observed in the seeds of all the plants investigated and has been found to increase in the course of their growth[186,187].

Among all the plant acylases, only three enzymes have been partly purified and investigated: from rape seeds[188], from tobacco leaves[184], and from the seeds of palo verde (*Parkinsona Aculeata L.*).[189]

The enzyme from rape seeds hydrolyses enantioselectively the acetyl derivatives of aromatic and aliphatic aminoacids and methionine, the D-form not only not undergoing enzyme cleavage but also showing no influence on the hydrolysis of the L-form. The acetyl derivatives of glycine, glutamic acid, and proline as well as the benzoyl and benzyloxycarbonyl derivatives of aminoacids are resistant to the action of the enzyme. The pH optimum of the catalytic activity is in the range 7.2–8.8. Cobalt(II) ions activate the acylase approximately by a factor of 50, but those of calcium(II), iron(II), magnesium(II), nickel(II), and zinc(II) are 10 times less effective. The enzyme activity is inhibited by *p*-chloromercuribenzoate (PCMB), but *o*-phenanthroline , 8-hydroxyquinoline, or *N*-ethylmaleimide do not influence the catalytic activity[188].

An enzyme with a much narrower substrate specificity, which hydrolyses only *N*-acetyl-L-arginine, *N*-acetyl-L-cysteine, *N*-acetyl-DL-methionine, and, significantly less effectively, *N*-formyl-DL-methionine, has been partially purified from tobacco leaves. The pH optimum of its catalytic activity is displaced to the alkaline region compared with the pig kidney acylase I and depends on the nature of the substrate hydrolysed. Cobalt(II) ions activate the tobacco leaf acylase, the degree of activation increasing during purification. PCMB fully inhibits the enzyme activity at a concentration of 0.5 mM, but iodoacetate (1.5 mM) and EDTA (20 mM) do not influence the catalytic properties of tobacco leaf acylase[184].

The acylase activity in the palo verde seeds is concentrated mainly in the embryo and in the cotyledons. The acylase has been purified by a factor of 75 and its properties have been found to resemble closely those of the acylase isolated from rape seeds[189]. The acylase from the palo verde seeds effectively cleaves a wide set of acetyl and formyl derivatives of aromatic and aliphatic aminoacids, *N*-formyl-L-methionine being the best substrate. The acyl derivatives of D-aminoacids are hydrolysed to only a very slight extent. The pH optimum of the catalytic activity depends on the nature of the substrate hydrolysed and is located in the range 7.0–9.0. Cobalt(II) ions activate the enzyme approximately by a factor of 1.5 and greatly increase its stability during isolation. On the other hand, it has been noted that the acylase retains its activity after dialysis and electrophoresis, i.e. it is most probably not a metalloenzyme, because the metal ion is bound very firmly to the protein globule. The acylase from the palo verde seeds is inhibited by SH reagents like the other acylases considered[189].

The properties of the acylases under examination are close to those of the pig kidney acylase I. Like acylase I they exhibit a high stereospecificity (methionine derivatives are hydrolysed most effectively), are activated by cobalt(II) ions, and are sensitive to the action of SH reagents. A characteristic difference between plant acylases and acylase I is the displacement to the alkaline region of the pH optimum of the catalytic activity and the fact that the plant acylases are capable of hydrolysing the derivatives of not only aliphatic but also aromatic aminoacids. Such broad substrate specificity makes possible the employment of plant acylases for the resolution of aminoacid racemates. However, one should note that even greater prospects, which have already been embodied in practice, are associated with the acylases isolated for various micro-organisms (see Table 4).

