# Micellar enzymology: methodology and technique

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The methodological procedures employed both for serving investigations in the field of micellar enzymology and developed directly in micellar enzymology and being of general significance, such as an estimation of molecular masses and sizes of biocatalysts, titration of active sites of enzymes, chemical modification of proteins (enzymes), conjugation and nanogranulation, are reviewed. Potentialities of using and informativity of various techniques are analyzed.

**Key words:** reverse micelles, proteins, enzymes, structure and function, methods for research, micellar enzymology.

Studies of proteins (enzymes) in systems of reverse micelles (or, more generally, in three-component systems water—surfactant—organic solvent) are being intensely carried out for more than 30 years and have resulted to the moment in the formation of a broad independent area of knowledge named "micellar enzymology." The progress and state-of-the-art of research in this area can be traced from reviews.[1—12]

In addition to a rich fundamental material, which enlarge concepts about the nature of enzymes and biocatalysis, the powerful methodical basis has been created for serving and development of studies in the field of micellar enzymology. This contains novel methods for manipulation with proteins (enzymes) and revealing their characteristics. Among them are new methods for the isolation and purification of proteins (enzymes), their chemical modification, titration of active sites, and designing of complexes, conjugates, and supramolecular ensembles. In the present work, it is to this methodical and technical aspects of micellar enzymology that the main attention is given.

First, note that unlike traditional homogeneous aqueous solutions, micellar systems in organic solvents are microheterogeneous (pseudohomogeneous), although they remain completely transparent and suitable for studying by classical instrumental methods. In other words, micellar systems combine the properties of standard homogeneous solutions* and particles, *viz.*, micelles, which exist in dynamic equilibrium. During solubilization, enzymes are incorporated into micelles, and it is micelles (micellar matrix) that are the basic factor of action upon, and regulation of, solubilized enzymes.

* Micellar systems are diphilic in nature and allow the operation with both hydrophilic (water-soluble) and hydrophobic (soluble in organic solvents) components.

## 1. The main experimental methods

### 1.1. Procedures for the incorporation of proteins (enzymes) into reverse micelles

Proteins (enzymes) can be incorporated into reverse micelles of surfactants in organic solvents using one of three following methods.

The first method, the so-callled "injection,"[13] is presently used most widely. Small amount (several percent v/v) of an aqueous solution of a protein is introduced into a solution of a surfactant in an organic solvent (anhydrous or little hydrated). The particular ratio of the amounts of the aqueous solution to the organic one is determined by experimental conditions, first, by the value of the required degree of hydration of the surfactant ($w_o$ = [$H_2O$]/[Surf]). The resulting mixture is vigorously shaken (for seconds or tens of seconds) until an optically transparent solution is formed. This method is in fact simple and efficient. Some problems appear when it is used, such as the questions about the state of equilibrium in the system obtained (see, *e.g.*, Ref. 14), processes and reactions that occur during the preparation of the system. The question about equilibrium can be answered by comparison with other methods for the achievement of this state (since the equilibrium state is independent of the way of its achievement). Some undesirable side reactions (leading, in particular, to partial inactivation of the solubilized enzyme) can be excluded by selection of the order of adding and mixing of the components. For example, solutions of the enzyme and substrate with the same final degree of hydration are prepared separately and incubated (equilibrated) for some time. Then the reaction is initiated by mixing of the stored solutions in a required proportion (we specially emphasize that in this

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case the degree of hydration of the surfactant, which is often the critical factor in the manifestation of the nonequilibrium character and side impeding phenomena, is not virtually changed in the final stage, *viz.*, initiation of the enzymatic reaction).

The second method proposed by Menger and Yamada[15] consists, first, in addition of the required amount of water (an aqueous buffer solution) to a solution of a surfactant in an organic solvent for the desired degree of hydration ($w_o$) to be achieved followed by dissolution of a dry (for example, lyophilized) protein preparation in the resulting micellar solution with vigorous shaking (stirring). The time required for the dissolution of dry protein is usually much longer than that for the solubilization of aqueous solutions (from several min to tens of h). In this procedure, the protein exists for a comparatively long time in contact with the surfactant and organic solvent and, as a rule, it is partially denatured. However, micellar solutions with a higher concentration (up to the saturation concentration) of the protein compared with that achieved in the first (injection) method can finally be obtained (with some loss of the protein). To decrease the protein losses, it is reasonable to perform dissolution in several stages: first, a small excess of the dry protein is taken; after several h of stirring of the suspension, the supernatant is separated, a new portion of the dry protein is added, the dissolution procedure is multiply repeated, and the content of the dissolved protein is determined in the supernatant. When using this procedure, one should keep in mind that a partial loss of the surfactant and water because of precipitantion can occur during the formation of the system. This circumstance needs to be additionally monitored, *e.g.*, by chromatography.[16] The surfactant concentration can easily be determined in a micellar solution due to its high content, for example, gravimetrically from the mass of the dry residue after the evaporation of the solvent from an aliquot of the analyzed solution. The water content in micellar system can conveniently be monitored by $^1$H NMR using the chemical shift value.[17] In discussion of problems of control of the water content, note the problem of characterizing the hydration of the starting surfactant preparations (the latter should be taken into account for the correct calculation of the working degrees of hydration). With this purpose, we successfully used IR spectroscopy and monitored the water content at a frequency of 3420 $cm^{-1}$ (the procedure is described in detail in Ref. 18).

It should be emphasized that when the second method of solubilization (with the dissolution of a dry protein preparation in a micellar solution) is used, the content of both protein and low-molecular-weight (in particular, salts) admixtures can vary in the resulting solution.

