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DETECTION AND DYNAMIC LOCALISATION OF ESTRADIOL–RECEPTOR COMPLEXES IN INTACT TARGET CELLS BY IMMUNOFLUORESCENCE TECHNIQUE

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SUMMARY

A systematic study has been made of an immunofluorescence technique for the kinetic demonstration of estradiol-17β in target cells. An experimental system suited for monitoring the steroid dynamics in cell suspensions has been developed. Pertinent control tests of the receptor binding and of the immune specificity have been carried out.

Fluorescent antibody was able to specifically detect estradiol bound to receptors in various cell compartments (cytoplasm, nuclear chromatin and nucleolus) at different times and temperatures of incubation. Lack of cytoplasmic receptors and some defects of the two-step mechanism could also be demonstrated in mixed cell subpopulations in human breast cancer.

INTRODUCTION

Extensive studies on biodynamic aspects of steroid–cell interactions have brought about an increased understanding of the biochemical effects of steroid hormones. Several relationships between the mechanism of steroid–cell interaction and both the physiological response [1–4] and some pathological situations [3, 5, 6] of target cells, have recently been identified.

The intracellular kinetics of steroids in target tissues have been studied mainly in whole animals, tissue slices and tissue homogenates. As an ultimate approach, steroid–cell interactions should be analyzed at the cell level; in this way some problems of steroid physiopathology could be resolved. Compartmental analysis of steroid distribution in individual cells has been carried out by autoradiographic [7] and fractionation techniques [8]. It has been recently suggested [9, 10] that the fluorescent antibody technique could be useful in order to trace the intracellular steroid kinetics. Specific steroid antisera resulted in significant progress in the understanding of steroid physiology [9, 11]. Steroid antibodies labelled with a fluorescent tracer might provide information, unobtainable with other methods and moreover extend the biochemical observations.

The present work, carried out on a dispersed cell system, is mainly concerned with immunofluorescence detection and dynamic localisation of estradiol bound to specific cell receptors. An experimental design involving live cell suspensions, short term incubation with temperature changes and time course studies, has been devised.

MATERIALS AND METHODS

Tissue samples. Tumour tissue specimens were obtained from 40 human primary breast cancers; 2 endometrial carcinomas; 1 lung and 1 gastric cancer. Two normal human spleens, two mouse livers and one breast from a 5-month pregnant woman, were also processed.

Preparation of cell suspensions. Tissue samples were transferred to a cold flask containing phosphate-buffered balanced salt solution (BBSS, Dulbecco or Hanks) and immediately processed. The tissue was minced and a cell suspension was made with a loose-fitting glass homogenizer in cold BBSS containing 2% (w/v) human serum albumin (HSA); the suspension was then filtered through a gauze filter to remove large clumps and fibrous strands. Dispersed cells were harvested by drawing off the fluid with a fire-polished Pasteur pipette, then were centrifuged and washed three times with cold BBSS-HSA.

In some experiments, cell suspensions were obtained using trypsin or collagenase, but the enzymatic treatment was not usually required since tumour cell dissociation was directly attainable.

Viability of dispersed cells was determined by the dye-exclusion test: 90 $\pm$ 3% of the cells excluded Trypan Blue.

Incubation procedure. For incubation, aliquots of dispersed cells were resuspended (3 to 5×10^6 cells/ml) in BBSS containing 2×10^{-7} M Estradiol-17β, 1% (w/v) HSA, 0.2% (v/v) ethanol, final concentrations.

Incubation was usually performed with occasional

gentle shaking at 4°C for 1 h; in some other experiments, incubation was carried out at room temperature (20–22°C). After incubation, cells were thoroughly washed in frequently-changed cold BBSS-HSA (total washing time about 1 h).

In order to study the temperature-dependent estradiol redistribution and to monitor its retention and release, after incubation and washing in the cold media, the cells were resuspended in minimal essential medium TC 199 fortified with 10% calf serum, and post-incubated in a 37°C water bath. The monitoring was achieved by sampling cells at different times of incubation. After the warm incubation, the cells were cooled in ice water and washed in cold BBSS-HSA. In every sample there was a negligible loss in viability, controlled by dye-exclusion and cytological examination.

