Localization of Erythrocyte Membrane Sulfhydryl Groups Essential for Glucose Transport

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ABSTRACT The reactions of three organic mercurial compounds, chlormerodrin, parachloromercuribenzoate (PCMB), and parachloromercuribenzenesulfonate (PCMBS) with intact red blood cells, hemolyzed red cells, hemoglobin solutions, and hemoglobin-free ghosts have been characterized. Both PCMB and PCMBS react with only 2 to 3 sulfhydryl groups per mole of hemoglobin in solution, whereas chlormerodrin reacts with 6 to 7. In hemoglobin-free ghosts, however, all three reagents react with a similar number of sulfhydryl groups, approximately 4×10^{-17} moles per cell, or about 25 per cent of the total stromal sulfhydryl groups, which react with inorganic mercuric chloride. In the intact cell the membrane imposes a diffusion barrier; chlormerodrin and PCMB penetrate slowly, whereas PCMBS does not. Kinetic studies of chlormerodrin binding to intact cells reveal that the majority of stromal sulfhydryl groups is located inside the diffusion barrier, with only 1 to 1.5 per cent (or 1 to 1,400,000 sites per cell) located outside of this barrier. Reaction of PCMBS with intact cells is limited to this small fraction on the outer membrane surface. All three reagents are capable of inhibiting glucose transport in the red cell. With chlormerodrin and PCMBS it was demonstrated that the inhibition results from interactions with the sulfhydryl groups located on the outer surface of the membrane.

INTRODUCTION

The present investigation was undertaken to characterize the reactions of several organic mercurial compounds with various fractions of human erythrocytes, and to relate the interactions to inhibitory effects on the transport of glucose through the cell membrane. The compounds tested were parachloromercuribenzoate (PCMB),[1] parachloromercuribenzenesulfonate

[1] Although PCMB is a mixture of parachloromercuribenzoate and parahydroxymercuribenzoate, in the present study a sufficiently high concentration of chloride was present to shift the equilibrium toward parachloromercuribenzoate.

(PCMBS), and chlormerodrin. The specificity and utility of monofunctional compounds such as PCMB for titration of sulfhydryl groups in proteins have been discussed by Benesch and Benesch (1). Velick (2) has indicated that substitution of a sulfonic acid group for the carboxyl of PCMB to yield para-chloromercuribenzenesulfonate (PCMBS) increases the aqueous solubility of the compound. It was therefore assumed that the increased hydrophilic properties of PCMBS would minimize its penetration through the lipid phase of the cell membrane and produce effects related only to the sulfhydryl groups of its outer surface. The third organic mercurial reagent, chlormerodrin, is a diuretic compound particularly suited for binding studies, as it has been found by Clarkson *et al.* (3) to have a minimal tendency to liberate inorganic mercury in solution, in contrast to several other organic mercurial compounds.

Sugar transfer by erythrocytes is very sensitive to agents which react with sulfhydryl groups. LeFevre (4) in 1948 first described inhibition of glucose transfer in erythrocytes, produced by PCMB, while Dawson and Widdas (5) have recently described the kinetics of inhibition by *N*-ethylmaleimide. Weed, Eber, and Rothstein, in studies of inhibition of glucose transport by $HgCl_2$ (6), called attention to the importance of correlating the physiological effect with the actual binding of the inhibitor to the cell, rather than with the concentration added. In the present investigation, the reactions of PCMB, PCMBS, and chlormerodrin with intact red cells, hemoglobin-free ghosts, and hemolyzed red cells, were correlated with studies of inhibition of glucose transport in order to localize and characterize the sulfhydryl groups essential for this membrane function. For comparative purposes, some data concerning inorganic mercury are also included from a previous paper (6).

METHODS

Human blood was collected from hematologically normal adult donors, utilizing heparin as the anticoagulant. After centrifugation, the plasma and buffy coat were discarded, and the red blood cells washed three times with 1 per cent NaCl. All experiments were started immediately after the third wash.

Fresh solutions of the sulfhydryl agents were prepared every 4 days, to avoid the possibility of their breakdown with liberation of free inorganic mercury into the solution. For example, the ultraviolet absorption of a 10^{-3} M PCMBS solution at 236 mμ decreased 23 per cent after storage for 12 days at room temperature, indicating the instability of this compound in solution. Experiments were performed both in unbuffered saline (final pH ranging from 6.8 to 7.0) and in saline buffered at pH 7.1 by 10 mM tris. No significant differences were found between the two series of experiments.

