

IN SITU ADSORPTION OF BOVINE SUBMAXILLARY MUCIN AT THE MICA/AQUEOUS SOLUTION INTERFACE

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ABSTRACT

An in situ adsorption measurement method at the mica/protein solution interface is described.

An apparatus especially constructed for this purpose permits direct and continuous measurement of total adsorption (reversible and irreversible) of ^{14}C -labelled proteins.

Bovine submaxillary mucin (BSM), extracted from salivary glands, was acetylated with $(\text{CH}_3, ^{14}\text{C})_2\text{O}$. The results show the increase of BSM adsorbed on mica surfaces with its concentration in solution and adsorption time. Pseudo plateaux are obtained for all concentrations studied, indicating the formation of thick layers. The loosely bound fraction of adsorbed mucin is proportional to the bulk concentration in solution. The amount of BSM adsorbed increases in the neighbourhood of the isoelectric point of BSM (pH 3).

INTRODUCTION

During recent years various ceramics have been applied as medical and dental implant materials [1,2]. The common feature of all these bioceramics is that SiO_2 forms their primary network. Adding Al_2O_3 or P_2O_5 to a glass composition can result in formation of alumina-silicate or calcium phosphate layers on top of a silica-rich substance. These protective layers are supposed to increase the durability of bioglasses in alkali and acid solutions [3].

When such ceramics are used as biomaterials they interface with the tissue so that the interactions with different physiological constituents such as collagen, mucopolysaccharides and mineral salts take place. All these interactions determine whether an implanted material may be considered as bio-compatible or not in a given physiological environment.

The concept of this work is to study adsorption at the mica-bovine submaxillary mucin solution interface, to get some insight into the behaviour of certain bioglasses in contact with mucosal surfaces.

Muscovite mica is a layer structured aluminosilicate and its basic chemical formula is $\text{KA}l_2(\text{AlSi}_3\text{O}_{10})(\text{OH})_2$ [4].

Mucins, glycoproteins in the mucous secretions of submaxillary glands, have been shown to consist of a polypeptide chain with many relatively small

oligosaccharide side chains linked to the peptide through glycosidic bonds [5]. Their function is to lubricate epithelial cells in the mouth, respiratory, gastro-intestinal and reproductive tracts and to protect them from intimate contact with the external environment [5].

Bovine submaxillary mucin (BSM) is a large, highly asymmetric, rod-like molecule, with a molecular weight of 4×10^6 [6].

In this paper, which is an extension of our earlier investigation of protein adsorption on polymer surfaces [7,8], we describe a new technique of in situ adsorption/desorption measurements of ^{14}C -labelled proteins on mica surfaces.

Direct relationship of these experiments with the measurements of forces between two mica surfaces separated by mucin aqueous solution, which are now being carried out in our laboratory using Israelachvili technique [9], can be anticipated.

EXPERIMENTAL

BSM isolation and radiolabelling

BSM was isolated from fresh salivary glands obtained at a slaughter house by a procedure described by Tettamanti and Pigman [10]. The lyophilized mucin was analysed to determine the sialic acid content which specifically characterizes glycoproteins. Using the periodate-resorcinol method [11], the mucin was found to contain about 30% sialic acid, a value in good agreement with that reported in Ref. [10].

Radiolabelling of the lyophilized mucin by acetylation with [$1\text{-}^{14}\text{C}$] acetic anhydride is described elsewhere [12]. The amount of NH_2 groups acetylated during this reaction and determined by a colorimetric ninhydrin reaction according to Yemm and Cocking [13] was found to be 40%. The total protein concentration after dialysis of acetylated mucin found by the Lowry method [14] was 0.55 mg/ml. Finally its specific activity, estimated by comparison with [^{14}C] hexadecyltrimethyl ammonium bromide solution of known specific activity, was about $4.4 \mu\text{Ci}/\text{mg}$ of dry protein.

The surface tension measurements of acetylated and nonacetylated mucin show that acetylation did not change the surface activity of BSM [12].

