OESTROGEN RECEPTORS AND PROLACTIN IN RAT MAMMARY TUMOUR DEVELOPMENT

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE
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MARINUS ALOYSIUS BLANKENSTEIN
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Promotor:	Prof. Dr. H.J. van der Molen
Coreferenten:	Prof. Dr. D.W. van Bekkum
	Prof. Dr. F.J. Cleton

De in dit proefschrift beschreven experimenten werden uitgevoerd op de Afdeling Biochemie II (Hoofd: Prof. Dr. H.J. van der Molen) van de Faculteit der Geneeskunde der Erasmus Universiteit Rotterdam. Er werd nauw samengewerkt met het Radiobiologisch Instituut TNO te Rijswijk (Direkteur: Prof. Dr. D.W. van Bekkum). De experimenten werden mede mogelijk gemaakt door de toekenning van een fellowship en een projektsubsidie (RBI 78-3) door het Koningin Wilhelmina Fonds te Amsterdam.

Aan mijn ouders,
Louise,
Ingeborg
en
Rianne.

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CONTENTS

VOORWOORD

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Chapter I

SCOPE OF THIS THESIS

Mammary cancer has been a challenge to researchers for many years, because of the continuous menace of this disease to (wo)mankind. Since it is difficult to study the mechanism of mammary gland carcinogenesis experimentally in the human, model systems had to be devised. "Spontaneous" mammary tumours in rats are quite rare, or occur only after a very long period (Burek & Hollander, 1977; Burek, 1978). Therefore, induction of tumours has been used to obtain large amounts of tumour tissue. Two tumour model systems which are relevant to the work presented in this thesis will be described in some detail.

Induction of mammary tumours by oestrogens and/or ionizing radiation was used for most of the experiments reported in this thesis. This tumour model has received much attention since it became clear, that ionizing radiation could cause mammary cancer in human females e.g. after the exposure to radiation from atomic bombings at the end of World War II (Wanebo et al., 1968; Land & McGregor, 1979) or after exposure to therapeutic and diagnostic doses of X-rays (Baral et al., 1977; Boice & Monson, 1977; Shore et al., 1977). There were several indications that in rodents treatment with oestrogens may initiate mammary gland carcinogenesis (Lacassagne, 1932; Meites, 1972; Noble et al., 1975; Rudali et al., 1975; Lemon, 1977). It is thought, however, that oestrogens would act partly through an increase in pituitary prolactin release (Mühlbock & Boot, 1967) and partly synergistic with prolactin (Meites, 1972). Furthermore, in the rat a synergistic action of oestrogens and radiation has been observed (Segaloff & Maxfield, 1971; Shellabarger et al., 1976). This finding, together with the wide-spread use of oestrogen-containing oral contraceptives (Rinehart & Piotrow, 1979) and an increased

exposure to radiation (Upton, 1975), point to the possibility that part of the mammary tumours observed in women could also have arisen, or will arise as a result of a synergistic interaction between oestrogens and ionizing radiation.

The aim of the studies presented in this thesis was to gain a better understanding of the contribution and mechanism(s) of action of oestrogens and prolactin in the development of mammary cancer. The presence of oestrogen receptors in human and rat mammary tumours and the correlation between the presence of receptors and the rate of success of endocrine therapy strongly suggest that the (co)carcinogenic action of oestrogens on the mammary gland is mediated by the oestrogen receptors. Since oestrogens are capable of inducing their own receptors (Sarff & Gorski, 1971; Mester & Baulieu, 1975; Baudendistel et al., 1978), the first event in oestrogen-mediated mammary gland carcinogenesis could be an increase in the mammary gland oestrogen receptor content. To detect such a possible increase, oestrogen receptors were estimated in mammary glands during the process of carcinogenesis rather than in tumours which had developed after the administration of carcinogenic stimuli, as has been done in all experiments reported in the literature sofar. Since administration of oestrogens to rats is known to increase the release of prolactin from the pituitary (Chen & Meites, 1970; Van der Gugten et al., 1970), plasma prolactin levels were estimated in an attempt to correlate effects on prolactin with other effects of oestrogen.

The first part of this thesis deals with the induction of mammary tumours and the method used for the administration of oestrogen to rats to obtain slightly elevated plasma oestrogen levels. Subsequent sections concern the method used for the estimation of oestrogen receptors in tissues from oestrogen-treated animals and the results of the estimation of oestrogen receptors in mammary glands and plasma prolactin of rats exposed to amounts of oestrogen and radiation which were expected to induce mammary carcinogenesis.

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Chapter 2

INTRODUCTION

2.1. Effects of hormones on the mammary gland

For proper differentiation, development and functioning, the mammary gland is dependent on several hormones (R.R. Anderson, 1974). Insulin accelerates the growth of the primary sprouts and insulin and prolactin act synergistically to stimulate further growth and development. For differentiation of the fetal gland into the ductal system, aldosterone is required in addition. Prolactin and progesterone are required for further growth and secretory activity (Ceriani, 1970). The allometric growth of the mammary tissue, which occurs before puberty, is thought to be caused by cyclic production of oestrogens by the ovary (Sinha & Tucker, 1969). During pregnancy, a further growth of the mammary tissue is observed, which requires prolactin, growth hormone, adrenal corticoids and thyroid hormone (R.R. Anderson, 1974). Finally, for lactation the presence of oxytocin, prolactin, growth hormone, thyroxine, adrenal corticoids and parathyroid hormone is necessary (Turner, 1974). For maintenance of mammary epithelial cells in culture, insulin and corticoids are required (Welsh et al., 1976; Hallowes et al., 1977).

2.2. Effects of hormones on mammary gland carcinogenesis in the rat

Mammary cancer in laboratory rats can develop "spontaneously" or after exposure of the animals to chemical or physical carcinogenic stimuli. Among the factors known to contribute to neoplastic transformation of the mammary gland are ionizing radiation (Segaloff & Maxfield, 1971; Shellabarger et al., 1976 and Broerse et al., 1978) and chemical carcino-

gens like dimethyl-benzanthracene (DMBA) (Huggins et al., 1959), N-nitrosobutylurea (Takizawa, 1973) and N-nitrosomethylurea (Chan et al., 1977). The response to carcinogenic agents is strongly dependent on the nutritional and endocrine status of the animals. A high dietary fat intake promotes the induction of mammary tumours by N-nitrosomethylurea (Chan et al., 1977) and DMBA (Hill et al., 1977), whereas ovariectomy greatly reduces the carcinogenic action of DMBA (Dao, 1962) and ionizing radiation (Broerse et al., 1978). Ovarian hormones apparently play a permissive role in rat mammary gland carcinogenesis. On the other hand it has been shown, that oestrogens may act synergistically with ionizing radiation in the induction of mammary cancer in the rat (Segaloff & Maxfield, 1971; Shellabarger et al., 1976 and Broerse et al., 1978). The mechanism through which oestrogens exert this synergistic action is still unknown.

Also for prolactin a cocarcinogenic action in rat mammary gland carcinogenesis has been demonstrated (Van der Gugten et al., 1973).

2.3. Oestrogen receptors and mammary cancer

The presence of oestrogen receptors in human mammary tumours has been studied extensively. Approximately 60% of primary mammary tumours in women contain detectable amounts of oestrogen receptors (McGuire et al., 1975; Koenders, 1979). The significance of the presence of oestrogen receptors in mammary tumours as a means of predicting the response of the tumour to "endocrine therapy", has been strongly emphasized. In a collaborative study of eight cancer institutes 54% of "oestrogen receptor positive" mammary tumours regressed after endocrine therapy, whereas only 5% of "oestrogen receptor negative" tumours showed a regression (McGuire et al., 1975).

Also in DMBA-induced rat mammary tumours oestrogen receptors have been demonstrated (e.g. Nicholson et al., 1976; Vignon & Rochefort, 1976; Tsai & Katzenellenbogen, 1977).

These tumours also regress after ovariectomy (Mobbs & Johnson, 1974; Fiebig & Schmähl, 1977). The correlation between the presence of oestrogen receptors and the result of endocrine therapy is beyond doubt, but the mechanism(s) through which the different endocrine therapies which have been used cause(s) the therapeutic effect, is ill understood, as is the role which oestrogen receptors could play in carcinogenesis.

2.4. The mechanism of action of oestrogens

It is generally accepted that oestrogens act on their target tissues through specific receptor molecules present in the target cells. Several recent reviews on this topic are available (e.g. King & Mainwaring, 1974; Chan & O'Malley, 1976; Van Beurden, 1977; De Boer, 1977; O'Malley & Birnbaumer, 1978), but within the context of this thesis it appears appropriate to discuss briefly some general aspects of the mechanism of oestrogen action in general and the possible role of oestrogen receptors in the action of oestrogens on the mammary gland and in mammary carcinogenesis. Most of the work done to elucidate the mechanism of action of oestrogens has been performed on chicken liver and oviduct and on calf and rat uterus, but for the present discussion the information available is considered to be valid for other oestrogen target tissues as well.

In the circulation, oestradiol is present mainly bound to plasma proteins, which have a large binding capacity, a moderate to high binding affinity ($K_d \approx 10^{-6}$ M), but a low binding specificity (Westphal, 1971). In human plasma sex hormone binding globulin (SHBG) is present which binds testosterone and oestradiol with high affinity, but low capacity (Murphy, 1968; D.C. Anderson, 1974). A comparable high affinity binder for oestradiol has not been observed in adult rat plasma (Murphy, 1968). In young rats, however, a high affinity oestradiol binding protein, α-fetoprotein, is present (Nunez et al., 1971). Such a protein has also been found in mammary

tumours (Nakao et al., 1978).

The free oestradiol present in the plasma can pass the cell membrane by simple diffusion (Müller et al., 1979). In the plasma of target cells the oestradiol encounters proteins which have a limited number of high-affinity, oestrogen-specific binding sites. These proteins are termed "Receptors". In this regard, α-fetoprotein is not identical to the oestradiol receptor (Radanyi et al., 1977). After binding of oestradiol to its cytoplasmic receptor the oestradiol-receptor complex undergoes a heat- and temperature-dependent conformational change which activates the complex to translocate to the nucleus (Gorski et al., 1968; Jensen et al., 1968; DeSombre et al., 1975; Weichman & Notides, 1977). The nucleus is thought to contain at least two types of binding sites for the oestradiol-receptor complex. In the model proposed by Yamamoto & Alberts (1975) it is suggested that the oestradiol-receptor complex exerts its effects by binding to a limited number of high affinity acceptor sites on the chromatin. In addition, the oestradiol-receptor complex would have a low affinity for non-specific DNA sequences, which would outnumber the specific binding sites. Translocation of oestrogen receptors to the nuclei will result in an increase of mRNA synthesis (Means et al., 1975; Schimke et al., 1975; Woo & O'Malley, 1975). Palmiter et al. (1976) have suggested that oestrogen-receptor complexes which bind to the high affinity DNA binding sites move along the genome, in a manner comparable to ribosome translocation along mRNA, until they encounter a locus where they can influence mRNA production. In contrast, oestrogen-receptor complexes which bind to the non-specific DNA sequences are not capable of such a translocation and exert no effect on the cell. The mRNA formed as a result of the interaction of the oestrogen-receptor complex with the genome is translated on the ribosomes and new proteins are synthesized which may cause a change in the metabolic activity of the cell. Among the effects oestrogens have on target tissues are the increased syntheses of the so-called "Induced Protein"

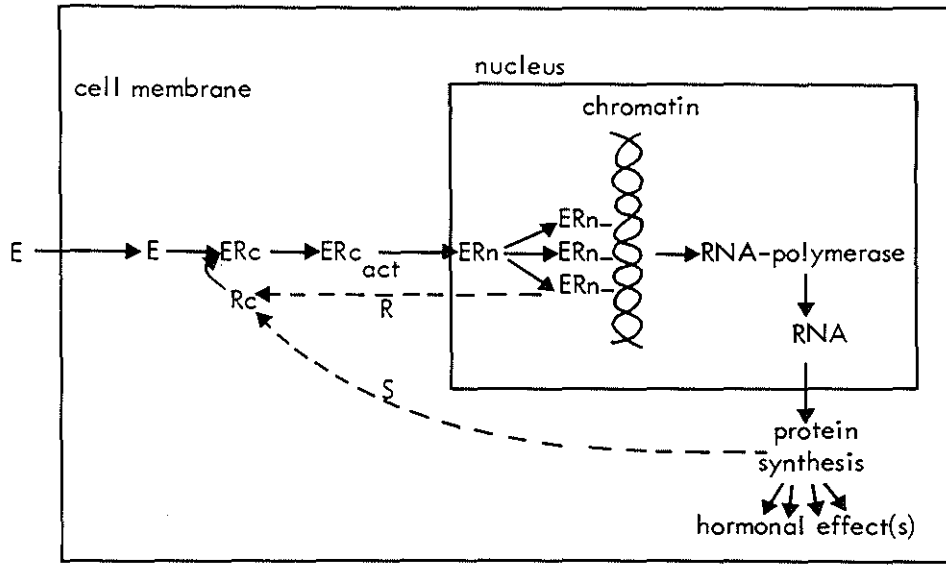

Figure 2.1. Simplified scheme for the action of oestrogens (E) on their target cells.
Free oestrogens (E) enter the cell, bind to the cytoplasmic oestrogen receptor (Rc) to form the oestrogen-receptor complex (ERc). This ERc migrates into the nucleus (ERn). Binding of ERn to the chromatin triggers RNA synthesis which ultimately results in the hormonal effect. Receptors in the cytosol could be replenished by synthesis (S) and/or reactivation (R) as discussed in section 2.4.

(I.P.) (Notides & Gorski, 1966; Means et al., 1975; Schimke et al., 1975), of ovalbumin (Palmiter et al., 1976) and of several nuclear proteins (Hemminki, 1977).

After completing its task, the oestradiol-receptor complex has to dissociate from its nuclear binding site. It is still unclear whether the receptor is degraded or partly reused for binding of oestradiol. A simplified scheme of oestrogen action on its target cell is given in Figure 2.1.

The results of several recent investigations have raised some questions concerning the general validity of the described model of oestrogen action depicted above. The demonstration of unoccupied nuclear oestrogen receptors in human

mammary tumours (Garola & McGuire, 1977; Zava et al., 1977) and pig uterus (Jungblut et al., 1978) makes an obligatory role of oestradiol on receptor activation and translocation questionable. In addition, Linkie & Siiteri (1978) have suggested that the translocation of the steroid-receptor complex would be a process of simple diffusion and that the transformation of receptors would occur in the nucleus. A third complicating factor is the presumed presence of more than one oestradiol receptor in target tissues (Clark et al., 1978; Eriksson et al., 1978). In spite of these apparently contradictory findings, there is ample information that justifies for the time being to accept the scheme depicted in Figure 2.1 as a working hypothesis for the biochemical mechanisms involved in hormone action.

2.5. The modulation of oestrogen receptor levels

Based on the model of hormone action presented in Figure 2.1, the effect of a hormonal stimulus is believed to depend on the number of hormone-receptor complexes which are bound to the target cell chromatin, hence a response would directly depend on the number of receptor sites available for hormone binding. From data obtained by Sarff & Gorski (1971) and Mester & Baulieu (1975) it appears that the number of receptor sites available for hormone binding, i.e. the number of cytoplasmic receptor sites, would be under the control of two processes. Following depletion of receptor sites from the cytoplasm by translocation to the nucleus a "reactivation" process, not requiring protein synthesis, as well as a cycloheximide-sensitive "synthetic" process would replenish the cytoplasmic receptors. The reactivation process would be triggered only when cytoplasmic oestrogen levels decrease by 50% or more, whereas the "synthetic" process would account for replenishment of smaller amounts of depleted oestrogen receptors. Indirect evidence that oestrogens influence the concentration of their own receptors comes from experiments

in which the receptor levels of target tissue have been studied throughout the oestrus cycle. It has been demonstrated that cytoplasmic and nuclear oestrogen receptor levels in rat uterine tissue are correlated with the concentration of circulating oestrogen (Hawkins et al., 1977; Thrower et al., 1977; Myatt et al., 1978; Shih & Lee, 1978). More direct evidence on this matter has come from the investigations of Sutherland et al. (1977) and Baudendistel et al. (1978), who have shown that oestrogens can induce the synthesis of their own receptors.

Prolactin is also thought to be involved in oestrogen receptor modulation. The decrease in rat liver oestrogen receptor following hypophysectomy could be partly prevented by prolactin administration (Chamness et al., 1975). In DMBA-induced rat mammary tumours, prolactin has been found to increase the number of oestrogen receptors (Leung & Sasaki, 1975; Sasaki & Leung, 1975; Hawkins et al., 1977; Asselin & Labrie, 1978). No effect of prolactin has been observed on uterine oestrogen receptor levels (Asselin & Labrie, 1978).

Summarizing the data presented in this section, it would appear that oestrogen as well as prolactin are capable of increasing oestrogen receptor binding capacity.

2.6. Oestrogen receptors in the mammary gland

The mammary gland, similar to other oestrogen target tissues, contains oestrogen receptors. Sander & Attramadal (1968) demonstrated by autoradiography a selective accumulation of intravenously injected radioactive oestradiol in mammary gland epithelium of adult virgin rats and of pregnant and lactating rats. Uptake of ^{3}H-oestradiol by isolated quiescent rat mammary parenchymal cells has also been demonstrated (Grubbs & Moon, 1974). Using a more refined autoradiographic technique, Sar & Stumpf (1976) reported that after *in vivo* exposure of mammary tissue from lactating rats to radioactive oestrogens, the label was present mainly in the nuclei

of epithelial cells, of acini and ducts. Wittliff et al. (1972) showed that the cytosol of lactating rat mammary gland contains a macromolecule which specifically binds ^{3}H-oestradiol with high affinity. This protein has a sedimentation coefficient of 8 S at low salt concentrations. This was further substantiated by Gardner & Wittliff (1973), who showed that the macromolecule was a protein and that the sedimentation coefficient changed from 8 S to 4-5 S when the protein was brought from a low to a high salt concentration. Leung et al. (1976) demonstrated the presence of the oestradiol receptor also during the post-lactation involution of the mammary gland. Park & Wittliff (1977), using a DNA-cellulose binding assay, demonstrated that the cytoplasmic oestradiol receptor from lactating rat mammary gland undergoes an oestradiol- and temperature-dependent transformation analogous to oestrogen receptors from other oestrogen target tissues. Unlike the uterine oestrogen receptor, however, the mammary gland oestrogen receptor did not show a 4 S - 5 S change in the sedimentation coefficient upon activation, but remained completely in the 4 S form.

The presence of oestrogen receptors, also in non-neoplastic mammary tissue, is not generally accepted. From autoradiographic experiments, the concentration of oestrogen receptors appears to be lower in normal, quiescent mammary tissue than in mammary tissue from pregnant and lactating rats or in mammary tumours (Grubbs & Moon, 1974). In normal human mammary tissue and benign mammary tumours, the oestrogen receptor concentration was also found to be lower than in malignant tumours (Wagner & Jungblut, 1976). This probably accounts for the extensive use of stimulated mammary glands (lactating or pregnant) in most of the studies discussed above. The apparently low oestradiol receptor concentration in non-neoplastic, quiescent mammary tissue could, on the other hand, also be due to the presence of endogenous unlabelled oestradiol, which would compete with the radioactive oestradiol added to detect the receptor sites (Fishman

et al., 1977). This methodologic problem will be dealt with in Chapter 5, concerning the "Estimation of oestrogen receptor sites".

2.7. References

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Chapter 3

INDUCTION OF MAMMARY TUMOURS IN FEMALE RATS

3.1. Introduction

It has been suggested that carcinogenesis is a two-step process (Rous & Kidd, 1941; Berenblum, 1947). Initiation of transformation of normal cells to latent tumour cells would be followed by promotion of proliferation of these cells, which would ultimately result in the formation of the actual tumour. Initiators of neoplastic transformation are found among chemical and physical agents, such as dimethylbenzanthracene (DMBA) (Huggins et al., 1959), N-nitroso compounds (Singer, 1979) and ionizing radiation (Kellerer & Rossi, 1975). Promotors of tumour growth are found among chemical agents, immunological and nutritional factors and hormones (Sivak, 1979).

3.2. Experiments on the induction of mammary tumours in female rats by oestrogen and ionizing radiation

The mechanism of the synergistic interaction of oestrogens and radiation in rat mammary carcinogenesis is still unknown. However, the model might be useful as an experimental counterpart of human mammary cancer (cf. Chapter 1). Therefore, it was decided to characterize this model with respect to type and dosage of radiation, which will be effective in causing mammary tumour formation, as well as with respect to the endocrine factors possibly associated with the carcinogenesis. This tumour model could easily fit the two-step mechanism proposed by Berenblum (1947). The primary carcinogenic transformation would then be initiated by ionizing radiation (Kellerer & Rossi, 1975), whereas oestrogen and/or prolactin would promote the further growth of the transformed

cell(s) and would act as the postulated "promotors". As mentioned in Chapter 1, the experiments described in this thesis will focus on the endocrine aspects (oestrogen receptors, prolactin) during carcinogenesis. These experiments were part of a larger project on mammary gland carcinogenesis and have been carried out in collaboration with the Radiobiological Institute TNO, Rijswijk, The Netherlands (Dr. J.J. Broerse, Dr. D.W. van Bekkum), where the radiation aspects are studied.

