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Enzyme Catalysis in Organic Synthesis

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The present state of enzyme catalysis and the prospects for its introduction in organic synthesis are examined. The physicochemical approaches whereby the yield of the desired product can be increased under conditions favourable for biocatalysis (at the optimum of the catalytic activity and stability of the enzyme) are analysed. Together with classical equilibrium and kinetic preparative methods, the thermodynamic features and general methodological aspects of a new approach—enzymatic synthesis in two-phase systems comprising water and a water-immiscible organic solvent—are discussed in detail.
The bibliography includes 170 references.

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I. INTRODUCTION

The application of biological catalysts (enzymes) in organic synthesis appears to be extremely promising and in many instances has a number of significant advantages over the classical methods of synthetic chemistry. This is due primarily to the fact that enzymes exhibit a unique specificity (selectivity), i.e. the reactions which they catalyse proceed as a rule via a single pathway without side processes. Furthermore, the unsurpassed catalytic activity of enzymes ensures synthesis under mild conditions. Consequently the practical application of biocatalysis might result in the saving of energy and resources and in addition might decrease the damage caused by modern chemical technology to the environment.

Despite the evident advantages of enzyme catalysis, the decisive steps in this field were outlined only the last 10–15 years. This is due, firstly, to the general progress in the study of the properties, structure, and functions of enzymes (see, for example, Refs.1–4), and, secondly, to the introduction of immobilised enzymes[5,6]. As a result of immobilisation (for example, the covalent attachment of the enzyme to a water-insoluble particle, the encapsulation of the enzyme in a gel, and the adsorption of the enzyme on a carrier), it is possible to separate the heterogeneous catalyst from the reaction medium and to use it again. Furthermore, immobilisation constitutes a method of the specific modification (improvement) of practically important properties of enzymes[5-7] and in the first place of their stability[8-11]. Thirdly and finally, enzymes have become available in large amounts in connection with the development of methods for their microbiological synthesis and isolation[12,13].

The successes achieved using biocatalysis in preparative organic synthesis as well as the likely prospects for their applications have been discussed in a number of reviews[5,6,14-27]. The vast majority of enzymatic processes have been developed only on a laboratory scale: typical examples are presented in two publications by the authors of this review[8,9]. However, certain processes have already been applied on an industrial scale[14,15].

Nevertheless there exists one factor which limits the application of enzymes in preparative organic synthesis on a wider scale. The point is that enzymes retain their unique catalytic properties only over a fairly narrow range of conditions, at neutral or nearly neutral pH and at not unduly high temperatures. However, the equilibria in many reactions of practical importance are displaced towards the initial reactants under these conditions. In other words, when biocatalysis is employed, a situation frequently arises where the conditions necessary for the retention of the catalytic properties of the enzyme are incompatible with the optimum thermodynamic conditions for the attainment of a high yield in the catalysed reaction.

The conflict between the relatively "severe" conditions under which the chemical reaction should take place and the "fragile" nature of the biocatalyst (its relatively labile structure) can be resolved in principle. One of the ways involves the stabilisation of the enzyme in relation to the inactivating influences of increased temperature, extreme pH values, organic solvents, etc. Significant advances have already been made on these lines[8-11] and the relevant studies are being continued (see, for example, Martinek et al.[16]). It is to be hoped therefore that in the future the classical approaches of preparative organic synthesis (alteration of temperature and pH, selection of reaction medium) will become common also in enzymatic reactions. This review deals with fundamentally different solutions of the given problem, namely physicochemical approaches which make it possible to increase the yield of the desired product without the stabilisation of the enzyme, directly under conditions favourably for biocatalysis.

II. EQUILIBRIUM APPROACHES

The approaches whereby the yield of the desired product (in enzyme-catalysed thermodynamically unfavourable synthetic reactions) can be improved by a shift of the chemical equilibrium will be referred to as equilibrium approaches.

1. The Method Based on the Low Solubility of the Product

The shift of the chemical equilibrium in a favourable direction may be caused by the poor solubility in water of one or several final products (under the reaction conditions), which

precipitate. The physicochemical essence of this phenomenon will be considered in relation to the reaction

$$A + B \rightleftharpoons C\downarrow (ppt). \quad (1)$$

The effective equilibrium constant $K_{eff} = [C]_{soln} + [C]_{ppt}/[A][B]$ is

$$K_{eff} = \frac{K}{[C]_{soln}} \left\{ [A]_0 (K/[C]_{soln})^{1/2} - 1 \right\}, \quad (2)$$

provided that the true equilibrium constant (in the absence of the precipitate) is given by $K = [C]/[A][B]$ and the material balance equation is of the form $[A]_0 = [A] + [C]_{soln} + [C]_{ppt}$ for $[A]_0 = [B]_0$, where $[A]_0$ and $[B]_0$ are the initial concentrations of the initial reactants, [A], [B], and [C] are the equilibrium concentrations of the reactants in solution, $[C]_{soln}$ is the maximum concentration of the dissolved product (solubility), and $[C]_{ppt}$ the concentration of the precipitated reactant (referred to the volume of the entire system). Eqn.(2) is valid only in the presence of a precipitate, i.e. for $[A]_0 > [C]_{soln} + [A] = [C]_{soln} + \{[C]_{soln}/K\}^{1/2}$.

It follows from Eqn.(2) that the shift of equilibrium(1) observed when the product precipitates should be more pronounced at high concentrations of the initial reactant and naturally for a lower solubility of the final product.

Applications. The method described is usually employed for the synthesis of the amide (peptide) linkage via the general mechanism

$$R_1COO^- + R_2NH_3^+ \rightleftharpoons R_1CONHR_2 + H_2O.$$

In this reaction the solubility of the product is as a rule significantly lower than those of the initial reactants because two charges disappear during the synthesis (see, for example, the synthesis of anilides[28-31], aminoacid phenylhydrazides[30,32,33], and oligopeptides with a specified sequence[29,30,34-43]). Various proteolytic enzymes are used as catalysts in this instance: α-chymotrypsin, trypsin (see, for example, Isowa et al.[38]), bacterial metalloproteinases[40], papain[43], etc.

Limitations of the method. The method suffers from two significant disadvantages. Firstly, the requirement for a low solubility of the final product greatly limits the choice of compounds which can be synthesised in this way[37]. In order to ensure a sufficiently low solubility of the corresponding product, sometimes the ionogenic groups in the initial reactants must be protected[36,43]. Secondly, the formation of the precipitate in the reaction hinders the application of water-insoluble enzyme derivatives as catalysts.

2. The Application of Consecutive Reactions

The yield of the reaction increases if one of the products is involved in a consecutive (enzymatic or non-enzymatic) thermodynamically favourable reaction, for example:

$$A + B \overset{K}{\rightleftharpoons} C + D, \quad (3)$$

$$D \overset{K_T}{\rightleftharpoons} F. \quad (4)$$

Since reaction (4), additionally introduced into the system, must be thermodynamically favourable, i.e. the condition $\Delta G_T < 0$ must hold, the equilibrium constant of the overall reaction $A + B \rightleftharpoons C + F$ must be greater than the equilibrium constant of the initial synthetic reaction (3):

$$K_\Sigma = \exp\left(-\frac{\Delta G^0 + \Delta G_T^0}{RT}\right) > K = \exp\left(-\frac{\Delta G^0}{RT}\right). \quad (5)$$

Reactions with formation of inorganic pyrophosphate. This method proved to be particularly fruitful in relation to reactions of the type

$$A + B \rightleftharpoons C + PP_i,$$

where PP_i is the organic pyrophosphate. Subsequent hydrolysis of the pyrophosphate, catalysed by the addition of inorganic pyrophosphatase added to the system (II),

$$PP_i \overset{II+H_2O}{\rightleftharpoons} 2P_i, \quad (6)$$

is a thermodynamically very favourable reaction; for example, at pH 7 the free energy of reaction (6) is $\Delta G_T^\circ = -5.27$ kcal mol^{-1}.[44] This ensures a significant shift of the equilibrium of the overall reaction towards the products. For example, in the synthesis of the nucleotide orotidine 5′-monophosphate (OMP) from orotic acid (OA) and 5-phospho-α-D-ribosyl diphosphate (PRPP),

$$OA + PRPP \rightleftharpoons OMP + PP_i, \quad (7)$$

catalysed by phosphoribosyltransferase, it was possible[45] to raise the yield from 54 to 100% by the introduction of the additional process (6). This method, based on the removal of the pyrophosphate side product, has been used in the enzymatic synthesis of NAD,[46] labelled galactoses[47], nucleic acids[48], and β-UDP-acetylglucosamine[49].

Regeneration of the cofactor of the enzymatic reaction. Many enzymatic reactions proceed with participation of cofactors:

$$A + Cof \overset{enzyme}{\rightleftharpoons} B + Cof', \quad (8)$$

where Cof and Cof′ are the initial and "spent" forms of the cofactor respectively[50,51]. The consumption of the cofactor greatly increases the cost of the products and ultimately restricts the wide-scale application of enzymes in organic synthesis. In recent years much effort has therefore been directed to the creation of the so-called cofactor regeneration systems. The regeneration usually takes place as a result of a coupled enzymatic or non-enzymatic reaction (or set of reactions) of the type

$$C + Cof' \rightleftharpoons D + Cof \quad (9)$$

(see the relevant reviews[6,9]). Consequently, in the regeneration of the cofactor, the yield of the product B may be determined by the equilibrium of the overall reaction $A + C \rightleftharpoons B + D$. This has been shown clearly[52] for the modification of steroids:

$$\text{steroid} + NAD \overset{K=2.6\cdot10^{-8}}{\rightleftharpoons} \text{steroid} + NADH, \quad (10)$$

(the steroid fragments are CH–OH and C=O, respectively)

catalysed by 20β-hydroxysteroid dehydrogenase.

