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Structural Investigation of Biological Material in Aqueous Environment by Means of Infrared-ATR Spectroscopy

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Abstract. Infrared attenuated total reflection (ATR) spectroscopy may be used to investigate biological material (e.g., membranes, proteins, erythrocytes etc.) under biological conditions provided that adhesion of the sample can be achieved in aqueous environment. Uncharged lipid multilayer model membranes can be attached by hydrophobic interaction when hydrophobic internal reflection plates (e.g., ZnSe, CdTe) are used. However, if an electric field is applied across the menbrane, germanium reflection elements would be preferred because of their low electric resistance ($\sim 50\ \Omega$cm). This material can also be used if cells or proteins are linked chemically to the ATR plate because of the hydrophilic surface which is similar to that of glass and, thus, enables chemical modification by silanization. It has turned out that good adhesion of uncharged and negatively charged model membranes to germanium plates is achieved when they are coated with a monomolecular layer of aminopropylsilane. There is some evidence that erythrocytes remain more stable when adsorbed to a polymerized aminosilane coating (organic silanization) rather than to the corresponding monolayer (aqueous silanization). Negatively charged germanium surfaces have been obtained by succinylation of the aminosilane coating. Furthermore it has been demonstrated that proteins can be bound to the aminosilane coating by means of carbodiimide. Immobilized acetylcholinesterase was still enzymatically active.

Key words: Infrared-ATR-spectroscopy – Biomembranes – Silanization – Adsorption.

1. Introduction

A prerequisite for the application of infrared attenuated total reflection (ATR) spectroscopy is the close contact of sample and internal reflection plate. Generally this is achieved easily when the biological material (membrane, protein, cell etc.) are in the dry or hydrated state. However, detachment of the sample may occur on addition of liquid water, especially when the surface of the reflection plate is hydrophilic like that of germanium. In this case immobilization of biological material can be

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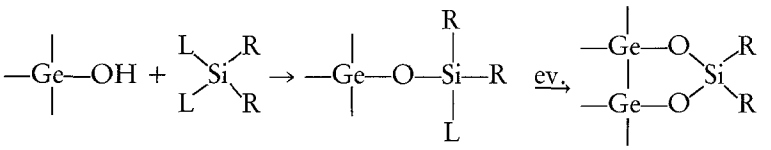
achieved by chemical modification of the germanium surface. Like, glass, germanium-plates are covered with a film of hydroxylic groups. Thus methods for immobilization on glass should be successful on germanium, too. Immobilizations on glass have been described by Weetall (1969a, b), Robinson et al. (1971), Baum et al. (1972) and Jacobson et al. (1978). The first step of such a procedure consists always in a silanization reaction. The silane compounds generally used possess at least one leaving group (*L*) and one functional group (*R*). The latter is of importance for the properties and further use of the plates.



L = leaving group; R = functional group

Sylanization with hydrophobic functional groups (*R*) enables adsorption of the sample by hydrophobic interaction. It may be applied for example to the binding of uncharged lipid bilayer membranes for investigation in aqueous environment. This adsorption effect is obtained again by means of hydrophobic internal reflection plates such as zinc selenide (ZnSe) and cadmium teluride (CdTe) as demonstrated on the one hand by a model membrane consisting of the lipid extract of the electric organ of *Torpedo marmorata* doped with the enzyme acetylcholinesterase (Fringeli, 1976) and on the other hand by a model membrane consisting of lecitin multibilayers doped with the peptide antibiotic alamethicin (Fringeli and Fringeli, 1979). In the latter case the membrane was in contact with liquid water for several days without showing significant loss of lecithin. However, if membranes, proteins or cells with a negative surface charge (e.g., erythrocytes) shall be adsorbed, the group R must be selected in such a way that the surface of the Ge-ATR-plate becomes positively charged. This can be achieved when *R* is an amine which is protonated at biologically reasonable pH-values. On the other hand a negatively charged Ge-surface can be obtained by the reaction of the aminosilylated surface mentioned above with succinic anhydride, resulting in $-COO^-$ as a functional group.

Furthermore the use of silylated Ge-ATR-plates enables the investigation of the adsorbed biological system under the influence of an electric field. Since field strengths in the order of 10^5 V/cm may easily be achieved in aqueous environment these experiments open a new field of biologically most interesting experiments.

2. Materials and Methods

2.1. ATR-IR *Spectroscopy*

The spectra were scanned with a Perkin Elmer Mod 325 infrared spectrometer, equipped with a KBr-grid polarizer. Single pass germanium internal reflection plates (50 × 20 × 1 mm) obtained from Harrick Sci. Corporation, Croton Dam Road, Box 867, Ossining, NY 10562, were used with an angle of incidence of 45 degrees. For a

detailed discussion of ATR-IR-spectroscopy the reader is referred to Harrick (1967).

2.2. Silanization

The plates have been polished on a nylon cloth by means of diamond spray (¼ μ) followed by ultrasonic cleaning with ethanol and short plasma cleaning. After silanization ultrasonic cleaning with ethanol was repeated in order to remove loosly bound silane. The purity of the plates and the amount of the silanization were determined spectroscopically.

