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ESTRADIOL AND PROGESTERONE RECEPTOR ACTIVITIES IN STORED LYOPHILISED TARGET TISSUE

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SUMMARY

Estradiol (ER) and progesterone receptors (PgR) were analysed in lyophilised target tissues. During lyophilisation of DMBA-induced mammary tumors, human breast cancer tissue and calf uterus tissue no deterioration of ER and PgR occurred. Moreover, no loss of ER and PgR was observed when lyophilised calf uterus tissue was stored for 2–3 months at 4°C. During storage for 3 weeks at room temperature PgR remained unchanged, whereas ER decreased to a minor extent. Lyophilisation did not influence dissociation constants (K_D) or ligand specificities of ER and PgR binding sites.

INTRODUCTION

Several investigators [1–3] have emphasized that during storage of target tissue, even at a temperature of −70°C, estradiol receptor activity decreases. For that reason storage of the tissue in liquid nitrogen [4] has been recommended. However Raynaud *et al.* [5] showed that even at the temperature of liquid nitrogen, mammary tumor biopsies may lose 20% of their progesterone receptor activity when stored for a two month period. The present study was designed to evaluate the influence of lyophilisation and of subsequent storage at 4°C and room temperature on estradiol and progesterone receptor activity in calf uterus tissue and in biopsies of human breast cancer and DMBA-induced rat mammary tumors.

MATERIALS AND METHODS

Materials. Estradiol-17β-2,4,6,7-^{3}H (105 Ci/mmol) was obtained from New England Nuclear, Boston, MA. Unlabeled 17β-estradiol (E_2) oestron, 5α-dihydrotestosterone and progesterone were purchased from Steraloids, Pawling, NY. Dexamethasone, α-monothioglycerol, bovine serum albumin (BSA), diethylstilbestrol (DES) and triamcinolone acetonide were obtained from Sigma Chemicals Co. Cyproterone acetate was kindly provided by Schering A.G., Berlin, Germany and medroxyprogesterone acetate and clomiphene by Farmitalia. Unlabeled 17,21-dimethyl-19-nor-pregna-4,9-diene-3,20 dione (R5020), 6,7-^{3}H-R5020 (56,5 Ci/mmol and 11β-methoxy-17-ethynyl-1,3,5(10)-estratriene-3,17β-diol (Moxestrol or R2858) were kindly supplied by Roussel-Uclaf, France. Tamoxifen (Nolvadex) and 16α-ethyl-21-hydroxy-19-norpreg-4-ene-3,20-dione (02058) were gifts from I.C.I., England and Organon, Oss, Holland respectively. Dithiothreitol (DTT), dextran T 70 and aqua luma were obtained from British Drug House (BDH), Pharmacia and Lumac respectively. All other products were reagent grade and purchased from Merck.

The following buffers were used: 1. Tris–HCl 10 mM, EDTA 1.5 mM, DTT 0.5 mM, pH 7.4 (for determination of the estrogen receptor). 2. Tris–HCl 10 mM, EDTA 1.5 mM, α-monothioglycerol 10 mM, 10% glycerol (vol./vol.) pH 7.4 (for determination of the progesterone receptor). The dextran-coated charcoal suspension contained 0.25% charcoal and 0.025% dextran T 70 in buffer 1.

The microtiterplates (v shape, 96 holes), the A.M.69 Microshaker and the centrifuge plate carriers were obtained from Cooke Instruments.

Preparation of lyophilised powder. Tissue of calf uterus, DMBA-induced rat mammary tumors and human breast cancer biopsies was frozen in liquid nitrogen and pulverized in the frozen form by means of a microdismembrator (Braun, Melsungen, Germany). A part of this pulverised tissue was immediately homogenized in cold buffer (wt/vol. = 1:4) and analysed for estradiol and progesterone receptor activity, whereas the remaining part was lyophilised in glass vials overnight (Modulyo Freeze dryer, Edwards, England). The vials were stoppered whilst under vacuum. This lyophilised powder was analysed for receptor activity after storage for different periods at 4°C or at room temperature.

