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Cytochemical methods of hormone assay

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Initially polypeptide hormones were assayed by their biological activity. Such conventional bioassays, however, are remarkably insensitive. For example, the Lipscomb and Nelson (1962) bioassay of corticotrophin (ACTH) cannot measure a concentration of less than about 100 pg/ml, while the normal circulating levels of the bioactive form of this hormone range from about 5 to 60 pg/ml. The introduction of radioimmunoassay increased the sensitivity with which hormones could be assayed quite remarkably, that of ACTH being able to measure as little as 10 pg/ml. Moreover, the number of assays which could be done in unit time by radioimmunoassay was far greater than could be achieved by conventional bioassay. The fact that radioimmunoassay fundamentally measures the number of specific molecules (or antigenic determinants) present rather than the functional activity of the hormone might be regarded as an improvement on bioassay, representing a precise chemical rather than a biological analytical tool.

However, so long ago as 1967 it was recognised that immunoassays measure a composite of antigenic activity which is not necessarily related to the biological activity of the hormone. A special committee, convened by the World Health Organization, noted a need for 'micro-bioassays' which would have at least the same sensitivity as radioimmunoassay and which could be done in parallel with it (World Health Organization, 1975). Since then reports concerning the dissociation of immunoactivity and bioactivity (for example, Besser *et al.*, 1971) have increased alarmingly. Thus gastrin has been shown to occur in a number of forms which show various biological activities (Yalow, 1974).

Apart from this problem of assaying molecules which may have little or no biological activity, the sensitivity of radioimmunoassay may be insufficient to measure low normal circulating levels of some hormones or pathologically low levels that might occur in some diseases. Thus the population survey of Tunbridge *et al.* (1976) and the other studies of Petersen *et al.* (1975) clearly show that the circulating level of TSH in a considerable proportion of normal people may be below 1·0 μU/ml or even below 0·5 μU/ml. Yet this is the sensitivity level of even the best radioimmunoassays currently available for this hormone.

Therefore there is clearly a need for a functional (that is, biological) assay system at least 10 times more sensitive than the current radioimmunoassays. Such bioassays could be performed in parallel with the more conventional radioimmunoassays to test whether the molecules detected by the latter were functional. Their greater sensitivity would also be of value when only small samples were obtainable, as in investigations of hormones in neonates (Holdaway *et al.*, 1973) or when many serial samples were required (Daly *et al.*, 1974a), and when the hormonal levels were below the limits of precise measurement by radioimmunoassay.

Organ maintenance culture

The effects of hormones can be demonstrated and investigated by maintaining the target organ in an organ culture system and observing the effect of adding the specific hormone (Gaillard and Schaberg, 1965; Fell and Rinaldini, 1965; Lasnitzki, 1965). Such investigations, while of immense value in their own context, were unsuitable for the measurement of very low concentrations of hormones. Probably, however, low levels could be assayed if instead of a morphological response a change in biochemical activity induced by the hormone in such organ cultures could be measured exclusively in the most responsive cells of the target tissue.

Quantitative cytochemistry

The major technological advance in the past ten years to make this aim possible is the development of cytochemistry as a precise form of cellular biochemistry (Chayen and Bitensky, 1968; Chayen *et al.*, 1974). Sections of unfixed, chilled tissue can be cut without producing a measurable ice artifact or change in biochemical activity, and the sections can be subjected to a histochemical reaction without loss of material, including 'soluble' enzymes, from the cells. The coloured and precipitated product

produced by controlled 'histochemical' procedures (Chayen *et al.*, 1973) is examined in a scanning and integrating microdensitometer which accurately measures chromophores even when precipitated in an optically heterogeneous fashion (Bitensky *et al.*, 1973).

