

Oestrogen receptor assay of cryostat sections of human breast carcinomas with simultaneous quantitative histology

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SUMMARY Cryostat sections of unfixed human breast carcinomas were assayed for oestrogen receptor (ER) content using an isoelectric focusing method to separate the receptor-bound oestradiol. Adjacent sections from the same tissue block were stained so that the tumour content could be estimated by point counting and the ER concentration adjusted to compensate for variations in cellularity. Elastosis was also assessed. The results confirm a positive correlation between ER values and both cellularity and elastosis. The measurement of ER in cryostat sections is relatively simple and rapid, is applicable to small tissue samples, and permits histological identification of the nature and composition of the assayed sample. The method is directly applicable to oestrogen receptor analyses of breast carcinomas in clinical laboratories with facilities for cryostat microtomy.

Oestrogen receptor (ER) concentrations in breast carcinomas are usually assayed in cytosol fractions prepared from tissue homogenates. Although an adjacent piece of tissue is often taken for histological confirmation, the two portions are not exact replicates because of tumour heterogeneity and sampling variability. Furthermore, the conventional cytosol assays for ER suffer from problems due to contamination of tumour homogenates with stromal elements; this varies with cellularity—that is, epithelial cell content. Attempts to circumvent these problems by localising ER histologically by labelled-ligand or ligand-antibody methods have met with criticism on biochemical and other grounds.¹⁻⁷

We have endeavoured to eliminate some of these difficulties by adapting a sensitive isoelectric focusing method of ER assay^{8,9} for use with cryostat sections, thus permitting simultaneous quantitative histology and ER assay on near facsimile samples. Our method utilises the observation that cytosolic constituents, including ER, will readily diffuse from unfixed cryostat sections into a surrounding aqueous medium^{6,7,10} without the need for mechanical homogenisation.

We report our experience with this technique and our initial observations on the correlation of recep-

tor content with tissue morphology in sections of human breast carcinomas. The method is readily applicable for use in diagnostic laboratories equipped for cryostat microtomy.

Material and methods

TISSUE COLLECTION AND STORAGE

Eighteen human breast carcinomas were obtained immediately after surgical removal and small samples (0.5–1 cm diam) suitable for cryostat microtomy taken and trimmed of fat and obvious necrotic tissue. After rapid freezing the tissue sample was stored at -70°C . Uterus for positive control tissue was obtained fresh from normal or oophorectomised (1–2 days previously) rats and similarly stored.

CRYOSTAT SECTIONS

A sufficient number of sections to give a total section area of $>1\text{ cm}^2$ were cut from the frozen tissue at $40\text{ }\mu\text{m}$ in a cryostat at constant cutting speed, collected on a small glass coverslip, and transferred to a small plastic tube for ER assay. This was done in duplicate for each sample. Two sections for histology were then cut from the same surface of the tissue block at $10\text{ }\mu\text{m}$ with minimal trimming: one was stained with haematoxylin and eosin, the other with Miller's elastic/van Gieson. Elastosis was subjec-

tively assessed to be either present or absent, without knowledge of the ER assay results.

QUANTITATIVE HISTOLOGY

The cellularity of the assayed sections with respect to their neoplastic cell content was estimated by point counting¹¹ on the adjacent sections taken for light microscopy. A graticule with 81 regularly distributed points was mounted in the focal plane of a $\times 10$ eyepiece; a $\times 10$ objective was used (total magnification $\times 100$).

The fraction of points coincident with tumour cells or cell aggregates was recorded for each field until the entire section had been covered; the mean value was calculated and the result expressed as a percentage estimate of the sectional area occupied by tumour profiles.

