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THE EFFECT OF LYOPHILIZATION ON STEROID RECEPTOR BEHAVIOR, AS DEFINED BY SUCROSE DENSITY GRADIENT ANALYSIS

G. R. JANES, A. J. KOENDERS and Th. J. BENRAAD.
Department of Experimental and Chemical Endocrinology, Medical Faculty, University of Nijmegen, The Netherlands

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SUMMARY

The effects of lyophilization of both cytosol and tissue powder were examined by sucrose density gradient analysis (SDGA) of uterine estrogen receptor (ER) and progesterone receptor (PgR) from pig and calf. Cytosol prepared from lyophilized material (both cytosol and tissue powder) was compared to that prepared concomitantly from frozen, pulverized powders of the same tissue. It was found that both ER and PgR responses to SDG ionic strength and the ability of ER to demonstrate temperature-dependent 4S → 5S transformation were unimpaired by lyophilization. In addition, lyophilized cytosol and lyophilized tissue powder gave equivalent results. A few experiments examining lyophilization effects on steroid receptors from human breast biopsies did note some diminution of the 8S form of both ER and PgR following lyophilization, although this effect was variable in extent. It is nevertheless concluded that much lyophilized material exhibits many of the same responses as frozen, and may be used in lieu of the latter, at least within the confines of the parameters described here.

INTRODUCTION

Receptor assays for estradiol receptor and progesterone receptor are now routinely performed on breast biopsy material, as an aid in the selection of appropriate treatment regimes for patients with advanced breast cancer [1–3]. In addition, there is increasing interest in the relationship between steroid receptor status and uterine malignant and pre-malignant disease. There have been several studies on the relationship between endometrial ER and PgR levels and pathologic processes associated with long-term estrogen administration to post-menopausal women [4, 5]. In addition, progestins have been used successfully in the treatment of advanced endometrial cancer [6, 7] and progesterone has been shown to effect ER and E_2DH (estradiol dehydrogenase) levels in both normal and neoplastic endometrial tissue, thus suggesting that assay of endometrial response to progestin treatment may help define tumor hormone sensitivity [5]. Recently several investigators [8–10], studying soluble ER and PgR levels in human endometrial adenocarcinoma, concluded that knowledge of receptor content contributed significantly to prognostic ability. Thus steroid receptor status and its clinical assay play an ever more important role in the analysis and management of breast and uterine carcinoma.

As the clinical significance of soluble ER and PgR levels has become clearer, particularly in the management of patients with advanced breast cancer, so has the importance of inter-laboratory quality control programs to compare methodologies and assay results [11–14], as the variety of methodologies employed by different institutions and companies can result in a relatively wide spectrum of values reported for a given sample [11, 12]. Because of its stability to temperature and prolonged storage intervals, lyophilized target tissue appears well suited as a reference preparation for use in such quality control programs [15, 16]. Following lyophilization of both human breast tumor tissue and calf uterus, assayed levels of cytosolic ER and PgR do not decline [17]. In addition, the ligand specificity spectrum of both ER and PgR has been shown to be unaltered by lyophilization of calf uterine cytosol [15]. Finally, when stored at 0–4°C, ER levels appear stable for at least six months in both human and calf lyophilized tissue and neither ER nor PgR from calf is significantly lowered following 1–2 weeks maintenance at room temperature [18]. Lyophilized target tissues thus appear to be very suitable reference preparations and are, in fact, already used as such in several quality control programs. However, there is still relatively little known of the extent to which the sedimentation behavior of cytosolic ER and PgR in these reference samples mimics that of fresh or frozen material. We have undertaken this study of the effects of lyophilization, of pig, calf and human breast tumor tissue, on the response of both ER and PgR, as defined by SDGA, to short-term warming and alterations in ionic strength. In so doing, we hoped to better characterize tissue powders used for quality control. In addition, however, if it could be shown that lyophilization leaves unchanged the full spectrum of responses seen with soluble ER and PgR from fresh or frozen material, lyophilized target tissue might also be used

for experimental purposes. This would allow the analysis of a single piece of tissue by multiple assays over a prolonged period of time and would therefore be quite advantageous.

