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EFFECT OF HEXACHLOROPHENE ON MONOVALENT CATION TRANSPORT IN HUMAN ERYTHROCYTES A MECHANISM FOR HEXACHLOROPHENE-INDUCED HEMOLYSIS

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SUMMARY

Hexachlorophene-induced hemolysis, as studied by phase contrast microscopy, appeared to be a result of osmotic swelling. Both swelling and subsequent hemolysis were markedly delayed by addition of the non-penetrating solute sucrose to the incubation mixture. Binding studies indicated that hexachlorophene is associated primarily with the erythrocyte membrane, the remainder being found in the cytoplasm. Hexachlorophene induced a dose-dependent, first-order efflux of Na^+ and K^+ from red cells. The rates of hemolysis and K^+ efflux induced by hexachlorophene were much greater than would be expected if this compound were acting simply as a metabolic inhibitor and/or an inhibitor of (Na^+–K^+–Mg^{2+})-ATPase. It is suggested that hexachlorophene induces the efflux of Na^+ and K^+ from red cells by directly altering the permeability of the cellular membrane. Further, hexachlorophene-induced hemolysis is probably a secondary event resulting from osmotic swelling subsequent to increased membrane permeability.

INTRODUCTION

The toxicity of hexachlorophene has been the subject of active investigation during the last few years. This work has revealed a number of toxic effects for this chlorinated bisphenol [1–4]. Unfortunately, relatively little is known about the biochemical mechanism(s) for its toxicity although hexachlorophene has been found to be a potent uncoupler of oxidative phosphorylation [5–7] and to inhibit a number of enzymes [5, 8].

Rather extensive studies on the mechanism for the bacteriocidal action of hexachlorophene have been reported [9–11]. These studies show that this compound induces the leakage of low molecular weight material from *Bacillus megaterium* and that it induces the lysis of protoplasts prepared from these bacterial cells. In addition, treatment of *B. megaterium* with high concentrations of hexachlorophene lead to detectable cytological changes [9].

It has also been shown [12–14] that hexachlorophene is a potent hemolytic agent, inducing hemolysis of washed red cells in vitro from a variety of sources. Recent studies have shown (Flores, G. and Buhler, D. R., unpublished) that hexachlorophene associates with both the cellular membrane and cytoplasm.

The present work is an attempt to elucidate the mechanism of hexachlorophene-induced hemolysis. To this end, we have undertaken a study of the effect of hexachlorophene on the morphology of red cells and on changes in membrane permeability as reflected by the efflux of monovalent cations. It is hoped that the results of these studies will contribute in a more general way to an understanding of the biochemical mechanism(s) for hexachlorophene toxicity.

MATERIALS AND METHODS

Erythrocyte preparation

Human blood samples were obtained from normal adult males, using acid–citrate–dextrose (15 ml/100 ml blood) as an anticoagulent. Immediately after collection, the blood was centrifuged at $1500 \times g$ for 10 min, and the plasma and buffy coat were carefully removed by aspiration. The cells were washed 4 times by suspension in about 6 vol. of 0.15 M NaCl followed by centrifugation. The cells from the last wash were suspended to a 50% hematocrit by adding isotonic saline, and the suspension was made 5.0 mM in glucose and 0.5 mM in $MgCl_2$. All of the experiments described below were performed within 7 days from collection using this stock red cell suspension (stored at 4 °C). Prior to each set of experiments, a portion of the stock red cell suspension was allowed to stand at room temperature for about 4 h.

Reagents

Hexachlorophene, 2,2′-methylene-bis-(3,4,6-trichlorophenol), donated by the Givaudan Corp., Clifton, N. J., was twice crystallized from isopropanol–water (m.p. 165–166 °C). [Methylene-^{14}C]hexachlorophene (spec. act. 3.52 Ci/mole) was obtained from Mallinckrodt Nuclear Co., St. Louis, Mo. Trizma base (reagent grade) and ouabain octahydrate (strophanthin C) were purchased from Sigma Chemical Co., St. Louis, Mo. The ^{22}NaCl in 0.5 M HCl (2.56 mCi/ml) was obtained from New England Nuclear, Boston, Mass. All other chemicals were of ACS reagent grade.

