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EFFECT OF METHYL METHACRYLATE ON THE STRUCTURAL STABILITY OF PROTEINS AND ERYTHROCYTE MEMBRANES

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ABSTRACT
PURPOSE: To study the concentration dependence of the effect, produced by methyl methacrylate (MMK) on acid and thermal hemolysis of human erythrocytes and on thermal denaturations of fibrinogen and major proteins of human erythrocyte membrane. METHODS: spectrophotometric assay of acid and thermal hemolysis; thermal analysis of erythrocyte suspension impedance; UV spectrophotometry of protein denaturation. RESULTS: At low concentrations (0.02 - 0.05 % (v/v)) MMK increase the resistance of human erythrocytes against HCl-induced hemolysis (about 20 %) and against the thermal hemolysis at 54,2°C (about 70 %). At higher concentrations, however, MMK accelerated both acid and heat – induced hemolysis. Depending on the MMK concentration similar biphasic effect was produced on the structural stability of an isolated protein, fibrinogen, and of the major proteins of isolated erythrocyte membrane, spectrin and the anion exchanger. The maximal stabilization effect (between 1 and 2°C increase in the denaturation temperature of the protein) occured at MMK concentration of about 0.03% (v/v). CONCLUSION. The present findings substantiate that MMK, at low concentrations, produces stabilizing effect on proteins and plasma membranes similar to that specific for the anti-inflammatory and antirheumatic drugs and herb extracts.

Key words: methyl methacrylate, anti-inflammatory drugs, thermal hemolysis, acid hemolysis

INTRODUCTION

Methyl methacrylate (MMK), or the methyl ester of methacrylic acid, is a colorless liquid, slightly soluble in water (1.5% w/v at 30°C) and very good, practically unlimited solubility in organic solvents. MMK easily polymerizes forming polymethylmethacrylate (PMMK), which is widely used. Industrial PMMK is produced by radical polymerization of MMK at moderate temperature in the presence of initiators or UV light. MMK and its polymer are becoming more widely used in medicine; in prosthetics as soft tissue filler, cosmetic surgery, dentistry for dental crowns and bridges, ophthalmology for hard contact lenses, etc.

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MMK is a volatile synthetic chemical that is used to produce polymer materials for the industry, medicine and everyday life. The majority of MMK pollution is predicted to be emitted to air, with small amounts being released into water and soil. The acute toxicity of MMK is low. MMK was not found carcinogenic in rats and mice exposed by inhalation. MMK is a mild respiratory and skin irritant in humans and has the potential to induce skin sensitization in susceptible individuals (1). Inside human body MMK is rapidly metabolized and hydrolyzed to methanol, methacrylic acid, formic acid and other end products.

In an extensive study Prakova (2) has found that the pH of blood plasma of workers in the production and processing of MMK was reduced. In addition, the concentration of hemoglobin and number of erythrocytes were both reduced depending on the extent of

exposure. This state was defined as metabolic acidosis and anemia, related to the end products of MMK hydrolysis (3). However, the direct impact of MMK on human erythrocytes was not investigated.

The main objective of this report was to elucidate the concentration dependence of the direct effect MMK elicits on the structural stability of a model protein (fibrinogen) and model plasma membrane (the membrane of human erythrocytes). Relatedly, we studied the effect of MMK on the denaturation temperatures of fibrinogen and of the erythrocyte membrane major proteins, as well as the effect of MMK on the acid and thermal resistance of human erythrocytes.

MATERIALS AND METHODS

Chemicals, erythrocytes and erythrocyte membranes

Methyl methacrylate, DIDS, EDTA and EGTA were purchased from Sigma (St. Louis, MO, USA). Fibrinogen was isolated from human blood plasma. Human erythrocytes were isolated from fresh citrated blood of healthy donors. The blood was centrifuged at 1500 rpm for 10 minutes and the upper layer of cediment that contained white blood cells and platelets was discarded. Prior to usage the erythrocytes were thrice washed in excess volume of cold NaCl saline and stored at 4° C for less than a day. Resealed erythrocyte membranes (EMs) were obtained from human erythrocytes as described earlier (4). The resealing was verified obtained the Boyle van't Hof linear relation of ghost volume with inverse of osmotic pressure (5). The anion exchange diffusion in erythrocyte membranes was inhibited by DIDS which is highly specific, powerful covalent inhibitor of the band 3 protein, the anion exchanger (6).

