process which exhibits the strongest inhibition (table), since feedback mechanisms exist between sulfation and elongation processes of the polysaccharide chain and, in addition, between chondroitin sulfate side-chain synthesis and the formation of the side chain protein linkage region[11,12]. Biosynthesis of total protein is also inhibited as proteoglycans represent about one half of cartilage dry weight[13]. The effect appears to be specific for antiproteoglycan serum, since it abolishes after absorbing the antibodies with proteoglycan, but not with cartilage collagen. Data presented could indicate possible receptors at the cell surface of cartilage cells acting with these antibodies or with soluble proteoglycan immune complexes, as has been suggested for the proteoglycan-hyaluronate complex[14]. At present it is still unclear which kind of antigen from the proteoglycan complex[10] produces the antibodies causing this effect.

The decreased biosynthesis capacity runs obviously parallel with an increased anaerobic glycolysis: during incubation rates of CO_2 production increase significantly ($p < 0.001$) up to 125 to 150% of controls (2.23 μmoles/g wet weight); the effect abolishes after absorbing the antiserum with proteoglycan, but not with cartilage collagen. Only after longer incubation (5.5 h) the activity of lactate dehydrogenase rises slightly to 120% of controls in the medium indicating cell damage. The content of carbazole-positive material (probably solubilized proteoglycans) as well as the activity of acid phosphatase exhibit minimal changes in the medium.

The data resemble findings observed with human rheumatoid arthritis: in synovial fluids a rise in pCO_2 and in concentration of lactic acid[15,16] as well as elevated cytoplasmic enzyme activities[17] (e.g. lactate dehydrogenase). The production of proteoglycans appears to be diminished with human rheumatoid synovial cells[18] and in joint cartilage of experimental hyperergic arthritis with later states[19]. However, in human rheumatoid arthritis, antiproteoglycan antibodies have not yet been detected, although a potential cell-mediated immune response to proteoglycan antigen has been demonstrated[20]. Finally, it has to be stated that early in vitro effects of antiproteoglycan serum on cartilage cells differ from the changes observed with animal osteoarthritis, which shows increased proteoglycan biosynthesis and loss of proteoglycan from articular cartilage only after repeated injections of antiproteoglycan immune globulin[2].

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The hemolytic activity of heterocyclic N-alkyl amine oxides

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Summary. The N-alkyl derivatives of morpholine-, pyrrolidine-, piperidine- and perhydroasepine-N-oxides caused the rapid, temperature-dependent, hemolysis of human red blood cells. The most hemolytic were the amine oxides with alkyl groups having 14–18 carbon atoms.

The N-alkyl derivatives of saturated heterocyclic amine oxides represent the biodegradable nonionic amphiphiles[1,2], possessing a significant antimicrobial activity[3,4]. Our recent studies on the mode of action of these relatively nontoxic compounds[5] revealed that disorganization of membrane structure after interaction of cells with amine oxides is primarily responsible for their antimicrobial activity[4]. This activity was found to be significantly dependent on the chain length of the hydrophobic alkyl, while it was only slightly influenced by other substituents of polarized N-oxide group[4]. In this paper we describe the effects of homologous series of 4-alkylmorpholine-N-oxides and some other amine oxides with an identical side chain but different basic structures on the stability of plasma membrane of human red blood cells.

Materials and methods. Washed red cells were prepared by diluting fresh human blood with 3 vol. of cold 0.154 M sodium chloride and centrifuging the suspension for 10 min at 1000 × g in a refrigerated centrifuge. The resulting pellet was washed twice and the final pellet of red cells was diluted to about the original volume with 0.154 M sodium chloride.

For measurement of hemolytic activity, the standard incubation mixture at 37 °C contained 0.154 M NaCl, 10 mM Tris-HCl, erythrocytes ($20–40 \times 10^6$ cells per ml) and amine oxide (at indicated concentration), final pH 7.45. At the time indicated an aliquot of mixture was centrifuged at 1000 × g for 2 min and the degree of hemolysis was evaluated by determining of the amount of hemoglobin released in the supernatant liquid spectrophotometrically at 537 nm[6]. The hemoglobin concentration in supernatant of red cells lysed in 10 mM Tris-HCl

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pH 7.45 represented 100% hemolysis. The hemolytic end-point corresponding to complete lysis (no turbidity) in standard incubation mixture at the temperature indicated was observed directly by eye in tubes of fixed size using a well defined black-on-white background for viewing[7].

Results and discussion. Figure 1 shows the effect of 4-dodecylmorpholine-N-oxide on the stability of human red blood cells membrane. It was found that, depending on the amine oxide concentration, an induction period of variable length preceded a period of rapid hemolysis. A significant degree of hemolysis was observed at 0.6 mM amine oxide concentration, which is very close to the minimal concentration of 4-dodecylmorpholine-N-oxide inhibiting the growth of various microbial cells[4].

