SH-Groups Essential for Estrogen Uptake and Retention in the Mouse Uterus

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SUMMARY

The effect of various sulfhydryl reagents on the uptake and retention of estrogens by the mouse uterus *in vitro* has been studied. It was found that *N*-ethylmaleimide and *p*-chloromercuribenzoate blocked the uptake and retention of 17β-estradiol by the uterus *in vitro* while iodoacetate blocked the uptake but not the retention of 17β-estradiol. *N*-Ethylmaleimide also blocked the uterine uptake and retention of the synthetic estrogen *meso*-hexestrol. On the other hand, it had no effect on the uptake and retention of the less estrogenic stereoisomer *racemic*-hexestrol. Estrogen concentration in a "nontarget" tissue, the diaphragm, was essentially unaffected by the sulfhydryl inhibitors.

INTRODUCTION

Estrogenic compounds are more readily accumulated and retained in target organs such as the mammalian uterus and vagina than in nontarget organs (1–6). Some insight into the structural requirements of a molecule for accumulation at these principal sites of biological action has been gained (5, 7–8). Several authors have made approaches to a biochemical characterization of binding sites in target organs. The current evidence is that a major fraction of the accumulated estrogen is associated (but not covalently bound) to protein (9).

A preferential accumulation of estrogens in the uterus can be demonstrated also *in vitro* (10, 11). In a series of such experiments some metabolic inhibitors were included. This report describes the effect of SH-inhibitors[1] on the uptake and retention of estrogens by the immature mouse uterus. The experiments with the SH-inhibitors were carried out *in vitro* because of their general toxicity. A blockade by iodoacetamide and cyanide of 17β-estradiol uptake *in vitro* in the rat uterus has been shortly reported by others (12).

[1] Abbreviations: SH, sulfhydryl; 17β-estradiol, estra-1,3,5(10)-triene-3,17β-diol; hexestrol, 3,4-bis-(*p*-hydroxyphenyl)-*n*-hexane.

MATERIALS AND METHODS

Tritiated 17β-estradiol was supplied by New England Nuclear Corporation. Its specific activity was 125 μC/μg. The preparation of the tritiated hexestrol isomers has been described (8). Their specific activities were 140 μC/μg. The radiochemical purity of the radioactive compounds was controlled by thin-layer chromatography (8) and was better than 97% at the time of use. *N*-Ethylmaleimide, *p*-chloromercuribenzoate, iodoacetic acid sodium salt, and 2,3-dimercaptopropanol were obtained from Sigma Chemical Company, sodium arsenite (reagent grade) from Merck.

Albino mice of the NMRI strain were used. They were 15–17 days old and weighed 9–11 g. They were killed by a blow on the head and their uteri and diaphragms immediately cut out. The uteri were divided at the cervix into two identical parts and from the diaphragms two 3–4 mg strips were cut out. The tissues were incubated in flasks containing Krebs-Ringer phosphate buffer, pH 7.4 and with

air as gas phase. Most flasks also contained 2% (w/v) bovine albumin (Cohn fraction V, Sigma B grade). Aliquots of alcoholic stock solutions of radioactive as well as nonradioactive estrogens were taken to dryness and redissolved in the buffer. Each incubation flask contained tissues from two animals; one half-uterus from an animal was shaken in the control flask, the other in the experimental flask. The flasks were shaken in a Warburg apparatus at 37°.

After incubation, the tissues were gently blotted, weighed wet, and individually solubilized in Hyamine (Packard Co). Finally the radioactivity was measured with the liquid scintillation technique (11). At least 10,000 counts were recorded for most samples; however, no sample was measured longer than 50 min. Counting efficiency was around 25% as determined by addition of internal standard. The recorded activities were transformed to disintegrations per minute and unit wet weight (dpm/mg). It has been found that the tissue radioactivity is identical with the original compounds by chromatography and crystallization according to (8).

