Biochimica et Biophysica Acta, 657 (1981) 277—294
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BBA 69177

THE PRINCIPLES OF ENZYME STABILIZATION

VI. CATALYSIS BY WATER-SOLUBLE ENZYMES ENTRAPPED INTO REVERSED MICELLES OF SURFACTANTS IN ORGANIC SOLVENTS *

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(Received January 9th, 1980)
(Revised manuscript received July 15th, 1980)

Key words: Catalysis; Entrapped enzyme; Organic solvent; Surfactant; Stabilization

Summary

1. The possibility of stabilizing water-soluble enzymes against the inactivating action of organic solvents by means of surfactants has been studied. Several enzymes (α-chymotrypsin (EC 3.4.21.1), trypsin (EC 3.4.21.4), pyrophosphatase (EC 3.6.1.1), peroxidase (EC 1.11.1.7), lactate dehydrogenase (EC 1.1.1.27) and pyruvate kinase (EC 2.7.1.40)) were used to demonstrate that enzymes can be entrapped into reversed micelles formed by surfactants (Aerosol OT, cetyltrimethylammonium bromide, Brij 56) in an organic solvent (benzene, chloroform, octane, cyclohexane). The enzymes solubilized in this way retain their catalytic activity and substrate specificity.

2. A kinetic theory has been put forward that describes enzymatic reactions occurring in a micelle-solvent pseudobiphasic system. In terms of this theory, an explanation is given for the experimental dependence of the Michaelis-Menten equation parameters on the concentrations of the components of a medium (water, organic solvent, surfactant) and also on the combination of the signs of charges in the substrate molecule and on interphase (++, +—, ——).

3. The results obtained by us may prove important for applications of enzymes in organic synthesis and for studying the state and role of water in the structure of biomembranes and active centres of enzymes.

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* For Part V see Ref. 66.

Introduction

The choice of a reaction medium and a catalyst is of prime importance for the realization of any kinetic process. To date, enzymes are by far the best catalysts. Their exceedingly high catalytic activity under mild conditions and exceptionally high substrate specificity [1] have made possible a wide use of biocatalysis in technological processes [2,3]. The catalytic properties of enzymes can also be employed in fine organic synthesis, in production of drugs, in the synthesis of important biochemical compounds and a number of enzymatic processes have by now been realized on a technological or plant pilot scale (for review see Ref. 4). But further application of enzymes is impeded by the fact that nature has mostly designed them for functioning in aqueous solution. And it is a known fact that many chemical reactions can thermodynamically favour the synthesis of a desired end product only in certain organic solvents. This is associated both with specific solvation effects and solubility of the individual components of the reaction; in other cases, in addition to the end product water is formed and in aqueous solutions the equilibrium is shifted towards the starting substances. Unfortunately, replacement of water as a reaction medium by an organic solvent is usually accompanied by either a total denaturation of the enzyme or dramatic decrease of its catalytic activity and loss of its substrate specificity (see review Ref. 4).

To make enzymes function in systems with a low water content, the following approaches were suggested. First, for the reactions that proceed in homogeneous aqueous-organic mixtures, an organic solvent is chosen, in which solvophobic interactions would be realized as in water [5]. An it is solvophobic (hydrophobic) interactions of some fragments of the polypeptide chain that are known to be responsible for the maintenance of the native structure of the protein and hence its catalytic activity. Second, the reaction is run in a water-water immiscible organic solvent biphasic system, the enzyme being localized in the aqueous phase [6,7]. In this case, the content of water in the system may be sufficiently low. Finally, a molecule of the enzyme (its catalytically active conformation) is protected from the unfavourable action of an organic solvent by means of surfactants [8] (see also reviews Refs, 4, 9).

The third approach may at first glance seem surprising, as in aqueous solutions surfactants are commonly used as denaturing agents [10]. But it is noteworthy that biomembranes that contain many enzymes, consists of none other than surfactants [11]. In other words, nature itself prompts us to stabilize active conformations of enzymes by entrapping them into aggregates of surfactant molecules.

Associates of the 'reversed' micelles type in organic solvents are built in such a way that polar ionic groups in a molecule of a surfactant make up the nucleus of the associate and the hydrocarbon fragments the external layer, see Fig. 1a. It is known that reversed micelles of a surfactant are capable of solubilizing ions, polar organic substances as well as a considerable amount of water (several dozens of H_2O molecules/molecule of a surfactant) [12—15]. In a preliminary communication [8] we demonstrated for the first time * that one can, with the

* It was previously established (see Ref. 16 and Refs. therein), that in organic solvents in the presence of hydrated reversed micelles, lipolytic enzymes display catalytic activity. This is not surprising since lipases function just on the aqueous-organic interphase.

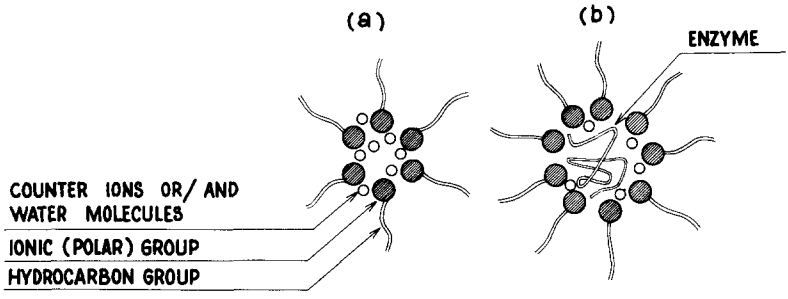

Fig. 1. A schematic representation of a reversed micelle of a surfactant.

help of a micelle-forming surfactant, solubilize in organic solvents relatively high concentrations of enzymes up to 1 mg/ml, which corresponds to $1 \cdot 10^{-5}$ M active centres (the molecular mass of the enzyme was not more than 100 000) and exceeds by far the required level of the 'catalytic concentration' of the majority of enzymes. The water-soluble (non-membrane) enzymes solubilized in this fashion retain their catalytic activity [8]. We explain this phenomenon in the following way.

A molecule of an enzyme that is entrapped in a reversed micelle (see Fig. 1b) is protected against denaturation (unfolding) in that the surface of the 'interphase' between the protein globule and the organic solvent is stabilized by molecules of the surfactant. As a result, the biocatalyst can avoid direct contact with the unfavourable organic medium because it is enclosed in a sort of a microreactor containing a rather limited amount of water, i.e., several hundreds or dozens of H_2O molecules/molecule of the enzyme; this corresponds to less than 1% (v/v) content of water in an organic solvent-surfactant system.

