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RIJKSUNIVERSITEIT LIMBURG TE MAASTRICHT

**STUDIES ON THE DETERMINATION OF
CYTOPLASMIC OESTRADIOL RECEPTOR
IN HUMAN BREAST CANCER**

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor in de
geneeskunde
aan de Rijksuniversiteit Limburg te
Maastricht
op gezag van de rector magnificus
Prof. Dr. W.H.F.W. Wijnen
Hoogleraar in de faculteit der Geneeskunde,
volgens besluit van het college van Dekanen
in het openbaar te verdedigen
op donderdag 14 mei 1981 te 16.00 uur
in de aula van de universiteit
Tongersestraat 53

Philippe Guy Fernand Arthur Hector Ralet

geboren in Charleroi

promotores: Prof. Dr. P.J. Brombacher

Prof. Dr. H.C. Hemker

referenten: Prof. Dr. H. Tuchmann-Duplessis

Prof. Dr. J.M. Greep

A Emmy et Didier

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CONTENTS

<i>chapter 1</i>	Introduction	1
<i>chapter 2</i>	The use of coated charcoal in the determination of oestrogen receptor activity	19
<i>chapter 3</i>	Coating of charcoal by dextran	31
<i>chapter 4</i>	Oestrogen receptor determination after protamine sulphate precipitation	45
<i>chapter 5</i>	Protein determination in mammary tumour cytosols . .	57
<i>chapter 6</i>	Plasma proteins in mammary tumour cytosols	81
<i>chapter 7</i>	Influences of buffer composition, temperature and tumour tissue on the pH of cytosols.	93
<i>chapter 8</i>	Characterisation of oestrogen receptor protein . . .	107
<i>chapter 9</i>	General survey and conclusions	139
	Summary.	141
	Samenvatting	143
	Résumé	145
	Zusammenfassung.	147

chapter 1

INTRODUCTION

OESTROGEN HORMONES AND RECEPTOR PROTEINS

In the study of cancer of the breast much attention has been paid in recent years to procedures interfering with the endocrine status of the patient. Both surgical treatment and hormonal therapy have been used extensively (1). Various response rates have been observed but few if any patients with metastases could be cured, although much progress has been made in the understanding of treatment.

Since the first observation (2) of endocrine influence on the growth and recurrence of breast cancer, many investigators have tried to find a better pathophysiological basis for the treatment of this type of malignancy. The recent introduction of reliable laboratory assays for hormone receptors has simplified the selection of therapy in many patients (3). The idea that hormones act through receptor proteins present in some tumours and not in others has now been generally accepted (4, 5) and this has been confirmed by the fact that the response to endocrine therapy is much better in patients whose tumours contain oestrogen receptors than in the oestrogen receptor negative patients (6). It can be stated that the greatest value of the determination of oestrogen receptor in tumour tissue is the ability of such tests to identify patients who can be spared either unnecessary ablative surgery of endocrine glands or resultless administration of hormonal drugs. Evidence has been presented (7, 8, 9) suggesting that patients with oestrogen receptor positivity have a better prognosis than those with receptor negativity. It is now certain that the symptoms of advanced breast

cancer can be relieved, sometimes for up to 10 years, by the administration of hormones, endocrine ablation, chemotherapy or combinations of these approaches (1). Moreover, after oophorectomy or the administration of oestrogens, the fraction of cells synthesising DNA falls in patients who respond to endocrine therapy, but not in patients who fail to respond (10), which is another support for the influence of oestrogen steroids on tumour protein synthesis in the presence of oestrogen receptors. In clinical studies of the response rates or failure of endocrine therapy a number of criteria for objective remission have been used, such as considerable decrease in the size of measurable lesions, no progress of other lesions and roentgenographic evidence of healing of osteolytic metastases (11). Unsatisfactory correlation between oestrogen receptor determinations *in vitro* and remission rates led to the introduction of the determination of progesterone receptors in cytosols from the same tissue in which oestrogen receptors had been determined. As far as remission was concerned the predictive value of these combined receptor data was clearly better than for oestrogen receptor values alone. This can perhaps be explained by the hypothesis that the synthesis of the progesterone receptor protein is positively influenced by the presence of oestrogen receptor complexes (12). The indication of remission in clinical studies can be given in several ways. A first possibility, generally used, is to calculate the rate of response to therapy in different groups of patients/tumours, classified according to oestrogen receptor positivity (ER+) or negativity (ER-) in combination with progesterone receptor positivity (PgR+) or negativity (PgR-). It has been reported that in patients with ER+/PgR+ tumours good remission rates (77%) were observed in contrast to ER+/PgR- tumours (27%) and ER-/PgR- tumours (11%) (13). More sophisticated schemes for analysis of clinical data have also been proposed giving "dose-response" curves in a four-parameter logistic model (14). Here the hormone receptor values were not just given as positive or negative but it appeared that the experimentally determined receptor concentrations are important for the prognosis of treatment.

Progesterone receptor values were studied at different levels of oestrogen receptor values and *vice versa* in relation to remission on treatment. Altogether, there is ample evidence now that the determination of oestrogen receptors in tumours of the breast is very important for the treatment of patients afflicted with these tumours. In a special report of a "consensus-development meeting" on "Steroid Receptors in Breast Cancer" (National Institutes of Health, 1979) it is stated (3): "Only with the recent study of the oestrogen receptor (ER) content of breast cancer tissue has there been any success in identifying those patients most likely to benefit from endocrine therapy Thus, results of ER assays provide valuable information for making the clinical decisions on the type of therapy to be employed" Results presented at that meeting indicated generally good agreement in receptor assays of primary and metastatic tumour samples from the same patient but changes in sequential receptor assay occurred from receptor positivity to negativity.

From the clinical importance of the determination of oestrogen hormone receptors it follows that attention has to be paid to the mechanism of action and the very nature of these proteins. All known hormone receptors are proteins that contain a site or sites to which the hormone can bind. The role of a receptor is in the first place to distinguish a particular signal from the specific hormone for which the cells concerned are targets and next to relay this signal in such a way that the appropriate cellular response follows. In general, the conformation of the receptor is changed by binding the hormone. Steroid hormone receptors are found in the cell cytoplasm but as soon as the steroid is bound the hormone receptor complexes migrate to the nucleus where they are bound to the chromatin and protein synthesis is greatly influenced (4). It is not clear whether the hormone originally penetrates the cell membranes simply by passive diffusion or if specific uptake mechanisms are active (15). An essential characteristic of an oestrogen target cell, as found in mammary gland or uterine tissue, is the capacity to bind oestradiol specifically and with high affinity. Steroid receptors

figure 1-1

Current concept of the role of receptor proteins in oestrogen hormone action.

SHBG : sex hormone binding globulin

A : albumin

E : oestrogen

P_C : cytoplasmic protein

R_C : cytoplasmic receptor

R_C-E : cytoplasmic receptor - oestrogen complex

R_C act-E : activated cytoplasmic receptor - oestrogen complex

R_N-E : nuclear receptor - oestrogen complex

RNA : ribonucleic acid

mRNA : messenger ribonucleic acid

: non specific binding protein

: specific binding protein (receptor)

have been determined in cytosols prepared from target tissues. The receptor hormone complex of the mammary gland sediments at 8-9 S (Svedberg units) on sucrose gradients with low salt concentrations. Increasing the salt content to solutions with greater than physiological ionic strength results in the sedimentation at 4-5 S (molecular weight about 70,000). The true molecular size of the receptor in the target cell is not known although some authors prefer the 4 S form (16). It has been reported that $3 \times 10^3 - 100 \times 10^3$ receptors per cell can be found.

There are some fundamental differences between the intracellular receptor proteins and steroid binding proteins found elsewhere, e.g. sex hormone binding globulin (SHBG) and albumin in blood plasma. The most striking difference is perhaps that the equilibrium constant of dissociation (K_d) is about 10 to 100 times smaller for receptor proteins than for SHBG and corresponding extracellular binding proteins. In cytoplasmic receptors K_d 's are commonly found in the order $10^{-9} - 10^{-11}$ mol/l. Oestrogens bound to extracellular proteins

provide a vast pool in equilibrium with a small amount of free hormones. The latter by passive diffusion pass the cellular membrane and are captured in the cytoplasm by specific receptor proteins and non-specific cytoplasmic binding proteins. It is believed that the primarily formed oestrogen receptor complex undergoes aggregation to larger entities or activation before binding of the complex to the cell nucleus occurs (17) and the nuclear membrane is passed. It is not fully known what this activation entails. Probably the conformation of the oestrogen receptor complex is changed so that a nuclear binding site on the receptor is exposed and binding to nuclear receptors occurs (R_N -E, 5 S). The nuclear proteins and the DNA of chromatin appear to participate in the binding process. It has been found in oestrogen responsive systems that there are more nuclear receptors than cytoplasmic steroid receptors, so the nuclear binding sites are never saturated in the intact cell (18, 19). It is understood that after binding of the oestrogen receptor complex to specific chromatin sites (generally considered to consist of non-histone proteins) in the nucleus, DNA transcription is induced by RNA polymerase. A specific mRNA is formed which migrates to the ribosomes in the cytoplasm where the code is translated resulting in the synthesis of specific protein. Whether steroid receptor chromatin complexes affect transcription of specific genes and thus influence protein synthesis or steroid receptor complexes themselves act as, or upon, enzymes that modify chromatin proteins is still unclear. The fact that there is a specific and pronounced influence on protein synthesis, however, is now generally accepted (figure 1-1).

BINDING OF STEROIDS TO RECEPTOR PROTEINS

The models used in the biochemical analytical procedures for the study of binding phenomena of steroid hormones to receptor proteins have been adapted following general principles of kinetics of protein ligand interactions (20). As non-specific binding proteins are likely to interfere in the analytical procedures a number of

parameters must be established and evaluated. Amongst these are binding specificity, binding affinity, binding capacity and the molecular form of the receptor proteins.

Binding specificity means that the receptor can discriminate signal from noise, in other words that the structural differences between various steroid hormones are recognised at the molecular level by the receptor proteins. This specificity can be estimated *in vitro* by determining the relative competition between different types of steroid hormones for binding sites on a specified receptor protein.

The binding affinity of the hormone for the receptor needs to be sufficiently high as concentrations of circulating hormones are in the range of 10^{-8} - 10^{-10} mol/l. In the study of these affinities it must be considered that the binding is non-covalent involving electrostatic interactions as hydrogen bonding and also hydrophobic interactions. The hydrophobic interactions to some extent drive the reaction on account of the presence of hydrophobic regions on the receptor protein and the non-hydrophilic character of steroids. The final binding is primarily the result of hydrogen bonds between charged groups on the hormone and oppositely charged groups at the complementary site on the receptor. These receptor sites also determine to some extent the binding specificity.

$$K_d = \frac{k_2}{k_1} = \frac{[H][R]}{[HR]}$$

The mathematics of the binding reaction can be conveniently derived from the general equations given here where $[H]$ is the concentration of hormone, $[R]$ is the concentration of receptor, $[HR]$ is the concentration of hormone receptor complex, k_1 is the rate constant of the association reaction, k_2 is the rate constant of the dissociation reaction and K_d is the equilibrium dissociation constant. The reciprocal of the latter is the equilibrium association constant

figure 1-2a

Binding of tritiated oestradiol by receptor positive mammary tumour cytosol (DCC-method).

T : total binding

N-Sp : low affinity high capacity non specific binding

Sp : specific (receptor) binding calculated by $T - N-Sp$

cap. : broken line indicates maximum specific binding capacity, i.e. receptor content per ml cytosol

$\frac{1}{2} s$: half maximum (saturation) binding

aff. : free hormone concentration at half saturation, equals K_d

figure 1-2b

Scatchard analysis of data shown in figure 1-2a.

cap. : maximum binding capacity per ml cytosol

K_d : minus inverse of slope

The protein concentration in the cytosol was 4.1 mg/ml, hence the

binding capacity is calculated as follows: receptor sites

0.220 pmol/ml cytosol or 220 fmol / 4.1 mg protein equals

54 femtomol/mg protein.

K_a (1/mol); K_d is expressed in mol/l.

From the above equations, $[R]$ and $[HR]$ can be cancelled if the concentrations are equal, that is, if half the receptor sites are occupied by hormones. In that case, $K_d = [H]$. This leads to an useful working definition of affinity, saying that the free concentration of hormone necessary to half-saturate receptors equals the equilibrium dissociation constant. The lower this concentration is, the higher the affinity of binding (figure 1-2a).

Binding is usually studied over a range of hormone concentrations from 1/10th to 10 times the half-saturating concentration. For the interpretation of data obtained in this way, the Scatchard plot (21) is mostly used. Linear plots are obtained permitting the determination of the the dissociation constant and the total concentration of binding sites which is also called the binding capacity.

If $n_{\max}$ is the total concentration of binding sites then

$$n_{\max} = [HR] + [R]$$

or
$$[R] = n_{\max} - [HR]$$

Substituting $[B]$ (concentration of bound hormone) for $[HR]$ and $[F]$ (concentration of free hormone) for $[H]$, then

$$[B] / [F] = [R] / K_d$$

and

$$\frac{[B]}{[F]} = \frac{n_{\max} - [B]}{K_d} = \frac{-[B]}{K_d} + \frac{n_{\max}}{K_d}$$

This is the equation for a straight line. From the graphical representation of $[B] / [F]$ vs. $[B]$, the K_d (minus the inverse of the slope) and $n_{\max}$ (the abscissa of the intercept of the line with the X-axis) can easily be read (figure 1-2b).

This description of binding phenomena is employed successfully in the determination of oestrogen receptors. Unfortunately, a cytosol from human tissue always contains non-specific macromolecular hormone binding components, e.g. tissue proteins and often serum proteins

(human serum albumin). There are differences, however, between the true hormone receptor and non-receptor binding proteins. The dissociation constants for non-specific hormone protein complexes are much higher than for the hormone receptor complexes. On the other hand, non-specific binding proteins are often abundantly available leading to a large binding capacity in the cytosol. Scatchard analysis performed on a series of concentrations of the hormone shows the existence of specific and non-specific binding (*figure 1-2a*). By adding considerable excess of radio-inert hormones to the assay system the binding of tracer by the true receptor is considerably depressed by competition. In this way the degree of non-specific binding in a given system can be determined. The binding capacity of hormones is expressed by the molar amount of hormone bound per mg protein or per μg DNA, the latter expression being used for receptor concentration in homogenates of cell nuclei.

The last parameter to be considered is the molecular form of the receptor protein. Although most receptor proteins have been found in gradient centrifugal analysis to sediment as 8 S macromolecules the occurrence of 4 S proteins has also been reported. The sedimentation constant found appeared to be dependant on the ionic strenght of the medium used in the analysis. There is no firm agreement on the actual native form of the receptor protein (22). It has been reported that the receptor changes from low affinity 4 S protein towards high affinity activated (perhaps 5 S) receptor protein just by the binding of oestradiol (23). The possibility is not excluded that tumours demonstrating hormone binding only in the 4 S region are less hormone dependant than those containing 8 S receptor proteins (24).

TECHNIQUES FOR THE DETERMINATION OF CYTOPLASMIC OESTROGEN RECEPTORS

The presence of the cytoplasmic receptor proteins in target tissues can be demonstrated both in tissue slices and in cytosols. The latter are protein extracts prepared by removing cellular debris and nuclei

from tissue homogenates by centrifugation at low temperature and carefully chosen pH to avoid irreversible damage to the receptor proteins.

Tissue slice analysis is performed by incubating tissue slices in a suitable medium containing radioactive oestradiol. The steroid is bound to the receptor proteins *in situ* and can be detected there. Detection of non-specific binding is performed by adding excess of competitor ligands, e.g. "cold" oestradiol or synthetic steroids. Another possibility is the demonstration of receptor sites by immunofluorescence techniques avoiding the necessity to use radioactive tracers. A drawback of these procedures is the impossibility of quantitative analysis. Furthermore it is difficult to find tissue slices which are truly representative for the whole tissue from which they have been taken (25, 26).

A number of techniques for the quantitative determination of the concentration of the specific receptor in cytosols has been described. The principles of all methods are the binding of radioactive oestradiol (tracer) by the receptor protein, the removal of excess tracer and the estimation of the ratio of specific to non-specific binding. Several techniques are used of which the most important are listed below:

Dextran coated charcoal (DCC) analysis (27, 28).

This is probably the most often used method in so called protein binding analysis. In receptor assay it consists of the incubation of cytosol samples with radioactive oestradiol which is completed after some hours by adding dextran coated charcoal. The latter is supposed to adsorb only small molecules, excess tracer, leaving undisturbed the oestradiol receptor complex. Removing the charcoal by centrifugation leaves only the protein-bound tracer in the solution and the radioactivity of the tracer-protein complex can be determined. The method is elegant and can easily be performed. However, the separation of bound and free tracer often deviates from what theoretically was expected, as will be described later on.

Sucrose density gradient assay (SGD) (29).

The sucrose density gradient ultracentrifuge assay is based on the separation of proteins due to sedimentation. Aliquots of cytosols are incubated with radioactive oestradiol with or without an excess of unlabelled competitive ligand (e.g. diethylstilbestrol or another oestrogen-like compound) to determine total and non-specific binding. Excess steroids are either removed by adding DCC and subsequent centrifugation, or the reaction media are directly submitted to ultracentrifugation. In the first case aliquots of the remaining supernatant are then layered on top of a cold linear gradient of sucrose. After ultracentrifugation (about 300,000 *g*) during 16 hours the tubes are punctured at the bottom and the contents taken in small fractions for the measurement of radioactivity. The fraction containing considerable tracer-protein complex is supposed to be the "receptor-fraction", found by comparison of experiments with and without excess cold steroid to detect non-specific binding.

Protamine precipitation assay (30, 31).

Another method having considerable advantage at first sight is the use of protamine sulphate to precipitate receptor protein. Samples of cytosol are mixed with a protamine sulphate solution. Upon centrifugation coprecipitation of the latter with certain protein fractions (amongst which all receptor proteins) occurs, forming a thin film on the bottom of the tube. After decanting the supernatant a buffer containing the tracer is added to the tubes. Radioactive oestradiol is bound to receptor proteins during subsequent incubation and the excess tracer is removed by decanting the contents of the tube and rinsing with buffer. Radioactivity remaining in the tubes is bound to the precipitated protein film and is easily measured, giving an indication of the receptor concentration. A disadvantage is that the necessary rinsing of the tubes brings the risks of loosing protein and dissociation of receptor-tracer complex. These phenomena could cause errors in the analysis, i.e. underestimation of receptor content. Nevertheless, the method seems to be competitive with the DCC technique.

Gel filtration (32).

Gel filtration is another general procedure used for separation and isolation of proteins from low molecular weight material in solutions. The principle of this technique has also been applied in the determination of oestrogen receptor protein in cytosols. Incubation of the cytosol with tritium-labelled oestradiol under suitable experimental conditions leads to saturation of the receptor sites. Proteins with adhering tracer can then be separated from excess tracer by gel filtration. Problems arise in some experiments on account of chromatographic adsorption effects of the column medium resulting in loss of tracer-receptor complex, and also in the necessary differentiation of complexes of binding proteins with radioactive steroid in the macromolecular fraction separated in this technique. Furthermore dissociation of the oestradiol receptor complex may cause serious errors.

Electrophoretic techniques (33).

The last techniques to be mentioned here are agar gel electrophoresis and polyacrylamide gel electrophoresis. These procedures are sometimes used to separate the proteins in the cytosol whereupon the electropherogram is either incubated with tracer as such or cut into thin slices containing the different protein fractions which were studied separately. Another possibility is to separate receptor-tracer complex from free receptor by electrophoresis after prior incubation. Severe problems arise on account of considerable dissociation of these complexes and the denaturation of the labile receptor proteins during the electrophoretic procedure.

