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Quantitative Assessment of Steroid Hormone Binding Sites by Thaw-Mount Autoradiography[1,2]

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A procedure for the quantitative assessment of nuclear receptors for steroid hormones—and other substances—in individual cells is presented. Thaw-mount autoradiography, a procedure developed earlier in our laboratory, is utilized. The silver grain yield (specific activity) is 16.6 disintegrations per silver grain as determined for tritium in guinea pig uterine tissues. An integrated formula is presented and applied for ^{3}H-estradiol, ^{3}H-diethylstilbestrol, and ^{3}H-aldosterone in sampled tissues. A comparison with data derived from the literature that are based on the homogenization of whole uteri and biochemical analysis shows comparable values with the autoradiographic data if the latter are pooled. The pooled data indicate 12-14,000 molecules of ^{3}H-estradiol per uterine nucleus, while subpopulations of target cells vary between 5,000 and 28,000 per nucleus.

KEY WORDS: Autoradiography, Quantitative technique; Steroid hormones; Receptors.

Dry-mount and thaw-mount autoradiography for the localization of diffusible substances were introduced by Stumpf and Roth in 1966 (9). Thaw-mount autoradiography, which has been adapted to routine applications by Stumpf (10) and Stumpf and Sar (11), but needs to be controlled against chemographic artifacts by dry-mount autoradiography, is based on the use of unfixed, unembedded frozen tissue that is sectioned in a cryostat and thaw-mounted on photographic emulsion-coated slides. For certain diffusible compounds, such as steroid hormones, perfusion fixation under specific conditions is feasible (Stumpf and Sar, unpublished) and may be used in conjunction with controls against diffusion artifacts and chemographic artifacts. Thaw-mount autoradiography has provided numerous first observations, some of which may not have been obtainable otherwise, for example, the mapping of nuclear receptor sites for various steroid hormones in the brain and pituitary, the discovery of estrogen target cells in the heart and walls of blood vessels, testis, ovary, thymus, pineal, skin, kidney (13), or the recent discovery of target cells for 1,25 $(OH)_2$ vitamin D_3 in the skin, stomach, anterior and posterior pituitary, parathyroid (13), and pancreas (3).

Quantitative differences in nuclear steroid hormone uptake have been noted in target cells of the same organ and of different organs. Also, evidence has been provided that quantitative changes in hormone uptake occur due to hormonal pretreatment and the developmental stage of the animal. These changes are selectively affecting certain populations or subpopulations of target cells and may show increase or decrease, or both, side by side within the same organ. These variations, as well as the ubiquitous cell heterogeneities in organs, impose limitations on the value of data derived from tissue homogenization and necessitate the use of histological approaches.

Certain requirements for quantitative autoradiography must be met. These include:

1. The results which are used for quantitative evaluation must be authentic, that is, the information contained in the autoradiogram must reflect the distribution in the tissue at the time of sacrifice or termination of treatment (in vitro), without translocation, redistribution, and leaching of the radioactively labeled compound(s) or tissue constituents.
2. Artifacts must be excluded which are due to latent image formation or changes of the latent image independent of the radiation from the tissue, as derived from positive and negative chemography, mechanical interaction, heat, humidity, and others.
3. Chemical purity of the injected radioactively labeled material must be high, possibly near 99%.

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4. The chemical nature of the radioactivity in the tissue structures or compartments of interest, must be characterized and its specificity established by competition studies. Chemical identification of the radioactivity in topographical tissue extracts are useful and may be performed in the same or another laboratory that is equipped for this purpose.
5. Autoradiograms with clumping of silver grains must be excluded. Clumping does not permit counting of silver grains and results in nonlinear recording of radiation due to coincidence quenching. Densitometric approaches for quantitative autoradiography that are based on measurements of light absorpotion in structures or compartments where aggregation and clumping of silver grains exist, produce imprecise or fallacious data and, therefore, are not recommended.
6. Constancy of experimental conditions, including section thickness (consider β-self-absorption and superimposition of structures), photographic exposure and processing must be maintained.
7. Corrections for background silver gains of the emulsion and "nonspecific" tissue silver grains may be necessary.