Organic Silanization. The reaction mixture was composed of 1% silane in 180 ml anhydrous toluene which was dried by molecular sieve (4 Å). The reaction time at 100° C was 1 to 2 h for aminopropyltriethoxysilane (aminopropyl Ge plates) and 70 h for dimethyldiethoxysilane (dimethyl Ge plates) as well as for ethyltriethoxysilane (ethyl Ge plates), respectively.

Aqueous Silanization. The reaction was carried out in a 10% aqueous solution of silane according to a modified method of Weetall (1976). After addition of silane the pH was adjusted to 4.5 with 5*n* HCl. The reaction was performed at 90° C for 24 h.

2.3. Covalent Coupling of Acetylcholinesterase to Aminopropyl Germanium Plates

The coupling was carried out according to a modified method of Baum et al. (1972). An aminopropyl germanium plate was placed in 22 ml 0.2 M phosphate buffer at pH 4. This solution was degassed by evacuation. 250 μl of dissolved acetylcholinesterase (15 I.U./μl) and 24 mg N-cyclohexyl-N′-[2-(4-morpholinyl)-ethyl]-carbodiimidemethyl-p-toluene-sulfonate were added. The reaction mixture was kept at 4° C for 16 h. Now the plate was washed with buffer solution and distilled water and dried at 95% humidity. The amount of coupled enzyme was determined spectroscopically in a N_2-flow saturated with D_2O(g) in order to avoid overlapping of the amid I band with the H_2O bending absorption band of bound water.

2.4. Succinylation of Aminopropyl Germanium Plates

An aminopropyl germanium plate was placed in 180 ml anhydrous acetone containing 9 g succinic anhydride (50 g/l) and 9 ml pyridin (5%). Drying was performed by means of molecular sieve (4 Å) during about 24 h. The reaction mixture was kept at 70° C for 6 h. Afterwards the plates were thoroughly cleaned with acetone and ethanol. The extent of succinylation was determined spectroscopically.

2.5. Adsorption of Erythrocytes to Aminopropyl Germanium Plates

Human erythrocytes of CPD-blood were washed three times with isotonic phosphat-buffer, packed by centrifugation and diluted to 25% hematocrit[1]. This solution was poured into the ATR cell (aminopropyl Ge plate), replacing isotonic solution. After a few minutes the non-adsorbed erythrocytes were washed out with isotonic buffer-solution.

3. Results and Discussion

3.1. Silanization

A. Neutral Hydrophobic Surface: Dimethyldiethoxysilane and Ethyltriethoxysilane (Organic silanization). The spectrum of bound dimethyldiethoxysilane shows only three weak absorption bands at 2930/2960 cm^{-1} which are assigned to CH_3-stretching vibrations and at 1265 cm^{-1} resulting from $Si\text{-}CH_3$-rocking vibration, respectively (Bellamy, 1966, Fig. 1).

As demonstrated by Figure 2 dimethyldiethoxysilane binding to a Ge surface levels off thus indicating saturation of surface covering.

Although the Ge surface appears macroscopically completely hydrophobic it must be assumed from Figure 3 that the monomolecular ethyl- or methylsilane layer

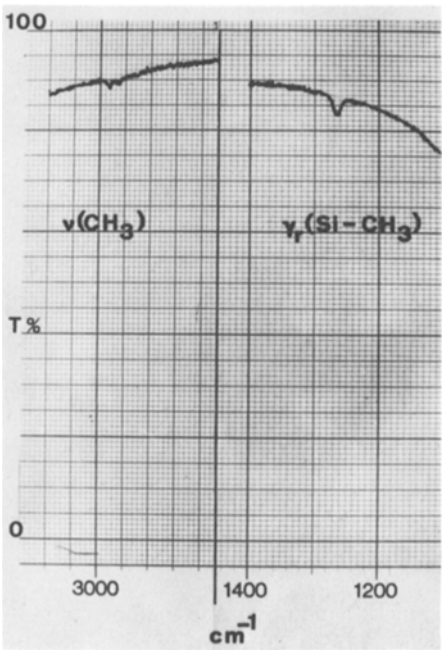


Fig. 1. Infrared-ATR spectrum of a germanium internal reflection plate silanized with dimethyldiethoxysilane for 70 h. Parallel polarized incident light, angle of incidence 45°, number of internal reflections: 50

1 The erythrocytes were prepared by H. Müller, Laboratory for Biochemistry, Swiss Federal Institute of Technology, CH-8092 Zürich

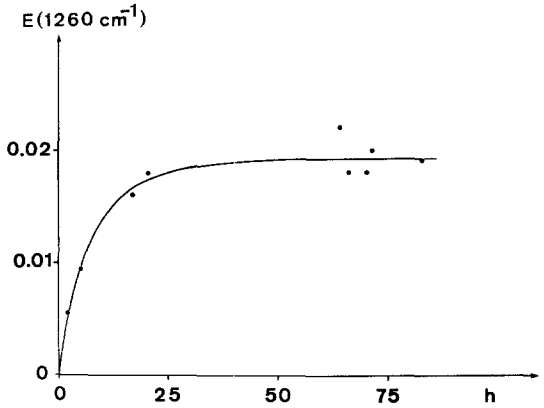


Fig. 2. Amount of bound dimethyldiethoxysilane as a function of the reaction time monitored via the CH_3-rocking vibration, $\gamma_r(CH_3)$ at 1260 cm^{-1}

