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Effect of mercuric ions on human erythrocytes. Relationships between hypotonic swelling and cell aggregation

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Abstract

This study was undertaken to verify the hypothesis that the haemolytic effect of mercuric ions on human erythrocytes is strongly decreased under swelling conditions (relative to isotonic suspensions). In fact, interaction of Hg^{2+} with swollen erythrocytes yields a rapid and cooperative cell aggregation, a phenomenon that appears to prevent penetration of mercuric ions into the cells and, accordingly, to avoid any haemolytic effect induced by the Hg^{2+} entrance. Since in vivo erythrocytes undergo big shape changes (swelling being a kind of shape modification) related to mechanical or (in some animals) osmotic stresses, the reported observations turn out to be also of some relevance for the understanding of certain toxicological effects of mercuric ions. © 1997 Elsevier Science B.V.

Keywords: Mercuric ions; Erythrocyte aggregation

1. Introduction

In a previous paper [1], some aspects of the toxicology of mercuric ions has been related more to Hg^{2+} interactions with cytoplasmatic components [2,3] than concerned with the direct alterations of cell membrane, the latter being only one the first site of action for the ions. In fact, binding of mercuric ions to the outside of erythrocyte membrane induces [1] a transient increase of the cell resistance to haemolysis for about five minutes, a time interval necessary for the going of the metal ions through the membrane into the cytoplasm; afterwards, in about 30 min the membrane recovers its normal fragility. In particular, haemolytic processes appear to be a consequence of metal ion inhibition of cytoplasmatic enzymes (such as superoxide dismutase, catalase and glutathione peroxidase [3–5], and production of activated forms of oxygen by Hg^{2+}-haemoglobin complexes [6]; disruption of the cytoskeleton, described only for organic mercurials, cannot be excluded [7].

As already observed [8,9], therefore, adducts between mercuric ions and erythrocyte membrane proteins are quite labile (in kinetic terms), even though

Abbreviations: ACD: Anticoagulant solution (Adenine, Citrate Natrium salt, Citric acid, Dextrose); DTT: 1,4-Dithiothreitol; EDTA: Ethylenediaminetetraacetic acid; IHP: Inositolhexakisphosphate Natrium salt; *p*CMB: *p*-Chloromercuribenzoate acid; *p*CMBS: *p*-Chloromercuribenzenesulfonic acid

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the thermodynamic stability of these complexes in biological system may be large. Such transient interactions have also been reported [1] for other heavy metal ions and strongly related to the chemical properties and stereochemistry of both metal ions and membrane components. Because of that, it appeared of interest to investigate the interaction of some heavy metal ions (in particular, Hg^{2+}) with human erythrocytes in slightly hypotonic solutions. In fact, under these experimental conditions an increase in the number and exposure (i.e., accessibility) of reacting sites for Hg^{2+}, as well as a change in their microenvironment relative to isotonic solutions is expected. Accordingly, hypotonicity should improve or, at least, prolong the membrane stabilization instead of favouring the metal ion toxicity (i.e., erythrocyte lysis). Therefore, the present study was designed to verify such hypothesis. The reported results indicate that swollen erythrocytes tend to aggregate rather than to haemolise [see Ref. [1]] in the presence of mercuric ions.

2. Material and methods

Fresh human blood, drawn from healthy donors in the presence of ACD[1] medium as anticoagulant (14 ml/100 ml blood; composition: citric acid, 26.3 g l^{-1}; dextrose, 3.87 g l^{-1}; NaH_2PO_4, 2.22 g l^{-1}; adenine, 0.275 g l^{-1}), was used throughout the experiments. The erythrocytes were washed three times with isotonic phosphate buffered saline (5 mM, pH 7.4). *p*-Choromercuribenzenesulfonic acid, mercuric chloride, cadmium chloride and zinc chloride were purchased from Sigma (St. Louis, MO, USA). All other chemicals (Carlo Erba, Milan, Italy) were of analytical grade and used without further purification.

