Isolation, Characterization, and Amino Acid Sequence of Melanotropins from Camel Pituitary Glands†

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ABSTRACT: The isolation of two β-melanotropins and two α-melanotropins from camel pituitary glands has been described, and their amino acid sequences have been determined. Two of them are identified as α-melanotropin and deacetylated α-melanotropin. There are also two β-melanotropins whose structures are identical with the bovine hormone except that one has glycine in position 2 and the other glycine in position 2 as well as glutamine in position 8. The melanocyte-stimulating and lipolytic activities of these four camel melanotropins have been investigated by *in vitro* assay procedures.

Two melanocyte-stimulating hormones have been identified in extracts from the posterior-intermediate lobes of pituitary glands (Lee and Lerner, 1956), and are known as α-MSH[1] and β-MSH. α-MSH was first isolated by Lerner and Lee (1955) from the porcine pituitary and its primary structure subsequently determined by Harris and Lerner (1957; Harris, 1959a). The same hormone was later found and chemically identified in bovine (Lo *et al.*, 1961), ovine (Lee *et al.*, 1963), equine (Dixon and Li, 1960), and macaque (Lee *et al.*, 1961) pituitaries. A closely related melanotropin has also been isolated from dogfish pituitary glands by Lowry and Chadwick (1970). β-MSH was first isolated from porcine pituitary glands (Geschwind *et al.*, 1956; Geschwind and Li, 1957; Porath *et al.*, 1955) and its amino acid sequence determined by Harris and Roos (1956, 1959) and Geschwind *et al.* (1956, 1957a). Subsequently, slightly different β-MSH molecules were isolated and their amino acid sequences determined from bovine (Geschwind *et al.*, 1957b), ovine (Lee *et al.*, 1963), human (Dixon, 1960; Harris, 1959b; Pickering *et al.*, 1963), equine (Dixon and Li, 1961), macacus (Lee *et al.*, 1961), and dogfish (Bennett *et al.*, 1974) pituitary glands. This article describes the isolation and primary structures of α-MSH, deacetylated α-MSH, and two β-melanotropins from camel pituitary glands (*Camelus dromedarius*).

Experimental Section

Acid acetone extract of 500 whole camel pituitaries (650 g) was obtained as previously described (Li, 1952). The resulting acid acetone powder (20 g) was dissolved in 1 l. of water at pH 3 and the solution was brought to 0.06 saturation with respect to NaCl. The NaCl precipitate was removed by centrifugation to yield 10 g of crude prolactin and the supernatant was brought to saturation with NaCl. The saturated NaCl precipitate was thoroughly dialyzed using the "hollow fiber device" (Bio-Rad, Richmond, Calif.) and the insoluble material was centrifuged off. The clear supernatant was lyophilized and yielded approximately 3.2 g (designated fraction D).

Fraction D was next submitted to chromatography on a CMC[1] column (1.5 × 45 cm) that had been equilibrated with 0.01 M NH_4OAc buffer of pH 4.6. Elution was performed initially with the same buffer. After three–four hold-up volumes (4 ml/tube) had been collected, a gradient with respect to pH and concentration was started by introducing 0.1 M NH_4OAc buffer of pH 6.7 through a 500-ml mixing flask containing the starting buffer. Later, the gradient was increased by substitution of 0.2 M NH_4OAc of pH 6.7 as the solution flowed into the mixing flask. Those fractions, which contained tryptophan as detected by the color test (Smith, 1953), were examined for homogeneity by paper electrophoresis. Further purification of these fractions was achieved by exclusion chromatography on Sephadex G-25 (fine).

Electrophoresis on Whatman No. 3MM paper was performed either in pyridine–HOAc buffer of pH 3.7 (pyridine–HOAc–H_2O, 4:40:1150 v/v) or in collidine–HOAc buffer of pH 6.7 (γ-collidine–HOAc–H_2O, 89:31:9800 by volume) for 2 hr at 400 V. The peptide bands as revealed by ninhydrin were eluted from the paper with 0.1 M HOAc and the eluates were dried in a desiccator for amino acid, end group, and sequence analyses. Amino acid analyses were carried out according to the method of Spackman *et al.* (1958) in an automatic amino acid analyzer (Model 120 or 120B, Beckman Instruments). Tryptophan determinations were performed by the procedure of Liu and Chang (1971). The NH_2-terminal residue was obtained by the dansyl procedure (Gray, 1967; Woods and Wang, 1967), and the dansyl Edman method was employed for sequence analysis as previously described (Li *et al.*, 1970).

