ENZYMES IN PREPARATIVE ORGANIC SYNTHESIS: THE PROBLEM OF INCREASING THE YIELD OF PRODUCT

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Dedicated to the memory of Dr Karel Jošt.

Approaches and methods increasing yield of the desired products in enzymatically catalyzed thermodynamically unfavourable syntheses are reviewed. Attention is paid especially to reactions in biphasic systems (water–water-immiscible organic solvent) and their modifications (micellar systems, pseudo-biphasic systems, aqueous biphasic systems, etc.). The dependence of chemical equilibrium and conversion of enzymatically catalyzed processes on the volume ratio of organic and aqueous phases and on the partition coefficients is described. Enzymatically catalyzed syntheses of peptides, amides, esters, NAD-dependent oxidations of hydroxy groups, etc. are given as examples. Various approaches to enzymatically catalyzed processes and their prospects are discussed.

1. INTRODUCTION

During the last 15–20 years enzymes have been widely utilized as catalysts in organic preparations both on the laboratory and industrial scale (see reviews[1–36]). This explosive increase of interest in biocatalysts among organic chemists is due to the

general progress in enzymology on the one hand and the still improving methods of isolation, purification and immobilization of enzymes on the other hand.

The main advantage of enzymes, attractive to synthetic chemists, consists in their high, often absolute, stereo- and regiospecificity. Thanks to this property, chemical transformations of polyfunctional and chiral compounds can be performed without formation of side products, which simplifies the synthesis and isolation (particularly, reduces the number of protective and deprotective steps) and saves the reagents. Another important feature of enzymatic processes, including synthetic ones, is that they proceed under mild physiological conditions, making the destruction of labile compounds less possible.

Unfortunately, under the conditions required for enzyme activity the equilibrium of many important chemical reactions prefers the starting reagents. As an example we can mention the proteases-catalyzed synthesis of a peptide bond (*A*):

$$\mathrm{R{-}COO^- + H_3N^+{-}R' \rightleftarrows R{-}CONH{-}R' + H_2O} \qquad (A)$$

$$\Delta G^0(\mathrm{pH\ 7{\cdot}0}) = +9{\cdot}2\ \mathrm{kJ/mol}$$

or the oxidation of alcohols, catalyzed by dehydrogenases (*B*):

$$\mathrm{{>}CH{-}OH + NAD^+ \rightleftarrows {>}C{=}O + NADH + H^+} \qquad (B)$$

$$\Delta G^0(\mathrm{pH\ 7{\cdot}0}) = +25{\cdot}1\ \mathrm{kJ/mol}\,.$$

As seen from the ΔG^0 values, in neutral aqueous solutions the equilibrium concentration of products is negligible. The usual methods of organic synthesis, such as working in non-aqueous media or at extreme pH values, cannot be applied because the biocatalyst would be inactivated.

Recently, a great number of papers appeared suggesting various methods how to achieve higher preparative yields in synthetic enzymatic reactions with simultaneous retention of the enzyme activity. In the present review we try to compare, evaluate and classify such approaches, paying attention particularly to synthetic enzymatic reactions in heterogeneous (biphasic) or pseudoheterogeneous systems of the "liquid–liquid" type in which the principal aspect is partition of the starting compounds and products between two phases.

2. METHODS OF INCREASING THE YIELD OF DESIRED PRODUCTS

The yield of the product can be increased both by the chemical equilibrium shift (Part 2.1) and by the kinetic approach. In the latter case the high concentration of product does not correspond to the thermodynamic equilibrium and exists for a certain time for purely kinetic reasons (Part 2.2). In addition to these two types,

there are systems in which the reason for the higher concentration of product is still unknown because of our incomplete understanding of the underlying processes (Part 2.3).

2.1 *Thermodynamic Approaches (Shift of Chemical Equilibrium)*

The chemical equilibrium can be shifted using the following methods.

A) The reaction is performed with concentrated solution of the substrates[37] or with large excess of one of the reagents[38–44].

B) The reaction is carried out under conditions so as one (or several) final product precipitates from the reaction mixture. Since the pioneering work of Max Bergmann[45], this is one of the most popular methods how to achieve equilibrium shift towards the final products in enzymatic syntheses of peptide and amide bonds[2,7,10,13,14,17,21,27,31,36]. Later, the method was used in the synthesis of model peptides[46–50], protected aspartam[51], angiotensin[52], and also in the fully enzymatic synthesis of enkefalin[53].

Even recently, this method has not lost its importance in enzymatic synthesis of peptides and amino acid derivatives. Using this principle, we prepared phenylhydrazides of all coded N-acyl-L-amino acids (except proline) with papain as catalyst[54]. 2-Naphthylamides[55], 4-methoxy-2-naphthylamides[56] and other aromatic amides[55] of N-acyl-L-amino acids were prepared analogously. Papain was also successfully used in the resolution of benzyloxycarbonyl-γ-carboxy-DL-glutamic acid[57]. The arising benzyloxycarbonyl-γ-carboxy-L-glutamic acid α-phenylhydrazide, which precipitated from the reaction mixture at pH 4·8, was isolated in a 40% yield. Papain-catalyzed synthesis of dipeptides* Z-L-Gla-L-Xaa-N_2H_2Ph, where Xaa is Leu, Phe, Met, Val, Ala, Asn, started from Z-DL-Gla and L-Xaa-N_2H_2Ph. After the enzymatically catalyzed condensation, the dipeptides Z-L-Gla-L-Leu-N_2H_2Ph and Z-L-Gla-L-Phe-N_2H_2Ph were isolated in the respective yields 43% and 33% (ref.[58]). The principle of precipitation of products from the reaction mixture also proved to be very advantageous in the enzymatically catalyzed synthesis of protected oxytocin[59] and vasopressin[60] fragments. The use of bulky hydrophobic groups for protection of α-amino and α-carboxy groups of amino acids and peptides and sulfhydryl groups in the cysteine moieties are a good illustration of this strategy (under these conditions no protection of the tyrosine side chain is necessary). Protected hexapeptides Z-Cys(Bzl)-Tyr-Xaa-Gln-Asn-Cys(Bzl)-N_2H_2Ph, where Xaa is Ile or Phe, were synthesized by the 2 + (2 + 2) scheme, using papain, α-chymotrypsin (or elastase)

