72. Amide-Bond Syntheses Catalyzed by Penicillin Acylase

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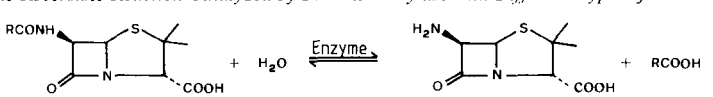
The enzymatic synthesis of amide bonds catalyzed by penicillin acylase is investigated both in H_2O solution and in organic solvents containing reverse micelles.The specificity of the reaction is rather high on the side of the acyl component, practically only phenylacetic acid gives sizeable yields. On the contrary, a variety of amino-acid esters, dipeptides, and tripeptides can be used as amino component, *e.g.* serine methyl ester, methionine ethyl ester, tyrosine ethyl ester, Gly–Asp, Ala–Tyr, Gly–Tyr–Gly *etc.* However, many other amino-acid residues do not react, and the possible reasons for this are discussed. Yields vary in the range 10–80%. A systematic study to optimize yields by varying the solvent composition is presented for one model reaction. The enzyme is also able to couple certain D-amino-acid residues (*e.g.* D-methionine ethyl ester or Gly-D-Asp) though at lower rate. Reverse micelles formed by the cationic surfactant cetyltrimethylammonium bromide (CTAB) in $CHCl_3$/isooctane are used to host penicillin acylase and to perform amide synthesis in which the product is preferentially soluble in the organic solvent mixture.The reaction is studied as a function of pH and certain micellar parameters, *e.g.* w_o ($w_o = [H_2O]/[CTAB]$). A new membrane enzyme reactor is utilized to separate the product from the enzyme-containing micelles. The advantages and the limits of this approach are discussed.

1. Introduction. – The enzyme penicillin acylase (E.C. 3.5.1.11) catalyzes the reversible hydrolysis of penicillin as indicated in *Scheme 1*. In the literature, this enzyme is also known as penicillin amidohydrolase. Three types of penicillin acylase have been described: penicillin-V acylase, penicillin-G acylase, and ampicillin acylase, depending on

Scheme 1. *The Reversible Reaction Catalyzed by Penicillin Acylase with Different Types of Penicillins*



Benzylpenicillin (Penicillin G), R = $PhCH_2$
Phenoxymethylpenicillin (Penicillin V), R = $PhOCH_2$
Ampicillin, R = $PhCH(NH_2)$

6-Aminopenicillanic acid (6-APA)

Carboxylic acid

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the type of penicillin which is preferentially hydrolysed [1]. The enzyme is produced in bacteria, fungi, yeasts, and actinomycetes. It is industrially used to produce 6-aminopenicillanic acid (6-APA) from benzylpenicillin. The 6-APA is then, chemically or enzymatically with penicillin acylase, converted into semi-synthetic penicillins. The enzyme is not only specific for penicillin, but it can hydrolyse certain cephalosporines or acylamino acids, amides, and esters [2]. It can also hydrolyse *N*-phenylacetyl-D-alanine [3] or phenylacetyl–D-Asp–L-Phe–OMe [4]. For penicillin acylase from *E. coli,* the pH optimum for the hydrolyis of benzylpenicillin is *ca.* 8.5 and for the synthesis pH 5.0 [5].

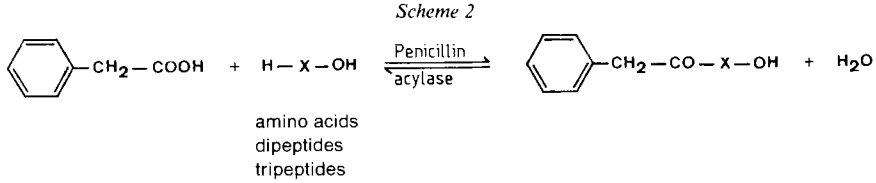
An interesting question concerns the specificity of the synthetic reaction, in particular of whether different acyl components can be coupled to 6-APA; and, more in general, whether and to what extent also aminopenicillanic acid and derivatives can be substituted by other compounds. *Cole* [6] has studied the coupling reaction with 6-APA, glycine, and hydroxylamine as amino components and a variety of acids, acid derivatives like esters, amides, or *N*-acetylglycine derivatives. He found that (*p*-hydroxyphenyl)acetic acid was the best substrate for the synthesis of penicillin from 6-APA. Recently, *Fuganti* and *Grasselli* have reported that aspartame can be prepared by enzymatic hydrolysis of the *N*-phenylacetyl protecting group [4].