Acid hemolysis

For this method, three stock solutions were initially prepared: 1) 1 % (v/v) stock solution of MMK in NaCl saline; 2) 2 % (v/v) stock suspension of erythrocytes in NaCl unbuffered saline; and 3) 2 % (v/v) HCl acid in distilled water. The working erythrocyte suspension was prepared adding 20µl stock suspension of intact or DIDS - inhibited erythrocytes (hematocrit 2 %) to 1,8 ml unbuffered NaCl saline with or without MMK, to obtain the optical density at 700 nm, OD_{700}, of 0.7. The acid hemolysis of cells was induced by injecting 20 µl HCl-load to that suspension at continuous stirring. The final pH was determined with a pH- meter in a separate experiment. The hemolysis was followed by recording the changes in OD_{700} (Spekol 21, Carl Zeiss Jena, Germany) on chart (7). At this wavelength, the light absorption of hemoglobin is nil and the measured optical density can be attributed mainly to the light scattering of cells still remaining intact. Acid resistance of cells is defined as the time needed to lyse 50% of cells according to the corresponding change in OD_{700}.

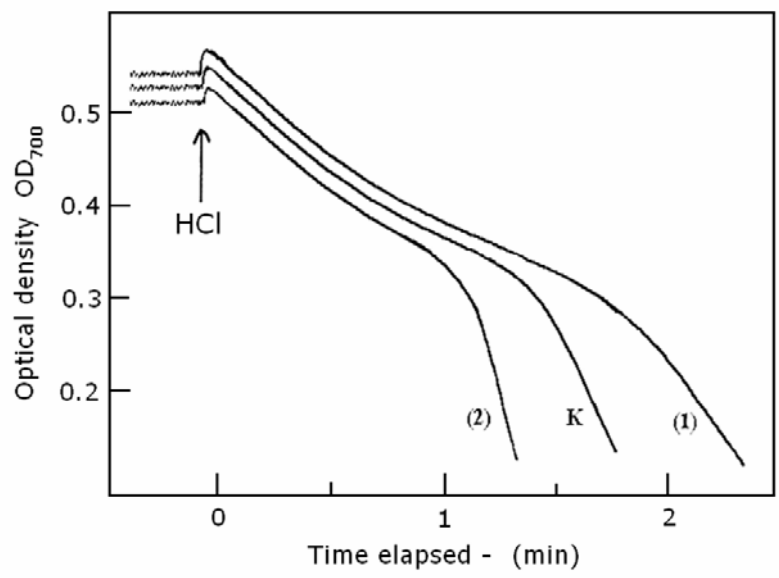

Figure 1. Time - course of HCl-induced hemolysis of human erythrocytes in the presence of MMK. The MMK concentration was zero (K), 0.03% (1) and 0.10% (2). Arrow indicates the addition of acid load (final pH 3.2).

Thermal hemolysis

To determine the rate of thermal hemolysis, 0.25 ml packed cells were suspended in 3 ml of NaCl-saline that contained 3 mM EDTA and incubated at 54,2˚C (8). The time course of thermohemolysis was followed by measuring the optical density at 700 nm, OD_{700}, of 0.05 ml aliquots periodically taken and diluted to 1.8 ml NaCl-saline. The thermal resistance of erythrocytes was defined as the time, needed to lyse 50 % of cells according to the corresponding change in OD_{700}.

Determination of the denaturation temperatures of spectrin and integral proteins of erythrocyte membranes (9).

It was carried out through derivative thermal analysis of suspension impedance (SOLARTON SI 1260 Impedance-Gain Phase Analyser, England). Briefly: intact erythrocytes or resaled EMs (inside contend 150 mM NaCl) were suspended (hematocrit 7 %) in an isotonic medium of 50 mM NaCl and sucrose, thus imposing an outward gradient of ion concentration across the membranes. 0.25 ml of this suspension was heated with constant heating rate (2˚C/min) and data for the temperature (t ^{o}C) and suspension impedance (Z, 10 kHz) were collected in computer. The first derivative of impedance against temperature was further obtained and presented as a function of temperature. The temperature differentiation was applied in order to compensate for the strong Boltsmann dependence of the suspension conductivity on the temperature.