RESULTS

The effect of various SH-inhibitors on the uptake *in vitro* of tritium-labeled 17β-estradiol by the uterus and diaphragm is illustrated in Fig. 1. The tissues were preincubated with the inhibitors in Krebs-Ringer phosphate buffer and then transferred to fresh medium which contained 17β-estradiol-^{3}H, albumin, and inhibitors. All the investigated inhibitors caused a marked depression of the preferential uterine uptake of 17β-estradiol at concentrations around 10^{-3} M. *N*-Ethylmaleimide was highly active also at 10^{-4} M concentration. The diaphragm content of radioactivity was not much affected by the inhibitors; however, a slight augmentation by

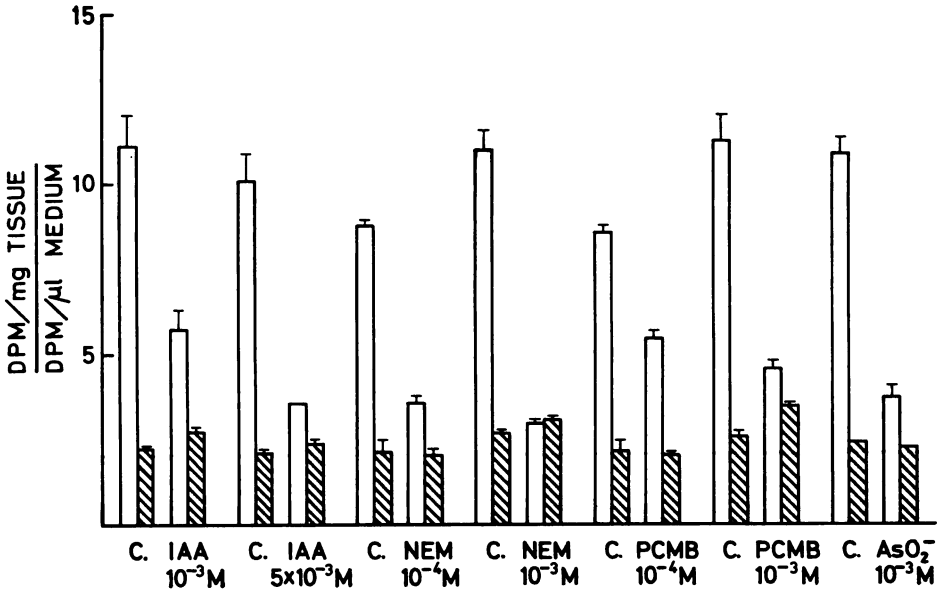

FIG. 1. *Effect of SH-reagents on the uptake of tritium-labeled 17β-estradiol by the mouse uterus and diaphragm*

The tissues were (a) incubated for 15 min with 3 ml buffer (controls) or 3 ml buffer with SH-reagent (experimentals); (b) incubated for 1 hour in other flasks containing 3 ml buffer with albumin and 0.0015 µg tritiated 17β-estradiol; the experimental flasks also contained SH-reagent.

There were tissues from 4 animals per group. Open columns represent the uterus, and the cross-hatched columns the diaphragm. Vertical bars denote one standard error of the mean (SEM); C, control; IAA, iodoacetic acid; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoate.

TABLE 1

Effect of N-ethylmaleimide on the uptake of tritiated hexestrol isomers by the mouse uterus and diaphragm

The tissues were (a) incubated for 15 min with 3 ml of buffer (controls) or 3 ml of 10^{-3} M *N*-ethylmaleimide in buffer (experimentals: *Exptl*); (b) incubated in other flasks for 1 hr in 3 ml of buffer with albumin 2% (w/v) and 0.003 μg of one of the tritiated hexestrol isomers; the flasks of the experimental groups also contained 10^{-3} M *N*-ethylmaleimide. Tissues were taken from 4 animals per group. Mean values ± SEM (standard error of the mean) are given.

Radioactive estrogen	Treatment	Content of radioactivity			Percent of control	
		Uterus: medium	Diaphragm: medium	Uterus: diaphragm	Uterus	Diaphragm
meso-Hexestrol	Control	2.1 ± 0.1	1.0 ± 0.04	2.1		
	Exptl.	0.74 ± 0.05	1.07 ± 0.02	0.69	35	103
racemic-Hexestrol	Control	6.7 ± 0.3	12.3 ± 0.6	0.54		
	Exptl.	5.8 ± 0.3	13.0 ± 0.5	0.45	88	106

10^{-3} M *p*-chloromercuribenzoate was observed.

