Surface-enhanced Raman scattering and its application to the study of biological molecules

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Work on surface-enhanced Raman scattering (RS) of light by molecules near a metal surface is reviewed. The experimental conditions for obtaining surface-enhanced Raman scattering spectra in different molecule-metal systems are examined. The basic characteristics and mechanisms of the effect are discussed. Special attention is devoted to applications of the method of surface-enhanced Raman scattering spectroscopy for structural-functional study of biological molecules: DNA, proteins, supramolecular complexes. It is pointed out that the large enhancement of the RS cross section makes it possible to reduce the concentration of the substances under study by three orders of magnitude—down to 10^{-8}–10^{-9} M. The short range of the enhancement mechanism in some systems makes it possible to obtain Raman scattering spectra for groups of atoms located directly adjacent to the surface of the metal and thereby to study the topography of biological macromolecules and the kinetics of their behavior at an interface. The prospects for applications of enhanced Raman scattering as a new method of vibrational spectroscopy of biopolymers are discussed.

CONTENTS

1. INTRODUCTION

Extensive experimental and theoretical information on surface-enhanced Raman scattering (SERS) of light by molecules located near the surface of a metal has now been accumulated. There are several reviews[1–6] and two monographs[7,8] largely devoted to clarifying the mechanisms of SERS. It apparently should be regarded as proven that there exist two mechanisms for the enormous (in many cases up to 10^{10}) increase in the Raman scattering (RS) cross section of adsorbed molecules: electromagnetic, which is associated with the intensification of the local electromagnetic field near the surface, and molecular, which is determined by the formation of new excited states of the molecule-metal complex. At the same time an increasing number of articles devoted not so much to the study of the physical aspects of the phenomenon of SERS as to the practical applications of the method for studying applied problems in the physics of surfaces, analytical chemistry, biophysics, etc., is appearing.

One of the most promising applications of SERS is to the study of the structural-functional characteristics of molecules of biological significance (see the review of Ref. 9). About 100 original papers devoted to the study of different classes of biological molecules [amino acids, nucleic bases,

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water-soluble, membrane, and photosensitive proteins, nucleic acids, and supramolecular complexes (see Table I)] have appeared in just the last five years. In the application of SERS spectroscopy to the study of complex molecules the question of the relative contribution of the electromagnetic and molecular mechanisms to the observed enhancement of the RS cross section is of special interest. To answer the question it is sufficient to know the characteristic distance from the surface of the metal at which the enhancement effect is observed. For conditions under which the long-range component of the RS enhancement mechanism is realized it is obviously possible to obtain RS spectra that are identical to those of nonadsorbed molecules but with a significantly lower detection threshold, while the shrot-range mechanism enables analysis of the topography of macromolecules and determination of the nature of the groups that can come into direct contact with the surface of the metal. The question of the conservation of the conformation of macromolecules accompanying adsorption on the surface of a metal is also extremely important. The enhancement of the RS cross section by the electromagnetic mechanism is apparently the least disruptive method, since in this case chemisorption with the formation of a metal-molecule complex is not a necessary condition, as it is in the case of the molecular mechanism. The optimal system for obtaining SERS spectra of biomolecules by means of long-range electromagnetic enhancement is a metal surface with regular roughness features, each feature having the same dimensions,[7,8] enabling efficient excitation of a surface electromagnetic wave in the metal. The molecular enhancement mechanism predominates under conditions when molecules are adsorbed on electrodes that are loosened up by anodic polarization in an electrolytic solution. There are many examples in which in this system adsorption does not change the conformational and functional properties of biopolymers,[11,12,22,23] but this question must be specially studied in each specific case. Thus the most important problem in the application of the SERS effect to the study of biomolecules is the choice of conditions for selective realization of the electromagnetic or molecular enhancement component.

It should be noted that some features of the structural organization of biopolymers make these molecules an exceptionally convenient tool for studying the mechanism of SERS. The study of proteins, nucleic acids, and model compounds whose three-dimensional structure is known from x-ray structural analysis has made it possible to determine the conditions under which the long- and short-range (compared with the wavelength of the exciting radiation) RS enhancement mechanisms are realized accompanying adsorption. It has been demonstrated convincingly in experiments with DNA molecules and some proteins that under conditions of adsorption on loosened up silver electrodes and silver hydrosols (coagulating when an adsorbate is added) the enhancement mechanism has a short range—the SERS spectrum vanishes when the distance between the group of interest and the surface of the metal exceeds 5 Å. In the study of

TABLE I. Biomolecules studied by the method of SERS spectroscopy.

	Type of compound	Name (abbreviation)	References
1.	Amino acids	Phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp), histidine (His), α and β alanine (Ala), glycine (Gly), asperagine (Asn), glutamic (Glu) and aspartic (Asp) acids, cysteine (Cys), methionine (Met), serine (Ser)	14, 33, 49, 115–118
2.	Dipeptides	Gly–Phe and Phe–Val	33, 116
3.	Water soluble proteins and polypeptides	Leucine-isoleucine-valine binding (LIV) and leucine specific, proteins from the periplasmic space of *E. Coli*	11–13
		Cytotoxin I from *Naja naja oxiana* cobra nucleus	116
		Lysozyme, bovine serum albumin	9
4.	Retinene-containing membrane proteins and their chromophores	Completely trans-retinene	35
		Bacteriorhodopsin in purple membrane (BR)	19, 20, 35
		Visual rhodopsin in membrane of photoreceptor disk	21, 119
5.	Porphyrin-containing substances	Porphyrins, porphins, and their derivatives	18, 61–63
		Biopigments (biliverdine and its derivatives)	120–126
		Metal porphyrins (Fe(III)-protoporphyrin), protoporphyrin IX	127, 128
		Heme-containing proteins (cytochromes c, cd_1, b_5, hemoglobin, myoglobin)	15, 16, 37, 42
		Chlorophyll-a, bacteriochlorophyll-a, bacteriopheophytin	24
6.	Flavins and flavoproteins	Proflavin, flavin-anion, riboflavin, lumoflavin, flavin-mononucleotide (FMN), flavin-adenine nucleotide (FAD), glucose-oxidase, riboflavin binding protein	26, 50, 134, 135
7.	Components of DNA and RNA	Nitride bases, nucleosides, nucleotides, polynucleotides, ribose, dysoxyribose	10, 22, 31, 51, 117, 118, 136–147
8.	Nucleic acids	Native and modified DNA, DNA-Pt complexes; DNA, subjected to thermal and γ-ray induced denaturation	84, 85, 135, 147–149
9.	Other biologically important compounds	Nicotine-amide-dinucleotide (NAD^+), citrate ion, anabolic medicines; benzoic, p-, n-amino benzoic, two-, three-, four-acetylbenzoic acids	23, 24, 45, 53, 56–60, 69, 147, 150–160
10.	Supramolecular complexes	Chromosomes, cells, photoreaction centers of bacteria and plants	17, 20, 28, 161

membrane fragments with a characteristic size of $\sim 1\ \mu m$ it turned out that the adsorption of unaggregated silver sol micelles ~ 100 Å in diameter causes the short-range molecular enhancement mechanism to predominate, while after aggregation of the sol coagulates $\sim 1{,}000$ Å in size the contribution of the electromagnetic mechanism increases sharply. Finally, it was discovered in experiments with biomembranes that the simplest system in which the electromagnetic enhancement mechanism predominates is apparently a "smooth" silver electrode—a metallic surface that has not been loosened up by anodic polarization in an electrolytic solution so as to increase the concentration of point surface defects that play the role of centers for the formation of molecule-metal chemical complexes. Loosening up the electrode sharply increases the contribution of the molecular enhancement mechanism, which has a markedly short-range character (see Sec. 4).

Analysis of SERS spectra of biomolecules containing groups of different physical nature has made it possible to determine the conditions under which molecule-metal complexes form and to clarify the chemical nature of such complexes. It turned out that groups with a developed π-electron system play the main role in adsorption on point defects of a metal surface. Groups forming σ complexes with the surface, owing to unshared electron pairs of the corresponding atoms, also make a significant contribution to SERS. Finally, the contribution of electrostatic interactions between the molecule and metal to molecular enhancement mechanisms is vanishingly small. Thus it has been demonstrated that chemisorption plays a determining role in the appearance of "molecular" SERS.

It follows from the foregoing discussion that the method of SERS spectroscopy has a number of unique possibilities, which make it very promising for studying a wide class of biological molecules. This is primarily attributable to the high sensitivity of the method. SERS spectra of biopolymers have been recorded at concentrations three to four orders of magnitude lower than in the traditional RS spectroscopy. This advantage is fundamental, since many biologically important compounds are available only in limited quantities; in addition, very often biopolymers cannot be dissolved at high concentrations. We also note that at concentrations significantly exceeding physiological values aggregation of biomolecules, leading to a change in their structure, can occur. Unlike other methods of optical spectroscopy the method of SERS spectroscopy makes it possible to study the state of separate groups of biological macromolecules and complexes located on the surface. This is especially important, since an enormous number of cullular biochemical processes occur precisely at an interface. Under certain conditions charged metallic surfaces can serve as convenient models for studying membrane processes. In addition, by varying the potential on the surface of a metal it is possible to monitor the state of groups of atoms in biomolecules, whose oxidation-reduction properties change in the process of their functioning. It is also possible to record electro- and photochemical transformations of some biomolecules adsorbed on the surfaces of electrodes or hydrosols; this enables the study of separate functional stages of such biological compounds under steady-state conditions.

Molecular biophysics has thus acquired a new method of vibrational spectroscopy. In this connection it is desirable to analyze data on SERS, concentrating on the physical features of this effect from the standpoint of the possibility of obtaining new spectroscopic information about the structure of biological molecules and their properties on the surface of a metal. The experimental procedures employed for obtaining SERS spectra and the basic characteristic features of the molecular and electromagnetic enhancement mechanisms are briefly examined in Secs. 2 and 3; special attention is devoted to biomolecules as a specific class of compounds studied by the SERS method. Section 4 is devoted completely to specific applications of the method for studying the different types of biomolecules.

2. EXPERIMENTAL METHODS FOR OBTAINING SURFACE-ENHANCED RAMAN SCATTERING SPECTRA

Intense SERS has heretofore been observed on specially prepared, rough metallic surfaces.[6] Different forms of surface roughness are obtained either by roughening up the surface of a metal [for example, electrodes in an electrochemical cell after an oxidation-reduction cycle (ORC), metallic films loosened up mechanically and chemically as well as with the help of ion bombardment or irradiation with hard ultraviolet radiation] or by creating special types of surfaces, such as spheres in coloidal suspensions, metallic island films, diffraction gratings, axisymmetric protuberances on a metallic coating, odered two-dimensional surfaces from metallic particles prepared with the help of microlithography, and by other methods.[6–8] The fine structure of the organization and the large sizes of the biomolecules, however, substantially restrict the number of systems in which these objects can be studied with the help of SERS spectroscopy. SERS and SERRS spectra of biological molecules have been obtained in electrochemical cells and systems of small isolated particles (metal hydrosols) as well as on the surfaces of silver films and coatings with regular nonuniformities.[7,9,23,24]

SERS spectra are recorded with the help of standard apparatus employed for obtaining RS spectra. Laser lines in the visible range are employed for excitation. To prevent photodestruction of the samples the laser radiation power in the cell compartment, as a rule, does not exceed 50 mW. Rotating cells, flow methods,[25] and multichannel detection systems[24,26] are also employed.

Interesting information enabling the solution of a number of biophysical problems has been obtained by incorporating a microscope in the RS spectrometer.[27,34] This enabled the study of the surface of supramolecular biological complexes in order to find nonuniformities in them and clarify their nature. Focusing the laser beam with the help of the objective lens of the microscope into a spot $1\ \mu m$ across makes it possible to obtain SERS spectra of very small volumes of matter. The laser beam is scanned over the surface or directed at a definite point, chosen with the help of a standard microscope. As the beam is scanned over the surface the two-dimensional spatial distribution of SERS for each wave number is obtained by reading off a vidicon. The use of a microscope in SERS spectroscopy has enabled the study of separate sections of chromosomes.[28] This method will undoubtedly be widely employed in the future for the analysis of the surface of cells and supracellular structures.

2.1. Metallic electrodes in an electrochemical cell

Figure 1a shows a diagram of an electrochemical cell employed for obtaining SERS spectra of biomolecules adsorbed on metallic electrodes. The cell, containing three electrodes—the working, comparison (usually silver), and auxiliary (usually platinum) electrodes—is connected into a potentiostat circuit. The working electrode is placed at the center of the cell in order to reduce the effect of diffusion processes and electrochemical reactions. The electrolyte consists of a solution of alkali-metal salts. The anions play the main role in the appearance of SERS.[7,29] The SERS signal enhancement in the presence of different anions decreases, as a rule in the following sequence: $I \gg Br = Cl > SCN > HPO_4 > SO_4 > ClO_4$.[30] Electrochemical cells with a working volume ranging from 15 ml to 50 μl have now been developed.[31–44] The typical electrochemical cell consists of two coaxial cylinders, one of which contains the working electrode and the solution of biomolecules under study, while the other contains the comparison and auxiliary electrodes in an electrolytic solution (Fig. 1c). Rough grinding of the contact surface between the inner cylinder and the cover glass ensures wetting by the electrolyte solution and conduction between the solutions in the inner and outer cylinders. The angle of incidence of the laser beam on the surface of the working electrode usually equals 45°, while the scattered light is collected at an angle of 90°. In separate cases the temperature of the cell is regulated and the orientation of the working electrode relative to the incident beam can be changed.[23,24] To obtain reproducible measurements of SERS special care must be taken to make sure that the reagents and the water are pure. Solutions are usually prepared with doubly and triply distilled water, which is degassed by passing helium or nitrogen through it for 20–30 min.