4. ε-*N*-Acyl-L-lysine Amidohydrolase

Their exists an enzyme capable of hydrolysing only the L-isomer when it acts on racemic ε-*N*-acyl-DL-lysine (ε-*N*-acyl-L-lysine amidohydrolase, EC 3.5.1.17). It is of interest to note that in this case ε-lysine acylase exhibits stereospecificity in the hydrolysis of a bond which is comparatively remote from the asymmetric centre in the substrate molecule. The ε-lysine acylase activity was first detected in the KT 83 soil bacteria[190]. Later the ε-lysine acylase activity was found in a number of micro-organisms: moulds (particularly *Aspergillus*), the *Rhodotorula glutinis* yeast[191], and the *Achromobacter* and *Pseudomonas* bacteria (particularly *Achromobacter pestifer*[192]). The optimum conditions for the growing of *A. pestifer* as a producer of ε-lysine acylase activity have been elucidated[193] and the enzyme itself has been purified as far as the electrophoretically homogeneous state[194]. The enzyme hydrolyses all possible ε-acyl derivatives of lysine: acetyl, chloroacetyl, di- and tri-chloroacetyl, iodoacetyl, n-butyryl, caproyl, caprylyl, glycyl, benzoyl, phenylacetyl, benzyloxycarbonyl, *p*- and *m*-nitrobenzoyl, and naphthoyl derivatives, but neither α-acyl- nor diacyl-L-lysine undergoes enzymic hydrolysis. The replacement of a hydrogen atom by an alkyl group in the ε-NH_2 group also renders the substrate non-hydrolysable[195]. The Michaelis constants for the hydrolysis of the ε-chloroacetyl, ε-acetyl, and ε-benzoyl derivatives of L-lysine are respectively 0.29×10^{-3}, 0.5×10^{-3}, and less than 0.1×10^{-3} M.[196] The enzyme is inhibited by silver(I), mercury(I), and mercury(II) ions, PCMB, chloroacetone, and iodoacetate. The inhibition by PCMB is fully removed by the addition of cysteine. EDTA has little effect, while iron(III) and aluminium(III) ions (at a concentration of 1 mM) and nickel(II), cobalt(II), manganese(II), calcium(II), strontium(II), and cadmium(II) ions (at a concentration 10 mM) activate ε-lysine acylase to the extent of 20–30%. The pH optimum of the activity of the enzyme from *A. pestifier* is 4.8–5.2, which differs from that of the ε-lysine acylases from *Pseudomonas* and *Aspergillus oryzae*, for which the pH optima are respectively 6.0 and 8.2–8.4.[197]

An enzyme which hydrolyses stereoselectively the *N*-δ-acyl derivatives of ornithine, has been detected in the cells of the *Pseudomonas* KT 80 strain[198], but it has been scarcely investigated.

Table 4. The acylase activities of various micro-organisms.

