[CONTRIBUTION FROM THE HORMONE RESEARCH LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY, CALIFORNIA]

The Isolation and Structure of α-Melanocyte-stimulating Hormone from Horse Pituitaries

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RECEIVED FEBRUARY 23, 1960

It has been possible to isolate α_e-MSH in highly purified form from the posterior lobes of horse pituitaries by a new and simplified method which makes use of counter-current distribution in a solvent system consisting of 0.1% acetic acid, *n*-butanol and pyridine, mixed in the volume ratio of 11:5:3. In this solvent system, the hormone distributes with a partition coefficient of 1.30. By the frog-skin assay method, α_e-MSH possesses an activity of 1.0×10^{10} units per gram. In addition, this molecule has been characterized by means of terminal residue and amino acid analyses as well as on the basis of the results of enzymic digestion. In every respect, α_e-MSH was shown to be identical with α-MSH from beef pituitaries. A partial amino-acid sequence has been proposed for α_e-MSH; it appears very probable that the equine α-MSH has a structure identical to that previously proposed for α-MSH isolated from beef and pig pituitaries.

The achievements of a variety of workers leading to the isolation and structural elucidation of the various melanocyte-stimulating polypeptide hormones from porcine and bovine pituitary glands have recently been reviewed.[1] A melanocyte-stimulating hormone, β-MSH, from pig pituitaries, has been shown to consist of a single peptide chain[2-5] containing 18 amino acid residues in the sequence H-Asp.Glu.Gly.Pro.Tyr.Lys.Met.Glu.-His.Phe.Arg.Try.Gly.Ser.Pro.Pro.Lys.Asp-OH. Bovine β-MSH[6,7] has a composition identical with that of the hormone from pig glands except that a glutamyl residue (position 2) is replaced by a seryl residue. Another melanocyte-stimulating peptide (α-MSH), also in the form of a single polypeptide chain but containing only 13 amino acid residues, has been isolated from both pig[8,9] and beef pituitary glands.[10] The complete removal of contaminating material from α_p-MSH[13] has presented considerable difficulties (see, for example, ref.[14]). The amino sequence of α_b-MSH was shown to be identical with that determined for α_p-MSH,[15,16] namely, acetyl.Ser.Tyr.Ser.Met.Glu.His.Phe.Arg.Try.Gly.-Lys.Pro.Val.NH_2. The adrenocorticotropic hormone (ACTH) preparations from pig, beef and sheep glands[17-19] also possess a melanocyte-stimulating activity but of a lower magnitude than that of the MSH peptides.[1,20] All these polypeptide hormones that exert melanocyte-stimulating activity contain an identical internal amino acid sequence which extends through seven amino acid residues (Met.Glu.His.Phe.Arg.Try.Gly).

The isolation and structural investigation of the MSH polypeptides from horse pituitaries have been undertaken in an effort to determine whether the similarity hitherto found to exist among the melanocyte-stimulating hormones extends to yet another species. Details of the study[21] on α-MSH from horse glands (α_e-MSH) are presented herein.

Experimental

Assays for melanocyte-stimulating activity were performed either on hypophysectomized *Rana pipiens*[23] or on isolated frog skins.[24] The early steps employed in isolating α_e-MSH from the posterior lobes of horse pituitaries followed closely those previously published for the MSH preparations from the other species.[1] The melanocyte-stimulating activity was adsorbed onto oxycellulose in the usual manner and was in turn eluted from the oxycellulose with 0.1 *N* HCl. Deacidification of this eluate was accomplished by batchwise treatment with the Amberlite anion exchange resin IR-4B in the hydroxide form. Lyophylization of the deacidified solution yielded a white powder which possessed the MSH activity.

Countercurrent distribution (c.c.d.) was carried out in a solvent system consisting of 0.1% acetic acid, 1-butanol and pyridine mixed in the volume ratios of 11:5:3.[25] Reagent grade *n*-butanol was used directly, but the pyridine was distilled through a Vigreux column under nitrogen and reduced pressure; only the middle fraction, which has a constant boiling point, was used to prepare the solvent for countercurrent distribution. All distributions were carried out in the 240-tube all-glass countercurrent apparatus[26]; each cell in the distribution train contained 5 ml. of lower phase. To insure that the two solvent phases would be in equilibrium during the actual distribution of material, upper phase was allowed to travel down the machine until each tube contained upper phase, then the apparatus was set to recycle the upper phase and, finally, the polypeptide material was added to the first tubes in the train. After the completion of the desired number of transfers, the distribution pattern was obtained by determining Folin-Lowry color yield[27] on suitable aliquots.