Prior to adsorption of the biomolecules the surface of the electrode is mechanically polished, degreased, and polarized with a cathodic current in order to remove oxides from the surface. To intensify the SERS signal the surface is roughened by subjecting it to one or several ORC. In so doing, first a positive potential and then a negative one relative to the saturated silver-silver chloride electrode, is applied to the working electrode; this leads to oxidation of the metal followed by its reduction on the surface of the electrode in the form of clusters ~ 100 nm in size, as confirmed by scanning electron microscopy.[2,8] Mechanical polishing without subsequent ORC is a much less effective preliminary treatment of the surface, though SERS spectra of biomolecules have been observed with the molecules adsorbed on "smooth" silver electrodes.[35] To prevent conformational changes in the biomolecules in the course of ORC they are added to the electrolyte after the surface of the electrode is loosened up with an anodic current.

It has been shown in a number of investigations that the presence of certain compounds (for example, pyridine, bipyridine, and others) in the electrolytic solution intensifies the SERS signal of the molecules under study owing to the formation of Ag-Py (01) and Hg-PyPy (01) complexes on the surface of the electrodes.[36,37]

The adsorption process, the change in the electrode impedance and capacitance of the double layer, and the total charge passing through the electrochemical cell during the ORC are monitored with the help of voltammetry. A typical ORC employed in experiments with biomolecules is performed in the course of changing the potential, consisting of a triangular pulse.[9] The potential scan rate and the range of variation of the potential are determined by the magnitude of the charge passed. This makes it possible to monitor the degree of anodizing and to estimate the degree of variation of the surface structure.[6,38] Detachment of Ag^+ ions from the electrode is detected by the appearance of current on the voltammogram (Fig. 1b). Usually the dependence of the current not on the electrode-electrolyte potential difference but rather on the potential difference between the working electrode and the saturated silver-silver chloride electrode (the potential of the electrode is constant relative to the electrolyte) is measured.

The parameters of ORC and the conditions for which maximum enhancement of the RS signals is observed depend on the type of electrode metal, the chemical nature of the molecules, and the electrolyte employed.[8,38] As shown in Refs. 36 and 39, illumination of an electrode with a laser beam during the ORC encourages loosening up of the metal surface and intensifies the SERS of the adsorbate molecules. The shape of the working electrode can be very diverse. Differential SERS spectroscopy with a rotating disk electrode, one-half of which was made of silver and the other half of platinum, was first employed by Shelnutt and Ginley[40] to detect small (≈ 0.6 cm^{-1}) frequency shifts in the SERS

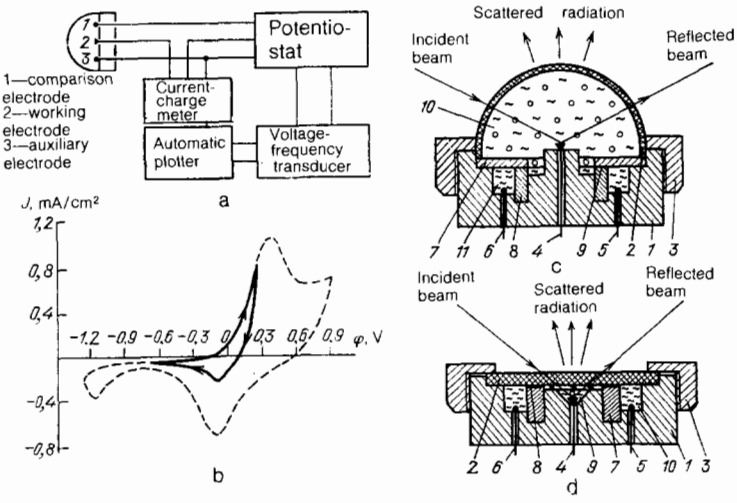

FIG. 1. a) Diagram of attachment to RS spectrometer for obtaining SERS spectra of molecules on silver electrodes in an electrochemical cell. b) Current-voltage characteristic of a silver electrode in a 0.1 M KCl electrolyte, containing 0.1% of the organic compound under study, recorded in an electrochemical cell with high scattered light collection efficiency. c) Electrochemical cell with high scattered light collection efficiency for obtaining SERS spectra of molecules on the surface of electrodes; 1) fluoroplastic housing; 2) quartz cap; 3) union nut; 4) working electrode; 5) auxiliary electrode; 6) comparison electrode; 7), 8) glass rings, separating the working volume of the electrolyte from the volume with the auxiliary electrode and comparison electrode; 9) electric contact through two ground glass surfaces; 10) working volume of electrolytes; 11) volume with auxiliary electrode and comparison electrode. d) Electrochemical microcell, intended for obtaining SERS spectra of molecules on the surface of electrodes; 1–7) as in c); 8) electric contact through two ground glass surfaces; 9) working volume of electrolyte; 10) volume with auxiliary electrode and comparison electrode.[33]

spectra of oxidized and reduced states of protoporphin. The electrochemical cell with a pressed working electrode proposed in Ref. 41 permits obtaining SERS spectra of biomolecules in the solid state. Hildebrandt and Stockburger[42] employed a cylindrical rotating electrode in order to prevent slow diffusion of the adsorbate from affecting the SERS intensity; this made it possible to record a signal with a constant equilibrium concentration of the molecules of interest.

The SERS spectra obtained from electrodes are temporally unstable. This is manifested as a constant decrease in the intensity of all bands; the rate of decrease depends on the potential on the electrode.[43] The most stable spectra are obtained with the potential at which there is zero charge for the electrode metal in the given electrolyte, i.e., when the field of the double layer is minimum and there are no electrochemical reactions.[5] The potential for which the strongest SERS spectra are obtained, however , is determined by the chemical nature of the molecules, the type of electrolyte, and the electrode metal.[5,8]

In spite of the relative simplicity of obtaining SERS spectra from electrodes in electrochemical cells this system is not very convenient for studying biochemical processes at the electrode/electrolyte interface, since there are no effective methods for monitoring the microstructure of the surface during ORC; electrochemical processes at the interface make it much more difficult to study and model the interaction of biomolecules with the surface of a metal. In addition, an electrode in an electrochemical cell is regarded as an inadequately "pure" system, since even minimal quantities of impurities, unavoidably present in the cell, change significantly the adsorption properties of the metal surface.

2.2. Systems of small isolated metallic particles

Another system in which SERS of biomolecules located near the surface of a metal can be observed is a system of small (compared with the wavelength of light) isolated particles. The enhancement of RS in this case can reach 10^6–10^7.[7] Stable dispersed systems consisting of silver, gold, or copper particles 10–100 nm in diameter are prepared by reducing dilute solutions of simple salts of these metals. The sols obtained have one or several extinction maxima in the visible region; their wavelengths depend on the size and shape of the particles and can vary depending on the preparation procedure as well as in time owing to particle aggregation or growth.

Salts with Ag^+ or $AuCl_4^-$ ions usually serve as sources of silver or gold for preparing such colloids; in addition, different reducing agents, including citrate and oxylate ions, hydroxylamine, sodium borohydride, and ethylene diamine tetraacetate ions (EDTA), are employed.[44–48] Transmission electron microscopy is employed to monitor the sizes and shapes of the colloidal particles (Fig. 2).[7,14]

Gold hydrosol, containing particles of uniform size and a regular spherical shape, are usually prepared by reducing $HAuCl_4$ with a solution of trisodium citrate. In so doing a colloid, consisting of gold spheres with a diameter of about 20 nm, with an extinction band in the region 500–550 nm is formed. By varying the relative concentrations of the metal salt and the reducer it is possible to control the sizes of the metal particles, depending on the purpose of the experiment.[45]

Silver hydrosol for SERS of biomolecules is usually prepared by reducing silver nitrate with sodium borohydride.[46] A hydrosol with peak extinction near 390 nm is formed. Electron microphotographs of the sol and the sol-adsorbate complex are shown in Fig. 2. The sol micelles obtained by the described method have a characteristic size of 12–16 nm. In the study of some amino acids[49,50] and DNA[51] silver hydrosols were prepared by reduction of silver nitrate by EDTA and citrate.

In these cases the metal particles had characteristic sizes of 320 and 300–150 nm, respectively. As shown in Ref. 52, silver hydrosol can be fractionated according to particle size with the help of centrifuging and chromatography.

When small biomolecules (amino acids and dipeptides, nucleic bases, etc.) are added to a hydrosol, aggregation of micelles occurs (Fig. 2b). For high concentrations of the adsorbate the coagulation can occur very rapidly and lead to complete precipitation of the complexes, if the colloid is not stabilized by the addition of a protector (sodium dodecylsulfate, gelatin, polyvinyl alcohol, glycerin, etc.).[7,42,53,54]

Important advantages of SERS spectroscopy of colloidal systems over the electrochemical cell method are the simplicity of preparation of hydrosols as well as the possibility of controlling the size and shape of the particles. Since hydrosols scatter light weakly it is quite easy to obtain ab-

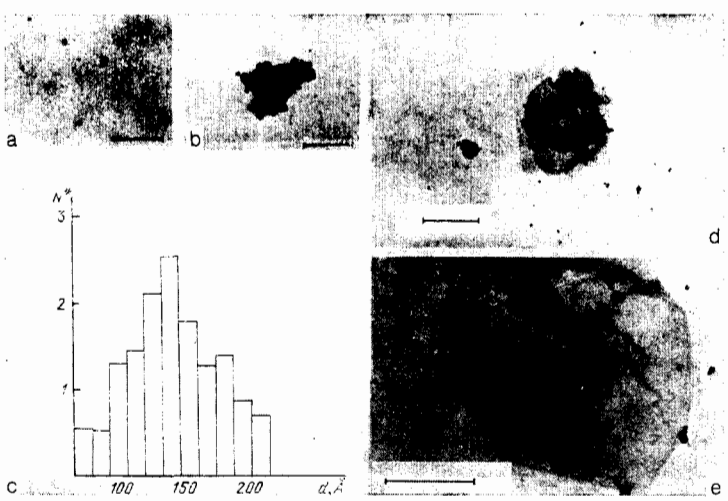

FIG. 2. Electron-microscopic photographs of silver hydrosol (a) and sol-phenylalanine complex (b) and histogram of the size distribution of sol micelles (c).[14] The hydrosol was prepared by the method of Ref. 46. Complexes: sol-photoreceptor disk (see d)[119] and sol—purple membrane (see e).[20] The horizontal scale equals 1 μm.

sorption, luminescence, and circular dichroism spectra; this makes it possible to extract additional information about the structure of the adsorbate studied by the SERS method. SERS spectra of biomolecules adsorbed on metallic colloids are obtained in standard quartz cells for liquid samples.

Surface-enhanced Raman scattering spectra of trace quantities (10^{-10} g) of organic compounds, adsorbed on substrates of silica gel,[22] filter paper,[23] and chromatographic plates,[55] treated with silver hydrosol, were recently recorded. This method for recording SERS will undoubtedly find wide application in analytical applications, especially in chromatography.

The method of SERS spectroscopy on metallic colloids has the following drawbacks: sedimentation of the sol occurs during prolonged storage, the reliability of the control of the surface potential of micelles (which is important for biomolecules undergoing photo- and electrochemical transformations) is inadequate, and the size and shape of the particles depend on the conditions under which the sol is stored.

2.3. Other systems

Aside from the widely employed systems of electrodes in electrochemical cells and metal colloids, SERS spectra have also been recorded for biomolecules adsorbed on silver island films,[24,56–58] on layers of metal oxides in tunnel junctions,[59,60] on ordered two-dimensional metal coatings,[23] and on the surface of silver roughened by deposition of CaF_2.[61–64] In the process the adsorption of biomolecules and recording of the SERS spectra were conducted in air.

Intense SERS spectra were obtained by Cotton *et al.*[24] from chlorophyll-a, bacteriochlorophyll-a and bacteriopheophytin-a, which were incorporated into inert polymer matrices and deposited in the form of monolayers on thin (≈ 5 nm) silver island films. The island films were prapared in a vacuum by means of slow (0.1 nm/sec) deposition of silver on a clean glass substrate; the thickness of the metal layer was monitored with the help of ellipsometry. The metal islands obtained were 20–40 nm in diameter. Monolayers containing inert fill with built-in biological chromophores were deposited on the silver surface by the Langmuir-Blodgett method. To prevent photodestruction and oxidation of the samples the preparation of the monolayers and their transfer onto a plate were conducted in an inert gas atmosphere in weak light, using short exposure times to excite SERS. This method makes it possible to model the distance of the chromophore from the surface of the biomolecule or membrane by depositing different numbers of monolayers.

The SERS spectrum of 4-aminobenzoic acid, adsorbed on a silver island film deposited on a hemicyclindrical prism (Al_2O_3) for efficient excitation of surface plasmons, was obtained by Dornhaus, Benner, and Chang.[56] The monolayer coating was prepared by depositing drops of the substance under study on a substrate, which was then rotated at a rate of 2,000 rpm. The thickness of the layer of adsorbed molecules was determined from the SERS spectra. Chen *et al.*[57] employed gold island films to study isonicotinic and benzoic acids.