ER ASSAY OF CRYOSTAT SECTIONS

Duplicate sets of cryostat sections ($40\ \mu\text{m}$) to give a total section area $>1\ \text{cm}^2$ were collected on small glass coverslips and dropped into paired $70 \times 9\ \text{mm}$ plastic tubes containing $100\ \mu\text{l}$ ET buffer ($1.5\ \text{mmol}$ disodium EDTA, $10\ \text{mmol}$ Tris-HCl, 0.05% bovine serum albumin, 0.02% sodium azide; pH 7.4). The coverslips were then gently crushed. A further $100\ \mu\text{l}$ of ET buffer containing $2 \times 10^{-9}\ \text{M}$ (2,4,6,7-³H)-oestradiol ($110\ \text{Ci}/\text{mmol}$; Radiochemical Centre, Amersham) was added and the tubes kept on ice for 1 hour with agitation every 15 min. The tubes were centrifuged at $1000\ g$ for 10 min at 4°C to sediment the sections and glass debris. The supernatant, containing cytosol which had diffused from the cryostat sections, was gently aspirated into a disposable plastic pipette tip and transferred to clean plastic tubes. The protein content in $50\ \mu\text{l}$ was determined immediately by the Coomassie blue method of Bradford¹² against a blank containing $50\ \mu\text{l}$ ET buffer alone. A small volume (approx $10\ \mu\text{l}$) of trypsin solution¹³ ($1\ \text{mg}/\text{ml}$) (type III; $10\ 000$ – $13\ 000$ BAEE units/mg protein; Sigma) and calcium chloride to give a final concentration of $2\ \text{mmol}$ was added to the remaining supernatant. The precise volume of trypsin to be added was gauged from the protein estimation (typically $5\ \mu\text{l}$ was added for an OD 0.15 at $595\ \text{nm}$ in the Bradford protein assay). Incubation was continued for 30 min at 10°C . Receptor-bound oestradiol was separated from free and non specifically bound hormone by subjecting the supernatant to isoelectric focusing.^{8,9}

COMPETITIVE BINDING STUDIES

To investigate the specificity of the high affinity oestradiol binding, incubations of section cytosols with $10^{-9}\ \text{M}$ ³H-oestradiol were conducted either alone

or in the presence of a 100-fold molar excess of either diethylstilboestrol (Sigma), progesterone (Sigma), testosterone (Sigma), or hydrocortisone (Sigma). After one hour on ice the assay tubes were centrifuged and the amount of receptor-bound ³H-oestradiol measured in the trypsinised supernatant by isoelectric focusing.

ISOELECTRIC FOCUSING

Trypsin-treated supernatant ($100\ \mu\text{l}$) from the incubation of cryostat sections with ³H-oestradiol was transferred to a plastic sample application frame ($10 \times 6 \times 2\ \text{mm}$) placed approximately 1 cm from the cathodal electrode strip on the surface of a thin-layer polyacrylamide gel ($245 \times 110 \times 1\ \text{mm}$; 2.4% (wt/vol) ampholine, pH 3.5–9.5; LKB). The baseplate was perfused with refrigerated water; the entire apparatus was enclosed in a refrigerated cabinet (Colora Messtechnik) at 4°C . The focusing conditions were similar to those employed by Gustafsson *et al.*⁹ At the end of the focusing period of 90 min the gel was cut into 5 mm slices and each dropped into a scintillation vial to which was added 10 ml of Instagel (Packard). The vials were then agitated for 1 h in a 50°C waterbath.

CALCULATION OF RECEPTOR CONCENTRATION

Radioactivity in the vials was determined by scintillation counting with correction for efficiency using the external standard technique. A graph of radioactivity against slice number was drawn for each sample and the cumulative height of the points in the single peak attributable to receptor-bound oestradiol, typically located approximately 6 cm from the cathodal electrode strip, was measured above the diagrammatic base line. The amount of oestradiol within this peak was calculated from the specific activity of the radioactive ligand and the result finally expressed in fmol/mg of section cytosol protein. All determinations were done in duplicate.

DIRECT TISSUE ISOELECTRIC FOCUSING (HISTOISOELECTRIC FOCUSING)

With two tumours, fresh tissue slices (approx 2 mm thick) were placed in approximately 20 volumes of $10^{-9}\ \text{M}$ (2,4,6,7-³H)-oestradiol in phosphate-buffered saline (PBS) for 2 h at 4°C with gentle agitation. They were then washed in 0.5% bovine serum albumin in PBS. Cryostat sections were cut at $40\ \mu\text{m}$, picked up on a glass coverslip and directly inverted onto the polyacrylamide gel surface for isoelectric focusing.¹⁴ The focusing conditions and sampling of the gel were as previously described. Replicate $40\ \mu\text{m}$ sections were assayed for protein content by the method of Lowry *et al.*¹⁵; adjacent 10

μm sections were stained with haematoxylin and eosin and Miller's elastic/van Gieson.

