J. Membrane Biol. 118, 251–257 (1990)

The Journal of
Membrane Biology

Effect of Magnesium Ions on Red Cell Membrane Properties

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Summary. Spectrin forms aggregates in solution when incubated at relatively high concentrations (several millimolar) of divalent cations. According to the evidence of electron microscopy, aggregates of globular appearance and rather uniform size are cooperatively formed from spectrin dimers, no intermediate structures being seen. Inter-dimer chemical cross-linking of spectrin in intact red cell membranes is enhanced if magnesium ions at a concentration of 0.5 mM or more are present. On the other hand, the elimination of magnesium from the interior of intact cells causes no significant change in shear elastic modulus, measured by micropipette assays, nor is there any dependence of membrane filtration rate on intracellular free magnesium concentration in the range 0–1 mM. Magnesium-depleted cells are, however, converted into echinocytes within a short period, in which control cells, exposed to ionophore and external magnesium ions, remain completely discoid. Magnesium-depleted cells also undergo structural changes on heating below the temperature at which vesiculation sets in. These reveal themselves by the transformation of the cells to a unique characteristic shape, by grossly reduced filtrability, and by extensive agglutination of the cells when treated with a bifunctional reagent. Magnesium ions thus regulate the stability, but not to any measurable extent the gross elasticity, of the red cell membrane.

Key Words erythrocytes · membrane elasticity · micropipette · membrane skeleton · spectrin · magnesium

Introduction

The elastic properties and the stability of the red cell membrane appear to be at least partly separable features, both controlled by the membrane skeleton (Mohandas, Chasis & Shohet, 1983; Chasis & Mohandas, 1986; Shields et al., 1987). Thus oxidative cross-linking of spectrin molecules in the network and dissociation of spectrin tetramers (the main structural unit) into dimers both lead to a large rise in shear modulus (Fischer et al., 1978). Cross-linking of spectrin by antibodies also increases membrane rigidity (Nakashima & Beutler, 1978). Reduction of spectrin concentration on the membrane by contrast primarily affects the stability of the cell under shear; loss of up to 40% of the spectrin by proteolysis had essentially no effect on the measurable elastic properties (Shields et al., 1987). On the other hand, in hereditary spherocytosis, a condition associated with spectrin deficiency, Waugh and Agre (1988) found a correlation between spectrin concentration and membrane shear modulus and bending stiffness.

The elastic properties of the red cell membrane have been described in terms of an entropy-spring model, based on the constraint of the end-to-end distance of the spectrin tetramers by the lattice (Stokke, Mikkelsen & Elgsaeter, 1986*a*; Kozlov & Markin, 1987). This model presents some difficulties, for example in respect of the temperature-dependence of the elasticity: the shear modulus increases with increasing temperature, thus violating the criterion for an entropy-dependent mechanism (Waugh & Evans, 1979; Englehardt & Sackmann, 1988). Vertessy and Steck (1989) have given persuasive arguments in favor of intra- and/or intermolecular interactions of the spectrin molecules as the source of elastic energy.

It has been shown that spectrin in solution is aggregated and precipitated by divalent cation concentrations of several millimolar (Elgsaeter, Shotton & Branton, 1976; Beaven & Gratzer, 1980). Given the high density of spectrin molecules on the membrane surface, the possibility that the intracellular free magnesium concentration is sufficient to generate spectrin-spectrin interactions of the kind seen in solution must be considered. We have examined further the effects of high magnesium ion concentrations on spectrin and the relation of intracellular magnesium concentration to the mechanical characteristics of the membrane.

Materials and Methods

Spectrin dimers were prepared from human red cells by extraction at low ionic strength, followed by column chromatography on

Sepharose 4B (Gratzer, 1982). The protein was precipitated with ammonium sulphate, redissolved in 0.1 M sodium chloride, 20 mM Tris, 1 mM sodium azide, pH 7.5, and dialyzed against the same buffer. The concentration, determined spectrophotometrically, was adjusted to 1–2 mg/ml.