In the present paper, as a continuation of our studies on the enzymatic synthesis of peptide bonds [7], we show that the enzyme penicillin acylase is able to catalyze the synthesis of certain amide bonds, and we investigate the specificity of this reaction for both the amino and the acyl components. In view of the claim that benzylpenicillin can be formally viewed as an L,D-dipeptide (namely L-Cys–D-Val) and that the conformation of D-Ala–D-Ala is similar to that of the penicillin molecule [8] in the transition state, we also investigate the use of D-amino acids and dipeptides containing D-amino acids as amino components.

Recently, the enzymatic peptide synthesis was extended to organic solvent with the help of reverse micelles [9]. This technique is useful for the case of H_2O-insoluble reagents or products. In this paper, we discuss the synthesis of the $CHCl_3$-soluble *N*-(phenylacetyl)tyrosine ethyl ester and the separation from the reagents with a membrane reactor.

2. Results and Discussion. – 2.1. *Enzymatic Synthesis in Aqueous Buffer or in Organic Solvent/Aqueous Buffer.* The reaction catalyzed by penicillin acylase involves practically the hydrolysis of an amide bond *Scheme 1.* Our strategy for the study of the specificity of the enzyme in the synthetic direction was based on systematic structural variations of both the amino and the acyl component. The results of our investigation can be summarized with the statement that the specificity is rather high on the side of the acyl component, whereas it is rather low for the amino component. In particular, we have found (see *Scheme 2*) that a number of amino acids or peptides (H–X–OH) can substitute for

Scheme 2



6-APA, whereas phenylacetic acid is about the only structure which can be effectively used as the acyl acceptor.

The reactions in citrate buffer without addition of organic solvent were carried out at pH 6.0. The pH changes during the synthesis for the coupling of phenylacetic acid with H–Tyr–OEt and with H–Gly–Leu–OH *(Table 1)* were found to be within 0.2–0.3 units and have, therefore, little influence on the enzyme activity. The reaction with amino acids proceeded with serine methyl ester, methionine ethyl ester, and tyrosine ethyl ester in 20–80% yield, but not with the free amino acids glycine, alanine, leucine, aspartic acid, proline, serine, and methionine. When unprotected dipeptides were used for the reaction with phenylacetic acid, the synthesis proceeded with dipeptides of the type H–Gly–X–OH with X = Gly, Asp, and other amino-acid residues (see *Table 1*). With dipeptides of type H–X–Tyr–OH, a reaction was detected with X = Gly, Ala, and Ser,

Table 1. *Penicillin Acylase Catalyzed Reaction of Phenylacetic Acids with Amino Acids, Dipeptides, and Tripeptides*

Starting material		Yield [%]	Time [h] to reach equilibrium	Amino-acids analysis of product	
Amino-acid esters	Ser–OMe[a])	11	40		
	Met–OEt[a])	70–75	40	n.d.	
	Tyr–OEt[b])	77	n.d.		
Dipeptides[b])	Gly–Gly	10–20	30		
	Gly–Asp	7–10	40	Gly/Asp	1:1.18
	Gly–Tyr	15	40	Gly/Tyr	1:1.02
	Gly–Leu	25	40	Gly/Leu	1:1.08
	Gly–Met	30–35	70	Gly/Met	1:1.03
	Gly–Ser	15	40	Gly/Ser	1:1.02
	Gly–Pro	10	24	Gly/Pro	1:1.02
	Ala–Tyr	10	90	Ala/Tyr	1:1.14
	Ser–Tyr	20–25	90	Ser/Tyr	1:1.15
Tripeptides[c])	Gly–Tyr–Gly	15–20	64	Gly/Tyr	1.75:1
	Gly–Leu–Tyr	20–25	64	Gly/Leu/Tyr	1:0.94:0.95

[a]) In aqueous citrate buffer/1,4-butanediol 1:1.
[b]) In aqueous citrate buffer.
[c]) In aqueous citrate buffer/1,4-butanediol 3:1.

