J. steroid Biochem. Vol. 24, No. 4, pp. 795–800, 1986
Printed in Great Britain.

0022-4731/86 $3.00 + 0.00

HORMONE INDEPENDENT ACTIVATION OF RAT UTERINE ESTROGEN RECEPTOR BY EXPOSURE OF ISOLATED UTERI TO ANAEROBIC CONDITIONS*†

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(*Received* 25 *May* 1985)

Summary—Incubation of isolated rat uteri under anaerobic conditions, which consisted of either an atmosphere of carbon monoxide or nitrogen, caused an increase in nuclear estrogen binding which was not dependent on added estrogen. The incubation of uteri in the absence of added estrogen under aerobic conditions (atmosphere of oxygen or oxygen–carbon dioxide [95–5%]) did not increase uterine nuclear estrogen binding levels. High salt (0.5-M KCl) extracts of the nuclear estrogen binding moiety induced by anaerobiosis were shown to possess a sedimentation coefficient on sucrose–glycerol gradients of 4.8S, a binding specificity restricted to estrogens and an apparent affinity constant of 1.35 nM. These data confirm that the nuclear binding moiety induced by anaerobiosis possesses the characteristics of an estrogen receptor. The enhanced nuclear estrogen receptor retention induced under anaerobic conditions could be accounted for by a significant increase in nuclear receptor extracted by high salt (0.5 M KCl) and by ethanol (salt resistant fraction). Furthermore, sequential extraction of nuclear estrogen receptor from uteri exposed to aerobic conditions in the presence of added estradiol paralleled the results obtained with anaerobiosis. Total receptor retained under anaerobiosis represented 25% of that observed under aerobic conditions in the presence of estrogen. These results indicate that anaerobic conditions can cause an activation of uterine estrogen receptor. This activation process represents a pathway for receptor activation which does not require steroid.

INTRODUCTION

Current models of steroid hormone action include a scheme whereby binding of the hormone to a specific receptor protein in the target cell leads to enhanced affinity (activation) of the steroid–receptor complex for nuclei [1, 2]. The necessity for steroid-induced translocation of receptor to the nuclear compartment has been questioned recently [3, 4]. However, regardless of whether the steroid binds to cytoplasmic or nuclear receptor, this binding results in an enhanced affinity of the complex for nuclei.

Nuclear retention of estrogen receptor has been shown to be required for the maintenance of uterine growth in the rat [5, 6], egg white protein gene transcription in the chick [7, 8] and recently, estrogen receptor complexes have been shown to mediate increases in RNA polymerase II activity in oviduct nuclei [9]. Because of the importance of estrogen receptor and nuclear retention of receptor in hormonal response, many studies have been directed towards an understanding of factors which can influence steroid-receptor activation *in vitro* [see 10, 11 for reviews]. These factors include heat, salt treatment, pH and dilution.

During preliminary studies with rat uterine explants we observed that *in vitro* incubation of uteri under an atmosphere of carbon monoxide caused an apparent enhanced retention of nuclear estrogen receptor in the absence of added estrogen§. Subsequent experiments with an atmosphere of nitrogen and in the absence of estrogen yielded similar results. The following experiments were initiated to demonstrate this phenomenon and to study whether anaerobiosis could provide a hormone independent pathway for receptor activation.

EXPERIMENTAL

Materials and animals

The following compounds were purchased: [2,4,6,7-$^3H_4$]17β-estradiol [85–100 Ci per mmole] (Amersham, Arlington Heights, Ill, U.S.A.); Tris and grade C dextran (Schwarz/Mann Orangeburg, N.Y., U.S.A); EDTA (Mallinckrodt, Inc., Paris, Ky, U.S.A.); activated charcoal, DNA-cellulose, bovine serum albumin, monothioglycerol, diethylstilbestrol,

*This study was supported by United States Public Health Service Grants ES00834 (D. K.) awarded by the National Institute of Environmental Health Sciences and HD-19414 (WCO) awarded by the National Institute Child Health and Human Development.

