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A PRELIMINARY REPORT ON THE USE OF IMMUNOPEROXIDASES TO STUDY BINDING OF ESTROGENS IN RAT UTERI

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SUMMARY

This work presents the results of a preliminary study using immunoperoxidases in light and electron microscopy for the detection of estrogen binding sites in rat uteri with estradiol and a synthetic estrogen (R 2858), highly specific to estrogen receptors. The presence of estradiol (or of R 2858) was detected 90 min. after injection in the endoplasmic reticulum, in the nuclear envelope and in chromatin. Four hours after injection nucleolar labeling was noted. R 2858 was particularly useful in detecting specific binding since its use greatly reduced background binding to non-specific proteins. Furthermore the validity of the immunological reaction was confirmed by numerous control experiments. In all our experiments, nuclear labeling was always observed even at 4°C, cytoplasmic labeling at 37°C was also observed but far less marked. Although these initial results are extremely encouraging, the technical constraints imposed (in order to preserve the estrogen receptors) lead to certain cytological alterations which necessitate prudence in interpretation.

INTRODUCTION

The use of immunofluorescence techniques [1] to detect estradiol (E2) binding sites in target tissues may lead to new knowledge of fundamental importance to the understanding of steroid hormone action. These techniques, which can be performed more rapidly than autohistoradiography, are highly interesting because of their relative simplicity as compared to biochemical methods and their possible application to very small biologic samples. Above all it is hoped that steroid binding sites will be localized at the infracellular level by using immunoperoxidases and electron microscopy. However, the use of these techniques raises questions concerning the specificity of the observed phenomenon and its relationship to the receptor-specific system as evidenced by well-established biochemical studies [2]. This paper attempts to answer these questions.

MATERIALS AND METHODS

Reagents. The following were used: TC 199 was from Institut Pasteur; Saline phosphate buffer 0.01 M: phosphate buffer pH 7.45, 0.15 M NaCl (9%) (PBS); Protamine sulfate was from Choay laboratory, 0.1% in saline phosphate buffer; Diaminobenzidine was from Sigma, 0.02% in trimaleate buffer (DAB); 0.05 M trimaleate buffer pH 7.6 (TMB); 2% osmium acid in 0.1 M phosphate buffer pH 7.4; Epon Epikote 812; PLP fixative (paraformaldehyde, lysine and potassium periodate) was from Merck. Concentration was 4% PLP in 0.037 M phosphate buffer, pH 6.9; Anti E2 serum and anti R 2858 serum was from Roussel-Uclaf; normal porc serum, anti rabbit-Ig serum and PAP were from Sebia; Fluoresceine labeled anti-rabbit immunoglobulins were from Sebia; Inhibition of peroxidases: pure methanol and crystallized acetic acid were from Prolabo, Sodium nitroferricyanide was from Sigma; Estradiol and R 2858 were from Roussel-Uclaf.

Equipment. Cryostat: International equipment Rua; Zeiss photomicroscope; Siemens Elmiskop 101 electron microscope.

Animals. Female Sprague–Dawley rats were from Iffa Credo. Feeding was supplied by Sourisserat. Castration was performed by bilateral oophorectomy with tubal ligation. The rats were sacrificed by dislocation. Gonads were examined histologically. Plasma E2 levels were measured in order to verify the absence of E2 in the plasma of oophorectomized rats [3].

Table 1. Experimental samples

Liquid nitrogen:	A1	B1	C1
4°C:	A2	B2	C2
37°C:	A3	B3	C3
		Incubation in ABE_2	

Groups D and E (intraperitoneal injection):	
D incubated with ABE_2:	D
D incubated with AB 2858:	DT
E incubated with AB 2858:	E
E incubated with ABE_2:	ET
E incubated with AB 2858, immunofluorescence technique:	EIF

Table 2. Immunocytochemical procedure

I. IMMUNOPEROXIDASE

A. FROZEN SECTION—Microtome—dt 30°C picked up on prepolymerized epon slides

B. INCUBATION AT 4°C

1a. *Untreated Aliquot* → 2.

1b. *Protamine sulfate* (0.1% 3 min) one aliquot
↓ Wash PBS (5 × 1 min at 4°C) (all washes are performed at 4°C)

2. *Normal pig serum* (1/20 PBS 10 min)
↓ Wash PBS (3 × 10 min)
3. *Specific anti serum* (1/100 in PBS 1 h)
↓ Wash PBS (3 × 10 min)
4. *Fixation PLP* (1 h 4°C)
↓ Wash PBS (3 × 10 min)
5. *Inhibition of endogenous peroxidase* (20 min at **18°C**)
↓ Wash PBS (3 × 10 min)

C. IMMUNOPEROXIDASE STAINING

Anti Rabbit serum Ig 1/20 in PBS (30 min) 4°C
↓ Wash PBS (4 × 15 min) 4°C
PAP 1/20 in PBS 20 min 4°C
Wash PBS (2 × 10 min) 4°C
↓ Wash in **TMB** (Buffer used for DAB) 2 × 10 min at **18°C**
DAB (in TMB) 20 min at **18°C**
DAB + H_2O_2 (in TMB) 0.01% 5 min at 18°C
Wash in TMB (3 × 10 min) 4°C
↓ Wash P.B. (pH 7.4 0.1 M) 2 × 5 min 4°C