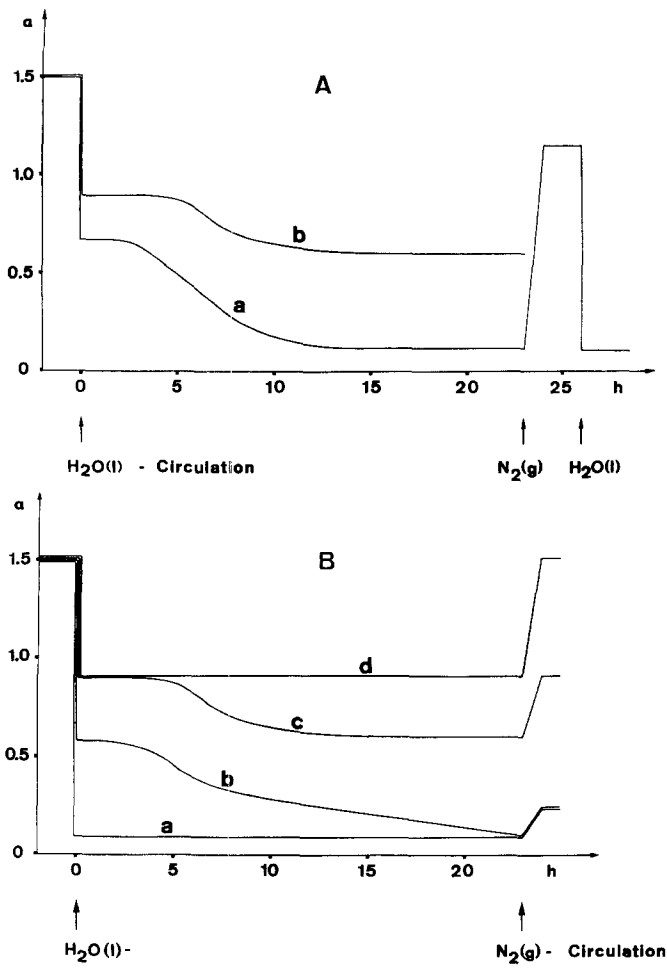


Fig. 3. Amount of lecithin adsorbed to silanized Ge-plates as a function of the time of interaction with circulating liquid water (flow rate: 1.3 cm/s, 25° C). **A** Adsorption behaviour of lecithin on two germanium plates which have been exposed to ethyltriethoxysilane for 20 h (*a*) and 70 h (*b*) at 100° C in toluene. **B** Adsorption behaviour of lecithin on germanium which was untreated (*a*), treated with dimethyldiethoxysilane (*b*), ethyltriethoxysilane (*c*) for 70 h at 100° C in toluene, and exposed to aminopropyltriethoxysilane (*d*) for 24 h at 90° C in aqueous solution, respectively

is not completely compact on a microscopic level. Figure 3 shows plots of absorption coefficients $\alpha = -\ln T$ of C = O stretching vibrations [ν(C = O)] of the lecithin membrane as depending on the time of interaction with circulating liquid water. The flow through the ATR cell was 2.0 cm^3/min resulting in a flow rate of 1.3 cm/s in the cell (cross-section 0.026 cm^2). Figure 3B shows typical adhesion behaviour of the model membrane on untreated, hydrophilic Ge surface (*a*), methyl- (*b*), ehtylsilanized (*c*) and aminopropyl-silanized (*d*) Ge-ATR plates, respectively. Figure 3A shows the influence of the duration of ethylsilane treatment of Ge-ATR plates on membrane adhesion. It demonstrates that adsorption of lecithin multilayers is significantly increased when the treatment is extended from 20 h (*a*) to 70 h (*b*). However, mass-loss could not be completely avoided by extending the reaction time. Furthermore it should be noted that the sudden change of the absorption coefficient by a factor 0.6 is generally observed after addition of liquid water to the adsorbed sample. This effect must be ascribed primarily to the fact that the magnitude of the components of the IR electric field in the rarer medium (E_x, E_y, E_z) depends on the refractive indices and on the ultrastructure of the sample (Harrick, 1967; Fringeli, 1979). On the other hand mass-loss is indicated by a further decrease of the absorption coefficient. In order to get information on the amount of mass-loss during interaction with liquid water the sample was dried again at the end of each experiment.