The binding of the sulfhydryl agents to intact red cells and to ghosts was usually calculated from determinations of the amount of the agent left in the supernatant

after centrifugation. Chlormerodrin[2] and inorganic mercury labeled with Hg^{203} were determined by measuring the radioactivity in a well-type scintillation counter. In some cases the amount of chlormerodrin bound to the cells was determined directly, by measuring the radioactivity taken up by the cells, after washing the cells twice in 30 volumes of ice cold saline. The results of the two procedures were in close agreement, so the loss of bound chlormerodrin during the washing procedure appeared to be negligible. PCMB[2] and PCMBS were usually determined spectrophotometrically, at 250 and 236 mμ respectively, utilizing a Beckman spectrophotometer. Since the reaction of these mercurials with sulfhydryl groups increases their ultraviolet absorption considerably (1), an excess of cysteine (final concentration 2×10^{-4} M) was added to the supernatants, prior to determination of the absorbancy. Appropriate blanks were measured simultaneously to correct for any ultraviolet-absorbing material that might have leaked out of the cells. The chemical estimations of PCMB and PCMBS binding were confirmed by the use of Hg^{203}-labelled PCMB and S^{35}-labeled PCMBS,[2] with the radioactivity of the supernatant analyzed with either a well-scintillation or a liquid-scintillation counter.

In order to determine the maximal number of cellular sulfhydryl groups with which each of these agents can react, the cells were hemolyzed in 50 volumes of distilled water. To measure the binding of the agents by hemoglobin, the hemolyzed cells were centrifuged at $9{,}000 \times g$ for 15 minutes to eliminate the stromal phase. The supernatant was dialyzed for 24 hours against 1 per cent NaCl, to eliminate glutathione and any other filterable thiols. The hemoglobin concentration of the remaining soluble protein fraction was determined. The sulfhydryl content of this soluble protein fraction is virtually equal to the sulfhydryl content of the hemoglobin (7). The binding of chlormerodrin to hemolyzed cells or to hemoglobin was calculated from the amount of radioactivity left in the supernatant, after precipitation of the bound chlormerodrin with the Ba-Zn reagent of Somogyi (8). Free chlormerodrin is not precipitated under these circumstances but the chlormerodrin bound to the ghosts, or to the soluble protein fraction of the cells is completely precipitated. Although chlormerodrin, bound to smaller molecules such as cysteine and glutathione, is not precipitated when only these molecules are present in the solution, with proteins in the solution this fraction is almost completely coprecipitated. The maximal chlormerodrin binding by the sulfhydryl groups of hemolyzed cells and of hemoglobin was measured by a Scatchard type analysis, previously described for $HgCl_2$ (6). For example, a typical experiment for the interaction of chlormerodrin with hemoglobin-free erythrocyte ghosts is illustrated in Fig. 1. The binding of PCMB to hemolyzed cells and to hemoglobin was measured with the titration technique described by Benesch and Benesch (1). Measurement of the absorption spectrum of PCMBS with and without cysteine revealed that a similar titration technique at a wave length

[2] Chlormerodrin labeled with Hg^{203} was obtained from Abbott Laboratories, North Chicago, Illinois; $Hg^{203}Cl_2$ was obtained from Oak Ridge. PCMBS (labeled with S^{35}) and PCMB (labeled with Hg^{203}) were obtained from the Radiochemical Centre, Amersham, England. Unlabeled chlormerodrin was kindly supplied by Dr. Harris L. Friedman, Lakeside Laboratories, Milwaukee, Wisconsin. Unlabeled PCMB and PCMBS were obtained from Sigma Chemical Company, St. Louis, Missouri.

of 236 mμ could be used to measure the binding of PCMBS to hemolyzed cells and to hemoglobin. In calculating the number of sulfhydryl groups per molecule of hemoglobin that react with each of the agents, the molecular weight of hemoglobin was assumed to be 66,800 (9).

Hemoglobin-free[3] ghosts were prepared by osmotic lysis as previously described (10) with the following modification. In lieu of the initial dialysis step, 1 volume of a 25 per cent suspension of red cells in 1 per cent NaCl was added to 7 volumes of 30 milliosmolar phosphate buffer, pH 7.4. Centrifugation and subsequent washes were carried out as previously described. Hemoglobin was measured by the method of Crosby, Munn, and Furth (11), or, when very small amounts of hemoglobin had to be measured, by the benzidine method of Bing and Baker (12), as modified by Dacie (13). Red blood cell and ghost counts were performed with a model B Coulter elec-