†A portion of this work was presented at *The 69th Annual Meeting of FASEB*, April, 1985, Anaheim, California (Abstract no. 6215).

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§The *in vitro* effect of anaerobiosis on uterine estrogen receptor was discovered by serendipity during the development of an assay for assessing whether a compound that is estrogenic *in vivo* is estrogenic *per se* or is a proestrogen which is metabolically activated [12, 13]. When we attempted to use an atmosphere of carbon monoxide (an inhibitor of hepatic microsomal monooxygenase activity) to block estrogen formation, we observed that carbon monoxide had the effect on uterine estrogen receptor reported in this study.

estrone, progesterone, testosterone, cortisol (Sigma Chemical Co., St Louis, Mo. U.S.A); 17β-estradiol (Steraloids, Inc., Wilton, N.H., U.S.A.); dithiothreitol (Calbiochem, LaJolla, Calif., U.S.A); glycerol (Matheson Coleman and Bell, Norwood, Ohio, U.S.A.); Aquasol, Liquifluor (New England Nuclear, Boston, Mass. U.S.A.); Parafilm (American Can Co., Greenwich, Conn. U.S.A.); 99.50% pure oxygen, USP grade 95–5% oxygen–carbon dioxide and 99.97% pure nitrogen (Linde Co., Danbury, Conn. U.S.A.); 99.99% pure carbon monoxide (Matheson, East Rutherford, N.J., U.S.A.). All other compounds were of reagent grade quality. Female Sprague–Dawley CD rats were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, Mass. U.S.A.).

Preparation of uterine explants for incubation

Immature female rats (20–22 days old) were sacrificed by cervical dislocation. The uteri were removed and dissected free from surrounding mesentery. Each uterus was cut through the fused region and six of the resulting horns, randomized, were added to a 30 ml serum vial containing 3 ml of Krebs–Ringer solution [12] at 2–4°C. The uterine explants were exposed to an atmosphere of either carbon monoxide, nitrogen, oxygen, or oxygen–carbon dioxide in the following manner. The appropriate gas was delivered through a 21 gauge 1.5 inch hypodermic needle (gassing needle) which was placed directly into the Krebs–Ringer solution containing the uteri. After vigorous gassing for 3 min, the vial was sealed with a rubber stopper containing a septum and the stopper was secured with a band of parafilm. To ensure that the space above the liquid in the vial contained only the desired gas, the following flushing procedure was carried out after the initial gassing. Additional gas was added to the vial through the gassing needle by passing the needle through the septum of the stopper. The septum was then pierced by a venting needle (21 gauge 1.5 inch) and the desired gas was washed through the vial for 1 min after which time the venting needle was removed. About 10 s later (after a positive pressure build up) the gassing needle was removed. All gassing procedures were carried out at 0–2°C. When estradiol was added, it was added in 5 μl of ethanol before gassing. After gasssing the vials were incubated for 1 h at 37°C in a water bath shaker.

Preparation of uterine cytosol and nuclei

Followng incubation the uterine explants (6 horns) from each vial were removed, washed in ice-cold buffer A (10 mM Tris–HCl, pH 7.4, containing 1.5 mM EDTA) and were homogenized in 2 ml of buffer A with a motor-driven 3 ml size Kontes Duall glass–glass tissue grinder (Kontes, Vineland, N.J., U.S.A.). The homogenate was centrifuged at 800 *g* for 20 min at 2–4°C. The resulting supernatant and pellet were used to prepare cytosol and nuclei. Cytosol was prepared from the supernatant by dilution to 3 ml with buffer A containing dithiothreitol (0.5 mM final concentration) and by centrifugation at 20,000 *g* for 20 min at 2–4°C. To prepare nuclei, the contaminating cytosolic estrogen receptor was removed from the 800 *g* pellet by washing the pellet 3 times with buffer A. Each washing was accomplished by adding 4 ml of buffer A, vortexing to suspend the pellet, and by repelleting by centrifugation at 800 *g* for 10 min at 2–4°C.