Materials and Methods

^{3}H-estradiol-17β, specific activity 91.8 Ci/mM, was obtained from New England Nuclear, with a radiochemical purity greater than 99% assessed by paper chromatography on Whatman No. 1 treated with 30% formamide in acetone using the solvent system benzene saturated with formamide. ^{3}H-estradiol was dissolved in 10% ethanol-isotinic saline and injected intravenously (iv) into a 35-day-old intact guinea pig, body weight 360 g, at 0.5 μg/100 g body weight. One hour after injection, the animal was decapitated, the uterus dissected and mounted on tissue holders for subsequent freezing in liquefied propane at −180°C. Two, 4, and 6 μ sections were cut and thaw-mounted onto dried photographic emulsion (Kodak NTB3)-coated slides. The slides with the mounted sections were placed in lightproof desiccator boxes and exposed at −15°C for 15, 30, 45, or 60 days.

Serial sections were cut in a Wide Range Cryostat (Harris Mfg. Co., Billerica, MA). The first two sections were freeze-dried and sealed in an ampul for subsequent combustion and radioactivity counting with a proportional counter: automatic gas-counter for quantitative microdetermination of tritium (Wallace Oy, Turku, Finland), counting efficiency 75%. The subsequent 2 sections were used for autoradiography. For each section thickness this sequence was repeated 30 times.

Quantification of radioactivity in autoradiograms. For determination of section area, the indirect paper weight method was used. The uterine cross section was projected on tracing paper and the circumference traced and cut out. The weight of the uterine area was related to the paper weight of a projected square of known size. The ratio defined the topological area as a fraction of the known square on the paper sheet:

$$\text{Area of section} = \frac{\text{total area of paper} \times \text{paper weight of section area}}{\text{weight of total area of paper}}$$

Repeated determinations showed variations in weight of less than 3%. In order to assess the total silver grain number of a given section, silver grains were counted with a × 100 objective (optovar 1.25 or 1.6) through an eyepiece grid within a field of 90 × 9 μ^2. Between 40–120 fields were counted randomly within a section by advancing in a meander fashion and selecting every 10th field. In order to obtain the total number of silver grains per section, the average number of silver grains per unit area was determined and multiplied according to the total area of a given section as determined earlier by the paper weight method. The results obtained using 40 fields per section agreed with those obtained with 120 fields per section.

Results and Discussion

Results are depicted in Tables 1 and 2. Table 1 shows that the silver grain yield varies with the section thickness and is optimal with the thinner sections. This has to do with the energy and range of the β-particles of tritium and related self-absorption in tissue. In the 6 μ sections, even when partially collapsed after melting, self-absorption probably is much more effective than in 4 μ sections. In addition, 6 μ sections may produce desensitation of emulsion due to increased amount of tissue fluid and extended interaction of tissue components with the emulsion (unpublished observation). Different exposure times give linear data, as long as coincidence quenching does not occur. The silver grain yield is similar at exposure times between 15 and 60 days (Table 2), even though clumping of silver grains appeared in certain cell nuclei at 60 days of exposure. During counting, the small clusters of silver grains were broken up and regarded as composed of individual grains. Apparently, this is feasible—but unsafe—at early stages of clumping.

According to these computations the average silver grain yield is 16.6 disintegrations per silver grain. From the present results as well as the results from similar studies published in the literature, it is apparent that the silver grain yield is specific for the experimental condition and may vary with section thickness, type of embedding material, radiation energy, and type of photographic emulsion. Table 3 compiles some of the relevant data from the literature. Accordingly, the present results appear to be in the expected range.

Computation of Nuclear Uptake of ^{3}H-Estradiol

Selection of cell population. Based on morphological or histochemical criteria, the cell type is determined and a histiogram of the nuclear diameters of all cells of this kind is plotted. From the histiogram it can be ascertained whether this is one cell population or includes different cell populations. Also, the maximal nuclear diameter of a given cell type can be determined.