* The following abbreviations are used: NAD nicotinamideadenine dinucleotide, Z benzyloxycarbonyl, BOC tert-butyloxycarbonyl, N_2H_2Ph phenylhydrazide, Gla γ-carboxyglutamic acid, Tyr(SO_3H) O^4-sulfotyrosine, ATP adenosine triphosphate, P_i inorganic phosphate, OBu^t tert-butyl ester, OMe methyl ester, Bzl benzyl.

and thermolysin. The final thermolysin-catalyzed condensation (step 2 + 4) proceeded very rapidly (about 90% conversion in 2 min) and the protected hexapeptides were isolated in yields up to 85%. In all the synthetic steps the molar excess of the amino component was not higher than 10%, the (experimentally determined) amount of the enzyme being relatively low. The thermodynamic approach, based on precipitation of the product from aqueous reaction medium, was also successfully applied to the synthesis of dipeptides containing O^4-sulfotyrosine as one of the amino acid components[61]. The presence of this highly hydrophilic amino acid required a special approach in the synthesis of the dipeptide Boc-Tyr(SO_3 . K)-Leu-N_2H_2Ph: Boc--Tyr(SO_3 . K)-OH was condensed with two equivalents of Leu-N_2H_2Ph in the presence of thermolysin. The desired O^4-sulfodipeptide precipitated from the reaction mixture as the salt Boc-Tyr(SO_3H)-Leu-N_2H_2Ph.Leu-N_2H_2Ph; in this way the negative charge of the sulfonyl group and its solubilizing effect on the synthesized dipeptide was compensated. A similar principle was used in the thermolysin-catalyzed synthesis of protected aspartam[51] and of the protected carboxy-terminal dipeptide cholecystokinin[62]. The mentioned reactions led to Z-Asp-Phe-OMe.Phe-OMe and Z-Asp-Phe-NH_2.Phe-NH_2, respectively.

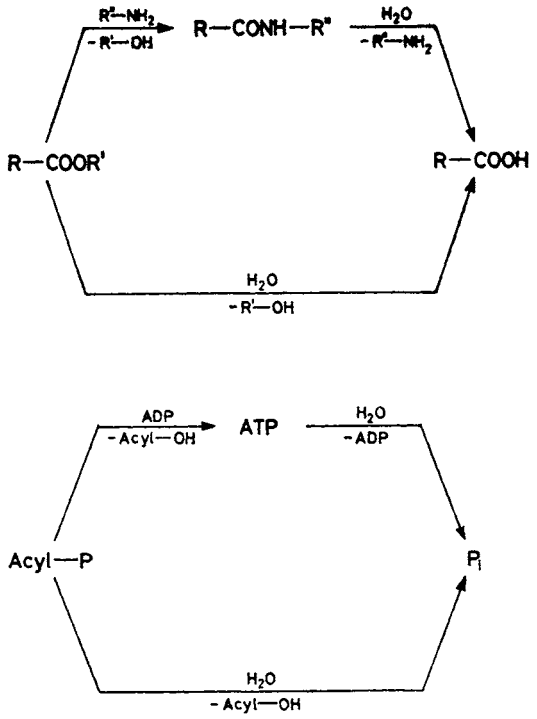
C) Method of consecutive reactions (processes). The reaction equilibrium can be shifted into the desired direction if one or several reaction products react further. Such consecutive reactions may be enzymatic reactions (hydrolysis of a pyrophosphate[63–65], cofactor regeneration[66–68]) or chemical reactions (reaction of acetaldehyde with semicarbazide[69], reaction antigen–antibody[9], enzyme–protein inhibitor[70] and other types of protein–protein interactions[71], complex formation[72]). Water, as one of the condensation products, can be removed from the reaction system by molecular sieves[73].

D) In the synthesis of esters, amides and peptides by reversed hydrolysis, water arises as one of the reaction products. To shift the equilibrium to the desired side, the reaction is performed in systems in which water is more or less replaced by a water-miscible solvent[13,21,23,26,27,39–44,73–86]. A model synthesis of dipeptides has shown that a substantial equilibrium shift may also be achieved by change in pK of the reacting groups[84]. Recently, Randolph and collaborators[87] suggested to replace water by liquid carbon dioxide rather than organic solvents.

2.2 *Kinetic Approach*

At present, the kinetic approach has found its widest and fruitful application in enzymatic synthesis of peptides and β-lactam antibiotics[2,7,9,10,13,14,17,21,23,27,31,35,36,79,88–95] and in regeneration of ATP[96–98]. The method is based on using such starting carboxylic components (esters or amides for peptides and antibiotics or anhydrides for ATP) whose hydrolysis energy is higher than that of the synthesized

product. The enzyme chosen as catalysts, must also exhibit transferase activity. The situation is illustrated in Scheme 1 for the synthesis of peptides or ATP.



SCHEME 1

The desired product is formed as an intermediate in hydrolysis of the starting activated compound and the dependence of concentration of R—CONH—R″ or ATP on time is represented by a curve with a maximum. Obviously, the maximum yield of the product depends on the ratio of the hydrolytic and transferase reaction rates which in turn depends both on thermodynamic parameters and on kinetic properties of the catalyst. It is worth notice that in the thermodynamic approach (2.1) the enzyme only reduces the time required for obtaining the maximum yield, without affecting this yield.