Estrogen receptor assay

The exchange assay for determination of cytosolic or nuclear estrogen receptor was essentially as described by Anderson *et al.*[14] and Clark *et al.*[15]. Estrogen receptor levels in the above cytosolic (20,000 *g*) preparations were determined by allowing the exchange to occur at 30°C for 30 min in the presence of 10 pmol of [^{3}H]estradiol in 0.3 ml of buffer B (10 mM Tris–HCl, pH 7.4, containing 1.5 mM EDTA and 0.5 mM dithiothreitol) which contained cytosol equivalent to 1/4 of a uterus. The exchange was terminated by immersion of the samples in an ice-water bath. The free [^{3}H]estradiol was removed by shaking for 15 min at 2–4°C in the presence of DCC (1% charcoal, 0.05% dextran, 10 mM Tris–HCl, at pH 8). The DCC was removed by centrifugation at 800 *g* for 10 min at 2–4°C and the radioactive content in 0.5 ml of the supernatant was determined by liquid scintillation counting employing 5 ml of Aquasol as a sample medium. Each sample was assayed in duplicate. The amount of estrogen receptor in the above nuclear preparations was determined by allowing an exchange to take place at 37°C for 1 h in the presence of 10 pmol [^{3}H]estradiol in 0.7 ml of buffer A which contained a suspension of washed nuclei equivalent to half a uterus. The exchange was terminated by adding 2 ml of ice-cold buffer A to the samples and by placing the samples in an ice bath. The samples were centrifuged (800 *g*, 2–4°C, for 10 min) and the supernatant was discarded. Unbound [^{3}H]estradiol was removed from the resulting pellets by washing 3 times with ice-cold buffer A. Each washing was accomplished by adding 2 ml of buffer A, vortexing to suspend the pellet and by repelleting the nuclei by centrifugation (800 *g*, 2–4°C, for 10 min). The bound [^{3}H]estradiol was extracted from the final pellet by suspending the nuclei in 2 ml ethanol (vortexing for 0.5 min) and by incubating at 30°C for 15 min. After incubation, the nuclei were pelleted by centrifugation (800 *g*, 10 min), the ethanol fraction was added to 10 ml Liquifluor, and the radioactive content was determined. Each sample was assayed in triplicate. Results from the determination of either cytosolic or nuclear estrogen receptor levels were corrected for nonspecific [^{3}H]estradiol binding by subtraction of values obtained from parallel determinations to which diethylstilbestrol (100 times the concentration of [^{3}H]estradiol) had been added.

Sequential extraction of nuclei

The incubation of uterine explants, preparation of nuclei, and exchange assay were carried out as described above with the following exception. During the exchange assay the nuclei were carried through the normal washing procedure to remove unbound [^{3}H]estradiol. However, after the last wash with buffer A the resulting nuclear pellets were extracted sequentially with aqueous KCl. The samples were placed in an ice-bath, 1 ml of 0.15 M KCl (2–4°C) was added, and the pellets were suspended by vortexing. Incubation was carried out by placing the ice-bath in a cold room (2–4°C) for 1 h with sample vortexing every 20 min. After incubation samples were centrifuged (800 *g*, 2–4°C for 15 min), the KCl extract (supernatant) was removed, an 0.8 ml aliquot was added to 10 ml Aquasol and the radioactive content was determined. The nuclear pellets resulting from the 0.15 M KCl extraction were subsequently extracted with 0.5 M KCl. The extraction procedure was identical to the one described for 0.15 M KCl. Following the 0.5 M KCl extraction the resulting pellets were subjected to a terminal extraction with 2 ml of ethanol. Ethanol extraction was identical to the procedure described above for the nuclear exchange assay. All determinations were in triplicate. Results were corrected for nonspecific binding of [^{3}H]estradiol by subtracting values obtained from parallel preparations containing diethylstilbestrol (100 times the concentration of [^{3}H]estradiol).