These quantitative techniques have been validated in a number of ways. For example, Altman (1972) found the same activity in two 'soluble' dehydrogenases in rat liver whether he measured them by conventional homogenate biochemistry or by quantitative cytochemistry (see also Chayen, 1977) Butcher (1970) found the same K_m/K_i ratio for succinate dehydrogenase activity in sections as had been reported from biochemical studies. The final evidence for the sensitivity and precision of quantitative cytochemistry has been the development of these cytochemical bioassay systems (Chayen *et al.*, 1976). All of them depend on the precise measurement of a change in biochemical activity induced by the hormone acting on its specific target cells. I will use the assay of two polypeptide hormones—namely, ACTH and TSH—to illustrate the procedures.

Cytochemical bioassay of ACTH

The first cytochemical bioassay was that for corticotrophin (Chayen *et al.*, 1972). It is based on the ascorbate-depletion bioassay of Sayers *et al.* (1948). The procedure is as follows.

The two adrenal glands are removed from a guinea-pig and each is cut into three segments. Each segment is maintained *in vitro* in non-proliferative maintenance culture (Trowell, 1959) for five hours in the presence of a synthetic culture medium (Trowell's T8 medium) reinforced with 10^{-3}M ascorbate. During this period the cells recover from the trauma of the excision of the gland, they are freed from the effects of endogenous hormone, and they replenish their content of ascorbate (Chayen *et al.*, 1972, 1976). At the end of the culture period the culture medium around each segment is replaced with identical medium containing one of a graded series of concentrations of ACTH (4 segments) or of the plasma diluted 1/100 or 1/1000. The hormone is allowed to act for four minutes, which is sufficient to give the maximal response at the concentrations used in these assays. The segments are then chilled to -70°C, sectioned at -25°C with the knife cooled to below -70°C with solid CO_2, and the sections are treated to demonstrate their ascorbate content. The reaction requires the use of a mixture of ferrocyanide and ferric ions so adjusted as to enhance its response to ascorbate rather than to other reducing moieties (Loveridge *et al.*, 1975). The resulting Prussian blue colour is measured in the cells of the zona reticularis by scanning and integrating microdensitometry.

The results from the four segments treated with graded concentrations of the standard preparation of the hormone show a negative linear correlation between the concentration of ACTH applied (5×10^{-15} to 5×10^{-12} g/ml) and the amount of reaction-product (Prussian blue) in unit area of the zona reticularis. The variation between serial sections from any segment treated with any one concentration of the hormone is not more than $\pm 4\%$. A similar variation is found between segments treated independently, each with the same concentration of ACTH (Daly *et al.*, 1974b). The concentration of ACTH in the plasma can be measured from this calibration regression line; after correction for dilution the two readings (at 1/100 and 1/1000 concentration) should agree to within $\pm 15\%$.

The index of precision, between 0·05 and 0·09, and the fiducial limits of a number of assays ranging from 87-115% to 98-102% (Daly *et al.*, 1974b), are achieved largely because of the cytochemical precision. The fact that it is a 'within-animal' assay also contributes to this precision.

The assay is highly specific for ACTH (Holdaway *et al.*, 1974). Tested on samples of pooled plasma to which known amounts of ACTH were added it gave the same results as the Lipscomb-Nelson bioassay, which measures corticosterone release in rats *in vivo*, and as radioimmunoassay (Rees *et al.*, 1973b). However, when the secretion of endogenous ACTH changes rapidly, as in the insulin-induced hypoglycaemia test, bioactivity and immunoactivity are sharply dissociated, probably owing to the presence of immunologically active but biologically inert fragments of ACTH, the half lives of which are longer than that of the complete hormone (Fleisher *et al.*, 1974). It is for such situations that it is important to be able to use a micro-bioassay to check radioimmunoassay. Moreover, this cytochemical bioassay distinguishes between biologically active ACTH and the other forms of immunologically measurable ACTH that occur in certain lung tumours (Bloomfield *et al.*, 1977).