The *meso*-isomer of the synthetic estrogen hexestrol is about as estrogenic as 17β-estradiol, while the *racemic* isomer is 100 times less active. *meso*-Hexestrol is much more readily taken up by the mouse uterus *in vivo* and *in vitro* than by the diaphragm (8, 11). The *racemic* isomer, on the other hand, is concentrated more markedly by the diaphragm. The following experiment illustrates the effect of *N*-ethylmaleimide on the uptake *in vitro* of the hexestrol isomers by uterus and diaphragm (Table 1). The uterine uptake of *meso*-hexestrol was depressed by *N*-ethylmaleimide while the diaphragm uptake was not. On the other hand, *N*-ethylmaleimide had no substantial effect on the very considerable uptake of *racemic*-hexestrol neither by the uterus nor by the diaphragm.

Under the above-mentioned conditions, interaction of the inhibitors with SH-groups in albumin might have changed the amount of estrogen available in solution. In a separate experiment the inhibitors were present only in the preincubation medium. After preincubation with the inhibitor the tissues were washed and then incubated in a buffer containing albumin and tritiated 17β-estradiol but not containing inhibitor. The uptake of radioactivity by the uterus was depressed about as much in this experiment as in the previous one (Fig. 2). It was also tested whether 2,3-dimercaptopropanol could reverse the uptake blockade by sodium arsenite as well as by *p*-chloromercuribenzoate (not shown in the figure). There was no reversal by 2,3-dimercaptopropanol.

Following incubation *in vitro* or following systemic injection 17β-estradiol and *meso*-hexestrol are much more strongly retained by the uterus than by the diaphragm in washout experiments. *Racemic*-hexestrol is retained to a high degree both by the uterus and the diaphragm. The effect of some SH-inhibitors on the retention of the estrogens by these tissues was investigated (Table 2). The concentrations of the SH-inhibitors were those which were previously found to inhibit the uptake by the uterus of 17β-estradiol and *meso*-hexestrol. *N*-Ethylmaleimide as well as *p*-chloromercuribenzoate strongly accelerated the washout of 17β-estradiol from the uterus. The effect of iodoacetate was exceptional; while this compound inhibited the uterine uptake of 17β-estradiol, it had no effect on the uterine retention at the same concentrations. *N*-Ethylmaleimide strongly depressed the retention of *meso*-hexestrol by the uterus, but the high retention of *racemic*-hexestrol by the uterus and the diaphragm was not much affected by treatment with *N*-ethylmaleimide.

Table 3 depicts the effect of *N*-ethylmaleimide on the retention *in vitro* of estrogens accumulated *in vivo*. 17β-Estradiol and *meso*-hexestrol were much more read-

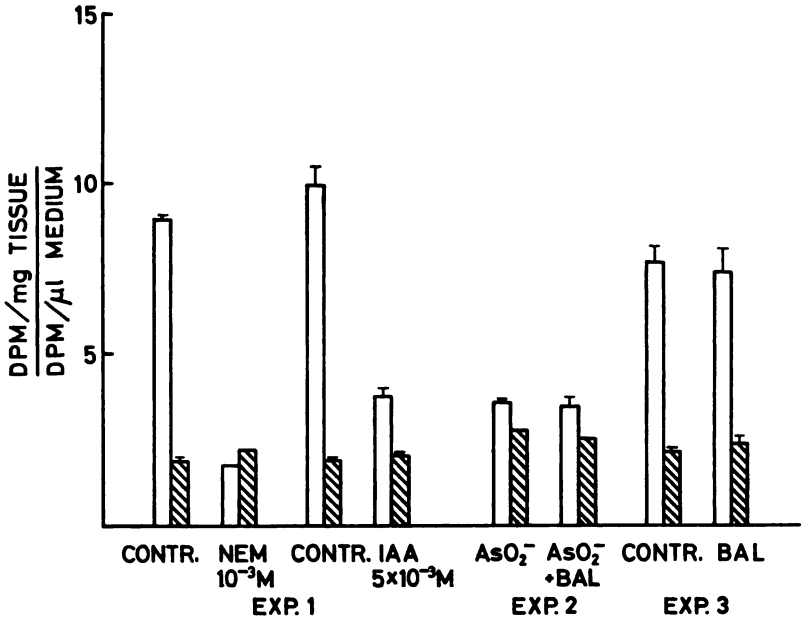

FIG. 2. *Effect of preincubation with SH-reagents (Exp. 1) or 2,3-dimercaptopropanol (Exp. 3) and effect of 2,3-dimercaptopropanol after arsenite treatment (Exp. 2) on the uptake of tritiated 17β-estradiol by the mouse uterus and diaphragm*