The surprising behaviour of lecithin multilayers adsorbed to both dimethyl- and ethylsilanized Ge ATR plates (duration of silanization 20 h) should be mentioned. These samples exhibit a continuous decrease of the signal intensity which levels off after about 20 h, indicating that a considerable amount of the membrane has detached from the ATR plate and is no longer within the penetration depth of the IR-electric field in the rarer medium. Under the given conditions the penetration depth is calculated to be 0.4 μ (Harrick, 1967). The thickness of a typical membrane was 10—15 double layers, i.e., 0.07—0.1 μ in aqueous environment. From the decrease of intensity of the ν(C = O) band [Fig. 3A (*a*)] one has to conclude that after an initial periode of 12 h up to 90% of the membrane is located outside of a distance of 1 μ from the surface of the ATR plate. (At 1 μ the electric field intensity in the 6 μ wavelength region is only about 8% of that at the surface of the reflection element). However, the strong intensity decrease cannot be explained only by desorption and solubilization of lecithin multilayers because a considerable amount of ν(C = O) intensity is regained after drying of the sample. Any further addition of water suddenly leads to desorption of the same amount as observed immediately before drying, c.f. Figure 3A (*a*). In a typical experiment $\sim$ 30% of the lecithin membrane detached completely after 20 h of exposure to liquid water in a closed cycle. This amount was enhanced to $\sim$ 50% after 81 h, although no significant decrease of the ν(C = O) intensity was detectable in liquid water within 20 and 80 h. These observations support the interpretation that membrane fragments with extensions of at least several microns detach partially from the ATR plate predominantly by mechanical interaction with the water flow. One should assume that the slow process of membrane detachment could be ascribed to the separation of the first lecithin monolayer (hydrocarbon chains) from the hydrophobic surface of the Ge plate. Furthermore, rearrangement of the generated hydrophobic membrane surface should be expected in order to get a hydrophilic surface contacting with liquid water. Such a

molecular rearrangement would prevent readsorption to the methyl silane treated surface during the first hours after drying, resulting in rapid detachment of the membrane after addition of liquid water in contrast to the behaviour of the original membrane, c.f. Figure 3A (*a*). Under certain conditions rearrangement of oriented assemblies of lipid bilayers may occur as reported by Kopp et al. (1975). It should be noted, however, that the detachment of large membrane areas (up to 0.5 cm^2) could not be observed with germanium plates treated for 70 h with dimethyldiethoxysilane or ethyltriethoxysilane, c.f. Figure 3B (*c*). That means that enhanced surface hydrophobicity prevents the detachment of large membrane fragments. The increase of the absorption coefficient α on drying after 24 h of liquid water circulation can be ascribed to the same phenomenon as the reduction of α upon addition of water at time zero, see above. Nevertheless one has to conclude that ethylsilylated Ge ATR plates should only be used if contact of the membrane with liquid water is limited to a few hours and if there is no water circulation required.

However, if the Ge ATR plate is coated with an aminopropylsilane monolayer, c.f. Sect. 2.2. the adhesion of the first lecithin monolayer is enhanced by electrostatic interaction. The corresponding multibilayer membrane does not show any mass-loss when exposed to circulating water for 24 h [Fig. 3B (*d*)]. Since aminopropylsilane