The early studies on the synergistic action of oestrogens and ionizing irradiation (Segaloff & Maxfield, 1971; Shellabarger et al., 1976) were carried out with diethylstilboestrol (DES) and X- and neutron-irradiation in A x C rats. In these studies the DES was administered through 20 mg pressed cholesterol pellets containing 5 mg of DES. In this way Segaloff & Maxfield (1971) as well as Shellabarger et al. (1976) were able to demonstrate the synergism between DES and radiation in rat mammary gland carcinogenesis. The main disadvantages to the use of DES are that it has to be used in high doses, which cause unwanted side effects, like loss of hair and impaired growth, and that DES does not occur in the rat under physiological circumstances. In addition, a reliable assay for DES in plasma was not available at the time the present studies were planned. As possible alternatives to the use of DES we considered the use of oestradiol and 17α-ethinyloestradiol (EE). EE, like DES, does not occur physiclogically, but it might be more relevant than DES, since it is widely used in oral contraceptive preparations.

We have studied the way in which oestradiol and ethinyloestradiol had to be administered to female rats in order to achieve a sustained release of oestrogen from the depot resulting in a moderately elevated plasma level of oestrogen over a prolonged period of time. The results of these studies are given in Appendix Paper I. From these results we concluded that subcutaneous implantation of oestrogen-cholesterol-paraffin pellets prepared by melting a mixture of the com-

pounds would meet our demands for both oestrogens.

The ultimate goal of animal model studies is to extrapolate the results to the human situation. When studying effects of chemicals, the comparative metabolism of the compounds should be taken into account. In the human female, oestrone and oestradiol are readily interconvertible and are metabolized mainly to 2-, 6-, 16α- and 16β-hydroxylated oestrogens, which are secreted into the urine (Fotherby, 1974). Rats metabolize oestradiol in a similar way, although the major route of oestrogen elimination in this species is via the bile and the faeces (Ball et al., 1974; Fotherby, 1974). Metabolism of ethinyloestradiol in the human also involves the hydroxylation reactions mentioned above, but in addition, fifteen to eighteen percent of the urinary metabolites of EE are de-ethinylated (Williams et al., 1975). In the rat, such de-ethinylation has not been observed (Ball et al., 1973; Helton et al., 1977). Furthermore, the conjugation of EE in man and rats differs considerably (Ghatei & Fotherby, 1979).

Based on the results of the experiments described in Appendix Paper I and the considerations above, we have chosen to perform all experiments with oestradiol which was implanted subcutaneously in cholesterol-paraffin fused pellets.

3.3. Experiments on the induction of mammary tumours in the rat with dimethylbenzanthracene

3.3.1. Introduction

The measurement of oestrogen receptors in tissues from oestrogen-treated rats is influenced by the occupation of receptor sites by the oestradiol administered via the pellet. Hence a technique had to be devised which would make it possible to estimate oestrogen receptors irrespective of the receptor occupancy. This technique will be described in Chapter 5. For proper evaluation of such a technique it was

necessary to use tissue which would closely resemble the mammary gland tissue and which would contain oestrogen receptors. It was decided to use DMBA-induced mammary tumour tissue for this purpose. The DMBA-induced rat mammary tumour was originally described by Geyer et al. (1951, 1953), Huggins et al. (1959, 1961) and Huggins & Yang (1962). The behaviour of this tumour in response to therapy closely resembles that of many human mammary tumours (Mobbs & Johnson, 1974; Fiebig & Schmähl, 1977).

A small survey of literature data on the carcinogenic response of two different rat strains to DMBA is given in Table 3.1. Fractionated gastric administration of the carcinogen gives a shorter latency period in tumour development than a single administration of the total dose (Takizawa, 1973). Therefore, this approach was tested on rats from three different strains.

3.3.2. Materials and Methods

Sprague-Dawley rats were obtained from C.R. France S.A., St. Aubin-le-Boef, France (F) and from the "Institut für Versuchstierzucht, Hannover, Germany (I.V.H.). Fisher rats were bred in our own colony from parents obtained from C.R. France S.A., St. Aubin-le-Boef, France. Wistar rats (R-Amsterdam substrain) were also bred in our own colony.

Dimethylbenzanthracene (DMBA) was purchased from Fluka, Basel, Switzerland and was dissolved in peanut oil to a concentration of 10 mg/ml. Gentle heating was applied when necessary. The carcinogen (10 mg) was administered via a stomach tube to 54-61 days old animals under light ether anaesthesia. A second and third dose of 10 mg DMBA were given one and two weeks later. The animals were palpated weekly and the occurrence of tumours was recorded.

Table 3.1.

LITERATURE DATA ON THE INDUCTION OF MAMMARY TUMOURS IN THE RAT WITH DMBA

strain, route of administration	dosage schedule	time between first treatment and tumour appearance (months)	tumour incidence (%)	total number of tumours (number of rats)	reference
Wistar					
gastric	1x30 mg	7-8	100		Takizawa, 1973
	2x15 mg	3-4	100		
	6x5 mg	2-3	100		
intravenous	3x2 mg	2	100	56 (16)	Feuer et al., 1976
Sprague-Dawley					
gastric	1x16 mg	1.5-2	100		Sasaki & Leung, 1975
	1x20 mg	1	100		Huggins et al., 1961
	1x20 mg	3-4	100		Jordan, 1976
	2x3x1.5 mg	5	90		Geyer et al., 1953
intravenous	1x5 mg	2.5	100	87 (13)	Kledzik et al., 1976

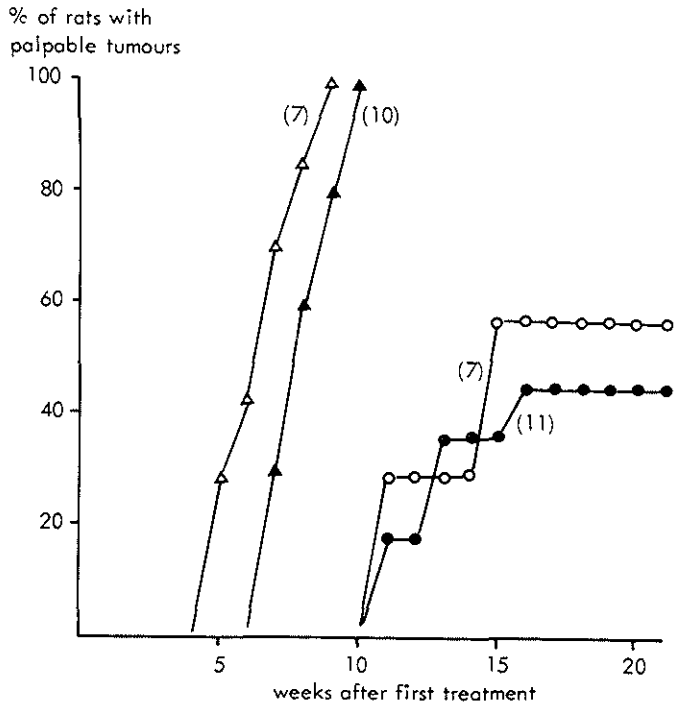

Figure 3.1. Occurrence of mammary tumours in rats of different strains following intra-gastric administration of DMBA.
Δ-Δ Sprague-Dawley rats IVH; ▲-▲ Sprague-Dawley F; o-o Fisher; ●-● Wistar R-Amsterdam. The numbers in parentheses refer to the total number of rats for each strain.

3.3.3. Results

The occurrence of mammary tumours in the rats of different strains is depicted in Figure 3.1. From the strains tested, rats from the Sprague-Dawley strain give the best results when a large number of tumours is required in a relatively short period. In contrast to the rats of the Fisher and Wistar strains, all Sprague-Dawley rats developed mammary tumours. Sprague-Dawley rats developed 8-15 tumours per animal, whereas in the other strains only single tumours were observed. In addition, the tumours appeared much faster in the Sprague-Dawley rats. Based on these results, we have

chosen Sprague-Dawley rats to be the hosts of our DMBA-induced mammary tumours. Since the mammary tumour incidence did not differ for the two Sprague-Dawley strains, rats from C.R. France S.A. were used for our further studies, because it was easier to obtain these rats than the German rats.

3.4. Summary

In this chapter, two models have been described for the induction of mammary tumours in the rat. The first model, in which mammary tumours are induced by the synergistic action of oestrogens and ionizing radiation, will be the subject of the studies described in Chapter 6 and Appendix Paper III. In this chapter the mode of administration and the nature of the oestrogen to be administered have been evaluated. It was concluded that administration of oestradiol through cholesterol-paraffin fused pellets can be used for a sustained release of oestrogen, resulting in moderately elevated plasma oestradiol levels.

The tumorigenic response of three rat strains to DMBA was investigated in order to obtain a large number of mammary tumours, required to evaluate an oestrogen receptor assay which would not be affected by the presence of endogenous oestrogen. After three intra-gastric applications of DMBA, Sprague-Dawley rats developed more tumours with a shorter latency period than Wistar and Fisher rats.

3.5. References

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Chapter 4

MATERIALS AND METHODS

4.1. Preparation of oestradiol pellets

In Chapter 2 and Appendix Paper I a general outline is given of the method for the preparation of the oestrogen pellets which were used for implantation. The oestrogen pellets used in the experiments to be described in Chapter 6 were prepared as follows:

- 5400 mg Paraffin (Merck, Darmstadt, Germany, m.p. 69-73°C) were placed in a 20 ml round bottomed tube which was heated to 180°C in an oil bath.
- 1800 mg Cholesterol (Sigma, St. Louis, MO, U.S.A.) were added.
- After homogenization of the melt, 800 mg of oestradiol (a gift from Diosynth, Oss, The Netherlands) were slowly added. The oestradiol dissolved in the melt under evolution of vapour bubbles.
- The melt was aspirated in silicone tubing Ø I.D. 3 mm, and allowed to cool overnight at room temperature (18-20°C).
- The steroid-filled tubing was cut in slices of 3 mm with the aid of a mould and the tubing was removed from the pellets.
- Pellets weighing 19.5-20.5 mg were selected for implantation.

For the total number of 152 pellets the mean weight was 20.11 ± 0.27 mg (s.d.). It should be noted that cholesterol and paraffin were used in a 1 : 3 ratio (w/w) rather than in a 1 : 10 (w/w) ratio as described in Appendix Paper I, since oestradiol dissolved faster in a melt containing a higher amount of cholesterol (K.J. van den Berg, personal communication). This change had no effect on plasma levels of oestra-

diol measured at different times after implantation (Chapter 6, Appendix Paper III).

4.2. Irradiation of the animals

The total dose of ionizing radiation used was administered as a single dose of 2 Gy (200 rad) X-rays. During the irradiation the animals were kept in small nylon containers rotating coaxially with the radiation beam axis (Broerse et al., 1978). The irradiations were performed by Dr. J.J. Broerse at the Radiobiological Institute T.N.O., Rijswijk, The Netherlands.

4.3. Radioimmunoassay of prolactin

For estimation of prolactin levels in the plasma samples, a homologous double antibody radioimmunoassay was used, which is based on the method described by Kwa et al. (1969). Rabbit antisera against rat prolactin and rat prolactin preparations for iodination were kindly provided by Dr. H.G. Kwa (Netherlands Cancer Institute, Amsterdam, serum K/RP-280) and Dr. A.F. Parlow (National Institute of Arthritis, Metabolism and Digestive Diseases, Rat Pituitary Hormone Distribution Program, N.I.H., Bethesda, MD, U.S.A., serum NIAMD-Anti-Rat Prolactin-S-6), who also supplied us with a rat prolactin reference preparation (RP-1).

A commercially available iodinated rat prolactin preparation (New England Nuclear, Dreieich, Germany) was used as a tracer in the prolactin assay. The specific activity of this preparation was 42.1 $\pm$ 1.4 µCi/µg (mean $\pm$ s.e.m., n = 6). Immediately upon arrival the label was diluted to a final concentration of 10,000 cpm/50 µl (0.5 ng) with 0.1% Bovine Serum Albumin (BSA) in 0.01 M phosphate-buffered saline (PBS) pH 7.6, containing 0.05% sodium azide. Stock solutions (1%) of antisera were prepared in PBS, containing 0.25% Normal Rabbit Serum (NRS). Working solutions of the antisera were prepared by dilution of the stock solutions with 1% NRS in PBS.

Stock solutions of the reference preparation (RP-1) were prepared in PBS, containing 1% BSA. This buffer was also used to adjust the final volume in the assay tubes to 0.8 ml.

Precipitation of the antigen-antibody complexes was achieved by incubation with 0.2 ml of a 5% solution of Donkey Anti Rabbit serum (Wellcome, Beckenham, U.K.) in PBS containing 0.5% BSA. The supernatant was separated from the precipitate after centrifugation at 4°C for 30 min at 1500xg in a MSE PL-100-A Coolspin centrifuge. The precipitate was washed once with ice-cold PBS and counted in a Searle Model 1195 gamma-counter. All incubations were performed at 6°C. Incubation periods of 48 hours with the first antibody, and 24 hours with the second antibody were used.

The binding of the label to different dilutions of the antisera was evaluated. The results are depicted in Figure 4.1. From these results it was concluded that the titer of the K-RP-280 serum was 4 times higher than that of the NIH-S-6 serum. Routinely, the K-RP-280 was used in a 1 : 10,000 dilution (final dilution 1 : 40,000). Standard curves were constructed with 0.8 - 50 ng of RP-1. The label is displaced from the antiserum by prolactin in rat plasma and pituitary cell culture medium in a dose-dependent way, parallel to RP-1 (Figure 4.2). Samples were assayed in at least four dilutions. The amounts of prolactin in the samples were calculated from the linear part of the ln-logit transformed standard curve*. Characteristics of the performance of the prolactin radio-immunoassay are given in Table 4.1.

Prolactin levels in plasma obtained at 09.00 h from our Sprague-Dawley rats throughout the oestrus cycle are shown in Figure 4.3, together with the oestradiol levels, which were assayed with the method described by De Jong et al. (1973).

*The computer program was written by W.F. Clotscher (Department of Biochemistry II, E.U.R.).

Table 4.1.

Characteristics of rat prolactin radioimmunoassay (means ± s.e.m., n = 8).

% initially bound tracer (B_o)	51 ± 1
mass at 50% inhibition	2.0 ± 0.2 ng RP-1
correlation coefficient standard curve	0.995 ± 0.001
intra-assay variation	4.7 ± 1.2%
inter-assay variation	14.9%

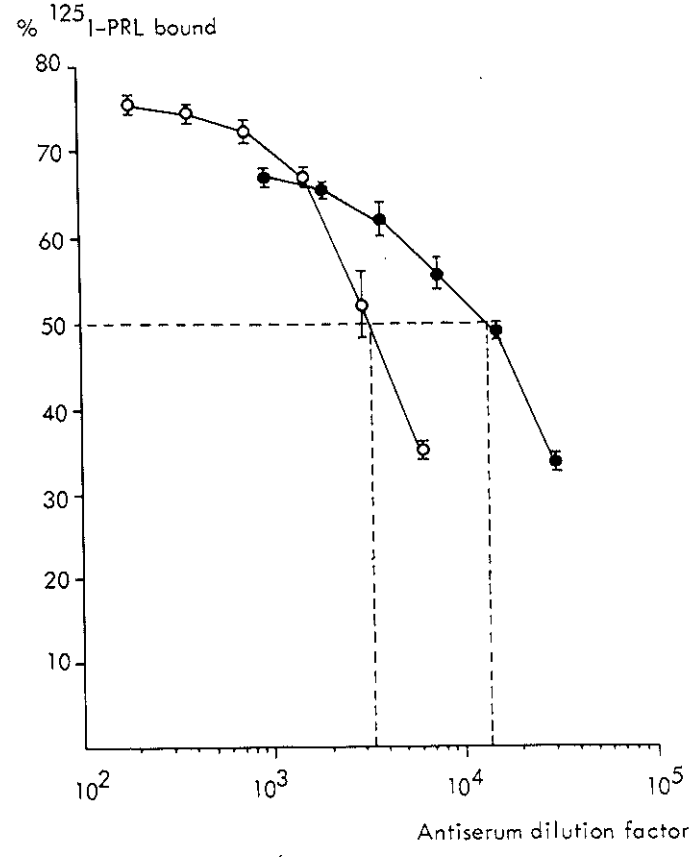

Figure 4.1. Antiserum titration curves with ^{125}I-prolactin (^{125}I-PRL). o——o NIH-S-6, •——• K-RP-280. Results are means ± s.d. (n = 4). The abcissa gives the dilution of antisera added to the tubes; the final dilution in the tubes is 4 times as high.

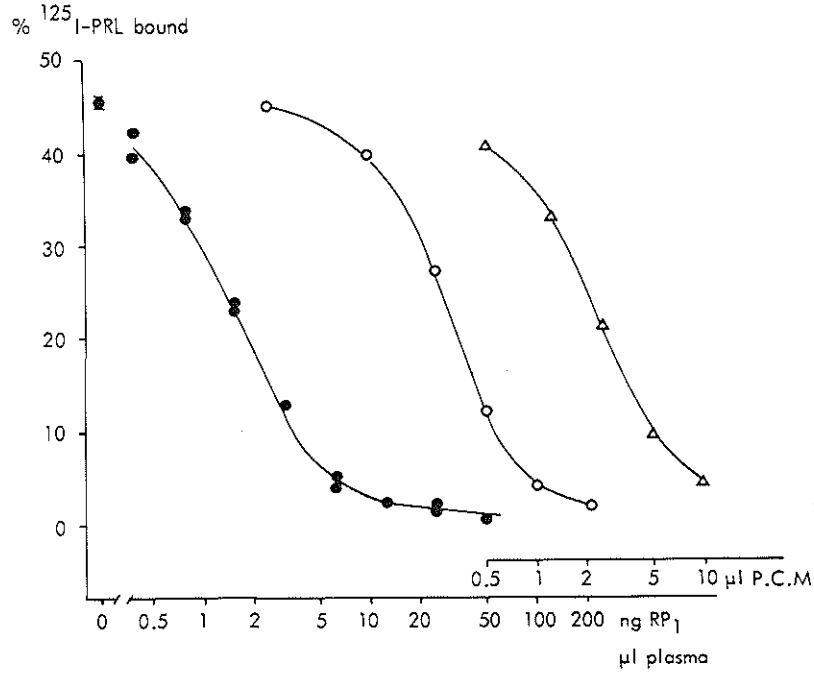

Figure 4.2. Displacement of ^{125}I-prolactin (^{125}I-PRL) bound to 1 : 40,000 diluted K-RP-280 antiserum by RP-1 (•——•), rat plasma (o——o) and pituitary culture medium (PCM) (Δ——Δ).

4.4. Estimation of DNA

DNA was estimated by the method of Giles & Myers (1965) in nuclear pellets, extracted with 0.4 M KCl, obtained from mammary gland tissue (Chapter 5). The pellets were dissolved in 1 N KOH by heating to 60°C for 30 minutes. Remaining fat was separated from the solution by centrifugation at 30,000xg for 30 min. The fat-free solution was obtained by piercing the bottom of the centrifuge tubes and aspirating the solution. DNA and protein were precipitated by incubation with 1 ml 1.2 M perchloric acid for 30 min at 6°C. Subsequently, the DNA was hydrolized by heating the tubes for 15 min at 70°C. To all tubes 1.2 ml of 3% diphenylamine solution and

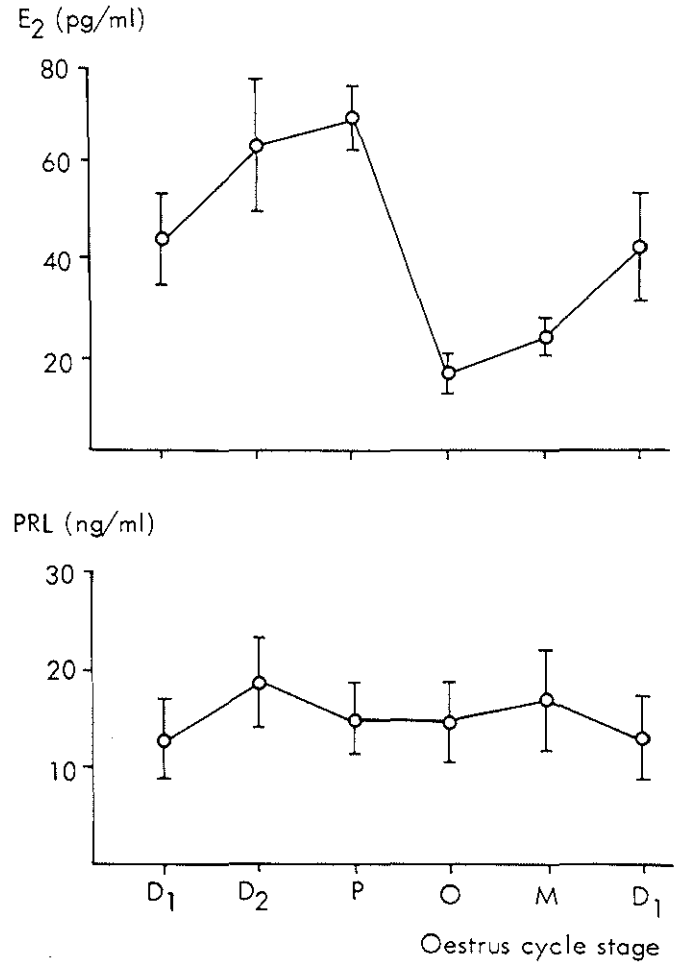

Figure 4.3. Plasma oestradiol (E_2) and prolactin (PRL) in Sprague-Dawley rats at 09.00 h at different days through the oestrus cycle (results are means ± s.e.m., n = 5). P = proestrus; O = oestrus; M = metoestrus; D_1 = dioestrus-day 1; D_2 = dioestrus-day 2.