Drops of washed cells were then either smeared or flattened with a cytocentrifuge on slides; lastly, the cells were allowed to dry in the cold.

Immunofluorescence staining. An indirect (sandwich) immunofluorescence technique has been used. All procedures were carried out on unfixed dried cells in the cold (4°C). Cells on a slide were first treated for 30 min in a moist chamber with diluted rabbit anti-estradiol antiserum; a dilution of 1/200 with phosphate buffered saline (PBS) was chosen, after several dilutions had been tried. Cells were then washed three times in PBS and allowed to react for 30 min with Fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin antiserum. A polyvalent antiserum against IgG, IgA and IgM was tested and used as previously stated [12]. After three final washes with PBS, preparations were mounted in buffered glycerol, 50% in PBS. Some preparations were counterstained with Evans blue (0.06%) or Methyl green–Eriochrome black to eliminate nonspecific background and to enhance cellular details.

Blockage and control experiments. "Estradiol binding blockage". Duplicate samples from cell suspensions were pre-incubated in a cold medium lacking in estradiol and supplied with 10^{-4} M Nafoxidine hydrochloride (U-11,100A), or 10^{-3} M N-ethylmaleimide. Moreover, 10^{-4} M Nafoxidine or 10^{-3} M N-ethylmaleimide were added to some incubations (with estradiol) and washing media.

Immune specificity control. To assure the specificity of the two immune reactions involved in the sandwich immunofluorescence assay, various control experiments were carried out.

(A) Incubation of the dispersed cells in a medium lacking in estradiol, at 4° or 37°C.

(B) Cells incubated in the estradiol-containing medium were treated as follows:

(1) before the fluorescein-conjugated antirabbit antiserum was applied, incubation with: (a) normal rabbit serum; (b) estradiol-unrelated rabbit antiserum; (c) anti-estradiol antiserum previously twice absorbed with estradiol in excess (3 mg/ml); (d) unlabelled antirabbit antiserum after cells had reacted with rabbit estradiol antibodies.

(2) Incubation with fluoresceinated antiserum alone without any pretreatment.

(3) Incubation with fluorescein-conjugated antiserum against antibody-unrelated rabbit protein, after the cells had reacted with anti-estradiol antibodies.

Microscopy. Preparations were examined with a Leitz Ortholux II microscope equipped with an OSRAM high pressure Xenon lamp XBO 75 W and an OPAK–FLUOR vertical illuminator. This illuminator and appropriate combination of exciting filters (1.5 mm BG12 plus S470 AL), dichroic mirror (TK510) and barrier filters (K515 plus S525 AL) allow the selective visualisation of fluorescein.

Photographs were recorded on Kodak Ektachrome high speed daylight color film and on Kodak Rayoscope black and white film.

Reagents. High specificity anti-estradiol-17β rabbit antiserum, raised to estradiol-6-carboxymethyloxime-BSA (lot 507–4°), was obtained from Serono Biodata