To obtain intense SERS by biomolecules adsorbed on silver films, in many cases, the surface of the silver was roughened by depositing CaF_2 on it.[61–64] Monolayers of the substances studied (tetramethyl porphin[61–63] or benoic acid[64]) were deposited, by means of centrifuging, from a solution onto an Al_2O_3 film, after which a separating layer 15 nm thick was created. The surface-roughening CaF_2 layer with a "mass thickness" of 40 nm creates significant roughness features up to 50 nm deep. The silver layer (20–60 nm) deposited on CaF_2 reproduces the wavy structure of the surface (Fig. 3).

Many workers[59,60] have observed SERS spectra form molecular layers of *n*-aminobenzoic and 4-acetylbenzoic acids, adsorbed on an oxide layer in metal tunnel junctions $Al–Al_2O_3–X–Ag$, where X is the molecule under study. Even though only a monolayer of the substance was present and the molecules were separated from the incident beam by a silver film 20 nm thick, significant (up to $5\cdot10^4$) enhancement of RS was observed. The samples were prepared by depositing an aluminum film 40 nm thick on a substrate under a pressure of 10^{-7} torr. The geometry of the surface nonuniformities was controlled by evaporation through metallic masks. The aluminum films were oxidized in air, after which the silver layer was deposited as a second electrode.

As is shown in Ref. 60, SERS can also be observed on smooth metal films 100 nm thick. Based on data from scanning electron microscopy the irregularities on such surfaces do not exceed 30 nm in size.

In the last few years increasing attention has been devoted in SERS spectroscopy to the creation of metal surfaces consisting of sequences of submicron isolated silver particles with a uniform shape.[7,65] Both the microlithographic technique[7] and some simpler methods are employed to create such systems.[23,65] Vodinh *et al.*[23] observed SERS by biomolecules of benzoic acid adsorbed on a silver surface in the form of uniform hemispheres with controllable sizes ranging from 40 to 1000 nm. The substrate was prepared by depositing polystyrene latex on filter paper followed by centrifuging, drying, and deposition of a layer of silver (200 nm). This made it possible to detect SERS signals for molecules deposited on a substrate with concentrations of up to 10^{-10} M. The highest RS enhancement factor was recorded with organic molecules adsorbed on ultrathin substrates, consisting of small columns of silicon oxide of the same shape and coated with a silver layer.[65] The SiO_2 columns consisted of hemiellipsoids, separated by 300–500 nm, with axes about 60 and 30 nm long. The stages in the substrate preparation pro-

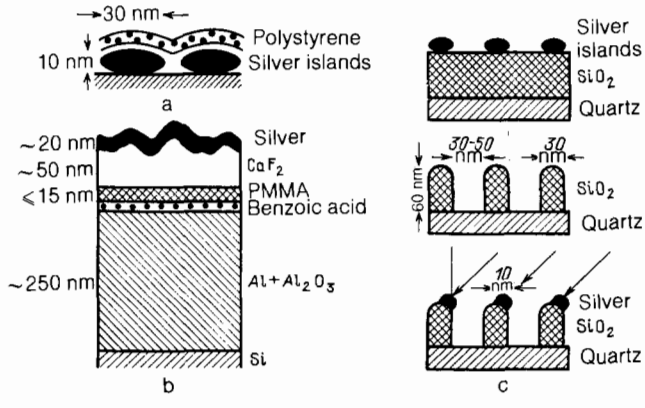

FIG. 3. Creation of regular microstructures on the surface. a, b) experiment on determining the dependence of RS enhancement on the distance between the molecule and the metal.[7,64] c) stages in the creation of regular nonuniformities giving maximum enhancement of the RS signal.[65]

cess are shown in Fig. 3. The silicon plate, on which the SiO_2 layer is deposited, is subjected to plasma etching using a silver island film as a mask. The surface prepared in this manner is analogous to the microstructures obtained with the help of microlithography,[7] but the technology is simpler.

Surface enhanced Raman scattering spectroscopy on metal surfaces with artificially created ordered microstructures is especially promising for analytical applications.

3. BASIC CHARACTERISTICS AND MECHANISMS OF SURFACE-ENHANCED RAMAN SCATTERING

Surface-enhanced Raman scattering has a number of features that distinguish it from standard RS. First of all, the RS cross sections for the vibrational modes of adsorbed molecules are at least 10^2–10^6 times larger than the analogous values for nonabsorbed molecules. Second, the enhancement depends on the excitation frequency and the degree of roughness of the substrate according to a law specific to SERS. In addition, both subatomic-scale roughness and roughness on scales of several tens and hundreds of angstroms play a determining role. Third, SERS spectra of many molecules differ markedly from the corresponding RS spectra of molecules in the free state. This is manifested in the selective enhancement of certain vibrations, as well as in the appearance of new bands in the SERS spectrum. In addition, when molecules are adsorbed on the surface of a metal luminescence, absorption, a number of nonlinear effects, as well as induced optical activity appear.[1–8]

Two obvious circumstances must be taken into account in studying the question of enhancement of RS of adsorbed molecules. First, near the surface of a metal the incident and scattered radiation will be stronger than in the bulk. This is caused by resonance excitation of surface electromagnetic waves on a rough surface. In addition, local resonances associated with photoexcitation of collecive electronic oscillations will exist in separate structures on the surface as well as in small isolated particles. This will increase the induced dipole moment of the molecules near the metal. Second, if in the case of an isolated molecule RS is a consequence of the vibrational modulation of the electronic polarizability of the molecule, then under conditions of adsorption the polarizability of the molecule-metal system must be studied, which means that new excited states will appear owing to the possibility of both charge transfer and local changes in the electron charge density near the surface, arising as a result of chemical bonding or tunneling of electrons in the metal to the location of the molecule. In this connection, two types of mechanisms for adsorption-induced enhancement of RS are distinguished: electromagnetic mechanisms, associated with the intensification of the local electromagnetic field near the surface, and molecular mechanisms, in which enhancement occurs owing to the formation of new excited states of the molecule-metal complex.[5,7]

In applications of SERS spectroscopy to the study of complex molecules the most important question is what is the relative contribution of the electromagnetic and molecular mechanisms to the enhancement of the RS spectrum. Here it is sufficient to know the range of each mechanism without determining its nature in detail. Thus long-range mechanisms make it possible to obtain RS spectra that are almost identical to those of nonabsorbed molecules, the difference being that their intensity is significantly higher than for the free molecules. Short-range mechanisms make it possible to obtain enhanced RS spectra from groups of atoms, located close to the surface of a metal, and thus to study the topography of macromolecules.

3.1. Surface-enhanced Raman scattering excitation spectra

In virtually all systems in which SERS has been observed the dependence of the intensity of the enhanced RS signal on the frequency of the exciting light differs from the ν^4 law characteristic for standard RS. Studies for different substances on electrodes,[66–68] in hydrosols,[69–71] on metallic films,[72,73] and in systems with controllable roughness[74] are in good agreement with one another and indicate that the dependence of the SERS intensity on the excitation wavelength is of a resonant nature and has a maximum in the range from 500 to 800 nm. On silver electrodes the maximum in the SERS excitation spectrum of most substances lies in the range 1.8–2.5 eV. For copper, gold, and platinum electrodes strong growth (stronger than for silver) in the intensity of the SERS signal was observed as λ_{exc} was increased up to 670 nm.[5,66] For metal hydrosols the maximum in the SERS excitation spectrum lies in the visible region and coincides with the maximum in the absorption spectrum of the complexes. The position of the maximum depends on the metal, the size and shape of separate paticles, and the chemical nature of the adsorbed molecules. The peak excitation of SERS on smooth and metal island films as well as on sinusoidal gratings depends on the sizes of the surface irregularities, the angle of incidence of the light, and the dielectric properties of the medium.[75]

The dependence of the excitation spectra on the nature of the metal and the state of the surface could indicate that intensification of local electromagnetic fields makes a definite contribution to the overall enhancement. In some cases, for example, for sinusoidal gratings, it has been established quite reliably that the SERS excitation spectrum is determined by excitation of surface electromagnetic waves.

3.2. Dependence of Raman scattering enhancement on the molecule-metal distance

The most important feature of SERS, both for clarifying the physical aspects of the phenomenon and for different applications, is the dependence of the enhancement of RS on the distance between the molecule and the surface of the metal. The SERS spectrum of pyridine, adsorbed on a loosened up surface obtained with photoreaction of iodine on silver, was observed by Seki and Philpott.[76] Analysis of the spectrum intensity as a function of the exposure of the pyridine, which was found from the carbon density determined with the help of Auger spectroscopy, showed that the observed dependence can be explained on the basis of intensification of local electromagnetic fields. This conclusion is based on the fact that significant SERS signal growth was observed for coatings extending beyond the first monolayer. The same dependence was also observed in other experiments,[77] where deuterated pyridine was adsorbed on the surface of pyridine, as well as by Rowe *et al.*,[78] who employed a polymer undercoating for adsorption of pyridine. On the other hand, Eesly[79] showed that the SERS signal from pyridine is generated primarily by the first monolayer, deposit-

ed on polycrystalline surfaces cleaned by ion bombardment. The dependence of the intensity of SERS by pyridine, adsorbed on silver island films, on the size of the coating was measured by Seki.[80] The authors believe that the enhancement occurs only for the first molecular layer, and only molecules adsorbed on some active centers create most of the enhancement. Pockrand and Otto[81] also conclude that there exist active centers, responsible for all the enhancement or at least for a large part of it. Measurements of the dependence of the SERS on the coverage of silver films deposited at low temperatures showed that the active adsorption centers are adatoms (atomic-scale roughness).

The dependence of the SERS intensity on the thickness of a pyridine coating on the (111) surface of an Ag single-crystal with weak periodic modulation of the surface profile was studied by Sanda *et al.*[82] Two types of enhancement were distinguished: the RS signal from physiosorbed pyridine (10^2), associated with the intensification of the local electromagnetic field near a molecule, and the RS signal from chemisorbed pyridine (10^4), which is multiplied by the enhancement associated with the local field.[83]

Convincing data supporting the short-range mechanism of RS enhancement accompanying adsorption of biomolecules on silver electrodes treated with the help of ORC as well as on silver hydrosols were obtained by Koglin *et al.*[84,85] These data will be presented in Sec. 4.

The construction of a complete theory of inelastic scattering of light by molecules adsorbed on the surface of a metal is at the present time a quite difficult problem.

This is attributable primarily to the fact that the optical properties of molecules change strongly on adsorption, and in order to describe them it is necessary to have a microscopic model of the molecule-metal interaction. The main theoretical difficulties arise when the characteristic distance between the molecule and the substrate becomes comparable to atomic dimensions. In this case the properties of the metal cannot be described with the help of a macroscopic model, since local electronic states of the surface play the main role in the interaction.[86] Not only the crystalline structure of the metal, but also the presence of surface defects, which largely determine the adsorption properties of the surface, must be taken into account. The description of the molecule must start from the actual charge distribution in it and the electron density distribution, and it must not be limited to the representation in the form of a point dipole or some other simplified form. In addition, the microscopic model of adsorption must be self-consistent, i.e., it must take into account the reciprocal effect of the metal surface accompanying the adsorption of the molecule. This effect consists of the redistribution of the electronic density in the metal and the broadening and shift of the ground, and especially, the excited energy levels of the molecule. The interaction of the molecule with the metal can be divided into two basic types: electrostatic interaction with electrons and ions of the metal and a specific electronic interaction owing to overlapping of the wave functions and hybridization of the electronic systems.

The next stage in the creation of the theory of SERS consists of a complete description of the interaction of light with an adsorbed molecule. Here the molecule is studied in the electromagnetic field formed when the light interacts with the real surface of a metal. Under certain conditions surface waves of collective excitations of the electron density, the so-called surface plasmons (SP), exist on metal surfaces.[87] Surface plasmons are excited when the component of the momentum of the incident photon parallel to the surface is equal to the momentum of the plasmon under conditions when quasimomentum is conserved. In addition, when the surface of a metal is exposed to light electron-hole pairs can form.[88] Finally, the real surface of a metal is coated with microscopic-scale irregularities. Photoinduced oscillations of the electron density (plasma oscillations)—local resonances[89]—can be excited in separate structural elements of the relief. Thus the theory of light scattering by the surface of a solid must include a microscopic model of adsorption, and it must also take into account all aspects of the interaction of light with the metal in the presence of adsorbed molecules.

A complete theory capable of explaining simultaneously all observed properties of SERS does not exist, though attempts of this kind have been made.[90,91] Thus Pandey and Schatz[91] calculated by the Hartree-Fok method the polarizability of a hydrogen molecule adsorbed on the surface of a metal as a function of time, the frequency of light, and the distance to the substrate. In this approach all problems associated with the finiteness of the size of the molecule, the distance between the molecule and the substrate, the type of molecule-metal interaction, and the dielectric response of the metal are solved within the framework of the proposed method. The results obtained demonstrate that it is possible to construct microscopic models by the method of *ab initio* calculation.

3.3. Electromagnetic mechanisms of enhancement

Electromagnetic mechanisms are those interactions between a molecule and a substrate that increase, with and without an external electromagnetic field, the induced dipole moment of the molecule under conditions when the overlapping of the electronic wave functions of the molecule and that metal is negligibly small or zero. Electromagnetic resonances that can increase the intensity of the local field, if the component of the momentum of the incident photon parallel to the surface equals the momentum of the SP, exist on a metal surface.