PURPOSE OF THE PRESENT STUDY

From the preceding paragraphs it is clear that much was left undiscussed. The necessity of determination of oestrogen-receptor concentration in mammary gland tumours is generally agreed. The procedure to be chosen is a difficult problem. Some methods were less

suitable for quantitative determination of receptor concentration in cytosols (gel filtration and gel chromatography, electrophoresis) but were useful in the characterization of the receptor proteins (33). In other methods great care had to be taken to avoid denaturation of receptor proteins due to changing ionic strength and local heat development in electrophoretic techniques. It is not surprising that the DCC method is still the procedure of choice in many laboratories whilst other investigators tend to use the sucrose density gradient procedure or the protamine sulphate precipitation technique.

In our laboratory the DCC method was used during some years but full satisfaction was not found. The main purpose of the present investigation therefore was the intensive study of parameters involved, such as the efficiency of separating bound and free tracer by dextran coated charcoal and the cause of loss of radioactivity supposed to be protein bound. Another fundamental problem appeared to be the accurate and reproducible determination of very low protein concentrations in sometimes rather small cytosol samples. Furthermore in the standardization of the determination of oestrogen receptors the importance of the pH of the reaction medium was often neglected. This was a reason for close investigation too.

In our opinion the predictive value of the determination of hormone receptors in cytosols from human tissues can most probably be improved by rigorous standardization of the technical aspects of the assay paying attention to all relevant parameters.

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chapter 2

THE USE OF COATED CHARCOAL IN THE DETERMINATION OF OESTROGEN RECEPTOR ACTIVITY

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ABSTRACT

Oestrogen receptor activity in mammary tumour and healthy tissues was determined by using a method of incubation with coated charcoal. It appears that both increased charcoal concentration and prolonged incubation lead to erroneous results, which are probably due to loss of receptor protein. It is concluded that receptor activity assays must be performed under strictly standardized experimental conditions.

INTRODUCTION

The separation of bound and free tracer is a crucial point in every radio-immunoassay. On this subject several methods have been published (1, 2) of which the dextran coated charcoal method and solid phase systems are most often used.

The principle of coating charcoal for the separation of bound from free tracer is the partial inactivation of the active charcoal so that large molecules can no longer be adsorbed, whereas smaller molecules can still penetrate to the charcoal surface and be adsorbed

there.

One of the weaker points in the procedures described is that a coating excluding all large molecules and leaving the possibility for all small molecules to be bound, is rather difficult, if not virtually impossible, to achieve. It often happens that even larger molecules, because of the imperfect coating, are still bound whilst more thorough coating leads to incomplete adsorption of the free tracer. For this reason the use of coated charcoal leads to decreased reliability in the method that we use for the estimation of oestrogen receptor in mammary tumour tissue cytosols. In this paper we demonstrate the influence of time of incubation and amount of coated charcoal used on the outcome of the analysis.

In the estimation of oestrogen receptors in cytosols from human tissue homogenates we followed the principles of the EORTC breast cancer co-operative group (3, 4). In this group it has been indicated that the charcoal adsorption technique has to be retained as the standard procedure, with the option of using more refined techniques when they become available. For this reason we studied the influence of coated charcoal in our estimations and we were rather disappointed to see the great influence on the outcome of the analysis by changing the conditions of the assay, especially concerning the amount of charcoal and time of incubation. We used cytosols from the following samples:

- a. pool of lyophilised calf uterus ($n=43$);
 - b. frozen calf uterus that afterwards has been homogenised and a cytosol prepared;
 - c. frozen human mammary carcinoma tissue, oestrogen receptor positive;
 - d. pool of 36 frozen mammary cancers, oestrogen receptor positive;
 - e. pool of healthy human (glandular) breast tissue obtained from plastic surgery patients ($n=11$);
 - f. pool of 28 human mammary cancers, oestrogen receptor negative.
- A cytosol is defined as oestrogen receptor positive (ER+) when 9 fmol $^3\text{H-E}_2$ or more is specially bound per mg protein. This is

true for classes a-e. Cytosols from healthy mammary breast tissue do bind from 10 to 15 fmol $^3\text{H}-\text{E}_2$ / mg protein (class e), whereas ER- cytosols all bind less than 9 fmol/mg protein. For the cytosols mentioned above, the oestrogen receptor values were respectively a : 65.2, b : 54.0, c : 40.1, d : 77.7, e : 10.6 and f : 3.6 fmol/mg protein.

These values clearly demonstrate the difference between ER+ and ER- tissues. Of course, the individual examples within these groups can be expected to show a wide range of oestrogen receptor values and this is indeed found.

MATERIALS AND METHODS

A. Steroids

1. 2,4,6,7,16,17- ^3H oestradiol-17 β ($^3\text{H}-\text{E}_2$), specific activity 137 Ci/mmol, was obtained from New England Nuclear (Boston, U.S.A.).

The radiochemical purity was initially greater than 99% and was checked regularly by thin layer chromatography. When necessary, the product was purified by gel filtration on Sephadex LH 20 (Pharmacia, Uppsala, Sweden).

2. Unlabelled oestradiol-17 β (E_2) was purchased from Sigma Chemical Company (U.S.A.)

B. Other chemicals

All other chemicals used were commercial products of analytical grade.

C. Reagents

1. Tris-HCl buffer 10 mM, pH 7.5 at 20°C, containing 1 mM EDTA, 3 mM NaN_3 and 0.5 mM dithiothreitol (DTT).
2. Dextran-coated charcoal suspensions.

The three dextran-coated charcoal suspensions used all contained dextran 0.5 g/l (Dextran T 70, Pharmacia), gelatine 2 g/l

(Gelatine weiss, Merck), as well as 1.25 g/l, 2.5 g/l or 5.0 g/l charcoal (Charcoal activated, decolorizing powder, BDH Chemicals Ltd) in the buffer solution.

D. Tissue collection and storage

1. Human breast tumours.

Tumours were excised, freed from fat as completely as possible, frozen in liquid nitrogen and transported directly to the laboratory where they were stored at -95°C prior to assay. The residual cytosols after the estimations were pooled and frozen in such a way that a pool of ER+ and a pool of ER- cytosols was obtained. These pools were used for further systematic investigation and have already been mentioned in the Introduction.

2. Healthy human breast tissue.

The tissue was obtained by plastic surgery and immediately after excision it was placed on ice, dissected free of fat, sliced and rinsed with sterile ice-cold NaCl 0.9% solution and stored at -95°C .

Here, too, residual cytosols were pooled for further systematic investigations.

3. Calf uterus.

- a. The uteri of three-month-old calves were placed on ice immediately after excision and transported to the laboratory where they were washed with sterile ice-cold NaCl 0.9% solution, sliced and stored at -95°C .
- b. A part of these frozen uteri was crushed and lyophilised in a Christ lyophiliser. From the anhydrous tissue a powder was prepared in a Braun mixer at 4°C . This tissue powder was used to prepare the "pool" cytosol.

E. Preparation of homogenates and cytosols.

Preparation of homogenates and cytosols was performed according to a slightly modified EORTC procedure, i.e. adding DTT to the extraction buffer to protect sulphhydryl groups in the receptor protein. The protein content of the cytosol was adjusted to

approximately 6 g/l, where upon the determination was repeated.

F. Protein determination

Protein determination was done by a biuret method in a centrifugal analyser.

G. Incubation of cytosols with oestradiol

Cytosols (200 μ l) were incubated for 22 hours at 4°C with 300 μ l of buffer containing increasing quantities, viz. 0.15, 0.30, 0.60 and 1.20 pmol $^3\text{H}-\text{E}_2$, so that the final concentrations of $^3\text{H}-\text{E}_2$ in the medium were 0.3, 0.6, 1.2 and 2.4 nM. A reference for radioactivity was prepared using buffer solution instead of cytosol.

H. Addition of coated charcoal

After the cytosol-tracer incubation, 0.5 ml each of the three charcoal suspensions was added to the incubated cytosol samples, mixed on a Vortex mixer, then incubated for various periods from 5 to 45 min at 4°C; charcoal concentrations in the media of viz. 0.625 g/l, 1.25 g/l or 2.50 g/l are thus obtained. Afterwards the tubes were centrifuged and 0.5 ml of the supernatant was taken for radioactivity measurement (liquid scintillation). The radioactivity measured was corrected for blank values. The ratio of bound to free radioactivity and the amount of radioactivity bound per milligram protein was then calculated for each charcoal concentration used and for increasing concentrations of $^3\text{H}-\text{E}_2$ tracer in the reaction medium. The influence of incubation time with constant quantities of charcoal could also be calculated. It must be kept in mind that at this point no distinction was made between specific and non-specific binding of tracer. This could, however, be calculated from the Scatchard plots obtained (5).

RESULTS

Influence of varying amounts of charcoal on the amount of tracer bound in cytosols with high receptor activity and on the apparent affinity constants K_a for the binding reaction.

figure 2-1

Binding of tracer in ER+ cytosols. Effect of coated charcoal incubation time. Charcoal concentrations in the incubation medium a: 0.625 g/l, b: 1.25 g/l, c: 2.50 g/l. $^3\text{H}-\text{E}_2$ concentrations: — 0.3 nM, --- 0.6 nM, — — — 1.2 nM, ---- 2.4 nM.

These cytosols were prepared from calf uterus and ER+ human breast tumours as mentioned in the Introduction (classes a, b, c and d).

For each sample, the proportion of the added amount of $^3\text{E}-\text{H}_2$ bound to the cytosol receptor protein was calculated. This was done with the three different amounts of coated charcoal used for

adsorption of the free tracer in the reaction medium. These experiments were also performed with increasing times of incubation, 5 to 45 min, with the coated charcoal.

The results of these experiments are given together in *figure 2-1*. It is clear that the apparent binding percentage of $^3\text{E}-\text{H}_2$ to the receptor protein decreased when increasing amounts of coated charcoal were used. This appears to be true for media containing different concentrations of tracer, without exception. As could be expected the binding percentage was lower when the tracer concentration was increased.

Next it can be seen that the binding percentage appeared to decrease with prolonged incubation time. After about 10 to 15 min of incubation with coated charcoal a state of equilibrium was more or less reached.

table 2-1

Affinity constants K_a (g protein/mol) calculated by Scatchard plots. The given values are to be multiplied by 10^9 .

incubation time (min)	pool ER+ tumours			pool calf uterus		
	charcoal concentrations in the medium (g/l)					
	2.50	1.25	0.625	2.50	1.25	0.625
5	19.7	10.7	8.2	25.8	15.9	10.1
10	19.9	17.9	11.2	25.1	23.5	14.5
15	21.3	17.2	11.6	19.9	20.5	14.9
20	23.8	20.6	14.6	19.3	22.8	16.3
25	24.6	20.4	14.0	-	-	-
30	21.7	19.1	16.0	19.8	24.6	22.0
35	21.2	19.1	16.7	19.2	22.1	21.5
40	20.7	22.1	18.1	18.4	22.1	22.5
45	20.2	21.2	19.1	17.1	20.1	21.0

figure 2-2

Binding of tracer in healthy breast tissue cytosols. Effect of coated charcoal incubation time. Charcoal concentrations in the incubation medium a: 0.625 g/l, b: 1.25 g/l, c: 2.50 g/l. $^3\text{H}-\text{E}_2$ concentrations: — 0.3 nM, ---- 5.4 nM.

Similar results were obtained for cytosols either from calf uterus and from ER+ human breast tumours. Apparent affinity constants are given in table 2-1.

Influence of varying amounts of charcoal on the amount of tracer

figure 2-3

Binding of tracer in ER- cytosols. Effect of coated charcoal incubation time. Charcoal concentrations in the incubation medium a: 0.625 g/l, b: 1.25 g/l, c: 2.50 g/l. $^3\text{E}-\text{H}_2$ concentrations: — 0.7 nM, ---- 2.4 nM.

bound in cytosols with low and negative receptor activity.

These cytosols were prepared from either healthy human glandular breast tissue (class e) or receptor negative human mammary cancers (class f). The same investigations were performed as with the

oestrogen receptor positive cytosols. Results are given in *figure 2-2* and *figure 2-3*.

Binding of $^3\text{H}-\text{E}_2$ to the coated charcoal again increased with increasing amounts of the latter and with prolonged incubation time. However, an apparent equilibrium was reached after prolonged incubation with coated charcoal, i.e. about 20-35 minutes, cytosol from healthy glandular breast tissue taking longer to reach equilibrium.

The calculation of affinity constants gives no reliable values on account of very low amounts of tracer bound in the cytosols.

DISCUSSION

From these results the following conclusions can be made:

1. The amount of coated charcoal used for the separation of free tracer from receptor bound tracer apparently influences the result. This can only be explained by either an exchange of bound tracer between receptor protein and coated charcoal or by the binding of more than only free tracer to the charcoal. In this case we suppose that a certain amount of tracer-receptor complex is bound to the charcoal. This means that the coated charcoal has an avidity both for the smaller molecules $^3\text{H}-\text{E}_2$ and to a lesser extent for the receptor protein, the latter now being marked by $^3\text{H}-\text{E}_2$.
2. There is a clear difference between the binding rates of $^3\text{E}-\text{H}_2$ to protein in the ER+ cytosols from calf uterus and receptor positive tumours, and those found in ER- tumours and low ER activity mammary gland tissue.

With the latter cytosols an equilibrium is reached after a significantly longer period of incubation with coated charcoal. It might be concluded from these data that the binding of $^3\text{E}-\text{H}_2$ to ER+ tumour cytosol or equally ER+ calf uterus cytosol is stronger than with cytosols from receptor poor tissues. This is

in accordance with the values for K_a found when Scatchard analysis is applied to the results of the determination.

The apparent values found for the affinity constants as given in *table 2-1* do support the hypothesis that the receptor protein oestrogen complex is adsorbed to the coated charcoal. It appears that this process is more rapid in the presence of the higher charcoal concentrations. The result of the said adsorption is that diminished values for "bound" tracer are found after longer periods of incubation so that the apparent affinity constants are decreased.

It is to be concluded that the use of coated charcoal is only permitted under strictly standardised experimental conditions, otherwise inevitably it leads to erroneous results.

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chapter 3

COATING OF CHARCOAL BY DEXTRAN

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SUMMARY

With the aim of assessing the influence of the properties of the different constituents of a dextran coated charcoal suspension on the separation of bound from free tracer in the oestrogen receptor determination, we have studied the distribution of molecular weights constituting one dextran preparation (T-70 of Pharmacia). The influence exerted by the method of pretreatment of the charcoal used for the dextran coating has also been quantitatively and qualitatively investigated. It appears that for optimal coating with dextran the charcoal must be kept in vacuo prior to the coating procedure. The latter requires at least three hours incubation time. The lower molecular weight fraction in the dextran T-70 preparation is preferentially adsorbed.

INTRODUCTION

Dextran coated charcoal is often used in immunoassays for the separation of bound from free tracer (1, 2). Our investigation on the determination of oestrogen receptor protein in cytosols led us to investigate the nature of the coating of dextran coated charcoal. Several aspects of this procedure have to be considered, e.g. the

homogeneity of the dextran, the nature of the charcoal used in the procedures and the incubation time required for coating. It can be imagined that these factors influence the coating of the charcoal used with the selected dextran preparation.

Now the nature of the coated charcoal certainly has an influence on the separation of free from bound oestrogen in the receptor determination and perhaps a greater influence on the binding of oestrogen receptor complex to the coated charcoal. The latter, of course, should not happen but we have proved (3) that a certain amount of protein bound oestrogen is caught by the dextran coated charcoal preparations recommended generally (4). We have thus studied the mentioned aspects of the coating procedure.

A main motive too for our study was the unsatisfactory result of an interlaboratory investigation on the quality control of oestrogen receptor assays in human breast cancer (5). The different possible causes of the lack of agreement in the results from analyses in several laboratories necessarily need further study beyond the present contribution on the nature of the dextran coated charcoal.

MATERIALS

Chemicals

- Dextran T-70 (Pharmacia, lot 11073).
- Charcoal activated, decolorizing powder, BDH. Ltd., lot 6561070. Quantities of this charcoal were respectively:
 1. stored 80 hours under vacuum ("vacuum charcoal")
 2. exposed 80 hours as a thin layer on filter paper in the air at room temperature ("air charcoal")
 3. saturated with CO₂ for 80 hours in an Erlenmeyer flask, shaken periodically ("CO₂-charcoal").
- Sephadex^R G-100 and G-200, Sephacryl^R S-300 (Pharmacia).
- Low and high molecular weight gel filtration calibration kits (Pharmacia).

- All other chemicals used were commercial products of analytical grade.

Reagents

- Tris-HCl buffer 10 mM, pH 7.5 at 23°C, containing 1 mM EDTA, 3 mM NaN₃ and 0.5 mM dithiothreitol (TED buffer).

This buffer is also used for determination of oestrogen receptors.

- The dextran charcoal suspensions used contained 0.5 mg/ml of dextran T-70 and either 0.5 or 2.5 mg/ml of charcoal.

The contact between the dextran solutions and the different quantities of the three different charcoals was set at 3 hours, (vide infra) at 23°C with constant stirring. Each suspension was then centrifuged and aliquots of the supernatant assayed for dextran content or used for gel filtration.

Equipment for Gel Filtration

- 2 cm² x 100 cm column (Pharmacia K 16/100).
- Peristaltic pump Minipuls II (Gilson).
- Spectrochrom M photometric detector (Gilson).
- Fraction collector MTDC (Gilson).
- Recorder BDC, input 10 mV (Kipp & Zonen).

METHODS

Dextran Adsorption vs. Incubation Time

The incubation time required for optimal coating of charcoal with dextran was investigated as follows:

Several charcoal samples were placed in Erlenmeyer flasks and adequate volumes of dextran solution in TED buffer (0.5 mg/ml) were added. The contents of the flasks were constantly stirred (magnetic stirrer) at 23°C. At several predetermined times samples were removed and immediately centrifuged to separate charcoal from the supernatant liquid. In the latter dextran concentrations were determined by the anthrone method (see dextran assay). The percentage of dextran

adsorbed to the charcoal was then calculated.

Dextran Assay

We used the method described by T.A. Scott and E.H. Melvin (6) with some modifications: 1 ml of each dextran sample was cautiously pipetted into a glass tube (100 x 12.5 mm) containing 5 ml of the anthrone reagent (200 mg anthrone dissolved in 100 ml sulfuric acid 85%) and immersed in an ice bath. The tubes were then stoppered, inverted ten times and immediately placed in a shaking waterbath at 90°C for 12 minutes (we found a stable maximal absorption after 12 minutes reaction at this temperature).

The tubes were then immediately cooled in the ice bath and the absorbances were quickly read in 1 cm cells at 620 nm, using TED buffer as reference.

For the determination of the dextran content in the fractions from the gel filtration we used standards containing 0.01, 0.02, 0.03, 0.04, 0.05, 0.07, 0.1, 0.2 and 0.3 mg dextran T-70/ml TED buffer. For the other determinations: 0.1, 0.2, 0.3, 0.4, 0.45 and 0.5 mg dextran T-70/ml TED buffer.

Gel Filtrations

a. On Sephadex^R G-100/G-200

Column: K 16/100 with flow adaptors.

Bed dimensions: 2 cm² x 90.5 cm.

Gel: Sephadex^R G-100 and Sephadex^R G-200 in the proportion (weight) 2:1 (as described in reference 7).

Eluent: TED buffer (see reagents).

Gel filtrations: upward flow, performed at 23°C.

Sample volume for each run: 2.0 ml.

Flow rate: 4.35 ml·cm⁻²·h⁻¹.

Fraction volume: 125 drops ≈ 2.0 ml

The peak levels of blue dextran 2000 and the calibration proteins were detected spectrophotometrically (λ: 254 nm); the peaks of glucose and dextran were measured spectrophotometrically at 620 nm after the anthrone reaction.