Only cells that fall in the range of the maximal nuclear diameter ($d_{\max}$) of a given cell type are used for silver grain counting, since in 4 μ sections, counting of nuclei with diameters smaller than maximal may include a segment only of a larger nucleus (Figure 1) or nuclei of a cell population of smaller $d_{\max}$. Since this cannot be decided, unless serial sections are used, in this study only cells ($n > 10$) with $d_{\max}$ are evaluated. The average volume of the slices with $d_{\max}$ is computed according to the formulas in Figure 1, and its

Table 1. *Correlation between radioactivity measured by tritium gas counting and silver grain counting*[a] *in 2, and 4, 6 μ frozen sections*

Section #	Disintegrations per min (dpm) per section	Section #	Silver grains per min per section	Disintegrations per silver grains (sg)
2 μ sections				
1 + 2	148.98	3	12.39	12.02
4 + 5	158.78	6	10.49	15.14
7 + 8	140.58	9	11.68	12.04
Mean				13.07 ± 1.03 SE
4 μ sections				
1 + 2	345.94	3	17.28	20.03
4 + 5	353.08	6	19.79	17.85
7 + 8	337.08	9	21.15	15.94
Mean				17.94 ± 1.18 SE
6 μ sections				
1 + 2	503.55	3	14.29	35.24
4 + 5	484.10	6	14.08	34.37
7 + 8	470.01	9	11.65	40.34
Mean				36.65 ± 1.86 SE

[a]30-day exposure time.

Table 2. *Correlations between radioactivity measured by tritium gas counting and silver grain counting of 4 μ frozen thaw-mounted sections at different exposure times*

Section #	dpm/section	Section #	sg/min/section	disintegrations/sg
15 day exposure				
1 + 2	290.72	3	20.30	14.32
4 + 5	340.00	6	18.89	18.00
7 + 8	329.09	9	19.96	16.48
10 + 11	321.33	12	19.51	16.47
Mean				16.32
30 day exposure				
13 + 14	353.08	15	20.12	17.55
16 + 17	345.94	18	17.79	19.44
19 + 20	337.08	21	19.14	17.61
22 + 23	328.11	24	21.00	15.62
Mean				17.56
45 day exposure				
25 + 26	328.67	27	19.83	16.57
28 + 29	356.77	30	20.68	17.25
31 + 32	317.90	33	20.21	15.73
34 + 35	328.11	36	22.87	14.35
Mean				15.98
60 day exposure				
37 + 38	328.67	39	19.68	16.70
40 + 41	337.93	42	20.32	16.63
43 + 44	348.69	45	20.86	16.72
46 + 47	345.81	48	21.42	16.14
Mean				16.55
			Mean	16.60 ± 0.34 SE

Table 3. *Silver grain yield of tritium derived β-particles*

Disintegrations/sg	Photographic emulsion	Biological material	Ref.
5	Kodak AR 10	bacteria smear	4
20	Kodak AR 10	cell smear	
10.9	Kodak AR 10	cell smear	15
100–200	Kodak AR 10	3 μm paraffin embedded lung	6
16	Kodak AR 10	3 μm methacrylate-embedded liver	7
19.3	Kodak NTB 3	cell smear	15
5.8	Kodak NTB 3	spermatozoa smear	5
16.6	Kodak NTB 3	4 μ thaw-mounted frozen sections	this article

reciprocal value is entered into the integrated formula (Figure 2) as a multiplication factor for the whole nucleus. Different formulas are used, depending on whether $d > t$ (t = thickness of slice), or $d < t$. If the nucleus cannot be considered a sphere but rather an ellipsoid, then only cells are evaluated with d_{1max} and d_{2max}, except if serial sections are used. For ellipsoid nuclei the same formulas are used as for spheres and d_{1max}, the smaller diameter, is entered.

Background silver grains are assessed by counting outside of the section area in at least two different regions of the photographic emulsion. The background silver grain number per nuclear area is substracted from the average number of silver grains over nuclear slices. In addition, it may be desirable to establish the ratio of nuclear and extranuclear tissue radioactivity in a similar fashion. Whether or not a nucleus is considered "labeled" needs to be defined and usually implies a nuclear-extranuclear silver grain ratio above 1 (see also Discussion by Arnold, this issue, p. 207).

