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MEASUREMENT OF ENDOGENOUS SUBCELLULAR CONCENTRATION OF STEROIDS IN TISSUE

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Summary—A reliable method for the extraction of steroid hormones from human uterine tissue and the subsequent measurement of these hormones in the subcellular compartments by radioimmunoassay is described. Extraction of radioactive steroid hormones from *in vivo* labelled human uterine tissue by different methods reveals that an almost quantitative extraction of steroid hormones from the nuclear fraction is obtained by sonication in ethanol-acetone. Extraction of steroid hormones with diethylether from a high speed cytosol is incomplete. Using a more potent denaturating agent prior to extraction with diethyl ether leads to complete extraction of unconjugated steroids.

INTRODUCTION

As steroid hormones exert their biological activity within the cells of target tissues, it is of interest to know their intracellular concentration, their subcellular distribution and their nuclear concentration to understand the (patho)physiological state of a tissue.

There is evidence that the nuclear concentrations of oestrogens in endometrium, myometrium and vagina are higher than in the cytosol [1, 2]. Moreover, in the same patients, differences were found in the concentration of oestradiol between these target tissues. Therefore, plasma concentrations of oestrogens do not reflect concentrations in tissues. In addition, target tissues are able to metabolize steroid hormones taken up from the circulation. Human mammary cancer tissue is an example of a tissue showing this so-called paraendocrine behaviour [3, 4], which implies that a tissue is able to metabolize physiologically active precursors to inactive metabolites and *vice versa*. These considerations lead to the conclusion that the measurement of steroid hormone concentrations at the cellular level might be an approach to gain further insight into the biological effects of these hormones. This approach requests a reliable and sensitive method to measure endogenous hormone levels in subcellular fractions of tissues.

In this paper we describe such a method, using human uterine tissues labelled *in vivo* by a continuous infusion of tracer amounts of radioactive steroids.

MATERIALS AND METHODS

Chemicals

[6,7-^{3}H]Oestrone (E_1) (sp. act. 44 Ci/mmol) and [2,4,6,7-^{3}H]oestradiol (E_2) (sp. act. 108 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, U.K. Their purity was checked bimonthly by TLC.

Non-labelled steroids were purchased from Makor Chemicals, Jerusalem, Israel. Suc *d'Helix Pomatia* (β-glucuronidase-sulphatase) was obtained from Industrie Biologique Francaise, Clichy, France.

All other reagents were of Analar quality from BDH Chemicals Ltd., Poole, Dorset, U.K. Organic solvents were distilled twice before use.

In vivo *administration of labelled steroids*

[^{3}H]E_1 or [^{3}H]E_2 was infused at a constant rate for 10–12 h before operation in women who were scheduled for hysterectomy. These patients participated in a research project on the uptake, metabolism and subcellular distribution of oestrogens in target tissues [1, 2]. Immediately after extirpation, the uterus was chilled on ice and transported from the operation theatre to the pathologist. Parts of the histologically normal myometrium, endometrium and vaginal tissue were stored at −70°C until analysis.

Preparation of subcellular fractions

The frozen tissue was chilled in liquid nitrogen and subsequently pulverized with a Micro-Dismembrator (Braun, Melsungen, W. Germany). All subsequent handlings were performed at 0–4°C, unless stated otherwise. After pulverization the powder obtained was homogenized in 4 ml of Tris-buffer (0.01 M Tris–HCl, 0.001 M EDTA, 0.003 M NaN_3, pH 7.4) by intermittent vortexing. A high speed cytosol and a crude nuclear fraction were obtained by centrifugation in a Beckman ultracentrifuge (type L5-65, rotor SW 50.1) at 4°C for 30 min at 100,000 *g*.

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Liquid scintillation counting

Liquid scintillation counting was done in a Packard 2650 TRICARB, using 10 ml of Emulsifier Scintillation-299 from Packard Becker BV Chemical Operations, Holland. To measure the total activity per gram tissue, 0.2 g tissue was digested in 3 ml of Protosol (New England Nuclear Corporation, Boston, U.S.A.) for 18 h at 50°C. After addition of 10 ml of scintillation solution and 0.5 ml of acetic acid, the radioactivity was counted. To measure the efficiency of the extraction of steroids, the extracted nuclear pellet was thoroughly dried and subsequently digested as described for whole tissue.

Extraction of radioactivity from in vivo *labelled tissue*
Cytosol: extraction with ether

To high speed cytosols, 3 vol of freshly distilled diethyl ether were added and the ^{3}H-activity was extracted by vortexing. The cytosol was extracted 3 times, the ether fractions were combined in a counting vial, the ether was evaporated and the radioactivity was counted.