Procedure:

Run	Sample	Concentrations
1st	Blue dextran 2000	0.5 mg/ml buffer
	Glucose	0.5 mg/ml buffer
2nd	Dextran T-70	2 mg/ml buffer
3rd	Catalase	3 mg/ml buffer
	Albumin	7 mg/ml buffer
4th	Ferritin	0.5 mg/ml buffer
	Aldolase	2 mg/ml buffer
	Ovalbumin	7 mg/ml buffer
	Ribonuclease	10 mg/ml buffer
5th	Dextran T-70	0.5 mg/ml buffer
6th	Solution of dextran T-70 Agitated for 3 hours at 20°C with 0.5 mg/ml of vacuum stored charcoal	(0.5 mg/ml buffer)
7th	Solution of dextran T-70 Agitated for 3 hours at 20°C with 0.5 mg/ml of CO ₂ saturated charcoal	(0.5 mg/ml buffer)
8th	Blue dextran 2000	0.5 mg/ml buffer
	Glucose	0.5 mg/ml buffer

b. On Sephacryl^R S-300

Same procedure as with the Sephadex^R G-100/G-200 mixture (except that the gel filtration was descending and the flow rate was 7.25 ml.cm⁻².h⁻¹).

RESULTS AND DISCUSSION

Adsorption of Dextran vs. Incubation Time

In figure 3-1 the rate of adsorption of dextran to several charcoal samples is given.

figure 3-1

Influence of incubation time on adsorption of dextran to charcoal. The percentage of dextran adsorbed from the solution to charcoal is given as a function of incubation time (see text) for several charcoal samples being stored viz. in vacuo —●—●—, in air —■—■— or in a carbon dioxide atmosphere —▲—▲—. Dextran concentration before incubation 0.5 mg/ml. Charcoal added 2.5 mg/ml in the final volume.

It is clear that the charcoal coating procedure must be continued for at least 3 hours in order to reach a state of saturation. As could be expected the in vacuo stored charcoal bound the highest amount of dextran. Here again it is clear that especially carbon dioxide but air as well considerably diminish the binding capacity of charcoal towards dextran. Moreover, it is not improbable that the sometimes found poor reproducibility in the determination of oestrogen receptor is also due to the use of improper preparations of dextran coated charcoal. This certainly explains to some extent

the unwanted adsorption of receptor bound oestrogen which we found in some experiments (3).

Homogeneity of Dextran T-70

Contrary to some other dextrans (?) the dextran T-70 used in this study gave only a single peak by gel filtration on Sephadex^R G-100/G-200 (2:1) (figure 3-2) with $K_{av} = 0.091$ (figure 3-3). A similar peak was obtained using Sephacryl^R S-300.

figure 3-2

Gelfiltration of dextran T-70.

Bed dimensions: 2 cm² x 90.5 cm; gel: Sephadex^R G-100 and G-200 in the proportion 2:1; eluent: TED buffer (Tris-HCl buffer 10 mM, pH 7.5 at 23°C, containing 1 mM EDTA, 3 mM NaN₃ and 0.5 mM dithiothreitol); gel upward flow; sample 2.0 ml of dextran T-70 (2 mg/ml TED buffer); flow rate: 4.35 ml·cm⁻²·h⁻¹; fraction volume: 125 drops $\approx$ 2.0 ml.

Calibration peaks: blue dextran 2000 (A); ferritin (B); catalase (C); aldolase (D); albumin (E); ovalbumin (F); chymotrypsinogen A (G); ribonuclease A (H); glucose (I).

figure 3-3

Calibration curve using calibration proteins on Sephadex^R G-100/G-200 (2:1). Gel filtration conditions see figure 3-1.

According to the manufacturers' documentation the weight average molecular weight $\overline{M}_w$ is approximately 70,300 and the number average molecular weight $\overline{M}_n$ 36,000.

Although a single peak was found the molecular weight declared for the dextran (70,000) cannot be directly deducted from the calibration of the column. This is due to the fact that protein gel filtration is not simply comparable to polysaccharide gel filtration for which no calibrators were available. Nevertheless, column calibration is anyhow necessary to control and check the homogeneity

of the gel and the constancy of column properties.

Nature of Charcoal Used in the Determination of Oestrogen Receptors

It was described earlier (8) that charcoal pretreated either with CO₂ or just prepared by boiling in water was highly different as far as adsorptive properties and capacities are concerned. For example, the adsorption of vitamin B₁₂ could not be performed when charcoal was saturated beforehand with carbon dioxide. For this reason we compared charcoal treated in vacuo for 80 hours, charcoal saturated with CO₂ for the same period of time and charcoal kept in air for this time. It appeared that the adsorptive capacity of the charcoal kept in carbon dioxide was much less than that of the charcoal kept in vacuo, but only slightly less than that of the charcoal exposed to air. This suggests that active charcoal really has to be evacuated before using it for the preparation of dextran coated charcoal for radioimmunoassay and similar experiments. The results of our investigations are given in *figure 3-1*. We started from the two concentrations of charcoal which were used by the E.O.R.T.C. members (0.5 and 1.5 mg/ml) (5).

It appeared that to the 0.5 mg/ml of charcoal kept in CO₂ or in vacuum respectively 1% and 2.8% of the dextran was bound whereas, in contrast, the 2.5 mg/ml of charcoal kept in vacuo was able to bind 17.6% of the dextran present in the solution. This was much more than the 7.6% of dextran which was adsorbed from the solution by the CO₂ saturated charcoal. Similar considerations apply to the comparison of charcoal kept in air with charcoal kept under vacuum (*table 3-2*).

From *table 3-1* and *table 3-2* it can be concluded that a charcoal kept under carbon dioxide is even less than charcoal kept in air, although the difference is not great (see also *figure 3-1*).

Adsorption of Dextran to Charcoal

If we look upon the nature of the dextran fractions adsorbed to charcoal we obtain the results of Sephadex gel filtrations as given

table 3-1

Dextran content (mg/ml); remaining in the solution after adsorption to charcoal. Starting solution 0.5 mg dextran T-70 /ml TED buffer.

charcoal	vacuum treated	CO ₂ treated
0.5 mg/ml	0.486	0.495
2.5 mg/ml	0.412	0.462

table 3-2

Dextran content (mg/ml); remaining in the solution after adsorption to charcoal. Starting solution 0.5 mg dextran T-70 /ml TED buffer.

charcoal	vacuum treated	air treated
0.5 mg/ml	0.483	0.491
2.5 mg/ml	0.410	0.457

in figure 3-4. Very similar results were obtained by gel filtration on Sephacryl^R S-300. It appears from this figure that the "heavier fractions" of the dextran are not adsorbed under the conditions of the experiment whereas the "lower molecular weight fractions" of the dextran are adsorbed to the charcoal.

As expected, the amount of low molecular dextran adsorbed to the charcoal was highly dependent on the nature of the pretreatment of the charcoal used, e.g. either incubation in carbon dioxide, in air or in vacuo.

It appeared that the adsorption of the lower molecular weight fractions took place preferentially to that of the larger macromolecules of dextran. Practically none of the latter was adsorbed using various charcoal.

figure 3-4

Gel filtration on Sephadex^R G-100/G-200 (2:1).

Gel filtration conditions see figure 3-1.

- dextran T-70 0.50 mg/ml TED buffer
- dextran T-70 0.50 mg/ml TED buffer after action of 2.5 mg/ml of "CO₂ charcoal"
- ▲—▲ dextran T-70 0.50 mg/ml TED buffer after action of 2.5 mg/ml of "vacuum charcoal"

From the experiments presented here, it can be concluded that the preparation of dextran coated charcoal must be necessarily done in a highly reproducible way in order to compare results of experiments on one day with those on an other and to compare one preparation with an other.

Furthermore, the dextran coated charcoal is mainly coated by "light dextran fractions". Whether this is of influence on the specificity

for adsorption of proteins and/or smaller molecules cannot be stated at this moment.

CONCLUSION

The high variability found in comparing the results of determinations of oestrogen receptor proteins in cytosols both between series in the same laboratory and between different laboratories is probably to some extent due to the nature of the used dextran coated charcoal preparation. From the present study it appears that the coating of charcoal with dextran must be done under highly standardized conditions in order to obtain reproducible preparations. The latter are a *conditio sine qua non* in the difficult field of investigation of receptor proteins where so many known and perhaps hitherto unknown factors do influence the outcome of the analysis.

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chapter 4

OESTROGEN RECEPTOR DETERMINATION AFTER PROTAMINE SULPHATE PRECIPITATION

SUMMARY

The determination of cytosol oestrogen receptor proteins has been investigated after precipitation with protamine sulphate. No satisfactory results could be obtained on account of continuing loss of radioactivity in the necessary washing procedures. Substitution of the generally recommended dextran coated charcoal (DCC) routine procedure for the determination of oestrogen receptors by a protamine sulphate method could not be advised.

INTRODUCTION

For the determination of oestrogen receptor concentration in cytosols or nuclear fractions of target tissues a great variety of methods has been published. Difficulties generally encountered included the risk of losing specific oestrogen binding protein during the procedure. Incorrectly elevated results could be found due to imperfect separation between free and protein bound tracer (radioactively labelled oestrogen).

The application of the so called DCC method (separation of free from protein bound tracer by dextran coated charcoal adsorption) is often recommended (1). However, excessively low values have been reported on account of receptor loss by charcoal adsorption (2). Another procedure has been published using glass beads for the adsorption of

proteins including steroid-receptor complexes (3). Low receptor values were found, possibly due to the unsatisfactory adsorptive properties of the beads. It appeared that only 8S proteins were adsorbed onto the glass. Specific oestrogen receptor proteins, however, are also found in the 4S class proteins (4, 5).

Procedures avoiding adsorption techniques for the separation of free from bound tracer have also been published. Amongst these, sucrose density gradient centrifugation (6), gel filtration (7) and agar gel electrophoresis (8) are mentioned. Apart from problems arising from the possibility of denaturation of proteins with these methods, these techniques are not suitable for the rapid analysis of large series of samples on a routine basis.

Another approach is the isolation of receptor proteins by the precipitation with polycation compounds, e.g. protamine sulphate. According to some authors (9) only specific oestrogen binding receptor proteins both in the 8S and 4S classes are precipitated, leaving in solution the proteins that non-specifically bind oestrogens and proteins from other than target tissues. It has also been claimed that hormone-receptor complexes can be distinguished from free receptor proteins by selective precipitation at different temperatures (10). Moreover, precipitation methods using protamine sulphate appear elegant and easy to perform.

In this study the methods published by Chamness (10) and by Robel and coworkers (11) were both investigated. Slight modifications were introduced where necessary for smooth routine procedures. Both methods are based on the same principle, i.e. precipitation of oestrogen receptor proteins with protamine sulphate.

In the first procedure (10) the precipitate is "coated" onto the wall of the reagent tube by centrifugation, whereupon incubation of the precipitate with radioactive oestradiol was performed. After the disposal of excess tracer the radioactivity in the tubes was measured being an indication of the amount of oestradiol binding protein.

In the second procedure (11) the precipitate was isolated by filtration onto a glass fiber filter. The precipitate containing receptor protein was incubated on the filter with tracer. Excess tracer was easily removed by rapid suction of washing buffer through the filter. Rapid washing was reported to be an advantage avoiding the dissolution of protein with bound radioactivity and loss of receptor bound tracer in the washing procedure.

CHEMICALS

- 2,4,6,7,16,17-³H oestradiol-17 β ; (³H-E₂) specific activity 137 Ci/mmol (New England Nuclear)
- 16 α -¹²⁵I-3,17 β -oestradiol ; (¹²⁵I-E₂) specific activity 200 Ci/mmol (New England Nuclear)
- diethylstilbestrol ; (DES) (Sigma)
- protamine sulphate (art.nr. 10123, Merck/Darmstadt)
- all other chemicals used were analytical grade quality.

REAGENTS

- buffers are composed as indicated in *table 4-1*
- protamine sulphate solutions:
 - 1) 2 mg/ml in buffer nr.1 to be used in the centrifugation method
 - 2) 1.2 mg/ml in buffer nr.2 to be used in the filtration method
- tracer solutions:
 - ³H-E₂ and ¹²⁵I-E₂ in buffer nr.1 to be used in the centrifugation method;
 - ³H-E₂ in buffer nr.2 to be used in the filtration method

table 4-1

Concentrations (mol/l) of constituents of buffers used in protamine sulphate precipitation assays of oestrogen receptors

buffer nr.	tris	EDTANa ₂	NaN ₃	DTT	triton X-100	ethanol	pH at 0°
1	10	1	3	0.5	-	-	7.9
2	20	3	1.5	1	-	-	7.9
3	20	3	1.5	1	1%	7%	7.9

- all concentrations in mmol/l except otherwise indicated

CYTOSOLS

The cytosols were prepared as follows:

500 - 600 mg of frozen human breast cancer tissue or calf uterus tissue were pulverised in a stainless steel mortar immersed in liquid nitrogen. The obtained frozen rough powder was then vibrated during 45 seconds, three times, at 50 cycles/second in a teflon vial with a micro dismembrator (Braun, Melsungen/Germany). The resulting frozen fine powder was transferred to a 5 ml polypropylene ultracentrifugation tube whereupon 3 ml buffer (buffer nr.1; *table 4-1*) at 0° were added. The tube was stoppered and vibrated for another two minutes with the microdismembrator. The volume was then adjusted to 5 ml with the buffer and the contents of the tube thoroughly mixed. After 30 minutes ultracentrifugation (100,000 g; 1°) the tube was laterally punctured and the supernatant collected avoiding contamination with lipids from the surface of the liquid.

When this procedure was followed cytosols were obtained containing protein between 2 - 4 mg/ml. Protein concentration was determined by a biuret method with centrifugal analysis (*this thesis; chapter 5*) with correction for contaminating serum proteins.

figure 4-1

Special equipment for serial incubation and filtration (see text)

SPECIAL EQUIPMENT

A filtration device was constructed using plastic syringes (inner diameter 9 mm, height 55 mm, total volume 3 ml). At the bottom of the syringes stainless steel grid discs were placed as supports for Whatman GF/C glass fiber filters (10 mm diameter, rough side upwards). Each syringe was connected to a three way stopcock. A series of 24 of these so constructed filtration devices were coupled and the common outlet connected to the vacuum pump (*figure 4-1*).

CENTRIFUGATION METHOD

It must be marked that the whole procedure was performed at 1°.

The protamine sulphate precipitation procedure was performed in acid washed glass tubes (11.5 x 75 mm) by adding 250 µl of protamine sulphate solution nr.1 to a sample of 250 µl of cytosol. The mixture was vortexed, left for 5 minutes and next centrifuged (1000 g) for 5 minutes. The formed precipitate thus "coated" the bottoms of the tubes and the supernatant could easily be removed by suction. Tracer was then added to the precipitates in the tubes and the total incubation mixture volume made up to 250 µl with buffer (buffer nr.1; *table 4-1*); final concentrations of tracer were *viz.* 0, 5, 10, 15, 20 and 25.10⁻¹⁰ M. After incubation overnight (18 hours) the supernatant was removed by suction and the remaining precipitate washed three times with 2 ml portions each of buffer nr.1. Similar series of experiments were performed with both tracers, i.e. ³H-E₂ and ¹²⁵I-E₂. In the iodine tracer experiments the radioactivity of the tubes was measured directly after the washing procedures. In the tritium tracer experiments the precipitates in the tubes were extracted twice with 1 ml portions of tetrahydrofuran each at room temperature. The extracts from each tube were collected and combined in scintillation vials and after addition of scintillation liquid the radioactivity was measured.

FILTRATION METHOD

As with the centrifugation method the filtration procedure was also performed in a cold room at 1°.

After moistening the filters with 200 µl portions each of buffer (buffer nr.2; *table 4-1*) and removal of the buffer by suction through the filters the stopcocks were closed. Consecutively 90 µl of protamine sulphate solution nr.2, 90 µl of cytosol and another 90 µl of the same protamine sulphate solution were pipetted into the syringes. After vigorous shaking of the filtration device in order to mix the contents

of the syringes the precipitate was allowed to form for 15 minutes. The stopcocks were then opened and the solution removed by gentle suction. After closing the stopcocks tracer ($^3\text{H-E}_2$) was added to a series of six syringes with equal contents to obtain final tracer concentrations of viz. 0, 5, 10, 15, 20 and 25.10^{-10} M, with or without simultaneous addition of DES (200-fold concentration) to control non-specific protein binding. The total volume in the incubation mixture was made up to 200 μl by addition of appropriate amounts of buffer (buffer nr.2; *table 4-1*). The contents of the syringes were mixed by shaking and incubation was performed overnight (18 hours). Afterwards, the liquid was removed by suction and 3 ml of wash-buffer (buffer nr.3; *table 4-1*) was put in the syringes, left in place for 5 minutes and removed by suction. The precipitate was then rinsed four times with light suction by application of 2 ml portions each of buffer on each filter. These washings were done directly without incubation. Finally suction was applied for some minutes until filters were dry. The filters were then removed from the syringes and put in vials containing 10 ml of scintillation liquid, and the radioactivity was measured.

RESULTS AND DISCUSSION

The basic principle of the protamine sulphate precipitation methods for the determination of oestrogen receptors is the binding and the quantitative measurement of specifically protein bound tracer (i.e. labelled oestradiol). Correction for non-specific binding is made by addition of an excess of a competitive "cold" ligand (DES). The specific receptor proteins are then saturated with cold ligand and the formation of these receptors with radioactive tracer is prevented. Residual radioactivity must be contributed to the ongoing binding of tracer to non-specific proteins.

Reliable results in the receptor assay can only be expected if the

figure 4-2

Loss of radioactivity from protamine sulphate / receptor precipitate (see text); incubation without (broken line) and with DES (solid line)

removal of free tracer from the precipitate can quantitatively be performed. Loss of protein bound tracer inevitably results in unduly decreased values for oestrogen receptor. It has been reported that in a comparative study of procedures the lowest values for oestrogen receptors were found with a protamine sulphate precipitation technique (12). This might be explained either by loss of tracer-receptor complex as such in the washing procedure of the precipitate or by loss of free tracer due to dissociation of the complex during this washing procedure. Washing the precipitate can not be totally omitted on account of the presence of adhering free tracer on the precipitate after the tracer exchange incubation procedure. Other authors, however, published satisfactory results (13) especially in the determination of nuclear oestrogen receptor in adult rat uterus tissue (14). Elsewhere, oestrogen receptor values determined in

rabbit uterus cytosols were found higher with the protamine sulphate technique than with the dextran coated charcoal method, whilst the opposite was true for cytosols from human breast cancer in the same investigation (15).

These considerations suggested close investigation of possible loss of radioactivity during the washing procedures. In the centrifugation method the removal of tracer was measured. Continuous loss of radioactivity with every wash cycle was observed (*figure 4-2*). As repeated washings of the precipitate resulted in increasing loss of radioactivity, the oestrogen receptor values estimated in this procedure were apparently dependent on the number of wash cycles. It could not be established whether only free tracer was gradually washed out or that tracer-receptor complexes were dissolved in the washing buffer and eliminated in this way, and this perhaps together with unspecific tracer-protein complexes. A third possibility to be considered was that the tracer-receptor complex dissociated during repeated washings and the liberated tracer was then removed. Anyhow, the values of the receptor concentrations as found by this method could not be considered to be reliable which is in agreement with earlier cited literature (12). It had been suggested there that possibly considerable amounts of non-specific tracer protein complexes were washed away or that tracer dissociated easily from these complexes. This quite well is in accordance with our observations that after suppression of specific tracer binding with DES, the decrease in radioactivity with wash cycles was larger than when specific tracer-receptor complexes were formed in the absence of DES (*figure 4-2*). It must be concluded that although the dextran coated charcoal method for the determination of oestrogen receptor is not without problems (*this thesis, chapter 2*), no improvement could be achieved by adopting the protamine sulphate precipitation procedure with the centrifugation technique.