The third method[19–21] is based on the spontaneous transfer (distribution) of protein in a biphasic system composed of approximately equal volumes of an aqueous solution of the protein and an organic solvent containing the surfactant (the micellar system with a specified degree of hydration). The protein transfer occurs without stirring or with gentle stirring and takes a rather long time (from tens of min to days). During this time interval, the protein (enzyme) actively interacts with molecules of the surfactant and the organic solvent, it is concentrated at the interface and undergoes denaturation. This method is inferior to those described previously because of being labor-consuming and being accompanied by the difficulties indicated. On the other hand, the method discussed has an independent significance and, undoubtedly, is very promising for the separation and purification of proteins (see, *e.g.*, Ref. 22) because such processes as the incorporation of protein molecules into micelles and exit from micelles to an aqueous solution can conveniently be controlled by the variation of such parameters as pH and ionic strength. Conditions for the selective incorporation into micelles can be found for each protein, and the desired protein can thus be extracted from a mixture. Replacing an aqueous phase by a pure solution and establishing the required pH and ionic strength values, re-extraction is carried out, and a solution of the target (purified) protein in water is obtained. This procedure can also be used for the extraction of other water-soluble components, including reaction products, from micellar systems. This will be discussed in the next Section.

### 1.2. Separation of components of micellar systems, regeneration of the enzyme, and isolation of reaction products

Since micellar systems are microheterogeneous, it is rather difficult to isolate one or another component from them. This problem, most likely, has no general solution, and each particular case requires individual consideration. At the same time, the whole set of approaches to the solution of this problem is available, and we are going to consider it successively.

Both the simplest and the most elegant method is the reversible separation of phases under physical action. For example, upon a relatively slight temperature change (in some case, with the temperature increase, and in other cases, with its decrease),[23] the phase which is rich in the organic solvent and contains the hydrophobic product and almost no surfactant, is separated from the micellar solution. This method seems to be most promising, technologically acceptable, and appropriate for purposes of organic synthesis where the product can be obtained in the pure state by the evaporation of the organic solvent and the initial system is easily recovered by addition of the pure organic solvent.

A general method for the separation of a low-molecular-weight component from micellar systems without their destruction is the use of semipermeable membranes. This principle can form the foundation for designing continuous reactors.

Water-soluble components can be extracted from a micellar system on going to the macrobiphasic system (its type corresponds to the third method for solubilization) by mixing with water or water-salt solutions.

A micellar system can easily be destroyed by the addition of water-miscible organic solvents such as acetone or ethanol. This method is very efficient and convenient when the solubilized protein (enzyme) needs to be carefully extracted with the retention of its structure and physiological (including catalytic) activity.[24] Therefore, it is necessary to remind that, first, solvents that are added (ethanol and, especially, acetone) must be cool; second, for precipitation with acetone in neutral and alkaline media, the formation of Schiff´s bases and side modification reactions of the isolated protein (including the loss of the solubility in water by the protein) are possible.

Selecting the organic solvent and the ratio of volumes of the solvent and micellar system, one can achieve phase separation and obtain solutions enriched in a particular component, for example, the enzymatic reaction product.[25]

Reversible transitions in the working three-component system (surfactant—water—organic solvent), *e.g.*, from the micellar to liquid-crystalline states (including the macroheterogeneous state where the surfactant and the enzyme are in the condensed phase, and the reaction product is in the organic solvent) can be considered as a general procedure for the component separation. This approach (see, *i.e.*, Ref. 26) is very promising for aims of organic synthesis.

Note in conclusion that the procedures described can often be used in combination, for example, the temperature (pressure) effect on the micellar system can be combined with the addition of water (aqueous buffer solutions), salts or organic solvents.

### 1.3. Methods for determination of characteristics of solubilized proteins (enzymes)

The structures and parameters of protein-containing micelles are of principle importance for the determination of properties and, first of all, catalytic activities of the solubilized enzymes. Instrumental methods for studying "empty" and protein-containing micelles will be considered henceforth in a special section. Here we would like to note that the ultracentrifugation method turned out to be, in our opinion, one of the most informative. As a rule, fractions corresponding to empty and protein-filled micelles can distinctly be discriminated in the sedimentation patterns. The calculation of the sedimentation coefficients ($s_o$ and $s_p$, respectively) and their further analysis allow, in many cases, the estimation of the molecular mass ($M_p$) and sizes of the solubilized protein (enzyme) from the simple equation (1)[27,28]

$$M_p = M_o(s_p/s_o - 1)(1 - v\rho), \quad (1)$$

where $M_o$ is the molecular mass of the micelle, $v$ is the partial specific volume of empty micelles, and $\rho$ is the solvent density. These values are well known for systems of reverse micelles based on Aerosol OT (AOT).[29,30] As can be seen from Eq. (1), determination of the molecular mass of the protein (enzyme) solubilized in the system of reverse AOT micelles requires only that the ratio of sedimentation coefficients of empty and filled micelles, $s_p/s_o$, was determined experimentally. This method has well recommended itself in the case of water-soluble (non-membrane) proteins. The determination of the molecular masses of several proteins are documented.[27,28] For high degrees of hydration of surfactants (large sizes of micelles), the accuracy of determination of the molecular mass of the incorporated protein decreases (the weight fraction of the protein decreases in the micelles which become heavier), and a break is observed in the plot of the molecular mass *vs.* degree of hydration calculated by using equation (1) in the region of relatively low degrees of hydration. The break point corresponds to the conditions of equality of the sizes of the polar internal cavity of the micelle and the protein molecule (its longest axis in the case of a non-spherical molecule).

Reverse micelles are dynamic formations, and the substance exchange occurs promptly (the lifetime of the surfactant in the micelle is of an order of microseconds) both between individual micelles upon their collisions and between micelles and the medium. In the case of water-soluble substances, the mass transfer occurs predominantly due to collisions of micelles. As a rule, in most cases chemical and enzymatic reactions in micellar systems occur in the kinetic regime. However, intermolecular interactions can specially be retarded by increasing the medium viscosity, *e.g.*, by introducing a hydrophobic polymer into the organic solvent phase. With this purpose, we used[31] non-cross-linked isoprene resins, which are well soluble in octane, the bulk solvent of the AOT micellar systems. The idea of this procedure is the following. In a viscous medium with controlled exchange between micelles, only the part of the substrate which is localized in the enzyme-containing micelles undergoes enzymatic transformation. Thus, in the micellar system retarded by the resin, the fraction of the transformed substrate (under the assumption of its uniform distribution between micelles) allow the estimation of the fraction of enzyme-containing micelles, *i.e.*, their concentration or, more exactly, the concentration of the catalytically active enzyme. As is seen, this is a basically new, universal method for the titration of active sites of hydrophilic (non-membrane) enzymes, which does not need special titrants but rather makes use standard

chromophore-containing substrates or products of their enzymatic transformations.[31]

Of course, the main characteristics of the solubilized enzyme is its catalytic activity. Section 1.5 will be devoted to the determination and regulation of the catalytic activities of enzymes in micellar systems. Nevertheless, here we indicate one kinetic characteristics: micelle sensitivity (or, in other words, membrane activity). The membrane activity (membranotropism) of the enzymes that are studied can easily and reliably be determined from the existence of a dependence of the maximum rate of the enzymatic reaction on the surfactant concentration in the system and from the character of changes in the reaction rate on changing the type of micelles (for details, see Refs. 3, 4, 6, 32—34).