50 µl of 0.16% acetaldehyde were added. The tubes were incubated for 18 h in the dark and after centrifugation for 10 min at 1000xg the extinction at 595 nm was estimated. Calf thymus DNA (Sigma, St. Louis, U.S.A.) was used as a standard.

4.5. References

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Chapter 5

ESTIMATION OF OESTROGEN RECEPTOR SITES

5.1. Introduction

Part of our studies concerned a possible role of oestrogen receptors in the mechanism of oestrogen-stimulated carcinogenesis of rat mammary gland. In this respect it was necessary to estimate the content of oestrogen receptors in mammary glands of normal virgin rats. Since some groups of rats to be studied received exogenous oestradiol (Chapter 3), such a receptor assay should be independent of the amount of "endogenous" oestradiol present in the mammary tissue. In addition, it should be possible to measure nuclear receptors since at least part of the receptors was expected to be present in the nuclear compartment. Furthermore, the assay should permit to reach unequivocal conclusions about the possible presence of oestrogen receptors in normal mammary glands of adult virgin rats, which had not been demonstrated previously.

The experiments to be described in this chapter have been carried out with the purpose:

1) to demonstrate the presence of oestrogen receptors in normal virgin rat mammary glands;
2) to develop an oestrogen receptor assay for the measurement of total (free and occupied) oestrogen receptors in nuclear preparations of DMBA-induced rat mammary tumours and
3) to evaluate whether oestrogen receptors, present in normal rat mammary tissue, could be estimated routinely with this assay.

5.2. Measurement of steroid receptors

Receptors for steroid hormones are frequently measured by incubation of tissue or tissue fragments with a saturating

dose of radioactive steroid to label the receptors with steroid. Receptor-bound and free steroid are then separated and the amount of binding sites is calculated from the amount of receptor-bound radioactivity. In addition to binding to specific, high-affinity, low-capacity receptors, steroids can be bound also by non-receptor proteins (cf. Chapter 2). Such non-receptor proteins usually have a lower affinity and a larger capacity for binding of steroids and this type of binding is generally termed "aspecific binding". In assaying the amount of specific receptors, a correction has to be made for the aspecific binding.

In our experiments we have used parallel incubations with a 100-200 fold excess unlabelled oestradiol to correct for aspecific binding. Since steroid receptors have a low binding capacity, the amount of receptor-bound radioactivity in the second incubation will be very small. The non-receptor binding proteins generally have a high capacity, hence the amount of "aspecifically" bound radioactivity will not be decreased in the second incubation. The amount of receptor-bound radioactivity is then determined as the difference of the results of the two incubations. Clearly, this type of correction is only valid as long as the excess non-radioactive steroid is not saturating the non-receptor binding sites (Chamness & McGuire, 1976).

5.3. Demonstration of cytoplasmic oestrogen receptors in mammary tissue from adult virgin rats

5.3.1. Introduction

The presence of oestrogen receptors in mammary glands of lactating rats and in rat mammary tumours is supported by autoradiographic and biochemical evidence (see section 2.6). For the resting mammary gland in virgin rats, there is only autoradiographical evidence which suggests uptake of oestrogens in epithelial cells (Sander & Attramadal, 1968; Grubbs

& Moon, 1974). Biochemical evidence for the presence of oestrogen receptors in normal resting mammary tissue has not been reported yet. The results of Kothari et al. (1977) suggested the presence of very small amounts of oestrogen receptor in the cytosol from mammary glands of young rats. They could not demonstrate, however, the presence of the receptor in cytosols from 140 days old or older animals. This negative result might have been caused by occupancy of the receptor sites by endogenous oestradiol, especially since the level of oestrogen receptors in cytosols of uterine tissue from intact rats as reported by these authors was also very low.

Since our studies were designed to evaluate the possible role of oestrogen receptors in carcinogenesis of the mammary glands of adult virgin rats, the absence or presence of receptors in the mammary glands at risk had to be demonstrated.

5.3.2. Materials and methods

Cytoplasmic oestrogen receptors were measured in tissues from animals which were ovariectomized 3 days before the experiments to avoid receptor occupancy by endogenous oestradiol. Mammary and uterine tissues were isolated from the decapitated animals and were immediately immersed in liquid nitrogen. Tissues were homogenized in a chilled (-190°C) Mikrodismembrator vessel (B. Braun, Melsungen, Germany) for 45 s at maximum amplitude. Two volumes of 0.01 M Tris-HCl buffer containing 0.0015 M EDTA and 0.02% sodium azide (w/v) were added to the powdered tissue. After thawing at 0°C the tissue was suspended in the buffer by vortex mixing. The cytosol was obtained by centrifugation at 100,000 x g at 1°C. The cytosol was incubated overnight at 0°C with 2×10^{-9} M of ^{3}H-oestradiol either in the absence or presence of 4×10^{-7} M unlabelled oestradiol. Separation of bound and free steroid was achieved by agar-gel electrophoresis according to Wagner (1972). The gel was sliced and the amount

of radioactivity was determined in the individual slices (Van Beurden-Lamers et al., 1974).

5.3.3. Results and discussion

The results in Figure 5.1 demonstrate the presence of oestrogen receptor sites in the cytosols of uterine and mammary tissue from ovariectomized rats. Like Kothari et al. (1977) we were not able to demonstrate the presence of oestrogen receptors in the cytosol of mammary tissue from intact rats. This is probably due to the occupancy of oestrogen receptors by endogenous oestradiol and concomitant translocation of the receptors to the nuclear compartment. Therefore, the cytoplasmic oestrogen receptor assay was considered inadequate for a proper assessment of oestrogen receptors in intact and oestrogen-treated virgin female rats which had to be used for the evaluation of the possible role of oestrogen receptors in rat mammary carcinogenesis.

5.4. Assay of nuclear oestrogen receptors in mammary tissue of adult virgin rats

If receptors are involved in the biochemical mechanism(s) of steroid action(s), it may be expected that nuclear receptors are closest to the site of action on the DNA/chromatin. In this respect, the nuclear exchange technique may be the most advanced and theoretically most attractive technique for receptor assay. In this type of assay, free oestrogen receptors in the cytoplasm are translocated to the nuclei either *in vitro* or *in vivo* by unlabelled oestradiol. After isolation of a nuclear fraction the unlabelled oestradiol is exchanged with ^{3}H-oestradiol and receptors are quantified. Different investigators have used variety of exchange conditions for the assay of oestrogen receptors in normal and neoplastic tissues as summarized in Table 5.1. Also for the separation of receptor-bound and free steroid

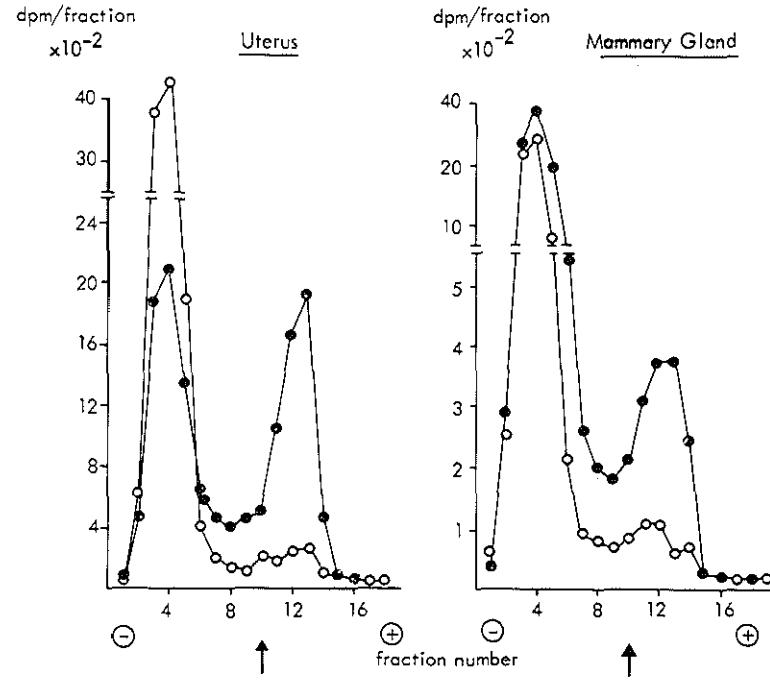

Figure 5.1. Oestrogen receptors in tissues from ovariectomized adult female rats.
Agar gel electrophoretic pattern of radioactivity in uterine and mammary tissue cytosols after incubation with 2 x 10^{-9} M ^{3}H-oestradiol, either in the absence (•——•) or presence (o——o) of 4 x 10^{-7} M unlabelled oestradiol. The arrows mark the application sites of the samples on the agar gel. Free steroids move to the cathode (-) whereas receptor-bound steroid migrates towards the anode (+).

different methods have been used (Table 5.1). The only method which, in addition to quantitative information, also gives a positive identification of the receptor is the nuclear exchange assay described by De Boer et al. (1977). In this method oestrogen receptors are identified by their sedimentation characteristics on sucrose gradients. We have evaluated whether this method could also be applied to the estimation of oestrogen receptors in rat mammary tissues. As a source of tissue we have used the DMBA-induced rat mammary tumour (section 3.3), since these tumours contain oestrogen receptors

Table 5.1. Nuclear exchange assays for oestrogen receptors in different target tissues

Reference	Tissue	Nuclear preparation	Exchange* conditions	Isolation of labelled steroid-receptor complex
Anderson et al. (1972)	immature rat uterus	800xg pellet	1 h 37°C TE-buffer	ethanol extraction
Zava et al. (1976)	rat uterus	protamine sulphate precipitated 0.6 M KCl extract of 800xg pellet	2 h 37°C TED-buffer	ethanol extraction
De Boer et al. (1977)	immature rat uterus	800xg pellet	1 h 37°C TEA-buffer 25% glycerol (v/v)	0.4 M KCl extraction and sucrose gradient centrifugation
	immature rat testis	500xg pellet	15 min 20°C TEA-buffer 25% glycerol (v/v)	
Tsai & Katzenellenbogen (1977)	rat uterus	800xg pellet	45 min 30°C TE-buffer	ethanol extraction
	rat DMBA-tumour	800xg pellet	4 h 15°C TE-buffer 45 min 30°C	ethanol extraction
Nicholson et al. (1977)	rat DMBA-tumour	800xg pellet + protamine sulphate	2 h 15°C TED-buffer	ethanol extraction
Laing et al. (1977)	human mammary carcinoma	5000xg pellet	18 h 4°C 0.15 M NaCl	not specified
Souter et al. (1979)	human endometrium	700xg pellet	18-24 h 4-10°C, 0.15 NaCl	filteration over Millipore filters

*The abbreviations used in the description of the exchange buffers are:
T: Tris-HCl 0.01 M; E: EDTA 0.0015 M; D: dithiotreitol; A: sodium azide.

and are readily available as a source of mammary epithelial tissue. The results of this evaluation are described in Appendix Paper II. The results show that the oestrogen receptor in DMBA-induced rat mammary tumours is unstable when kept at elevated temperatures for long periods. At elevated temperatures dissociation of steroid-receptor complexes will occur at a higher rate, whereas degradation may be kept to a minimum when a short incubation time is used. Exchange at 23°C for 15 minutes was shown to give the same results as exchange at 0-6°C for 1 hour or more. For routine purposes we have used an exchange temperature of 0-6°C.

For the application of the nuclear exchange assay to the estimation of oestrogen receptors in normal (non-neoplastic) rat mammary tissue, a slight modification of the assay procedure had to be introduced. For uterine and DMBA-tumour tissue, translocation of oestrogen receptor sites to the nuclei was successfully achieved by incubation of minced tissue with 10^{-8} M unlabelled oestradiol as described in Appendix Paper II. In non-neoplastic mammary tissue, however, the translocation could not be achieved in this way. This was probably caused by the presence in the virgin rat mammary gland of massive amounts of fat cells, which may inhibit proper penetration of the oestradiol into the tissue. This problem was circumvented by the use of *in vivo* translocation of receptors. Animals were injected subcutaneously with 25 µg of oestradiol. At the time of sacrifice, 1 h later, a plasma oestradiol level of 7.5 ± 1.0 ng/ml (mean ± s.e.m., n = 11) was reached. In this way, nuclear receptors could be detected in the mammary glands of adult virgin rats, whereas cytoplasmic receptors were undetectable. With this modification the amount of oestrogen binding sites in mammary gland tissue of normal adult virgin rats was estimated to be 64 ± 9 fmoles/mg protein in the nuclear extract (mean ± s.e.m., n = 6).

To demonstrate that the oestrogen receptor in normal rat mammary tissue is heat-sensitive, similar to the receptor in DMBA-tumour and uterine tissue (Appendix Paper II), we incu-

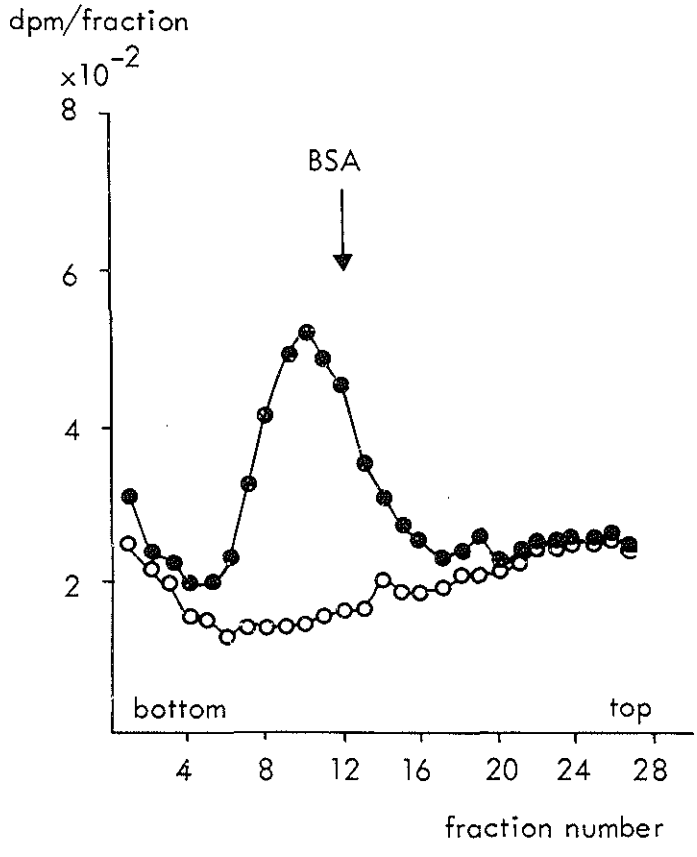

Figure 5.2. Instability of the oestrogen receptor from mammary glands of virgin rats at room temperature. Rats were injected with 25 µg of oestradiol 1 hour prior to sacrifice. Nuclear suspensions of mammary tissue were incubated for 18 h at 6°C (●——●) and 19°C (o——o) with 10^{-8} M ^{3}H-oestradiol. The gradient profiles obtained after incubation with 100-fold excess unlabelled oestradiol coincided with the profile for the 19°C incubation.

bated nuclear suspensions of normal mammary tissue for 18 h at 6°C and 19°C. The results in Figure 5.2 indeed show that the oestrogen binding capacity is destroyed after incubation at room temperature. For the assay of oestrogen receptors in normal rat mammary tissue we therefore also adopted 18 h and 0-6°C as standard conditions for the nuclear exchange.

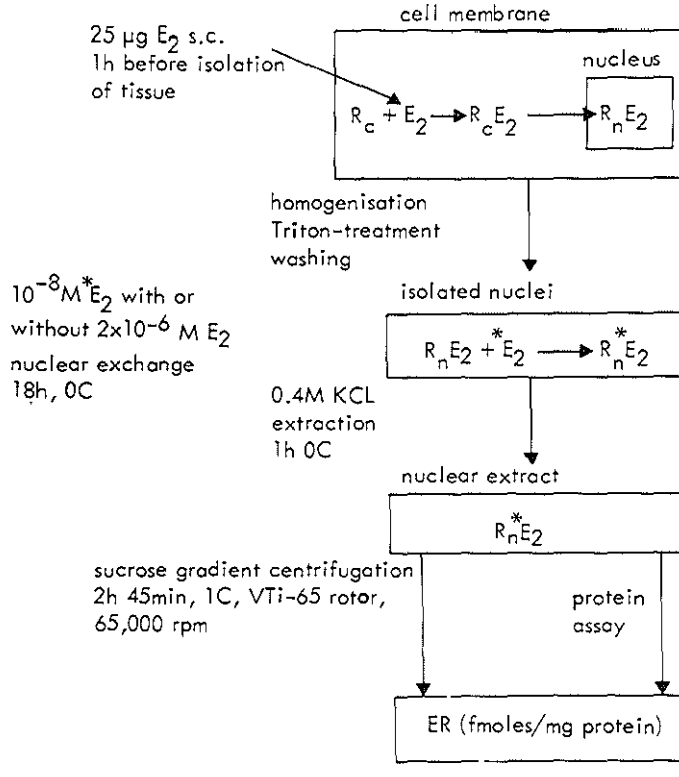

Figure 5.3. Flow-chart for exchange assay of nuclear oestrogen receptors.

A schematic outline of the method used is illustrated in Figure 5.3.

To further evaluate the nuclear exchange assay used, minced uteri of ovariectomized animals were incubated for 1 hour with either 10^{-8} M ^{3}H-oestradiol (direct labelling of receptors) or 10^{-8} M unlabelled oestradiol. Nuclear suspensions were prepared and ^{3}H-oestradiol-receptor complexes were extracted with 0.4 M KCl from the suspension obtained from the tissue incubated with ^{3}H-oestradiol. The nuclear suspension obtained from the tissues that were incubated with radioinert oestradiol were incubated overnight at 6°C with 10^{-8} M ^{3}H-oestradiol to exchange the receptor-bound oestra-

diol. After exchange a nuclear extract was prepared. Oestrogen receptors were estimated by protamine-sulphate precipitation and protein and DNA were measured in the extracts and the pellets respectively. The results of this experiment are presented in Table 5.2. Both receptor assays yield identical results when the binding was expressed as fmoles bound steroid/mg protein (P) in the nuclear extract. When the binding was expressed as fmoles/mg DNA in the pellet, the 32% decrease in the estimated DNA content after the exchange incubation resulted in a significantly higher estimated receptor level after exchange.

Table 5.2.

Comparison of exchange assay vs. direct labelling for estimation of nuclear oestrogen receptors in uteri from ovariectomized rats (means ± s.e.m. (n)).

parameter	exchange	direct	P*
binding			
total (fmole/mg P)	356± 12 (3)	302± 29 (3)	N.S.
aspec. (fmole/mg P)	95± 4 (3)	41±0.3 (3)	<0.01
spec. (fmole/mg P)	261± 13 (3)	261± 29 (3)	N.S.
total (fmole/mg DNA)	3943±228 (3)	2170±290 (3)	<0.01
aspec. (fmole/mg DNA)	1063± 72 (3)	260± 21 (3)	<0.01
spec. (fmole/mg DNA)	2880±150 (3)	1920±270 (3)	<0.05
Pellet DNA (µg)	215± 8 (6)	317± 23 (6)	<0.01
Extract protein (mg/ml)	2.39±0.02 (6)	2.11±0.07 (6)	<0.01

*Student's t-test.

The results in Table 5.2 suggest that the exchange procedure influences the DNA content of the nuclear pellet more

than the protein content of the nuclear extract. The most probable explanation for the observed effect appears to be that, during the exchange, some of the DNA, either intact or split by nuclease, goes into solution and is discarded together with the exchange medium. Also during the extraction of the nuclear pellet, some DNA may dissolve in the nuclear extract. To test this hypothesis, nuclear suspensions were prepared from mammary and uterine tissue, either directly (I) or after incubation of minced tissue for 1 h at 37°C (II). The suspensions obtained from the tissue which was not incubated (I) were incubated overnight at 6°C in exchange medium. All nuclear suspensions were extracted with 0.4 M KCl and DNA was estimated in the exchange media, nuclear extracts and pellets. The results in Table 5.3 indeed show that DNA is lost in the exchange and extraction buffers.

Table 5.3.

Loss of DNA from mammary and uterine nuclear pellets during exchange and direct labelling assays for nuclear oestrogen receptors

Assay	Fraction	DNA-content in different fractions (%, mean ± s.d., n = 3)	
		Mammary Gland	Uterus
direct	nuclear pellet	83.7 ± 2.3	96.2 ± 0.7*
	nuclear extract	16.2 ± 2.1	3.8 ± 0.7*
exchange	nuclear pellet	62.4 ± 2.5**	57.2 ± 6.8**
	exchange medium	19.5 ± 4.4	16.7 ± 1.3
	nuclear extract	18.0 ± 1.9	25.9 ± 6.7**

* $P < 0.05$ vs. incubation of minced tissue

** $P < 0.05$ vs. mammary tissue

5.5. Discussion and Conclusions

From the results of the experiments described in this chapter and in Appendix Paper II the following conclusions were obtained:

1) Oestrogen receptors are present in mammary tissue of adult virgin rats.
2) The rat uterine oestrogen receptor can dissociate its ligand at a low temperature, similar to the mammary gland oestrogen receptor.
3) The DNA content of nuclear pellets from mammary and uterine pellets is significantly lower after exchange assay than after direct labelling assay for nuclear oestrogen receptors.
4) The oestrogen receptors in rat mammary tissue can be estimated with the low-temperature nuclear exchange assay described in Appendix Paper II, provided that oestradiol was administered *in vivo* rather than *in vitro* for translocation of receptor sites from the cytoplasm to the nucleus.