The effect of mercuric ion binding to erythrocytes was investigated at 25°C by using a stopped flow method that follows light scattering changes of the system [1]. Rapid modifications in scattered light were followed as absorbance changes at 693 nm, a wavelength values at which haemoglobin does not absorb. A given volume of erythrocytes (suspended in 135 mM NaCl buffered with 5 mM Tris–HCl or in buffered isotonic saline) was mixed in a stopped-flow apparatus (Hi-Tech SF11; dead time = 50 ms) with an equal volume of 5 mM Tris–HCl, pH 7.4 containing mercuric ions at various concentrations; osmotic conditions were kept constant by addition of NaCl. In general, the experimental suspensions had the final haematocrit value equal to 0.5% v/v. For additional details, see Ref. [1].

The percentage of haemolysis was determined by the ratio between haemoglobin free in solution after incubation with mercuric ion solutions and total haemoglobin released after sonication of the erythrocytes.

Total haemoglobin after sonication was determined at 540 nm by the cyanomethemoglobin method, while the ferric derivative was measured at 630 nm by the cyanide method [10].

Previous studies [1,11] have shown that the intensity of the scattered light (measured by a stopped-flow apparatus) increased as erythrocytes undergo a shape transition from normal biconcave disc to swollen sphere, invaginated erythrocytes or cell clusters, whereas a decrease in the intensity of light scattering was observed in the case of haemolysis or of cell crenation. In order to check whether the optical changes observed in the reported experiments should mainly be associated to formation of cell aggregates and/or morphological changes of erythrocytes, the phenomenon induced by mercuric ions was investigated by video microscopy. A Zeiss Axiophot microscope, equipped with a 100 W epiillumination lamp, an electric shutter and an interference filter (band pass 400–450 nm), was used throughout. A solid state CCD camera (Type 558, by 12S, France) was connected to the microscope outlet and an Apple IIGS PC was used to control the camera and the shutter of the microscope. The assembly of the instrument and the necessary electronics is described elsewhere [12]. Image analysis was carried out on an Apple MacIntosh IIvx using the program 'Image'. The time course of erythrocyte aggregation after addition of mercuric chloride is shown in Fig. 1. At six fixed times a small volume (ca. 10 μl) of a mildly stirred, erythrocyte suspension (1×10^9 cells/1) in hypotonic buffered saline (e.g., 250 mOsm) was laid on a microscope slide and a coverslide was mounted on. On Hg^{2+} addition, the onset of aggregation was observed within seconds and the process followed for at least 60 s, when $> 90\%$ erythrocytes were recruited into aggregates ranging from doublets to sex-

