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Studies with Steroid–Fluorescein Conjugates on Oestrogen Target Tissues

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Abstract—*Steroid–fluorescein amine and steroid–BSA–fluorescein–isothiocyanate conjugates have been prepared and their ability to bind to oestrogen receptors assessed in competitive binding studies. The binding of all the fluorescent conjugates to uterine cytosol proteins was low when compared with either oestradiol or diethylstilboestrol. A comparative study was carried out to assess the relationship between oestrogen receptor content, determined biochemically, and histochemical localisation of the oestrogen binding components on thin sections of rat uteri, DMBA-induced mammary tumours and also human breast tumour tissue taken at mastectomy. The data indicate that in thin sections of tissue all of these conjugates appear to bind not to the classical oestrogen receptor moiety but to other oestrogen binding proteins.*

INTRODUCTION

NUMEROUS studies have been reported relating to the potential value of oestrogen–fluorescein conjugates to assess the oestrogen receptor content of tissue sections [1–4]. It has been envisaged, for example, that the use of these compounds may eventually complement, or even replace, the standard biochemical assay for the determination of oestrogen receptor status of breast tumours as a means of selecting patients for appropriate forms of therapy. Potentially the histochemical utilisation of oestrogen–fluorescein conjugates has two obvious advantages over the currently available biochemical assay involving the association of radiolabelled ligands to tumour cytosol preparations of the receptor protein *in vitro*. Firstly, since there is no *a priori* reason to believe that all cells of ER-positive breast tumours should contain oestrogen receptor protein, a histochemical assay would provide an assessment of the proportion of cells lacking receptors to be made. Secondly, when receptors were present, their intracellular location could be directly identified. Such information might prove not only a useful prognostic parameter, but might also provide valuable information relating to a better understanding of the endocrinology of tumour growth and development.

A wide spectrum of these conjugates have been assessed, including oestradiol–fluorescein amine [5], oestradiol/oestrone linked at varying positions and through different bridging groups to fluorescein [3, 6] and oestradiol linked to bovine serum albumin–fluorescein isothiocyanate (BSA–FITC). The structures of these various conjugates are illustrated in Fig. 1. Despite the apparent successful application of these conjugates [7, 8], their efficacy in 'localising' oestrogen receptors has been the subject of some controversy [9].

The present study, initiated three years ago, provides the experience from this laboratory on this subject and deals with some of the more controversial factors associated with the use of these fluorescent compounds. Special emphasis has been given to their specificity, purity and relative binding affinities (RBA) for oestrogen receptor preparations. Similarly, the histochemical data obtained after the treatment of oestrogen target tissues with both oestradiol-17β and the non-steroidal antioestrogen, tamoxifen, have been compared with the cellular location of oestrogen receptor proteins as determined using standard [^{3}H]-oestradiol exchange assays. The information gained from these studies has also been applied to the in-

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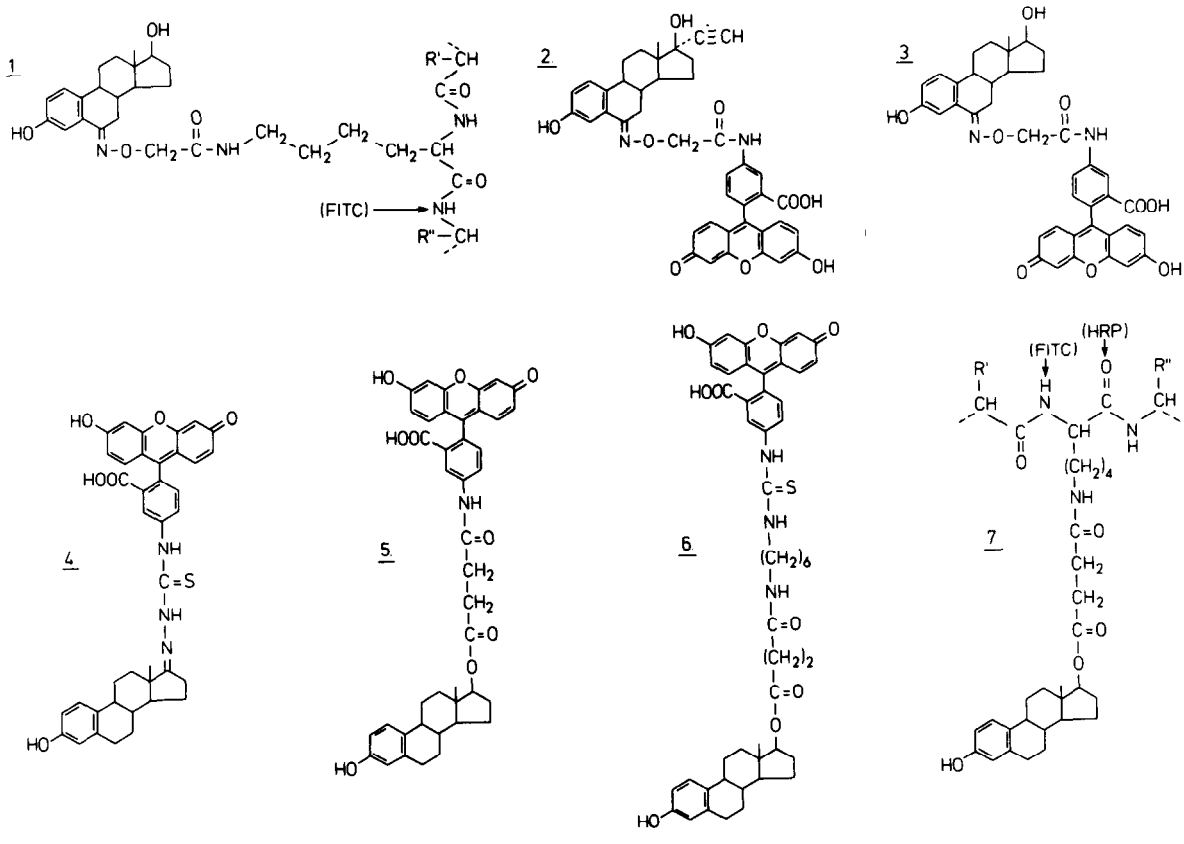


