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Influence of Estrogenic Pesticides on Membrane Integrity and Membrane Transfer of Monosaccharide into the Human Red Cell

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Some natural and synthetic estrogens inhibit carrier-mediated transport of glucose into human red blood cells and membrane vesicles from the placenta (LeFevre, 1959; Johnson and Smith, 1980, 1985). The inhibitory action of these estrogens on transport appears to be a direct effect at the membrane and does not involve receptor binding and protein synthesis. It is not clear, however, whether such inhibition is a common feature among estrogenic agents. Several chlorinated hydrocarbon pesticides have been shown to possess estrogenic activity and these include chlordecone (=Kepone), o,p'DDT, p,p'DDE, and methoxychlor. Chlordecone has been shown to bind to the estrogen receptors and possess estrogenic activity in the reproductive tracts of birds and mammals (Palmiter and Mulvihill, 1978; Bulger et al., 1979; Eroschenko, 1981). Estrogenic activity of o,p'DDT in the mammalian uterus and avian oviduct has also been demonstrated (Bitman et al., 1968; Bitman and Cecil, 1970; Stancel et al., 1980). p,p'DDE possesses some estrogenic activity as shown by its ability to promote the deposition of glycogen in a rat uterus (Bitman and Cecil, 1970), however, it does not inhibit the binding of estradiol to the rat testicular cytosolic estrogen receptor (Bulger et al., 1978). Finally, methoxychlor inhibits the binding of estrogen to receptors in the rat uterus (Nelson et al., 1978). Thus, these pesticides could have inhibitory effects on the human sodium-independent glucose transporter. Owing to the apparent importance of this membrane transporter in human tissues, direct interaction of hormones and xenobiotics with the glucose transporter is of fundamental significance.

Some pesticides have been shown to alter membrane structure directly and alter the passive permeability of membranes (Antunes-Madeira and Madeira, 1979; Buff and Berndt, 1981).

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chlordecone: decachlorooctahydro-1,3,4-mentheno-2H-cyclobuta[cd]pentalene-2-one
o,p'DDT: 1-[o-chlorophenyl]-1-(p-chlorophenyl)-2,2,2-trichloroethane
p,p'DDE: 2,2-bis-[p-chlorophenyl]-1,1-dichloroethylene
methoxychlor: 1,1,1-trichloro-2,2-bis[p-methoxyphenyl]ethane

Whether the estrogenic pesticides influence passive diffusion of sugars across membranes has not been established. Finally, preliminary observations (this laboratory) have suggested that some estrogens and pesticides have lytic effects on intact cells. Consequently, this study focuses on the ability of several estrogens and estrogenic pesticides to disrupt the cell membrane, influence the monosaccharide transporter, and alter the rate of monosaccharide permeation through the membrane by simple diffusion.

MATERIALS AND METHODS

Since storage of human red cells alters the kinetics of sugar transport (Weiser et al., 1983), human red cells were prepared for study within one hour of blood collection and cells were studied immediately after preparation. Heparinized blood was washed by centrifugation in phosphate buffered saline (PBS) (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 0.9 mM $CaCl_2$, 2.7 mM KCl, 0.5 mM $MgCl_2$, pH 7.4). Cells were suspended in PBS then centrifuged at 10°C for 5 min at 1000 X g. The supernatant and white blood cell layer were aspirated from the red cells, which were then resuspended and washed by centrifugation two additional times. The final pellet of red cells was suspended in PBS to a hematocrit of 25 ± 2%.

To examine the influence of estrogens and estrogenic pesticides on membrane integrity, the cell preparation was further diluted to a suspension hematocrit of 12.5%. Cholesterol, 17-β-estradiol, the synthetic estrogen, diethylstilbestrol (DES), o,p'DDT, p,p'DDE, chlordecone, methoxychlor, or ethanol alone (the vehicle in all exps.) were added to the red cell suspension to final ethanol concentration of 2%. This concentration of ethanol did not result in appreciable cell lysis. After 1 hr incubation at 20°C, 0.1 mL of cell suspension was added to 1 mL PBS and this final suspension was immediately centrifuged for 2 min at 13,000 X g in a microcentrifuge. The supernatant was diluted by 50% and the absorbance at 540 nm read for hemoglobin. This absorbance was related to the total amount of hemoglobin in an aliquot of red cells to determine the percentage of cells having lysed due to the various treatments. Incubation of the cells with the above compounds for an additional 2 hr resulted in no appreciable additional cell lysis.