but not, *e.g.* with bulkier X groups such as Val, Leu, Met, Pro, or Tyr. It is interesting to notice that both H–Met–OEt and H–Tyr–OEt gave a relatively high yield (see *Table 1*), whereas the dipeptide H–Met–Tyr–OH gave a low yield. This may be ascribed to steric hindrance which may prevent an effective binding of the bulky dipeptide with the enzyme active site. The reaction occurred also with H–Asp–Phe–OH and H–Ala–Leu–OH, but not with H–Tyr–Gly–OH and H–Leu–Leu–OH. Moreover, a coupling product was obtained from phenylacetic acid and the tripeptides H–Gly–Leu–Tyr–OH and H–Gly–Tyr–Gly–OH, but not from H–Ala–Leu–OH. In all cases, the product was detected in the solution equilibrium mixture (a product precipitation was observed only in the case of (phenylacetyl)tyrosine ethyl ester) by chromatographic techniques (see *Exper. Part*).

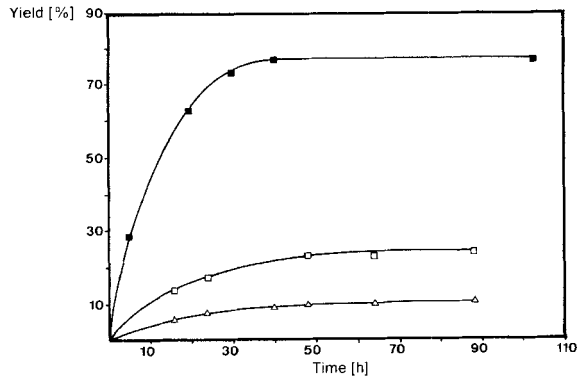


Fig. 1. *Time course for the coupling of phenylacetic acid with Met–OEt* (■), *Ala–Tyr*, (△), *and Ser–Tyr* (□)

A typical time course of the reaction with amino-acid esters or peptides is shown in *Fig. 1*. In the case of H–Ala–Tyr–OH and H–Ser–Tyr–OH, the equilibrium was reached after *ca.* 90 h; on the other hand, the reaction with methionine ethyl ester proceeded faster, and the yield reached the maximum (*ca.* 75%) in *ca.* 40 h.

No peptide-bond hydrolysis was observed during the coupling as shown by amino-acids analysis (in the limits of experimental errors).

While the results summarized in *Table 1* show that penicillin acylase can be used as the catalyst for the synthesis of certain amide bonds, it is also clear that generally the yield is rather low. Studies to increase the yield of the enzymatic synthesis of *N*-(phenylacetyl)peptides were carried out with the model H–Gly–Leu–OH (see *Scheme 2*, in H–X–OH = H–Gly–Leu–OH), which was chosen because of the good solubility and good yield in the aqueous buffer. Some typical results are shown in *Table 2*. The concen-

Table 2. *Influence of the Concentration and the Solvent Composition on the Reaction between Phenylacetic Acid and H–Gly–Leu–OH Catalyzed by Penicillin Acylase*

$PhCH_2COOH$ [mM]	H–Gly–Leu–OH [mM]	Composition of the solvent[a])	Yield [%]	Time [h] to reach equilibrium
150	100	aq. buffer	28	40
450	270	aq. buffer	30	40
150	100	aq. buffer/glycerol 1:1	47	40
150	100	aq. buffer/$Bu(OH)_2$ 1:1	70	64
150	100	aq. buffer/MeOH 3:1	48	24
200	133	aq. buffer/DMF 3:1	53	40
200	133	aq. buffer/MeCN 3:1	8	
115.4	76.9	aq. buffer/$Bu(OH)_2$/MeOH 46.1:34.6:19.3	60	60
125	83.3	aq. buffer/$Bu(OH)_2$/glycerol 41.7:33.3:25	55	40
125	83.3	aq. buffer/$Bu(OH)_2$/glycerol/MeOH 41.7:25:16.65:16.65	62	40
83.3	83.3	aq. buffer/DMSO/$Bu(OH)_2$ 58.3:10.4:31.3	44	40

[a]) In % (*v*/*v*); aq. buffer = aq. citrate buffer (50 mM, pH *ca.* 6.00), $Bu(OH)_2$ = 1,4-butanediol.

tration and the solvent composition were varied in an empirical way, trying to operate with the highest concentration in a given solvent. Occasionally, the solubilities were poor, as in the last four entries of *Table 2*.

Firstly, we studied the effect of increasing the concentration of the reagents. An increase of the concentration of phenylacetic acid and H–Gly–Leu–OH by a factor of *ca.* 3 in aqueous citrate buffer did not change the yield. At a concentration of phenylacetic acid higher than 1M (10 times higher than the one of H–Gly–Leu–OH), no product was detected, most likely because of enzyme inhibition.

