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Separation of ACTH and MSH Fractions from Pituitaries of Different Species.* (27992)

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We have shown that ACTH evokes a specific melanophore reaction in frogs which cannot be ascribed to contamination by infinitesimal amounts of MSH(1-3). This view was also held by Johnsson and Högberg(4) but was vehemently contested by Li and his collaborators(5). Recently our view was vindicated after the amino-acid sequence of ACTH(6-9), alpha-MSH(10,11) and beta-MSH(12,13) was determined. These 3 hormones contain a core of 7 amino-acids:

1 2 3 4 5 6 7
(Met-Glu-His-Phe-Arg-Try-Gly)

which elicits the melanophore effect both in man and frogs(6).

Thus, 3 melanophore hormones exist in the pituitary, one in the anterior lobe (ACTH), one in the intermediate lobe (alpha-MSH or intermedin), one in the posterior lobe (beta-MSH), which were separated by us biologically(14,15).

The present paper deals with the fractionation and chromatographic separation of the 3 melanophore hormones from pituitaries of 3 different species: oxen, sheep and pigs.

Materials and methods. Separation of ACTH, alpha-MSH and beta-MSH was carried out according to the method of Steelman *et al.*(17). The starting material was a concentrate obtained by purifying dry powders of the anterior and posterior lobes of the pituitary glands of oxen, sheep and pigs. These concentrates were partially purified by the Landgrebe and Mitchell method(18) after adsorption of ACTH with oxycellulose. Four ml concentrates were dissolved in 100 ml distilled water adjusted to pH 4 with 10% hydrochloric acid, and dialyzed for 24 hours against distilled water. The dialyzed solution was adjusted to pH 5.8 with 0.1 M ammonium hydroxide and centrifuged. The clear supernatant was placed on a column (12 × 400 mm) with sodium carboxy methyl cellulose (CMC) absorbent (0.4-0.5 meq./g).

The CMC was prepared by reaction of monochloroacetic acid with a suspension of cellulose in concentrated sodium hydroxide, then equilibrated with 0.01 M ammonium acetate (pH 5.8) after washing with about 400 ml 0.01 M ammonium acetate. Beta-MSH was obtained first by a fraction collector in 100 tubes containing 3 ml samples, alpha-MSH being retained on the column. Elution of alpha-MSH was accomplished at a gradient of 0.1 M ammonium acetate of pH 6.5 passing through a mixing chamber of 200 ml of 0.01 M ammonium acetate buffer. The alpha-MSH was obtained through the fraction collector in another 100 tubes.

The peptides were identified in the tubes by the method of Anderson *et al.*(19) with a

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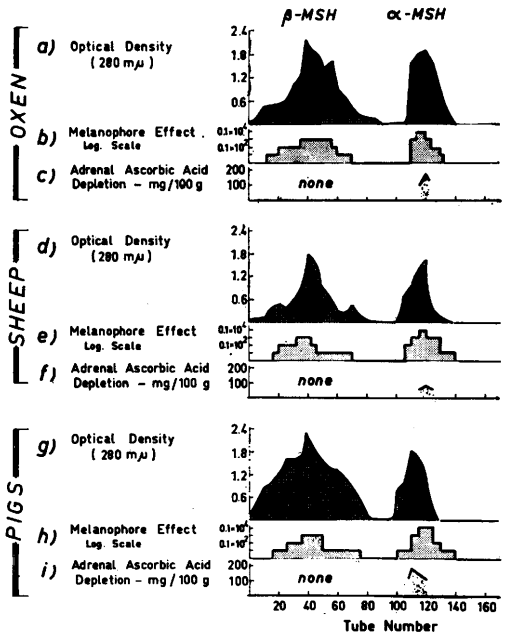

FIG. 1. Pattern of optical density, melanophore effect and adrenal ascorbic acid depletion activity of posterior pituitary lobe extracts from oxen, sheep and pigs.

Beckman DU Spectrophotometer, where 2 peaks were obtained in the ultraviolet spectrum (280 mμ).

Melanophoric activity of the 3 fractions was tested in tree-frogs *(Hyla aborea)* by the method of Sulman(20-22).

Samples which darkened Hyla frogs were tested for ascorbic acid depletion by the method of Hodges and Vernikos(23). Ascorbic acid determinations were performed by the dinitrophenyl-hydrazine method of Roe and Kuether(24).

Results. Posterior lobes. Fig. 1 compares 3 parameters of the extracts: (1) optical density, (2) melanophore effect, and (3) adrenal ascorbic acid depletion activity of posterior lobe extracts from oxen (a,b,c), sheep (d,e,f), and pigs (g,h,i).

1) Upon spectrophotometric reading of the optical density on the wave length (280 mμ) 2 peaks each appeared for the posterior lobes of oxen (a), sheep (d), and pigs (g). The first peak of beta-MSH appeared for oxen in tube No. 39 (a), for sheep in tube No. 40 (d), and for pigs in tube No. 38 (g). The second peak of alpha-MSH appeared for oxen in tube No. 120 (a), for sheep in tube No. 120 (d), and for pigs in tube No. 110 (g).

2) The melanophore effect for these 6 peaks (Table I) was obtained for oxen (b) in test tubes Nos. 12-70 and 105-142, for sheep (e) in test tubes Nos. 15-70 and 104-140, and for pigs (h) in test tubes Nos. 17-75 and 100-140.

3) Adrenal ascorbic acid depletion was negative for all species in the beta-MSH tubes (c,f,i). Positive results were obtained for alpha-MSH fractions in tubes Nos. 115-120 for oxen (c), Nos. 118-120 for sheep (f), and Nos. 110-120 for pigs (i).

