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HEMOLYTIC PROPERTIES OF HEXACHLOROPHENE AND RELATED CHLORINATED BISPHENOLS*

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Abstract—Although hexachlorophene (HCP) and other chlorinated bisphenols are not hemolytic *in vivo*, even when administered at lethal doses, incubation of these compounds with nucleated and non-nucleated erythrocytes *in vitro* produces hemolysis. Chlorinated bisphenols are effective hemolytic agents at concentrations of 2×10^{-5} to 2×10^{-4} M. The hemolytic effect of the chlorinated bisphenols is characteristic of the erythrocyte donor species rather than dependent on the presence or absence of nucleation in the red cells. Of all the species tested, fish erythrocytes are the most susceptible and turkey erythrocytes are the most resistant to hemolysis by HCP though both are nucleated. No correlation was found between the hemolytic activity of the chlorinated bisphenols and their lipophilic character or the degree of ionization (p*K*a). Whole plasma or solutions of human and bovine serum albumin, hemoglobin and other proteins protected the erythrocytes against hemolysis induced by chlorinated bisphenols. The protection varied somewhat with the chlorinated bisphenol in question and differed among proteins of the same type from different species.

HEXACHLOROPHENE [HCP, 2,2′-methylenebis (3,4,6-trichlorophenol)] and similar compounds have been widely used in a number of germicidal, fungicidal and antiparasitic applications.[1,2] The chlorinated bisphenols are known to be quite toxic to mammals,[3–6] and recent studies[5,6] have shown that chronic exposure of rats to HCP results in edematous changes in the brain. However, there is little information about the mechanisms for toxicity of HCP and related chlorinated bisphenols.

Previous work indicated that HCP and similar compounds alter the permeability of plant[7] and bacterial[8] membranes. Thorsell[2] demonstrated an increase of ATPase and decrease of succinate oxidase and cholinesterase activities when homogenized liver flukes (*Fasciola hepatica*) were incubated with HCP. Corbett and Goose[9] have suggested that the fasciolicidal properties of HCP are due to uncoupling of oxidative phosphorylation, and recent work has demonstrated potent uncoupling effects on oxidative phosphorylation in rat liver and brain mitochondria by low concentrations of HCP[10–12] and other chlorinated bisphenols.[12] Chlorinated bisphenols also inhibit the mammalian succinoxidase, cytochrome oxidase and lactic dehydrogenase systems.[11,13] In addition, hexachlorophene and related compounds have been shown to bind strongly to certain proteins[11,13] and polypeptides,[14] with a resulting diminution in their bactericidal[8] and uncoupling[11,12] capabilities.

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These characteristics suggest that much of the biological activity of the chlorinated bisphenols may result from their strong interaction with cellular or subcellular membranes. To explore this hypothesis, we have investigated the hemolytic properties of the chlorinated bisphenols. It has been shown previously that certain chemicals or drugs alter the permeability or integrity of the red cell membrane resulting in the destruction of these cells.[15,16] Since the degree of hemolysis can be quantified, erythrocytes, therefore, provide a simple and convenient model system *in vitro* for studying interactions between drugs and cellular membranes.[17]

Drugs can induce erythrocyte hemolysis either through a direct attack on the red cell membrane[16,18] or indirectly, by inhibition of energy metabolism or oxidation of cellular components.[15,16,18] Normal survival of the red cell *in vivo* depends on its ability to maintain its size and shape, primarily by regulating active cation transport, and by its capacity to counteract oxidative degradation of cellular components.[19] In non-nucleated erythrocytes, energy for preserving cellular morphology and protecting against oxidative damage is derived from the glycolytic and pentose cycle metabolism of glucose. In addition to these pathways, nucleated cells also contain all of the trichloroacetic acid (TCA) cycle enzymes and, hence, exhibit a substantial aerobic respiration.[20,21] Energy metabolism in both types of cells serves two principal purposes: maintenance of oxidation-reduction homeostasis through formation of reduced pyridine nucleotide and glutathione and generation of ATP to preserve red cell structure.

