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The Cytochemical Bioassay of ACTH

Quantitative cytochemical techniques have recently been applied to the assay of hormones (Alaghband-Zadeh *et al.* 1974, Daly, Loveridge, Bitensky & Chayen 1974).

Although assays for several hormones are being developed, most experience so far has been gained with corticotrophin (ACTH). Segments of guinea pig adrenal are maintained in non-proliferative organ culture (Trowell 1959) for 5 hours. This allows them to escape the influence of the guinea pig's own hormonal environment. They are then rapidly chilled to —70°C and sectioned at 20 μm in a cryostat. Each section is transferred to a microscope slide, and the slide immersed for 1 minute in an incubation medium containing appropriate dilutions of an ACTH standard or of unknown. The incubation medium contains a colloid stabilizer to prevent diffusion of intracellular enzymes from the section into the medium. By means of a suitable slide holder and segmented trough it is possible to react about 50 slides simultaneously. Normally these would include four tenfold dilutions of standard (5 fg/ml–5 pg/ml), and two tenfold dilutions of each unknown (1 in 100 and 1 in 1000 is usual, but 1 in 10 may be used if the level is expected to be very low). The slides are then immersed in a second trough containing a potassium ferricyanide–ferric chloride mixture. Prussian blue (ferric ferrocyanide) is precipitated in the section, proportionally to the concentration of reducing groups present in the tissue. The intensity of stain in the zona reticularis is measured by scanning and integrating microdensitometry. There is an inverse linear correlation between the intensity of staining and the log of the concentration of ACTH. Unknowns may be read from the standard graph, and duplicates should be within $\pm 15\%$. Values as low as 500 fg/ml can be determined on 200 μl of unextracted plasma, and even lower values can be assayed with larger plasma volumes. Because the standards and unknowns are assayed on the same animal and the adrenal is isolated in organ culture before being exposed to ACTH, hypophysectomy and rigorous environmental control of the assay animal are not necessary. The method has been applied in physiological and clinical studies (Daly, Fleisher, Chambers, Bitensky & Chayen 1974, Daly, Fletcher, Glass, Chambers, Bitensky & Chayen 1974).

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β-melanocyte Stimulating Hormone

It has recently been reported that β-melanocyte stimulating hormone (β-MSH) appears to be absent from the human pituitary. The 22 amino acid peptide, known widely as 'human β-MSH' may be an artifactual fragment (probably formed by an enzymic cleavage during certain extraction procedures) of a much larger pituitary peptide, which has been previously identified and termed β-Lipotrophin (β-LPH) (Scott & Lowry 1974). β-LPH has 91 amino acids and contains the complete sequence of 'human β-MSH' within the 37–58 portion of the molecule.

The purpose of the present study was to determine whether 'human β-MSH' was absent, or present, in plasma and in human tumours associated with the inappropriate production of β-MSH-like immunoreactive peptides. These are secreted in association with ACTH in the ectopic ACTH syndrome. Plasmas were fractionated on Biogel P6 and P10 in a saline/albumin buffer and tissues were acid extracted and subjected to gel filtration on a Biogel P6 column eluted with 6% acetic acid. Fractions were radioimmuno-assayed for β-MSH. Human β-LPH and γ-LPH both cross-react completely in this system.

In plasmas from 3 patients with Nelson's syndrome, and 1 normal subject following pyrogen administration, no immunoreactivity was found in the expected elution position of 'human β-MSH'. The bulk of the immunoreactive material eluted in the position expected for β-LPH (Fig 1). Only a minor portion ($<0.5\%$) of the immunoactivity eluted in fractions beyond the expected elution position of 'human β-MSH', but this may be artifactual. In all the plasmas studied, a substantial amount of the β-MSH immunoactivity was found in the void volume fractions (M.Wt $>20\,000$). However, we have not yet identified whether this is a possible precursor of β-LPH, released intact from the pituitary, or