In the region of enzymatic synthesis of peptides this approach is suitable particularly in cases where properties of the synthesized peptide prevent its precipitation from the reaction medium (high solubility) or no other possibility exists how to shift the chemical equilibrium toward synthesis. The choice of proteolytic enzymes is restricted only to serine and thiol proteases which are capable of cleaving both the amide and ester bonds. In the alkaline region of pH the esterase activity of these enzymes prevails

over the protease activity[93]. The peptide bonds of the enzymatically synthesized peptide, which during the reaction remains dissolved in the reaction medium, should not be further split by the enzyme. This property, typical for papain whose peptidase activity at pH 8 and higher is negligible, was utilized in the stepwise synthesis of Leu-enkefalin[99] and several shorter peptides[88]. In our laboratory we studied[61] the reaction of Boc-Tyr(SO_3.Na)-OMe with Leu-N_2H_2Ph, catalyzed with papain at pH 9. The arising dipeptide Boc-Tyr(SO_3.Na)-Leu-N_2H_2Ph remained dissolved in the reaction medium without being further attacked by the enzyme. Later, we extended this model reaction[62] to the preparation of the carboxy-terminal octapeptide of cholecystokinin (CCK-8) which in its sequence Asp-Tyr(SO_3^-)-Met-Gly-Trp-Met--Asp-Phe-NH_2 contains O^4-sulfotyrosine. The classical chemical synthesis of CCK-8, based on sulfation of free tyrosine hydroxyl in the protected octapeptide, meets some problems. To overcome them, we condensed the sulfated dipeptide Boc-Asp(OBu^t)--Tyr(SO_3.Na)-OMe with the hexapeptide Met-Gly-Trp-Met-Asp-Phe-NH_2 under catalysis with a very small amount of papain at pH 9. To synthesize another analogue of CCK-8, we reacted the sulfodipeptide with the hexapeptide Met-Pro-Trp-Met--Asp-Phe-NH_2. No cleavage of peptide bonds was observed and the protected octapeptides were isolated by preparative HPLC in the respective yields of 42% and 44%.

Factors affecting the maximum yield of the product are reviewed in detail, with many references, by Kasche[23] (exemplified by synthesis of peptides and antibiotics) as regards enzymatic mechanisms.

As an actual transferase reaction we can denote the intramolecular transformation of peptides, containing amino-terminal glutamine moiety or its derivatives, into pyroglutamyl peptides and pyroglutamic acid derivatives, catalyzed by glutaminyl tRNA-cyclotransferase (E.C.2.3.2.5), present in commercial samples of papain. Thus, on treatment of Gln-N_2H_2Ph, Gln-Leu-N_2H_2Ph and Gln-His-Pro-NH_2 with crude papain, Glp-N_2H_2Ph, Glp-Leu-N_2H_2Ph and Glp-His-Pro-NH_2 (thyrotropin releasing factor), respectively, were prepared on the preparative scale[100].

2.3 *Approaches of Unidentified Mechanism*

It has been shown[101-103] that α-chymotrypsin, immobilized in matrices of certain properties, catalyses the synthesis of esters[101,102] or peptides[103], the yield of the product being substantially higher than in water. Addition of natural (not immobilized) enzyme to the system decreased the product concentration to the level characteristic for water[101]. Change in the matrix properties by varying the water content also leads to changes in the product concentration[102,103]. Undoubtedly, properties of the matrix affect the equilibrium of an immobilized enzyme-catalyzed reaction; however, a physico-chemical mechanism of the observed behaviour is hitherto unknown.

3. ENZYMATICALLY CATALYZED SYNTHESIS IN BIPHASIC SYSTEMS

All the approaches mentioned above have only a limited application. Such limitations may be connected with properties of the catalyzed reaction, technological realization of the process or mechanism of the catalysis[17,26,27]. In 1977 an equilibrium approach has been suggested that obviously represents a more general solution of the problem of increasing yield in enzymatic preparative processes[104]. The method is based on performing the reaction in a biphasic system composed of water and a water-immiscible organic solvent. The enzyme is present in the aqueous phase and therefore no stabilization of the enzyme against denaturation by the organic solvent is necessary. Inactivation on the interface can easily be avoided using immobilization (see 3.3). The biphasic character of the system allows to vary the effective equilibrium constant practically for any reaction (see 3.2).

3.1 *Retrospect*

Reese and Mandels[105] have apparently been the first authors who described a biphasic "water–water-immiscible organic solvent" system as a medium for preparative synthesis. These authors prepared a column in which the stationary phase was aqueous solution of an enzyme (β-glucosidase or invertase) absorbed in cellulose and the mobile phase was butanol with dissolved substrates (and the corresponding reaction products). These experiments aimed at preparation of a catalyst, suitable for a continuous reactor, in another way than by adsorption. The method of Reese and Mandels could not compete with the later discovered and very widely used immobilization of enzymes and has been used only rarely[106].

A better fate has met another idea – to use biphasic systems for enzymatic transformations of water-insoluble organic compounds such as steroids, terpenes or hydrocarbons. The method can be applied to preparative-scale conversions in small volumes making the isolation easier and saving the enzyme. The most intensive investigations in this direction have been performed by Fukui[20], Antonini[15] and Lilli[107] and their coworkers.

Only in the late seventies (vide supra) a paper by Martinek's group has shown for the first time that chemical equilibria in biphasic systems can be controlled on the basis of different partition coefficients of starting compounds and products. This principle was illustrated by synthesis of N-acetyl-L-tyrosine ethyl ester from N-acetyl-L-tyrosine and ethanol in 100% yield[104].

3.2 *General Considerations on Chemical Equilibria in Biphasic Systems "Water–Water-Immiscible Organic Solvent"*

In this part we shall consider how the equilibria of various chemical reaction types change compared with those in homogeneous solutions, and also how they are

affected by the partition coefficients (i.e. by the organic phase character and volume ratios of the phases).