MATERIALS AND METHODS

Materials

The radioactive ligand 17β-[2,4,6,7-^{3}H]-estradiol (100 Ci/mmol) was obtained from Radiochemical Centre, Amersham, Buckinghamshire, U.K. The radioactive progestin used was [17α-methyl-^{3}H]-promegestone (90 Ci/mmol) from New England Nuclear, Boston, MA. On arrival, both ligands were diluted in ethanol to 100 nM concentration and stored at −20°C in the dark. [Methyl-^{14}C]-bovine serum albumin (20 μCi/mg) was ordered from New England Nuclear. Aqualuma was purchased from T. J. Baker Chemicals and Dextran T70 was obtained from Pharmacia. Charcoal (Aktivkohle für Analyse), EDTA (Triplex), sucrose and $NaHPO_4$ were purchased from Merck; reagents were of analytical grade. Sodium azide was obtained from BDH and monothioglycerol (α-thioglycerol; purity 98%) from Sigma.

Preparation of cytosols and lyophilization

Both calf and pig uteri were obtained from a local slaughterhouse; they were placed on ice immediately on removal from the animal. Human breast tumor tissue was derived from biopsy samples received for routine Scatchard analysis of cytosolic steroid receptor status [19]; it was deep frozen on removal (−70°C) and stored in liquid N_2 until time of assay (less than 3 weeks after biopsy). Calf and pig uteri were cleaned of fat and adherent connective tissue, minced on ice, and frozen in liquid N_2. All tissue was then pulverized to a fine, homogeneous powder by means of a stainless steel mortar precooled in liquid N_2 and a microdismembrator (Braun, Melsungen, FRG). Frozen material was stored at this step in liquid N_2. Cytosol was prepared for lyophilization by mixing tissue powder with buffer (0.02 M Na_2HPO_4/NaH_2PO_4, 1.5 mM EDTA, 3.0 mM NaN_3, pH 7.4; 1:4 w:v) and centrifuging at 105,000 *g* for 0.5 h at 2°C (MSE Prepspin 75). Cytosol and a portion of the tissue powder were measured into glass vials in liquid N_2 and lyophilized for 20 h (15, 17, 18; Modulyo Freezer Dryer; Edwards, England). Following this period, they were sealed under vacuum and stored at 4°C until use.

Sucrose density gradient centrifugation

Lyophilized cytosols were reconstituted with 10 mM α-monothioglycerol (and 10% glycerol, where indicated). Cytosols were prepared from frozen and lyophilized tissue by mixing with 0.02 M $NaHPO_4$ buffer (0.02 M Na_2HPO_4/NaH_2PO_4, 1.5 mM EDTA, 3.0 mM NaN_3, 10 mM α-monothioglycerol, pH 7.4; 10% glycerol added where indicated; 1:4 w:v for frozen tissue; 1:20 for lyophilized tissue), followed by repeated aspiration and discharge through a pasteur pipette and then centrifugation, as before. During the interval between preparation and analysis (not more than 14 days for pig and calf uteri), lyophilized tissue was stored, in the glass vials, at 0–4°C, and frozen material in liquid N_2. All cytosols were labeled with 10 nM [^{3}H]-estradiol or [^{3}H]-promegestone for 1 h at 0°C (except where indicated otherwise). Unbound steroid was removed by mixing and incubating with a dextran-coated charcoal pellet (0.25% charcoal, 0.025% dextran; 200 μl per 150 μl cytosol suspension, pelleted and supernatant removed). After 10 min at 2°C, the charcoal and unbound steroid were removed by centrifugation (5 min, 15,000 *g*; Heraeus Christ Varifuge) and 150 μl of supernatant was layered on a 5–20% sucrose density gradient, together with 10 μl of [^{14}C]-bovine serum albumin (BSA) as a marker protein. Gradients were prepared in 0.02 M $NaHPO_4$ buffer (with EDTA, NaN_3 and monothioglycerol, as before), with or without the addition of glycerol and/or 0.4 M KCl, as indicated, and were spun for 16 h at 240,000 *g* in a MSE Prepspin 75. Tubes were punctured at the bottom and 120 μl fractions were collected, mixed with 3 ml Aqualuma and counted in a liquid scintillation spectrometer (PRIAS, Packard PLD Tri-Carb). Tritium counts were corrected for ^{14}C overlap with the aid of appropriate ^{3}H/^{14}C standards run concurrently.

