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ENZYMATIC SYNTHESIS IN BIPHASIC AQUEOUS-ORGANIC SYSTEMS

I. CHEMICAL EQUILIBRIUM SHIFT

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Summary

A theoretical analysis of the causes of chemical equilibrium shift (i.e., change in product yield), accompanying replacement of water as the reaction medium by biphasic system ‘water-water-immiscible organic solvent’, is given. A model is described, which is based on equilibrium partition of reagents between the two phases and establishment of a chemical equilibrium. In terms of this model, the apparent equilibrium constant, K_{biphasic} which is evolved from the equilibrium concentrations of the reagents referred to the total volume of the biphasic system, should depend on the ratio of the volume of the organic and aqueous phases and the partition coefficients.

The theoretical dependences were verified experimentally. Firstly, it was shown that for oxidation of isobutanol into isobutyraldehyde, catalyzed by alcohol dehydrogenase, the equilibrium shift that takes place in a water-hexane biphasic system is determined by the partition coefficients of the reagents found in an independent experiment. Varying the composition of the organic phase (hexane or ethyl acetate and their mixtures), the equilibrium could be shifted (compared to the aqueous solutions) towards both the initial reagents and the end-products; thereby changing the apparent equilibrium constant by two orders of magnitude.

Secondly, α-chymotrypsin-catalyzed synthesis of ethyl ester of *N*-benzoyl-L-phenylalanine (from the respective acid and alcohol) in a biphasic system containing chloroform, benzene, carbon tetrachloride or diethyl ether, has been studied. The apparent equilibrium constant has been experimentally demonstrated to depend on the ratio of the aqueous and organic phase volumes in an extreme fashion, as has been predicted by the theory. The yield of the product in the reaction optimum amounts to 100%, whereas in water it is as low as 0.01%.

Introduction

Enzyme-catalyzed chemical conversions proceed under mild conditions, at high rates and usually very specifically. Hence the increasing use of enzymes in preparative organic synthesis [1—4]. However, in many reactions that are of practical interest, the equilibrium is shifted towards the initial compounds. To increase the yield of the end product, the reaction conditions (e.g., pH, temperature, solvent, etc.) should be changed. Unfortunately, enzymes retain their catalytic activity within very narrow limits, i.e., in aqueous solutions, at neutral or near-neutral pH values and at moderate temperatures. This means that in the presence of enzymes the equilibrium can hardly be shifted by traditional methods of organic chemistry.

Quite a lot has been done to stabilize enzymes against denaturing effects of high temperatures and extreme pH values (see reviews of Refs. [4—7]). Denaturation of enzymes in organic solvents can be avoided if the reaction is carried out in a 'water-water-immiscible organic solvent' biphasic system [8,9]. An enzyme, being localized in the aqueous phase, does not have an unfavourable contact with the organic medium and does not lose its catalytic activity. An enzymatic biphasic system can either be an emulsified aqueous solution of enzyme in an organic phase [10] or reverse micelles of a surfactant with an entrapped enzyme (Refs. 11, 12, see also reviews [4—7,13]). However, a suspension, in an organic medium, of particles (of porous glass or ceramics, hollow fibers, gel granules, microcapsules), impregnated with an aqueous solution of an enzyme is technologically much more effective (8, 9 and reviews to Refs. 4—7). The enzyme can be used both in free and immobilized states. The content of water in such systems, low as it may be (less than 1%, v/v), is however high enough to contain the required amount of enzyme.

Biphasic aqueous-organic systems are extensively used in organic chemistry as reaction media but normally for a different purpose, i.e., in phase-transfer catalysis: biphasic systems allow one not only to increase the rate of a certain reaction, but also to improve the specificity and other kinetic parametres of a catalyzed process (see reviews to Refs. 14—16). However, there has been no general discussion in the literature about the effect of the biphasic nature of media on the equilibrium of chemical reactions. Some specific cases have been dealt with in chemical analysis, where the separation (extraction) methods based on partition of a substance between immiscible phases are used [17]. That is why the matter will be discussed below in some detail.

If water as a reaction medium is replaced by a biphasic system with a low content of aqueous component, a pronounced shift in the chemical equilibrium is observed [8,9]. One of the reasons for this shift in a reaction of the $A + B \rightleftharpoons C + H_2O$ type (where one of the products is water) seems to be obvious, i.e., the lower content of the aqueous reagent. The aim of this work is to analyse the other, more general cause of the shift, i.e., a change in the apparent equilibrium constant which is due to the partition of the reagents between the phases.

We shall also dwell on the perspectives of the utilization of biphasic enzyme-containing systems in preparative organic synthesis.

