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Morphological demonstration and quantification of TSH binding sites in neoplastic and non-neoplastic thyroid tissues

An autoradiographic study using ^{125}I-labelled thyrotropin

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Summary. We have developed a morphological method to portray TSH binding sites in intact tissue specimens. Frozen sections were incubated with ^{125}I-labelled TSH so as to localise binding sites by autoradiography. The proof of specificity was substantiated by: the competitive inhibition of ^{125}I-TSH-labelling with cold TSH, the lack of binding in non-target tissues and a lack of binding in TSH target tissues after incubation with ^{125}I-hCG or free ^{125}I.

In applying this method to a total of 22 surgical specimens of thyroid, striking differences came to light in respect of the degree to which ^{125}I-TSH binding occurred in the various thyroid disorders. When compared with histologically normal tissue, labelling was generally decreased in toxic adenomas, non-functioning adenomas (cold nodules), and thyroids affected by Graves' disease, whereas non-toxic colloid goitre cases clearly exhibited denser binding. Medullary and anaplastic carcinomas exhibited no specific labelling whilst binding varied in the differentiated carcinomas between no effective binding or a level resembling that found in normal thyroid tissue.

Key words: Human thyroid – TSH receptor – ^{125}I-TSH – Autoradiography – TSH binding sites

Introduction

The growth and function of the thyroid gland is regulated by thyrotropin (TSH). The initial step of the action of TSH on thyroid tissue is binding of this hormone to specific receptors located on the thyrocyte plasma mem-

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brane. Clinical observations of tumour regression subsequent to TSH suppression therapy (Crile 1957) and of stimulation of iodine uptake in many tumours under elevated circulating levels of TSH (Thomas-Morvan et al. 1982) support the concept that the complex regulatory system which mediates TSH actions on the cells of the thyroid remains intact in neoplasia. Disturbances of this system may nevertheless lead to severe functional disorders of this organ: there is now compelling evidence that Graves' hyperthyroidism is due to antibodies to the TSH receptor which exhibit different degrees of TSH agonism (Rees Smith 1981).

The aim of the present study was to investigate whether or not there are differences in the density of TSH binding sites amongst various thyroid disorders. As far as we have been able to ascertain, analyses relating to this subject have previously been performed by various laboratories using membrane fractions of tissue homogenates, comparing normal and neoplastic human thyroid (Mandato et al. 1975; Ichikawa et al. 1976; Field et al. 1978; Clark and Castner 1979; Carayon et al. 1980; Abe et al. 1981; Saltiel et al. 1981; Thomas et al. 1984). In order to avoid measurements of heterogeneous cell populations, a potential source of error inherent to the *in vitro* assays, we developed a procedure to localise TSH binding sites morphologically in structurally intact tissue specimens and employed this method on a selection of different neoplastic and non-neoplastic human thyroid conditions.

Material and methods

A prerequisite for this study was the availability of highly purified bovine TSH (70 U/mg) labelled with ^{125}I using the iodogen method (source: Fa. Henning, Berlin[1]). The ^{125}I-TSH was dissolved in a buffer solution (10 mM Tris-HCl pH 7.5; 50 mM NaCl and 1 mg BSA/ml) at a concentration of 407 Becquerel (Bq), that is 3 nU ^{125}I-TSH/100 µl. In order to remove any free ^{125}I, the solution was dialysed against this buffer (12 h at 4° C) immediately prior to use.

Cryostat sections (snap frozen in isopentane cooled with liquid nitrogen within several minutes after surgical excision and stored at −80° C) were cut at 4–5 µm, fixed in acetone at 4° C for 10 min, dried at 4° C for 15 min, and rehydrated with PBS (pH 7.4). All 3 sections of each specimen were then incubated with 110 µl of the above-named TSH-dilution. The incubation was carried out for 3 h at 37° C in a humid atmosphere. The sections were then submerged in PBS (4 times, 5 min each time) and treated twice (each time for 5 min) with a chromalaum gelatine solution (0.5 g gelatine at 37° C in 100 ml double distilled water, 0.05 g $KCr(SO_4)_2 \times 12\ H_2O$ added after cooling to room temperature). Subsequently, the usual stripping film procedure (Schmiegelow et al. 1983) was applied for purposes of autoradiography. The film exposure time was 3 weeks. The sections were counterstained with haemalaum.

Reflection photometry (Microscopic photometer MPV III, Fa. Ernst Leitz) was used to quantify the autoradiograms (wave length 624 nm). The reflections of the different regions of interest (epithelium, colloid, interstitial connective tissue) and the backgrounds (areas outside the sections) were individually measured 20 times on each slide. The specific reflection was calculated (reflection of the region of interest minus the reflection of the background) and the mean values of triple tests were obtained.

These procedures were used on surgical specimens of thyroid glands revealing the following abnormalities diagnosed clinically, and histologically on paraffin sections:

[1] The authors thank Dr. M. Deparade and Dr. G. Bienhaus for their advice and assistance in scheduling the incubation procedures and for generously providing the radioligand

- toxic adenoma (=hot nodule[2]) ($n=4$),
- follicular adenoma (=cold nodule) ($n=5$),
- papillary carcinoma ($n=1$),
- follicular carcinoma ($n=2$),
- anaplastic carcinoma ($n=2$),
- medullary carcinoma ($n=2$).

In addition, measurements were made on areas of histologically inconspicuous thyroid tissue of normal appearance ($n=7$), such sections always being taken from extranodular parenchyma in cases where disease was non-diffuse.

