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CORRESPONDENCE

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Localizing estradiol and other diffusible hormones and drugs by autoradiography and immunocytochemistry

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In a recent article, Jungblut and Sierralta (1998) presented a technique for the identification of target cells by "immunohistochemical detection of covalently rearranged estradiol" in rehydrated paraffin sections. Localizing diffusible compounds at cellular–subcellular levels is notoriously difficult and fraught with pitfalls. Various techniques have been recommended during the past four decades. There are claims galore. Most had to be abandoned. As we have pointed out before, artifacts were sometimes misinterpreted as data and published as results (e.g., Pfaff 1968; Brökelmann 1969; Wezeman 1976). Now what appears to be the successful use of an acetic acid–formaldehyde mixture for the fixation and immobilization of estradiol should be applauded.

If estradiol, or any other diffusible steroid or drug, can be immobilized by liquid fixation and localized at its original in situ cellular and subcellular locus of deposition and binding without loss and translocation – and then withstand subsequent decalcification, graded alcohol dehydration, immersion in xylene, embedding in paraffin, and, after the tissue block is sectioned, renewed immersion in xylene for deparaffinization, graded alcohol rehydration, repeated incubation with antibodies for several hours, several washings, counterstaining, washing, and again dehydration – it would indeed be an extraordinary achievement and promises wide application. One wonders how a substance attached to protein by ionic and van der Waal forces can be visualized at its original in situ site without translocation when covalent linkage – though carefully expressed by the authors as "covalently rearranged" – is achieved during or after diffusion of an acid–formaldehyde solution. After covalent linkage has been completed, the subsequent use of several solvents should not matter, although it might.

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In the past, several investigators have applied different fixatives, alone or combined, that would covalently link the steroid molecule and have also tried photoaffinity labeling. All proved impractical. By contrast, the dry- and thaw-mount autoradiography techniques were developed to avoid the many steps and dangers associated with fixation, embedding, and repeated washings. Thaw-mount autoradiography is based on the premise of studying the unmolested tissue with minimal treatment, while achieving maximal structural integrity and high resolution. Freeze-mounting of unfixed and unembedded tissue, thin frozen sectioning, and thaw-mounting (controlled by dry-mounting) on dry photographic emulsion are crucial steps toward achieving authentic cellular–subcellular localization of diffusible compounds. By comparison, this procedure is neither "laborious" nor "labor-intensive", as sometimes misjudged, but simple, sensitive, and expedient as documented by the many successful applications and discoveries made with this technique (Stumpf 1998).

The authors state that their method is "less laborious" and has "the same functional relevance as autoradiography". While the latter may theoretically be the case, this remains to be further established. There appears to be some agreement with our published autoradiographic data, but there are also differences. In our autoradiograms with ^{3}H-estradiol, there is evidence for localization of radioactivity not only in granulosa cells of ovarian follicles, but also in theca and interstitial gland cells as well as in antral steroid-binding protein, all of which appear negative in the authors' immunohistochemical studies with antibodies to estradiol. Arterial walls in uterine tissue are stated by the authors to be unstained with estradiol antibodies, but we have shown them to be labeled with ^{3}H-estradiol in autoradiograms.

In the present studies with antibodies to estradiol, nuclei of the vaginal epithelium are stained much more strongly in diestrus (Fig. 3a) as compared to the periovulatory condition (Fig. 3b; Jungblut and Sierralta (1998). This is inconsistent with the current views that the amount of estrogen receptors and ligand occupation is expected to be much higher in the periovulatory state with plasma lev-

els of estradiol about five times higher than those in diestrus. Accordingly, one would expect reversed staining intensities for those shown in Fig. 3a and b. Of course, as a general matter, it needs to be considered that variations may be attributable in part to different endocrine status and species of the experimental animals and that cellular concentration of the receptor is not a constant parameter.

With autoradiography, quantification is easily possible, and even the number of molecules can be calculated (Stumpf et al. 1981). This is not so with immunohistochemistry. Through quantitative autoradiography, a hierarchy of ligand binding to receptor in different target tissues can be demonstrated. This appears to be of functional, therapeutic, and toxicological significance (Stumpf 1995).

In order to validate the technique of Jungblut and Sierralta, additional experiments with radiolabeled estradiol would be desirable. Possible loss of compound could be measured at each step, including the untreated tissue prior to fixation, the treated tissue after fixation, after alcohol dehydration, and after the treatment with antibodies. For instance, using ^{14}C-estradiol as an indicator, after fixation with cold picric acid–paraformaldehyde and a similar embedding–deembedding procedure followed by immunohistochemistry with antibodies to estradiol, the tissue sections contained between 7 and 27% of the total ^{14}C-estradiol determined to be initially present in rat paracervical ganglion after incubation in 7 nM ^{14}C-estradiol (Thompson et al. 1985). Possible translocation of estradiol could be detected through the parallel use of thaw-mount autoradiography. Some histochemical comparisons with parallel autoradiography may be essential for establishing further the utility of the technique recommended by Jungblut and Sierralta. Biochemical binding tests alone, even though valuable and successful, may not be enough. As opposed to the authors' covalent estradiol fixation en bloc, fixation of thin frozen sections may be examined. Thus problems of penetration and possible translocation of the compound, related to the advancement of the fluid phase in the tissue, can be minimized or excluded. With frozen sections, the dehydration–embedding and deembedding–rehydration steps would be avoided. The procedure would not only be simplified but also its sensitivity improved. If the "covalently rearranged" estradiol indeed remains precisely at its original site during penetration of the liquid fixative, electron microscopic localization at the ultrastructural level could be endeavored. A boon of new data could then be expected.

Ultimately, someone would need to compare results obtained with the different histochemical techniques: immunohistochemistry with antibodies to estradiol, autoradiography with radiolabeled estradiol, and, adding to that, immunohistochemistry with antibodies to estradiol receptor. Most likely, the results obtained with the different techniques will not be identical due to technique-related sensitivities as well as physiological conditions, such as the presence of unoccupied receptor protein and ligand accessibility and deposition.

There is a need for applying reliable cytochemical procedures for the identification of cellular and subcellular target sites of hormones and drugs, both for experimental and clinical diagnostic purposes. Amazingly, pharmaceutical companies are, in general, not held to utilizing histochemical approaches for cellular target identification in drug research and development. Accordingly, for most drugs precise information is lacking about in situ cellular sites of action, related effects, side effects, or toxicity. Information derived merely from the required routine radioassays with excised organs or tissue pieces and from whole body autoradiography is grossly deficient and may be useless or even misleading (Monro 1994; Stumpf 1995, 1996).

These latter considerations attribute further importance to the efforts of Jungblut and Sierralta. Decades of studies of steroid-receptor interactions with homogenized tissue components apparently have led these biochemists to the present development of a new histochemical approach. They have recognized the need to link biochemical in vitro and cytochemical in vivo information for a better understanding of mechanisms of action, and others should too. It is unfortunate that drug authorities seem unaware of the urgent need to request more precise and revealing research techniques. In this light Jungblut and Sierralta deserve high praise for their ardent efforts in this field.

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