Dextran-coated charcoal assay of receptor activity. The lyophilised powder was weighed and carefully homogenized in cold buffer (wt/vol. = 1:40) using a Ten Broeck homogenizer. All subsequent steps were performed at 0 to 4°C. The homogenate was centrifuged at 105,000 *g* for 60 min to prepare the cytosol.

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All the receptor assays were performed in microtiter-plates described for this purpose by Katzenellenbogen *et al.* [6]. Fifty microliter of cytosol was incubated with six different concentrations of tritiated steroid ranging between 10^{-9} and 10^{-8} M for R5020 and between 10^{-9} and 8×10^{-9} M for E_2 and bound hormone was counted (B_{tot}).

The nonspecific binding (B_{ns}) and the ratio of nonspecific bound to free ($^{B}ns/U_{ns}$) for R5020 and E_2 were determined in the presence of 10^{-6} M of nonradioactive R5020 and E_2 or DES respectively.

After equilibrium was reached (18–20h at 4°C), the unbound steroid (U) was removed by adding 0.1 ml dextran coated charcoal suspension. The plates were shaken for 10 min on a mechanical shaker and thereafter centrifuged for 20 min at 1.000g.

Aliquots (0.1 ml) of the supernatant were transferred to counting vials containing 3 ml of scintillation fluid. The radioactivity was counted with an efficiency of 46%. The amount of radioactivity bound was subtracted from the total amount of radioactivity present (T), giving the corresponding amounts of unbound steroid ($U = T - B$). The ratio $^{B}ns/U_{ns}$ was independent of the tritiated steroid concentration. This ratio was multiplied by ($T - B_{tot}$) giving the correct amount of nonspecific binding. The specific receptor binding (B_s) was calculated according to the following equation:

$$B_s = B_{tot} - (T - B_{tot})\,(B_{ns}/U_{ns})$$

The calculated values of B_s were plotted against the corresponding $B_s/T - B_{tot}$ ratios giving the Scatchard plot. Scatchard plot analysis was performed to determine the concentration of receptor binding sites (B) and the equilibrium dissociation constant (K_D). The concentration of binding sites was expressed as fmoles/mg protein. The protein content of the supernatant was determined by the method of Lowry [7] using BSA as a standard.

RESULTS

Nine biopsies of human breast cancer tissue and 15 DMBA-induced rat mammary tumors were analysed for estradiol receptor activity before and after the pulverized tissue was lyophilised. Subsequently the lyophilised specimen was stored at 4°C up to three days. Figure 1 shows the values obtained before and after lyophilisation: $Y = 1.05\,X - 4.9$, $r_{spearman} = 0.90$ and $P < 0.001$.

The results of progesterone receptor assays performed on 5 biopsies of human breast cancer and on 7 DMBA-induced rat mammary tumors, before and after lyophilisation of the tissue, are plotted in Fig. 2: $Y = 0.90\,X + 7.0$. $r_{spearman} = 0.96$ and $P < 0.001$. One of the human breast cancer tissues showed an oily residue after lyophilisation.The receptor values of this tumor, which decreased 50 to 60% after lyophilisation, are marked by an asterisk in Figs 1 and 2. Estradiol and progesterone receptor-negative

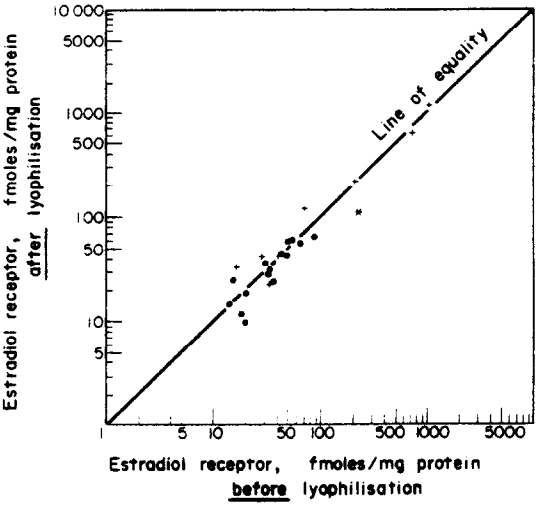

Fig. 1. Correlation between estradiol receptor binding sites before and after lyophilisation. DMBA-induced mammary tumors (●) and human breast cancer tissue (+). Human breast cancer biopsy, which showed an oily residue after lyophilisation (*).