The regeneration of the cofactor

$$NADH + \text{pyruvate} \overset{K_T=3.8\cdot10^{11}}{\rightleftharpoons} NAD + \text{lactate}, \quad (11)$$

catalysed by lactate dehydrogenase, is a thermodynamically favourable process. This is why the equilibrium of the overall process (10)–(11) is displaced towards the formation of the product, namely the keto-form of the steroid: $K_\Sigma = KK_T \approx 10^4$.

Other examples. In an analogous system with regeneration of the cofactor

$$\text{steroid} + NADH \xrightarrow{\text{20β-hydroxysteroid dehydrogenase}}$$

(steroid fragment C=O)

$$\rightarrow \text{steroid} + \text{NAD}, \quad (12)$$

$$\text{NAD} + C_2H_5OH \xrightarrow{\text{alcohol dehydrogenase}} \text{NADH} + CH_3CHO, \quad (13)$$

it was found[58] that the overall equilibrium constant is approximately unity. In order to shift the equilibrium towards the formation of the hydroxy-form of the steroid, the familiar reaction involving the strong binding of the acetaldehyde formed as a side product into the semicarbazone was used:

$$CH_3CHO + NH_2NHCONH_2 \rightleftharpoons CH_3CHNNHCONH_2 + H_2O. \quad (14)$$

Thus the additional process accompanying the synthetic reaction and responsible for the shift of the overall equilibrium to the right may be both an *enzymatic reaction* of type (6) and a *non-enzymatic* chemical reaction, for example reaction (14). In principle, this thermodynamically favourable auxiliary process can be also physical. Thus Laskowski and coworkers[54] observed the trypsin-catalysed synthesis of the peptide linkage in the active centre of the soya-bean trypsin inhibitor:

$$-^{64}\text{Lys} + {}^{65}\text{Ile} \rightleftharpoons -^{64}\text{Lys}-^{65}\text{Ile} + H_2O. \quad (15)$$

The equilibrium of the synthetic reaction is displaced to the right, because the product forms a stable complex (binding constant approximately 10^8) with trypsin.

3. Enzymatic Synthesis in Water–Organic Solvent Mixtures

The synthesis of compounds such as ethers, esters, amides, and anhydrides, etc. is usually carried out by the reversal of hydrolytic reactions, so that water is one of the products:

$$A + B \overset{K}{\rightleftharpoons} C + H_2O. \quad (16)$$

Under the conditions of the enzymatic reaction (in water), the equilibria of such reactions are therefore as a rule displaced to the left as a consequence of the high concentration of the aqueous component (55.5 M).

Physicochemical (sources) of the shift of the equilibrium. Evidently the yield of the product C can be increased by reducing the concentration of water in the system, because $[C] = K[A][B]/[H_2O]$. For this purpose, it is convenient to carry out the reaction not in water but in a water–organic solvent mixture with a high content of the non-aqueous component: dimethyl sulphoxide, formamide, dimethylformamide, dioxan, acetone, ethylene glycol, glycerol, monohydric alcohols, etc. (see the relevant reviews[9,11,55]).

Together with this trivial "concentration" effect, equilibrium (16) is influenced also by factors associated with the medium, such as the change in the dielectric constant or polarity, etc. Among specific effects of this kind, the shift of the equilibrium caused by the change in the pK of the acids (and/or) bases involved in the reaction must be regarded as extremely common.

We shall consider this phenomenon in relation to the synthesis of the amide linkage:

$$\begin{array}{ccccc} R_1COOH & + & R_2NH_2 & \rightleftharpoons & R_1CONHR_2 + H_2O. \\ \downarrow K_a & & \downarrow K_b & & \\ R_1COO^- & & R_2NH_2^+ & & \end{array} \quad (17)$$

In water (at any pH) the equilibrium in this reaction is displaced to the left primarily due to thermodynamically favourable ionisation processes involving at least one of the initial reactants. On passing to a water–organic solvent mixture with a high content of the non-aqueous component steady shifts of the observed pK_a and pK_b take place due to both electrostatic and solvation effects[56]. As a rule the pK_a for the acid changes most significantly: it increases, i.e. the ionisation equilibrium shifts towards the uncharged form. As a result, the equilibrium of the entire reaction (17) is displaced towards the product being synthesised[57,58]. For example, on passing from water to an 85% solution of butane-1,4-diol, the equilibrium constant for the synthesis of the peptide linkage increased by a factor of 80.[57]

The systems investigated. Water–organic solvent mixtures with a high content of the non-aqueous component have been used to synthesise the ethyl and glyceryl esters of *N*-acetyl-L-tyrosine[59], urea[60], glycerophosphate[61], aliphatic acid esters[62,63], dipeptides[57,64], and human insulin[64,65], as well as for the resynthesis of the hydrolysed peptide linkage in ribonuclease[58]. An unsuccessful attempt has also been made to synthesise sucrose from glucose and fructose[66].

Limitations of the method. A significant disadvantage of the method is that, in the presence of high concentrations of organic solvents, an appreciable impairment of important characteristics of enzymes such as catalytic activity, specificity, and stability is as a rule observed (see paper of Klibanov et al.[61] and the references therein as well as Butler's review[55]). Frequent attempts have therefore been made to protect the enzyme from inactivation by organic solvents via immobilisation (see the discussion in the papers by Martinek and coworkers[67,68]). However, this approach has still not let to sufficiently stable enzyme preparations. For this reason, investigators usually select simply by trial and error conditions (organic solvent and its concentration) such that the shift of the equilibrium towards synthesis is sufficient and at the same time an appreciable inactivation of the enzyme is not observed.

In contrast to these empirical approaches, a criterion, based on scientific principles, has been recently formulated[61] for the selection of the organic solvent as the component of the medium in enzymatic (including synthetic) reactions. Nevertheless, even under optimum conditions, it is not possible to reduce the concentration of water in the system by a factor greater than 10–20, which naturally restricts the scope of the method.

Criterion for the selection of the optimum solvent (from the standpoint of its influence on the enzyme). Such a criterion can be justified on the basis of general ideas concerning the relative role of the forces maintaining the native structure of the protein molecules in aqueous solutions: "hydrophobic interactions" play a dominant role[69,70] (despite recent doubts[71,72]). Consequently, a "good" solvent for the protein should not affect such interactions.

In order to answer the question which solvents have this property, we turned[61] to the results obtained for the information of micelles by surfactants in various solvents. Thus Ray[73] concluded that hydrophobic interactions constitute merely a special case of solvophobic interactions.

In terms of their capacity to undergo solvophobic interactions, all solvents can be divided into three classes: the first class includes water, glycerol, ethylene glycol, aminoethanol, formamide and certain others, the second class includes methylformamide and dimethylformamide, and the third includes methanol, ethanol, and toluene. Solvophobic interaction occur to the greatest extent in the solvents belonging to the first class (thus detergent micelles are formed only in these solvents); they are manifested much less in the solvents of the second class and are virtually

absent in solvents of the third class. According to Ray[73], this difference is caused by the fact that solvents of the first class contain in their molecules at least two or three fragments capable of forming hydrogen bonds. As a result of intermolecular interactions, hydrogen bonds give rise to rigid (thermodynamically unfavourable) skeletal solvent structures around the solvophobic solute molecules. This leads to solvophobic (or, in the special case of water, hydrophobic) interactions. In a series of solvents of the first class solvophobic interactions occur most effectively (this has been inferred from the critical micelle-formation concentrations) in water and glycerol; in the remaining solvents this capacity is significantly smaller.

These data enabled us to formulate[61] a criterion for the selection of the optimum solvent. The criterion is universal, because the effectiveness of solvophobic as well as hydrophobic[74] interactions is not determined by the nature of the interacting molecules but depends primarily on the nature of the solvent. Consequently, if it is assumed that the main role in the maintainence of the structure of the protein molecules is that of hydrophobic interactions, one must recognise that the best solvents for enzymatic reactions must be those of the first class and among them water (which is used in nature) and glycerol. In this connection it is appropriate to mention that in certain studies it has indeed been found experimentally that solvents of the first class, particularly glycerol and to a lesser extent ethylene glycol, denature proteins to a much lesser extent than others. This empirical finding has found a physicochemical explanation[61,73].

In order to confirm the above views, we investigated[61,75] the dependence of the catalytic activities of certain enzymes (acid phosphatase, α-chymotrypsin, formate dehydrogenase, and penicillin amidase) on concentration in aqueous solutions of certain organic solvents. It was found[61,75] that glycerol and ethylene glycol (which belong to the first class) inactivate enzymes to a lesser extent than dimethylformamide (second class) or ethanol (third class). The same conclusion follows from a comparative study carried out recently by Butler[55] on acid phosphatase in aqueous solutions of ethylene glycol (first class) and methanol (third class). These facts are in good agreement with the concept developed by ourselves[61], according to which the native structure of protein molecules (and hence their catalytic activity) are maintained in those solvents which are capable of solvophobic interactions. At the same time one should note that the three-dimensional structure of protein molecules is maintained not only by hydrophobic interactions but also by other types of bonding—electrostatic or hydrogen, etc.[69]; therefore the replacement of water by another solvent cannot be totally "ignored" by the protein. This also probably explains the decrease of the catalytic activity at an unduly high glycerol concentration[61,75] (see also the discussion of the criteria for the selection of the solvent in Butler's review[55]). Nevertheless, in many instances water–glycerol mixtures (or mixtures of water and a solvent of the first class) are entirely suitable as media for synthetic reactions[59,61,76–82]. We shall consider one example.