The introduction of the filtration method was based on the intention to prevent or reduce loss of radioactivity from the tracer-receptor

table 4-2

Comparison of oestrogen receptor values in human breast cancer cytosols; determination by protamine sulphate precipitation techniques and by the dextran coated charcoal assay.

cytosol nr.	protamine sulph. techniques		dextr.c.charcoal assay
	$^{125}\text{I-E}_2$	$^3\text{H-E}_2$	$^3\text{H-E}_2$
1	84	62	129
2	199	170	274
3	401	433	525
4	-	22	31
5	-	15	11
6	-	132	180
7	-	24	83

- cytosol nrs 1 - 3: protamine sulphate (centrifugation) technique
- cytosol nrs 4 - 7: protamine sulphate (filtration) technique

complex in the washing procedures. It was supposed that the absence of centrifugation after incubation and exchange of tracer permits a rapid washing out of free tracer without loss of specific material (11). Unfortunately we could not confirm this. The loss of radioactivity was less than with the centrifugation method but still with each wash cycle tracer was removed. With the first washing the disappearance of tracer from the filter was about 8% equal to the loss in the centrifugation procedure. This was regarded to concern

the washing out of free tracer. The radioactivity decrease on the filter with following rinse cycles was less, *viz.* totalling 11%, 15% and 18% for the second, third and fourth cycle. Here too the loss of activity was larger in the presence of DES suggesting that unspecific activity was removed with each rinse cycle or that free tracer after dissociation of the tracer receptor complex, or the latter as such, was washed away. Loss of radioactivity in the presence of DES was after first and following washings *viz.* 11%, 16%, 22% and 29%.

Finally some cytosols were analysed both with protamine sulphate precipitation procedures and with the dextran coated charcoal method (*table 4-2*). No agreement between the procedures was found. It must be concluded that with our cytosols the applied protamine precipitation techniques offered no real advantage over the dextran coated charcoal technique as recommended by the European Organisation for Research and Treatment of Cancer (*1*).

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PROTEIN DETERMINATION
IN MAMMARY TUMOUR CYTOSOLS

SUMMARY

The various methods for the determination of the concentration of total proteins in mammary tumour cytosols were investigated. The different procedures for this determination of the usually low levels of protein produce different values. This is explained by differences in the underlying reaction mechanisms and by the use of various protein standards in the methods described. A proposal is made for the standardisation of both the analytical procedure and the reference protein mixture (horse serum) to be used as a standard. It appears that the biuret reaction serves best in the standardisation of protein determination. An analytical procedure has been developed in which centrifugal analysis is performed and whereby the sensitivity and precision of the biuret reaction are improved. The importance of an unambiguous analysis of total protein is stressed, as this is the basis for the estimation of the oestrogen receptor concentration in the cytosol.

INTRODUCTION

The expression of oestrogen receptor activity in tissues meets a number of problems where the concentration of receptor protein is concerned. The ideal situation would exist if the concentration of receptor protein in terms of specific receptor binding capacity per cell (together with the number of cells per quantity of tissue) could

be given. Although attempts in this direction have been made (1) it is very difficult to produce accurate data in this way. Another approach is to refer to the receptor protein in terms of concentration *vs.* total protein either in the tissue itself or in a cytosol prepared from the tissue (2). Expression of the receptor protein concentration with reference to nucleic acid concentration was reported in nuclear fractions of tissue homogenates (3). The oestrogen receptor concentration is now generally expressed in terms of oestrogen binding capacity in femtomoles per mg total protein in the cytosol prepared from the tissue under investigation (4). It must be kept in mind, however, that not only the receptor concentration but also the affinity of the receptor protein towards oestrogens are important characteristics. A correction for so-called non-specific binding is made.

In the Netherlands, 17 laboratories are participating in a quality control programme on the measurement of oestradiol receptor concentration in tissue from mammary gland tumours. Initially, the results produced by different laboratories could hardly be compared, which was to some extent due to the divergent data given for protein concentrations in the cytosol preparations distributed (5). It is obviously quite difficult to obtain "true" values for very low concentrations of proteins as found in these cytosols. The problem is complicated by the fact that several procedures for the protein determination unfortunately lead to different results. Although much work has been done on the comparison of methods, no general agreement has been reached (6, 7, 8, 9). For this reason some of the protein determination procedures used in the earlier mentioned quality control scheme were compared by us in order to determine which method could best be recommended.

It should be noted that the E.O.R.T.C. (European Organisation for Research and Treatment of Cancer) advise a protein determination method based on the affinity of the dye Coomassie Brilliant Blue for protein, using a commercial human serum albumin preparation as a standard (Kabi^R standard, ten fold diluted) (10). Although the

choice of this dye-binding procedure is understandable in view of its reported sensitivity, a simple biuret reaction was thought to be preferable on account of its general applicability to any protein. In this reaction all peptide bonds are measured almost independent of the protein composing amino acids. The sensitivity of the biuret reaction is reported to be further enhanced by the introduction by Lowry of the Folin phenol reagent.

Thus, three procedures were considered, the wellknown biuret reaction which is still widely used (11), the Lowry method using the Folin phenol reagent especially developed for low protein concentrations (12), and the determination of protein concentration by measuring the binding of the dye Coomassie Brilliant Blue (13).

Biuret method: The well-known biuret reaction is perhaps next to the Kjeldahl analysis the most widely used procedure for determination of proteins in solution. Its non-selective character is generally an advantage because the method can be used for every protein solution. Important advantages are found in the determination of low protein concentrations in small sample volumes when a centrifugal analyser is used. The introduction of this technique enables analyses to be performed on a scale which hitherto has not been possible. This technical improvement can be applied to the classical biuret reaction. The specific problem was the quantification of total protein present in cytosols in the range of 0.5 to 6.0 mg/ml.

Lowry method: The Lowry method for protein determination combines the biuret reaction for the detection of peptide bonds with the Folin Ciocalteu reaction for the detection of phenolic OH-groups (12). In this way the residues from phenolic amino acids in the protein solution provide an extra contribution to the final colour of the reaction mixture.

Coomassie Brilliant Blue method: The use of the so-called protein dye Coomassie Brilliant Blue in the quantification of proteins has been suggested by Bradford (13). Although the procedure described appears to be rapid and reproducible, it is only of limited analytical value because it shows a wide variability in its sensitivity to various

proteins (14). Incorrect values have been reported when human serum albumin or bovine globulin were used as the standard (15). This is altogether not too surprising because in dye-binding methods only polypeptide moieties of proteins are evaluated, so that lipo- and glyco-proteins are relatively underestimated. Moreover, various proteins are not equally rich in dye-fixing basic groups which explains different sensitivity for different proteins. Human serum albumin is perhaps the most sensitive for dye fixation. Nothing can be predicted about the affinity of the dye for specific oestrogen receptor proteins.

The determination of tumour protein is further complicated by the fact that in cytosols prepared from tumour tissue a certain amount of serum protein is always found. In extreme cases up to 50% of the total protein content is found to consist of human serum albumin. It goes without saying that correction for this non-tumour protein must be made in order to make a correct estimate of the receptor protein, expressed in terms of oestrogen binding capacity *vs.* total protein.

MATERIALS

All chemicals used were commercial products of analytical grade.

1. TED-buffer.

Tris-HCl buffer 10 mM, pH 7.5 at 23°C containing 1 mM EDTA, 3 mM NaN_3 and 0.5 mM dithiothreitol.

2. Biuret reagent.

The concentrated biuret reagent (Roche Diagnostica) consisted of an aqueous solution containing potassium sodium tartrate 200 mmol/l, copper sulphate 120 mmol/l, potassium iodide 100 mmol/l and sodium hydroxide 2 mol/l.

3. Lowry reagents.

a. Sodium carbonate 180 mmol/l in sodium hydroxide 100 mmol/l.

b. Copper sulphate 20 mmol/l in potassium sodium tartrate 35 mmol/l.

c. Carbonate copper solution: 50 ml of reagent a and 1 ml of reagent b were mixed before use.

d. Phenol reagent of Folin Ciocalteu.

A mixture consisting of 100 g of sodium tungstate ($\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$), 25 g of sodium molybdate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$), 700 ml of water, 50 ml of 85% phosphoric acid, and 100 ml of concentrated hydrochloric acid in a 1.5-l flask was refluxed gently for 10 hours. Thereafter 150 g of lithium sulphate, 50 ml of water, and a few drops of bromine water were added and the mixture was boiled for 15 minutes without condenser to remove excess bromine. After cooling, the solution was diluted to 1 l, and filtered. The reagent had no greenish tint. (The acid concentration of the reagent was determined by titration with 1 N NaOH to a final pH = 9.0.) The acidity of the reagent had to be about 1 N. (The reagent is also commercially available in a concentrated solution, Merck 9001, which after dilution with an equal volume of water is ready for use.)

4. Coomassie Brilliant Blue reagent.

100 mg of Coomassie Brilliant Blue G-250 (C.I. 42655, Sigma B 5133) was dissolved in 50 ml 95% ethanol. To this solution 100 ml phosphoric acid 85% (w/v) were added. The resulting solution was diluted to a final volume of 1 liter. Final concentrations in the reagent: Coomassie Brilliant Blue G-250: 0.01% (w/v), ethanol 1 M, and phosphoric acid 1.48 M. The dye reagent had always to be filtered (over Whatman no. 1 filter paper) just before use. (The reagent is also commercially available as a dye reagent concentrate, Bio Rad, which can be used after dilution with four volumes of water.)

5. Protein standards.

a. Seronorm (lyophilised horse serum, Nyegaard & Co, Oslo, Norway), lot no. 138; containing 67.0 mg total protein/ml (biuret method), 26.0 mg albumin/ml; 10.77 mg protein nitrogen/ml by Kjeldahl analysis after dissolving in 10.0 ml of H_2O (apparently a protein factor of 6.22 is used).

- b. Kabi Diagnostica Protein standard (human serum albumin dissolved in physiological saline, Kabi Stockholm, Sweden), lot no. 57739; containing 103.8 mg human albumin/ml (Kjeldahl-analysis, protein factor 6.4); 16.22 mg protein nitrogen/ml.
 - c. Bio-Rad Protein Assay standard I (bovine plasma globulin lyophilised, Bio Rad Lab., Richmond, California, U.S.A.), lot no. 17893; containing 1.39 mg bovine globulin/ml after addition of 20.0 ml H₂O.
6. Working standards.
- a. For the biuret method the following protein working standards were prepared by dilution with H₂O and TED-buffer:
reconstituted horse serum (Seronom) 0.94, 1.86, 3.79, 6.09, 8.38, 9.57 mg protein/ml;
reconstituted human serum albumin solution (Kabi standard) 1.30, 2.60, 3.89, 5.19, 6.23, 7.74, 10.38 mg protein/ml;
reconstituted bovine globulin solution (Bio-Rad standard) 1.39, 2.78, 5.56, 8.34, 11.12, 13.9 mg protein/ml.
 - b. For the more sensitive Lowry method, the first two standards were diluted ten fold, respectively in H₂O and TED-buffer, in order to obtain sufficiently low concentrations.
 - c. For the Coomassie Brilliant Blue method five fold dilutions of the working standards sub a. were made.

METHODS

Biuret method. Procedure: In order to enhance the sensitivity of the method and to reduce the amount of sample needed a procedure for centrifugal analysis* was developed, in which concentrated reagent could be used. This procedure is specified in *table 5-1*.

Lowry method. Procedure: Samples and standards were diluted ten fold before use. To 0.40 ml of diluted sample, diluted standard, water or TED-buffer (blanks), 2.0 ml of reagent 3.c were added, mixed and allowed to stand for 10 minutes at room temperature; then

* Cobas Bio; Hoffmann - La Roche

table 5-1

Scheme for the determination of proteins in solution by the biuret reaction (centrifugal analysis procedure). Samples and standards are used undiluted. Horse serum protein standards are dissolved in TED-buffer.

temperature	30	°C
type of analysis *	7	
wave length	546	nm
sample volume	40	μl
diluent volume (H ₂ O)	40	μl
reagent volume (H ₂ O)	200	μl
incubation time	30	sec
starting reagent volume (biuret)	40	μl
time interval (reaction time)	300	sec
standard 1 concentration	1.86	mg/ml
standard 2 concentration	6.09	mg/ml
standard 3 concentration	9.57	mg/ml

* machine code indicating a method with blank subtraction

0.2 ml of reagent 3.d was quickly added and the contents of the tube were immediately mixed. After 30 minutes the absorbance of the samples, standards and blanks was read against water at 520 nm in a spectrophotometer using a 1 cm cuvette.

Coomassie Brilliant Blue method. Procedure: Samples and standards were diluted five fold with either water or TED-buffer before use. To 0.1 ml of samples (diluted), standards (diluted), water or TED-buffer 5.0 ml of Coomassie Brilliant Blue G-250 reagent were added and mixed. After 10 minutes, optical densities were read at 595 nm in a spectrophotometer using 1 cm cuvettes against reagent blank, water blank or TED-buffer blank. After each reading, the cuvettes were rinsed with methanol and water to eliminate any trace of dye.

CHOICE OF A PROTEIN STANDARD

The determination of total protein in cytosols prepared from human breast tumours is of fundamental importance as long as oestrogen receptor activity is expressed per mg protein. The total protein content serves as a parameter for the amount of tumour tissue represented by the prepared cytosol. One of the problems is that most determination procedures are based on the use of so-called standard solutions containing a known amount of protein (8, 9, 16). The latter preferably has to be of the very same nature as the proteins in the sample to be analysed. This, however, is virtually impossible in the estimation of oestrogen receptor protein or total proteins in the cytosol. Receptor proteins are not available in a highly purified state and the protein pool in cytosols is composed of different tissue proteins and a variable amount of plasma proteins. It is, therefore, not surprising that the composition and nature of the protein standards used have a marked influence on the results of the determination of oestrogen receptor concentrations. These effects are, of course, stronger when the reaction by which the protein is determined is more specific for special proteins. It can easily be understood that a "general" reaction (i.e. one without preference for specific characteristics of certain protein molecules) is more suitable when a mixture of different species of protein is to be investigated. The biuret reaction serves this purpose quite well, while a more sensitive reaction such as the one described by Lowry is more dependent on the amount of phenolic amino acid residues in the proteins. These phenolic groups are found to a different extent in various proteins. Finally dye fixation is also dependent on the nature and amino acid composition of the proteins concerned (17). As was mentioned in the "introduction" several misleading results have been reported when this analytical procedure has been used. A standard composed of 70% human serum albumin and 30% human serum globulin was recommended for use in CSF investigation (15). No special standard can be recommended for tumour proteins, although from an analytical point of view bovine globulin has been found to

figure 5-1

Protein calibration curves by the Coomassie Brilliant Blue method; the dye reagent was used as a blank; —▲— human serum albumin; —●— horse serum proteins; —■— bovine serum globulin. All proteins were dissolved in water.

be preferable (figure 5-1) for use with Coomassie Brilliant Blue.

If a choice of standard has to be made from what has just been considered, a mixture of proteins seems to be the most suitable and there are no strong indications that any available specific protein is preferable. Therefore, a whole serum preparation which is easily obtained in such quantities that use of the same batch can be guaranteed for years is to be preferred. From the three standards suggested the lyophilised equine full serum standard (Seronom^R) has been chosen. The two other available standards are both incomplete preparations from blood, either human serum albumin or bovine plasma globulin.

figure 5-2

Protein calibration curves by the biuret method (centrifugal analysis); absorbance read against water; —▲▲ human serum albumin; —●● horse serum proteins; —■■ bovine serum globulin. All proteins were dissolved in water.

Summarising the reasons for preferring the complete horse serum (Seronom^R) preparation:

- It is a complete serum.
- It is lyophilised and can easily be dissolved in water or in TED-buffer.
- Its composition is regularly checked by about 170 clinical chemistry laboratories in the Netherlands so that a good quantity of a standardised preparation is available. This is really an important reason to confirm the choice of this standard.

figure 5-3

Protein calibration curves by the Lowry method; TED-buffer in reagent was used as a blank; —▲— human serum albumin; —●— horse serum proteins; —■— bovine serum globulin. All proteins were dissolved in TED-buffer.

- In the biuret method the photometric sensitivity for the three standards studied is only slightly different (figure 5-2) whilst in the Lowry method the optical density obtained for a certain amount of protein is highest for complete horse serum (figure 5-3).

INTERFERENCES OF THE TED-BUFFER CONSTITUENTS ON THE QUANTITATIVE DETERMINATION OF PROTEINS

It is well known that many substances have an influence on the protein determination methods referred to in this investigation (18). Special attention was paid to the constituents of the buffer used, i.e. interference from Tris (13, 19, 20, 21, 22) and (to a lesser extent) also from EDTA (13, 23). A number of procedural modifications were studied which were more or less successful in eliminating this interference (24, 25).

Biuret method for protein determination in centrifugal analysis.

It has been reported (20) that increasing the Tris concentration in

figure 5-4

Calibration curves by the biuret method (centrifugal analysis) of horse serum proteins dissolved in TED-buffer (solid line) or in water (broken line). Absorbance read against water.

the buffer, both increased the optical density in the biuret reaction and decreased the slope of the protein calibration curve. Nevertheless, the absorbance in the presence of Tris was proportional to the protein concentration. In order to check this, calibration curves of horse serum (Seronorm^R) were made in the range 0-10 mg protein/ml when the contribution of the TED-buffer was found to equal approximately 0.5 mg protein/ml (figure 5-4). The final molarity of Tris in the

figure 5-5

Calibration curves of horse serum proteins dissolved in TED-buffer by the Lowry method. Absorbance read against a blank containing either TED-buffer (solid line) or water (broken line) in reagent.

in the biuret reaction was 1.25. Similar results were obtained with other protein standards, including human serum albumin and bovine globulin. In the protein concentration range 0-10 mg/ml there was a distinct contribution from TED-buffer in biuret reagent to the optical density, but no decrease in the slope of the curve was found. Reading the samples against a blank containing biuret reagent and buffer resulted in a calibration curve through zero. The influence of the TED-buffer on the determination of proteins in biuret reagent could not be denied, but interference could easily be compensated.

Lowry method. Using the Lowry method similar interference phenomena were seen. This was no surprise because the contribution of Tris was due to complex formation with the copper in the reagent. In this

figure 5-6

Calibration curve of horse serum proteins dissolved in TED-buffer by the Coomassie Brilliant Blue method. Absorbance read against a blank containing either TED-buffer (—Δ—Δ—) or water (—●—●—) in the dye reagent.

reaction the final molarity of Tris was 1.54. The calibration graph in the range up to 1.2 mg protein / ml was slightly curved. Another source of interference might have been reduction of the Folin - Ciocalteu reagent by EDTA (23). Again the use of TED-buffer as a blank fully eliminated the influence of the interference on the result of the analysis (*figure 5-5*).

Coomassie Brilliant Blue method. In the commonly used binding method with Coomassie Brilliant Blue, no interference has been seen either from Tris or EDTA as used in the TED-buffer (*figure 5-6*).

Similar investigations as with Tris have been performed with EDTA in the concentration (1 mM) as used in the buffer. It appeared that in this rather low concentration no influence whatsoever could be noted. These results were in agreement with those in the literature (13, 16). It must, therefore, be recommended that the blank in the protein determination consist of the full TED-buffer containing all constituents except protein.

EVALUATION AND COMPARISON OF THE THREE CONSIDERED PROTEIN DETERMINATION METHODS

In order to choose the best procedure for the determination of the protein concentration in cytosols to express oestrogen receptor concentration, the three methods described above were compared. As a standard lyophilised horse serum (Seronorm^R) reconstituted in TED-buffer was used.

The biuret reaction in centrifugal analysis was compared to the other procedures. Protein concentrations in the cytosols in the range 1.0 mg/ml to 4.5 mg/ml were determined. The protein calibration curve in the biuret reaction was perfectly linear.

