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ENZYMATIC SYNTHESIS IN BIPHASIC AQUEOUS-ORGANIC SYSTEMS

II. SHIFT OF IONIC EQUILIBRIA

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Summary

Ionic equilibria in 'water-water-immiscible organic solvent' systems have been studied. It has been shown that in such systems shift of the apparent p*K* value of acids and bases takes place (compared to aqueous solutions), the value of the shift being rather high, up to 5 and more pH units (with 2,4-dinitrophenyltryptophan as an acid and neutral red as a base).

The p*K* shift of ionogenic reagents observed in biphasic systems can be used in preparative organic synthesis for increasing the yield of end products in enzyme-catalyzed reactions. In connection with this, the physico-chemical reasons for the equilibrium shift in a chemical reaction that involves one or two ionogenic reagents are theoretically analyzed.

The above approach has been tested with two α-chymotrypsin-catalyzed reactions, i.e., synthesis of *N*-benzoyl-L-phenylalanine ethyl ester (from NBz-LPhe-OH and ethanol) and synthesis of *N*-acetyl-L-tryptophanyl-L-leucine amide (from NAc-LTrp-OH and LLeu-NH_2). In water the equilibria in these reactions are shifted almost entirely towards the starting reagents with the yield of end product being negligibly low. In biphasic systems consisting of chloroform + 5% (v/v) water or ethyl acetate + 2% (v/v) water, the yield of both the ester and the dipeptide reaches 100%.

Introduction

'Water-water-immiscible organic solvent' biphasic systems have recently been suggested for use as media for enzyme-catalyzed chemical reactions [1,2]. In such systems there occurs a rather large shift of chemical equilibrium, that is, the equilibrium constant changes by several orders of magnitude. This allows the yield of the end product to be raised in thermodynamically unfavourable reactions while the microenvironment of the catalyst (enzyme) is maintained fit for its functioning. In our previous communication [3], we made a quantitative analysis of the regularities of equilibrium shift (increase in the product yield) of chemical reactions proceeding in biphasic water-organic systems compared to aqueous solutions in the reactions of type $A \rightleftharpoons B$, $A + B \rightleftharpoons C + D$ and $A + B \rightleftharpoons C + H_2O$. Some relatively simple reactions as, for example,

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

or

$$A + B \overset{K_{eq}}{\rightleftharpoons} C + D\,, \qquad \text{where } B + H^+ \overset{K_b}{\rightleftharpoons} BH^+$$

which are hindered by ionization of the reagents are not infrequent in practice. If water as a reaction medium is replaced by a biphasic system, it should be expected that not only the equilibrium constant (K_{eq}) of the chemical reaction proper will change, as demonstrated previously [1—3], but also the degree of ionization of the ionogenic reagent. As a result, the equilibrium shift of the total process, that is characterized by two apparent equilibrium constants, K_{eq} and K_a, or respectively, K_{eq} and K_b, will in all probability depend on the pH.

The aim of the present work was, first, to analyse the physico-chemical reasons for change in the ionic equilibria constants in biphasic water-organic systems compared to aqueous solutions, second, to analyse the pH dependence of the chemical reaction involving reagents, and finally, to outline the perspectives for increase in the product yield in enzyme-catalyzed synthesis.

Theory

Ionic equilibria shift. On ionization of an acid in a biphasic aqueous-organic media,

$$\begin{array}{l} (HA \overset{K_a}{\rightleftharpoons} H^+ + A^-)_{\text{in water}} \\ P_{HA}\Big\Updownarrow \\ (HA)_{\text{in organic solvent}} \end{array} \qquad (1)$$

we shall assume that only its nondissociated form * is soluble in the organic phase; K_a is the ionization constant of the acid in water, P_{HA} is the partition coefficient of the noncharged form of the acid between the organic and the aqueous phases. The apparent ionization constant of an acid in a biphasic system is determined by the relation

$$pK_{a,\text{biphasic}} = pH - \log \frac{[A^-]_t}{[HA]_t}$$

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* This assumption is usually valid [4]; the only exception being highly polar solvents that are capable of extracting from water-charged compounds with counterions in the form of ionic pairs [5].