This is the first report in which the presence of oestradiol receptors in mammary glands of adult virgin rats has been demonstrated biochemically. Earlier attempts of Kothari et al. (1977) failed, which is in agreement with our experiments carried out in the presence of endogenous oestradiol. In tissue from intact rats, receptors could only be demonstrated with the use of the nuclear exchange assay and after *in vivo* translocation of the receptors from the cytoplasm to the nucleus. Therefore, it appears that many, if not all, oestrogen receptors in mammary glands are normally occupied with endogenous oestradiol.

The observation that mammary gland, mammary tumour and uterine oestrogen receptors can exchange their ligand even at low temperatures, is in contrast to the results of Anderson et al. (1972), Zava et al. (1976), De Boer et al. (1977), Nicholson et al. (1977), Tsai & Katzenellenbogen (1977) and

Souter et al. (1979). The observations of Laing et al. (1977) with human mammary tumours and of Traish et al. (1979) with isolated rat uterine cells are in agreement with our observations. The reason for this discrepancy is still unclear. It has been suggested that the use of Triton in the preparation of the nuclear pellets might cause the effect, since Triton, even at low concentrations, would increase the dissociation constant of the oestrogen-receptor complex (W. de Boer, private communication, 1979). Preliminary experiments, however, have shown that the exchange of the steroid from the uterine oestrogen receptor occurs also if Triton is omitted from the buffer used to wash the nuclear pellet. This could be explained if the receptor were protected from the Triton as long as it is incorporated in the nuclear pellet. Hence, it appears unlikely that the dissociation of the oestrogen-receptor complex at low temperatures is caused by the use of Triton.

The results in Tables 5.2 and 5.3 show that DNA is lost from the nuclear pellet during the exchange procedure. Since DNA is thought to play an essential role in nuclear binding of the steroid-receptor complex, it is peculiar, that no concomitant decrease in oestrogen receptor concentration was observed (Table 5.2). When a comparison was made between mammary gland oestrogen receptor concentrations expressed per mg of protein in the nuclear extract and per µg of DNA in the nuclear pellet, a statistically significant correlation was observed ($r = 0.667$, $n = 31$, $p < 0.01$). In view of the data in Tables 5.2 and 5.3, it is unclear whether this statistical significance reflects a true biological significance. Therefore, we have expressed the oestrogen receptor level relative to the protein concentration in the nuclear extract (Chapter 6 and Appendix Paper III).

The nuclear exchange assay described in this chapter yields the same results as the <u>in vivo</u> assay in which tritiated oestradiol was injected in ovariectomized animals (Appendix Paper II). Therefore, we have concluded that the nuclear

exchange assay is suitable for the estimation of oestrogen receptors in tissues from intact animals.

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Chapter 6

RAT MAMMARY GLAND OESTROGEN RECEPTORS AND PLASMA PROLACTIN DURING MAMMARY CARCINOGENESIS INDUCED BY OESTROGEN AND RADIATION

Oestrogens and ionizing radiation can act synergistically in the development of mammary tumours in female rats (Segaloff & Maxfield, 1971; Shellabarger et al., 1976; Broerse et al., 1978). The mechanism through which oestrogens enhance radiation-induced mammary carcinogenesis has not been elucidated yet. It is generally accepted that oestrogens exert their effect(s) on target tissues through specific receptors (cf. Chapter 2). Oestrogens can influence the synthesis of oestrogen receptors (Sarff & Gorski, 1971; Mester & Baulieu, 1975) and oestrogens are also known to stimulate pituitary prolactin release (Chen & Meites, 1970). From these observations at least two mechanisms could be considered to explain the effect of oestrogens in rat mammary tumour development. Firstly, oestrogens could influence their interaction with the mammary tissue through inducing the synthesis of their own receptors. Secondly, since prolactin appears to play a major role in mammary tumour development in the rat (Meites, 1972), oestrogens could exert their co-carcinogenic action via an increase in the prolactin concentration in the plasma.

The experiments described in this chapter were performed to relate the synergistic interaction of oestrogen and ionizing radiation in rat mammary tumour development with possible changes in the content of nuclear oestrogen receptors in the mammary glands and the plasma prolactin concentration. For this purpose, nuclear oestrogen receptors were estimated with the nuclear exchange assay, described in Chapter 5 and Appendix Paper II, in mammary tissue of female rats for a period of 14 months after administration of 2 mg of oestradiol (Chapter 3) and exposure to 2 Gy of X-rays. As a result of

this treatment, approximately 50% of the rats was expected to develop mammary tumours (Broerse et al., 1978). Prolactin was estimated in the plasma of the same rats.

The results, described in Appendix Paper III, revealed that oestradiol administration caused a decrease in the oestrogen receptor content of the mammary glands and an increase in the plasma prolactin concentration. Radiation had no additional effect on the parameters studied. During the progress of the experiments it appeared that as a result of the treatment regimen used in the present study, 50% of the rats could be expected to develop a mammary tumour. In addition, it turned out that the synergistic interaction of oestrogens and radiation in mammary tumour development applies only to malignant tumours which accounted for 50% of the total tumour incidence (Van Bekkum et al., 1979). It could be envisaged, that the expected increase in the oestrogen receptor content of the mammary tissue occurs only in those rats which ultimately would develop a malignant mammary tumour (25%). A concomitant decrease in the oestrogen receptor content of mammary tissue of rats which will not develop such tumours could then result in the observed decrease in the mean receptor concentration. If this were true, the frequency distribution of the estimated oestrogen receptor levels should show the existence of two populations of rats, one with a low, and the other with a high content of oestrogen receptors in the mammary tissue. The actual frequency distribution, which is shown in Figure 6.1, does not indicate the existence of two such populations. In contrast, the results in Figure 6.1 once more reflect the general decrease of the estimated content of oestrogen receptors of the mammary tissue observed in oestrogen-treated rats.

The decrease in the oestrogen receptor content of the mammary tissue and the increase in the prolactin concentration of the plasma observed in oestradiol-treated rats occur well before mammary tumours become manifest (Broerse et al., 1978). Therefore it is suggested, that oestrogens exert their cocar-

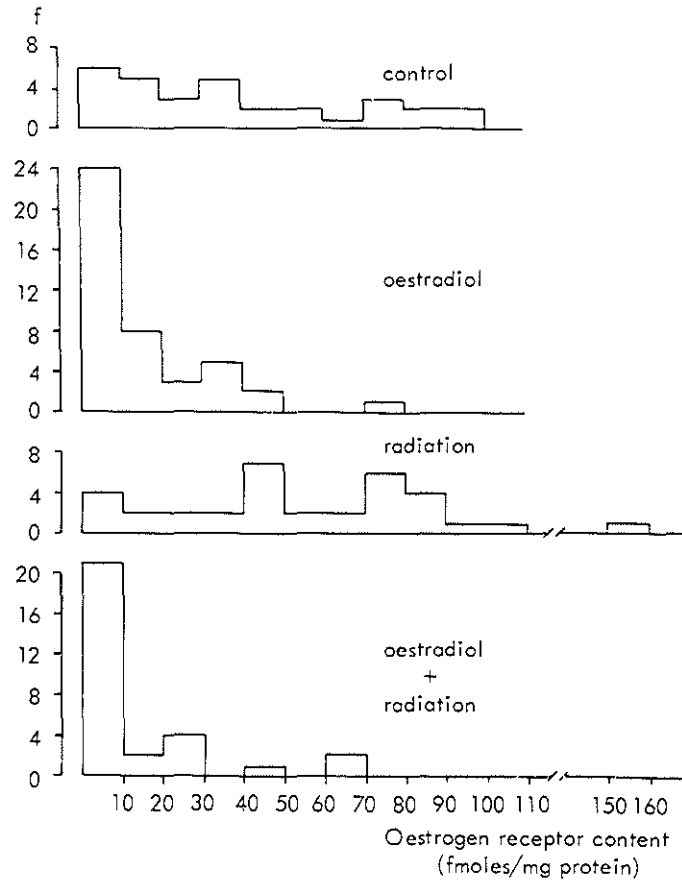

Figure 6.1. Frequency (f) of oestrogen receptor contents estimated in mammary tissue of control rats and of rats treated with oestradiol and/or radiation, from 2-14 months after treatment.

cinogenic effect, if any, through either or both of these changes.

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CHAPTER 7

GENERAL DISCUSSION

The experiments described in the preceding chapters were carried out in an attempt to detect possible changes in the number of oestrogen receptors in mammary tissue and in plasma prolactin before mammary tumours become macroscopically manifest. It was hoped to obtain more insight in the suggested role of oestrogens (Segaloff & Maxfield, 1971; Shellabarger et al., 1976; Broerse et al., 1978; Segaloff & Pettigrew, 1978; Van Bekkum et al., 1979; Holtzman et al., 1979) and prolactin (Meites, 1972; Welsh & Nagasawa, 1977) in the mechanism of mammary gland carcinogenesis. The experiments described in this thesis differed from the approach which is generally used, in that the parameters under study were estimated during the process of carcinogenesis, and not after the tumours had developed. The model system used was the oestrogen- and radiation-induced rat mammary cancer as discussed in Chapters 1 and 3. This model appeared also relevant for the human situation, because of the possibility that the risk for human females to develop mammary cancer might increase as a result of more frequent exposure to ionizing radiation in diagnostic medicine (Upton, 1975) and the concomitant use of oestrogen-containing oral contraceptives by an increasing number of females (Rinehart & Piotrow, 1979).

The main conclusions from the results described in Chapter 6 and Appendix Paper III show, that after oestrogen and radiation treatment the oestrogen receptor content of mammary tissue was decreased and plasma prolactin concentrations were increased. These changes are the result of the administration of exogenous oestradiol, and occur well before mammary tumours are known to develop in these animals. It was suggested that oestrogens exert their effect(s) in tumour development through either one or both of these changes. It has been shown, that oestradiol as such is not carcinogenic in the Sprague-Dawley

rats used in the present study (Van Bekkum et al., 1979). Prolactin is known to stimulate the growth of mammary tumours in the rat (Meites, 1972). Therefore, oestradiol might exert at least part of its enhancing effect in radiation-induced mammary carcinogenesis through an increased prolactin secretion.

In the first section of this chapter the possible involvement of oestrogen receptors in mammary tumour development will be discussed. In subsequent sections, the model system used will be evaluated, and a comparison of rat and human mammary cancer will be made.

7.1. The possible role of oestrogen receptors in rat mammary tumour development

Based on the generally accepted mechanism of steroid hormone action (Chapter 2), several mechanisms could be suggested for the involvement, if any, of oestrogen receptors in the co-carcinogenic action of oestrogens on mammary tissue.

Firstly, the continuous release of oestradiol from the pellet implanted might cause the receptors in the mammary tissue to be permanently translocated into the nuclear compartment. The expression of the effects of oestrogens might depend on the period during which the oestradiol-receptor complex is bound to the chromatin. It has been suggested, for instance, that anti-oestrogens act initially as oestrogens in translocating cytoplasmic receptors to the nucleus, and that the anti-oestrogenic effect of these compounds would reside in their capability to prolong the retention of the receptor in the nucleus and thus interfere with the recycling of the receptors from the nucleus to the cytosol (Clark et al., 1973; Katzenellenbogen et al., 1977). If the continuous presence of oestradiol in our rats would have resulted in a continuous nuclear localization of the receptors in the mammary tissue, thus interfering with the proper recycling of the receptors to the cytoplasm, then oestradiol would be able to

diminish its own effect on mammary tissue. Evaluation of these suggestions will require detailed knowledge of the processing of nuclear oestradiol-receptor complexes, which is presently not available.

A second possibility for the involvement of oestrogen receptors in mammary carcinogenesis could be that oestrogens influence the synthesis of oestrogen receptors in the tissue at risk. From the results obtained in relatively short-term experiments reported by Sarff & Gorski (1971) and Mester & Baulieu (1975), it was suggested (Chapter 2) that oestrogens can stimulate the synthesis of oestrogen receptors in the mammary tissue. It appears from the results reported in Chapter 6 and Appendix Paper III, however, that this does not apply for long-term experiments, such as used in the present study. A decrease was observed in the mammary gland oestrogen receptor content, whereas the uterine receptor content was not affected. Katzenellenbogen et al. (1977) suggested from their studies on the mechanism of action of anti-oestrogens, that prolonged retention of oestrogen receptors in the nucleus could not only interfere with the recycling of receptors, but also prevent de novo synthesis of receptors. This situation might also prevail in the mammary tissue of the rats in our study.

Finally, since normal mammary tissue is considered to contain less oestrogen receptors than mammary tumour tissue (Wagner & Jungblut, 1976), the effect of oestrogens on mammary tumour development might be mediated through a specific increase of the receptor content of cells in preneoplastic tissue or in microtumours. This suggestion of Wagner & Jungblut (1976), however, was based on measurements of cytoplasmic receptors, rather than nuclear or total receptor content. This possibility is supported by the observation that in the cytosol of normal mammary tissue from intact adult female rats no oestrogen receptors could be detected (Kothari et al., 1977; Chapter 5), whereas rat mammary tumours do contain receptors in the cytoplasm (Nicholson & Golder, 1975).

Using the nuclear exchange assay described in Chapter 5, it was possible to demonstrate the presence of oestrogen receptors in normal mammary tissue as well as in mammary tumours (Chapters 5 and 6). The oestrogen receptor level in DMBA tumours appears to be comparable to that measured in normal mammary tissue, when expressed relative to the protein concentration in the nuclear extract. Also in tumours induced with oestradiol and radiation, the receptor content, 19 $\pm$ 7 fmoles/mg nuclear extract protein (mean $\pm$ s.e.m., n = 8, range 4-60), does not exceed that of normal mammary tissue. It would be of interest to compare with the nuclear exchange assay the oestrogen receptor content of human mammary tumours to that of normal mammary tissue. The suggestion, that oestrogen treatment would cause an increase in receptor levels specifically in preneoplastic or microtumour cells, is not consistent with the considerations given above, nor with the overall decrease in receptor content of the mammary tissue as reported in Chapter 6. Such a specific increase in receptor levels also would result in an inhomogenous frequency-distribution of receptor levels, since only 25% of rats given 2 mg of oestradiol and exposed to 2 Gy of X-rays develop a malignant mammary tumour (Van Bekkum et al., 1979). The results in Figure 6.1 do not support an inhomogenous frequency distribution, and therefore it seems unlikely that the action of oestrogens on mammary tumour development is mediated through a specific increase in the oestrogen receptor content of cells that will ultimately develop into a tumour.

Summarizing the considerations given in this section, it appears, that the development of mammary tumours in female rats exposed to exogenous oestradiol, is preceded by a prolonged decrease in the content of nuclear oestrogen receptors in the mammary tissue. This decrease, in combination with a possible interference in receptor replenishment and/or *de novo* receptor synthesis, is suggested to result in a decreased sensitivity to oestradiol of the mammary tissue. It remains to be investigated whether a decreased sensitivity to oestrogens plays a role in the growth of mammary tumours.

7.2. Evaluation of the rat mammary carcinogenesis model used in the present study

The model for mammary carcinogenesis described in the present study is based on the observation that oestrogens and ionizing radiation can act synergistically to increase the occurrence of mammary tumours in female rats (Segaloff & Maxfield, 1971; Shellabarger et al., 1976; Broerse et al., 1978). While our experiments were in progress, it appeared that this synergism was not observed in all rat strains. Moreover, the amount of oestrogen implanted appeared to be important (Holtzman et al., 1979). In Table 7.1 the present knowledge about the simultaneous action of oestrogens and radiation in rat mammary carcinogenesis is summarized. The data in this table show, that, in at least three rat strains (AxC, Wistar and Sprague-Dawley), the action of oestrogen and radiation is accompanied also by an increase in the plasma prolactin concentration and the development of pituitary tumours. In the Sprague-Dawley substrain studied by Shellabarger et al. (1978), no cocarcinogenic effect of DES was observed, whereas the rise in plasma prolactin concentration was "only" 5-fold and no pituitary tumours were observed. Since no cocarcinogenic effect of DES was observed, it is tempting to speculate from this observation, that only a large increase in plasma prolactin concentration as a result of oestrogen administration would be effective as cocarcinogenic stimulus. The published data do not permit a firm conclusion about this aspect, since the observation period in these experiments was rather short (214 days). It would be of interest to estimate also in this Sprague-Dawley substrain the oestrogen receptor content of the mammary gland after DES implantation. If implantation of DES in female Sprague-Dawley rats of the substrain used by Shellabarger et al. (1978) would not cause a decrease of the mammary gland oestrogen receptor content, the involvement in carcinogenesis of the decrease in the receptor content observed in the present

Table 7.1.

Interaction of oestrogens and radiation in rat mammary carcinogenesis

Rat Strain	Type of radiation	Amount and quality of implanted oestrogen	Synergism observed	Rise in plasma prolactin	Pituitary tumour development	References
A x C	X-rays	5 mg DES	yes	n.r.	n.r.	Segaloff & Maxfield, 1971 Segaloff & Pettigrew, 1978
A x C	neutrons	5 mg DES	yes	30x	yes	Shellabarger et al., 1976 Stone et al., 1979
S.D.	neutrons	5 mg DES	no	5x	no	Shellabarger et al., 1978 Stone et al., 1979
S.D.	neutrons X-rays	2 mg E_2	yes	25x	yes	Van Bekkum et al., 1979 This Thesis, Chapter 6
Wistar (WAG/Rij)	neutrons X-rays	2 mg E_2	yes	25x	yes	Van Bekkum et al., 1979 Blankenstein, unpublished
F 344	X-rays	0.98 mg DES 1.6 mg DES 2.6 mg DES 3.9 mg DES	no no yes no	n.r.	n.r.	Holtzman et al., 1979

Abbreviations used:

DES: diethylstilboestrol; E_2: oestradiol; S.D.: Sprague-Dawley; n.r.: not reported.

study (Chapter 6) would become more likely. A disadvantage of such a study, however, would be that the effect of implantation of DES does not necessarily have to be similar to the observed effect of oestradiol. An alternative could be a study on the effect of oestradiol on the mammary gland oestrogen receptor content in prolactin-deprived rats of our Sprague-Dawley strain.

A practical disadvantage related to both studies suggested above is the long latency period (over 12 months) between the exposure of the rats to the carcinogenic stimuli and the manifestation of mammary tumours. Furthermore, large groups of animals are required, since not all rats will develop mammary carcinoma. This disadvantage might be circumvented by the use of DMBA, rather than radiation, as a primary carcinogen. Administration of DMBA to female rats of the proper strain will result in the development of more mammary tumours in a relatively short time (2-3 months, Chapter 3). With respect to the latency period and to tumour incidence, the DMBA model system might, however, be less similar to human mammary cancer than the radiation model.

In summary, it appears necessary to investigate the possible involvement of a decrease in the mammary gland oestrogen receptor content in rat mammary tumour development with the aid of the radiation model in prolactin-deprived rats of the Sprague-Dawley strain used in the present study.

7.3. Extrapolation of data obtained in rat model systems to human mammary gland carcinogenesis

Since the human mammary gland is relatively unaccessible to experimental study of carcinogenesis, it is important to use suitable animal model systems. For a proper extrapolation of data obtained in such model systems to the human situation, a high degree of similarity between the model and human tumours is a prerequisite. A number of factors which enhance the risk for breast cancer has been recognized from epidemio-

logic studies. Some of these risk factors have parallels in rat mammary tumour models, e.g. ionizing radiation (Van Bekkum et al., 1979; Land & McGregor, 1979), dietary fat intake (Carrol, 1975; Chan et al., 1977; Cave et al., 1979; Hopkins & Carrol, 1979). A possible similar role for prolactin in the development of human mammary cancer is less clear. Based on the important role which prolactin could play in rodent carcinogenesis, Boot (1970) stressed the need for an extensive analysis of the relationship between human prolactin and breast cancer. This analysis became possible with the development of a radioimmunoassay for human prolactin (e.g. Kwa et al., 1973), but a specific role for prolactin in human breast cancer has not been found yet (Franz, 1978; Nagasawa, 1979). Kwa et al. (1974) found no differences in plasma prolactin levels between breast cancer patients and control women, but Cole et al. (1977) reported significant differences at certain stages of the oestrus cycle (e.g. on the fifth day preceding the midcycle oestradiol peak, during the follicular and preovulatory phases and among the highest midcycle levels). Recently, Kwa et al. (1978) and Tarquini et al. (1978) reported an abnormal rise in plasma prolactin concentration in the early evening in nulliparous and obese women as well as in nulliparous women with benign or malignant breast disease. Malarkey et al. (1977) reported a change in the nocturnal variation of plasma prolactin in women with breast cancer. These results suggest that prolactin could play a very subtle role in human mammary carcinogenesis.