The precision of the three methods was compared by determination of standard deviations (from duplicate analysis) and coefficients of variation over the range studied. It must be kept in mind that both for the Lowry procedure and the Coomassie Brilliant Blue procedure

table 5-2

Comparison of three methods for protein determination.

method		biuret	Lowry	Coomassie B.B.
range (mg/ml)		1.0-4.5	0.10-0.45	0.20-0.90
intraseries	n	32	20	22
	$\bar{x}$	3.11	0.394	0.760
	s.d.	0.034	0.010	0.011
	c.v.	0.95 %	2.7 %	1.4 %
interseries	n	42	28	32
	$\bar{x}$	3.28	0.393	0.555
	s.d.	0.056	0.017	0.038
	c.v.	1.5 %	4.3 %	6.8 %
detection limit		0.07	0.02	0.02

- protein concentrations are given in mg/ml
- standard deviations were determined from duplicate analysis either intraseries or between series
- detection limits were determined as twice the standard deviation of the blank

extra dilutions of samples and standards had to be made. Detection limits of the methods have been determined as twice the standard deviation for the blank. The results of this investigation are given in table 5-2. From these figures it can be seen that the more sensitive Lowry procedure as compared with the biuret procedure had a lower degree of precision, which might perhaps be expected. The comparison between intra series coefficient of variation and inter series coefficient of variation in both methods was quite acceptable. This was not true for the Coomassie Brilliant Blue procedure. It appeared that the reproducibility (as expressed by coefficient of variation between series) of this procedure was less acceptable than

for the others. It can readily be understood that variation in dye fixation is more susceptible to changes on account of the complexity of dye binding and the possible variation of the reagents after filtration from day to day. For these reasons, the dye binding procedure was regarded by us as the least to be preferred.

A choice between the biuret reaction as such and the modification including a reaction on phenolic groups (Lowry procedure) was made on the basis of difference in precision and simplicity of performance. It should also be kept in mind that a greater influence of endogenous reducing substances can be expected in the Lowry procedure. The biuret method was easily performed because a procedure using a centrifugal analyser had been developed. The use of this type of analyser, although preferable on account of precision and the possibility of using small sample volumes, was not practicable for the Lowry method. The reaction scheme for the latter method, requiring two incubations with an interval of about 15 minutes, took too much analyser time. Moreover, the second incubation itself required another 30 minutes and such a scheme was unacceptable in a busy laboratory. The necessity for predilution of samples and standards for the Lowry procedure was another disadvantage.

It can be concluded that the simple biuret method without predilution has the best precision and can be used satisfactorily in a centrifugal analyser. This method is, therefore, recommended.

CORRELATION OF THE DIFFERENT METHODS

The comparison of protein concentration measurements in 17 cytosols by the biuret method using a Cobas Bio centrifugal analyser and by the Coomassie Brilliant Blue procedure, was then studied. Lyophilised horse serum (Seronorm^R) reconstituted in TED-buffer was used as a standard. The results of this study are given in *figure 5-7*.

Although the coefficient of correlation ($r=0.972$) and the regression line ($y = 1.1058x + 0.0374$) were quite acceptable, it

figure 5-7

Comparison of protein concentration measurements of 17 cytosols (●) and 3 "haemolytic" cytosols (■) by the biuret method (Cobas Bio centrifugal analyser) and by the Coomassie Brilliant Blue procedure; $y = 1.1058x + 0.0374$; $r = 0.972$; broken line $y=x$. As a standard a solution of horse serum proteins in TED-buffer was used.

should be noted that no valid results could be expected with the Coomassie Brilliant Blue procedure if the cytosol contained traces of haemoglobin. In contrast to others (16), we have found a much stronger haemoglobin interference in this method than in the biuret method. In order to check this, a very dilute hemoglobin solution was added to some cytosols and grossly exaggerated values were then found in the Coomassie Brilliant Blue procedure (figure 5-7). Most

figure 5-8

Comparison of protein concentration measurements of 23 cytosols by the biuret method (Cobas Bio centrifugal analyser) and by the Lowry procedure; $y = 1.2317x + 0.0341$; $r = 0.987$; broken line $y=x$. As a standard a solution of horse serum proteins in TED-buffer was used.

tumours contain some blood which is almost impossible to wash fully out. This is another reason for preferring the biuret reaction to the Coomassie Brilliant Blue method.

A similar comparative study was made on 23 cytosols where the protein content was determined both by the biuret method and the Lowry procedure (figure 5-8). Again, lyophilised horse serum (Seronom^R) reconstituted in Ted-buffer was used as a standard. A satisfactory coefficient of correlation ($r = 0.987$) was found and the linear regression was $y = 1.2317x + 0.0341$. The fact that the

values found by the Lowry method were significantly higher than those found by the biuret method could be explained by apparently greater content of phenolic groups in the cytosol proteins than in the horse serum protein standard. Another possibility was the interference of non-protein substances in the cytosols. It has been reported (22, 26) that this type of interference could be overcome in the Lowry method by precipitating proteins prior to the determination. A certain risk, however, of losing protein, e.g. in a necessary washing procedure, is then introduced which is even more serious considering the low protein concentration in the cytosols. For this reason too, we prefer the simple biuret reaction for determination of protein in cytosols from human mammary tumours.

DISCUSSION

From the investigation performed it can be concluded that no single method for the determination of total protein in cytosols from mammary tumours can be recommended as being superior. For reasons of rationalisation and in order to promote comparability of results from various laboratories, however, the simple biuret method which measures peptide bonds in all proteins is proposed. In our opinion this is an advantage so long as the structure and properties of purified receptor protein have not been elucidated.

Of considerable importance also is the improved precision of the biuret method, which is reported to be 3% for the normal serum protein concentration of 60-80 mg/ml (11, 17, 27). In contrast, the method using centrifugal analysis yields a coefficient of variation of 1.48% even in the range of low protein concentrations in the cytosol (1.0 - 4.5 mg/ml) with a detection limit of 0.07 mg/ml.

The biuret method is preferred to the Lowry method because the latter detects varying amounts of phenolic and sulphhydryl groups as well as peptide bonds in the various proteins. Because it is not

certain that proteins in cytosols from different tumours are always of the same amino acid composition and because of the different reactivity towards protein standards, the special features of the Lowry method will lead to less uniformity in this field of protein determination (9, 12, 17). That the influence of the amount of certain amino acids on the result of the protein determination is important is illustrated by the fact that the tyrosine content of different proteins in human serum may vary by a factor of five or more (18, 28). Using the Lowry method, this can lead directly to considerable differences in the apparent protein concentration in serum. The same considerations concerning the standard proteins to be used also lead to a preference for the biuret method over the Lowry modification.

From the correlation between the biuret reaction and the modified procedure as described by Lowry it can be seen that results obtained with the latter method were approximately 23% higher than with the biuret reaction. On account of all these complications, preference is given to the simple biuret reaction.

Comparing these procedures with the Coomassie Brilliant Blue dye binding method for determination of protein concentration shows that the latter method has certain clear disadvantages despite its reported sensitivity (13). The qualitative composition of the dye is neither well defined nor guaranteed, and its binding properties are also dependent on many factors. The coefficient of variation between series is larger than for both biuret methods. Another great disadvantage, however, is the strong disturbing interference from haemoglobin in the cytosol preparations which leads to exaggerated protein values (*figure 5-7*).

From this study of oestrogen receptor concentrations it was concluded that the original biuret method performed in a centrifugal analyser was the method of choice for the determination of proteins in mammary tumour cytosols. It must be stressed once more that reliable protein determination is of the utmost importance. When the protein concentration is wrongly reported too high this means that the ratio

of specific oestrogen binding capacity to total protein content is found to be too low. As a consequence, the decision might be taken that hormonal treatment of the patient concerned should not be started. When, however, this decision has been wrongly made, an important treatment for the patient is thus withheld.

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chapter 6

PLASMA PROTEINS IN MAMMARY TUMOUR CYTOSOLS

SUMMARY

The determination of serum albumin in mammary tumour cytosols is described. Both radial immunodiffusion assay and turbidimetric analysis can be used. It is concluded that in the calculation of oestrogen receptor concentration the protein content of the cytosols must be corrected for the presence of serum proteins.

INTRODUCTION

In the expression of oestrogen receptor concentration as binding capacity in fmols of oestrogen per mg protein in cytosols prepared from tissues, a number of difficulties are involved. These have been described in detail in a study on protein determination in mammary tumour cytosols (*chapter 5*). However, once a suitable method for the protein determination in the concerned cytosols has been found, another problem is that not all protein in the cytosol is really tumour protein. It can easily be understood that in most tumours a certain contamination with blood is present. Although this is sometimes very small it leads to an elevated protein content based on the presence of plasma proteins in the cytosol. It appears that the method of preparation of the cytosol does not fully eliminate plasma protein. Sometimes fairly large amounts (even up to 40%) of serum albumin are reported to be found in tumour cytosols (1).

A correction for these non-tissue proteins can be made by performing specific determination of contaminant plasma proteins by immunochemical methods. For this reason the serum albumin content was determined immunochemically using a so-called monospecific albumin antiserum. Several procedures can be used for this determination which have to be compared and should lead to the same results.

In this study radial immunodiffusion (2) has been compared with a rapid immunochemical albumin determination performed by centrifugal analysis (3).

MATERIALS AND METHODS

1. Human serum albumin (H.S.A.) standards. Purified human albumin (Behring Werke AG, Marburg/Lahn, Germany; lot nr. 55263) was dissolved in water to make standard solutions of the appropriate concentrations.
2. Anti-H.S.A. serum (LN rabbit antiserum to human albumin, Behring Werke, lot nr. A 3903 F) was obtained commercially and had a titre of 2.0 g/l. This titre is expressed as the quantity in grams of the particular protein antigen (here: human serum albumin) precipitated by 1 litre of antiserum in the single radial immunodiffusion test (4).
3. Radial immunodiffusion (R.I.D.) determinations were done using commercially available agarose plates (LC-Partigen^R, Behring Werke). H.S.A. standards (albumin-LC standards, Behring Werke) were used with concentrations of 0.076 g/l, 0.193 g/l and 0.390 g/l. Linear relations were always obtained between precipitations area surfaces and concentrations.

Another series of R.I.D. experiments was performed using agarose plates prepared in the laboratory: To 5 ml of a 2% (w/v) agarose (Agarose L, Behring Werke, lot nr. 252007 B) solution in water

table 6-1

Scheme for the immunochemical turbidimetric determination of albumin in cytosols (diluted 1:25) by centrifugal analysis.

temperature	30 °C
type of analysis *	7
wave length	340 nm
sample volume	50 µl
diluent volume (H ₂ O)	50 µl
reagent volume (PEG in buffer)	200 µl
incubation time	30 sec
starting reagent volume (antiserum)	50 µl
time interval (reaction time)	600 sec
albumin standard 1	0.3 mg/ml
albumin standard 2	1.0 mg/ml
albumin standard 3	2.0 mg/ml

*machine code indicating a method with blank subtraction
(Cobas Bio Analyzer, Hoffmann La Roche)

were added 0.5 ml of antiserum (rabbit anti-H.S.A.), 2.2 ml of a 15% (w/v) solution of polyethylene glycol-6000 and 2.3 ml of a barbital-acetate buffer, pH 8.2. This buffer was prepared by dissolving 8.92 g barbital sodium (5,5 diethylbarbituric acid sodium salt) with 5.89 g sodium acetate trihydrate in water, adding about 120 ml 0.1 M hydrochloric acid and adjusting to pH 8.2 whereupon water was added to make up a total volume of 100 ml. Final data for the agarose plates: agarose 1%, antiserum titre 0.1 g/l (dilution 1:20), PEG-6000 concentration 3.3%, buffer molarity for sodium barbital 0.022 M and for sodium acetate 0.036 M, pH 8.2, ionic strength $\mu = 0.05$.

4. Centrifugal turbidimetric analysis was performed using a method

originally described for immuno globulins (3) i.e. fixed time analysis with blank subtraction. Cytosols were diluted with buffer 1 : 25. The description of the procedure is given in *table 6-1*. Polyethylene glycol (PEG-6000) was added to the reaction mixture in order to accelerate formation and insolubilisation of the albumin-anti H.S.A. complex. H.S.A. standards were prepared with concentrations of 0.5 g/l, 1.0 g/l and 2.0 g/l.

5. Immuno electrophoresis was done in agar gel (2% w/v) (Agar purum, Behring Werke, lot nr. 2103 A) in the barbitol/acetate buffer already described.

The method was based on a technique by Grabar and Williams (5). The same antiserum was used as above.

RESULTS

Comparison of the results of determination of human serum albumin in mammary tumour cytosols with the techniques described did not show significant differences for freshly prepared cytosols (*figure 6-1*). In cytosols which had been kept at 4°C for at least three months ("aged" cytosols) the values found by turbidimetric analysis were also equal to those resulting from single radial immunodiffusion (*figure 6-2*). In this case, however, a double ring was observed in the latter procedure where the larger zone was washed out with physiological saline solution. This phenomenon was observed both in commercially agarose plates (Partigen^R-LC) and in plates prepared in laboratory. The latter plates contained PEG-6000 (see Materials and Methods section) in order to create circumstances as similar as possible to the reaction mixture in turbidimetric analysis. The presence of PEG, however, did not enhance these phenomena (*table 6-2*). Perhaps ageing of the cytosols had caused some proteins to precipitate aspecifically in the agarose without becoming irreversible insoluble.

For further investigation immunoelectrophoresis was performed. Sometimes double precipitation lines voor H.S.A. were observed both

figure 6-1

Correlation of the radial immunodiffusion assay (R.I.D.) and turbidimetric analysis of human serum albumin in fresh mammary tumour cytosols, $n=36$, $r=0.986$ (figure 6-1) and in "aged" cytosols, $n=25$, $r=0.993$ (figure 6-2).

figure 6-2

table 6 - 2

Comparison of values (mg/ml) obtained in albumin determinations, in fresh and "aged" cytosols, by immunochemical turbidimetric analysis (ITA) and by radial immunodiffusion without (RID) and with (RID/PEG) addition of polyethylene glycol.

cytosol	y	x	a	b	r	n
fresh	ITA	RID	0.98	0.09	0.986	36
fresh	ITA	RID/PEG	0.92	0.10	0.988	19
fresh	RID/PEG	RID	1.06	0	0.988	19
"aged"	ITA	RID	0.91	0.11	0.993	25
"aged"	ITA	RID/PEG	1.00	0.08	0.995	19
"aged"	RID/PEG	RID	0.92	0.03	0.993	17

- between all methods there is linear correlation characterized by the equation $y = ax + b$
- the number of paired data (n) and the linear correlation coefficient (r) are also given

in fresh and "aged" cytosols. These, however, consisted of insoluble material which did not disappear during the washing and subsequent drying procedures. Apparently the cytosols contained albumin-like proteins or perhaps partly degraded serum albumin which might have arisen during the cytosol preparation procedure. Moreover, immuno-electrophoresis of tumour cytosols using polyvalent anti human serum antiserum demonstrated the presence of a large number of serum proteins in the cytosol (figure 6-3).

It should also be noted that in cytosols from calf uterus a remarkably high albumin content was found. These cytosols are widely used in oestrogen receptor analysis as positive controls and were subjected to the same experiments and analyses as the human tumour cytosols. It appeared that the commercially obtained monovalent

figure 6-3

Immuno-electrophoresis of mammary tumour cytosols.

- A upper pattern: fresh cytosol;
centre well: monospecific anti H.S.A. antiserum;
lower pattern: pooled human serum.
- B upper pattern: "aged" cytosol;
centre well: monospecific anti H.S.A. antiserum;
lower pattern: pooled human serum.
- C upper pattern: fresh cytosol;
centre well: polyvalent anti human serum antiserum;
lower pattern: pooled human serum.

figure 6-4

Immunoelectrophoresis of calf uterus cytosol and other proteins with monospecific anti H.S.A. serum (centre well in all patterns).

- A upper pattern: calf uterus cytosol;
lower pattern: pooled human serum.
- B upper pattern: calf uterus cytosol;
lower pattern: bovine serum albumin.
- C upper pattern: calf uterus cytosol;
lower pattern: equine serum.

rabbit antiserum against human serum albumin cross reacted with bovine serum albumin as was demonstrated in immunoelectrophoresis (*figure 6-4*). With this antiserum equine serum also yielded cross reactions. The antiserum apparently was not species-specific.

DISCUSSION

From the experiments described it is clear that tumour cytosols contain albumin and other serum proteins in addition to tissue proteins. This is not surprising and is in accordance with data reported by others (1).

As no significant differences were found between the radial immunoassay and albumin determination by immunochemical centrifugal analysis, the latter procedure is recommended on account of its good feasibility in the routine laboratory and the short time required for the analysis. The consequence of this finding is that a correction must be made for non tumour protein concentration. This may well result in a higher incidence of receptor positive tumours than hitherto found. Another consequence is that the criterion used for the distinction between "positive" and "negative" tumours has to be reconsidered.

Definitions of positive and negative may vary (6, 7, 8) between authors. In our laboratory tumours having an oestrogen receptor concentration under 3 fmol/mg protein are considered negative whilst those over 9 fmol/mg protein belong to positive tissues. There is certainly a "grey zone" which in practice, however, appears to be of little significance when good corrections for non-specific binding of oestradiol are inherent in the determination procedure.

The selectivity of commercially available antisera against human serum albumin is another aspect which needs to be reconsidered. Apparently there is a high degree of cross reactivity in these antisera towards albumin of mammalian origin, i.e. man, cow and horse. This simplifies the determination of the oestrogen receptor

concentration in the commonly used calf uterus cytosol (positive control) and explains why in some positive controls only a weak oestrogen binding is found, i.e. when a considerable part of the protein in these cytosols consists of bovine serum proteins. No cross reaction was seen with serum proteins other than albumin. This suggests the hypothesis that the probability of reaction with human tumour tissue protein may be very low or absent. If this is true the possibility is introduced of making due correction for the presence of serum proteins in the cytosol.

The proposition (9) is supported that the cytosol protein concentration should be corrected by subtraction of the amount of the serum protein calculated from the albumin estimated immunologically. This serum protein calculation is then based on the assumption that roughly 60% of the protein content of serum consists of albumin.

From the present study it is concluded that in the determination of the protein concentration in tumour cytosols a correction has to be made by subtracting serum protein estimated as 1.7 times the albumin concentration. This corrected tumour protein content in the cytosol is to be used for the calculation of oestrogen receptor concentration. New attention must be paid to the establishment of criteria for positivity and negativity of oestrogen receptor in tissues.

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chapter 7

INFLUENCE OF BUFFER COMPOSITION, TEMPERATURE AND TUMOUR TISSUE ON THE pH OF CYTOSOLS

SUMMARY

The influences of buffer composition, temperature of pH adjustment of the buffer, tumour tissue protein and lactate content on the pH of breast cancer cytosols has been studied. Moreover, the results obtained by oestrogen receptor determination in cytosols prepared with different buffers have been compared. The conclusion from these investigations was that standardization of reaction conditions and the use of 0.05-0.1 M TED-buffer should be recommended.

INTRODUCTION

Many different buffers have been used in the preparation of tumour tissue cytosols and the subsequent determination of steroid receptor concentration in these cytosols (1,2,3). The problem is even more complicated because the temperature at which the pH of the buffer is adjusted, is often not mentioned. This causes much uncertainty on the actual conditions of the assay as some of the buffers used have an important temperature coefficient.

In the present investigation several parameters concerned with the pH of the reaction medium have been studied, such as buffer composition, temperature of initial pH adjustment, protein content of the cytosol and the lactate content of the latter. It is remarkable that information on the lactic acid content of tumour tissues is almost never reported

table 7-1

Composition of TED buffers and phosphate buffers

nr.	tris-HCl	EDTA	NaN ₃	DTT	tglyc	glyc	pH 7.5 adj.	pH 7.9 adj.
1	10	1	3	0.5	-	-	0°	-
2	10	1	3	0.5	-	-	25°	-
3	10	1	3	0.5	10	10%	0°	-
4	10	1	3	0.5	10	10%	25°	-
5	50	1	3	0.5	-	-	-	0°
6	100	1	3	0.5	-	-	-	0°
7	200	1	3	0.5	-	-	-	0°
	phosph.	EDTA	NaN ₃		tglyc	glyc	pH 7.5 adj.	
8	10	1.5	3		10	-	0°	-
9	10	1.5	3		10	-	25°	-
10	10	1.5	3		10	10%	0°	-
11	10	1.5	3		10	10%	25°	-

- all concentrations are given in mmol/l (except glycerol conc.)
- glyc : glycerol concentration in % volume/volume
- tglyc : α -monothioglycerol
- phosph. : phosphate buffers are made by mixing appropriate volumes of 10 mM solutions of KH₂PO₄ and K₂HPO₄
- pH was adjusted to the predetermined value at the temperature mentioned in the last columns

in this context although lactic acid production in malignant tumours can be quite important and rather weak buffers are used in the assays of steroid receptor protein concentration. The use of weak buffers prevents the influence of increased ionic strength on the molecular form of receptor but gives less pH stability.