Microscopy

An aliquot of the stock red cell suspension was diluted about 40-fold with 0.14 M NaCl–0.01 M potassium phosphate buffer (pH 7.4). A portion of the resulting suspension was made $1 \cdot 10^{-4}$ M in hexachlorophene, and a drop was placed between two plastic coverslips supported on a standard glass slide. The red cells were observed under a Nikon Model S-Ke microscope equipped with Koehler illumination, a dark green filter, and Nikon phase-contrast optics. Photomicrographs were recorded on Ektachrome (EX-135) film with the aid of a semi-automatic photomicrographic attachment (Nikon Microflex Model EFM).

Measurement of K^+ efflux

An aliquot (2.0 ml) of the stock red cell suspension was washed 3 times with 10.0 ml of isotonic LiCl–Tris buffer (120 mM LiCl–55 mM Tris–HCl buffer (pH 7.4

at 25 °C), containing 5 mM glucose and 0.5 mM $MgCl_2$). The packed cells from the final wash were diluted with 10.0 ml of the same buffer (10% hematocrit) and incubated in a thermostated vessel at 25.0 °C.

Changes in extracellular K^+ concentration with time were measured essentially as previously described [15]. The protocol for a typical experiment was as follows. Using the above suspension of washed red cells, the change in K^+ concentration with time in the absence of hexachlorophene was monitored for 5–10 min to obtain a base line. An appropriate aliquot of a stock solution of hexachlorophene ($2.48 \cdot 10^{-2}$ M, in 95% ethanol) then was added to the rapidly stirred suspension, while the cation electrode response was continuously recorded. The efflux of K^+ was monitored for a period of 20–30 min. For each set of experiments, a suspension of red cells was treated with a large amount of stock hexachlorophene (approx. 120 μl). Within 1.5 h the K^+ concentration ceased to change with time, and it is this final value for K^+ concentration that is used as K_∞^+ in the calculation of first-order rate constants as described below. The effect of ethanol alone on K^+ efflux was measured in a manner analogous to that for hexachlorophene.

The degree of hemolysis during the course of each experiment was determined by measuring the amount of hemoglobin in supernatant fractions by the benzidine method [16]. Quantitation of the number of red cells, to allow correlation of different experiments, was accomplished by determining the hemoglobin in a given red cell suspension by the cyanomethemoglobin method [17].

The data were analyzed with the aid of a computer program which, from values of K^+ concentration versus time, returned plots of $\log [K_\infty^+/(K_\infty^+ - K_t^+)]$ versus time and the first-order rate constant k (min^{-1}) from a linear least squares fit of the data points.

Measurement of Na^+ efflux

22 ml of the stock red cell suspension was allowed to equilibrate with 250 μCi $^{22}NaCl$ in the cold for about 20 h. The suspension was warmed to room temperature and allowed to stand for 3 h, then centrifuged and washed 5 times with isotonic NaCl, and finally diluted to a 50% hematocrit. For measurements of $^{22}Na^+$ transport, 2.0 ml of the suspension was washed with isotonic LiCl–Tris buffer as described above for measurements of K^+ efflux. Following the addition of hexachlorophene, aliquots (0.25 ml) were removed at timed intervals (over a period of 20–30 min) and centrifuged immediately in a Beckman Minifuge for 15 s. A portion of each supernatant fraction (about 0.2 ml) was immediately withdrawn and placed aside for later radioactivity measurement. The total time for removal of an aliquot of the suspension, centrifugation, and collection of the supernatant was less than 30–45 s. Na_∞^+ was determined from measurements of the total $^{22}Na^+$ concentration in the suspension. The above procedure was followed for each hexachlorophene concentration. For two experiments (hexachlorophene concentrations of $9.9 \cdot 10^{-5}$ and $16.1 \cdot 10^{-5}$ M), K^+ concentration was monitored simultaneously to allow correlation of $^{22}Na^+$ and K^+ efflux. Before addition of hexachlorophene, a background sample was taken and centrifuged as above.

For measurement of radioactivity, 0.10-ml aliquots of each supernatant fraction and total cell suspension were diluted with 0.10 ml water, and 20 μl 55% trichloroacetic acid was added. After standing for 1 h in the cold, the solution was

centrifuged to remove precipitated protein. A portion of the supernatant fraction (0.10 ml) was added to 3.5 ml Aquasol (New England Nuclear, Boston, Mass.) for counting in a Packard (Packard Instrument Co., Donners Grove, Ill.) Model 3375 liquid-scintillation spectrophotometer. The instrument was optimized for counting ^{22}Na in Aquasol. After correction for the blank, the data were analyzed by computer as described above for K^+ efflux.