Reaction A ⇄ B. In a biphasic system the reagents are distributed between water and an organic solvent:

$$\begin{array}{ccc} (\mathrm{A} & \overset{K_{aq}}{\rightleftharpoons} & \mathrm{B})_{aq} \\ \Big\uparrow\Big\downarrow P_A & & P_B\Big\uparrow\Big\downarrow \\ (\mathrm{A} & \underset{K_{org}}{\rightleftharpoons} & \mathrm{B})_{org} \end{array} \qquad (C)$$

The apparent equilibrium constant in reaction (*C*), which equals the ratio of total concentrations of A and B, referred to the total volume of the biphasic system, i.e. $K_{app} = [B]_{tot}/[A]_{tot}$, can be written[104,108–111] as:

$$K_{app} = K_{aq}\frac{1 + \alpha P_B}{1 + \alpha P_A} \qquad (1)$$

or

$$K_{app} = K_{org}\frac{1 + 1/\alpha \,.\, P_A}{1 + 1/\alpha \,.\, P_B}, \qquad (2)$$

where K_{aq} and K_{org} are the equilibrium constants of reaction (*C*) in water and in the organic solvent, respectively, $\alpha = V_{org}/V_{aq}$ is the volume ratio of the organic and aqueous phases, and $P_A = [A]_{org}/[A]_{aq}$, $P_B = [B]_{org}/[B]_{aq}$ are the respective partition coefficients of the reagents A and B. Here and in the following text the subscripts org and aq relate the given quantity to the solvent and aqueous phase, respectively.

As seen from Eq. (*1*), the value of K_{app} changes monotonously with the change of α and, depending on the organic solvent, it either increases (for $P_A < P_B$) or decreases (for $P_A > P_B$). At sufficiently high values of α (small volume of the aqueous phase) the equilibrium constant nears a limit value, $K_{aq}\,P_B/P_A \equiv K_{org}$. Partition coefficients of organic compounds between water and commercially available organic solvents range usually[112] from 10^{-4} to 10^{+4} and therefore the maximum equilibrium shift (K_{app}/K_{aq}) can in principle be quite considerable.

As an example, let us consider the oxidation of 2-methylpropanol, catalyzed with alcohol dehydrogenase:

$$(CH_3)_2CHCH_2OH + NAD^+ \rightleftarrows (CH_3)_2CHCHO + NADH + H^+ \qquad (D)$$

This reaction has been well studied in water[113]. In a biphasic system[104,108–111] the equilibrium is described practically by an equation analogous to Eq. (*1*) because neither the oxidized (charged) nor the reduced form of the employed cofactor diffuses from water into the organic solvent and, consequently, for NAD^+ and NADH the partition coefficients are practically zero. Thus, we write

$$K_{app} \approx K_{aq} \frac{1 + \alpha P_{ald}}{1 + \alpha P_{alc}}, \qquad (3)$$

where the subscripts ald and alc refer to the aldehyde and alcohol, respectively. The experimental results are given in Fig. 1. Two points are worth notice. First, the studied reaction represents a system in which both the starting and the final compounds are of almost the same hydrophobicity. Thus, in the "classical" system water–octanol the partition coefficients for the aldehyde and the alcohol differ only by 12% (ref.[112]). Nevertheless, in some other solvents (Fig. 1) these partition coefficients differ sufficiently (e.g. due to specific solvation effects). As a result, the apparent equilibrium constant can be varied within rather wide limits (almost two orders of magnitude) by variation of the organic phase. Second, as seen from Fig. 1, the theoretical curve *1* (constructed according to Eq. (*3*), using separately determined[110] partition coefficients for 2-methylpropanol and 2-methylpropanal) agrees satisfactorily with the experiment.

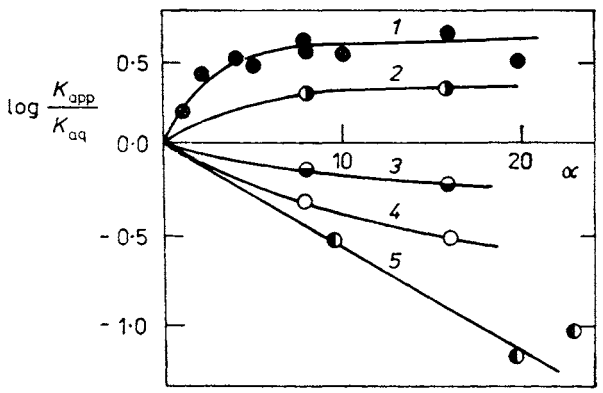


FIG. 1

Dependence of the apparent equilibrium constant, K_{app}, on the volume ratio of the phases, α, and on the organic phase for alcohol dehydrogenase-catalyzed oxidation of 2-methylpropanol in biphasic water-organic systems. Organic phase: *1* hexane, *2* hexane–ethyl acetate (87·5 : 12·5), *3* hexane–ethyl acetate (50 : 50); *4* hexane–ethyl acetate (25 : 75), *5* ethyl acetate. The curve *1* is constructed according to Eq. (*3*) using independently obtained partition coefficients[110]

Reaction A + B ⇄ C + D. The apparent equilibrium constant depends on the volume ratio of the organic and aqueous phases (α) in a more complex manner[104,108–111] than for the above-mentioned first-order reaction:

$$K_{app} = K_{aq} \frac{(1 + \alpha P_C)(1 + \alpha P_D)}{(1 + \alpha P_A)(1 + \alpha P_B)} . \qquad (4)$$

Typical cases are given in Fig. 2.

In principle, the maximum equilibrium shift, observed in a biphasic system at very low water content, may be even greater than that for a simple system A ⇄ B (vide supra), because the expression for the maximum shift, $K_{app}/K_{aq} = P_C P_D / P_A P_B$ (for $\alpha \to \infty$), contains a greater number of partition coefficients. A more important feature of chemical equilibria, involving second-order reactions, is that the dependence of K_{app} on α at the given ratios of partition coefficients may have an extremum (cf. Fig. 2). In other words, in a system of two immiscible solvents the apparent equilibrium constant may exceed the limit values (upper as well as lower) for the equilibrium in each phase.