Fig. 1. *Structures of the various fluorescent-oestrogen conjugates prepared*: 1. *Oestradiol-6-OCMO-BSA-FITC.* 2. *Ethynyl-oestradiol-6-OCMO-FA.* 3. *Oestradiol-6-OCMO-FA.* 4. *Oestrone-17-FITC.* 5. *Oestradiol-17-FA.* 6. *Oestradiol-17-HMD-FA.* 7. *Oestradiol-17-BSA-FITC (HRP).*

terpretation of the binding of fluorescent conjugates to a series of oestrogen receptor-positive and negative human breast tumours.

MATERIALS AND METHODS

Materials

All reagents and organic solvents were analytical grade with the exception of dioxan, dimethylformamide and tributylamine, which were distilled and stored over molecular sieves (4A) until required. Fluorescein isothiocyanate (FITC), fluorescein amine (Isomer I) and hexamethylenediamine (HMD) were purchased from Aldrich Chemical Co. Ltd., Gillingham, Dorset, Bovine serum albumin (BSA, Cohn Fraction V), oestradiol-3-17β-diacetate, oestradiol-17β-hemisuccinate and 11α-hydroxyprogesterone were all obtained from Sigma Chemical Corp. Ltd., Poole, Dorset; all other steroid derivatives, unless stated otherwise, were purchased from Steraloids Inc., Wilton, NH. Isobutyl chloroformate was purchased from Eastman-Kodak Ltd., Kirkby, Liverpool, and Sephadex G-25 (fine) and G200 from Pharmacia Ltd., Hounslow, Middlesex. Thin-layer (TLC) precoated silica chromatography plates (20 × 20 cm) were obtained from Camlab Ltd., Cambridge. Preparative TLC plates were prepared using Kieselgel $HF_{253+346}$ (E. Merck, Darmstadt, W. Germany) and a Corning platemaker.

Animals

Mature virgin female Sprague–Dawley rats, bred in the Tenovus Institute animal unit, were used throughout the study. The animals were housed in a 12 hr light/12 hr dark enviroment and were fed food and water *ad libidum*. Two types of tissue were used: (a) uteri from 7-day ovariectomised animals to which were administered i.v. either (i) saline vehicle (100 μl), (ii) oestradiol-17β (5 μg) 2 hr prior to sacrifice or (iii) tamoxifen (300 μg) 16 hr prior to sacrifice; (b) dimethylbenzanthracene (DMBA)-induced mammary tumours from intact animals treated as in (a). Immediately after sacrifice, a representative portion of either mammary tumour tissue or uterus was taken for histochemistry. The remaining tissue was separated into cytosol and nuclear fractions [10] and assayed for oestrogen receptor content. The methods used in the determination of total and accessible oestrogen receptor sites together

with competitive binding procedures have been described previously [11].

Conjugate preparation

(a) *Steroid–BSA–FITC conjugates.* (i) *Oestradiol 17β–BSA–FITC.* Oestradiol-17β–BSA–FITC was prepared by a modification of Erlanger's mixed anhydride procedure [12] developed by Gaetjens and Pertschuk [13]. This method produced low molar incorporation ratios of steroid: BSA. Briefly, oestradiol-17β-hemisuccinate (60 μmol) was dissolved at 11°C in anhydrous dioxan (1000 μl), tributylamine (60 μmol) and isobutyl chloroformate (60 μmol) and added to BSA (3.9 μmol) in 50% (w/v) aqueous dioxan (32 ml). The steroid–protein conjugate was then dialysed against 10mM ammonium bicarbonate and freeze-dried. When required, aliquots of the conjugate (50 mg) were dissolved in carbonate buffer (0.05 M, pH 9.5) at 4°C, FITC (2 mg) added and the mixture allowed to stand at 4°C for 7 hr. The resulting conjugate was dialysed extensively against ammonium bicarbonate and then purified by gel-exclusion chromatography on Sephadex G-25. Fractions containing the steroid–BSA–FITC conjugate were pooled, freeze-dried and stored at 4°C until required. The incorporation of steroid and FITC into the BSA was determined by spectrophotometry, as described by Gaetjens and Pertschuk [13]. The ratio of steroid to protein was 3.5:1 whilst the incorporation of FITC was 2.9:1. A derivative to progesterone-11α-hemisuccinate was prepared in the same manner.