Adsorption of BSM at the mica/aqueous solution interface

A new apparatus and technique have been used to measure the adsorption of radiolabelled proteins. The principle of the apparatus is illustrated in Fig. 1. A specially constructed circular glass container with fixing screws enables one to form a cell with a mica window at the bottom. The mica windows are freshly cleaved under a laminar flow cabinet and their thickness is ca. $10 \mu\text{m}$. Molten paraffin is spread onto the flat ground part of the glass cell and the glass container is tightly sealed by means of Viton "O" rings. The cell is filled with ^{14}C -labelled protein (ca. 3 ml) and placed in a special support above the

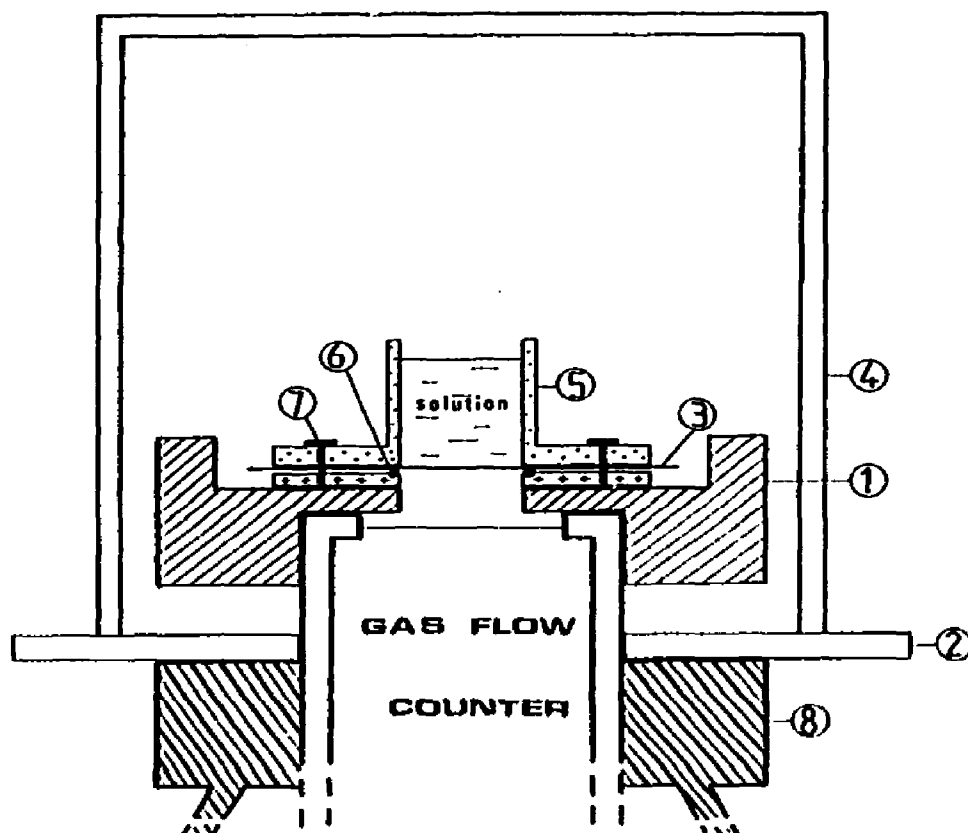


Fig 1. In situ adsorption measuring device. (1), (2), (8) supports ensuring reproducibility of geometrical conditions; (3) mica window; (4) cover; (5) glass cell; (6) "O" ring; (7) cell assembling screws.

gas flow counter which measures the radioactivity and displays it on a recorder as a function of time.

Thus, the in situ adsorption/desorption of proteins on solid surfaces can be measured. Contrary to other commonly used techniques to measure protein adsorption on solid surfaces, our method enables one to distinguish between the reversibly and irreversibly adsorbed fractions of the protein. Two calibrations are required to know the quantity of adsorbed protein molecules. Because the mica windows varied in thickness it was necessary, before each measurement, to determine how much radiation they absorb. This was done using the solid polymer source, in our case [^{14}C] poly(methyl methacrylate).