Simultaneously with our experiments with micelles [8], Douzou et al. [17] found that enzymes retain their catalytic activity in microemulsions with 1—5 μm drops. The latter systems are apparently less efficient, as, first, they contain high amounts of water (commensurable with the content of oil) and secondly, they are not transparent and hence defy spectrophotometric measurements *.

The present paper reports the results of a kinetic study of the reactions catalyzed by enzymes (chymotrypsin **, trypsin, pyrophosphatase, peroxidase, lactate dehydrogenase and pyruvate kinase) in 'organic solvent-water-surfactant' systems.

Theory

The kinetics of an enzymatic reaction proceeding in a pseudo-biphasic system consisting of 'reversed' micelles of a surfactant in an organic solvent

Micellar effects in organic (non-enzymatic) reactions have found quite a

* When this manuscript was being prepared for publication Douzou et al. [66] reported having used our systems [8] for studying enzymatic reactions at low temperatures.

** When this manuscript was submitted for publication, Menger and Yamada [69] reported that α-chymotrypsin, solubilized by Aerosol OT in heptane, catalyzed hydrolysis of *N*-acetyl-L-tryptophan methyl ester.

comprehensive explanation in terms of a kinetic theory [18—20], see also review [21]. The kinetic theory, which was originally suggested for aqueous solutions of surfactants, is also valid for systems with reversed micelles [22]. Here we shall consider a reaction between enzymes E and substrate S that obeys the Michaelis kinetics,

$$E + S \rightleftharpoons ES \rightarrow E + \text{products} \tag{1}$$

and proceeds in an 'organic solvent-water-surfactant' system.

Let us assume that (i) a solution of a surfactant consists of two phases [23], i.e., of a bulk 'phase' of an organic solvent and a 'phase' of micelles wetted by water. Let us assume further that (ii) the substrate is distributed evenly between the two phases [24]:

$$(S)_{\text{in the bulk phase}} \overset{P_s}{\rightleftharpoons} (S)_{\text{in micelles}} \tag{2}$$

the partition coefficient being presented in the following way:

$$P_s = \frac{[S]_{mic}}{[S]_b} \tag{3}$$

Here and below indices mic and b refer to the micellar and bulk phases, respectively.

We shall not take into account the distribution of the enzyme, as (iii) proteins are practically insoluble in hydrophobic solvents [25]; besides, in non-aqueous media enzymes usually undergo denaturation (see review Ref. 4). Therefore, we shall assume that all the catalytic activity is confined to the micellar phase.

Let us assume that (iv) the rate of the reaction proceeding in the micelles obeys the Michaelis-Menten equation. Then during some initial period (when the concentration of the products is negligibly small compared to the initial concentration of substrates) with excess of the substrate over the enzyme and under steady-state conditions, the apparent reaction rate (relative to the volume of the entire system) can be expressed in the following way:

$$v = \frac{k_{cat,mic}[E]_{0,mic}[S]_{0,mic}}{K_{m,mic} + [S]_{0,mic}} \cdot \theta \tag{4}$$

where θ is the volume proportion of the micellar phase and index 0 shows initial concentrations.

Let us assume that (v) the exchange of the substrate molecules between the phases is sufficiently fast, i.e., enzymatic reaction Eqn. 1 does not violate equilibrium Eqn. 2. * Then the concentration of the reagents can be found from

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* Although this assumption seems to be quite reasonable, it should be kept in mind that no quantitative rate studies have been performed so far. It has been reported for certain, not numerous, systems that low molecular weight compounds, on being dissolved in an organic solvent, are instantaneously found in the micellar micromedium [26,27]; the rate of the exchange between micelles (as a result of their direct contact on collision) is also rather high [28] (see also Ref. 66). On the other hand, it was found in our laboratory that on addition of water to a triple system 'organic solvent-water-surfactant' the equilibrium under certain conditions establishes rather slowly (several minutes) [29], see also Experimental.

Eqn. 3 and the material balance equations:

$$[S]_{0,t} = [S]_{0,mic} \cdot \theta + [S]_{0,b}(1 - \theta) \quad (5)$$

$$[E]_{0,t} = [E]_{0,mic} \cdot \theta$$

It should, however, be kept in mind that Eqn. 3 is only valid (vi) for sufficiently diluted solutions; hence the concentration of the reagents should be much lower than that of the surfactants.

Substituting Eqns. 3 and 5 into Eqn. 4 we shall have:

$$v = \frac{k_{cat,app}[E]_{0,t}[S]_{0,t}}{K_{m,app} + [S]_{0,t}} \quad (6)$$

where

$$k_{cat,app} = k_{cat,mic} \quad (7)$$

and

$$K_{m,app} = K_{m,mic} \frac{1 + \theta(P_s - 1)}{P_s} \quad (8)$$

In the case of a charged substrate, Eqn. 8 could be simplified if one assumed that (vii) the molecules of the substrate are confined to the aqueous-micellar phase, i.e., not only $P_s >> 1$, but $P_s\theta >> 1$. Then

$$K_{m,app} = K_{m,mic} \cdot \theta \quad (9)$$

Let us analyze the quantitative difference between the observed and the true Michaelis constants. If $P_s = 1$, then, whatever the θ, $K_{m,app} = K_{m,mic}$. If, however, the substrate is hydrophobic, i.e., poorly soluble in an aqueous-micellar phase ($P_s << 1$) and is, therefore, almost totally present in the organic phase, then the difference between the observed and the true Michaelis constants, $K_{m,app}$ and $K_{m,mic}$, largely depends on P_s. That is, if $P_s \to 0$, then $K_{m,app}/K_{m,mic} \approx 1/P_s \to \infty$. In reality P_s, for a host of low molecular compounds, can be as low as $1 \cdot 10^{-4}$, as it follows from the partition coefficients in the systems of water-organic solvent or water-micelles, see, for instance, reviews [21,69]. On the other hand, for the substrates well solubilized by wetted micelles, i.e., when $P_s >> 1$, Eqn. 9 holds true. So the difference between the observed and true Michaelis constants can be characterized only by θ. In effect, $\theta < 1$; the θ lowest limit is determined by those minimal quantities of water and a surfactant that should be used to solubilize the enzyme in its lowest concentration that can yet be detected by the kinetic method used. In the present investigation, in particular, the minimal θ values were as low as $1 \cdot 10^{-3}$ (cf. Fig. 5). The observed $K_{m,app}$ can, thus, markedly differ from the true $K_{m,mic}$ at least by a factor from $1 \cdot 10^4$ (see above) to $1 \cdot 10^{-3}$.