The physico-chemical basis for the existence of an extremum can be illustrated by the following example. Suppose that one of the starting reagents (A) is present entirely in one phase (water) whereas the other (B) is entirely in the other phase (organic solvent). In this case the reaction equilibrium in a biphasic system will be almost completely shifted to the starting compounds ($K_{app} \to 0$), regardless of the

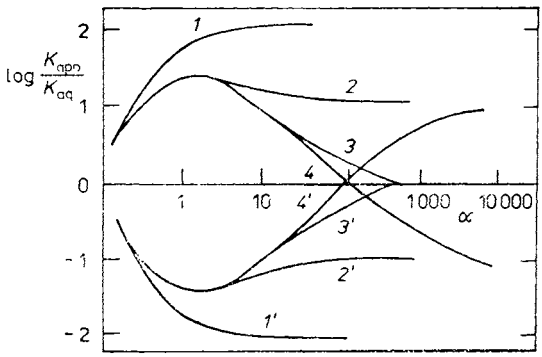


FIG. 2

Dependence of the apparent equilibrium constant, K_{app}, on the volume ratio, α, for reaction A + B ⇄ C + D in a water-organic system and for the following partition coefficients: curves *1–4*: $P_A = P_B = 1$, $P_C = 100$, $P_D = 1$ (*1*), $P_D = 10^{-1}$ (*2*), $P_D = 10^{-2}$ (*3*), $P_D = 10^{-3}$ (*4*); curves *1′–4′*: $P_C = P_D = 1$, $P_A = 100$, $P_B = 1$ (*1′*), $P_B = 10^{-1}$ (*2′*), $P_B = 10^{-2}$ (*3′*), $P_B = 10^{-3}$ (*4′*)

equilibrium constants in the individual phases. The reason is that the compounds A and B are separated and cannot interact with each other. On the other hand, the reaction equilibrium will be shifted completely to the product side ($K_{app} \to \infty$; irrespective of the equilibrium constants in one or another phase) if the final products are fully separated from each other (if one product (say C) is dissolved exclusively in water whereas the other one (D) in the organic solvent).

The extremum character of equilibrium in a biphasic system has been indeed observed (when varying the volume ratio of the organic and aqueous phases) for the α-chymotrypsin-catalyzed synthesis of N-benzoyl-L-phenylalanine ethyl ester from ethanol and N-Bz-L-Phe (refs[110,111]) (Fig. 3). Whereas in water the ester arised in negligible yield[114], in biphasic systems (at optimum volume ratio of the organic and aqueous phases) the yield was 80%, 64%, 62% and 26% for chloroform, benzene, tetrachloromethane and diethyl ether, respectively[110,111]. Thus, the use of a biphasic system raised the yield of ester from practically zero to a preparative level.

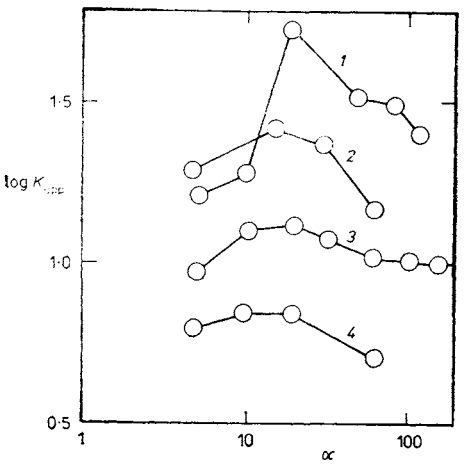


FIG. 3

Dependence of apparent equilibrium constant, K_{app}, on the volume ratio of the phases, α, and on the character of the organic phase for the synthesis of N-benzoyl-L-phenylalanine ethyl ester, catalyzed with α-chymotrypsin, in biphasic water-organic systems. Organic phase: *1* chloroform, *2* benzene, *3* tetrachloromethane, *4* diethyl ether. (K_{app} in water is 10^{-4} (not shown))

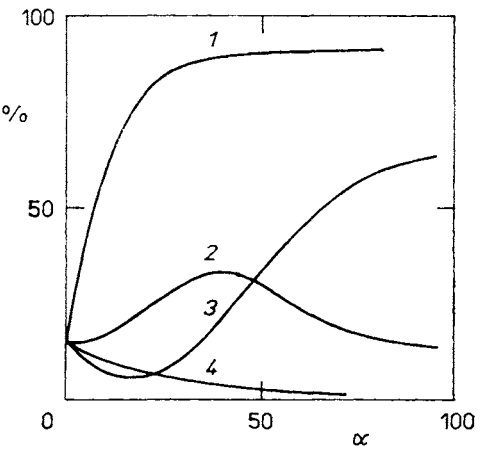


FIG. 4

Plot of product yield in reaction A + B ⇄ C + H_2O vs volume ratio of phases, α, for some values of partition coefficients: *1* $P_A = P_B = 1$, $P_C = 50$; *2* $P_A = P_B = 10^{-1}$, $P_C = 10^{-2}$; *3* $P_A = 10$, $P_B = 10^{-2}$, $P_C = 1$; *4* $P_A = P_B = 1$, $P_C = 10^{-1}$. In all cases $K_{aq} = 10$, [A] = [B] = 1 (refs[110,111])

Reduction of water content. In some reactions such as synthesis of ethers, esters, amides, peptides, etc. water is one of the reaction products:

$$A + B \rightleftarrows C + H_2O \qquad (E)$$

Therefore, in aqueous solutions the equilibrium of such reactions is shifted toward the starting reagents because the concentration of water in the system is high (55·5 mol l^{-1}). On transition to biphasic systems the water content decreases and therefore the yield of the product C should, in principle, increase. However, in a biphasic system the amount of C depends on the water content (aqueous phase) in two ways: according to the law of mass action

$$[C]_{tot} = K_{app}[A]_{tot}[B]_{tot}/[H_2O]_{tot} \qquad (5)$$

and via the equilibrium constant, which is determined by a function of the type (*4*) and, consequently, depends on α. We can thus write[110,111]:

$$[C]_{tot} = K_{app}\frac{[A]_{tot}[B]_{tot}}{[H_2O]_{tot}} = K_{aq}\frac{[A]_{tot}[B]_{tot}(1 + \alpha P_C)(1 + \alpha)}{55{\cdot}5(1 + \alpha P_A)(1 + \alpha P_B)}, \qquad (6)$$

where

$$[H_2O]_{tot} = \frac{[H_2O]_{aq}}{1 + \alpha} = \frac{55{\cdot}5}{1 + \alpha}.$$

According to Eq. (*6*) the dependence of [C] on the composition of the aqueous phase may be quite complicated (Fig. 4). Some values of partition coefficients may lead even to reduction of the yield of product C, notwithstanding the lower concentration of water as the second product. This is the result of the fact that an increase in α results in greater decrease of the apparent equilibrium constant than of the total water concentration in the system (Eq. (*6*)).