RESULTS

The sedimentation behavior of soluble steroid receptors from pig and calf were examined, before and after lyophilization, in response to two basic parameters: salt and temperature. The effect of lyophilization on sedimentation patterns of both ER and PgR, under conditions of varied ionic strength, is presented in Figs 1 and 2. In each case, data from lyophilized samples are compared to those from frozen, pulverized uterine tissue; the lyophilized tissue was prepared directly from this frozen tissue powder. Figure 1 compares the distribution of estradiol binding sites in cytosol prepared from frozen and lyophilized pig uterus, analyzed on gradients with and without added 0.4 M KCl. The two 4S peaks on the high-salt gradient shown on the left are nearly identical, running just behind the 4.6S BSA marker. The lyophilized tissue peak is slightly smaller, reflecting the proportionally lower protein concentration of this cytosol (data not shown). On the right, the same cytosols are examined on a sucrose density gradient prepared without added KCl; again the two peaks are nearly identical. The dominant form of the receptor is the 8S, running well ahead of the BSA, but a small 4S component is also visible in fraction 18. When cytosols from similarly prepared calf uterine tissue (lyophilized cytosol, lyophilized tissue, and frozen tissue; data not shown) were labeled and analyzed, the results were the same. Lyophilization did not affect the salt-

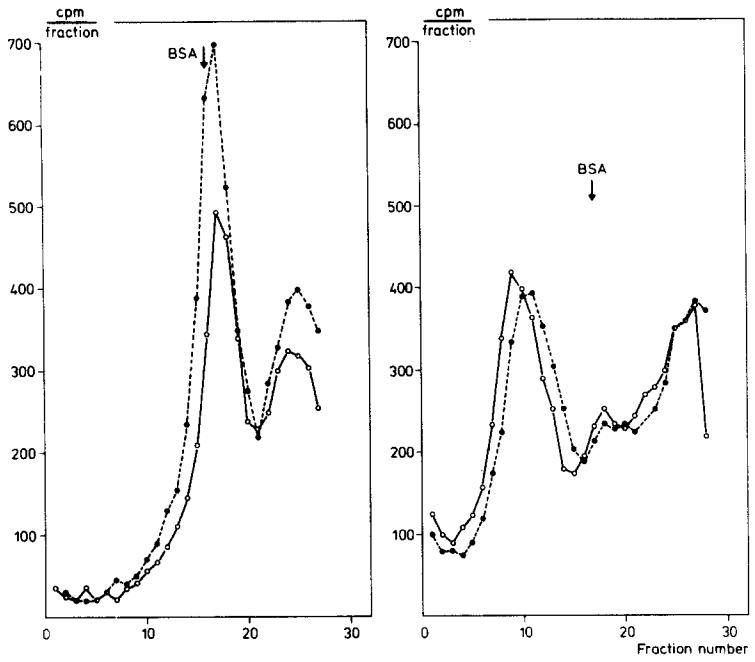

Fig. 1. Sedimentation patterns of estradiol receptors analyzed in cytosol from frozen (●——●) and lyophilized (○——○) pig uterine tissue: Response to salt. Tissue powders were prepared from frozen, minced uterus as described in Materials and Methods. One portion was stored in liquid nitrogen (frozen tissue) and one lyophilized overnight. Cytosols were prepared by mixing tissue with phosphate buffer (0.02 M Na_2HPO_4/NaH_2PO, 1.5 mM EDTA, 3.0 mM NaN_3, 10 mM monothioglycerol; pH 7.4) followed by centrifugation. Cytosols were equilibrated with 10 nM [^{3}H]-estradiol for 1 h at 0°C, adsorbed with dextran-coated charcoal (DCC) and layered on 5–20% sucrose gradients in phosphate buffer with (left) and without (right) 0.4 M KCl. The samples were centrifuged in a MSE (Ti) swing-out rotor at 240,000 *g* for 16 h at 0°C. The bottoms were pierced and 30 fractions were collected directly into counting vials. The radioactivity was determined in a Packard PRIAS PLD Tri-Carb spectrometer; tritium counts were corrected for ^{14}C overlap. BSA (↓); migration of [^{14}C]-bovine serum albumin marker. All gradients are displayed with the top at the right.