In the case of the incidence of light from a vacuum on a smooth metal surface the dispersion equation of SP has the form

$$k^2 = \left(\frac{\omega}{c}\right)^2 \frac{\varepsilon}{\varepsilon+1}, \qquad (1)$$

where ω is the plasmon frequency, equal to the frequency of the incident light, ε is the permittivity of the metal; and, c is the velocity of light.[92] In the region of existence of the surface plasmon RE $\varepsilon = \varepsilon' < 0$

$$|k| > |k_t|,$$

whence it follows that the excitation of SP by a light wave incident from a vacuum onto a smooth metal surface is impossible because quasimomentum is not conserved. When the surface of the metal is perturbed by a grating with a definite vector q or a random roughness, having a Fourier component with vector q, quasimomentum is conserved and the SP can be excited by the light wave, which is equivalent to an increase in the effective, i.e., local, field of the wave. The local field is intensified owing to the focusing effect: plane waves incident on such a grating are compressed into

surface waves, which have extension in one direction. Excitation of SP can be realized with the help of prisms with a definite refractive index[93] (disrupted total internal reflection), or by creating on the surface a diffraction grating,[94] owing to which the momentum component parallel to the surface will change as a result of diffraction. The enhancement of RS owing to excitation of SP on the surface of diffraction gratings was studied theoretically in Refs. 95 and 96. The local field is calculated in a manner analogous to the derivation of the Fresnel equations, but with the difference that the refracted wave is added to the incident, reflected, and transmitted waves. The amplitudes of the refracted waves are calculated with the help of perturbation theory, in which the quantity $\xi\omega/c$ is the small parameter (ξ is the mean height of the grating).

As a result the dispersion equation acquires the form

$$\left(\frac{\omega}{c}\right)^2 \sin^2\theta + 2\frac{\omega}{c}\sin\theta\cdot\sin^2\varphi\,\frac{2\pi n}{L} + \left(\frac{2\pi n}{L}\right)^2 = \left(\frac{\omega}{c}\right)^2 \frac{\operatorname{Re}\varepsilon(\omega)}{1+\operatorname{Re}\varepsilon(\omega)}, \quad (2)$$

where the square of the wave vector of the SP stands on the right side and the square of the component of the wave vector of the diffracted photon parallel to the surface stands on the left side. If the frequency of the laser (ω), the material, and the wavelength of the relief pattern (L) are given, then this equation can be satisifed by properly choosing the angles of incidence θ and φ. The maximum value of the local field is proportional to the ratio $\operatorname{Re}\varepsilon(\omega_R)/\operatorname{Im}\varepsilon(\omega_R)$; therefore for a small imaginary part of the permittivity at the resonance frequency ω_R the metal becomes a good enhancer. In the presence of a grating the SP can enhance not only the incident radiation, but also the dipole radiation at the Stokes frequency.[97] All the arguments for the incident radiation also hold for the scattered radiation, and the intensity of the RS will exhibit a resonance as a function of the angle of detection.

Surface plasmons can exist not only on regular structures of the grating type, but also on the surface of a metal with random roughness. The theory of enhancement of RS owing to the excitation of SP on surfaces with random roughness was developed in a number of papers (see the reviews Refs. 1–8). The enhancement of RS in this case will be less than on regular gratings, since any additional random roughness, added to the sinusoidal grating, will give rise to damping of the SP, broadening of the resonance, and reduction in the intensity of the local field at the surface. It should be noted that the RS enhancement factor does not eceed 10^4, even for regular gratings.[3,98]

If the surface contains separate particles of metal with a characteristic size of 5–50 nm, then its optical properties cannot be described by the model of a smooth surface with small perturbations. The optical properties of very rough silver films, island films, and colloidal suspensions are determined by resonances of the optical conductivity or collective excitations of electrons in separate protuberances of metal—by local resonances (see the reviews of Refs. 1–8). When the frequency of the incident light equals the frequency of the local resonance polarizability of the protuberance becomes significant and the proturberance becomes a source of an electric field. In addition, enhancement of RS can occur owing to excitation of local resonances at the Stokes frequency. In this case, the protuberance acts like an antenna.

The enhancement of RS owing to the "glowing tip" effect—a separate strongly elongated protuberance on whose tip lies a molecule (classical dipole)—was studied in Refs. 99–101. The corresponding collective electronic resonance with frequency in the visible region of the spectrum is a Mie dipole resonance.[102] When the frequency of the incident light is in resonance with the dipole plasma oscillations, $\varepsilon(\omega) = -\varepsilon_R$, where ε_R determines the dipole resonance of an elongated protuberance. The local electric field at the tip of the protuberance is enhanced by a factor of $(\varepsilon - 1)/(\varepsilon + \varepsilon_R)$. Thus for silver with $\lambda_{exc} = 500$ nm the field enhancement factor is ~ 30, so that the local intensity of the field at the surface of the tip of the protuberance is 900 times higher than the field intensity far from the surface. The local field on the lateral surface of the protuberance far from the tip is a factor of almost $|\varepsilon|$ lower in amplitude (about 10 for silver with $\lambda_{exc} = 500$ nm).

Thus electromagnetic enhancement of RS is determined by three factors: 1) classical enhancement, caused by SP on a rough surface; 2) local resonances of the electron density in separate structural elements of the surface; and, 3) the "glowing tip" effect. Enhancement owing to SP, as already mentioned, is more efficient on regular gratings and can reach 10^4.[3,98]

3.4. Molecular mechanisms of enhancement

The term "molecular mechanisms of enhancement" refers to effects that, owing to the overlapping of the wave functions of the molecule and the metal, change the matrix element for RS of an adsorbed molecule. These mechanisms have a short range, since in order for them to operate efficiently direct contact between the molecule and the metal is required. Enhancement of RS occurs owing to the formation of a new resonance transition, associated with charge transfer from the metal to the molecule.[4]

Coulomb interaction of the molecule and the metal broadens and shifts the ground and, especially, the excited states of the molecule. As a result, if the standard RS occurs in the free state of the molecule, then, owing to broadening of the levels in the adsorbed state, a band of states forms between which resonance absorption of a light quantum and, correspondingly, RRS is possible. This hypothesis was first stated before the discovery of SERS,[103] and was later developed in Refs. 104 and 105.

The simplest model of RS enhancement owing to charge transfer (including an explanation of the enhancement of the RS cross section by a factor of 10^6) was proposed by McCall and Plazman.[106] Part of the contribution of the electrons in the metal to the RS cross section could be due to the effect of oscillations of the nuclei on charge transfer from the metal to the molecule.

Let V be the volume of the metal participating in charge transfer and let χ be the susceptibility of the metal. Then the polarizability of the metal $\alpha_M = V\chi$. A shift in the position of the molecule bound to the metal (δ_r) will result in the transfer of charge $(\partial_q/\partial r)\delta_r$, which changes the polarizability by an amount

$$\frac{\partial\alpha_M}{\partial r}\delta_r \equiv \frac{\partial(V\chi)}{\partial q}\frac{\partial q}{\partial r}\delta_r. \quad (3)$$

The susceptibility has the form

$$\chi = (\varepsilon - 1)/4\pi, \quad (4)$$

where $\varepsilon = 1 - (\omega_p/\omega)^2$ (Drude's formula), $\omega_p^2 = 4\pi ne^2/m$, and $n = q/V$. A change in q is accompanied by a change in ω_p, determined by the derivative

$$\frac{\partial \omega_p}{\partial q} = \frac{4\pi e^2}{mV}.$$

Correspondingly

$$\frac{\partial \alpha_M}{\partial r}\delta_r = \frac{e}{m\omega^2}\frac{\partial q}{\partial r}\delta_r, \tag{5}$$

whence the RS cross section is easily found[120]:

$$\frac{\partial \sigma}{\partial \Omega} = \frac{1}{3}\frac{e}{m\omega^2}\left(\frac{\partial q}{\partial r}\right)^2 (\Delta r)^2, \tag{6}$$

where $(\Delta r)^2$ is the square of the matrix element $\langle 0|\delta_r|1\rangle$, where $|0\rangle$ and $|1\rangle$ are the wave functions of the ground and excited vibrational states. In this model charge transfer does not lead to a resonance transition, so that the enhancement factor is small (about 20).

Pettinger *et al.*[107] have proposed that charge transfer occurs after the formation of a complex that can absorb light in the visible region of the spectrum. As a result RRS occurs and the enhancement factor can reach 10^6.

In most models of the microscopic interaction of a molecule with a metal (direct charge transfer,[106] electron tunneling,[108] interaction with electron-hole pairs) the surface is assumed to be ideal and the presence of surface defects is ignored. Such defects, however, lower the potential barrier on the surface of the metal and thereby facilitate adsorption (see the reviews of Refs. 2, 4, and 7). Vacancies and points of emergence onto the surface, adatoms, and clusters of adatoms act as active centers of adsorption. These defects are also centers of photon-electron interaction.[7] On the basis of the study of resonance transitions of a molecule through an adatom accompanying adsorption it is assumed[5,7] that the absorption band of the complex arises owing to optical excitation of an electron from a quasilevel of the atom into the free states of the conduction band of the metal. If, in the process, the molecule forms a complex with the adatom by means of partial transfer of the electron charge from the adatom to the adsorbed molecule, then strong modulation (by vibrations of the molecule) of the adatom-metal transition probability bcomes possible, and this should be manifested in the appearance of RRS. According tp Persson[108] and Otto *et al.*[109,110] an adatom lowers the symmetry of the lattice, as a result of which intraband optical transitions in the metal become allowed, and this in turn gives rise to absorption in the adatom-metal system. Resonance enhancement of RS can be expressed, in this case, as[108]

$$\sigma_v(\omega) = \left|\sum \frac{\langle 0|H_{ep}|j\rangle\langle 1,\ j|H_{ev}|0,\ i\rangle\langle i|H_{ep}|0\rangle}{(\omega-\omega_v-\omega_j-iY_j)(\omega-\omega_i-iY_i)}\right|^2, \tag{7}$$

where $\sigma_v(\omega)$ is the scattering cross section, H_{ev} is the Hamiltonian describing the interaction of the electrons in the metal with vibrations of the molecule with frequency ω_v, H_{ep} is the electron-photon interaction Hamiltonian, ω is the frequency of the incident light, $\langle 0|$ is the initial state of the electron, $|i\rangle$ and $|j\rangle$ are intermediate states, Y_i and Y_j are decay constants, $\hbar\omega_i$ and $\hbar\omega_j$ are the energies of 0–i and 0–j transitions, respectively, and 0 and 1 are the ground and excited vibrational states. In the presence of a symmetry-lowering adatom transitions with any energy $\hbar\omega_i$ become possible.[108]

The effect of an adatom on the electronic structure and optical properties of the surface of a metal was studied theoretically by Brodsky *et al.*[111,112] They showed that the scattering matrix has a pole at the energy of an electron in the metal equal to the energy of the bound state of the adatom. According to Refs. 109 and 110 electrons in the metal can absorb light in intraband transitions only in the case when momentum is not conserved, which happens only for electrons scattered by adatoms. The probability for scattering is maximum for electrons with energies close to the energy of the bound state of the adatom.[111,112] For this reason, in Ref. 113, because the electronic states of the metal with energy close to the quasienergy level of the adatom are partially localized on the adatom, while the excited states of the metal can lie above the Fermi level, the absorption band of the complex is interpreted as a charge transfer band in the atom-metal system. Some basic properties of SERS were explained in Ref. 113: the resonant dependence of the spectrum intensity on the frequency of the exciting light ω, the dependence of the ratios of the intensities of different lines on ω, the dependence of the ratio of the intensities of the Stokes and anti-Stokes lines on ω, etc. Estimates of the RS enhancement factor give a value of $\sim 10^4$.

There exists one other group of models[2,4,6,7] that are based on the excitation of electron-hole pairs in the metal by the incident light. A model in which the adatoms are centers of strong photon-electron interaction and their excitations are precisely electron-hole pairs is studied in Ref. 114. Here the excitation is transferred from the metal to the molecule and vice versa, so that a photon at the Stokes frequency is emitted as a result of pair recombination. As demonstrated in Ref. 2, this process depends on the fourth power of the electron-photon interaction constant.

In conclusion it should be noted that although molecular mechanisms for RS enchancement can give an increase in the intensity of the spectra by a factor of 10^4 and higher, their realization requires quite specific conditions: either a suitable electronic structure of the molecules (for reduced RRS) or the presence of sufficient numbers of active centers (for formation of a molecule–active center–metal resonance transition). Since in real experiments RS enhancement is observed for molecules having different electronic properties and chemical nature, it is obviously impossible to construct a complete theory of SERS based solely on molecular mechanisms, though such mechanisms can play a very significant role in the overall effect.

4. APPLICATION OF SURFACE-ENHANCED RAMAN SCATTERING TO THE STUDY OF BIOLOGICAL MOLECULES

Table I gives the main types of biological molecules for which it has been possible to obtain SERS spectra. Thus far the basic advantages of the method have been determined on the example of these compounds and the range of problems that can be solved with the help of SERS spectroscopy has been identified. At the same time it has turned out that using biomolecules as the object of study makes it possible to clarify some features of the mechanism of SERS that are important for applications of the method: the long-range nature of the enhancement in different systems, the role of chemisorption, the nature of groups of atoms whose vibrations exhibit the largest enhancement, etc. We shall examine the basic results of the structural-functional study of biomolecules by the SERS method, as well as the analytical applications of the method.