### 1.4. Modification of solubilized proteins

It should be noted first that systems of hydrated reverse micelles in organic solvents can serve as "diphilic" solvents, *i.e.*, they can dissolve (solubilize) both hydrophobic and hydrophilic substances (reactants). The former substances will predominantly be localized in the phase of an organic solvent, whereas the latter will be localized in the polar cavity of micelles. Proteins (enzymes) are predominantly solubilizzed in the polar cavities of micelles, as a rule, in the 1 : 1 stoichiometric ratio (different ratios and forms of oligomeric proteins are discussed below). Thus, the reverse micelle can be considered as a unique molecular reactor with controlled and tunable sizes in which a protein molecule can be incorporated and subjected to the action of reactants (modifiers) dissolved in the system. The reactants can be either hydrophilic or hydrophobic, either low- or high-molecular. The described principle for the modification of solubilized proteins (enzymes) is exemplified in this section.

**Hydrophobization of proteins.** Micellar systems are very convenient for the controlled introduction of hydrophobic reactants poorly soluble in water (one—two hydrophobic residues per protein molecule) into the protein.[35] This approach was successfully employed for the introduction of residues of long-chain aliphatic acids,[35—37] phospholipids,[38] hormones (thyroid and steroidal),[39,40] and organometallic compounds (for example, ferrocene)[41] into protein molecules.

**Designing of protein ensembles with specified stoichiometry.** A protein molecule is incorporated into the internal cavity of a reverse micelle, whose size can be varied by changing the degree of hydration of the surfactant. As a rule, simple proteins (enzymes) form protein-containing micelles in a ratio of 1 : 1. In some cases, a greater number of protein molecules can be incorporated into one micelle when very high (higher than 10 mmol $L^{-1}$) protein concentrations are used.[42] Oligomeric proteins (enzymes) are different. When micelle sizes are varied, a whole set of various oligomeric forms of oligomeric enzymes (monomers, dimers, tetramers, *etc.*) can be obtained for many individual enzymes[43—57] and for their mixtures.[58] Using a micellar matrix, researchers succeeded in the formation of unusual (from the point of view of classical "aqueous" enzymology) protein-protein complexes, for example, a compact non-covalent dimer of chymotrypsin or a stable (it dissociates in aqueous solutions only in the presence of 8 *M* urea) non-covalent complex of chymotrypsin with peroxidase.[59]

**Preparation of protein complexes with polymers.** In a homogeneous solution, "cross-linking" of two macromolecules is a very serious problem because of a possibility of numerous intermolecular reactions resulting in the formation of the cross-linked block-copolymer. In a micellar medium, it is quite possible to restrict the reaction sphere by the intrinsic space of one micelle and completely suppress (or completely exclude if necessary) reactions at the level of intermicellar interactions. In other words, the use of classical cross-linking reagents in micellar systems allows one to "cross-link" (chemically fix) complexes in the "intramicellar" regime. This idea has been implemented, in particular, in studies[60—62] on the formation of complexes and conjugates of proteins and synthetic polymers with various stoichiometry. It was shown that at low degrees of hydration where micelles are small the protein and polymer molecules are localized in different micelles and do not virtually interact with each other. With an increase in the sizes of the micelles, after some critical value of the degree of hydration of the surfactant had reached (this value is determined by the sizes of the formed complex), the formation of a complex occurs, which can chemically be cross-linked in an almost 100% yield. Further increase in the micelle sizes (the degree of hydration of the surfactant) results in the formation of protein-polymer complexes with a greater stoichiometry.[60—62]

**Nanogranulated (nanoencapsulated) proteins (enzymes).** A polymeric shell around a protein molecule can be formed either from the pre-existing polymer or by polymerization of the corresponding monomers in a medium of reverse micelles. Speiser[63,64] was the first to carry out the polymerization of monomers solubilized by reverse micelles with the purpose of nanoencapsulation and nanogranulation of biologically active substances (see also the reviews[65—67]). In our studies on the copolymerization in the systems of reverse micelles, we used proteins (enzymes) chemically modified by acryloyl chloride as the macromonomers.[67—69] The copolymerization of monomers and macromonomers solubilized in systems of reverse micelles form polymeric particles (nanogranules), which can easily be isolated by the destruction of the system using, *e.g.*, precipitation with acetone. The particles thus obtained are nearly monodisperse, their sizes are of an order of tens of nanometer (they are real nanoparticles), and they contain the covalently incorporated (immobilized), highly stable

enzyme. The particles can be dissolved in water or suspended in an organic solvent to be used as biocatalysts. The solubility in organic solvents with the purpose for performing there biocatalytic reactions can be enhanced by the incorporation of hydrophobic fragments into nanogranules, for example, by copolymerization with surfactants containing double bonds in their hydrophilic parts.[69—71] Nanogranules in systems of reverse micelles can also be obtained from the existing polymers, *e.g.*, gelatin[72] or poly(vinyl alcohol) (by the formation of a cryogel).[73] Lecithin-based organogels are studied and described in detail.[74—76] An example of a block-copolymer with structural elements of the type of reverse micelles is presented in Ref. 77.