sensitive distribution of 4 and 8S forms of the estrogen receptor.

Similarly, the distribution of PgR (analyzed in pig and calf; cytosols prepared from fresh and lyophilized tissue) in response to salt and/or glycerol addition to sucrose gradients (Fig. 2) was minimally affected by lyophilization. Progesterone receptor from calf migrated as a discrete 4S peak in the presence of 0.4 M KCl (upper two graphs), and as a somewhat broader but predominantly 8S receptor when salt was deleted (lower two graphs). The presence of (10%) glycerol in incubation and gradient buffer did not have a pronounced effect on the ability to discriminate the 8S PgR form in calf uterus, although this appeared to be species dependent. When PgR from frozen and lyophilized calf uterus was again analyzed on high (upper right) and low salt (lower right) gradients without added glycerol, 8S receptor was still evident under low-salt conditions, albeit smaller than that seen when glycerol was present (lower left). In contrast, PgR analyzed in pig uterus cytosol prepared from frozen tissue, from lyophilized tissue and in reconstituted, lyophilized cytosol, migrated only as 4S receptor regardless of gradient salt concentration unless glycerol was added. In the presence of 10% glycerol, PgR appeared in predominantly the 4S form but with a clear 8S component. This phenomenon was unaffected by lyophilization (data not shown).

Figures 3 and 4 demonstrate the effect of mild heating on both ER and PgR derived from pig and from calf uterus, respectively, prepared as fresh and lyophilized material. All gradients contained 0.4 M KCl. On the left in Fig. 3, cytosolic ER from frozen pig uterus, prelabeled for 1 h at 0°C (●), migrated as a 4S component with a 5S shoulder. When an aliquot of this prelabeled cytosol was then warmed to 26°C for 15 min (○), much of the 4S component was lost and replaced by a macromolecule, presumably 5S receptor in combination with some larger aggregates, sedimenting just ahead of BSA. When the experiment was repeated with reconstituted, lyophilized pig cytosol (Fig. 3, right), the transition from 4S (unheated prelabeled cytosol) to 4S (prelabeled, heated) was even clearer than it had been using unlyophilized material; similar data was obtained with calf uterus (data not shown). In Fig. 4, PgR in cytosol from frozen and lyophilized calf uterus tissue was also warmed following a 1 h prelabeling period at 0°C. Neither PgR from