Commercial enzyme preparations of trypsin (Serva, Lot No. 3725A) and leucineaminopeptidase (Worthington, LAP Lot. No. 54A 313) were used without further purification. Digestions employing LAP (E/S = 1/25) and trypsin (E/S = 1/50) were performed in 0.05 M Tris-HCl buffer of pH 8.5 containing 0.01 M $MgCl_2$ at 37° for 16 hr.

Melanocyte-stimulating activity was determined *in vitro* by the frog skin assay (Shizume *et al.*, 1954; Ramachandran, 1970) using the four-points design. Lipolytic activity was determined *in vitro* with rabbit fat cells as previously described (Ramachandran *et al.*, 1972). For these assays,

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[1] Abbreviations used are: MSH, melanocyte-stimulating hormone, melanotropin; CMC, carboxymethylcellulose; α_s-ACTH, sheep adrenocorticotropin; β_b-MSH, bovine β-MSH; β_m-MSH, monkey β-MSH.

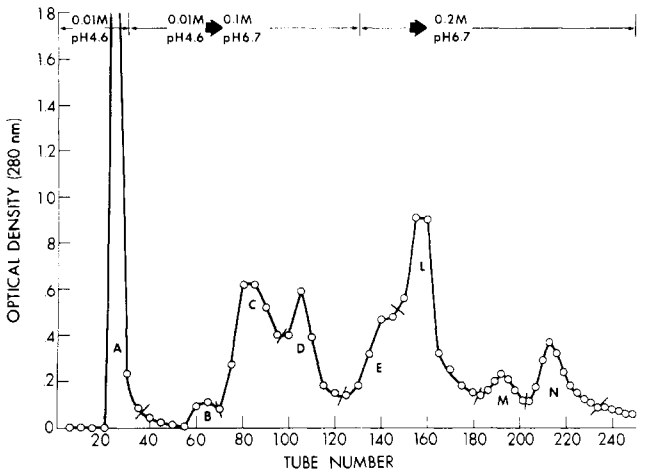

FIGURE 1: CMC chromatography of 800-mg fraction D: column size, 1.5 × 45 cm; flow rate, 12 ml/hr; 4 ml/tube. See text for conditions.

either synthetic monkey β-MSH (Wang *et al.*, 1973) or sheep ACTH (Li *et al.*, 1955) was employed as the standard for comparison.

Results

Figure 1 presents the chromatographic pattern when 800 mg of fraction D was submitted to a CMC column. Seven components were obtained and only components C, D, L, and N were found to contain tryptophan. After lyophilization, yields of the components were: C, 130 mg; D, 44 mg; L, 52 mg; and N, 22 mg.

β_{C1}-MSH. Forty-four milligrams of component L (Figure 1) was submitted to gel filtration on a Sephadex G-25 column in 0.1 M HOAc. As shown in Figure 2, five peaks were obtained, but only peak C was found to contain tryptophan. The content in peak C was lyophilized, yielded 14 mg, and was designated L-C. When L-C was examined in paper electrophoresis at pH 3.7 and 6.7, it was homoge-