Recently, Halling[115] suggested that, because the vapour pressure of water over biphasic systems is practically equal to that over pure water, the thermodynamic activity of water in a biphasic system should be 55·5 at any volume ratio of the phases α. His statement implicitely uses the Raoult's law which, as known, relates directly the activity of a component with its partial vapour pressure over the solution at equilibrium. Apparently, in the given case the assumption of Halling is not correct. The Raoult's law holds only for ideal solutions and is not obeyed by most real systems. Moreover, the existence of biphasic systems alone represents an example of the so-called positive deviation from the Raoult's law. The separation of liquids (formation of biphasic systems) occurs just because in a certain concentration range

the theory predicts decrease of the vapour pressure of a component with increase of its concentration in a homogeneous binary mixture. In actual fact, however, this is not the case and the homogeneous system is unstable and separates into two phases[116].

Shift of ionic equilibria. Many reactions involve ionization of the reagents, e.g.:

$$HA + B \overset{K_{eq}}{\rightleftharpoons} C + D, \quad \text{where} \quad HA \overset{K_a}{\rightleftharpoons} H^+ + A^- \qquad (F)$$

or

$$A + B \overset{K_{eq}}{\rightleftharpoons} C + D, \quad \text{where} \quad B + H^+ \overset{K_B}{\rightleftharpoons} BH^+ \qquad (G)$$

On transition from water to biphasic systems the equilibrium constant of not only the chemical reaction (K_{eq}) but also of the ionization processes changes because the partition coefficients for the ionized and non-ionized species are different. The equilibrium shift for the whole process will undoubtedly reflect the changes in all these constants.

We shall now analyse the changes in ionization constants in biphasic water-organic systems relative to aqueous solutions and evaluate the possible ways how to increase yields in enzyme-catalyzed synthetic preparative processes.

Let us consider an ionization process, e.g. ionization of an acid, in a biphasic water-organic system[117,118]:

$$\begin{array}{l} (HA \overset{K_a}{\rightleftharpoons} H^+ + A^-)_{aq} \\ \quad \Big\updownarrow P_{HA} \\ (HA)_{org} \end{array} \qquad (H)$$

We can justifiably assume[112] that the organic phase dissolves only the uncharged form of the acid (except strongly polar solvents that may extract charged compounds as ion pairs from the aqueous phase).

The acid ionization constant in a biphasic system can be determined according to the Henderson–Hasselbach equation:

$$pK_{a,app} = pH - \log \frac{[A^-]_{tot}}{[HA]_{tot}}, \qquad (7)$$

where $[A^-]_{tot}$ and $[HA]_{tot}$ are the respective total concentrations of the ionized and nonionized acid, based on the total volume of the biphasic system; the pH value

relates to the aqueous phase. Thus[117,118]

$$pK_{a,app} = pK_a + \log(1 + \alpha P_{HA}) . \qquad (8)$$

Analogically, the protonation of a base B in a biphasic system:

$$\begin{array}{l} (B + H^+ \overset{K_B}{\rightleftharpoons} BH^+)_{aq} \\ \quad \Big\updownarrow P_B \\ (B)_{org} \end{array} \qquad (I)$$

is characterized by the apparent equilibrium constant[117,118]

$$pK_{B,app} = pK_B - \log(1 + \alpha P_B) . \qquad (9)$$

As seen, in biphasic water-organic systems the pK_a value of an acid is higher by the increment $\log(1 + \alpha P_{HA})$ whereas the pK_B value of a base is lower by $\log(1 + \alpha P_B)$. Since the partition coefficients for organic acids and bases can reach values of $10^2 - 10^4$ (ref.[112]), in a system containing e.g. 1 vol.% of water (i.e. $\alpha = 100$) the effective pK shift may amount to 4–6 pH units.

Ionization equilibria for 2,4-dinitrophenyltryptophan (acid) and neutral red (base) as models in water-organic systems have been studied[117,118]. It has been found that: *1*) compared with aqueous solutions, the effective pK in such systems does shift, the shift being 5 and more pH units, *2*) for neutral red, the experimental plot of $pK_{B,app}$ against α shows a good fit with the theoretical curve constructed according to Eq. (*9*), using the independently determined partition coefficient P_B.

Let us consider, how these pK shifts can affect the equilibria of chemical reactions in biphasic water-organic systems and how the yield of the desired product can be increased.

Matching the pH *optimum of the catalyst with the reaction equilibrium.* Often the pH optimum of the enzyme stability and catalytic activity differs substantially from pH values thermodynamically favourable for the synthesis of a given product. Preparation of β-lactam antibiotics (thermodynamic optimum at pH 3–5, depending on the character of the compound)[24], *p*-nitroanilides (optimum pH 2–3) and esters of N-substituted amino acids (optimum pH < 3) may serve as an example (refs[114,119]). On the other hand, most enzymes catalyzing synthetic reactions (penicillin amidase, trypsin, α-chymotrypsin and other proteases) are active and stable only at neutral or nearly neutral pH values.

This contradiction can be solved by using biphasic water-organic systems instead of water. The method is illustrated by the α-chymotrypsin-catalyzed synthesis of

N-benzoyl-L-phenylalanine ethyl ester:

$$\begin{array}{l} \mathrm{R{-}COOH} + \mathrm{R'OH} \rightleftharpoons \mathrm{R{-}COOR'} + \mathrm{H_2O} \qquad (J) \\ \quad \Big\uparrow\Big\downarrow K_a \\ \mathrm{R{-}COO^-} \end{array}$$

The pH-dependence of the equilibrium constant for this reaction in water[114] (curve *1*) is given in Fig. 5. As seen, only pH values lower than 3 are favourable for the synthesis whereas at about neutral conditions (highest activity of α-chymotrypsin) the equilibrium is shifted practically completely to the starting compounds. Important for this situation is that one of the reagents (acid) is dissociated under these conditions. In water, therefore, this enzymatic reaction cannot be of preparative importance.