Measurement of the degree of hemolysis

A suspension of red cells (2.5% hematocrit) was prepared from an appropriate aliquot of the stock red cell suspension as described for measurement of K^+ efflux. Sucrose was then added to the desired concentration. Hexachlorophene was added to the rapidly stirred suspension and a zero-time aliquot was immediately withdrawn and centrifuged in a Beckman Minifuge for 15 s. Other aliquots were taken at the times indicated in Fig. 3. Hemoglobin in the supernatant fraction was determined by the cyanomethemoglobin method.

Determination of hexachlorophene binding to red cells

The amount of hexachlorophene bound to red cells at different hexachlorophene concentrations was measured in the following manner. An aliquot of the stock red cell suspension was washed and diluted as for measurements of K^+ efflux. The resulting suspension (10% hematocrit) was submitted to continuous stirring. A 0.25-ml aliquot was centrifuged and the supernatant fraction was saved as a background sample. [*Methylene*-^{14}C]Hexachlorophene ($2.5 \cdot 10^{-2}$ M; 3.52 Ci/mole) was added and a 0.25-ml aliquot was removed and treated as above. This process was repeated for each hexachlorophene addition. An aliquot (10 μl) of the cell suspension after each addition served as a measure of total [^{14}C]hexachlorophene. An aliquot (10 μl) of each supernatant fraction and total cell suspension was dissolved in 3.5 ml Aquasol and counted in a Packard Model 3375 liquid-scintillation spectrophotometer. The percent of the total [^{14}C]hexachlorophene bound at each hexachlorophene concentration was computed directly from the background corrected data.

The amount of hexachlorophene associated with the cytoplasm and bound to the cell membrane was determined in the following way. A washed suspension of red cells was prepared as above and a 1.0-ml aliquot was taken for each hexachlorophene concentration. To the stirred aliquot [^{14}C]hexachlorophene was added and a 0.25-ml aliquot was removed and treated as above. 2 min after the addition of hexachlorophene, a 25-μl aliquot of the suspension was hemolyzed in 0.25 ml distilled water. After 1 min, a 10-μl aliquot of the hemolysate was removed for counting, and the remainder was centrifuged for 5 min in a Beckman Minifuge to separate red cell ghosts from the cytoplasm. A 100-μl aliquot of the supernatant fraction was taken for counting in Aquasol. The amount of [^{14}C]hexachlorophene bound to the membrane and associated with the cytoplasm was computed from the data after correction for the amounts of unbound [^{14}C]hexachlorophene.