Results

RECEPTOR ANALYSIS OF CRYOSTAT SECTION CYTOSOLS

Receptor-bound ^3H -oestradiol was represented by a single peak on isoelectric focusing in which the maximum radioactivity occurred approximately 6 cm from the cathodal end of the gel. This peak was absent when sections were incubated in the presence of 10^{-7} M DES which fully saturates the high affinity receptor. The peak obtained with breast carcinomas coincides with the position of the saturable peak seen with cryostat sections of control rat uterus, the height of which is greater in oophorectomised animals (Fig. 1). The radioactive peak on the cathodal side of the receptor is attributable to non-specific binding; it is not saturable with DES.

Incubation of breast carcinoma cryostat sections

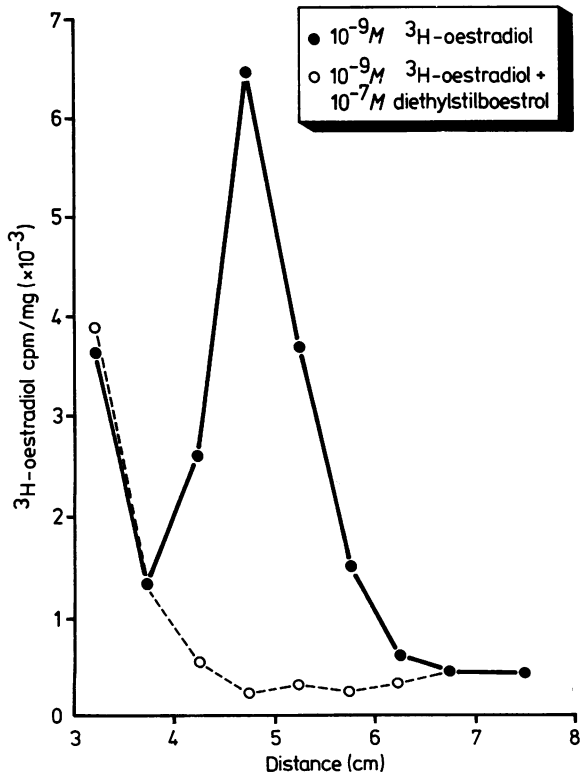


Fig. 1 Isoelectric focusing profile of supernatant from oophorectomised rat uterus cryostat sections ($40\mu\text{m}$) after incubation with either 10^{-9} M ^3H -oestradiol alone (\bullet) or 10^{-9} M ^3H -oestradiol in the presence of 10^{-7} M diethylstilboestrol (\circ).

with ^3H -oestradiol in the presence of a range of unlabelled steroid hormones or analogues as competitors for the receptor showed a high degree of specificity for oestrogens. A 100-fold molar excess of DES totally saturated the receptor so that no detectable specific binding was evident as a characteristic peak on isoelectric focusing. Other steroids in a 100-fold molar excess showed significantly less competition for the receptor: with progesterone, testosterone, and hydrocortisone the specific binding was 70%, 70% and 97% respectively of the controls incubated with ^3H -oestradiol alone.

Diffusion of the receptor from cryostat sections occurs rapidly. ER is detected in substantial quantities 15 min after immersion in buffer and reaches the maximum concentration in the supernatant at one hour (Fig. 2).

Typical isoelectric focusing profiles of ^3H -oestradiol labelled cryostat section supernatants from representative breast carcinomas are shown in

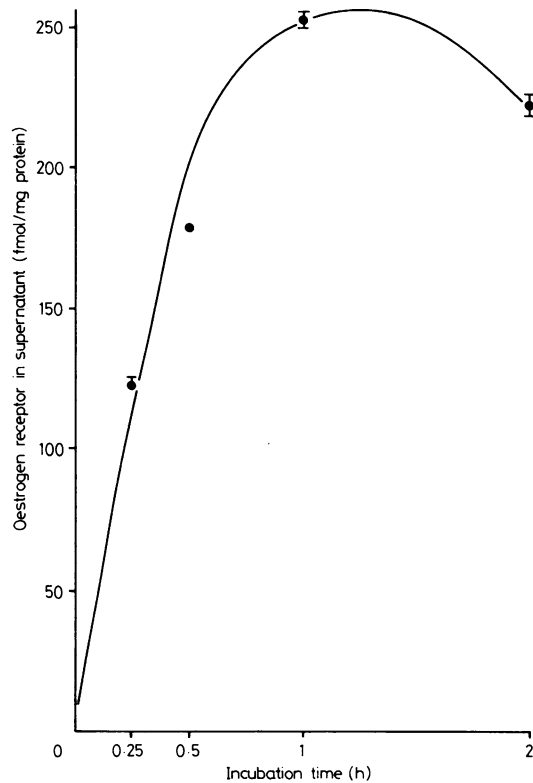


Fig. 2 Rate of diffusion of ER from replicate cryostat sections of a breast carcinoma immersed in ET buffer containing 10^{-9} M ^3H -oestradiol. After times indicated, sections and supernatant were separated by centrifugation and the receptor content of the latter determined by isoelectric focusing.