(ii) *Oestradiol-17β-6-(O-carboxymethyl)-oxime–BSA–FITC.* Oestradiol-6-(*O*-carboxymethyl)-oxime was prepared by the method of Lee [2]. Oestradiol-3-17β-diacetate was oxidised in glacial acetic acid with chromium trioxide and the 6-keto derivative saponified in methanolic potassium hydroxide and coupled to aminooxyacetic acid hemihydrochloride to produce the 6-(*O*-carboxymethyl)-oxime derivative. The oxime was then coupled to BSA and FITC as described above. The incorporation of both oestradiol-6-(*O*-carboxymethyl)-oxime and FITC were determined as described above. The ratios of steroid: protein and FITC: protein were 9.4:1 and 3.5:1 respectively.

(b) *Steroid–fluorescein conjugates.* Fluorescent-labelled derivatives of oestradiol-17β-hemisuccinate, oestradiol-6-(*O*-carboxymethyl)-oxime, ethynyl oestradiol-6-(*O*-carboxymethyl)-oxime, testosterone-11α-hemisuccinate, testosterone-17-hemisuccinate, 5α-dihydrotestosterone-17-hemisuccinate, 5α-dihydrotestosterone-1-carboxyethylthioether and 11α-hydroxy progesterone-11-hemisuccinate were prepared using a modification of the method described by Dandliker *et al.* [14]. Briefly, aliquots of steroid derivative (100 μl, 4 mg) in dry dimethylformamide were mixed with fluorescein amine (80 μl, 50 mg/ml), dicyclohexylcarbodiimide (40 μl, 100 mg/ml) and acidified acetone (1 ml) and maintained at 4°C for 48 hr. The products were purified [14] by preparative TLC using the solvent, chloroform: ethanol: water (54:12:1 v/v), eluted and stored in ethanol at 4°C until required.

(c) *Steroid–hexamethylene–fluorescein.* Oestradiol-17β-hemisuccinate was linked to FITC, incorporating a hexamethylenediamine 'bridging' group at position C-17 by a modification of the method of Daxenbichler *et al.*, [15]. Briefly, a 30-molar excess of hexamethylenediamine (360 mg) was reacted with FITC (40 mg) in dimethylformamide (15 ml) at 0°C. The supernatant was decanted and the precipitate washed with dimethylformamide (10 ml), dissolved in water (10 ml) and acidified to pH 5.5 with dilute hydrochloric acid. The precipitate (13.5 mg) was then linked to oestradiol-17-hemisuccinate (6.6 mg) by the mixed anhydride procedure of Erlanger *et al.* [12]. The final product was obtained after addition of water (5 ml) and dilute hydrochloric acid (to pH 5.5) to the reaction mixture, producing a precipitate which was extracted with ethyl acetate (2 × 5 ml) and then lyophilised. The product was re-dissolved in methanol and purified by TLC as described by Daxenbichler *et al.* [15].

(d) *Steroid–hydrazone–fluorescein.* Oestrone-17–FITC [4, Fig. 1] was prepared by the method of Dandliker *et al.* [6].

(e) *Additional purification of conjugates.* 'Free' steroid in the BSA conjugates was removed by treatment with dextran-coated charcoal (4°C, 20 min). Non-steroidal impurities were removed by re-purifying the conjugates using gel-exclusion chromatography on Sephadex G200–120 (55 × 1.5 cm column). The effect of these additional purification steps on the characteristics of the BSA conjugates was determined in the cytosol assay and histochemical procedure.

Histochemical procedures and fluorescence assessment

All tissues were routinely mounted in Tissue-Tek II O.C.T. Mountant (Miles Labs. Inc., IL), frozen on solid carbon dioxide and stored at −70°C until required. Sections (4 μm) were cut onto subbed [16] slides using a cryostat at ap-

proximately −25°C. The frozen sections were processed by two methods.

(1) Conjugates were prepared in PBS (0.01 M, pH 7.4) containing 10% ethanol (v/v) and incubated (2 hr, 25°C) with the tissue sections in a humidity chamber. After incubation the sections were rinsed thoroughly in PBS at 4°C, mounted in buffer and examined for fluorescence ($\lambda = 490$ nm) on a Leitz Orthoplan microscope (Leitz Instruments, W. Germany) fitted with a mercury lamp and a Ploemopak incident beam fluorescence unit.