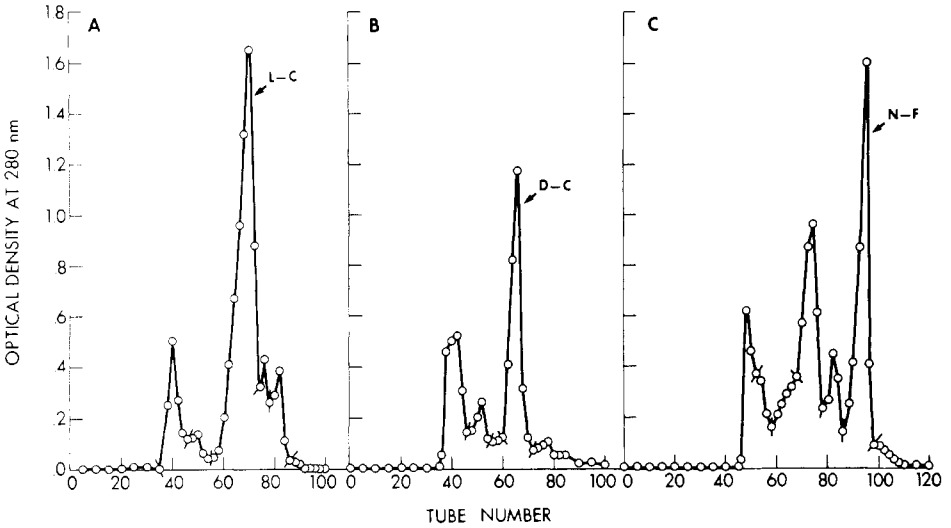

FIGURE 2: Gel filtration of components L, D, and N (see Figure 1) on a Sephadex G-25 (fine) column (3 × 70 cm) in 0.1 M HOAc: flow rate, 9 ml/hr; 3 ml/tube; (A) L, 44 mg; (B) D, 52 mg; and (C) N, 66 mg.

Table I: Amino Acid Composition[a] of Melanotropins from Camel Pituitaries.

Amino Acid	L–C[b]	D–C[c]	β_b-MSH[d]	N–F[e]	F–C[f]	α-MSH[g]
Lys	1.9 (2)	2.0	2	1.0	1.0	1
His	0.9 (1)	0.9 (1)	1	1.1 (1)	0.9 (1)	1
Arg	1.0	0.9 (1)	1	1.1 (1)	0.8 (1)	1
Asp	1.9 (2)	2.1 (2)	2	0	0	0
Ser	0.8 (1)	1.0	2	1.5 (2)	2.0 (2)	2
Glu	0.9 (1)	1.1 (1)	1	1.0	1.0	1
Pro	2.7 (3)	3.0	3	1.1 (1)	1.0	1
Gly	2.8 (3)	3.1 (3)	2	1.1 (1)	1.0	1
Val	0	0		0.9 (1)	1.2 (1)	1
Met	1.0	1.0	1	0.9 (1)	0.8 (1)	1
Tyr	1.0	1.0	1	1.9 (2)	0.7 (1)	1
Phe	1.0	1.2 (1)	1	0.9 (1)	0.9	1
Trp	1.0	1.1 (1)	1	1.0	1.0	1
Total	18	18	18	13	13	13
NH_2-terminal	Asp	Asp	Asp	Ser	None	None

[a] Expressed in molar ratio. [b] See Figure 2A. [c] See Figure 2B. [d] Taken from Geschwind *et al.* (1957b). [e] See Figure 2C. [f] See Figure 4B. [g] Taken from Harris and Lerner (1957).

Table II: Amino Acid Composition[a] of Tryptic Peptides from Camel Melanotropins.

	L-C[b]			D-C[b]			N-F[b]		F-C[b]	
Amino Acid	1	2	3	1	2	3	1	2	1	2
Lys	0.9	1.0		1.1	1.1			1.0		1.0
His			1.0			1.1	1.2		0.8	
Arg			1.0			1.0	1.0		1.0	
Asp	1.0	0.9		1.0	1.0					
Ser		0.8			1.0		1.8		2.0	
Glu			1.1			0.9	0.9		0.8	
Pro	1.2	2.0		1.0	1.8			0.9		0.9
Gly	2.0	1.1		2.0	1.1			1.0		1.1
Val								1.1		0.9
Met			0.9			0.8	0.8		1.0	
Tyr	1.0			1.0			1.0		1.0	
Phe			1.1			1.2	1.2		1.0	
Trp[c]			(1)			(1)		(1)		(1)

[a] Expressed in molar ratio. [b] For amino acid contents of L-C, D-C, N-F, and F-C, see Table I; tryptic peptides were separated by paper electrophoresis; see Figure 4. [c] Indicated by the Ehrlich test.

Table III: Structural Investigations on Tryptic Peptides Derived from Camel Melanotropins.