The absorption coefficient for mucin windows varied within 30–50% range of total radioactivity originating from the source.

Allowance has also to be made for the radioactivity originating from the bulk protein molecules, nonadsorbed at the mica/solution interface but con-

tributing to the total measured radioactivity. This value is proportional to the BSM concentration and may be obtained when instead of [^{14}C]BSM a nonadsorbing substance containing the same radioactive element ^{14}C is used. Aqueous solutions of potassium thiocyanate (K^{14}CNS) which were isotopically diluted to give the same specific activity as that of [^{14}C]BSM were used for this purpose. At low BSM concentrations $< 5 \times 10^{-2}$ mg/ml the bulk radioactivity contribution to the total measured radioactivity is negligible and for adsorption at the highest bulk concentration studied (2×10^{-1} mg/ml) this contribution represented about 20% of the total adsorption value.

The total amount of BSM adsorbed on the mica sample was calculated by means of a calibration graph, previously determined by depositing and drying on mica surfaces known amounts of labelled protein which, when counted, yielded a factor per unit amount of BSM. Knowing the conversion factor and the area of the mica window, the amount of BSM adsorbed ($\mu\text{g}/\text{cm}^2$) was obtained.

All experiments were carried out in triplicate with each sample being counted three times; an overall mean value was then taken.

The amount of mucin irreversibly adsorbed on the mica surfaces was obtained by replacing the BSM solution with water or with a buffer solution in the measuring cell.

It has been recently shown [15] that the reductive methylation of chicken eggwhite lysozyme with $^3\text{HCHO}$ has significant effects on its adsorption behaviour on hydrophilic and hydrophobic surfaces — for example, on both surfaces the labelled material adsorbs preferentially to the unlabelled. The feasibility of using ^{125}I - and $^{99\text{m}}\text{Tc}$ -labelled albumin for adsorption studies on different surfaces has also been questioned [16]. To test if such artefacts

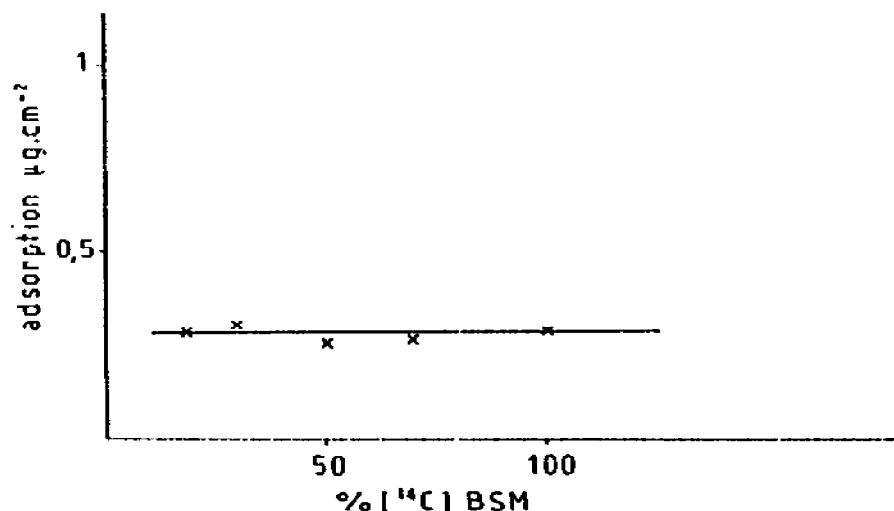


Fig. 2. Test showing the equivalence of adsorption of labelled and unlabelled BSM. Total BSM concentration of the labelled/unlabelled mixture in solution 0.1 mg/ml; $[\text{NaCl}] = 10^{-3} \text{ M}$; adsorption time 5 h.

are involved in the adsorption measurements which we have performed on mica, the percentage of labelled protein was varied from 20 to 100% with constant bulk concentration of 0.1 mg/ml and 0.15 M NaCl.