Membrane transfer of monosaccharide was followed by measuring the uptake of tritiated 3-O-methyl-D-glucose (3-O-MG) or tritiated L-glucose by the red cell suspension at 20°C. [3-O-MG is a D-glucose analog which is transported by the human sodium-independent glucose transporter but is not metabolized (Campbell and Young, 1952). As L-glucose has an extremely low affinity for the transporter (LeFevre, 1961), L-glucose uptake is taken to represent uptake by simple diffusion.] Prior to uptake experiments, red cells were incubated for 1 hr with cholesterol, estrogen, or pesticide. Influx experiments were initiated by adding 40 µL of 6 mM monosaccharide (labeled plus unlabeled

monosaccharide and without estrogen or pesticide) to 40 µL of the red cell suspension (Hct: 25%). Transport of monosaccharide was measured as 3-0-MG taken up in about 3 sec and membrane transfer by simple diffusion was measured as L-glucose uptake in 1 hr in the absence or presence of 5 µM cytochalasin B or 125 µM phloretin. Transport of 3-0-MG was timed with a Kwik-set Labcron timer with foot-pedal control (Lab-Line Instruments, Inc., Melrose Park, IL). Uptake was terminated by the addition of 1.0 mL ice-cold PBS containing 250 µM phloretin; phloretin was not used to terminate the uptake of L-glucose. Upon dilution with cold PBS, the entire sample was immediately pipetted onto 250 µL silicone oil (General Electric Co., Waterford, NY; product: F50, specific gravity: 1.025) and the cells were separated from the aqueous medium by centrifugation for 2 min in the micro-centrifuge. The labeled aqueous fluid was aspirated off and replaced with PBS; PBS and silicone oil were then removed from the red cell pellet. The pellet was recovered with the bent end of a pipe cleaner which was cut off and placed into a scintillation vial with 1.0 mL water. After complete lysis of the red cells, 10 mL of Kodak Ready-To-Use III scintillator solution (Eastman Kodak, Rochester, NY) was added and the sample counted with a United Technologies Packard Minaxi-beta liquid scintillation counter. Radioactivity trapped external to the pelleted red cells was estimated by adding the control cell suspension to the ice-cold, phloretin-containing PBS before adding the labeled monosaccharide solution. The trapped radioactivity has been eliminated from all data presented and all data are expressed as uptake per liter packed cell volume.

Chlordecone (99% purity) was obtained from Chem Service (West Chester, PA), o,p'DDT, p,p'DDE (99% purity), and cytochalasin B were from Aldrich Chem. Co. (Milwaukee, WI), methoxychlor (98% purity), 17-β-estradiol, DES, cholesterol, and phloretin were from Sigma Chem. Co. (St. Louis, MO). [^{3}H]3-0-Methyl-D-glucose and [^{3}H]L-glucose were from New England Nuclear (Boston, MA).

RESULTS AND DISCUSSION

It was noted that exposure of red cells to several of the compounds under study led to apparent hemolysis. Therefore, lysis was estimated by determining the amount of hemoglobin released from the red cells after an hour of exposure to the substances under study. As shown in Fig. 1, cholesterol, methoxychlor, p,p'DDE, and o,p'DDT exhibited no hemolytic effect on cells at a concentration of 1 mM. However, at this concentration, DES, estradiol, and chlordecone induced hemolysis to 12, 50, and 100%, respectively, of control values. To examine the influence of these substances on membrane transfer of monosaccharide, concentrations of estrogens or estrogenic pesticides which resulted in less than 7% hemolysis were subsequently used.