At a steady-rate heating, the obtained termogram appeared as a horizontal line. Upon passing through a specific temperature interval, particular protein of erythrocyte membrane incurs conformation change causing change in passive electric properties of membrane. At 49,5˚C (T_A) the peripheral protein spectrin denatures (10, 11) causing a decrease in the membrane capacity (12), while the membrane ion permeability became strongly activated at 62^{o}C (T_g) due to a pre-denaturational change in the conformation of major integral protein, band 3 protein (13). Hence, two thermally induced membrane alterations could be detected as sharp peaks centered at T_A and T_g on the termogram (Fig. 3). The top temperatures of each peak had practically the same values in both intact erythrocytes and isolated EMs (9). During repeated heatings of different portions of same suspension, the variations of T_A and T_g lay in the range ±0.2 ˚C. The method is assumed not sensitive to changes in the cells shape according to the data presented earlier (14).

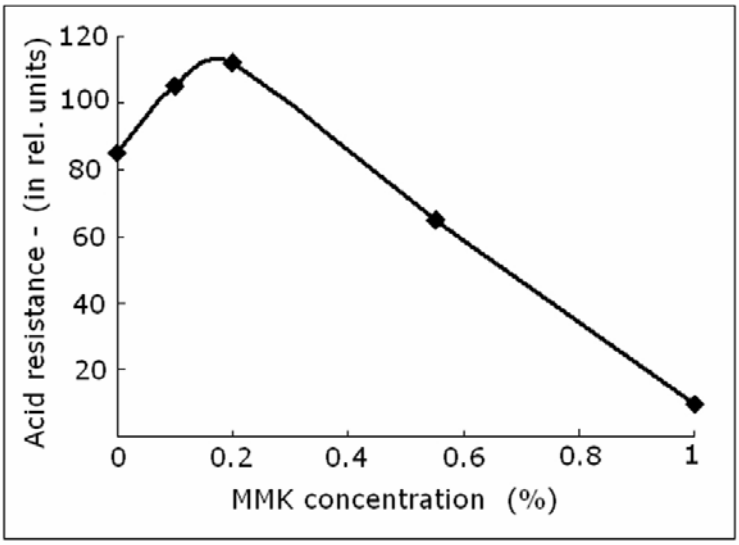

Figure 2. Biphasic effect of MMK concentration on the acid resistance of human erythrocytes.

Spectrophotometric assay of the thermal denaturation of fibrinogen

The denaturation of fibrinogen was followed spectrophotometrically at 270 nm using UV spectrophotometer (Milton Roy Spectronic 21D, USA). The working medium was well deaerated (100^{o}C, 5 min) solution of 130 mM NaCl, 20 мМ phosphate buffer, pH 7.4, and the indicated concentration of MMK. At 270 nm the light absorption of MMK is minimal. At first, the optical transmittance at 270 nm (T_{270}) of the cuvette containing 2.8 ml working medium was adjusted to 100 %. Fibrinogen, freshly prepared as a stock solution (10 mg/ml)

in the working medium, was then added reducing the T_{270} to about 40 %. The protein solution was then heated with 2°C/min and the obtained data for temperature and T_{270} were collected in computer. The first derivative of T_{270} against temperature was further obtained and presented as a function of temperature. During the heating the optical transmittance T_{270} kept a slight downhill trend, however, about the temperature of thermal denaturation, T_d, the T_{270} sharply decreased depicting a peak. The temperature of denaturation was determined as the top temperature of the peak.

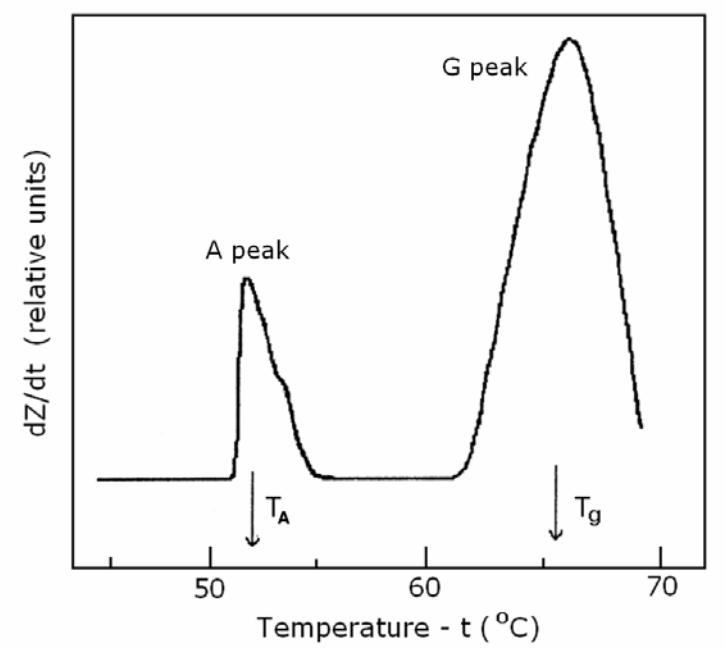

Figure 3. Temperature profile of the temperature derivative dZ_s/dt of the impedance of erythrocyte membrane suspension. The A peak corresponds to the heat denaturation of spectrin, while the G peak is related to pre-denaturational change in the anion exchanger of EMs.