Theory

Reaction of $A \rightleftharpoons B$ type In a biphasic system there is the following partition between water and the organic solvent:

$$\begin{tikzcd}
(A \arrow[r, shift left, "K_w"] \arrow[d, shift right, "P_A"'] & B)_{\text{in water}} \arrow[l, shift left] \arrow[d, shift left, "P_B"] \\
(A \arrow[u, shift right] \arrow[r, shift left, "K_{org}"] & B)_{\text{in organic solvent}} \arrow[l, shift left] \arrow[u, shift left]
\end{tikzcd} \qquad (1)$$

Let us determine the dependence of apparent equilibrium constant, K_{biphasic}-$[B]_{\text{total}}/[A]_{\text{total}}$, on the partition coefficients P_A and P_B and the ratio of the volumes of the aqueous and organic phases. The analysis of equilibrium reaction 1 will be similar to that of the reaction system involving micelles of a surfactant [18,19]. The total concentrations of reagents A and B referred to the total volume of a biphasic system can be derived from the material balance equations:

$$[A]_{\text{total}}(V_w + V_{\text{org}}) = [A]_w \cdot V_w + [A]_{\text{org}} \cdot V_{\text{org}}$$

and

$$[B]_{\text{total}}(V_w + V_{\text{org}}) = [B]_w \cdot V_w + [B]_{\text{org}} \cdot V_{\text{org}}$$

if the expression for the partition coefficients,

$$P_A = [A]_{\text{org}}/[A]_w$$
$$P_B = [B]_{\text{org}}/[B]_w,$$

and for the equilibrium constant in one of the phases,

$$K_w = [B]_w/[A]_w$$

or

$$K_{\text{org}} = [B]_{\text{org}}/[A]_{\text{org}}$$

are taken into consideration.

Then

$$K_{\text{biphasic}} = K_w \frac{1 + \alpha \cdot P_B}{1 + \alpha \cdot P_A} \qquad (2)$$

or

$$K_{\text{biphasic}} = K_{\text{org}} \frac{1 + 1/\alpha \cdot P_A}{1 + 1/\alpha \cdot P_B} \qquad (3)$$

where $\alpha = V_{\text{org}}/V_w$ is the volume ratio of the organic and water phases.

As is evident from Eqns. 2 and 3, the value of K_{biphasic} changes continuously as α increases, that is, depending on the organic solvent used, it either increases (with $P_A < P_B$) or decreases (with $P_A > P_B$). If the value of α is sufficiently high (when the content of the aqueous phase is sufficiently low) the equilibrium constant tends to a limit which is equal to $K_w \cdot P_B/P_A \equiv K_{\text{org}}$. In Fig. 1 the characteristic dependences of K_{biphasic} on α with certain ratios of the partition coefficients are given.

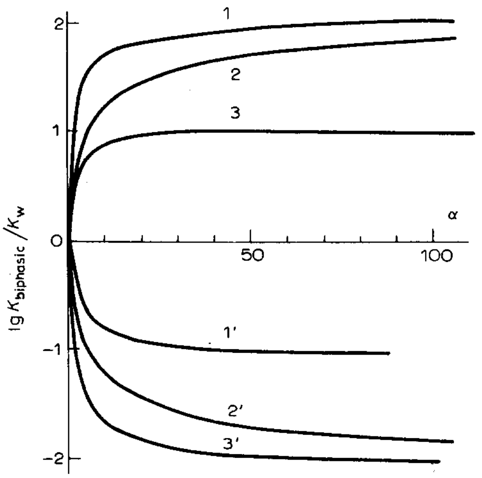


Fig. 1. Apparent equilibrium constant of an A ⇌ B type reaction in a biphasic aqueous-organic system vs. phase volume ratio (α) at certain values of partition coefficients: 1. $P_A = 0.1$, $P_B = 10$, $P_B/P_A = 100$; 2. $P_A = 0.02$, $P_B = 2$, $P_B/P_A = 100$; 3. $P_A = 0.2$ $P_B = 2$, $P_B/P_A = 10$; 1′. $P_A = 2$, $P_B = 0.2$, $P_B/P_A = 0.1$; 2′. $P_A = 2$, $P_B = 0.02$, $P_B/P_A = 0.01$; 3′. $P_A = 10$, $P_B = 0.1$, $P_B/P_A = 0.01$.

Bimolecular reaction of type $A + B \rightleftharpoons C + D$. For this reaction going in a biphasic system:

$$\begin{tikzcd}
(\mathrm{A} \arrow[d, shift left, "P_A"] & +\,\mathrm{B} \arrow[rr, shift left, "K_w"] \arrow[d, shift left, "P_B"] & & \mathrm{C} \arrow[ll, shift left] \arrow[d, shift left, "P_C"] & +\,\mathrm{D})_{\text{in water}} \arrow[d, shift left, "P_D"] \\
(\mathrm{A} \arrow[u, shift left] & +\,\mathrm{B} \arrow[rr, shift left, "K_{\text{org}}"] \arrow[u, shift left] & & \mathrm{C} \arrow[ll, shift left] \arrow[u, shift left] & +\,\mathrm{D})_{\text{in organic solvent}} \arrow[u, shift left]
\end{tikzcd} \tag{4}$$

the apparent equilibrium constant is

$$K_{\text{biphasic}} = K_w \frac{(1 + \alpha \cdot P_C)(1 + \alpha \cdot P_D)}{(1 + \alpha \cdot P_A)(1 + \alpha \cdot P_B)}. \tag{5}$$

As in the previous case, with sufficiently high values of α (when the content of the aqueous phase is low), the value of K_{biphasic} tends to a limit that is equal to $K_w P_C P_D / P_A P_B \equiv K_{\text{org}}$. However, with the moderately high values of α, the $K_{\text{biphasic}}(\alpha)$ function has two new, rather important properties.