where $[A^-]_t$ and $[HA]_t$ are the total concentrations of the dissociated and nondissociated forms of the acid, respectively, calculated with respect to the whole system; pH characterises the aqueous phase. The values of $[A^-]_t$ and $[HA]_t$ can be found by solving the equation for the dissociation constant of the acid in water,

$$pK_a = pH - \log \frac{[A^-]_w}{[HA]_w}$$

together with the equation for the partition coefficient of the nonionic form of the acid,

$$P_{HA} = [HA]_{org}/[HA]_w$$

and also the material balance equation for the nondissociated form of the acid,

$$[HA]_t(V_w + V_{org}) = [HA]_w \cdot V_w + [HA]_{org} \cdot V_{org}$$

Then

$$pK_{a,\text{ biphasic}} = pK_a + \log(1 + \alpha \cdot P_{HA}) \qquad (2)$$

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

It follows from Eqn. 2 that in biphasic aqueous-organic systems, the apparent pK_a value of the acid increases by the value of $\log(1 + \alpha \cdot P_{HA})$. The partition coefficients, P_{HA}, for many organic acids lie in the range of 10—10^3 [4]. This means that if the content of the aqueous phase is say, 1% (v/v) (i.e., $\alpha = 100$) the pK_a shift should be 3—5 units. Of course, if the content of water is still lower ($\alpha > 100$) and the solvent has $P_{HA} > 10^3$, the pK_a shift could be still higher.

The protonization of a base in a biphasic aqueous-organic system,

$$\begin{tikzcd} (B + H^+) \arrow[r, shift left, "K_b"] \arrow[d, shift left] & BH^+)_{\text{in water}} \arrow[l, shift left] \\ (B)_{\text{in organic solvent}} \arrow[u, shift left, "P_B"] \end{tikzcd} \qquad (3)$$

is described by a function that is similar to Eqn. 2:

$$pK_{b,\text{biphasic}} = pK_b - \log(1 + \alpha \cdot P_B) \qquad (4)$$

where K_b and $K_{b,\text{biphasic}}$ are the ionization constants for base B in water and in a biphasic system, respectively; P_B is the partition coefficient of base B. It is obvious that in biphasic compared to aqueous sytems, the apparent value of pK_b decreases by a value of $\log(1 + \alpha \cdot P_B)$.

Reactions involving one ionogenic group. This type of reaction is exemplified by the synthesis of an ester bond:

$$\begin{tikzcd} RCOOH + R'OH \arrow[r, shift left, "K_{\text{nonionic},w}"] \arrow[d, shift left] & RCOOR' + H_2O \arrow[l, shift left] \\ RCOO^- \arrow[u, shift left, "K_a"] \end{tikzcd} \qquad (5)$$

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* Under the conditions when not only the neutral but also the charged forms of the acid dissolve in the organic phase, the shift of the ionic equilibrium will also take place, but the pK_a vs. α function will be somewhat more complicated.

The apparent equilibrium constant of this reaction in water (K_w) depends on pH and equals

$$K_w = \frac{[\text{ester}]\,[H_2O]}{[\text{RCOOH} + \text{RCOO}^-]\,[\text{R'OH}]} = \frac{K_{\text{nonionic,w}}}{1 + K_a/[H^+]} \tag{6}$$

where $K_{\text{nonion,w}}$ is the equilibrium constant of reaction (5) in water calculated for the nonionized form of the acid. At neutral or subneutral pH value (i.e., under the conditions that are optimal for the functioning of the enzyme capable of catalysing the synthesis) the acid is as a rule completely dissociated and hence the total equilibrium of reaction (5) is almost entirely shifted to the left [6—10].

When biphasic aqueous-organic media are used instead of water, the equilibrium of reaction (5) is also determined by the partition of uncharged reagents between the phases:

$$\begin{tikzcd}
(\mathrm{RCOOH} \arrow[d, shift right, "P_{\mathrm{acid}}"'] \arrow[d, leftarrow, shift left] & + & \mathrm{R'OH} \arrow[d, shift right, "P_{\mathrm{alc}}"'] \arrow[d, leftarrow, shift left] \arrow[r, shift left, "K_{\mathrm{nonionic,org}}"] \arrow[r, leftarrow, shift right] & \mathrm{RCOOR'} \arrow[d, shift right, "P_{\mathrm{ester}}"'] \arrow[d, leftarrow, shift left] & + & H_2O)_{\text{in organic solvent}} \arrow[d, shift right, "P_{H_2O}"'] \arrow[d, leftarrow, shift left] \\
\Big(\mathrm{RCOOH} \arrow[d, shift right, "K_a"'] \arrow[d, leftarrow, shift left] & + & \mathrm{R'OH} \arrow[r, shift left] \arrow[r, leftarrow, shift right, "K_{\mathrm{nonionic,w}}"'] & \mathrm{RCOOR'} & + & H_2O\Big) \\
\mathrm{RCOO^-} & & & & & _{\text{in water}}
\end{tikzcd} \tag{7}$$