Aggregation of spectrin in solution was followed turbidimetrically. As shown previously, aggregation and eventually precipitation of the protein at physiological ionic strength is brought about (depending on protein concentration) by millimolar concentrations of calcium ions or somewhat higher magnesium ion concentrations (Beaven & Gratzer, 1980); a divalent cation concentration of 20 mM caused extensive aggregation in a few minutes. Observations were made in a Perkin Elmer Hitachi MPF 3L spectrofluorimeter with both monochromators set at 350 nm. The samples were thermostatted at 35°C. At intervals aliquots were withdrawn, diluted as required with buffer and applied to carbon-coated grids for electron microscopy. The spectrin was negatively stained with 1% uranyl acetate and examined in a Philips 200 or Jeol 200CX electron microscope.

For cross-linking of spectrin *in situ*, dimethyladipimidate was used. This reagent does not readily pass through the membrane. Washed red cells were accordingly lysed with 5 mM sodium phosphate, and the ghosts were recovered by centrifugation. They were suspended in 0.1 M sodium chloride, 20 mM triethanolamine, pH 7.8, with 0–5 mM magnesium chloride, and kept on ice to prevent resealing. Dimethyladipimidate was dissolved in the buffer at 10 mg/ml and immediately added to the ghost suspension to give a reagent concentration of 1 mg/ml. After 1 hr on ice, the ghosts were collected by centrifugation, washed once with buffer and resuspended once more in the buffer. SDS was added to a concentration of 1% and after heating at 100°C for 5 min, the ghost proteins were separated by gel electrophoresis in the buffer system of Laemmli (1980). The proportion of spectrin cross-linked was estimated by densitometry of the gels, stained with Coomassie blue R250.

To remove magnesium ions from intact cells, the washed cells were suspended at a hematocrit of 4% in phosphate-buffered isotonic saline; the ionophore A23187 in ethanol solution was added to give a concentration of 5 μg/ml, together with 2.5 mM EDTA. For controls, the ionophore was added with 0.15 mM magnesium chloride and 1 mM EGTA, and higher magnesium concentrations were also used. The suspensions were incubated at 37°C and aliquots were taken at intervals up to 2 hr.

For magnesium analysis cells were collected from samples of the supernatants by centrifugation through a cushion of 2 : 1 dibutylphthalate : dinonylphthalate. They were lysed with 10 volumes of water and the lysates were deproteinized with 5% trichloroacetic acid. The supernatant after centrifugation was made 10 mM in sodium chloride and analyzed for magnesium content by flame absorption photometry. For morphological examination in the light microscope, samples of the cell suspension were first diluted with an equal volume of 2% glutaraldehyde in phosphate-buffered isotonic saline.

To determine thermal stabilities of treated cells, suspensions were heated for 10 min in a water bath. Aliquots were fixed with glutaraldehyde and monitored for vesiculation or shape changes in the light microscope. The mechanical effects of heat treatment were also tested by measuring cell filtrability (*see below*). Membrane elasticity was measured directly by the micropipette method (Evans, 1973). A pipette with internal diameter 1.5 μm was used to aspirate a small portion of the membrane. The shear elastic modulus was determined from the linear relation between the length of the membrane "tongue" in the pipette and the applied pressure. Overall cellular resistance to deformation was assessed by a filtration method (St. George's Filtrometer, Carri-Med, Dorking, UK). The incubated suspensions at known hematocrit were passed through 5 μm pore filters at a set pressure. The device determines a series of three flow rates and delivers the initial flow rate (relative to the suspending medium) and the rate of decrease of flow rate (clogging rate, CR). The red cell transit time (RCTT) is determined from the initial relative flow rate and the hematocrit.