Micro-organisms	Substrate specificity		Enzyme inhibitors and activators	pH optimum of activity	Refs.
	hydrolyses	does not hydrolyse			
Str. caespitosus	Ac-L-aminoacids	D-isomers	NI : bivalent cations, EPTA	7.0—8.0	[199]
Bact. cadaveris	Form-Gly, -Tyr Ac-Tyr	Ac-, Clac-Asp		7.0—8.0	[200]
Micrococcus n. sp.	ditto	ditto	—	7.0—8.0	[200]
Hydrocarbon oxidising micro-organisms	Ac-Leu, -norLeu, -Val, -Met, Bz-Leu	Ac-Trp, Bz-Asp	—	—	[201]
Bacillus brevis *Bacillus subtilis* *Bacillus megaterium* Micrococcus lysodeckticus	Ac-*L*-Met	Ac-*D*-Met	—	—	[202]
Corynebacterium glutamicum	Ac-*L*-Glu	Ac-*D*-Glu	—	—	[203]
E. coli B	Form-L-Met, Ac-L-Met, Ac-L-aminoacid		A : Co(II)(5×10^{-5}M), Ni(II)(10^{-5}M), Fe(II)(10^{-2}M), Mn(II)(10^{-2}M); I : Zn(II), EDTA	6.5—6.6	[204]
E. coli 160-37	Form-L-aminoacids		EA : Mn(II)(10^{-3}M), Co(II) (10^{-4}M)	7.2	[204]
E. coli ATCC 9637	Ac-Met, -Orn	Ac-Ala, -Leu, -Val, -Glu, Clac-Tyr, Bz-Arg	A : Co(II); I : Cu(II), Zn(II), Ni(II), EDTA	7.0	[205]
E. coli	Phac-*L*-Ala, -*L*-Val, -*L*-Ambut, -*L*-Leu, -*L*-Phe	D-isomers	—	—	[206, 207]
Ditto	Ac-*L*-Ala, -*L*-Met, Clac-*L*-Ala, -*L*-Met, -*L*-Asp, -*L*-Leu, -*L*-Phe	D-isomers Clac-L-Tyr, -L-Trp	A : Co(II)	—	[208]
Neursopora crassa	Phac-L-aminoacids				[209]
Mycobacterium smegmatis	Palmityl-L-Asp, -L-Val, L-Phe, decanoyl-L-Asp, lauryl-L-Asp	Ac-, butyryl-, hexanoyl-, octanoyl-L-Asp	A : alkali metal ions; I : Cu(II), Hg(II)	5.7—7.3	[210, 211]
Ditto	Ac-*L*-Val, -*L*-Asp, Clac-*L*-Phe		A : bivalent cations; I : Cu(II), Hg(II)	—	[210, 211]
Mycobacterium avium	palmityl-L-Asp, -L-Val, -L-Phe		NI : Cu(II), Hg(II)	6.2	[212]
Ditto	Ac-*L*-Val, *L*-Asp, Clac-*L*-Phe	—	I : Cu(II), Hg(II)	7.4	[212]
Mycobacterium phei	lauryl-L-Phe, -L-Ala, -L-Ambut, -L-Met, -L-Glu, Bz-L-Ala, -L-Ambut	—	—	—	[213]
Staphylococcus oureus	Bz-Gly, -Phe	—	—	—	[213]
Brewer's yeast	Ac-*L*-Met, Clac-*L*-Ala, -*L*-Leu, -*L*-Phe, -*L*-Trp, Gly-*L*-Met	—	—	—	[214]
Pseudomonas ATCC 11299B	Form-Asp, Clac-Asp	Ac-Met, Form-Leu	EA : Co(II); A : Fe(II)	7.0	[215]
Ditto	Form-, Ac-, Clac-Asp	Ac-Met, Form-Leu	EA : Co(II); A : Fe(II)	8.0	[215]
Soil pseudomonads	Ac-L-Asp, -L-Asn, Bz-L-Asp, dipeptides with L-Asp or L-Asn at the C-end	D-isomers, Ac-, Bz-aminoacids	Ea : Co(II), Zn(II)	—	[216, 217]
Ditto	Ac-*L*-Ala, Bz-*L*-Ala	ditto	Ditto	—	[218]
»	Ac-L-Glu, -L-Gln, Bz-L-Glu, -L-Gln, dipeptides with L-Glu or O-Gln at C-end	—	—	—	[219]
Pseudomonas KT83	Bz-D-Phe, -D-Phg, -D-*p*-nitro-Phe, -D-β-phenylSer, aromatic diClac-D-aminoacids	L-isomers	—	—	[220, 221]
Pseudomonas KT 84	diBz-*L*-Lys **, Bz-*L*-Ser, -*L*-Thr, -*L*-Orn, -*L*-Met, -*L*-Glu, -*L*-Asp, -*L*-Leu, -*L*-Val	D-isomers	—	—	[222, 223]