The apparent constant of the total process can be presented as follows:

$$K_{\text{biphasic}} = \frac{[\text{ester}]_t \cdot [H_2O]_t}{[\text{RCOOH} + \text{RCOO}^-]_t \cdot [\text{R'OH}]_t} = \frac{K_{\text{nonionic,biphasic}}}{1 + \dfrac{K_{\text{a,biphasic}}}{[H^+]}} \tag{8}$$

where $[H^+]$ is the concentration of H^+ in the aqueous phase and $K_{\text{a,biphasic}}$ is determined by Eqn. 2; $K_{\text{nonionic,biphasic}}$ is the equilibrium constant in a biphasic system calculated [3], for the nonionised form of the acid:

$$K_{\text{nonionic,biphasic}} = K_{\text{nonionic,w}} \frac{(1 + \alpha \cdot P_{\text{ester}})(1 + \alpha \cdot P_{H_2O})}{(1 + \alpha \cdot P_{\text{acid}})(1 + \alpha \cdot P_{\text{alc}})} \tag{9}$$

where P_{ester}, P_{H_2O}, P_{alc}, P_{acid} are the partition coefficients of the ester, water, alcohol and the nondissociated form of the acid, respectively.

Let us analyse Eqn. 8. As in biphasic systems the apparent value of pK_a of an acid can only increase compared to water, see Eqn. 2, the pH dependence of the total reaction (7) should shift towards pH higher values. As has been shown above, see the analysis of Eqn 2, the value of this shift can easily reach (depending on the P_{HA} value of the acid in question and the content of the aqueous phase in the system) 3—5 units. Fig. 1 shows the pH dependence for the apparent equilibrium constant of process (7). The curves are calculated according to Eqn. 8 for the typical values of $K_{\text{nonion,w}} = 7$ and p$K_a = 3$, see, for example, the data for system NAc-LPhe and EtOH [8,9] with P_{acid} being assumed to be equal to 10^2 [4]. As can be seen from the Fig. 1 biphasic systems make it possible, by choosing the appropriate value of α, to operate both in the pH range that is thermodynamically favourable for the synthesis of the end product (i.e., the range where the equilibrium constant reaches its highest value) and where the catalytic activity of the enzyme is the highest.

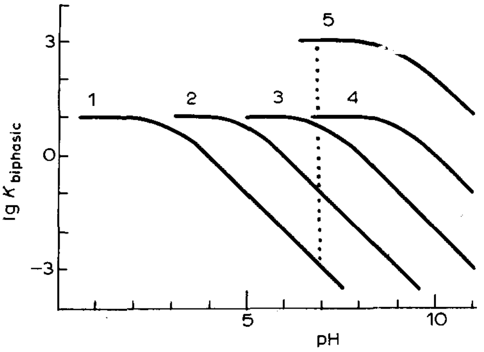
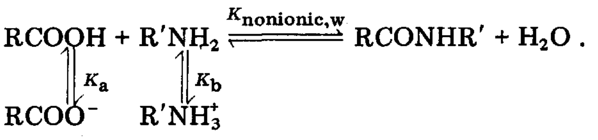


Fig. 1. pH dependence of the apparent equilibrium constant of reaction (7) in water (curve 1) and in a biphasic water-organic system (curves 2—5). Curves 1—4 are calculated with Eqn. 8 using the assumption that $K_{nonion,w} = K_{nonion,biphasic} = 7$ for $pK_a = 3$, $P_{acid} = 10^2$ and $\alpha = 0$ (curve 1), $\alpha = 1$ (curve 2), $\alpha = 10^2$ (curve 3) and $\alpha = 10^4$ (curve 4). Curve 5 is calculated with Eqns. 8 and 9 for $K_{nonion,w} = 7$, $pK_a = 3$, $P_{acid} = 10^2$, $P_{alc} = 0.1$, $P_{ester} = 10^5$, $P_{H_2O} = 10^{-2}$, $\alpha = 10^4$.

For example, if in water, it is the acid pH values that are most favourable for the synthesis in water (curve 1), by decreasing the content of water in a biphasic system, the pH-independent range of the highest value of the equilibrium constant can be shifted much further to the neutral or alkaline pH values, where this or that enzyme are most efficient. As a result of such a shift (with the values of α approx. 10^4, which are quite realistic) the change in the equilibrium constant that is observed, for example, at pH 7, amounts to four orders of magnitude, cf. curves 1 and 4 in Fig. 1 (broken line).

Moreover, a biphasic water-organic medium has an additional source of equilibrium shift. There is that in a biphasic system apparent value of pK_a that characterizes the dissociation of the starting acid, change can also occur in the apparent equilibrium constant of the synthesis itself that involves a non-dissociated form RCOOH, see Eqn. 7. The contribution of the pH-independent effect to the total equilibrium constant, $K_{biphasic}$, is described by Eqn. 9. In Fig. 1 the value of this additional pH-independent contribution to the total equilibrium shift amounts to two orders of magnitude. This is obvious from the shift of curve 5 upwards compared to curve 4; curve 5 was calculated with Eqn. 8 taking into account Eqn. 9 and the typical values of $K_{nonion,w} = 7$, $pK_a = 3$ [8,9], $P_{acid} = 10^2$, $P_{alc} = 0.1$, $P_{ester} = 10^5$, $P_{H_2O} = 10^{-2}$ [4]. As a result, at neutral pH values (approx. 7), the total shift of the equilibrium constant toward the synthesis amounts to six orders of magnitude with the realistic values of α that correspond to the aqueous phase content of 0.01% (v/v) in a biphasic system (cf. curve 1 and 5, broken line).