tumors remained negative after lyophilisation ($n = 8$). The dissociation constants, calculated from the Scatchard plots, did not change systematically by the lyophilisation procedure. ($P > 0.1$, Wilcoxon test for paired observations).

Pulverized powder of lyophilised calf uterus tissue was analysed for receptor activity after different storage periods at 4°C or at room temperature. The results of these experiments are collected in Table 1. As can be seen from this table, storage of lyophilised tissue at 4°C did not influence estradiol and progesterone receptor activities. At room temperature progesterone receptor activity remains unchanged

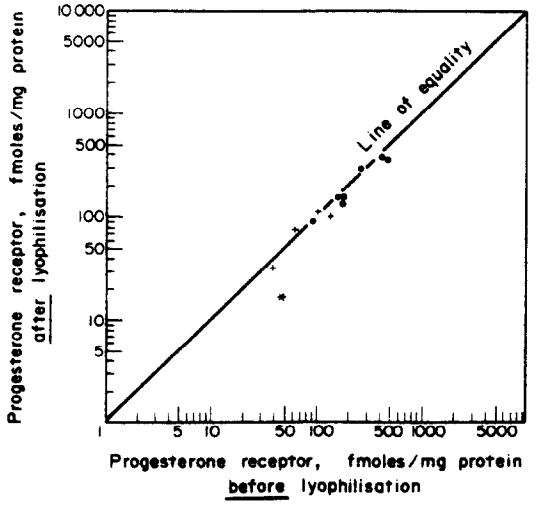

Fig. 2. Correlation between progesterone receptor binding sites before and after lyophilisation. DMBA-induced mammary tumors (●) and human breast cancer tissue (+). Human breast cancer biopsy, which showed an oily residue after lyophilisation (*).

Table 1. Effect of lyophilisation and subsequent storage at 4°C or at room temperature on oestradiol and progesterone receptor activities in calf uterus tissue

	Estradiol receptor (fmol/mg protein)	Progesterone receptor (fmol/mg protein)
Before lyophilisation	367	785
After lyophilisation days, 4°C		
3	372	808
10	287	750
17	352	805
24	329	856
38	353	839
44	287	—
51	—	901
65	—	804
80	273	—
94	392	—
After lyophilisation days, room temperature		
1	391	752
7	378	739
14	335	768

whereas estradiol receptor activity decreased to a minor extent.

Cytosols of lyophilysed calf uterus tissue prepared and analysed at the same day showed a variation of 632 ± 14 fmol/mg protein (mean ± 1 S.D.) (range 614–649 fmol protein, $n = 7$) and 1085 ± 112 fmol/mg protein (range 923–1230 fmol/mg protein, $n = 7$) for the determination of estradiol and progesterone receptor binding sites respectively.

The measured dissociation constants (K_D), using [^{3}H]-E_2 and [^{3}H]-R5020 as the ligands, did not show systematical changes during lyophilisation and during storage of lyophilised calf uterus tissue at 4°C or at room temperature. The mean ± 1 S.D. of the dissociation constants of the experiments collected in Table 1 was 0.29 ± 0.19 nM and 2.5 ± 1.0 nM for the estradiol and the progesterone receptor respectively.