Cytosol: extraction with ethanol–acetone

One volume of a high speed cytosol was added dropwise to 4 vol of ethanol–acetone (1:1, v/v), under gentle vortexing. the denatured proteins were spun down and the pellet was washed once with 2 vol of ethanol–acetone. The supernatants were combined, the ethanol–acetone was evaporated and the radioactivity counted.

In the final extraction procedure the residue of the evaporated supernatants was redissolved in 1 ml of Tris-buffer after addition of 25 μl of ethanol (99%). Subsequently the ^{3}H-activity was extracted 3 times with 3 vol of freshly distilled ether.

Nuclear fraction

To isolate the ^{3}H-activity from the nuclear fraction, the crude nuclear pellets were sonicated in 4 ml of Tris-buffer (method I), in 5 ml of ethanol (99%) (method II) or in 5 ml of ethanol–acetone (1:1, v/v) (method III). The suspension in Tris-buffer was centrifuged for 30 min at 100,000 ***g*** and the supernatant was extracted 3 times with 3 vol of freshly distilled ether. The two other suspensions were centrifuged for 10 min at 3.000 ***g*** and the pellets were washed once with 5 ml of ethanol (99%) or ethanol–acetone (1:1, v/v), respectively. The supernatants were combined, evaporated and counted. In the extracted nuclei the residual radioactivity was measured.

Chromatography of extracted radioactivity

In vivo labelled human endometrium was processed as described above. The high speed cytosol was divided in two equal parts (a and b). From both parts radioactivity was extracted with ethanol–acetone (1:1, v/v) as described. From cytosol b the extracted radioactivity was redissolved in 3 ml of distilled water plus 0.3 ml of 0.1 M acetate-buffer, pH 5.0. Hydrolysis of conjugates was done for 48 h at 37°C after addition of β-glucuronidase and sulphatase (500 and 4,000 U per ml, respectively). Subsequently the radioactivity was extracted with freshly distilled diethyl ether and the ether was evaporated. An aliquot of the extracted hydrolysed mixture was counted. To both residues (a and b) 40 μg unlabelled E_2 was added as a marker and the residues were applied to TLC plates (Silicagel 60F-254, Merck, Darmstadt, W. Germany), with the system chloroform–ethanol (9:1, v/v). Subsequently zones of 1 cm were scraped off, put into counting vials and radioactivity was counted.

RESULTS AND DISCUSSION

We have studied the steroid hormone concentration in two subcellular compartments of the cell, the high speed cytosol and the crude nuclear fraction. The high speed cytosol contained no intracellular particles but may have contained enzymes and other proteins from intracellular organelles which might have been lysed by the freezing-thawing procedure or during cell fractionation. The so-called nuclear fraction would also have contained some subcellular particles, fragments of ruptured cells and other cell debris. A problem inherent to this method is the possibility that steroids migrate from one cell compartment to another during the preparation of the cell fractions. We have considered including the estimation of marker enzymes to monitor damage of subcellular particles. However we feel that the distribution of these enzymes does not necessarily reflect the migration of steroid hormones. As far as we know, there are no adequate means to check this migration. Therefore these fractions prepared *in vitro* probably do not represent entirely the intracellular distribution *in vivo*. We have taken all precautions to reduce this possible migration: direct cooling of tissue and homogenization at low temperature after cooling in liquid nitrogen.

To study tissue hormone concentrations it is necessary to use a procedure that enables quantitative extraction of all endogenous steroids present in the tissue. The availability of *in vivo* labelled tissues stimulated us to compare several extraction methods. Table 1 summarizes the data concerning the concentration of radioactivity of *in vivo* labelled human tissue, the distribution over the subcellular fractions and the percentage of activity which could be extracted with the different methods used. It appeared that the total radioactivity per g of tissue is not homogenously distributed over the tissue and consequently we did not know exactly for each individual sample the amount of radioactivity processed.

Extraction of radioactivity from cytosol with freshly distilled ether is far from complete; about 35–50% of radioactivity remains in the cytosol even after repe-

Table 1. Concentration, distribution and extraction of ^{3}H-activity *in vivo* labelled human uterine tissue

	CYTOSOL					NUCLEAR FRACTION				
		Extracted with:					Extracted			Total recovery
	Radioactivity %	Ether %	%	Ethanol–acetone %	%	Radioactivity %	%	%	Method	%
E_1	86	47	(54)			25	9	(35)	I	111
	59	30				21	7	(35)		80
	89	n.d.				55	19	(79)	II	114
	63	n.d.				22	18	(83)		85
E_2	44	24	(56)			44	25	(57)	I	88
	44	28	(64)			40	22	(55)		84
	49			48	(99)	50	41	(83)	II	99
	48			47	(98)	46	41	(89)		94
	44	28	(64)			53	50	(95)	III	97
	44	28	(62)			57	56	(98)		101
	22	n.d.				76	75	(98)	III	98
	30	n.d.				83	79	(95)	III	113
	58	n.d.				81	78	(96)	III	139
	26	n.d.				75	69	(92)	III	101