Peptides[a]	Structure[b]
LC-1	Asp-Gly-Gly-Pro-Tyr-Lys-
-2	Trp-Gly-Ser-Pro-Pro-Lys-Asp
-3[c]	Met-Gln-His-Phe-Arg
DC-1	Asp-Gly-Gly-Pro-Tyr-Lys
-2	Trp-Gly-Ser-Pro-Pro-Lys-Asp
-3[c]	Met-Glu-His-Phe-Arg
NF-1[c]	Ser-Tyr-Ser-Met-Glu-His-Phe-Arg
-2[d]	Trp-Gly-Lys-Pro-Val-NH_2

[a] See Table II for amino acid analysis. R_F values relative to Lys at pH 6.7 are: LC-1, 0.07; LC-2, 0.07; LC-3, 0.43; DC-1, 0.07; DC-2, 0.07; DC-3, 0.21; NF-1, 0.31, NF-2, 0.85. [b] →, dansyl-Edman procedure; →, LAP digestion. [c] LAP digests of LC-3, DC-3, and NF-1 gave the following amino acid compositions (molar ratios): LC-3, $Met_{0.9}Gln_{0.7}His_{1.0}Phe_{1.0}Arg_{1.0}$; DC-3, $Met_{0.8}Glu_{0.9}His_{1.1}Phe_{1.2}Arg_{1.0}$; NF-1, $Ser_{1.8}Tyr_{1.0}Met_{0.8}Glu_{0.9}His_{1.2}Phe_{1.2}Arg_{1.0}$. [d] Digestion of NF-2 with LAP gave $Trp_{1.0}Gly_{0.8}Lys_{0.8}$; the remaining material was submitted to the dansyl-Edman degradation to give the proposed sequence.

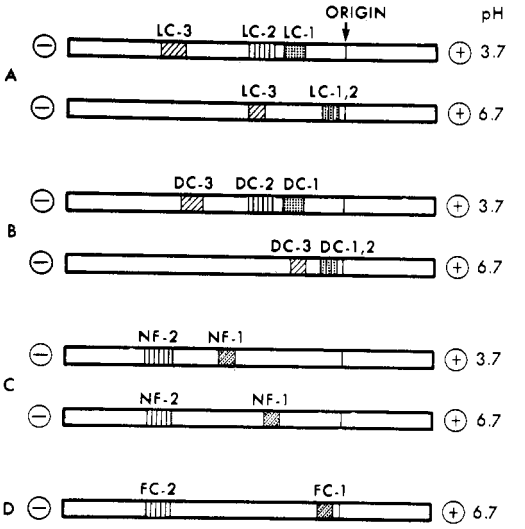

FIGURE 3: Paper electrophoresis of tryptic digests of L-C, D-C, N-F, and F-C at pH 3.7 and/or pH 6.7. Positive color tests: (▥) Trp; (▨) His; (▩) Tyr.

neous with R_F values relative to lysine at 0.54 and 0.32, respectively. NH_2-terminal analysis gave only aspartic acid as the end group. The amino acid content of L-C, summarized in Table I, indicates that it is identical with the composition of bovine β-MSH (Geschwind *et al.*, 1957b) with one more residue of glycine and one less residue of serine. A sample of L-C was analyzed by the dansyl-Edman method for its amino acid sequence and gave the following result:

Asp-Gly-Gly-Pro-Tyr-Lys-Met-Glx-His-Phe-

Tryptic digests of L-C were submitted to paper electrophoresis at pH 3.7 and gave rise to three components (LC-1, LC-2, and LC-3) as shown in Figure 3A. Color tests showed that LC-2 contained tryptophan. Eluates of the peptide bands from the paper were submitted to amino acid and sequence analyses, and the results are summarized in Tables II and III. From the amino acid sequence of NH_2-terminal residues of L-C as mentioned above, it is evident that the sequence of tryptic peptides of L-C is LC-1 → LC-3 → LC-2. Thus, the complete amino acid sequence of L-C is:

H-Asp-Gly-Gly-Pro-Tyr-Lys-Met-Gln-His-Phe-
1 5 10
Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH
15

L-C is designated as β_{C1}-MSH or [Gly^2,Gln^8]-β_b-MSH.