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(2) I. I. Geschwind, C. H. Li and L. Barnafi, THIS JOURNAL, **78**, 4494 (1956).
(3) J. I. Harris and P. Roos, *Nature*, **178**, 90 (1956).
(4) I. I. Geschwind, C. H. Li and L. Barnafi, THIS JOURNAL, **79**, 620 (1957).
(5) J. I. Harris and P. Roos, *Biochem. J.*, **71**, 431 (1959).
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(7) I. I. Geschwind, C. H. Li and L. Barnafi, *ibid.*, **79**, 6394 (1957).
(8) A. B. Lerner and T. H. Lee, *ibid.*, **77**, 1066 (1955).
(9) T. H. Lee and A. B. Lerner, *J. Biol. Chem.*, **221**, 943 (1956).
(10) Although detailed reports on the isolation and structure of α_b-MSH have not appeared, results of these investigations have been mentioned in two recent reviews.[11,12]
(11) I. I. Geschwind, in "Columbia University Conference on Comparative Endocrinology," John Wiley and Sons, Inc., New York, N. Y. 1958, pp. 421–443.
(12) C. H. Li, *Laboratory Investigation*, **8**, 574 (1959).
(13) Analogous to a system of terminology proposed for ACTH preparations [C. H. Li, *Science*, **129**, 969 (1959)], we refer to bovine α-MSH as α_b-MSH and porcine α-MSH as α_p-MSH, throughout this paper, and we designate the equine hormone as α_e-MSH.
(14) S. L. Steelman, R. N. Andersen and R. M. McGregor, *Biochim. Biophys. Acta*, **33**, 256 (1959).
(15) J. I. Harris and A. B. Lerner, *Nature*, **179**, 1346 (1957).
(16) J. I. Harris, *Biochem. J.*, **71**, 451 (1959).
(17) P. H. Bell, THIS JOURNAL, **76**, 5565 (1954).
(18) C. H. Li and J. S. Dixon, *Science*, **124**, 934 (1956).
(19) C. H. Li, I. I. Geschwind, A. L. Levy, J. I. Harris, J. S. Dixon, N. G. Pon and J. O. Porath, *Nature*, **173**, 251 (1954).
(20) C. H. Li, *Adv. Prot. Chem.*, **11**, 101 (1956).
(21) A preliminary report of this work has appeared.[22]
(22) J. S. Dixon and C. H. Li, *Fed. Proc.*, **18**, 818 (1959).
(23) L. Hogken and D. Slome, *Proc. Roy. Soc. (London)*, **B108**, 10 (1931).
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(26) L. C. Craig, W. Hausmann, E. H. Ahrens and E. J. Harfenist, *Anal. Chem.*, **23**, 1276 (1951).
(27) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

Quantitative amino acid determinations of acid hydrolysates of the hormone of peptide fragments derived from enzymic digests of the intact hormone were carried out by the paper-DNP method of Levy.[28] The tyrosine and tryptophan content were determined spectrophotometrically.[29]

All enzymic digestions of α_e-MSH and all steps involved in the structural elucidation of the resulting peptides were carried out in parallel with identical experiments in which α_b-MSH[30] was used as the substrate; *i.e.*, separate samples of the α-MSH from the two species were submitted to digestion at the same time under identical conditions and the various analyses made on the two digestion mixtures were performed side by side. Enzyme that had been treated with diisopropyl fluorophosphate was used for the digestion with carboxypeptidase, which was carried out in an enzyme to substrate ratio of $^1/_{20}$ for 24 hr. at 38° in 1% $NaHCO_3$. Quantitative determination of the amino acids released by the carboxypeptidase was performed by the paper-DNP method[28] as previously described.[31]

For the chymotryptic digestion the ratio by weight of enzyme to substrate was 1:30. Crystalline chymotrypsin (Armour, Lot 381-092) and α-MSH were dissolved in 1.5 ml. of NH_4OH solution of *p*H 8.5 and digestion was allowed to proceed for 24 hr. at 39°. The reaction mixture was then frozen and lyophylized. For the tryptic digestion the ratio by weight of enzyme to substrate was 1:100. α-MSH and crystalline trypsin (Armour Lot 181) in NH_4OH solution (1.5 ml., *p*H 8.5) were incubated for 30 minutes; as in the case of the chymotrypsin, the products of the reaction were recovered by lyopilization.