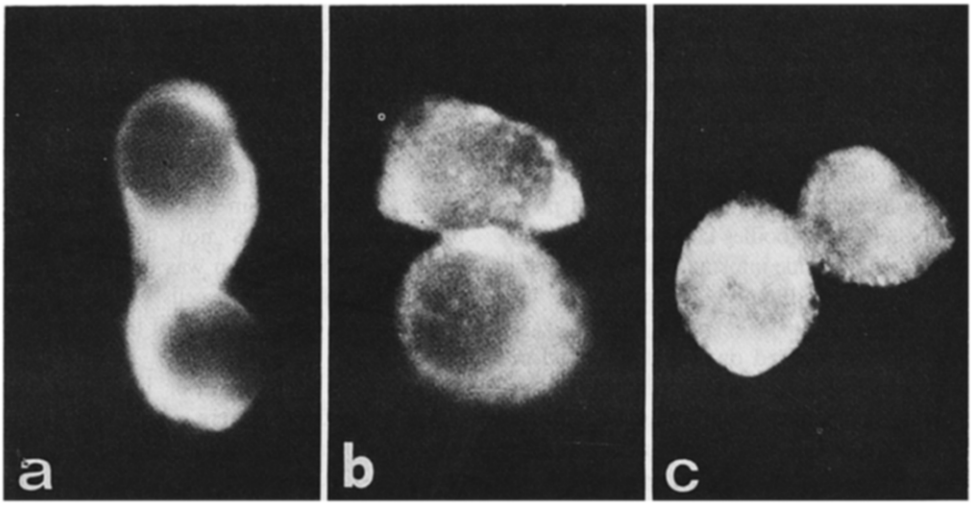

Fig. 1. Temperature-dependent fluorescent patterns of the estradiol distribution in cells from human breast cancer at 4°C (a), 22°C (b), 37°C (c).

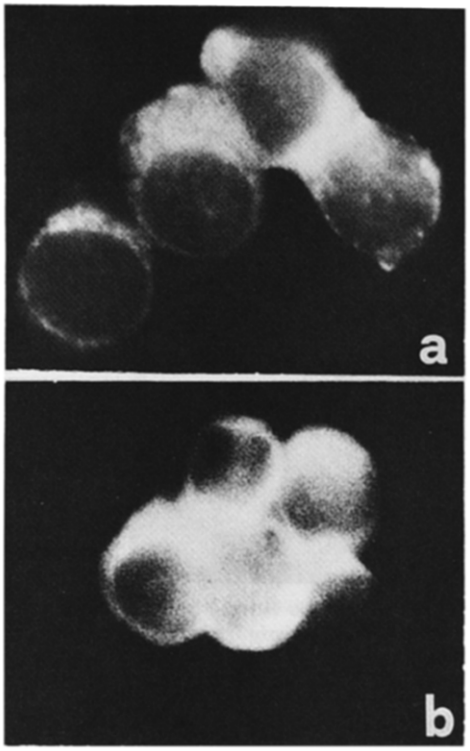

Fig. 2. Breast tissue cells from a pregnant woman display detectable (endogenous) estradiol before incubation (a). Fluorescence appears to be enhanced after incubation in estradiol-containing medium (b).

Division, Rome, Italy. Estradiol-17β (from Ikapharm, Ramat-Gan, Israel), Nafoxidine hydrochloride (U-11,100A, gift from The Upjohn Co., Kalamazoo, Mich., U.S.A.), N-ethylmaleimide (from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A.), TC Medium 199 (from Difco Lab., Detroit, Mich., U.S.A.), Fluorescein labelled antirabbit immunoglobulin antisera (from Behringwerke, Marburg–Lahn, Germany and from Biochemical Check Up, Milan, Italy) have been used.

RESULTS

The fluorescence staining showed three general patterns depending on the different experimental conditions (Figs. 1, 3b, 3c, 3d).

The cells incubated in the cold mostly displayed a bright, homogeneously diffuse fluorescence of the cytoplasm; the nuclear area was usually unstained. In many breast tumour samples, the fluorescence intensity was not uniformly bright and there was also a variable percentage of negative cells (Fig. 4). The possibility that negative staining was due to cell damage was ruled out by dye-exclusion test. Moreover, the negative cells often displayed more atypical cytological features than the positive ones.

The second pattern of fluorescence was exhibited by the cells incubated at room temperature: together with a more marked cytoplasmic staining, we could observe a bright-fluorescent, light stippling of the nuclear area.

The third staining pattern was seen after the slow post-incubation warming up to 37°C: in addition to the cytoplasmic fluorescence, increasing nuclear staining occurred which, lastly, filled the whole nuclear area. In some breast tumours, a double cell population was recognized; one did not show any nuclear labelling after warming, despite the clear cytoplasmic fluorescence (Fig. 5). These subpopulations did not display any manifest cytological differences.