Secondly, we attempted to increase the yield by varying the nature of the solvent. Since H_2O is the by-product of the synthesis, H_2O-miscible organic solvents can, in principle, be used to increase the yield of the enzymatic reaction (by reducing the overall concentration of H_2O). An other effect of the organic-solvent addition is the decrease of the equilibrium constant for the H^+ transfer from the COOH to the NH_2 termini of the substrate [10]. Although organic solvents may increase the yield of enzymatic syntheses, they may also have a denaturing effect. One has to find then the organic-solvent concentration which optimizes the yield without causing a sizeable denaturation. In our case, the addition of 25% of MeCN to the aqueous phase lowered the yield to *ca.* 8%; however, with 25% of MeOH or DMF, the yield was higher than in aqueous buffer (48 and 53%, resp.). The 1,4-butanediol and especially glycerol stabilize the enzyme. Thus in an aqueous buffer solution containing 50% of glycerol, no precipitation of denatured enzyme was observed for over 100 h, and the yield was *ca.* 47% *(Table 2)*. A yield as high as 70% was observed, when the reaction was carried out with 50% of 1,4-butanediol. As shown in *Table 2,* the reaction time was rather long (25–60 h). Several reactions were performed with mixtures of three or more solvents. A few typical examples are reported in the last four entries of *Table 2;* the best yield was *ca.* 62%.

As mentioned in the *Introduction,* benzylpenicillin can be viewed as an L,D-dipeptide [4] and actually some studies are present in the literature (see *e.g.* [2–4]) to test the enzyme specificity with D-amino acids. In this respect, it was of interest to study the enzymatic synthesis by penicillin acylase with D-amino-acid residues or peptides. The enzyme catalyzed the coupling of phenylacetic acid with H–D-Met–OEt and H–D-Tyr–OEt, but the yields were rather low compared with the ones of the corresponding L-isomers. In the case of H–D-Met–OEt, the 10% yield for reaction in aqueous citrate buffer/1,4-butanediol 1:1 could be increased to 25% by adding 0.5 mg/ml of enzyme after 200 h. For H–D-Tyr–OEt in aqueous citrate buffer, the yield was *ca.* 7% after 44 h. The dipeptides H–Gly–D-Asp–OH and H–Gly–D-Leu–OH were also suitable substrates, giving similar yields than H–Gly–L-Asp–OH and H–Gly–L-Leu–OH. Thus, there is no specificity for an L- or a D-amino acid in the second position of a dipeptide. No reaction was detected with H–D-Ala–D-Ala–OH and H–D-Ala–L-Leu–OH.

The low yield with D-amino acids could in principle be explained by assuming that D-amino acid or H–D-Ala–D-Ala–OH (an analogous of penicillin) are inhibitors of the enzyme. To clarify this question, we investigated the effect of D-amino acids on the rate of the reaction between phenylacetic acid and H–L-Met–OEt. In particular, 10 mol-% of H–D-Met–OEt, H–D-Tyr–OEt, or H–D-Ala–D-Ala–OH were added to H–L-Met–OEt in three separate experiments. No differences in the time course and in the yield between these reactions and a blank reaction with only phenylacetic acid and H–L-Met–OEt were observed.

The reaction rates and the yields with D-residues could be improved by increasing the enzyme concentration. All this indicates that the lower yields in the case of D-components are due to kinetic factors, *i.e.* the enzyme catalytic activity is indeed affected by the configuration at C(α) of the concerned amino acid.

All reactions described until now involved phenylacetic acid as the acyl component. Studies were performed with a series of phenylacetic-acid analogs, including aromatic amino acids, and H–Gly–Tyr–OH as the amino component, but yields were generally modest: In pure aqueous citrate buffer, the reaction with (+)-L-mandelic acid proceeded with a 5% yield (15% with phenylacetic acid), whereas in aqueous citrate buffer/1,4-butanediol 1:1, the yield was 48% with phenylacetic acid and only 18% with *trans*-3-hexenoic acid. With (4-hydroxyphenyl)acetic acid and (4-aminophenyl)acetic acid, the products were characterized by analytical HPLC and ^{1}H-NMR, but it was not possible to determine the yields. No reaction was detected with *trans*-2-hexenoic acid and homophthalic acid. The same applied to amino acids structurally related to phenylacetic acid such as (*R*)-2-phenylglycine and phenylalanine. It should be considered that in the case of (4-aminophenyl)acetic acid, (*R*)-2-phenylglycine, and phenylalanin, the starting acid can act as amino as well as acyl component.