In order to assess further the characteristics of the ^{125}I-TSH binding, more incubations were carried out using:

1. ^{125}I-hCG (222 Bq; 2.2 Bq; 0.02 Bq)	on: normal thyroid tissue (guinea pig, hog), normal liver (human) and epididymal fat pad tissue (guinea pig)
2. free ^{125}I (222 Bq; 2.2 Bq; 0.02 Bq)	on: normal thyroid tissue (guinea pig, hog), normal liver (human) and epididymal fat pad tissue (guinea pig)
3. ^{125}I-TSH in the presence of excess unlabelled TSH (Sigma, 5,000 μU; 500 μU; 50 μU; 5 μU; 500 nU; 50 nU each/190 μl buffer)	on: normal thyroid tissue (guinea pig, hog)
4. ^{125}I-TSH (814 Bq; 407 Bq; 40 Bq; 4 Bq; 0.4 Bq)	on: normal thyroid tissue (guinea pig, hog) normal liver (human) and epididymal fat pad tissue (guinea pig)

Results

Preliminary experiments using hog and guinea pig thyroids showed an intermediate density of ^{125}I-TSH binding with follicle cells, whereas faint traces of silver grains in each area in the colloid cavities and in the interstitial connective tissue, as measured by reflection photometry, did not differ from the background. Incubation of guinea pig epididymal fat pad tissue resulted in strong membrane-bound labelling. Neither of the aforementioned tissues showed any specific binding when incubated with free ^{125}I or ^{125}I-hCG. Normal human liver tissue yielded negative results when incubated with any of these 3 radioactive preparations.

Incubation of hog thyroid cryostat sections with ^{125}I-TSH in the presence of different concentrations of unlabelled TSH revealed the labelling to be significantly inhibited by cold TSH in a dose-dependent manner[3]. Reflection photometry performed on autoradiograms of hog thyroids showed the grain density to increase with increasing concentrations of ^{125}I-TSH[4]. No significant difference, however, was detected when values of the 2 highest radioligand concentrations were compared.

[2] Upon preoperative scintigraphy, performed in 11 of the cases, ^{99}mTc had been used each time

[3] Presence of 50 nU unlabelled TSH: ca. 85% labelling; 500 nU: ca. 75%; 5 μU: ca. 60%; 50 μU: ca. 45%, 500 μU: 10%, 5,000 μU: no significant labelling. Similar binding values were obtained using membrane homogenates (H.W. Müller, unpublished results)

[4] 0.4 Bq: no significant labelling; 4 Bq: <5% labelling; 40 Bq: approximately 10% labelling (as compared to 407 Bq: 100%)

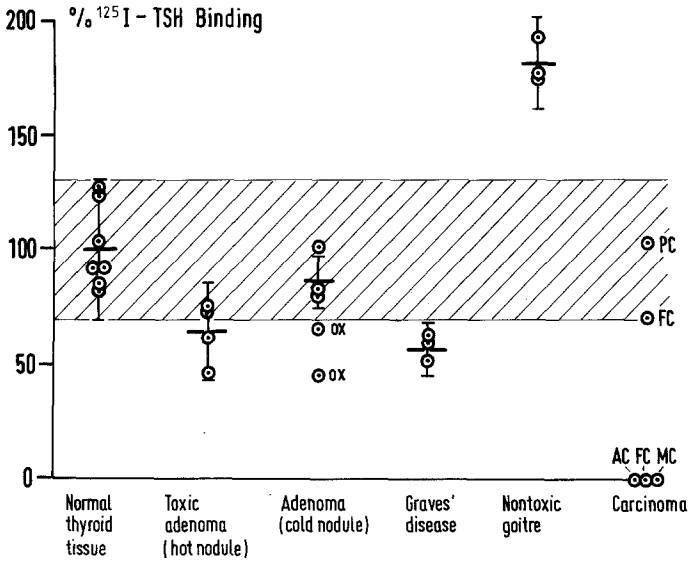

Fig. 1. Relative values and standard deviations of ^{125}I-TSH binding to follicle cells in different thyroid disorders (standard deviation of histologically normal thyroid parenchyma marked by hatching) (ox = oxyphilic; PC, FC, MC, AC = papillary, follicular, medullary, anaplastic carcinoma)

The mean values of ^{125}I-TSH binding measurements as determined by reflection photometry upon examination of a total of 22 human thyroids are shown in Fig. 1. To obtain evidence of the relative density of TSH binding in different thyroid disorders, the mean value from measurements of 7 specimens containing histologically normal thyroid as well as diseased parenchyma was normalised to 100%, the remaining measurements being related to this 100% value. Measurements of ^{125}I-TSH binding with these 7 specimens, however, varied considerably (82%–127%), and no clear-cut correlation was apparent between the degree of labelling and the kind of thyroid disease witnessed in the remainder of these glands (papillary and follicular carcinoma each $n=1$, toxic adenoma $n=2$, follicular adenoma $n=3$).

Follicle cells of toxic adenomas (hot nodules) ($n=4$), follicular adenomas ($n=5$), and Graves' disease ($n=3$) all showed less ^{125}I-TSH binding than those of histologically normal parenchyma. Typical examples of the 2 former lesions are given in Figs. 2 and 3. Among follicular adenomas, the 2 oxyphilic tumours exhibited lower values than the remaining 3 of orthoplastic appearance. Labelling of the 7 specimens of toxic adenoma and Graves' disease revealed lower binding values altogether than normal thyroid tissue or orthoplastic adenomas.