(i) As the content of the organic phase in the reaction system 4 increases, the function (5) may have an extreme. Let us find the conditions for this extreme. From the expression for the first derivative,

$$\frac{\partial K_{\text{biphasic}}}{\partial \alpha} = K_w \left[(P_C + P_D) - (P_A + P_B) + 2\alpha (P_C P_D - P_A P_B) + \alpha^2 P_A P_B P_C P_D \left(\frac{1}{P_A} + \frac{1}{P_B} - \frac{1}{P_C} - \frac{1}{P_D} \right) \right] [(1 + \alpha \cdot P_A)^2 (1 + \alpha \cdot P_B)^2]^{-1} \tag{6}$$

it follows that function (5) is continuous only if

$$\begin{cases} P_C + P_D \geqslant P_A + P_B \\ 1/P_C + 1/P_D < 1/P_A + 1/P_B \end{cases} \tag{7}$$

or

$$\begin{cases} P_C + P_D > P_A + P_B \\ 1/P_C + 1/P_D \leqslant 1/P_A + 1/P_B \end{cases} \tag{8}$$

for the case when $K_{\text{biphasic}} > K_w$, and

$$\begin{cases} P_C + P_D \leqslant P_A + P_B \\ 1/P_C + 1/P_D > 1/P_A + 1/P_B \end{cases} \tag{9}$$

or

$$\begin{cases} P_C + P_D < P_A + P_B \\ 1/P_C + 1/P_D \geqslant 1/P_A + 1/P_B \end{cases} \tag{10}$$

for the case when $K_{\text{biphasic}} < K_w$. If the inequalities

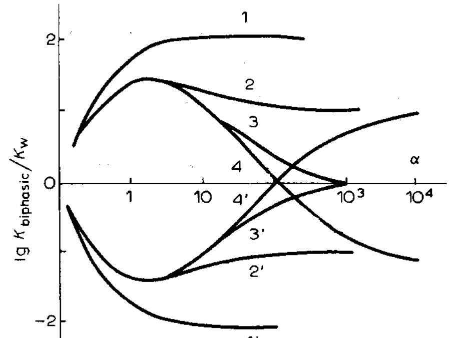
$$\begin{cases} P_C + P_D > P_A + P_B \\ 1/P_C + 1/P_D > 1/P_A + 1/P_B \end{cases} \tag{11}$$

are valid, the $K_{\text{biphasic}}(\alpha)$ function has a maximum. Some examples are shown in Fig. 2. On the other hand, function (5) will have a minimum if

$$\begin{cases} P_C + P_D < P_A + P_B \\ 1/P_C + 1/P_D < 1/P_A + 1/P_B \end{cases} \tag{12}$$

A more detailed analysis of Eqns. 5 and 6 shows that (with non-negative values of α) the $K_{\text{biphasic}}(\alpha)$ dependence for a reaction of type (4) has no more than one extreme (if at all).

The presence of the extreme should be interpreted to mean that the apparent equilibrium constant in a biphasic system can be higher (or lower) than the



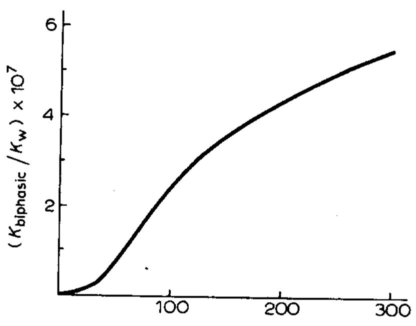


Fig. 2. Apparent equilibrium constant of an $A + B \rightleftharpoons C + D$ type reaction in a biphasic aqueous-organic system vs. phase volume ratio (α) at certain values of partition coefficients. Curves 1—4: $P_A = P_B = 1$, $P_C = 10^2$, $P_D = 1$ (1), $P_D = 10^{-1}$ (2), $P_D = 10^{-2}$ (3), $P_D = 10^{-3}$ (4). Curves 1'—4': $P_C = P_D = 1$, $P_A = 10^2$, $P_B = 1$ (1'), $P_B = 10^{-1}$ (2'), $P_B = 10^{-2}$ (3'), $P_B = 10^{-3}$ (4').

Fig. 3. An example of an S-shaped dependence of apparent equilibrium constant for an $A + B \rightleftharpoons C + D$ type reaction in a biphasic aqueous-organic system vs. phase volume ratio (α) at the following values of partition coefficients: $P_A = P_B = 10^{-2}$, $P_C = P_D = 10^2$. Note that nonlogarithmic spacing is used.

equilibrium constants of the same reaction in either of the phases! This is shown in Fig. 2.

(ii) The other peculiarity of reactions of type (4) in biphasic systems is that the dependence of the apparent equilibrium constant, K_{biphasic}, on the phase volume ratio (α) can give a sigmoid curve (in nonlogarithmic co-ordinates). The conditions (i.e., partition coefficient ratio) under which the $K_{\text{biphasic}}(\alpha)$ function must be an S-shaped curve, are difficult to express analytically. Fig. 3 shows just one example. In the case of a monomolecular reaction of type 1 such an effect does not take place (compare Figs. 1 and 3). It is obvious that the curves in the logarithmic co-ordinates, i.e., $\log K_{\text{biphasic}}(\log \alpha)$ will always be S-shaped for both reactions regardless of the partition coefficients.

Reactions of type $A + B \rightleftharpoons C$. The apparent equilibrium constant equals

$$K_{\text{biphasic}} = K_w \frac{(1 + \alpha \cdot P_C)(1 + \alpha)}{(1 + \alpha \cdot P_A)(1 + \alpha \cdot P_B)} \tag{13}$$

that is, can be described by the same equation as reactions of type (4), if one assumes that $P_D = 1$; hence it will have the same properties.