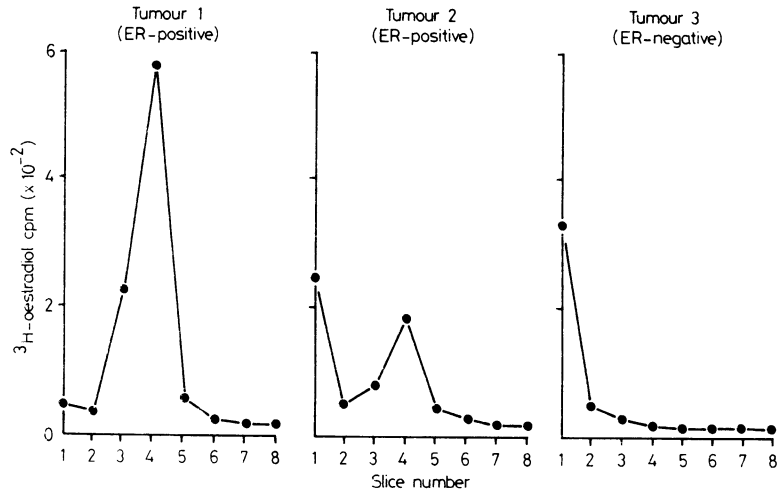


Fig. 3 Representative isoelectric focusing profiles of supernatants from cryostat sections from 3 breast carcinomas incubated with 10^{-9} M 3 H-oestradiol. Tumours 1 and 2 would be regarded as E positive; tumour 3 is negative.

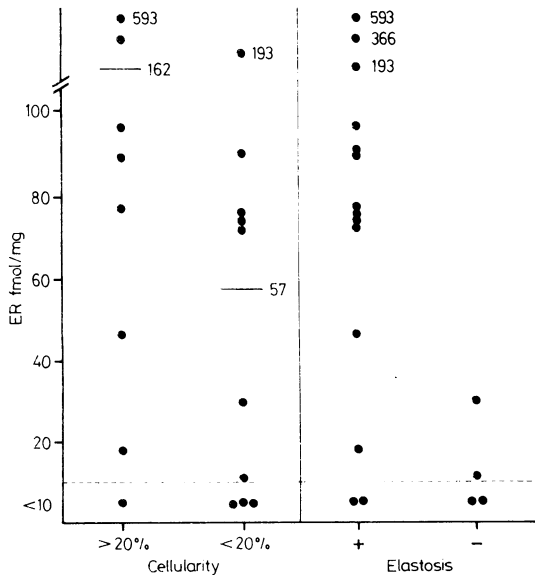


Fig. 4 Correlation between ER concentration and histology of adjacent cryostat sections from breast carcinomas. The trend towards higher ER concentrations in more cellular tumours is not significant at the 5% level; correlation with periductal elastosis is significant ($p < 0.01$).

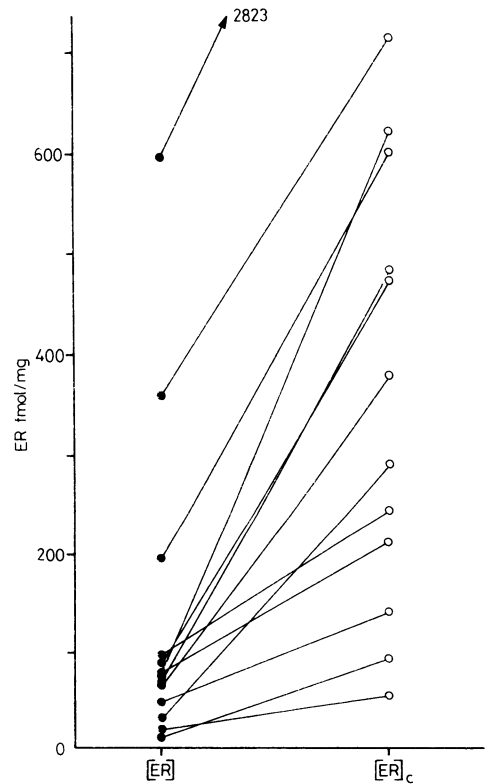


Fig. 5 Assayed ER concentrations ($[ER]$) in cryostat sections from breast carcinomas corrected for cellularity ($[ER]_c$) estimated by point counting on near facsimile sections.