In contrast to this, the 3 non-toxic goitre cases demonstrated a powerful binding with a mean value of 180% when compared with normal thyroid tissue (Fig. 4). Hyperplastic microfollicular areas, seen in one of these lesions, showed a slight decrease in ^{125}I-TSH density when compared to

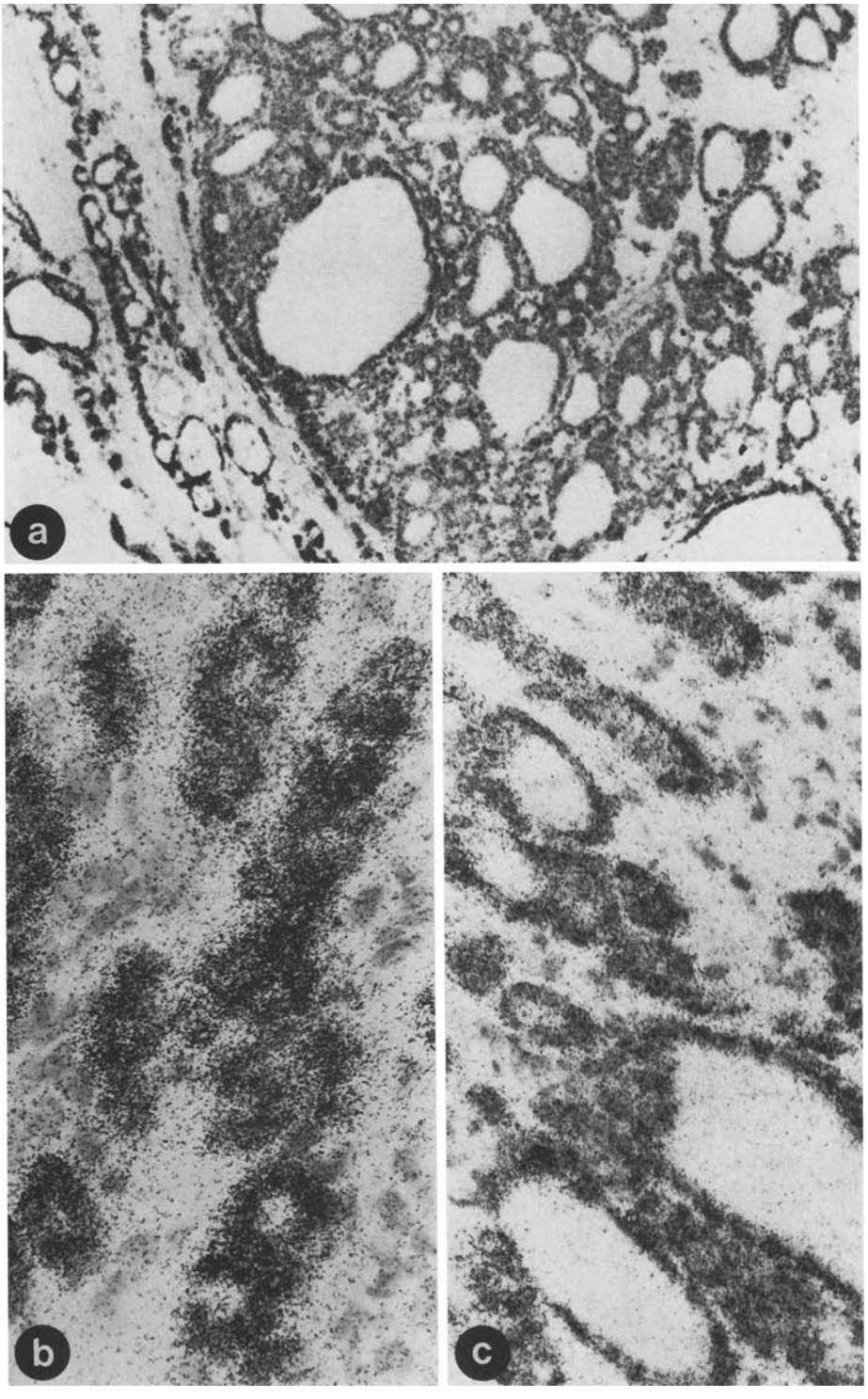

Fig. 2a–c. ^{125}I-TSH autoradiogram (frozen section) of toxic adenoma (hot nodule) with decreased binding of thyrocytes within the nodule as compared to extranodular parenchyma: **a** low power magnification, ×75; **b** residual parenchyma and **c** toxic adenoma, ×460