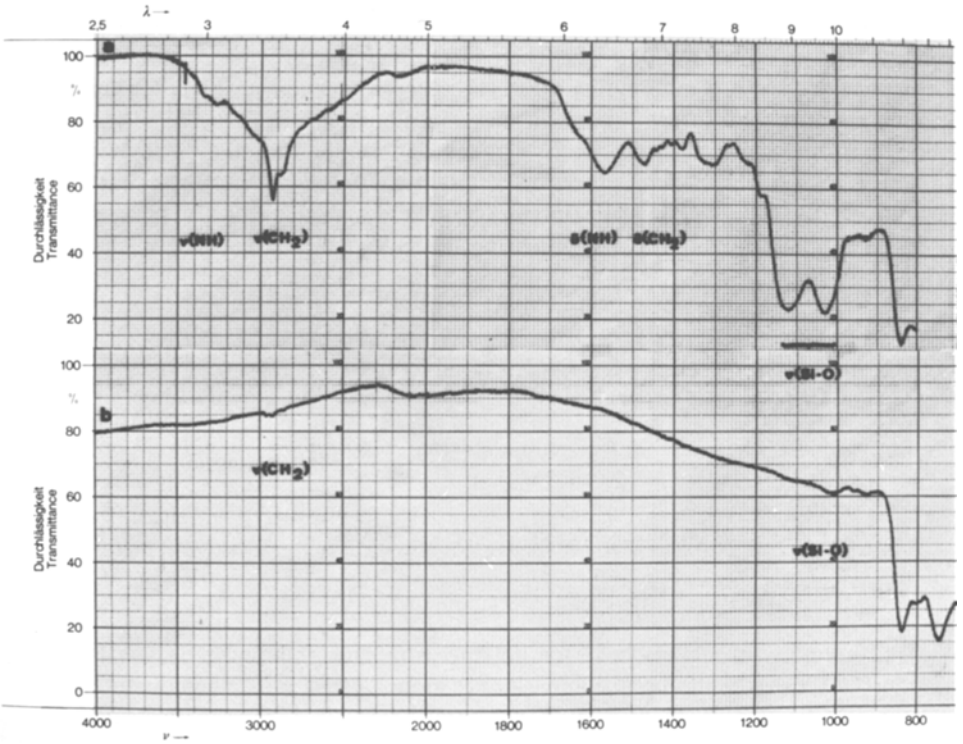


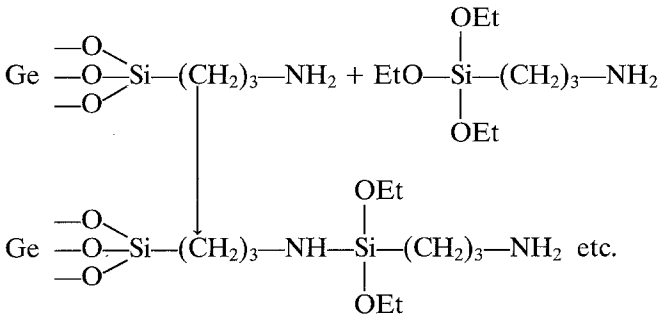
Fig. 4. Infrared ATR spectrum of a germanium internal reflection plate silanized with 1% aminopropyltriethoxysilane in toluene for 1.5 h at $T = 100°$ C (*a*) and with 10% aminopropyltriethoxysilane in aqueous solution, pH 4.5, for 24 h at 90° C (*b*). Parallel polarized incident light, angle of incidence: 45°, number of internal reflections: 50

exhibits a positive surface charge under most biologically relevant pH-values it can furthermore be used to adsorb negatively charged membranes, e.g., erythrocytes, which generally cannot be attached to neutral surfaces in aqueous environment. This subject will be discussed in the following section.

Ge-ATR Plate with Surface Charge. In the preceding section use of hydrophobic interaction was made in order to adsorb biological materials to ATR plates. If, however, a membrane exhibits a significant surface charge, adsorption may not necessarily be performed either to a hydrophobic or to a hydrophilic surface. In such a case the surface of the ATR plate must be chemically modified in order to get a surface charge of opposite sign thus resulting in electrostatic binding of the membrane.

A positively charged germanium surface may be obtained by silanization with aminopropyltriethoxysilane resulting in bound $-NH_3^+$ groups at biologically relevant pH values. On the other hand, a negatively charged germanium surface may be obtained by succinylation of amino groups already bound to the surface, thus resulting in free $-COO^-$ groups under the most biologically relevant pH conditions.

B. Positive Surface Charge: Aminopropyltriethoxysilane. Organic Silanization. The IR-ATR spectrum of a germanium plate which was silanized for 1.5 h in toluene (c.f. Sect. 2.2.) is presented in Figure 4a. There is a remarkable difference in the reaction behaviour of dimethyldiethoxysilane and aminopropyltriethoxysilane because in the latter case in much shorter time much more substance is bound to the germanium surface. Furthermore no saturation is obtained as observed with dimethyldiethoxysilane (Fig. 2). One must expect therefore that aminopropyltriethoxysilane reacts not exclusively with the –OH groups of the germanium surface but also with $-NH_2$ groups of bound aminopropylsilane, as proposed in the reaction scheme below.



Furthermore strong evidence for polymerization of aminopropylsilane is also obtained via an estimation of the intensity per monolayer of the antisymmetric CH_2-stretching vibration at 2925 cm^{-1}. It is assumed that all three ethoxygroups have reacted with the germanium surface, i.e., only the $-CH_2$-groups of the aminopropyl chain are involved in this band. The area per molecule is estimated from a CPK molecular model to be ~ 25 Å^2. Using a standardized absorption coefficient for the CH_2- anti-symmetric stretching vibration of M_0^2 [ν_{as} (CH_2), 25° C] =

$4.1 \cdot 10^{-19}$ cm^{-2} · functional group^{-1} · reflection^{-1} one can estimate the corresponding absorption coefficient per monolayer by means of straightforward procedure described by Fringeli (1980). It is found for parallel polarized incident light to be $\alpha_{\parallel}[\nu_{as}(CH_2)] = 2.46 \cdot 10^{-2}$ per monolayer aminopropylsilane ($\theta = 45°$, 50 reflections), resulting in a transmission $T^{\parallel} = 97.6\%$. Comparing this value with the actual transmission of $\nu_{as}(CH_2)$, which is < 80% (Fig. 4a) one concludes that considerable polymerisation must have occurred. Earlier Weetall (1976) obtained some evidence for polymerisation of aminopropyltriethoxysilane in organic solvent since he observed much higher loadings of ethylamine than by aqueous silanization (see below). Furthermore, the silane appeared patchy and was found to be less resistant. On the other hand Jacobson et al. (1978) reported that the ethylamine coupling to glass beads in organic solvent reaches saturation after 6 days. However, in the view of the observations mentioned above the latter result seems open to be question. Rather, it appears that the bound material is highly polymerized after such a long reaction time.