Pseudomonas KT 85	Bz-*L*-β-phenylSer, -*L*-isoLeu	D-isomers	—	—	[224, 225]
Pseudomonas KT 104	Ac-*L*-Trp	Ac-*D*-Trp	—	—	[226]
Pseudomonas KT 202, KT 205	Bz-*L*-Thr, diBz-*L*-Lys **, Ac-*L*-Trp, -*L*-Met	D-isomers			[227]
Pseudomonas KT 218	Bz-, Ac-L-aminoacids with straight C_6, C_8, C_{12}, or C_{14} chain; Bz-L-Thr	D-isomers	—	—	[228]
Pseudomonas KT 224	Ac-*L*-Met	Ac-*D*-Met	—	—	[229]
Pseudomonas KT 230, KT 231, KT 232, KT 233	diBz-L-Lys**; Bz-L-Phe, -L-Thr, -L-aminoacids (aliphatic)	D-isomers	—	—	[230, 235]
Pseudomonas KT 251, KT 256	Ac-*L*-Trp	Ac-*D*-Trp	—	—	[235]
Pseudomoans KT 301	diBz-*L*-Lys **	diBz-*D*-Lys	—	—	[236]
Pseudomonas KT 303 KT 305	Bz-*L*-Trp	Bz-*D*-Trp	—	—	[233]
Pseudomonas KT 313	diBz-*L*-Lys **, α-Phac-ε-Bz-*L*-Lys	D-isomers	—	8.0	[237]
Pseudomonas KT 801	Bz-, Phac-, diClac-octanoyl-L-aminoacids*	D-isomers	A : Mg(II)*; I : Co(II), Cu(II), Mn(II), *o*-phenanthroline		[238]
Pseudomonas AAA 6020	Phac-L-Val, -L-Leu, -L-Glu, -L-Ala, -L-Met, -D-aminoacids	Ac-, Bz-aminoacids	I : Hg(II)*, Zn(II)*, Co(II) (10^{-3}M)*	8.0	[239]
Pseudomonas AAA6029	Bz-, Ac-D-Phe-, -D-Met, -D-Leu, -D-Ala, -D-Val	Phac-D- and -L-aminoacids, Bz-, Ac-L-Phe, -L-Met, -L-Leu, -L-Ala, -L-Val, Ac-D-L-amino-acids	A : L-Cys(3×10^{-3}M); I : Hg(II), Zn(II)(10^{-3}M), PCMB(10^{-3}M); NI ; Ca(II), Co(II), Mg(II) (10^{-3}M)	10.5 (Bz-*D*-Phe) 6.8 (Ac-*D*-Phe)	[240]
Micrococcus agilis	Ac-Phe-, -Met, -Val, -Leu, Clac-Phg, -Tyr, -Glu	Ac-Asp, Ac-*D*-Phe	I : Ag(I)*, Mn(II), Pb(II), Cu(II), Cd(II), Hg(II)(10^{-3}M), iodo-acetamide, PCMB, *o*-phenanth-roline, mercaptoethanol	7.0	[241]
Streptomyces tuirus IFO 13418	Ac-*D*-Phg, -*D*-Leu, -*D*-Ala, -*D*-Met, -*D*-Val, -*D*-Phe, -*D*-Tyr, -*D*-Trp, -L-Met	L-isomers	I : Ag(I), Hg(II)(10^{-5}M); NI : NaN_3, NaCN	7.5	[242]
Pseudomonas aeruginosa	Ac-L-Glu, Form-L-Glu, fluoroacetyl-L-Glu, propionyl-L-Gly, Ac-L-Gln, -L-Met, -L-Ala, -Gly	butyryl-L-Glu, Form-L-Met*, Ac-L-Ser*, -L-Asn*, -L-His*, -L-Asp*,	EA : Co(II), Zn(II), Mn(II), Mg(II) (10^{-3}M); I : PCMB (10^{-5}M); NI : *N*-ethylmaleimide	6.5	[243]
Penicillium vinaceous	aromatic Ac, Clac-L-amino-acids	D-isomers	A : Co(II)(10^{-3}M) I : Hg(II), Mn(II)	5.6—5.8 (Ac-amino-acids)	[244—247]
Penicillium oxalicum	diAc-*L*-Lys, Ac-*L*-Met, -*L*-Ala, -*L*-Glu, *L*-Leu *, -*L*-Val *, -*L*-isoLeu *, -*L*-His *, -*L*-Asp *, Clac-*L*-Ser, -*L*-Val *	—	—	7.6-7.8 (Clac-amino-acids)	
Aspergillus oryzae	ε-Bz-α-Ac-L-Lys**, Clac, Ac-aminoacids	D-isomers	A: Co(II), Zn(II) *, Cd(II) *, Mn(II) *, Cu(II) * (10^{-3} *M*)	7.5	[248—251]