In the integrated formula (Figure 2) are also entered the silver grain yield, the exposure time, the specific activity of the labeled compound, and Avogadro's number in relationship to the molecular weight. This computation of the number of molecules per nucleus, or other tissue compartment, is useful only if the identity of the ratioactivity is clarified.

Table 4 shows the results of the computation of estrogen nuclear binding sites in uterine tissues. It is apparent from these data that the uterus must be considered not as a whole, but as an organ that is composed of many cell types, such as, uterine epithelium, glandular epithelium, substantia propria, inner and outer muscle (in addition, there are endothelial cells, pericapillary cells, leukocytes, eosinophils, basophils, and macrophages). There is evidence for heterogeneous populations in a seemingly uniform type of tissue: subluminal stroma may vary from deep stroma, and glandular cells may contain from a few to many estrogen binding sites, depending on the position in the stroma. The data presented here are, therefore, only an initial indication and much more work needs to be done. It is needless to emphasize that data on estrogen uptake capacity need to be correlated with other histochemical or biochemical parameters in order to arrive at an understanding.

For comparison, Table 5 lists data from the literature on the biochemical assessment of estrogen binding sites. The quantitative data obtained from autoradiograms are comparable with those obtained from tissue homogenization, if the data from different uterine components are averaged. A comparison of the autoradiographic data between rat and guinea pig uteri shows a two times higher number of estrogen binding sites in the guinea pig.

Here are some other preliminary data derived from autoradiograms: In a Swiss albino mouse, 2 months old and

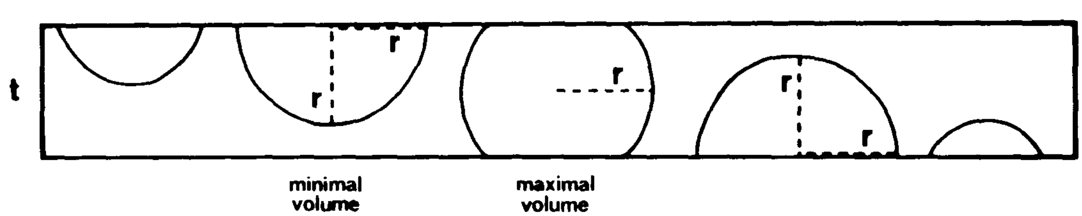

Figure 1. Computation of nuclear volume from tissue slices. Example for $t > r$ where t = 4 μm; r = 3 μm. First r_{max} is assessed then the average volume of nuclear slices with d_{max} (r_{max}) is calculated according to the following formulas: for $t > r$: $1 - (3/8\ (r/t))$ and for $t < r$: $3/4(t/r) - (1/8\ (t/r)^3)$.

$$\text{molecules per cell nucleus} = \frac{\text{sg per nuclear}^1}{\text{slice } (d_{max})} \times \frac{\text{factor to obtain}^2}{\text{sg per nuclear volume}} \times \frac{\dfrac{\text{disintegrations per sg}^3}{\text{exposure time in min}} \times \dfrac{\text{molarity lab. comp.}}{(\text{spec. act.}) \text{ in } \mu g} \times \dfrac{\text{Avogadro's No.}^4}{\text{mol wt in } \mu g}}{\text{spec. act. in } \mu C \times \text{dpm per } \mu C}$$

[1]Silver grains(sg) are counted over nuclei with maximal diameter (d_{max}) of a homogeneous cell population and the average sg value is used.

[2]Depending on slice thickness(t) and radius (r) the following formula is used:

$$\text{for } t > r: \frac{1}{1 - \frac{3}{8}\left(\frac{r}{t}\right)} \qquad \text{for } t < r: \frac{1}{\frac{3}{4}\left(\frac{t}{r}\right) - \frac{1}{8}\left(\frac{t}{r}\right)^3}.$$

[3]Average 16.6 disintegrations yield one silver grain. This silver grain yield applies only to specific experimental conditions: Four µm thaw-mounted frozen sections, exposed at −15°C on Kodak NTB-3 emulsion, use of Kodak D-19 developer and Kodak fixer under defined concentration, temperature and time, and other factors.

[4]6.0225×10^{23}.