Fig. 3. The lower limit of detection of this assay is approximately 10 fmol/mg section cytosol protein.

With 12 breast carcinomas separate samples were also assayed for ER content in another laboratory by the dextran-coated charcoal method using cytosols prepared from tumour homogenates. Although the overall correlation was excellent ($r = 0.98$) this was largely attributable to a relatively small number of tumours with very high or very low ER concentrations in which there was substantial agreement between the two methods. No agreement was seen in the 15–80 fmol/mg range: for these samples the correlation coefficient was 0.02.

ER VALUES AND HISTOLOGY OF CRYOSTAT SECTIONS

There was tendency towards higher ER values in more cellular sections. Cellularity in this series of tumours ranged from 6.5 to 40%. Comparison of periductal and stromal elastosis with ER values

showed a positive correlation in both instances. This trend appeared most marked with periductal elastosis but relatively few tumours in this series lacked this feature (Fig. 4)

CORRECTION OF ER CONCENTRATION FOR CELLULARITY

ER values were adjusted to compensate for varying cellularity as follows:

$$[\text{ER}]_c = \frac{[\text{ER}] \times 100}{T}$$

where $[\text{ER}]_c$ = ER value "corrected" for cellularity.

$[\text{ER}]$ = assayed ER concentration.

T = mean percentage area of section occupied by tumour profiles.

When the measured ER values were thus adjusted for cellularity there was, of course, a general tendency for the calculated values to be higher (Fig. 5). Some tumours adopted new positions in the overall ranking of results.

DIRECT TISSUE ISOELECTRIC FOCUSING

Cryostat sections cut from fresh tissue that had been incubated in ^3H -oestradiol gave a peak of radioactivity, when subjected to direct isoelectric focusing, in a position on the gel identical to that obtained by the section cytosol method (Fig. 6).

Discussion

The method of ER analysis described in this paper has a significant advantage over the traditional methods using cytosol fractions of tumour homogenates: the presence of tumour in a facsimile of the assayed sample can be confirmed and cellularity quantitatively estimated. Also, isoelectric focusing is an ideal procedure for separating receptor-bound oestradiol from that which is non-specifically bound, particularly when working with small volumes such as the cytosol-enriched supernatant from cryostat sections. As Gustafsson *et al*⁹ have shown, it is unnecessary to treat the samples with dextran-coated charcoal to remove free steroid and a parallel incubation with diethylstilboestrol can be safely omitted because the isoelectric focusing profile of non-specific binding closely approximates the diagrammatic baseline beneath the peak obtained with tritiated oestradiol alone.

The cryostat section method of ER assay eliminates the need to produce a classical cytosol preparation by tissue homogenisation and centrifugation. ER appears to share with certain intracellular

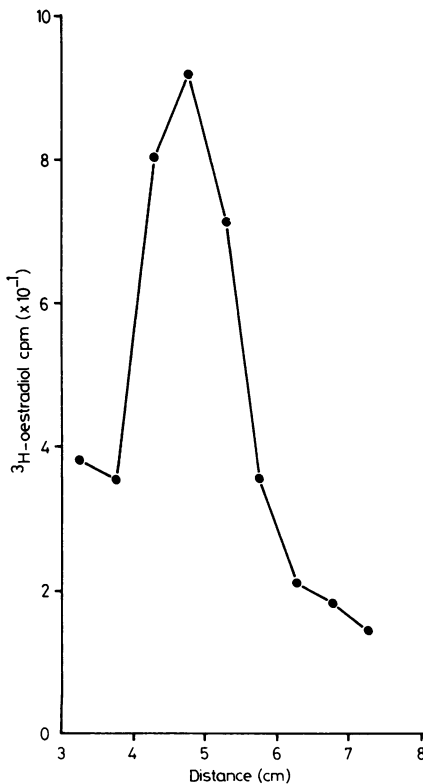


Fig. 6 Isoelectric focusing profile from a cryostat section of an ER positive breast carcinoma placed directly on gel. The fresh tissue had first been incubated in 10^{-9} M ^3H -oestradiol. Note the broad peak due to the unavoidable omission of limited proteolysis with trypsin.