We have, therefore, examined the hemolytic activities of the chlorinated bisphenols, especially HCP, on mature erythrocytes from several species. Both nucleated and non-nucleated erythrocytes have been employed in our studies to help identify the mechanism of hemolysis. The role of plasma and specific proteins in protecting against hemolysis has also been examined. Preliminary reports of parts of this research have appeared earlier.[22,23]

MATERIALS AND METHODS

The chlorinated bisphenols 2,2′-methylenebis (3,4-dichlorophenol); 2,2′-methylenebis (3,5-dichlorophenol); 2,2′-methylenebis (4,6-dichlorophenol); and hexachlorophene [2,2′-methylenebis (3,4,6-trichlorophenol)] were kindly donated by the Givaudan Corp., Clifton, N.J. Dichlorophene [2,2′-methylenebis (4-chlorophenol)] and bis (2-hydroxyphenyl) methane were purchased from the Aldrich Chemical Corp., Milwaukee, Wisc.; bithionol [2,2′-thiobis (4,6-dichlorophenol)] was purchased from Pflatz & Bauer Co., Flushing, N.Y.; 2,4-dinitrophenol, glutathione and most of the proteins and enzymes were purchased from Sigma Chemical Co., St. Louis, Mo., and Nutritional Biochemicals Corp., Cleveland, Ohio; and the pentachlorophenol was obtained from Eastman Organic Chemicals, Rochester, N.Y. Inorganic chemicals were purchased from Mallinckrodt Chemical Co., St. Louis, Mo.

Fresh blood with non-nucleated erythrocytes from humans, Wistar rats, sheep and rabbits or nucleated erythrocytes from rainbow trout, grass frogs, chickens and turkeys was collected in heparin. Washed erythrocytes were obtained by centrifuging blood for 5 min at about 2000 rev/min in a clinical centrifuge, removing the supernatant (plasma) and buffy layer and washing the resulting cells three times with Ringer's solution. The washed erythrocytes were then made up to the original blood volume with 0·154 M saline buffered with H_2CO_3–$NaHCO_3$, pH 7·4.

Hemolysis experiments were carried out with whole blood and washed erythrocytes. One-tenth ml of blood or washed cell suspension was incubated for 1–2 hr with 3 ml of a solution of chlorinated bisphenol in isotonic saline, buffered to pH 7·4 with H_2CO_3–$NaHCO_3$. Bisphenol concentrations ranged between 6×10^{-6} M and 2×10^{-4} M. Each experimental set consisted of five or six tubes containing different bisphenol concentrations and a control tube with blood or erythrocyte suspension alone. Fish and frog erythrocytes were incubated at room temperature (22 ± 1°) and experiments with rat, human, sheep, rabbit, chicken and turkey erythrocytes were conducted at 37°. At the end of the incubation period, the tubes were centrifuged and the hemoglobin concentration in the supernatant solution was determined spectrophotometrically by the cyanmethemoglobin method.* Per cent hemolysis was calculated by comparing the hemoglobin content of the supernatant solution with the total found in the 0·1 ml of blood or erythrocyte suspensions used in the incubations. The 50 per cent hemolysis values were obtained from a graphic plot of the data. Hemolysis in the controls ranged between 1 and 5 per cent.

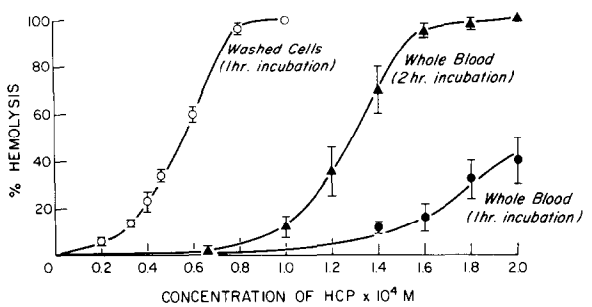

FIG. 1. Hemolytic effect of hexachlorophene in human blood and washed erythrocytes. Each experimental point represents the mean of from five to ten determinations and the bars represent ± one standard deviation from the mean value. Washed erythrocytes, 1-hr incubation (—○—○—); whole blood, 2-hr incubation (—▲—▲—) and 1-hr incubation (—●—●—).

RESULTS

Although the chlorinated bisphenols did not produce hemolysis *in vivo* even when administered to experimental animals at lethal doses,[6] incubation of erythrocytes in the presence of concentrations of chlorinated bisphenols ranging from 2×10^{-5} M to 2×10^{-4} M leads to partial or complete hemolysis. The hemolytic effect of HCP in human blood and washed erythrocytes is shown in Fig. 1. This plot of bisphenol concentration versus hemolysis is representative of the type of experiment conducted from each of the chlorinated bisphenols reported here.