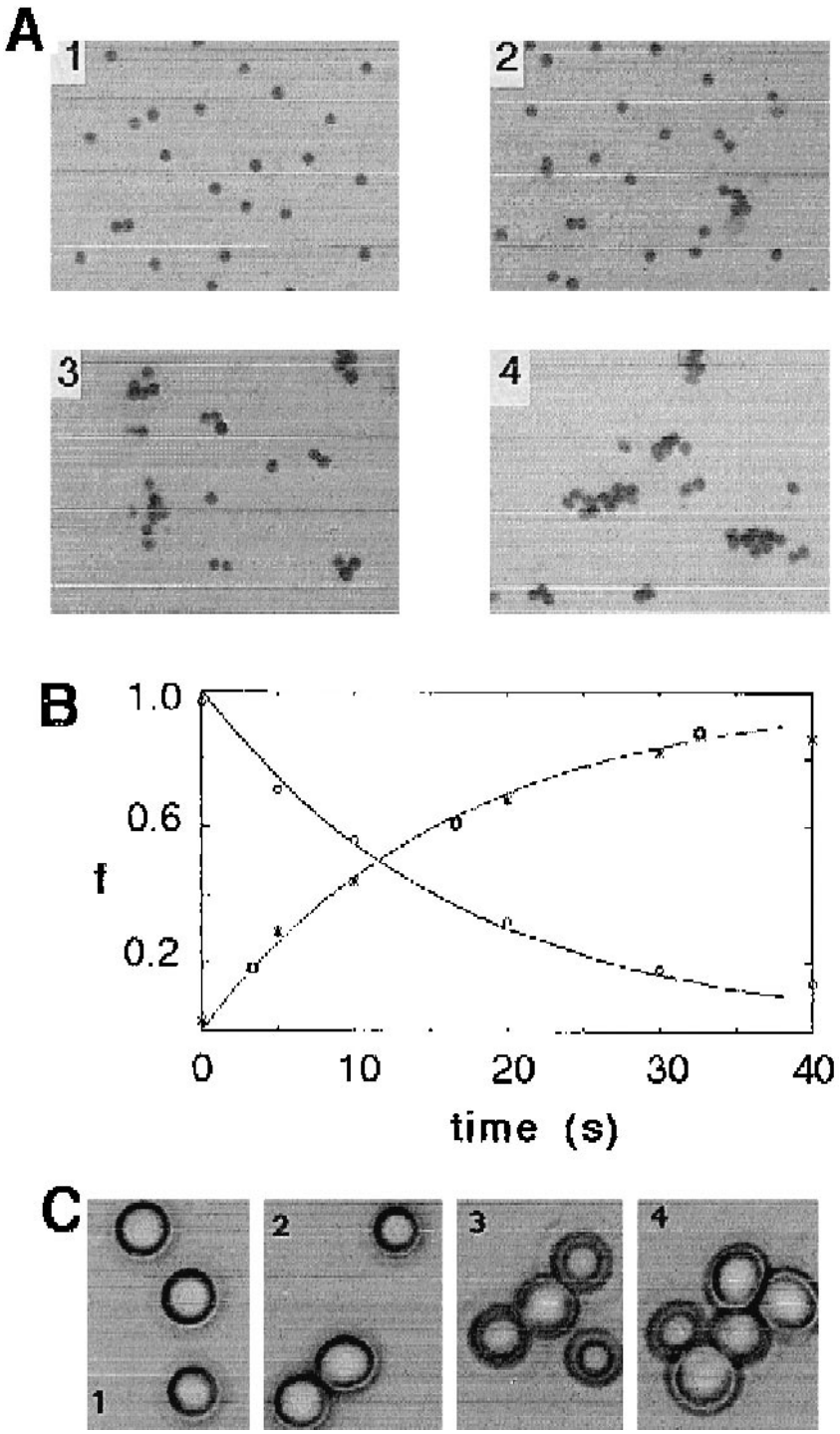

Fig. 1. Human erythrocyte aggregation (Panels A and B) and aggregation morphology (Panel C) induced by mercuric ions (0.1 mM) in hypotonic buffered saline (250 mOsm at pH 7.4 and 20°C), observed under light microscope at various times (s) ($A_1 = 0$; $A_2 = 5$; $A_3 = 20$: $A_4 = 40$; $C_1 = 0$; $C_2 = 5$; $C_3 = 20$; $C_4 = 40$). Panel B reports the fraction (f) of erythrocytes recruited in aggregates (*) and the changes in singlet concentration (○) during the experiment; three values (□), taken from the time course of the very same system monitored with the stopped flow apparatus, are also indicated. For more details, see Section 2.

tuplets and larger aggregates (Fig. 1A,C): doublets were mainly formed in the first 5 s (Fig. $1A_2$); in the subsequent time interval the doublets appeared to grow in size, leading to the formation of triplets, quadruplets and larger aggregates (Fig. $1A_3$–A_4). Thus, there was a sequential rise of aggregates of increasing size after Hg^{2+} addition. The distribution of singlets and aggregates was measured by gating on each particle size (singlet to sextuplets and larger aggregates) present at each fixed time in 20 low enlargement fields and normalizing by the total number of particles detected. The extent of aggregation (ordinates in Fig. 1B) was quantitated from the fraction of single erythrocytes that were recruited into aggregates as defined by the following equation:

$$f = \left(1 - \frac{2}{S + 2D + 3T + 4Q + 5P + 6S^{+}}\right)$$

where the aggregate sizes are given by S (singlets), D (doublets), T (triplets), Q (quadruplets), P (pentuplets) and S^{+} (sextuplets and larger aggregates). In the absence of mercuric ions, erythrocytes placed in hypotonic solutions remained as singlets (ca. 99%) during the time of the aggregation measurements. Under the experimental conditions chosen (e.g., those in Fig. 1), the aggregation process was mostly (more than 80%) monophasic. The good correspondence (Fig. 1B) of the aggregation kinetics monitored by video microscopy and by stopped flow measurements supports the view that both methods record the same phenomenon. In other words, changes in the intensity of the scattered light are closely related with cell aggregation and not with cell morphological transformation. The observed clustering was fully reversible by adding to aggregated erythrocytes different chelating agents (at 50 mM final concentration), such as EDTA, IHP and cysteine. Moreover, the aggregation phenomenon was investigated in details under microscope and by stopped flow apparatus at various metal ion concentrations (0.10 to 0.50 mM), Hg^{2+} being (as expected on the basis of its higher affinity for thiols relative to other cations [13]) more effective than Zn^{2+} and Pb^{2+}; however, in the presence of Pb^{2+} any quantitative analysis was impaired by the light scattering induced by the formation of inorganic precipitate (i.e., $PbCl_2$).