The yield of product C in a reaction of $A + B \rightleftharpoons C + H_2O$ type. It has been shown above how the apparent equilibrium constant changes if the content of water and the organic phase are varied. In preparative organic synthesis it is the yield of products that is the most important characteristic of the reaction. But usually it is sufficient to analyse the equilibrium constants, since in most reactions this value and the yield of products change in parallel. A special case is the reaction involving water, for example:

$$\mathrm{A + B \rightleftharpoons C + H_2O} \tag{14}$$

In this case the yield of product C in a biphasic system depends in two ways on the concentration of water, i.e., via the apparent equilibrium constant (that is determined by functions of type (5) and consequently depends on α) and in accordance with the principle of active mass:

$$[\mathrm{C}]_{\text{total}} = K_{\text{biphasic}} \frac{[\mathrm{A}]_{\text{total}}[\mathrm{B}]_{\text{total}}}{[\mathrm{H_2O}]_{\text{total}}} \tag{15}$$

The value of $[\mathrm{H_2O}]_{\text{total}}$ can be evaluated from the material balance equation:

$$[\mathrm{H_2O}]_{\text{total}}(V_w + V_{\text{org}}) = [\mathrm{H_2O}]_w V_w + [\mathrm{H_2O}]_{\text{org}} V_{\text{org}}$$

considering that $[\mathrm{H_2O}]_w = 55.5$ and $[\mathrm{H_2O}]_{\text{org}}/[\mathrm{H_2O}]_w = P_{\mathrm{H_2O}}$. Then

$$[\mathrm{H_2O}]_{\text{total}} = \frac{(1 + \alpha \cdot P_{\mathrm{H_2O}})\ 55.5}{1 + \alpha} \tag{15a}$$

Having substituted Eqns. 5 and 15a into Eqn. 15 we shall have:

$$[\mathrm{C}]_{\text{total}} = K_w \frac{[\mathrm{A}]_{\text{total}} \cdot [\mathrm{B}]_{\text{total}}}{55.5} \frac{(1 + \alpha \cdot P_C)(1 + \alpha)}{(1 + \alpha \cdot P_A)(1 + \alpha \cdot P_B)} \tag{16}$$

This equation has α^2 both in the numerator and denominator; therefore, the

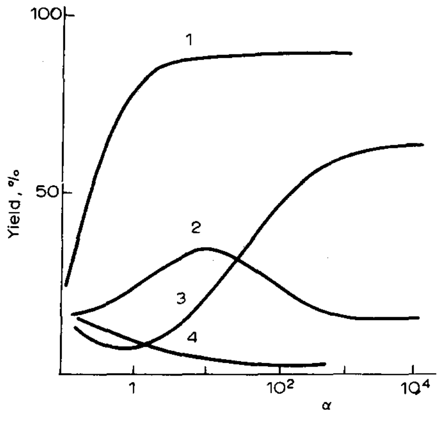
$[C]_{total}$ vs. α dependence can be rather complex (Fig. 4). For example, with some ratios of partition coefficients, the yield of product C even decreases if the concentration of the second end product, water, decreases (Fig. 4, curves 2—4). This amazing phenomenon is due to the fact that as α increases, the apparent equilibrium constant decreases more than the total concentration of water in the system, cf. Eqn. 16.

General remarks and some methodological recommendations. A theoretical analysis allows one to make the following conclusions.

(i) The apparent equilibrium of a chemical reaction in a biphasic system is determined by the equilibrium constant of this reaction in one of the phases, the partition coefficients of the starting reagents and end products and the volume ratio of the organic and aqueous phases. This means that, if one knows the partition coefficients (from the literature or from independent experiments), one can derive K_{biphasic} and hence the yield of the reaction products. It should be noted, however, that the information about the values of the partition coefficients for various substances is very scanty (cf. Ref. 20).

It is very important to be able to predict the direction and the magnitude of an equilibrium shift in a biphasic system compared to water, because the dependence of the K_{biphasic} value on the phase volume ratio (α) can be very complex. This can then make the elucidation of the reaction conditions for the synthesis difficult. Two items can be important here, these are described in (ii) and (iii).

(ii) The value of K_{biphasic} for reaction (4) in a biphasic system can be much higher (Fig. 2, curves 2—4) or lower (Fig. 2, curves 2′—4′) than the equilibrium constants of the same reaction in each of the phases. Let us analyse this phe-



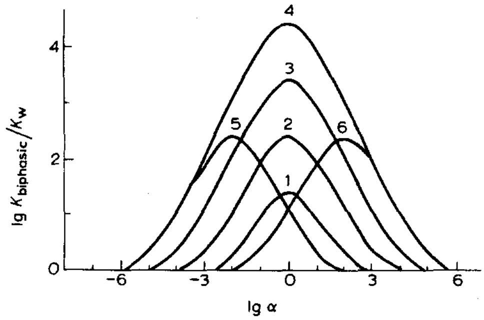


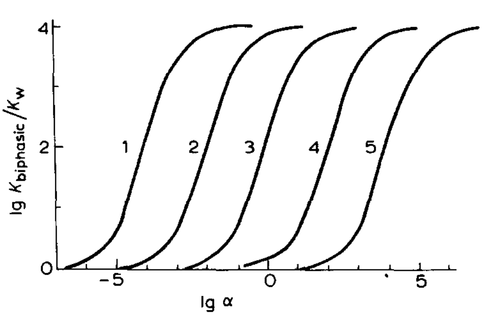
Fig. 4. Product yield C in a $A + B \rightleftharpoons C + H_2O$ type reaction in a biphasic aqueous-organic system vs. phase volume ratio (α) at certain values of partition coefficients. Curves: 1. $P_A = P_B = 1$, $P_C = 50$; 2. $P_A = P_B = 0.1$, $P_C = 0.01$; 3. $P_A = 10$, $P_B = 0.01$, $P_C = 1$; 4. $P_A = P_B = 1$, $P_C = 0.1$; in all cases $K_w = 10$.