Density gradient sedimentation analysis

Sucrose (5–20%)-glycerol (10%) gradients were formed in polyallomer tubes suited for use in a Beckman SW 50.1 rotor and were allowed to equilibrate for 24 h at 2–4°C. The sucrose–glycerol was prepared in 10 mM Tris–HCl buffer (pH 7.4) containing 0.4 M KCl, 1 mM EDTA, and 12 mM monothioglycerol. Samples were layered on top of the gradients and were centrifuged at 170,000 *g* for 22 h at 2°C. The tubes were pierced at the bottom and 26 three-drop fractions were collected. The radioactive content of each fraction was determined by liquid scintillation counting. Sedimentation coefficients were determined by the method of Martin and Ames[16] using bovine serum albumin (4.6S) as the standard.

RESULTS

In an initial observation, rat uteri from immature animals were incubated with *no* added estrogen under an atmosphere of either low purity carbon monoxide or oxygen. The carbon monoxide caused a marked depletion of uterine cytosolic estrogen receptor and an enhanced retention of nuclear estrogen binding compared to oxygen (data not shown). Because these results suggested an "estrogen-like" action for carbon monoxide, the phenomenon was investigated further. High purity carbon monoxide (99.99%) was used to eliminate the possibiity of an estrogen-like contaminant in the gas. The results with high purity carbon monoxide demonstrated a significant ($P \leq 0.005$) increase in nuclear estrogen binding and concomitant decrease in cytosolic estrogen binding ($P \leq 0.005$) compared to oxygen alone (Table 1, experiment 1). In a subsequent experiment, essentially the same results were obtained by replacing the carbon monoxide atmosphere with one of nitrogen (Table 1, experiment 1), suggesting that the observed activity was caused by anaerobic conditions and was not the result of properties intrinsic to the 2 gases. Furthermore, anaerobiosis caused a significant decrease in the total amount of uterine estrogen binding (Table 1, experiment 1).

To determine the characteristics of the receptor under anaerobic conditions, the experiments depicted in Table 2 and Fig. 1 were conducted. No significant difference was observed between the displaceable binding or the K_A values determined for nuclear estrogen binding (0.5 M KCl extract) obtained from uteri incubated under anaerobic conditions in the absence of added steroid or under aerobic conditions in the presence of estradiol (Table 2). Moreover, high salt extracts obtained from the two treatment groups

Table 1. *In vitro* effect of different gasses on the distribution of estrogen receptor in cytosolic (Rc) and nuclear (Rn) compartments of uteri from immature rats

Experiment	Addition (20 nM)	Atmosphere	[^{3}H]Estradiol bound per uterus fmol (Mean ± SE) Rc	Rn	Rc + Rn
1	None	O_2	1047.0 ± 60.2	137.6 ± 6.6	1184.7 ± 58.3
	None	CO	315.5 ± 26.1	305.3 ± 34.6	620.8 ± 59.7
	None	N_2	325.2 ± 36.0	315.4 ± 21.9	640.6 ± 55.1
2	None	O_2	1003.9 ± 62.8	108.5 ± 5.9	1112.4 ± 68.6
	E_2	O_2	78.8 ± 14.5	722.4 ± 29.2	801.2 ± 15.7
	None	O_2–CO_2	816.2 ± 44.7	92.8 ± 17.8	909.0 ± 49.5
	E_2	O_2–CO_2	46.6 ± 10.6	788.0 ± 32.2	834.7 ± 22.5

Isolated uteri were incubated under the designated atmosphere for 1 h at 37°C. After incubation, the uteri were homogenized and the cytosolic and nuclear estrogen receptor levels were determined. See Experimental for detailed procedures. E_2 = 17β-estradiol. Statistical analysis by Student's *t*-test. Number of incubations (*n* value) per group equals 4. *Experiment 1.* N_2 or CO vs O_2; Rc, Rn, Rc + Rn: $P < 0.005$. *Experiment 2* (1) O_2 (no E_2) vs O_2 (E_2); Rc: $P < 0.001$, Rn: $P < 0.001$, Rc + Rn: $P < 0.005$. (2) O_2–CO_2 (no E_2) vs O_2–CO_2 (E_2); Rc: $P < 0.001$, Rn: $P < 0.001$, Rc + Rn: (NS).