In men, as in rodents, prolactin secretion appears to be stimulated by oestrogens (Frantz et al., 1972; Yen et al., 1974; Adu-Fadil et al., 1976; Dericks-Tan & Taubert, 1976; Jones et al., 1977). Lind et al. (1978), however, did not observe a significant change in plasma prolactin levels during postmenopausal oestrogen replacement therapy, although the amounts of oestrogen administered were sufficient to cause decreased lutropin and follitropin levels in peripheral plasma. On the other hand, symptoms of amenorrhoea and galac-

torrhoea concomitant with hyperprolactinaemia have been associated with the use of oral contraceptive agents (Fossati et al., 1976; Healy et al., 1977; Tollis, 1977).

It has also been suggested that the occurrence of prolactin-secreting pituitary tumours is related to the use of oral contraceptive preparations (Sherman et al., 1978). Annegers et al. (1978) reported an increase in the incidence rate of pituitary adenomas in 15-44 years old women in Olmsted County, Minesota. Since this increase was not observed in males, the use of oral contraceptives was considered as a possible etiologic factor, but no significant association was found. Therefore it was suggested that the increase in the observed incidence rate of pituitary adenomas might be due to improved diagnostic methods (Annegers et al., 1978; Shearman et al., 1978).

It is tempting to speculate that the pituitary tumours observed in the experiments described in this thesis (Appendix Papers I and III) are comparable to the prolactinomas observed in human females. The symptoms associated with the occurrence of these tumours in rats, i.e. infertility, secretory activity of the mammary gland and hyperprolactinaemia, are quite comparable to those seen in women. If prolactin is indeed capable of acting as a promotor of mammary tumour growth in women, the increase in the occurrence of pituitary adenomas should lead to an increase in the incidence of mammary carcinoma. Such an increase has not been reported yet, but the study of this possibility certainly deserves greater attention.

For the time being, it remains important to unravel the mechanism of mammary carcinogenesis in animal models as far as possible, and to identify the role of environmental factors known to be related to the disease. A better understanding of animal carcinogenesis would certainly offer a better model for comparison with the human situation.

7.4. References

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SUMMARY

Oestrogens and ionizing radiation can act synergistically in the development of mammary carcinoma in female rats. It is still unknown how oestrogens influence the development of radiation-induced tumours. It is well known, that prolactin can promote the growth of rat mammary tumours, and one possibility to explain the effect of oestrogens is through an oestrogen-mediated increase of prolactin in the plasma. Alternatively, it could be possible, that oestrogens affect the synthesis or the availability of their own receptors in mammary tissue, thus influencing the sensitivity of mammary tissue to oestrogens.

It was the aim of the study described in this thesis to detect possible changes in the oestrogen receptor content of the mammary tissue and the prolactin concentration in the plasma of rats exposed to low doses of exogenous oestrogen and/or radiation (Chapter 1). If such changes would occur before the manifestation of mammary tumours, they might be related to the induction or the development of the tumours. Such conditions could also occur in the human through the widespread use of oestrogen-containing oral contraceptives and the increase in environmental radiation.

The method which had to be developed for the administration to female rats of a depot of oestradiol with a sustained release of the hormone, has been the subject of the first part of Chapter 3 and Appendix Paper I. It was concluded, that a satisfactory small increase of oestradiol in peripheral plasma can be obtained after administration of the oestradiol by subcutaneous implantation of a pellet prepared by melting a mixture of oestradiol, cholesterol and paraffin. The exact procedure used in the present study for the preparation of these pellets and other techniques, including methods for the estimation of prolactin in plasma and the DNA content of mammary tissue preparations, are described in Chapter 4.

For a reliable evaluation of the content of oestrogen receptors in tissues obtained from intact animals treated with exogenous oestrogen, a receptor assay was developed which is not influenced by the occupancy of receptors by endogenous or exogenous oestrogen. This assay, which uses exchange of receptor-bound oestradiol with radioactive ligand in nuclear preparations of mammary tissue, is presented in Chapter 5 and Appendix Paper II. To evaluate this assay, it was necessary to use a tissue which is similar to mammary gland tissue and which is known to contain oestrogen receptors. For this purpose we used mammary tumours induced in female rats by oral administration of dimethylbenzenthracene as described in the second part of Chapter 3.

The main experiment in the present study is described in Chapter 6 and Appendix Paper III. In this experiment, rats of the Sprague-Dawley strain were divided among four groups, and were given either no treatment, oestradiol treatment, radiation treatment, or oestradiol treatment followed by radiation treatment. The amounts of oestradiol implanted (2 mg) and the dose of X-rays delivered (2 Gy) were known to result within 14 months in the development of mammary tumours in about 50% of the rats. In addition, these treatments are known to act synergistically in the occurrence of malignant mammary tumours. With intervals of 2 months, the oestrogen receptor content of the mammary tissue and the prolactin concentration in the plasma were estimated in 6 rats of each group for a period of 14 months. The following results were obtained:

1. Oestradiol treatment caused a prolonged reduction of the estimated number of oestrogen receptors in the mammary gland, in spite of the continuous presence of oestradiol, which was expected to stimulate oestrogen receptor synthesis.
2. Oestradiol treatment caused a prolonged increase in the prolactin concentration in peripheral plasma.
3. The oestrus cycle disappeared in animals treated with oestradiol.

4. Radiation had no effect on the parameters studied, in spite of the reported synergistic interaction with oestradiol treatment on the occurrence of malignant mammary tumours.
5. The changes caused by oestradiol treatment in the oestrogen receptor content of the mammary tissue, as well as in the plasma prolactin concentration and the oestrus cycle, were already present at 2 months after treatment, which is well before the time that mammary tumours become manifest (10-12 months after treatment).

From these results it cannot be concluded whether the effect of oestrogens on mammary tumour development is related to one or more of the changes observed. Further investigations will have to determine, whether and which, if any, of these changes are involved in rat mammary carcinogenesis.

Finally, in Chapter 7, the possible role of oestrogen receptors in mammary tumour development is discussed, the experimental model for mammary carcinogenesis used in this study is evaluated, and brief reference is made to a possible extrapolation of data obtained in experimental models to human mammary cancer. It is emphasized, that it is important to unravel the mechanism of mammary carcinogenesis in rodents, since a better understanding of animal carcinogenesis will certainly offer a better model for comparison with the human situation.

SAMENVATTING

Wanneer ratten die met oestrogenen behandeld zijn, worden blootgesteld aan neutronen- of röntgenstraling, ontwikkelen zich meer maligne mammatumoren dan op grond van de afzonderlijke effekten van oestrogenen en straling verwacht zou worden. Deze zogenaamde synergistische werking van oestrogenen en straling is beschreven voor de oestrogenen diethylstilboestrol en oestradiol, en diverse doses hoog- en laagenergetische neutronenstraling en röntgenstraling. De manier waarop oestrogenen het ontstaan of de ontwikkeling van mammatumoren bij de rat beïnvloeden, is niet bekend. Uit de literatuur was bekend, dat toediening van oestrogenen aan ratten de afgifte van prolaktine door de hypofyse kan stimuleren, terwijl prolaktine de groei van mammatumoren in knaagdieren kan stimuleren. Op grond van deze gegevens is overwogen, of het waargenomen synergisme tussen oestrogenen en straling zou kunnen berusten op een stijging van de prolaktinekoncentratie in het plasma, waardoor de groei van mammatumoren wordt gestimuleerd. Anderzijds kan echter een direkt effekt van de oestrogenen op het melkklierweefsel niet bij voorbaat worden uitgesloten door de aanwezigheid van receptoren voor oestrogenen in het melkklierweefsel.

Volgens het algemeen aanvaarde werkingsmechanisme van steroïdhormonen, oefent oestradiol zijn werking op doelwitweefsels uit door middel van binding aan cytoplasmatische eiwitten met een hoge bindings-affiniteit voor het hormoon. Deze eiwitten, "receptoren" genaamd, binden het hormoon, zodra dit de doelwitcel binnengaat. Het ontstane hormoon-receptorkomplex begeeft zich van het cytoplasma naar de celkern, waar het gebonden wordt aan acceptorplaatsen op het chromatine. Hier stimuleert het komplex de synthese van mRNA, hetgeen uiteindelijk leidt tot het waargenomen effekt van het hormoon op de doelwitcel. Op deze wijze kunnen oestrogenen de groei van het weefsel, of de synthese van bepaalde eiwitten stimuleren. Eén van de effekten van oestradiol zou

de stimulering van de synthese van de eigen receptor kunnen zijn. Door beïnvloeding van òf het aantal, òf de beschikbaarheid van de oestrogeenreceptoren, zouden oestrogenen hun eigen effekten op het melkklierweefsel kunnen beïnvloeden.

De in dit proefschrift beschreven experimenten werden uitgevoerd met het doel om mogelijke veranderingen in het gehalte aan oestrogeenreceptoren van het melkklierweefsel en in de prolaktinekoncentratie van het plasma aan te tonen in ratten die behandeld werden met oestrogenen en al dan niet bestraald werden (Hoofdstuk 1). Wanneer deze veranderingen vooraf zouden gaan aan het manifest worden van mammatumoren, dan zouden zij een rol kunnen spelen bij het ontstaan of de ontwikkeling van de tumoren. Het werkingsmechanisme van steroïdhormonen, de hormonale invloeden op normaal en neoplastisch melkklierweefsel en het belang van de aanwezigheid van oestrogeenreceptoren in mammatumoren zijn beschreven in Hoofdstuk 2.

De methode die gebruikt is om een continue, geringe verhoging van de oestradiolconcentratie in plasma te verkrijgen is beschreven in het eerste deel van Hoofdstuk 3 en Appendix Paper I. Implantatie van een oestradiol-bevattend tablet op cholesterol- en paraffinebasis gaf de beste resultaten. De tabletten werden bereid door een homogene smelt van de drie stoffen af te laten koelen in een siliconenrubberslang, en deze vervolgens in plakjes van 3 mm dikte te snijden. Het protocol voor de bereiding van de tabletten en de methoden gebruikt voor de bepaling van prolaktine in plasma en DNA in kernen van melkklierweefsel, zijn beschreven in Hoofdstuk 4.

Bepaling van het gehalte aan oestrogeenreceptoren in een weefselmonster geschiedt in het algemeen door de bepaling van de hoeveelheid radioaktief oestradiol die aan de receptoren gebonden kan worden. In weefsels afkomstig van met oestradiol behandelde, intakte dieren zou een dergelijke bepaling echter beïnvloed worden door bezetting van de receptoren door endogeen of exogeen oestradiol. Hierdoor zouden slechts de niet-bezette receptoren radioaktief oestradiol

binden, zodat het totaal aantal aanwezige receptoren wordt onderschat. Daarom werd een methode voor de bepaling van het totaal aantal (bezette en onbezette) receptoren ontwikkeld, welke niet gestoord wordt door de aanwezigheid van niet-radioaktief oestradiol. Bij deze methode, beschreven in Hoofdstuk 5 en Appendix Paper II, wordt het eventueel aanwezige niet-radioaktieve oestradiol uitgewisseld tegen radioaktief oestradiol. Voor de evaluatie van de toepasbaarheid van deze bepaling werd gebruik gemaakt van mammatumoren welke ontstaan in vrouwelijke ratten na toediening van dimethylbenzanthraceen, zoals beschreven in het tweede deel van Hoofdstuk 3.

Het belangrijkste experiment uit deze studie is beschreven in Hoofdstuk 6 en Appendix Paper III. Sprague-Dawley ratten werden behandeld met oestradiol, met röntgenstraling, of met oestradiol en bestraling. Ratten uit een kontrôlegroep werden niet behandeld. De geïmplanteerde hoeveelheid oestradiol (2 mg) en de dosis toegediende straling (2 Gy) werden zodanig gekozen, dat binnen 14 maanden ontwikkeling van mammatumoren bij ongeveer 50% van de ratten te verwachten was. Bovendien was bekend, dat deze behandelingen een synergistisch effekt hebben op het ontstaan van maligne mammatumoren. Met tussenpozen van 2 maanden werden in 6 ratten van elke groep het gehalte aan oestrogeenreceptoren in het melkklierweefsel en de koncentratie van prolaktine in het plasma bepaald. De volgende resultaten werden verkregen:

1) Na behandeling met oestradiol was het gehalte van oestrogeenreceptoren in melkklierweefsel langdurig verlaagd, in tegenstelling tot de verhoging welke verwacht werd op grond van de voortdurende aanwezigheid van oestradiol.
2) De prolaktinekoncentratie in het plasma van dezelfde dieren was langdurig (10-50 x) hoger dan het prolaktinegehalte in plasma van dieren uit de kontrôlegroep.
3) Dieren die met oestradiol behandeld waren, vertoonden geen oestruscyclus meer.
4) Bestraling had geen additioneel effekt op de bestudeerde

parameters, ondanks de synergistische werking met oestradiol op het ontstaan van maligne mammatumoren.

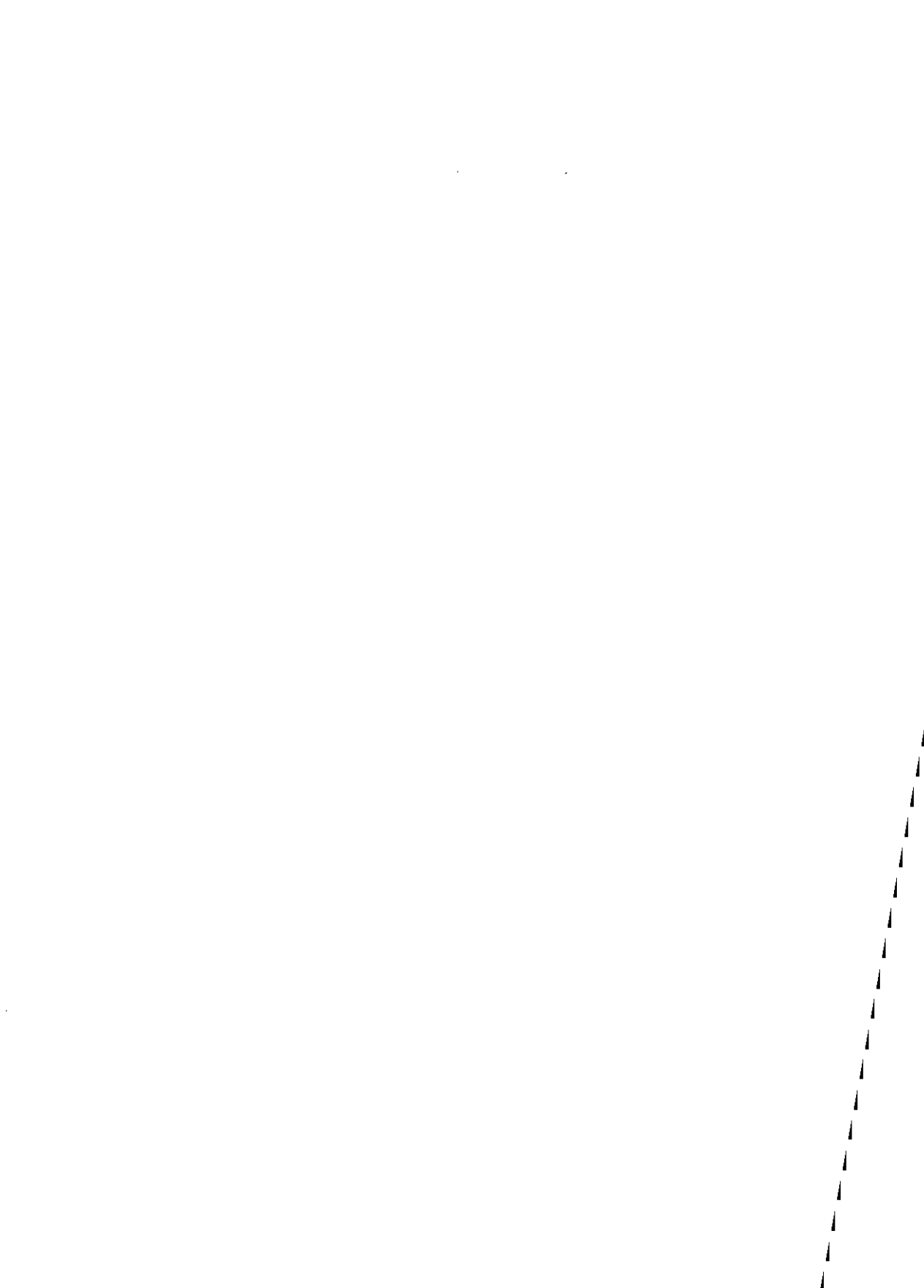
5) De onder 1 t/m 3 genoemde veranderingen manifesteerden zich reeds 2 maanden na het implanteren van het oestradioltablet. Dit tijdstip ligt ver voor het tijdstip waarop de eerste mammatumoren worden waargenomen (10-12 maanden na behandeling).

Op grond van deze resultaten lijkt het mogelijk dat het effekt van oestradiol op het ontstaan van mammatumoren bij de rat gerelateerd is aan één of meer van de na 2 maanden reeds waargenomen veranderingen. Verder onderzoek is nodig om vast te kunnen stellen óf, en zo ja welke van, deze veranderingen een rol spelen in de carcinogenese van de melkklier.

De algemene diskussie in Hoofdstuk 7 betreft o.a. de rol die oestrogeenreceptoren zouden kunnen spelen bij de ontwikkeling van mammatumoren bij de rat. Tevens is geprobeerd het in deze studie gebruikte model te evalueren en tenslotte werd aandacht besteed aan extrapolatie van gegevens uit dier-experimentele studies naar de situatie bij de mens. Het blijft belangrijk om met behulp van diermodellen een zo volledig mogelijk inzicht te krijgen in alle faktoren en processen die leiden tot het ontstaan van mammatumoren. Opheldering van het mechanisme van de carcinogenese in diermodellen zal ongetwijfeld een leidraad bieden voor de opheldering van het mechanisme van de carcinogenese bij de mens.

LIST OF ABBREVIATIONS

BSA	- bovine serum albumin
^{o}C	- degrees Celsius
DES	- diethylstilboestrol
DMBA	- 7,12-dimethyl-benzanthracene
DNA	- deoxyribonucleic acid
EE	- 17α-ethinyloestradiol
ER	- oestrogen receptor
g	- relative centrifugal force
^{3}H	- tritium
K_d	- dissociation constant
mRNA	- messenger RNA
M	- moles per litre
n	- number of observations
NRS	- normal rabbit serum
PBS	- phosphate buffered saline
r	- correlation coefficient
RNA	- ribonucleic acid
S	- Svedberg unit
s.d.	- standard deviation
s.e.m.	- standard error of the mean
SHBG	- sex hormone binding globulin
w/v	- weight/volume
w/w	- weight/weight
X-rays	- Röntgen rays



LIST OF TRIVIAL NAMES

Trivial name used in this thesis	Systematic name
aldosterone	- 4-pregnen-11β,21-diol-3,18,20-trione
cholesterol	- 5-cholesten-3β-ol
diethylstilboestrol	- 3,4-bis(4-hydroxyphenyl)-3-hexene
17α-ethinyloestradiol	- 17α-ethinyl-1,3,5(10)-oestratriene-3,17β-diol
oestradiol	- 1,3,5(10)-oestratriene-3,17β-diol
oestrone	- 3-hydroxy-1,3,5(10)-estratriene-17-one
progesterone	- 4-pregnene-3,20-dione
testosterone	- 17β-hydroxy-4-androsten-3-one

CURRICULUM VITAE

Op 8 maart 1950 ben ik te Utrecht geboren. In juni 1968 behaalde ik het HBS-B-diploma aan het St. Bonifatiuslyceum aldaar. In september van dat jaar begon ik de chemie-studie aan de Rijksuniversiteit Utrecht. Het kandidaatsexamen S 2 werd in mei 1972 afgelegd. Het doctoraal-examen (hoofdvak Analytische Chemie, bijvak Klinische Chemie) werd afgelegd in mei 1974. Vanaf 1 juni 1974 was ik als wetenschappelijk medewerker verbonden aan de afdeling Biochemie II van de Erasmus Universiteit Rotterdam, waar de in dit proefschrift beschreven studie, in samenwerking met het Radiobiologisch Instituut TNO te Rijswijk, werd verricht. Van 1 januari 1976 tot en met 31 december 1978 werd mij een Research-Fellowship toegekend door het Koningin Wilhelmina Fonds, dat ook door toekenning van een projektsubsidie in belangrijke mate aan dit onderzoek heeft bijgedragen.

Appendix Paper I

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The Effect of Subcutaneous Administration of Oestrogens on Plasma Oestrogen Levels and Tumour Incidence in Female Rats*

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Abstract—*A study on a possible synergistic effect of oestrogens and ionizing radiation on mammary carcinogenesis required the use of female rats with elevated plasma levels of oestrogens in a longitudinal experiment. Following subcutaneous implantation of oestrogen pellets, plasma oestrogen levels were monitored as a function of time after administration, procedure of pellet preparation, chemical nature of the oestrogen and concentration of the oestrogen in the pellets. 17β-oestradiol (E_2), 17β-oestradiol 3-benzoate (E_2B) and 17α-ethynyloestradiol (EE_2) were studied in this respect. In initial studies, 5–20 mg E_2B were implanted; this resulted in extremely high peripheral plasma levels and caused deterioration in the physical condition of the animals. After a latent period of 10–12 months, pituitary and mammary tumours were observed in a considerable number of animals.*

Experiments with pellets containing tritium labelled 17β-oestradiol were performed in order to compare the kinetics of hormone release from pellets produced by various techniques. Based on the results of these experiments, implantation of oestrogen in a paraffin-cholesterol pellet was adopted for all further experiments. Peripheral plasma levels of oestrogen are reported for 17β-oestradiol and 17α-ethynyloestradiol at various time intervals after administration of pellets containing 1.0 and 2.5 mg of oestrogen to intact and ovariectomized rats.