2.2. *Enzymatic Synthesis in Reverse Micelles.* In *Chapt. 2.1,* enzymatic syntheses have been carried out in H_2O solution. A few methods have been proposed for reactions involving reactants not soluble in H_2O, *e.g.* the use of biphasic systems [11]. More recently, enzymes solubilized in organic solvents with the help of reverse micelles have been proposed [12–14]. Using α-chymotrypsin, we have been able to prepare the H_2O-insoluble, hydrocarbon-soluble tripeptide Z–Ala–Phe–Leu–NH_2 [9]. Now, we describe the enzymatic synthesis of *N*-(phenylacetyl)tyrosine ethyl ester from phenylacetic acid and tyrosine ethyl ester with the help of penicillin acylase in reverse micelles. This reaction is an example of compartmentalization of reactants and products, in the sense that both substrates are more soluble in H_2O than in the organic phase, whereas the product is

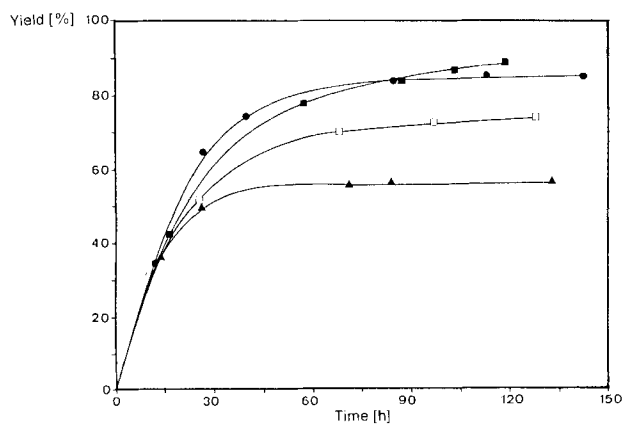


Fig. 2. *Time course for the reaction with penicillin acylase in reverse micelles at different pH values.* Conditions: 0.1M CTAB in $CHCl_3$/isooctane 1:1; $w_o = 12.5$; H_2O-pool concentrations: $[H–Tyr–OEt]_{wp} = 0.1$M, $[PhCH_2COOH]_{wp} = 0.15$M, $[\text{penicillin acylase}]_{wp} = 1.0$ mg/ml; r.t. yields in % of the starting concentration of the ester; pH 5.6 (■); pH 6.1 (□); pH 6.7 (●); pH 7.2 (▲).

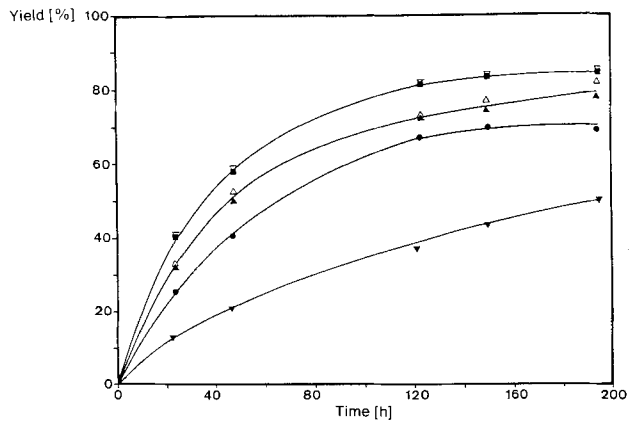


Fig. 3. *Yields at different w_0 values.* Conditions as in *Fig. 2* at pH 6.0; for w_0 = 6.7–12.5, citrate buffer was used, for w_0 = 12.5–17.8, unbuffered H_2O was used; w_0 = 6.7 (▼), w_0 = 10.0 (●), w_0 = 12.5 (citrate; ▲), w_0 = 12.5 (water; △), w_0 = 15.0 (■), w_0 = 16.1 (▽).

better soluble in the organic phase; it should be, therefore, preferentially expelled from the micelle into the organic phase, after synthesis. Enzyme and reagents were first prepared in an aqueous stock solution at a given pH and then added with a microsyringe to 0.1M cetyltrimethylammonium bromide (CTAB) in $CHCl_3$/isooctane 1:1. We first determined the pH optimum for this reaction in H_2O as well as in reverse micelles to be at pH 5.5–6.0. In *Fig. 2*, the time course of the reaction is illustrated for different pH values for H_2O in the H_2O pool. Under the chosen conditions, the equilibrium was reached in *ca.* 100 h, with a normal hyperbolic behavior. No by-product could be detected by HPLC.