(2) Conjugate solutions were prepared in Earle's balanced salt medium [17] and incubated (30 min, 25°c) with the tissue sections in a humidity chamber. After rinsing thoroughly in buffer (4°C) the sections were mounted and examined for fluorescence.

Transparencies were taken with a Leitz Orthomat-W camera attachment on 160 ASA tungsten Ektachrome film uprated to 320 ASA using an automatic exposure system.

Fluorescence on the tissue sections was assessed 'semi-quantitatively' on a scale of intensity from negative to positive (± to 5+) at a specific magnification of ×440. Specificity of fluorescence was determined by incubating in the presence and absence of various competitors (50-fold molar excess), including diethylstilboestrol (DES), tamoxifen, certain naturally occurring steroids and also in the presence of a number of 'non-specific' controls, comprising *N*-acetyl-fluorescein amine, BSA–FITC (without steroid attached), steroid BSA conjugates (without FITC) and buffer alone.

Competitive binding studies

A selection of oestrogen conjugates, listed in Table 1, were incubated as competitors to [^{3}H]-oestradiol-17β in a standard rat uterine cytosol assay [11]. Their ability to displace tritiated oestradiol was determined over a wide range of concentrations. The relative binding affinity of each conjugate was determined where possible and compared with data on various unconjugated steroidal competitors. In the case of the BSA conjugates, the relative binding affinity was calculated before and after treatment with dextran-coated charcoal (DCC) and after column chromatography on Sephadex G200 (Table 3). For comparative purposes the steroids listed in Table 2 were also included to indicate their value as competitors or specificity 'controls' in the histochemical procedure.

Table 1. Relative binding affinities (RBA) of various oestrogen–fluorescein conjugates in the rat uterine cytosol assay for oestrogen receptor

Competitor	Relative binding affinity
Oestradiol-17β	100
Diethylstilboestrol	94
Tamoxifen	5.0
Oestrone-17–FITC	2.8
Oestradiol-17–FA	1.4
Ethynyl oestradiol-6–FA	0.6
Oestradiol-17–HMD–FA	0.6
Oestradiol-6–FA	0.2
Oestradiol-6–BSA–FITC	0.01
Oestradiol-17–BSA–FITC	0.01

FA, Fluorescein amine; HMD, hexamethylenediamine; BSA–FITC, bovine serum albumin–fluorescein isothiocyanate.

RESULTS

Competitive binding studies

Table 1 provides data on the relative binding affinity of the various fluorescein-labelled oestrogens for the cytoplasmic oestrogen receptors from rat uterus. Although all conjugates tested displayed some degree of competition with [^{3}H]-oestradiol for the receptor protein, their binding affinity, however, was low in comparison with either unlabelled oestradiol or DES. This was especially evident with those fluorescein conjugates linked to oestradiol *via* BSA molecules.

Introduction of a hexamethylenediamine bridging group at C-17 of oestradiol, a modification designed to increase the distance between fluorescein and the oestradiol molecule, reduced the affinity of the conjugate for the receptor protein. Ethynyloestradiol-6–FA bound to the oestrogen receptor with approximately three times greater affinity than oestradiol-6–FA. Of the conjugates with fluorescein directly linked to the oestrogen molecule, oestrone and oestradiol-17–FA showed the highest affinity for the receptor protein, with RBA's of 2.8 and 1.4% respec-

Table 2. Relative binding affinities of various androgen and progesterone–fluorescein conjugates in the oestrogen cytosol receptor assay

Competitor	Relative binding affinity(%)
Oestradiol-17β	100
Diethylstilboestrol	94
Tamoxifen	5
Testosterone-11α–FA	0.01
Testosterone-17–FA	0.01
5α-Dihydrotestosterone-17–FA	0.01
5α-Dihydrotestosterone-1-CET–FA	0.01
Progesterone-11α–FA	0.001
Progesterone-11α–BSA–FITC	0.001

Table 3. The effect of dextran-coated charcoal treatment on the RBA of oestradiol and progesterone–BSA–FITC conjugates

Competitor	Relative binding affinity (%)	
	− DCC	+ DCC
Oestradiol-6–BSA–FITC	0.01	< 0.001
Oestradiol-17–BSA–FITC	0.01	< 0.001
Progesterone-11α–BSA–FITC	< 0.001	< 0.0001

tively (Table 1). The affinity of molecules in which fluorescein had been conjugated to testosterone, 5α-dihydrotestosterone or progesterone was low compared to oestradiol (Table 2), as might be expected from the known specificity of this receptor protein [18, 19]. Pretreatment of the BSA conjugates with dextran-coated charcoal immediately prior to their use in the competitive binding assays decreased the apparent affinity of the receptor for the conjugates (Table 3), suggesting the presence of noncovalently bound steroid. Re-purification of both the steroid–BSA–FITC and steroid–fluorescein amine conjugates by gel exclusion chromatography and thin-layer chromatography respectively had no appreciable effect on their affinities with respect to the receptor protein or upon the pattern of fluorescence observed in the histochemical procedure.