Results

Depending on protein concentration, temperature and the concentration and identify of the added divalent cation, spectrin solutions become turbid over a period of minutes to hours. As described previously (Beaven & Gratzer, 1980), the turbidity progress curve ultimately reached a plateau and then gradually a precipitate formed. The effects of calcium and magnesium ions were qualitatively similar, but calcium induced aggregation at lower concentrations, or caused it to proceed more rapidly at the same concentration. The phenomenon is clearly a general one and has low specificity, for it was also brought about by strontium and barium ions (*data not shown*). Examination of the protein by electron microscopy at various stages of aggregation revealed that it behaved as a two-state system. Protein sampled immediately after addition of the divalent cation showed only separate dimers (Fig. 1*a*). Thereafter large aggregates of essentially amorphous appearance, but relatively uniform size, appeared. At the plateau of the turbidity progress curve all the spectrin had been converted into the large aggregates (Fig. 1*b* and *c*). The aggregation is thus cooperative.

To determine whether this self-association process has a counterpart in spectrin on the membrane, unsealed ghosts at physiological ionic strength were exposed to the covalent cross-linking reagent, dimethyladipimidate. The ghost protein was solubilized in SDS and examined by gel electrophoresis. It was found that the $\alpha\beta$ spectrin dimer units were internally cross-linked with high efficiency, as often noted before, to give a product of apparent molecular weight about 500,000. In the absence of divalent cations there was further cross-linking, to the extent of about 50% in the conditions of these experiments, between dimers. The inter-dimer cross-linked species do not arise from the head-to-head contacts between the pairs of dimers making up the tetramer, since spectrin tetramers, subjected to cross-linking in solution, yield no significant amounts of cross-linked species other than the dimer. When magnesium ions were present at concentrations above about 0.5 mM the extent of inter-dimer cross-linking rose (Fig. 2). Thus lateral spectrin-spectrin contacts evidently prevail in the membrane under all condi-