We have also investigated the influence of the H_2O content of the micellar solution, expressed as $w_0 = [H_2O]/[CTAB]$, on the yield of the same reaction. Results are shown in *Fig. 3*: the higher the H_2O content, the higher is the yield and the faster is the equilibrium reached. This is at variance with the behavior of most enzymes in reverse micelles for which the maximal activity is found at a small w_0 value, usually within the range 6–12 [13][15–17]. In all the experiments reported until now, enzyme and reactant concentrations were kept constant in the H_2O pool. This means that the higher the overall concentration, the higher is the w_0 value. Note that the yield was 85% at w_0 = 15.0 or 16.1 which is somewhat higher than in H_2O (where, under the best conditions, the yield was *ca.* 77%).

We also investigated further the question of suitable enzyme reactors for use with micellar solutions. Our first experiments of enzymatic peptide synthesis in reverse micelles were carried out using a hollow fiber reactor [9]. This reactor was not suitable for producing preparative amounts of product. Thus, a new ultrafiltration membrane reactor was set up (see *Fig. 4*): reservoir 1 contained the micellar solution with the enzyme and the reactants, reservoir 2 initially only the organic phase. The semipermeable membrane (resistant to organic solvents, *e.g.* $CHCl_3$) between the two reservoirs allowed the separation of the reaction product accumulating in reservoir 2 from the large micellar aggregates. The formation of product was followed again by HPLC. *Fig. 5* shows some typical results.

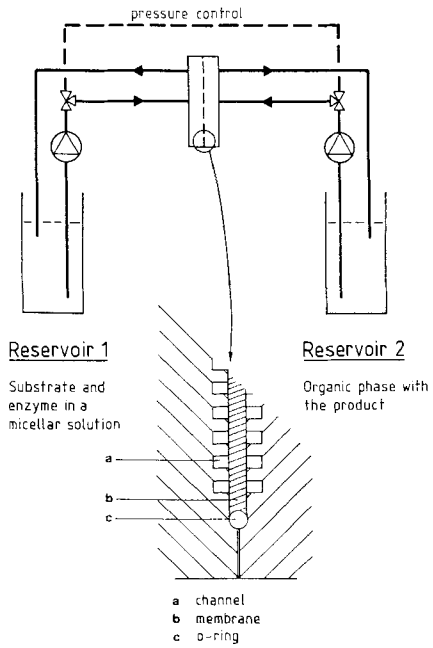


Fig. 4. *Membrane reactor.* See *Exper. Part* and *Fig. 5* for detailed description.

The solutions in both reservoirs remained clear during the whole experiment, and a total yield of > 60% could be achieved. However, the product was distributed between the two reservoirs, *i.e.* 72% in reservoir 1 and 28% in reservoir 2, which actually is the major problem in this reactor configuration. Insufficient diffusion of the product through

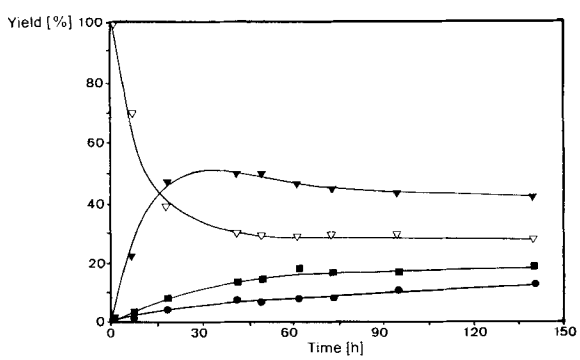


Fig. 5. *Reaction with penicillin acylase in the membrane reactor.* Conditions: *reservoir 1*: 0.1M CTAB in 30 ml of $CHCl_3$/isooctane 1:1; H_2O pool concentration: $[H-Tyr-OEt]_{wp} = 0.1M$, $[PhCH_2COOH]_{wp} = 0.15M$; [penicillin acylase]$_{wp}$ = 1.5 mg/ml; $w_o = 15.0$; dist. H_2O pH 6.0; *reservoir 2:* 30 ml of $CHCl_3$/isooctane 1:1; r.t., *BM-10* membrane (*Berghof GmbH*, Tübingen, Germany) with a mol.-wt. cut-off of 1000; H–Tyr–OEt in reservoir 1 (▽); H–Tyr–OEt in reservoir 2 (●); product in reservoir 1 (▼); product in reservoir 2 (■).