Cellular localisation of oestrogen receptors and steroid–fluorescein binding components

Characteristically, when assayed *in vitro*, preparations of uteri removed from non-treated intact rats contained high levels of cytoplasmic oestrogen receptor protein and low concentrations of nuclear oestrogen binding sites (Table 4). When thin sections of the same samples of tissue used in the biochemical assay were incubated with the oestrogen–fluorescein conjugates listed in Table 1, only a predominant cytoplasmic fluorescence was evident, primarily localised in epithelial elements (both glandular and lumenal) (Fig. 2a, b). The fluorescence was only partially displaceable by a 50-fold molar excess of DES. This particular fluorescent pattern was achieved with 0.5–2 μM concentrations of conjugates directly linked to oestradiol or oestrone and 10–25 μM for those linked via BSA or HRP. These patterns were also observed with tissue from rat mammary tumours, with fluorescence being mainly localised in the cytoplasm of epithelial cells. Of interest was that incubation of thin sections of either rat uteri or DMBA-induced mammary tumours with fluorescein-labelled androgens or progesterone also showed this pattern of cytoplasmic binding.

Treatment of experimental animals with tamoxifen 16 hr prior to removal of mammary tumours or uteri resulted in a shift in the cellular distribution of oestrogen binding components from the cytoplasm to the nucleus, a change that was monitored by the biochemical assay (Table 4). Incubation of these same tissues with the oestrogen-fluorescein conjugates showed a differential response. Only those conjugates linked through BSA produced nuclear fluorescence (Fig. 3a, b), the remainder binding preferentially to cytoplasmic components. Furthermore, increasing the concentration of the conjugates 4-fold failed to produce any appreciable nuclear binding, merely enhancing the background and cytoplasmic staining. Similar, although often less intense, patterns of fluorescein binding were observed in tissue from animals pre-treated with oestradiol. Interestingly, incubation of thin sections of rat uteri from animals treated with either tamoxifen or oestradiol with progesterone linked to fluorescein via BSA also showed nuclear fluorescence similar to that observed with the oestrogen–BSA–fluorescein conjugates. Inclusion of dithiothreitol in the incubation mixtures markedly decreased the fluorescein binding to all sections.

The results obtained using Earle's balanced salt medium were strictly comparable with those observed with the PBS, although with the

Table 4. Cellular distribution of oestrogen receptors

Treatment	Cytoplasm (fmol/uterus)	Nucleus (fmol/uterus)
Control	813 ± 133	203 ± 56
Oestradiol	233 ± 41	774 ± 73
Tamoxifen	329 ± 43	532 ± 49

latter, noticeably less fluorescence was present in the stromal elements and in necrotic tissue.

Incubation of thin sections of a series of human primary breast tumours with either oestradiol–BSA–FITC or oestradiol–fluorescein showed only cytoplasmic binding, and no nuclear fluorescence was evident in the tumours examined (Figs. 4–7). The distribution of binding components was also more variable than observed in either rat mammary tumour or rat uterus, and no real correlation was evident between oestrogen receptor values determined from the biochemical assay and the fluorescent patterns in thin sections. The data obtained using the BSA conjugate indicated a 38% correlation ($n = 26$), whereas with the oestrone-17–FITC and oestradiol-17–FA conjugates the correlations were 65 ($n = 20$) and 29% ($n = 14$) respectively with respect to the cytosol receptor assay. These data clearly indicate a lack of correlation between the cytoplasmic oestrogen receptor assay and this fluorescence localisation method. It was noted, however, that taking a separate piece of tissue for receptor analysis than that used for histochemical localisation occasionally gave anomalous results, the two pieces being assessed as one receptor-positive and the other receptor-negative. Processing the same piece of tissue through the receptor assay after cutting sections for the histochemical procedure eliminated these anomalies and improved the correlation by between 2 and 4%, a relatively small change, leaving the overall conclusion unaltered.

DISCUSSION

The potential value of fluorescein-labelled oestrogens as agents for assessing the oestrogen receptor status of human breast tumour tissue has received much attention in recent research literature [3, 7, 9]. Invariably it has been stated that such conjugates bind to oestrogen proteins [7, 8] and translocate these proteins from the cytoplasm to the nucleus of oestrogen target cells [4]. Significant correlations have been reported between receptor content and fluorescein-labelled oestrogen binding to thin sections of breast tumours [7, 8], the inference being that these derivatives localise oestrogen receptors. The present study, however, casts some doubt on the generality of these conclusions. Whilst all of these fluorescein-linked compounds apparently bind to the cytoplasmic oestrogen receptor protein in competitive binding studies *in vitro*, only the BSA conjugates bind to the nuclei of tissue sections associated with receptor translocation by tamoxifen and oestradiol, as demonstrated biochemically. Similarly, this nuclear binding was also localised by progesterone-11α–BSA–FITC, but was inhibited by the reducing agent dithiothreitol, a compound normally used to 'activate' oestrogen receptor proteins in standard biochemical assays [20].