Enzymatic synthesis of glycerophosphate. In water (at pH 5) the equilibrium constant "does not favour" the synthesis of glycerophosphate[83]. For example, at glycerol and inorganic phosphate concentrations of 2 mM the yield of glycerophosphate should be approximately only 0.01%. The situation is different in 95% glycerol. Fig.1 (curve *3*) illustrates the kinetics of the accumulation of glycerophosphate in its enzymatic synthesis from glycerol and phosphate[61]. The same level of glycerophosphate in the system can be reached also in the solvolysis of α-glycerophosphate (curve *1*) or β-glycerophosphate (curve *2*) by acid phosphatase, which shows that the level of accumulation of glycerophosphate attained corresponds to true thermodynamic equilibrium in the system and is not limited, for example, by the inactivation of the enzyme in the water–organic solvent mixture. Fig.1 shows that the yield of glycerophosphate in 95% glycerol is approximately 35%, which is entirely acceptable for preparative purposes.

III. NON-EQUILIBRIUM (KINETIC) APPROACHES

In certain cases it is possible to attain a concentration of the desired product so high that it does not correspond to the thermodynamic state of the system and arises and persists for a specific time as a result of purely kinetic factors. This approach is widely used to increase the yield of products in enzyme-catalysed thermodynamically unfavourable synthetic reactions.

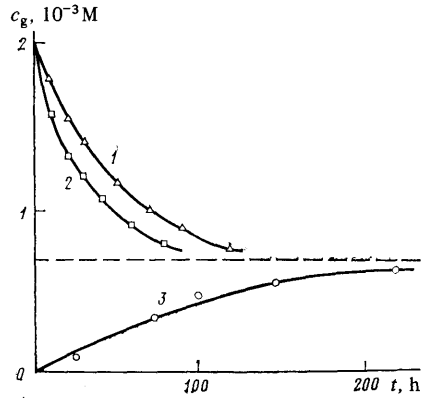


Figure 1. Kinetic curves for the acid phosphatase-catalysed hydrolysis (curves *1* and *2*) and synthesis (curve *3*) of glycerophosphate in 95% glycerol (c_g = glycerophosphate concentration): 1) α-glycerophosphate; 2) β-glycerophosphate. The dashed line represents the equilibrium glycerophosphate concentration[61].

1. Transfer Reactions Catalysed by Hydrolytic Enzymes

If the equilibrium in the reaction

$$\text{Acyl—OH} + \text{Nucl—H} \rightleftharpoons \text{Acyl—Nucl} + H_2O$$

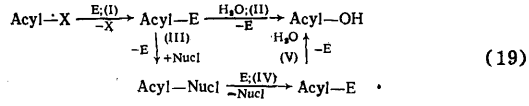
is displaced towards the initial reactants, the yield of the product Acyl–Nucl can be increased significantly with the aid of the kinetic procedure described above.

The essential physicochemical nature of the method. For example, suppose that it is necessary to synthesise a derivative Acyl–X, which, together with the inevitable hydrolysis, can interact effectively with a nucleophile (Nucl–H):

$$\begin{array}{l} \text{Acyl—X} \xrightarrow{H_2O} \text{Acyl—OH} + \text{HX}, \\ \quad -\text{XH}\downarrow\ \ +\text{Nucl—H} \\ \text{Acyl—Nucl} \xrightarrow{H_2O} \text{Acyl—OH} + \text{Nucl—H}. \end{array} \qquad (18)$$

The desired product Acyl–Nucl will then be formed in the reaction mixture as an intermediate, which subsequently hydrolyses. Evidently the time variation of the concentration of the intermediate Acyl–Nucl should be represented by a curve with a maximum (see the kinetics of consecutive reactions[84]). It is important that, for certain ratios of the concentrations of the reactants and the rate constants for the individual stages, the maximum concentration of Acyl–Nucl may prove to be significantly higher than the equilibrium value.

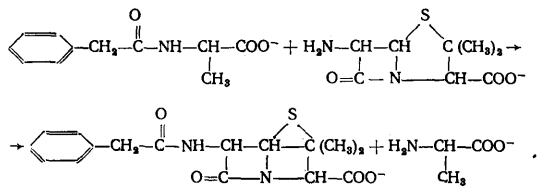
We shall consider reaction (18) in greater detail under catalytic conditions (with participation of a hydrolase). The mechanism of catalysis by many enzymes of the class of hydrolases includes the formation of an intermediate covalent compound comprising the acyl component of the substrate and the enzyme—the acylenzyme[3,85]. In subsequent stages the acylenzyme decomposes under the influence of water with regeneration of the catalytically active enzyme (E) and the formation of the corresponding acid [stages (I) and (II)]:



(19)

However, when a nucleophile (Nucl), capable of competing effectively with water, is present in the system, there is a possibility of a parallel reaction [(I) and (III)] and the intermediate Acyl–Nucl will be formed in the reaction mixture and will be subsequently hydrolysed enzymatically in stages (IV) and (V).[3,86]

The kinetics of the accumulation and hydrolysis of the product Acyl–Nucl are described by a system of differential equations which cannot be solved in the general case in an analytical form and its solution requires the application of numerical integration methods on a computer. The kinetics of the enzymatic transfer of the acyl residue to the nucleophile have been investigated in very great detail[87,88]. Fig. 2 shows as an example how benzylpenicillin accumulates in the reaction mixture in its synthesis from phenylacetylglycine and 6-aminopenicillanic acid:



(20)

The reaction was catalysed[87] by penicillin amidase. Fig. 2 shows that the concentration of the product at the kinetic optimum greatly exceeds the equilibrium level (dashed line).

For the further development of the given kinetic method, it is important that the experimental results are fully described by the theoretical curve plotted using kinetic and equilibrium constants determined from independent experiments[87,88], which leads to the possibility of predicting the maximum yield and hence of optimising the synthetic process.

Application. Botvinnik and coworkers were some of the first investigators to apply the above method in the stereospecific synthesis of oligopeptides with a specific sequence (using α-chymotrypsin as the catalyst)[89,90]; later, the synthesis of peptides was investigated in detail by Japanese workers[91,92]. The method has also been used for the penicillin amidase-catalysed synthesis of β-lactam antibiotics[87,88,93], phosphatidylserine[94], and phosphatidylethanolamine[95] (using phospholipase as the catalyst).

Limitations of the method and prospects. The application of the method is limited by the fact that it is necessary to select (and synthesise) an initial substrate Acyl–X which is hydrolysed fairly slowly via the catalytic pathway (II) [see reaction (19)] and reacts vary effectively (rapidly) with the added nucleophile [stage (III)]. The maximum yield of the product Acyl–Nucl is also limited by its subsequent hydrolysis [stages (IV) and (V)]. In principle, both these hydrolytic "obstacles" to the given method can be at least partially suppressed by carrying out the synthesis under conditions ensuring the precipitation of the product[89–92]. In future investigations an attempt may be made to reduce the undesirable hydrolysis of the initial reactant Acyl–X and the product Acyl–Nucl by carrying out the synthesis in a water–organic solvent mixture, with a high content of the non-aqueous component, rather than in water. For the same purpose, to increase the kinetically controlled yield, the application of two-phase systems comprising water and a water-immiscible organic solvent can also prove useful (see below).

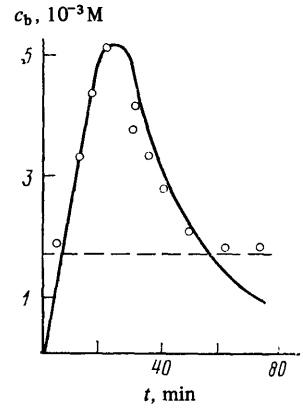


Figure 2. Kinetic curve for the accumulation of the product in the synthesis of benzylpenicillin (c_b = benzylpenicillin concentration) from phenylacetylglycine and 6-aminopenicillanic acid catalysed by penicillin amidase. Circles—experimental data; continuous curve—the result of the solution of a system of differential equations by numerical integration on a computer[87].

2. Kinetically Controlled Equilibrium

An extremely interesting phenomenon has been observed recently[96]: the hydrolysis of the ethyl ester of *N*-acetyl-L-tyrosine catalysed by α-chymotrypsin immobilised on charged carriers (Sephadexes) does not go to completion at pH 8 (only to the extent of 60–90% depending on the conditions). Furthermore, the synthesis of the ester from the corresponding acid and alcohol takes place under the same conditions (in water in the presence of an enzyme immobilised on a carrier):

N–Acet–*L*-TyrOH + EtOH ⇌ N–Acet–*L*-TyrOEt + H_2O

(Fig. 3). As a result, it is possible to attain the same composition of the reaction mixture both from the side of synthesis and from the side of hydrolysis. This limiting composition does not change for a long time. At the same time, the above

state of the system does not correspond to the true thermodynamic equilibrium. Thus, when a certain amount of free (non-immobilised) enzyme is added to the system, the concentration of the ester falls almost to zero.

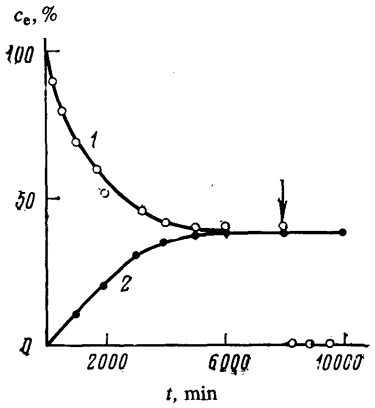


Figure 3. Kinetic curves for the hydrolysis (curve *1*) and synthesis (curve *2*) of the ethyl ester of *N*-acetyl-L-tyrosine (c_e = ester concentration) catalysed by α-chymotrypsin immobilised on charged carriers (sephadexes). The arrow denotes the instant of the addition of the non-immobilised enzyme[96].

It is believed[96] that the phenomenon is associated with the fact that the free energy of the chemical reaction changes in the electrical double layer (EDL) on the surface of the carrier (relative to the free energy of the same reaction in the absence of the electric field) due to the orientation energy of the reactant dipoles. Ultimately it may turn out that within the EDL the reaction equilibrium favours the synthesis of the product to a greater extent than in water. Naturally the volume of the EDL on the surface of the carrier constitutes only a fraction of the total volume of the system and the shift of the equilibrium in it should have a very insignificant effect on the overall equilibrium composition of the reactants throughout the entire system. However, if the rates of reaction in the bulk phase of the system are low compared with the corresponding rates in the EDL, i.e. if the reaction equilibrium in the entire system is established solely via the EDL, the observed equilibrium composition of the reactants may be kinetically controlled by the equilibrium of the reaction in the electrical double layer. This is why the authors[96] proposed the term "kinetically controlled equilibrium" for phenomena of this kind.

