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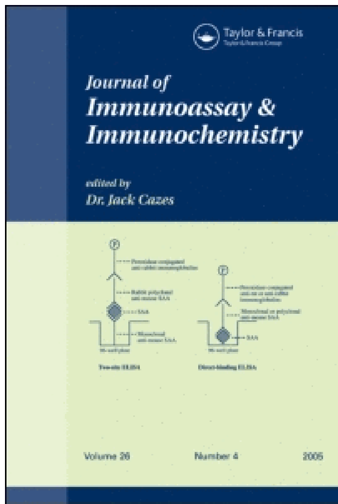

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A CYTOCHEMICAL SECTION-BIOASSAY FOR THYROTROPIN

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ABSTRACT

A cytochemical section-bioassay of thyrotropin has been developed which retains the sensitivity of the earlier segment-assay (10^{-4} μU/ml) and allows a considerable increase in the rate at which these within-animal bioassays can be done. The index of precision (n=23) was 0.11±0.06; intra-assay variation was 1.3%; inter-assay variation was 10.9±6%. Fiducial limits (p=0.95) ranged from 65-150% to 78-127%. The system is also capable of detecting thyroid-stimulating immunoglobulins.

The cytochemical segment-assay of thyroid stimulators (1) is well established (e.g. 2, 3, 4). It depends on measuring the labilization of the lysosomal membranes in the follicle-cells of guinea-pig thyroid maintained *in vitro*, in short-term organ culture and exposed to graded concentrations of the stimulator. The change in the functional state of the lysosomal membranes is quantified by microdensitometric measurement of the increased permeability of these membranes to the

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chromogenic substrate, leucine 2-naphthylamide, which is cleaved by the intralysosomal arylamidases (5). The limit of sensitivity of the cytochemical segment-assay of thyrotropin, of about 10^{-4} µU/ml, is at least 1000 times as great as that of the equivalent radioimmunoassay (about 0.5 µU/ml) so that it has been used to assay low normal circulating levels (0.1-0.5 µU/ml) as well as to explore the pathophysiology of sub-normal levels (2, 4). Thus the advantages of this assay are: (i) it can measure low normal circulating levels of thyrotropin (TSH) that are currently below the limit of detection by radioimmunoassay; (ii) it measures the biologically active hormone only, so allowing the distinction of immunoreactive (but biologically inactive) hormone in certain cases of hypopituitary hypothyroidism (6, 7, 8); (iii) it can be used to detect thyroid stimulators such as thyroid-stimulating immunoglobulins (TsI) even if they are not of the type known as LATS (3, 9).

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The drawback to this segment-assay is that, being a within-animal assay, four segments are required for establishing the calibration dose-response graph for the thyroid tissue of that animal, and two more must be used for each sample to be assayed, at two different concentrations, to establish parallelism of response. Yet it is rare to be able to cut more than six segments, containing uniform and normal follicles, from the thyroid gland of a normal guinea-pig. Making such glands goiterous is not helpful, because the cells become less responsive. The poor through-put of the segment-assay (2 or 3 a week) stultified its application to the many clinical and research problems to which it was uniquely suited.

Furthermore, a much faster assay was needed for the bioassay of thyrotropin-releasing hormone for which a two-stage, sensitive assay, based on the assay of TSH released from segments of a pituitary gland *in vitro*, had been devised (10).

This communication describes a method, and its validation, for a cytochemical section-assay of thyroid stimulators developed along the lines of the earlier section-assay for corticotropin (11).

METHOD

The method which was finally devised was as follows. The thyroid gland was removed from a female guinea-pig which had been killed by asphyxiation in nitrogen. The animals were of the Hartley strain, weighing about 300 g, the thyroid gland of heavy animals becoming too cystic and unresponsive. Gently and expeditiously, the two lobes were freed of connective tissue and fat. Each was bisected and placed on the lens-tissue on the metal-mesh table used for Trowell adult-organ maintenance culture (12, 13); the normal Trowell T-8 medium, pH 7.6, was added to the vitreosil dish, in which the table stood, to reach up to the level of the lens-tissue. In this way the thyroid segments were fed with culture-medium by capillarity through the lens-tissue, without being immersed in the medium. The culture-pots were sealed and the atmosphere inside the pots was replaced by 95% oxygen:5% carbon dioxide. The thyroid segments were maintained *in vitro* at 37°C for 5 h.