4.1. Amino acids, peptides, and model compounds

Polypeptides and proteins consist of residues of amino acids. The general formula for 20 of the most common amino acids has the form

$$RCH(NH_3^+)COO^-.$$

The side chains of the amino acids (R) can have a different chemical nature—they are aromatic rings with a developed π-electron system (phenylalanine, tyrosine, tryptophane, histidine), uncharged polar groups (glycine, serine, etc.), charged (with neutral pH) polar groups (lysine, arginine) or nonpolar groups (alanine, leucine, etc.). Thus the SERS spectra of amino acids are interesting not only because it is necessary to obtain reference frequencies in order to analyze the SERS spectra of proteins and supramolecular complexes, but also because information about the nature of groups whose vibrational bands are maximally enhanced by adsorption can be obtained.

The SERS spectra of amino acids as well as some dipeptides and model compounds adsorbed on silver electrodes and silver hydrosols were recently obtained.[14,33,115,116] Adsorption is accompanied by the appearance of a low-frequency band (200–250 cm^{-1}), corresponding to the oscillation of the molecule-metal chemical bond[7] and indicating chemisorption of the amino acids. The square of the frequency of this low-frequency vibration is a linear function of the potential on the working electrode. Analysis of the SERS spectra of amino acids with side chains of different chemical nature has shown that chemisorption can occur through unshared electron pairs of nitrogen and oxygen atoms, as well as owing to the formation of π-electron complexes of the molecule with the metal.[115,116] The most intense SERS is characteristic for amino acid molecules having a developed π-electron system—aromatic amino acids tyrosine (Tyr), tryptophan (Trp), and phenylalanine (Phe) (Fig. 4). The enhancement factor for the RS cross section under conditions of adsorption, owing to unshared pairs, turns out to be several orders of magnitude smaller. In the study of chemically modified amino acids, whose charged groups were neutralized by uncharged groups, it turned out that the intensity and shape of the SERS spectra are identical to the spectra of the corresponding unmodified amino acids, indicating that the contribution of electrostatic interactions accompanying adsorption is insignificant.

The SERS spectra of aliphatic amino acids are very similar to one another (Fig. 5). Only the vibrational bands of the carboxyl and amino groups are manifested; in addition, the RS enchancement factor is about two orders of magnitude lower than for aromatic amino acids. The SERS spectra of equimolar mixtures of aromatic (Phe) and aliphatic (Ala) amino acids exhibit only Phe bands, indicating that the aromatic amino acid is the one predominantly adsorbed

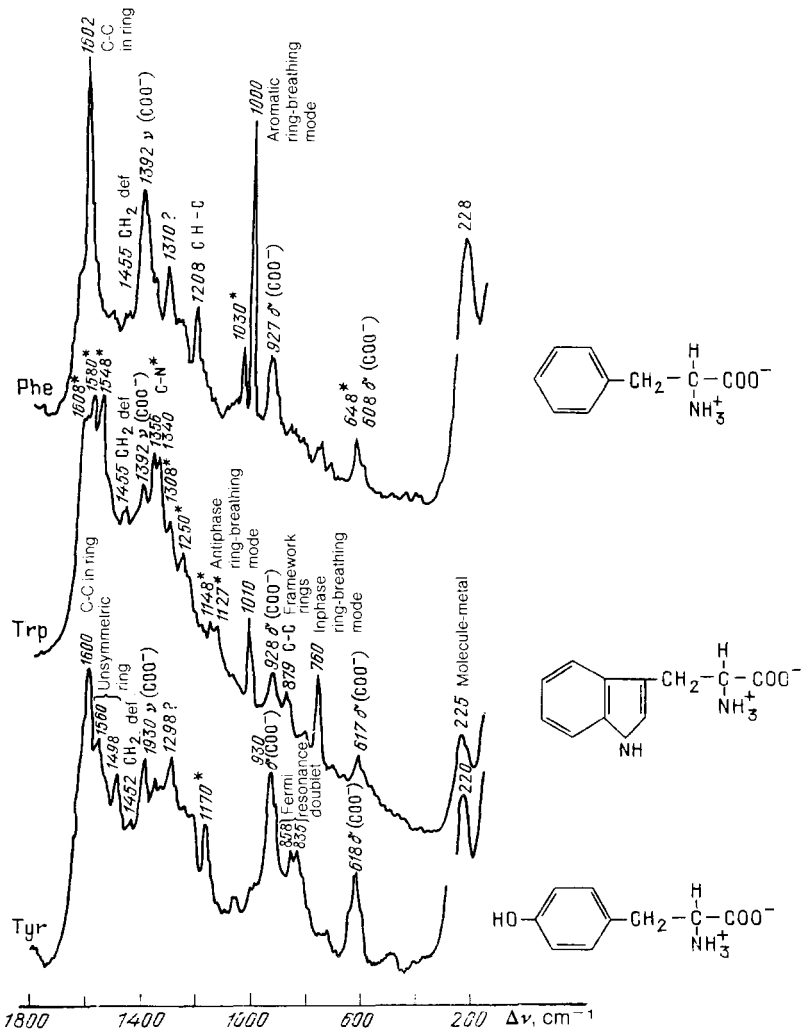

FIG. 4. SERS spectra of aromatic amino acids phenylalanine (Phe), tryptophan (Trp), and tyrosine (Tyr), adsorbed on silver hydrosols.[14] The structural formulas corresponding to the amino acids are shown on the right. The concentration of the compounds equals 0.1 mg/ml; $\lambda_{exc} = 514.5$ nm.

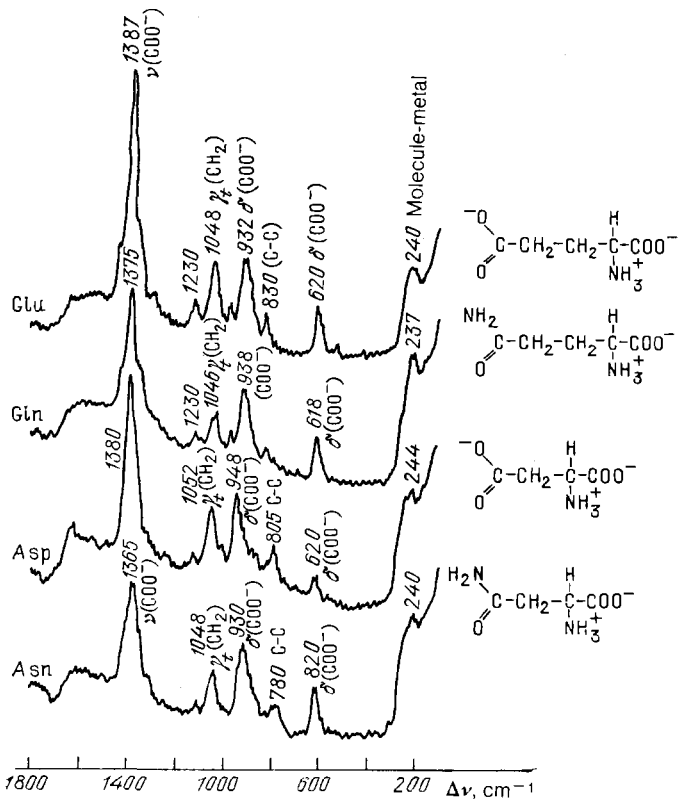

FIG. 5. SERS spectra of aliphatic amino acids glutamine (Gln), aspergine (Asn), glutamic acid (Glu) and aspartic (Asp) acid.[33] The structural formulas of the corresponding amino acids are shown on the right. The concentration of the compounds equals 0.3 mg/ml; $\lambda_{exc} = 514.5$ nm.

in the case of competing bonding. Analogously, only bands of the aromatic residue are observed in the SERS spectra of dipeptides consisting of residues of aliphatic and aromatic amino acids. All this indicates that the π-electron systems of aromatic residues have the highest affinity to the metal. The interaction with the metal through a σ bond with the participation of unshared electron pairs of nitrogen and oxygen atoms is several orders of magnitude weaker.[115]

The absorption spectrum of the complex silver-hydrosol–phenylalanine (like the absorption spectra of all other amino acids) contain two bands with maxima at 395 and $\approx$570 nm. The 395 nm band is characteristic for the absorption spectra of silver hydrosol without the adsorbate and corresponds to the plasma resonance of a spherical silver particle with a diameter of $\approx$20 nm.[46] The position of the maximum of this band depends both on the type of metal and on the size and shape of the particles. The long-wavelength band appears after the adsorbate is added to the sol. The position of the maximum and the half-width of the band are not strictly determined. With time the maximum is shifted toward lower energies and the band is broadened; in addition, the rate of the shift is proportional to the adsorbate concentration. Creighton *et al.*[46] conjecture that this band is associated with the scattering of light by aggregates of silver micelles with a specific shape and a characteristic size of 100–200 nm. Such aggregates are indeed formed with coagulation of a hydrosol, associated with the addition of amino acid (see Fig. 2b).[14] The coagulation of a hydrosol, however, can be induced by adding salt and in this case a distinct long-wavelength maximum does not appear in the absorption spectrum; only an asymmetric broadening of the 395 nm band into the long-wavelength region occurs. A distinct maximum also does not appear when the amino acid glycine is added to the hydrosol. This indicates unequivocally that the chemical nature of the compound forming the complex with silver is important for the appearance of a long-wavelength maximum in the adsorption spectrum.

The parameters of the absorption bands of a new electronic transition, responsible for RRS of the complex, if such exists, were calculated in Ref. 14 from an analysis of the relative intensities of the Stokes and anti-Stokes bands in the SERS spectrum of the sol-Phe complex. It turned out that the maximum of such a hypothetical absorption band coincides to within 1 nm with the maximum of the long-wavelength absorption band appearing when amino acids are adsorbed on silver hydrosol, while the half-width of the observed band is several times longer than the half-width of the expected electronic transition band.

The appearance of a long-wavelength band in the absorption spectrum of the silver hydrosol complex with amino acids and its parameters are apparently determined by the superposition of the following effects. First, the chemisorption of a molecule on the surface of silver is accompanied by an electronic transition associated with charge transfer from the metal to the molecule. The absorption band corresponding to this transition has a maximum in the visible region of the spectrum. Second, aggregation and the concomitant Mie scattering give rise to significant broadening of the electronic transition band.[162] In addition, the aggregation of silver hydrosol affects the process of chemisorption of a separate micelle on the surface and, therefore, the parameters of the metal-molecule charge-transfer band.[14]

The excitation spectrum of SERS of sol–amino-acid complex has a resonance character; the maximum coincides with that of the long-wavelength absorption band (Fig. 6). However, the dependence of the RS enhancement factor on the excitation wavelength is different for different vibrational bands in the SERS spectrum. Thus the 1600 cm^{-1} band of

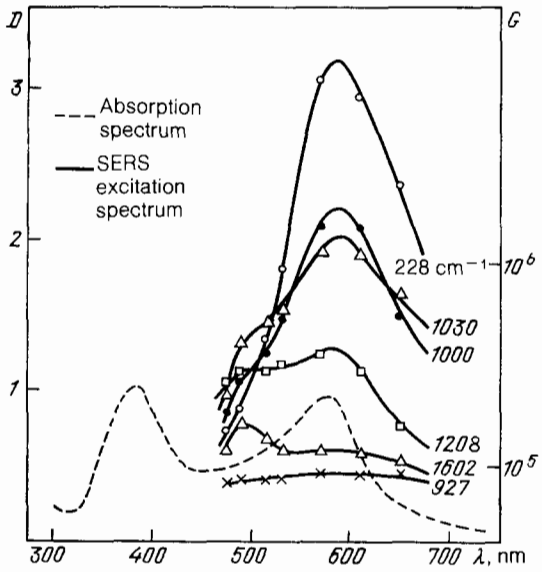

FIG. 6. Absorption spectrum and SERS excitation spectrum of *L*-phenylalanine adsorbed on silver hydrosols.[116] The SERS excitation spectra are presented for different vibrational bands of the complex.

phenylalanine does not have a distinct maximum in the region 500–700 nm, while the intensity of the 228 cm^{-1} band at 570 nm is more than 10 times higher than its intensity at 488 nm.

The indicated dependence of the RS enhancement factor on the excitation wavelength as well as its high (up to $\sim 10^6$) value indicate the existence of at least two enhancement mechanisms[7]: electromagnetic (without a distinct resonance character) and molecular (owing to chemisorption and formation of a complex with charge transfer).

It is quite difficult to interpret the SERS spectra of amino acids adsorbed on silver electrodes because wide structureless bands with maxima near 1350 and 1590 cm^{-1} as well as in the region 2800–3000 cm^{-1} appear after the electrodes are "activated" with the help of ORC. These bands are associated with the graphitization of carbon, reduced from carbon dioxide gas dissolved in the water,[163] on the metal (Fig. 7). Graphitization of carbon obviously intensifies the RS signal, since after the solutions are degassed by passing helium through them, in addition to a reduction of the intensity of these bands, the SERS signals of the compounds under study also become weaker.[115] The frequencies of the main characteristic bands in the SERS spectra of amino acids adsorbed on silver electrodes are nonetheless very close to the bands observed with adsorption on hydrosols and can be employed as references in the analysis of SERS spectra of water soluble and membrane proteins.