( ) The radioactivity is expressed as a percentage of the total radioactivity in the cytosol or in the nuclear fraction. For methods I, II and III see Materials and Methods section.

ated extractions (Table 1). Ether-extraction of steroids added to plasma yields much higher recoveries. After addition of [^{3}H]E_1, [^{3}H]E_2, [^{3}H]DHEA (dehydroepiandrosterone) and [^{3}H]Adiol (5-androstene-3β,17β-diol) to myometrial or breast tumour cytosol, 80–100% of the added steroids can be extracted with ether. With ethanol–acetone (1:1, v/v) nearly 100% of the activity in the cytosol could

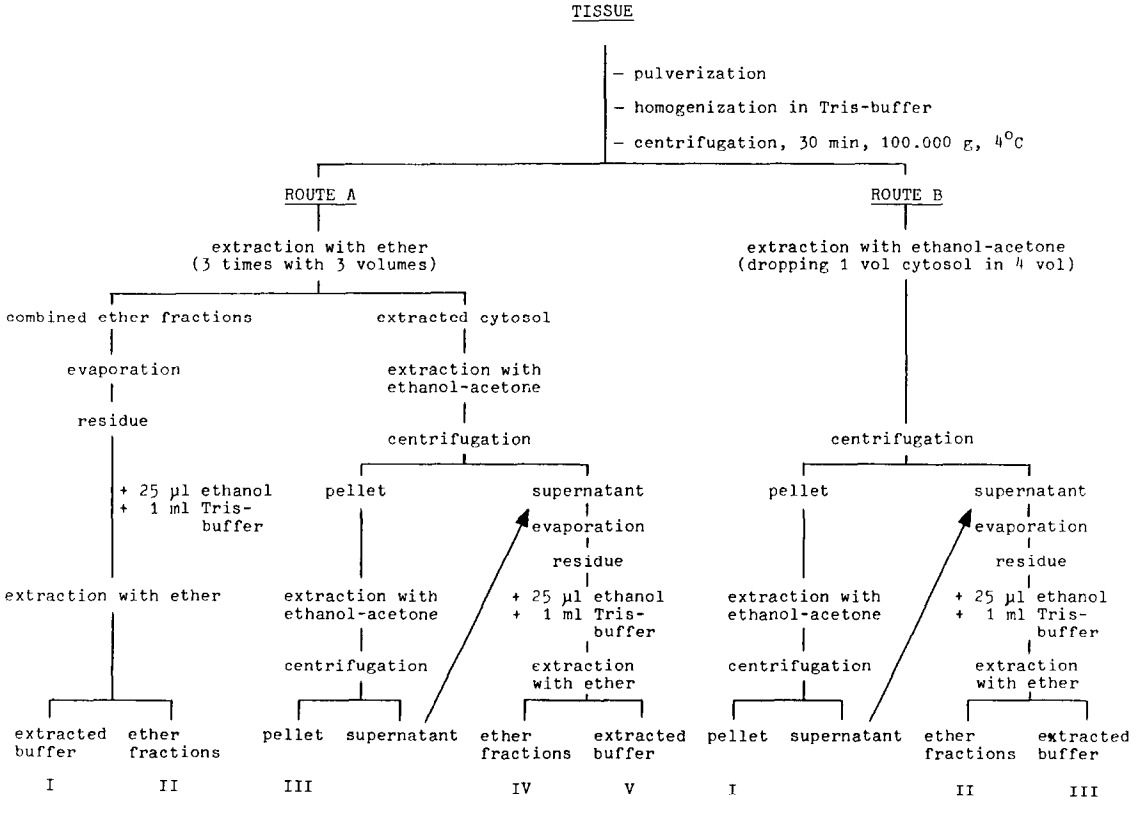

Fig. 1. Procedure to test several methods for the extraction of radioactivity from *in vivo* labelled human myometrium.