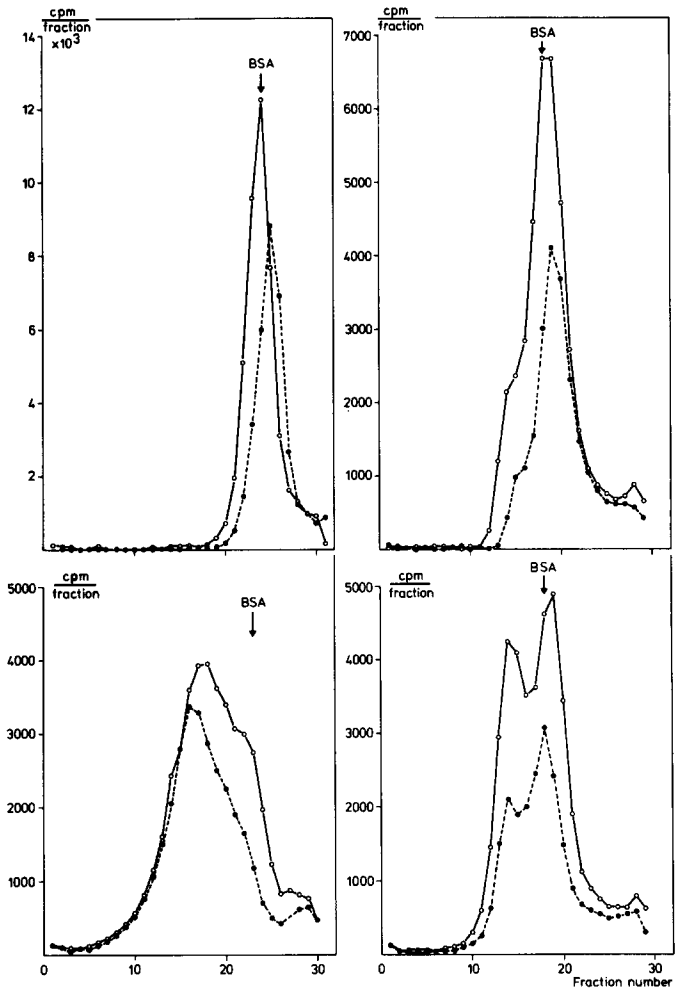

Fig. 2. Sedimentation patterns of progesterone receptors analyzed in cytosol from frozen (●——●) and lyophilized (○——○) calf uterine tissue: Response to salt and glycerol. Cytosols equilibrated with 10 nM (^{3}H]-promegestone for 1 h at 0°C were adsorbed with DCC and layered on 5–20% sucrose gradients in phosphate buffer with (left) and without (right) 10% glycerol, and with (upper) and without (lower) 0.4 M KCl. The samples were centrifuged and counted as described for Fig. 1.

frozen (left) nor lyophilized (right) material responded to warming (28°C, 15 min). Progesterone binding components from both preparations (frozen and lyophilized) migrated in high-salt gradients (in the presence of glycerol) as a discrete 4S band both before and after warming. The results from pig uterus were similar but data are not shown here.

Occasionally, the quantity of cytosol prepared from frozen human breast tumor specimens for routine, clinical ER/PgR analysis was sufficient to allow lyophilization of several aliquots. Analysis by SDG could then be used to estimate the effect of lyophilization on sedimentation patterns; the results from four such analyses are presented in Table 1 and Fig. 5. Numbers 1–3 in Table 1 represent results using pooled cytosols from 2–3 patients (cytosols mixed prior to lyophilization) whereas No. 4 consisted of cytosol from only 1 patient. Examining the ER data first, it is clear that in every case shown, lyophilization resulted in a detectable loss of 8S receptor, though of variable degree. In turn, the percentage of counts distributing as 4S receptor increased in all cases. Subsequent storage of lyophilized samples for up to 6 weeks indicated greater lability of the 4S than the 8S form of ER in lyophilized samples. The 8S receptor showed very little additional deterioration during storage; however, the 4S receptor, which appeared to increase its relative concentration immediately following lyophilization, returned to values closer those noted prior to lyophilization. It could be speculated that lyophilization induces some transient clumping of small molecular species, perhaps sub-fragments of the ER, which then break down relatively quickly with time. As with ER, PgR sedimenting in the 8S form also seemed labile to lyophilization; the values decreased in all three samples (Nos 1–3). The relative concentration of the 4S species was remarkably stable to lyophilization. The responses of both the 4S and the