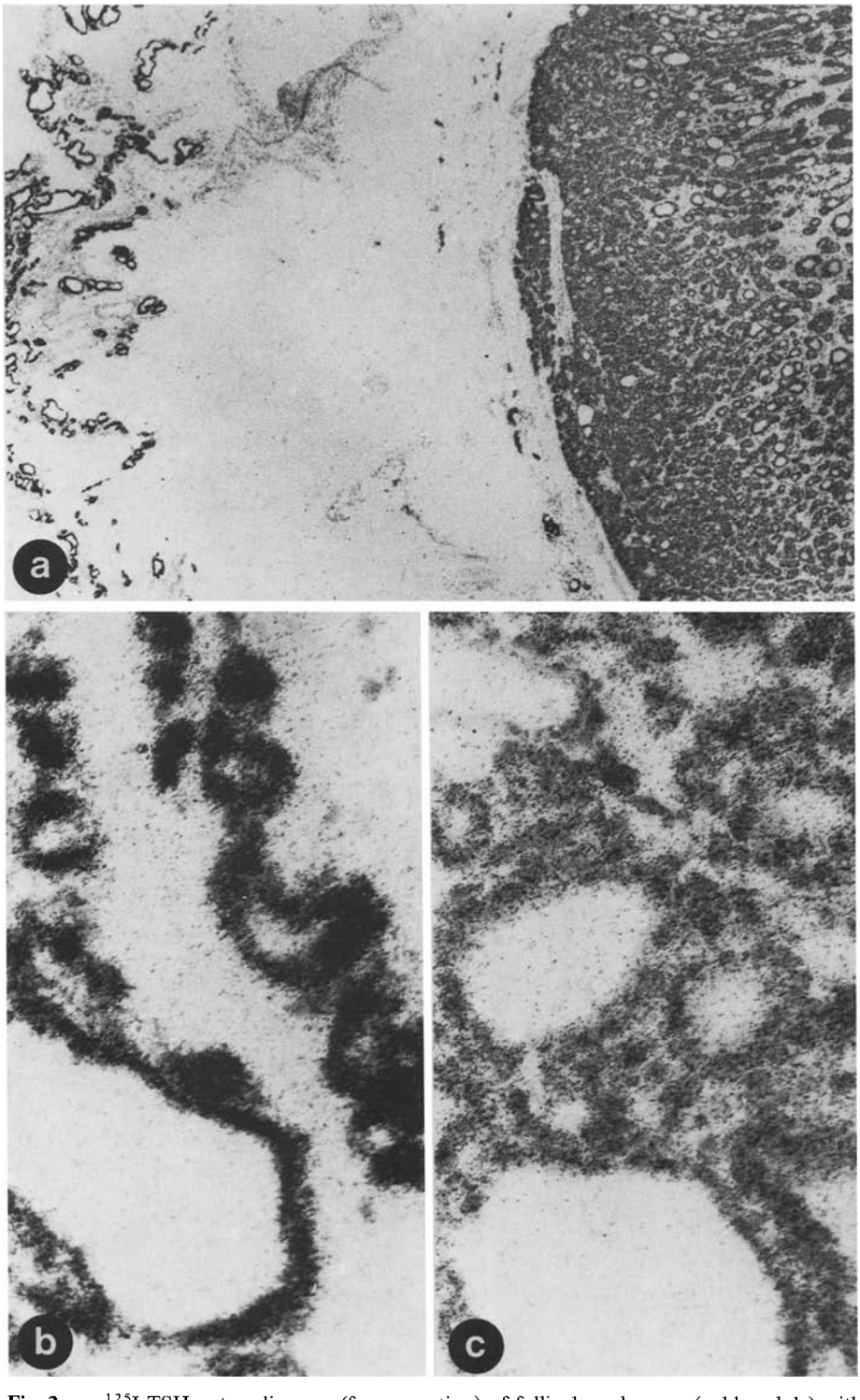

Fig. 3a–c. ^{125}I-TSH autoradiogram (frozen section) of follicular adenoma (cold nodule) with reduced binding of labelled hormone as compared to residual parenchyma: **a** low power magnification, ×29; **b** residual parenchyma and **c** follicular adenoma, ×460

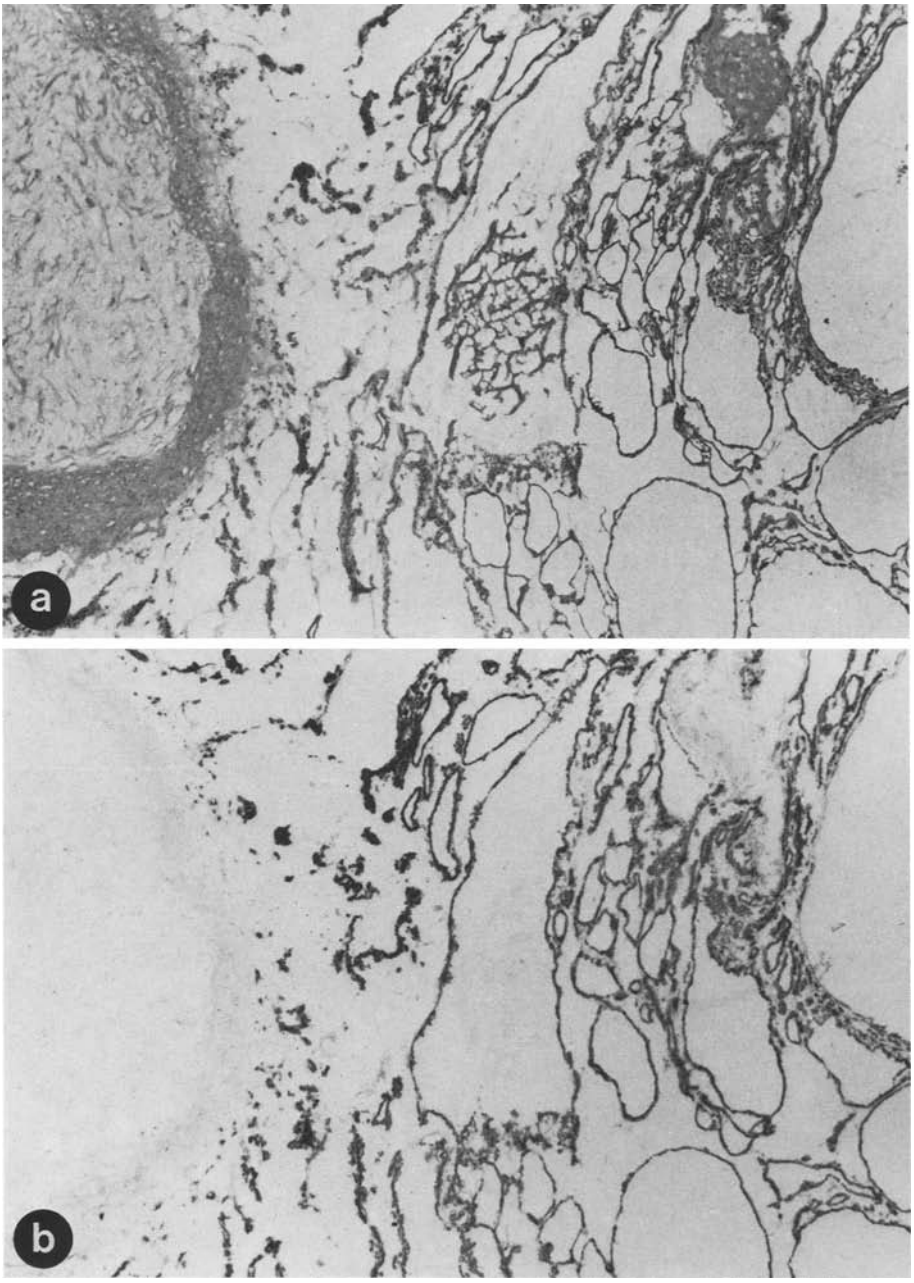

Fig. 4a, b. Frozen sections of nontoxic colloid goitre case with degenerative change (scar to be seen on the left): **a** H&E and **b** ^{125}I-TSH autoradiogram, × 29