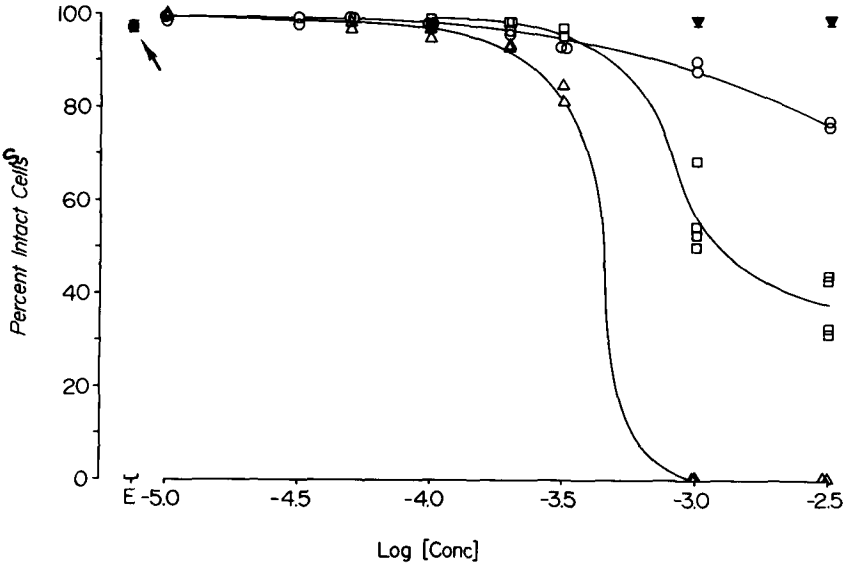

Figure 1. Percent intact red cells as a function of the concentration of the estrogenic agent. Solid circle (arrow, above E on the abscissa) represents the influence of 2% ethanol, the vehicle used in these experiments (mean ± SD, n=4). The solid inverted triangle represents the range of influence of cholesterol, o,p'DDT, p,p'DDE, or methoxychlor. Most pronounced are the actions of DES (○), estradiol (□), and chlordecone (△) on the membrane integrity of the cell.

As previously shown with placental membrane vesicles (Johnson and Smith, 1980, 1985), estradiol and DES inhibited the rapid uptake of 3-O-MG across the cell membrane by the glucose transporter (Fig. 2 and Table 1). At 0.16 mM, they inhibited the initial rate of uptake by about 70 and 85%, respectively. Cholesterol, which served as a control, had relatively little effect on this transport. At 0.16 mM, methoxychlor, p,p'DDE, and o,p'DDT showed inhibition of 3-O-MG uptake by about 40 to 50%. Thus, these pesticides, previously reported to possess estrogenic activity, mimic the actions of estradiol and the synthetic estrogen, DES, on membrane transport. Chlordecone, at concentrations which did not induce hemolysis, did not affect 3-O-MG uptake. Desaiah (1981) showed that chlordecone is particularly potent in inhibiting membrane Na^{+}-K^{+}-ATPase activity in the brain and membrane oligomycin-sensitive Mg^{++}-ATPase activity in the heart with apparent inhibitory constants of 2 μM and 50 nM, respectively. Relative to the potent action of chlordecone on those ATPase activities, the results of the present study indicate that the estrogens and estrogenic pesticides in this investigation exhibit relatively weak inhibitory actions on monosaccharide uptake via the carrier-mediated mechanism. The physiological significance of such weak inhibition remains to be determined.

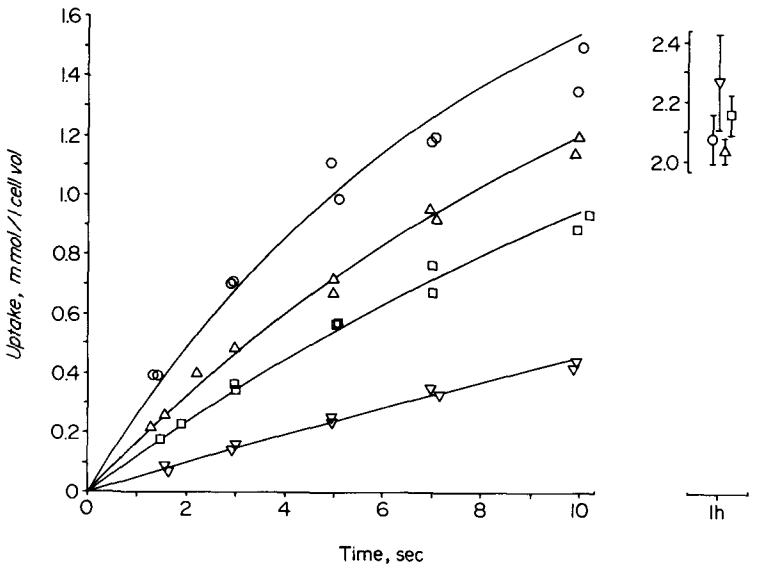

Figure 2. The uptake of 3.0 mM 3-0-MG at 20°C in the absence (○) and presence of 0.16 mM methoxychlor (△), o,p'DDT (□), and DES (▽). Curves are drawn according to integrated rate equation:

$$-kt = \ln[(N_E - N_t)/N_E]$$

where t is time in sec, N_E and N_t are concentrations of 3-0-MG taken up at equilibrium (1 hr) and at t, respectively, and k is the first order rate constant taken to be: control = 0.130/sec, methoxychlor = 0.083/sec, o,p'DDT = 0.059/sec, and DES = 0.024/sec. Data at 1 hr are means ± SD, n = 3.