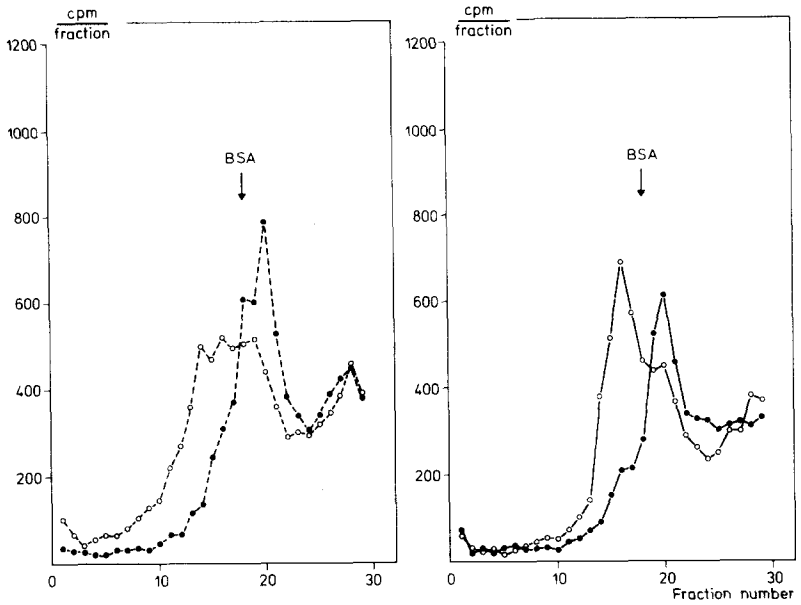

Fig. 3. Soluble estrogen receptor in pig uterus, analyzed in cytosol prepared from frozen tissue (– – –) and from reconstituted lyophilized cytosol (——): Effect of temperature. Reconstituted, lyophilized cytosol was prepared by initially mixing phosphate buffer (without monothioglycerol) with frozen, minced uterus. Following centrifugation, an aliquot was snap-frozen and lyophilized overnight. This was reconstituted by addition of 10 mM monothioglycerol. Cytosols were equilibrated with 10 nM [^{3}H]-estradiol at 0°C for 1 h; an aliquot was then transferred to a 26°C waterbath for 15 min. Samples of the heated (○) and unheated (●) cytosols were adsorbed with DCC, layered on 5–20% sucrose gradients in phosphate buffer with 0.4 M KCl and centrifuged.

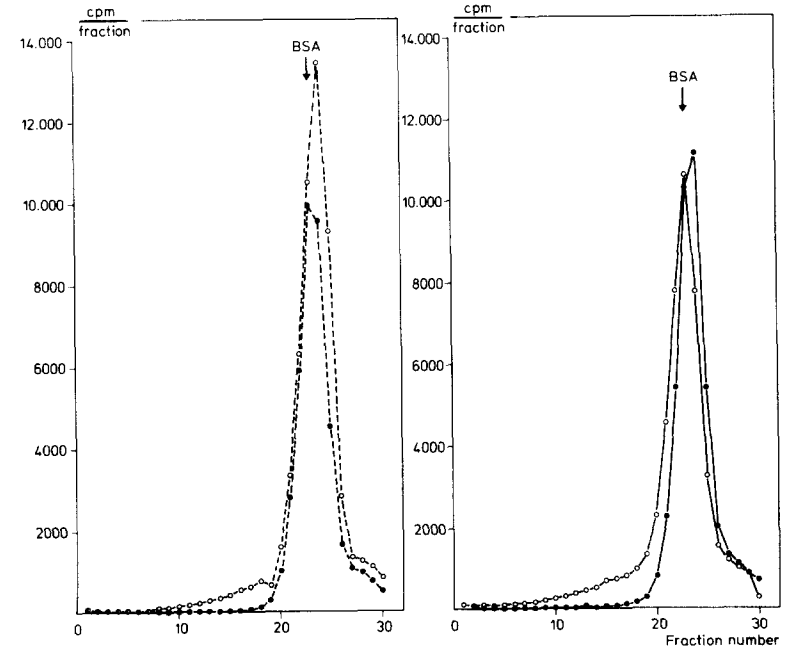

Fig. 4. Soluble progesterone receptor analyzed in cytosol prepared from frozen (– – –) and from lyophilized (——) calf uterine tissue: Effect of temperature. Cytosols were equilibrated with 10 nM [^{3}H]-promegestone at 0°C for 1 h. An aliquot was then transferred to a 28°C waterbath for 15 min. Samples of the heated (○) and unheated (●) cytosols were adsorbed with DCC, layered on 5–20% sucrose gradients in phosphate buffer with 0.4 M KCl and 10% glycerol, and centrifuged.