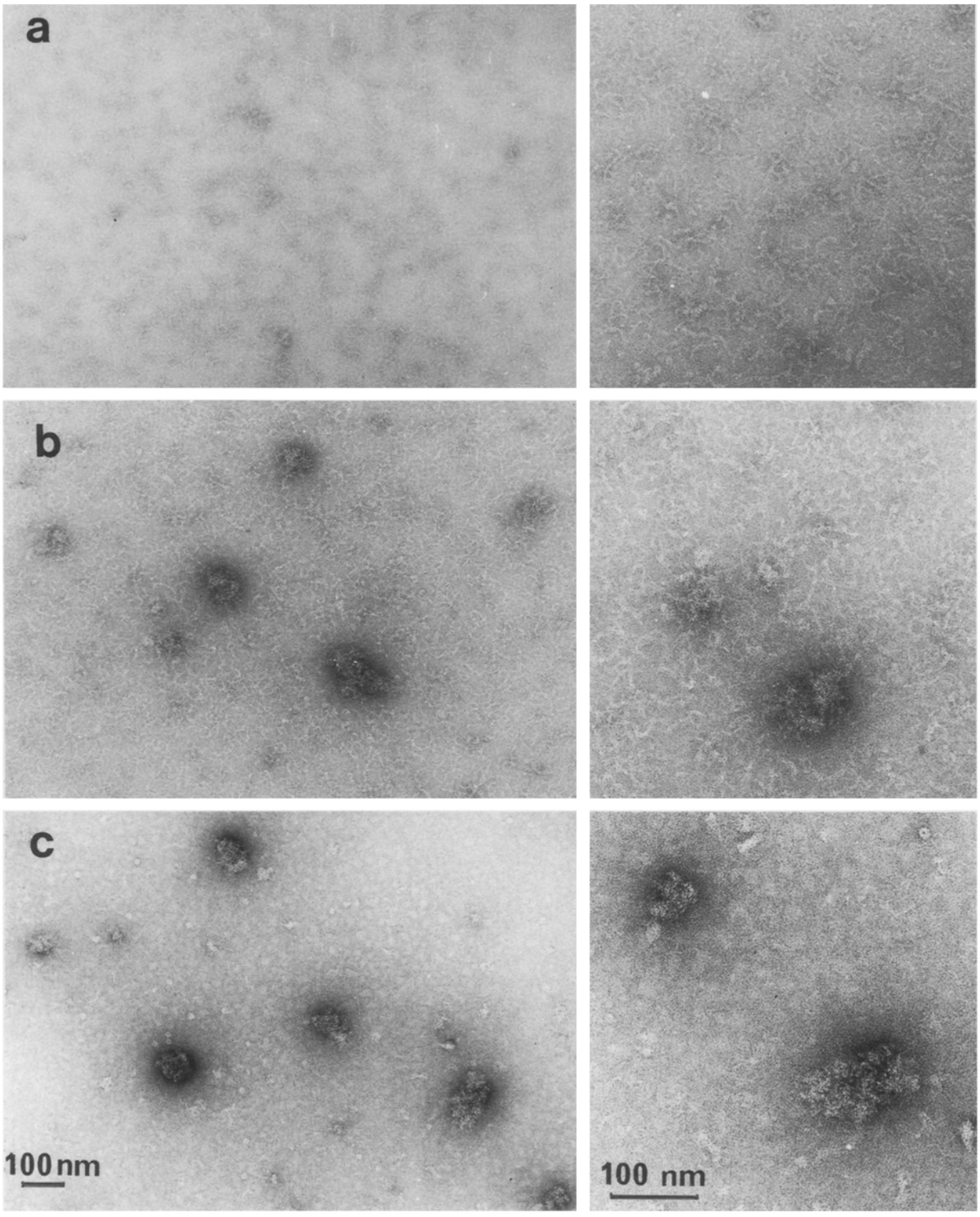

Fig. 1. Electron micrographs of spectrin dimers undergoing polymerization by divalent cations (in this case 20 mM calcium ions): (*a*) immediately after mixing, showing absence of aggregated protein; (b) partly polymerized, showing dimers in coexistence with high aggregates; (*c*) fully polymerized, showing only high aggregates

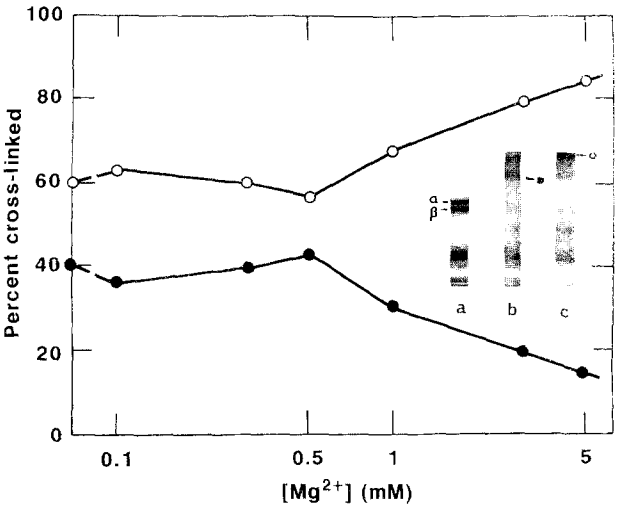

Fig. 2. Yield of inter-dimer cross-linked spectrin, resulting from treatment of membranes with dimethyladipimidate, as a function of magnesium ion concentration. The ordinate expresses the percentage of the total spectrin that is in the form of cross-linked $\alpha\beta$ dimer (filled circles) and of the inter-dimer cross-linked products (open circles), to which the cross-linked dimer is progressively converted with increasing magnesium ion concentration. Points at the ordinate represent samples with no added magnesium. The inset shows representative SDS-polyacrylamide gels from such an experiment: *a* shows the spectrin in untreated ghosts, *b* that in ghosts cross-linked with dimethyladipimidate (1 mg/ml) in the absence of added magnesium ions, and *c* that in ghosts cross-linked in the presence of 5 mM magnesium ions. Separate spectrin chains are denoted α and β, the cross-linked dimer by a filled circle and the higher cross-linked species by an open circle