### 1.5. Kinetics of reactions catalyzed by enzymes in systems of reverse micelles and its regulation

As in the classical (aqueous) enzymology, the kinetics of enzymatic reactions in micellar media obeys, in most cases, the classical Michaelis equation with the only exception that the kinetic parameters determined by this equation are effective and depend on the degree of hydration and concentration of micelles. A detailed analysis of the kinetics of enzymatic reactions that obey the Michaelis equation in systems of hydrated reverse micelles of surfactants in organic solvents and interpretation of the observed parameters are given in Refs. 78 and 79. Here we only mention that the observed $K_m$ values and other parameters, whose dimensionalities include the concentration, in (micro)heterogeneous systems depend on the local concentrations of the corresponding reactants, which, in turn, are controlled by the ratio of volumes of the phases and the type of substance distribution between the phases. The catalytic constant $k_{cat}$, whose dimensionality includes only time and, hence, these systems are prone to concentrating effects, are preferential as the characteristics of the reactivity of enzymes in micellar systems. Nevertheless, the $k_{cat}$ values observed in micellar systems depend on many factors, including the structures (types) of micelles and their sizes.

As a rule, the plot of $k_{cat}$ *vs.* degree of hydration of the surfactant (size of micelles) is bell-shaped[80] (see also the reviews[1—12]). If the reaction occurs in the kinetic regime (it is not violated by diffusional limitations) and possible pH effects are taken into account, the optimum of the enzyme activity is observed, as a rule, where the sizes of the initial (empty) micelles and the molecule of the incorporated protein-biocatalyst are equal (the principle of geometric correspondence is fulfilled) (see, *e.g.*, Refs. 6, 9, and 80). The optimum degree of hydration, $w_{o,opt}$, is related to the molecular mass of the enzyme, $M_p$, by the simple equation (2):

$$w_o^{opt} = 0.47\sqrt[3]{M_p} - 2.7. \quad (2)$$

The inverse problem can also be solved: the molecular mass of the enzyme, $M_p$, can be estimated using Eq. (3) from the degree of hydration where the optimum of the catalytic activity is observed.

$$M_p = (2.14w_o^{opt} + 5.7)^3. \quad (3)$$

In the case of non-spherical molecules, it should be kept in mind that calculations use the value of the long axis, whose size controls the size of optimum micelles by the principle of geometrical correspondence. In the case of oligomeric enzymes and enzyme complexes, the plot of $k_{cat}$ *vs.* $w_o$ is wave-shaped and characterized by several optima, each reflecting functioning of these or other particular oligomeric forms.[43—59]

Water in the polar cavity of reverse micelles can partially or completely be replaced by a water-miscible organic solvent (see, *e.g.*, Refs. 9, 81, and 82). The profiles of the plots $k_{cat}$ *vs.* $w_o$ are narrowed and shifted to the region of "drier" micelles because the principle of geometrical correspondence continues to be strictly fulfilled and the activity of the solubilized enzyme (for example, chymotrypsin) increases due to an increase in the rigidity of micelles and enhancement of the stabilizing effect of the micellar matrix on the structure of the incorporated protein.[9,83,84] In the limit, one can obtain a highly reactive enzyme in an almost "dry" (anhydrous) medium.[9,83,84] In this system, one can successfully shift the equilibrium of several reactions, first of all, hydrolytic reactions, and synthesize the nitroanilide substrate of chymotrypsin in a high yield from the hydrolysis products.[85] In the fundamental aspect, such systems open a new way for studying the role of water in enzymatic catalysis and refining mechanisms of enzymatic reactions. In particular, we have demonstrated the formation of an intermediate of the presumably acyl-enzyme type in the hydrolysis of amide and anilide substrates by chymotripsin.[9]

## 2. Instrumental research methods

### 2.1. Spectral methods

As has already repeatedly been mentioned, an advantage of systems of reverse micelles in organic solvents providing easy operation with them is their optical transparency. This property allows the use of diverse spectral methods. Let us consider some examples.

**Absorption spectroscopy** in both the visible region and the ultraviolet wavelength regions is widely used for most of routine experiments in which enzymatic reactions are followed by monitoring the accumulation of the reaction product which absorbs in certain spectral region or the consumption of the substrate. However, analysis of the data obtained requires account of the fact that molar absorption (molar extinction) coefficients of many compounds in the system of reverse micelles can

substantially differ from those in aqueous solutions. Moreover, in the reverse micellar system, this parameter can substantially change with a change in the water content. For example, $\varepsilon_{335}$ for cinnamoylimidazole changes in the system of reverse AOT micelles in octane from 2500 to 4750 $mol^{-1}$ L $cm^{-1}$ when the degree of hydration of the surfactant varies from 0 to 70 and does not reach the value observed in an aqueous solution (9360).[42] In the case of *p*-nitroaniline, changes in $\varepsilon_{380}$ in the reverse micellar system do not substantially depend on the degree of hydration of the surfactant and of the presence of considerable concentrations of water-miscible organic solvents in the internal cavity of the micelles. The $\varepsilon_{380}$ values are 9000—11000 $mol^{-1}$ L $cm^{-1}$, which differs from that observed in an aqueous solution (14500). In the case of NADH*, which is the coenzyme of many enzymatic reactions and is convenient to be followed by monitoring either its formation or consumption at a wavelength of 340 nm, the molar absorption coefficient remains virtually unchanged either on going from aqueous solutions to the system of reverse AOT micelles in octane or with a change in the water content in this system. Various factors can influence the change in the molar absorption coefficient, such as the local shift of the pH in the internal cavity of micelles, distribution of the substance between the aqueous, micellar, and organic phases, a change in pK of ionogenic groups of the compound used, *etc.*