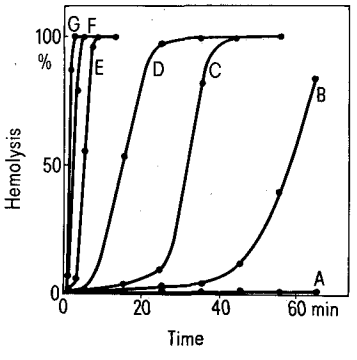

Fig. 1. Effect of incubation time and the concentration of 4-dodecylmorpholine-N-oxide on the hemolysis of red blood cells. Amine oxide concentrations: *A* 0–0.4 mM; *B* 0.5 mM; *C* 0.6 mM; *D* 0.7 mM; *E* 0.8 mM; *F* 0.9 mM; *G* 1.0 mM.

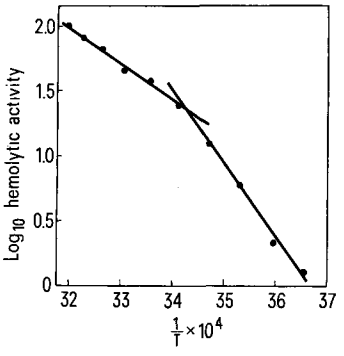

Fig. 2. Arrhenius plot of hemolytic activity of 4-dodecylmorpholine-N-oxide. 1 mM amine oxide was added to suspension of red blood cells incubated in standard medium at the indicated temperature (°K) and the time t_T (min) required to cause the complete hemolysis was determined. The hemolytic activity is conveniently expressed as value $100/t_T$ corresponding to percent hemolysis per min incubation time at indicated temperature (T).

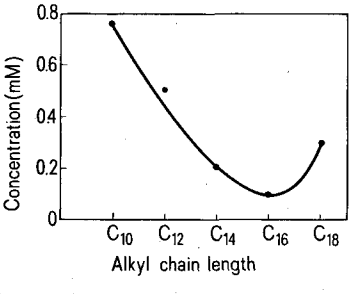

Fig. 3. Dependence of hemolytic concentration of 4-alkylmorpholine-N-oxide required to cause complete hemolysis of red cells on the length of the hydrophobic alkyl chain. Temperature 37 °C, time of interaction 30 min.

The hemolytic activity of 4-dodecylmorpholine-N-oxide was found to be remarkably dependent on the temperature. The time needed for amine oxide (1 mM) induced complete lysis of red blood cells at 0 °C was 60fold longer than that at the 37 °C. Data obtained from hemolytic activity of 4-dodecyl-morpholine-N-oxide subjected to Arrhenius kinetic demonstrated a biphasic curve with a transition point at 18 °C (figure 2). Since erytrocyte membrane lipids undergo a transition at 18–20 °C[8], these findings indicate that the changes in the physical state of the lipid phase of erytrocyte membrane[9] after its interaction with amine oxide may be intimately involved in mechanism of amine oxide-induced hemolysis.

The study of the relationship between hemolytic activity and chemical structure of amine oxides revealed that cytolytic activity in homologous series of 4-alkylmorpholine-N-oxides was dependent on the length of the hydrophobic alkyl (figure 3). In a tested series of amine oxides, the 4-hexadecyl-morpholine-N-oxide was found to be the most active. The amine oxides with alkyl groups having 2–8 carbon atoms exhibited very poor hemolytic activity. The slightly higher hemolytic concentration of 4-octadecylmorpholine-N-oxide might probably be the result of its decreased solubility in water as compared to amine oxides with shorter alkyl chain. In the series of amine oxides having the same alkyl chain (C_{12}) but differing in the structure of heterocyclic ring, the following order of compounds according to their decreasing hemolytic activities was established: 1-dodecylperhydroasepine-N-oxide, 1-dodecylpiperidine-N-oxide, 1-dodecylpyrrolidine-N-oxide, 4-dodecylmorpholine-N-oxide and N,N-dimethyldodecylamine-N-oxide. In this case, however, the relative difference in hemolytic concentrations of amine oxides did not exceed a factor 3. Thus, the general structure activity relationships for hemolytic activity by amine oxides are very similar to those for antimicrobial activity[4] or acute toxicity in mammals[5].

The results of this paper demonstrate the hemolytic activity of some heterocyclic N-alkyl amine oxides. This activity, as well as the recently described antimicrobial activity[4], were found to be dependent on the temperature, relative concentration and chemical structure of amine oxides. It is evident that in both cases the biological membranes which, after interaction with amine oxides, undergo changes in molecular organization, osmotic and permeability properties are the site of action of these compounds.

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