The kinetic control of the equilibrium can be achieved, for example, *catalytically* if the catalyst is incorporated within the EDL. In this case the rate of reaction within the EDL is naturally higher than in the bulk phase of the system and for this reason (if the equilibrium within the EDL favours synthesis) it is possible to achieve a fairly high yield of the product, which naturally does not correspond to equilibrium in the bulk phase of the entire system. This situation is believed[96] to occur precisely in the example discussed above.

From the same standpoint, it is apparently possible to explain also the finding that the hydrolysis of the same substrate (the ethyl ester of *N*-acetyl-L-tyrosine) takes place only to the extent of 60% when the reaction is catalysed by α-chymotrypsin immobilised on a spiropyran matrix[97]; the synthesis from the acid and alcohol takes place correspondingly to the extent of 40% (Fig.4). It is of interest to note that this effect occurs only under illumination; in the dark the synthetic reaction does not occur, while the hydrolysis proceeds to the extent of 100%. Fig.4 shows that, as a consequence of this, there is a possibility of regulating the position of the "kinetically controlled equilibrium" with light. Japanese workers explain this phenomenon by the fact that the properties of the matrix, which apparently determine the chemical reaction equilibrium near or within it, are different in the dark and in the light[97].

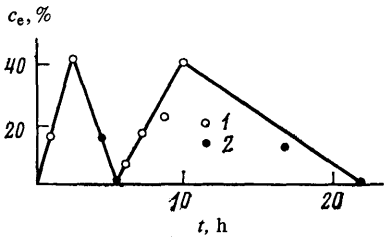


Figure 4. Kinetic curves for the synthesis (curve *1*) and hydrolysis (curve *2*) of the ethyl ester of *N*-acetyl-L-tyrosine catalysed by α-chymotrypsin immobilised on a spiropyran matrix: 1) illuminated system; 2) system in the dark[97].

Both systems were discovered quite recently (they were reported in 1978 and 1979 respectively), have been comparatively little investigated (the mechanism and reaction of the second system are still not entirely clear), and it is therefore difficult to assess their advantages and disadvantages and also the potential possibilities of their applications in enzymatic organic synthesis.

IV. ENZYMATIC SYNTHESIS IN TWO-PHASE SYSTEMS COMPRISING WATER AND A WATER-IMMISCIBLE ORGANIC SOLVENT

Each of the methods described above, which makes it possible to displace the equilibrium (or in the general case to increase the yield of the desired product) in enzyme-catalysed thermodynamically unfavourable synthetic reactions, is applicable in only a limited number of instances. An equilibrium approach has been proposed recently, which apparently constitutes a more general solution of the problem. Its essential feature is that the reaction is carried out in a two-phase system water–water-immiscible organic solvent[67,68]. The enzyme is present in the aqueous phase and it is therefore unnecessary to solve the traditional problem of stabilising the enzyme with respect to denaturation by the organic solvent. The added substrates, dissolved in the organic phase, diffuse from the latter into water, react chemically under the influence of the enzyme, and the products diffuse from water back to the organic phase. Thus, the thermodynamic equilibrium in the given reaction is established throughout the water–organic solvent system via water (with the aid of the enzyme present in water). The volume fraction of the organic phase can be in principle as close to unity as is necessary (the water content must be only sufficient to dissolve the required amount of the enzyme with retention of catalytic activity). Consequently the equilibrium conditions

in such a system can be as close as required to the equilibrium in the pure organic medium.

An enzymatic two-phase system can be created either in the form of two immiscible layers, or as an emulsion of an aqueous solution of the enzyme in the organic phase, or, finally, by the incorporation of the enzyme in the inverted micelles of the surfactant[16,98] (see also the relevant reviews[8-11]) or in liquid membranes[99]. However a suspension of particles (porous glass or ceramic, whole fibres, gel grains, microcapsules, etc.) impregnated with an aqueous solution of the enzyme is much more convenient for technological purposes. The enzyme can be used in both free and immobilised states.

The essential physicochemical features of the shift of the chemical equilibrium. On passing from water (as the reaction medium) to a two-phase system with a low content of the aqueous component, a significant shift of the chemical equilibrium was observed[67,68]. One of the causes of the shift of the equilibrium, observed in reactions of the type $A + B \rightleftharpoons C + H_2O$ (where one of the products is water) is self-evident; this is a concentration effect due to the decrease of the concentration of the aqueous reactant in the two-phase system. However, there exists yet another more general source of the shift, namely the change in the effective equilibrium constant as a result of the partition of the reactants between the phases. We shall consider the quantitative characteristics of this phenomenon in relation to certain simple reactions.

The reaction $A \rightleftharpoons B$. In a two-phase system the reactants are partitioned between water and the organic solvent:

$$\begin{array}{ccc} (A & \overset{K_w}{\rightleftharpoons} & B)_{aq} \\ \Big\downarrow P_A & & \Big\downarrow P_B \\ (A & \underset{K_o}{\rightleftharpoons} & B)_{org} \end{array} \qquad (21)$$

Here the subscripts w and o refer respectively to water and the organic solvent. The effective equilibrium constant of reaction (21), which is equal to the ratio of the overall concentrations of reactants A and B referred to the volume of the entire two-phase system, i.e. $K_{eff} = [B]_{tot}/[A]_{tot}$, can be formulated as follows[100-102]:

$$K_{eff} = K_w \frac{1 + \alpha P_B}{1 + \alpha P_A}, \qquad (22)$$

or

$$K_{eff} = K_o \frac{1 + 1/\alpha P_A}{1 + 1/\alpha P_B}, \qquad (23)$$

where K_W and K_O are the equilibrium constants of reaction (21) in water and in the organic solvent respectively, $\alpha = V_O/V_W$ the ratio of the volumes of the organic and aqueous phases, $P_A = [A]_O/[A]_W$ and $P_B = [B]_O/[B]_W$ are the partition coefficients of the reactants between the phases.

Eqn.(22) shows that K_{eff} changes monotonically with increase of α, namely, depending on the choice of the organic solvent, it can either increase (for $P_A < P_B$) or decrease (for $P_A > P_B$). For fairly high values of α (when the content of the aqueous phase is extremely low), the equilibrium constant tends to a limit $K_W P_B/P_A \equiv K_O$. The partition coefficients of organic compounds between water and commercially available organic solvents are usually between 10^{-4} and 10^4.[103,104] Consequently the limiting shift of the equilibrium (K_{eff}/K_W) can be very considerable in principle.

As an example we shall consider the oxidation of isobutyl alcohol catalysed by alcohol dehydrogenase:

$$\begin{array}{ccc} \{(CH_3)_2CHCH_2OH + NAD \rightleftharpoons (CH_3)_2CHCHO + NADH\}_{water} & & \\ \Big\downarrow P_{alcohol} \qquad\qquad \Big\downarrow P_{aldehyde} & & \\ \{(CH_3)_2CHCH_2OH \qquad\qquad (CH_3)_2CHCHO\}_{org} & & \end{array}$$

This reaction has been thoroughly investigated in water[105]. In a two-phase system[100-102] the position of the equilibrium in this reaction is described by an equation analogous to Eqn.(22), because the cofactor employed does not pass from water to the organic solvent in either the oxidised or in the reduced form (being electrostatically charged).

Consequently the partition coefficients of NAD and NADH are virtually zero. Thus

$$K_{eff} \simeq K_w \frac{1 + \alpha P_{aldehyde}}{1 + \alpha P_{alcohol}}. \qquad (24)$$

The experimental results are presented in Fig.5. Two factors should be noted for the system considered. Firstly, the reaction investigated constitutes a system in which the difference between the hydrophobic properties of the initial and final reactants is very slight. Thus in the water–octanol classical system the partition coefficients of the aldehyde and alcoholic groups differ only by 12%.[103] Nevertheless, it is possible to select a solvent (see Fig.5) such that the partition coefficients of the initial and final reactants differ fairly considerably (for example, due to specific solvation effects). As a result, by varying the nature of the organic phase, it was possible to alter the effective equilibrium constant within very wide limits (by a factor of approximately 100). Secondly, Fig.5 shows that the theoretical curve *1* [plotted on the basis of Eqn.(24) using the partition coefficients of isobutyl alcohol and isobutyraldehyde found in an independent experiment] is in satisfactory agreement with experiment.

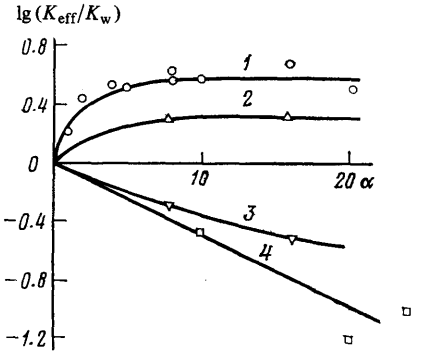


Figure 5. Dependence of the effective constant (K_{eff}) of the reaction involving the oxidation of isobutyl alcohol to isobutyraldehyde catalysed by alcohol dehydrogenase in a two-phase water–organic solvent system on the phase volume ratio α and on the composition of the ethyl acetate–hexane organic phase (ethyl acetate, %): 1) 0; 2) 12.5; 3) 50; 4) 100 (Semenov et al.[100,101]).

