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# Liposome aggregation in presence of the sweeteners cyclamate and saccharine

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The interaction of the sweeteners saccharine and cyclamate with large unilamellar liposomes and planar bilayer lipid membranes (BLM) was studied. Application of the methods of light scattering and sound velocimetry showed that saccharine induces aggregation of liposomes, while cyclamate probably caused increase of the hydration of liposome surface. The sweeteners induced changes of BLM compressibility in a direction perpendicular to the membrane plane. The cyclamate induced considerably larger decreases in the elasticity module than saccharine. The obtained results show that both saccharine and cyclamate interacts with the surface of lipid bilayer and could modify the physical properties of lipid membranes.

## 1. Introduction

The nonnutritive sweeteners saccharine and cyclamate are widely used in foods for diabetics. These sweeteners have been extensively evaluated for genetic activity. Particularly for calcium cyclamate and its major matabolite cyclohexylamine, there is no evidence for direct genotoxicity [1]. The sweeteners may have, however, cancer-promoting or carcinogenic activity. Additional studies and development of well validated systems have been recommended in order to monitor the possible of site binding the sweeteners and to determine whether there is an increasing risk of cancer in humans that are long-term users of sweeteners [2].

One of the possible targets of sweeteners is the cell surface. In analogy with the effect of saccharides and polysaccharides [3], which cause aggregation of liposomes, this effect is also expected for sweeteners. In this work, we studied whether saccharine and cyclamate can induce aggregation of large unilamellar liposomes composed either of egg yolk phosphatidylcholine (eggPC) or dioleoylphosphatidylcholine (DOPC) and whether these sweeteners change the mechanical properties of the membrane. Under normal physiological conditions (pH 7.4), saccharine is a neutral molecule, while cyclamate is negatively charged. In the past it has been shown that in addition to turbidity [4], the method of measurement of sound velocity in a liposome suspension [5] is an effective method to study aggregation of liposomes. The latter method is also useful to study interactions of various low or high molecular compounds with membranes [6]. In addition we used the method of electrostriction on planar bilayer lipid membranes (BLM) [6] to determine whether the sweeteners induce changes of BLM compressibility in direction perpendicular to its plane.

## 2. Investigations, results and discussion

Fig. 1 shows the changes of decadic turbidity of the suspension of liposomes composed of DOPC as a function of saccharine (curve 1) and cyclamate (curve 2) concentrations. The opposite effect of sweeteners on the light dispersion is observed. While saccharine induced decreases in decadic turbidity (corresponding to increase of light dispersion), cyclamate had the opposite effect. In addition, while the changes of turbidity induced by saccharine were monotonous, those induced by cyclamate were biphasic. At relatively low concentrations (0–0.3 mM) the turbidity

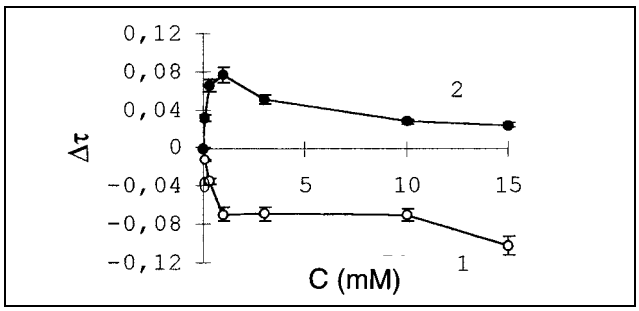


Fig. 1.: Dependence of decadic turbidity, τ, of liposomes composed of DOPC on concentration of 1-saccharine and 2-cyclamate. $\tau = -(1/L)\log(I/I_0)$ (L is the optical path length, I-intensity of light measured at certain concentration of sweetener, $I_0$-light intensity without sweetener). Results represents mean ± S.D. obtained in three independent experiments for each sweetener

increases, while at $c > 0.3$ mM it decreases and reaches saturation at $c \sim 15$ mM. In contrast to saccharine, the turbidity of liposome suspensions in the presence of cyclamate is positive at all concentration ranges studied. The effect of saccharine could be explained by formation of liposome aggregates. The effect of cyclamate is not ambigous from the turbidity study. We therefore investigated how the sweeteners influence the sound velocity in liposome suspension. The changes in the so-called concentration increment of the sound velocity: $[u] = (u - u_0)/cu_0$ were determined, where $u_0$ is the sound velocity in buffer and u is the sound velocity in liposome suspension at a certain concentration of sweetener, c. The results of the experiments are presented on Fig. 2a, b. Fig. 2a shows the plot of the value [u] as a function of saccharine concentration in the liposome suspension composed of eggPC (curve 1) and DOPC (curve 2). It was found that for both liposome compositions saccharine induces decreases in the concentration increment of sound velocity. A more pronounced effect of saccharine on eggPC liposomes in comparison with DOPC liposomes could be associated with the larger inhomogeneity of the lipid bilayer of these liposomes and with the content of polyunsaturated fatty acids, that enhance liposome aggregation [5]. An opposite effect on the [u] value was observed for cyclamate (Fig. 2b). Cyclamate induced an increase in the [u] value. The decrease in the [u] value induced by saccharine supports the conclusion of liposome aggregation derived from turbidity measurements. As previously shown [5], due to liposome