On transition to biphasic water-organic systems[117,118,120,121] the apparent pK_a value of an acid increases (Eq. (*8*)); consequently, the pH-dependence of the equilibrium constant shifts towards neutral or basic region of pH. The curve 2 in Fig. 5 depicts this dependence for the reaction (*J*) in a biphasic system water–chloroform (5 : 100, v/v). As seen, the p*K* value of the acid increases by more than 3 units compared with aqueous solutions (curve 2 is shifted to the right along the abscissa). As a result, the thermodynamically favourable pH value matches that of catalytic activity and stability of α-chymotrypsin. Moreover, the limit (i.e. pH-independent)

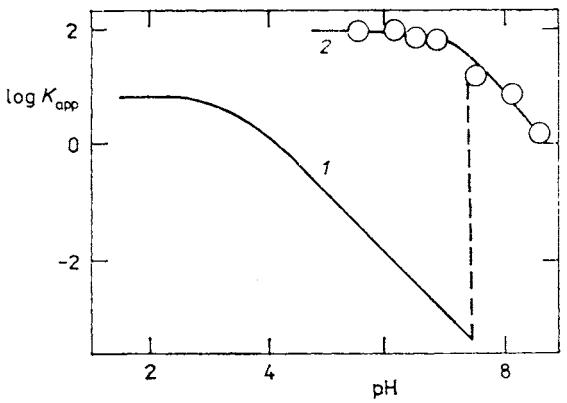


FIG. 5

Plot of apparent equilibrium constant, K_{app}, for synthesis of N-benzoyl-L-phenylalanine ethyl ester vs pH in water (*1*) and in a biphasic water–chloroform system ($\alpha = 20$; *2*). Curve *1* is calculated according to the relation $K_{aq} = K_{nonion,aq}/(1 + K_a/[H^+])$ using $K_{nonion,aq} = 7$ (ref.[123]) and $pK_a = 3{\cdot}35$ (refs[117,118])

value of the equilibrium constant increases by more than one order of magnitude (shift of curve 2 higher along the ordinate axis) as the result of extraction of the product ester from the aqueous into the organic phase. Thus, the overall change of the equilibrium constant at pH 7·5 (optimum catalytic activity and stability of α-chymotrypsin) amounts to almost 5 orders of magnitude. Thanks to this fact it has been possible to reverse the hydrolysis reaction practically completely to the side of synthesis at neutral pH values: the yield of the ester in the given system is about 80–100% (depending on the conditions) whereas in water it does not exceed 0·01%.

Synthesis of peptides. A reaction, involving two ionogenic groups, can be exemplified by synthesis of a peptide bond:

$$\begin{array}{ccccc} \mathrm{RCOOH} & + & \mathrm{R'{-}NH_2} & \rightleftarrows & \mathrm{RCONHR'} + \mathrm{H_2O} \qquad (K) \\ \Big\uparrow\Big\downarrow K_a & & \Big\uparrow\Big\downarrow K_b & & \\ \mathrm{RCOO^-} & & \mathrm{R'{-}NH_3^+} & & \end{array}$$

The pK_a values of carboxylic acids usually range from 3 to 4 and the pK_B of amines from 8 to 10 (refs[114,122]); therefore in water one of the starting reagents exists practically completely in the charged form (Fig. 6, curves *1* and *1'*) over the whole pH range and the overall reaction equilibrium is shifted much toward the starting reagents[123].

In biphasic water-organic systems, the apparent pK_a value of an acid usually increases and the pK_b of a base decreases. This means that at neutral pH the proportion of uncharged species increases both for acids and amines (Fig. 6, curves 2 and 2'), shifting the equilibrium of reaction (*K*) toward the synthesis. Usually, the uncharged peptide is more soluble in the organic solvent than the starting compounds and this better solubility is another reason for the equilibrium shift. Finally, the third cause is that the equilibrium yield of the reaction product in a water-organic system should increase also due to the reduced amount of water.

This approach has been applied[117,118,124–127] to the α-chymotrypsin-catalyzed synthesis of N-acetyl-L-tryptophyl-L-leucinamide:

$$\text{N-Ac-L-Trp} + \text{L-LeuNH}_2 \rightleftarrows \text{N-Ac-L-Trp-L-LeuNH}_2 + \mathrm{H_2O} \qquad (L)$$

In the biphasic system ethyl acetate–2 vol.% water the equilibrium constant increased by more than three orders of magnitude (Fig. 7, curves *1* and 2) compared with the aqueous medium, making the synthesis of the dipeptide practically quantitative. In water, under otherwise the same conditions, the yield was not higher than 0·1%.

Simultaneously with the Martinek's group[117], Jakubke and coworkers independently utilized biphasic water-organic systems for enzymatic synthesis of peptides[21,128], employing, however, mainly the kinetic approach.