Table 4 (cont'd).

Micro-organisms	Substrate specificity: hydrolyses	Substrate specificity: does not hydrolyse	Enzyme inhibitors and activators	pH optimum of activity	Refs.
Aspergillus avamori 78-2 *Streptoverticillium*	Ac-Trp, -Met Ac-*L*-Met, -*L*-Leu, -*L*-Phe, -*L*-Trp, -*L*-Phg	D-isomers	— —	7,0	[252] [253]

Notation: A = activators, I = inhibitors, NI = no influence on activity, EA = essential for activity, Form = formyl, Clac = chloroacetyl, norLeu = norleucine, Ambut = α-aminobutyric acid, Phg = α-aminophenylacetic acid (phenylglycine), and isoLeu = isoleucine.

*Weakly hydrolyses or activates.

**The hydrolysis of the diacyl derivatives of lysine takes place only at the α-amino-group (the hydrolysis of the ε-amino-group was examined in Section VI).

Table 5. The preparation and properties of immobilised acylases.

Methods of immobilisation, enzyme	Retention of activity, %	Change in properties of immobilised enzyme relative to native enzyme	Refs.
Pig kidney acylase I			
Adsorption on A-50 DEAE-sephadex	—	IT	[262]
on silica gel or C-80 silochrome	50	shift of pH optimum by 1 to alkaline region	[263,264]
on silica gel coated by a layer of lecithin, cholesterol, or inosine	25—45	ditto	[264]
on DEAE-cellulose	—	— —	[264]
on ceramic carriers based on Al_2O_3, SiO_2, and TiO_2	H	pH optimum becomes very broad (7.5-9.5)	[265]
on silochrome coated with a protein or a synthetic polymer	15	—	[266,267]
Bonding to "Enzacryl-AA"	30	shift of pH optimum to alkaline region by 0.4; IT	[268]
to silochrome by means of γ-aminopropyltriethoxysilane and glutaraldehyde	H	—	[269]
to DEAE-cellulose via triazine dyes	H	—	[269]
to silochrome via diazotised *m*-phenylenediamine	H	—	[269]
to insoluble polyamide (copolymer of acrylamide, methyl acrylate, and *N,N'*-methylenebisacrylamide) via an amide linkage	50—100	IT	[270]
to COOH-containing gels via soluble carbodi-imides	—	—	[271]
to water-soluble dialdehyde-epidex and dialdehyde-dextran	L	—	[272]
Incorporation in polyacrylamide gel by oxidative-reductive polymerisation or photopolymerisation	6—12	—	[266]
Incorporation in polyacrylamide gel by radiopolymerisation	25—45	change in substrate specificity	[273]
Acylase from *Aspergillus ochracens*			
Covalent bonding to cell mycelia with the aid of albumin and glutaraldehyde	80	Increase of resistance to action of inhibitors; IT	[274]
Acylase from *Aspergillus oryzae*			
Adsorption on activated charcoal, acid, neutral, and alkaline alumina, and silica gel	<1		[275]
on tannin bonded to aminohexylcellulose	20—25		[276]
on PAB-cellulose	0	shift of pH optimum to acid region by 0.5-1; IT	[275,277, 278]
on TEAE-cellulose	51.5		
on DEAE-cellulose	55.2		
on C-50 KM-sephadex	0	shift of pH optimum to acid region by 0.5-1; IT	
on A-25 and A-50 DEAE-sephadex	56—59		
on IRC-50, IR-4B, and RI-45 amberlites	0		
Covalent bonding to diazotised PAB-cellulose	5.3		[279,280, 281,282]
to diazotised arylamino-glass	43.5		
to diazotised "Enzacryl-AA"	3.6		
to CM-cellulose azide	0		
to BrCN-activated cellulose	1.0		
to BrCN-activated sephadex	1.2		
to chloroacetycellulose	11.3	shift of pH optimum to alkaline region by 0.5-1; IT	
to bromoacetylcellulose	28 0		
to iodoacetylcellulose	39.0		
to arylamino-glass	20—40	shift of pH optimum to acid region by 0.5; IT	
Cross-linking on carrier (AE-cellulose) by a bifunctional reagent			[283]
1,4-dibromobutene	0.4		
1,4-dichlorobutene	0.4		
dicyclohexylcarbodi-imide	1.2		
di-iodomethane	0.3	—	
glutaraldehyde	0.6		
hexamethylene di-isocyanate	1.6		
tolylene di-isocyanate	0.2		
Cross-linking by a bifunctional reagent			[283]
glutaraldehyde	14.7	—	
tolylene di-isocyanate	1.3		
Incorporation in polyacrylamide gel	52.8	shift of pH optimum to acid region by 0.5; IT	[284]
Incorporation in carrageenan gel	50	IT	[285]
Microencapsulation			
in nylon	36.0	—	[284]
in polycarbamide	15.0		
in ethylcellulose	10.4		

Table 5 (cont'd).