Reactions involving two ionogenic groups. This type of reaction can be exemplified by the synthesis of an amide:



$$\begin{tikzcd}[column sep=small] \mathrm{RCOOH} \arrow[d, shift left, "K_a"] & + & \mathrm{R'NH_2} \arrow[d, shift left, "K_b"] \arrow[r, shift left, "K_{nonionic,w}"] & \mathrm{RCONHR'} + \mathrm{H_2O}\,. \arrow[l, shift left] \\ \mathrm{RCOO^-} \arrow[u, shift left] & & \mathrm{R'NH_3^+} \arrow[u, shift left] & \end{tikzcd}$$

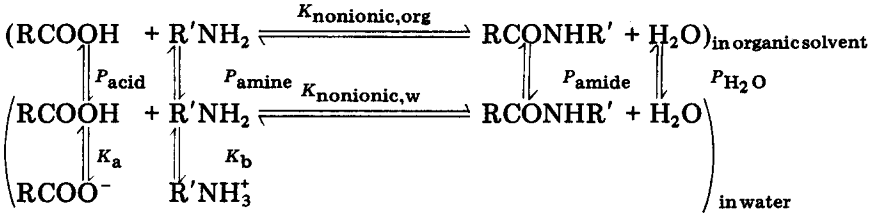
(10)

The equilibrium constant of this reaction in water is equal to

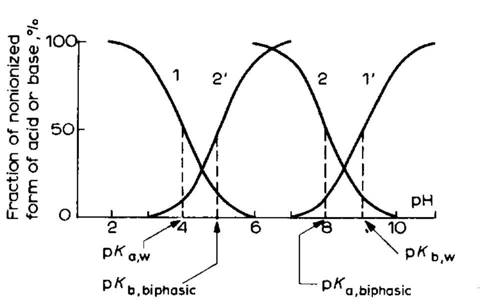
$$K_w = \frac{K_{\mathrm{nonionic,w}}}{1 + \frac{[\mathrm{H^+}]}{K_b} + \frac{K_a}{[\mathrm{H^+}]} + \frac{K_a}{K_b}} \tag{11}$$

The values of pK_a for carboxylic acids usually lie in the range of 3—4 and pK_b values for amine are approx. 8—10 [11,12]. This means that in water at least one of the reagents is in the entirely charged state over the whole pH range (see Fig. 2, curves 1 and 1'). As a result, the equilibrium of the total reaction (10) is shifted to the left, that is, towards the hydrolysis [6,10,13—15]. In other words, even at the optimal pH (see Fig. 3, curve 1) the total value of the equilibrium constant unfavourably differs from the K_{nonion} (calculated for the nonionized forms of the acid and the amine) by a factor of $K_b/K_a \sim 10^{-4}$—10^{-5}. This follows from Eqn. 11 if one proceeds from the most commonly used characteristics of the acidity of the starting reagents.

In a biphasic aqueous-organic media, the equilibrium of a reaction will be more complicated, as the partition of the nonionic reagents between the phases should be taken into consideration:



$$\begin{array}{lcl} (\mathrm{RCOOH} + \mathrm{R'NH_2} & \overset{K_{\mathrm{nonionic,org}}}{\rightleftharpoons} & \mathrm{RCONHR'} + \mathrm{H_2O})_{\text{in organic solvent}} \\ \Updownarrow P_{\mathrm{acid}} \qquad \Updownarrow P_{\mathrm{amine}} & & \Updownarrow P_{\mathrm{amide}} \qquad \Updownarrow P_{\mathrm{H_2O}} \\ \left(\mathrm{RCOOH} + \mathrm{R'NH_2}\right. & \overset{K_{\mathrm{nonionic,w}}}{\rightleftharpoons} & \mathrm{RCONHR'} + \mathrm{H_2O} \\ \Updownarrow K_a \qquad \Updownarrow K_b & & \\ \mathrm{RCOO^-} \qquad \mathrm{R'NH_3^+} & &)_{\text{in water}} \end{array} \tag{12}$$