Table 1. Cytosols from human breast tumor: distribution of ER and PgR following lyophilization and subsequent storage

No.	Time after Lyophilization (d = days)	% ER 4S	% ER 8S	% PgR 4S	% PgR 8S
1	Pre	32.4	44.3	64.0	24.6
	Post*	50.7	30.2	67.9	15.1
2	Pre	38.2	41.5	56.8	30.5
	Post*	60.6	17.3	54.8	23.8
	5 d	45.1	20.8	52.0	24.5
3	Pre	36.2	48.5	47.4	46.2
	Post*	42.2	40.8	50.9	39.8
	15 d	34.1	50.3	35.6	57.8
4	Pre	—	—	—	—
	Post*	56.9	15.5	64.3	13.9
	17 d	47.8	11.2	62.8	7.9
	45 d	64.7	11.6	55.0	12.3

* Values determined within 48 h after lyophilization.

Cytosols from human breast cancer biopsies were prepared as described in Materials and Methods. For each experiment, cytosols from one to three patients were pooled, analyzed for ER and PgR distribution by SDGA, and then several aliquots were lyophilized overnight. Lyophilized samples were stored under vacuum at 4°C for the time period indicated, before reconstruction and SDGA, as described in Figs 1 and 3, respectively. Values for the 4S and 8S forms of both ER and PgR represent the area under the respective curves, as a percentage of total counts (% c.p.m.) on the gradient.

8S form of PgR to the varying storage intervals were inconclusive. Figure 5 shows the sucrose density gradient analyses from No. 1 in Table 1. The decrease in 8S species of both ER (left) and PgR (right) after lyophilization (●---●) is clear. While the 4S form of PgR appears smaller after lyophilization, the percentage of total counts sedimenting as 4S is unchanged.

DISCUSSION

Lyophilization is, at present, a convenient and frequently used technique for the preparation of reference samples for the quality-control of steroid receptor assays [11]. The stability of binding activity within lyophilized samples has been documented by both sedimentation analysis [16] and dextran-coated charcoal assay [15, 17]. In order to better characterize the biochemical responsiveness and sedimentation behavior of lyophilized material, we examined the effects of salt and of warming on lyophilized uterine tissue powder and lyophilized cytosol. Lyophilized samples, primarily from pig and calf, were compared to tissue powders prepared from the same material but stored in liquid N_2. Figures 1 and 2 demonstrate that there was little effect of lyophilization on the response of either ER or PgR to 0.4 M KCl during sedimentation analysis. ER sedimented as a 4S species in high-salt gradient, and as 8S in low-salt, as previously reported for both calf [20, 21] and pig [22]. The sedimentation behavior of PgR was also essentially unaltered by lyophilization; however, it was noted that the dependence of the 8S molecular form on glycerol addition described by Faber *et al.*[23] and by Philibert and Raynaud[24], was somewhat species dependent. In