D. ELECTRON MICROSCOPY PREPARATION

Osmium acid 2% in P.B. 20 min 4°C
Alcohol deshydration
Epon embedding
Polymerization ⟶ ultrathin section

II. IMMUNOFLUORESCENCE

A. FROZEN SECTION

Picked on gelatine coated slides

B. INCUBATION at 4°C

idem A until step 4
4. Fixation PLP 1 h 4°C
Wash PBS (3 × 10 min)

C. IMMUNOFLUORESCEINE STAINING

Anti Ig Rabbit serum (fluoresceine labelled)
Wash PBS (4 × 10 min)
Counter staining (Toluidine blue)
Glycerin mounting

Immunocytochemical study. The first study was performed on a series of six female rats: two prepubertal rats aged 18 days (A), two pubertal rats aged 30 days (B) at dioestrus stage and two pubertal rats aged 30 days oophorectomized 8 days prior to removal of the uterus (C). Histologic examination was performed on the ovaries of all animals. In the prepubertal group follicular growth was observed and poorly developed but functional interstitial cells were seen. The animals were sacrificed and the uteri removed at 4°C and submitted to the following treatment. One part of each uterus was immediately frozen in liquid nitrogen: (AI,

BI, CI). Another part was cut into small pieces and incubated from 1 h at 4°C with 10^{-7} M E2 in Saline phosphate buffer or in TC 199, rinsed in the same buffer and then frozen in liquid nitrogen (A2, B2, C2). The remainder was similarly treated, but before being frozen in liquid nitrogen was incubated in TC 199 for 4 h at 37°C (A3, B3, C3).

In the second study two sets of two pubertal rats were oophorectomized and adrenalectomized 8 days prior to receiving either 1 μg E2 or 0.1 μg of R 2858 by intraperitoneal injection [4–7]. The uteri were removed 90 min. after the injection and were frozen in liquid nitrogen (D and E respectively), 5 μ thick frozen sections at −30°C were made from the different samples, picked up on polymerized Epon slides, and treated by the "PAP" method of Sternberger[8] which was slightly modified to localize the E2 or R 2858 cell receptor sites.

Sections were incubated with specific antiserum. (Prior to incubation one control aliquot was incubated for 3 min in 0.1% protamine sulfate). The specific antiserum was either 1% anti E2 (ABE2) in saline phosphate buffer or 1% anti R 2858 (AB 2858) in saline phosphate buffer.

After incubation in specific antiserum, the sections were fixed for 1 h in paraformaldehyde-lysine-periodate (PLP) according to the technique of McLean and Nakane [9]. PLP fixation was followed by inhibition of endogenous peroxidase as described by Straus [10]. Peroxidases were developed using diaminobenzidine in H202, followed by superfixation of sections in osmium acid. Sections were deshydrated in alcohol and Epon embedded.

In some experiments (group E uteri) immunofluorescence was performed according to the technique of Nenci[1].

The samples obtained have been listed in Table 1 and the immunocytochemical technique in Table 2.

The following immunologic controls were performed: 1. incubation with specific antiserum was omitted. 2. specific antiserum was replaced by nonimmune rabbit serum. 3. specific antiserum was replaced by antihemoglobin immunoglobulins, 4. incubation with antirabbit-Ig porcine serum was omitted, 5. incubation in PAP was omitted, 6. the antiserum was charged with the corresponding estrogen added at 10^{-6} M, 4 h prior to incubation, 7. the immunocytochemical technique was performed on non-target tissue (rat striated muscle tissue), 8. the validity of the diaminobenzidine reaction was confirmed by incubation some samples in medium devoid of H_2O_2, 9. sections of uterus from a castrated female rat were incubated in AB 2858. Samples were analyzed by light microscopy. Microphotographs were obtained from epon-embedded sections Electron micrographs were obtained from samples A, C, D, DT, E and ET.

Measurement of nuclear E2 binding sites. Nuclear E2 receptors were measured in purified nuclei from the uteri of 3 prepubertal rats aged 18 days according to the technique described in (11–14).

RESULTS

Immunocytochemistry

Light microscopy with immunoperoxidases (Figs 1 to 8). The results obtained from the first series of rat uteri are summarized in Table 3. When present, nuclear labeling was well evident whereas no cytoplasmic labeling distinguishable from background staining was observed. Table 4 summarizes results from the second series of animals. Both E2 and R 2858 were recognized only by their corresponding antiserum. Once again, no clear cytoplasmic labeling was seen. Controls (Table 3) were negative (Figs. 7 and 8) except for the presence of variable but generally weak background labeling and the presence of positive nuclear labeling in control no. 6, which was particularly pronounced with test sample A1 (Fig. 6).