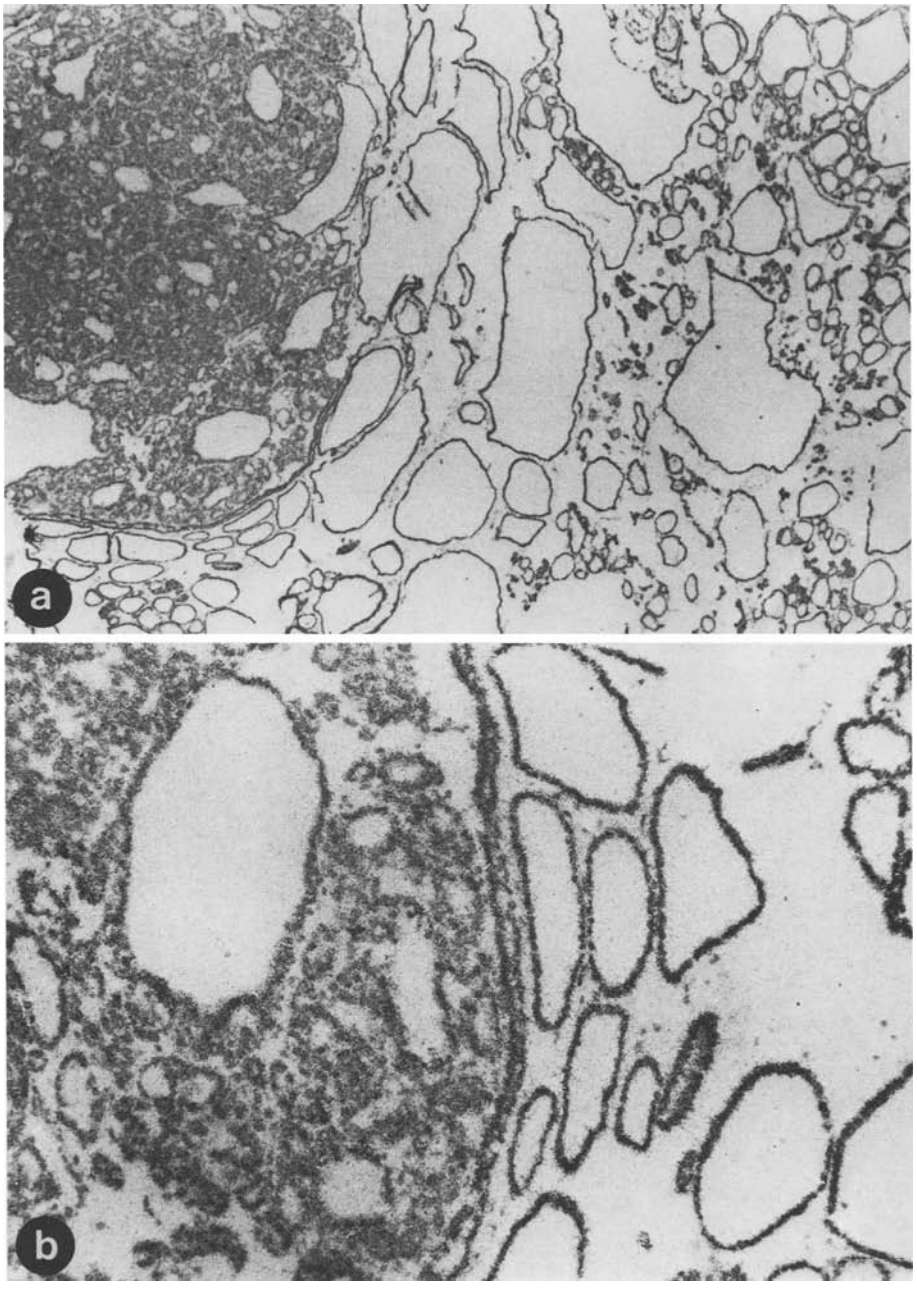

Fig. 5a, b. ^{125}I-TSH autoradiogram (frozen section) of nontoxic colloid goitre case with reduced binding of hyperplastic microfollicular areas: **a** ×29; **b** ×115