Aqueous Silanization. A drastic reduction of the degree of polymerization is obtained when silanization is performed in aqueous solution as demonstrated by Figure 4b. It should be noted that the transmission of $\nu_{as}(CH_2)$ at 2925 cm^{-1} is $T^{\parallel} = 97.4\%$ which is close to the theoretical value of 97.6%, see above. At present an accurate assignment of the two bands at 1125 cm^{-1} and 1030 cm^{-1} is not available. However, it is know that Si–O–C, Si–O–Ge and N–Si–O stretching are involved (Bellamy, 1966).

C. Negative Surface Charge: Succinylation. Figure 5b shows the ATR-IR spectrum of a succinylated aminopropyl germanium plate. The amide I band at 1635 cm^{-1} and the amide II band at 1550 cm^{-1} as well as the C = O stretching band of –COOH at 1725 cm^{-1} and the absorption band at 1260 cm^{-1} which is typical for the carboxylic group (Bellamy, 1966) give evidence for successful succinylation. This reaction opens two possibilities of immobilisation. On the one hand positively charged particles can be adsorbed since in aquous solutions the carboxylic group is negatively charged under most biologically relevant conditions. On the other hand coupling of proteins can now be achieved by selective chemical activation, of the carboxylic groups of the silane. This method has the advantage that cross-linking of protein molecules can be excluded which is not the case when the carboxylic groups of the enzyme itself are chemically activated in order to react with an amino silylated surface.

3.2. Covalent Coupling of Acetylcholinesterase (AChE) *to Aminopropyl Germanium Plates*

AChE was covalently bound to the alkylamine surface of germanium by means of carbodiimide activation of carboxylic groups of the enzyme (c.f. Sect. 2.3.). In order to avoid complete drying the sample was kept under a nitrogen flow of ~ 90% relative humidity at 25° C. The amount of bound enzyme can easily be checked via the intensities of the amide I (~ 1650 cm^{-1}) and amide II (~ 1540 cm^{-1}) bands,

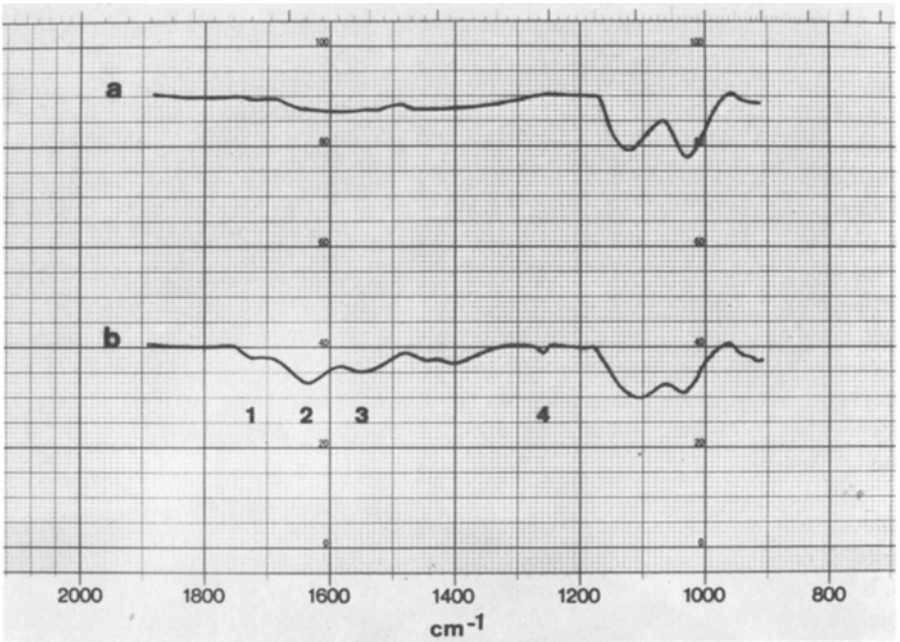


Fig. 5. Infrared-ATR spectrum of a germanium internal reflection plate silanized with aminopropyltriethoxysilane in toluene (*a*) and of the same plate after succinylation for 8 h at $T = 70°$ C (*b*) in acetone. Parallel polarized incident light, angle of incidence: 45°, number of internal reflections: 50. Assignment: $\nu(C=O)_{COOH} = 1$; Amide I = 2; Amide II = 3; $\nu(CO)_{COOH} + \delta(OH)_{COOH} = 4$