Immunofluorescence. The EIF sample did not display marked homogeneous fluorescence in the cytoplasm of myometrial cells as described by Nenci[1]. We only observed a very fine network of cytoplasmic

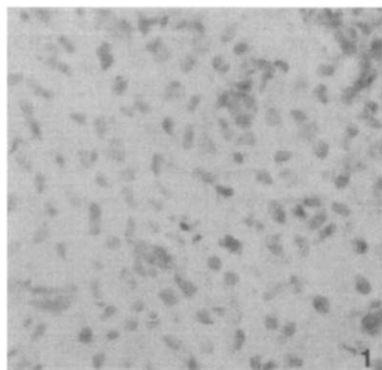


Fig. 1. Nuclear labeling in myometrium from oophorectomized and adrenalectomized rat having received intraperitoneal E2. Sections incubated with ABE2 (×400).

Fig. 2. Absence of nuclear labeling in myometrium of oophorectomized and adrenalectomized rat having received intraperitoneal E2. Sections incubated with AB2858 (×200).

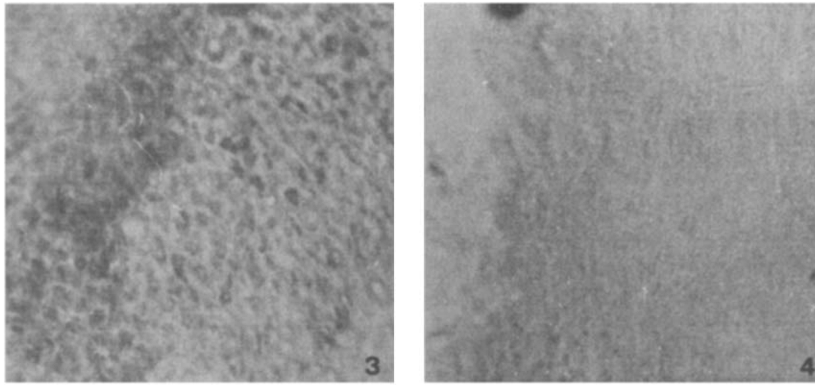


Fig. 3. Nuclear labeling in myometrium of oophorectomized and adrenalectomized rat having received intraperitoneal R-2858. Sections incubated with AB2858 (×310).

Fig. 4. Absence of nuclear labeling in myometrium of oophorectomized and adrenalectomized rat having received intraperitoneal R-2858. Sections incubated with ABE2 (×210).

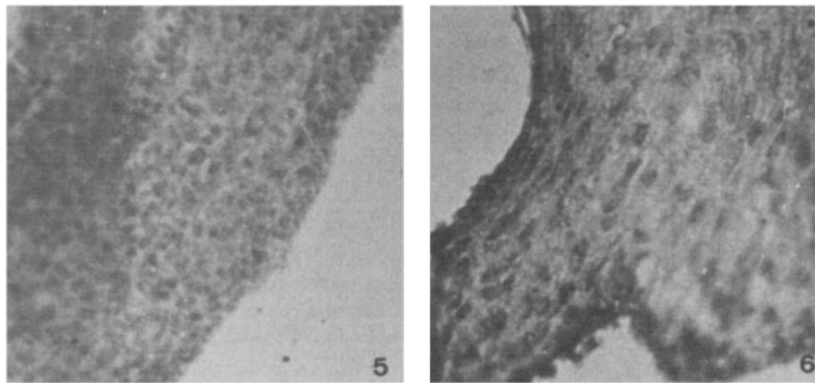


Fig. 5. Nuclear labeling in pubertal rat myometrium. Pieces of uterus were incubated with E2 at 4°C. Sections incubated with ABE2 (×280).

Fig. 6. Nuclear labeling in prepubertal rat myometrium. Sections incubated in 1% ABE2 + 10^{-6} M E2 (×400).

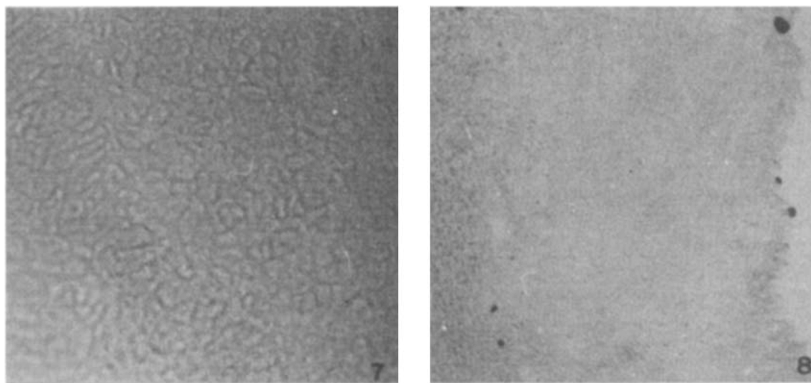


Fig. 7. Prepubertal rat uterus incubated with E2 at 37°C. Absence of labeling after incubating sections with normal rabbit serum (×370).

Fig. 8. Uterus from oophorectomized rat. Sections incubated with AB2858. Labeling is absent (×150).