Notation. H = high, L = low, and IT = increase of thermal stability.

5. Industrial Utilisation of Microbial Acylases

Utilisation of microbial acylases for the resolution of amino-acid racemates into optical isomers constitutes at present the only method which has found an industrial application. For this reason there is an unusually great interest in micro-organisms capable of hydrolysing *N*-acylated aminoacids. The available literature data on microbial acylases are compiled in Table 4.[199-253]

It is seen from the data in Table 4 that microbial acylases exhibit a virtually no substrate specificity and hydrolyse all possible acyl derivatives of all the natural aminoacids except proline, hydroxyproline, and a number of synthetic α-amino-acids. It is of interest to note that the majority of acylases exhibit a pronounced stereospecificity and hydrolyse selectively only the acyl derivatives of the L-isomers of amino-acids, but, among the mutants of soil micro-organisms, it has been possible to detect an enzyme which catalyses the hydrolysis of acylated D-aminoacids with a fairly broad substrate specificity[220,221,240]. The presence of acylases stereospecific

to both L- and D-isomers gives rise to the hope that it will be possible to select a scheme for the aminoacid racemate resolution process depending on which of the two optical isomers has to be obtained.

However, despite the very large number of studies devoted to the conditions under which the enzyme producers are cultivated, the isolation and partial purification of acylases, the development of methods for the preparative resolution of racemates with the aid of the preparations obtained, the immobilisation of acylases from micro-organisms (the immobilisation of acylases will be considered in detail below), and the action of reactors based on an immobilised enzyme, hitherto neither the acylase from *Aspergillus oryzae* nor the acylase from soil micro-organisms have been obtained in a sufficiently pure form; nothing is also known about the mechanism of the action of these enzymes. Undoubtedly the knowledge of the mechanism of the enzyme action would assist in the search for optimum process conditions and in the selection of the type of catalyst for the industrial resolution of racemates using acylases from micro-organisms.

A whole series of methods for the resolution of aminoacid racemates into optical isomers with the aid of soluble acylases of microbial origin have now been patented[254-261], but there is no doubt that the future in this field belongs to immobilised enzymes or cells.

The extensive technological possibilities for the application of immobilised biocatalysts, in particular aminoacid acylases, have led to numerous studies on the immobilisation of the pig kidney acylase I and particularly the acylases from micro-organisms. The available literature data on the methods of preparation and properties of immobilised aminoacid acylases are compiled in Table 5.[262-285] The table shows that a high retention of the activity of the microbial acylase on immobilisation is achieved in the following methods: adsorption on DEAE- and TEAE-cellulose or DEAE-sephadex, covalent bonding to diazotised arylamino-glass or halogenocellulose, bonding via glutaraldehyde to arylamino-glass, incorporation in a polyacrylamide gel, and microencapsulation using nylon. However, the simplicity of preparation, low-cost, high operational stability, and the possibility of regeneration by the adsorption on the carrier of a fresh portion of enzyme corresponding to the loss of activity[286] (Fig.3) make acylase sorbed on DEAE-sephadex the most suitable biocatalyst for the practical resolution of racemates[287]. An industrial apparatus for the resolution of alanine, methionine, phenylalanine, valine, and tryptophan has been devised on the basis of this biocatalyst (Fig.4).[288] A 0.2 M solution of acetyl-DL-methionine, containing 5×10^{-4} M cobalt(II), is passed through a 1000 litre column containing the acylase from *Aspergillus oryzae* immobilised on DEAE-sephadex at a rate of 2000 litre h^{-1} at 50 °C and pH 7.0. The free L-methionine is precipitated when the eluate is evaporated. The yield is 91% and $[\alpha]_D^{25} = +23.4°$ after recrystallisation from water. The acetyl-D-methionine in the mother liquor is heated at 60 °C with acetic anhydride for racemisation and acetyl-DL-methionine is isolated and reused as the substrate[287].