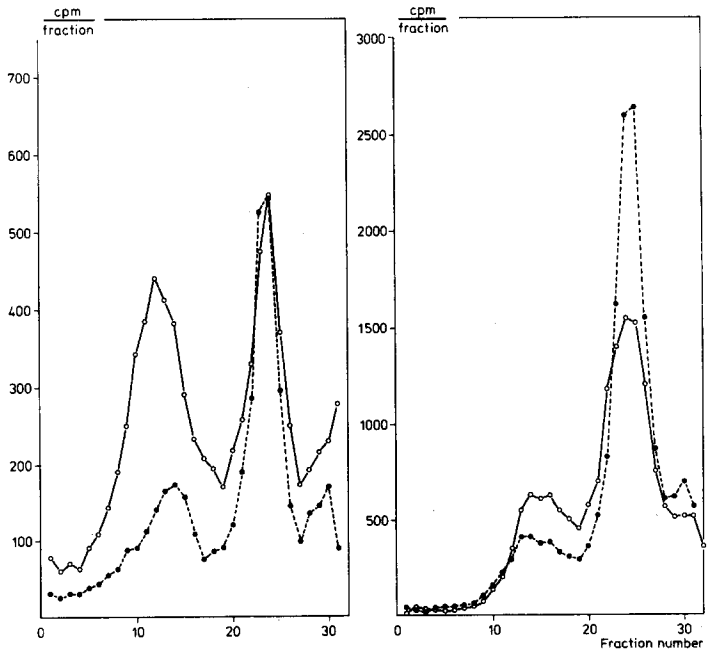

Fig. 5. Cytosols from human breast tumor tissue: Effect of lyophilization on the distribution of ER (left) and PgR (right) following SDGA. Cytosols were prepared from frozen biopsy material; aliquots were analyzed both before (○——○) and immediately following (●---●) lyophilization. Aliquots were equilibrated with 5 nM [^{3}H]-estradiol or [^{3}H]-promegestone for 4 h at 0°C, treated with DCC and centrifuged on 5–20% sucrose gradients without added glycerol or KCl.

pig, the 8S form of the receptor was only observed in the presence of glycerol; in calf tissue the 8S peak was clearly identifiable, albeit smaller, even without glycerol. This may simply reflect small, species-related variations in the intrinsic association constant of R 5020 (promegestone) for 8S receptor in uterine cytosol or in the activity of cytosolic proteases acting on the 8S form, as proposed for ER by several authors [20, 25]. Again, this phenomenon appeared unaffected by lyophilization.

Warming of uterine cytosols is known to elicit ER transformation from the 4S to the 5S [22, 26]. This transformation was observed equally in lyophilized and unlyophilized samples from both pig and calf. The relatively short time interval (15 min) used for cytosol warming generally allowed only the development of a 5S shoulder and a decrease in the 4S peak; however, cytosol both from frozen and lyophilized tissue proved occasionally unstable over longer periods of heating. This corresponds with the work of Buchi and Villee[27], who found that receptor activation, defined by nuclear uptake, began to decrease after 10 min at 25°C, and postulated that inactivation and receptor aggregation might be the cause. As expected [28], the sedimentation coefficient of PgR was unchanged by warming, regardless of preparation technique or the presence of glycerol in the sucrose gradients.

Although the majority of this experimental series was devoted to uterine tissue from pig and calf, a few experiments also considered human material. The 8S components of both ER and PgR from these human breast cytosols seemed to be sensitive to lyophilization. In the case of ER, the decrease in the 8S form was accompanied by an increase in 4S, suggesting a breakdown of 8S components into subcomponents sedimenting at or around 4S. In contrast, however, PgR demonstrated the same 8S lability but without any apparent sensitivity of the 4S species to lyophilization. Freedman and Hawkins[29] noted that the 8S form of ER predominates in low-salt sucrose density gradient analysis (of breast cytosols) but they and other groups [30] also reported the 8S as unstable over time. Our data also suggest that the 8S form of both ER and PgR in human breast tumor cytosol may be unstable to manipulation, at least in the form of lyophilization, a point of considerable interest to laboratories which assay the 8S form as an indicator of functional steroid receptor.

In conclusion we suggest that lyophilization not only increases the stability of steroid receptor binding activity over time, as previously reported, but also leaves basically unchanged the responses of ER and PgR to warming and manipulations of ionic strength, as defined by sedimentation analysis. In addition, it should be noted that throughout our study, both lyophilized tissue and cytosol were examined, in addition to frozen material. In no instance was a difference in the results from the two types of lyophilized material noted. Finally, lyophilized material from pig and calf examined in this study had been stored (at 4°C under vacuum) for up to 14 days after lyophilization; within this time interval, there appeared to be no loss of response to heat or salt. The data from human breast tumor did indicate that lyophilized samples of this tissue may be more labile and should be approached with caution.

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