Table 3. Results of the first series: light microscopy with immunoperoxidases

			Without exogenous estradiol		Exogenous estradiol 4°C		Exogenous estradiol 37°C	
Endogenous estradiol	Uteri A (Prepubertal)	Nucleus	∓	Sample A1	+	Sample A2	+	Sample A3
		Cytoplasm	−		∓		∓	
	Uteri B Pubertal	Nucleus	+	Sample B1	+	Sample B2	+	Sample B3
		Cytoplasm	∓		∓		∓	
	Uteri C (Oophorectomized)	Nucleus	−	Sample C1	+	Sample C2	+	Sample C3
		Cytoplasm	−		∓		∓	

Peroxidase staining was graded as follows: − no staining, ∓ slight staining not easily distinguished from background staining, + well-defined staining. All samples were examined blind by four different experimentators.

fluorescence at the limit of light microscopic resolving power, thus rendering both observation difficult and microphotography very uncertain. The nuclei were weakly and non-homogeneously labeled (Fig. 9).

Electron microscopy (Figs. 10 to 15). The following observations were made on tissue conservation: more or less satisfactory preservation of nuclear chromatin and nucleolar structures, more or less marked lysis of the nuclear envelope and endoplasmic reticulum alterations in mitochondria, and fairly pronounced lysis of myofibrils.

As regards labeling with peroxidases, in the nucleus labeling appeared grain-like and dispersed on the chromatin. This aspect was particularly evident in samples D and E (Fig. 12) where nucleolar labeling was not detected. On the other hand, in sample A3 incubated at 37°C for 4 h nucleolar labeling was rather frequent whereas chromatine labeling was much weaker (Figs. 10 and 11). Labeling was also observed in the intact nuclear envelope and endoplasmic reticulum. This labeling was not the result of endogenous peroxidases since effective inhibition of the latter was confirmed in the controls using electron microscopy. Other immunologic controls demonstrated that binding of the peroxidases was related to the specific antibody. Protamine sulfate was used at 4°C to precipitate, possibly stabilize, E2 receptor as in *in vitro* receptor assays [15, 16]. Nor clear-cut difference was noted in these compared whatever the concentration and timing used.

Measurement of free E2 binding sites. Measurement of free E2 binding sites in isolated nuclei from the uteri of three prepubertal rats revealed a concentration of 870 ± 110 fmol/mg nuclear proteins, *i.e.* 4.3 ± 0.5 fmol/mg tissue [13, 14].

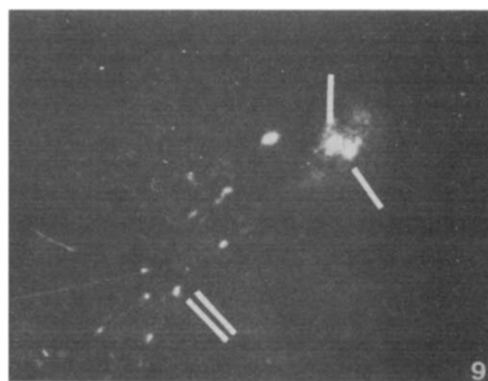
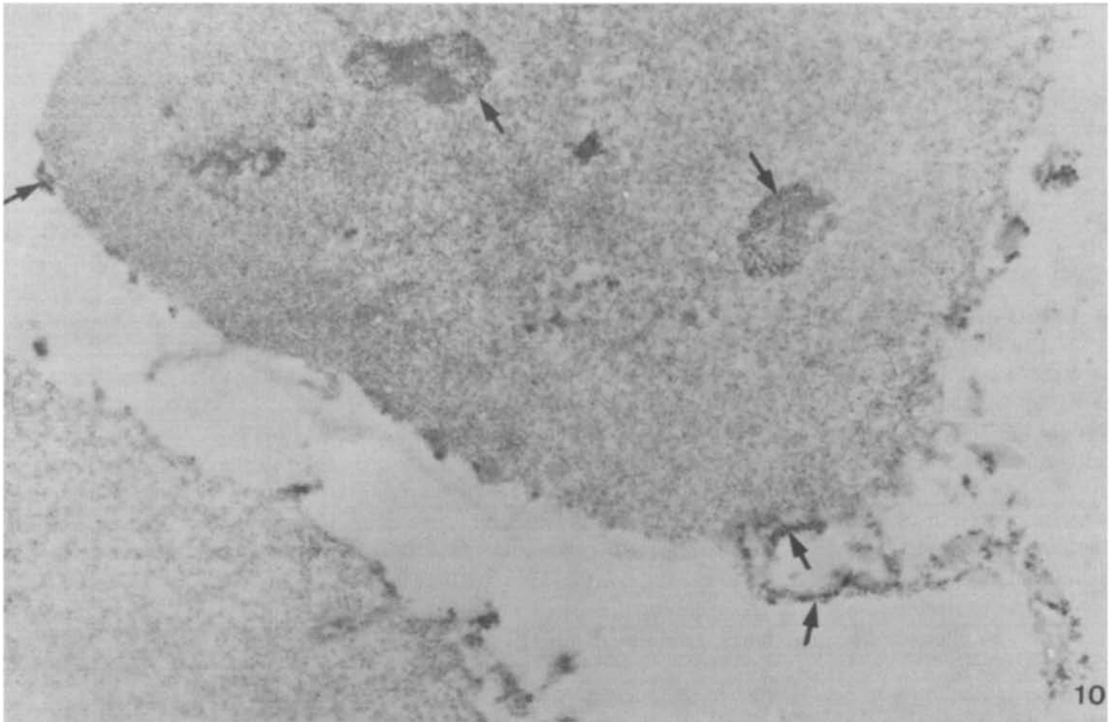