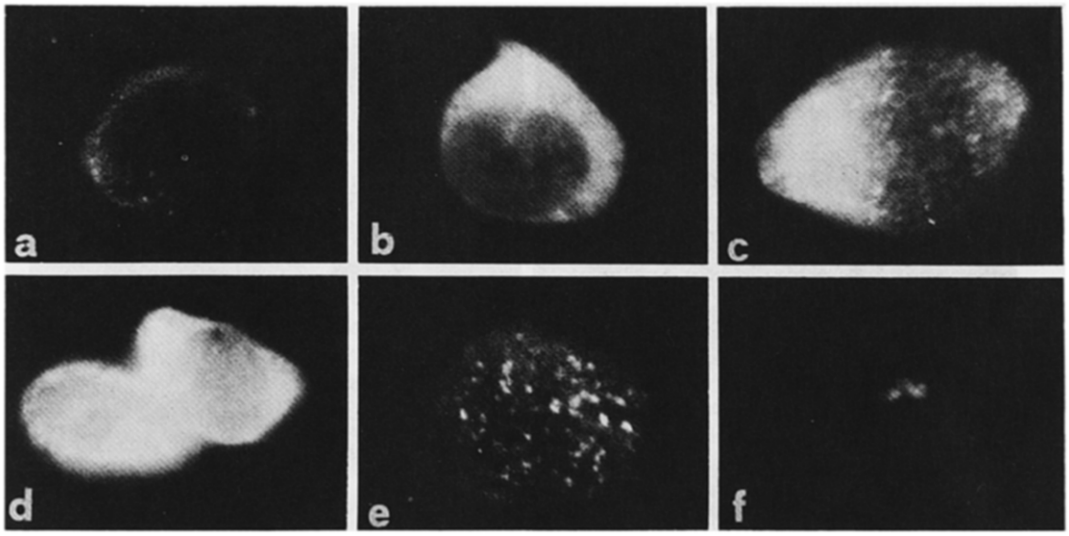

Fig. 3. Dynamic time-course of estradiol binding, redistribution and retention in cells from a pre-menopausal breast cancer. (a) Endogenously bound estradiol. (b) Total specific binding capacity at 4°C. (c) Translocation to the nucleus of the bound estradiol at 22°C. (d) Nuclear estradiol loading at 37°C. (e) Estradiol bound to nuclear chromatin at the 5th h of post-incubation. (f) Final retention of estradiol by nucleoli at the 15th h of post-incubation.

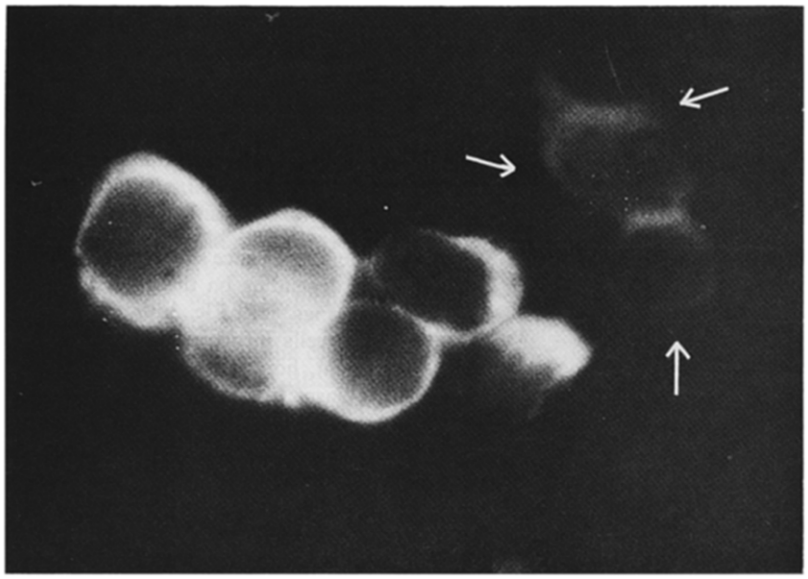

Fig. 4. In some breast cancers, besides cells displaying specific cytoplasmic estradiol-binding at 4°C, cell subpopulations exhibited a minimal binding capacity (*arrows*).

Sampling the cells at different times of warm post-incubation showed the virtual disappearance of cytoplasmic fluorescence. After 5–6 h the nuclear staining was concentrated on nuclear chromatin in small fluorescent dots (Fig. 3e), which disappeared after 12–15 h. At that time, one or rarely two roughly spherical, bright nuclear bodies were still stained. Nucleoli were identified as the site of this late fluorescence by phase contrast and cytological stainings.