MATERIALS

- buffers studied in this investigation have been listed in *table 7-1*. Most of these have been used by different authors, those containing glycerol being especially recommended for the progesterone receptor determination (1,2).
- lyophilised horse serum (Seronorm^R, Nyegaard, Oslo/Norway)
- fresh human serum obtained from blood which was left at ambient temperature for clotting during one hour
- all other chemicals used were analytical grade quality
- pH meter (Research pH meter PHM 64; Radiometer, Copenhagen/Denmark)

CYTOSOLS

The cytosols were prepared as follows:

500 - 600 mg of frozen human breast tumour tissue or calf uterus tissue were pulverised in a stainless steel mortar immersed in liquid nitrogen. The obtained frozen rough powder was then vibrated during 45 seconds three times at 50 Hz in a teflon vial with a micro dismembrator (Braun, Melsungen/Germany). The resulting frozen fine powder was transferred to a 5 ml polypropylene ultracentrifugation tube whereupon 3 ml buffer (generally TED buffer nr. 2 in *table 7-1*), at 0° were added. The tube was stoppered and vibrated for another two minutes with the microdis-membrator. The volume was made up with the same buffer to 5 ml and the contents of the tube thoroughly mixed. After 30 minutes ultracentrifugation (100,000 g; 1°) the tube was laterally punctured and the supernatant collected avoiding lipid contamination from the surface of the liquid.

Following this procedure cytosols were obtained with protein content within 2 - 4 mg/ml, in accordance with the cytosols generally used in our laboratory for steroid receptor determination.

figure 7-1

Influence of temperature on TED buffers (tris buffers containing EDTA and DTT (dithiothreitol)).

a : buffer adjusted at pH 7.5 at 0° (table 7-1; buffer nr.1)

b : buffer adjusted at pH 7.5 at 25° (table 7-1; buffer nr.2)

- buffers were heated and cooled with temperature changes of 0.3°/minute

METHODS

Lactate determination. The lactic acid content of 0.5 ml of the samples was determined enzymatically with lactate dehydrogenase. A lactate calibration solution (1.0 M; Boehringer, Mannheim/Germany) was used to prepare standard solutions of lactic acid. NADH absorbance was read at 340 nm.

figure 7-2

Influence of temperature on phosphate buffers.

a : buffer adjusted at pH 7.5 at 0° (table 7-1; buffer nr.8)

b : buffer adjusted at pH 7.5 at 25° (table 7-1; buffer nr.9)

- buffers were heated and cooled with temperature changes 0.3°/minute

Protein determination. Protein was determined by a biuret method by centrifugal analysis (this thesis; chapter 5).

Serum albumin was determined by radial immuno diffusion in Partigen^R-LC plates (Behring Werke, Marburg/Germany).

pH measurements were performed as follows:

A buffer sample was placed in a 250 ml beaker and a precision thermometer and the combination electrode of the pH meter were immersed. The

beaker was placed in a water bath. The contents of this bath and the sample were both continuously stirred with a magnetic stirrer. Increase or decrease of temperature was adjusted at 0.3° per minute. For each sample the pH variations were measured twice, i.e. during increasing and during decreasing temperature over the range 0° to 25° *vice versa*.

RESULTS

The pH values of TED buffers with the concentrations as indicated for the determination of oestrogen receptors (generally 0.01 M) appeared to be considerably temperature dependent. Increasing the temperature provoked pH decrease (*fig. 7-1*). The temperature coefficient for the TED buffers was 0.016 pH units per degree centigrade.

The pH values of 0.01 M phosphate buffers changed in opposite direction with temperature variation (*fig. 7-2*). A positive temperature coefficient of 0.008 - 0.004 pH units per degree centigrade was measured, the variation being dependent on the initially adjusted pH and temperature.

In both types of buffers glycerol addition (10% volume/volume) had no influence on the pH values. This addition is often recommended for the preparation of cytosols for the determination of progesterone receptor protein.

According to generally used procedures cytosols for the determination of oestrogen receptors were prepared with TED buffer (buffer nr.2 ; *table 7-1*). The pH of the cytosols was measured at the working temperature 0° . The diversity of results of pH measurements is illustrated in *figure 7-3*. Mean and median pH value was 7.65; range 6.4 to 8.2

The lactate concentration in the cytosols has also been determined. No direct relation with the pH values could be established. Concentrations ranged from 0.08 to 3.2 mmol/l (*fig. 7-4*). Mean concentration 1.06 mmol/l; median concentration 0.8 mmol/l.

In the cytosols that were studied not much influence of the protein

figure 7-3 pH values of mammary tumour cytosols prepared with TED buffer (buffer nr.2; table 7-1)

figure 7-4 lactate concentration in mammary tumour cytosols prepared with TED buffer (buffer nr.2; table 7-1)

concentration on the pH could be observed. These concentrations varied between 2.4 mg/ml and 4.0 mg/ml. This is not surprising because in the preparation of cytosols standardization of the wet tissue weight per volume buffer is respected.

Studies on the influence of addition of lactic acid or/and proteins to TED buffers were also performed. Values of pH found after varying amounts of lactic acid added to TED buffers with different concentrations of tris (buffers nrs 2, 5, 6 and 7; table 7-1) are given in figure 7-5. All buffers contained a same amount of added equine serum proteins. This was added in order to obtain protein containing buffers comparable

figure 7-5

Influence of addition of increasing amounts of lactic acid on the pH of TED buffers after addition of a constant amount of equine serum protein

—○— TED buffer 0.01 M tris (buffer nr.2; table 7-1)

—●— TED buffer 0.05 M tris (buffer nr.5; table 7-1)

—□— TED buffer 0.10 M tris (buffer nr.6; table 7-1)

—■— TED buffer 0.20 M tris (buffer nr.7; table 7-1)

★, pH 7.9 adjusted at 0° before protein addition for all four buffers

P, addition of equine serum protein to a final concentration of 2 g/l in each buffer

○, pH of all buffers after addition of protein

all pH values were measured at 0°

figure 7-6

Influence of addition of increasing amounts of equine serum protein on the pH of TED buffers after addition of a constant amount of lactic acid; pH values were all measured at 0°

- buffer concentrations as given in figure 7-5

★: pH 7.9 adjusted at 0° for all buffers before lactic acid addition

L: addition of lactic acid to a final concentration of 0.0026 M in each buffer

to cytosols with the lowest protein concentration (2 mg/ml). The addition of this amount of protein only slightly changed the pH of the buffers which had initially been adjusted at 7.9 at 0°. As could be expected the greatest pH change was observed in the buffer with the lowest tris concentration.

In a comparable series of experiments increasing amounts of protein (a concentrated solution of lyophilized blood serum) were added to TED buffers after addition of a constant amount of lactic acid. Not surprisingly the pH decrease was strongest in the least concentrated buffer. This decrease could only partially be restored by adding protein in increasing amounts. More concentrated buffers were less influenced (*fig. 7-6*). Both human and equine serum proteins were used; similar results were obtained.

A last series of experiments have been the determinations of oestrogen receptors in cytosols prepared in different buffers (*nrs 2, 5, 6 and 7; table 7-1*). The same calf uterus tissue was used for the preparation of the cytosols. Results are given in *table 7-2*.

DISCUSSION

A number of variable factors in the reaction circumstances of the determination of oestrogen receptor concentration in tissue cytosols have been studied. Considerable influence of the temperature of initial pH adjustment on the final pH in the reaction medium was found. This was equally true for TED buffers and for phosphate buffers. It seems to be indispensable therefore, that accurate information be given on the preparation of the buffers and the cytosols including temperature of pH adjustment. And this although it has been reported that the cytosol receptor protein activity is insensitive to pH changes within the range 7 to 9 (4). However, disappointing results found in interlaboratory comparative studies (5) suggest at least strict standardization of reaction conditions when improvement of interlaboratory correlation is aimed at.

A considerable influence of tumour tissue constituents on the final pH of the reaction medium in TED buffer was also found. The pH of the TED buffers significantly changed upon lactic acid addition. This pH decrease was somewhat counteracted by protein. However, the use of the

table 7-2

Oestrogen receptor determination of calf uterus cytosol in different TED buffers

sample nr.	wet tissue weight	tris conc.	pH of cytosol	lactate conc.	protein conc.	E.R.	$K_d \cdot 10^{-9}$
1	510	0.01	7.61	1.17	2.25	436	0.32
2	502	0.05	7.80	1.10	2.27	493	0.37
3	517	0.10	7.85	1.24	2.32	422	0.31
4	508	0.20	7.86	1.45	2.55	424	0.47

- the tissue (mg wet weight) is processed in 5 ml buffer
- tris concentration in the TED buffers is given in mol/l
- lactate concentration in the cytosols is given in mmol/l
- protein concentration in the cytosols is given in mg/ml
- oestrogen receptor concentration (E.R.) is given in fmol/mg protein
- the pH of the buffers was adjusted at 7.9 at 0°
(buffers are specified in *table 7-1*, nrs 2, 5, 6 and 7)

0.01 TED buffer might result in considerably depressed pH values, this being especially possible when the protein concentration in the cytosol is low and the lactate concentration is relatively high. In order to avoid too low results in the oestrogen receptor determination it seems advisable to recommend the use of more concentrated buffers, e.g. the 0.05 or even the 0.100 M TED buffers. This is even more necessary as the optimum for oestrogen receptor activity is pH 8 (6).

No direct relation between pH depression and lactic acid content in the cytosols was found. Apparently other constituents from the tumour tissue also contribute to the final cytosol pH.

Finally the influence of buffer molarity on the result of the oestrogen receptor determination was studied. Although some variations were found no absolute preference for any of the four different buffer could be established. This could perhaps be explained by the fact that the low protein content of the cytosols prevents a number of non-specific binding reactions and the inherent complications in the calculation of the results (7). Another reason might have been the fact that this was investigated in calf uterus cytosols; although significant lactate concentrations were found, the composition of the tissue is still different from mammary tumours of human origin. A problem in many of the studies as described here is that quite often the human breast cancer tissue samples which were submitted for investigation were quite small.

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chapter 8

CHARACTERISATION OF OESTROGEN RECEPTOR PROTEINS

SUMMARY

For the characterisation of oestrogen receptor proteins a number of techniques have been used: gel filtration on Sephadex^R G-150 and on Sephacryl^R S-300, sucrose density gradient ultracentrifugation and polyacrylamide gel electrophoresis. The molecular weight class of the receptor proteins was estimated. In the cytosol both specific and non-specific binding proteins were detected and distinguished. Moreover, two forms of receptor proteins were demonstrated, both being able to bind labelled oestradiol specifically. On account of distinct fundamental differences in the techniques, it could hardly be expected that results would be identical in the three procedures. Receptor proteins of "low" molecular weight were invariably found and aggregation phenomena were always observed. It is highly probably that characterisation of native receptor proteins cannot be performed by the usual technical procedures because of inherent influences of the detection methods on the structure of these receptors.

INTRODUCTION

An interesting aspect of the determination of steroid hormone binding in cytosols prepared from human tissue is the characterisation of the binding proteins. A number of attempts have

been made in this field using techniques suitable for the separation and identification of macromolecules, such as gel filtration (1, 2) electrophoresis (3) and density gradient centrifugation (4). These analytical procedures provide information on the proteins involved whilst separation between free hormones and steroid protein complexes is also accomplished. Binding phenomena can thus be studied such as the binding of a particular steroid to one or more proteins in the same cytosol and the specificity of tracer binding in the presence of a large excess of radio-inert ligand.

The binding of oestradiol to receptor proteins in human mammary tumour cytosols was studied, in order to gather information on the properties of the binding proteins. This investigation was performed as part of a study of the causes of divergent analytical results obtained in the routine determination of oestrogen receptor concentrations in different laboratories. This divergence is annoying as results of determinations are important for the decision whether hormonal therapy is to be applied in a specific case or denied because no success can be expected.

GEL FILTRATION

PREPARATION OF CYTOSOLS FROM HUMAN MAMMARY TUMOUR TISSUE

Cytosols were prepared as described earlier (5, 6). No variations in this procedure were allowed during the present study. This method for cytosol preparation was similar to the one that has been recommended by the E.O.R.T.C. (5) and is used in many laboratories.

CHEMICALS

- 16 α - 125 Iodo - 3,17 β - oestradiol (specific activity: $\approx$ 200 Ci/mmol) (New England Nuclear) (125 I-E₂).

- Sephadex^R G-150 (Pharmacia).
- Sephacryl^R S-300 (Pharmacia).
- Low and high molecular weight gel filtration calibration proteins (Pharmacia kits).
- Human serum albumin (Nordic Pharmaceuticals).
- All other chemicals used were commercial products of analytical grade.

REAGENTS

- Tris-HCl buffer 10 mM, pH 7.5 at 25°C, containing 1 mM EDTA, 3 mM NaN₃ and 0.5 mM dithiothreitol (TED buffer).

SAMPLES

Human breast cancer tissues were homogenised in TED buffer. In order to obtain cytosol protein concentrations of about 3 mg/ml tissue samples of about 500 - 600 mg wet weight were processed in 5 ml buffer. Tumour cytosol was obtained from a pool of five tumours. Oestrogen receptor concentration of this cytosol was 174 fmol/mg protein. Aliquots of cytosol were incubated with labelled oestradiol (¹²⁵I-E₂); final concentration 25×10^{-10} M with or without addition of diethylstilboestrol (DES); final concentration 50×10^{-8} M as competitive "cold" ligand. Incubation was performed overnight in melting ice, whereupon samples (1.5 - 2.0 ml) of the reaction mixture were placed in the column.

GEL FILTRATION EQUIPMENT

- 2 cm² x 100 cm column (Pharmacia K 16/100) packed with Sephadex^R G-150.
- 2 cm² x 100 cm column (Pharmacia K 16/100) packed with Sephacryl^R

Sephacryl^R S-300.

- Peristaltic pump Minipuls II (Gilson).
- Spectrochrom M photometric detector (Gilson).
- Fraction collector MTDC (Gilson).
- Recorder BDC, input 10 mV (Kipp & Zonen).
- γ -Counter NE 1621 (Nuclear Enterprise).

GEL FILTRATION PROCEDURE ON SEPHADEX^R G-150

Bed dimensions: 2 cm² x 94.5 cm.

Eluent: TED buffer (see reagents).

Gel filtrations: upward flow performed at 2° ± 1°C.

Sample volume for each run: 2.0 ml.

Flow rate: 3.3 ml . cm⁻² . h⁻¹.

Fraction volume: 100 drops $\simeq$ 1.8 ml.

Absorbance of the effluents: continuously monitored at 280 nm.

The analytical procedure is outlined in *table 8-1*. For reasons of efficiency results are included in this table.

GEL FILTRATION PROCEDURE ON SEPHACRYL^R S-300

Bed dimensions: 2 cm² x 89 cm.

Eluent: TED buffer (see reagents).

Gel filtrations: upward flow performed at 2° ± 1° C.

Sample volume for each run: 1.5 ml

Flow rate: 13.2 ml . cm⁻² . h⁻¹.

Fraction volume: 100 drops $\simeq$ 1.8 ml.

Absorbance of the effluents: continuously monitored at 280 nm.

The analytical procedure is outlined in *table 8-2*. For reasons of efficiency results are included in this table.

table 8-1

Gel filtration on Sephadex^R G-150.

run	sample 2 ml	conc. mg/ml	molecular weight	RESULTS	
				peak fr.nr	K _{av}
1st	blue dextran 2000 glucose	2		40	
		1		103	
2nd	cytosol incubated with ¹²⁵ I-E ₂			45	0.07
				67	0.42
				115	-
3rd	human serum alb.	1	69,000	71	0.48
4th	cytosol incubated with ¹²⁵ I-E ₂ and DES			47	0.10
				117	-
5th	catalase	3	232,000	56	0.25
	bovine albumin	7	67,000	73	0.51
	chymotrypsinogen A	3	25,000	89	0.77
6th	ferritin	0.5	440,000	43	0.04
	aldolase	2	158,000	64	0.36
	ovalbumin	7	43,000	79	0.61
	ribonuclease A	10	13,700	96	0.88
7th	blue dextran 2000 glucose	2		41	
		1		104	

ESTIMATION OF MOLECULAR WEIGHTS BY GEL FILTRATION ON SEPHADEX^R G-150
AND SEPHACRYL^R S-300

For each column packing a calibration curve was obtained by plotting the K_{av} values of reference standard proteins *vs.* the logarithms of their molecular weights. The K_{av} values (available partition

table 8-2

Gel filtration on Sephacryl^R S-300.

run	sample 2 ml	conc. mg/ml	molecular weight	RESULTS	
				peak fr.nr	K _{av}
1st	blue dextran 2000 glucose	2 1		35	
				89	
2nd	cytosol incubated with ¹²⁵ I-E ₂			36	0.02
				40	0.09
				56	0.39
3rd	thyroglobulin	2	660,000	38	0.06
	catalase	3	232,000	46	0.20
	bovine albumin	7	67,000	63	0.52
	chymotrypsinogen A	3	25,000	74	0.72
4th	ferritin	0.5	440,000	41	0.11
	aldolase	2	158,000	51	0.30
	ovalbumin	7	43,000	70	0.65
	ribonuclease A	10	13,700	85	0.93
5th	cytosol incubated with ¹²⁵ I-E ₂			37	0.04
				41	0.11
6th	blue dextran 2000 glucose	2 1		35	
				89	

coefficients) were calculated as follows:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where V_e was the elution volume corresponding to peak elution of the standard protein or labelled oestradiol peak, V_o was the void volume of the column, determined with blue dextran 2000 and V_t was the total volume of the packed bed, measured by glucose elution. The

peak levels of blue dextran 2000 and the calibration proteins were detected by absorbance at 280 nm; the peak of glucose elution was measured spectrophotometrically at 620 nm performing the anthrone reaction on aliquots of the fractions (?).

RESULTS

By gel filtration of cytosol samples previously incubated with $^{125}\text{I-E}_2$ (tracer), on Sephadex^R G-150 several fractions appeared to contain protein bound iodine-125. No linear correlation of radioactivity with the protein concentration in the fractions could be observed (*figure 8-1*). A first broad peak (fractions 43-46) was followed by an even more extended range of radioactivity (fractions 63-75). Both elution ranges coincided with the elution of protein. A last and wide range of radioactivity was eluted between fractions 90-160 with a distinct peak around fraction nr. 115. Protein elution was absent from fraction 118 and onwards. This last elution of radioactivity must be attributed to free tracer. Incubation of cytosol with tracer and two hundred fold excess DES led to disappearance of radioactivity in some elution fractions. In the first range radioactivity could still be detected (fractions 44-47) whilst no radioactivity at all was found in the fraction range 63-75. No decrease in elution of radioactivity in fractions 90-160 was observed.

As the only difference between these experiments was the addition of an excess of radio-inert DES to the cytosol-tracer mixture in the second incubation, it could be concluded that in fractions 44 and 47 (peak 1, *figure 8-1*) both specific receptor proteins and non-specific binding proteins were eluted. On the contrary, in the fractions around nr. 67 (peak 2, *figure 8-1*) only specific receptor protein was present. A first conclusion can be that the specific binding protein in peak 2 is to be considered as the smaller receptor protein. The specific part of binding protein in peak 1 corresponds

figure 8-1

figure 8-1

Gelfiltration of cytosols on Sephadex^R G-150:

- protein absorption (280 nm)
- radioactivity of fractions from cytosol incubated with $^{125}\text{I-E}_2$ (25.10^{-10}M)
- radioactivity of fractions from cytosol incubated with $^{125}\text{I-E}_2$ (25.10^{-10}M) and DES (50.10^{-8}M)

Peaks 1 and 2 : binding proteins.