**IR spectroscopy** is also used in reverse micellar systems because the water content there is usually low, being not more than 10%. In particular, this method turned out to be convenient for the determination of the content of water present in the initial preparation of the surfactant.[42] The relative intensity of light transmission ($I/I_0$) is measured at 3420 $cm^{-1}$, the obtained linear plot of the absorption of the solution *vs.* the amount of water introduced into the system ($D = \log(I_0/I)$) is extrapolated to the zero absorption, and the value indicating the amount of the "background" water in the initial preparation is obtained from the cut in the abscissa. Normally, the AOT specimens available from various companies contain from 0.4 to 2.5 water molecules per surfactant molecule, and this should be taken into account in the calculation of the total water content in the system under study. IR spectra were used to determine the relative content of α-helices, β-structures, and disordered regions of the structure in the α-chymotrypsin molecule incorporated into the ternary AOT—water—octane system using intensities of various amide bands, for example, the "amide I" band in the 1600—1700 $cm^{-1}$ region.[86] It was found that the incorporation of α-chymotrypsin into reverse micelles resulted in the shift of the maximum and a decrease in the intensity of the band at 1653 $cm^{-1}$ corresponding to the α-helix regions in the protein molecule, and the shift and a decrease in the intensity of the band at 1638 $cm^{-1}$ corresponding to the β-structures. The Fourier analysis of the spectra[86] using their second derivatives has led to a conclusion that the number of disordered regions and β-turns increase when the protein is incorporated into the reverse micelles with a low water content ($w_o$ 6—10) where the internal cavity was smaller than the size of the incorporated protein. It should be emphasized that, under these conditions, the water differs substantially in properties from the water added. Different types of water at different degrees of hydration were also observed by IR spectroscopy.[87,88] A substantial disadvantage of the method is the necessity to use very high protein concentrations, which are often beyond the solubility limits in micellar systems.

**Fluorescence spectroscopy** is successfully used for studying various proteins in reverse micellar systems. Fluorescence spectra depend on the environment to a greater extent than the absorption spectra. Lesser amounts of a substance are needed to detect the fluorescence parameters, *i.e.*, the high sensitivity is an evident advantage of the method. Fluorescence provides data on the conformation, binding sites, interactions with the solvent, the degree of flexibility, and coefficients of rotational diffusion of macromolecules as well as information about the localization of substance in live cells. Different variants of fluorescence have been used to characterize the dynamics and microenvironment of various probes, including proteins, in reverse micellar systems.[89—93] Fluorescence data can differ for different proteins and peptides due to the influence of the micellar environment on the protein structure and interactions of the protein with the surface of the internal micellar cavity and the solvent. In many cases, the incorporation of proteins into micelles is accompanied by a decrease in the time of rotational relaxation of the protein in the region corresponding to side chains of the tryptophan residues.[89—93] Data from phosphorescence of horse liver alcohol dehydrogenase suggest higher flexibility (mobility) of the coenzyme-binding domain in the reverse micellar system compared with an aqueous solution.[94] Similarly, experiments on the decay of fluorescence anisotropy of the single tryptophan residue (Trp140) in staphylococcus nuclease showed its high mobility in the reverse micellar system compared with an aqueous solution.[95] Studying fluorescence of proteins in reverse micellar systems, one should keep in mind that the microenvironment of a fluorescent probe has a significant effect on the results obtained, and this environment, in turn, can substantially depend on the nature of the surfactant, water content in the system, *etc.* Fluorescence along with other methods has been used for studying processes associated with the denaturation and renaturation, dissociation and association of proteins in reverse micellar systems.[96—101] Note that the kinetics of chemical and enzymatic reactions can be monitored using fluorescent substrates or products and

* NADH is the reduced form of nicotinamide adenine dinucleotide.

measuring the light intensity in chemi- and bioluminescence reactions in which the light is one of the products.[102,103]

**Circular dichroism spectroscopy** is widely used for studying the conformation of proteins in reverse micellar systems as it is carried out in aqueous solutions. CD spectra in the long-wave ultraviolet region (250—320 nm) usually have many overlapping bands belonging to tryptophan, tyrosine, phenylalanine, and disulfides. Therefore, the most valuable information about the protein structure is obtained from the spectra in the short-wave ultraviolet region (190—250 nm) (peptide bonds). However, many surfactants and other components of reverse micellar systems absorb in this region. This problem is solved in its own way for each particular case, for example, by decreasing the AOT concentration or replacing cetyltrimethylammonium bromide by chloride. Conformational effects manifested in the CD spectra of proteins in reverse micellar systems are discussed in reviews.[4,5,89] Here we mention only several essential points. It was with the use of CD spectra that a surprising property of reverse micelles has been discovered, namely, their ability to effect compactization of biopolymers,[5,89] which has been used for protein folding from their denatured preparations.[89,98,104,105] For example, it has been shown for the myelin protein, which is highly flexible in aqueous solutions with the locally ordered regions of β-sheets, that its incorporation into reverse micelles changes the CD spectra: these demonstrate a more ordered compact structure containing about 20% of α-helices along with β-breaks and β-turns.[11,89] Moreover, the incorporation of high-molecular-weight DNA into the system of reverse micelles is accompanied by an increase in the ellipticity and the appearance of a new band at 300 nm in the CD spectrum, indicating a higher spiralization of DNA.[106] A similar phenomenon of globulization or spiralization was observed when polyelectrolytes were incorporated into reverse micelles.[62] At high degrees of hydration (the size of the internal cavity of the micelle is larger than that of the incorporated protein), in the absence of specific interactions between the protein and micellar matrix, many enzymes did not exhibit substantial changes in the CD spectra (see examples in Ref. 11).

Among specific interactions we can mention, *e.g.*, the loss of ellipticity in the near-UV (with no changes in the far-UV) observed in the CD spectra of trypsin in reverse AOT micelles. In the authors′ opinion, this loss is related to the removal of calcium due to its capability of strong binding with the AOT molecule.[107] Interesting results were obtained in studying lysozyme in the reverse AOT micellar system in isooctane.[108] It was found that the enzyme incorporated into the reverse micelles remained catalytically active but its CD spectrum differed basically from that of lysozyme dissolved in water. It turned out that this difference was associated with the substrate, which favored the conformational rearrangement of the enzyme. The CD spectra also demonstrated a change in the conformation of alcohol dehydrogenase in the reverse AOT micellar system in octane upon coenzyme binding.[109]