We have analyzed a simple pseudophasic model which is based on the assumption that all the reagents are uniformly distributed over the whole volume of the water-wetted micelles. Generally speaking, the model must be sufficiently comprehensive to take into account the heterogeneity of the structure of reversed micelles; among other things, a surface layer and an aqueous nucleus could be discerned in a micelle [26,30] (see also Refs. to Refs. 12—15).

However, no data are so far available in the literature that could help estimate even the overall size of a reversed micelle containing a solubilized enzyme. Unfortunately, the numerous published data characterizing 'empty' micelles can not be used to this end. First, Menger and Yamada [69] suppose that enzyme molecules 'create' their own micelles in an organic solvent rather than occupy empty ones already present. Second, we have found (unpublished results) by the methods of sedimentation and low angle X-ray scattering that the size of a reversed micelle can really change as a result of the protein solubilization and depending on concentrations of a surfactant and water.

Nevertheless, it can be stated even now that the volume proportion of the micellar phase in system (θ) can be varied not only at the expense of the concentration of the surfactant but also by changing the concentration of water in reversed micelles. If the concentrations of both components that form the micellar phase are varied with their molar ratio being constant, i.e. $[H_2O]/[\text{surfactant}] = \text{constant}$, it can be assumed in the first approximation that the volume ratio of the surface layer and micellar nucleus remains constant as well. On the other hand, by varying the concentration of the surfactant and maintaining a constant concentration of water (or vice versa) one can alter the internal structure (and other characteristics) of micelles more drastically. Both these approaches have been tested by us in the present work for studying the reactivity of enzymes under both sets of conditions.

Experimental

Materials

Most of the enzymes used by us were commercial preparations, i.e., α-chymotrypsin (EC 3.4.21.1) was a product of the Leningrad meat packing plant (U.S.SR.); trypsin (EC 3.4.21.4) of the Olain chemical reagents plant (Latvian S.S.R.); horse radish peroxidase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) from Sigma. Pyrophosphatase (EC 3.6.1.1) was isolated from baker's yeast by Avaeva et al. [31,32] as described by Kunitz [33]. Chymotrypsinogen was obtained from the Leningrad meat packing plant.

All the surfactants used were commercially produced. Sodium salt of di(2-ethyl-hexyl)sulphosuccinic acid (Aerosol OT) manufactured by Cyanamide Co, kindly given to us by Professor F.M. Menger (U.S.A.), was purified as described [27]; cetyltrimethylammonium bromide (CTAB) was a product of Chemapol (Czechoslovakia) which was recrystallized using the method of Duynstee [34]; cetylpoly-(10)-ethyleneglycol (Brij 56) from Honeywill-Atlas Limited, OK or Atlas Chemical Industrial, NY, through Atlas-Seppic, Paris, France was a kind gift of Professor F. Puisieux (France).

Organic solvents (octane, benzene, cyclohexane, chloroform) of reagent grade were purified by distillation.

All the substrates were commercial preparations, i.e., *N*-glutaryl-L-phenylalanine *p*-nitroanilide from Serva, *N*-benzoyl-D,L-arginine *p*-nitroanilide from Sigma, *N-trans*-cinnamoylimidazole from Reanal (Hungary), *p*-nitrophenylguanidinebenzoate from Merck, phospho*enol*pyruvate, ADP and NADH from Sigma, pyrogallol from Wako.

Preparation of the stock solutions of the enzymes. α-Chymotrypsin or tryp-

sin was dissolved in a buffer solution (0.02 M phosphate/borate/acetate) at pH 7.0 or 8.0. The concentration of the active centres of α-chymotrypsin was determined by titration with *N-trans*-cinnamoylimidazole as described [35]. The concentration of the active centres of trypsin was determined by titration with *p*-nitrophenylguanidinebenzoate as described [36].

Peroxidase was dissolved in a buffer solution (0.02 M phosphate/borate/acetate) at pH 7.0 and its concentration was determined spectrophotometrically (403 nm) using the molar absorption value of $9.1 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ [37].

Lactate dehydrogenase and pyruvate kinase were dissolved in a buffer solution (0.05 M phosphate/2 mM EDTA/5 mM $MgCl_2$/1 mM dithiothreitol/1 M ammonium acetate) at pH 6.8.

Pyrophosphatase was dissolved in a buffer solution ($5 \cdot 10^{-3}$ Tris-HCl) that contained $1 \cdot 10^{-2}$ M $MgCl_2$ at pH 7.0.

A typical experiment. This was as follows: 2 ml of a 0.1—0.5 M surfactant dissolved in an organic solvent was supplemented with 0.01 ml (or less) of a concentrated solution of enzyme in an aqueous buffer, vigorously shaken, then supplemented with 0.01 ml (or less) of a solution of substrate in water or acetonitrile, shaken again; then the rate of the enzymatic reaction was measured in the homogeneous (optically transparent) solution.

On addition of an aqueous solution to an organic solvent containing a surfactant, the system becomes transparent rather rapidly, within several seconds (or less). However, it takes much longer, sometimes many minutes, to achieve equilibrium distribution [29]. It is possible that this is due to slow structural rearrangements of micelles on the alteration of their hydration degree. These slow structural-solvational rearrangements that occur in a transparent (not turbid) micellar solution can be detected, for example, as attenuating oscillations of the absorbance (410 nm), which arise on addition of an aqueous solution of *p*-nitrophenolate to a solution of Aerosol OT in octane [29].

To rule out the effect of such phenomena on the kinetics of enzymatic reactions in systems involving reversed micelles, we sometimes employed, in addition to the above-described techniques, some other mode of mixing the reagents, i.e., aliquots of stock solutions of the enzyme and substrate were solubilized (separately) in 1 ml of an organic solvent and the resulting solutions (with the same concentrations of the surfactant and equal $[H_2O]/[surfactant]$ molar ratios) were incubated for 10—30 min and only then mixed in a spectrophotometric cuvette.

Measurements of the rates of enzymatic reactions. The rate of hydrolysis (under the action of α-chymotrypsin or trypsin) of the *p*-nitroanilide substrates was followed spectrophotometrically, by the formation of *p*-nitroaniline. For this purpose molar absorption of *p*-nitroaniline at 380 nm had been determined in the required micellar system. For example, in a micellar system that is described in the caption to Fig. 2 this value proved to be 13.500 $M^{-1} \cdot cm^{-1}$. In the buffer (without the surfactant or octane) *p*-nitroaniline has a similar molar absorption value, i.e. 14.500 $M^{-1} \cdot cm^{-1}$.