These data, together with the ability of oestradiol-17–FA and oestrone-17–FITC to bind within the cytoplasm, under conditions whereby oestrogen receptors have been largely translocated to the nuclei of both rat mammary tumour and rat uterine tissues, implies that fluorescein-labelled oestrogens do not specifically localise oestrogen receptors. It now seems likely that these ligands bind differentially to other cellular proteins similar to the type II oestrogen binding proteins reported by Eriksson *et al.* [21]. Type II binding sites have been reported to be present in cytoplasmic and nuclear fractions, with the nuclear form being stimulated by oestradiol in rat uterine lumenal epithelial cells [22]. Moreover, these proteins, unlike oestrogen receptors, are inhibited by thiol reagents such as dithiothreitol and have a high capacity for oestrogens.

Treatment of the steroid–BSA–FITC conjugates with a suspension of charcoal, whilst markedly reducing the relative binding affinities and suggesting the presence of non-covalently bound steroid, did not affect either the intensity or pattern of fluorescence produced in the tissues from rats treated with tamoxifen or in sections of human breast tumours.

The relatively poor correlation observed between oestrogen receptor status and the histochemical localisation of fluorescein-labelled oestrogen binding components in sections of both rat and human tissue samples, coupled with the very low relative binding affinities observed with these conjugates, emphasises the need to exercise extreme caution when trying to assess 'receptor status' with this technique. A real evaluation of the clinical usefulness of such assays may well have to await the preparation of a conjugate with a reasonably high RBA. At present, any critical assessment of the value of such available fluorescent conjugates should be made in relation to not only the biochemical receptor analysis, but also to the clinical status of patients with breast cancer and the relationship between the histochemical pattern and the subsequent course of the disease.