β_{C2}-MSH. Component D (52 mg, Figure 1) was put on a Sephadex G-25 column in 0.1 M HOAc, and the chromatographic pattern may be seen in Figure 2B. The main peak

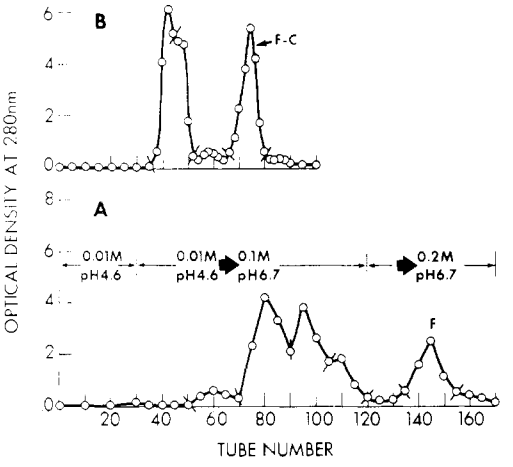

FIGURE 4: (A) CMC chromatography of 130 mg of material from peak C (Figure 1). Conditions are the same as in Figure 1. (B) Gel filtration of the material in peak F on Sephadex G-25 (fine) column. Same conditions as Figure 2.

was lyophilized and yielded 9 mg (designated D-C). Paper electrophoresis of D-C gave a single spot at pH 3.7 with an R_F value of 0.19 relative to lysine. Both amino acid and NH_2-terminal sequence analyses are identical with those for β_{C1}-MSH as summarized in Table I. The dansyl-Edman degradation of D-C gave the following result:

Asp-Gly-Gly-Pro-Tyr-Lys-Met-Glx-His-Phe-

One of the tryptic peptides of D-C, DC-3 (Figure 3B), migrated slower than that of LC-3 (Figure 3A). Amino acid compositions of the three tryptic peptides of D-C are the same as those of L-C (Table II). Sequence analyses of these tryptic peptides indicate that the only difference between D-C and L-C is the presence of glutamic acid in the former and glutamine in the latter peptide. Thus, the complete amino acid sequence of D-C is:

H-Asp-Gly-Gly-Pro-Tyr-Lys-Met-Glu-His-Phe-

1 5 10

Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH

15

D-C is designated β_{C2}-MSH or [Gly^2]-β_b-MSH.

des-Ac-α-MSH. By similar procedures for the isolation of L-C and D-C, 10 mg of N-F was obtained from 66 mg of component N (Figure 1)[2] after gel filtration on Sephadex G-25 in 0.1 M HOAc as shown in Figure 2C. The peptide behaved as a homogeneous substance when tested by paper electrophoresis as well as by NH_2-terminal and amino acid analyses (Table I). Tryptic digests of N-F gave two fragments as revealed by electrophoresis on paper at two pH values (Figure 3C). Amino acid composition and sequence analyses of these two fragments are summarized in Tables II and III. It is evident that N-F has the following primary structure:[3]

[2] Studies with sheep pituitary glands (Pickering *et al.*, 1963) indicated that ACTH occurs in component N. Isolation and structure of peptides related to ACTH from camel pituitaries will be reported elsewhere.

[3] The conclusion that the COOH-terminal valine was amidated was derived from the mobilities of peptides NF-2 and FC-2 at pH 6.7 (see Figure 3). Since these peptides had two net plus charges and since amino acid sequences of the peptides were known, it was assumed that the carboxyl-terminal residue was amidated.

Table IV: Melanocyte-Stimulating Activity of Camel Melanotropins.

MSH	Dose (ng)	Response[a]	Potency[b] %	95% Confidence Limit	λ
Synthetic β_m-MSH	3	40.3 ± 2.4			
	9	51.4 ± 3.2			
α-MSH[c]	1	43.4 ± 3.4	386	118–3660	0.4
	3	53.0 ± 6.3			
Deacetylated α-MSH	3	32.0 ± 0.8	57	27–91	0.20
	9	46.4 ± 3.5			
β_{C1}-MSH[d]	3	39.0 ± 1.9	122	75–221	0.22
	9	56.2 ± 4.1			
β_{C2}-MSH	3	29.2 ± 4.3	64	27–111	0.25
	9	49.4 ± 4.3			

[a] MSH activity expressed as per cent decrease in reflectance. Five skins used for each assay. Values in mean ± standard error. [b] Compared with the potency of synthetic β_m-MSH. [c] Relative potency to deacetylated α-MSH, 684% with 95% confidence limit of 323–404 and λ = 0.34. [d] Relative potency to β_{C2}-MSH, 163% with 95% confidence limit of 98–349 and λ = 0.23.

Table V: Lipolytic Activity of Camel Melanotropins.