Table 2. Results of the extraction of radioactivity from cytosol from *in vivo* labelled human myometrium

	Fractions	Sample 1 Recovery%	Sample 2 Recovery%	Sample 3 Recovery%
	I extracted buffer	3	1	1
	II combined ether fraction	31	35	34
Route A	III pellet	7	6	10
	IV combined ether fraction	25	27	27
	V extracted buffer	33	31	28
	I pellet	4	5	5
Route B	II combined ether fraction	63	62	67
	III extracted buffer	33	33	27

be extracted (data not shown). The question arises why so much of the *in vivo* infused radioactivity could not be extracted with ether. It is possible that during *in vivo* labelling the infused tracer is metabolized to more polar compounds or to conjugates, sulphates or glucuronidates, which resist ether-extraction.

Therefore, we estimated the extractability of radioactivity with ether from *in vivo* labelled human myometrium without (route A) and with (route B) prior precipitation in ethanol–acetone (1:1, v/v) (Fig. 1). The results of this experiment are summarized in Table 2. With ether 31–35% of the radioactivity could be extracted directly. An additional 25–27% became ether-extractable after treatment of the ether extracted cytosol with ethanol–acetone (route A2; IV). Thus the total percentage of activity which could be extracted after pretreatment is 62–67% (route B; II). Tissues were processed as shown in Fig. 1, fraction numbers being the same as in that figure. Extraction was done in triplicate. Results are given as percentages of total radioactivity in the cytosol.

This experiment supports the supposition that extraction of endogenous steroid hormones from human tissue with ether alone, as has been performed by several investigators [5–8] is not quantitative. Obviously ethanol–acetone is a much more potent denaturating agent than ether and also liberates steroid hormones which are tightly bound to high affinity proteins such as receptors. Still with these extraction routes 27–33% of the activity stayed in the cytosol and appeared to be not ether-extractable. To investigate the nature of this radioactivity, all activity

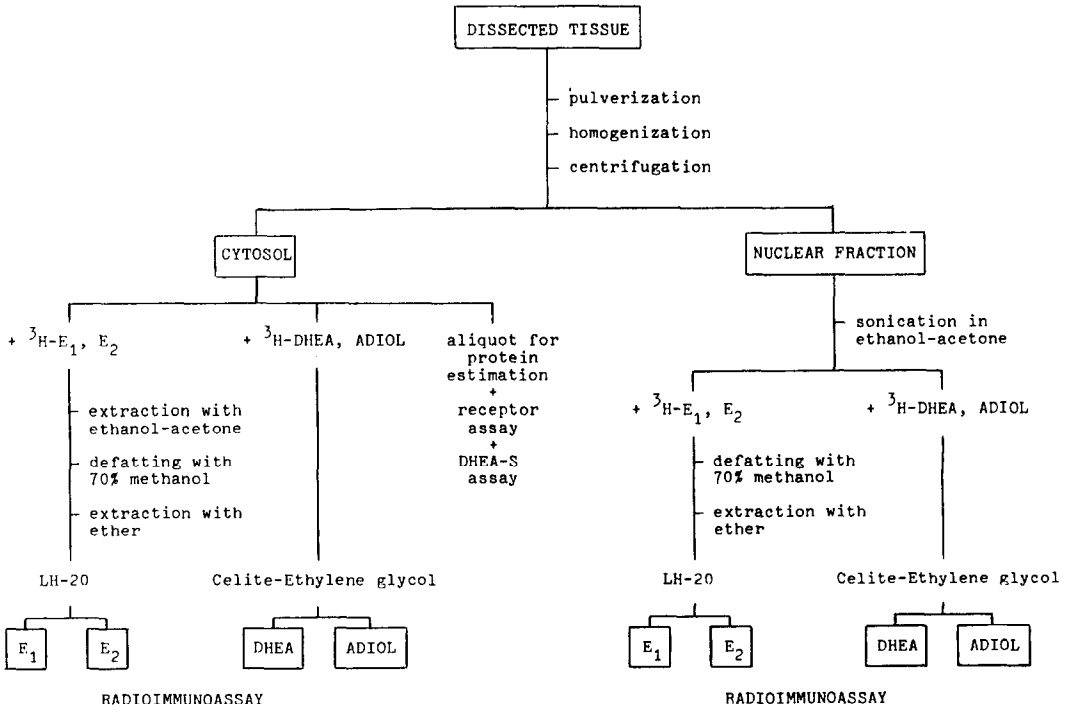

Fig. 2. Procedure for the extraction of steroid hormones from human tissue and their quantitation by specific radioimmunoassays.