Incubation of washed human erythrocytes for 1 hr at 37° produced 50 per cent hemolysis at a HCP concentration of $0{\cdot}6 \times 10^{-4}$ M (Fig. 1). It is evident that components in plasma protected erythrocytes against the bisphenol since a HCP concentration almost 4-fold greater was required in whole blood preparations to produce a similar degree of hemolysis as that observed in washed cells. Even with a 2-hr incubation, $1{\cdot}3 \times 10^{-4}$ M HCP or a 2-fold increase in concentration was necessary to produce 50 per cent hemolysis with whole blood.

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TABLE 1. HEMOLYTIC EFFECTS OF HEXACHLOROPHENE ON ERYTHROCYTES FROM DIFFERENT SPECIES

Type of erythrocyte	Species	Concn of HCP ($\times 10^{-4}$ M) producing 50 per cent hemolysis: Washed erythrocytes	Whole blood
Nucleated*	Fish	0·20	0·70
Nucleated	Chicken	0·85	1·65
Nucleated	Frog	1·20	1·60
Nucleated	Turkey	1·90	2·0 (11%)†
Non-nucleated‡	Rat	0·40§	0·90
Non-nucleated	Human	0·60	1·30
Non-nucleated	Sheep	0·70	2·00
Non-nucleated	Rabbit	1·20	2·0 (20%)†

* Samples of blood or erythrocytes were incubated for 1 hr at 23° for fish and frog and at 37° for chicken and turkey. Hemolysis in controls ranged between 1 and 5 per cent.

† Only 11 and 20 per cent hemolysis at the maximum concentration tested, 2×10^{-4} M HCP.

‡ Incubation of the samples at 37° for 2 hr except for rat blood which was incubated for 1 hr.

§ Incubation for 1 hr at 23° gave 50 per cent hemolysis at a HCP concentration of $0{\cdot}8 \times 10^{-4}$ M.

The difference among species in susceptibility to the hemolytic effect of HCP for whole blood and washed erythrocytes is shown in Table 1. Presence or absence of nucleation in erythrocytes was apparently unrelated to hemolytic tendency, because fish erythrocytes hemolyzed most readily in the presence of HCP while turkey erythrocytes were the least susceptible to hemolysis. In all species, plasma protected against lysis. The incubation temperature influenced the rate of hemolysis elicited by

TABLE 2. EFFECT OF VARIOUS PROTEINS ON HEXACHLOROPHENE-INDUCED HEMOLYSIS OF WASHED HUMAN ERYTHROCYTES*

Protein	Assumed molecular weight	Per cent hemolysis: 1×10^{-4} M HCP	2×10^{-4} M HCP
Human α,β-globulins	125,000	2·5	14·5
Human serum albumin	69,000	2·0	20·0
Bovine serum albumin	69,000	2·0	23·6
Bovine hemoglobin	68,000	9·0	30·0
Human plasma†	100,000	12·0	41·7
Equine myoglobin	17,000	66·0	94·0
Equine cytochrome c	12,384	67·0	94·0
Human transferrin	90,000	89·0	94·0
Bovine γ-globulin	150,000	94·0	94·0
Chicken ovomucoid	28,000	96·0	98·0
Reduced glutathione‡	307	94·0	98·0

* Erythrocytes were incubated at 37° for 1 hr with 1×10^{-4} M and 2×10^{-4} M HCP containing 5×10^{-4} M protein or peptide. Hemolysis in controls was 1 per cent for blood and 2 per cent for washed erythrocytes.

† Human washed erythrocytes incubated with HCP in the presence of 5×10^{-4} M fresh human plasma.

‡ Reduced form of the tripeptide γ-glutamyl-cysteinyl-glycine.

the chlorinated bisphenols since the concentration of HCP required for 50 per cent hemolysis of washed rat erythrocytes doubled when incubations were carried out at 23° rather than the normal 37° (Table 1).

The capability of other proteins and glutathione to influence the HCP hemolytic process has also been investigated (Table 2). The proteins examined fall into two groups, differing widely in effectiveness. Of the weakly protective proteins, myoglobin and cytochrome c gave a degree of protection, while the remaining proteins had practically no effect on HCP-induced cell lysis. Proteins with an apparent high affinity for HCP, however, such as albumin,[11] offered strong protection. This differential anti-hemolytic effect is more readily apparent when the proteins were tested at the higher HCP concentration of 2×10^{-4} M.