Deacylation of *trans*-cinnamoyl-α-chymotrypsin was followed spectrophotometrically (335 nm) by the rate of consumption of *trans-N*-cinnamoylimidazole (at saturation of the enzyme by this substrate).

Oxidation of pyrogallol catalyzed by peroxidase was followed spectrophoto-

metrically by the formation of purpurogallin (420 nm). The molar absorption of this product in a micellar system described in the caption to Fig. 7 is the same as in the buffer ($6.300\ M^{-1} \cdot cm^{-1}$).

The catalytic activity in the pyruvate kinase-lactate dehydrogenase system, see Eqn. 10, was followed spectrophotometrically by the consumption of NADH. The molar absorption of NADH (340 nm) in the micellar system described in the caption to Fig. 8 is the same as in the buffer ($6.500\ M^{-1} \cdot cm^{-1}$).

The rate of hydrolysis of pyrophosphate catalyzed by pyrophosphatase was determined by the amount of the inorganic phosphate formed in the reaction; to this end, aliquots were taken at certain intervals of time from the reaction mixture. Determination of inorganic phosphate in the aliquots was carried out as described in [38] and modified by us [39].

Spectrophotometric measurements were performed in Hitachi-Perkin-Elmer model 124 and Cary-16 spectrophotometers in thermostatted cuvettes.

Results and Discussion

Catalysis by α-chymotrypsin and trypsin entrapped in reversed micelles of ionogenic surfactants (Aerosol OT or CTAB) in octane or octane + chloroform mixture (1 : 1). The concentration of these proteolytic enzymes does not change as a result of their solubilization in organic solvents. We have demonstrated it by titration of the active centres both for α-chymotrypsin (with *N-trans*-cinnamoylimidazole [35] used as a titrant) and trypsin (titrated with *p*-nitrophenylguanidinebenzoate [36]), solubilized in systems II and VI, respectively (see the Table I).

Moreover, these enzymes display a catalytic activity towards their specific substrates. For example, α-chymotrypsin solubilized by Aerosol OT in octane catalyzes the hydrolysis of *N*-glutaryl-L-phenylalanine *p*-nitroanilide, see Fig. 2. The rate of the reaction (liberation of *p*-nitroaniline) is proportional to the concentration of the enzyme (from $1 \cdot 10^{-6}$ to $1.4 \cdot 10^{-5}$ M, for other conditions see Table I).

N-Glutaryl-L-phenylalanine *p*-nitroanilide is a specific substrate of α-chymotrypsin [40]; this makes us think that catalysis involves the active centre. In the same conditions, chymotrypsinogen, that is a structural analogue and the precursor of α-chymotrypsin containing no active centre, hardly ever affects the rate of hydrolysis of the same substrate. This means that the apparent rate of the enzymatic reaction should not have been affected by nucleophilic groups of the protein (cf. Ref. 41).

The kinetics of the enzymatic reactions proceeding in organic solvents used by us can be described (like in water) by the Michaelis-Menten equation, see Fig. 2. It is obvious from this data that the rate of the enzymatic reaction can be even higher than in water (at the same total concentration of the enzyme).

(i) The 'true' parameters of the Michaelis-Menten equation. For a comparative analysis it is necessary to study the dependence of the apparant rate of an enzymatic reaction on the volume ratio of the bulk (organic solvent) and the aqueous-micellar phases, see Theory. Varying the value of θ (see Eqns. 4—8), we kept constant the molar $[H_2O]/[surfactant]$ concentration ratio, to be able

TABLE I

KINETIC PARAMETERS OF α-CHYMOTRYPSIN AND TRYPSIN CATALYSIS AT 26°C

For systems with reversed micelles, the values of true kinetic parameters calculated according to Eqns. 6—9 and characterizing the reaction in an aqueous-micellar phase are presented. True kinetic parameters, k_{cat} and K_m, were calculated according to Eqns. 7 and 9 from dependences of $k_{cat,app}$ and $K_{m,app}$ on (an example is given in Fig. 3). The experimental $k_{cat,app}$ and $K_{m,app}$ values, in turn, were determined by the Lineweaver-Burk method (Fig. 2). To this end, the initial steady-state (v) of the enzymatic reaction was measured for not less than five concentrations of the substrate. The root-mean-square error (calculated by means of a computer program designed to carry out least-squares evaluation) was not over 30% for all the kinetic parameters. The concentration of α-chymotrypsin (per the total volume of the system) was varied from $1 \cdot 10^{-6}$ M to $1.4 \cdot 10^{-5}$ M. The concentration of trypsin (per total volume of the system) was varied from $1 \cdot 10^{-6}$ M to $5 \cdot 10^{-6}$ M. The concentration of *N*-glutaryl-L-phenylalanine *p*-nitroanilide (per total volume of the system) was varied from $5 \cdot 10^{-5}$ M to $5 \cdot 10^{-4}$ M. The concentration of *N*-benzoyl-D,L-arginine *p*-nitroanilide (per total volume of the system) was varied from $7 \cdot 10^{-5}$ M to $2.5 \cdot 10^{-3}$ M. n.d., no determination.

System	Enzyme	Substrate	Reaction medium	Charge of substrate	Charge of surfactant	k_{cat} s^{-1}	K_m M^{-1}	k_{cat}/K_m $M^{-1} \cdot s^{-1}$
I	α-Chymotrypsin	*N*-glutaryl-L-phenylalanine nitroanilide	water	—	without surfactant	$7 \cdot 10^{-3}$	$1 \cdot 10^{-3}$	7
II			Aerosol OT (0.04—0.3 M)/ water/octane at $[H_2O]$/ [surfactant] = 20	—	—	$9 \cdot 10^{-4}$	$3 \cdot 10^{-3}$	$3 \cdot 10^{-1}$
III			CTAB (0.1—0.8 M/water/ 1 : 1 chloroform + octane at $[H_2O]$/[surfactant] = 25	—	+	n.d.	n.d.	$4 \cdot 10^{-3}$
IV	Trypsin	*N*-benzoyl-D,L-arginine *p*-nitroanilide	water	+	without surfactant	$3 \cdot 10^{-1}$	$1 \cdot 10^{-3}$	$3 \cdot 10^{2}$
V			Aerosol OT (0.04—0.3 M)/ water/octane at $[H_2O]$/ [surfactant] = 20	+	—	n.d	n.d.	$1 \cdot 10^{-3}$
VI			CTAB (0.1—0.8 M)/water/ 1 : 1 chloroform + octane at $[H_2O]$/[surfactant] = 25	+	+	$4 \cdot 10^{-2}$	$1 \cdot 10^{-2}$	4

* An aqueous buffer solution (0.02 M acetate/phosphate/borate) at pH 8.0 was used.