The electrophoretic separations on paper were carried out in the Durrum type apparatus (Spinco Div., Beckman Instruments Co., Belmont, Calif.). The digests of α_e-MSH and α_b-MSH were applied to the paper (Whatman 3 MM filter paper) as thin bands and subjected to electrophoresis in a γ-collidine-acetic acid-water buffer[32] of *p*H 7 at 4° for 6 hr. under a potential gradient of 11 volts per cm. Guide strips cut from the paper after drying and treated with ninhydrin or with the appropriate reagent[33] revealed the positions of tryptophan-, histidine-, tyrosine- and arginine-containing peptides. Those areas on which the peptides were disclosed were cut out and the material was eluted either with 1% NH_3 or with 0.1 *N* acetic acid.

Results

Isolation by C.c.d.—The crude MSH concentrate obtained from the oxycellulose eluate was shown to possess a melanocyte-stimulating activity of 3.8 × 10^8 u./g. by the *in vitro* method.[34] For the countercurrent distribution the crude material was dissolved in solvent from the first three cells in the train, and distribution was carried out for 239 transfers, at the rate of about 12 transfers per hour. Some emulsification occurred during the first few distributions, and throughout the experiment the solvent phases separated more slowly in the first few tubes than in the remainder of the distribution train. A typical distribution pattern is shown in Fig. 1a. The material in Tubes 132 to 172 (K = 1.5) was pooled as shown and recovered by removal of solvent with the rotary evaporator followed by lyophylization. Such material has an MSH activity of 4.4 × 10^9 u./g. The material from three such experiments was combined, dissolved and again lyophilized; the lyophilized product was redistributed for 1095 transfers in the same solvent system, to give the distribution pattern shown in Fig. 1b. It might seem from this distribution pattern that the material with the lower partition coefficient travels faster than the component with the higher partition coefficient; this, of course, is not the case. Because the upper phase solvent has been recycled through the apparatus several times, the faster-moving component (α_e-MSH) has already made a complete circle and passed the slower-moving material to reach a new relative position in the distribution train. The presence of two peaks indicates that a small amount of material has been separated from the main component by the second distribution process. Furthermore, the distribution of this main component (K = 1.3)[34] follows closely that to be expected from a pure component. The contents in Tubes 97 to 176 were combined and lyophylized. The product (α_e-MSH) was assayed and found to contain 1.0 × 10^{10} u./g. A single dose of 0.001 μg. injected into hypophysectomized *Rana pipiens* caused darkening to the extent that a melanophore index of 2–3+ was reached within 1 hr. When solutions of α_e-MSH (1 mg./ml.) in 0.1 *M* NaOH were kept in a boiling water-bath for 15 minutes, the duration of the darkening effect of the treated hormone on the skin of hypophysectomized frogs was greatly prolonged, a phenomenon characteristic of ACTH and MSH peptides as well as synthetic peptides related to these hormone

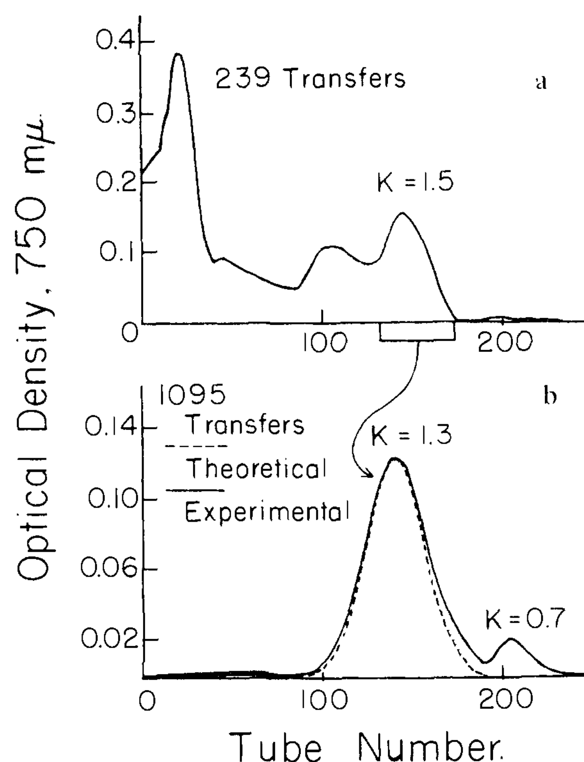

Fig. 1.—(a) Counter-current distribution (239 transfers) of MSH concentrate obtained from the oxycellulose step. Solvent system, 0.1% acetic acid, *n*-butanol and pyridine in the volume ratios of 11:5:3. (b) Counter-current distribution (1095 transfers) of the component with K = 1.5 obtained above. Solvent system same as above.

(28) A. L. Levy, *Nature*, **174**, 126 (1954).

(29) T. W. Goodwin and R. A. Morton, *Biochem. J.*, **40**, 628 (1946).