The hemolytic activities of six related chlorinated bisphenols in blood and erythrocytes of fish, frog, rat and human are reported in Table 3. As observed previously with HCP, fish erythrocytes were the most sensitive and those of the frog the least sensitive to bisphenol-induced hemolysis. The protective effect of plasma against hemolysis in whole blood was observed for all chlorinated bisphenols and all species studied. Some differences in the relative protective effect of plasma were observed with the various bisphenols tested.

The hemolytic effectiveness of the various chlorinated bisphenols varied appreciably with the position and number of chlorine substituents on the aromatic ring. While 2,2′-methylenebis (3,5-dichlorophenol) was as active as HCP in causing red cell destruction, the isomeric 2,2′-methylenebis (3,4-dichlorophenol) was the least effective compound tested. Similarly, bithionol [2,2′-thiobis (4,6-dichlorophenol)] was a more potent hemolytic agent than the corresponding methylenebisphenol compound.

Related phenols such as pentachlorophenol, 2,4-dinitrophenol and the non-chlorinated bisphenol, bis-(2-hydroxyphenyl) methane, were completely inactive against washed fish, frog, rat and human erythrocytes at the maximum concentration of 2×10^{-4} M tested.

DISCUSSION

Lysis of erythrocytes by drugs is thought to occur by either of two basic mechanisms.[15,16,18] The first involves direct association of the drug with the red cell membrane, resulting in changes in membrane structure, increased permeability, osmotic swelling and hemolysis. In the second mechanism, the drug or its metabolite first penetrates into the cell interior where it or its oxidation products then interfere with cellular metabolism ultimately resulting in membrane damage and hemolysis.

Ansel and Cadwallader[24] have reported that a number of antibacterial preservatives including phenols exhibit hemolytic activity against human and rabbit erythrocytes. They concluded that the antibacterial and hemolytic properties of such chemicals result from similar mechanisms involving their alteration of the permeability and/or integrity of the erythrocyte and bacterial membranes.

Previous observations on the modification of the plant[7] and bacterial[8,25] membranes by HCP and its effect on membrane-bound enzyme systems in mitochondria[9–12] and the endoplasmic reticulum* support the conclusion that hemolysis is

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TABLE 3. HEMOLYTIC EFFECT OF CHLORINATED BISPHENOLS*

Compound	Concn × 10^{-4} M producing 50 per cent hemolysis							
	Fish		Frog		Rat		Human	
	Blood	Washed cells	Blood	Washed cells	Blood	Washed cells	Blood	Washed cells
2,2′-Methylenebis (4-chlorophenol)	1·44	0·72	†	†	†	†	†	†
2,2′-Methylenebis (3,4-dichlorophenol)	†	2·00‡	†	†	†	†	†	†
2,2′-Methylenebis (3,5-dichlorophenol)	1·20	0·20	1·60	0·80	2·00	0·75	2·00§	0·80
2,2′-Methylenebis (4,6-dichlorophenol)	1·00	0·30	1·85	1·30	1·85	1·05	†	1·30
2,2′-Methylenebis (3,4,6-trichlorophenol), (HCP)	0·70	0·20	1·60	1·20	0·90	0·40	1·30	0·60
2,2′-Thiobis (4,6-dichlorophenol)	1·00	0·22	1·75	1·30	1·60	0·80	2·00	0·75

* Blood and washed erythrocytes were incubated in buffered saline solutions at 23° for fish and frog and at 37° for rat and human. Human blood samples were incubated for 2 hr; all other samples were incubated for 1 hr. Hemolysis in controls was: fish blood 5 per cent; fish erythrocytes 8 per cent; frog blood 4 per cent; frog erythrocytes 6 per cent; rat blood 3 per cent; rat erythrocytes 5 per cent; and human blood and erythrocytes 2 per cent.

† Less than 20 per cent hemolysis at the maximum bisphenol concentration of 2×10^{-4} M tested.

‡ Only 43 per cent hemolysis at a bisphenol concentration of 2×10^{-4} M.

§ Only 34 per cent hemolysis at a bisphenol concentration of 2×10^{-4} M.

the result of a direct interaction between the chlorinated bisphenols and the plasma membrane of the erythrocytes. This hypothesis has been verified in subsequent studies with ^{14}C-labeled HCP* which show that low, nonhemolytic concentrations of HCP are primarily bound to the red cell membrane. The nature of this association is not known but may involve membrane proteins, since HCP has been shown to strongly bind other types of proteins.[11,12]

A hemolytic mechanism involving oxidative damage[15,16,18] to erythrocytes is relatively unlikely since no significant metabolism of HCP has been detected *in vivo*.[26] Furthermore, interaction of HCP with whole erythrocytes or lysates does not produce any appreciable oxidation of Fe (II) hemoglobin to Fe (III) hemoglobin.* These results suggest[27] that hemolysis does not result from a disruption of oxidation-reduction processes in the red cell.