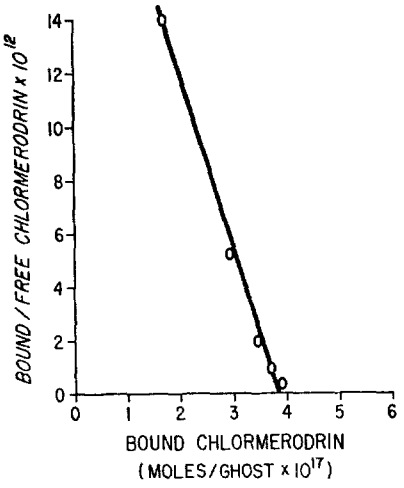


FIGURE 1. Mass law plot of chlormerodrin binding data from a representative experiment. The abscissa is the number of moles of chlormerodrin bound per hemoglobin-free red cell ghost. The ordinate is the ratio of the number of moles of chlormerodrin bound per ghost to the concentration of unbound reagent. The equation is

$$B/F = \frac{B_t}{K} - \frac{B}{K}$$

where B is the bound agent, B_t the maximum number of binding sites, F the free agent, an K the dissociation constant. The intercept on the abscissa represents B_t.

tronic particle counter using a 50 μ orifice. The instrument was set at a lower threshold of 60, upper threshold of infinity, amplification of ¼, and aperture current of 1.

Glucose uptake was measured by following volume changes in the red cells with the microhematocrit technique. After incubation with one of the sulfhydryl-reacting agents, the erythrocytes were spun down and most of the supernatant was removed. The cells were resuspended to a 50 per cent cell suspension. Sufficient glucose was added to give an initial concentration in the medium of 5 per cent. Glucose uptake was approximated by measuring the change in the hematocrit value over an interval of 10 minutes and comparing these values with the hematocrit value of a sample of the same suspension, to which sufficient NaCl was added to give the medium the same initial osmolarity as the glucose-containing medium. More exact measurements of glucose transfer were performed by measuring glucose efflux at 6°C. After centrifugation the cells were cooled to 6°C and resuspended at a hematocrit value of 6

[3] Determination of residual hemoglobin in the stromal fraction (10) indicated that the hemoglobin, retained in the ghosts, could not account for more than 1 per cent of the measured chlormerodrin, PCMB, or PCMBS binding, by those ghosts.

per cent in a solution of varying concentrations of the sulfhydryl agents in 1.8 per cent NaCl, chosen to be approximately isoosmotic with the cell contents after equilibration with 5 per cent glucose. Glucose efflux from the cells was evaluated by measuring the glucose concentration in the medium at frequent intervals, utilizing the glucose-oxidase method as modified by Washko and Rice (14).

RESULTS

A. *The Total Number of Sulfhydryl Groups Reacting with Chlormerodrin, PCMB, and PCMBS, in Hemolyzed Cells, Ghosts, and Hemoglobin*

The results of experiments designed to measure the total number of sulfhydryl groups in hemolyzed cells, ghosts, and hemoglobin, reacting with chlormerodrin, PCMB, PCMBS, and inorganic mercury are summarized in Table I.

The total number of binding sites for PCMB and PCMBS were the same, approximately 1000 $\times$ 10^{-18} moles/cell, but for chlormerodrin the number was three times as high, and for inorganic mercury, four times as high. The differences must be attributed primarily to the reactivity of the sulfhydryl groups of hemoglobin which contains 90 per cent of the cellular sulfhydryl (6). The only other sulfhydryl component of consequence besides the stroma is the reduced glutathione which amounts to 260 $\times$ 10^{-18} moles/cell and which reacts with each of the reagents (1, 6). It has previously been demonstrated that inorganic mercury reacts with hemoglobin within intact cells to the extent of 8.5 moles/mole hemoglobin (6). From the present experiments it can be calculated that chlormerodrin reacts with 6 to 7 and PCMB and PCMBS with 2 to 3 moles/mole of hemoglobin.

Less than 5 per cent of the sulfhydryl groups of the cell are located in the hemoglobin-free stroma (10) and titration of hemoglobin-free ghosts with inorganic mercury yields a value of 182 $\times$ 10^{-18} moles/cell (6). The organic mercurials react with fewer sulfhydryl groups in hemoglobin-free ghosts but they all titrate the same maximal number, 38 to 41 $\times$ 10^{-18} moles/cell, or approximately 22 per cent of the total number of sulfhydryl groups (based on Hg titration).

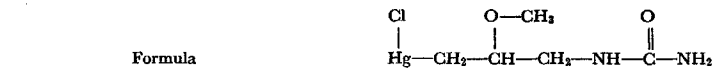
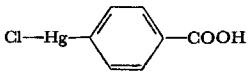
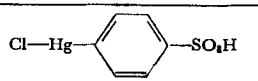
The reaction of chlormerodrin, PCMB, and PCMBS with hemolyzed cells, hemoglobin, and ghosts is a prompt one. Incubation for 60 minutes produces no more binding than incubation for 5 minutes. At 4°C the reaction is complete within 10 minutes. This is of importance in considering the binding of these sulfhydryl agents by intact cells.