Calibration peaks: blue dextran 2000 (a), ferritin (b), catalase (c), aldolase (d), human serum albumin (e), bovine albumin (f), ovalbumin (g), chymotrypsinogen A (h), ribonuclease A (i) and glucose (j).

with a larger receptor protein. In both experiments non protein-bound tracer was eluted in the same fraction range.

For a number of calibration proteins of known molecular weight the K_{av} values for this column were calculated (table 8-1). A graphical representation of the calibration of the column with proteins of known molecular weights against the calculated K_{av} values was made (figure 8-2). From this graph it was possible to estimate roughly the molecular weight of the receptor proteins. These were found to lie around 390,000 (small amount) and around 100,000 (the larger amount).

By performing the same experiments with Sephacryl^R S-300 different elution patterns were obtained (figure 8-3). This was true both for protein elution and for elution of radioactivity. A final separation in the high molecular weight range was observed where two distinct peaks were eluted (fractions 36 and 40). The first of these appeared to consist largely of specific binding protein whilst the second peak was to a larger extent still found after incubation with excess DES. As in the Sephadex gel filtration the low molecular weight receptor activity was to larger extent suppressed in the presence of DES (peak 3, figure 8-3). The high molecular weight

figure 8-2

Calibration of Sephadex G-150 column. Open circles 1 and 2 correspond with binding proteins (see figure 8-1 and text).

binding protein fraction in the Sephacryl filtration very clearly consisted of two different peaks (peaks 1 and 2, figure 8-3) of which the first was much suppressed in the presence of DES, indicating that this fraction contained predominantly specific receptor protein.

Furthermore, a pronounced adsorption chromatographic effect of the Sephacryl towards the free tracer was observed. The latter was eluted in fractions 363-421 which was far beyond the glucose

figure 8-3

Gel filtration of cytosols on Sephacryl^R S-300:

—— protein absorption (280 nm);

----- radioactivity of fractions from cytosol incubated with ¹²⁵I-E₂ (25.10⁻¹⁰ M);

———— radioactivity of fractions from cytosol incubated with ¹²⁵I-E₂ (25.10⁻¹⁰ M) and DES (50.10⁻⁸ M).

Peaks 1, 2 and 3: binding proteins.

Calibration peaks: blue dextran 2000 (a), thyroglobuline (b), ferritine (c), catalase (d), aldolase (e), bovine serum albumin (f) and ovalbumin (g).

fraction (nr 89). The elution volume for the free tracer thus was nearly four times the total volume of the column which must have been caused by adsorption of the free steroid to the Sephacryl.

figure 8-4

Calibration of Sephadex^R S-300 column. Open circles 1, 2 and 3 correspond with binding proteins (see figure 8-3 and text).

Calibration of the column with reference proteins (figure 8-4) allowed a rough estimate of the molecular weight of receptor proteins. The two first elution peaks corresponded to molecular weights of 860,000 (specific receptor peak) and 460,000 (non-specific binding protein) and the small peak (fraction 56) contained specific receptor protein of molecular weight around 115,000. The latter value was in rough accordance with the value of 100,000 found in the Sephadex^R G-150 experiment. The very large protein (860,000 molecular

weight) may have been an aggregate of smaller receptor proteins.

Iodine labelled oestradiol was used as a tracer to obtain high sensitivity. Similar experiments were started using $^3\text{H-E}_2$ as a tracer. This technique was soon abandoned on account of low sensitivity of measurement of radioactivity. First results were in good agreement with the experiments where the $^{125}\text{I-E}_2$ tracer was used.

SUCROSE DENSITY GRADIENT ULTRA CENTRIFUGATION (SDG)

PREPARATION OF CYTOSOLS FROM HUMAN MAMMARY TUMOUR TISSUE

Cytosols were prepared as described earlier (5, 6). No variations in this procedure were allowed during the present study. This method for cytosol preparation was similar to the one that has been recommended by the E.O.R.T.C. (5) and is used in many laboratories.

CHEMICALS

- 16α - ^{125}I do-3,17 β -oestradiol (specific activity $\approx$ 200 Ci/mmol) (New England Nuclear).
- Diethylstilbestrol (Sigma).
- Sucrose (Analar).
- Charcoal activated (BDH, decolorizing powder).
- Dextran T-70 (Pharmacia).
- Gelatin weiss (Merck).
- All other chemicals used were commercial products of analytical grade.

REAGENTS

- Tris-HCl buffer 10 mM, pH 7.5 at 25°C, containing 1 mM EDTA,

- 3 mM NaN_3 and 0.5 mM dithiothreitol (TED buffer).
- Dextran coated charcoal (DCC) suspension, prepared by adding 0.5 g of charcoal to 100 ml of TED buffer containing 0.5 g of dextran T-70 and 0.2 g of gelatin.

SAMPLES

Cytosol samples were prepared and incubated as described for gel filtration (*vide supra*). In some cases the free oestradiol and DES were removed by stirring a volume of the incubated sample with a pellet obtained by centrifugation of an equal volume of DCC suspension. The mixtures were agitated for 20 minutes at 2°C and centrifuged at 2000 g. Aliquots of the supernatant were layered on the sucrose gradient.

SDG EQUIPMENT

SDG equipment consisted of:

- Beckman ultracentrifuge type L2-65B with swing out rotor type SW 60 Ti was used with poly allomer tubes 5 ml. The tubes were punctured using a fraction recovery system (Beckman).
- Modified multi-channel gradient former (Cenco).
- Spectrochrom-F photometric detector (Gilson) connected with a recorder (BDC, input 100 mV. Kipp & Zonen).
- Fraction collector MTDC (Gilson),
- Peristaltic pump Minipuls II (Gilson).
- γ -Counter NE 1621 (Nuclear Enterprise).

PROCEDURE

- Linear sucrose concentration gradients of 5 - 25% or 5 - 30% sucrose in TED buffer were prepared at 2°C in poly allomer

ultracentrifugation tubes of 4.4 ml volume.

- Samples (volume 100 - 200 μ l) were cautiously layered on top of the sucrose gradients. Bovine serum albumin was used as a marker protein (4.6 S).
- The tubes were placed in cold rotor buckets (2°C) and centrifuged for 17 hours at 300,000 g.
- The bottoms of the tubes were punctured and a solution of sucrose (45% containing 1% blue dextran 2000) was pumped into the bottom of the gradient tubes displacing the protein containing gradient solution.
- The peak levels of proteins and blue dextran 2000 were continuously monitored at 280 nm.
- Fractions were collected and their radioactivity measured.

RESULTS

A first series of experiments was performed where free hormones were not removed with a DCC pellet. Three tracer binding peaks were found, *viz.* in fractions 8 (peak 1), 15 (peak 2) and 32 (peak 3) (*figure 8-5*). After suppression of specific binding with DES peak 1 disappeared almost completely indicating the presence of 4 S receptor protein in the tumour cytosol. Peak 2 disappeared to some extent leaving a certain degree of unspecific binding as was also reported elsewhere (8). Peak 3 appeared to consist of non-specific binding protein. At the bottom of the tubes a small amount of non-specifically bound tracer was always detected (fraction 61). Tracer in this fraction must have been bound either to very high molecular weight or to strongly aggregated proteins.

In another series of experiments the influence was studied of removing the free hormones with a DCC pellet after incubation of the cytosol with hormones. Simultaneous runs of samples either without or with DCC treatment were analysed (*figure 8-6*). It was observed that treatment with coated charcoal before SDG analysis removed not

figure 8-5

Sucrose density gradient centrifugation of human mammary tumour (ER+) cytosol:

- — — protein absorption (280 nm);
- radioactivity of fractions from cytosol incubated with ¹²⁵I-E₂ (25.10⁻¹⁰M) (peak 1, 2 and 3);
- radioactivity of fractions from cytosol incubated with ¹²⁵I-E₂ (25.10⁻¹⁰M) and DES (50.10⁻⁸M).

Sucrose gradient: 5-25% sucrose in TED buffer.

Sample size: 0.2 ml.

Centrifugation: 17 hours, 300,000 g, 2°C.

Fraction volume: 75 µl (5 drops).

only free tracer but also a considerable amount of protein bound tracer. The residual total radioactivity in the protein containing fractions was greatly diminished and the free tracer radioactivity (top of the tube) disappeared. Furthermore, it could be observed that

figure 8-6a

figure 8-6b

figure 8-6

SDG analysis of human mammary tumour (ER+) cytosol:

a. without treatment of incubate with dextran coated charcoal

b. with treatment of incubate with dextran coated charcoal.

----- Radioactivity after incubation with $^{125}\text{I-E}_2$ ($25 \cdot 10^{-10}\text{M}$).

———— Radioactivity after incubation with $^{125}\text{I-E}_2$ ($25 \cdot 10^{-10}\text{M}$) and DES ($50 \cdot 10^{-8}\text{M}$).

Sucrose gradient: 5-30% sucrose in TED-buffer.

Sample size: 0.15 ml.

Centrifugation: 17 hours, 300,000 g, 2°C .

Fraction volume: 30 μl (3 drops).

figure 8-7a

figure 8-7

SDG analysis of human mammary tumour (ER+) cytosol:

- ultracentrifugation in sucrose gradient in TED buffer
- ultracentrifugation in sucrose gradient in TED buffer containing 0.1 M KCl.

----- Radioactivity after incubation with $^{125}\text{I-E}_2$ ($5 \cdot 10^{-10}\text{M}$).

———— Radioactivity after incubation with $^{125}\text{I-E}_2$ ($5 \cdot 10^{-10}\text{M}$) and DES ($10 \cdot 10^{-6}\text{M}$).

Sucrose gradient: 5 - 30% sucrose.

Sample size: 0.2 ml.

Centrifugation: 20 hours, 300,000 g, 2°C.

Fraction volume: 75 μl (5 drops).

relatively less radioactivity in the 4 S region (peak 1 in figure 8-6a and in figure 8-6b) remained than in the higher molecular weight fraction (peak 2 in figure 8-6a and in figure 8-6b). This is possibly the result of stronger adsorption of the smaller receptor proteins to DCC (9). Here good agreement was found with earlier data

figure 8-7b

on the adsorption of receptor-tracer complex by DCC (6, and chapter 2 of this thesis). Moreover, it has been reported (10) that in different tumour cytosols changing patterns of receptor proteins can be found, ranging from only pure 8 S proteins to predominantly 4 S proteins and combinations in all possible proportions.

In a third series of experiments the influence of adding KCl to the cytosol was investigated. It has been reported (11) that dissociation of 8 S receptor proteins into 4 S proteins could be observed in the presence of KCl (0.4 M). In order to study this salt effect KCl was added in a final concentration of 0.1 M in the sucrose gradient solution. From figure 8-7 it can be learned that no obvious shift from larger to smaller receptor proteins occurred during ultracentrifugation. However, a certain decrease of specific binding activity was observed. Whether this was due to denaturation of receptor protein during the procedure could not be established.

POLYACRYLAMIDE GRADIENT GEL ELECTROPHORESIS (PAGE)

PREPARATION OF CYTOSOLS FROM HUMAN MAMMARY TUMOUR TISSUE

Cytosols were prepared as described earlier (5, 6). No variations in this procedure were allowed during the present study. This method for cytosol preparation was similar to the one that has been recommended by the E.O.R.T.C. (5) and is used in many laboratories.

CHEMICALS

- 16α - 125 Iodo-3,17 β -oestradiol (specific activity: $\approx$ 200 Ci/mmol) (New England Nuclear) (125 I-E₂).
- Diethylstilbestrol (Sigma).
- All other chemicals used were commercial products of analytical grade.

REAGENTS

- Tris-HCl buffer 10 mM, pH 7.5 at 25°C, containing 1 mM EDTA, 3 mM Na₃N and 0.5 mM dithiothreitol (TED buffer).
- Electrophoresis buffer: tris 90 mM, borate 80 mM, pH 8.2 at 0°C, containing 3 mM Na₂EDTA.
- Staining solution: 0.5% Coomassie Brilliant Blue dissolved in 5% methanol in 21% acetic acid solution.
- Destaining solution: 0.5% methanol in 21% acetic acid solution.
- Surface autoradiography enhancer EN³HANCETM spray (NEN).

SAMPLES

Mammary tumours cytosols were prepared and incubated as described

for SDG analysis. The sample density was increased by dissolving sucrose to obtain a 25% sucrose concentration. Unbound tracer was in some cases removed by DCC pellets. The applied sample volumes were 10 to 50 μ l. High and low molecular weight calibration proteins (Pharmacia) were dissolved in TED buffer (100 μ l / vial); 5 μ l samples were used.

EQUIPMENT

- Polyacrylamide gradient gel slabs, concave gradient of 4% to 30% (separation range for globular proteins 50,000 - 2,000,000 molecular weight) (Pharmacia PAA 4/30).
- Gel electrophoresis apparatus: Uniscil / Gradipore Electrophoresis unit (Tempus) for 4 slabs.
- Electrophoresis power supply LKB 2103 (with constant voltage).
- Gel slicer (3 mm).
- Film: Definix medical (Kodak).
- γ -Counter NE 1612 (Nuclear Enterprise).

ELECTROPHORESIS PROCEDURE

- The whole procedure was performed at 1°C.
- After pre-equilibration of the gel slabs (30 min, 100 V) with circulating buffer, the samples (cytosols and calibration proteins) were applied and pre-electrophoresis was applied for 20 min at 80 V (without circulating buffer) until the proteins moved into the gel.
- The circulation of the buffer was then re-established and the electrophoresis was performed for 16 hours at 130 V (constant voltage).
- After electrophoresis the staining procedure was performed with the staining solution (20 min) and excess of dye was removed by diffusion for 24 hours in the destaining solution.
- Samples were routinely applied to two gel slabs; after

electrophoresis the first was used for the staining procedure and calibration of the molecular weight curve; the second was used for determination of radioactivity of the slices.

MOLECULAR WEIGHT ESTIMATION

The molecular weights of the radioactivity binding proteins were estimated by comparison of their electrophoretic mobility with those of the calibration proteins. A calibration curve was made by plotting the R_f (relative mobility) of the calibration proteins vs. the logarithm of their molecular weight. By means of the determined R_f values of the radioactivity binding proteins the molecular weights were estimated.

RESULTS

Polyacrylamide gradient gel electrophoresis was performed on cytosol samples after incubation with labelled oestradiol ($^{125}\text{I-E}_2$), either with or without excess diethylstilboestrol (DES). After electrophoresis the gel slabs were cut into 3 mm thick slices ("fractions") and the radioactivity was measured. From stained duplicates the position of calibration proteins on the electropherograms could be indicated.

In a first series of experiments several peaks of radioactivity were observed (*figure 8-8*). A first peak of very high activity at the starting point of the electropherogram indicated the spot where free tracer was found. A "shoulder" peak next to the first one could not be suppressed by excess DES. It must be assumed that very high molecular weight proteins / protein aggregates non-specifically bound some tracer. However, two other peaks of radioactivity (peak 1 and peak 2 in *figure 8-8*) were to a major extent suppressed when DES was added during the incubation. It must be concluded that specific

figure 8-8

Polyacrylamide gradient gel electrophoresis of cytosol proteins.

- Human breast tumour cytosol (ER+) was incubated with $^{125}\text{I-E}_2$ without (broken line) or with (solid line) addition of DES.
- Peaks 1 and 2 contain specific receptor proteins.
- Calibration peaks: thyroglobulin (a), ferritin (b), catalase (c), lactate dehydrogenase (d), phosphorylase-b (e), bovine serum albumin (f).

figure 8-9

Calibration of polyacrylamide gradient gel corresponding to figure 8-8.

receptor proteins were found here. From the protein calibration curve (figure 8-9) molecular weights in the ranges of 205,000 for peak 1 and 66,000 for peak 2 could be calculated.

In a second series of experiments the excess tracer was removed with a DCC pellet after incubation. In the electropherogram (figure 8-10) no free tracer was observed. Again non-specific high molecular weight binding proteins were found. As in the first experiments two fractions (peaks 1 and 2 in figure 8-10) contained specific binding proteins in similar molecular weight ranges, i.e. 190,000 and 72,000 (figure 8-11). It could be remarked that residual radioactivity in the protein fractions was considerably lower than without DCC treatment. Autoradiography of the electropherogram was attempted. No

figure 8-10

Polyacrylamide gel electrophoresis of cytosol proteins.

- Human breast tumour cytosol (ER+) was incubated with $^{125}\text{I-E}_2$ ($5 \cdot 10^{-9}\text{M}$) without (broken line) or with (solid line) addition of DES (10^{-6}M). The incubation mixture was treated with dextran coated charcoal before electrophoresis.
- Peaks 1 and 2 contain specific receptor proteins.
- Calibration peaks: thyroglobulin (a), ferritin (b), catalase (c), lactate dehydrogenase (e), phosphorylase-b (f) and bovine serum albumin (g).

figure 8-11

Calibration of polyacrylamide gradient gel corresponding to figure 8-10.

valuable results could be obtained. Short contact showed no detectable stains on the film whilst longer exposition (1 month) produced diffuse spots from which no conclusion could be made.

DISCUSSION

In the present study several oestradiol binding proteins were found in breast cancer tissue cytosols. Obviously some of these proteins had a rather limited binding capacity and could be saturated with an excess of "cold" hormone (DES) whereupon no further uptake of labelled oestradiol occurred. These proteins must be considered as specific oestrogen receptors. The continuing uptake of labelled

tracer even in the presence of considerable excess of diethylstilboestrol must be attributed to the large binding capacity of unspecific proteins.

It could also be concluded that gel filtration is a valuable and rather simple (and inexpensive) procedure to detect specific oestrogen receptor proteins. Especially the use of Sephacryl^R S-300 could be recommended because separation time was much shorter than with the more classical Sephadex^R columns. Attention must be paid, however, to the retention of free tracer on the Sephacryl^R column, which must be eluted completely before a following run could be started, in order to avoid contamination. Moreover, Sephacryl^R S-300 is sold as a pre-swollen preparation so that equilibration of columns packed with it, is rapidly performed.

The existence of different molecular forms of oestrogen receptor proteins has often been reported (12, 13, 14, 15). This is sometimes attributed to aggregation of receptor as such or with other proteins due to the influence of the medium (temperature, pH and ionic strength), although the suggestion has also been made that larger receptor molecules might dissociate during extraction and isolation. Specific binding proteins having sedimentation coefficients of 4 S and 8 S are often found, with corresponding molecular weights. In gel filtration higher values were reported than in sucrose density gradient analysis (16, 17). Our results were in good agreement with these data from the literature.

In gel filtration a considerable amount of lower molecular weight receptor protein with a roughly estimated molecular weight of about 100,000 - 115,000 (*figure 8-1* and *figure 8-2*) was invariably found. This corresponds quite well with the 4 S receptor protein found in sucrose density gradient analysis (*figure 8-5*). That the molecular mass for this receptor was higher in gel filtration than in SDG analysis may well be explained by the hypothesis that probably in the process of gel filtration aggregation of proteins occurred as has also been reported by others (13, 14). In the determination of molecular weight of receptor proteins by gel filtration a number of

different values have been reported in connection with several processes to which the native cytosols were subjected. Amongst these fractionation by ammonium sulphate precipitation, affinity chromatography and isoelectric focussing must be mentioned. Values ranging from about 70,000 (close to albumin) to about 440,000 (close to ferritin) have been reported (15).