Figure 2. Computation of number of molecules from autoradiograms.

ovariectomized for 3 days, 1 hr after subcutaneous injection of 1.0 µg/100 g body weight of ^{3}H-estradiol-17β, in the arcuate nucleus of the hypothalamus, a population of large target neurons (d = 10.8 µ) contains 88,300 molecules per nucleus; a population of slightly smaller neurons (nuclear d = 10.4 µ) contains 25,509 molecules per nucleus. In the preoptic nucleus of the brain of a pregnant mouse (16th day of pregnancy) at 3 hr after the injection of ^{3}H-diethylstilbestrol, 8,000 molecules per nucleus were found, while the number in the same region of a fetus was 2,783. ^{3}H-aldosterone in 33-day-old adrenalectomized female Holtzman rat, 30 min after iv injection of 1 µg/100 g body weight, 80,837 molecules per nucleus are found in neurons in area CA_{3b} of the hippocampus, 7,368 in neurons of the dentate gyrus and 5,917 in glial cells.

Conclusions

The present study is a first attempt to utilize autoradiography for the quantitative assessment of steroid hormone receptors in target tissues. The silver grain yield of 16.6 disintegration per silver grain is a statistical value derived from uterine tissues. The silver grain yield needs to be confirmed and assessed with other tissues. Adjustments may become necessary for tissues with different densities. The great variety of nuclear uptake and retention, even within the same organ, is impressive and argues for further application and sophistication of the present technique. The unified formula should facilitate the procedure and use of the computer. New insights into kinetics of uptake and turnover of steroid hormones and other substances for individual cell populations can be expected.

If it can be corroborated that 16.6 disintegrations are required to yield one silver grain, the sensitivity of the thaw-mount autoradiographic technique for tritium with 4 µ thick sections and Kodak NTB 3 emulsion can be defined as follows: for a compound that has every molecule tritium labeled in six positions, theoretically 68 years of exposure would be required for one molecule to yield one silver grain. Accordingly, if 340 molecules exist in 1 μ^3 of tissue, 5 silver grains can be expected after 1 year of exposure. One thousand molecules would require 4 months exposure to yield 5 silver grains. Thus, a specific concentration of 300–500 molecules per 1 μ^3 is required for detectability under the conditions outlined. If ^{125}I instead of ^{3}H were used as a marker, with a short half-line of 60 days and a similar but somewhat higher β energy, 4–7 molecules could be detected.

Table 4. *Estrogen nuclear binding sites in uterine tissues assessed by quantitative autoradiography*[a]

	Rat 115 g 32-day-old castr. 5 days 37-day exposure		Rat 203 g 68-day-old castr. 4 days 44-day exposure		Guinea pig 30-day-old intact 15-day exposure	
Lumenal epithelium	5,245 d_{max} 5.5 µ	60/µ^3 sg 4.19	8,253 d_{max} 5.35 µ	102/µ^3 sg 7.9	14,320 d_{max} 7.45 µ	66/µ^3 sg 4.1
Glandular epithelium	10,229 d_{max} 5.0 µ	156/µ^3 sg 8.43	8,290 d_{max} 5.85 µ	179/µ^3 sg 16.9	18,410 d_{max} 6.85 µ	110/µ^3 sg 5.5
Stroma, small cells			13.005 d_{max} 4.35 µ	302/µ^3 sg 13.18		
Stroma, large cells	23,210 d_{max} 6.3 µ	177/µ^3 sg 17.6	28,055 d_{max} 7.56 µ	124/µ^3 sg 23.06	54,110 d_{max} 6.55 µ	370/µ^3 sg 16.5
Muscle, inner	12,780 d_{1max} 5.2 µ d_{2max} 10 µ	45/µ^3 sg 10.4	12,227 d_{1max} 4.2 µ d_{2max} 10 µ	132/µ^3 sg 12.5	29,410 d_{1max} 7 µ d_{2max} 12 µ	96/µ^3 sg 8.7

[a]All animals received iv 0.5 µg/100 g body weight of [2,4,6,7-^{3}H] estradiol-17β and were killed 1 hr afterwards. sg = average number of silver grains per nuclear slice with maximal nuclear diameter (d_{max}). The upper line of each horizontal column contains the number of molecules per nucleus (left) and per µ^3 of nuclear volume (right).