the membrane or the fact that the product is not quantitatively released from the micelles may be responsible for this. Another problem is the enzyme stability: the enzyme was somewhat less stable in the reactor than in a batch reaction. This might be ascribed to a stressing of the reverse micelles containing the enzyme and the reactants by pumping.

The ultrafiltration membrane reactor was also used in a continuous reaction process: both reactants were added from time to time as solids to the reaction solution in reservoir 1 containing the enzyme in reverse micelles at a given w_0 value. In this way, *ca.* 32 mg (yield *ca.* 74%) of product (sum of reservoir 1 and 2) could be obtained after 114 h.

3. Concluding Remarks. – Penicillin acylase is able to efficiently catalyze the coupling to give certain amide bonds. Although this is not its normal reaction, the hydrolysis of penicillin does involve a kind of amide bond. Thus, our study illustrates again the case in which an enzyme specificity can be extended to a larger family of compounds having a functional similarity with the original substrate.

The enzyme specificity for amide synthesis is, however, rather high. This is particularly true for the acyl component where, *e.g.* phenylacetic acid can effectively be used and phenylalanine not. However, strangely enough, hexenoic acid and mandelic acid do react. This indicates stringent structural prerequisites on this substrate moiety, *i.e.* it must have a certain critical length. Concerning the amino component, glycine is not accepted as free amino acid, whereas a glycyl residue is a prerequisite for the coupling of dipeptides and tripeptides. An other interesting point is that some residues bulkier than glycine such as alanine, serine, or aspartic acid can not fit in the active site when they are part of a dipeptide (in particular the dipeptides Met–Tyr or Tyr–Gly do not react), whereas the amino acids Met–OEt or Tyr–OEt are good substrates and provide high yields. We have also noticed that all the dipeptides with a glycyl residue can act as substrate and that the second amino-acid residue of dipeptides of the type Gly–X has an influence only on the yield. All this information points to strict definitions concerning the dimensions and configuration of the active site of the enzyme. As the X-ray structure is still lacking, our data cannot be properly matched with considerations about the active-site binding and mechanism of action. The same holds for the *per se* interesting observations about the partial specificity with D-amino acids.

The studies in reverse micellar system have to be considered as model reactions, the main aim being the gathering of more data on the two most important aspects for bioorganic enzymatic reactions: *i*) the efficiency of micellar compartmentalization in the case of H_2O-insoluble products; *ii*) the efficiency of enzyme reactors which are compatible with the reverse micellar system. The present data contribute to some progress on both issues, although further work appears to be necessary, particularly concerning the enzyme reactors.

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Experimental Part

Materials. Penicillin acylase (E.C. 3.5.1.11) from a modified strain of *Escherichia coli* (ATCC 11 105) was a gift from Dr. *Sauber, Hoechst AG*, Frankfurt, Germany. Amino acids and dipeptides were purchased from *Serva* (Germany), *Bachem, Senn Chemicals*, or *Novabiochem* (Switzerland), *trans*-hexenoic acid (97%) from *Aldrich* (Germany). Cetyltrimethylammonium bromide (CTAB) was a product of *Serva* and was recrystallized from MeOH/Et_2O before use. All org. solvents were UV-grade and were purchased from *Fluka* (Switzerland). All other chemicals were from *Fluka* at the highest available quality and used without further purification.

Enzymatic Synthesis in Aqueous Buffer or in Organic Solvent/Aqueous Buffer. The general conditions for the synthesis were: 0.05M citrate buffer (pH 6.0), 0.1M amino component (*e.g.* Met–OEt or Gly–Leu), 0.15M acyl component (*e.g.* phenylacetic acid), and 0.5 mg/ml of enzyme. When aq./org. solvent mixtures were used, the conc. are given as overall (*e.g.* referred to the total volume of H_2O plus org. solvent); the pH was measured before mixing the buffer with the org. solvent and assumed not to change. The reactions were carried out with 1 ml of soln. at r.t. and with shaking at 400 rpm. To verify whether the plateau of the product conc. was due to a reached equilibrium and not to enzyme denaturation, 0.5 mg/ml of enzyme were added to some of the solns. after 66 h, and no change in the yield was observed.