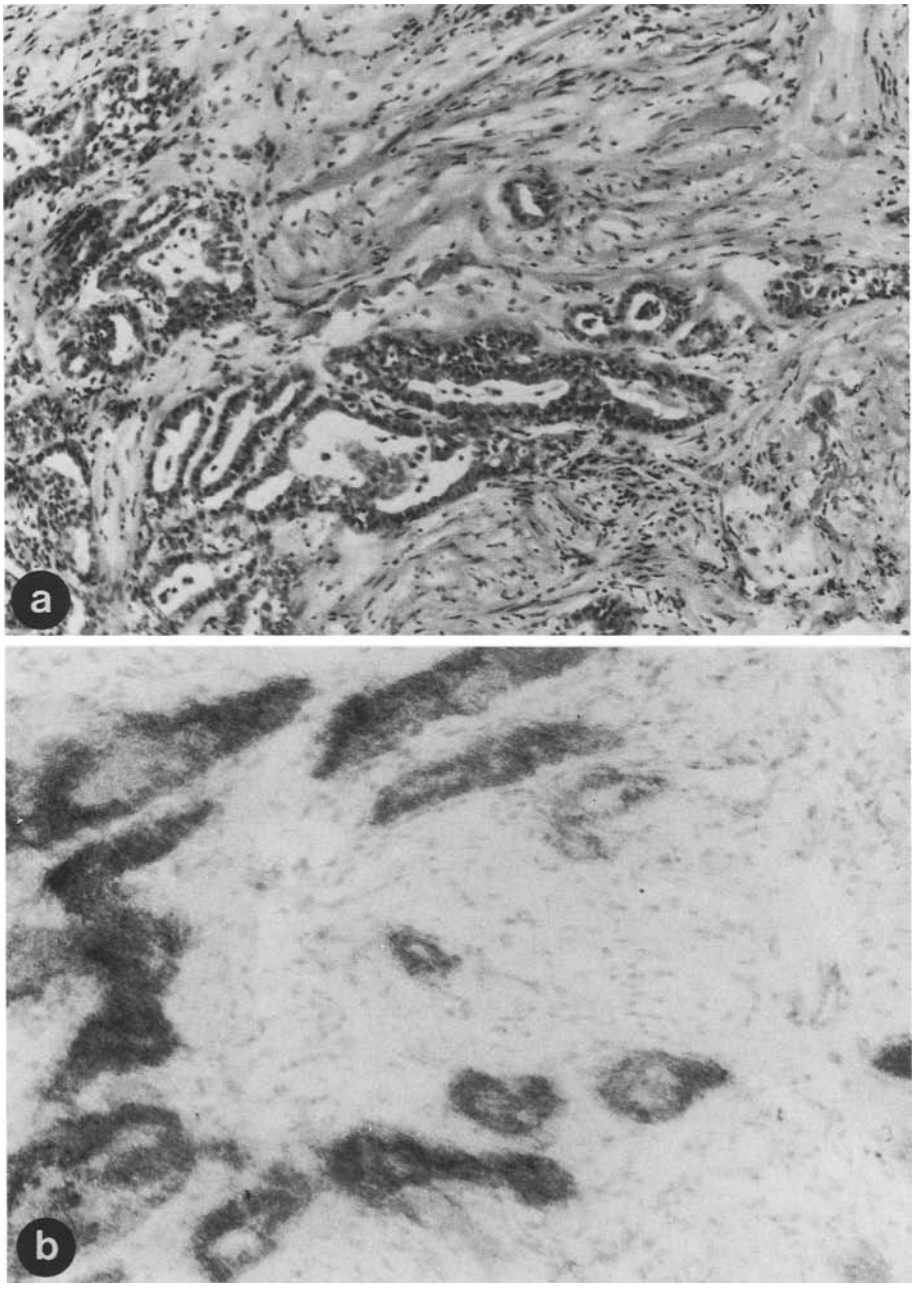

Fig. 6a, b. Frozen sections of follicular structured papillary carcinoma with intensive labelled neoplastic follicle cells: **a** H&E and **b** ^{125}I-TSH autoradiogram, ×115

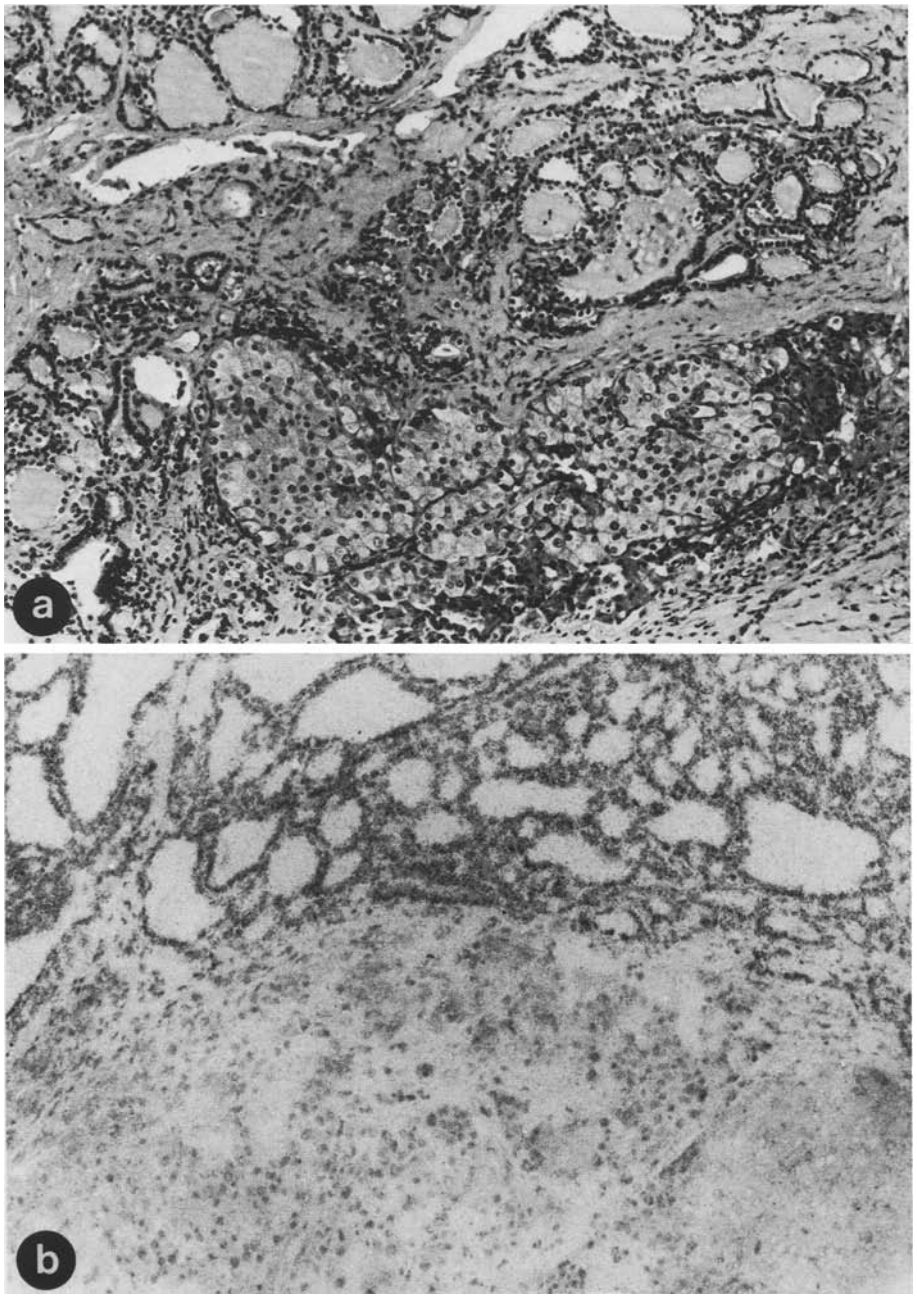

Fig. 7a, b. High grade follicular carcinoma without specific TSH-binding as compared to residual parenchyma: **a** paraffin section, H&E, and **b** ^{125}I-TSH autoradiogram, frozen section, ×115