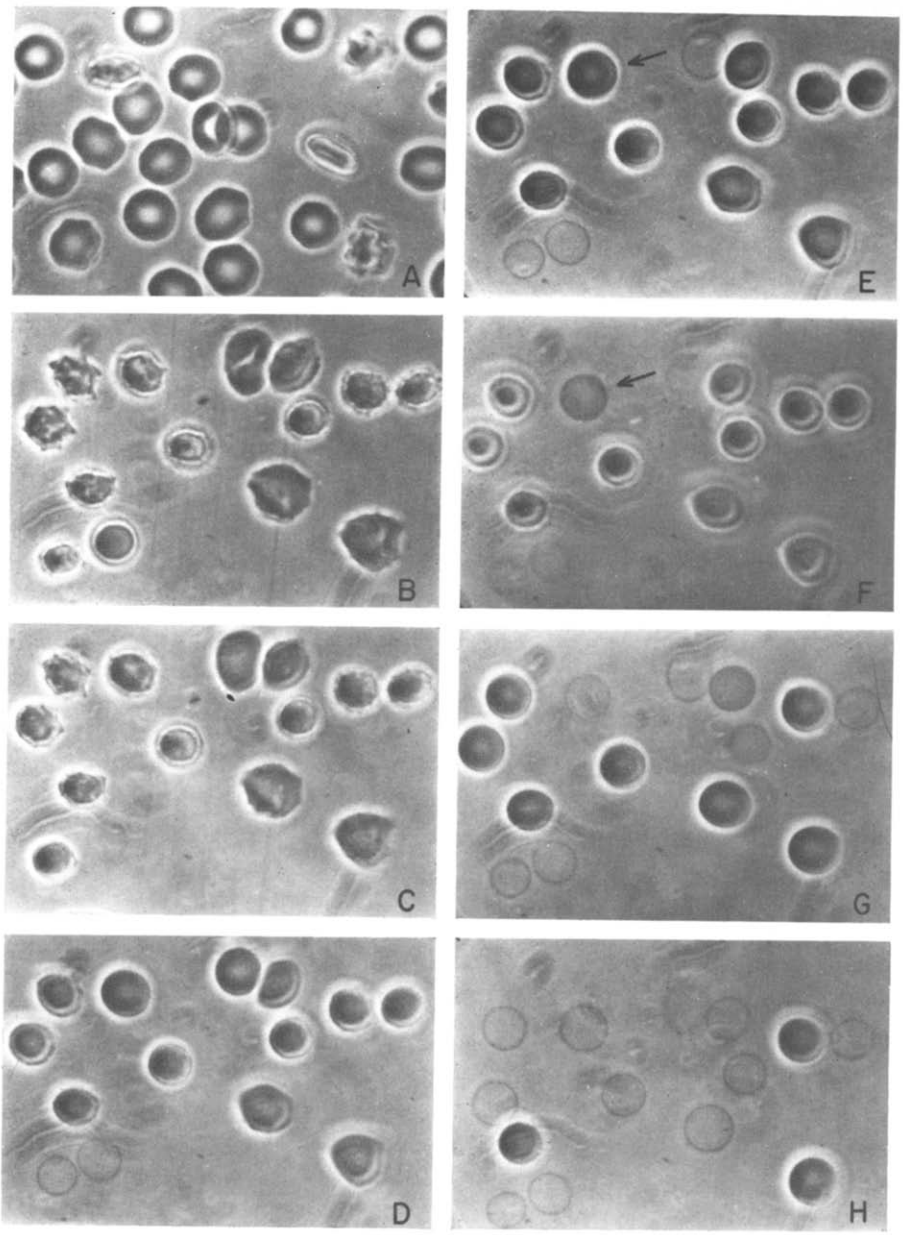

Fig. 1. Phase contrast microscopy (× 1000) of hexachlorophene-treated and untreated human red cells. The cells (2.5 % hematocrit) were incubated at room temperature in 0.14 M NaCl–0.01 M potassium phosphate buffer (pH 7.4), containing $1 \cdot 10^{-4}$ M hexachlorophene. (A) Control cells before hexachlorophene addition. (B–H) Cells treated with hexachlorophene. Photomicrographs of same field and recorded at times indicated as time after hexachlorophene addition: (B), 1 min, note crenated cells; (C), 24 min; (D), 62 min, note ghosts and prelytic spheres; (E), 76 min, note prelytic sphere (arrow); (F), 82 min, note partially hemolyzed cell (arrow); (G), 106 min; (H), 123 min. Control cells incubated in 0.14 M NaCl–0.01 M potassium phosphate buffer (pH 7.4) for the duration of the above experiment were identical to the cells shown in (A).

RESULTS

Microscopy

Results of phase contrast microscopy are shown in photomicrographs reproduced in Fig. 1. Examination of these photomicrographs and of others recorded at shorter time intervals reveals that hexachlorophene induces visual shape changes in the red cells which lead to eventual hemolysis. The sequence of changes can be summarized as follows: (1) immediately upon addition of hexachlorophene (i.e. within 1 min), the red cells become extensively crenated, then (2) they gradually swell to smooth then to prelytic spheres, and, finally, (3) they undergo colloid–osmotic hemolysis yielding erythrocyte ghosts. A similar sequence of shape changes has been observed in red cells treated with other hemolytic agents [18].