Cells from endometrial carcinomas displayed similar cytoplasmic diffuse fluorescence in the cold. Studies on redistribution and retention in the warm have not yet been carried out.

No fluorescence positive cells were found in preparations from human spleen and non-target tumours and from mouse liver.

All the fluorescent stainings were always prevented by pre-incubation and washing in media containing Nafoxidine and N-ethylmaleimide. Every control experiment confirmed the immune specificity of the detection of estradiol in our experimental conditions.

Staining generally occurred only in the preparations exposed to estradiol. The cell suspension from breast tissue of a pregnant woman displayed a moderate cytoplasmic and faint nuclear fluorescence even before the incubation with estradiol, after which

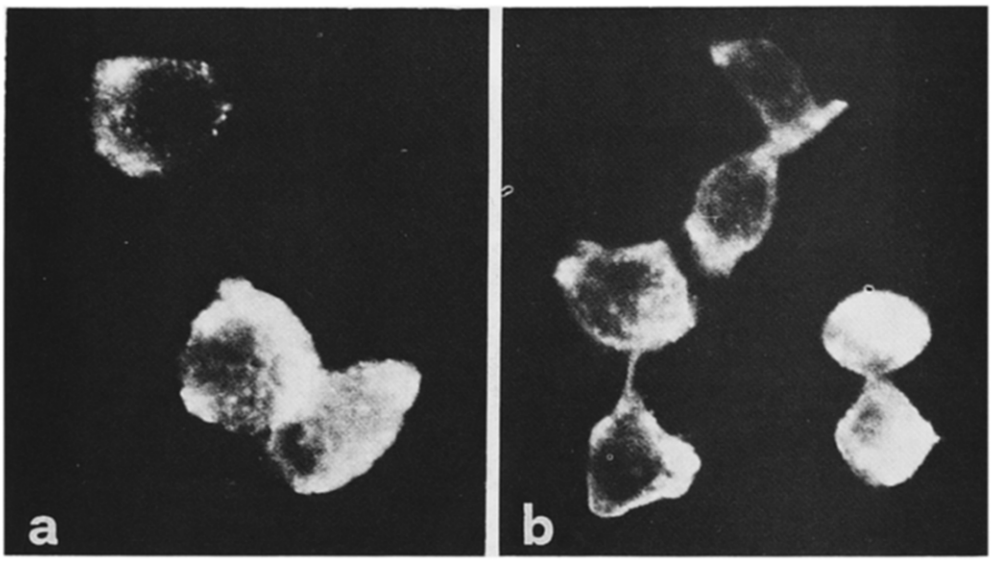

Fig. 5. In some tumours, mixed cell populations were identified, one of which displays an impaired nuclear translocation of the cytoplasmic bound estradiol at 22°C (a) and at 37°C (b).

the cytoplasmic staining was reinforced (Fig. 2a, b). Also cells from some pre-menopausal breast cancers exhibited an occasional fluorescence without any exposure to estradiol (Fig. 3a).

DISCUSSION

To gain useful information on kinetic aspects of estradiol–cell interactions at the cell level, an experimental design suited for studying steroid dynamics in intact target cells, has been devised. Isolated cell preparations from breast tumours have been used, since it has been shown that cells from many breast tumours behave as typical estradiol-target cells and contain variable amounts of specific estradiol–receptors [6, 16–20]. An indirect (sandwich) immunofluorescence technique has been employed for the *in situ* estradiol localisation. Similar to the double antibody radioimmunoassay technique [21], it involves the reaction of the first specifically bound anti-estradiol antibody with a second anti-immunoglobulin antibody, labelled with a fluorescent tracer.

It should be pointed out that our experimental conditions involved a hyperphysiological estradiol concentration. The purpose of this study was to detect the total binding capacity of the cell receptors, which are present in reserve amounts [3, 14, 22], and not only the fraction required to elicit physiological responses. A reasonably good distinction between high and low affinity binding is automatically obtained in intact cell systems like our own; contribution of non-specific binders to steroid uptake and retention may be disregarded both *in vivo* and related *in vitro* conditions, provided that an adequate washing is carried out [1, 8]. The receptor specificity of the estradiol binding in our experimental system has been established chiefly by blocking experiments. Comparison of hormone uptake in the presence and absence of blocking compounds (Nafoxidine, N-ethylmaleimide) is indeed considered to allow a good distinction between specific interaction and non-specific binding [14, 16, 23, 24]. The specificity of immune reactions at the different steps of sandwich immunofluorescence has been deeply examined. Various kinds of control tests have provided compelling evidence of the reliable specificity of the immunohistochemical reaction. The blocking and control experiments together indicate that the estradiol traced by fluorescent antibody is bound to its specific cell receptors.