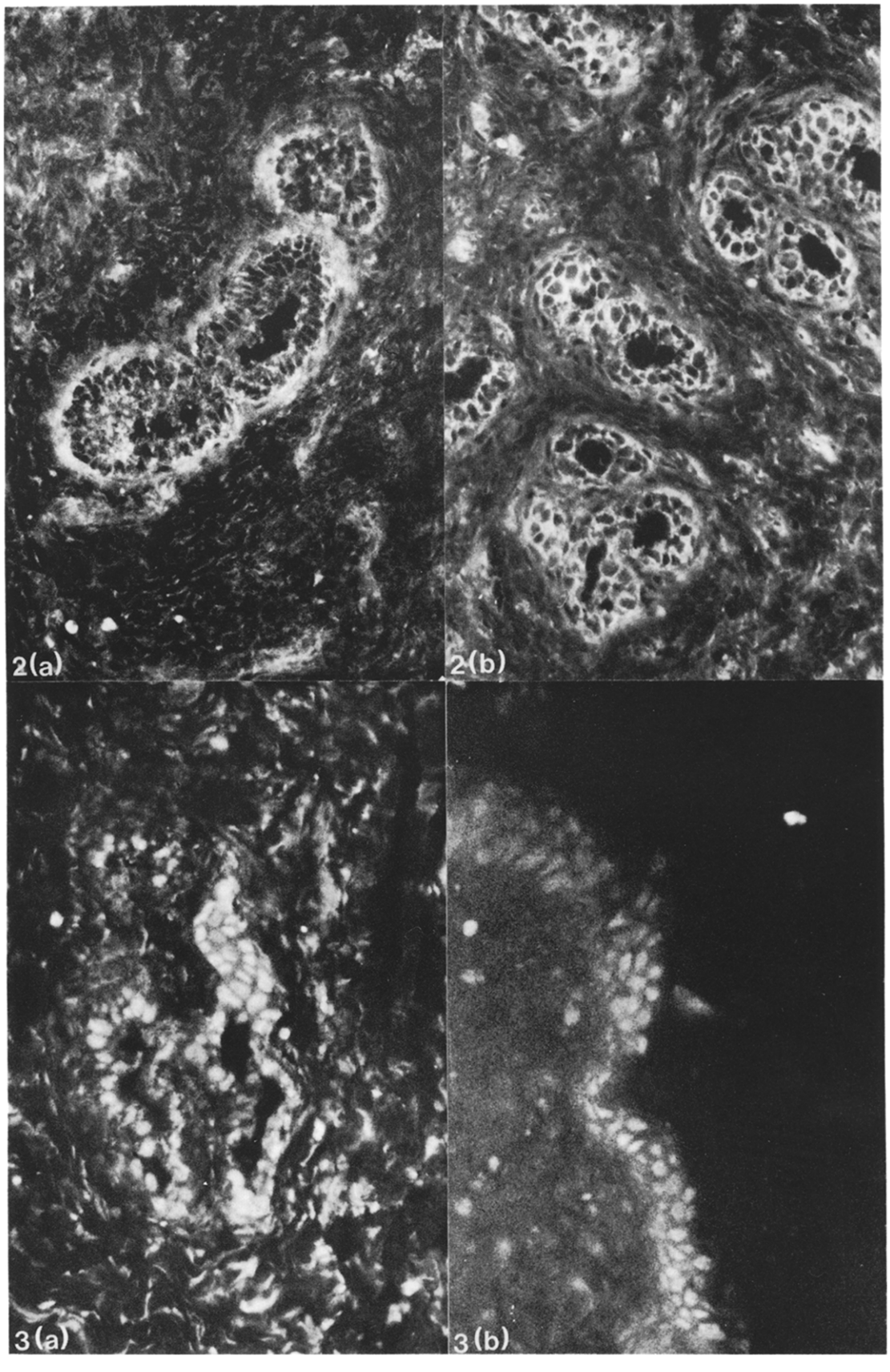
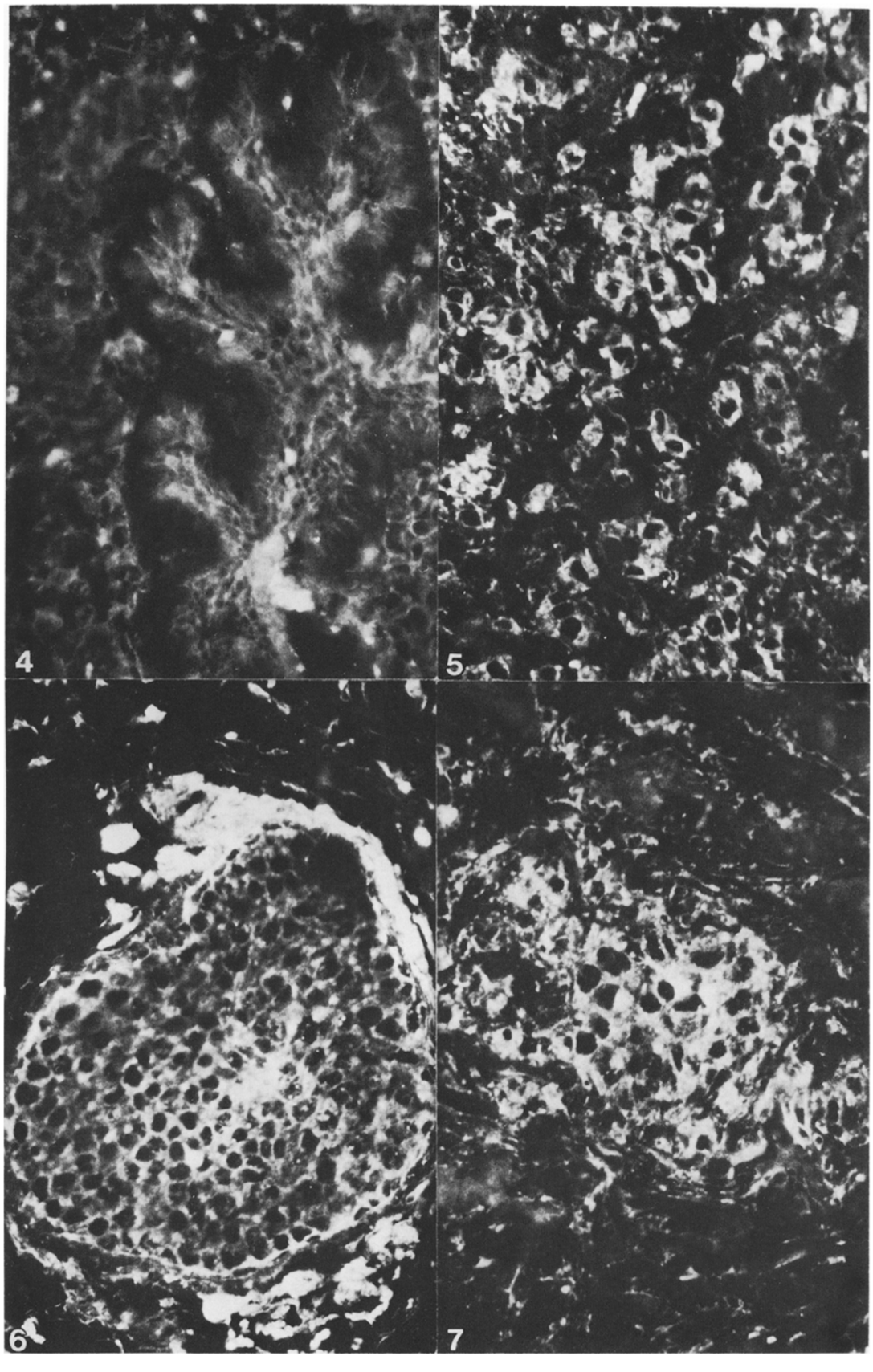


Fig. 2a, b. Cytoplasmic localisation of oestrogen binding components in sections of rat uteri from control animals using the oestradiol-17–FA conjugate. (*magnification* ×440).

Fig. 3a, b. Nuclear localisation of oestrogen binding components in sections of rat uteri from animals treated with tamoxifen using the oestradiol-17–BSA–FITC conjugate (*magnification* ×440).