At the end of this period the segments were chilled to -70°C in n-hexane ('free from aromatic hydrocarbons' grade, boiling range +67-70°C); after 30 sec, or up to 60 sec, the chilled segments were

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removed from the hexane with cold forceps and placed in pre-cooled glass tubes for storage at -70°C. This chilled tissue could be stored for up to 2 days; thereafter it showed marked loss of responsiveness to thyroid stimulators. During this time it was sectioned at 12 μm in a Bright's cryostat equipped with an automatic cutting device which controlled the rate at which the sections were cut and so ensured a constant thickness of sections (14, 15). This thickness was found to be optimal: it was just larger than the height of the follicle-cells (and therefore assumably the thickness of these cells) so increasing the possibility of having intact cells within the sections. It was found helpful to cut a ribbon of three sections and mount this triad on the slide, the middle section being free of distortion. The methods of chilling, sectioning and transferring the sections on to glass slides were those described in detail by Chayen *et al*. (16).

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The sections had to be processed as soon as possible after they had been cut. They were dried at 37°C for 10 min, during which time they were clipped in duplicate, back-to-back, into a loose-fitting lid of a hormone-reaction chamber (Fig. 1). The clips were so arranged that, when the lid was placed in position on the chamber, each pair of slides fitted into an individual well cut in the Perspex base of the chamber. A groove cut into the vertical back of the lid, next to each clip, facilitated the precise orientation of the slides. It was helpful to mount the sections towards one end of the slides to ensure that the sections fitted into the well and so would be covered by the medium.

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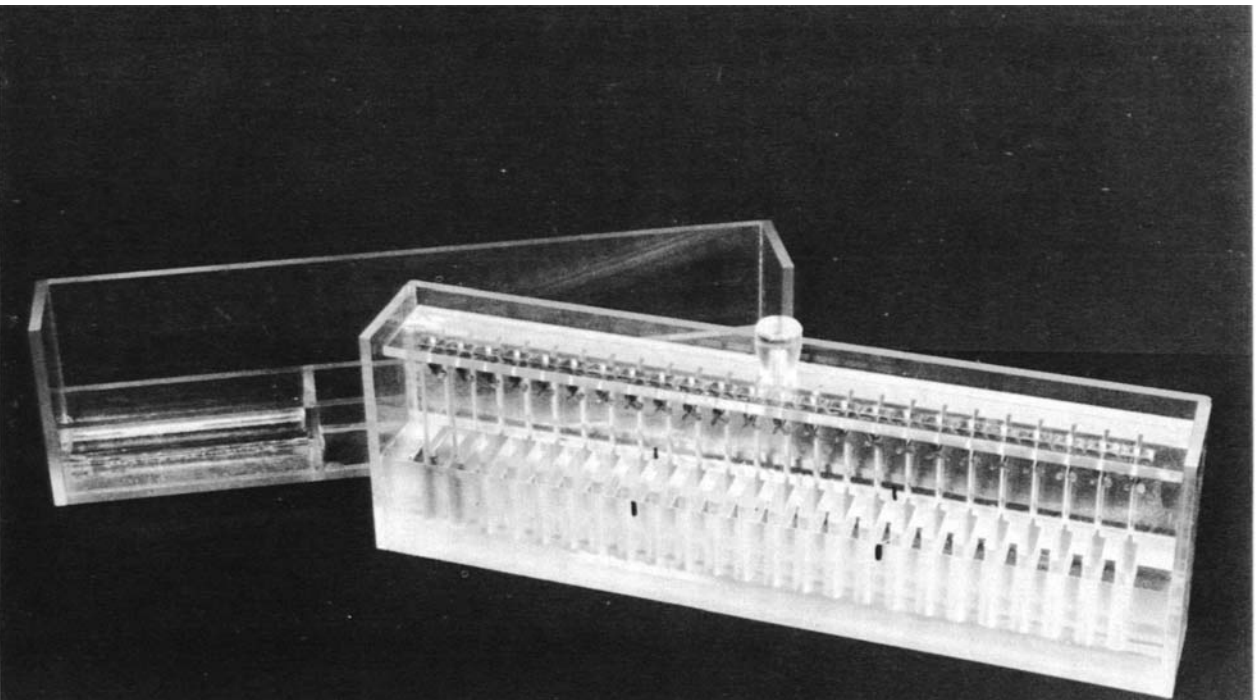