Table 5. *Number of estrogen binding sites in uterine cells or nuclei as obtained from homogenized whole uteri and pooled quantitative autoradiography data*

Number of binding sites	Status of animal	Authors
	Biochemical	
5,000 per cell	day 1 immature Holtzman	1
20,000 per cell	day 10 rat	
16,000 per cell	day 22	
16,000 per cell	ovariectomized adult rat	8
10,000 per nucl.		14
	250–350 g virgin Purdue-Wistar rat	
1,000 per nucl.	estrus-metestrus	2
3,500 per nucl.	metestrus-diestrus	
5,000 per nucl.	proestrus	
	Autoradiographical	
12,900 per nucl.	115 g Holtzman rat ovariectomized, 32-day old	this article
14,000 per nucl.	203 g Holtzman rat ovariectomized, 68-day old	this article
29,000 per nucl.	375 g guinea pig intact, 30-day old	this article

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Literature Cited

1. Clark JH, Gorski J: Ontogeny of the estrogen receptor during early uterine development. Science 169:76, 1970
2. Clark JH, Anderson JN, Peck EG Jr: Nuclear receptor estrogen complexes of rat uteri. Adv Exp Med Biol 36:15, 1973
3. Clark SA, Stumpf WE, Sar M, DeLuca HF, Tanaka Y: Target cells for 1,25 dihydroxyvitamin D_3 in the pancreas. Cell Tissue Res. 209:515, 1980
4. Hughes WL, Bond VP, Brecher G, Cronkite EP, Painter RB, Quastler H, Sherman FG: Cellular proliferation in the mouse as revealed by autoradiography with tritiated thymidine. Proc Natl Acad Sci USA 44:476, 1958
5. Hunt WL, Foote RH: Efficiency of liquid scintillation counting and autoradiography for detecting tritium in spermatozoa. Radiat Res 31:63, 1967
6. Kisieleski WE, Baserga R, Vaupotic J: The correlation of autoradiographic grain counts and tritium concentration in tissue sections containing tritiated thymidine. Radiat Res 15:341, 1961
7. Maurer W, Primbsch E: Grösse der β-selbstabsorption bei der ^{3}H-autoradiographie. Exp Cell Res 33:8, 1964
8. Notides A: Binding affinity and specificity of the estrogen receptor of the rat uterus and anterior pituitary. Endocrinology 87:987, 1970
9. Stumpf WE, Roth LJ: High resolution autoradiography with dry-mounted, freeze-dried, frozen sections. Comparative study of six methods using two diffusible compounds, ^{3}H-estradiol and ^{3}H-mesobilirubinogen. J Histochem Cytochem 14:274, 1966
10. Stumpf WE: Autoradiographic techniques for the localization of hormones and drugs at the cellular and subcellular level. Acta Endocrinol Suppl 153:205, 1971
11. Stumpf WE, Sar M: Autoradiographic techniques for localizing steroid hormones. In Methods in Enzymology, Vol. XXXVI, Hormone Action, Part A. Steroid Hormones. Academic Press, New York, 1975, p 135–156
12. Stumpf WE, Sar M: Autoradiographic localization of estrogen, androgen, progestin and glucocorticosteroid in "target tissues" and "non-target tissues." In Receptors and Mechanism of Action of Steroid Hormones. Edited by J Pasqualini. Marcel Dekker, New York, 1976, p 41–84
13. Stumpf WE, Sar M, Reid FA, Tanaka Y, DeLuca HF: Target cells for 1,25-dihydroxy-vitamin D_3 in intestinal tract, stomach, kidney, skin, pituitary and parathyroid. Science 206:1188, 1979
14. Toft D, Gorski J: A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterizations. Proc Natl Acad Sci USA 55:1574, 1966
15. Wimber DE, Quastler H, Stein OL, Wimber DR: Analysis of tritium incorporation into individual cells by autoradiography of squash preparations. J Biophys Biochem Cytol 8:327, 1960