** Molar concentration ratio.

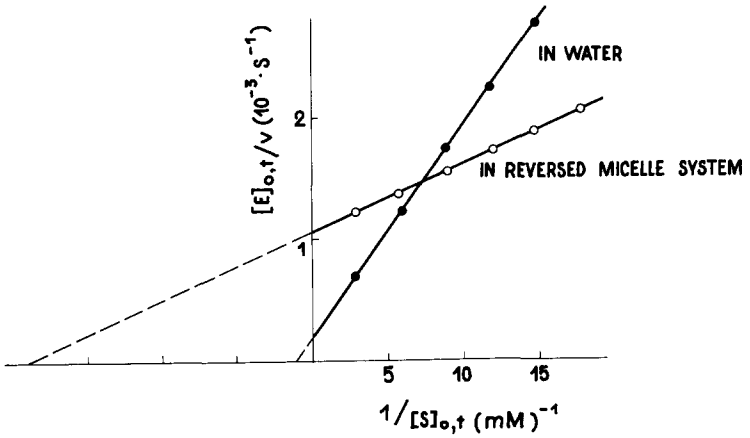

Fig. 2. Lineweaver-Burk plots for hydrolysis of *N*-glutaryl-L-phenylalanine *p*-nitroanilide catalyzed by α-chymotrypsin. Experimental conditions: 26°C, $[E]_{o,t} = 7 \cdot 10^{-6}$ M, ●———●, in a system of 0.1 M Aerosol OT + aqueous buffer (0.02 M phosphate/borate/acetate, pH 8) + octane; molar $[H_2O]/[surfactant] = 20$; ○———○, in water (0.02 M phosphate/borate/acetate, pH 8).

to operate at a fixed degree of hydration of micelles. We assumed θ to be equal in the first approximation to the volume of solubilized water.

In kinetics of enzymatic reactions proceeding in reversed micelles one can expect various pH effects [67,69]. However, the way of varying the θ we used, involved a constant hydration of micelles. So one may think that the change in the θ does not alter the microenvironment of the solubilized enzyme. We can, thus, suppose that even if the pH in the reversed micelles differs from the pH of the buffer used, the difference in question is constant at all θ values. Furthermore, these experiments were run at high hydration, $[H_2O]/[surfactant] = 20$ or 25 (see Table I), were the pH shift should not be too great. We shall analyze this aspect below by the example of the simple stage of the enzymatic process, deacylation of *N-trans*-cinnamoyl-α-chymotrypsin.

Fig. 3 shows by way of example the apparent parameters of tryptic hydrolysis of specific [42,43] substrate, i.e., *N*-benzoyl-D,L-arginine *p*-nitroanilide. The enzyme was entrapped into reversed CTAB micelles in an 1 : 1 chloroform/octane mixture. It is seen from Fig. 3 that the value of $k_{cat,app}$ does not depend on the volume ratio of the aqueous-micellar and the bulk phases. Consequently, according to Eqn. 5, this value may be believed to be a true characteristic of the reaction in the aqueous-micellar phase, $k_{cat,mic}$.

At the same time, according to Eqn. 9, the value of the apparent Michaelis constant should depend linearly on θ; this proved to be the case in the experiments, see Fig. 3. The slope of the linear dependence of $K_{m,app}$ on θ is the true Michaelis constant for the reaction in the aqueous-micellar phase ($K_{m,mic}$). The relevant data for various systems are listed in Table I.

(ii) Acceleration of the enzymatic reaction at the expense of the concentrating of the reagents in the micellar phase. As seen in Table I, the true parameters of the Michaelis equation that characterize the catalysis by both α-chymotrypsin and trypsin in the aqueous-micellar phase are somewhat lower than for an enzymatic reaction running in water. It could, therefore, seem surprising that

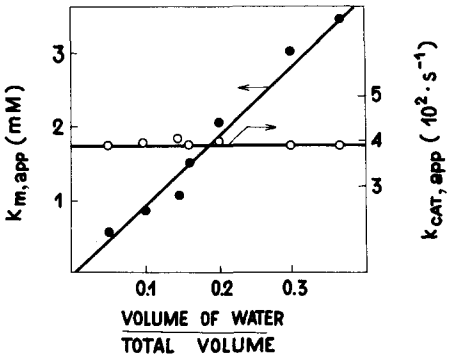

Fig. 3. Hydrolysis of *N*-benzoyl-D,L-arginine *p*-nitroanilide catalyzed by trypsin solubilized in a system of CTAB + aqueous buffer (0.02 M acetate/phosphate/borate, pH 8.0) + 1 : 1 chloroform octane mixture). The Michaelis parameters $K_{m,app}$ are calculated depending on the volume proportion of water in the system (at $[H_2O]/[CTAB] = 25$, with the concentration of the surfactant being varied from 0.1 to 0.8 M). Experimental conditions: 26°C, $[E]_{o,t} = 5 \cdot 10^{-6}$ M.

the total rate of the reaction that is observed in a biphasic system is sometimes higher than the rate in water *, see for example, Fig. 2. This can be explained by the fact that the reagents (enzyme and substrate) that do not dissolve in octane, concentrate in the reversed micelles. This leads to a considerable decrease in the apparent Michaelis constant (that depends on the value of θ, see Theory and Fig. 3) and to an acceleration of the reaction (under conditions where the enzyme is not yet saturated with the substrate). The phenomenon of the concentrating of reagents in micelles has been well documented previously for organic (non-enzymatic) reactions (see review Ref. 21 and our recent paper [22]). The concentration effects of this kind are also involved in the reactions that are catalyzed by enzymes immobilized in (or on) supports, (see reviews [44—46]).

(iii) The role of the signs of charges in the molecules of a substrate and a surfactant. Two moments can be discerned after a more detailed analysis of the true parameters of the Michaelis equation, see Table I.