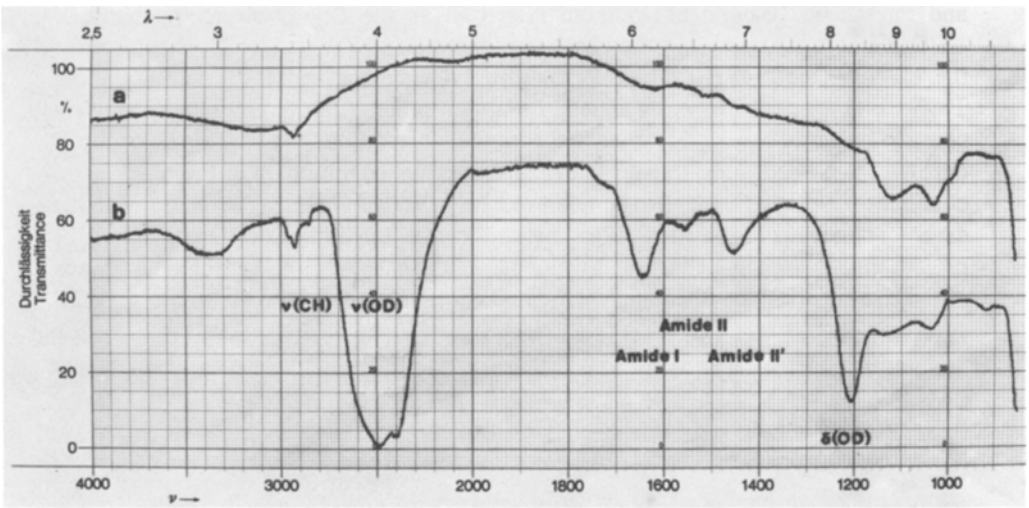


Fig. 6. Infrared-ATR spectrum of a germanium internal reflection plate silanized with aminopropyltriethoxysilane in toluene (*a*) and of the same plate after coupling of acetylcholinesterase by means of carbodiimide for 16 h at $T = 4°$ C in phosphate buffer at pH 4 (*b*). Parallel polarized incident light, angle of incidence: 45° C, number of internal reflections: 50, $T = 25°$ C. Sample kept in $N_2/D_2O(g)$ atmosphere at ~ 90% relative humidity

respectively. However, it must be kept in mind that in the case of $N_2/H_2O(g)$ flow about $1/3$ of the intensity of the amide I band results from the H_2O bending vibration. For that reason AChE was hydrated in a $N_2/D_2O(g)$ flow, Figure 6b. The ATR-IR spectrum presented in Figure 6b was scanned after $\sim$ 12 h of sample exposure to a $N_2/D_2O(g)$ atmosphere of $\sim$ 90% relative humidity at 25° C. The practically complete exchange of hydrogen by deuterium is manifested by the shift of the amide II vibration ($\sim$ 60% N–H bending) from $\sim 1540\ cm^{-1}$ to $\sim 1430\ cm^{-1}$. In order to determine the quantity of bound enzyme, knowledge of the standardized absorption coefficient per functional group, per internal reflection and per cm^2 is required, c.f. Fringeli (1980). For the amide I absorption band one obtains a value of M_0^2 (amide I, 25° C) $= 1.0 \cdot 10^{-16}\ cm^{-2} \cdot$ functional group$^{-1} \cdot$ reflection^{-1} (Fringeli and Fringeli, 1979). On the other hand the absorption coefficient of the amide I′ vibration of the bound AChE was found to be α (amide I′ $= -\ln T = 0.187$ for perpendicularly polarized light. Assuming a molecular weight of 260,000 for AChE (Leuzinger et al., 1969) and a mean molecular weight of 100 per amino acid the number of peptide bonds per AChE molecule is estimated to be $\sim$ 2600. Based on these assumptions the surface concentration of AChE bound to alkylsilane is $\sim 50 \cdot 10^{-13}\ mol/cm^2$, i.e., $\sim 3 \cdot 10^{11}\ molecules/cm^2$. The uncertainty of these values is estimated to be smaller than $\pm\ 2 \cdot 10^{-13}\ mol/cm^2$. The resulting area per AChE molecule is $3.3 \cdot 10^4\ Å^2$ which corresponds to a circular area with 200 Å diameter provided that homogeneous binding of the enzyme has occurred. This, however, is not necessarily the case, because the coupling technique applied also leads to cross-linking between protein

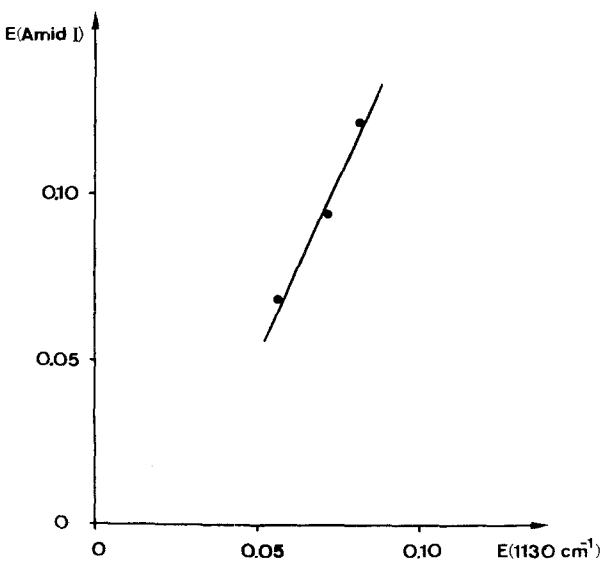


Fig. 7. Amount of bound acetylcholinesterase as a function of the amount of bound aminoalkylsilane (organic silanization, degree of polymerization, c.f. Sect. 3.1.B). E (1130 cm^{-1}): Extinction coefficient of C–O-stretching vibration of the ethoxy group; E (amide I′): Extinction coefficient of the amide I′ band at 1635 cm^{-1}. Germanium internal reflection plate, parallel polarized incident light, angle of incidence: 45°, number of internal reflections: 50, $T = 25°$ C. Sample kept in $N_2/D_2O(g)$ atmosphere at $\sim$ 90% relative humidity