Fig. 5. Extreme character of the dependence of the apparent equilibrium constant in an $A + B \rightleftharpoons C + D$ type reaction in a biphasic aqueous-organic system on phase volume ratio (α) at certain values of partition coefficients. Curves: 1. $P_A = P_B = 1$, $P_C = 10^2$, $P_D = 10^{-2}$; 2. $P_A = P_B = 1$, $P_C = 10^3$, $P_D = 10^{-3}$; 3. $P_A = P_B = 1$, $P_C = 10^4$, $P_D = 10^{-4}$; 4. $P_A = P_B = 1$, $P_C = 10^5$, $P_D = 10^{-5}$; 5. $P_A = P_B = 10^2$, $P_C = 10^5$, $P_D = 10^{-1}$; 6. $P_A = P_B = 10^{-2}$, $P_C = 10$, $P_D = 10^{-5}$.

nomenon in greater detail using as a case in point a biphasic system where $K_w = K_{org}$, i.e., let us assume that in water and in an immiscible organic solvent the equilibrium constant has one and the same value. The values of the partition coefficients will be varied in the most reasonable range from 10^{-5} to 10^5 (cf. Ref. 20). Fig. 5 shows how $K_{biphasic}$ changes depending on the phase volume ratio (α). One can see that the maximal value of $K_{biphasic}$ for reaction (4) in a biphasic system can be 10^4 or more times as high as the respective equilibrium constants in each of the phases. This means that the biphasic systems can prove exceptionally advantageous for increasing the yield of an end product in the reactions (not necessarily enzymatic) where the equilibrium is not shifted to a desirable degree in any of the solvents that are available or usable for the reaction in question.

(iii) When choosing the conditions for the synthesis one has to take into consideration the fact that the $K_{biphasic}(\alpha)$ function can be S-shaped (even with nonlogarithmic co-ordinates, see Fig. 3). In other words, within a certain range of α values the equilibrium of the reaction is insensitive to the change in the medium. This can be seen in Figs. 5 and 6 where we analyse the case when $10^4 \cdot K_w = K_{org}$. It is obvious that, depending on the partition coefficients, which were varied by us within the reasonable range from 10^{-5} to 10^5, (cf. Ref. 20), the shift of the chemical equilibrium does not take place both with a negligibly low content of an organic phase ($\alpha = V_{org}/V_w \sim 10^{-5}$, curve 1), and with comparable contents of the organic and aqueous phases ($\alpha \sim 1$, curve 3) or even when the system is utterly devoid of water ($\alpha \sim 10^5$, curve 5).

(iv) Biphasic systems can be devised in the following way. (a) For systems with a low content of the organic phase ($\alpha << 1$), one can use for example an aqueous solution of a micelle-forming surfactant; the inner (hydrophobic) region of the micelles will make the organic phase [18,19]. (b) For systems with a low content of water ($\alpha >> 1$), the enzyme should be entrapped in 'reverse' micelles of a surfactant in an organic solvent [11,12]. (c) Systems with



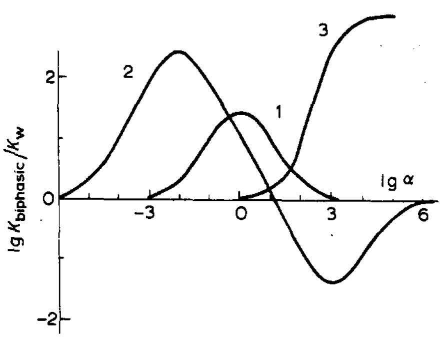


Fig. 6. Apparent equilibrium constant in an $A + B \rightleftharpoons C + D$ type reaction in a biphasic aqueous-organic system vs. phase volume ratio (α) at certain values of partition coefficients. Curves: 1. $p_A = p_B = 10^3$, $p_C = p_D = 10^5$; 2. $p_A = p_B = 10$, $p_C = p_D = 10^3$; 3. $p_A = p_B = 10^{-1}$, $p_C = p_D = 10$; 4. $p_A = p_B = 10^{-3}$, $p_C = p_D = 10^{-1}$; 5. $p_A = p_B = 10^{-5}$, $p_C = p_D = 10^{-3}$.

Fig. 7. Apparent equilibrium constant in an $A + B + C \rightleftharpoons D + E + F$ type reaction in a biphasic aqueous-organic system vs. phase volume ratio (α) at certain values of partition coefficient. Curves: 1. $p_A = p_B = p_C = p_D = 1$, $p_E = 10^2$, $p_F = 10^{-2}$; 2. $p_A = p_B = 10^2$, $p_C = 10^{-5}$, $p_D = 10^5$, $p_E = p_F = 10^{-3}$; 3. $p_A = p_B = p_C = 10^{-3}$, $p_D = p_E = p_F = 10^{-2}$.

comparable amounts of the aqueous and organic phases can operate, as is indicated above (see Introduction), on the basis of an emulsified aqueous solution of an enzyme in an organic solvent or as a suspension in this solvent of porous particles impregnated with an aqueous solution of the enzyme.