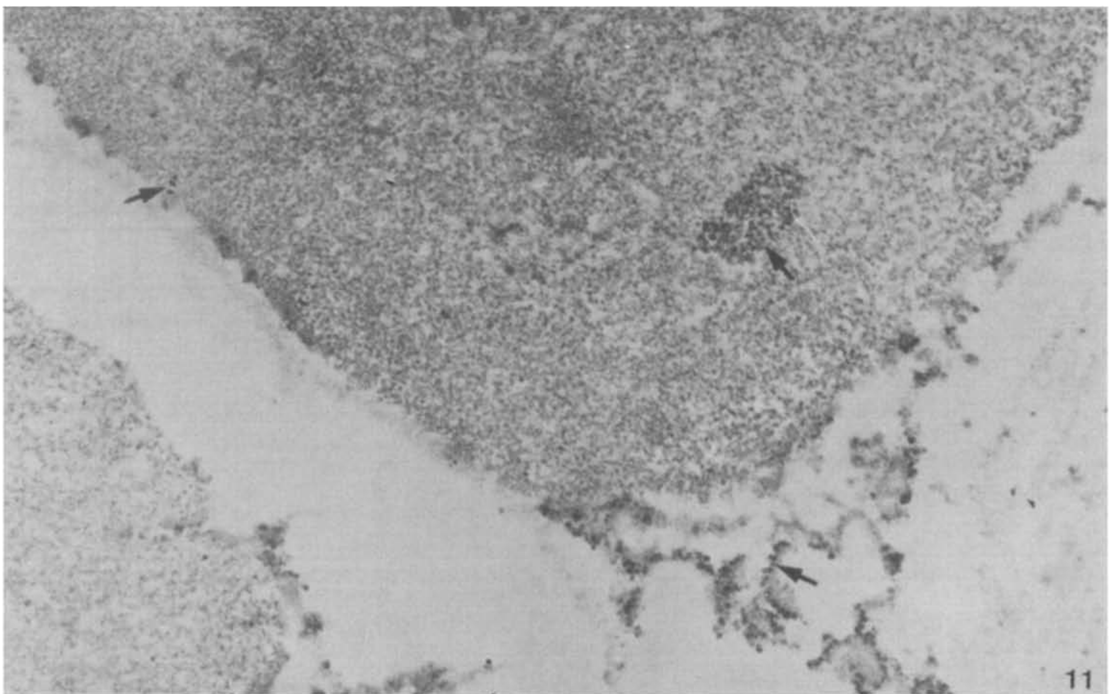
Fig. 9. Detection of R-2858 by immunofluorescence in rat myometrium (sample EIF). The nucleus is weakly and irregularly labeled (/). A very fine fluorescent network located nearby probably corresponds to cisternae of endoplasmic reticulum (//) (× 2,500).

Table 4. Results of the second series: light microscopy with immunoperoxidases

		+ABE2		+AB2858	
Rat D oophorectomy adrenalectomy + Intraperitoneal estradiol	Nucleus	+	Sample D, Fig. 1	−	Sample DT, Fig. 2
	Cytoplasm	∓		−	
Rat E oophorectomy adrenalectomy + Intraperitoneal R 2858	Nucleus	−	Sample ET, Fig. 4	+	Sample E, Fig. 3
	Cytoplasm	−		∓	

For legend, see footnote to Table 3.
The animals were oophorectomized and adrenalectomized. Each rat D received 1 μg E2 by intraperitoneal injection. Each rat E received 0.1 μg R 2858 by intraperitoneal injection. It can be seen that E2 and R 2858 were recognized only by their corresponding antiserum. Once again, cytoplasmic labelling was not obvious.





Figs. 10 and 11. Electron micrographs showing labeling of peroxydases in the nucleus and endoplasmic reticulum (↗). Ultrathin uncontrasted sections from sample A3 (prepubertal rat uterus incubated with E2 at 37°C). Micrographs are from the same cell sectioned at different levels. Acceleration potential was 60 kV (Fig. 10: × 28,000; Fig. 11: × 32,000).