Synthesis of ionic products. It might seem that the biphasic approach is not applicable to reactions leading to ionic products because they are insoluble in the organic phase and thus cannot be distributed throughout the whole system. However,

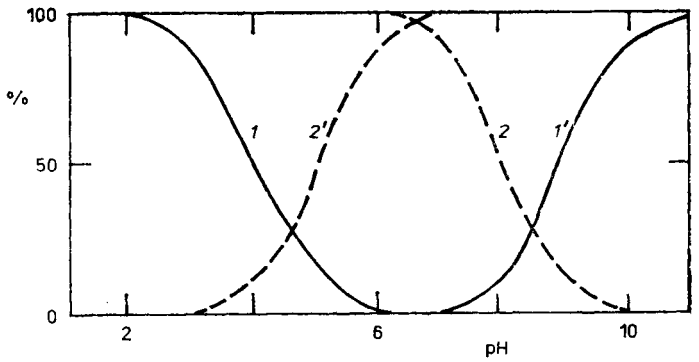


FIG. 6

Theoretical titration curves for the carboxylic (curves *1* and *2*) and amine (curves *1'* and *2'*) components in reaction (*K*) in water (curves *1* and *1'*) and in a biphasic water-organic system (curves *2* and *2'*). Calculated for $\alpha = 100$ and $P_{acid} = P_{amine} = 10^2$ (refs[117,118])

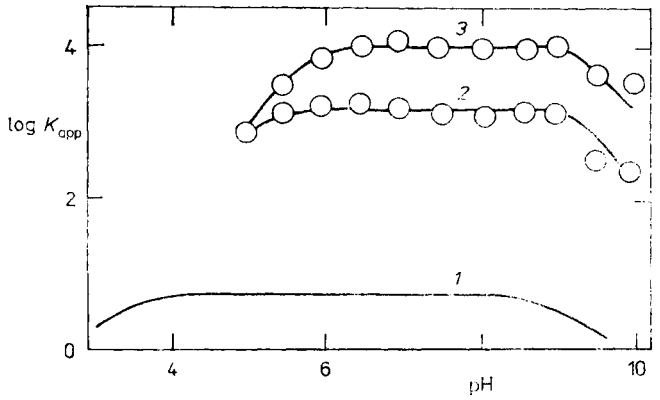


FIG. 7

Plot of apparent equilibrium constant versus pH for synthesis of N-Ac-L-Trp-L-Leu-NH_2 in water (*1*) and in a biphasic water–ethyl acetate system at $\alpha = 5$ (*2*) and $\alpha = 50$ (*3*) (refs[117,118])

this hindrance can be overcome by employing hydrophobic counterions. The approach was tested with the synthesis of glycerophosphate from glycerol and an inorganic phosphate[108], and benzylpenicillin from 6-aminopenicillanic and phenylacetic acids[129]. In both bases, the negatively charged products diffused into the organic phase (chloroform) thanks to the presence of the hydrophobic tetrabutylammonium counterion, the yields being higher than in aqueous solutions.

3.3 *Methodical Aspects*

This part deals with the published methods of preparing biphasic (or pseudo--biphasic) water-organic systems.

1. The reaction vessel contains two immiscible liquids: water (a buffer solution) and an organic solvent. A soluble form of the enzyme is present in the aqueous phase, the starting reagents are introduced into the aqueous and/or the organic phase. To accellerate the reaction, the mixture can be stirred.

2. Method 1 involves the danger of inactivation of the biocatalyst at the liquid/liquid interface. Such inactivation can be avoided by immobilization[127,128,130] or by impregnation of a porous carrier with the aqueous phase, which also sharply reduces the contact of the catalyst with the interface[17,104].

3. The inactivation of the enzyme on the interface may also be suppressed by a porous membrane[131].

4. Preparatively very attractive are pseudoheterogeneous (microheterogeneous) micellar systems in which one phase is the inner region (core) of a surfactant micelle. Micellar pseudo-biphasic systems can be realized in two ways: *1*) a solution of the surfactant in water[132] (water as the hydrophilic phase, the inner micellar region, consisting of hydrocarbon moieties of the surfactant, as the hydrophobic phase) and *2*) a solution of the surfactant in an organic solvent (the organic solvent as hydrophobic phase, the inner region of the micelle, consisting of polar segments of the surfactant and solubilized water, as the hydrophilic phase). The second alternative (reversed micelles) is of particular interest because it allows to decrease the water content in the system to several % or even less. This is very promising with regard to the equilibrium shift of water-forming condensations[133,135].

5. Microheterogeneous systems (microemulsions) may arise and exist indefinitely (i.e. be thermodynamically stable) even in the absence of surfactants. As has been shown recently, in such stable microemulsions the catalytic activity of enzymes is retained and, consequently, such systems can be utilized preparatively[136]. Isolation of the product in the absence of surfactants is substantially simpler than with the micellar systems.

6. In principle, all the above-mentioned considerations concerning equilibrium shifts in biphasic water-organic systems hold also for other heterogeneous systems,

consisting e.g. of two aqueous phases[137]. To achieve heterogeneity, polymers or salts (such as polyethylene glycol, dextran or phosphates) are added to the system.

7. During the last years a series of methods have been devised that allow to perform enzymatic processes (including syntheses) in practically anhydrous organic solvents. The enzymes are present in the reaction mixture as an insoluble lyophilisate powder[33,138] or in an immobilized form[139] and activity of such preparations is comparable with that of enzymes in aqueous solutions. The monolayer of water on the surface of the protein molecules in the lyophilized preparations is evidently sufficient for the catalytic activity.

8. Enzyme molecules, modified by covalent attachment of high-molecular polyethylene glycol, are soluble in organic solvents and their activity is preserved[140]. It is possible to attach iron particles to the polyethylene glycol chains making thus the catalyst magnetic[141] and thus well separable after the reaction.

As seen, the choice of methods for performing preparative enzymatic reactions in biphasic (or pseudo-biphasic) water-organic or water-polymeric systems is quite large. The selection of a particular system depends on the activity and stability of the catalyst in the given system, solubility of the starting compounds and products, and product isolation. However, of prime importance is the consideration of partition coefficients of the starting and arising compounds (strictly speaking, the difference between these partition coefficients because it determines the magnitude of the equilibrium shift to the product side, i.e. the yield.)

4. CONCLUSION

Enzymes are more and more utilized in the everyday practice in the synthesis, modification or protection (deprotection) of peptides, proteins, nucleic acids, steroids, lipids, antibiotics and other types of compounds. Many processes have already been employed on an industrial scale or in pilot plant production. The synthetic potentialities of enzymes in laboratory or industry are still far from being exhausted and their introduction will undoubtedly be promoted by elaboration and improvement of methods and approaches increasing the yield of products.

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