The reaction $A + B \quad C + D$. The dependence of the effective equilibrium constants on the ratio of the volumes of the organic and aqueous phases (α), i.e.

$$K_{eff} = K_w \frac{(1 + \alpha P_C)(1 + \alpha P_D)}{(1 + \alpha P_A)(1 + \alpha P_B)}, \qquad (25)$$

is more complex[100-102] than for the previous first-order reaction. Typical instances are presented in Fig.6.

The limiting shift of the equilibrium observed in a two-phase system in the presence of an extremely low water content can be in principle even greater than in the simple reaction A ⇌ B (see above). This is evident because the expression for the limiting shift $K_{eff}/K_w = P_C P_D / P_A P_B$ (at $\alpha \to +\infty$) includes a large number of partition coefficients. However, a more important feature of chemical equilibria involving second order reactions is that the dependence of K_{eff} on α for specific ratios of the partition coefficients can have an extremum (Fig.6). In other words, in a system of two immiscible solvents the effective equilibrium constant may be greater (or correspondingly smaller) than the limiting values characterising the equilibria in each phase. This factor must be taken into account in selecting the optimum conditions for the chemical synthesis (for further details, see below).

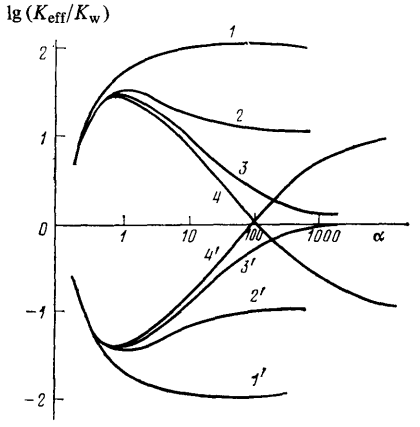


Figure 6. Dependence of K_{eff} for a reaction of the type A + B ⇌ C + D in a water–organic solvent two-phase system on α for certain values of the partition coefficients: 1)–4) $P_A = P_B = 1$, $P_C = 10^2$ [P_D: 1) 1; 2) 10^{-1}; 3) 10^{-2}; 4) 10^{-3}]; 1')–4') $P_C = P_D = 1$, $P_A = 10^2$ [P_B: 1') 1; 2') 10^{-1}; 3') 10^{-2}; 4') 10^{-3}] (Taken from Refs.100–102).

The essential physicochemical nature of this phenomenon (the presence of an extremum) can be discovered very clearly in relation to the following example. We shall assume that the initial reactants are distributed between the phases in such a way that one of them (A) is located fully in one of the phases (water), while the other (B) is located fully in the other (organic solvent). Then the reaction equilibrium in the two-phase system is completely displaced to the left ($K_{eff} = 0$) whatever the equilibrium constant in each phase separately. The reason for this is evidently that the reactants A and B, being separated in space, cannot interact. On the other hand, the equilibrium is fully displaced to the right ($K_{eff} \to \infty$ regardless of the final equilibrium constants in a particular phase) if the final products prove to be completely separated from one another, i.e. when one of the products (for example C) is concentrated entirely in water, while the other (D) passes to the organic solvent.

The extremum of the equilibrium in the two-phase system has been observed experimentally (by varying the ratio of the volumes of the aqueous and organic phases) in relation to the α-chymotrypsin-catalysed synthesis of the ethyl ester of *N*-benzoyl-L-phenylalanine (from ethanol and the corresponding acid)[100-102]. In water the yield of the ester is negligible[106,107]. At the same time in a two-phase system (for the optimum ratio of the volumes of water and the organic solvent) the yield was 80, 64, 62, and 26% for chloroform, benzene, carbon tetrachloride, and diethyl ether respectively[100-102]. Thus, as a result of using two-phase systems, it is possible to increase the yield of the ester virtually from zero to a preparative level.

Decrease of the concentration of the aqueous reactant. In reactions such as the synthesis of ethers, esters, amides, peptides, etc. of the type A + B ⇌ C + H_2O one of the products is water. Therefore in aqueous solution their equilibrium is displaced towards the initial reactants as a consequence of the high concentration of water in the system (55.5 M). On passing to two-phase systems, the water content diminishes; as a result of this, the yield of the product C should in principle increase.

However, one should bear in mind that in a two-phase system the yield of the product C depends on the concentration of water in two ways: not only via the Law of Mass Action, i.e. $[C]_{tot} = K_{eff}[A]_{tot}[B]_{tot}/[H_2O]_{tot}$, but also via the equilibrium constant determined by a function of the type of (25) and hence depending on α. Ultimately we have[100-102]

$$[C] = K_{eff}\frac{[A][B]}{[H_2O]} = K_w\frac{[A][B](1+\alpha P_C)(1+\alpha)}{55.5(1+\alpha P_A)(1+\alpha P_B)}. \quad (26)$$

According to this equation, the dependence of [C] on α can be very complex (Fig.7). In particular, for certain ratios of the partition coefficients there is actually a decrease of the yield of the product C with decrease of the concentration of the second product (water) in the system. This is because the effective equilibrium constant diminishes to a greater extent with increasing α than the overall concentration of water in the system [see Eqn.(26)].

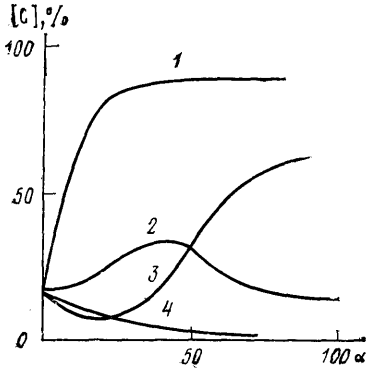


Figure 7. Dependence of the yield of the product C in a reaction of the type A + B ⇌ C + H_2O in a two-phase water–organic solvent system on α for the following partition coefficients: 1) $P_A = P_B = 1$, $P_C = 50$; 2) $P_A = P_B = 0.1$, $P_C = 0.01$; 3) $P_A = 10$, $P_B = 0.01$, $P_C = 1$. 4) $P_A = P_B = 1$, $P_C = 0.1$; it was assumed that $K_W = 10$ (from Martinek et al.[102]) in all cases.

The shift of ionic equilibria. The occurrence of many reactions involves the ionisation of the reactants, for example,

$$\left.\begin{aligned} HA + B &\underset{K'_{eq}}{\overset{K_{eq}}{\rightleftharpoons}} C + D, \text{ where } HA \overset{K_a}{\rightleftharpoons} H^+ + A^- \\ A + B &\underset{K'_{eq}}{\overset{K_{eq}}{\rightleftharpoons}} C + D, \text{where } B + H^+ \overset{K_b}{\rightleftharpoons} BH^+. \end{aligned}\right\} \quad (27)$$

On passing from water to two-phase systems, a change is to be expected not only in the equilibrium constant of the chemical reaction proper (K_{eq}) but also in the equilibrium constants of the ionisation processes (K_a or K_b). As a result, the shift of the equilibrium of the overall process will be evidently determined by the changes in all the constants and will therefore depend on the pH.

In this Section we shall analyse the physicochemical causes of the changes in the constants of ionic equilibria in two-phase water–organic solvent systems relative to aqueous solutions and we shall also consider the possibilities arising in this instance for increasing the yield of products in enzyme-catalysed thermodynamically unfavourable synthetic reactions.

We shall consider, for example, the ionisation of an acid in a two-phase water–organic solvent system[108,109]:

$$\begin{array}{l}(HA \underset{}{\overset{K_a}{\rightleftharpoons}} H^+ + A^-)_{aq} \\ \downarrow P_{HA} \\ (HA)_{org}\end{array} \quad . \tag{28}$$

Here we shall assume that virtually only the uncharged form of the acid or base dissolves in the organic phase. This hypothesis is as a rule valid (see for example, Leo et al.[103]). The only exceptions are the highly polar solvents which are capable of extracting from water charged compounds in the form of ion pairs[110].

The ionisation constant of an acid in a two-phase system will be defined in the usual way by the Henderson–Hasselbach equation:

$$(pK_a)_{eff} = pH - \lg \frac{[A^-]_{tot}}{[HA]_{tot}},$$

where $[A^-]_{tot}$ and $[HA]_{tot}$ are the overall concentrations of the ionised and unionised forms of the acid respectively, calculated relative to the volume of the entire two-phase systems, and the pH characterises the aqueous phase. We then have[108,109]

$$(pK_a)_{eff} = pK_a + \lg(1 + \alpha P_{HA}). \tag{29}$$

Similarly for the protonation of a base B in a two-phase system we have

$$\begin{array}{l}(B + H^+ \underset{}{\overset{K_b}{\rightleftharpoons}} BH^+)_{aq} \\ \downarrow P_B \\ (B)_{org}\end{array} \tag{30}$$

and we obtain[108,109] the following expression for the effective equilibrium constant:

$$(pK_b)_{eff} = pK_b - \lg(1 + \alpha P_B). \tag{31}$$

Thus in two-phase water–organic solvent systems, the pK_a for the acid increases by an amount $\lg(1 + \alpha P_{HA})$ and the pK_b for the base decreases correspondingly by an amount $\lg(1 + \alpha P_B)$. The partition coefficients of organic acids and bases can reach[103,104] ($10^2 - 10^4$). Consequently, in a system containing, for example, 1 vol% of water ($\alpha = 100$) the shift of the effective pK can amount to 4–6 pH units.

The ionisation equilibria of the model compounds 2,4-dinitrophenyltryptophan (acid) and Neutral Red(base) have been investigated[108] in two-phase water–organic solvent systems. It has been shown that, firstly, the effective pK in such systems is indeed displaced relative to aqueous solutions and the experimental shift reaches five or more pH units. Secondly, the experimental dependence of $pK_{b,two\text{-}phase}$ of Neutral Red on α is described satisfactorily by the theoretical curve based on Eqn.(31) using the partition coefficient P_B found in an independent experiment.

We shall now consider how the shift of the pK of the reactants can influence the equilibria of the chemical reactions occurring in two-phase water–organic solvent systems and what possibilities arise in this instance for increasing the yield of the desired product.