INTRODUCTION

In view of the relatively high incidence of mammary cancer in the human, it is of great importance to unravel the mechanism of mammary gland carcinogenesis in experimental models. Previous studies have suggested a relationship between the occurrence of mammary tumours in the human and several factors, including radiation [1], nutritional status [2] and oestrogen use [3, 4]. Furthermore, it has been shown [5, 6] that, in the rat, oestrogen (diethylstilboestrol, DES) and ionizing radiation (X-rays and neutrons) act synergistically to produce mammary tumours. In these studies, DES was administered to female A × C rats via subcutaneous implantation of hand pressed DES-cholesterol pellets containing 5 mg DES and 15 mg cholesterol. However, the authors provided no data on the actual levels of DES in peripheral plasma after implantation. For a proper evaluation of the possible carcinogenic effect of the administered oestrogen, it is important to know the relationship of the oestrogen plasma level after oestrogen administration to the physiological oestrogen level.

The present study was designed to define the hormonal status of experimental animals during combined oestrogen and radiation treatment in terms of oestrogen levels in peripheral plasma, rather than in terms of the amount of oestrogen implanted in the animal. In this respect we have studied 17β-oestradiol (E_2), 17β-oestradiol-3-benzoate (E_2B) and 17α-ethynyloestradiol

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(EE_2), since these are either naturally occurring, therapeutic or contraceptive oestrogens.

MATERIAL AND METHODS

Animals

Female Wistar WAG/Rij rats either intact or after hystero-ovariectomy (Ovex) were used throughout this study. The animals were kept in rooms with controlled temperature, light and humidity conditions and received tap water and standard dry pellets *ad libitum*. Oestrogen pellets were implanted subcutaneously in the dorsal region of the neck under light ether anaesthesia. Blood was taken from the tail under light ether anaesthesia at regular intervals after pellet implantation. The blood was collected in heparinized tubes and centrifuged within 2 hr to obtain plasma, this was stored at −20° C until assay.

Preparation of oestrogen pellets

Oestrogen pellets were prepared by one of the following methods:

Method A

The oestrogen was mixed with an appropriate amount of cholesterol. The mixture was pressed with a small hand-operated press to give pellets weighing 21.8 ± 2.3 mg (mean ± S.D., $n = 109$) with a cross section of approximately 4 mm.

Method B

The oestrogen was mixed with cholesterol. The mixture was melted in an oil bath at 190°C and was aspirated into silicon tubing of 3 mm cross section in 0.5 ml portions. After cooling the tube at ambient temperature, segments of 2–3 mm were cut.

Method C

A mixture of oestrogen, cholesterol and paraffin was made and pellets were prepared as described for method B. Cholesterol and paraffin were used in a 1 : 10 (w/w) ratio [7]. The average pellet weight was 20.3 ± 1.3 mg (S.D., $n = 100$).

Pellets selected for implantation weighed 19–21 mg. Tritiated oestrogens were included in the pellets in some experiments and steroid release was monitored by liquid scintillation counting of small blood samples taken at regular intervals after pellet implantation. In a counting vial, 0.2 ml of heparinized blood, 0.2 ml of isopropyl alcohol and 0.2 ml of a 30% solution of hydrogen peroxide were mixed and incubated at 60° C for 1 hr to decolorize the blood. The residue was dissolved after incubation with 2 ml of Soluene (Packard) for another 2 hr at 60° C. Finally, 15 ml of Permablend scintillation cocktail (Packard) were added and the radioactivity was determined in a Nuclear Chicago Mark II liquid scintillation counter with a counting efficiency of approximately 10%.

Steroids

17β-Oestradiol and 17α-ethynyloestradiol for implantation were gifts from Organon, Oss, The Netherlands. 17β-Oestradiol 3-benzoate was purchased from Sigma, St. Louis, U.S.A. 17β-Oestradiol and 17α-ethynyloestradiol for use as standards in radioimmunoassay were obtained from Steraloids, Pawling, New York, U.S.A. (2,4,6,7-^{3}H)-17β-Oestradiol was obtained from the Radiochemical Centre, Amersham, United Kingdom and had a specific activity of 85 Ci/mmole. (6,7-^{3}H)-17α-Ethynyloestradiol with a specific activity of 40 Ci/mmole was purchased from New England Nuclear, Frankfurt am Main, Germany. Radioactive steroids were purified by paper chromatography using a system light petroleum:benzene:ethanol:water, in the ratio of 5:5:7:3 by volume. Purity of radioactive steroids was checked every two months.

Steroid estimations

Plasma 17β-oestradiol was assayed as previously described by De Jong, Hey and van der Molen [8]. Cross reaction of 17β-oestradiol 3-benzoate in the 17β-oestradiol radioimmunoassay was 5%. Plasma 17α-ethynyloestradiol was measured essentially as described by Warren and Fotherby [9], but the method was adapted to the protocol for oestradiol radioimmunoassay.

E_2 and EE_2 were not separated by the chromatographic procedure used. We do not expect, however, that endogenous 17β-oestradiol interfered with the assay of EE_2 in the EE_2 treated rats, because the cross-reaction of E_2 with the EE_2 antiserum was 3.5% and because it appears reasonable to assume that only very little E_2 was present after administration of EE_2 which will suppress pituitary lutropin secretion and ovarian E_2 secretion.

Scoring of tumours

Animals which developed mammary tumours were killed either when tumour size was 5–6 cm, or as soon as indicated by the physical condition of the animals. Autopsy was performed on all animals and the occurrence of tumours was registered. Pituitary tumours were classified as such after histological examination.

RESULTS

In an initial experiment E_2B-cholesterol pellets were prepared according to method A.

These pellets contained 5 mg E_2B and 15 mg cholesterol. From 1 to 4 pellets were implanted in intact and ovariectomized rats. Blood samples were taken at day 0, 3, 7, 14 and 30 days after implantation and E_2 was assayed in the plasma. The results for the intact animals are given in Table 1. The results obtained for the ovariectomized animals were essentially the same. The condition of all animals was affected by the oestrogen administration and loss of hair and a decrease in body weight were generally observed. Furthermore within a latent period of 10–12 months, almost all rats (92%) developed pituitary tumours; some of the intact animals also developed mammary tumours. The tumour incidence in this group of animals is shown in Table 2; control animals were still tumour free. In view of the high peripheral plasma E_2 levels measured after administration of E_2B, which reflect a pharmacological rather than a physiological condition, it was decided to decrease the amount of oestrogen to be administered to the animals. Consequently, the second experiment was designed to compare three different methods of pellet preparation. Two doses of E_2 which were lower than the amounts of E_2B used in the first experiment were used. The results obtained are presented in Fig. 1 and show that implantation of the pressed E_2-cholesterol pellets (method A) tends to give higher plasma levels than the other two methods, especially during the first 40 days after implantation of the pellets. This observation was confirmed by the results of the measurements of radioactivity in the blood after implantation of pellets containing tritiated E_2; these are summarized in Table 3. The half-life of radioactivity in the blood was calculated from disappearance curves of plasma radioactivity. The part of the curve after the onset of the decrease in plasma E_2, which normally occurred about 4 days after implantation, was used for this calculation.

In a third experiment, a comparison was made of the oestrogen release from pellets prepared by method C for implantation of E_2 and EE_2 in intact and ovariectomized rats. The results of this experiment are shown in Fig. 2. There appears to be no difference between the rate of release of E_2 and EE_2 in intact and ovariectomized rats.

DISCUSSION

The present results show that, in the rat, an elevated level of plasma oestrogen can be effectuated and maintained by the subcutaneous

Table 1. *Plasma 17β-oestradiol in intact female rats after implantation of pellets containing* 5 mg *17β-oestradiol 3-benzoate and* 15 mg *cholesterol. Results are given as means ± S.E.M. (n). For n = 2 individual results are given. All results are expressed as* pg/ml

Time after implantation (days)	17β-oestradiol (pg/ml) number of pellets implanted 0	1	2	3	4
0	56 ± 12 (5)				
3	29 ± 3 (3)	650 – 1000	1160 – 2250	1438 ± 238 (3)	3720 – 2080
7	44 – 69	389 ± 74 (3)	882 ± 32 (3)	973 ± 90 (3)	1162 ± 190 (3)
14	103 ± 14 (5)	484 ± 38 (4)	639 ± 63 (5)	1088 ± 137 (5)	1292 ± 133 (5)
30	71 ± 9 (6)	285 ± 18 (4)	404 ± 48 (5)	841 ± 97 (5)	1032 ± 190 (6)

Table 2. *Tumour incidence in intact and ovariectomized rats after implantation of 17β-oestradiol 3-benzoate (E_2B) pellets*

Number of pellets implanted	Total amount of E_2B implanted (mg)	Total number of animals	Latent period (months)	Number of pituitary tumours	Rats with mammary tumours
1	5	intact 5	10	5	4*
		ovex 4	10	4	—
2	10	intact 5	10	5	2†
		ovex 4	12	3	—
3	15	intact 4	11	4	—
		ovex 5	10–11	4	—
4	20	intact 5	8–10	4	1†
		ovex 5	10	5	—

*3 Multiple, 1 single tumour observed.
†Single tumours observed only.

considerations, it is quite possible that the carcinogenic effect of oestrogens on the mammary gland reported by Segaloff and Maxfield [5]; Shellaberger *et al.* [6] and found in the present study, is an oestrogen mediated prolactin effect rather than a direct effect.

The occurrence of mammary tumours in intact rats and the absence of such tumours in ovariectomized rats in spite of oestrogen administration could suggest the existence of another ovarian factor which is involved in the induction of mammary tumours. In mice and rats a synergistic action of progesterone and oestrone on prolactin-induced mammary tumours has been described [20, 21]. In contrast, others reported that progesterone may protect the mammary gland from becoming neoplastic [22]. Experiments with larger groups of animals are in progress to confirm our observations on the effect of ovariectomy on mammary tumour development and to study the role of prolactin and progesterone in this respect.

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Appendix Paper II

(J. Steroid Biochem. 13 (1980) in press)

ESTIMATION OF TOTAL OESTROGEN RECEPTORS IN DMBA-INDUCED RAT MAMMARY TUMOURS BY EXCHANGE OF NUCLEAR BOUND LIGAND AT LOW TEMPERATURE; A COMPARISON WITH RAT UTERUS

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SUMMARY

A nuclear exchange assay for the estimation of oestrogen receptors in dimethylbenzanthracene (DMBA)-induced rat mammary tumours is described. In brief, the method consists of translocation of receptor sites to the nuclei with oestradiol-17β (E_2); isolation of Triton-washed nuclear preparations; exchange of ligand by incubation of nuclear pellets with radioactive E_2 for 1 hour at 0 C and extraction of E_2-receptor complexes with 0.4 M KCl.
The optimum temperature for nuclear exchange appeared to be 0 C when a 1 hour incubation period was used. When nuclear exchange incubations were carried out at temperatures of 15 C or higher, the steroid-receptor complex appeared to be unstable. This instability is not the result of trypsin-like proteolytic activity, nor of dissociation of the steroid-receptor complex during the assay procedure. Exchange of E_2 at 0 C was also demonstrated for uterine nuclear pellets.
Quantitative aspects of the nuclear exchange assay were studied in ovariectomized rats. No differences were found between *in vivo* and *in vitro* labelling of receptor sites. Comparison of extraction of nuclei-bound E_2 with ethanol and extraction of steroid-receptor complexes with 0.4 M KCl showed that less radioactivity was extracted from the nuclear preparations with 0.4 M KCl. Ethanol extraction released much aspecifically bound E_2 from the nuclear preparations.

INTRODUCTION

Quantitative estimation of receptors for oestradiol-17β (E_2) is generally performed by labelling receptor sites with tri-

tiated E_2. The results of such estimations may be influenced by the presence of unlabelled ligand, which may bind to the receptors, thus preventing the binding of the radioactive form of the ligand to the receptors. It has been shown, that endogenous steroid bound to nuclear receptor sites will exchange with exogenously added labelled steroid. This observation has been applied to estimation of nuclear receptor sites through labelling of nuclear receptor preparations with radioactive ligand. Assays for steroid receptors in which this concept is used, are generally called "nuclear exchange assays", and such assays for E_2-receptors have been reported for rat uterus (1-4), dimethylbenzanthracene (DMBA)-induced mammary tumours (4,5) and testicular tissue (6). In these assays either crude nuclear preparations (1,2,4,6) or protamine-sulphate precipitated steroid-receptor complexes (3,5) are incubated with radioactive E_2. Following the exchange of labelled steroid, two procedures for the quantification of receptors have been used most frequently. In the experiments of Zava et al. (3), Tsai & Katzenellenbogen (4) and Nicholson et al. (5) excess free steroid is removed by repetitive washings of the nuclear preparation or the protamine sulphate precipitate followed by extraction of receptor-bound radioactive E_2 with ethanol. Mester & Baulieu (2) and De Boer et al. (6) have used extraction of steroid-receptor complexes from the nuclear preparation with 0.4 M KCl, followed by sucrose gradient centrifugation to identify and quantify the receptors. It is generally agreed that the nuclear exchange of ligand should be carried out at moderately high temperatures, e.g. 15-37 C, because the rate of exchange at temperatures below 10 C is considered to be negligible.

It was the purpose of the present study to compare different methods for the quantification of oestrogen receptors following exchange of nuclear bound ligand. In this respect, sucrose gradient centrifugation following KCl extraction of nuclear E_2-receptor complexes and determination of radioactive E_2 following ethanol extraction were studied. The results show,

that nuclear exchange of E_2 occurs even at temperatures as low as 0 C, both for uterine and for DMBA-induced mammary tumour tissue. In addition, it will be shown, that ethanol extraction of nuclear-bound tritiated E_2 released high amounts of E_2 which are not specifically bound to the nuclear preparation. This may cause inaccurate results for receptor levels in tissues with low amounts of specifically bound steroid.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats were used in this study. They were housed in a light- (14 h light, 10 h darkness) and temperature- (20-22 C) controlled room and received standard dry pellets (Hope Farms, Woerden, The Netherlands) and tap water ad libitum. Mammary tumours were induced in 54-61 days old rats by three intragastric feedings of 10 mg dimethylbenzanthracene (DMBA, Fluka, Basel, Switzerland) with one week intervals. The first tumours appeared 5 weeks after the first DMBA feeding, whereas at 10 weeks tumour incidence was 100%. The number of tumours per rat varied from 7-15. Histologically 80% of the tumours were classified as adenocarcinoma, 8% as carcinoma, 8% as sclerosing papillary cystadenoma. The remainder of the lesions was classified as hyperplastic nodules. Ovariectomy was performed 40 hours before sacrifice under light ether anaesthesia through lateral incisions. Animals were killed by decapitation.

Methods for the estimation of nuclear oestrogen receptors

a) Nuclear exchange assay

Uterine and mammary tumour tissue were collected and trimmed free of fat; tumour capsules were removed. Necrotic tumours were not used. Tissue samples (up to 1 gram) were minced with scissors and incubated under constant agitation in scintillation vials containing 2-3 ml of Krebs Ringer Bicarbonate Buffer, pH = 7.4, containing 0.2% (w/v) of glucose (KRBG). Incubations were carried out for 1 hour at 37 C in an atmosphere of 95% oxygen and 5% carbon dioxyde in the presence of 2×10^{-8} M of unlabelled E_2 (Steraloids, Pawling, New York, U.S.A.) in order to translocate free receptors to the nuclei. In some experiments the potent protease inhibitor di-isopropylfluorophosphate (DFP, BDH, Poole, England) was present in a concentration of 1 mM (7). After the incubation, the vials were placed on ice. The incubation medium was separated from the tissue fragments by centrifugation at 700 x g for 10 min at 6 C. Unless stated otherwise, all subsequent centrifugations were performed under these conditions. Tissues and

General procedures

Protein was measured by the method of Lowry et al. (9) using bovine serum albumin as a standard. For protein estimation, nuclear pellets were dissolved in 1 ml of 1 N sodium hydroxyde by heating for 30 minutes at 65 C.
DNA was measured according to Giles & Myers (10). As a sedimentation marker for sucrose gradient centrifugation Bovine Serum Albumin (4.6 S, BSA) was used.
The BSA was centrifuged on a separate gradient and detected in the fractions by its adsorption at 280 nm.
Protamine-sulphate precipitation of receptors was performed according to Zava et al. (3). Nuclear extracts were diluted to a KCl concentration of 0.08 M prior to addition of the protamine sulphate (Organon, Oss, The Netherlands).

RESULTS

Evaluation of the nuclear exchange assay

In Figure 1 the result of a gradient centrifugation of the KCl extract of a DMBA-induced mammary tumour nuclear pellet is presented. After nuclear exchange a small 4 S-peak is usually present in addition to the main peak (at 5 S). For calculation of receptor concentrations both peaks were used.
A comparison of the nuclear exchange assay using incubation of the nuclear pellet with tritiated E_2 for one hour at 20 C (6), and the direct nuclear receptor assay (8) using tumour tissue obtained from an intact rat is shown in Figure 2. From these results it appeared that with the direct assay no receptors could be demonstrated, whereas with the nuclear exchange assay a receptor peak was clearly visible (fractions 8-16). This observation leads to the conclusion that in the direct assay, in which minced tissue of intact animals is incubated with radioactive steroid, the ratio of the concentration of endogenous and radioactive steroid is unfavourable at radioactive steroid concentrations known to be effective in ovariectomized animals. When exchange is performed on washed nuclear pellets from intact animals, this concentration ratio is more favourable.
Results of nuclear exchange assays depend on the temperature at which the exchange reaction is carried out and we have investigated the effect of incubation temperature and incubation

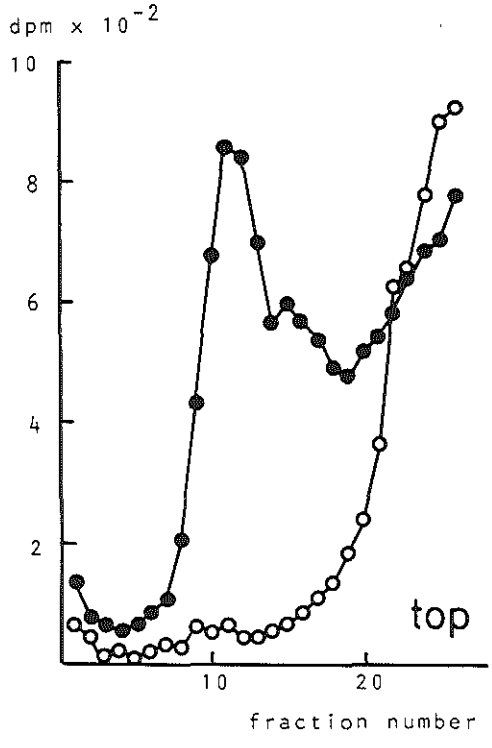

Fig. 2 Comparison of sucrose gradient centrifugation patterns obtained after direct in vitro labelling o——o and nuclear exchange following translocation •——• for the assay of oestrogen receptors in DMBA-induced rat mammary tumour tissue obtained from an intact rat. Nuclear exchange was carried out for 1 hour at 20 C.