Effect of hexachlorophene on K^+ and Na^+ efflux

The efflux of K^+ and Na^+ from red cells in the presence of different concentrations of hexachlorophene was found to follow first-order kinetics, as evidenced by the linear relationship obtained when log $[K^+_\infty/(K^+_\infty - K^+_t)]$ or log $[Na^+_\infty/(Na^+_\infty - Na^+_t)]$ was plotted against time. The first-order rate constants, k_{K^+} and k_{Na^+}, computed from these plots, are shown in Fig. 2 as a function of the concentration of hexachlorophene. It is clear that an increase in the rate of K^+ and Na^+ efflux attends an increase in hexachlorophene concentration, albeit k_{K^+} is greater than k_{Na^+} at hexachlorophene concentrations higher than about $5 \cdot 10^{-5}$ M. Experiments with ethanol alone (no hexachlorophene) revealed that this solvent in the concentrations used had a negligible effect on K^+ efflux from red cells. It should be noted that during the time course of the experiments in isotonic LiCl–Tris buffer less than 2% hemolysis was observed at the highest hexachlorophene concentrations studied.

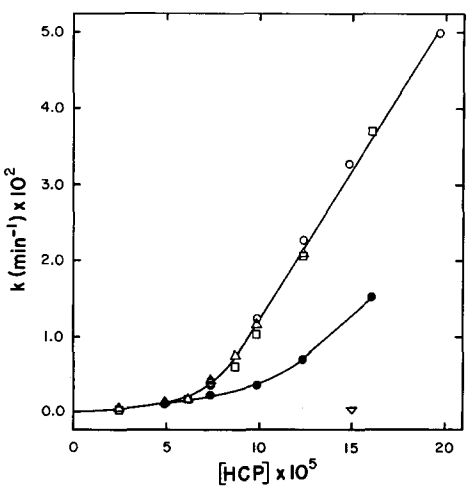

Fig. 2. The effect of hexachlorophene (HCP) concentration on the first-order rate constants for K^+ and Na^+ efflux. K^+ efflux measured from three different red cell preparations (▽, □, ○); Na^+ efflux (●). K^+ efflux in the presence of $15 \cdot 10^{-5}$ M ouabain (▽).

Measurement of the efflux of Na^+ from red cells was based on the loss of $^{22}Na^+$ from cells which had been loaded with this isotope. It has been previously shown [19] that the specific activity of cellular $^{22}Na^+$ is the same as that of $^{22}Na^+$ leaving the cell.

Shown also in Fig. 2 is the effect of $1.5 \cdot 10^{-4}$ M ouabain on K^+ efflux in the absence of hexachlorophene. The first-order rate constant for K^+ efflux under these conditions is $3 \cdot 10^{-4}$ min^{-1} as compared with k_{K+} equal to $3.2 \cdot 10^{-2}$ min^{-1} in the presence of $1.5 \cdot 10^{-4}$ M hexachlorophene.

Hexachlorophene-induced hemolysis

The results of measurements of percent hemolysis against time, in various solutions containing $3.0 \cdot 10^{-4}$ M hexachlorophene, are shown in Fig. 3. In a red cell suspension of 2.5% hematocrit, hemolysis in isotonic LiCl–Tris buffer occurs about twice as rapidly as in isotonic NaCl–phosphate buffer. In both buffers, the non-penetrating solute sucrose (150 mM) markedly delays hexachlorophene-induced hemolysis. In the presence of $3.0 \cdot 10^{-4}$ M hexachlorophene and 300 mM sucrose, complete hemolysis required about 16 h. No hemolysis was observed in cells suspended for 24 h in isotonic NaCl–phosphate buffer containing $1.5 \cdot 10^{-4}$ M ouabain or in isotonic LiCl–Tris buffer alone.

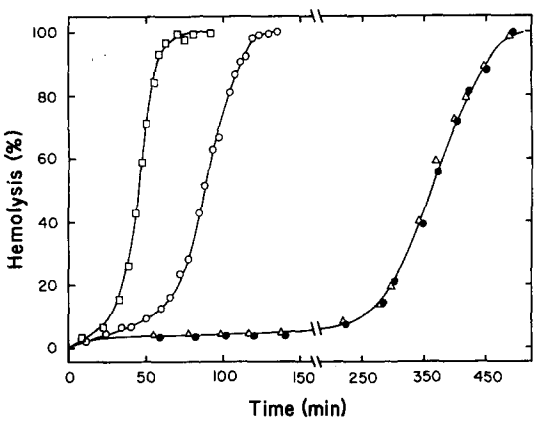

Fig. 3. The time course of hexachlorophene-induced hemolysis of red cells incubated in different media at 25.0 °C. Hexachlorophene concentration was $3.0 \cdot 10^{-4}$ M for all experiments. Incubation in: isotonic LiCl–Tris buffer (□); 0.14 M NaCl–0.01 M potassium phosphate buffer (pH 7.4) (○); isotonic LiCl–Tris buffer containing 150 mM sucrose (△); isotonic saline–phosphate buffer containing 150 mM sucrose (●). Note change in time scale.