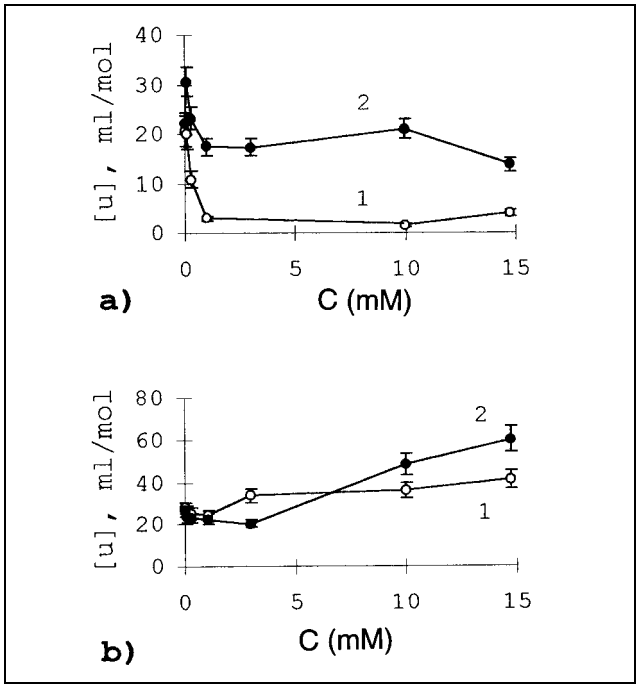


Fig. 2.: Dependence of concentration increment of sound velocity, [u], on concentration of a) saccharine; b) cyclamate in liposome suspension composed of 1-egg PC and 2-DOPC. Results represent mean ± S.D. obtained in three independent experiments for each sweetener.

aggregation decrease in hydration of the liposomes takes place. Under the conditions of the experiments (T = 20 °C), the hydrated shell that covers liposomes is well ordered. It is known that decreases in the ordering of hydrated shell or its lost result in decreases in the [u] value. The opposite effect of cyclamate on the [u] value, i.e. its increase with increasing concentration of sweetener, could be evidence for two possible effects: an increase in hydration of liposomes due to binding of cyclamate on the liposome surface, or an increase in the ordering of the lipid bilayer [6, 7].

In order to elucidate the possible mechanism of the effects of sweeteners on the lipid bilayer, the electrostriction of BLM composed of eggPC and cholesterol (3 : 1 w/w) was studied. Cholesterol was used to stabilize BLM. Unmodified membranes were characterized by elastic module $E = (1.90 \pm 0.38) \times 10^7$ Pa (the value of E represents mean ± S.E. obtained for 5 independently measured BLM). Addition of saccharine resulted in a slight decrease of E (Fig. 3a, curve 1), while cyclamate induced considerably larger decreases in the elasticity module (Fig. 3a, curve 2). The effect of cyclamate was more reproducible and almost identical changes in elasticity module were obtained on two independently examined BLMs. In contrast to cyclamate, saccharine induced both decreases, slight increases or even no changes of BLM compressibility. Changes in elastic properties of BLM are a clear evidence for interaction of sweeteners with BLM. More pronounced changes of elasticity module induced by cyclamate suggest a stronger interaction with the BLM surface. Due to the negatively charged cyclamate, repulsive forces appear at polar parts of the membrane. This repulsion might disrupt the membrane structure. Lower ordering of polar parts could then be the reason of higher compressibility of the membrane (i.e. lower value of elasticity modulus, E). To investigate this assumption, experiments on BLM composed of soy bean phosphatidylcholine (SBPC) and cholesterol (3 : 1 w/w) were performed. These phospholipids bear