(1) When the charges in a substrate molecule and on the interphase (in a surfactant molecule) are of the same sign (systems II and VI), the differences in k_{cat} and K_m values for water and reversed micelles, respectively, are not so significant (not more than one order of magnitude). This can be ascribed to the alteration in the character of the aqueous medium that surrounds the solubilized enzyme in a reversed micelle. It was in fact shown previously [27,47] that water enclosed in reversed micelles differs from water in the macrophase and is similar in properties to an aqueous-organic mixture. On the other hand, it is known that addition of organic water-miscible solvents to aqueous solutions of α-chymotrypsin, weakens enzyme-substrate complex formation, i.e., causes an increase in the Michaelis constant (see, for example, [48,50]). Besides, in a reversed micelles micromedium the conformation of the protein globule and/or

* When this paper was being prepared for publication, Wolf et al. [67] reported that ribonuclease solubilized, as described by us [8], in reversed micelles of Aerosol OT in octane also display a higher catalytic activity than in water. However, they did not present any explanation for the fact.

solvation state of the catalytically active groups of the enzyme, and hence their reactivity, may have changed. All these question should be studied in the future. Analysis of optical absorption, fluorescence and circular dichroism data of solubilized protein [51] can prove very useful in such an investigation.

(2) The situation is different when the charge in a molecule of a substrate and on the interphase (in a molecule of a surfactant) are of the opposite sign (systems III and V). Then in reversed micelles the decrease in the total second-order rate constant (k_{cat}/K_m) is more pronounced, i.e., three to five orders of magnitude, compared to the reaction in water. As we failed to determine individual parameters of the Michaelis equation (in the Lineweaver-Burk coordinates) at the operative concentrations of the substrates owing to its poor solubility, we are apt to ascribe the effect to the higher Michaelis constant. If this is the case, the reason for the apparent decrease in the catalytic efficiency should be the concentration factor: the substrate molecules electrostatically interact with the oppositely charged surface layer of the micelle and concentrate in it. So there may be very little or no substrate in the vicinity of the enzyme molecule, which, being surrounded with water, fails to have contact with the interphase. Such concentration effects are usually associated with immobilization of enzymes on (or in) polyelectrolyte carriers [52] (see also reviews [44—46]).

It goes without saying that the above mechanism should be substantiated with experimental evidence. It is obvious, however (see Table I) that the combination of charges on a micellar 'matrix' and in a substrate molecule is an important factor that allows one to influence the efficiency of enzymatic catalysis in reversed micelles.

(iv) Deacylation of trans-cinnamoyl-α-chymotrypsin. For studying the reactivity of solubilized water we used (as a model reaction) hydrolysis of the acylenzyme which is an intermediate in α-chymotrypsin catalysis [1,53]. The best compound for this purpose is *trans*-cinnamoyl-α-chymotrypsin. The kinetics of hydrolysis of this enzyme in water is well documented see [53] and refs. therein).

Fig. 4 shows the pH-dependence of deacylation reaction in reversed micelles of Aerosol OT in octane. Whatever the water content in the micelles, the pH profile for the apparent value of the first-order rate constant is described by an S-shaped curve, as in the relevant bulk-water reaction [56]. The sigmoidal rate-pH profile of chymotrypsin catalysis originates from the ionization of an imidazolium ring at the active site. Fig. 4 shows that incorporation of the acylenzyme into a reversed micelle entails a shift in the observed pK_a of this functional group towards more alkaline pH values; we shall point out, for comparison, that the pK_a characterizing the deacylation of *trans*-cinnamoyl-α-chymotrypsin is 7.15 [56]. Recently Wolf and Luisi [67] demonstrated a substantial agreement between the chemical reactivity of ribonuclease in pH 4.5 aqueous solution and in the Aerosol OT micelles prepared with pH 7.1 buffer. Furthermore, our result agrees with the data of Menger and Yamada [69] for the α-chymotrypsin-catalyzed hydrolysis of the specific substrate, *N*-acetyl-L-tryptophan methyl ester. Apparently, the negatively charged surface layer of the Aerosol OT micelles created by the sulfonate groups, stabilizes the imidazole conjugate acid at the α-chymotrypsin active site, so that it becomes less acidic.

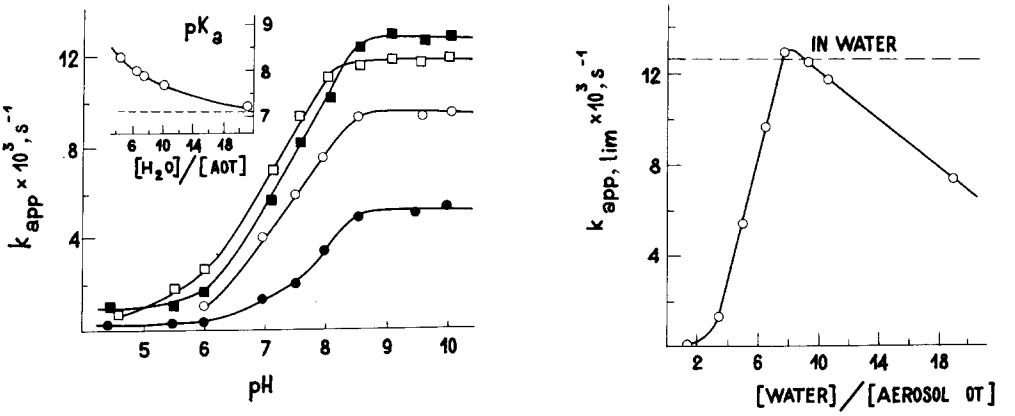

Fig. 4. pH-Dependence of the rate constant of the *trans*-cinnamoyl-α-chymotrypsin deacylation in the system of 0.1 M Aerosol OT + aqueous buffer (0.05 M Tris-acetate for pH 4.5—8.5 and 0.04 M Tris-borate for pH 8.0—11) + octane. Molar concentration ratio of $[H_2O]/[AOT]$ is ●———●, 4.8, ○———○, 6.4, ■———■, 7.6, □———□, 10.3. Conditions: 26°C; $[E]_{o,t} = 1 \cdot 10^{-5}$ M; [*N*-*trans*-cinnamoylimidazole]$_{o,t}$ $= 2.5 \cdot 10^{-4}$ M. Broken line is for pK_a in aqueous solution.

Fig. 5. Dependence of the limit (pH independent) rate constant of the *trans*-cinnamoyl-α-chymotrypsin deacylation ($k_{app,lim}$) on the content of water solubilized in a system of 0.1 M Aerosol OT + aqueous buffer (0.02 M phosphate/borate/acetate, pH 7.0) + octane. Experimental conditions: 26°C, $[E]_{o,t} = 1 \cdot 10^{-5}$ M, [*trans*-*N*-cinnamoylimidazole]$_{o,t} = 1 \cdot 10^{-4}$ M.