Larger molecular weight receptor proteins were also invariably found in our experiments which might correspond to the 8S receptors often reported in the literature. It may be assumed that the higher values found in gel filtration (*figure 8-3*, peaks 2 and 1) correspond to 8S receptor protein and aggregations of it. Molecular weights even as large as 860,000 were found (*figure 8-4*). Similar phenomena were observed in SDG analysis (*figure 8-5*) and this has also been reported elsewhere (10). In order to prevent aggregation of non-specific to specific binding proteins or aggregation of receptor proteins as such treatment of the cytosol with small amounts of proteolytic enzymes such as trypsin (18) or addition of Ca^{++} -ions or other chaotropic salts (13, 19) or heparin (17) have been suggested. In our opinion, however, this could easily provoke artefacts. Otherwise it has been suggested that the prevalence of 4S receptor protein might be an artefact due to endogenous proteolytic activity in the cytosol (20). Whatever it is the smaller receptor proteins were always found in our investigation and the larger and very large molecular weight forms were encountered to a variable extent.

In the polyacrylamide gradient gel electrophoresis two distinct specific receptor protein fractions were found in the molecular weight ranges of 230,000 and 70,000. A highly aggregated or high molecular weight binding protein was also found which was no surprise on account of the denaturing properties of the gel medium employed (21). In general, the electrophoresis experiments confirmed the results of the gel filtration and sucrose density gradient studies, including the binding of protein-tracer complexes by dextran coated charcoal.

As a tracer in most experiments iodinated oestradiol was used.

Although steric effects might change the behaviour of this compound compared to oestradiol, no difference was observed between the results of this tracer and tritiated oestradiol (22). As iodine¹²⁵ is a γ -emitter the measurement of radioactivity with this isotope is easier than with tritium, a β -emitter. This makes such an investigation also possible for laboratories where no liquid scintillation equipment is available. Moreover, the sensitivity of γ -measurement is generally greater which improves the detection limit of the tracer-receptor complex.

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GENERAL SURVEY AND CONCLUSIONS

From the foregoing chapters it is clear that the determination of oestrogen receptor proteins is of great importance for the clinical treatment of patients with breast cancer. A number of aspects are to be considered amongst which are the presence or absence of specific oestrogen binding proteins in the tumour tissue and the presence or absence of progesterone receptor tissue. The correct techniques for these determinations of course are of extreme importance because so much is contributed by the results of these analyses to the possibilities of treatment and prognosis for the patients. For this reason the foregoing study was performed and several aspects are to be considered.

It can now be stated that the use of coated charcoal in the determination of oestrogen receptor concentration is still the method of choice for several reasons. In the first place, it is a convenient and easily applied technique, which from this point of view can only be compared to the protamine sulphate precipitation method. The latter, however, presents still a number of difficulties. This is also true for the dextran coated charcoal procedure and several aspects of this technique have been extensively studied. It can be concluded that strict standardisation of the technical procedure is obligatory in order to obtain reliable and reproducible results. This standardisation is not only required for the receptor determination itself, but also for the preparation of the reagents used in the estimation, e.g. the coating of charcoal by dextran.

Another important feature of the determination of oestrogen proteins is the determination of unspecific protein in mammary

tumour cytosols. Always, a certain amount of non-cytoplasmic protein is present of which the greater part consists of plasma protein. A reasonable estimate of the concentration of the latter can be made by specific immunochemical determination of the human serum albumin content in the cytosols. In our opinion it is strictly necessary to make a correction for these non-tumour proteins and to reassess the criteria for oestrogen receptor positivity (ER+) or oestrogen receptor negativity (ER-).

Furthermore, the influence of buffer composition, temperature and pH in the cytosols and in the buffers which are used in the determination, seems to be important. Although the oestrogen binding activity of the receptor proteins within a certain pH range seems to be uninfluenced, it is also true that at elevated pH values too much non-receptor protein is extracted and on the other hand, at too low pH values the receptor protein activity is greatly reduced.

Characterisation of the receptor proteins confirmed some studies published elsewhere. It appeared not to be possible to isolate and fully characterise the specific receptor proteins. Especially the question whether the so-called 4S proteins must be considered to be an artefact, produced from 8S proteins by proteolytic activity within the cytosols, could not be answered unambiguously.

It must be concluded that the clinically very important determination of oestrogen receptor proteins has to be standardised as far as the technique of determination is concerned. It must be assumed that this standardisation should also be applied to the excision of tumour tissue and the treatment of the latter before it is presented for analysis, e.g. cooling after excision, short time between excision and transport to the laboratory, etc. The dextran coated charcoal method as recommended by the E.O.R.T.C. can quite well be used, although further study is necessary to improve the procedure or to replace it by more specific methods.

SUMMARY

A general introduction and survey of the available literature on the clinical importance of the determination of oestrogen receptors is given in *chapter 1*. Techniques for the determination of these receptors are also shortly reviewed. The purpose of the present study was concluded from these introductory considerations: the investigation of several aspects of the determination of oestrogen receptor proteins in mammary tumour tissue cytosols.

In *chapter 2* the use of coated charcoal in the determination of oestrogen receptor activity is extensively studied. It appeared that the coated charcoal did not adsorb only free tracer but also protein bound tracer. This of course is a serious disadvantage of the procedure which could perhaps be overcome by strict standardisation of the method, such as incubation time, pH of the reaction medium, temperature, etc.

In *chapter 3* the charcoal study is continued by considering several aspects of the coating procedure. Here, too, it is concluded that perfect coating is virtually impossible, but much was gained by the use of standardised preparations of both charcoal and dextran.

The eventual substitution of the much used dextran coated charcoal method by a technically easy procedure (protamine sulphate precipitation technique) is considered in *chapter 4*. From the results obtained in this study there are no reasons to replace the generally recommended dextran coated charcoal procedure by this protamine sulphate precipitation technique. Further investigation might be necessary.

The protein content in the mammary tumour cytosols is very important because the receptor activity is expressed as the capacity to bind labelled oestradiol (fmoles) per mg total cytosol protein. In *chapter 5* a method for a simple, precise and reproducible protein

determination in small cytosol samples has been proposed.

An improvement in the indication of receptor activity is the expression of oestradiol binding capacity per mg tumour tissue protein in the cytosol. This requires a correction for the presence of serum proteins in the total cytosol protein. This correction can be made by specific immunochemical determination of human serum albumin. Cytosol tumour tissue protein is calculated by subtraction of the serum protein content from the total protein concentration (*chapter 6*).

In *chapter 7* the influence of buffer composition, temperature and protein content and lactate concentration of the cytosol and the pH of the reaction medium was studied. Within certain limits no influence of the variation of the pH could be found on the results of the receptor analysis. Nevertheless strict standardisation of conditions seems to be advisable including the use of buffers with sufficient capacity.

Characterisation of oestrogen receptor proteins by several protein separating techniques was studied in *chapter 8*. The receptor proteins always appeared in at least two molecular forms probably corresponding to the 4 S and 8 S proteins as reported elsewhere.

Considering the different aspects of the determination of oestrogen receptor proteins and the importance of the results of this type of analysis for the clinical treatment and the prognosis of the course of the disease, it is strongly recommended that strict standardisation of the determination should be performed (*chapter 9*). No unambiguous method for the determination of these receptors can be advised but the E.O.R.T.C. recommended dextran coated charcoal procedure appears to be the method of choice at the present time.

SAMENVATTING

In *hoofdstuk 1* is van de beschikbare literatuur een algemeen overzicht gegeven. Met name is hier ingegaan op de klinische betekenis van de bepaling van oestrogeenreceptoren en ook op de verschillende technieken die voor deze bepaling zijn beschreven. Voortvloeiend uit deze inleidende beschouwingen is dan het doel van het onderhavig onderzoek geformuleerd: het bestuderen van verschillende aspecten van de bepaling van oestrogeenreceptor-eiwitten in cytosolen van mammatumorsewefsel.

Hoofdstuk 2 is de weergave van een uitgebreide studie over het gebruik van dextran-kool bij de bepaling van oestrogeenreceptoren. Er wordt gerapporteerd dat niet alleen vrije gemerkte hormonen (kleine moleculen) maar ook hormoon-eiwit complexen door de kool worden geadsorbeerd. Hoewel dit als een ernstig bezwaar van de beschreven techniek beschouwd wordt is het niettemin mogelijk door een hoge mate van standaardisering van de reactieomstandigheden toch de bepaling met redelijke betrouwbaarheid uit te voeren. (Dit onderzoek werd eveneens gepubliceerd in Eur. J. Nucl. Med., 1981.)

In *hoofdstuk 3* zijn de verschillende aspecten beschreven die betrekking hebben op de "coating" van koolstof met dextran. Voorbehandeling van kool en de samenstelling van het dextranpreparaat (samengesteld uit verschillende fracties met uiteenlopend molecuulair gewicht) blijken van belang te zijn, evenals de reactietijd. Voor het bereiden van dextran-kool waarmee reproduceerbare resultaten kunnen worden verkregen, is standaardisatie van de coating noodzakelijk. (Gepubliceerd in NucCompact, 1980.)

Gezien de bezwaren, inherent aan de gecoate koolstof methode, is een andere qua techniek voor de routine geschikte, analyse (protamine-sulfaat precipitatie techniek) nader onderzocht (*hoofdstuk 4*). De verkregen resultaten rechtvaardigen niet de vervanging van de over

het algemeen aanbevolen dextran-koolstof methode door deze protamine-sulfaat precipitatie techniek.

Aangezien de activiteit van de receptor-eiwitten in cytosolen wordt uitgedrukt als de specifieke bindingsactiviteit voor gemerkt oestradiol (fmol) per mg cytosol-eiwit, is de bepaling hiervan van eminent belang. In *hoofdstuk 5* wordt een eenvoudige en nauwkeurige methode voor de totaal-eiwitbepaling in kleine cytosol-monsters beschreven. Het totale cytosol-eiwit bevat naast tumoreiwitten steeds ook serumeiwitten. Een gecorrigeerde cytosol-eiwit waarde wordt verkregen door het totale gehalte te verminderen met het gehalte aan serumeiwitten. Met behulp van een specifieke immuno-chemische bepaling van humaan serumalbumine in cytosol-monsters kan genoemde correctie worden aangebracht (*hoofdstuk 6*).

De invloed van buffersamenstelling, temperatuur en eiwit- en lactaatgehalte van de cytosol en van de pH van het reactiemedium werd eveneens bestudeerd (*hoofdstuk 7*). Gebleken is dat binnen bepaalde grenzen variatie van de pH niet noemenswaard van invloed is op de oestrogeenreceptor bepaling. Nochtans lijkt ook hier standaardisering van de reactie-omstandigheden geboden.

Karakterisering van oestrogeenreceptor-eiwitten met behulp van verschillende scheidingstechnieken, zoals die bij eiwitonderzoek worden gebruikt, is beschreven in *hoofdstuk 8*. Steeds werden tenminste twee moleculaire vormen van receptor-eiwit gevonden, waarschijnlijk overeenkomend met de in de literatuur vermelde 4 S en 8 S eiwitten.

Voor de behandeling en prognose van patiënten met mammacarcinoom is de bepaling van oestrogeenreceptoren van eminent belang. In de praktijk blijkt dat de nauwkeurigheid van deze analyses veelal te wensen overlaat, waarvoor verschillende redenen zijn aan te voeren. Standaardisering en uniformering van de bepalingen wordt met klem aangeraden (*hoofdstuk 9*). Het voorgaande in acht nemende kan gesteld worden dat de door de E.O.R.T.C. aanbevolen dextran-kool methode, mits stringent gestandaardiseerd, momenteel de meest bruikbare is.

RESUME

Le *chapitre 1* donne un aperçu général de la littérature traitant de l'importance clinique de la détermination des récepteurs des oestrogènes, décrit brièvement les techniques de dosage de ceux-ci et définit le but de ce travail comme étant l'étude de divers aspects du dosage des récepteurs des oestrogènes dans la fraction soluble cellulaire (cytosol) des tumeurs mammaires.

Le *chapitre 2* étudie de manière approfondie l'action du charbon-dextran dans le dosage; il apparaît que la suspension de charbon-dextran adsorbe non seulement l'hormone marquée mais aussi une certaine quantité des complexes formés par les protéines et l'hormone. Cet inconvénient non négligeable pourrait être réduit par une standardisation rigoureuse de l'action de la suspension de charbon-dextran sur le cytosol préincubé (durée de contact, pH et température du milieu).

L'étude du charbon-dextran se poursuit dans le *chapitre 3* où sont examinés plusieurs paramètres de l'enrobage du charbon par le dextran. Il est peu probable qu'un enrobage idéal puisse être obtenu mais l'emploi d'une préparation standardisée utilisant des constituants rigoureusement définis améliorera la reproductibilité du dosage.

Le *chapitre 4* envisage la possibilité de remplacer la méthode utilisant le charbon-dextran par une autre technique aisée pour les analyses de routine et faisant appel à une précipitation des protéines par le sulfate de protamine. Les résultats obtenus n'apportent pas d'argument décisif pour ne plus employer la méthode charbon-dextran généralement utilisée; un grand nombre d'expériences est nécessaire pour départager les deux méthodes.

L'activité des récepteurs (concentration de sites) s'exprime le plus souvent comme étant la quantité d'oestradiol (fmol) liée

spécifiquement par milligramme de protéines totales du cytosol; le *chapitre 5* propose une méthode simple, précise et reproductible de dosage des protéines dans les cytosols et n'exigeant qu'un faible volume d'échantillon.

Il semble plus exact d'exprimer les résultats par milligramme de protéines tissulaires de la tumeur. Cette valeur est obtenue en soustrayant des protéines totales du cytosol les protéines sériques contaminant l'échantillon; les dernières protéines étant calculées à partir de la valeur de la sérum albumine. Le *chapitre 6* décrit une méthode valable de dosage spécifique de la sérum albumine humaine dans les cytosols.

Le *chapitre 7* s'attache à l'influence de divers paramètres modifiant le pH du milieu avant l'incubation avec l'hormone (composition des tampons, température d'ajustage de leur pH, taux en lactate et en protéine des cytosols). Bien qu'aucune influence de la variation de pH n'ait été observée sur les résultats des dosages des récepteurs d'oestrogène pour autant que celle-ci reste en deçà de certaines limites, il semble préférable de la réduire autant que possible par l'emploi d'un tampon standardisé de capacité suffisante.

La caractérisation des récepteurs de l'oestradiol par différentes techniques de séparation des protéines est exposée dans le *chapitre 8*; les récepteurs semblent toujours se présenter sous au moins deux formes moléculaires correspondant vraisemblablement aux protéines sédimentant à 4 S et 8 S décrites dans la littérature.

Au vu des différents aspects du dosage des récepteurs de l'oestradiol, il semble qu'il soit nécessaire de produire un effort de standardisation très stricte, l'importance des résultats de ce dosage n'étant plus à démontrer tant en ce qui concerne le traitement clinique que le pronostic d'évolution du cancer mammaire (*chapitre 9*). Il n'existe pour le moment aucune méthode idéale de dosage mais l'Organisation Européenne pour la Recherche et le Traitement du Cancer (E.O.R.T.C.) recommande la méthode utilisant le charbon-dextran, laquelle semble être actuellement la plus satisfaisante.

ZUSAMMENFASSUNG

Im *Kapittel 1* wird eine allgemeine Einleitung und ein Übersicht der vorhandenen Literatur über die klinische Relevanz der Oestrogen-Rezeptor Bestimmung gegeben. Auch die Bestimmungsmethoden werden kurz gestreift. Das Ziel der vorliegenden Studie wurde dann folgendermassen formuliert: Die Untersuchung von einigen Aspekten der Oestrogen-Rezeptor Bestimmung in Cytosolen aus Mamma-Tumorgewebe.

Kapittel 2 befasst sich ausführlich mit der Verwendung von beschichteter A-Kohle bei der Bestimmung der Oestrogen-Rezeptor Aktivität. Es stellte sich dabei heraus, dass die beschichtete A-Kohle nicht nur freie sondern auch proteingebundene Markierungssubstanz adsorbiert. Dies ist ein wesentlicher Nachteil der Methodik, welche möglicherweise durch strikte Standardisierung der A-Kohle Inkubation (Inkubationszeit, pH des Reaktionsmediums, Temperatur, u.s.w.) beseitigt werden kann.

Im 3. *Kapittel* wurden verschiedene Aspekte der A-Kohlebeschichtung näher untersucht. Es wurde auch hier festgestellt, dass eine perfekte Beschichtung wahrscheinlich nicht möglich ist, wobei jedoch Vieles gewonnen wird durch die Verwendung von standardisierten A-Kohle- und Dextran-Präparationen.

Es wurde versucht — wie in *Kapittel 4* beschrieben — die vielfach angewendete sog. "Dextran beschichtete A-Kohle Methode" durch eine technisch einfache Prozedur (die Protaminesulphat Fällung) zu ersetzen. Damit wurden jedoch keine besseren Ergebnisse erzielt. Weitere Untersuchungen werden hier notwendig sein.

Weil die Rezeptoraktivität als Bindungskapazität für markiertes Oestradiol (fmol) per mg Cytosol-Protein angegeben wird, ist der Proteingehalt in Mamma-Tumor Cytosolen sehr wichtig. Eine genaue und reproduzierbare Methode zur Bestimmung des Gesamteiweisses in kleinen

Cytosolproben wurde beschrieben (*Kapittel 5*).

Es ist essentiell dass das gemessene Protein Tumor-Eiweiss ist. Dies ist nicht immer der Fall. Es wurde deshalb vorgeschlagen in den gleichen Cytosolproben auch spezifisch das humane Serumalbumin zu bestimmen. Korrektur des Gesamteiweisses für die kontaminierenden Serumproteine wird strikt empfohlen (*Kapittel 6*).

Im *Kapittel 7* wird der Einfluss der Pufferzusammensetzung, der Temperatur, des Protein- und Lactatgehaltes im Cytosol und des pH-Wertes im Reaktionsmedium untersucht. Innerhalb bestimmter Grenzen wurde kein Einfluss des pH gefunden, obwohl strikte Standardisierung der Reaktionsbedingungen notwendig zu sein scheint.

Die Charakterisierung der Oestrogen-Rezeptor Proteine wurde mit verschiedenen Protein-Trennverfahren studiert (*Kapittel 8*). Die Rezeptorproteine traten immer in mindestens zwei molekularen Formen auf, die offenbar korrespondierten mit den an anderer Stelle beschriebenen 4 S-Faktor und 8 S-Faktor Proteinen.

In Anbetracht der unterschiedlichen Aspekte der Oestrogenrezeptor-Bestimmung und der Wichtigkeit der Ergebnisse für die klinische Behandlung und Prognose des Krankheitsverlaufs bei Patientinnen mit Mamma Tumoren ist Strikteste Standardisierung der Bestimmungsmethode zu empfehlen (*Kapittel 9*). Obwohl es bis heute noch keine eindeutige Methode zur Bestimmung dieser Rezeptoren gibt, scheint die von der E.O.R.T.C. empfohlene sog. "Dextran beschichtete A-Kohle Methode" zur Zeit die Methode der Wahl zu sein.

CURRICULUM VITAE

Philippe Guy Fernand Arthur Hector Ralet werd op 6 juni 1945 geboren in Charleroi (België), waar hij in 1963 het eindexamen Humanités Latin-Sciences aflegde en het diploma ontving aan het Athénée Royal de Charleroi.

Vervolgens studeerde hij farmacie aan de Rijksuniversiteit te Luik waar hem op 6 juli 1970 het diploma van apotheker werd uitgereikt.

Na een jaar als zodanig werkzaam te zijn geweest, werkte hij gedurende twee jaar als militair-apotheker in het medisch centrum van het belgische leger in Aken.

Daarna verbreedde hij zijn kennis door het volgen van verschillende post-doctorale stages bij de Universiteit te Luik, en wel bij de Service de Transfusion Sanguine en het Centre de Toxicologie Clinique et Médico-Légale.

Van 1975 tot december 1978 werkte hij in Duitsland als gespecialiseerd industrie-apotheker in een geneesmiddelenfabriek waar hij de verantwoordelijkheid had voor de registratie van geneesmiddelen voor Latijns Amerika en de franstalige landen in Europa en Afrika.

Sinds december 1978 is hij werkzaam geweest op de afdeling klinische chemie (hoofd Prof. Dr. P.J. Brombacher) van het De Wever-Ziekenhuis te Heerlen, waar hij ook zijn proefschrift bewerkte.