Table 2. Binding specificity of rat uterine nuclear estrogen receptor under anaerobic and aerobic conditions

Competitor	Displaceable binding (%)	
	Anaerobic	Aerobic + Estradiol
Estradiol	100%	100%
Diethylstilbestrol	108 ± 3.3	99 ± 1.0
Estrone	101 ± 3.6	95 ± 1.4
Progesterone	0	0
Testosterone	0	0
Cortisol	0	0
	K_A ($\times 10^9 M^{-1}$)	
	Anaerobic	Aerobic + Estradiol
	1.35 ± 0.13	1.79 ± 0.43

Immature rat uteri were incubated under anaerobic conditions which consisted of an atmosphere of nitrogen (no added steroid) or under oxygen in the presence of radioinert estradiol (20 nM). Uterine nuclei were prepared (as described in Experimental) and extracted with 0.5 M KCl (1 uterine equivalent per 0.6 ml) [see Experimental]. Nuclear extracts (0.3 ml) were incubated in duplicate with either 5 nM [^{3}H]estradiol in the presence or absence of 500 nM competitor (specificity assay) or with 0.18–5.0 nM [^{3}H]estradiol in the presence or absence of a 100-fold excess (relative to [^{3}H]estradiol) of DES (K_A determination). All additions were in 0.1 ml of buffer A. Incubation was for 1 h at 30°C followed by 10 min at 1–4°C. Unbound [^{3}H]estradiol was removed with 0.5 ml dextran coated charcoal. Displaceable binding was expressed as a percentage of the binding displaced by unlabeled estradiol (100%). K_A values were obtained from Scatchard [17] plot analysis. All values are the mean ± SEM ($N = 3$).

demonstrated identical sedimentation pattern when analyzed by sucrose–glycerol density gradient centrifugation (Fig. 1). These data confirmed that the nuclear binding moiety induced by anaerobiosis possesses the characteristics of an estrogen receptor.

Experiment 2 in Table 1 depicts the effect of incubation of uterine explants in the presence or absence of added estradiol under an atmosphere of oxygen or the more physiological mixture of oxygen–carbon dioxide (95–5%). No discernible difference was observed in the estrogenic action of estradiol under either atmosphere. When the two sets of experiments reported in Table 1 are contrasted, a striking parallelism between the effects of anaerobiosis and the estradiol on the distribution of estrogen receptor in uterine explants becomes apparent. In both cases the depletion of cytosolic estrogen receptor and enhanced retention of nuclear receptor are dramatic. The parallelism between the two sets of the above data suggests an *in vitro* activity common to both estradiol and anaerobiosis.

The following experiments were conducted to examine more rigorously whether the nuclear retention phenomenon observed with anaerobiosis represented