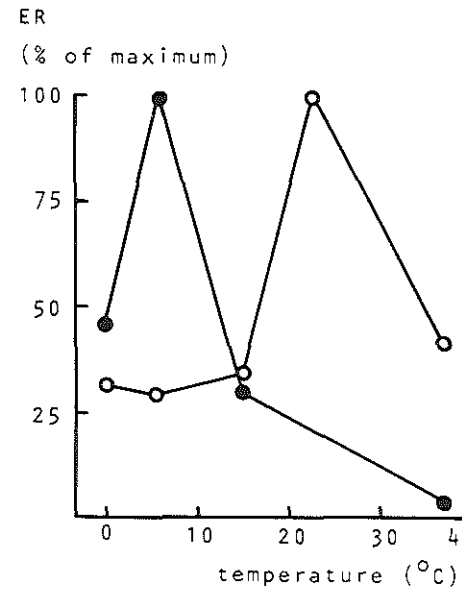

Fig. 3 Effect of exchange temperature on estimated oestrogen receptor levels (ER) in nuclear extracts of DMBA-induced rat mammary tumour. Exchange was performed for 30 min (o——o) or 1 hour (•——•) at different temperatures.

time on the result of the exchange assay. The results in Figure 3 suggest that the E_2-receptor complex in nuclear preparations of DMBA-induced rat mammary tumours is not stable at elevated temperatures. To further evaluate this suggestion, DMBA-induced mammary tumour tissue from ovariectomized animals was incubated with ^3H-E_2 to translocate all receptor sites to the nuclei. Nuclear preparations of this tissue were kept in

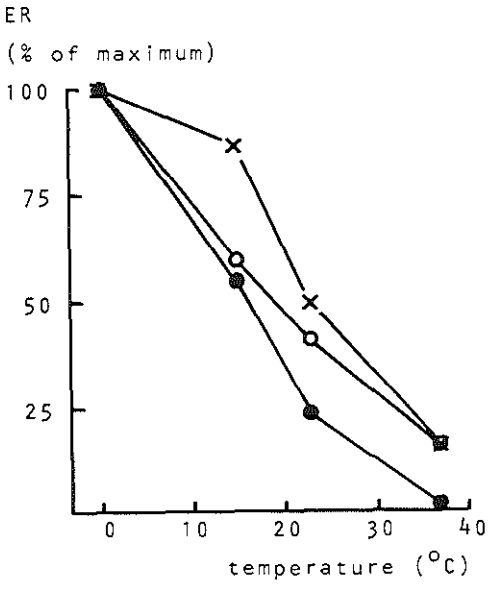

Fig. 4 Effect of di-isopropylfluorophosphate (DFP) and radioactive E_2 in isolation and incubation media on the stability of the 17β-oestradiol-receptor complex (ER) from DMBA-induced mammary tumour tissue. Tissue was obtained from ovariectomized animals, receptors were labelled by incubation of the tissue with radioactive 17β-oestradiol. Nuclear fractions were kept in exchange buffer for 1 hour at different temperatures. •——• no additions, o——o DFP, x——x DFP + ^3H-E_2.

exchange medium for 1 hour at different temperatures. In a parallel set of incubations di-isopropylfluorophosphate (DFP) was added to all media. DFP is known to inhibit trypsin-like proteolytic activity even at 4 C (7). In a third set of preparations radioactive E_2 was present in addition to the DFP to minimize dissociation of the E_2-receptor complex during the preparation and the handling of the nuclear fraction. From the results of these experiments (Figure 4) it can be concluded, that the receptor-E_2 complex in nuclear preparations of DMBA-induced rat mammary tumours is unstable when kept for 1 hour at temperatures of 15 C or higher. This instability is probably not caused by trypsin-like proteolytic activity, since incubation with DFP showed only a minor effect at temperatures above 20 C. Addition of excess radioactive E_2 to all media to protect the steroid-receptor complex from dissociation had only a limited effect at temperatures lower than 23 C.

For serial estimations of oestrogen receptors in mammary tumours an exchange period of 1 h at 0 C was adopted. At this temperature longer incubation did not increase the amount of

^3H-E_2 bound to the receptors (data not shown).
The nuclear exchange assay was compared to the direct *in vitro* nuclear receptor assay and the *in vivo* nuclear receptor assay. The nuclear exchange assay was performed on tumour tissue from intact animals. For the direct *in vitro* and *in vivo* assay of nuclear receptors tissue from ovariectomized rats was used, since these assays require the absence of endogenous E_2. The data in Table 1 show that the results of the nuclear exchange assay are equivalent to those obtained with both the *in vivo* injection and the direct *in vitro* assays.

Table 1

OESTROGEN RECEPTORS IN DMBA-INDUCED RAT MAMMARY TUMOURS MEASURED WITH DIFFERENT ASSAYS

Assay procedure		KCl-extractable oestrogen receptor sites (fmoles/mg nuclear extract protein)
a. Ovariectomized animals		
- *In vivo* labelling of receptors by injection of ^3H-E_2		44
- *In vitro* labelling of mammary tissue with ^3H-E_2		49 49 54
b. Intact animals		
- Exchange with ^3H-E_2 in nuclear preparations of mammary tissue	15' 23 C	49 60
	30' 23 C	67 49
	60' 0 C	44

Low temperature ligand exchange in uterine nuclear pellets

A uterus of a DMBA-tumour bearing rat was incubated for 1 h at 37 C with 10^{-8} M unlabelled E_2. Nuclei were incubated with tritiated E_2 at either 0 C or 15 C. The results in Figure 5 reflect that exchange of E_2 from its receptor sites at low temperature occurs also in uterine nuclei.

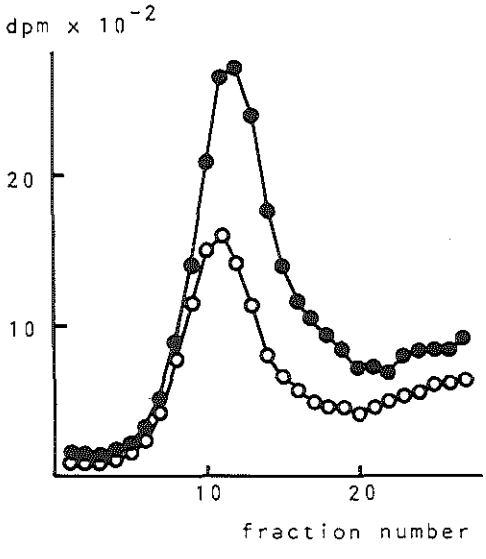

Fig. 5 Sucrose gradient analysis of nuclear extracts from uterine tissue following exchange for 1 hour at 0 C (o——o, 1.7 mg protein/ml extract) and 15 C (•——•, 2.5 mg protein/ml extract). The uterus was taken from an intact rat carrying DMBA-induced mammary tumours.

In contrast to the results obtained for mammary tumour tissue, in uterine nuclei the optimum temperature for exchange during 1 hour was 15 C rather than 0 C. The amounts of receptor expressed as femtomoles/mg nuclear extract protein were 145 and 180 for exchange at 0 C and 15 C respectively.

Measurement of oestrogen receptors in uterine and DMBA-induced mammary tumour tissue using ethanol extraction of receptor-bound ^3H-E_2

The experiments described in this section were performed with uterine and DMBA-induced mammary tumour tissues obtained from ovariectomized rats. Receptor sites were labelled either directly *in vitro* or by nuclear exchange for one hour at different temperatures following translocation of receptor sites

to the nuclei with unlabelled E_2. Following labelling of receptors excess ligand was washed away and receptor-bound radioactive E_2 was extracted with ethanol. The results in Table 2 show that the amount of aspecific binding is unacceptably high in all cases, except when receptors in uterine tissue were labelled directly in vitro. The low figures obtained with mammary tumours after exchange at 37 C indicate that lower exchange temperatures should be used.

DNA content of nuclear preparations after exchange

Since some authors (3,5) have used the DNA content of their preparations to express the nuclear receptor content, we have also measured the DNA content of our preparations. The DNA/protein ratio of nuclear preparations of DMBA-induced mammary tumours decreased when the exchange was carried out at 37 C (Table 3). The protein content of the pellets did not change with changes in incubation temperature. From Table 3 it appears that the DNA-content of nuclear preparations is not suitable for the expression of results of nuclear exchange assays for oestrogen receptors in this type of tissue when exchange is carried out at temperatures above 20 C.

Efficiencies of KCl and ethanol extraction

The efficiencies of ethanol and KCl extraction of tritiated E_2 and E_2-receptor complexes respectively from nuclear preparations of uterine and DMBA-induced mammary tumour tissue following different labelling procedures, are presented in Table 4. Less radioactivity was extracted with KCl following nuclear exchange, whereas the efficiency of ethanol extraction of labelled hormone was not affected by the labelling procedure.

Evaluation of charcoal treatment of nuclear extracts

Since charcoal can remove oestradiol from its receptor, we also measured receptor sites in uterine nuclear preparations omitting the charcoal treatment. Quantification of receptor sites was performed using the protamine-sulphate precipitation

Table 2

RESULTS OF NUCLEAR EXCHANGE ASSAY OF OESTROGEN RECEPTORS USING ETHANOL EXTRACTION OF RECEPTOR-BOUND OESTRADIOL-17β

Tissue	Assay Conditions	n	Total Bound Radioactivity (fmole/mg protein)	% Aspecific Binding	Specifically Bound Radioactivity (fmole/mg protein)
Uterus	direct in vitro	6	263 ± 20§	33 ± 5	174 ± 12
	exchange 0 C	4	254 ± 56	54 ± 17¶	109 ± 19¶
	20 C	3	328 ± 8	57 ± 20¶	137 ± 35
	37 C	3	285 ± 13	58 ± 22¶	116 ± 30
DMBA-tumour	direct in vitro	5	431 ± 74	65 ± 21¶	164 ± 62
	exchange 0 C	3	527 ± 144	70 ± 3¶	159 ± 53
	6 C	3	544 ± 88	72 ± 7¶	146 ± 24
	20 C	3	604 ± 150	76 ± 16¶	114 ± 37
	37 C	3	314 ± 12	88 ± 11¶	38 ± 19Ψ

§ means ± s.e.m.

¶ significantly different from direct in vitro labelling of uterus ($p < 0.05$)

Ψ significantly different from direct in vitro labelling of DMBA-tumour ($p < 0.05$)

Table 3

DNA/PROTEIN RATIO OF UTERINE NUCLEAR PELLETS AFTER OESTROGEN RECEPTOR ASSAY

Assay conditions	DNA/protein ratio	
	uterus	DMBA-tumour
direct in vitro	116 ± 6 (12)	231 ± 28 (12)
exchange[§] 0 C	85 ± 7 (6)[¶]	238 ± 27 (6)
6 C	n.d.	238 ± 25 (6)
20 C	100 ± 9 (6)	232 ± 19 (6)
37 C	95 ± 18 (6)	82 ± 13 (6)[¶]

§ exchange time: 1 hour

¶ $p < 0.05$ compared to corresponding direct in vitro labelling

n.d. = not determined

Table 4

EFFICIENCIES OF KCl EXTRACTION OF OESTRADIOL-17β-RECEPTOR COMPLEXES AND ETHANOL EXTRACTION OF RECEPTOR-BOUND OESTRADIOL-17β FROM NUCLEAR PREPARATIONS OF RAT UTERINE AND DMBA-INDUCED MAMMARY TUMOUR TISSUE

Receptor assay	KCl extraction (% radioactivity extracted)		Ethanol extraction (% radioactivity extracted)	
	Uterus	DMBA-tumour	Uterus	DMBA-tumour
in vivo	75 (2)§	46 (2)	93 (2)	-
direct in vitro	72 ± 11 (4)	-	88 ± 4 (10)	-
nuclear exchange	48 ± 9 (6)¶	35 ± 1 (6)	84 ± 10 (15)	93 ± 12 (12)

§ means = s.e.m. (n) or means (n = 2)

¶ significantly different from in vitro receptor assay

technique (3) which, in contrast to the sucrose gradient centrifugation, is insensitive to the presence of excess free steroid. For quantitative precipitation of receptors nuclear extracts were diluted to a KCl concentration of 0.08 M (3). We compared the results obtained after sucrose gradient centrifugation of 10 charcoal-treated uterine nuclear extracts with those after protamine sulphate precipitation of the same, yet untreated, extracts for quantification of oestrogen receptor sites. The results of the protamine-sulphate precipitation assay were 102 ± 3% (mean ± s.e.m., n = 10) of that of the sucrose gradient assay. Apparently under these experimental conditions charcoal does not strip oestradiol from its receptor.

Interference by non-receptor proteins

In mammary tumour tissues, α-fetoprotein (α-FP) may be present (11). To evaluate if αFP would interfere with the present nuclear exchange assay, we assayed oestrogen receptors in uterine tissue with or without addition of 50% (v/w) immature rat plasma prior to homogenization. The plasma, which was obtained from 8-days old rats, was found to contain 11 nmoles of oestrogen binding sites/ml. The uterine oestrogen receptor level was not increased after addition of the serum and therefore we concluded that the oestrogen receptor assay as presented is not sensitive to the presence of α-fetoprotein in the tissue to be assayed. Therefore, both oestradiol and diethylstilboestrol can be used as competitors in this present assay.

DISCUSSION

The results of this study show that exchange of ligand from E_2-receptors in Triton-washed nuclear preparations of rat uterus and DMBA-induced mammary tumours is possible even at temperatures as low as 0 C (Figures 3-5). The highest values for the number of specific oestrogen receptor sites in DMBA-induced mammary tumours were obtained using a combination of

a long incubation time and a low temperature or a short incubation time and an elevated temperature (Figure 3 and Table 1). Prolonged incubation at elevated temperatures decreased the number of measurable receptor sites (Figure 3). This decrease could only in part be explained by protease activity or dissociation of the steroid-receptor complex during the procedure (Figure 4). Because of the unfavourable effect of a higher exchange temperature on the stability of the steroid-receptor complex we have adopted the incubation for 1 hour at 0 C for serial estimations of oestrogen receptors in mammary tumour tissue.

For DMBA-induced mammary tumour tissue results of the nuclear exchange assay were compared to those obtained with an *in vivo* injection assay and direct *in vitro* assay. In the *in vivo* injection assay, radioactive E_2 is injected in an ovariectomized animal, in the direct *in vitro* assay tissue from an ovariectomized rat is incubated with 10^{-8} M ^{3}H-E_2 in order to translocate all receptors to the nucleus. After one hour, tissue is isolated and E_2-receptor complexes are extracted from a nuclear preparation. The *in vivo* injection assay is considered to give the best reflection of the true receptor level of a tissue, because receptors are labelled *in vivo* in the absence of endogenous E_2. Furthermore, the handling of the tissue is minimal in this assay, since no incubations have to be carried out to obtain translocation of receptors to the nuclei or nuclear exchange. Results of the *in vivo* injection assay did not differ from results obtained with either the *in vitro* labelling or the nuclear exchange assay (Table 1). Garola & McGuire (12) demonstrated that translocation of receptors from the cytoplasm to the nuclei *in vivo* is incomplete after surgical removal of a part of the tumour. This is possibly due to changes in vascularization as a result of the operation. Therefore tissues of different animals of the same litter were used in the comparison presented in Table 1.

It has been reported that the level of oestrogen receptor in

target tissues decreases after ovariectomy. However, in view of the large half life of oestrogen receptors (5-6 days (13, 14)) and the relatively short period used in our experiment between ovariectomy and receptor assay (40 hours), the effect of ovariectomy on receptor levels in our experiments was considered negligible.

The optimal conditions found in the present study for nuclear exchange assay of oestrogen receptors in DMBA-induced rat mammary tumours are in contrast to those reported in the literature. Tsai & Katzenellenbogen (4) used crude nuclear preparations of DMBA-induced mammary tumours, a 45 minutes exchange period at 30 C and ethanol extraction of bound ^{3}H-E_2. Nicholson et al. (5) added protamine sulphate to a crude nuclear suspension of the tumour tissue to prevent solubilization of receptors and exchange for 2 hours at 15 C. They also used ethanol extraction for receptor quantification.

The discrepancy between our results and those reported in the literature cannot be explained by the ethanol extraction (Table 2). In addition in our hands the ethanol releases an unacceptably high amount of aspecifically bound E_2 from the nuclear pellets. The expression of results per DNA content of the pellets also does not account for this discrepancy, since the DNA content is not affected by incubation temperatures of 20 C or lower (Table 3).

For uterine tissue optimum exchange is not achieved after 1 hour incubation at 0 C (Figure 5), which is in agreement with earlier findings (3,6). In contrast to those findings we did measure an appreciable amount of exchange when we incubated uterine nuclear pellets at 0 C.

Both for uterine and DMBA-induced mammary tumour tissue the efficiency of KCl extraction of steroid-receptor complexes following nuclear exchange is lower than after *in vivo* or *in vitro* labelling of receptor sites (Table 4). This might reflect a change in compartmentalization of the E_2-receptor complex during the different incubations used in nuclear exchange assays. It has been suggested (15) that the tightly

bound receptor sites which cannot be extracted with 0.4 M KCl are the most important ones in the action of steroid hormones on the target cell. Repeated extractions with 0.6 M KCl, however, appear to release more than 90% of the steroid-receptor complexes from the nuclei (16,17). The extraction efficiency for a single KCl extraction following direct in vitro labelling of uterine E_2 receptors reported by these authors is in agreement with our findings (Table 4). Although we do not extract all steroid-receptor sites from the nuclear preparations, the reproducibility of a one step 0.4 M KCl-extraction of such preparations is within acceptable limits (Table 4).
Data from a preliminary communication by Laing et al. (18) suggest that for human mammary tumours incubation of crude nuclear pellets with radioactive E_2 for 18 hours at 4 C as well as at 20 C and at 37 C would result in complete exchange. They also reported that the rat uterine receptor does not exchange its ligand at low temperature, which is in contrast to our findings (Figure 5). Kiang (19), however, suggests that the nuclear receptors measured at low temperature by Laing et all (18) would be in the free form and would not contribute to the hormone-dependent nature of a tumour. Garola & McGuire (12) demonstrated that free receptors do not occur in nuclei of DMBA-induced mammary tumours.
It is generally accepted that cytoplasmic oestrogen receptors are activated upon binding of E_2 and that the sedimentation coefficient of the receptor shifts from 4 S to 5 S during this process (20). Recently, Linkie & Siiteri (21) have presented evidence that this activation could also occur in uterine nuclei within 5 min after exposure to tritiated oestradiol-17β. After a labelling period of one hour, however, the 4 S component accounted for only 13% of the total amount of receptors. As shown in Figure 1, a small 4 S peak could also be detected in the nuclear extract of DMBA-induced mammary tumours.
Eriksson et al. (22) and Clark et al. (23) have proposed the presence of two types (I and II) of oestrogen receptors in

uterine cytosol and nuclei. It was suggested, that the type II cytoplasmic receptor was different from the type II nuclear receptor. The type I nuclear receptor, which would be the type I cytosol receptor translocated to the nuclei would be physiologically active and would not be readily labelled at low temperature. Notides et al. (24) and Traish et al. (25), however, observed only a single oestrogen receptor in uterine nuclear extracts, which sedimented at 5 S. No essential differences in labelling at 0 C and 37 C were observed, but equilibrium was reached slower at 0 C than at 37 C. Therefore it appears that the presence of two separate types of nuclear oestrogen receptors in uterine tissue is still a matter of debate. Furthermore, for other oestrogen target tissues, as the DMBA-induced rat mammary tumour used in the present study, there is no evidence for the possible presence of two types of oestrogen receptors. Since the present assay measures predominantly 5 S receptors which are considered to be the physiologically active sites (20), we conclude that the nuclear exchange assay as described offers a reliable estimation of the total amount of receptors.

In conclusion, from the present results a nuclear exchange assay employing a 1 hour 0 C exchange period for oestrogen receptor in DMBA-induced rat mammary tumours has been developed, which also gives good results for normal mammary tissue (to be published). The advantages of this assay are the distinct sedimentation patterns of steroid-receptor complexes after sucrose-gradient centrifugation and the absence of interference by endogenous E_2 or non-receptor oestrogen binding proteins.

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Appendix Paper III

(submitted for publication)

OESTROGEN RECEPTORS IN MAMMARY TISSUE AND PLASMA PROLACTIN CONCENTRATIONS DURING MAMMARY CARCINOGENESIS IN RATS INDUCED BY OESTROGEN AND IONIZING RADIATION

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SUMMARY

The amount of nuclear oestrogen receptors in mammary tissue and plasma concentrations of prolactin were studied in female rats for a period of 14 months after administration of a cholesterol-paraffin pellet containing 2 mg of oestradiol, and/or exposure to 2 Gy (200 rad) of X-rays. Oestrogen receptors were estimated after *in vivo* translocation of free receptors to the nucleus with nuclear exchange assay at low temperature. Oestrogen treatment resulted in a decrease of the content of oestrogen receptors of the mammary tissue of both irradiated rats and non-irradiated rats. In oestrogen-treated rats plasma prolactin was elevated 10-50 times and pituitary tumours were observed. Radiation had no additional effect on the mammary tissue oestrogen receptor content and the plasma prolactin concentration. The changes in the oestrogen receptor content of the mammary tissue and the prolactin concentration of the plasma preceded the development of mammary tumours. The results obtained suggest that the effect of oestrogens in the synergistic interaction of oestrogens and radiation on rat mammary tumour development is related to the large increase in the plasma prolactin concentration and/or the decrease in the oestrogen receptor content of the mammary tissue.

INTRODUCTION

Oestrogens and radiation can act synergistically in the development of mammary tumours in female rats (Segaloff & Maxfield, 1971; Shellabarger, Stone & Holtzman, 1976; Broerse, Knaan, Van Bekkum, Nooteboom, Hollander & Van Zwieten, 1978).

The mechanism through which oestrogens enhance radiation-induced mammary carcinogenesis has not been elucidated yet. It is, however, generally accepted, that oestrogens exert their effect(s) on target tissues through specific receptors present in the cytosol, followed by translocation of the oestrogen-receptor complex from the cytosol to the nucleus, interaction with the chromatin and induction of mRNA synthesis, which ultimately leads to the observed effect of the hormone. One of the effects of oestrogens would be the stimulation of oestrogen receptor synthesis (Sarff & Gorski, 1971; Mester & Baulieu, 1975). Oestrogens are also known to stimulate pituitary prolactin release (Chen & Meites, 1970).
From these observations at least two mechanisms can be considered to explain the effect of oestrogens in rat mammary carcinogenesis. Firstly, oestrogens could influence mammary tissue by influencing the synthesis of their own receptorss. Secondly, since prolactin appears to play a major role in mammary tumour development in the rat (Meites, 1972), oestrogens could exert their cocarcinogenic action via an increase in the plasma prolactin concentration.
It was the aim of the present investigation to relate the synergistic action of oestradiol and ionizing radiation on rat mammary tumour development to possible changes in the mammary gland oestrogen receptor content and/or the plasma prolactin concentration. Therefore, mammary gland oestrogen receptors and plasma prolactin were estimated in rats at different times after the administration of oestradiol and/or exposure to X-rays. It will be shown that during the period of mammary tumour development the content of oestrogen receptors in the mammary gland decreased, whereas the plasma prolactin concentration was greatly increased.