Binding of hexachlorophene to red cells

Measurements of its binding to red cells show (Fig. 4) that greater than 90% of the [^{14}C]hexachlorophene added to the red cell suspension is associated with red cells, the remainder being free in solution. Of the amount bound to red cells, the major portion is associated with the cellular membrane (measured as the difference between total amount bound and the amount in the cytoplasm), with the remainder being found in the cytoplasm. These results must be tempered by the realization that, during hemolysis and centrifugation, redistribution of [^{14}C]hexachlorophene from

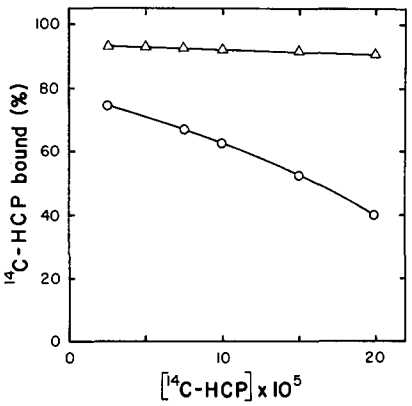

Fig. 4. The amount of [^{14}C]hexachlorophene (HCP) bound to red cells and red cell membrane as a function of [^{14}C]hexachlorophene concentration. △, % bound to red cells; ○, % bound to membrane. The amount in the cytoplasm is taken to be the difference between the amount bound to the cell and the amount bound to the membrane.

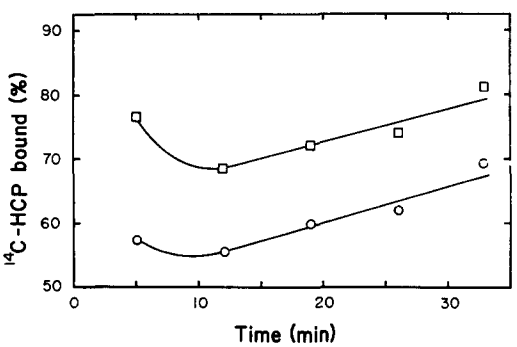

Fig. 5. The effect of incubation time on the amount of [^{14}C]hexachlorophene (HCP) bound to the cellular membrane. Hexachlorophene concentration: $7.5 \cdot 10^{-5}$ (□) and $15 \cdot 10^{-5}$ M (○).

membrane to cytoplasmic constituents, or vice versa, may have occurred. However, the results shown in Fig. 5 indicate that the distribution of [^{14}C]hexachlorophene between membrane and cytoplasm is relatively time independent, with perhaps the amount associated with the membrane increasing with time of incubation.

DISCUSSION

The results of experiments presented in this paper indicate that hexachlorophene induces: (1) osmotic swelling of red cells followed by hemolysis and (2) a dose-dependent increase in the prelytic efflux of the monovalent cations, Na^+ and K^+. Also, hexachlorophene-induced hemolysis is delayed by addition of the non-penetrating solute sucrose. The protective effect of non-penetrating solutes (e.g. sucrose) has been observed for hemolysis induced by other hemolytic agents, and it is generally attributed [20–22] to a balancing of the osmotic pressure of cellular hemoglobin by

the extracellular solute. The results presented here are characteristic of hemolysis occurring by a colloid–osmotic mechanism, i.e. hemolysis is thought to result from disruption of the normal mechanism of osmoregulation of cell volume, allowing the colloid–osmotic pressure of cellular hemoglobin to be expressed [20, 21, 23].

Osmoregulation in the red cell is currently thought [22, 24] to be accomplished by a balance between active and passive transport of Na^+ and K^+, and any disruption of this balance can be expected to eventually lead to colloid–osmotic hemolysis. Such disruption of cation transport can result from the following: (1) direct inhibition of (Na^+–K^+)-activated, Mg^{2+}-dependent ATPase, (2) inhibition of energy yielding metabolic reactions leading ultimately to oxidative destruction of the red cell, and (3) a direct increase in the permeability of the cellular membrane to monovalent cations.