molecules (c.f. Sect. 2.3. and 3.1.B). The latter assumption is also supported by preliminary electron micrographs which reveal a homogeneous distribution of spherical particles with diameters between 150 Å and 1500 Å. Furthermore it was found that the amount of enzyme bound under equal conditions to aminoalkyl layers depends significantly on the degree of polymerisation of the silane, Figure 7. Since the number of free $-NH_2$ groups should not alter during polymerisation, one is lead to the conclusion that the efficiency of protein coupling is increased when the distance of the amino group from the surface is increased, evidently due to less steric hinderance.

3.3. Adsorption of Erythrocytes to Aminopropyl Germanium Plates

Until recently the application of ATR-IR spectroscopy in membrane research was limited to lamellar membrane systems which, due to their oriented structure, are optimum for structural studies on a molecular level (Fringeli et al. 1972; Fringeli, 1977; Fringeli and Fringeli, 1979). Such membrane systems are generally not suited for studies of transport across the membrane. However, if it becomes possible to adsorb closed membrane systems, such as vesicles or erythrocytes to an internal reflection plate, ATR-IR spectroscopy offers a promising tool for direct observation of membrane transport processes. Using conventional ATR-technique the smallest detectable concentration of a small solute molecules is in the order of $c_{min} = 10^{-3}$ M. Because the penetration depth of the infrared beam into the rarer medium is in the order of microns (Harrick, 1967) only that part of the solution which is in direct contact with the ATR plate can be monitored, i.e., a very small fraction of the total volume of the ATR cell. Therefore a substance S which is enclosed in vesicles or

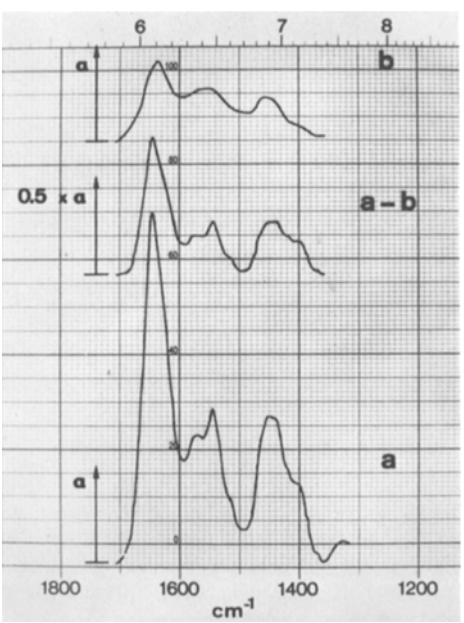


Fig. 8. Infrared-ATR spectrum of human erythrocytes in isotonic D_2O solution, pH 7.4, adsorbed to a aminopropylsilylated germanium internal reflection plate. Absorption spectra: $\alpha = -\ln T$. (*a*) Native erythrocytes; (*b*) Erythrocytes after hemolysis by osmotic shock; (*a–b*) Difference spectrum between (*a*) and (*b*), i.e., absorption spectrum of hemoglobin in the native environment. Scale expansion: × 0.5. Parallel polarized incident light, angle of incidence: 41°, number of active internal reflections: 45, $T = 25°$ C

erythrocytes with a concentration $c_s > c_{min}$ can be detected as soon as the closed membrane system is adsorbed to the ATR plate. However, when S leaves the interior of the closed system, e.g., by diffusion, the corresponding infrared signal will vanish because outside the dilution is so high that $c_s \ll c_{min}$. The following results are part of a more extended investigation which will be published elsewhere (Fringeli et al., 1980). In this experiment human erythrocytes have been adsorbed to an aminopropyl germanium ATR plate by pouring isotonic buffered solution of erythrocytes in D_2O in a closed cycle for several minutes into the ATR cell. Adsorption was directly monitored via the amide I band which appeared very intense because of the large amount of hemoglobin enclosed. As soon as the band intensity remained constant the non-adsorbed erythrocytes were washed out by an isotonic buffered solution in D_2O. Afterwards the spectrum was scanned in the amide I/II region, Figure 8 (*a*). It should be noted that partial *H-D* exchange of the enclosed hemoglobin has already occurred as revealed by the shift of the amide II band from $\sim 1550\ cm^{-1}$ to $\sim 1430\ cm^{-1}$ Figure 8 (*a*), (*a–b*). In order to demonstrate that the IR signals of substances enclosed in the erythrocytes with $c_s \gg c_{min}$ vanish as soon as the substance leaves the interior, hemolyses was initiated by an osmotic shock. Figure 8 (*b*) shows the IR-ATR spectrum of empty erythrocytes, whereas Figure 8 (*a–b*) shows the difference spectrum of 8 (*a*) and 8(*b*), i.e., the spectrum of hemoglobin in the native environment of the intact erythrocyte.

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