METHYLMERCURY-INDUCED HEMOLYSIS AND BINDING OF THE MERCURY BY ERYTHROCYTE MEMBRANE

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Summary.....Binding of methylmercury by human erythrocyte membranes was examined in association with the mercury-induced hemolysis. Organic mercurials such as *p*-chloromercuribenzoate and methylmercury showed less potent hemolytic activity than HgCl_2 . Entry of methylmercury into the erythrocytes was very rapid. Over 95 % of methylmercury was taken up by the cells within 5 min over the concentration range studied. Percent binding by the membrane of methylmercury was lower than HgCl_2 . Affinity of the intact membranes as well as of the isolated membranes was found to be lower for methylmercury than for HgCl_2 . Analysis by means of the Scatchard plot indicated a variety of binding sites of the erythrocyte membranes for the mercurials. The critical level of methylmercury required for hemolysis was about 200 nmole/mg membrane protein. No close correlation was observed between membrane sulfhydryl inhibition and hemolysis. The results presented here suggested that affinity of the erythrocyte membranes for the mercurials is one of the determining factors in hemolysis.

Methylmercury is well known to cause mainly neurological signs (Suzuki 1969 ; Takeuchi 1972 ; Klein et al. 1972 ; Richardson and Murphy 1974) and also considered to affect the stability of the liver lysosomal membrane (Pekkanen 1971). Although the molecular basis of methylmercury toxicity has not been defined clearly, one possible target site might be the plasma membrane. On the other hand, distribution studies revealed that the ingested methylmercury is highly concentrated in the red cells (Suzuki et al. 1971 ; Swensson et al. 1959). Based on these findings, it is assumed that the erythrocyte membrane is one of the primary cellular organelles most vulnerable to the external toxic agent.

In the present experiments, methylmercury-induced hemolysis as manifestations of structural destruction of the erythrocyte membrane was investigated in association with the mercurial binding to the membrane. Since a mercurial compound, either as an inorganic or organic form, is characterized by a marked chemical affinity for sulfhydryl groups, the alterations in the membrane sulfhydryl contents induced by methylmercury were also studied.

METHODS

Human blood was drawn from a healthy volunteer and centrifuged to separate off the plasma and buffy coat. The packed red cells were washed with buffered saline (154 mM sodium chloride, 11.2 mM sodium phosphate, pH 7.4) until the supernatant became colorless, and resuspended to make 25% hematocrit. When hemolytic potencies were compared between mercurials, 0.1 ml of the red cell suspensions were added to 4.9 ml of the buffered saline containing a test compound in varying concentrations. The mixture was incubated at 37°C and shaken at 70 cycles/min for varying periods of time. After centrifugation, the percent hemolysis was measured by determining hemoglobin in the supernatant colorimetrically at 540 nm in a Hitachi 124 spectrophotometer.

Binding studies were carried out using radiolabelled compounds. One ml of ^{14}C -labelled methylmercuric chloride (MMC) or ^{203}Hg -labelled HgCl_2 solution in the buffered saline was added to 2 ml of the red cell suspensions. After incubation and centrifugation, the packed red cells were hemolyzed with 25 volumes of ice-cold 15 mM Tris-HCl buffer (pH 7.4) and centrifuged for 20 min at 10,000 rpm in a Hitachi 20 PR refrigerated centrifuge. After removal of hemolysate, the pellets of the cell membranes were washed twice with the Tris buffer. The radioactivity in the membrane and intracellular fractions was determined with either a Nuclear Chicago Mark II liquid-scintillation or an Aloka JDC 751 well-scintillation counter. The percent uptake of the mercurials by the cells was calculated from determination of the amount of the agent left in the bathing media.

In order to examine binding of MMC or HgCl_2 by the isolated membranes, 0.5 ml of the radiolabelled mercurial solutions were added to an equal volume of the membrane suspensions corresponding to 500–600 μg protein. Binding of the mercurials by the isolated membranes was tested by the approximately same procedures as described above. The numbers of sulfhydryls in the membrane fraction were estimated by the method described by Ellman (1959). Protein content was determined by the method of Lowry et al. (1951).

RESULTS

Hemolytic potencies of mercurials Among the mercurials tested, MMC showed the least hemolytic effect on the human erythrocytes (Fig. 1). Inorganic mercury was 7- to 10-fold as potent as the organic mercurials, 50% hemolysis of 0.5% cell suspension being given by 25 μM HgCl_2 , 180 μM *p*-chloromercuribenzoate (PCMB) and 250 μM MMC at 3 hr.