Table 3. Reliability criteria of the radioimmunoassays

	E_1			E_2			DHEA			Adiol			DHEA-S		
	conc. fmol*/2ml	C.V. %	*n*	conc. fmol*/2ml	C.V. %	*n*	conc. fmol*/2ml	C.V. %	*n*	conc. fmol*/2ml	C.V. %	*n*	conc. pmol/ml	C.V. %	*n*
Intra-assay precision	189	5.4	5	210	3.4	5	6428	3.7	5	912	3.2	4			
	659	3.4	5	790	12.5	5								14.5	5(5)
		14.3	20(1)		18.0	20(1)		12.7	20(3)		13.9	20(3)		6.7	20(6)
		5.2	20(2)		5.7	20(2)		5.4	20(4)		5.2	20(4)		9.6	20(7)
Inter-assay precision	248	19.9	6	232	9.9	6	6893	7.6	5	1476	8.8	4	11076	7.2	4
	674	5.1	6	827	5.9	6	12238	6.3	4	1838	10.2	4	2492	7.4	3
Sensitivity†	7.4 ± 1.33 fmol			7.0 ± 1.07 fmol			30 ± 11 fmol			27 ± 9 fmol			0.59 ± 0.15 pmol		
	n = 5			*n* = 5			*n* = 14			*n* = 8			*n* = 4		

*2 ml of pooled high speed cytosol were extracted.

†Sensitivity was calculated from twice the SD of the initial binding.

(1)–(7) Intra-assay precision calculated from measurements in duplicate of one extract of cytosol (E_1, E_2, DHEA and Adiol) or one cytosol dilution (DHEA-S) read from the standard curve between 0–37 fmol/ml (1), 37–185 fmol/ml (2), 0–100 fmol/ml (3), 100–1000 fmol/ml (4), 0–2.6 pmol/ml (5), 2.6–26 pmol/ml (6) and 26–130 pmol/ml (7).

extracted with ethanol–acetone was chromatographed on thin layer plates before and after incubation with the enzymes β-glucuronidase and sulphatase. After incubation, all activity could be extracted with ether and chromatography revealed that all activity had a chromatographic mobility similar to E_2. No activity stayed on the origin. Before treatment with β-glucuronidase and sulphatase about 30% of the applied radioactivity appeared to have a polarity too high to run from the origin. This percentage is comparable to the non ether-extractable radioactivity mentioned before. It is unlikely that this conjugated E_2 (30% of total tissue activity) is formed during the incubation, because all manipulations are done at 0–4°C over a short period of time. It is reasonable to assume that the conjugates are formed *in vivo* after the infusion of [^{3}H]E_2.

Extraction of radioactivity from the nuclear fraction by sonication in Tris-buffer (method I) is a very incomplete method, as appears from Table 1. Extraction by sonication in absolute ethanol (method II) and especially in a mixture of ethanol–acetone (1:1, v/v) (method III), gives much better results. With the last method the extraction is almost complete: 96% ± 4 (SD) of total nuclear activity is obtained (Table 1).

From these experiments the following conclusions can be drawn: (1) An almost quantitative extraction of steroid hormones from the high speed nuclear fraction is obtained with ethanol–acetone. Over 95% of the activity from the *in vivo* labelled nuclear fraction could be extracted. (2) Extraction of steroid hormones from high speed cytosol of human uteri with ether is incomplete and may give rise to an underestimation of steroid tissue concentrations even when [^{3}H]steroids are added to calculate the recovery. Using a more potent denaturating agent prior to extraction with ether leads to complete extraction of unconjugated steroids. Therefore, denaturation in ethanol–acetone followed by extraction with ether is preferable.

Measurement of endogenous steroids

Based on the data described we designed a procedure to measure both oestrogens and androgens in normal and neoplastic human breast tissue. An outline of this procedure is given in Fig. 2. As the nuclear pellet cannot be divided exactly into two parts and the extraction of steroid hormones with ethanol–acetone is practically complete, ^{3}H-labelled steroids were added to the extract.

The extraction of steroids from cytosols and nuclear fractions of human breast tissue results in residues which were contaminated with fat. Therefore, the residues were redissolved in 3 ml of 70% methanol in water and stored at −20°C overnight. After centrifugation in the cold, the supernatant was evaporated and the residue was redissolved in Tris-buffer to be extracted subsequently with ether.

After extraction oestrogens were chromatographed on LH-20 columns (eluted with toluene–methanol) and androgens on Celite–ethylene glycol (eluted with ethyl acetate in iso-octane). Quantitation of the individual steroids was done in duplicate by radioimmunoassay, using highly specific antisera, as described earlier [4].

DHEA-S was estimated directly in the cytosol, using a kit from Radioassay Systems Laboratories, Inc., Carson, California. The reliability criteria of the radioimmunoassays are summarized in Table 3.

With this method, summarized in Fig. 2, we have investigated the subcellular distribution of oestrogens and androgens in normal and neoplastic human breast tissue. Results from that investigation will be reported separately.

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