B. *The Binding of Chlormerodrin, PCMB, and PCMBS to Intact Cells*

The uptake of the agents, shown in Fig. 2, is quite different from that by hemolyzed cells. First, the uptake of PCMB and of chlormerodrin by intact cells, although slow, continues for over 3 hours, whereas the reaction with

TABLE I

THE BINDING OF SULFHYDRYL AGENTS BY RED BLOOD CELLS

	Chlormerodrin	PCMB	PCMBS	Mercury
Formula				Hg
Total binding sites (hemolyzed cells), moles/cell × 10^{18}	2900±110	1080±80	1070±120	4100±240
Total binding sites per ghost, moles/cell × 10^{18}	39.8±6.1	41.0±5.0	38.2±7.3	182±17.5
Binding sites outside diffusion barrier, moles/cell × 10^{18}	2.1±0.2		2.3±0.3	
Inhibition of glucose transfer	+	+	+	+

The values presented are mean values ± the standard deviation obtained in studies of cells from ten donors each in the cases of PCMB and PCMBS, and from twelve different donors with chlormerodrin. The mercury values are taken from previously published data (6).

hemolyzed cells was complete in less than 10 minutes. Second, PCMB and PCMBS react with an equal number of sites in hemolyzed cells, but in the experiment represented by Fig. 2, little or no uptake of PCMBS by intact cells was measurable. It is apparent that the rate of uptake by intact cells is limited by a membrane that is somewhat permeable to chlormerodrin and PCMB but which is relatively impermeable to PCMBS at the concentrations employed in these experiments ($\leq 10^{-4}$ M).[4]

A more detailed analysis of the binding of chlormerodrin revealed some complications. At low concentrations of the agent, the binding curve (Fig. 3) shows a toe similar to that reported previously for mercury binding to eryth-

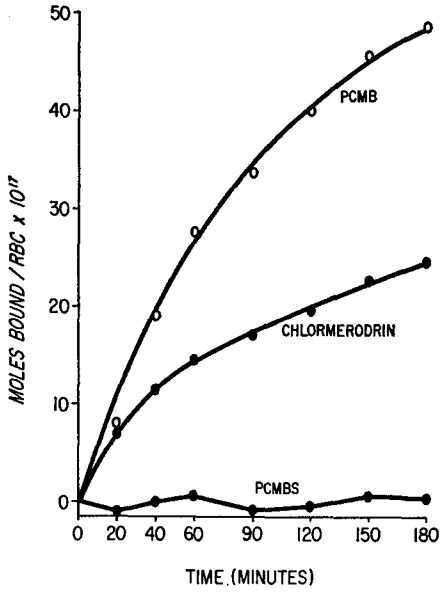


FIGURE 2. The binding of chlormerodrin, PCMB, and PCMBS to intact erythrocytes. Experiments were conducted at 24°C, using a 1.25 per cent red cell suspension. The initial concentration of reagent in the medium was 10^{-4} M.

rocytes (Fig. 4 in reference 6). As in the case of mercury, this toe is caused by the release of small amounts of chlormerodrin-complexing material from the cells: it increases in size with the time of incubation of the erythrocytes prior to testing with chlormerodrin and also on resuspending the cells in a medium that originally contained a higher percentage of cells. Seventy per cent of this toe disappears when the supernatant is treated with the Ba-Za reagent and the results are corrected for the precipitated chlormerodrin. In a typical experiment the concentration of chlormerodrin-complexing material that is precipitated with the Ba-Zn reagent from the supernatant of a 6 per cent

[4] Although the uptake of PCMBS by intact red cells at room temperature is minimal and increases very little with time, the uptake at 4° is somewhat less, suggesting a minimal penetration at room temperature.

cell suspension increased from 0.12×10^{-5} M (immediately after washing the cells) to 1.30×10^{-5} M, after 3 hours.

A second complication is seen at higher concentrations of chlormerodrin after 90 to 180 minutes. The binding seems to reach a maximum value at a level considerably below the number of titratable sulfhydryl groups. Simultaneously, small but increasing amounts of hemoglobin are detectable in the medium. Some of the cells hemolyze and release chlormerodrin previously taken up, and also release soluble binding substances into the medium. When appropriate corrections are made for bound chlormerodrin in the medium, it appears that the uptake of the agent proceeds until all is bound or until the cells are completely hemolyzed.