4.2. Water soluble proteins

The possibility of using the method of SERS spectroscopy for studying water soluble proteins was first demonstrated for the proteins of the periplasmic space of *E. coli*.[11,12] The SERS spectra of water soluble proteins are a superposition of the SERS spectra of aromatic amino acid residues present in the molecules under study. The observed differences in the frequencies and relative band intensities as well as the dependence of the SERS spectra on the potential are associated with the different orientation of the side chains of the amino acid residues relative to the surface of the electrode.

Figure 8 shows the SERS spectra of two water soluble proteins—lysozyme and albumin—adsorbed on electrodes with the potential at which there is a zero charge on the silver electrode.[116] We note that the spectra were obtained with concentrations $\sim 10^2$–10^3 times lower than the minimum necessary for recording RS spectra in solution. Based on an analysis of the relative intensities of the bands in the SERS spectra as well as data from high-resolution x-ray structural analysis it was concluded that the enhancement of RS is observed only for groups of atoms that are located on the surface of a protein globule and interact with the metal.[3] This confirms the short-range character of SERS under these experimental conditions.

In RS spectroscopy of proteins the vibrations of peptide groups (amide I and amide III) are especially informative.[27,164] These vibrations, hwoever, could not be observed in the SERS spectra of water soluble proteins. This is appar-

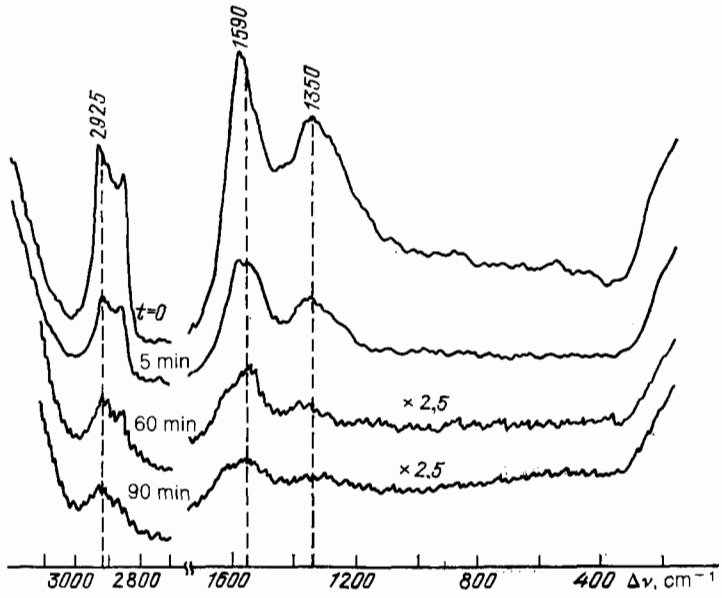

FIG. 7. SERS spectra of graphitized carbon on the surface of a silver electrode, obtained in pure electrolyte (0.1 M Cl) and recorded at different times, during which degassing of the solution was conducted.[33]

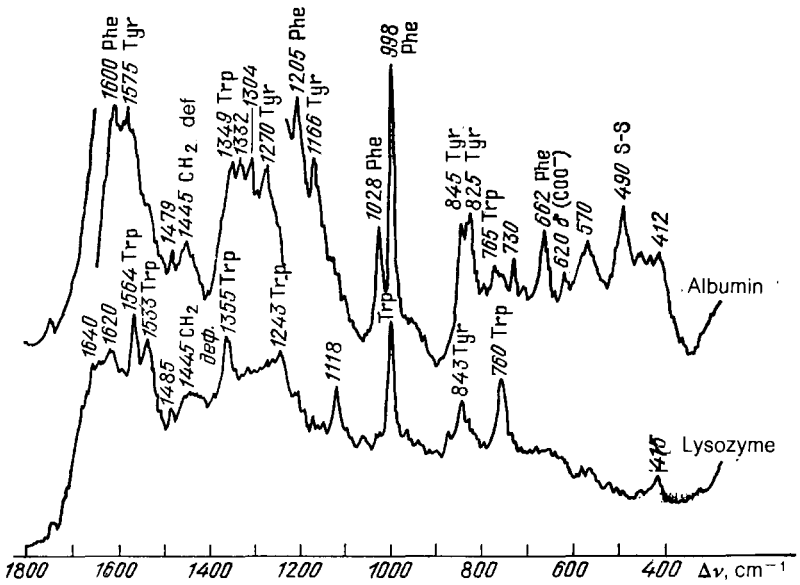

FIG. 8. SERS spectra of lysozyme and bovine serum albumin adsorbed on silver electrodes with the potential corresponding to zero charge for the silver electrodes.[116] The concentration of the adsorbate equals 10^{-6} M; λ_{exc} = 514.5 nm.

ently attributable to the screening of the peptide groups by the side chains of amino acide residues which increases their distance from the surface of the metal and impedes efficient enhancement of the corresponding vibrations.

The fact that under the experimental conditions employed adsorption has virtually no effect on the conformation of macromolecules is important for the SERS spectroscopy of biopolymers,[12–14,134] though this question must be specially examined in each specific case. Thus when LS protein is adsorbed on a silver electrode its capacity to bind with the substrate is conserved,[12] while flavoproteins, as Spiro *et al.* showed,[134] retained 86% of their functional activity when adsorbed on silver hydrosol and this percentage increased to 96% after the protein was desorbed. Analysis of the circular dichroism spectra of cytotoxin I from Naja naja oxiana cobra venom on a silver hydrosol (Fig. 9) shows that the confirmation of the polypeptide chain remains unchanged, while significant changes in the region of absorption of aromatic amino acid residues indicates that the aromatic side chains interact directly with the silver.[13] Spiro *et al.*[130] also showed that for some heme-containing proteins adsorbed on silver the microenvironment of the heme groups can be destroyed and the heme-protein bonds can be broken.

To conserve the conformation of adsorbed biopolymers the substance under study must be added after ORC, and the conditions for reduction of the silver salts by excess reducing agent when obtaining hydrosols must be met precisely in order to remove the silver ions, which affect the structure of the molecules under study (see Sec. 2).

4.3. Photosensitive membrane proteins and pigment-protein complexes

The possibilities of SERS spectroscopy for studying photosensitive membrane proteins and pigment-protein complexes are best illustrated for bacterio- (BR) and visual (R) rhodopsins.[19–21,35,119]

Bacteriorhodopsin from purple membranes of *Halo-*

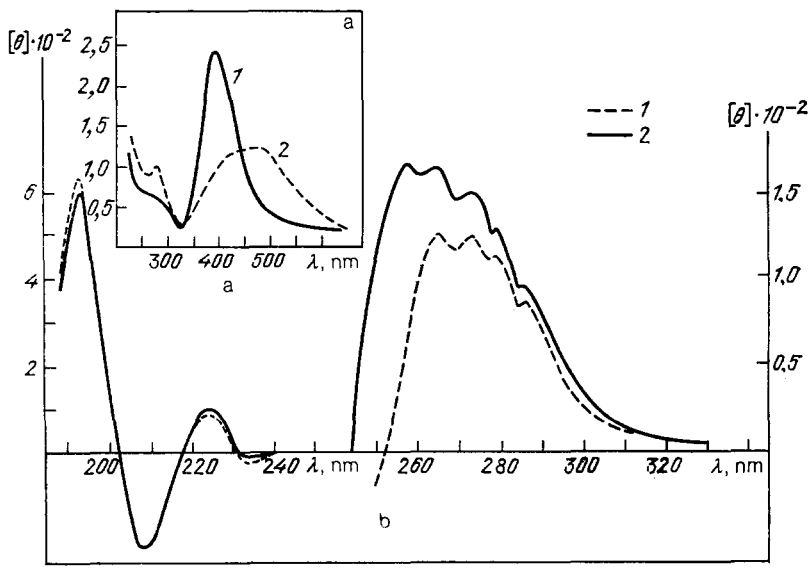

FIG. 9. a) Absorption spectra of silver hydrosol (1) and the sol-cytotoxin complex (2). b) circular dichroism spectra of cytotoxin (1) and the sol-cytotoxin complex (2) in peptide (190–240 nm) and aromatic (340–240 nm) regions.[14]

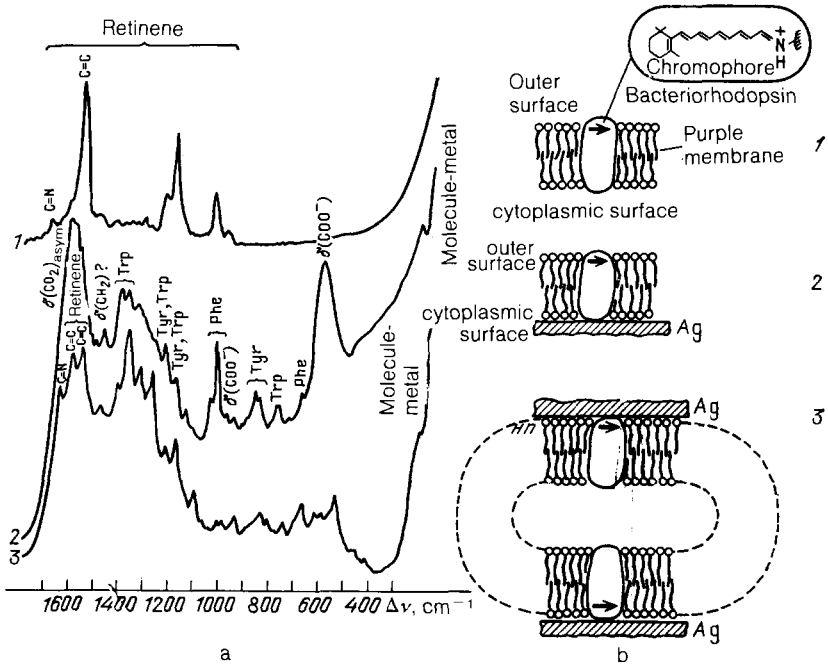

FIG. 10. a) RRS spectra of a water suspension of purple membranes (1) and SERS spectra of purple membranes adsorbed on silver electrodes (2), and adsorbed shadows of cells of *Halobacterium halobium* (3). b) purple membrane (1), adsorbed purple membrane (2), and adsorbed ghost of a cell of *Halobacterium halobium*, closed with the cytoplasmic side inwards (3).[20]

bacterium halobium is a unique photosensitive protein that realizes transmembrane proton transfer.[165] To understand the molecular mechanism of the functioning of BR the topography of its chromophoric center (the site of the retinene residue) must be determined. The method of SERS spectroscopy was employed to establish the arrangement of the retinene relative to the surface of the membrane.[19,20] To this end SERS spectra were obtained of purple membranes, of ghosts of the cells of *Halobacterium halobium* (closed with the cytoplasmic surface inwards), and of analogs of BR adsorbed on silver hydrosols and electrodes.

When adsorbed on electodes with the potential corresponding to zero charge for the silver electrode the purple membranes are oriented with their inner side toward the electrode.[20] There are no bands of the chromophore in the SERS spectrum. The wide structureless band with a maximum at $\approx 1600\ cm^{-1}$ is associated with the graphitization of the metal surface due to carbon dioxide gas dissolved in the electrolyte (see Sec. 4.1).[163] At the same time the SERS spectra of the ghosts of cells, which can come into contact with the surface of the metal only via their outer side, contain a fine structure, associated with the enhancement of the

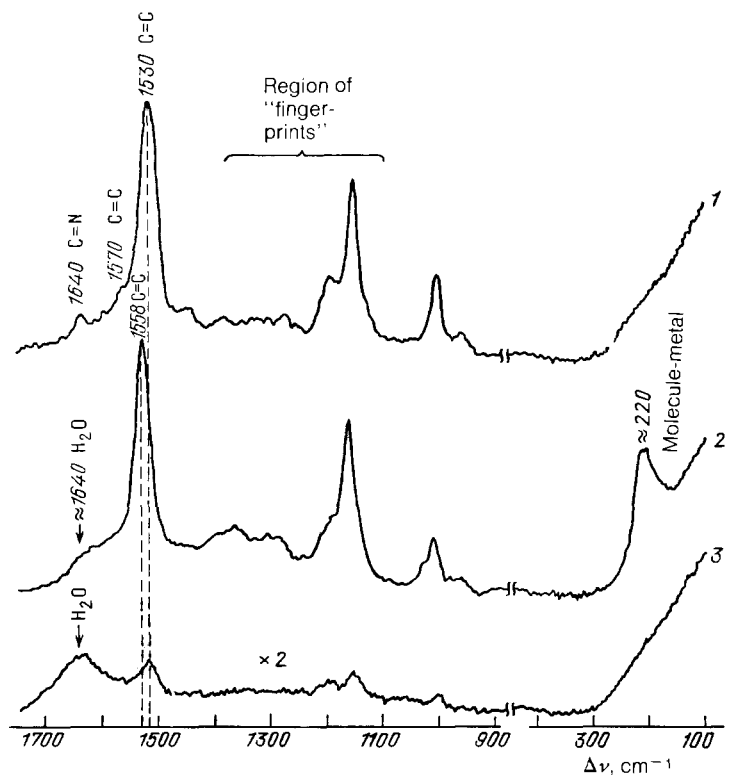

FIG. 11. RRS spectra of a water suspension of purple membranes (the protein concentration equals 10^{-5} M) (1), SERS of purple membranes adsorbed on silver hydrosol with a concentration of 10^{-7} M (2) and RRS of a water suspension of membranes with a concentration of 10^{-7} M (3).[20] In spectrum 3 the sensitivity is doubled.

vibrational bands of retinene (Fig. 10), against the background of a structureless carbon band. This implies that the π-electron system of retinene lies close to the outer side of the purple membrane.