RESULTS AND DISCUSSION

MMK produced specific effect on the time-course of HCl-induced hemolysis of intact human erythrocytes **(Figure 1)**. The onset of hemolysis and the time for 50% hemolysis were both markedly postponed in the presence of low concentrations of MMK. At high MMK concentrations, however, the acid hemolysis was accelerated. Similar results were obtained with DIDS-treated cells (not shown). Thus, a biphasic effect of MMK on the HCl - induced hemolysis was observed, i.e. protection of erythrocytes against the hemolytic action of HCl at low concentrations and sensitization of erythrocytes towards acid hemolysis at higher MMK concentrations **(Figure 2)**. The MMK concentration offering maximum protection of the cells was about 0.03% (v/v).

The structural stability of each protein can be expressed by the temperature of its thermal denaturation; the higher is the temperature of denaturation, the more stable is the protein structure. **Fig. 3** shows the temperature derivative of suspension impedance during transient heating as affected by the thermal denaturations of major membrane proteins. The suspension contained resealed erythrocyte membranes under strong transmembrane gradient of ion concentration. This termogram demonstrates the thermal denaturations of undermembrane peripheral protein spectrin at T_A (11) and the integral protein at T_g (13). MMK, present in the suspension media, elicited biphasic effect on the structural stability of these membrane proteins producing stabilization at low concentrations and destabilization at greater concentrations. For example, the T_A and T_g were both increased by about 2°C at the presence of 0.15% (v/v) of MMK and decreased by 1 to 2°C at the presence of 0.30% MMK (not shown).

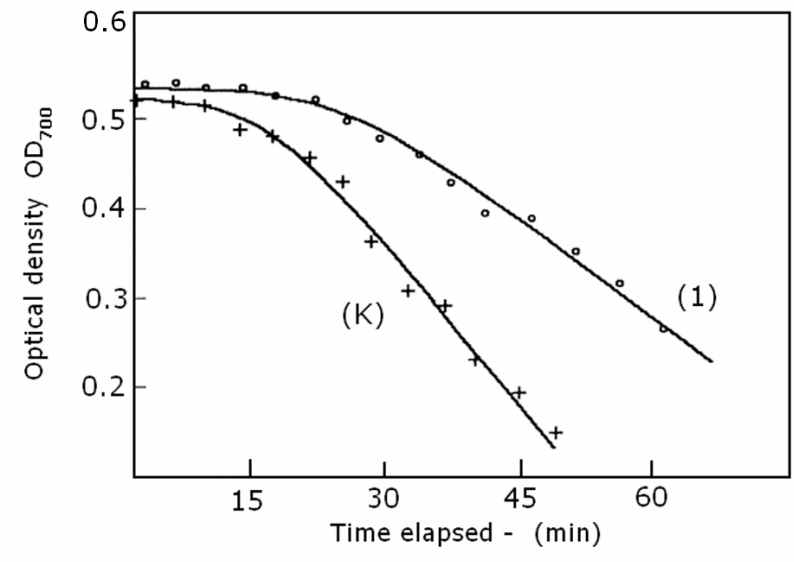

Figure 4. Effect of MMK on the time - course of thermal hemolysis of human erythrocytes exposed to 54.2°C. The MMK concentration was zero (K), and 0.05% (1).

Previous reports have demonstrated that the T_g temperature of erythrocyte membrane corresponds to the ability of erythrocytes to retain cytosolic ions at hyperthermia (9) and related this temperature to the resistance of erythrocytes against thermal hemolysis (8). The increase in T_g leads to an increase in the thermal resistance of erythrocytes and vice versa. **Figure 4** displays that MMK at concentration of 0.05% increased by about 70 % the time for 50% hemolysis under thermal stress, the thermal resistance of erythrocytes.

Finally, we present similar effect of MMK on the structural stability of an isolated, single protein, fibrinogen **(Figure 5)**. MMK, present at low concentration (up to 0.05%), increased the temperature of heat denaturation by about 1°C, while at greater concentration the latter was linearly reduced.