Hormone	Dose (ng)	Response[a]	Potency[b] %	95% Confidence Limit	λ
α_s-ACTH	55	2.54 ± 0.06			
	166	6.26 ± 0.18			
β_{C1}-MSH[c]	55	3.37 ± 0.19	118	104–134	0.03
	166	6.44 ± 0.02			
β_{C2}-MSH	55	2.66 ± 0.18	98	85–112	0.03
	166	5.99 ± 0.09			
α-MSH[d]	55	6.84 ± 0	381	326–455	0.02
	166	8.49 ± 0.06			
Deacetylated α-MSH	55	7.92 ± 0.24	513	430–630	0.02
	166	9.69 ± 0.05			

[a] Micromole of glycerol production per gram of cells per hour. Determinations in triplicate. Values in mean ± standard error. [b] Per cent potency in terms of α_s-ACTH. [c] Relative potency to β_{C2}-MSH, 120% with 95% confidence limit of 106–141 and λ = 0.03. [d] Relative potency to deacetylated α-MSH, 53% with 95% confidence limit of 49–57 and λ = 0.06.

H-Ser-Tyr-Ser-Met-Glu-His-Phe-

1 5

Arg-Trp-Gly-Lys-Pro-Val-NH_2

10

Thus, N-F is deacetylated α-MSH (Harris and Lerner, 1957; Harris, 1959a,b).

α-MSH. When 130 mg of component C (Figure 1) was rechromatographed on the CM-cellulose column as shown in Figure 4A, according to the color test only peak F con-

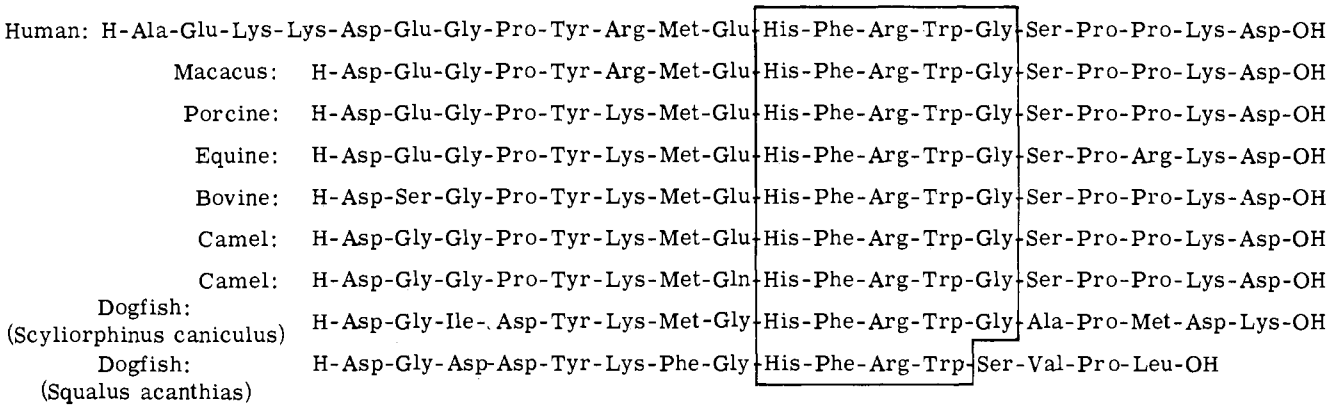

FIGURE 5: Amino acid sequences of β-melanotropins from various species.

tained tryptophan. The material in peak F (42 mg) was then lyophilized and submitted to gel filtration on Sephadex G-25 (fine) in 0.1 M HOAc. The tryptophan-containing component was located in peak F-C (Figure 4B); after lyophilization it yielded 4 mg. Paper electrophoresis of F-C at pH 6.7 indicated that it migrated as a single component with a mobility of 0.43 relative to lysine. No NH_2-terminal residue was detected by the dansyl method. Amino acid analysis of F-C showed its composition was identical with N-F (see Table I). Hydrolysis of F-C with trypsin gave two fragments (FC-1 and FC-2) as shown in Figure 3D. One fragment had the same mobility as NF-2 (Figure 3C) and the other migrated slower than NF-1. In addition, FC-1 was positive to color tests for histidine and tyrosine but negative to the ninhydrin test. Amino acid contents of FC-1 and FC-2 are identical with the tryptic fragments of N-F (Table II). These data, together with bioassay results (see below), substantiate the assumption that F-C is α-MSH with the structure[3] proposed by Harris and Lerner (1957).