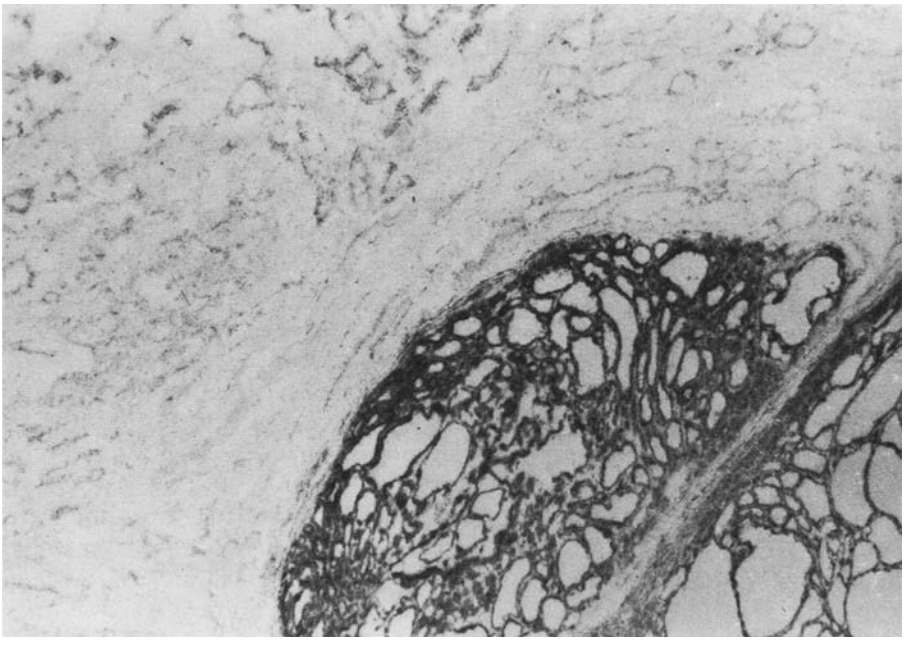

Fig. 8. ^{125}I-TSH autoradiogram of an intensively labelled non-toxic colloid goitre containing a medullary carcinoma (*left side*) without specific TSH-binding, × 29

the surrounding macrofollicles (Fig. 5). This difference of up to 20% was also observed with reflection photometry.

Neoplastic follicle cells of the only papillary carcinoma (Fig. 6) and one of the 2 follicular carcinomas featured in this study showed a ^{125}I-TSH binding equivalent to the mean value and the mean value minus standard deviation of inconspicuous thyroid parenchyma, respectively. Upon thyroglobulin immunohistochemistry, both lesions showed an intensive diffuse cytoplasmic reaction of some 40% of the tumour cells. In the remaining follicular carcinoma where metastases of the brain had been in evidence initially, no specific ^{125}I-TSH binding could be detected (Fig. 7). In contrast with the brain metastasis removed previously, the primary lesion was entirely devoid of thyroglobulin immunoreaction. In this respect, therefore, this high grade follicular carcinoma paralleled our findings with the 2 anaplastic and 2 medullary carcinomas (Fig. 8) under investigation, all of which lacked any evidence of ^{125}I-TSH binding.

Discussion

The interaction of TSH with a specific receptor on the thyroid cell surface is presumed to stimulate the adenylate cyclase system and initiate a cascade of cAMP kinase-regulated metabolic processes (Kohn et al. 1984). The ability of TSH to stimulate thyroid cell proliferation is also a well-recognized phenomenon, which has been confirmed by experimental studies (Nitsch

and Wollman 1980; Valente et al. 1983a, b). Trophic TSH effects, however, appear to utilize a different receptor domain or transmission system or both, not mediated by a cAMP signal (Valente et al. 1982). It was thus that Kohn et al. (1984) recommended the definition of membrane molecules, important to the cell surface recognition process, in binding studies rather than in functional response assays.

Moreover those investigations studying TSH receptors in terms of their adenylate cyclase response have yielded contradictory results which probably cannot be explained only by the use of different in vitro conditions. Evidence produced by in vitro studies is, moreover, restricted on account of the possibility of non-homogeneity of the tissue assayed: Takahashi et al. (1978) in contrast to other investigators demonstrated small numbers of binding sites in medullary carcinomas, and suspected this finding was due to the contamination of preparations with minute segments of normal thyroid tissue. Yet, of course, this reservation holds true for all TSH receptor assays which use membrane preparations.

The aim of the present study, therefore, was to establish a morphological method for the detection of the TSH binding site density in thyroid tissue which was structurally intact, combining it with the opportunity of associating the binding values ascertained to specific cell populations.

In the preliminary stage, we attempted to employ an immunoperoxidase staining sequence on the lines of Witorsch's (1979) methodology for the visualization of prolactin binding sites in human prostate tissue. Exposing both cryostat and paraffin sections of thyroid tissue to varying concentrations of TSH and then to human TSH antisera, however, failed to produce any immunospecific staining. Hence, we selected an autoradiographical method using ^{125}I-labelled bovine TSH for the portrayal of TSH binding sites. Our investigations were thus analogous to recently published studies on the localisation of receptors for somatostatin (Maurer and Reubi 1985), the corticotropin-releasing factor (De Souza et al. 1984), the putative neurotransmitter substance P (Mantyh and Hunt 1985a), and the hormone TRH (Mantyh and Hunt 1985b) by incubating tissue sections with the respective radiolabelled substances.