The results of competition studies, performed on calf uterus tissue before and after lyophilisation, with increasing concentrations of various nonradioactive compounds are collected in Table 2. The results were expressed as the relative binding affinity (RBA), which is defined as the relative concentrations of competitor and radioinert ligand required to displace 50% of bound radioligand from its receptor binding sites. Comparison of RBAs determined in calf uterus tissue before and after lyophilisation revealed no essential differences. 02058, medroxyprogesterone acetate and progesterone were very potent inhibitors of [^{3}H]-R5020 binding, whereas cyproterone acetate and triamcinolone acetonide were less active in displacing [^{3}H]-R5020. Dihydrotestosterone competed only to a minor extent and estradiol, dexamethasone and cortisol were amost completely ineffective in displacing [^{3}H]-R5020 from its binding sites. Tritiated estradiol was only displaced from its binding sites by estrogens or anti-estrogens. Moxestrol and DES most effectively displaced [^{3}H]-E_2 and oestron was less effective in displacing [^{3}H]-E_2. Tamoxifen and clomiphene exerted only weak competitive effects. Dexamethasone, 5α-dihydrotestosterone and progesterone did not compete with [^{3}H]-E_2 for its receptor binding sites even when present at 1000-fold molar excess.

In contrast to lyophilised calf uterus, receptor activity decreased strongly, as would be expected, when pulverized calf uterus was stored at room temperature without lyophilisation. After storage for 24 h at room temperature a decrease of ER and PgR was observed from 1031 to 35 fmol/mg protein and from 1217 to 39 fmol/mg protein respectively.

DISCUSSION

It has been the experience of many investigators that during storage of target tissues at −20°C or

Table 2. Competition for [^{3}H]-R5020 and [^{3}H]-E_2 receptor binding in cytosol of calf uterus tissue before and after lyophilisation.

	[^{3}H]-R5020		[^{3}H]-E_2	
	Before	After	Before	After
R5020	100	100		
02058	~1000	~1100		
Medoxyprogestrone acetate	500	540		
Progesterone	214	151	<0.01	<0.01
Cyproterone acetate	38	42		
Triamcinolone acetonide	19	17		
5α-Dihydrotestosterone	2.5	1.8	<0.01	<0.01
Estradiol	0.2	0.5	100	100
Moxestrol			35	44
DES			19	17
Oestron			2.5	3.1
Tamoxifen			0.12	0.15
Clomiphene			0.012	0.016
Dexamethasone	0.15	0.23	0.01	0.01
Cortisol	<0.01	<0.01		

−70°C steroid hormone receptor proteins deteriorate [1–3, 8–10]. However, some tumor samples appeared to be much more sensitive to storage conditions than other tumor samples. Wittliff and Savlov reported that storage of a breast tumor biopsy at −70°C for one month resulted in a loss of 75% of estradiol receptor activity [3]. During the Bethesda Workshop (18–19 July, 1974 in Bethesda, Maryland U.S.A.) most investigators agreed that if the receptor assay could not be immediately performed on fresh tissue, freezing the whole tissue in liquid nitrogen would minimize the loss of ER upon storage. However, even when tumors are stored in liquid nitrogen, progesterone receptor levels may decline to a considerable extent as was reported by Raynaud and co-workers [4] and was also observed in our laboratory.

From the present study it appeared that during lyophilisation of pulverised target tissue oestradiol and progesterone receptor levels did not decline. Furthermore it was observed that during storage of lyophilised calf uterus tissue in the course of about 2–3 months at 4°C no loss of binding capacity for estradiol and the progestin R5020 occurred. Storage at room temperature was employed for only three weeks, a period long enough for sending specimens to other laboratories. In the course of these three weeks there was no or only a minor decrease of estradiol and progesterone receptor activity.

Currently a number of laboratories analyse lyophilised calf uterus tissue for interlaboratory control.

The performed competition studies indicated that during lyophilisation the ligand specificity of the estradiol and progesterone receptor binding sites remained essentially unchanged. In this study progesterone was 1.5 to 2.0 times more potent than R5020 in displacing [^{3}H]-R5020 from the progesterone receptor binding sites present in cytosol of calf uterus tissue. However, in our [11] as well as other laboratories [12,13] R5020 was about 5 times more potent than progesterone when the displacement studies were performed in DMBA-induced mammary tumors or in biopsies of human breast cancer. The reason for this discrepancy is not clear, but will be subject of future investigations.

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