With (a) and (b) we can hardly speak of biphasic systems proper as the interface is practically absent; it is more correct to call them 'pseudobiphasic systems' [19]. Nevertheless, all that has been discussed above concerning the equilibrium shift, will hold for such systems [18,19,21].

(v) We have so far dealt with mono- and bimolecular reactions. In more complex reactions, the $K_{\text{biphasic}}(\alpha)$ function may be more complicated. But the principle will remain the same. By way of example, let us consider reaction $A + B + C \rightleftharpoons D + E + F$. Fig. 7 shows that the $K_{\text{biphasic}}(\alpha)$ function can have an extreme (curve 1); in principle there should be two extremes, a maximum and a minimum (curve 2); finally, as α increases, K_{biphasic} does not start changing immediately, a certain value of α is to be achieved (curve 3).

(vi) When dealing with the chemical equilibrium shift in biphasic aqueous-organic systems, we have not imposed any liminations on the nature of the organic phase, except that it should be immiscible with water. This means that virtually any substance can be used as an 'organic phase' that is capable of forming a biphasic (or pseudobiphasic) system. It can be, for example, a water-insoluble polymer [22,23]. The same regularities should hold for a system of two immiscible organic solvents.

(vii) We have discussed no limitations with regard to the nature of the catalyst. Its role is actually to help a fast creation of the equilibrium. This means that the equilibrium shift in a biphasic system will take place with chemical catalysts as well. The reaction can also be a noncatalytic one.

Experimental

Materials

Bovine α-chymotrypsin (EC 3.4.21.1) was manufactured at the Olain chemical reagents plant (Latvian, U.S.S.R.). Alcohol dehydrogenase (EC 1.1.1.1) from horse liver (spec. act. 2 units), NAD and NADH were commercial preparations of Reanal. *N*-Benzoyl-L-phenyl alanine was the product of Serva. Large-pore silica gel was obtained from the All Union Research Institute of Petroleum Industry; its specific surface area was 38.8 m^2/g, pore diameter 600 Å and grain size not more than 0.5 mm. The mineral salts, alkali and organic solvents were of analytical grade and manufactured by 'Soyuzkhimreactive'.

Methods

Enzymatic synthesis of N-benzoyl-L-phenylalanine ethyl ester. Synthesis was carried out in the following way: 1.5 g silica gel were kept under low pressure in 0.5 phosphate buffer (pH 7.0), then the excess of buffer solution was removed, the wet silica gel weighed and supplemented with 0.5 ml 0.05 M solution of *N*-benzoyl-L-phenylalanine in water that contained 10 mg α-chymotrypsin. This preparation was stirred for 10 min and supplemented with a certain amount (5—200 ml) of chloroform containing 0.1 M ethanol. The biphasic system obtained was continuously shaken overnight (at 20°C). We have ascertained that the reaction time sufficiently exceeds the time needed for attaining the equilibrium. Then the water-silica gel phase was filtered off and the resulting organic solution was evaporated to dryness in a rotary evaporator under vacuum. The resulting precipitate was practically 100% ethyl ester of *N*-benzoyl-L-phenylalanine. The degree of the ester synthesis was determined with the pH-stat technique (as described in Refs. 8, 9).

The value of the apparent equilibrium constant, $K_{\text{biphasic}} = [\text{ester}] \cdot [H_2O]/[\text{acid}] \cdot [\text{ethanol}]$, was determined on the basis of the equilibrium concentrations of the reagents compared to the total volume of the biphasic system. For this purpose the equilibrium concentrations of the reagents were evolved from the initial concentrations of *N*-benzoyl-L-phenylalanine and ethanol and the experimentally found equilibrium concentration (in the organic phase) of the ester. When determining the concentration of the water reagent (which was also compared to the volume of the entire system), not only the volume of the aqueous phase was taken into consideration, but also the water that is dissolved in the organic phase (in the organic solvent); to this end use was made of the data of Leo et al. [20].

Oxidation of isobutanol catalyzed by alcohol dehydrogenase. 0.4 ml 0.1 M pyrophosphate buffer (pH 8.4) was supplemented with 0.1 ml of the stock solution of NAD (10 mg/ml in water), 0.03 ml of isobutanol, 0.01 ml of a crystalline suspension of alcohol dehydrogenase (16 mg/ml) in an aqueous solution of $(NH_4)_2SO_4$ and a certain amount of an organic solvent (1—10 ml). The biphasic system was stirred at 20° C until the equilibrium was established (i.e., for about 20—40 min), we ascertained that this time is sufficient for the highest (equilibrium) concentration of NADH to be reached. For this purpose, 0.3 ml of the aqueous phase were collected, placed in a cuvette containing 2 ml 8 M urea, and the absorbance was determined at 340 nm. The measurements were carried out in a Beckman model 25 spectrophotometer. The molar absorption of NADH was measured to be 6200 [24].