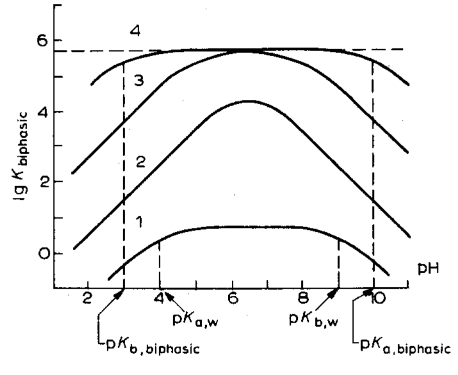


Fig. 2. Titration curves of the carboxy (curves 1 and 2) and amine (curves 1' and 2') components involved in the synthesis of an amide (10) in water (curves 1 and 1') and in a biphasic aqueous-organic system (curves 2 and 2'). Calculations are made using Eqns. 2 and 4 for $P_{\mathrm{acid}} = P_{\mathrm{amine}} = 10^2$, $\alpha = 10^2$, $pK_a = 4$, $pK_b = 9$.

Fig. 3. pH dependence of the apparent equilibrium constant of reaction (10) in water (curve 1) and in a biphasic aqueous-organic system (curves 2—4). The curves are calculated using Eqn. 13 with the assumption that $K_{\mathrm{nonion,w}} = K_{\mathrm{nonion,biphasic}} = 5 \cdot 10^5$ for $pK_a = 4$, $pK_b = 9$, $P_{\mathrm{acid}} = 10^2$, $P_{\mathrm{amine}} = 10^2$, $\alpha = 0$ (curve 1), $\alpha = 1$ (curve 2), $\alpha = 10^2$ (curve 3) and $\alpha = 10^4$ (curve 4).

The equilibrium constant in this case will look like

$$K_{\text{biphasic}} = \frac{[\text{amide}]_t \cdot [\text{H}_2\text{O}]_t}{[\text{RCOOH} + \text{RCOO}^-]_t \cdot [\text{R}'\text{NH}_2 + \text{R}'\text{NH}_3^+]_t}$$

$$= \frac{K_{\text{nonionic,biphasic}}}{1 + \dfrac{[\text{H}^+]}{K_{\text{b,biphasic}}} + \dfrac{K_{\text{a,biphasic}}}{[\text{H}^+]} + \dfrac{K_{\text{a,biphasic}}}{K_{\text{b,biphasic}}}} \qquad (13)$$

where the apparent values of the acidity constants are determined by Eqns. 2 and 4, and the equilibrium constant calculated for the nonionized forms of the reagents on the analogy with Eqn. 9 (see also previous work [3]) equals

$$K_{\text{nonionic,biphasic}} = K_{\text{nonionic,w}} \frac{(1 + \alpha \cdot P_{\text{amide}})(1 + \alpha \cdot P_{\text{H}_2\text{O}})}{(1 + \alpha \cdot P_{\text{amine}})(1 + \alpha \cdot P_{\text{acid}})} \qquad (14)$$

As is obvious from scheme (12), the partition of the nonionic reagents between the phases should affect both the ionization degree of the acid and the amine, and the equilibrium of the synthesis of the amide from the nonionized forms of these starting reagents. It follows from Eqn. 13 that each of these contributions can be analyzed separately.

Thus, in biphasic aqueous-organic media, compared to water, the apparent pK_a value for an acid always increases, and pK_b of a base always decreases, see Eqns. 2 and 4. This means that at neutral pH values the proportion of the noncharged forms of both an acid and an amine increases to a greater or lesser degree (see Fig. 2, curves 2 and 2′); hence the equilibrium of reaction (10) should shift to the right, i.e., towards the synthesis. To estimate the value of this pH-dependent effect, let us see how much the apparent equilibrium constant can change in terms of only the denominator of the right-hand part of Eqn. 13. We shall use the typical values of $pK_a = 4$, $pK_b = 9$, $K_{\text{nonion,w}} = 5 \cdot 10^5$, [10] and $P_{\text{acid}} = P_{\text{amine}} = 10^2$ [4]. Fig. 3 shows, for several values of α, the pH dependence of the value of K_{biphasic}. It is tentatively assumed thereby (to analyse separately the pH-dependent and the pH-independent effects) that $K_{\text{nonion,biphasic}} = K_{\text{nonion,w}}$. One can see that as the content of water in the system decreases, the apparent pK values of the starting reagents become closer and finally their inversion takes place. At the expense of this, the optimal value of the total equilibrium constant increases; thereby the value of the K_{biphasic} tends (as the result of the inversion of the acidity characteristics of the initial amine and acid) to the value of $K_{\text{nonion,biphasic}}$. In other words, finally, when the content of water becomes very low, the ionic equilibria of the starting reagents are totally shifted towards their nonionic forms. At the realistic values of $\alpha = 10^2$—10^4, the respective changes in the equilibrium constant will be five orders of magnitude, cf. curves 1 and 4 in Fig. 3.