Uptake of MMC and HgCl_2 by the red cell and their distributions within the cell The entry of 0.01–1 mM MMC into the erythrocyte was very rapid and most of the mercury was cleared from the bathing media (Table 1). Over 95% of MMC was taken up by the cell within 5 min and this uptake level did not change further with time. The passage pattern of HgCl_2 into the erythrocyte was compared with that of MMC at the low non-hemolytic concentration of 10 μM for 60 min. The rate and extent of uptake

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Fig. 1. Hemolytic effects of mercurials on the human erythrocytes. Erythrocyte suspensions of 0.5% hematocrit were incubated with mercurials at 37°C and shaken at 70 cycles/min. After centrifugation of the cell suspensions, the degree of hemolysis was determined by measuring hemoglobin in the supernatant colorimetrically at 540 nm. Comparisons were made at the constant concentration of 100 μM (*upper*) and at a given time of 3 hr (*lower*). Each point represents the mean of 5 or 6 determinations and standard error of the means is within ±5%.

of HgCl₂ were comparatively lower than that of MMC as shown Fig. 2. Within 15 min about 75% of HgCl₂ entered into the erythrocyte and the distribution between the cell and medium reached to equilibrium.

Analytical data concerning MMC distribution within the cell are demonstrated as the percent binding by the membrane in the last column in Table 1. Over a 100-fold concentration range, the percentage bound was almost constant and about 4 % of the mercury taken up by the cell. Accumulation of HgCl₂ in the membrane, however, was

Table 1. MMC uptake by erythrocytes and its distribution within the cell.

Concentration (mM)	Time (Min)	% Uptake	% Binding by the membrane
0.01	5	95.0	4.5
	15	98.0	4.4
	60	96.7	4.7
0.1	5	96.4	4.2
	15	98.2	4.5
	60	96.2	4.5
1	5	98.7	2.6
	15	97.6	2.9
	60	95.9	4.0

Erythrocyte suspensions of 16.6% hematocrit were incubated with MMC. % Uptake represents the percent against the amount of MMC added and % binding by the membrane the percent against the amount of MMC taken up by the cell. Each value represents the mean of 3 or more determinations and standard error of the means is about $\pm 10\%$.

Fig. 2. Uptake of mercurials by erythrocytes. Erythrocyte suspensions of 16.6% hematocrit were incubated with $10 \mu\text{M}$ of mercurials [—○—, HgCl_2 ; —●—, MMC]. Each point represents the mean of 3 determinations with standard error. No hemolysis was observed for 60 min.

Fig. 3. Distribution of mercurials within the cell. The ordinate represents the percentage bound by the membrane of mercurials [$-\bigcirc-$, HgCl_2 ; $-\bullet-$, MMC] taken up by the cell. Each point represents the mean of 3 determinations with standard error.

Fig. 4. Hemolytic effect and binding by the membrane of [MMC. Erythrocyte suspensions of 16.6% hematocrit were incubated with MMC for 12 hr. Each point represents the mean of 3 or more determinations with standard error.

about 3-fold higher than that of MMC at the concentration of $10 \mu\text{M}$ (Fig. 3). When the mercurial amount bound to the membrane was expressed on the basis of nmole/mg protein, the average value of HgCl_2 was 1.00 and that of MMC 0.45.

Hemolytic effect and binding by the membrane of MMC When the red cell suspensions of 16.6% hematocrit were exposed to MMC at the concentration of below

3 mM, little or no hemolysis was observed within a few hr. Hemolysis began to occur at the concentrations of over 2 mM in a 12hr-incubation period (Fig.4). On the contrary, the mercurial amount bound to the membrane increased proportionally to increasing concentrations and the critical level for hemolysis was found to be about 200 nmole of MMC/mg membrane protein. Two to 3 mM MMC was so rapidly taken up by the cell as in the case of below 1 mM and over 95% of the mercury entered into the cell within 5 min. With regard to the mercurial distribution within the cell, the percentage in the membrane fraction was only slightly higher than that shown in Table 1.

The reversibility of the mercurial binding by the cell and membrane was studied in connection with the hemolytic effect. After contact with 3 mM MMC for 30 min, erythrocytes were washed 3 times with buffered saline containing 10 mM cysteine and continued to be incubated in the fresh saline. This washing procedure required about 30 min and eliminated over 90% of MMC from the cell. The mercurial amount still remained in the membrane was less than that found after treatment with 0.3 mM MMC and no hemolysis was observed.