Figure 1. The apparatus for the section-assay. Foreground: the reaction-chamber, divided into separate wells so that each pair of duplicate sections, held-back-to-back in the lid, can be located in its relevant well. The vertical back of the lid is grooved to orientate the sections precisely. Background: the cytochemical-reaction trough.

Each of the first four wells contained one of four logarithmically graded concentrations of the standard preparation of the hormone or other stimulator (e.g. MRC Research A standard at 10^{-4} to 10^{-1} μU/ml inclusive). The next two wells contained the dilution of the first plasma to be assayed (e.g. at 1/100 and 1/1000 dilutions); the next two wells contained dilutions of the second plasma, and so on. A quality-control sample could be included in another two wells.

The main problem in developing this section-assay was to preserve the integrity of the sections at pH 7.6 while not overstabilizing the cells so that they, and the lysosomal membranes, could not respond to the stimulator. Consequently all the samples, whether of the standard preparation of the stimulator or of the plasma to be assayed, were diluted in the following medium. Firstly, a 0.1% aqueous solution of gum tragacanth (Hadassah Export Co., P.O. Box 614, Teheran, Iran) was prepared and left to stand overnight at room temperature, with constant stirring, to achieve effective 'solution' or dispersion of the gum. To 120 ml of T-8 medium were added 30 ml of this solution, to achieve a final concentration of the gum tragacanth of 0.02%. Anhydrous sodium acetate (615 mg) was added to these 150 ml of solution, giving a final molarity of 0.05 M. Both of these were included to help stabilize the sections.

Trowell's T-8 medium contains sufficient bicarbonate to equilibrate with 5% carbon dioxide to a pH of 7.6. Because the section-assay medium will be used in the normal atmosphere prevailing in the hot-room, some of the bicarbonate must be removed so that the medium equilibrates to the same pH against the atmosphere in the laboratory. This is achieved

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by the addition of hydrochloric acid. Generally it sufficed to add 2.4 ml of 1N hydrochloric acid to the 150 ml of medium (which contained 120 ml of T-8 medium: this applied to the Trowell's medium supplied by Gibco Europe, and probably to that from other sources). This addition of acid to the medium caused the indicator, in the medium, to change to a yellow colour. The medium was left at 37°C in a large conical flask, with a cotton-wool plug as a stopper, and agitated occasionally to assist the equilibration. After about 30 min the colour of the indicator reverted to normal, showing that the pH had stabilized at about pH 7.6. This was then checked by the use of a pH-meter.

When the sections were clipped in the lid, and the wells filled appropriately, the lid was placed on the Perspex base so that all the sections were immersed simultaneously into the appropriate medium. This was done in a hot-room, at 37°C. After the required time (90 sec for the assay of TSH) the lid was lifted and placed on the cytochemical-reaction trough, so plunging the sections simultaneously into the cytochemical chromogenic reaction-medium. This medium consisted of the following:

0.1 M acetate buffer, pH 6.1	100 ml
0.85% sodium chloride	80 ml
0.02 M potassium cyanide	10 ml
leucine 2-naphthylamide hydrochloride (Sigma)	80 mg
dissolved in 10 ml of distilled water.	