The results shown in Fig. 2 indicate that neither the labelled nor the unlabelled mucin molecules are preferentially adsorbed.

RESULTS AND DISCUSSION

Rates of adsorption have been determined for various mucin concentrations in bulk solution at constant ionic strength $[\text{NaCl}] = 10^{-3} M$. The results are represented in Fig. 3. The first meaningful points of adsorption kinetics registered with our experimental technique are those after about 15 min from the beginning of adsorption. The curves in Fig. 3 show also that initial rates of adsorption, for all concentrations studied, are high and then a more gradual increase of adsorption takes place. In all cases the adsorption process did not attain the steady-state values and the pseudo plateau was observed on all curves. If the surface concentrations of these adsorption experiments were plotted versus square root of adsorption time, they would yield the curves shown in Fig. 4.

The dashed portions of Fig. 4 are the extrapolation of the curves to the zero adsorption time. Assuming the diffusion-controlled process, the diffu-

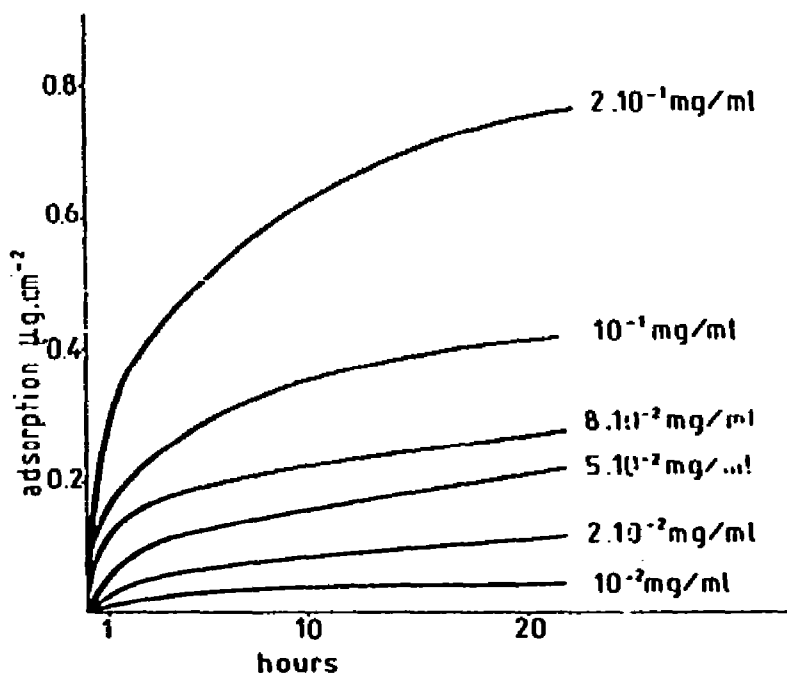


Fig. 3. Kinetics of BSM adsorption on mica for various mucin concentrations $[\text{NaCl}] = 10^{-3} M$.