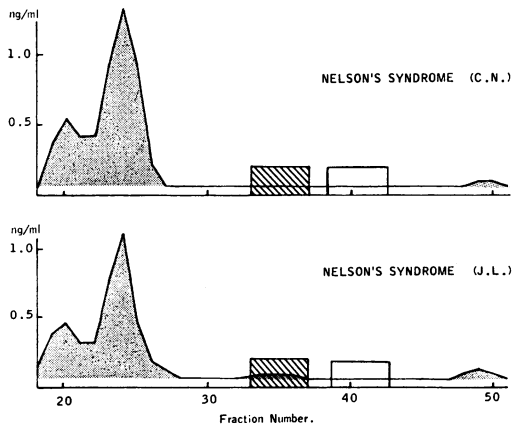

Fig 1 *Fractionation of plasma from 2 patients with Nelson's syndrome on Biogel P6/P10 column eluted with Frog-Ringer/albumin buffer. (Column dimensions: P6 100 cm × 1 cm; P10 33 cm × 1 cm). The shaded peaks represent β-MSH immunoreactive material. The cross-hatched block indicates the elution position of ACTH and the open block that of synthetic human β-MSH*

β-LPH bound non-covalently to a plasma protein.

It has previously been shown that extracts of tumours associated with the ectopic production of ACTH contain, in addition to intact ACTH, COOH–and NH_2–terminal fragments of the ACTH molecule which are normally only found in the pituitaries of animals possessing a distinct pars intermedia. We believed it possible that these tumours might also contain, in addition to intact β-LPH, fragments of the β-LPH molecule such as γ-LPH and β-MSH which are also associated with the presence of a pars intermedia. A peptide, eluting on Biogel P6 gel filtration in an identical position to bovine β-MSH, was identified in a glacial acetic acid extract of an ovarian metastasis of a bronchial carcinoid tumour. Material resembling β-LPH was also found. An HCl/acetone extract of a medullary carcinoma of the thyroid contained immunoreactive peptides with similar molecular weights to β- and γ-LPH. No peptide resembling 'human β-MSH' was identified in any tumour.

Thus, while the situation in some human tumours appears to differ from that found in the human pituitary, it is still not clear whether this is due to the presence of specific cleavage enzymes in the tumours, or is merely the result of increased catabolic activity in rapidly growing, necrosing tissue.

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Hormonal Effects of Synthetic ACTH Analogues

It is well established that patients with diseases such as rheumatoid arthritis and bronchial asthma may respond clinically to high levels of plasma corticosteroids, which may be achieved either by oral synthetic steroid, such as prednisone or by subcutaneous or intramuscular injection of corticotrophin (ACTH). In recent years there has been a trend away from the use of steroids which in daily divided dosage, in excess of the equivalent of 7.5 mg prednisone, usually cause the suppression of the hypothalamo-pituitary-adrenal axis with subsequent failure of the patient to respond to stress (Treadwell *et al.* 1963, Landon *et al.* 1965, Livanou *et al.* 1967, Jasani *et al.* 1967). On the other hand, both animal ACTH preparations and synthetic ACTH analogues, such as α1–24 ACTH zinc (tetracosactrin depot), will give rise to therapeutic levels of plasma corticosteroids without suppressing the brain-adrenal axis if used in appropriate dosage (Bacon *et al.* 1968, Carter & James 1970, Irvine *et al.* 1971).

Recently, a new polypeptide has been synthesized, consisting of the first 18 amino-acids of natural ACTH, substituted at positions 1, 17 and 18 by d-serine, lysine and lysine respectively (Desaulles *et al.* 1969). Termed substituted α1–18 ACTH, it has a prolonged corticotrophic action (Keenan *et al.* 1971, Irvine *et al.* 1974) resulting from the modification of the peptide sequence and has the advantage over α1–24 ACTH Zn of subcutaneous administration. Irvine *et al.* (1973) showed substituted α1–18 ACTH in a dose of 0.25 mg subcutaneously to give rise to therapeutic levels of plasma 11-hydroxycorticosteroids (11-OHCS) for most of the day in 8 healthy subjects, and in 6 of the 8 allowed the endogenous secretion of ACTH in the early hours of the morning as evidenced by plasma 11-OHCS being higher at 0800 hours than 0300 hours on the day following the injection. In theory, at least, the daily use of such a preparation should not suppress the brain-adrenal axis in the majority of patients.

In a further study (Irvine *et al.* 1974) the effect of 1 mg of each of α1–24 ACTH Zn and substituted αl–18 ACTH i.m. on adrenocortical, anterior pituitary and testicular hormone levels over 24 hours in dexamathasone-suppressed males was reported. Each synthetic ACTH analogue had a prolonged action of 24 hours or more on plasma 11-OHCS measured by com-