The pH was adjusted to pH 6.5 and the medium warmed to 37°C. Then, just before the solution was poured into the trough, 100 mg of Fast blue B (zinc salt; Serva) were dissolved in the medium.

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The sections were reacted at 37°C for 6 min and then plunged into a second trough containing 0.85% sodium chloride. They were then transferred, for about 5 min at room temperature, to a bath containing an 0.1 M solution of copper sulphate to chelate the reaction-product. Each section was then mounted under a cover-slip in the water-soluble Farrants' solution which had been adjusted to pH 6.5 with sodium acetate. (The method has been described by Chayen _et al_., 16, and Bitensky and Chayen, 5.)

The reaction-product in individual thyroid-follicle cells, normally one cell from each of at least ten follicles per section, was measured in the middle section of each triad, by means of either a Vickers M85 or a Barr and Stroud GN2 scanning and integrating microdensitometer (as discussed by Bitensky, 17) at 550 nm, with a x100 oil-immersion objective; the scanning spot was 0.2 μm in the plane of the specimen.

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RESULTS

The time-course of the response of the cells, in sections, to TSH at 10^{-1} and to 10^{-2} μU/ml showed maximal lysosomal naphthylamidase activity at 90 sec exposure to the hormone (Fig. 2). Samples of sera, obtained from patients with Graves' disease, and tested at 1:100 dilution, showed maximal stimulation at 3.5 min and minimal activity at 90 sec. In sections exposed to the medium alone, the activity decreased over the first 60 sec and remained low during the subsequent 2.5 min.

With a standard period of exposure to the hormone of 90 sec, TSH gave a linear log-dose response over the range of 10^{-4} to 10^{-1} μU/ml.

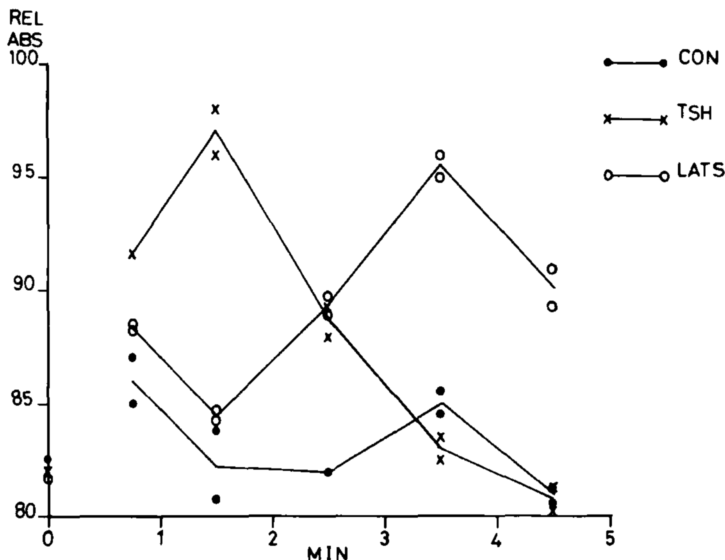

FIGURE 2. Time-course of the lysosomal naphthylamidase response in sections exposed to a single concentration of either (i) TSH (crosses) or (ii) a serum (diluted $1:10^2$) containing LATS (open circles), compared with the response to the vehicle alone (filled circles).

The mean naphthylamidase activity, denoted by the mean relative absorption (Rel Abs) recorded in 20 thyroid follicle cells, is shown in each of the duplicates used for each time.

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Dilutions of normal plasma, at 1:100 and 1:1000 concentrations, gave parallel responses (Fig. 3). The addition of an antibody specific to human TSH reduced the concentration of TSH-activity in the plasma from 0.4 µU/ml to 0.003 µU/ml.