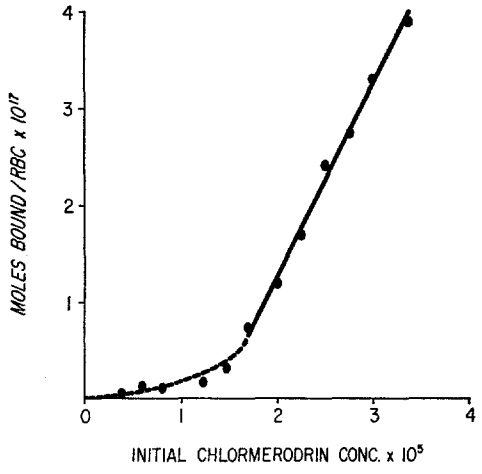


FIGURE 3. Chlormerodrin binding as a function of concentration at low concentrations of the agent. Temperature, 26°C; hematocrit reading, 6 per cent; incubation time, 80 minutes.

With the above complications avoided or taken into account, the kinetics of uptake of chlormerodrin were measured at eight different concentrations ranging from 2 to 20×10^{-5} M (with a hematocrit value of 7 per cent). In each case, the uptake decreased continuously with time, but the amount of agent taken up at any particular time was always directly proportional to the concentrations of agents added. Thus in Fig. 3, the percentage of the added chlormerodrin which is taken up as a function of time falls within narrow limits and could be fitted by a single curve for all concentrations tested. Furthermore, the data for individual concentrations or the composite curve (Fig. 4) can be converted into a straight line by assuming first order kinetics with a half-time of 90 minutes (Fig. 5). The proportionality between the rate of uptake and the concentration of agent added, and the first order kinetics during the course of the uptake, suggest a diffusion-limited process, presumably the permeation of the membrane.

At low temperatures (4°C) the uptake of chlormerodrin is markedly re-

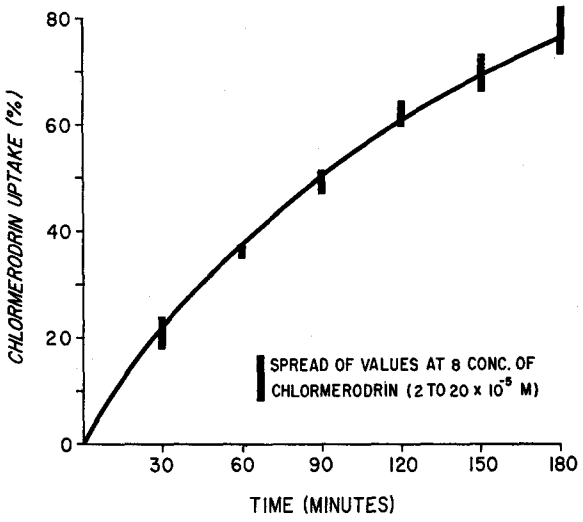


FIGURE 4. Percentage uptake of added chlormerodrin as a function of time at different initial concentrations. Experiments were conducted at 20°C using a 9 per cent red cell suspension. Initial chlormerodrin concentrations in the medium, 2, 4, 6, 8, 10, 12, 16, and 20 $\times$ 10^{-5} molar.

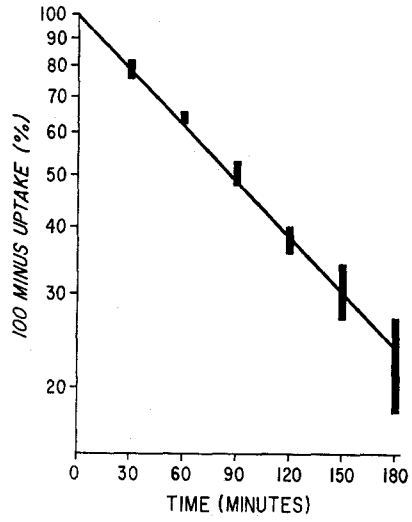


FIGURE 5. First order plot of uptake data of Fig. 4.

duced (Fig. 6). Since the binding of chlormerodrin to hemolyzed cells is not appreciably slowed by lowering the temperature to 4°C, the marked influence of temperature must be related to the diffusion of chlormerodrin into the cell. The initial velocity of chlormerodrin penetration into the cell at 24°C is about 10 times higher than at 4°C. Thus the penetration of chlormerodrin into the cell shows a Q_{10} of about 3; this figure is in accord with

the assumption that chlormerodrin has to penetrate through a lipid diffusion barrier (15).