The attainment of the coincidence of the pH optima of the efficiency of the catalyst and of the equilibrium of the catalysed reaction. The situation frequently arises where the pH optimum of the stability and catalytic activity of the enzyme is significantly removed from the pH values which favour thermodynamically the synthesis of the given product. As an example, one may point to the synthesis of β-lactam antibiotics (the thermodynamic pH optimum is in the range 3–5),[88] 4-p-nitroanilides (with a pH optimum of 2–3), and esters of *N*-substituted aminoacids whose synthesis is favoured by pH values below 3.[106,107,111] At the same time the majority of enzymes capable of catalysing these synthetic reactions (penicillin amidase, trypsin, α-chymotrypsin, and other proteases) are active and stable only at neutral (or nearly neutral) pH. This contradiction can be resolved by employing two-phase water–organic solvent systems rather than water as the reaction medium.

As an example, we shall consider the synthesis of the ethyl ester of *N*-benzoyl-L-phenylalanine catalysed by α-chymotrypsin:

$$\begin{array}{l}R{-}COOH + EtOH \rightleftharpoons R{-}COOEt + H_2O \\ \downarrow K_a \\ R{-}COO^-\end{array} \quad . \tag{32}$$

Fig.8 illustrates the pH dependence of the equilibrium constant of this reaction in water (curve *1*).[106] Evidently the synthesis is favoured only by pH values below 3, while at neutral and nearly neutral pH (i.e. under the conditions where α-chymotrypsin functions) the equilibrium is almost wholly displaced towards the initial substances; the fact that one of the reactants (the acid) is dissociated under the given conditions plays a significant role in this instance. Thus in water the enzymatic reaction is of no preparative value.

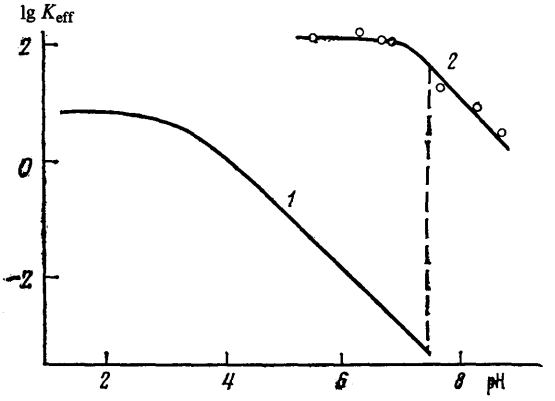


Figure 8. pH dependence of the equilibrium constant of the reaction involving the synthesis of the ethyl ester of *N*-benzoyl-L-phenylalanine in water (curve *1*) and in the chloroform—5 vol.% of water two-phase system (curve *2*)[108,112].

On passing to two-phase water–organic solvent systems[108,112,113], the effective pK_a for the acid increases [see Eqn.(29)], so that the pH dependence of the equilibrium constant shifts towards neutral or alkaline pH. Fig.8 illustrates the pH dependence of the equilibrium constant of reaction (32) in the chloroform +5 vol.% of water two-phase system (curve 2).

Evidently, compared with aqueous solutions, the pK for the acid has increased by more than three units (see the shift of curve *2* to the right along the abscissa axis compared with curve *1*). As a result, it was possible to achieve the coincidence of the pH thermodynamically favourable for the synthesis and the pH optimum of the catalytic activity and stability of α-chymotrypsin (see the dashed line, pH 7.5). In addition, the limiting, i.e. pH-independent, equilibrium constant has also increased (by more than an order of magnitude) (see the upwards shift of curve *2* along the ordinate axis). The reason for this is that the product (the ester) is preferentially extracted from water into the organic phase. Ultimately the overall change in the equilibrium constant at pH 7.5 (dashed line in Fig.8) is by approximately five orders of magnitude. This is why it has become possible to drive the hydrolytic reaction almost wholly towards synthesis at neutral pH. Thus the yield of the ester in the given system is 80–100% (depending on the reactant concentrations) while in water it does not exceed 0.01%.

Peptide synthesis. An example of reactions involving di-iogenic compounds may be the synthesis of the peptide linkage:

$$\begin{array}{ccc} R_1COOH + R_2NH_2 & \rightleftharpoons & R_1CONHR_2 + H_2O \\ \downarrow\uparrow K_a \quad\quad \downarrow\uparrow K_b & & \\ R_1COO^- \quad R_2NH_2^+ & & \end{array} \qquad (33)$$

The pK_a for carboxylic acids are usually in the range 3–4 and the pK_b for amines are 8–10.[50,107,114] Consequently, in water, over the entire pH range, at least one of the initial reactants exists almost entirely in a charged form. As a result, the equilibrium of the overall reaction is almost wholly displaced to the left, towards hydrolysis[107].

On passing to two-phase water–organic solvent systems, the effective pK_a for the acid as a rule increases and the pK_b for the base diminishes (see above), which implies that at neutral pH the fraction of the uncharged forms of both the acid and the amine increases to some extent[108,109]. Consequently the equilibrium of reaction (33) should be displaced to the right, towards synthesis. The second source of the shift of the equilibrium is that the peptide is as a rule extracted from water to the organic solvent more effectively than the initial reactants. Thirdly and finally, the equilibrium yield of the product in reaction (33) in a two-phase water–organic solvent system should also increase owing to the decrease of the water content.

We tested this approach[108,109,115,116] in relation to the α-chymotrypsin-catalysed synthesis of *N*-acetyl-L-tryptophanyl-L-leucine amide:

$$\text{N—Ac—}L\text{-Trp} + L\text{-LeuNH}_2 \rightleftharpoons \text{L—Ac—}L\text{-Trp—}L\text{-LeuNH}_2 + H_2O.$$

On passing from water to the two-phase system ethyl acetate + 2 vol.% of water, the equilibrium constant of the synthetic reaction increased by more than three orders of magnitude (cf. curves *1* and *3* in Fig.9). As a result, the reaction equilibrium was displaced considerably to the right, which made it possible to synthesise the dipeptide almost quantitatively in water the yield does not exceed 0.1% other conditions being equal.

Synthesis of ionic products. It would appear that the two-phase approach to preparative organic synthesis is inapplicable to reactions where the products are ions because they would not pass from water to the organic phase and hence would not be distributed throughout the system. However, this difficulty can be overcome by selecting for the ionic reactants hydrophobic counterions, as is done, for example, in the chemistry of nucleotides[117] and has also proved useful in the radioactive analytical method[118]. We tested this approach in relation to the synthesis of glycerophosphate from inorganic phosphate and glycerol[67]. The reaction was catalysed by alkaline phosphatase using tetrabutylammonium as the hydrophobic counterion for the phosphates. In the chloroform–water two-phase system the yield of glycerophosphate was more than 30%,[67] while in water (in the absence of the organic phase but otherwise under the same conditions) the yield was negligible[83].

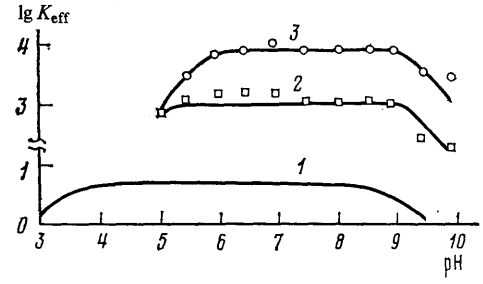


Figure 9. pH dependence of the equilibrium constant of the reaction involving the synthesis of *N*-acetyl-L-tryptophanyl-L-leucine amide in water (curve *1*) and in the ethyl acetate–water two-phase system containing 17 vol.% (curve *2*) and 2 vol.% (curve *3*) of water[108,115].

The enzymatic two-phase method is also applicable to other processes of practical importance involving ionic products, such as the modification of antibiotics[119], the synthesis of a dipeptide from free aminoacids[120], and the separation of a racemic mixture of free aminoacids[121].

General remarks concerning the choice of experimental conditions. Theoretical study[100–102] of the equilibria of chemical reactions in two-phase water–organic solvent systems permits the following conclusion: the equilibrium of a chemical reaction in a two-phase system is in general determined by the equilibrium constant of this reaction in one of the phases, the partition coefficients of the initial and final reactants, and the ratio of the volumes of the organic and aqueous phases. Consequently, knowing the equation describing the equilibria of the reactions in the two-phase systems and the partition coefficients (from the literature or from independent experiments), it is possible to calculate K_{eff} and hence the yield of the reaction products [see, for example, Eqns.(22), (25), and (26)]. Unfortunately the amount of information in the literature about the partition coefficients of various substances is at present inadequate and the available information is not always reliable[103,104]. In addition one must bear in mind that at high concentrations of reactants the latter can themselves alter the reaction medium and the distribution of the reactants (and hence the effective reaction equilibrium) will exhibit deviations from a simple (ideal) model[122].

It is important to be able to predict the mode and extent of the change in the equilibrium in a two-phase system relative to water because the dependence of K_{eff} (and of the yield of the chemical reaction products) in a two-phase system on the volume ratio of the phases (α) can be fairly complex. Consequently it can hinder the selection of the experimental conditions for the synthesis. In this connection, we draw attention to two factors.

1. For certain ratios of the partition coefficients, K_{eff} for reaction (4), observed in a two-phase system, can be significantly greater (Fig.6, curves 2–4) or correspondingly smaller (Fig.6, curves 2′–4′) than the equilibrium constants of the same reaction in each phase. We shall analyse this phenomenon in greater detail (quantitatively) in relation to a two-phase system where $K_W = K_O$, i.e. we shall assume that the equilibrium constant is the same in both immiscible solvents. The partition coefficients will be varied in the range of reasonable values from 10^{-5} to 10^5 (cf. Leo et al.[103]).