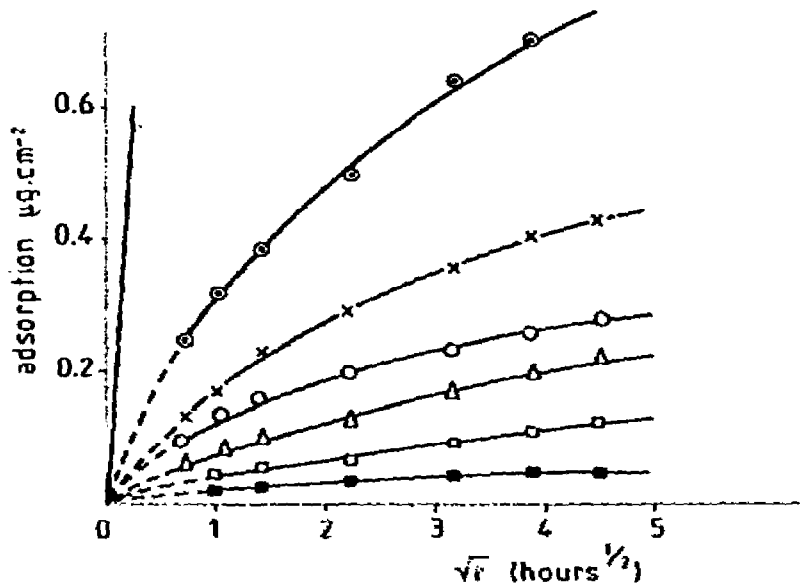


Fig. 4. Adsorption of BSM as a function of $t^{1/2}$ on mica surfaces. BSM concentrations: (●) 2×10^{-1} mg/ml; (×) 10^{-1} mg/ml; (○) 8×10^{-2} mg/ml; (△) 5×10^{-2} mg/ml; (□) 2×10^{-2} mg/ml and (●) 10^{-2} mg/ml in $10^{-3} M$ NaCl. The straight line represents the calculated initial slope for a diffusion coefficient of $2.3 \times 10^{-11} m^2/s$ given by Holly [19] for BSM spread monolayers at 2×10^{-1} mg/ml bulk.

sion coefficient can be calculated from Fig. 4 and from the following equation

$$A = 2c(D/\pi)^{1/2} (t)^{1/2}$$

where c is the protein concentration in solution, D the diffusion coefficient and t the adsorption time.

For a given protein concentration, the initial slope of the curve A vs. $t^{1/2}$ is used to determine the diffusion coefficient D .

As illustrated in Fig. 5 the obtained values for D change with the BSM solution concentration. Such concentration dependence of D may be related to the highly pronounced tendency towards aggregation of mucin molecules in solution. Previous particle size measurements performed with a Coulter Nano-Sizer instrument [12] show that the mean radius of BSM macromolecules at pH 7.1 and $0.15 M$ NaCl increases from 130 nm for 10^{-1} mg/ml to 1000 nm for 4×10^{-1} mg/ml.

Also, other researchers [17,18] found that the size and molecular weights of mucins may be considerably different, depending on their origin, concentration, ionic strength and pH.

From surface tension measurements of BSM saline aqueous solutions, Holly [19] reported a value of $2.3 \times 10^{-11} m^2/s$ for the apparent diffusion coefficient and described this value as a realistic one for macromolecules of

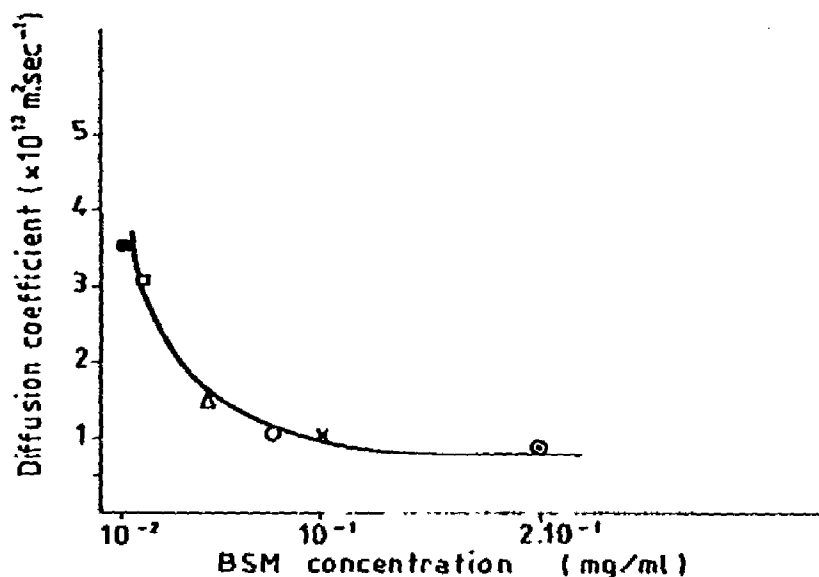


Fig. 5. Concentration dependence of diffusion coefficient of BSM in $10^{-2} M$ NaCl.

this size. Shogren et al. [17], using a light scattering technique, measured the diffusion coefficient of porcine submaxillary mucin in solution and obtained values ranging from $0.4-2.5 \times 10^{-12} \text{ m}^2/\text{s}$ depending on the protein concentration in solution.