Distribution of the bound chlormerodrin between the stromal and non-stromal phases was measured by preparing hemoglobin-free ghosts at 4°C from intact cells that had reacted with chlormerodrin. Whereas the fraction of chlormerodrin-reactive sulfhydryl groups in ghosts is only 1.4 per cent of the total in the cell (Table I), the per cent of bound chlormerodrin recovered from the ghosts was 5.2 to 9 per cent (average: 6.4 per cent) in different experiments. These data indicate that during the uptake of chlormerodrin by intact cells, the per cent saturation of the stromal phase is at least four times higher than the per cent saturation of the non-stromal phase. This is a minimal

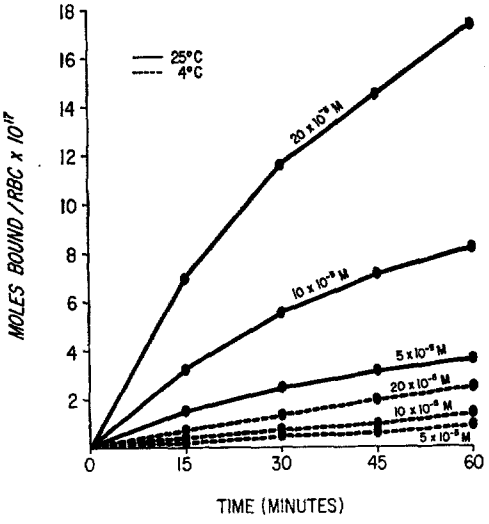


FIGURE 6. The influence of temperature on chlormerodrin uptake by RBC.

figure, because during the process of preparation of ghosts, some redistribution of the bound reagent from the stromal to the non-stromal phase takes place.[5] The relatively higher degree of saturation of the stromal phase of intact cells may be attributed either to a relatively stronger affinity of the stromal sulfhydryl groups or to their more superficial localization. The latter conclusion is probably correct because studies of chlormerodrin distribution between ghosts and ghost-free hemolysate indicated that the affinity of both classes of sulfhydryl groups for chlormerodrin is about the same under these experimental conditions. Furthermore, the percentage of bound chlormero-

[5] When hemoglobin-free ghosts were incubated with chlormerodrin, washed free of unbound reagent, and then resuspended in a fresh hemoglobin solution chosen so as to equal the hemoglobin concentration in the supernatant during the first step of ghost preparation, and recentrifuged, the amount of chlormerodrin recovered from the ghosts after one wash was only 40 per cent of the amount originally bound.

drin, recovered from the stromal phase, is higher after very short incubation times (5 minutes), and also after incubation with the agent at a reduced temperature (4°C): In either case, up to 20 per cent of the bound chlormerodrin can be recovered from the ghosts.

The per cent of cellular chlormerodrin recovered from the stromal phase at a given time is relatively constant, even though the concentration of added reagent may vary over a tenfold range. It may therefore be concluded that the binding of chlormerodrin to the stromal phase as well as to the intact cells proceeds according to diffusion kinetics. This suggests that the majority of stromal sulfhydryl groups is located within the diffusion barrier. This was proved by the following experiment: Washed red blood cells were divided into two portions; one portion was suspended in 1 per cent NaCl, and from the other cells, hemoglobin-free ghosts were prepared. The ghosts were suspended in 1 per cent NaCl, adjusting the number of ghosts per milliliter so as to equal the number of cells per milliliter in the cell suspension. The ghosts and the red cells were incubated with the same concentration of chlormerodrin, at 4°C. After 10 minutes the chlormerodrin binding was measured. A binding of 0.25×10^{-17} moles/red cell and of 4.04×10^{-17} moles/ghost was found. In fact, the ghosts were saturated with chlormerodrin, whereas the intact cells took up only 6 per cent of the amount of chlormerodrin taken up by the ghosts. This indicates that the majority of stromal sulfhydryl groups with which chlormerodrin reacts readily in ghosts is not easily accessible in intact cells, presumably because of localization within or at the inner face of the diffusion barrier.

C. *Inhibition of Glucose Transfer Caused by Sulfhydryl Agents*

Studies on glucose entry by the microhematocrit method revealed that chlormerodrin, PCMB, and PCMBS caused a considerable inhibition of this process. For example, incubation of a 6 per cent cell suspension for 20 minutes with 10^{-4} molar solutions of each of these agents resulted in an inhibition of about 80 per cent. The inhibition of glucose entry by chlormerodrin showed two peculiarities. In the first place notable differences in sensitivity among blood samples from different donors were observed. These differences were especially pronounced, when the cells were incubated with low chlormerodrin concentrations. In four different blood samples exposure of the cells to 0.5×10^{-4} M chlormerodrin caused an inhibition of glucose entry of 30, 60, 60, and 85 per cent respectively.