Binding of MMC and HgCl₂ by the isolated membrane The relationship of MMC or HgCl₂ binding by the isolated membrane to the mercurial concentrations added up to 3 mM could not be represented as a saturation type of curve. It was clearly shown, however, that HgCl₂ had a somewhat higher affinity for the membranes than MMC. Analysis by means of the Scatchard plot indicated more than 2 segments instead of a single straight line, suggesting several classes of the mercurial binding sites in the membranes (Fig. 5). By extrapolation of the segments to the x-axis, the maximal numbers of binding sites were estimated. Primary binding sites for MMC and HgCl₂ were 43 and 210 nmole/mg protein respectively. Subsidiary sites for MMC and HgCl₂ were 96 and 400 nmole/mg protein respectively.

Membrane sulfhydryl inhibition and hemolytic effect Since the mercurial compounds are characterized by the high affinity and selectivity for sulfhydryl groups in small molecules as well as in proteins, it was attempted to study the relationship between the extent of hemolysis and membrane sulfhydryl inhibition. Within 5 min 1 mM MMC blocked membrane sulfhydryl by 40% and no further increase in inhibition rate was observed with time (Fig. 6). In contrast to this rapid time course, in the presence of 30 mM N-ethylmaleimide (NEM) a sulfhydryl alkylating agent, the inhibition rate of membrane sulfhydryls was gradually increased up to 100%. In spite of the complete blockade of membrane sulfhydryls, NEM could bring about little or no hemolysis after a period of 60 min. These findings suggested a small and slow entry of NEM into the cell as compared with that of MMC. Unpublished work in this laboratory has shown that only 15% of ¹⁴C-labelled NEM entered slowly into the human erythrocytes over a 1000-fold concentration range.

The concentration of MMC required for about 50% hemolysis of 16.6% red cell suspensions for 60 min was 7.4 mM. Uptake of this considerably high dose of MMC was also rapid and about 90% of the mercurial entered into the cell within 5 min. During this very short period the membrane sulfhydryls were completely blocked, but no hemolysis

Fig. 5. Scatchard plot of MMC and $HgCl_2$ binding by the isolated membranes. The isolated membrane suspensions containing 500–600 μg of protein/ml were incubated with radiolabelled mercurials for 60 min. Scatchard plot is based on the equation: $r/Df = nk - rk$, where Df is the concentration of free agent, r the bound agent, n the maximum number of binding sites, and k the association constant.

Fig. 6. Time course of membrane sulfhydryl inhibition. Erythrocyte suspensions of 16.6% hematocrit were incubated with 1 mM MMC [—○—] and 30 mM NEM [—●—]. No hemolysis was observed for 60 min. Each point represents the mean of 3 determinations with standard error.

Table 2. Time course of membrane sulfhydryl inhibition and hemolysis by MMC.

Time (Min)	% Inhibition of membrane sulfhydryls	% Hemolysis
5	100	0
60	100	51.8
90	100	75.2
90*	100	60.5

Erythrocyte suspensions of 16.6% hematocrit were incubated with 7.4 mM MMC.

*After 5 min-incubation with MMC, the erythrocytes were washed three times with buffered saline. This washing procedure required about 30 min.

Each value represents the mean of 3 determinations and standard error of the means is about $\pm 10\%$.

occurred (Table 2). Hemolysis of human erythrocytes induced even by a high dose of MMC was shown to be preceded by a lag period. After erythrocytes exposed to MMC for 5 min were washed 3 times with the buffered saline alone, completion of membrane sulfhydryl blockade and damage leading to hemolysis seemed not to be influenced. The present results revealed that the mercurial binding to the erythrocyte constituents was strong and steady but reversible in the presence of sulfhydryl agents such as cysteine as mentioned above.