Fig.5 presents a comparison of the usual method with a soluble acylase and the continuous process based on the immobilised enzyme. Firstly, there is a sharp reduction of the consumption of the biocatalyst and, secondly, there is an appreciable decrease of the amount of labour required and the purification procedure is simplified, which makes it possible to obtain a larger amount of the final product from the same amount of the starting materials. All these factors taken together make it possible to decrease by a factor of 2 the cost of the manufacture of L-aminoacids when an immobilised acylase is used.

The examples presented show that the enzymic conversion of racemates into optically pure aminoacids is already a method which has found technological applications, together with other procedures, for the preparation of these compounds. It is noteworthy that there is at present no universal method for the manufacture of the entire set of essential aminoacids. In each specific instance the procedure for the manufacture of a particular aminoacid must therefore be determined individually as a function of the raw materials, the amount required, the required purity, the availability of the necessary biocatalysts, and many other factors. However, there is no doubt that biotechnological processes employing enzymes capable of achieving the enantioselective conversion of the simplest aminoacid derivatives will find, in combination with the chemical synthesis of the starting materials, still more extensive applications in the immediate future.

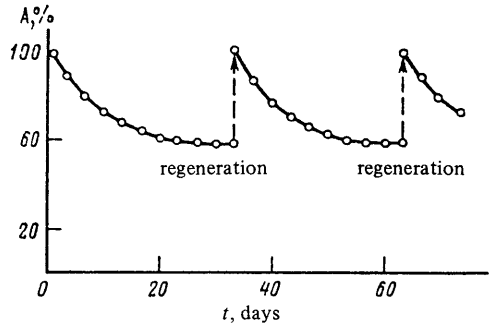


Figure 3. The stability and regeration of a column containing the acylase from *Aspergillus oryzae* immobilised on DEAE-sephapez; *A* = activity.

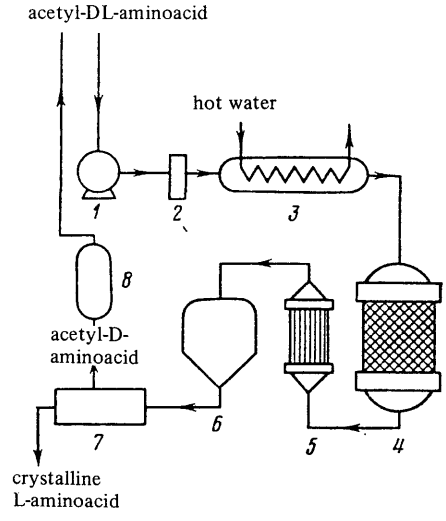


Figure 4. Industrial apparatus for the resolution of aminoacid racemates based on the acylase from *Aspergillus oryzae* immobilised on DEAE-sephadex[283]; 1) pump; 2) filter; 3) heat exchanger; 4) reactor; 5) evaporator; 6) crystalliser; 7) separator; 8) racemiser.

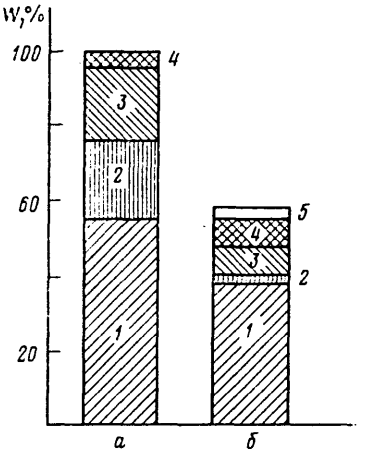


Figure 5. The relative costs W in the industrial resolution of aminoacid racemates with the aid of the acylase from *Aspergillus oryzae*, both soluble and immobilised on DEAE-sephadex: *a*) periodic process (soluble enzyme); *b*) continuous process (immobilised enzyme)[283]; costs of different process components: 1) substate; 2) enzyme; 3) labour; 4) fuel; 5) carrier for immobilisation.

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