In this regard, we must mention that this conclusion apparently disagrees with some results recently obtained [25–28] by an immunochemical reaction. By this method, steroid bound to its own receptor does not appear to be recognized by specific antibody which can, instead, remove the free and the aspecifically bound steroid. At present we cannot give a satisfactory explanation of this discrepancy. However, one should bear in mind that the reaction kinetics of an immunochemical system does not necessarily occur in the same way in an immunohistochemical one, since the experimental conditions are very different.

This study also showed that specific receptors filled by endogenous estradiol were detected simultaneously with the unfilled sites; this seems a noteworthy advantage of the immunohistochemical approach.

An expected finding from this study was that the intracellular distribution of bound estradiol, seen by its reaction with specific fluorescent antibody, was not fixed but drastically altered by changing the temperature of the incubation media. A redistribution of the traceable material by warming was readily obtained. Cells incubated in the cold displayed a diffuse cytoplasmic pattern of fluorescent staining; when post-warmed up to 37°C, a nuclear fluorescence gradually appeared. These findings are in close agreement with the two-step mechanism of the steroid–cell interaction [1, 3, 4, 14, 15], of which our pictures give a direct visual representation.

Time-course experiments designed for monitoring the estradiol nuclear incorporation, retention and release indicate that nuclear fluorescence progressively decreased during the first few hours. After the bulk of the label had been lost, some fluorescent spots persisted on the nuclear chromatin. The re-uptake of estradiol, following its release into the washing medium, appeared to be negligible. These preliminary results (to be published in detail) agree with similar observations from *in vivo* and *in vitro* studies [3, 14, 22, 29].

A striking unexpected phenomenon detected by time-course monitoring was the late nucleolar positivity. Nucleoli are the last structures in the nuclear area to retain the estradiol, after its complete release from nuclear chromatin. Autoradiographic pictures have shown that nucleoli bind very little or none of the radioactive estrogen [30], even if it has also been claimed that a part of the nuclear radioactivity can be recovered in the nucleolar fraction by centrifugal fractionation techniques [31–33]. Nucleolus or nucleolar-associated chromatin [3] have been sometimes suggested to be a major site of estradiol retention in target cells. Whether the ultimate retention of estradiol by the nucleolus is a key event or has some other meaning is still open to question.

CONCLUSION

The possibility of analyzing the intracellular kinetics of estradiol in intact cells is of great interest and instrumental not only in the understanding of some steroid–cell interactions, but also in the evaluation of the estrogenodependence of human breast cancer. The experimental system developed here appears indeed to be able to characterize different cell subpopulations in a given tumour which differ with regard to the preservation of the receptor mechanism (Figs. 4, 5). Lack of cytoplasmic receptors, impaired translocation to the nucleus of estradiol–receptor complexes, failure in their chromatin binding and kinetic differences in their nuclear retention and release, have all been demonstrated by the immuno-

fluorescence technique in cells from human breast cancer (extensive manuscript in preparation). The role of these alterations in the progression of the tumour from a responsive to an unresponsive state and in predicting the patient's response to hormonal therapies must still be ascertained.