DISCUSSION

The present experiments confirmed that the kinetics of organic mercurial hemolysis are generally characterized by a lag period followed by a slow hemolysis (Fig. 1). HgCl_2 proved to have a greater hemolytic potency than the organic mercurials in consistence with previous observations (Tsen and Collier 1960 ; Arbuthnott 1962). An effort to elucidate the reasons for differences of the hemolytic effects between inorganic mercury and MMC, was made by examining of the binding of these 2 mercurials by the erythrocyte constituents. Over the concentration range studied, a very rapid entry of MMC through the membrane barrier into the cell (Table 1 and Fig. 2) was probably due to its high lipid-solubility as compared to HgCl_2 . The opposite relationship, however, was observed with respect to the mercurial binding by the membrane. The affinity of erythrocyte membranes was higher for HgCl_2 than for MMC (Fig. 3). These findings suggested that the mercurial permeation through and binding to the cell membrane are separate phenomena. A rapid and virtually complete uptake of MMC by erythrocytes has been already reported by White and Rothstein (1973), suggesting the quite different distribution from that of inorganic mercury across the membrane at equilibrium (Weed et al. 1962). van Steveninck et al. (1965) indicated a large asymmetry in the distribution of mercurial-binding groups in the erythrocyte membrane, that is, 95% of chlormerodrinreactive groups located within membrane and/or on its inner face. If this distribution pattern could be extended to almost mercurial compounds, it may be assumed that the mercurials which penetrate the membrane as a diffusible form, will be then bound possibly by the intracellular hemoglobin and the membrane. Weed et al. (1962) reported that the percentage

of cellular HgCl_2 bound to the membrane was 31% at $1\ \mu\text{M}$, 16% at $10\ \mu\text{M}$ and 3% at $100\ \mu\text{M}$. Such an inverse relationship between the percentage bound by the membrane and the concentration used was not observed in the case of MMC as demonstrated in Table 1.

As seen in Fig. 4, the amount of MMC bound to the membrane increased linearly with the concentration up to 3 mM. However, distinct hemolysis began to occur at around 2 mM. It was shown that a critical amount of MMC bound to the membrane was about 200 nmole/mg protein. This value was in good agreement with the total number of sulfhydryl groups in the erythrocyte membrane estimated by the method of Ellman (1959). This method is based on the reaction of 5, 5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) with sulfhydryls. The resulting 5-thio-2-nitrobenzoic acid anions give an intense yellow color which can be used to measure sulfhydryls. Of course, there is no evidence for complete reaction of DTNB with all sulfhydryls in the membrane. If the reaction of DTNB with the membrane sulfhydryls is incomplete, the mercurials might be able to bind with sulfhydryl groups inaccessible to DTNB or other heavy metal-binding groups.

No level of saturation of the mercurial binding by the isolated membranes was observed at the concentrations of HgCl_2 or MMC used in this study. Graphical treatment by means of the Scatchard plot as depicted in Fig. 5 suggested the existence of a number of binding sites each with a different affinity for the mercurials. Binding capacity of the intact membranes as well as of the isolated membranes was much higher for HgCl_2 than for MMC. The maximum number of binding sites for MMC estimated in the isolated membranes was about 100 nmole/mg protein. This value was of the roughly same order as reported by Komatsu et al. (1975) who calculated it from the linear plot of the amount of MMC bound to the membrane against the hematocrit values. The maximum number found in the isolated membranes was, however, rather below the critical level for hemolysis examined in the intact membranes. The reason for this disparity is now little known. One possible explanation is that the MMC concentration used was insufficient to determine the maximum number exactly. Another one is that small amounts of hemoglobin might be avidly bound to the membranes prepared from the cells which were previously exposed to the high concentration of MMC.

The concentration which produced 50% hemolysis of 16.6% erythrocyte suspensions at 1 hr was 7.4 mM of MMC in contrast to about 1 mM of HgCl_2 . With this high concentration, the amount of MMC bound to the membrane must be quite beyond the critical level for hemolysis in view of a rapid entry of MMC into the cell and complete blockade of membrane sulfhydryls within 5 min. The present results denote that the mercurial hemolysis requires a lapse of time in spite of instantaneous reaction of the critical level of MMC with the cell membrane.

The importance of sulfhydryl groups in the maintenance of the integrity of the erythrocyte membrane was demonstrated by a number of papers (Benesch and Benesch 1954; Jacob and Jandl 1962; van Steveninck 1965). This concept has been drawn from the observed disturbances of a particular function by using sulfhydryl inhibitors such as

the mercurials and NEM. Unfortunately, however, there has been no determination of the actual contents of membrane sulfhydryls. The present results suggested no direct parallelism between hemolysis and membrane sulfhydryl inhibition as far as the sulfhydryls were estimated by the reaction of DTNB. More detailed studies regarding the relationship between the degree of hemolysis and membrane sulfhydryl blockade will be reported otherwise.

On the basis of the results presented in this paper, it is conceivable that affinity of the red cell membrane for the mercurials is one of the determining factors in hemolysis and the subsequent process of the membrane damage is mainly time-dependent. The diversity of sulfhydryl groups in the membrane or the existence of binding sites other than sulfhydryls which is suspected in this experiment must be clarified by further studies.

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