The reactions were followed at 230, 260, or 280 nm by anal. HPLC with a *Perkin-Elmer-Series-4* liquid chromatograph with a UV detector *LC-90 UV*, an integrator *LCI-100*, and a *Brownlee Lab. RP-18-Spheri-10* column. The column was eluted with MeCN (HPLC-grade) and 0.01M H_3PO_4, the most used conditions being gradient elution, 4 min with 1% MeCN in 0.01M H_3PO_4 (constant), then linear to 75% MeCN and 25% 0.01M H_3PO_4 within 9 min, flow rate 1.5 ml/min, small changes of these conditions were used to perform a better separation. For analysis, 10–20 µl of the reaction solns. were diluted with 0.2 ml of MeCN/0.1M HCl 1:1, 10 µl of the dil. soln. injected, and the yields determined from the HPLC.

The products were purified by prep. HPLC with a *Perkin-Elmer-Series-2* liquid chromatograph and a *Perkin-Elmer* prep. *C-18* column eluted with MeCN and 0.01M H_3PO_4 or by extraction with AcOEt from an acidic aq. phase and washing of the residue of the dried org. phase with Et_2O. All products were analysed by ^{1}H-NMR (DMSO or $CHCl_3$) with a *Bruker HXS 360*.

Enzymatic Synthesis in Reverse Micelles. The syntheses in reverse micelles, with or without the membrane reactor, were carried out in 0.1M CTAB in $CHCl_3$/isooctane 1:1 (*v/v*) with $w_o = 6.7–17.8$. The H_2O pool conc. were equal to that used for the syntheses in the aq. citrate buffer, namely 0.05M citrate (pH 6.0), 0.1M tyrosine ethyl ester and 0.15M phenylacetic acid. An enzyme concentration of 1–1.5 mg/ml was used.

For the synthesis in the membrane reactor, a recirculating thin channel ultrafiltration reactor was used. It was machined in two sections from stainless steel in the workshop of the Polymer Institute of the ETH Zürich. Both pieces contained channels of semi-spherical cross-section with dimensions of 1023 mm length and 2 mm diameter. A 77-mm-diameter *Berghof-BM-10* membrane (mol.-wt. cut-off 1000) separated these tow pieces. The joints between the two sections of the reactor were sealed with *Viton* O-rings. The pressure could be measured at the inlet side of both membrane sides. The total reactor volume was 3.2 ml, and the membrane area available for filtration was 20.6 cm^2, whereas the volume of both reservoirs was 30 ml. Reservoir 1 was filled with 30 ml of a reverse micellar soln. containing the substrates and reservoir 2 with 30 ml of $CHCl_3$/isooctane 1:1. A run with this reactor was started by injecting an aq. soln. of the enzyme in reservoir 1 and then pumping the two solns. through the reactor. Normally, the flow rate during a reaction was 2–4 ml/min. Two *Ismatec* peristaltic pumps were used to ensure accurate delivery of the liquids. All parts of the reactor, including valves and pumps which were in contact with the liquids, were either stainless steel, *Viton*, or PTFE. All reactions were followed by HPLC using the same instruments and column as described before for the syntheses in aq. buffer. Conditions: gradient elution, 4 min with 1% MeCN in 0.01M H_3PO_4 (constant), then linear to 100% MeCN within 20 min, flow rate 2 ml/min; 10–20 µl of micellar reaction soln. were injected and the reaction was followed at 280 nm spectrophotometrically.

Amino-Acid Analysis. The *N*-phenylacetyl derivatives of di- and tripeptides were hydrolysed *in vacuo* at 110° with 1ml 6M HCl and 20 µl of 5% (*w/v*) aq. phenol for 2–8 h. Derivatives of the products were prepared by reaction with dabsyl chloride (= (dimethylamino)azobenzenesulfonyl chloride) for 10 min at 70°; these were analyzed with a *Gilson* HPLC provided with a *Marchery-Nagel Nucleosyl C18* column which was eluted with 0.025M NH_4OAc (pH 6.3)/DMF (40 ml/l) and MECN/DMF (40 ml/l); detection with a *Gilson Holochrome* at 420 nm and a *Hewlett-Packard 3392 A* integrator.

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