An additional source of the total equilibrium shift is that change may occur in the apparent equilibrium constant of the synthesis itself that involves nonionized forms of the reagents, see Eqn. 14 [3]. The value of this pH-independent effect can reach several orders of magnitude depending on the partition coefficients ratios.

To sum up the above, biphasic aqueous-organic systems make it possible for us to shift the equilibrium in the reactions of type (10) towards synthesis.

Experimental

Materials

Use was made of bovine α-chymotrypsin (EC 3.4.21.1), grade A, of the Olaine chemical reagents plant (Latvian, U.S.S.R.); *N*-acetyl-L-tryptophan and L-leucine amide from Reanal (Hungary); 2,4-dinitrophenyltryptophan, neutral red, mineral salts, organic solvents, analytical grade from Soyuzkhimreactiv.

Methods

*pK_a values of N-benzoyl-*L*-phenylalanine and N-acetyl-*L*-tryptophan.* Values were determined in the following way. 0.1 ml 0.008 M stock solution of the respective acid was mixed with 2 ml 0.5 M phosphate buffer and at a pH from 1 to 7 the absorption at 250 nm for *N*-benzoyl-L-phenylalanine and at 293 nm for *N*-acetyl-L-tryptophan was determined. The reference cuvette was filled with the solution of the same acid at the same concentration at pH 1. The absorption vs. pH curve was drawn and pK_a was determined from the inflection point.

The pK_a value of 2,4-dinitrophenyltryptophan in a water-diethyl ether system. This was determined in the following way. 5 ml $3 \cdot 10^{-5}$ M of 2,4-dinitrophenyltryptophan dissolved in diethyl ether were supplemented with 0.1—50 ml of buffer ($NaH_2PO_4/CH_3COONa/H_3BO_3$, 0.1 M of each), pH from 1 to 11, and mixed until an equilibrium was established. Then the content of the noncharged form of the acid in the organic phase was determined by the absorption at 340 nm. We ascertained that at highly alkaline pH values of the aqueous solution (pH about 12), no extraction of 2,4-dinitrophenyltryptophan into diethyl ether solvent occurs. This means that the charged form of the acid is insoluble in the organic phase. Then the pH dependence of the portion of the noncharged form of the acid was determined (taking into account that the content of the noncharged form of the acid in the aqueous phase is negligibly low), to find the pK_a value from the inflection point of this curve.

pK_b value of neutral red in a water-chloroform system. This was determined in the following way. 0.05 ml of the $5 \cdot 10^{-3}$ M of the stock solution of neutral red in 60% ethanol was mixed with 0.95 ml of buffer ($NaH_2PO_4/CH_3COONa/H_3BO_3$, 0.1 M of each), pH from 1 to 9, and from 0.1 to 30 ml chloroform were added. The solution was mixed until an equilibrium was established and the content of the charged form of neutral red in the aqueous phase was determined by the absorption at 520 nm. We ascertained that at highly acid pH values of the aqueous solution (pH about 1—2), no extraction of the dye into chloroform occurs; this means, that the charged form of the base is insoluble in the organic phase. Then the pH dependence of the charged portion of the dye was determined, to determine the pK_b value from the inflection point.

The partition coefficient of neutral red. This was determined in the following way. 5 ml $5 \cdot 10^{-3}$ M neutral red dissolved in chloroform were supplemented with an equal volume of 0.1 M phosphate buffer (pH 9). The system was mixed, left to stand for some time and the absorption of the aqueous phase at 450 nm was determined. Then 0.5 ml of the organic phase was collected, evaporated to dryness, the precipitate was dissolved in 20 ml of the same buffer and the absorption (at 450 nm) was also determined. The partition coefficient was calculated as the ratio of the two absorption values with dilution being taken into account.

Enzymatic synthesis of N-benzoyl-L-phenylalanine ethyl ester. This was carried out as previously [1—3].

Enzymatic synthesis of N-acetyl-L-tryptophanyl-L-leucine amide. This was carried out in the following way. 10 mg α-chymotrypsin were dissolved in 1 ml 0.03 M of the solution of L-leucine amide in water, supplemented with 0.5 ml 0.5 M phosphate buffer (pH 5—10) and a certain amount of water-saturated ethyl acetate (7.5—75 ml) containing $4 \cdot 10^{-4}$ M *N*-acetyl-L-tryptophan. The system was mixed at 20°C, 2—5 ml aliquots of the organic phase were collected and evaporated to dryness in a rotary evaporator. The yield of the product (*N*-acetyl-L-tryptophanyl-L-leucine amide) was determined. To this end, the product was isolated chromatographically and identified spectrophotometrically; the procedures are as described [16].