Organochlorine pesticides have been shown to alter the structure of lipid bilayers and influence membrane fluidity (Buff and Berndt, 1981; Buff et al., 1982; Duxbury and Thompson, 1987). They have also been shown to alter the permeability of liposome membranes (Antunes-Madeira and Madeira, 1979). Consequently, the influence of estrogens and estrogenic pesticides on membrane permeability by simple diffusion was examined. The uptake of L-glucose was taken to represent uptake by simple diffusion. Relative to the influence of the ethanol vehicle alone, the estrogens and estrogenic pesticides appeared to reduce L-glucose uptake (Table 2). Furthermore, the percent reduction of L-glucose uptake due to these agents was similar to their effect on 3-0-MG uptake. This similarity could be due to an effect of these estrogenic agents on membrane structure. However, this phenomenon could also be due to an effect on the glucose transporter where the transporter has a low but finite affinity for [^{3}H]L-glucose or where a transportable [^{3}H]contaminant was in the L-glucose preparation. To explore these possibilities,

Table 1. Uptake of 3.0 mM 3-O-MG (based on uptake at about 3 sec) in the presence of cholesterol or estrogenic agent expressed as a percentage of the control value. Rate of 3-O-MG uptake in the control was 170 ± 10 μmole/L cell vol/sec, n = 4 (mean ± SD). (Uptake in the presence of 2% ethanol was 98.2 ± 5.4% of control, n = 4.)

Agent	Uptake*	Agent	Uptake*
Cholesterol		Methoxychlor	
0.16 mM	88.8 ± 2.7%	0.16 mM	59.0 ± 4.4%
0.5 mM	92.4 ± 5.8%	0.5 mM	66.6 ± 1.7%
Estradiol		o,p'DDT	
0.16 mM	31.9 ± 2.0%	0.16 mM	46.4 ± 4.4%
DES		0.5 mM	41.0 ± 3.3%
0.16 mM	14.7 ± 1.6%	p,p'DDE	
		0.16 mM	60.4 ± 4.0%
		0.5 mM	55.5 ± 2.8%
		Chlordecone	
		0.05 mM	111 ± 14%

* n = 4 for all except estradiol and DES where n = 8

Table 2. Uptake of 3.0 mM L-glucose (based on uptake at 1 hr) in presence of the ethanol vehicle, cholesterol, or estrogenic agent expressed relative to the control value. Studies were run with or without 5 μM cytochalasin B or 125 μM phloretin. Rate of L-glucose uptake in the control was 255 ± 25 μmole/L cell vol/hr (mean ± SD, n = 4, all exps.).

Agent	Uptake, no inhibitor	Uptake with Cytochalasin B	Uptake with Phloretin
Ethanol (2%)	74.5 ± 5.1%	2.1 ± 0.9%	5.8 ± 0.7%
Cholesterol (0.5 mM)	82.7 ± 1.9%	4.8 ± 2.4%	5.6 ± 2.3%
Estradiol (0.16 mM)	23.0 ± 2.0%	7.7 ± 1.9%	5.7 ± 1.0%
DES (0.16 mM)	10.6 ± 2.3%	11.0 ± 4.2%	7.4 ± 0.9%
Methoxychlor (0.5 mM)	49.1 ± 3.2%	6.8 ± 2.3%	6.4 ± 1.6%
o,p'DDT (0.5 mM)	25.2 ± 1.9%	6.2 ± 2.3%	3.8 ± 1.8%
p,p'DDE (0.5 mM)	35.0 ± 2.7%	7.9 ± 3.0%	5.4 ± 1.2%
Chlordecone (0.05 mM)	96.1 ± 9.2%	7.4 ± 1.7%	7.1 ± 0.9%

labeled L-glucose uptake was measured in the presence of cytochalasin B or phloretin, inhibitors of the human sodium-independent glucose transporter (LeFevre, 1959; Johnson and Smith, 1980, 1985). The results shown in Table 2 suggest that the observed actions of the estrogenic agents on L-glucose uptake, in the absence of inhibitors, were due to the activity of the glucose transporter: due either to a low affinity for L-glucose or due to the presence of a transportable and tritiated contaminant. In the presence of cytochalasin B or phloretin, these estrogenic agents had no discernible effect on membrane transfer of monosaccharide by simple diffusion.

The results of this study on human red cells therefore show that some estrogenic agents have lytic effects on cell membranes and inhibitory actions on membrane transport of monosaccharide at high concentrations. However, whether these actions are physiologically significant remains to be determined. The results also suggest that these agents have little, if any, influence on membrane transfer of monosaccharide by simple diffusion.

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