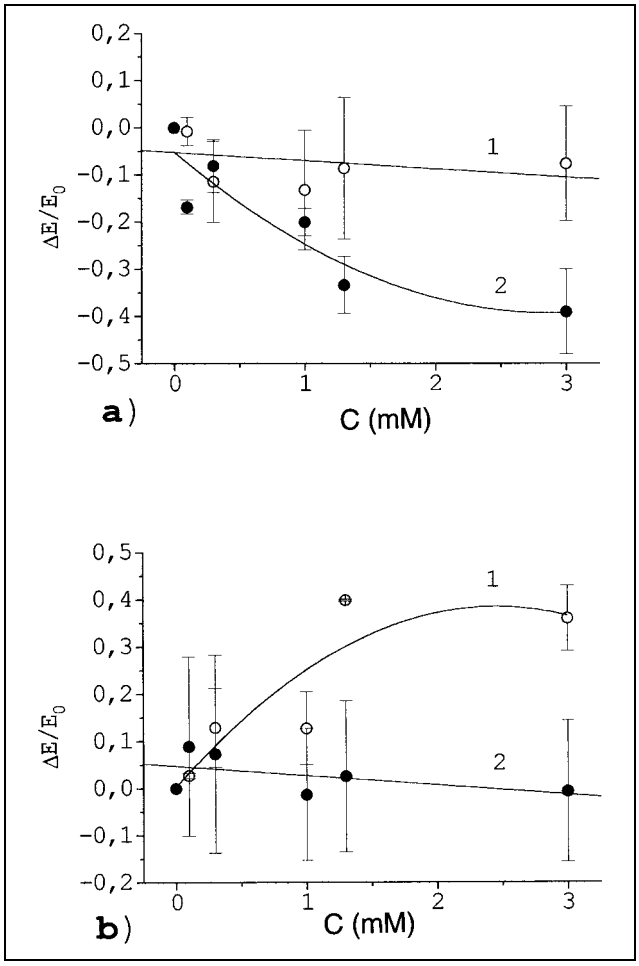


Fig. 3.: Plot of relative changes of elasticity modulus, $\Delta E/E_0$, of BLM on concentration of 1-saccharine and 2-cyclamate for BLM composed of egg PC + cholesterol (a) or SBPC + cholesterol (b). ($\Delta E = e + E_0$, where $E_0$ is elasticity modulus prior addition of sweetener and E is steady-state value of elasticity modulus after addition at certain concentration. The steady state value of E was usually established after 5–10 min following addition of sweetener). Results represent mean ± S.D. obtained on 2–3 BLMs

certain negative charges due to the presence of phosphatidylglycerols. The results of these experiments are presented on Fig. 3b. It is shown, that in this case the most pronounced changes of elasticity module take place for saccharine (curve 1), while only slight changes of elasticity module were observed for cyclamate (curve 2). The results are in considerable contrast with those obtained with eggPC BLMs. Weaker effects of cyclamate on BLM composed of SBPC could be due to the negatively charged surface of SBPC, which makes adsorption of negatively charged molecules of cyclamate more difficult. On the other hand, increases of elasticity module following addition of saccharine could be caused by the shielding of negative surface charges of BLM by saccharine molecules, and thus weaken the repulsive forces at the membrane plane. Certainly, the elastic module of unmodified SBPC BLM $E = (1.03 \pm 0.08) \times 10^7$ Pa was about 1.8 times lower than that for eggPC BLM. We suspect that the lower value of E for negatively charged BLM composed of SBPC might be caused in particular by the presence of negative charges at the membrane surface. The changes of elasticity module for both lipid compositions were not accompanied by substantial changes in the BLM electrical capacitance, as measured by means of amplitude of first current harmonic ($C = I_1/2\pi f U_0$). These changes following addition of sac-

charine or cyclamate at the concentration range studied (0–3 mM) were not higher then 8.7 ± 1.8 %.
We conclude that the sweeteners have surface effects on the liposomes. While saccharine induces aggregation, cyclamate probably caused increases in the hydration of the liposome surface. The study of BLM electrostriction showed an interaction of sweeteners with the lipid bilayer caused by changes in the compressibility of the membrane.

## 3. Experimental

### *3.1. Formation of liposomes, BLM and chemicals*

Large unilamellar liposomes (diameter ~100 nm) were prepared by the extrusion method according to MacDonald et al. [8] using LiposoFast (Avestin Inc., Canada). The liposomes were formed from the mixture of egg phosphatidylcholine (eggPC) or dioleoylphosphatidylcholine (DOPC) (Sigma, USA). The concentration of lipids was 2 mg/ml. The liposomes were prepared in 10 mM KCl + 2 mM HEPES (pH 7.4). Bilayer lipid membranes (BLM) were formed according to the method of Mueller et al. [9] on a circular hole (diameter ~ 0.7 mm) in a wall dividing a Teflon cup into two identical compartments of 3 ml volume each. The lipid solution used was eggPC and cholesterol (Fluka, Switzerland) (3 : 1, w : w) or soy bean phosphatidylcholine (SBPC) (P-1 Biochemicals, USA) and cholesterol (3 : 1, w/w) dissolved in n-heptane (Fluka) (20 mg/ml). The cup was filled with 0.1 M KCl + 2 mM HEPES (pH 7.4). KCl and HEPES (analytical grade) were from Merck (Germany) and Sigma, respectively. Cyclamate was from Aldrich (USA) and saccharine was from Sigma (USA). All experiments were performed at temperature T = 20 ± 1 °C.