enzymes a marked tendency to diffuse from cryostat sections into the surrounding aqueous medium;^{10,16} indeed this observation is the basis of an important criticism of published methods claiming to localise ER in unfixed cryostat sections.^{6,7}

Cell membranes are rendered porous by ice crystal damage during the freezing of tissue and the freeze/thaw cycle of cryostat microtomy. Also, the cytoplasm of many cells on the section surface will actually be in direct contact with the surrounding medium. The ER-rich cytosol rapidly diffuses into the medium, reaching equilibrium in less than 1 h. These findings are contrary to those of Lee who claims that high affinity oestrogen binding sites are retained within frozen sections.¹⁷ Furthermore, Lee's results suggest that a 100-fold molar excess of DES totally abolishes binding of oestradiol at a concentration of 10^{-9} M in cryostat sections; this is unexpected because some degree of non-specific oestradiol binding must be inevitable. We remain sceptical of any histochemical method for ER detection and localisation utilising "a diffusible substance to localise a soluble protein in an aqueous medium on a substrate rendered porous by freezing or fixation".² This diffusibility of ER facilitates the preparation of a receptor-enriched cytosol from cryostat sections simply by immersion in buffer. Coupled with a highly sensitive assay method based on isoelectrophoretic separation, and the opportunity to perform simultaneous histology on near facsimile tissue sections, this would seem to be an ideal method for analysis of ER and other cytosolic receptors.

Histological examination of near facsimile sections from each tumour studied confirmed the presence of tumour in the assayed sample. With conventional assays using tissue homogenates there is no absolute guarantee that the assayed sample contains viable tumour, despite the presence of tumour in an adjacent tissue block. Negative or low ER assay results may be due to the absence of viable tumour from the assayed sample. Desmoplastic tumours contain a considerable proportion of stromal connective tissue; tumour cells are relatively sparse. If a highly desmoplastic carcinoma contains ER-rich tumour cells, the assayed ER value may be misleadingly low because of dilution of the homogenate by stromal constituents. Conversely, a highly cellular tumour comprising neoplastic cells of low ER content might give a relatively high assayed ER value. Simultaneous quantitative histology allows some adjustment to be made for these variables. In many breast carcinomas the viable and actively growing peripheral zone contains numerous stromal fibroblasts, myofibroblasts and variable numbers of inflammatory cells. If most of the ER in a breast

carcinoma is within the tumour cell compartment (though this assumption may be questionable), there would seem to be legitimate grounds for "correcting" the assayed ER values to compensate for varying cellularity.¹⁸ Whether the "corrected" ER values will have greater clinical significance than the assayed values remains to be determined.

The lack of correlation in the 15–80 fmol/mg range between the results of the cryostat section method and DCC assays of separate homogenised samples from the same tumours may be explained by heterogeneity. This may not be a significant problem in tumours of very high or negligible ER content, but in the borderline range tumour heterogeneity may lead to inconsistencies in the measured ER content. Even when multiple samples from a single tumour are assayed by the same method similar inconsistencies have been found.¹⁹

Our preliminary observations on correlations between tumour histology and ER content in replicate sections indicate a trend towards higher mean ER values in tumour samples showing elastosis. This accords with the experience of other investigators.²⁰ A more thorough appraisal of histology and ER concentrations in cryostat sections is in progress.²¹

Direct tissue isoelectric focusing (histoisolectric focusing) in agarose²² or polyacrylamide gel¹⁴ has not been employed previously for receptor analysis. We have established the feasibility of assaying the ER content of tissues by this method. Although the fresh tissue must first be incubated with tritiated oestradiol, in other respects the method is simpler: the sections are taken directly from the cryostat to the isoelectric focusing stage. But there are some disadvantages: the labelled hormone may not fully penetrate the tissue; unless different isotopes are used, only one hormone can be used for each piece of tissue; and it is difficult to use trypsin to resolve the receptor into a single sharp peak on isoelectric focusing. This attractive method of section analysis is, nevertheless, worthy of further examination.

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