Section assay of ACTH

The tissue is preserved during chilling and sectioning sufficiently to allow the cells in sections to respond to ACTH with the same sensitivity as when tested in segments of adrenal tissue. Thus cytochemical section assay for this hormone is possible (Alaghband-Zadeh *et al.*, 1974). Probably all cytochemical bioassays will be converted to section-assays, because these are much quicker procedures.

In practice, segments of the adrenal gland are

maintained *in vitro* for five hours, for the reasons I have already explained. This tissue is then 'primed' with ACTH at a concentration ($0{\cdot}5 \times 10^{-15}$ g/ml), which has no other detectable effect in the assay than to maintain full sensitivity of response. The segments are then chilled and sectioned at 20 μm, a thickness which is just greater than the largest dimension of the cells of the zona reticularis. The sections are then exposed in duplicate either to the graded concentrations of the hormone or to samples of plasma each diluted 1/100 and 1/1000 in the T8-ascorbate medium containing a colloid stabilizer which is used in the other methods of quantitative cytochemistry to preserve the integrity of sections. The time to achieve maximum response is now only one minute. The cytochemical reaction for ascorbate and its measurement are as for the segment assay.

One worker can make 30-40 section assays a week. The procedure is therefore comparable in output with radioimmunoassay.

Cytochemical bioassay of thyrotrophin

This assay depends on the ability of TSH to increase the permeability of the lysosomal membranes of the thyroid follicular cells concomitant with the endocytosis of colloid. This is demonstrated by cytochemical measurement of the activity of an intralysosomal enzyme by means of a chromogenic substrate which diffuses through the lysosomal membrane. The activity of the enzyme is expressed as a percentage of its maximal activity measured after the membrane has been rendered fully permeable to the substrate by a suitable procedure (Bitensky *et al.*, 1973; Bitensky and Chayen, 1977). Such methods indicate the remarkable degree of preservation in the tissue sections of even these organelles, which are very sensitive to slight degrees of damage.

The procedure is to maintain segments of the thyroid gland of one guinea-pig for five hours *in vitro* in T8 medium (without added ascorbate), as in the assay of ACTH. Each segment is then exposed for seven minutes to one of a series of graded concentrations (10^{-4} to 10^{-1} μU/ml) of the standard preparation (MRC A standard) of thyrotrophin (TSH) or to the plasma diluted 1/100 or 1/1000. The tissue is chilled, sectioned, and treated to show lysosomal naphthylamidase activity. The colour so generated is measured in individual thyroid follicle cells by scanning and integrating microdensitometry. Increased permeability of the lysosomal membrane, induced by increasing concentrations of TSH, is recorded as increased amount of reaction product produced in unit time of the cytochemical reaction. This assay is 10 000 times more sensitive than the equivalent radioimmunoassay (Bitensky *et al.*, 1974). Moreover, by extending the time of exposure to the hormone and the plasma the activity of thyroid-stimulating antibodies may be measured (Bitensky *et al.*, 1974; Petersen *et al.*, 1975; McKenzie and Zakarija, 1977).

As in the case of ACTH, this cytochemical bioassay shows good agreement with radioimmunoassay when there is no rapid flux of the hormone or alteration in the hormone molecule (Petersen *et al.*, 1975). However, there have been a number of cases recently where, in agreement with Krieger's (1974) findings, discrepancies between radioimmunoassay and bioassay, including cytochemical bioassay, have been found (Belchetz, 1976).

Other hormones

The fundamental principle of the cytochemical bioassay system can be applied to the bioassay of any biologically active molecule. It has been used to assay luteinizing hormone (Rees *et al.*, 1973a; Kramer *et al.*, 1974), gastrin (Loveridge *et al.*, 1974), and thyrotrophin-releasing hormone (Gilbert *et al.*, 1975). Preliminary studies (Chambers *et al.*, 1976) indicate a possibility of a cytochemical bioassay of parathyroid hormone reaching a sensitivity of 1×10^{-15} g/ml. The established methods are already widely used in Britain and elsewhere, as are the micro-bioassay systems recommended by the World Health Organization (World Health Organization, 1975).

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