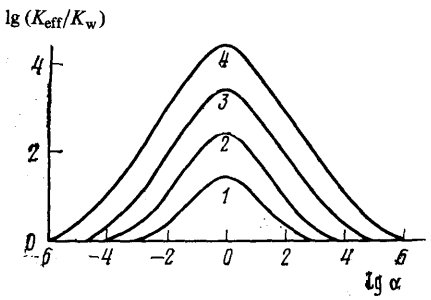


Figure 10. The extremum in the relation between K_{eff} for a reaction of the type A + B ⇌ C + D in the water–organic solvent two-phase system on the phase volume ratio (α) for the following partition coefficients: 1) $P_A = P_B = 1$, $P_C = 10^2$, $P_D = 10^{-2}$; 2) $P_C = 10^3$, $P_D = 10^{-3}$; 3) $P_C = 10^4$, $P_D = 10^{-4}$; 4) $P_C = 10^5$, $P_B = 10^{-5}$ (from Martinek et al.[102]).

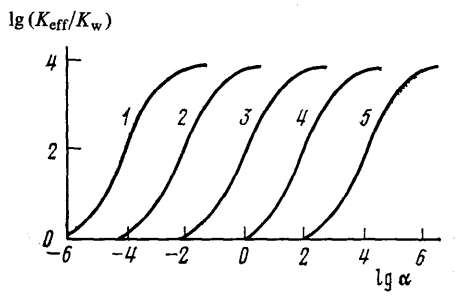


Figure 11. Examples of the S-shaped relation between K_{eff} for a reaction of the type A + B ⇌ C + D in a two-phase water–organic solvent system on α for the following partition coefficients: 1) $P_A = P_B = 10^3$, $P_C = P_D = 10^5$; 2) $P_A = P_B = 10$, $P_C = P_D = 10^3$; 3) $P_A = P_B = 0.1$, $P_C = P_D = 10$; 4) $P_A = P_B = 10^{-3}$, $P_C = P_D = 0.1$; 5) $P_A = P_B = 10^{-5}$, $P_C = P_D = 10^{-3}$ (from Martinek et al.[102]).

Fig.10 shows how the effective value of K_{eff} varies in a particular instance with the phase volume ratio α. Evidently the maximum effective equilibrium constant, observed for a reaction of the type A + B ⇌ C + D in a two-phase system, can exceed by a factor greater than 10^4 the corresponding equilibrium constants for the same reaction in each of the phases. Consequently the application of two-phase systems as reaction media can be particularly useful for increasing the yield of the desired product in reactions (not necessarily enzymatic) where the equilibrium is not displaced in the required direction in any of the solvents which are available and suitable for the given reaction.

2. In selecting the conditions for the synthesis, one should also bear in mind that the dependence of K_{eff} on α may be S-shaped (even in terms of non-logarithmic coordinates)[102], i.e. over a certain range of values of α the reaction equilibrium is as it were insensitive to a change in the medium. This can be seen from Fig.11, where we analysed the case where $10^4 K_W = K_O$. Evidently, depending on the partition coefficients (which were varied in the range of reasonable values from 10^{-5} to 10^5),[103] the shift of the chemical equilibrium is observed both for a negligible content of the organic phase ($\alpha = V_O/V_W \cong 10^{-5}$, curve 1) and for comparable amounts of the phases ($\alpha \cong 1$, curves 3), or even, finally, in the absence of water from the system ($\alpha \cong 10^5$, curve 5). In each case it is convenient to employ different methodological procedures to establish the two-phase system. Thus two-phase systems can be formed in the following way.

(a) For systems with a low content of the organic phase ($\alpha \ll 1$), this can be, for example, an aqueous solution of a surfactant forming micelles†; the internal (hydrophobic) region of the micelles can serve as the organic phase[123].

(b) For systems with a low water content ($\alpha \gg 1$), the enzymes should apparently be enclosed in "inverted" micelles of surfactants in an organic solvent[16,98,125].

(c) Systems containing comparable amounts of the organic and aqueous phases can be set up, as already mentioned above (see Section I), in the form of an emulsion of an aqueous solution of the enzyme in an organic solvent or as a suspension in the same solvent of porous particles impregnated with an aqueous solution of the enzyme. When an emulsion is used, the question of the specific interface is extremely important: the greater the specific interface, the faster the diffusion of the reactants through the interface, which has a favourable influence on the reaction kinetics; on the other hand, under these conditions the inactivation of the catalyst (enzyme) can be accelerated as a result of its adsorption at the interface[126]. In this instance one can employ a simple two-layer system.

Generally speaking, any substance capable of forming a two-phase (or pseudo-two-phase system) can be used as the "organic" phase. For example, a water-insoluble polymer can be used for this purpose[127,128].

V. THE PRESENT STATE OF ENZYME CATALYSIS AND THE PROSPECTS FOR ITS INTRODUCTION INTO ORGANIC SYNTHESIS

It is seen from the above survey that enzymes have been used very successfully without additional stabilisation (in relation to denaturation by the reaction medium) in a whole series of synthetic reactions. This has also been promoted by the development of physicochemical approaches whereby the yield of the product can be increased directly under conditions favourable (optimal) for biocatalysis.

It follows from the data in the present review that for the purposes of organic synthesis about 50 enzymes have already proved useful, mainly in the following processes (references are given only to reviews and the latest surveys).

†In the case of micellar systems it is possible to speak of the presence of two phases only as a matter of convention because there is no visible interface. It is therefore more appropriate to speak of "pseudo-two-phase" systems[123]. Nevertheless all the characteristics of the shift discussed above obtain in this instance (see, for example, Martinek and coworkers[124,125]).

1. The Synthesis of Oligo- and Poly-nucleotides

The methods for the chemical synthesis of the internucleotide linkage have been as yet inadequately developed and it is virtually impossible to synthesise a polynucleotide containing several tens of units by a purely chemical procedure[129]. Much attention is therefore devoted in the literature to enzymatic methods for the synthesis of oligo- and polynucleotides. In the synthesis of oligoribonucleotides and oligodeoxyribonucleotides use is therefore made of ribonuclease[24,129–132]:

$$N{>}p + N' \xrightarrow{\text{RNAase}} NpN', \qquad (34)$$

polynucleotide phosphorylase[24,129,130,133]:

$$I + NDP \xrightarrow{\text{PNPase}} IpN + P_i \qquad (35)$$

or RNA ligase[129,134]:

$$NpN' + pN''pN'''p \xrightarrow{\text{RNA ligase + ATP}} NpN'pN''pN'''p\ . \qquad (36)$$

Here N >p is the nucleotide 2′,3′-cyclic phosphate, N the nucleoside, NDP the nucleoside diphosphate, NpN′ the dinucleoside monophosphate, and I the initiator (primer) molecule (usually a dinucleotide).

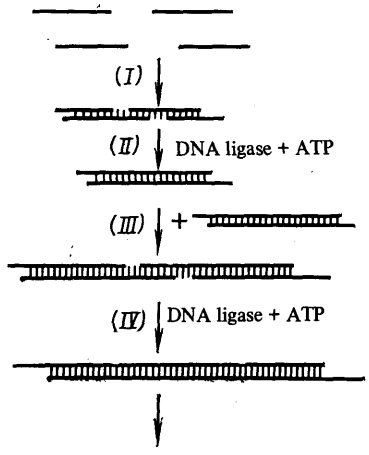


Figure 12. Illustration of the "method of sticky ends". Horizontal lines—sequence of nucleotides joined by covalent bonds; vertical lines—complementary non-covalent (hydrogen) bonds.

For reaction (34), preference is given to non-specific ribonucleases as the catalyst. This makes it possible to synthesise oligonucleotides containing a wide variety of nucleosides, including some which are not encountered in nature. Reaction (35) may involve the extremely undesirable polymerisation process and several approaches have been developed for its suppression[24]. Reaction (36) has been discovered comparatively recently and so far has been hardly used.

Khorana and coworkers[135,136] developed a method for the synthesis of double-stranded polynucleotides with a definite sequence, which has come to be called the "method of sticky ends". His idea can be understood from Fig.12. According to this method, in the first stage the initial oligonucleotides (with a length equivalent to 5–15 units) form a double-stranded complex as a result of complementary non-covalent interactions [stage (1)]. Next, the 3′- and 5′-ends in the resulting complex, which have approached one another in space, are joined by covalent linkages under the influence of DNA ligase [stage(II)]. All the fragments from which the final polynucleotide is to be made up are synthesised in this way and are linked initially via complementary non-covalent interactions [stage (III)] and then also by covalent bonds [stage (IV)]. Using this scheme, Khorana and coworkers synthesised the structural gene of alanine tRNA [135] and the complete gene of suppressor tyrosine tRNA.[136]

2. Regeneration of ATP

Adenosine 5′-triphosphate (ATP) is the substrate of many enzymatic reactions and is widely used in research and in recent years also in enzymatic organic synthesis. In connection with the latter factor, the problem of the regeneration of ATP from ADP and (or) AMP arose.

Among the fairly large number of systems proposed for this purpose, two reactions, catalysed either by acetylphosphokinase[137–139], i.e.

$$ADP + \text{acetylphosphate} \xrightarrow{\text{acetylphosphokinase}} ATP + \text{acetate},$$

or by carbamoyl phosphate kinase[140],

$$ADP + \text{carbamoyl phosphate} \xrightarrow{\text{carbamoyl phosphote kinase}} ATP + \text{carbamate},$$

must be recognised at present as the most suitable.

If AMP is used as the initial reactant, then adenylate kinase must also be introduced into the system[137]:

$$AMP + ATP \xrightarrow{\text{adenylate kinase}} 2ADP.$$

Laboratory devices for the regeneration of ATP have been constructed on the basis of acetylphosphokinase and adenylate kinase immobilised on Sepharose and carbamoyl phosphokinase immobilised on glass[138,140].