The value of the apparent equilibrium constant, $K_{\text{biphasic}} = [\text{isobutyraldehyde}] \cdot [\text{NADH}]/[\text{isobutanol}] \cdot [\text{NAD}]$ was calculated on the basis of the equilibrium concentrations of the reagents compared to the total volume of the biphasic system. The equilibrium concentrations of the reagents were determined on the basis of the initial concentrations of isobutanol and NAD and the experimentally determined equilibrium concentration of NADH (in the aqueous phase, see above).

The partition coefficients. These coefficients of isobutanol and isobutyraldehyde in the water-hexane system were determined as described [25].

Results and Discussion

In this work our primary concern was to experimentally prove that the equilibrium shift, observed when water as a reaction medium is replaced by a biphasic system (containing water and an organic solvent), is determined by the partition of the reagents between the phases as was predicted theoretically (see above). Secondly, we believed it necessary to provide experimental proof for another theoretical statement, i.e., that the dependence of K_{biphasic} on the phase volume ratio may have an extreme, and that the optimal value of

K_{biphasic} is considerably higher (or lower) than the values in each of the phases (water or an organic medium). Finally, we wanted to demonstrate that K_{biphasic} can change quite appreciably even when the starting reagent and the end product differ only slightly in hydrophobicity, determined as described by Hansch [20]. Our aims predetermined the choice of the systems.

Alcohol dehydrogenase-catalysed oxidation of isobutanol in a biphasic water-organic system

The apparent equilibrium constant of the reaction

$$(CH_3)_2CHCH_2OH + NAD \rightleftharpoons (CH_3)_2CHCHO + NADH + H^+$$

in a biphasic system equals (cf. Eqn. 5)

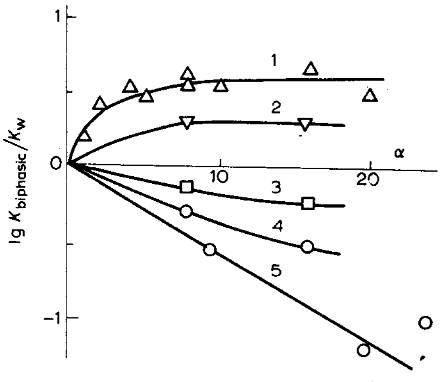
$$K_{\text{biphasic}} = K_{\text{w}} \frac{(1 + \alpha \cdot P_{\text{ald}})(1 + \alpha \cdot P_{\text{NADH}})}{(1 + \alpha \cdot P_{\text{alc}})(1 + \alpha \cdot P_{\text{NAD}})}$$

or

$$K_{\text{biphasic}} \approx K_{\text{w}} \frac{1 + \alpha \cdot P_{\text{ald}}}{1 + \alpha \cdot P_{\text{alc}}}$$

if one assumes that P_{NAD} and $P_{\text{NADH}} << 1$, as the cofactor used in the enzymatic reaction is charged both in the oxidized and reduced forms and consequently, is hardly soluble in the organic solvents used. We have provided some experimental (spectrophotometric) evidence that NAD and NADH are localised in the aqueous phase.

The experimental results that illustrate the change in the apparent equilibri-



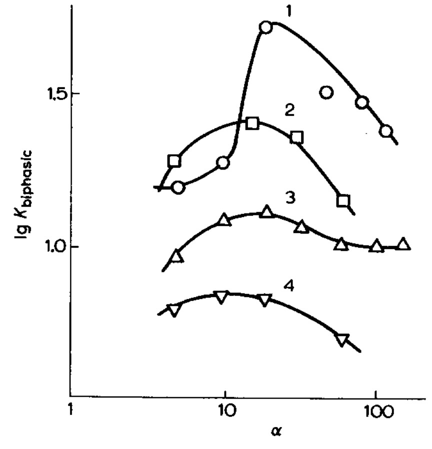


Fig. 8. Apparent equilibrium constant for the alcohol dehydrogenase-catalyzed oxidation of isobutanol in a biphasic aqueous-organic system vs. phase volume ratio (α) and the nature of the organic phase: 1. hexane; 2. 87.5% hexane + 12.5% ethyl acetate; 3. 75% hexane + 25% ethyl acetate; 4. 50% hexane + 50% ethyl acetate; 5. ethyl acetate.

Fig. 9. Extreme character of the dependence of the apparent equilibrium constant of the α-chymotrypsin-catalyzed synthesis of ethyl ester of *N*-benzoyl-L-phenylalanine in a biphasic aqueous-organic system vs. phase volume ratio (α) and the nature of the organic phase: 1. chloroform, 2. benzene, 3. carbon tetrachloride, 4. diethyl ether.

um constant in biphasic systems compared to water are presented in Fig. 8. The value of $K_w = 5.7 \cdot 10^{-4}$ (pH 8.4) [26].

This reaction gives a good example of a system in which the starting reagent and the end product hardly ever differ in hydrophobicity. For example, in terms of the classical biphasic system of Hansch (water-octanol), the partition coefficients of the alcohol and aldehyde groups differ by only 12% [20]. Nevertheless, we chose such solvents that the partition coefficients of the starting reagent and final product are sufficiently different, apparently owing to specific solvation effects. We used as the organic phase hexane (Fig. 8, curve 1), ethyl acetate (curve 5) or mixtures of these solvents (curves 2—4) and could, therefore, vary the value of K_{biphasic} for the reaction studied within two orders of magnitude.