In Exp. 1 (a) incubation for 15 min with buffer and SH-reagents (controls only buffer); (b) washing for 15 min in 3 ml fresh buffer; (c) incubation for 1 hr with 3 ml buffer with albumin and 0.0015 µg tritiated 17β-estradiol. In Exp. 2 both groups were (a) incubated with 10^{-3} M arsenite in 3 ml buffer for 15 min; (b) one group incubated with 10^{-3} M 2,3-dimercaptopropanol in buffer for 15 min, the other group only with buffer; (c) both groups incubated with tritiated 17β-estradiol as in Exp. 1. In Exp. 3 pretreatment for 15 min with buffer or 10^{-3} M 2,3-dimercaptopropanol in buffer was followed by incubation with tritiated 17β-estradiol as in Exp. 1.

There were tissues from 4 to 6 animals per group. Open columns represent the uterus, cross-hatched columns the diaphragm. Vertical bars denote 1 SEM; NEM, *N*-ethylmaleimide; IAA, iodoacetic acid; BAL, 2,3-dimercaptopropanol.

ily taken up by the uterus than by the diaphragm *in vivo*. *N*-Ethylmaleimide strongly accelerated the washout of these estrogens from the uterus. The low uptake of *racemic*-hexestrol relative to the diaphragm content, on the other hand, was not very sensitive to treatment with *N*-ethylmaleimide. The diaphragm content was substantially unaffected by *N*-ethylmaleimide in all experimental groups.

DISCUSSION

The reactions of so-called sulfhydryl inhibitors are not completely specific against sulfhydryl groups. It is established that, e.g., *N*-ethylmaleimide also can react with protein amino groups to a significant extent (13). However, the "SH-inhibitors" used represent chemically widely different substances, but with the reactivity against SH-groups in common. It is therefore likely that the blockade of the uterine uptake and retention of potent estrogens by these substances actually involves SH-groups.

The concentrations of the SH-reagents which were active in the experiments are more than a million times higher than that concentration of estrogen which saturates the uptake capacity of the uterus. The SH-reagents clearly must react with other molecules besides the hypothetical "receptor" molecules. One might therefore question whether the studied effects can be considered as effects on these receptors. From Tables 1 and 2 evidence emerges that at least the action of *N*-ethylmaleimide is

TABLE 2

Effect of SH-reagents on the retention in vitro of the radioactivity accumulated by uterus and diaphragm

The tissues were (a) incubated for 1 hr with 0.0015 μg of tritiated 17β-estradiol or 0.003 μg of one of the tritiated hexestrol isomers in 3 ml buffer with albumin[a]; (b) incubated in other flasks for 15 min with 3 ml buffer (controls) or buffer + SH-inhibitor (experimentals); (c) incubated in other flasks in 3 ml of buffer with albumin and 0.15 μg of the appropriate nonradioactive estrogen; the flasks of the experimental groups also contained stated concentrations of SH-inhibitor. Tissues were taken from 6 animals per group. Mean values ± SEM are given.

Radioactive estrogen	Inhibitor,[b] concentration	Content of radioactivity			Percent of control	
		Uterus: medium	Diaphragm: medium	Uterus: diaphragm	Uterus	Diaphragm
17β-Estradiol	None	6.5 ± 0.5	0.22 ± 0.01	30.2		
	NEM, 10^{-3} M	0.93 ± 0.04	0.22 ± 0.01	4.3	14	102
17β-Estradiol	None	3.8 ± 0.1	0.27 ± 0.01	14.4		
	PCMB, 10^{-3} M	0.90 ± 0.06	0.28 ± 0.02	3.2	24	106
17β-Estradiol	None	7.4 ± 0.3	0.27 ± 0.01	27.7		
	IAA, 10^{-3} M	7.2 ± 0.4	0.25 ± 0.01	28.8	97	94
17β-Estradiol	None	5.2 ± 0.4	0.22 ± 0.02	23.6		
	IAA, 5×10^{-3} M	6.2 ± 0.2	0.22 ± 0.02	28.8	120	100
meso-Hexestrol	None	1.4 ± 0.06	0.42 ± 0.04	3.4		
	NEM, 10^{-3} M	0.27 ± 0.03	0.31 ± 0.02	0.85	19	75
racemic-Hexestrol	None	2.1 ± 0.1	3.2 ± 0.1	0.64		
	NEM, 10^{-3} M	1.8 ± 0.07	3.8 ± 0.2	0.84	87	118

[a] This is the medium of the table heading.