**Nuclear magnetic resonance** is a powerful spectral method which can in principle provide information about the structure and dynamics of biopolymers. Modern instruments make it possible to get information about the ternary structure of proteins with the molecular masses up to 20 kDa from NMR spectra. Limitations appear for larger molecules. One of them is related to relaxation processes of mobile parts of molecules. At a fast motion, a possibility of the energy transfer by spin-spin relaxation diminishes because nuclei rarely exist in the appropriate relative orientation, the relation time is long (it is inversely proportional to the resonance linewidth), and the spectral lines are narrow. An increase in the molecular size of a protein results in shorter times of spin-spin relaxation, which deteriorates the resolution of the spectrum due to a decrease, in particular, in the signal/noise ratio. This problem could be solved when the protein was incorporated into reverse micelles in an organic solvent with a low viscosity.[110] This approach was accomplished for the small (9 kDa) protein, [$^{15}$N]-ubiquitin, incorporated into reverse AOT micelles in various organic solvents ranging from octane to liquid propane.[110] It was shown that the rate of spin-spin relaxation was proportional to the solvent viscosity. Several important points are noteworthy. First, it is possible to apply the NMR method to reverse micelles; second, proteins retain in reverse micelles for a long time (more than a week); third, it is possible to record NMR spectra in liquid propane under elevated pressure. In general, the NMR method is being widely used for studying the state of water in a system of reverse micelles, its mobility, and other properties at different degrees of hydration of the surfactant, temperatures in the presence of various additives, including proteins, *etc.*[17,111—120] It has been found by $^{1}$H NMR spectra[17,111,116] and $^{2}$H NMR spectra[121] that at low degrees of hydration water inside the micelle is strongly immobilized, and its mobility is lowered. However, the latter increases with an increase in the degree of hydration of the surfactant. An important question concerning the fate of water that fills the internal cavity of the micelle upon the incorporation of a protein (whether it is redistributed between other micelles or not) was also elucidated on the basis of results of NMR spectroscopy. It has been shown using $^{1}$H NMR that the presence of α-chymotrypsin even in high concentrations in the reverse AOT micellar system in octane has no effect on the position of the signal of water in the spectrum and its width[119]; the plots of the chemical shifts of protons in the spectrum *vs.* degree of hydration in the absence and in the presence of the protein (0.1 mmol $L^{-1}$) coincide. Likewise, the absence of changes in the chemical shifts upon the incorporation of α-chymotrypsin into reverse AOT micelles in octane was detected by $^{13}$C NMR.[119] However, determination of the spin-spin

relaxation time on $^{13}C$ nuclei has shown a change in the mobility of particular segments of the AOT molecule, namely, the $\alpha$-$CH_2$ groups due to their presumable hydration, upon protein incorporation into the system. Thus, the NMR spectroscopic data[119] have led to an important conclusion, which confirmed the hypothesis about the unchanged micellar sizes upon protein incorporation, which has earlier been proposed by us based on results of sedimentation analysis.[27]

$^{31}P$ NMR spectroscopy of reverse micelles from natural phospholipids provides information about the state of the head groups of phospholipids.[5,112,113] In addition, the appearance of an isotropic signal in the $^{31}P$ NMR spectrum revealed nonbilayer structures of the type of reverse micelles, which appear in the lipid bilayer under certain conditions, *e.g.*, with an increase in the concentration of $Ca^{+2}$ ions.[122,123] Based on these data, the authors[124] formulated a metamorphous-mosaic model of the biological membrane in which nonbilayer structures play an important role.

Finally, the dependence of the chemical shift of the signal for the phosphate anion in the $^{31}P$ NMR spectrum on the pH of the starting phosphate buffer solubilized in AOT micelles in isooctane was used as a method for the determination of the pH in the internal cavity of reverse micelles.[115]

**Electron paramagnetic resonance** turned out to be very useful and informative method for studying both the mobility of the protein molecule in the reverse micellar system and the microenvironment of the internal cavity of the micelle. As in an aqueous solution,[125—128] labels[89,93,129—135] or probes[129,131,136—138] covalently linked to certain parts of protein molecules can be used. Molecules containing the nitroxyl radical are most often used as spin labels and probes for studying reverse micellar systems, although numerous stable radicals have been described to date.[139] Analysis of ESR spectra gives the correlation time of the rotational mobility of the label. In addition, the polarity of the medium in which the label is present can be estimated from the linewidths in the spectrum and the distance between the lines.

Studies on reverse micellar systems by ESR revealed peculiarities similar to those mentioned above for other methods, namely, a lowered polarity and enhanced viscosity of water in the internal cavity of micelles compared with those of the bulk water.[12,131,136—138] The dependence of a distance (*a*) between the low-field and central lines in the ESR spectrum, which characterizes the polarity, on the degree of hydration of the surfactant was studied.[129,131,136] It was shown that *a* increases with the degree of hydration approaching the value characteristic of an aqueous solution.

The mobility of the probe also depends on the degree of hydration of the surfactant, it can either increase or decrease with an increase in the water content in the system, depending on the nature of the probe and its capability of interacting with the micellar matrix and/or redistribution between the aqueous and organic phases. The use of labels covalently linked with the protein allows characterization of the mobility of the protein molecule as a whole and its dependences on the water content in the system and micellar sizes or the mobility of particular regions of the protein molecule, for example, in the region of the active site. These dependences can substantially differ. For example, when the degree of hydration increased, the mobility of the label covalently bound to the surface of phosphorylase *b* increased, whereas the mobility of the label incorporated into the hydrophobic region of the active site of the protein molecule decreased.[131] The dependence of the rotational frequency of the label in the region of the active site of chymotrypsin or acidic phosphatase, depending on the degree of hydration of the surfactant, had a minimum coincided with the maximum catalytic activity of the enzyme.[129,130] The mobility of the label on the protein surface increased smoothly with the degree of hydration to reach the limiting value where the sizes of the internal cavity of the micelle and the sum of the sizes of the protein and label molecules coincided.[130] Similar conclusions about the relationship between the enzyme activity and label mobility were drawn from the analysis of the label mobility covalently bound to Cys25 of the active site of papain.[132]

Collisions of micelles may have a destabilizing influence on the structure and activity of the enzyme at low degrees of hydration where the sizes of the internal cavities of empty micelles are smaller than the sizes of protein-containing micelles. This question was specially studied by experiments with different surfactant concentrations.[130,133] It was shown that a decrease in the surfactant concentration, *i.e.*, a decrease in the number of collisions of micelles, changed the profile of the plot of the label mobility resulting finally in a constant value of the rotational frequency of the label in the active site. This value was the same as that where the sizes of the protein molecule and the internal cavity of the micelle matched and where the highest catalytic activity of the enzyme is observed. Thus, the ESR method allowed researchers to characterize the mobility of protein molecules incorporated into the system of reverse micelles and to reveal in particular the lowered mobility of the region of the active site of the enzyme, which is "adjusted" to perform catalysis.