### *3.2. Turbidity measurement*

Aggregation of vesicles under the influence of sweeteners was determined turbidimetrically. This method of determination of the degree of aggregation is based on the dispersion of light by colloid particles of the system. The larger the particles, the better the dispersion of light and the higher the intensity of light. The measured cell contained the vesicle suspension while the reference cell was filled with buffer. Sweetener was added to both, sample and reference cells. The measurements were carried out at a wave length $\lambda = 555$ nm using a spectrofluorimeter SD1000 (Ocean Optics Inc., USA). Under these experimental conditions no changes of intensity of light by sweeteners occurred. Using the measured value of light intensity without ($I_0$) and with (I) sweetener, we determined decadic turbidity

$$\tau = -(1/L)\log(I/I_0)\,, \qquad (1)$$

where L is the optical path length. The quartz optical cells (Helma, Germany) were thermostated by Ultrathermostat U4 (Germany) with accuracy 20 ± 0.1 °C.

### *3.3. Ultrasound velocimetry*

The measurement of the velocity of ultrasound allows for evaluation of the elastic properties of aqueous media, such as liposome or protein suspension. This evaluation is based on a simple relationship: $\beta = 1/(\rho u^2)$, where $\beta$ is coefficient of adiabatic compressibility and $\rho$ is the density. In the study of mechanical properties of solution, measuring a relative change in a physical characteristics per unit of solute concentration rather than its absolute value is often more important, precise and easier [10]. The so-called concentration increment of ultrasound velocity [u] is a convenient parameter that characterizes changes in compressibility of vesicles and can be easily determined experimentally. This value is defined by the relation

$$[u] = (u - u_0)/u_0 c \qquad (2)$$

where u and $u_0$ are the sound velocities in solution and buffer, respectively and c is the molar concentration of sweetener. The determination of [u] is based on the measurement of changes in resonance frequency of acoustic wave propagated in small cavity (0.7 ml) of the cell containing piezoelectric transducers. It has been shown, that the changes of resonance frequency are proportional to the changes of sound velocity [5, 7, 10] In these experiments, we used to resonance cell configurations. One cell was filled with sample – liposome suspension, the second one with buffer. The sweeteners were added to both cells so the direct effect of sweetener on liposomes was measured at a frequency of ca. 7 MHz. Since the intensity of the sonic signal in the sample liquid was very small (the pressure amplitude in the ultrasonic wave was less than $10^3$ Pa), any effect of the sound wave on a structural transition of the liposomes was avoided. Cells were thermostated at T = 20 ± 0.02 °C with a Lauda RK 8 CS ultrathermostat.

### *3.4. Electrostriction method*

Membrane compressibility in direction perpendicular to its plane can be induced by application of a voltage of small amplitude (~50–100 mV). The voltage will compress the membrane with pressure $p = C_s U^2/2h$ ($C_s$ is specific electrical capacity of membrane) and this will result in changes of membrane thickness, h. The elasticity in direction perpendicular to the membrane plane is characterized by the Young modulus of elasticity $E = -p/(\Delta h/h)$. The value of E is sensitive to ordering of hydrophobic part of the membrane and changes following interaction with BLM of various compounds [6]. In this work, the value E of BLM as a function of sweetener concentration was determined using an electrostriction method [6, 7]. Briefly, due to non linear dependence of membrane capacitance on amplitude of electrostriction voltage $U = U_0 \sin 2\pi ft$, ($U_0$ is amplitude and f-frequency of ac voltage): $C = C_0(1 + \alpha U^2)$, where $C_0 = C$ ($U = 0$) and $\alpha$ is coefficient of electrostriction, in current flowing across BLM, $I = d(CU)/dt$, in addition to the basic component of the current with amplitude $I_1$ and frequency f, also the third current harmonic with amplitude $I_3$ and frequency 3f will appear. The elasticity modulus can be determined using equation:

$$E = (3C_s U_0^2/4h)(I_1/I_3) \qquad (3)$$

The amplitude of first current harmonics allow to determine BLM electrical capacitance $C = I_1/2\pi f U_0$. In experiments, an ac voltage with amplitude $U_0 = 60$–$80$ mV and frequency f = 1 kHz was applied to the BLM through Ag/AgCl electrodes placed in salt agar bridges. For the calculation of E value $C_S = 0.4$ μF/cm$^2$ and h = 4.6 nm were used [6].

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