3. Results

Addition of bi- or tri-valent cations (all having chloride as counterion), at a final concentration of 20 mM in 5 mM Tris–HCl, pH 7.4, to human red blood cells (suspended in hypotonic buffered saline, i.e., 280 mOsm) induced haemolysis or erythrocyte aggregation, depending on the nature of metal ion used. In general, the apparent effectiveness of the cations investigated appeared to be largely a function of the ionic radius. Thus, from a phenomenological viewpoint, a rapid, nearly monophasic haemolysis was observed in the case of Cr^{3+} (ionic radius 0.063 nm), Cd^{2+} (0.097 nm)and Co^{2+} (0.072 nm); in particular, the time course in the presence of Cr^{3+} corresponded to a single exponential up to 90% of the process whereas the other two metal ions determined an event which diverged slightly from a simple one (Fig. 2). On the contrary, Hg^{2+} (0.11 nm), Pb^{2+} (0.12 nm), and Zn^{2+} (0.14 nm) induced a more complex phenomenon, an initial decrease in the light scattering being followed by a slow increase (Fig. 2). As a whole, these data indicate that all cations having ionic radii larger than 0.1 nm induce erythrocyte aggregation, whereas the smaller ones produce haemolysis. Of course, the mechanisms of the different biological actions carried out by the various cations is governed by kinetic and thermodynamic factors, the ion size controlling only the steric accessibility of these ligands to the proper binding sites.

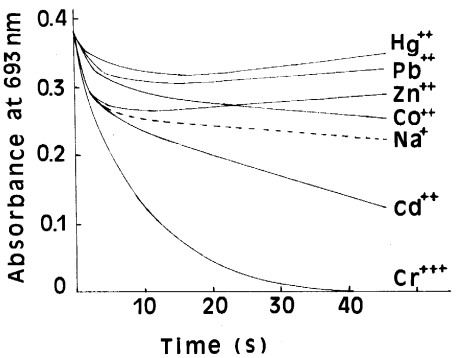

Fig. 2. Time course of light scattering changes observed at 693 nm and 25°C upon addition of 1×10^{-2} M (before mixing, in 5 mM Tris–HCl, pH 7.4) of heavy metal ions or NaCl (dotted line) to a suspension of human erythrocytes in hypotonic buffered saline (final osmotic concentration: 165 mOsm).

Fig. 3A reports time courses observed upon rapid addition of different Hg^{2+} concentrations (whose isoosmolarity was kept constant by NaCl) in 5 mM Tris–HCl, pH 7.4 (siringe 1) to a fixed number of suspended erythrocytes (8×10^9 cells/1) in buffered hypotonic saline (siringe 2); the fractional haemolysis observed 1 min after rapid mixing of the two syringes content is also indicated in the figure. It should be pointed out that for each experimental condition the final percentage of haemolysis closely corresponded to the relative amplitude for the decrease in the light scattering signal, giving confidence in the validity of the kinetic signal (i.e., decrease in optical density) as a measure of haemolysis. Moreover, it is relevant that at $[Hg^{2+}] = 0.1$ mM most erythrocytes were completely haemolysed, while at 0.25 mM only 30% lysis was observed and most erythrocytes turned out to be