Figs. 4–7. Cytoplasmic localisation of oestrogen binding components in sections taken from human breast tumours (magnification ×440) using the oestradiol-17–FA (Figs. 4 and 5) conjugate and oestradiol-17–BSA–FITC conjugate (Figs. 6 and 7).

REFERENCES

1. PERTSCHUK LP, GAETJENS E, CARTER AC, BRIGATI DJ, KIM DS, FEALEY TE. An improved histochemical method of detection of oestrogen receptors in mammary carcinoma. *Am J Clin Pathol* 1979, **71**, 504–508.
2. LEE SH. Cytochemical study of oestrogen receptor in human mammary cancer. *Am J Clin Pathol* 1978, **70**, 197–203.
3. BARROWS GH, STROUPE SB, RIEHM JD. Nuclear uptake of a 17β-oestradiol–fluorescein derivative as a marker of oestrogen dependence. *Am J Clin Pathol* 1980, **73**, 330–339.
4. NENCI I, DANDLIKER WB, MEYERS CY, MARCHETTI E, MARZOLA A, FABRIS G. Oestrogen receptor cytochemistry by fluorescent oestrogen. *J Histochem Cytochem* 1980, **28**, 1081–1088.
5. RAO BR, FRY CG, HUNT S, KUHNEL R, DANDLIKER WB. A fluorescent probe for rapid detection of oestrogen receptors. *Cancer* 1980, **46**, 2902–2912.
6. DANDLIKER WB, BRAWN RJ, HSU M-L *et al.* Investigation of hormone–receptor interactions by means of fluorescence labelling. *Cancer Res* 1978, **38**, 4212–4223.
7. PERTSCHUK LP, TOBIN EH, TANAPAT P *et al.* Histochemical analyses of steroid hormone receptors in breast and prostatic carcinoma. *J Histochem Cytochem* 1980, **28**: 799–810.
8. LEE SH. Cellular oestrogen and progesterone receptors in mammary carcinoma. *Am J Clin Path* 1980, **73**, 323–329.
9. CHAMNESS GC, MERCER WD, MCGUIRE WL. Are histochemical methods for oestrogen receptor valid? *J Histochem Cytochem* 1980, **28**, 792–798.
10. NICHOLSON RI, DAVIES P, GRIFFITHS K. Effect of oestradiol-17β and tamoxifen on nuclear oestradiol-17β receptors in DMBA-induced rat mammary tumours. *Eur J Cancer* 1977, **13**, 201–218.
11. NICHOLSON RI, SYNE JS, DANIEL CP, GRIFFITHS K. The binding of tamoxifen to receptor proteins under equilibrium and non-equilibrium conditions. *Eur J Cancer* 1979, **15**, 317–329.
12. ERLANGER BF, BOREK F, BEISER SM, LIEBERMANN S. Steroid–protein conjugates. I. Preparation and characterisation of conjugates of bovine serum albumin with testosterone and cortisone. *J Biol Chem* 1957, **228**, 713–727.
13. GAETJENS E, PERTSCHUK LP. Synthesis of fluorescein labelled steroid hormone-albumin conjugates for the fluorescent histochemical detection of hormone receptors. *J Steroid Biochem* 1980, **13**, 1001–1003.
14. DANDLIKER WB, HICKS AN, LEVISON SA, BRAWN IJ. A fluorescein-labelled derivative of oestradiol with binding affinity towards cellular receptors. *Biochem Biophys Res Commun* 1977, **74**, 538–545.
15. DAXENBICHLER G, GRILL HJ, MOSER E, DAPUNT O. Receptor bind of fluorescein labelled steroids. *J Steroid Biochem* 1980, **13**, 489–494.
16. PAPPAS PW. The use of a chromealum–gelatin (subbing) solution as a general adhesive for paraffin sections. *Stain Technol* 1971, **46**, 121–124.
17. EARLE WR. Methods in tissue culture. *J Natl Cancer Inst* 1943, **4**, 165–170.
18. DAVIES P, POWELL-JONES W, NICHOLSON RI, GRIFFITHS K. The specificity of the androgen receptor of DMBA-induced mammary tumours of the rat. *Eur J Cancer* 1977, **13**, 1421–1427.
19. NICHOLSON RI, DAVIES P, GRIFFITHS K. Interaction of androgen with oestradiol-17β receptor proteins in DMBA-induced mamary tumours—a possible oncolytic mechanism. *Eur J Cancer* 1978, **14**, 439–445.
20. MARKAVERICH BM, WILLIAMS M, UPCHURCH S, CLARK JM. *Endocrinology* 1981, **109**, 62–68.
21. ERIKSSON H, UPCHURCH S, HARDIN JW, PECK EJ, CLARK JH. *Biochem Biophys Res Commun* 1978, **81**, 1–7.
22. Differential cell stimulation by estrogen agonists and antagonists. In : SUTHERLAND RL, JORDAN VC, eds. *Non-steroidal Antiestrogens.* New York, Academic Press, 1982, 113–141.