Table 1. Shape transformations in magnesium-depleted red cells following heat treatment

Incubation time (min)	Discocytes	Echinocytes	
		Mild	Severe
0	98 ± 1	2 ± 1	0
30	89 ± 6	6 ± 3	5 ± 3
60	35 ± 16	14 ± 3	50 ± 16
120	7 ± 5	3 ± 2	90 ± 7
Control (120 min)	98 ± 1	2 ± 1	0

Values given are percentages ±SD (n = 8).
The controls were incubated with ionophore and 0.15 mM magnesium chloride and 1 mM EGTA.

Table 2. Membrane elasticity of magnesium-depleted red cells determined by micropipette assay

Shear elastic modulus of control cells	μ = 5.6 ± 1.0	(n = 44)
Shear elastic modulus of depleted cells	μ = 5.7 ± 1.45	(n = 48)
Discocytes	μ = 5.3 ± 1.3	
Mild echinocytes	μ = 6.3 ± 1.4	

Elastic moduli are expressed in 10^{-3} dynes cm^{-1}. Measurements represent means ±SD of sample means from five experiments; n is the number of cells measured.

tions, but their extent is significantly elevated when magnesium ions are present.

Red cells were depleted of cytoplasmic magnesium by treatment with the ionophore A23187 and external EDTA. As reported by Flatman and Lew (1980) the intracellular magnesium concentration fell essentially to zero in a matter of minutes. Cells that had been treated in this manner underwent rapid echinocytosis [*cf.* the period of hours required for echinocytosis induced by metabolic depletion—*see*, e.g., Backman (1986)]. The cells were scored as smooth, mildly spiculated or extensively spiculated, the last two types corresponding roughly to types II and III echinocytes in the nomenclature of Bessis (1973). Control cells (treated with the ionophore and magnesium, in place of EDTA) were about 98% discocytic after the same period. Results are summarized in Table 1. Thus magnesium ions stabilize the discoid form of the cell.

Elasticity measurements on magnesium-depleted and control cells were performed by the micropipette method. Any differences in membrane shear elastic modulus or in shear viscosity between magnesium-depleted and control cells were within the limits of experimental reproducibility. The results are given in Table 2. This shows furthermore that the shear modulus does not differ significantly between the discocytic and echinocytic populations of magnesium-depleted cells. Thus the elastic properties of the membrane are not grossly affected by intracellular magnesium ions. This conclusion is supported by the results of filtrability measurements, which showed no alteration in RCTT, following magnesium depletion. The same was true when the internal free magnesium concentration was made 1 mM, the cells retaining their normal discocytic morphology; even when the cells were loaded with 10 mM magnesium, there was only a small effect on the filtration properties.

The filter clogging rate of magnesium-depleted cells increased by 70% (average of six experiments, with $P < 0.01$). This effect could well be a consequence of the observed echinocytosis, which suggests that membrane stability may be impaired by the elimination of magnesium. The resistance to thermal breakdown of the membrane was therefore also examined. It was found that both the magnesium-depleted and control cells underwent vesiculation with a sharp onset at 49°C. However, evidence of structural changes in the membranes of the mag-