3. Synthesis of Labelled Compounds

Compounds containing heavy or radioactive atoms in specific positions within their molecules have found extensive applications in chemistry and biology for the investigation of the mechanisms of chemical and enzymatic reactions, metabolic pathways, molecular structures, etc. Enzymes have been used on an increasing scale in recent years for the synthesis of compounds of this kind. The application of enzymes has the following principal advantages.

Firstly, *specificity*. In the synthesis of labelled compounds it is extremely important to introduce the label into rigorously defined positions within the molecule. The unique specificity of enzymes as a rule permits this. Apart from the high purity of the desired product, the expensive radioactive material is not consumed on side reactions in enzymatic synthesis.

Secondly, *high rate*. At the present time it appears extremely promising to use as labels the so-called short-lived isotopes, for example ^{11}C (half-life $T_{1/2} = 20.3$ min), ^{13}N ($T_{1/2} = 10$ min), ^{15}O ($T_{1/2} = 2$ min), and others. However, as a consequence of the high rate of decomposition of such isotopes, purely chemical synthetic methods were frequently ineffective. The synthesis of compounds containing short-lived isotopes became possible as a result of the employment of enzymes[141].

Thirdly, *mild conditions*. Enzymatic reactions proceed under mild, as a rule, physiological conditions. This is significant, for example, in the synthesis of labelled amino-acids (there is no racemisation) or proteins (there is no denaturation).

Tens of examples of the successful employment of enzymes for the synthesis of various labelled compounds have now been described.

Proteins. The following system is normally used in the synthesis of labelled protein derivatives[142-144]:

$$\text{protein} + K^{125}I + H_2O_2 \xrightarrow{\text{peroxidase}} \text{protein} - {}^{125}I.$$

The enzyme is used in the soluble or (better) immobilised[144] form.

Aminoacids. The initial reactants for the synthesis of labelled aminoacids are α-ketoacids and $\overset{*}{N}H_3$:[141,145-149]

$$R{-}\underset{\underset{O}{\|}}{C}{-}COOH + \overset{*}{N}H_3 + NADH \rightleftarrows L\text{-}R{-}\underset{\underset{{}^*NH_2}{|}}{CH}{-}COOH + NAD^+.$$

The reaction is catalysed by glutamate dehydrogenase obtained from ox heart, which (in contrast to the enzyme from other sources) exhibits a broad substrate specificity.

Nucleoside triphosphates. The problem of the synthesis of nucleoside triphosphates containing ^{32}P in the α-, β-, or γ-positions only has been solved with the aid of enzymes[150-153].

Sugars. Enzymes have been used to synthesise certain commercially unavailable sugars from commercial preparations of labelled glucose[47,154].

4. Synthesis of Oligopeptides

In the 1930's Bergmann showed for the first time that proteolytic enzymes can affect not only the hydrolysis but also the synthesis of peptides. In recent years the number of publications on this topic has increased sharply, since enzymatic or combined (enzymatic plus chemical) methods came to be regarded as a serious alternative to purely chemical synthesis of the peptide linkage. The advantages of the enzymatic method include the following: (a) the absence of racemisation; (b) mild reaction conditions which rule out degradation of labile compounds; (c) the absence of the need to protect the functional groups in the aminoacid side chains: (d) the absence of the need to activate the reacting groups.

Apart from the large number of model oligopeptides[29,30,34-42,57,58,64,65,89-92], important biologically active compounds such as angiotensin[39], encephalins[42,155], and human insulin[64,65] have been synthesised with the aid of enzymes.

The range of enzymes employed has been greatly extended: whereas up to 1970 only papain and chymotrypsin were used as catalysts, nowadays trypsin, pepsin, subtilisin, ficin, bromelain, carboxypeptidase (for the removal of protecting groups[156]), thermolysin, and bacterial metalloproteinases have also found applications.

5. The Synthesis and Modification of β-Lactam Antibiotics

The enzymatic method for the modification of β-lactam antibiotics in order to improve their properties, developed on a wide scale in recent years, appears to be extremely promising. According to this procedure[5,15], in the first stage the natural β-lactam antibiotics are subjected to penicillin amidase-catalysed hydrolysis in order to obtain the so-called nucleus of the antibiotic: 6-aminopenicillanic acid (in the case of penicillins) or 7-aminocephalosporanic acid) (in the case of cephalosporins). In the second stage the nucleus of the antibiotic is acylated (using penicillin amidase as the catalyst):

$$R^1{-}\underset{\underset{O}{\|}}{C}{-}N{-}\overline{\text{nucleus}} \underset{-R^1COOH}{\overset{+H_2O}{\rightleftarrows}} H_2N{-}\overline{\text{nucleus}} \xrightarrow{+R^2COOH} R^2{-}\underset{\underset{O}{\|}}{C}{-}\underset{\underset{H}{|}}{N}{-}\overline{\text{nucleus}} \quad (37)$$

In the second, acylation stage the yield of the product can be increased by carrying out the reaction in a weakly acid medium (a pH of 4.5–5.0 is optimum for the synthesis in most instances) or using as the acylating agent not the free acid but its activated derivatives.

By varying the acyl group R^2, it is possible to improve specifically the properties of antibiotics—to extend the spectrum of their activity, to increase their stability in the organism, and to reduce their allergenic effect. Several tens of "semisynthetic" antibiotics have been obtained in this way[5,15,87,88,157].

6. Modification of Steriods

Enzymes, cells, or unpurified extracts are also used for the modification of steroids[158-164].

7. Industrial Processes

Together with the laboratory developments discussed above, a series of technological (industrial) processes involving immobilised enzymes have been set up recently: (a) the synthesis of L-aspartic acid using immobilised microbial cells containing aspartase[14]; (b) the synthesis of L-malic acid using immobilised microbial cells containing fumarase[15]; (c) the synthesis of 6-aminopenicillanic acid using immobilised penicillin amidase[15]; (d) the separation with the aid of immobilised aminoacylase of racemic mixtures of aminoacids (obtained by a chemical, non-enzymatic procedure)[15,19]; (e) the synthesis of D-aminoacid derivatives with the aid of immobilised hydantoinase[15].

There are also large-scale industrial processes which go beyond the classical framework of organic synthesis and are more in the nature of the industrial manufacture of food products; (f) the preparation of glucose–fructose syrups using immobilised glucose isomerase[165,166]; (g) decomposition of lactose (the preparation of lactose-free milk) with the aid of lactase[15].

Certain processes have been developed only on pilot apparatus: (h) the synthesis of glucose from partial starch hydrolysates using immobilised glucoamylase[167]; (i) the synthesis of glucose and (or) ethanol from cellulose using cellulase[168]; (j) the preparation of glucose–fructose syrups from sucrose with the aid of immobilised invertase[15].

The Table, which we took from Sweigart and Dale[14] and supplemented by Klesov's data[169], gives a list of organisations which have set up these industrial (or pilot) processes.

It may seem surprising that a review of organic syntheses includes also the "food" processes (f)–(j). The need for this combined approach to the analysis of the present state of the problem (the introduction of enzymes into industrial processes) arises partly from the fact that enzyme catalysts have long been used in the food industry and at the present time (1976–1979) the food industry is the main consumer of the manufactured enzymes[13]. However, the principal factors which led us to consider, as far as possible, the most complete picture of the technological processes catalysed by enzymes are as follows. Firstly, the use of enzymes is beginning to break up the usual framework of organic synthesis. Secondly, any enzymatic food process (processes) gives rise to

new prospects for the large-scale preparation of raw material for chemical engineering, namely ethanol (and possibly also glucose). Finally, on the basis of processes already introduced, it is easier to predict certain economic factors. Evidently modern microbiological industry is already (1976) able to ensure the manufacture of enzymes in amounts running into hundreds of tons annually (at a cost of approximately 150 million dollars)[13]. However, the contribution of the ensyme to the overall cost of the product is in many instances insignificant[13,14].

The use of immobilised enzymes in large-scale (industrial) (L) and pilot plant (P).

Firm	Process*									
	a	b	c	d	e	f	g	h	i	j
ANHEUSER BUSCH (BRD)	—	—	—	—	—	L	—	—	—	—
CLINTON CORN (USA)	—	—	—	—	—	L	—	—	—	—
CAR MI (USA)	—	—	—	—	—	L	—	—	—	—
CORNING GLASS (USA)	—	—	—	P	—	L	L	P	—	P
US ARMY NATICK CENTER (USA)	—	—	—	—	—	—	—	—	P	—
DENKI KAGAKU (Japan)	—	—	—	—	—	P	—	—	—	—
DIAMOND SHAMROCK (USA)	—	—	—	—	—	—	P	—	—	—
GIST-BROCADES (Netherlands)	—	—	—	—	—	L	P	P	—	P
GULF OIL (USA)	—	—	—	—	—	—	—	—	P	—
HAMEEN PERUNA (Finland)	—	—	—	—	—	L	—	—	—	—
ICI (England, USA)	—	—	—	—	—	L	—	—	—	—
LEHIGH UNIVERSITY (USA)	—	—	—	—	—	—	P	—	—	—
NOVO (Denmark)	—	—	—	—	—	L	—	—	—	—
SANMARSU (Japan)	—	—	—	—	—	L	—	—	—	—
SNAM PROGETTI (Italy)	—	P	L	P	L	P	L	P	P	—
TANABE SEIYAKU (Japan)	L	L	L	L	—	—	—	—	—	—
VALIO (Finland)	—	—	—	—	—	—	P	—	—	—

*For the classification of the processes, see text.

For the introduction of biocatalysis into industrial processes (in which economic factors play a decisive role) on a wider scale, it is naturally necessary to improve further processes involving the microbiological synthesis and isolation of enzymes, methods for the preparation of carriers and the immobilisation of enzymes on them, enzymatic reactors, etc. However, the key problems at the present stage are the stabilisation of enzymes (see, for example, Martinek and Berezin[170]) and then the regeneration of cofactors, the use of polyenzyme systems, and the immobilisation of micro-organisms.

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