To 0.8 ml of a plasma which had been assayed as containing TSH at 1.4 µU/ml were added an additional 10 µU of the MRC Research A standard preparation of TSH (0.2 ml containing 50 µU/ml). The plasma was then assayed again at dilutions of 1:100 and 1:1000; it was found to contain 12 µU/ml, so giving a recovery of the added TSH of 108%.

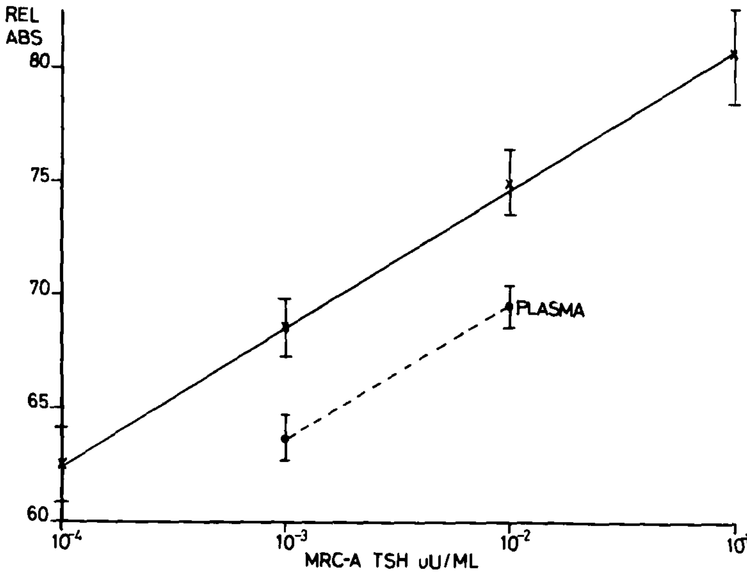

FIGURE 3. The standard calibration response-graph of the mean lysosomal naphthylamidase activity (Rel Abs) induced by increasing concentrations of a standard preparation of TSH (crosses). The plasma (filled circles), at $1:10^2$ and $1:10^3$ dilutions (not related to the calibration on the x-axis), gave a parallel response; the concentration of TSH in these two dilutions of the sample can be read off the calibration-graph. Bars indicate the mean value in each of the duplicate sections.

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The effect of a standard preparation of luteinizing hormone (LH; MRC standard 68/40) was tested in this assay system at concentrations of 7.7 mU/ml and 77 μU/ml. The former gave activity equivalent to that of TSH at $2x10^{-4}$ μU/ml which was neutralized by the antibody specific to human TSH; the activity of the lower concentration was undetectable.

To estimate intra-assay variation, five or six sections were measured for each point in four separate assays. The mean coefficient of variance in 23 samples was 1.3% with values ranging from 0.5 to 2.8%. The inter-assay variation (n = 17) was 10.9±6% (mean and standard

deviation). The index of precision (n = 23) was 0.11±0.06 (SD). The slope (b) was 3.8±0.8 (mean and standard deviation; n = 20). There was acceptable parallelism of the response to samples of plasma, tested at two dilutions, with the response to the standard preparation of the hormone, as shown by the coefficient of variance between the two dilutions, in 20 assays, being only 8.2±6.4%. The fiducial limits ($p = 0.95$) ranged from 65-150% to 78-127%.

DISCUSSION

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The new section-bioassay appears to retain all the advantages of the earlier segment-assay and allows many more samples to be assayed. Moreover, since each sample, as well as those for the standard calibration-graph, are all assayed on serial sections of the same piece of thyroid-tissue, it is feasible to use human tissue for this assay. Even though much human thyroid-tissue, removed at operation, may be too diseased to be suitable for such work, the specimens can be checked histologically to find one block of acceptable tissue. This can be of some advantage when it is required to test human plasma for the presence of thyroid-stimulating immunoglobulins which might be suspected of being species-specific. It is a further advantage of this assay, as of the earlier segment-bioassay, that thyroid-stimulators other than thyrotropin can also be detected and measured by the same procedure; at suitable concentrations the long-acting stimulators can be distinguished from TSH by the longer time required to express their activity (18).

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