In the second place it was observed that prolonged incubation of the cells (over 90 minutes) often resulted in a reversal of the inhibitory effect (from 85 per cent to 55 per cent in one sample). Two observations offer a possible explanation: (*a*) In the course of time a redistribution of the bound chlormero-

drin from the membrane to the inside of the cell may occur, transferring some of the agent from sulfhydryl groups involved in glucose transport to other sulfhydryl groups. (*b*) The release of chlormerodrin-complexing material from the cells may remove some of the agent from the stromal phase.

D. *Sulfhydryl Groups Essential for Glucose Transport*

Although each of the sulfhydryl agents inhibits glucose transport, the minimal binding associated with such inhibitors was observed with PCMBS. The binding was exceedingly small so that a measurable uptake by the spectrophotometric technique was obtained only at high hematocrit values (50 per cent). With the use of S^{35}-labeled PCMBS, however, estimates could be made

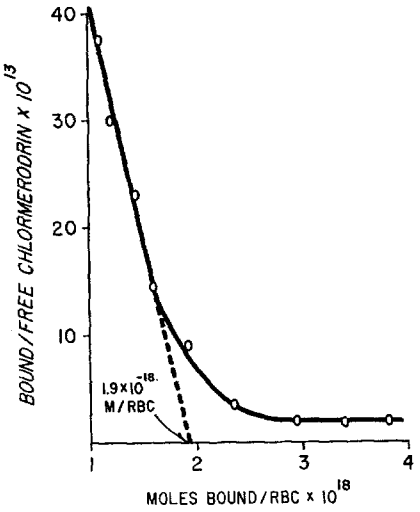


FIGURE 7. Mass law plot of chlormerodrin binding data at 4°C, after an incubation time of 10 minutes.

at lower hematocrit readings. The mean value for 10 experiments was 2.3×10^{-18} moles/cell $\pm$ 0.8×10^{-18} moles (SD) or only 6 per cent of the PCMBS-titratable groups of the hemoglobin-free ghosts (Table I).

With chlormerodrin, it was also possible to demonstrate that only a small fraction of the sulfhydryl groups of the membrane, presumably located on the outer surface, is involved in sugar transport. Chlormerodrin binding to intact cells was measured after exposure of the cells to varying, low chlormerodrin concentrations for a short time (*10 minutes*) at 4°C to minimize penetration through the diffusion barrier. A mass law plot of the results (Fig. 7) shows two components. The first segment, at the lowest concentrations of reagent, gives a straight line compatible with a mass law reaction with accessible sulfhydryl groups. The second segment at higher concentrations is not properly expressed in terms of the mass law plot, but may represent the slow penetration of chlormerodrin into the cell. By extrapolation of the first segment of the

curve, the number of sulfhydryl groups presumably outside the diffusion barrier can be estimated at 1.8×10^{-18} moles/cell. This value was remarkably constant in different blood samples (SD in 5 experiments: 0.2×10^{-18} moles/cell). To determine whether chlormerodrin binding to these accessible sulfhydryl groups is associated with inhibition of glucose transfer, all further experiments on glucose transfer were performed at 6°C, measuring the glucose efflux from preloaded cells. The low temperature minimized penetration of chlormerodrin into the cells and slowed glucose efflux to a rate conveniently measured by chemical methods. With a binding of chlormerodrin of 2.8×10^{-18} moles/cell, the inhibition of glucose efflux at 1 hour was 62 per cent.

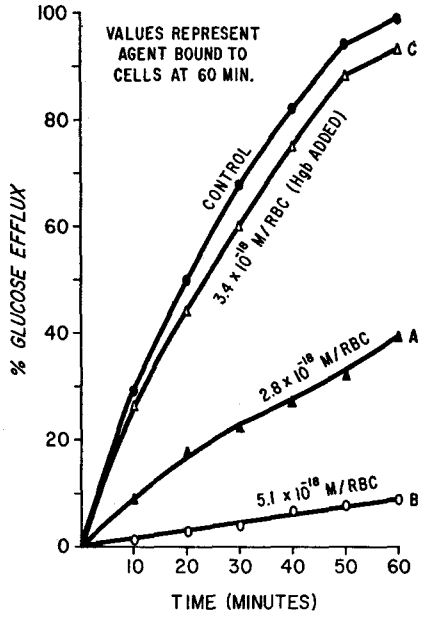


FIGURE 8. Inhibition of glucose efflux at 6°C from preloaded cells, caused by chlormerodrin. A, initial chlormerodrin concentration in the medium: 1.2×10^{-5} molar; B and C, initial chlormerodrin concentration in the medium: 4.6×10^{-5} molar; C, hemoglobin (5×10^{-4} M) was added after the cells were incubated for 10 minutes with chlormerodrin.