### 2.2. Light, neutron, and X-ray scattering

The methods using light scattering, X-ray, or neutron scattering, are widely applied to obtain information about sizes and shapes of protein-containing and "empty" reverse micelles. Small-angle neutron scattering seems to be a very powerful method, which allows revealing the structure, interactions, and phase transitions in micellar and microemulsion systems.[140—143] Small-angle X-ray[144—146] and light scattering[147—150] are also used.

The light scattering technique includes static light scattering, which measures the intensity of the light scattered by a system of particles in some interval of scattering angles, and dynamic light scattering, which is often named "quasi-elastic light scattering" and measures the spectral distribution of the light scattered at different angles.[147—150] In some studies, the authors use the technique of measuring the time of the process, which is named photon correlation spectroscopy and allows direct measurement of the correlation time of the fluctuating intensity of the light scattered by a system of colloidal particles in the Brownian motion.[147] It was this method that was used to study reverse AOT micelles in isooctane and a conclusion was made that the aggregation number of the system remains unchanged over a wide range of surfactant concentrations. This implies that the dilution of a system with an organic solvent changes only the number of micelles without changing their sizes.

Results of similar importance were obtained in studies where diverse scattering techniques have been used[140,141,143,145,147] which indicated that a system of reverse micelles of AOT molecules in an organic solvent is characterized by a very narrow size distribution for each specified degree of hydration (monodispersity). Similarly, the data on the system with various additives, including the incorporated protein,[142,144,149,150—156] were obtained by scattering methods. In this case, however, the results of different studies do not coincide. In studies based on scattering, a question is often discussed what changes in the system occur upon protein incorporation. Experimental results by different authors differ substantially. For example, when α-chymotrypsin was incorporated into the system of reverse AOT micelles in heptane, no noticeable change in parameters of either filled or empty micelles was observed by quasi-elastic neutron scattering.[151] This important result confirmed, in fact, our results obtained using ultracentrifugation.[27] Similar results on the retention of the micelle parameters upon protein incorporation for high degrees of hydration were obtained by quasi-elastic light scattering[152] for the basic myelin protein and also by laser light scattering for dehydrofolate reductase in cetyltrimethylammonium and dodecylammonium bromide micelles.[157] The different situation is observed for low degrees of hydration and in the presence of proteins capable of interacting with the micellar matrix.[152,153,156] In this case, large aggregates and several populations of micelles are observed, and in the case of, *e.g.*, cutinase, the bimodal distribution appears after 24 h of incubation of the enzyme in micelles presumably due to protein unfolding.[150] It is shown[156] that the radius of the micelle remains unchanged when the concentration of the incorporated cytochrome *c* changes to the values corresponding to two protein molecules per micelle, after which the sizes of the aggregates increase sharply due to the percolation of the micelles. Such diverse results can be explained by different experimental conditions and concentrations of the incorporated protein. In the methods discussed, the degree of filling micelles with protein plays an important role: the higher the degree of filling the more reliable the results. However, high degree of filling cannot always be achieved due to limited solubility; it can be assumed that, in some cases, the protein and/or surfactant precipitated (which was not always visible with the human eye) with a probable capture of some water during experiment.

### 2.3. Ultracentrifugation

The ultracentrifugation method, in which particles move under the centrifuge force and their concentration distribution over the length of a centrifuge tube is detected at certain time intervals, is a popular method for the characterization of macromolecules. Using this method, one can obtain information about the shape, molecular mass, and density of the molecule.[158,159] Since reverse micelles can be considered as particles (protein-containing or "empty") capable of moving in the centrifuge field, and the migration of the boundary can be monitored by photoelectric scanning at the appropriate absorption band, the method is also applicable for systems of reverse micelles. The sedimentation of micelles can be monitored by the absorption of the surfactant (in the case of AOT, this is the ultraviolet region below 280 nm) or by "coloring" reverse micelles, *e.g.*, by picric acid (400 nm).[160] In the case of micelles formed of AOT molecules in octane, one pronounced sedimentation boundary is observed in the sedimentation pattern, which suggests that sedimentated particles have the same shapes and sizes.[27,28,161—164] Of course, monodispersity of the reverse micellar system of AOT molecules was also established by other methods, for example, X-ray or neutron scattering discussed above. However, ultracentrifugation is both a simple and reliable method. The second boundary with a higher sedimentation coefficient (protein-containing micelles) appears in the sedimentation pattern, and the parameters of the light fraction ("empty" micelles) remain unchanged.[28,29] This behavior of the system allowed the development of the method for the determination of molecular masses of proteins from data of ultracentrifugation comparing "empty" and "filled" micelles and thus circumventing the necessity of the determination of the diffusion coefficient in the Svedberg equation.[27,30] The method can be used provided the size of the internal cavity of the micelle is greater than, or equal to, the size of the protein molecule. At too low degrees of hydration where the initial "empty" micelles are smaller than the incorporated protein molecule, the calculated value deviates from the true value because the conditions of unchanged parameters of micelles upon protein incorporation is violated.

As has been mentioned above, when oligomeric enzymes are incorporated into a reverse micellar sys-

tem, these enzymes often dissociate into functioning subunits. Sedimentation analysis turned out to be useful for the detection of individual oligomeric forms and their complexes.[48,50,56,58,163–165] It should be mentioned that the preparative separation of oligomeric forms of proteins with subunits substantially differing in molecular masses, for example, glutamyl transferase[46] and penicillin acylase,[50] was successfully carried out using the ultracentrifugation method.