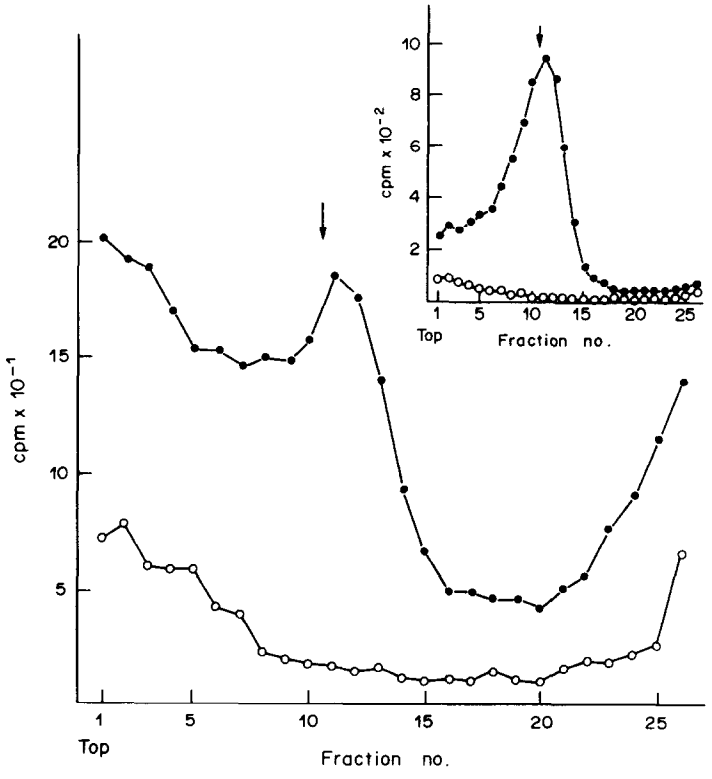

Fig. 1. Sucrose–glycerol density gradient sedimentation analysis of uterine nuclear binding moiety induced by anaerobiosis. High salt (0.5 M KCl) uterine nuclear extract (1 uterine equivalent per 0.6 ml) was prepared and incubated for 1 h at 30°C with 5 nM [^{3}H]estradiol (—●—) or 5 nM [^{3}H]estradiol in the presence of 0.5 μM radioinert diethylstilbestrol (—○—). Following incubation, unbound [^{3}H]estradiol was removed with dextran-coated charcoal and 0.3 ml of sample was layered on top of each gradient. All subsequent procedures were as described in Experimental. Arrow indicates location of Bovine Serum Albumin (4.6 S). Inset: conditions as described above, except nuclear extract was prepared from uteri: incubated under aerobic conditions in the presence of 20 nM radioinert estradiol. Gradients shown above are representative of 3 experiments.

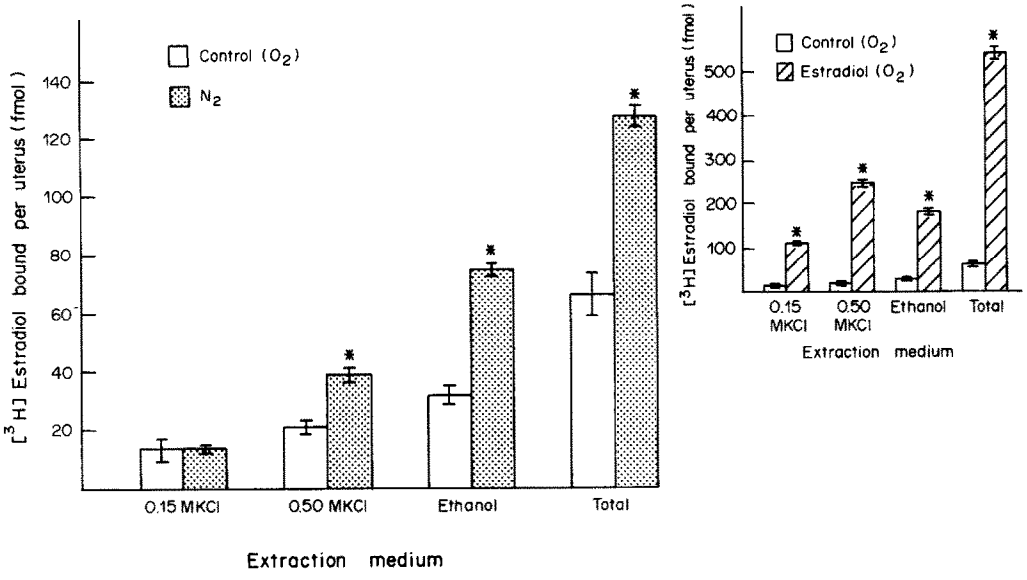

Fig. 2. Sequential extraction with salt and ethanol of nuclear estrogen receptor (ER_n) from rat uterine explants exposed to an atmosphere of O_2, N_2 or $O_2 + E_2$. Immature rat uterine explants were incubated at 37°C for 60 min under an atmosphere of O_2 or N_2 in the absence of added E_2. Uterine nuclear fractions were prepared as described in Experimental. ER_n was extracted sequentially with 0.15 M KCl, 0.5 M KCl, and finally ethanol. ER_n was assayed by ^{3}H-steroid binding assay as described in Experimental. The sequential extraction of ER_n from explants incubated in the presence of radioinert E_2 (20 nM) under an atmosphere of O_2 is shown in the inset. Values represent the mean ± SEM $N = 11$ (O_2), 10 (N_2), 7 ($O_2 + E_2$). Data were analyzed by ANOVA with Duncan's posteriori test. $^*P < 0.01$.