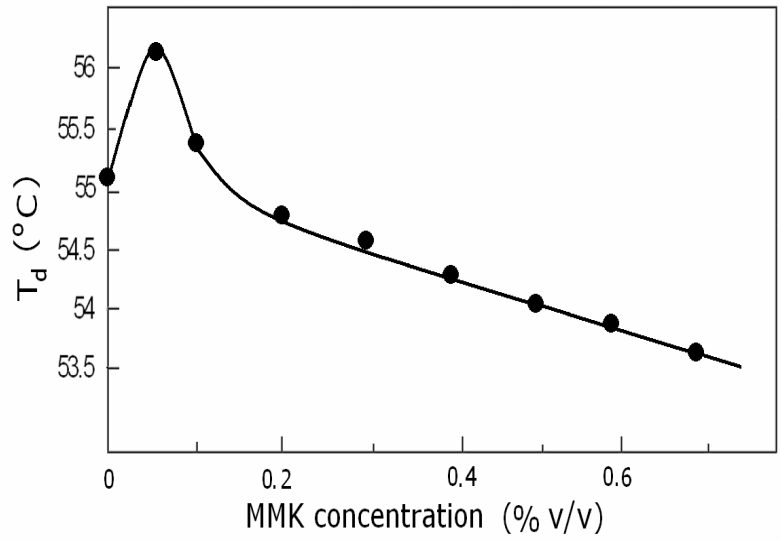

Figure 5. Effect of MMK concentration on the denaturation temperature, T_d, of fibrinogen.

The main finding of this study was that MMK, present at low concentrations, protects human erythrocytes from acid and heat-induced hemolysis and stabilize the structure of the major proteins of erythrocyte membrane and that of fibrinogen. This protective effect was demonstrated at MMK concentrations equal and higher than those found in the blood

plasma of humans exposed to MMK in MMK-producing and processing factories. These results are in line with the conclusion, expressed in other reports (2, 3), that not MMK itself but chiefly its metabolites produce harmful effects on those people.

The present findings substantiate that MMK produces stabilizing effect on proteins and plasma membranes similar to that produced by some anti-inflammatory and antirheumatic drugs. Data obtained by other authors have shown that heat-induced lysis of human erythrocytes and thermal denaturation of isolated proteins (albumin) were both inhibited to variable extent by a group of drugs, water extracts of herbs and natural bioactive substances that all possess anti-inflammatory, antirheumatic, antiarthritic and analgesic action. This group includes aspirin and diclofenac (15), indomethacin, ibuprofen, ibufenak, piroksikam, naproxen and phenylbutazone (16) and some water extracts of herbs (17, 18, 19). As a rule, similar effects are generally exhibited by the group of nonsteroidal anti-inflammatory drugs, NSAIDs, and especially those derived from the propionic and acetic acids (20). This is explained by the structure / effect relationship between the pharmacologic properties of medicines and their basic chemical structure. MMK is a derivative of methyl methacrylic acid which structurally is similar to the propionic and acetic acids **(Figure 6)**. This possibly explains, in addition to the lipophillic nature of MMK, the common stabilization effect, produced by MMK and these drugs, on proteins and plasma membranes.

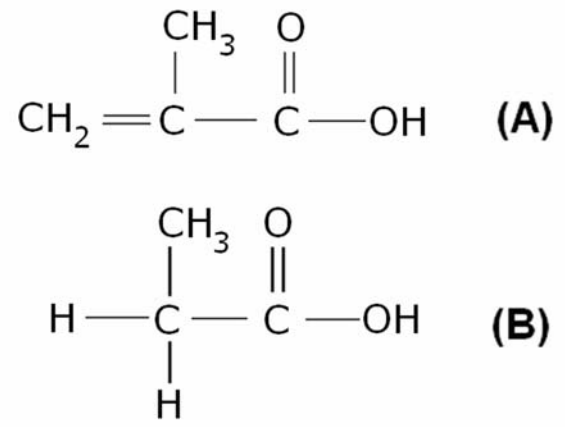

Figure 6. Chemical formulae of methyl methacrylic acid (A) and propionic acid (B).

CONCLUSSION

The obtained findings demonstrate that similar to NSAIDs, low concentrations of methylmetacrylate stabilized the structure of isolated protein, of major proteins of erythrocyte membrane, and enhanced the resistance of erythrocytes against the deleterious impact of heat and acid.

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