If the initial slope of BSM adsorption vs. $t^{1/2}$ were calculated with the apparent diffusion coefficient value given by Holly, then the straight line shown in Fig. 4 would be obtained. On the mica surfaces the adsorption process is much slower. According to Van Dulm and Norde [20], who studied the adsorption of albumin on different solid surfaces, the probable reason that the adsorption process does not follow the diffusion-controlled mechanism is due to the existence of a barrier (electrostatic, orientational or steric) against deposition on the already arrived molecules. They observed such slowing down of adsorption for the system where both the adsorbing surface and the protein molecules were negatively charged. This is also the case of our mica-mucin system. Furthermore, the first force/distance measurements between two mica surfaces covered with adsorbed BSM layers have shown that repulsion occurs in the initial stage of bringing together two mica surfaces. Such repulsion may be explained by electrostatic and steric hindrance [21]. Moreover, when comparing our results for BSM diffusion coefficients on mica surfaces with the value reported by Holly [19] for the BSM apparent diffusion coefficient, it should be borne in mind that the mucin used by Holly was Sigma commercial grade and as such should be different from our highly purified BSM.

In Fig. 6 the total amounts of adsorbed mucin are plotted against the solution concentration. These results demonstrate that thick mucin layers are

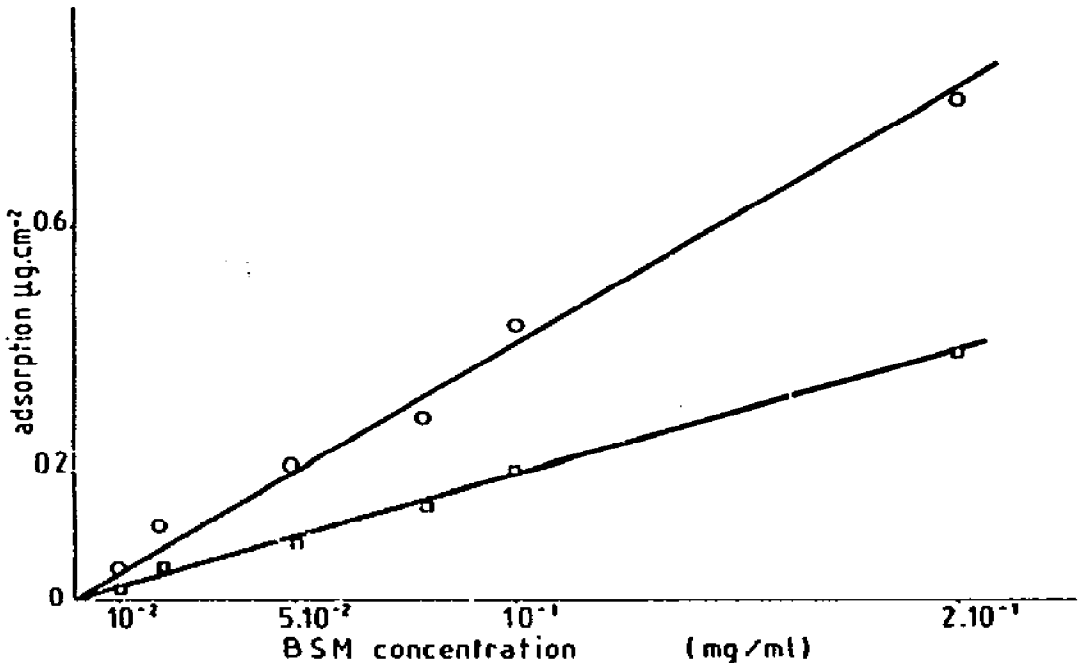


Fig. 6. Total (o) and irreversibly adsorbed (□) BSM on mica vs. BSM concentration; $[\text{NaCl}] = 10^{-3} M$; adsorption time 20 h.