Inhibition of both monovalent cation transport and energy metabolism would be expected to result in rates of hemolysis and monovalent cation efflux that are related to the rate of passive transport of Na^+ and K^+. Our measurements of passive K^+ efflux determined in the presence of the (Na^+–K^+)-ATPase inhibitor ouabain [25] (at $1.5 \cdot 10^{-4}$ M) yield a rate constant for the passive efflux of K^+ which is less than 0.01 of k_{K+} in the presence of $1.5 \cdot 10^{-4}$ M hexachlorophene. Further, no hemolysis is observed in red cells (2.5% hematocrit) suspended for 24 h in isotonic NaCl–phosphate buffer and treated with $3.0 \cdot 10^{-4}$ M ouabain, whereas complete hemolysis is observed in 2 h in the presence of $3.0 \cdot 10^{-4}$ M hexachlorophene. It can be concluded that hemolysis and K^+ efflux induced by hexachlorophene are not simply due to inhibition of (Na^+–K^+)-ATPase. Similarly, inhibition of red cell metabolism by iodoacetate [26] and depletion [27] of glucose and ATP stores does not result in rates of hemolysis and K^+ efflux that are greater than expected from the rate of passive monovalent cation transport. Also, treatment of red cells with *N*-ethylmaleimide at a concentration sufficient to completely inhibit glucose metabolism and to reduce the level of free cellular glutathione to zero results in only a slow efflux of K^+ and essentially no hemolysis [28]. It is clear from the results presented in this paper that hexachlorophene is much more effective at inducing hemolysis and monovalent cation efflux than would be expected if it were acting solely as a metabolic inhibitor.

We have left to consider the possibility that hexachlorophene functions directly at the membrane level by altering permeability. Our results show that hexachlorophene induces a rapid, prelytic loss of monovalent cations without concomitant loss of hemoglobin. These results suggest that this compound alters the permeability of the red cell membrane by inducing the formation of "pores" (in the context of the present discussion, the term "pore" is intended to refer to the permeability character of the cell membrane and not necessarily to the existence of permanent, physical holes in the membrane) of such a size as to allow ready exit of Na^+ and K^+ (hydrated radii: $Na^+ = 2.76$ Å, $K^+ = 2.32$ Å) while hemoglobin (mean diameter = 64 Å) is retained. One can also deduce that the size of these "pores" is close to the effective diameter of sucrose (about 9 Å), since sucrose, while having a protective effect, is eventually able to penetrate as indicated by the delayed hemolysis observed in its presence. Further, as shown in Fig. 2, the first-order rate constant for the efflux of K^+ is greater than k_{Na+}, suggesting that the "pore" size is such that there can be selectivity on the basis of the relative size of Na^+ and K^+. A prerequisite to direct alteration of perme-

ability is that hexachlorophene must interact with the membrane. Results presented in Fig. 4 show that [^{14}C]hexachlorophene binds largely to the plasma membrane, and, thus, a necessary, but not sufficient, condition is fulfilled.

On the basis of the results presented in this paper, we conclude that hexachlorophene induces the efflux of Na^+ and K^+ from red cells by altering the permeability of the cellular membrane to monovalent cations. Further, the hexachlorophene-induced hemolysis, at least in the range of hexachlorophene concentrations used here, is likely a secondary event resulting from osmotic swelling subsequent to increased membrane permeability.

In closing, an intriguing example of hexachlorophene toxicity is found in human infants [29, 30], rats [4, 31, 32], mice [32] and monkeys [33, 34] which have undergone chronic exposure to this bacteriocide. Examination of the brains of individuals so exposed reveal a cerebral edema limited to the white matter, apparently resulting from fluid accumulation within the myelin sheath [32, 33]. There is an analogy between this toxic effect of hexachlorophene and its hemolytic effect, namely, both result from an accumulation of fluid. It is not known if the mechanism of hexachlorophene-induced cerebral edema is similar to the mechanism of hexachlorophene-induced hemolysis as presented in this paper, but it is interesting to consider the possibility.