Similar pH effects were observed not only for solubilized protein but for non-protein acids as well, such as phenol red [69], cytidine 2′,3′-phosphate [67] and dinitrophenol [27] incorporated in reversed Aerosol OT micelles in heptane or octane; this problem was debated in more general terms in the recent paper by Fujii et al. [70].

It is of interest that, as the water content in the micelles grows, i.e., with the increase in the $[H_2O]/[AOT]$ concentrations the observed shift in the pK_a value progressively decreases and the observed pK_a tends to its limit, the 'true' value, characterizing the α-chymotrypsin active site in the bulk-water (Fig. 4, broken line). This implies that at sufficiently high concentrations of water in the reversed micelle the solubilized enzyme molecule gets detached from the surface layer and is totally buried within a water pool.

The nature of the pH effects in reversed micelles is relatively clear. A decisive role is played here by the fact that a 'local' concentration of H^+ in the surface layer of the micelle differs from that inside its water pool and can be lower or higher depending on the surface charge, see, for instance, recent publications [27,70]. This phenomenon has been well documented previously for organic (non-enzymatic) reactions in aqueous surfactant solutions, [21]. The H^+ concentration effects of this kind are also involved in the reactions that are catalyzed by enzymes immobilized in/on supports, see reviews [44—46].

We shall concentrate here on the pH-independent (limit) value of the apparent rate constant of the *trans*-cinnamoyl-α-chymotrypsin deacylation at high pH values (Fig. 4). Fig. 5 shows that this value of $k_{app,lim}$ largely depends on the content of water in micelles. The hydration degree of the micelles was varied by changing $[H_2O]/[$Aerosol OT$]$ molar ratio with the concentration of

the surfactant being constant. The following three points deserve consideration.

First, there is hardly any hydrolysis at a low water content in the micelles. This is in keeping with the point of view that the first portions of solubilized water firmly bind to surfactant molecules and form hydrate shells for the ionic groups [27,30,55].

Secondly, as the degree of hydration of micelles increases, the rate of hydrolysis goes up to achieve the level (broken line) that characterizes deacylation of *trans*-cinnamoyl-α-chymotrypsin in pure water [54,56,57].

Thirdly, further increase of the water content in micelles results in a rate of hydrolysis of the acylenzyme that is lower than that observed for the aqueous solution.

The existence of the optimal concentration of water in micelles (Fig. 5) seems to have the following reasons. Firstly, with a low content of water in micelles, its concentration is lower than in pure water (where it was equal to 55 M); this should reduce the rate of the hydrolytic reaction. Secondly, it is not excluded that in the microenvironment of micelles with a low content of water there occurs (at least partial) denaturation of an enzyme globule which is due to the fact that part of the water is spent on solvation of the surfactant molecules. If this is the case, the dehydration should affect the conformation of the protein and decrease its catalytic activity. But, as the content of water in the micelles increases, the protein becomes more and more hydrated and the globule will tend to acquire the native conformation. Decrease in the catalytic activity on further increase of the water content in the micelles seems to be due to conformational rearrangements in the micellar phase [29,58,60], which in principle can alter the conformation of the protein in such a way that its catalytic activity will decrease.

The question of the effect of water content on the state of protein entrapped in reversed micelles of course requires further study. It is obvious, however (see Fig. 5), that variation of the degree of hydration of water allows extensive regulation of the catalytic activity of entrapped enzymes.

Pyrophosphatase in reversed micelles of Brij 56 in cyclohexane. We have studied the dependence of catalysis on the degree of hydration of reversed micelles for another hydrolytic enzyme, pyrophosphatase. It is seen in Fig. 6 that, as in the case of chymotrypsin reaction (Fig. 5), the maximal rate of pyrophosphatase-catalyzed pyrophosphate hydrolysis has an optimum as the content of water in micelles increases. At a certain optimal degree of hydration of micelles, the enzyme solubilized in them is as effective as in water (broken line).

Catalysis by peroxidase entrapped into reversed Aerosol OT micelles in octane or benzene. We have found [8] that peroxidase, on being solubilized with the help of a surfactant in an organic solvent, retains its catalytic activity with respect to hydrogen peroxide oxidation of substrates of different chemical nature, e.g., potassium ferrocyanide [59] and pyrogallol [60].

We used oxidation of pyrogallol for a more detailed study of the peroxidase action. Analysis of the spectra showed that both in the case of an aqueous solution and an organo-micellar phase, purpurogallin is the product of enzymatic reaction; this means that chemically the direction of the reaction is the same in the two systems. However, the kinetic parameters of the reaction in water and

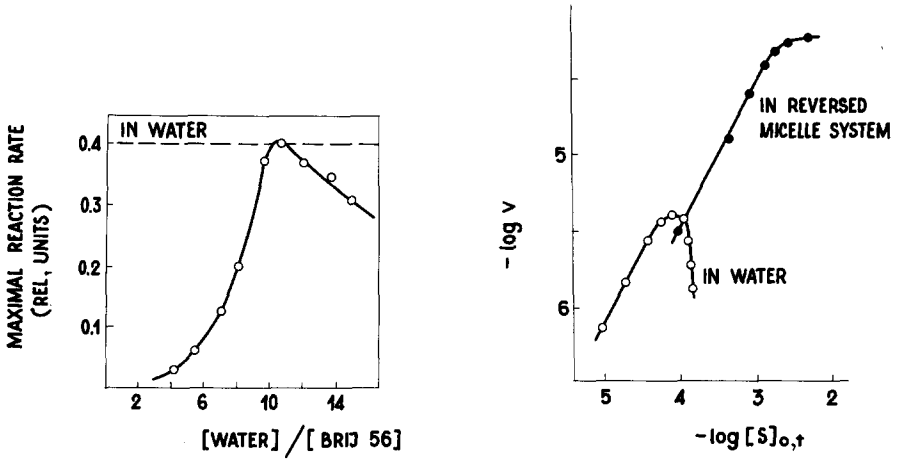

Fig. 6. Dependence of the maximal rate (V, relative units) of pyrophosphate hydrolysis catalyzed by pyrophosphatase, on the content of water solubilized in a system of 0.2 M Brij 56 + aqueous buffer (0.05 Tris-HCl, pH 7.0, 0.02 M $MgCl_2$) + cyclohexane. Experimental conditions: 26°C, $[E]_{o,t} = 3 \cdot 10^{-9}$ M, $[pyrophosphate]_{o,t} = 1.25 \cdot 10^{-4}$ M. For comparison, the value of V in the same buffer without the surfactant or organic solvent (broken line) is shown.