REFERENCES

1. Baulieu E. E., Alberga A., Jung I., Lebeau M. C., Mercier-Bodard C., Milgrom E., Raynaud J. P., Raynaud-Jammet C., Rochefort H., Truong H. and Robel P.: *Recent Prog. Horm. Res.* **27** (1971) 351–419.
2. Jensen E. V. and DeSombre E. R.: In *Biochemical Actions of Hormones* (Edited by G. Litwack). Academic Press, New York, Vol. 2 (1972) pp. 214–253.
3. King R. J. B. and Mainwaring W. I. P.: *Steroid–Cell Interactions.* Butterworths, London (1974).
4. Liao S.: *Int. Rev. Cytol.* **41** (1975) 87–172.
5. Dao T. L. (Editor): *Estrogen Target Tissues and Neoplasia.* The University of Chicago Press, Chicago (1972).
6. McGuire W. L., Carbone P. P. and Vollmer E. P. (Editors): *Estrogen Receptors in Human Breast Cancer.* Raven Press, New York (1975).
7. Stumpf W. E. and Sar M.: *Meth. Enzymol.* **36** (1975) 135–156.
8. Munck A. and Wira C.: *Meth. Enzymol.* **36** (1975) 255–264.
9. Midgley A. R., Niswender G. D., Gay U. L. and Reichert L. E.: *Recent Prog. Horm. Res.* **27** (1971) 235–286.
10. Jensen E. V., Liao S., Mohla S., Gorell T. A. and DeSombre E. R.: *J. steroid Biochem.* **5** (1974) 987–988.
11. Abraham G. E.: *Acta endocr., Copenh.* suppl. **183** (1974).
12. Nenci I.: *Lab Invest.* **32** (1975) 257–260.
13. Jensen E. V. and DeSombre E. R.: *Ann. Rev. Biochem.* **41** (1972) 203–230.
14. Jensen E. V. and DeSombre E. R.: *Science* **182** (1973) 126–134.
15. Jensen E. V., Brecher P. I., Mohla S. and DeSombre E. R.: *Acta endocr., Copenh.* suppl. **191** (1974) 159–172.
16. Jensen E. V., Block G. E., Smith S., Kyser K. and DeSombre E. R.: In *Estrogen Target Tissues and Neoplasia* (Edited by T. L. Dao). The University of Chicago Press, Chicago (1972).
17. Jensen E. V., Block G. E., Smith S. and DeSombre E. R.: *Recent Results Cancer Res.* **42** (1973) 55–62.
18. McGuire W. L., Chamness G. C., Costlow M. E. and Shepherd R. E.: *Metabolism* **23** (1974) 75–100.
19. Leclercq G., Heuson J. C., Schoenfeld R., Mattheiem W. H. and Tagnon H. J.: *Europ J. Cancer* **9** (1973) 665–673.
20. Leclercq G., Heuson J. C., Deboel M. C. and Mattheiem W. H.: *Brit. Med. J.* **1** (1975) 187–189.
21. Midgley A. R., Rebar R. W. and Niswender G. D.: *Acta endocr., Copenh.* suppl. **142** (1969) 247–254.
22. Anderson J. N., Peck E. J. and Clark J. H.: *Endocrinology* **92** (1973) 1488–1495.
23. Jensen E. V., Hurst D. J., DeSombre E. R. and Jungblut P. W.: *Science* **158** (1967) 385–388.
24. Terenius L.: *Acta endocr., Copenh.* **66** (1971) 431–447.
25. Fishman J. and Fishman J. H.: *J. clin. Endocr. Metab.* **39** (1974) 603–606.
26. Fishman J., Fishman J. H., Nisselbaum J. S., Menendez-Botet C., Schwartz M. K., Martucci C. and Hellman L.: *J. clin. Endocr. Metab.* **40** (1975) 724–727.
27. Castaneda E. and Liao S.: *J. biol. Chem.* **250** (1975) 883–888.
28. Castaneda E. and Liao S.: *Meth. Enzymol.* **36** (1975) 52–58.
29. Giannopoulos G. and Gorski J.: *J. biol. Chem.* **246** (1971) 2524–2530.
30. Stumpf W. E.: *Endocrinology* **85** (1969) 31–37.
31. Arnaud M., Beziat Y., Guilleux J. C., Hough A., Hough D. and Mousseron-Canet M.: *Biochim. biophys. Acta* **232** (1971) 117–131.
32. Arnaud M., Beziat Y., Borgna J. L., Guilleux J. C. and Mousseron-Canet M.: *Biochim. biophys. Acta* **254** (1971) 241–254.
33. Andress D., Borgna J. L., Cazaubon C. and Mousseron-Canet M.: *J. steroid Biochem.* **5** (1974) 895–903.