Once the experiment was carried out on a large scale to obtain about 100 mg of the product. In this case, when the equilibrium was achieved, the organic phase was washed with an aqueous buffer (pH 7) until the product in the organic phase proved chromatographically homogeneous. Then the organic phase (approx. 200 ml) was evaporated to dryness and the precipitate was identified by elementary analysis. It was found: C-64,24%; H-7,42%; N-15,41%. Calculated for $NAc\text{-}Trp\text{-}Leu\text{-}NH_2$: C-63,66%; H-7,31%; N-15,71%.

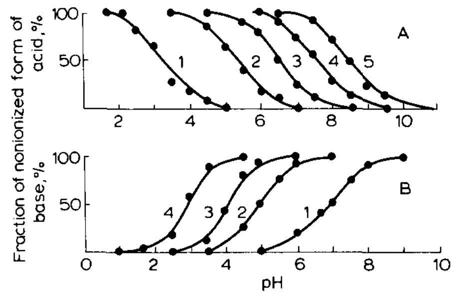
Calculation of the apparent equilibrium constant. Having determined the equilibrium yield of the product (ester or peptide), we determined the apparent equilibrium constant, K_{biphasic} = [ester or peptide] · [water]/[acid] · [ethanol or amine]. The concentration of all the reagents (including water) was compared to the total volume of the biphasic system. The amount of water in the system included not only water that forms a separate phase, but also water that is dissolved in the organic solvent. To estimate the latter value, the data of Leo et al. (Table in Ref. 4) was used.

Results and Discussion

Shift of acid-base equilibria

The shift of the pK_a and pK_b values predicted by us to take place in the biphasic system (see Theory) have been proved experimentally (Fig. 4). 2,4-Dinitrophenyltryptophan (acid) and neutral red (base), the substances whose ionization can be readily followed spectrophotometrically, were used as model compounds. It is obvious from Fig. 4 that if a biphasic system is used instead of water, a shift in the apparent p*K* value occurs, the value of which depends on the volume ratio of the aqueous and organic phases. The shift is as impressive as five units in the case of 2,4-dinitrophenyltryptophan (Fig. 4A, cf. curves 1 and 5) and is about four units for neutral red (Fig. 4B, cf. curves 1 and 4).

The p*K* vs. $\log\alpha$ function is linear (see Fig. 5), as it should be in conformity with Eqns. 2 and 4 if the content of water in the system is sufficiently low. We have found the partition coefficient for the noncharged form of the neutral red in a water-chloroform system in an independent experiment (see Experimental). It proved to be equal to 500. The theoretical dependence



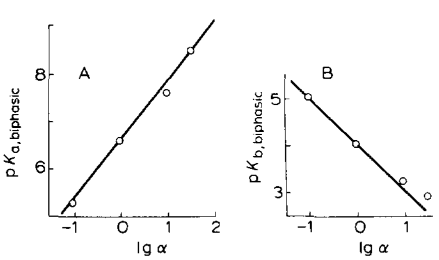


Fig. 4. Titration curves of 2,4-dinitrophenyltryptophan (A) and neutral red (B) in biphasic systems consisting of water-diethyl ester and water-chloroform, respectively. A : α = 0 (curve 1), α = 0.1 (curve 2), α = 1 (curve 3), α = 10 (curve 4), α = 50 (curve 5). B : α = 0 (curve 1), α = 0.1 (curve 2), α = 1 (curve 3), α = 30 (curve 4).

Fig. 5. Dependence of the apparent value of pK of 2,4-dinitrophenyltryptophan (A) and neutral red (B) in biphasic aqueous-organic systems, on the phase volume ratio (α).

derived from Eqn. 4 with the use of this value (log P_B = 2.7) is in good agreement with the experimental results, see Fig. 5B.

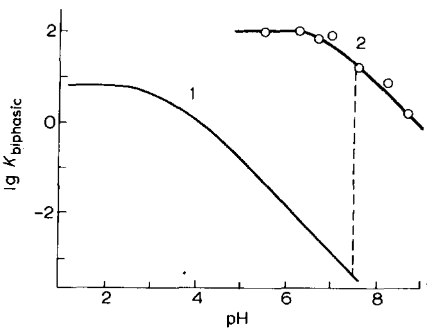
α-Chymotrypsin-catalysed synthesis of N-benzoyl-L-phenylalanine ethyl ester. Synthesis of *N*-benzoyl-L-phenylalanine ethyl ester from an acid and an alcohol can serve as an example of a pH-dependent reaction of type (7):

$$\text{NBz-LPhe} + \text{EtOH} \rightleftharpoons \text{NBz-LPhe-OEt} + H_2O \tag{15}$$