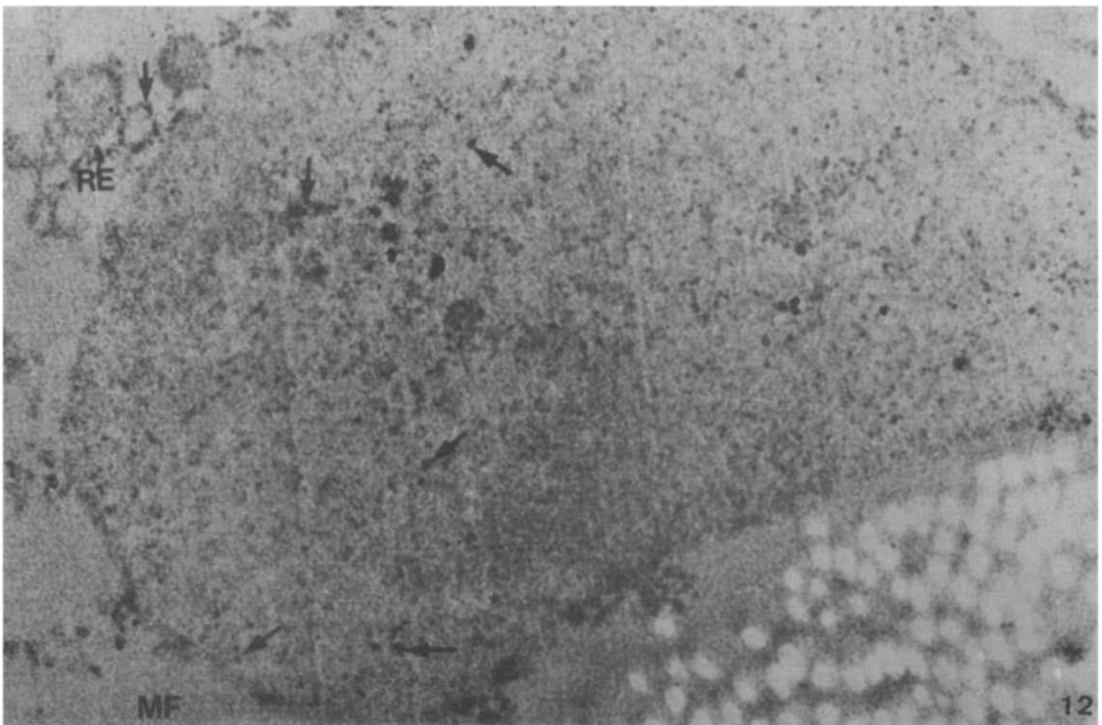


Fig. 12. Detection of R-2858 in myometrial cell nucleus (↗). Ultrathin uncontrasted sections from sample E. Acceleration potential was 60 kV (× 32,000).

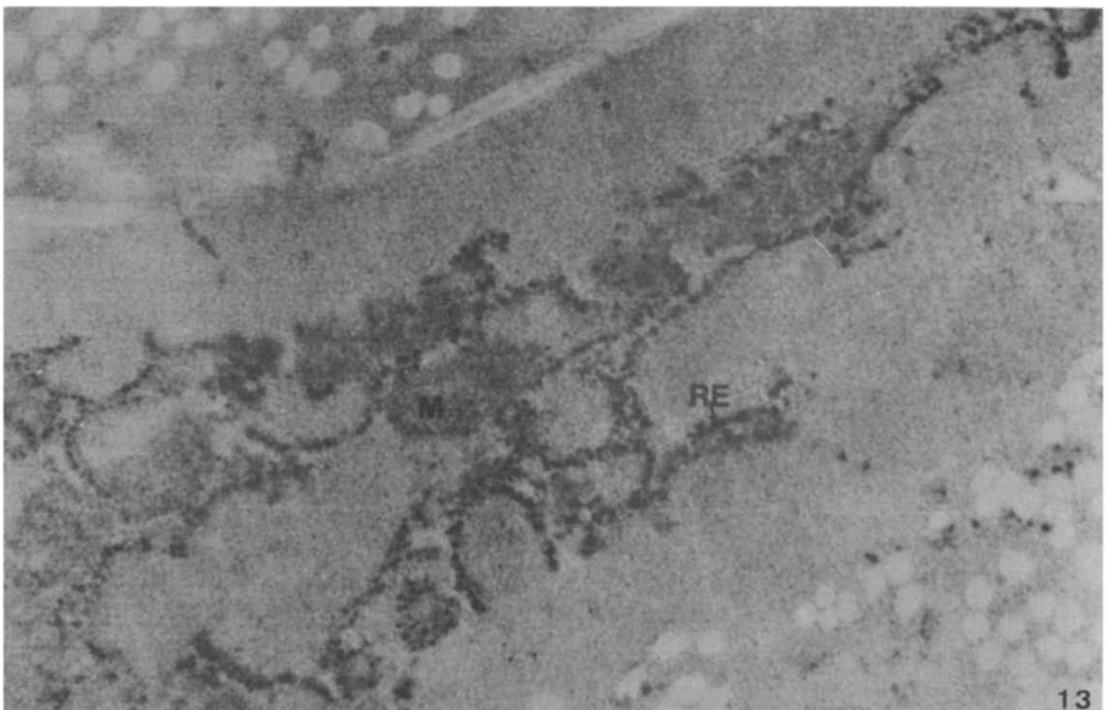


Fig. 13. Demonstration of R-2858 on membranes of endoplasmic reticulum (RE). M: mitochondria. Micrograph from uncontrasted sections of sample E. Acceleration potential was 60 kV (× 45,000).