estrogen receptor activation. The data shown in Fig. 2 depicts the results of sequential extraction of nuclei from uterine explants exposed to an atmosphere of oxygen or nitrogen. Parallel data for estradiol are given for purposes of comparison (Fig. 2, inset). No difference in specific binding of [^{3}H]estradiol to nuclear receptor was observed between 0.15 M KCl extracts from uteri incubated under oxygen or nitrogen. However, significant differences were observed between the two treatments (oxygen or nitrogen) in the 0.5 M KCl extracts and in the salt resistant ethanol extracts. In both of the latter cases the level of nuclear receptor was markedly higher in the explants subjected to anaerobiosis (nitrogen). As anticipated, a significantly higher level of nuclear receptor was observed in uterine explants incubated with estradiol under an atmosphere of oxygen compared to oxygen alone (Fig. 2, inset).

DISCUSSION

Retention of nuclear estrogen receptor has been associated with estrogen response [1–8]. In this study nuclear estrogen receptor retention was significantly increased in immature rat uteri exposed to anaerobic conditions (nitrogen or carbon monoxide) compared to aerobic conditions (oxygen or oxygen–carbon dioxide). These data paralleled those observed under aerobic conditions (oxygen) in the presence of estradiol (20 nM). The enhanced nuclear estrogen receptor retention observed under anaerobic conditions was not dependent on added estrogen. In addition, similar results were obtained with uteri from rats subjected to adrenalectomy and ovariectomy (data not shown), suggesting that endogenous estrogens were not involved in this phenomenon. The results of sequential extraction of nuclei from uterine explants exposed to an atmosphere of oxygen or nitrogen demonstrated: (a) that nuclear estrogen receptor extracted with low salt (0.15 M KCl) could not account for the difference in estrogen receptor retention observed between aerobic (oxygen) and anaerobic (nitrogen) conditions; (b) that the increase in nuclear estrogen receptor observed under anaerobic conditions could be accounted for by the significant increase in estrogen receptor extracted by 0.5 M KCl and ethanol. Thus, this phenomenon appears to represent receptor activation because of the resistance of the receptor to salt extraction. Furthermore, the binding properties and sedimentation pattern in sucrose–glycerol gradients of nuclear extracts (0.5 M KCl) obtained from anaerobic preparations were characteristic of an estrogen receptor and were essentially the same as the properties observed with extracts obtained from preparations incubated with estradiol under aerobic conditions.

Most studies which have addressed the influence of various factors on receptor activation have been done in the presence of added hormone [10, 11]. The present study is distinguished, however, by the fact that activation occurred in the absence of estradiol. Whether factors that influence activation in the pres-

ence of hormone are operable under anaerobic conditions in the absence of estradiol is presently unknown. Moreover, this activation process does not appear to be readily reversible; anaerobic conditions in this study (Table 1 and Fig. 2) were maintained for only 1 h, the subsequent assay and extraction procedures were carried out under ambient atmospheric conditions. Whether the significant loss of receptor induced by anaerobiosis (Table 1) is associated with this activation process remains to be established.

The nuclear estrogen receptor retention induced by anaerobiosis may represent a pathway for estrogen receptor activation that does not require estrogen. However, for this receptor activation pathway to have relevance, it is necessary to establish whether it can initiate a biological response. Studies of this nature are currently in progress.

Acknowledgements—The excellent technical assistance of Ms Jane E. Temple and Ms Frederica J. Nanni is gratefully acknowledged.

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