Previous studies have shown the bioactivity of the iodinated TSH to be comparable to that of unlabelled TSH (Azukizawa et al. 1977). The ^{125}I-TSH concentrations used in our procedure (30 nU/ml) were 30 times lower than those usually used in in vitro binding assays (e.g. Clark et al. (1981): 890 nU/ml) and 100 times lower than the physiological serum levels of hTSH (3 μU/ml) (Imagawa et al. 1982). In respect of pH value, temperature, and duration of incubation, our procedure resembled the in vitro assays.

The specificity of our method was demonstrated by the inhibition of labelling through the addition of increasing amounts of cold TSH; by furnishing evidence of ^{125}I-TSH binding in non-thyroidal tissues with the known presence of a TSH-responsive adenylate cyclase system (Kohn et al. 1985), namely guinea pig epididymal fat pad tissue (Teng et al. 1975), and by failure to detect specific labelling in tissues without TSH-binding being

demonstrated, using membrane preparations. ^{125}I-TSH appeared to bind saturably to hog thyroid cells, since labelling did not significantly increase when the radioligand concentration in routine use (407 Bq) was doubled. The fact that the procedure is reproducible was demonstrated by the detection of the identical silver grain density when sections of the same tissue samples were repeatedly incubated. However, we were not able to detect specific ^{125}I-TSH binding when paraffin sections of formalin or bouin fixated specimens were used.

The idea of using the autoradiographic TSH receptor visualisation on surgical specimens of thyroid glands was originally conceived to illustrate its applicability on human material. In various forms of thyroid disease we found reproducible deviations in the receptor labelling from normal tissue. Bearing in mind the small number of cases no conclusive statement can be made. However, our results should be examined in the light of in vitro findings in identical pathological conditions.

There were striking differences when measurements of different specimens of histologically normal thyroid tissue were compared, raising the question as to whether histologically inconspicuous thyroid tissue of normal appearance can be regarded as being functionally normal. Our results tie up with the in vitro findings which, apart from the between-assay variations, revealed a wide range of patient-to-patient variations (Carayon et al. 1980). Thus some authors have always compared the binding of tracer quantities to membrane preparations of neoplastic thyroid tissue with the binding to adjacent non-neoplastic tissue removed from the same patient (Clark and Castner 1979).

Our findings of a similarly reduced ^{125}I-TSH binding in hyperfunctioning "hot" nodules as well as in "cold" adenomas parallel the in vitro observations of Clark et al. (1981) who investigated both conditions, and of Takahashi et al. (1979) who described reduced binding in the particulate fraction in adenomas.

The reduced binding of thyroid tissues from patients with Graves' disease agrees with the findings of Takahashi et al. (1978) who demonstrated a significant decrease in the number of low affinity sites and a marginally significant decrease in high affinity sites. It is highly probable that this can be attributed to competition from other thyroid stimulators rather than from endogenous TSH, the immunoassayable TSH being suppressed in the patients.

The strong labelling of non-toxic colloid goitre requires further elucidation. The same applies to the reduced binding density in hyperplastic microfollicular areas, observed with one of these lesions. Our findings might possibly find confirmation in Clark's brief mention that "colloid goitre binds well" (discussion subsequent to address given by Clark et al. (1981)). Carayon et al. (1978), however, reported non-toxic multinodular goitres as having the same TSH binding site concentrations as normal thyroid.

The receptor capacity of differentiated thyroid carcinomas has been stated to be normal (Ichikawa et al. 1976; Abe et al. 1981) and markedly reduced (Clark et al. 1981) in different test series. These differences are

in accord with our own data and, since tissue preparation and incubation conditions were absolutely identical for all our cases under investigation, they would appear to be caused by tumour to tumour variation. Thus, documenting the presence or absence of TSH receptors may be clinically important, not only in predicting survival, but also in selecting patients who may benefit from TSH-suppressive oral replacement or who require more aggressive surgical and medical therapy.

Lack of specific binding in our medullary and anaplastic carcinoma cases parallels the findings of Carayon et al. (1980) and Abe et al. (1977, 1981). This seems to be the reason why TSH-suppressive therapy is ineffective in the treatment of these two conditions.

With its ability to associate data on TSH binding site density with certain histological features our procedure renders the simultaneous detection of different functional and structural variables possible in a given tissue specimen. Clark et al. (1985) recently described the coexistence of estrogen and TSH receptors in the cytosol and in a membrane particulate fraction of several neoplastic and non-neoplastic thyroid tissues. In employing both a histomorphological method of estrogen receptor visualization and TSH binding site identification, evidence as to whether these two kinds of receptors are located in the same cell population would the provided. Yet a comination of thyroglobulin-, T_3-, or T_4-immunohistochemistry and the autoradiographic demonstration of ^{125}I-TSH binding, does appear to be suited to detect the dissociation of different partial functions of neoplastic follicle cells. Thomas-Morvan et al. (1982) came to this conclusion in demonstrating TSH binding to be necessary, although not sufficient, for iodine metabolism. Thus, these authors suspected additional defects in some of these lesions which caused lack of either iodine uptake or organification.

Because of the limited resolution power of the light microscopical autoradiograms used in this study, we were unable to localise the site of ^{125}I-TSH binding within radiolabelled cells accurately. We are not, therefore, in a position to discuss Fahraeus-van Ree and Farid's (1984) findings in respect of TSH binding sites in nuclei and nuclear matrix of porcine thyrocytes. However, we do expect to obtain further evidence from the combined use of ^{125}I-TSH autoradiography with both cryo-ultramicrotomy of snap frozen thyroid tissue and electron microscopy of cultivated thyrocytes.

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