(30) The isolation of α_b-MSH from posterior lobes of bovine pituitaries was carried out exactly as described herein for the α_e-MSH.

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(32) I. M. Lockhart and E. P. Abraham, *Biochem. J.*, **58**, 633 (1954).

(33) R. J. Block, E. L. Durrum and G. Zweig, "A Manual of Paper Chromatography and Paper Electrophoresis," 2nd Ed., Academic Press, Inc., New York, N. Y., 1958, pp. 128–139.

(34) α_b-MSH has been shown to distribute in this solvent system with the same partition coefficient as that of α_e-MSH.

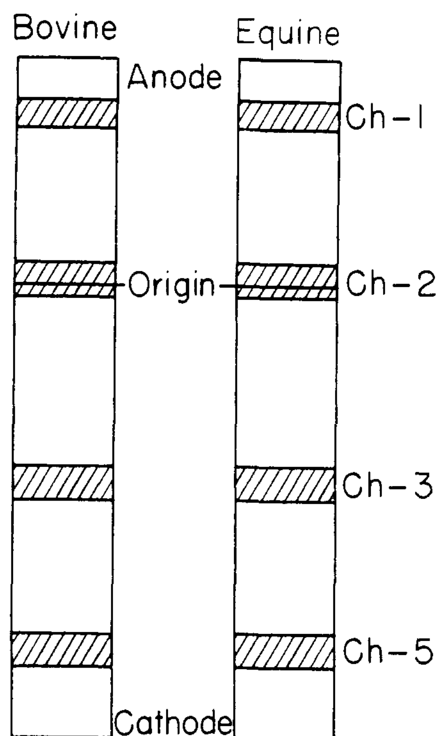

Fig. 2.—Resolution of chymotryptic digests of α_e-MSH and α_b-MSH by zone electrophoresis on paper; buffer, γ-collidine–acetic acid–water at pH 7.0 and 4°; 11 volts/cm. for 6 hr.

peptides.[1,12,20] From 1 kg. of posterior lobes of horse pituitaries, 0.058 g. of the hormone was isolated. Table I summarizes the yield and estimated MSH potency of various fractions obtained from each step of the procedure for the isolation of α_e-MSH.

TABLE I

YIELD AND POTENCY OF VARIOUS FRACTIONS OBTAINED FROM PROCEDURE FOR ISOLATION OF α_e-MSH

Procedure	Yield from 1 kg. fresh posterior horse pituitaries, g.	Estimated MSH potency, u/g.
Acetone-dried powder	180	1.1×10^7
Glacial acetic acid extract	33	6.5×10^7
Oxycellulose adsorption	0.99	3.8×10^8
Countercurrent distribution		
(a) 239 transfers	.125	4.4×10^9
(b) 1095 transfers	.058	1.0×10^{10}

Amino Acid Composition.—The amino acid composition of α_e-MSH is given in Table II, column 4. The preparation no longer contains detectable amounts of any of those amino acids, such as aspartic acid, alanine, leucine and cystine, which appeared in non-stoichiometric amounts (see Table II, column 3) in the sample obtained from the first c.c.d. step. The ratio of tyrosine to tryptophan in the hormone peptide as determined by the spectrophotometric method was almost unity (0.9:1), quantitative data on these two amino acids indicated that they were present in the amount of one mole per mole of the polypeptide hormone. The amino acid composition (expressed in molar ratios) of α_e-MSH (see Table II) was:

$Glu_1, Ser_2, Pro_1, Gly_1, His_1, Met_1, Val_1, Phe_1, Lys_1, Tyr_1, Try_1, Arg_1$

TABLE II

THE AMINO ACID COMPOSITION OF α_b-MSH AND α_e-MSH PREPARATIONS

Amino acid	α_b-MSH[a]	Purified α_e-MSH[b]	α_e-MSH
Glu	1	1.0	1.1
Asp		0.3	
Ser	2	2.1	2.1
Pro	1	1.3	0.9
Ala		0.1	
Gly	1	1.4	1.2
Cyss		0.06	
His	1	.8	0.5
Met	1	.7	1.3
Val	1	.9	1.0
Leu		.2	
Phe	1	1.3	1.0
Lys	1	1.1	0.8
Tyr	1	1.0	0.8
Arg	1	0.7	1.2
Try	1	1	1

[a] Taken from (11,12); results are expressed as molar ratios. [b] Obtained after first countercurrent distribution.