When adsorbed on silver hydrosol the purple membrance can interact with silver both via its outer and inner sides[20] (Fig. 11). The presence of the vibrational bands of the chromophore in the spectrum confirms the conclusion that the retinene lies close to the surface of the membrane. We note that the similarity of the RRS and SERS spectra of BR in the "fingerprint" region (1000–1400 cm^{-1}) indicates that when the protein is adsorbed the polyene chain of retinene retains the full trans- conformation characteristic for BR in a water suspension.[165]

To determine more accurately the distance between the retinene and the outer surface of the purple membrane the SERS spectra of analogs of BR, constructed from bacterioopsin, and analogs of retinene with a polyethylene chain of different length occupying the same section in the protein as the residue of the fully transretinene in the BR molecule, were obtained. It turned out that for the shortest analogs (with one and two double bonds) the SERS signal is virtually undetectable, and when the number of double bonds in the polyene chain of the analog increases intense SERS appears. This is attributable to the removal of the π-electron system from the surface of the metal accompanying shortening of the analog and not to the weakening of the π-electron system, since for free analogs of retinene adsorbed on a hydrosol an intense SERS signal is also observed for the shortest analogs. We assume that for analogs with three and four double bonds part of the π-electron system falls within the SERS layer 0.5 nm thick, while for the shortest analogs, built into the BR, this does not happen. The results obtained made it possible to determine the distance from the retinene to the surface of the membrane.[20]

The fact that the mechanism of RS enhancement accompanying adsorption of membrane proteins on hydrosols under our experimental conditions is of a short-range character was employed to determine the topography of the chromophore in another photosensitive membrane protein—bovine visual rhodopsin.[21,119] This pigment is located in the membranes of the photoreceptor disks in the outer segments of the rods in the retina and plays the key role in vision.[165] With the help of SERS spectroscoy it was shown that the retinene residue in R is located at a distance of 0.5–1 nm from the cytoplasmic surface of the membrane, and when it is adsorbed on silver hydrosol the 11-cis configuration of the polyene chain, characteristic for the native pigment is conserved in the chromophore. In addition, a study of photoreceptor disks, closed with the cytoplasmic surface inwards, and disks in which the cytoplasmic surface of the membrane was screened from the metal by nonoclonal antibodies (Fig. 12) confirmed that under the experimental conditions employed the mechanism of RS enhancement accompanying adsorption on silver hydrosol has a short range.

The simplest method for obtaining SERS spectra by the long-range (probably, plasmon) enhancement mechanism is to employ a "smooth" silver electrode.[35] A "smooth" electrode is an electrode that is not loosened up by anodic polarization in order to increase the concentration of point defects (playing the role of adsorption centers) on the surface. In this case microdefects up to 100 nm in size predominate on

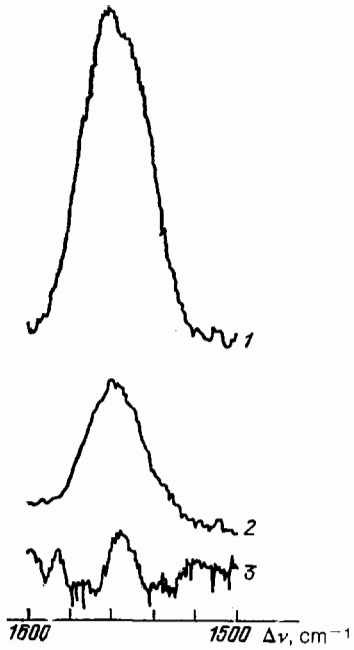

FIG. 12. SERS spectra of photoreceptor disks (1) and of the photoreceptor disk—monoclonal antibody to the C-end of the rhodopsin molecule complexes (2, 3).[119] The rhodopsin/antibody ratio equals 1/1 for (3) and 5/1 for (2).

the surface of the electrode. In such a system enhancement of the RS signal from adsorbed molecules occurs owing to the excitation of an elecromagnetic surface wave (see Sec. 3.3).[3] The magnitude of this enhancement, of course, cannot be very large owing to the irregularity of the macrodefects on "smooth" electrodes.[7]

The signal-to-noise ratio in the SERS spectrum of BR on a "smooth" electrode is about 50 times higher than in the RRS spectrum of a water suspension of purple membranes with the same protein concentration (Fig. 13a). Variation of the potential on the electrode produces appreciable changes in the spectrum. When the electrode potential is increased or decreased relative to the point of the potential corresponding to zero charge for the silver electrode the relative integrated intensity of the band corresponding to the stretching vibration of C = C with a maximum at $\simeq 1505\ cm^{-1}$, corresponding to the kinetic intermediate of the photocycle of BR with an absorption maximum at 610 nm (Fig. 13b), increases. The adsorbed and unadsorbed membranes are apparently in dynamic equilibrium, and the transition through the potential corresponding to zero charge for silver is accompanied by reorientation of the purple membranes near the surface of the "smooth" electrode.

After absorbing light quanta bacteriorhodopsin and rhodopsin undergo photo chemical transformations associated with successive changes in the electronic-conformational state of the chromophore and its protein environment. In BR such transitions are of a cyclic character (photocyclic BR), while in rhodopsin they give rise to photolysis of retinene.[165] Since adsorption on silver hydrosols is associated with chemisorption on point defects and interaction of the π-electron system of retinene with the metal in the electric double layer,[20] there arises the question of how this affects

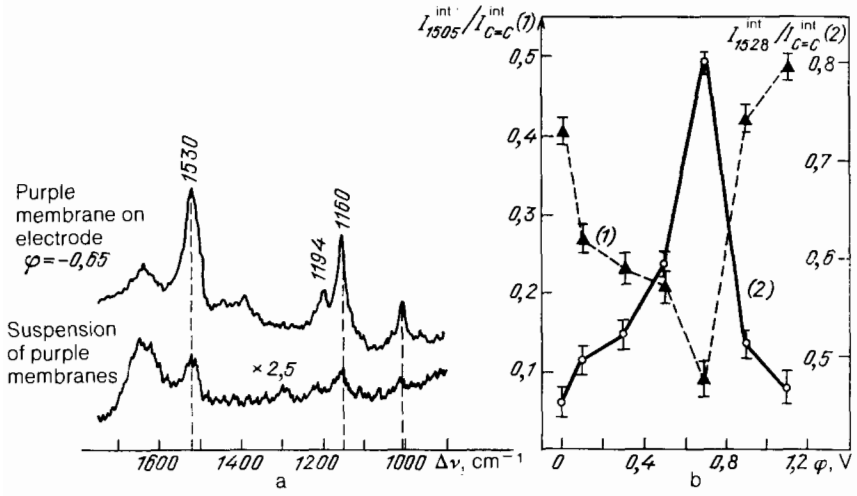

FIG. 13. a) RRS spectra of a suspension of purple membranes and SERS spectra of purple membranes adsorbed on a "smooth" silver electrode with the potential corresponding to zero charge for the silver electrode; the sensitivity is 2.5 times higher for the bottom spectrum. b) Dependence on the electode potential of the relative integrated intensities of bands with maxima at 1505 and 1528 cm^{-1} in the SERS spectra of purple membranes on a "smooth" electrode.[35]

the characteristics of the photochemical transformations of pigments. It turned out that rhodopsin molecules adsorbed on silver hydrosol are not subjected to photolysis.[119] An analogous result was also obtained for BR adsorbed on hydrosol, while for BR adsorbed on a "smooth" electrode at the potential corresponding to zero charge for silver all characteristics of the photocycle are preserved.[35] Adsorption on hydrosol and loosened up silver electrodes is accompanied by the formation of metal-adsorbate chemical bonds and gives rise to a significant redistribution of the electron density in the chromophore and groups of its protein environment.[119] This makes impossible subtle electron-conformational restructurings of the chromophoric centers.

The observed effect makes it possible to halt the photochemical processes at different stages and, therefore, to study the intermediate products of photoinduced transformations. This approach is especially promising for visual pigments, in which light induces photochemical changes associated with the loss by the protein of its functional activity.

The possibility of employing SERS spectrosocpy for solving another extremely important class of problems, associated with photosynthesis, was demonstrated by Cotton *et al.*[17,24] Photoreaction centers of bacteria contain, aside from protein, different pigments (bacteriochlorophylls, bacteriopheophytins, quinones) that give RRS spectra.[166] The vibrations corresponding to individual pigments cannot be separated in the analysis of RRS spectra of photoreaction centers.[17,166] At the same time it turned out that when photoreaction centers are adsorbed on the surface of silver electrodes and excitation is performed with different wavelengths, for certain potentials on the electrodes spectra characteristic for individual pigments are obtained (Fig. 14). Thus SERS makes it possible to study separate constituent chromophores of complex supramolecular biological structures. In so doing, the spectra of free pigments can be employed to interpret the SERS spectra as well as to determine the ratios of the different types of constituent chromophores of the photoreaction centers. Thus Cotton *et al.*[24] were able to record the SERS spectra of chlorophyll-a, bacteriochlorophyll-a, and bacteriopheophytin, adsorbed in the form of monolayers on silver island films.

It should be noted that the study of the constituent pigments of photoreaction centers with the help of RS spectroscopy is, as a rule, greatly impeded by the characteristic fluorescence of the compounds studied.[17] At the same time it turned out that adsorption on the surface of silver gives rise to strong quenching of fluorescence, which makes it possible to record SERS spectra with pigment concentrations as low as 10^{-7} M. This fact was employed to study, aside from photosynthetic pigments, a number of flavins and flavoproteins adsorbed on silver electrodes and silver hydrosols.[50,134,135] These investigations gave new data on the structure of flavins and sol-flavoprotein complexes,[26,50,134] as well as on the mechanism for the interaction of DNA with proflavin—acridine dye, exhibiting strong mutagenic action.[135]

Porphins, their derivatives (porphyrins), and porphyrin-containing proteins occupy an important place among biological chromophores and chromoproteins, studied with the help of SERS spectroscopy. Compounds of this

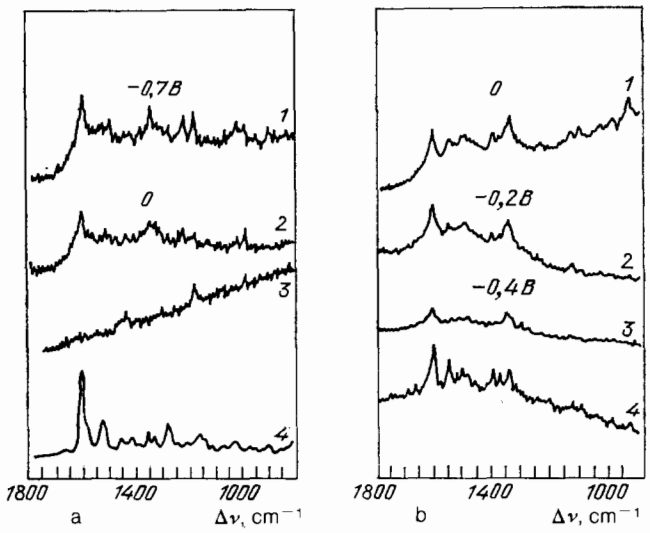

FIG. 14. a) SERS spectra of reaction centers of the bacteria *Rhodopseudomonas sphaeroides* as a function of the potential on the silver electrode: 1) 0.7 V; 2) 0 V; 3) buffer; 4) bacteriochlorophyll dissolved in CH_2Cl_2 with a corresponding quantity of pyridine for formation of the bacteriochlorophyll:pyridine complex; $\lambda_{exc} = 457.9$ nm. b) SERS spectra of reaction centers of the bacteria *Rhodopseudomonas sphaeroides* as a function of the potential on the silver electrode: 1) 0 V; 2) 0.2 V; 3) 0.4 V; 4) bacteriopheophytin in Ch_2Cl_2; $\lambda_{exc} = 530.9$ nm.[17]

type play a key role in oxidation-reduction reactions in cells (respiration, photosynthesis).[167] The SERS spectra of free porphins and porphyrins as well as the products of their decomposition (bile pigments) have been obtained on polycrystalline electrodes and hydrosols and in layered structures Ag/CaF_2.[18,61–63,120–126,128] It turned out that adsorption of tetraphenyl-porphin on the surface of silver electrodes depends strongly on the electropotential and the ionic state of SO_3^- and COO^- groups, bound to the porphyrin core. Depending on the potential, dissociation or aggregation of the constituent monomers of porphin as well as formation of a complex of the chromophore with silver atoms were observed. With adsorption of porphyrin chromophores in layered structures the exchange reaction between the Ni atoms of porphyrins and silver atoms was observed with the help of SERS spectroscopy.[61–63] We note that in this case the vibrations of all groups of the chromophore are intensified. This is obviously linked with the realization of the electromagnetic enhancement mechanism.