Fig. 7. Dependence of the initial rate (mol $\times$ l^{-1} min^{-1}) of peroxidase oxidation of pyrogallol on the concentration of this substrate. Experimental conditions: 26°C, $[E]_{o,t} = 3 \cdot 10^{-9}$ M, $[H_2O_2]_{o,t} = 2.5$ mM; •———•, in a system of 0.1 M Aerosol OT + 2% (v/v) aqueous buffer (0.02 M phosphate/borate/acetate, pH 7.0) + octane; o———o, in water (0.02 M phosphate/borate/acetate, pH 7.0).

in the micellar system proved to be entirely different. Peroxidase catalysis in an aqueous solution is inhibited by excess of the substrate [61]. As seen in Fig. 7, the effect of substrate inhibition does not take place in a reversed micelles medium. That is why in a micellar system, higher reaction rates can be realized than in water (Fig. 7).

A double-enzyme system (lactate dehydrogenase/pyruvate kinase) in reversed micelles of Brij 56 in cyclohexane. Pyruvate kinase (four subunits,

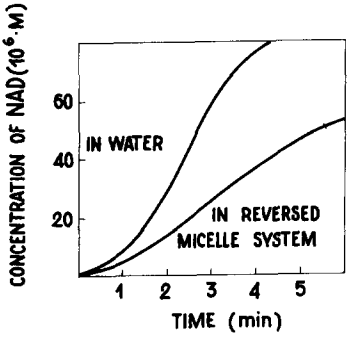

Fig. 8. A 'product vs. time' kinetic curve for accumulation of NAD in Eqn. 10 in a system of 0.2 M Brij 56 + 4% (v/v) aqueous buffer (0.05 M phosphate, pH 6.8, 2 mM EDTA/5 mM $MgCl_2$/1 mM dithiothreitol/1 M ammonium acetate) + cyclohexane. For comparison the data on the reaction in the same buffer without the surfactant and organic solvent are given. Experimental conditions: 35°C, $[pyruvate\ kinase]_{o,t} = 2 \cdot 10^{-2}$ mg/ml, $[lactate\ dehydrogenase]_{o,t} = 2 \cdot 10^{-4}$ mg/ml, $[phospho\textit{enol}pyruvate]_{o,t} = 0.5$ mM, $[ADP]_{o,t} = 0.2$ mM, $[NADH]_{o,t} = 0.2$ mM.

molecular mass 340 000) and lactate dehydrogenase (four subunits, molecular mass 145 000) exemplified quarternary structure enzymes. These two enzymes make up a double-enzyme system that catalyze coupled reaction:

$$\text{Phospho}\textit{enol}\text{pyruvate} \xrightarrow[\text{ADP}\quad\text{ATP}]{\text{Pyruvate kinase}} \text{Pyruvate} \xrightarrow[\text{NADH}\quad\text{NAD}]{\text{Lactate dehydrogenase}} \text{Lactate} \qquad (10)$$

As seen in Fig. 8, both enzymes retain their catalytic activity and specificity in carrying out chemical conversions Eqn. 10. Therefore, there is a great similarity between the kinetics in the reversed micelles system and those for the aqueous solution, other conditions being the same *.

As in water [64], in a reversed micelles system there is a lag-period in the action of the double-enzyme system. In terms of the theory [60] the duration of the lag-period depends on the parameters of the reaction catalyzed by the second enzyme, i.e., lactate dehydrogenase (reduction of pyruvate into lactate). We proved experimentally that, as in water [62] our reversed micelle system also produced the lag-period equal to the ratio of the Michaelis parameters (K_m/V = 1.3 min) for lactate dehydrogenase. This gives us grounds for believing that the double-enzyme system, entrapped in reversed micelles does not have diffusion hindrances in the exchange of low molecular weight reagents.

Conclusion

The six enzymes that we have studied belong to different classes: transferases (pyruvate kinase), oxidoreductases (peroxidase, lactate dehydrogenase) and hydrolases (chymotrypsin, trypsin, pyrophosphatase). Moreover, these enzymes greatly differ in structure: an enzyme molecule consists of one or several polypeptide chains or of several subunits. Finally, some of them have a prosthetic group (haem) or a coenzyme (ADP and NADH). Nevertheless, all the enzymes studied, when solubilized in organic solvents with the help of surfactants, retain their catalytic activity for quite long time (at least a week) **. For example, we found that α-chymotrypsin, on being solubilized ($1 \cdot 10^{-5}$ M) in a system of 0.1 M Aerosol OT + 1 vol.% aqueous buffer (0.02 M phosphate/borate/acetate, pH 8) + octane, has retained its catalytic activity for two years now.

These results are very important for applied studies. For example, a high stability of the enzymes solubilized according to our method [8] in organic solvents make them usable in organic synthesis, see reviews [4,9].

On the other hand, solubilization of enzymes in reversed micelles deserves a fundamental study. This is due to the fact that systems of reversed micelles in nonaqueous solvents allow one to strictly dose the quantity of water molecules

* A.V.L. is pleased to acknowledge the assistance of Dr. F. Seydoux, in whose laboratory the experiment took place.

** It should be noted that there are conditions (that depend on the ratio of the concentrations of a surfactant, water, and an enzyme) under which the catalytic activity in a system (say, involving trypsin) rapidly (within many minutes) disappears. It is surprising that the level of the residual activity of the enzyme essentially depends on the nature of the buffer and of the substrate. The question is under study now [63].

that surround a solubilized enzyme. This fact, as pointed out in [8], is very advantageous for studying some aspects of the mechanism of enzyme action. For example, there are grounds for believing that the use of reversed micelles as a reaction medium will allow one to study intermediates of enzymatic reaction that are labile in aqueous solutions. This can be inferred from the data in Fig. 5, where it is shown that in 'dry' micelles the acylenzyme should not hydrolyze. Again, reversed micelles-entrapped enzymes will be very helpful in studying the role of water in the maintenance of the catalytically active conformation of a protein; see above, discussion of Fig. 5. Finally, micellar systems are useful for studying the reactivity of water in the role of a chemical reagent. So far, H_2O molecules have been reported to have an exceedingly high reactivity in a non-enzymatic reaction (hydrolysis of picrylchloride in Aerosol OT reversed micelles in octane) [54].

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