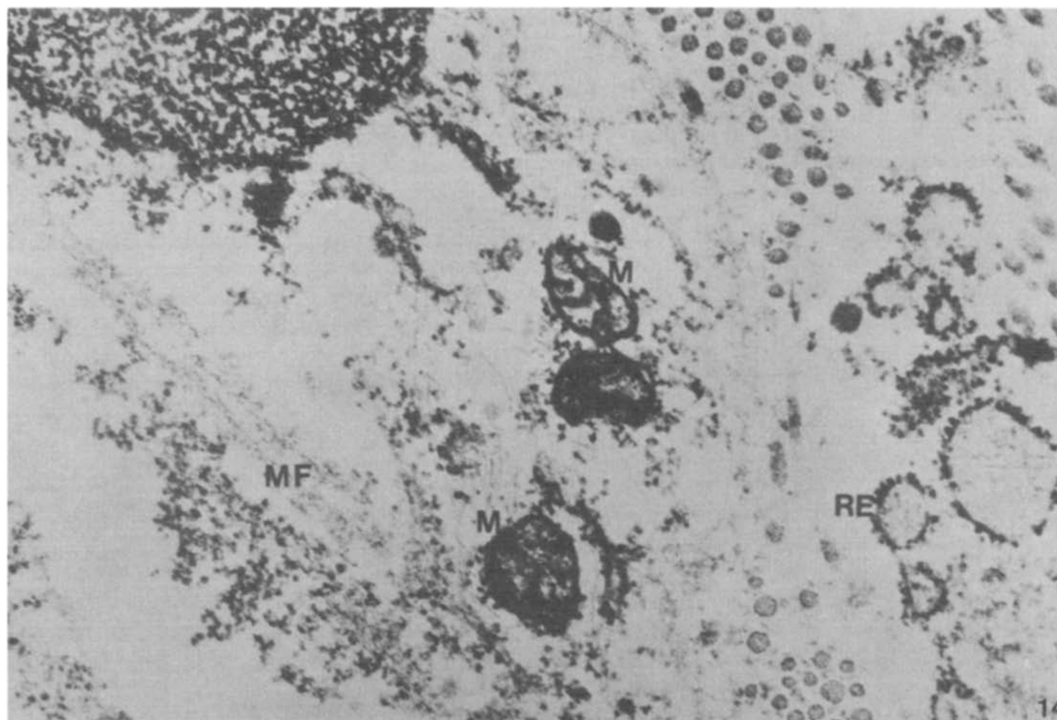


Fig. 14. Ultrathin section from sample E contrasted by uranyl acetate and lead citrate. Acceleration potential was 80 kV. Cell structures are poorly preserved but mitochondria (M), dilated and ruptured elements of endoplasmic reticulum (RE) and myofibrils (MF) can be recognized (×45,000).

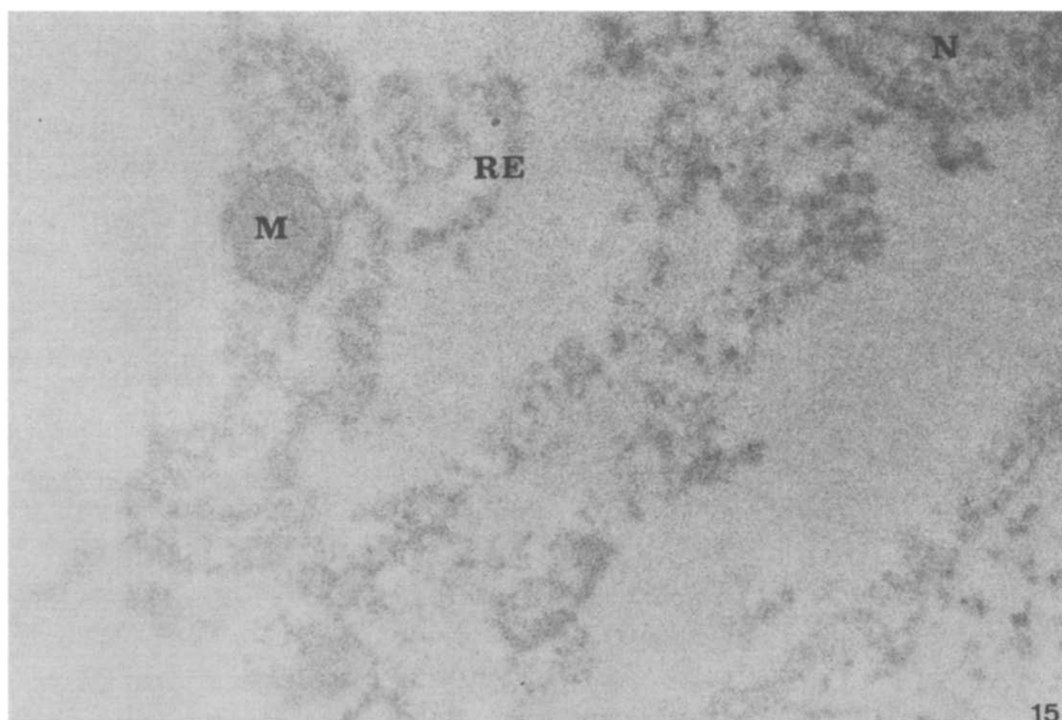


Fig. 15. Ultrathin uncontrasted section from sample ET. Acceleration potential was 40 kV. Anti-E2 antibody did not recognize R-2858 bound in the region of the nucleus (N) and endoplasmic reticulum (RE) M: mitochondrium (×60,000).