Metalloporphyrins and heme-containing proteins (myoglobin, hemoglobin, and cytochromes c, cd_1, and b_5) were studied in Refs. 15, 16, 37, 42, 127, and 129–133. These proteins contain porphyrin chromophores with an iron atom, and depending on the charge on the iron atom they participate in different biochemical oxidation-reduction reactions together with oxygen.[167] When the electrode potential (relative to the point of the potential corresponding to zero charge for silver) is reduced, together with a reduction of the intensity of the vibrational bands of Fe(III)-Ag, bands corresponding to vibrations of the Fe(II)-Ag bonds are recorded in the SERS spectra of protoporphyrins, myoglobin, and cytochrome-c; this indicates that on the surface of the electrode Fe(III) is reduced to Fe(II). This transition is observed at a potential of –0.6 V, and the reverse oxidation of iron occurs at a potential of -0.2 V.[127]

Another interesting application of SERS spectroscopy in the study of porphyrin-containing proteins is the possibility of recording the oxidation-reduction reactions on the surface of a metal with the participation of cytochromes—the most important proteins in the respiration and photosynthesis sytems. Cotton *et al.*[36] and Taniguchi *et al.*[37] showed that the adsorption of these proteins on silver electrodes in the presence of pyridine and bipyridine accelerates electron transfer between the electrode and the protein. It is significant that when the cytochrome molecule is adsorbed its characteristic native conformation is preserved.[15]

The possibility of selective change in the functional state of proteins, like the previously described effect of the potential on the parameters of the photochemical transformations of BR and R, opens up a new area of application of SERS to the study of structural-functional characteristics of biopolymers.

The possibility of selective change in the functional state of proteins, like the previously described effect of the potential on the parameters of the photochemical transformations of BR and R, opens up a new area of application of SERS to the study of structural-functional characteristics of biopolymers.

4.4. DNA and its components

The SERS spectra of native and denatured DNA, complexes of DNA with platinum, modified DNA, and DNA components were studied in detail in Refs. 9, 10, 31, 51, 84, 85, 117, 118, and 136–149. It turned out that for DNA and polydi- and trinucleotides the short-range mechanism of RS enhancement accompanying adsorption on silver electrodes and hydrosols is clearly manifested. Thus when polyadenine is adsorbed on silver hydrosol bands characteristic for adenine, which, according to x-ray structural data, is located about 0.5 nm from the surface of the molecule, are not manifested in the SERS spectrum.[167] When polyadenine is irradiated with γ-rays breaks appear in the polynucleotide chain, as a result of which adenine is able to come into direct contact with the surface of the metal. In the process there appears in the SERS spectrum a distict signal that becomes stronger as the dosage increases and threfore as the number of breaks increases (Fig. 15).[84]

Native DNA gives in the SERS spectrum 30–40 bands with frequencies characteristic for adenine, guanine, cytosine, and thymine (nucleotide bases appearing in DNA) as well as for groups of the sugar-phosphate core of the molecule.[9] It turned out that SERS spectroscopy is a very sensitive method for recording subtle conformational changes in DNA caused by ionizing radiation[84] (destabilization of the double helix of DNA can be detected even at dosages as low as 1 krad), mutagenic factors,[135] as well as thermal denaturation of the molecule.[148]

As an example Fig. 16 shows the SERS spectra of DNA before and after thermal denaturation.[148] Denaturation of DNA, which opens up the double-chain structure of the molecule, leads to the appearance of intense signals associated with vibrations of the residues of the nucleotide bases, in the SERS spectrum. SERS spectroscopy has been used to analyze the effect of mutagenic factors (methylation of nucleotide bases, interaction with dyes) on the structure of DNA.[134,144] Koglin *et al.*[144] recorded bands corresponding to vibrations of methyl guanine in the SERS spectrum of methylated DNA. In addition, it was concluded from an analysis of the relative intensities of the bands of native and modified DNA that methylation of the nucleotide bases leads to structural changes in their microenvironment.

Since enhancement of RS cross sections accompanying adsorption on loosened up silver electrodes and hydrosols is observed only for groups of atoms not more than 0.5 nm from the surface of the metal, the method of SERS spectroscopy gives a unique possibility for studying the arrangement of chromophoric groups both in native DNA and accompa-

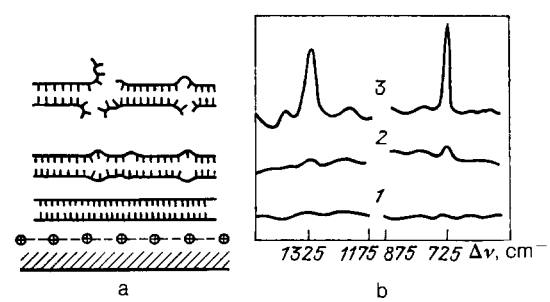

FIG. 15. Diagram of the formation of breaks in the double-strand structure of DNA irradiated with Co^{60} γ-rays (a) and SERS spectrum (b) with dosages of 0 krad (1; native DNA), 10 krad (2), and 100 krad (3).[84] The compounds were adsorbed on a silver electrode with a potential of -0.1 V.

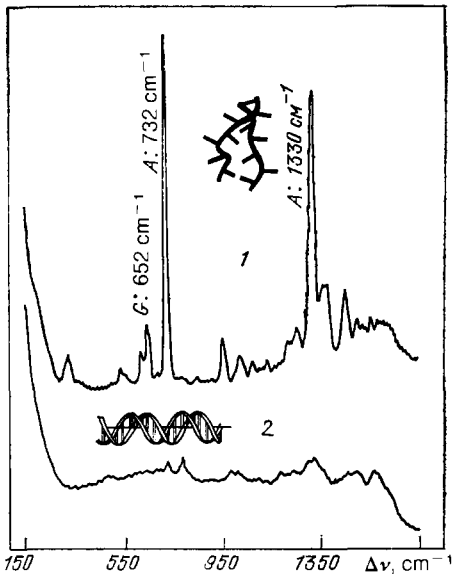

FIG. 16. SERS spectra of DNA subjected to thermal denaturation (1) and natural DNA (2).[148] The compounds were adsorbed on silver electrodes with the potential corresponding to zero charge for silver. A and G denote adenine and guanine, respectively.

nying the interaction of natural DNA with other chromophores. Examples are studies of binding of acridine dyes (strong mutagens)[135] as well as platinum compounds: cis-$Pt(NH_3)_2Cl_2$ and [Pt-(dien)Cl]Cl.[149] The first of these platinum compounds is a strong antitumor agent, while the second is inactive. When DNA binds with cis-$Pt(NH_3)_2Cl_2$ the SERS spectra of the complex and free DNA are identical; when the complex [Pt-(dien)Cl]Cl–DNA is adsorbed on silver the vibrational bands characteristic for the platinum compound appear in the SERS spectrum. The coordination compound of platinum in the first case obviously intercalates into the double-strand DNA molecule, while in the second case it interacts with the surface groups. It is very likely that this approach will be very useful in studying the molecular basis for the action of mutagens, medicines, and other DNA-specific agents.

A characteristic feature of the method of SERS spectroscopy is that the enhancement factor for the RS cross section of water is low, which makes it possible to record in the SERS spectrum of the adsorbate the C–H and C–D vibrational bands in the region 2800–3200 cm^{-1}. In the RS spectrum this region is overlapped by the intense band of O–H stretching vibrations of water. By observing the C–H and C–D vibrational bands with the help of SERS spectroscopy it is possible to study the deuteroexchange process in biopolymers, obtaining information about the topography and lability of separate functional groups. Deuteroexchange in 5′-AMP and guanine, adsorbed on silver electrodes, was studied in Ref. 137. It turned out that the vibrations of the C–H and C–D bands are clearly manifested in SERS spectra only after ORC. In addition, the intensity of the C–H and C–D bands was much higher than that of the water band.

SERS spectra of chromosomes adsorbed on silver electrodes were recently obtained.[28] If bands characteristic for both DNA and protein appear in the RS spectra of the chromosome, then adsorption enhances predominantly the bands corresponding to the nucleotide bases in the DNA of the chromosome. Thus the structural changes in chromosomes associated with genetic diseases can be analyzed with the help of SERS spectroscopy. In addition, it is possible to study the topography of the surface of chromosomes and clarify the nature of the groups participating in the binding of the chromosome with the nuclear membrane during cell division.

Extremely interesting analytical applications of SERS to the analysis of the derivatives of nucleotide bases were recently demonstrated by Koglin *et al.*[9,22] As is well known, the highly efficient method of liquid chromatogrpahy is widely employed in working with biomolecules to obtain individual chemically pure compounds. It turned out that treatment of silica gel plates having separate spots (2–4 mm in diameter), corresponding to individual types of nucleotide bases or their derivatives, with silver hydrosol produces intense SERS, making it possible to characterize the separate compounds. Biomolecules in quantities as small as 0.12 ng per spot have been detected.[9,22] The most intense SERS spectra were obtained for quantities of about 30 ng per spot (Fig. 17).

The highest RS enhancement factor for biologically important compounds was obtained with adsorption of benzoic acid on silver surfaces with artificially created regular nonuniformities.[23] Van Duyne *et al.*[168] recently proposed employing the method of SERS spectroscopy with spatial resolution in analytical applications. In preliminary experiments performed with the help of this method they obtained an SERS signal from pyridine, adsorbed on a silver electrode, in concentrations as low as 10^{-18} M. Such methods will undoubtedly find wide application in analytical fast analyses of biological compounds.

5. CONCLUSION. POSSIBILITY AND PROSPECTS FOR FURTHER PROGRESS IN SURFACE-ENHANCED RAMAN SCATTERING SPECTROSCOPY

In conclusion we note that based on the publications studied in this review it is obvious that SERS spectroscopy can be widely employed for studying very diverse biological processes. The SERS spectra obtained for trace quantities of biomolecules (in some cases at concentrations as low as 10^{-18} M) enables the use of this method in analytical applications. Experimental methods enabling selective realization of long-range ("smooth" silver electrodes, island films, surfaces with regular macrodefects) and short-range (unaggregated silver hydrosols, electrodes treated with the help of ORC) components of the SERS mechanism make it possible to reduce the limit of detection of RS spectra and to study the topography of macromolecules. The quenching of luminescence of biological chromophores accompanying adsorption on the surface of silver, discovered in experiments on SERS, has made it possible to obtain spectra of compounds whose characteristic luminescence strongly impeded such experiments. The functional state of molecules adsorbed on silver electrodes can be changed by varying the electrode potential, and in addition this change is, as a rule, quite "soft" and does not alter the conformational state of the macromolecule as a whole. In addition, SERS spectroscopy should be useful in biotechnology and medicine, where a large number of analyses of biological compounds must be performed. This method, aside from being extremely sensitive, is comparatively

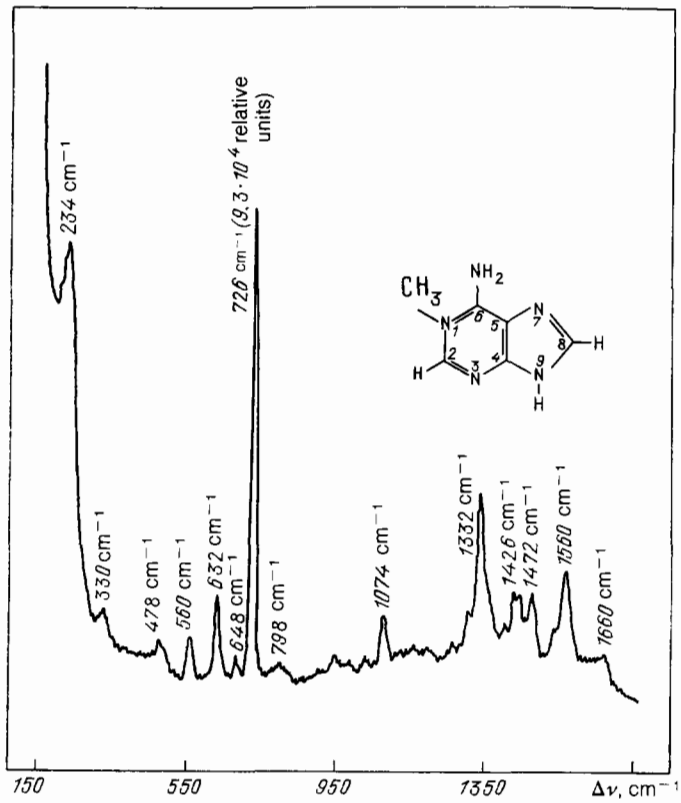

FIG. 17. Identification of spots on a chromatogram with the help of SERS spectroscopy. SERS spectrum of methyl adenine. The spot contains 30 ng of the substance. (E. Koglin, data in press).

simple, since it does not require the construction of specialized expensive apparatus—standard Raman scattering spectrometers are employed to obtain the SERS spectra.

SERS spectroscopy of biomolecules is developing very rapidly. The research work is directed primarily toward developing new, more convenient systems in which the advantages of this method (high RS signal enhancement factor, preservation of the natural conformation of the molecule on adsorption) would be realized most fully. The surface of silver with predeposited regular macrodefects has, in this respect, obvious advantages. It should also be noted that the use of picosecond laser pulses for the excitation of RS of compounds adsorbed on a metal surface is also promising. This approach enables obtaining RS spectra from surfaces that might appear to be unsuitable for obtaining RS enhancement.[7] Systems with multichannel detection of spectra, the use of microscopes and spatial resolution systems, as well as lasers with a wide range of tunable frequencies are being widely employed in SERS spectroscopy. All this promises that the data presented in this review are only the first step in the application of SERS to the study of biologically important molecules.

We are deeping grateful to V. F. Bystrov for his support of this work as well as to Dr. E. Koglin (Institute of Applied Physical Chemistry, Julich, West Germany) and Professor T. J. Spiro (Princeton University, USA) for providing to us the results of their work prior to publication.

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