built up on mica surfaces with increasing protein concentration in solution. Also the loosely bound fraction of the adsorbed mucin (the amounts corresponding to the difference between two curves in Fig. 6) increases with the protein concentration in the solution. Similar results were found by us for mucin adsorption on surface oxidized polyethylene films [12] and by Watkins and Robertson [22] for γ -globulin adsorption on silicon rubber using an internal reflection fluorescence technique.

The presence of loosely bound BSM on mica surfaces is also confirmed by force measurements between two adsorbed mucin monolayers on mica performed in our laboratory by means of Israelachvili's technique [9]: On pressing the two solid surfaces together, a part of the adsorbed mucin desorbs, and a part of it remains on the mica surface.

The influence of pH has been studied for the adsorption on BSM at constant ionic strength ($10^{-3} M$). The results in Fig. 7 show that the amount adsorbed depends on pH. It can be seen that BSM adsorption rises in the neighbourhood of the isoelectric point of BSM (pH ca. 3) [23]. Such increase of adsorption in the neighbourhood of the isoelectric point of proteins has already been reported by numerous authors studying protein adsorption on polymer films or polymer lattices [24–27]. It may be attributed to the fact that more BSM molecules can adsorb on a given surface due to their rearrangement to form more complex structures. The rearrangement

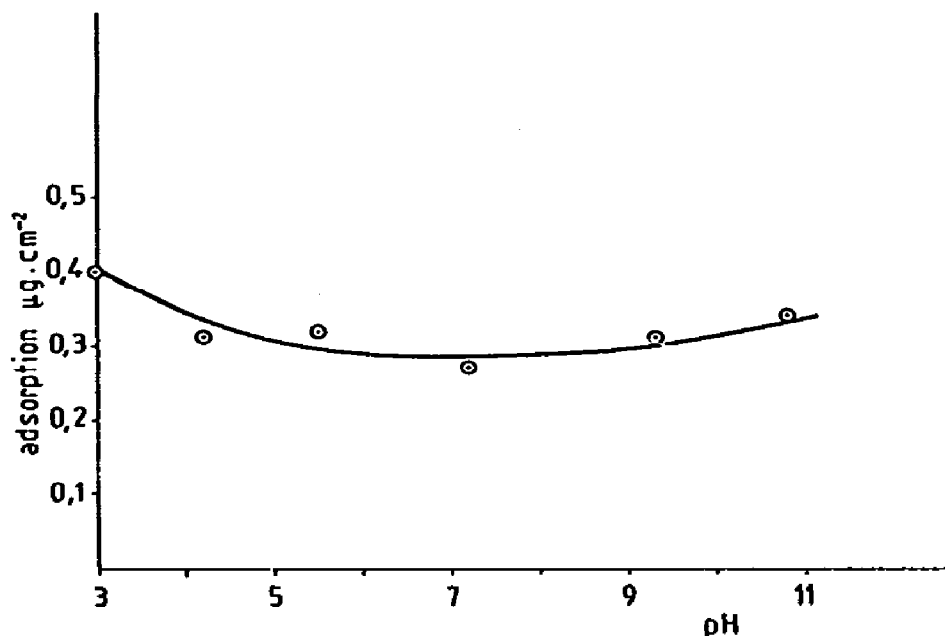


Fig. 7. pH dependence of BSM adsorption on mica. BSM concentration 0.1 mg/ml; [NaCl] = $10^{-3} M$; adsorption time 5 h.

of protein molecules near to the isoelectric point should be favoured by weakening of intramolecular and lateral interactions between adsorbing molecules. The same conclusions concerning the influence of pH on adsorption of BSM molecules were reached by Evans and Blank [28] in their study of mucin adsorption at the polarized mercury/water interface using ac polarography.

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