Corticotropic activity was thus found only with the alpha-MSH fraction.

Anterior lobes. The same method was used for separating fractions from active ACTH extracts of the anterior lobes of oxen, sheep and pigs, giving no peaks whatever (Table II). This shows that with this method of elution ACTH remained in the column and that alpha-MSH and beta-MSH are not contained in pituitary anterior lobe extracts of oxen, sheep, and pigs.

Discussion. While our work was under way, several investigators(6,9-13,18,25,37-39) have clarified the structure of the 3 hormones which govern the appearance of melanosis, and have also made possible their synthesis (Fig. 2):

(1) ACTH from the anterior lobe contains 39 amino acids; Nos. 1-13 are absolutely essential for eliciting a stimulating effect on the adrenal gland; Nos. 4-10 are indispensable for eliciting melanosis.

(2) Alpha-melanophore hormone from the

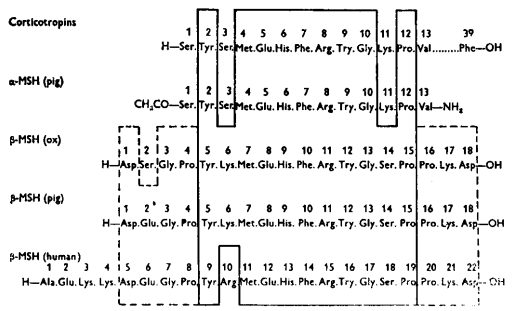

FIG. 2. Amino-acid sequences of ACTH, alpha-MSH and beta-MSH, according to Harris(39).

TABLE I. Comparative Activity of Posterior Pituitary Lobe Extracts of Oxen, Sheep and Pigs with Regard to Optical Density, Chromatophore Effect and Adrenal Ascorbic Acid Depletion.

Post. pituitary lobes	Tube No.	(1) Peak optical density at 280 mμ α-MSH	β-MSH	(2) Chromatophore effect in tree frogs, dose α-MSH	β-MSH	(3) Adrenal ascorbic acid depletion, mg/100 g tissue
Oxen	39		2.1		$.1 \times 10^3$	—
	120	1.93		$.1 \times 10^4$		180
Sheep	40		1.8		$.1 \times 10^3$	—
	120	1.64		$.1 \times 10^4$		80
Pigs	38		2.32		$.1 \times 10^3$	—
	110	1.82		$.1 \times 10^4$		180

intermediate lobe (intermedin) is composed of 13 amino acids which are identical with Nos. 1-13 of ACTH. This hormone may, therefore, cause the ACTH effect in the adrenal gland.

(3) Beta-melanophore hormone from the posterior lobe of the pituitary is composed of 18 amino acids (22 in man). Nos. 7-13 are identical with Nos. 4-10 of ACTH and are responsible for eliciting the melanophore reaction, but in Nos. 1-6 beta-melanophore hormone differs from ACTH to such an extent that it cannot stimulate the adrenal gland.

The results obtained by fractionation of anterior lobe extracts show that in oxen, sheep and pigs intermedin (alpha-MSH) does not belong to the anterior pituitary hormones. The reason is to be sought in the anatomical structure of the pituitaries of these species whose intermediate lobes are closely connected to the posterior lobes. This is not the case with humans where the remnants of the intermediate lobe, as far as they are discernible, are incorporated in the anterior lobe.

We have, therefore, concluded that the melanophore test may be used for identification of ACTH, alpha-MSH and beta-MSH, with the ascorbic acid depletion test providing additional differentiation between ACTH and alpha-MSH on one side and beta-MSH on the other.

We have made use of this fact for quantitative determination of ACTH where small quantities, *e.g.*, 0.01 γ, can be measured by the most sensitive frog method(22,26-31). Our results have been confirmed by several authors(32-36).

Summary. The pituitary anterior and posterior lobes of oxen, sheep and pigs were extracted and ACTH, alpha-MSH and beta-MSH were separated on oxycellulose and CMC columns. They were identified by their optical density at 280 mμ, adrenal ascorbic acid depletion in rats and the melanophore effect in tree frogs. ACTH and alpha-MSH elicited both ascorbic acid depletion and melanosis, and beta-MSH melanosis only. The significance of these overlapping reactions is discussed in the light of the recently

TABLE II. Comparative Activity of Anterior Pituitary Lobe Extracts of Oxen, Sheep and Pigs with Regard to Optical Density, Chromatophore Effect in Tree Frogs and Adrenal Ascorbic Acid Depletion.

Ant. pituitary lobes	Chromatophore effect of extracts before separation, ml	Tube No.	(1) Peak optical density at 280 mμ α-MSH	β-MSH	(2) Chromatophore effect, dose α-MSH	β-MSH	(3) Adrenal ascorbic acid depletion, ml
					ml		
Oxen	$.1 \times 10^4$	1-200	0	0	$.1 \times 10^2$ neg.	$.1 \times 10^2$ neg.	.5 neg.
Sheep	$.1 \times 10^3 - 10^4$	1-200	0	0	.1 neg.	.1 neg.	.5 neg.
Pigs	$.1 \times 10^4$	1-200	0	0	$.1 \times 10^2$ neg.	$.1 \times 10^2$ neg.	.5 neg.

discovered amino acid sequence of the 3 peptide hormones. The melanophore reaction is based upon the sequence of the 7 amino acids:

1 2 3 4 5 6 7
Met-Glu-His-Phe-Arg-Try-Gly.

The ascorbic acid depletion is based on at least 3 additional amino acids preceding the above in the chain: Ser-Tyr-Ser.

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