Biological Property. The melanocyte-stimulating activities of L-C, D-C, N-F, and F-C as assayed by the *in vitro* frog skin method are summarized in Table IV. Table V presents the lipolytic potency of the four melanotropins isolated from camel pituitary glands. In addition, biological activities of synthetic monkey β-MSH and α_s-ACTH are included in Tables IV and V, respectively.

Discussion

From 500 camel pituitary glands, 16 mg of α-MSH (F-C), 13 mg of des-Ac-α-MSH (N-F), 67 mg of β_{C1}-MSH (L-C), and 24 mg of β_{C2}-MSH (D-C) were isolated in highly purified form and their primary structures elucidated. As expected, the amino acid sequence of camel α-MSH is identical with that of porcine (Harris and Lerner, 1957; Harris, 1959a,b), ox (Lo *et al.*, 1961), ovine (Lee *et al.*, 1963), equine (Dixon and Li, 1960), and dogfish (Bennett *et al.*, 1974). It was somewhat surprising to isolate almost an equal quantity of deacetylated α-MSH. Bennett *et al.* (1974) very recently reported the presence of α-MSH with an unacetylated NH_2-terminal and partly amidated COOH-terminal group in dogfish (*Squalus acanthias*) pituitaries. Since the structure of α-MSH is identical with the NH_2-terminal tridecapeptide sequence of ACTH (Ramachandran, 1973), it is possible that the deacetylated hormone is derived either from degradation products of ACTH or incomplete biosynthesis of α-MSH itself, or both. At any rate, the occurrence of des-Ac-α-MSH in extracts of mammalian pituitary glands has not been previously reported.

There are two β-melanotropins[4] in camel pituitary glands. Figure 5 presents the amino acid sequence of known β-melanotropins. It is of interest to note that the residue in position 2 for all mammalian β-MSH is either glutamic acid or serine, with the exception of camel, where the residue is glycine. This is also true in the case of dogfish β-melanotropins. One of the camel β-MSH's has glutamine in position 8 instead of glutamic acid which occurs in all mammalian hormones. When the amino acid sequences of all known β-melanotropins are compared as shown in Figure 5, it is striking that the tetrapeptide His-Phe-Arg-Trp or the pentapeptide His-Phe-Arg-Trp-Gly has been conserved during the evolution from dogfish to man. Indeed, the synthetic His-Phe-Arg-Trp (Otsuka and Inouye, 1964) and His-Phe-Arg-Trp-Gly (Schwyzer and Li, 1958) possess melanocyte-stimulating activity.

It may be recalled that the octadecapeptide sequence of β-MSH is a part of the structure of β-lipotropin (Li *et al.*, 1965, 1966). It has also been suggested that β-MSH is formed from enzymic degradation of β-lipotropin (Chrétien *et al.*, 1973; Scott and Lowry, 1974). In the experiments reported here, we were not able to demonstrate the existence of β-lipotropin in the camel pituitary extracts. Is it possible that β-lipotropin has been completely degraded to β-MSH before we receive the glands from the slaughter house? Or is it possible that the camel pituitary does not synthesize β-lipotropin at all?

It may be seen in Table IV that β_{C1}-MSH is slightly more active than β_m-MSH, and has almost two times the activity of β_{C2}-MSH in frog skin assays. As expected, deacetylation of α-MSH markedly decreases the melanocyte-stimulating activity, which is in agreement with earlier reports (Guttmann and Boissonnas, 1961; Hofmann and Yajima, 1961). However, when compared with α-MSH (Table V) deacetylated α-MSH is more active in isolated rabbit fat cells. The replacement of glutamine in position 8 by glutamic acid causes a lowering of the melanotropic activity when the potencies of the two camel β-MSH's are compared. This is also true for the lipolytic activity (see Table V). A detailed investigation on the biological activity of camel melanotropins and their synthetic analogs, as well as various β-MSH's, will be reported elsewhere.

[4] It was pointed out by one of the referees that Glu-8 in β_{C2}-MSH could be derived from the deamidation of Gln-8 of β-$_{C1}$-MSH. This is unlikely because studies on model glutamine peptides by us and others (Gráf *et al.*, 1971; Riniker *et al.*, 1972) show that the deamidation of glutamine is not detectable under isolation procedures for ACTH.

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