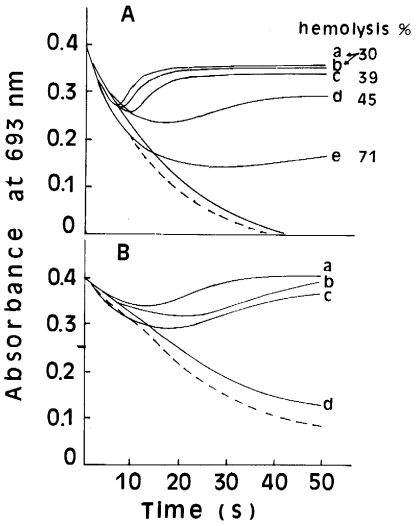

Fig. 3. Time courses of light scattering changes, at 693 nm and 25°C, of a suspension of human erythrocytes in hypotonic buffered saline upon mixing of $HgCl_2$ buffered (5 mM Tris–HCl, pH 7.4) solutions at different metal concentrations. The number of erythrocytes before mixing always was $8 \times 10^9/1$. Panel A reports data at the following mercuric ion concentration (after mixing): (a) 0.25; (b) 0.22; (c) 0.20; (d) 0.15; (e) 0.12; (f) 0.10 mM. The dashed line represents the time course recorded in the absence of mercuric ions. Near each curve the percentage of haemolysis, observed 1 min after mixing, is reported (for further details, see Section 2). Panel B refers to cells preincubated for 30 min at 37°C in the presence of 0.5 mM *p*CMBS, upon addition of buffered (5 mM Tris–HCl, pH 7.4) $HgCl_2$ solutions at different concentrations. The mercuric ion levels after mixing were: (a) 0.50; (b) 0.15; (c) 0.12; (d) 0.10 mM. The dashed line represents the time course recorded in the absence of mercuric ions.

Table 1
Effect of osmotic jump (defined as osmolarity of erythrocyte suspension minus final osmolarity after addition of hypotonic mercuric ion solution) on aggregation rate (expressed in terms of half time, $t_{1/2}$) of human erythrocytes at pH 7.4 and 20°C

Osmotic jump (mOsm)	$[Hg^{2+}]$ (mM)	$t_{1/2}$ (s)
30	0.15	20
	0.25	20
50	0.15	13
	0.25	13
90	0.15	8
	0.25	6

aggregated (as checked by microscope observation), as previously reported [1]. Thus, within a narrow range of Hg^{2+} concentration (0.1 to 0.25 mM), the effect of the metal ions passed from total haemolysis to aggregation of most cells. It is interesting to underline that the rate of aggregation (as measured by increase in optical density) was a function of the osmotic jump; in fact, when osmolarity of the mercuric ion solutions was increased by adding NaCl (from 10 to 200 mOsm), aggregation was observed as well but the rate slowed down (see Table 1), and was independent of Hg^{2+} concentration (at least over the 0.1–0, 25 mM range), suggesting that the swelling process might play a crucial role. As a further investigation, the rate of the process was measured in the presence of a constant Hg^{2+} concentration (0.25 mM), but varying the number of erythrocytes per unit volume (1 to 25×10^{-9} cells/1): the results (data not shown) indicated that the aggregation rate decreased as the number of red blood cells per litre increased, implying that this event was regulated by the ($[Hg^{2+}]$/cell ion binding sites) ratio.

Moreover, it is important to point out that under hypotonic conditions no haemolysis was observed in the aggregated systems, even after one hour incubation in the presence of 1 mM Hg^{2+}. This behaviour was different from that monitored for unswollen erythrocytes at the same Hg^{2+} concentration [1], in the latter case 20–30 min incubation having been enough to lead to extensive haemolysis. This evidence indicates that upon cell aggregation the damaging effect of Hg^{2+} is drastically reduced.