The chlorinated bisphenols are active hemolytic agents for reasons other than their effect[11,12] on respiratory metabolism, since other uncouplers of oxidative phosphorylation had no apparent effect on erythrocyte stability. The hemolytic tendency of HCP and related compounds toward erythrocytes varied with the animal species and appeared to be independent of the presence or absence of a nucleus. There are important differences between nucleated and non-nucleated erythrocytes in energy production[20,21] and in the behavior toward chemicals which alter glutathione and macromolecular SH metabolism.[28] In spite of these differences, nucleated cells were not unusually sensitive toward the bisphenols. In fact, among the cells tested, nucleated erythrocytes were the most (fish) and the least (turkey) susceptible toward hemolysis. These observations imply, therefore, that lysis of red cells by the chlorinated bisphenols probably does not result from an interference in cytoplasmic metabolism.

The basis for the relative differences in hemolytic tendency among species is not known but may be associated with variations in the protein and lipid composition of the red cell membrane.[29,30] Other factors such as cell size, volume and form, membrane thickness, hemoglobin type and content and differences in the viscoelastic properties of the red cell membranes have also been suggested[31] to influence the osmotic fragility of erythrocytes from various species.

Nakaue *et al.*[12] have correlated some physicochemical properties of chlorinated bisphenols, particularly lipid solubility, ionization state and number of chlorines, with the uncoupling activity of these chemicals and found a linear relationship between the effect on oxidative phosphorylation and the p*K*a. Uncoupling activity of the chlorinated bisphenols tended to increase with p*K*a and with each successive addition of chlorine to the aromatic ring. However, these workers found no relationship between the uncoupling effect and the lipophilic character of the chlorinated bisphenols.

The hemolytic properties and hydrophobic character of many classes of chemicals have been shown to be closely related.[32,33] Nevertheless, in the present study we failed to find a correlation between the hemolytic activities of the chlorinated bisphenols studied and their lipophilic character (Table 4). Moreover, there did not appear to be a direct relationship between the hemolytic potency and the p*K*a of these phenols, although hemolysis tended to increase with additional chlorine substitution. Ansel and Cadwallader[24] also observed an increase in the hemolytic activity of other phenols with addition of chlorine substituents.

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TABLE 4. HEMOLYTIC PROPERTIES, PARTITION COEFFICIENTS AND IONIZATION CONSTANTS OF THE CHLORINATED BISPHENOLS

Compound	Log P_{hexane}*	Log $P_{octanol}$†	pK_1‡	Concn × 10^{-4} M producing 50% hemolysis in washed cells: Fish	Human
2,2'-Methylenebis (4-chlorophenol)	0·70	5·28	9·2	0·72	§
2,2'-Methylenebis (3,4-dichlorophenol)	0·70	7·36	8·1	>2·00‖	§
2,2'-Methylenebis (3,5-dichlorophenol)	0·76	6·76	7·6	0·20	0·80
2,2'-Methylenebis (4,6-dichlorophenol)	1·34	7·58	7·0	0·30	1·30
2,2'-Methylenebis (3,4,6-trichlorophenol)	1·21	8·74	5·7	0·20	0·60
2,2'-Thiobis (4,6-dichlorophenol)	1·51	6·26	6·1	0·22	0·75

* Log of the hexane-pH 6·75 buffer partition coefficients (from Nakaue *et al.*[12]).
† Calculated according to Fujita *et al.*[34]
‡ From Nakaue *et al.*[12]
§ Less than 20% hemolysis at 2 × 10^{-4} M.
‖ Only 34% hemolysis at 2 × 10^{-4} M.

The protective effect of plasma against HCP-induced hemolysis apparently results from the binding of HCP to albumin in mammals and to other plasma proteins in fish and frog.* Bovine serum albumin also has been found to protect mitochondria against the uncoupling of oxidative phosphorylation by HCP.[11] Similar binding probably explains the protective influence of plasma against hemolysis elicited by the other chlorinated bisphenols and the effectiveness of other proteins in this regard (Tables 2 and 3).

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