This reaction has previously been studied [1—3] with only one pH value. Fig. 6 shows the pH dependence of the apparent equilibrium constant in a biphasic system containing chloroform and 5% (v/v) water (curve 2). For the sake of comparison, curve 1 is given which characterises the equilibrium in water and is calculated using Eqn. 6 with $K_{\text{nonion,w}} = 7$ [10], and $pK_a = 3.35$, which was determined in an independent experiment by spectrophotometric titration. One can see that when water was replaced by a biphasic aqueous-organic system, the apparent pK_a value shifted by more than three units (see the shift of curve 2 to the right along the abscissa compared to curve 1). Besides, the apparent value of the equilibrium constant for the synthesis involving the nonionic form of the acid ($K_{\text{nonion,biphasic}}$) has increased by more than one order of magnitude, see the shift of curve 2 upwards to curve 1. As the result, the total equilibrium constant shift at pH 7.5 (the optimum of the catalytic activity of α-chymotrypsin) amounts to about five orders of magnitude, (see broken line in Fig. 6).

So it has become possible at neutral pH values to turn the hydrolysis almost entirely towards synthesis; the yield of the ester in this system was about 80—90% (with the total concentrations of *N*-benzoyl-L-phenylalanine and ethanol of 10^{-3} M and 0.1 M, respectively). In water (in the absence of an organic phase, other things being equal) the yield is as low as 0.001% (cf. Ref. 10).

However, the high yield of the synthesis is not the major result of this investigation. The most important conclusion is that the replacement of water



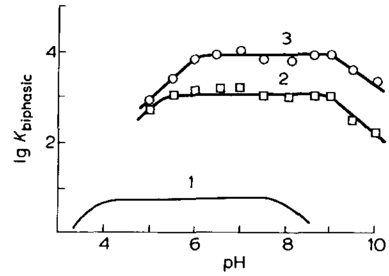


Fig. 6. pH dependence of the apparent equilibrium constant for the synthesis of *N*-benzoyl-L-phenylalanine ethyl ester in water (curve 1) and in a biphasic system consisting of chloroform and 5% (v/v) water (curve 2).

Fig. 7. pH dependence of the apparent equilibrium constant of the synthesis of *N*-acetyl-L-tryptophanyl-L-leucine amide in water (curve 1) and in a biphasic system consisting of ethyl acetate-water with different content of the latter, i.e., 17% (v/v) (curve 2) and 2% (v/v) (curve 3).

as the reaction medium by a biphasic aqueous-organic system allows one to make the pH range that is thermodynamically favourable for the synthesis coincident with the pH-optimum of the catalytic activity (broken line in Fig. 6).

α-Chymotrypsin-catalyzed peptide synthesis. The approach that we have developed was tested with the synthesis of *N*-acetyl-L-tryptophanyl-L-leucine amide:

$$\text{NAc-LTrp} + \text{L-LeuNH}_2 \rightleftharpoons \text{NAc-LTrp-L-LeuNH}_2 + \text{H}_2\text{O} \qquad (16)$$

The experimental results are presented in Fig. 7 (curves 2 and 3) in the form of the pH dependence of the apparent equilibrium constant of reaction (16) in a biphasic water-ethyl acetate system at various ratios of the organic aqueous phases. In the same figure is the pH dependence of the equilibrium constant of reaction (16) in water (curve 1) calculated from Eqn. 11 with $K_{\text{nonion,w}} = 5 \cdot 10^5$ [10], pK_a = 3.2 (determined in an independent experiment by spectrophotometric titration) and pK_b = 8 [17]. It is instead of water, an almost three unit shift of the pK_a of the carboxylic component (*N*-acetyl-L-tryptophan) occurs, the content of the aqueous phase being 2% (v/v); see the shift of curve 3 to the right compared to curve 1. Somewhat unexpected is the fact that the pK_b value of leucine-amide has hardly changed at all on replacement of water by the biphasic aqueous-organic system. This seems to be due to the fact that ethyl acetate, being a polar solvent or being involved in solvation interactions, extracts from water the noncharged and the charged forms of leucine-amide in approximately the same way.

Nevertheless, the total equilibrium constant of the synthesis has grown by more than three orders of magnitude. As the result, the equilibrium of reaction (16) has shifted considerably to the right, which has made possible a preparative synthesis of the dipeptide. The yield of the product in this water-ethyl acetate system (the content of water 2%, v/v) with the concentrations of the starting reagents $[\text{NAc-LTrp}]_t = [\text{L-LeuNH}_2]_t = 4 \cdot 10^{-4}$ M and pH = 7.0 was 44%. Moreover, if there is an excess of one of the reagents, the yield can reach 100%, whereas in water the yield under the same conditions is only 0.01% [10]. This means that the use of a biphasic system has allowed us to raise the yield from a negligibly low to a preparative level.

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