In one of the systems used (water-hexane), the partition coefficients of alcohol and aldehyde were determined in an independent experiment (see Experimental). They proved to be equal to 0.25 and 1.2, respectively. Using these values, and Eqn. 2, we determined the theoretical dependence of K_{biphasic}/K_w vs. phase volume ratio (α). It is obvious from Fig. 8 (curve 1) that the experimental results are in good agreement with theoretical predictions.

α-Chymotrypsin-catalysed synthesis of N-benzoyl-L-phenylalanine ethyl ester

Inequalities (11) and (12) desribing the conditions for the maximum in the $K_{\text{biphasic}}(\alpha)$ function for the reactions of $A + B \rightleftharpoons C + D$ type are valid if, for example, $P_C >> P_A \sim P_B >> P_D$. Such a relationship can be realised experimentally, if one of the products (D) is water, since its partition coefficient is usually low; it does not exceed 10^{-2} for the majority of the solvents [20]. Let us analyse, by way of example, the synthesis of *N*-benzoyl-L-phenylalanine ethyl ester from the respective acid and alcohol:

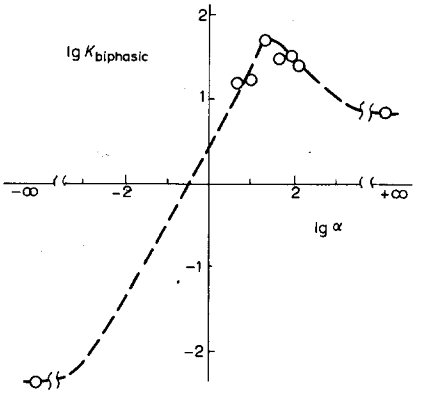
$$\text{N-Bz-Phe} + \text{EtOH} \rightleftharpoons \text{N-Bz-Phe-OEt} + H_2O$$

The $K_{\text{biphasic}}(\alpha)$ dependence observed experimentally (Fig. 9) proved to have a maximum for all the four solvents that were used for the organic phase (chloroform, benzene, carbon tetrachloride and diethyl ether). This results proves the theoretically predicted peculiarity of the biphasic systems, that the value of K_{biphasic} can be higher than both limiting values characterising the equilibrium in either of the phases. For comparison's sake, we shall mention that in water the equilibrium in the synthesis of *N*-benzoyl-L-phenylalanine ethyl ester is shifted entirely towards the starting compounds [27]; $K_w = 2 \cdot 10^{-3}$ (pH 7). The literature does not contain the figure for the equilibrium in an organic medium. To estimate the equilibrium constant, the following relationship could be used:

$$K_{\text{org}} = K_w \cdot P_{\text{ester}} \cdot P_{H_2O}/P_{\text{acid}} \cdot P_{\text{alc}}$$

which can be derived from Eqn. 5 if $\alpha \to \infty$. Semenov et al. [28] estimated the partition coefficients in the water-chloroform system were $P_{\text{ester}} = 4.1 \cdot 10^3$, $P_{\text{acid}} = 0.11$ (pH 7), $P_{\text{alc}} = 10^{-2}$. In the same system, the partition coefficient for water calculated with the use of data from Hansch et al [20], is equal to $1.2 \cdot 10^{-3}$. Hence $K_{\text{org}} \approx 9$.

In Fig. 10 one can see that both these values, K_w, i.e., K_{biphasic} at $\log \alpha \to$



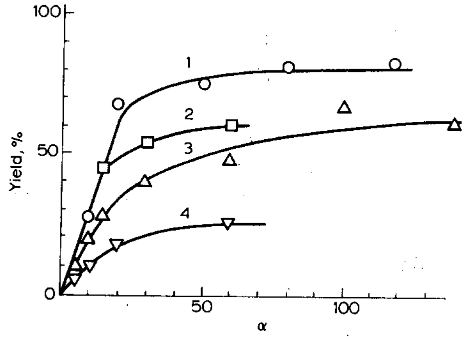


Fig. 10. Extreme character of the dependence of the apparent equilibrium constant of the α-chymotrypsin-catalyzed synthesis of *N*-benzoyl-L-phenylalanine ethyl ester in a chloroform-water biphasic system on the phase volume ratio (α). The curve was calculated with Eqn. 5 assuming $K_W = 2 \cdot 10^{-3}$ (pH 7) [27], $P_{ester} = 4.1 \cdot 10^3$, $P_{acid} = 0.11$ (pH 7), $P_{alc} = 10^{-2}$ [28], $P_{H_2O} = 1.2 \cdot 10^{-3}$ [20].

Fig. 11. Yield of *N*-benzoyl-L-phenylalanine ethyl ester in a biphasic aqueous-organic system vs. phase volume ratio (α) and the nature of the organic phase: 1. chloroform, 2. benzene, 3. carbon tetrachloride, 4. diethyl ether.

$-\infty$, and K_{org}, i.e., $K_{biphasic}$ at $\log \alpha \rightarrow +\infty$, that characterise the equilibrium in each of the phases, are much lower than the maximal value of $K_{biphasic}$ observed for the biphasic system.

This conclusion is very important for preparative organic synthesis. For example, in water the yield of the ester is as low as 0.01% [27]. In a water-organic solvent biphasic system the yield is 80, 64, 62 and 26% for chloroform, benzene, carbon tetrachloride and diethyl ether, respectively (Fig. 11). This means that the use of biphasic systems has allowed the yield of the product to be raised from zero to a preparative level.

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