### 3. Other approaches used in micellar enzymology

**Regulation by physical factors**, such as the temperature, pressure, and even the electromagnetic field, is applicable to enzymes in systems of reverse micelles. Here note that the effect in this case can be due to the direct influence of the factor on the enzyme or indirect, mediated by the micellar matrix. The behavior of the reverse AOT micellar system in isooctane has been studied in detail[147] for different degrees of hydration of micelles and different temperatures. A substantial change in the sizes of the surfactant aggregates with temperature was observed. Temperature dependences of the phase transitions of the system were discussed in detail.[10,166] Structural changes in the micellar matrix can affect, most likely, the temperature plots of various parameters of proteins incorporated into the system. In most studies, thermal stabilities of enzymes (proteins) and a possibility of the stabilization of preparations were considered, and the parameters were compared with those obtained in aqueous solutions.[167–169] Among the methods which allow monitoring changes in proteins and calculating the thermodynamic parameters of the thermoinactivation process, we emphasize differential scanning calorimetry, which was also applied to systems of reverse micelles with incorporated protein.[97,170] With ribonuclease T as an example, it was shown[97] that in the region of matching sizes of the protein and the micelle (for degrees of hydration of 5—12) the temperature-induced changes in the protein structure are reversible (native protein — unfolded protein). At higher degrees of hydration, the process becomes partially irreversible, a stage of the irreversible transition from the unfolded to the denatured state is supplemented. Note that where the protein and micelle sizes match the temperatures of transition and changes in enthalpy turned out to be equal to those observed in an aqueous solution, and the protein structure (CD and fluorescence methods) remained unchanged upon its incorporation into micelles.

The pressure is yet another factor capable of substantially influencing both the structure of the micellar matrix and the activity and structure of the incorporated enzyme.[171–176] For low surfactant concentrations and degrees of hydration in the region of reverse micelles, the elevated pressure can increase, most likely, the lifetime of one surfactant molecule in one micelle, *i.e.*, in some enhancement in the rigidity of the micelle, which, in turn, can result in the stabilization of the protein structure inside the micelle. The substantial stabilizing pressure effect against thermal inactivation was demonstrated for chymotrypsin in reverse AOT micelles in octane.[173] This effect was additive to the effect of glycerol,[177] whose capability of enhancing the rigidity of the micellar matrix is known.[178] In the case of oligomeric enzymes, elevated pressure favored stabilization of particular oligomeric forms and certain protein complexes involving the micellar matrix[176,179]; no such effect was observed in an aqueous solution.[176,179] At high degrees of hydration and high surfactant concentrations, the elevated pressure induced structural rearrangements in the micellar matrix, for example, it favored the phase transition from reverse micelles to lamellae.[172,180,181] The behavior of reverse AOT micelles under the elevated pressure was determined by the nature of the organic solvent: the critical concentration of micelle formation in chloroform substantially depended on the pressure, while in benzene it was pressure-independent.[182]

The electromagnetic field is yet another physical factor considered from the viewpoint of the influence on enzymatic reactions. The question about the interaction of biological molecules with non-ionizing radiation is disputed in the literature, in particular, due to a possible influence of the latter on the appearance and development of malignant tumors.[183] The influence of microwave fields with different intensities on acetylcholine esterase and NADH : cytochrome-P-450-reductase was studied in the reverse AOT micellar system in isooctane[184] because, as has already been mentioned, this system simulates the properties of the cellular environment of enzymes and also, which is significant, is transparent for the waves of the interval studied.

**The use of reverse micelles for protein fractionation** is one of the aspects that makes reverse micellar systems an attractive tool in biotechnology. Reverse micelles were used for the separation of mixtures of proteins. The extraction of particular components from water to the organic phase depended on the pH, the nature and concentration of the salt, and the nature of the surfactant and organic solvent.[185–191] A simple method for the purification of intracellular proteins by their extraction to reverse micelles from entire cells was based on same principle.[192] A surfactant with the opposite charge was proposed to be used when the inverse extraction of proteins to an aqueous phase was needed. In the case of negatively charged AOT used for direct extraction, dodecyltrimethylammonium bromide was used for inverse protein extraction.[188,193] Finally, bioaffinity additives to systems of reverse micelles were used for the selective separation of proteins. For example, the solubilized trypsin inhibitor helped isolation of trypsin from a mixture of several proteins[194] or concanavalin A was used to isolate horseradish peroxi-

dase,[195] *etc*. This aspect of using reverse micelles was considered in more detail in reviews.[185,196,197] Reverse micellar systems can be helpful in the mild reversible synthesis of apoproteins. The heme can almost completely be extracted from peroxidase and incorporated back changing the degree of hydration without applying drastic methods with the use of strong acids and organic solvents.[42]

**Crystallization from reverse micelles** is also an important aspect of using these systems. This approach was applied, in particular, to the improvement of morphological characteristics and solubility of crystalline forms of aspartame.[198] Protein crystallization is an important stage in the elucidation of the three-dimensional structure, *e.g.*, by X-ray diffraction analysis. However, proteins containing such components as sugars or lipids and hydrophobic parts are very complicated objects for obtaining crystals, and in some cases, these proteins are not crystallized at all. The use of reverse micellar systems allows the solution of this problem. In the case of a prionic protein, it is the structural rearrangements in the molecule that can be crucial in the mechanism of the appearance of prionic diseases, in particular, spongy encephalitis (cow rabies). However, crystallization of prionic proteins is always accompanied by polymerization, which precludes revealing distinctions in the structures of different forms of proteins.[199] The use of reverse AOT micelles in isooctane allowed the incorporation of the prionic protein into the micelles as monomers or dimers, and then two- and three-dimensional crystals of the protein were obtained.[199]

**Protein folding, problem of aggregation.** Researchers have to surmount the problem of aggregation for folding of gene-engineering proteins, which often form inclusion bodies. Attempts to extract proteins from these inclusion bodies result in the formation of insoluble aggregates. The use of reverse micelles allowed folding of ribonuclease A without aggregation even when the protein concentration was increased.[104,200] Here we also consider reverse micelles as a tool or a convenient matrix, whose shape favors folding of extended biopolymers. Therefore, varying the water content in the system, *i.e.*, sizes of micelles, one can assemble both supramolecular complexes and particular subunits. For triosephosphate isomerase[96] and formate dehydrogenase,[98] a possibility of obtaining (under specific conditions) folded monomeric or dimeric protein particles has been shown. Moreover, even polyelectrolytes incorporated into the reverse micelle can be folded to form compact particles, globules.[61,62]

In conclusion we would like attract the readers′ attention to the methodical wealth and sometimes uniqueness of micellar systems as a "tool" in studying enzymes. All material presented above allows us to expect that these systems will correctly be evaluated and used.

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