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REFERENCES

1 Gump, W. S. (1969) J. Soc. Cosmet. Chem. 20, 173–184
2 Kimbrough, R. D. (1971) Arch. Environ. Health 23, 119–122
3 Kimbrough, R. D. (1973) Pediatrics 51, 391–394
4 Nakaue, H. S., Dost, F. N. and Buhler, D. R. (1973) Toxicol. Appl. Pharm. 24, 239–249
5 Caldwell, R. S., Nakaue, H. S. and Buhler, D. R. (1972) Biochem. Pharm. 21, 2425–2441
6 Nakaue, H. S., Caldwell, R. S. and Buhler, D. R. (1972) Biochem. Pharm. 21, 2273–2277
7 Cammer, W. and Moore, C. L. (1972) Biochem. Biophys. Res. Commun. 46, 1887–1894
8 Gould, B. S., Frigerio, N. A. and Lebowitz, W. B. (1955) Arch. Biochem. Biophys. 56, 476–485
9 Silvernale, J. N., Joswick, H. L., Corner, T. R. and Gerhardt, P. (1971) J. Bacteriol. 108, 482–491
10 Joswick, H. L., Corner, T. R., Silvernale, J. N. and Gerhardt, P. (1971) J. Bacteriol. 108, 492–500
11 Corner, T. R., Joswick, H. L., Silvernale, J. N. and Gerhardt, P. (1971) J. Bacteriol. 108, 501–507
12 Flores, G. and Buhler, D. R. (1971) Fed. Proc. 30, 1199
13 Flores, G. and Buhler, D. R. (1972) Fed. Proc. 31, 520
14 Flores, G. and Buhler, D. R. (1974) Biochem. Pharm. 23, in press
15 Obaid, A. L., Rega, A. F. and Garrahan, P. J. (1972) J. Membrane Biol. 9, 385–401
16 Naumann, H. N. (1964) in Hemoglobin, Its Precursors and Metabolites (Sunderman, F. W. and Sunderman, Jr, F. W., eds) pp. 40–48, J. B. Lippincott Co., Philadelphia
17 Hainline, A. (1958) in Standard Methods of Clinical Chemistry (Seligson, D., ed.) Vol. 2, pp. 49–60, Academic Press, New York

18 Ponder, E. (1948) Hemolysis and Related Phenomena, Reissued edn., pp. 29–40, Grune and Stratton, New York
19 Garrahan, P. J. and Glynn, I. M. (1967) J. Physiol. 192, 159–174
20 Wilbrandt, W. (1941) Pflüg. Arch. Ges. Physiol. 245, 22–52
21 Whittam, R. (1964) Transport and Diffusion in Red Blood Cells, pp. 185–191, Edward Arnold, London
22 Tosteson, D. C. (1964) in The Cellular Functions of Membrane Transport (Hoffman, J. F., ed.), pp. 3–22, Prentice Hall, Englewood Cliffs
23 Ponder, E. (1948) Hemolysis and Related Phenomena, Reissued edn., pp. 242–243, Grune and Stratton, New York
24 Tosteson, D. C. and Hoffman, J. F. (1960) J. Gen. Physiol. 44, 169–194
25 Dunham, E. T. and Glynn, I. M. (1961) J. Physiol. 156, 274–293
26 Passow, H. (1964) in The Red Blood Cell (Bishop, C. and Surgenor, D. M., eds) pp. 110–118, Academic Press, New York
27 Chapman, S. J., Allison, J. V. and Grimes, A. J. (1971) Scand. J. Haematol. 8, 347–358
28 Jacob, H. S. and Jandl, J. H. (1962) J. Clin. Invest. 41, 779–792
29 Powell, H., Swarner, W., Gluck, L. and Lampert, P. W. (1973) J. Pediatr. 82, 976–981
30 Mullick, F. G. (1973) Pediatrics 51, 395–399
31 Kimbrough, R. D. and Gaines, T. B. (1971) Arch. Environ. Health 23, 114–118
32 Lampert, P., O'Brien, J. and Garrett, R. (1973) Acta Neuropathol. 23, 326–333
33 Caley, F. R. (1972) in 30th Annu. EMSA Meet. 1st Pacific Reg. Conf. Electron Microsc. (Arceneaux, C. J., ed.), pp. 338–339, Claitors Publishing Div., Baton Rouge
34 FDA Bulletin. Hexachlorophene and Newborns, Dec. 1971.