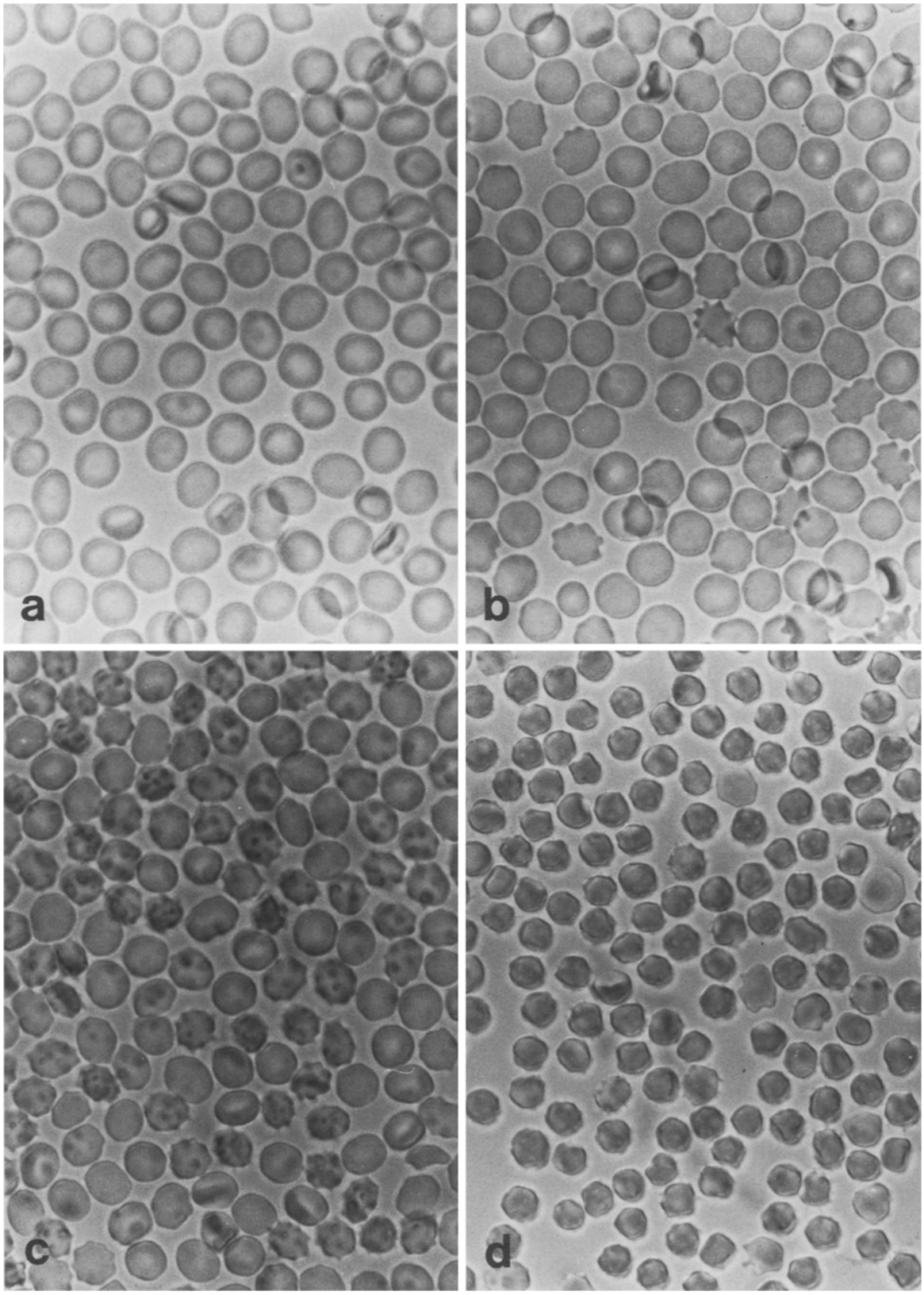

Fig. 3. Morphology of heat-treated red cells as a function of intracellular magnesium ions: (*a*) cells incubated at 37°C with ionophore A23157, 0.15 mM magnesium chloride and 1 mM EGTA; (*b*) the same cells after subsequent heating at 48°C; (*c*) cells incubated at 37°C with ionophore and 1 mM EDTA; (*d*) same cells as in *c* after subsequent heating at 48°C

nesium-depleted cells at lower temperatures could be obtained. After exposure to 45°C for 10 min, followed by cross-linking with glutaraldehyde, the magnesium-depleted, but not the control cells agglutinated to a stiff gel.