[b] NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoate; IAA, iodoacetic acid.

TABLE 3

Effect of N-ethylmaleimide on the retention of estrogens accumulated in vivo by the uterus and diaphragm

A dose of 0.01 μg tritiated 17β-estradiol or 0.02 μg of one of the tritiated hexestrols in 0.1 ml saline was injected subcutaneously and the animals were killed after 1 hr. One half-uterus and a piece of diaphragm were incubated *in vitro* for 15 min with 2 ml 10^{-3} M *N*-ethylmaleimide in buffer and then for 1 hr in another flask with 2 ml 10^{-3} M *N*-ethylmaleimide in buffer with albumin and 0.1 μg of the appropriate nonradioactive compound (experimentals: *Exptl.*). The other half-uterus and another piece of diaphragm were incubated under the same conditions except that the incubation media did not contain *N*-ethylmaleimide (controls).

Retentions were calculated individually, i.e., the animals were their own controls. There were tissues from 6 animals per group. NS means no significant counts.

Radioactive estrogen	Treatment *in vitro*	Content of radioactivity (dpm/mg wet weight)			Retention (% of control)	
		Uterus	Diaphragm	Uterus: diaphragm	Uterus	Diaphragm
17β-Estradiol	Control	522 ± 36	<10 (NS)	—		
	Exptl.	41 ± 5	<10 (NS)	—	7.8	—
meso-Hexestrol	Control	1267 ± 104	46 ± 3	27.8		
	Exptl.	495 ± 65	37 ± 4	13.4	39	82
racemic-Hexestrol	Control	343 ± 56	122 ± 10	2.8		
	Exptl.	213 ± 24	120 ± 10	1.8	70	100

quite specific. It has been found that as 17β-estradiol, *meso*-hexestrol but not the inactive estrogen *racemic*-hexestrol is more avidly taken up by the mouse uterus than by the diaphragm (11) (Table 2). *N*-Ethylmaleimide blocked the uptake of *meso*-hexestrol by the uterus and accelerated washout from its binding sites. On the other hand, *N*-ethylmaleimide if anything, only had a weak activity on the uterine uptake and retention of the inactive estrogen, *racemic*-hexestrol. The physicochemical properties of the stereoisomeric hexestrols are similar while the spatial arrangements are different. An unspecific destruction of the uterine tissue would not likely affect the accumulation of the two isomers differently. It would seem therefore that *N*-ethylmaleimide affects specific sites which are directly involved in estrogen accumulation. Other workers have isolated discrete protein fractions from rat and calf uterine tissue which are capable of binding 17β-estradiol (9). It is possible that the SH-inhibitors in some way change the conformation of such "receptor" proteins, in analogy to the known effects of organic mercurials on the conformation of glutamate dehydrogenase. This enzyme is "allosterically" inhibited by the synthetic estrogen stilbestrol, and this inhibition is reduced by organic mercurials (14).

N-Ethylmaleimide and *p*-chloromercuribenzoate blocked the uptake as well as the retention of 17β-estradiol in the uterus. Iodoacetate, while inhibiting uptake had no substantial effect on the retention of accumulated 17β-estradiol. It is possible that estrogen accumulation in the uterus involves two dissociated processes. From other experiments (Terenius unpublished) it is known that 5×10^{-3} M cyanide strongly inhibits the uterine uptake of 17β-estradiol *in vitro* but at the same concentration does not accelerate washout of accumulated 17β-estradiol. Furthermore, reduced incubation temperature inhibits the uptake of 17β-estradiol and *meso*-hexestrol. On the other hand, reduced incubation temperature does not accelerate washout from the uterine tissue. The mechanism for maintaining the concentration of estrogen in the uterus thus appears to be bimodal; after transport the estrogen becomes firmly bound.

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