DISCUSSION

The above results lead us to believe that immunoperoxidases in addition to immunofluorescence can be used to study E2 receptor sites in target tissues. The specificity of the reaction was confirmed by the results of immunologic controls and by cross-reaction experiments (shown in Tables 4 and 5). R 2858, which is highly specific for E2 receptors and E2 are recognized by only the corresponding antisera. Furthermore, the use of R 2858 confirmed that the technique reveals high affinity E2 binding sites only since R 2858 does not bind to other proteins, including plasma proteins [6, 7]. Under all experimental conditions even at 4°C, marked nuclear labeling was observed. However clear cytoplasmic labeling in the absence of nuclear labeling as reported by Nenci using immunofluorescence was not obtained at 4°C in the presence of hormone (A2, B2, C2) although such cytoplasmic labeling could have been expected at least in samples A2 and C2. Several possible explanations may be proposed to account for this failure: 1. our study was performed on myometrium where much of the cytoplasm is occupied by myofibrils. Thus, if E2-specific sites are not located on the myofibrils, then they must be localized and concentrated in a small cytoplasmic zone inaccessible to light microscopy in our experimental conditions. This hypothetical explanation is supported by results on immunofluorescence and electron microscopy in this study which demonstrated the presence of labeling in the endoplasmic reticulum only; 2. the myometrium may be relatively devoid of E2-specific binding sites; 3. finally, since the technique used was time consuming, the poor preservation of certain cytoplasmic cell structures, may account for a loss in binding sites.

Surprisingly, nuclear labeling was clearly visible in samples A2 (prepubertal rat uterus incubated with E2 at 4°C) and C2 (oophorectomized rat uterus incubated with E2 at 4°C). Since the immunologic controls were negative it appeared that free nuclear receptors might be present. Biochemical measurement confirmed this notion in prepubertal rats. Recently, autoradiographic studies performed by Sheridan[17] confirmed the nuclear and cytoplasmic localization of estrogen binding at 4°C. Furthermore it is reasonable to suppose that in both prepubertal and oophorectomized rats there might also be weak secretion of E2 or estrone (which is weakly estrogenic) leading to nuclear transfer of cytoplasmic receptors. The estrone which saturates the sites could then be displaced by E2 [18]. Finally, control no. 6 displayed nuclear labeling with sample A1 and this observation may be explained by the presence of free receptors binding the E2-antibody complex (Fig. 6).

This technique which was developed in order to achieve immunocytochemical evaluation of estradiol binding sites by electron microscopy seems to be less satisfactory than immunofluorescence: it is more time consuming, more uncertain and less discriminating since it is often difficult to distinguish between week specific labeling and background phenomena. Electron microscopy revealed the presence of labeling on the endoplasmic reticulum, nuclear envelope and chromatin. In experiments performed at 37°C nucleolar labeling was detected after 4 h. These interesting but preliminary results should be interpreted with caution since our cytological data were of poor quality due to the impossibility of obtaining tissue fixation prior to the action of specific antibodies. It is therefore difficult to establish definitively that the cytoplasmic E2 binding sites are exclusively located on the cell membrane system, since other binding sites may have disappeared, and retractile phenomena may have caused hyaloplasmic sites to be adsorbed onto the membranes. However, one of the most important functions of the endoplasmic reticulum is that of transport and it is not absurd to postulate *a priori* that E2 binding sites would be located on the cell membranes from which they can be extracted using biochemical techniques. This postulate could account for the lability of these receptor proteins when isolated using biochemical procedures and their relative stability in experimental conditions of immunofluorescence or immunoperoxidase techniques where binding to membranes renders them less accessible to structural changes and loss of function.

In agreement with biochemical data [19] labeling of chromatin was observed in the nucleus. The nuc-

Table 5. Results of control experiments

	A1–A2–A3	B1–B2–B3	C1–C2–C3
1. Specific antiserum omitted	Negative	Negative	Negative
2. Non immune rabbit serum	Negative	Negative	Negative
3. Anti-hemoglobin immunoglobulins	Negative	Negative	Negative
4. Anti-rabbit-Ig serum omitted	Negative	Negative	Negative
5. PAP omitted	Negative	Negative	Negative
6. I % ABE2 + 10^{-6} M E2	Positive + + for A1 only	Doubtful	Doubtful
7. Striated muscle incubated with: E2 at 4°C + ABE2 = Negative, E2 at 37°C + ABE2 = Negative.			
8. Diaminobenzidine reaction in medium without H202 = Negative			
9. Uterus from castrated rat + AB2858 = Negative			

Controls were negative except for the existence of a positive nuclear labeling in control No. 6 for sample A1.

leolar labeling, also found on serial sections, is an interesting phenomenon since it is known that E2 specifically increases the activity of nucleolar polymerase A [20] and that this step both occurs with relative delay (4–6 h) compared to overall chromatin staining and it is necessary for late reincrease in polymerase B activity which parallels the trophic action of estrogen. Additionally, certain authors described a limited number of nuclear receptor sites which undergo slow dissociation and seem to be those required for the trophic action of estrogenic compounds [21, 22].

New experiments using a slightly modificated technology are now underway in the hope that they will yield improved quality of cytological data, thus enabling confirmation or rejection of these preliminary results. If confirmation ensues, the technology outlined in this study may be used to localize steroid hormone receptor sites during cell kinetic studies, and to identify specific receptor sites in microscopic cell samples which cannot be satisfactorily manipulated *in vitro* using biochemical techniques.

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