The altered response to thermal perturbation was reflected also in filtrability measurements. Untreated, or control cells showed only small changes in filtrability after incubation at temperatures up to 48°C: the RCTT increased by 23% (average of six experiments), and the clogging time remained the same. By contrast, the magnesium-depleted cells, heated to the same temperature showed (four experiments) an increase in RCTT of between four- and twentyfold, while the clogging rate increased by a factor or 5–10. These cells also showed a unique morphology: whereas the control cells, after heating to 48°C, remained discocytic, the magnesium-depleted cells assumed a lobed spherical form with spicules (Fig. 3). Intracellular magnesium thus protects the cell against thermally induced structural changes.

Discussion

From the surface area of the red cell, the known contents of the membrane-skeletal constituents and the largely hexagonal symmetry of the network, it follows that the spacing between the network junctions is about 50 nm. This is about one quarter of the extended length of the spectrin tetramer, as revealed by the electron microscope (Shotton Burke & Branton, 1979) and half the RMS end-to-end distance of spectrin tetramers in solution, inferred from solution scattering data on dimers and tetramers (Elgsaeter, 1978; Reich et al., 1982) and hydrodynamic studies (Mikkelsen & Elgsaeter, 1978; Stokke & Elgsaeter, 1981). Thus the spectrin in the membrane is in a highly crowded and crumpled state. This is presumably the reason why chemical cross-linking can generate higher species than the $\alpha\beta$ dimer. The promotion of such cross-linking by magnesium ions presumably reflects the magnesium-dependent cooperative spectrin-spectrin interactions that we have observed in solution (Fig. 1).

Vertessy and Steck (1989) have given reasons for believing that the elasticity of the red cell membrane arises from protein-protein interactions, rather than from constraints on the configurational entropy of the spectrin tetramers. Alterations in spectrin-spectrin interactions, generated by intracellular magnesium, evidently do not affect the elastic properties of the membrane, as judged by our micropipette aspiration data. (We disregard the possibility, raised by the theoretical considerations of Stokke et al. (1986*b*), that a change in shear elastic modulus is offset by a precisely balancing perturbation of the modulus of area compression, since this would require an unrealistically large magnesium-dependent change in the latter, amounting to at least two orders of magnitude.) The shear elastic modulus is therefore by implication regulated by different types of interactions, which could possibly be between the two chains of each spectrin dimer or between spectrin and integral elements of the membrane, either proteins or lipids.

Magnesium ions must, nevertheless, exert some form of control over the material character of the membrane, for they stabilize the discoid shape, as well as structural features that are otherwise perturbed by exposure to temperatures below that at which the cell vesiculates. Our observations are thus consistent with the view that stability and elasticity of the membrane are separately controlled properties (Chasis & Mohandas, 1986; Shields et al., 1987). What the temperature-labile elements are is at this stage a matter of conjecture. One possibility is actin, which in the free state requires divalent cations to maintain the native structure and is also stabilized in the polymeric form by tropomyosin. The association of F-actin with tropomyosin is magnesium dependent, and it is known that the tropomyosin associated with the actin protofilaments in the red cell is lost in the absence of magnesium ions (Fowler & Bennett, 1984). Disruption of the continuity of the cytoskeletal network, resulting from heating in the absence of magnesium ions, might be expected to release constraints on the diffusion of transmembrane proteins, of which band 3 is the most abundant, and so permit clustering of intramembrane protein particles and agglutination of the cells on exposure to the bifunctional reagent.

We thank Dr. T.J.B. Simons for generously performing magnesium analyses for us. This investigation received the financial support of the UNDP/WHO Special Programme for Research and Training in Tropical Diseases.

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Received 14 February 1990; revised 7 May 1990