In order to check the possible dependence of the phenomenon reported in Fig. 3A on the water influx through erythrocyte membrane, similar experiments with *p*CMBS under the very same conditions were performed. It is known that this mercurial compound inhibits the osmotic permeability of human erythrocytes by about 90% and the diffusional permeability by about 50% by binding to sulphydryl groups of an abundant membrane protein, CHIP28k [14–16]. As expected, rapid addition of 0.5 mM *p*CMBS (in 5 mM Tris–HCl, pH 7.4) to erythrocytes was not followed by any aggregation. However, when Hg^{2+} (under the same experimental conditions of Fig. 2) were added to erythrocytes, previously incubated for 30 min with 0.5 mM *p*CMBS in buffered saline (a procedure that minimizes the water influx into red blood cells [17,18,1]), the initial rapid decrease in light scattering largely disappeared (see Fig. 3B in comparison with Fig. 3A) and the rate of aggregation slowed down. Therefore, in line with the results already reported in Ref. [1] it may reasonably be assumed that the first part of the curve (i.e., the decrease in light scattering) indeed represents the haemolysis phenomenon which starts immediately after mixing, while the second portion (i.e., the scattering increase) is likely to represent the aggregation which occurs to a significant extent only when the curve slope changes sign; as a consequence, the two portions of the curves obtained at higher Hg^{2+} concentration (Fig. 3A) may be analyzed separately. The haemolysis phenomenon (as observed in Fig. 3A) is reported by a semilogarithmic plot in Fig. 4A, while Fig. 4B shows the aggregation process in kinetic terms. In both cases, calculations were performed under the assumption of a first order kinetic process, the validity of which was supported by the linearity of the experimental points (see Fig. 4A). It must be pointed out that the rate constant of the haemolysis phase was roughly independent of metal concentration, differing only a 1.5 fold range; in contrast, the cell aggregation was strongly concentration dependent. Fig. 4B (inset) also shows the relaxation time $(1/t)$ for erythrocytes aggregation as a function of Hg^{2+} concentration. This dependence was not linear and at least two different slopes were observed: the rate of aggregation increased quickly in the range $2–3 \times 10^{-4}$ M, while a slower dependence was observed at higher Hg^{2+} concentration. Moreover, extrapolation to low $[Hg^{2+}]$ intercepted the *X*-axis: this evidence could suggest that the aggregation process requires a minimum Hg^{2+} concentration to occur. In addition, the narrow range of Hg^{2+} concentration, over which the whole phenomenon takes place, represents a strong indication for a mechanism involving some cooperative interaction among the groups favouring aggregation.

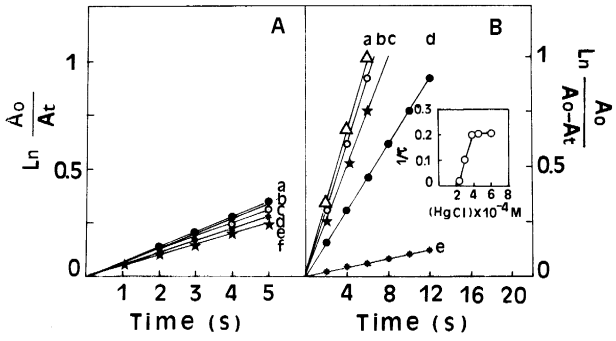

Fig. 4. Kinetic analysis of the light scattering signal following the addition of buffered (5 mM Tris–HCl, pH 7.4) solutions containing varying amounts of $HgCl_2$ (see legend to Fig. 3A). A_0 corresponds to the absorbance at 693 nm at time zero and A_t at the time *t*. Panel A represents the semilogarithmic plots of the first phase of the curves shown in Fig. 3A. Panel B refers to the increasing part of the curves shown in Fig. 3A, taking the plateaux as initial point. Near each curve the letter refers to the corresponding trace reported in Fig. 3A. Inset of panel B shows the dependence of relaxation time ($1/t$, i.e., the reciprocal of the apparent first-order rate constant) on Hg^{2+} concentration.

Finally, in order to gain further insight into the interaction of Hg^{2+} with the membrane binding sites, the aggregation effect induced by mercuric ions on erythrocytes (previously incubated for 5 min with other heavy metal ions (e.g., Zn^{2+} Pb^{2+} and Co^{2+}) or thiol reagents (such as cysteine, glutathione, DTT or *p*CMB)) was investigated (Fig. 5). In the case of pre-treatment with monocharged *p*CMB, a marked delay in the formation of aggregates was observed, accompanied by a much reduced (ca. 20-fold) rate for the scattering increase. Conversely, the other compounds reported in Fig. 5 did not appreciably delay the appearance of aggregates, though slowed down to various extends the apparent rate of scattering increase. As an additional detail, it appears interesting to notice that cell aggregation was not observed in the presence of the monovalent organic mercurials *p*CMB or *p*CMBS, suggesting that the cluster forma-