MATERIALS AND METHODS

Female Sprague-Dawley rats from the REPGO-strain were used in this study, because of their high susceptibility to radiation-induced mammary carcinogenesis (Broerse et al., 1978). They were housed, five to a cage, on wood shavings and had free

access to tap water and standard laboratory chow (Hope Farms, Woerden, The Netherlands). Oestrogen pellets were implanted subcutaneously in the dorsal region of the neck at the age of 7 weeks. Radiation was given at 8 weeks of age. The rats were divided among four groups, receiving either no treatment, oestrogen treatment, radiation treatment, or oestrogen plus radiation treatment.

Cholesterol-paraffin pellets containing 2 mg of oestradiol were prepared essentially as described previously (Blankenstein, Broerse, De Vries, Van den Berg, Knaan & Van der Molen, 1977). Cholesterol and paraffin were used in a 1:3 (w/w) ratio, rather than in a 1:10 (w/w) ratio, since oestradiol dissolved faster in a melt containing the higher amount of cholesterol. The oestradiol concentrations reached in peripheral plasma following implantation of pellets with a cholesterol-paraffin ratio of 1:3 (w/w) (see: Results section) were not different from those observed after implantation of pellets with a cholesterol-paraffin ratio of 1:10 (Blankenstein et al., 1977). Radiation treatment was given as a single dose of 2 Gy (200 rad) X-rays. When given this dosage, approximately 50% of the rats is expected to develop mammary tumours within 14 months (Broerse et al., 1978), and a synergistic interaction of oestradiol and radiation on mammary tumour development has been observed (Van Bekkum, Broerse, Van Zwieten, Hollander & Blankenstein, 1979). Vaginal smears were taken daily from a number of rats to assess the oestrus cycle stage. Oestradiol-treated rats showed a persistent oestrus. Animals which were not treated with oestradiol were used in the metoestrus phase of the cycle to circumvent possible fluctuations of oestrogen receptor levels during the cycle. With intervals of two months, six animals from each group were killed. Before killing the animals by decapitation, the rats were anaesthesized with ether and a 2-3 ml blood sample was collected in heparinized tubes after amputation of a 2 cm piece of the tail. Subsequently, the animals were injected subcutaneously with a solution of 25 µg of oestradiol in 0.1 ml of olive oil. This treatment resulted in an oestradiol concentration in the plasma of 7.5 $\pm$ 1.0 ng/ml (mean $\pm$ s.e.m., n = 1) and should cause the transfer of all cytoplasmic receptors to the nuclei. One hour after the injection, the animals were killed by decapitation and the six inguinal mammary glands were excised and placed on ice.

Oestrogen receptors were estimated in the mammary tissue with the low-temperature nuclear exchange assay as published elsewhere (Blankenstein, Aitken-Cook, Mulder & Van der Molen, 1978; Blankenstein, Peters-Mechielsen, Mulder & Van der Molen, 1980). Briefly, the method consists of translocation of free receptors to the nuclei through the injection of 25 µg of oestradiol, preparation of a Triton X-100 washed crude nuclear pellet, exchange of unlabelled oestradiol with 10^{-8} M ^{3}H-oestradiol, extraction of ^{3}H-oestradiol-receptor complexes with 0.4 M KCl, adsorption of excess free steroid to Dextran-coated charcoal, separation of free and bound steroid by centrifugation of the nuclear extract for 2 h and 45 min at

65,000 rpm in a Beckman VTi-65 rotor through linear 10-30% sucrose gradients, fractionation of the gradients and determination of radioactivity in the separate fractions. Oestrogen receptor values were expressed per mg of protein in the nuclear extract. Plasma prolactin was assayed with a homologous double antibody radioimmunoassay based on the method described by Kwa, Van der Gugten & Verhofstad (1969). Rabbit anti-rat-prolactin serum was a gift from Dr. H.G. Kwa (Netherlands Cancer Institute, Amsterdam). Prolactin reference preparation RP_1 was kindly provided by Dr. A.F. Parlow (Rat Pituitary Hormone Distribution Program, NIAMDD, NIH, Bethesda, MA, U.S.A.). Commercially available ^{125}I-rat-prolactin (New England Nuclear, Dreieich, Germany) with a specific activity of 1500 ± 50 kBq/µg (42.1 ± 1.4 µCi/µg) was used as a tracer. Donkey anti-rabbit serum (Wellcome, Beckenham, England) was used as precipitating serum. Plasma oestradiol concentrations were measured according to De Jong, Hey & Van der Molen (1973).

RESULTS

Macroscopic observations

The increase in body weight of oestradiol-treated rats was much slower than that of animals receiving no oestrogen (Table 1). Since radiation had no effect on the growth, the data from the groups not receiving oestradiol were pooled and

Table 1. Body weight of rats treated with oestradiol and rats given no oestrogen treatment at different intervals after the start of the experiment. Results are means ± s.e.m. (n).

Time after treatment (months)	Body weight (g)	
	oestrogen	no oestrogen
2	173 ± 3 (8)*	208 ± 6 (6)
4	201 ± 7 (13)*	237 ± 6 (10)
6	208 ± 7 (8)*	239 ± 5 (9)
8	213 ± 14 (5)*	241 ± 5 (11)
10	230 ± 6 (15)*	255 ± 6 (8)
12	254 ± 12 (12)	255 ± 8 (11)
14	278 ± 18 (8)	272 ± 13 (9)

*$P < 0.05$ vs. no oestrogen (Student's t-test)

compared with the pooled data from the two oestradiol-treated groups. A significant difference in body weight between the two groups was observed up to 10 months after the start of the experiment. In addition, oestradiol treatment caused the disappearance of the oestrus cycle and a massive stimulation of the mammary glands with the formation of large secretory vesicles. From 10 months after oestrogen treatment, pituitary tumours were found at autopsy in more than 50% of the animals. Irradiated rats showed pin point red dots all over the mammary tissue during the first 8 months after irradiation. Furthermore, at two months after irradiation small nodules were observed in the mammary glands of three rats out of five rats which were examined. One of these nodules was large enough to permit assay of oestrogen receptors, and was found to contain twice as many receptor sites as the surrounding normal mammary tissue (204 vs. 98 fmoles/mg protein). Pituitary tumours were found only occasionally in irradiated rats, starting 12 months after irradiation.

Mammary gland oestrogen receptors

Figure 1 shows levels of mammary gland oestrogen receptors measured in non-irradiated rats at different intervals after oestradiol administration. It appears that oestradiol treatment resulted in a decrease of the estimated content of mammary gland oestrogen receptors, although the difference was statistically significant only at 2, 4 and 12 months after implantation of the oestradiol pellet. The effect of oestradiol on oestrogen receptor levels was more pronounced in irradiated animals, as shown in Figure 2. At 2 months after irradiation, the mammary gland oestrogen receptor content was higher in irradiated rats that received no oestrogen than in control rats ($P < 0.05$). This could be due to the presence of the small, receptor-rich nodules mentioned before.

A longitudinal quality control for the receptor assay could not be performed, because this assay requires the use

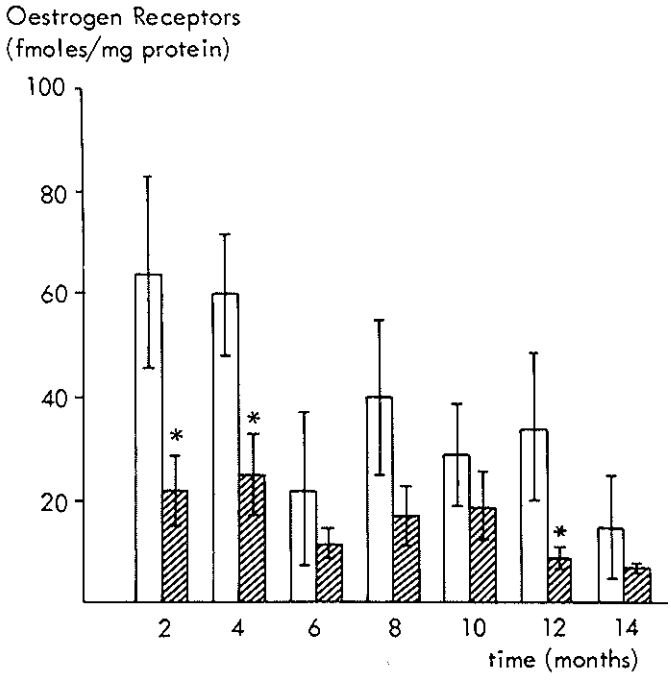

Figure 1. Levels of mammary gland oestrogen receptors in control rats (open bars) and in oestrogen-treated rats (shaded bars), measured at different intervals after the start of the oestradiol treatment. Results are means ± s.e.m., n = 4-8. *P < 0.05 vs. control rats (Wilcoxon's test).

of fresh tissue, which cannot be stored as a homogenous pool. Nevertheless, to exclude the possibility that the observed differences in receptor levels reflected experimental errors due to a possible incomplete exchange during the assay, in each receptor assay series oestrogen receptors were also estimated in the uterus of one of the animals used. The oestrogen receptor content of uterine tissue from oestrogen-treated animals was 257 ± 22 fmoles/mg protein (mean ± s.e.m., n = 31), as compared to 263 ± 26 fmoles/mg protein (mean ± s.e.m., n = 21) in tissue from animals which received no oestrogen pellet. From these results we have concluded, that the decrease in oestrogen receptor content observed in the mammary glands of oestrogen-treated animals is not caused by

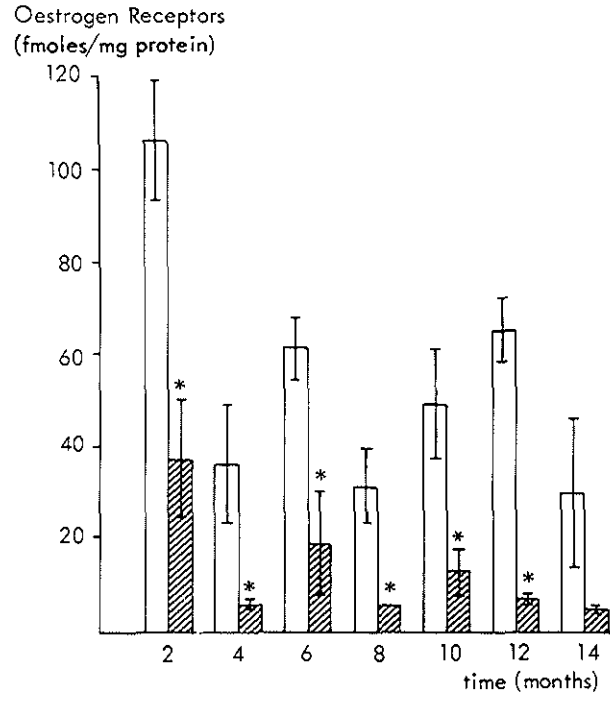

Figure 2. Levels of mammary gland oestrogen receptors in irradiated rats (open bars) and in irradiated rats treated with oestradiol (shaded bars), at different intervals after the start of the oestradiol treatment. Results are means ± s.e.m., n = 4-8. *P < 0.05 vs. irradiated rats (Wilcoxon's test).

incomplete exchange of oestradiol from its receptor.

Plasma prolactin

Plasma prolactin levels, measured at different time intervals after oestrogen treatment, are shown in Figure 3 for non-irradiated rats and in Figure 4 for irradiated rats. Oestradiol implantation caused a marked increase in prolactin levels. Radiation had no effect on plasma prolactin in oestrogen-treated rats, nor in rats which were not given an oestrogen pellet. From Figure 3 it is apparent, that the plasma prolactin concentration in control rats increases with ageing.

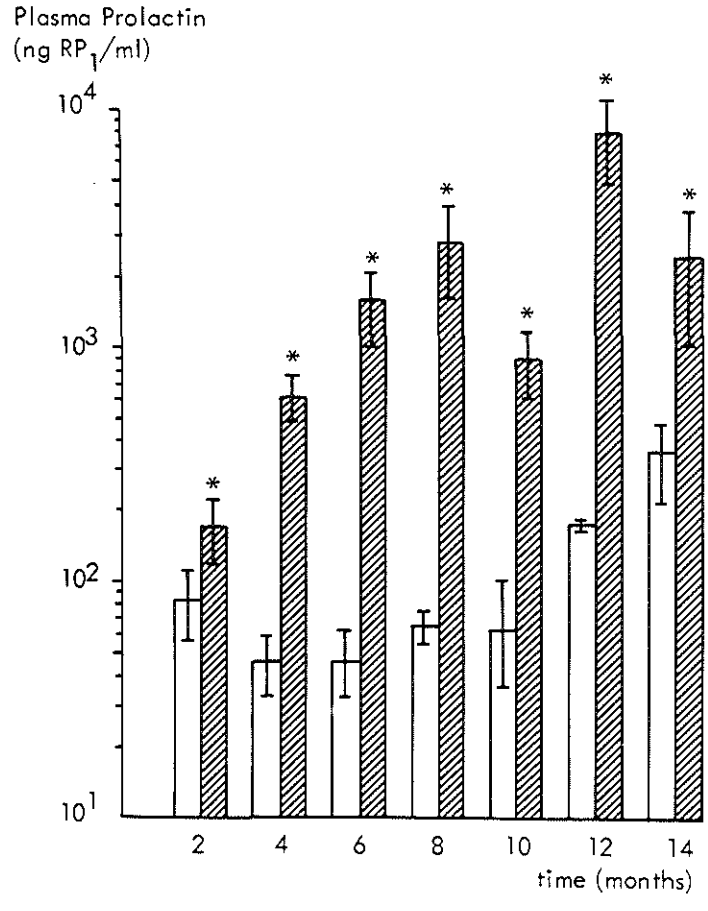

Figure 3. Plasma prolactin concentrations in control rats (open bars) and in oestradiol-treated rats (shaded bars) at different time intervals after the start of oestradiol treatment. Results are means ± s.e.m., n = 3-8. *P < 0.05 (Wilcoxon's test).

Plasma oestradiol

Plasma oestradiol concentrations were estimated to evaluate the release of oestradiol from the oestradiol pellet. The results given in Table 2 show, that oestrogen treatment caused no significant increase in the plasma oestradiol concentration, as compared to the levels estimated in the met-oestrus phase of the cycle in animals which were not treated

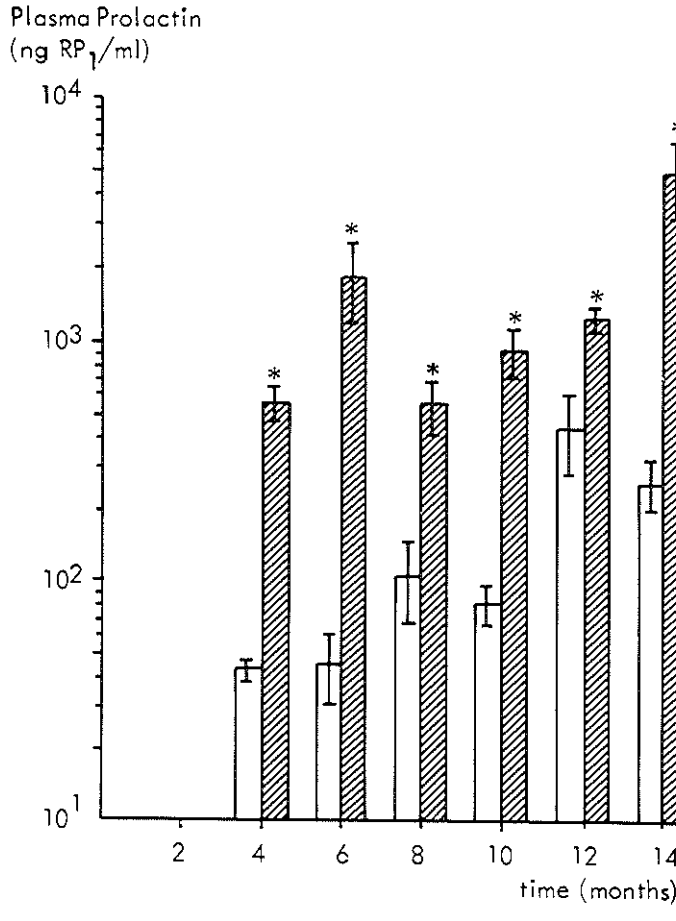

Figure 4. Plasma prolactin concentrations in irradiated rats (open bars) and in irradiated rats treated with oestradiol (shaded bars) at different time intervals after the start of the oestradiol treatment. Results are means ± s.e.m., n = 3-8. *$P < 0.05$ (Wilcoxon's test).

with oestradiol. Yet the oestradiol treatment was sufficient to stop the oestrus cycle, to retard the growth and to stimulate prolactin secretion. Plasma oestradiol was elevated in irradiated rats from 4 to 8 months after irradiation. Such an effect was not observed for oestrogen-treated irradiated animals.

Table 2. Plasma oestradiol concentration in untreated rats, oestrogen-treated rats, irradiated rats and oestrogen-treated irradiated rats at different time intervals after the start of the treatment. Results are means ± s.e.m. (n) or individual values (n = 1 or 2). In cyclic rats, plasma samples were taken in the morning of the day of metoestrus.

Time after treatment (months)	Plasma oestradiol (pg/ml)			
	untreated	oestrogen	radiation	oestrogen + radiation
2	46± 9 (6)	101-137 (2)	46±10 (5)	50±25 (3)
4	-	79± 9 (6)	233±66 (4)**	67± 8 (6)*
6	49± 6 (3)	96± 20 (4)	88± 7 (3)*	95±15 (6)
8	54± 7 (5)	92± 33 (3)	82± 8 (4)*	60± 9 (4)
10	54 (1)	77± 11 (3)	52± 6 (4)	61± 7 (6)
12	69±17 (4)	61± 23 (3)	33± 5 (5)	52± 6 (5)
14	48± 9 (4)	67± 25 (6)	38±12 (5)	141±26 (6)*

* $P < 0.05$ vs. corresponding control (Student's t-test)
** $P < 0.05$ vs. preceding value

DISCUSSION

The results of the present study demonstrate that subcutaneous oestradiol implantation causes a decrease of the content of oestrogen receptors in mammary glands of irradiated rats as well as in rats which were not irradiated (Figures 1 & 2). Radiation had no additional effect on oestrogen receptor levels, although the combined effects of the dosages of oestradiol and radiation used in this study are known to act synergistically in the development of malignant mammary tumours (Van Bekkum et al., 1979). The decrease in mammary tissue oestrogen receptor content theoretically could reflect occupancy of receptors by the oestradiol administered and incomplete exchange with the radioactive ligand. However, the results of estimations of oestrogen receptors in uterine

tissue obtained from the same oestradiol-treated rats did not show a decrease. From this observation we have concluded, that the decrease of the oestrogen receptor content of mammary tissue is not caused by incomplete exchange.
The data presented in Figures 3 & 4 clearly demonstrate, that implantation of an oestradiol-containing pellet early in life results in a prolonged increase in the concentration of prolactin in the plasma. In the present study, no attempt was made to demonstrate a possible (co-)carcinogenic action of prolactin on the mammary gland, but this has been suggested in the literature (e.g. Meites, 1972; Welsh & Nagasawa, 1977). The large increase in pituitary prolactin release observed in the present study is in contrast to observations reported by Stone, Holtzman & Shellabarger (1979). These authors observed only a small but significant increase in plasma prolactin levels in their Sprague-Dawley rats and detected no pituitary tumours during an observation period of 7 months. ACI rats, however, responded to diethylstilboestrol treatment with an increase in the plasma prolactin concentration comparable to the increase observed after oestradiol implantation in the Sprague-Dawley rats used in the present study. It appears from this observation, that large differences exist between the different substrains of Sprague-Dawley rats.
In untreated rats of the Sprague-Dawley substrain used in the present study, the incidence of malignant mammary tumours was 7%. In rats treated with oestradiol and in rats irradiated with 2 Gy of X-rays the observed incidences of malignant mammary tumours were 5% and 0% respectively, whereas in oestradiol-treated irradiated rats an incidence of 25% was observed (Van Bekkum et al., 1979). This synergistic effect of radiation and oestrogens in the occurrence of malignant mammary tumours was not accompanied by an increased oestrogen receptor content of the mammary tissue. Therefore, we have concluded that it is unlikely that the co-carcinogenic effect of oestrogens in rat mammary carcinogenesis is mediated through an in-

crease in the number of oestrogen receptors in the mammary tissue, as might have been expected from the observations of Sarff & Gorski (1971) and Mester & Baulieu (1975). These authors reported, based on short-term experiments with uterine tissue, that oestrogens can induce the synthesis of their own receptors. It appears from the results of the present study, that this does not apply for mammary tissue during long-term experiments.

From the present results, it would appear that the effect of oestrogens on mammary tumour development in the rat could be related to a decrease in the oestrogen receptor content of the mammary tissue, to increased prolactin secretion, or to the disappearance of cyclic fluctuations in the plasma oestradiol concentration. However, all these changes are present well before the manifestation of mammary tumours (Broerse et al., 1978) and further investigations will have to determine whether and which, if any, of these effects is involved in the action of oestrogens on rat mammary carcinogenesis.

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