With a binding of 5.1×10^{-18} moles/cell, the inhibition was 92 per cent (Fig. 8). In the latter experiment, hemoglobin was added to the medium after incubation of the cells with chlormerodrin. The inhibition of sugar transport was almost completely reversed, and the bound chlormerodrin was reduced to 3.4×10^{-18} moles/cell. Thus the inhibition must be associated with only 1.7×10^{-18} moles/cell of superficially bound reagent, that can be removed by an extracellular complexing agent. This removable chlormerodrin corresponds closely in amount to the maximal superficially bound agent demonstrated by the mass law plot in Fig. 7.

In another experiment, the chlormerodrin was added to the erythrocytes a few minutes before cooling. This resulted in a higher chlormerodrin uptake (13.2×10^{-18} moles/cell) and an inhibition of glucose efflux of 94 per cent.

Addition of hemoglobin reduced the chlormerodrin binding to 11.4×10^{-18} moles/cell, indicating removal of only 1.8×10^{-18} moles chlormerodrin, presumably bound at the outer surface of the cells. Yet, although 11.4×10^{-18} moles chlormerodrin remained bound per cell, the inhibition of glucose efflux was reversed (from 94 to 12 per cent).

DISCUSSION

Various sulfhydryl agents penetrate into red blood cells with greatly differing velocities. In contradistinction to the very fast uptake of inorganic mercury (3), PCMB and chlormerodrin penetrate very slowly, whereas PCMBS penetrates little if at all, with its reaction limited to a small fraction of the total cellular sulfhydryl content, located outside of the diffusion barrier. The large differences in permeation of PCMB and PCMBS can be accounted for by the substitution of the sulfonic acid group in PCMBS for the carboxyl group in PCMB which increases the hydrophilic character of this compound (2). The fact that a diffusion barrier is rate-limiting for the uptake of compounds such as chlormerodrin and PCMB is indicated by the rapid reaction with hemolyzed cells in contrast to the slow uptake by intact cells and by the fact that the slow uptake follows first order behavior in any single run, and that the initial rate is proportional to the initial concentration of the agent.

The large differences in the maximal number of cellular sulfhydryl groups reacting with each of the agents are consistent with known differences of reactivity of various sulfhydryl groups. This has been illustrated by the reaction of hemoglobin with sulfhydryl agents, such as *N*-ethylmaleimide, which reacts only with two sulfhydryl groups (16), while inorganic mercury reacts with eight and a half sites per molecule of hemoglobin (6). For PCMB at pH 7, a value of 2 to 3 was obtained, in good agreement with the figure of 2.85 mentioned by Huisman (9). PCMBS also reacts with two to three sulfhydryl groups per molecule of hemoglobin, whereas chlormerodrin reacts with six to seven sulfhydryl groups. The interactions of PCMB, PCMBS, and chlormerodrin with stromal sulfhydryl groups are, on the other hand, equal: Each reacts, at pH 7, with about 25 per cent of the total number of stromal sulfhydryl groups, as measured with mercury (6).

The experiments with the non-penetrating agent PCMBS and with chlormerodrin and PCMB at low temperature in short periods of incubation allow the localization of a small fraction of the stromal sulfhydryl groups on the outer surface of the lipid membrane of the cells. This fraction (approximately 1 to 1,400,000 groups per cell) constitutes as little as 1 per cent of the stromal, or 0.06 per cent of the total cellular sulfhydryl content, as measured with mercury (6). As these agents react with only 25 per cent of the total number of stromal sulfhydryl groups, the fraction of sulfhydryl groups on the outside of the membrane constitutes 5 per cent of the total chlormerodrin- or PCMBS-

reactive stromal sulfhydryl groups. These data indicate a large asymmetry in the distribution of sulfhydryl groups in the membrane, with 95 per cent of the PCMBS-chlormerodrin–reactive groups located within the membrane and/or on the inner face of the membrane. The inhibition of glucose transport by these reagents is associated with that small fraction of the stromal sulfhydryl groups located on the outer face of the membrane. Thus, the maximal number of sulfhydryl groups involved in glucose transfer is about 1,400,000 per cell. The real number may be only a fraction of these 1,400,000 groups per cell; this value is only an upper limit. It is noteworthy that LeFevre, approaching the problem of glucose transport by an entirely different technique, found as the upper limit for the number of sugar transport sites in red blood cells a number of 500,000 per cell (17).

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