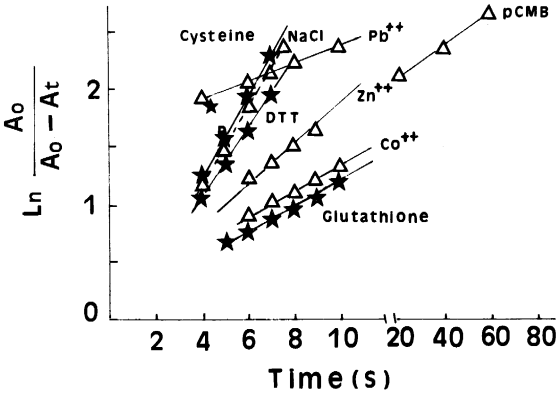

Fig. 5. Semilogarithmic plot of the increasing part of the light scattering signal recorded upon addition of $HgCl_2$ (2×10^{-4} M final concentration) to erythrocytes pre-incubated with different chemical agents for 5 min. Mixing starts at $t=0$, while the aggregation takes place differently for each system. concentration in the incubation vessel was 2 mM for all chemicals (i.e., cysteine, glutathione, DTT, *p*CMB, Zn^{2+}, Co^{2+} and Pb^{2+}).

tion was strictly related to inorganic bivalent mercuric cations. In particular, in the presence of *p*CMB the haemolysis was delayed for 4–5 min and then took place very slowly ($t_{1/2}=8$ min), probably due to the higher affinity of the aromatic mercury (with respect to inorganic Hg^{2+}) for the phospholipidic monolayer [19].

4. Discussion

The reported data show that the increased exposure and/or number of mercuric ion binding sites, accompanying the swelling process, may play a role in modulating the toxicological effect of Hg^{2+}.

Microscopic observation reveals that interaction of mercuric ions with the erythrocyte membrane, while cells are swelling, brings about cellular aggregation. The empirical observation that progress curves of light scattering increase are accurately described by a single exponential suggests that the rate-limiting step in cell aggregation is not the frequency of cell–cell collisions, but is an event which proceeds at a rate independent of the red blood cell number per unit volume. Moreover, the aggregation process is strongly dependent on Hg^{2+} concentration only when the osmotic jump is large (e.g., from 280 to 140 mOsm but not from 280 to 240 mOsm), indicating that the swelling indeed plays a crucial role. In fact, in *p*CMBS treated erythrocytes, where the water influx is partially inhibited, the shape transition is slowed down as well as the rate of aggregation.

Apparently, the aggregation process requires a minimum concentration of Hg^{2+} to occur. In particular, from the plot of $1/t$ vs. $[Hg^{2+}]$ (see inset of Fig. 4) the number of sites per cell available to mercuric ions can approximately be calculated turning out to be in the order of 10^8, in agreement with previous determinations with $^{51}Cr^{2+}$ [20]; moreover, the steepness of the line, in the narrow range of 0.2–0.35 mM mercuric ions, suggests that the aggregation process is driven by a cooperative mechanism, such that the formation of the first nucleus of aggregate could render the adhesion of additional cells more favourable, the whole process depending on the bivalent cations present. Therefore, at least two steps should be considered: an initial shape transition (i.e., swelling) of the erythrocytes, which is followed by mutual adhesion of cells forming large aggregates in the presence of bivalent cations (such as Hg^{2+}, Pb^{2+} and Zn^{2+}) bound to the external erythrocyte surface. Furthermore, the limited range of Hg^{2+} concentration, over which the overall phenomenon (i.e., swelling and aggregation) occurs, requires that during swelling erythrocytes go through a series of intermediate states which display higher reactivity for the metal cation than the resting cell and/or exposure of an increased number of binding sites, possibly reflecting a substantial structural transition at the level of the membrane during the hypotonic swelling. Interactions of Hg^{2+} with membrane proteins and/or anionic heads of phospholipids were taken into account for explaining the marked rigidification of the membrane fluidity, as observed by EPR measurements [5,21]. Even though other possibilities cannot be ruled out, this feature may account, at least qualitatively, for the quite sharp dependence of the degree of haemolysis, and thus of the percentage of aggregation on the Hg^{2+} concentration. Finally, the aggregation phenomenon seems to require large bivalent cations; in fact, the possibility of a bridge between two cells appears to be a key event in inducing the aggregation, this being consistent with the absence of an effect induced by *p*CMB or *p*CMBS (which are monovalent compounds).

From another viewpoint, even though the range of mercuric ion concentration used in this investigation

is certainly higher than the usual values found in the blood circulation system and, accordingly, out of the classical toxicological interest, the reported results appear useful for enlarging upon Hg^{2+} toxicity, in that they suggest the potential existence of a new type of pathological effects induced by mercuric ions, based on erythrocyte aggregation.

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