Terminal Residue Analysis.—Digestion of α_e-MSH with carboxypeptidase for 24 hr. at 38° failed to release any amino acids; however, digestion with carboxypeptidase carried out for 6 hr. on a sample of the equine hormone that had stood for 70 minutes in 1 N HCl at 80° released 0.47 mole of valine per mole of the peptide as well as appreciable quantities of other amino acids. Thus, it may be assumed that the C-terminal valine is in amide form, similar to oxytoxin and the vasopressins (in all of which the C-terminal amino acid is glycinamide).[35]

The intact hormone did not give a positive ninhydrin reaction,[33] indicating that it probably contains no free α-amino group. In addition, when α_e-MSH was allowed to react with fluodinitrobenzene,[36] no α-dinitrophenyl amino acids were formed; the only product was ϵ-dinitrophenyllysine. It was also shown that among the peptide fragments obtained from either the chymotryptic or tryptic digestions, there was one which did not react with ninhydrin. These observations may be taken to mean that the α-amino group in the hormone is blocked and hence not available for reaction with either ninhydrin or fluorodinitrobenzene.

Chymotryptic Digest.—In Fig. 2 the electrophoretic pattern obtained with the chymotryptic digest of α_e-MSH is compared directly with the pattern obtained from digested α_b-MSH. Both digests separated in an identical fashion. Band Ch-1 showed no ninhydrin color but did give a strong positive reaction (violet color) with the Pauly reagent,[33] indicating that this peptide had no free amino nitrogen but contained either tyrosine or histidine, or both. Amino acid analysis of this peptide fragment showed that serine and tyrosine were present in approximately an equimolar ratio (Table III), in view of the known specificity of chymotrypsin, it may be concluded that Ch-1 has the structure R-Ser.Tyr. Similarly, peptide Ch-2

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(36) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

TABLE III

THE AMINO ACID COMPOSITION[a] OF THE VARIOUS PEPTIDES RESULTING FROM THE DIGESTION OF HORSE AND BEEF α-MSH

Amino acid	Peptide: Ch-1		Ch-2		Ch-3		Ch-5		Tr-1		Tr-2	
	Horse	Beef	Horse	Beef	Horse	Beef	Horse	Beef	Horse	Beef	Horse	Beef
Ser	1.0	1.0	0.9	1.1					2.0	2.6		
Tyr	0.5	0.4							0.7	0.7		
Met			0.5	0.2					0.9	0.7		
Glu			0.8	0.8					1.0	1.0		
His			1.1	1.1					0.5	0.4		
Phe			1.0	1.0					1.0	1.0		
Arg					1.0	1.0			0.9	1.1		
Try					(1)	(1)					(1)	(1)
Gly							1.2	1.0			1.0	1.4
Lys							0.9	0.9			1.0	0.9
Pro							1.0	0.9			0.8	0.6
Val							1.0	1.0			1.0	1.0

[a] Values are expressed as molar ratios.

gave a positive reaction (cherry red) with the Pauly reagent, as well as a positive ninhydrin reaction. N-terminal residue analysis[28,36] showed serine at the N-terminus, stepwise degradation from N-terminus by the Edman procedure[37,38] gave serine, methionine, glutamic acid and histidine. From this information, together with the amino-acid content (Table III), the structure for peptide Ch-2 may be formulated as Ser.Met.Glu.-His.Phe.

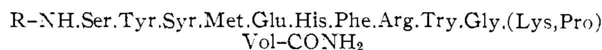
The Ch-3 band gave positive reactions with ninhydrin, and with the Sakaguchi and Ehrlich reagents,[33] indicating the presence of arginine and tryptophan. Amino acid analysis of peptide Ch-3 (Table III), as well as N-terminal residue analysis[28,36] of the peptide, showed only arginine. Since the material gave a positive reaction for tryptophan, it can be concluded that this peptide has the composition Arg.Try.

Peptide Ch-5 initially gave a yellow color with ninhydrin; when the guide strip was allowed to stand at room temperature for some time, the yellow area turned gray. Such a response is given by peptides with N-terminal glycine residues. Quantitative amino acid analysis of peptide Ch-5 was performed to give the composition, expressed in molar ratios, shown in Table III. From these data, peptide Ch-5 was assumed to have the structure Gly.(Lys,Pro,Val).

Comparison of the results of amino acid analyses for the various peptides reveals that the peptides resulting from the chymotryptic digestion of the α-MSH's isolated from the two species (horse and ox) are identical.

Tryptic Digest.—The products resulting from the tryptic digestion of an α_e-MSH sample were separated by the same procedures as those employed for the chymotryptic digests. Electrophoresis on paper of these tryptic digests gave the patterns shown in Fig. 3. Only two main bands were obtained. Band Tr-1 was ninhydrin negative but gave a strong Pauly reaction, indicating the presence of tyrosine and/or histidine, whereas Band Tr-2 gave a strong color with ninhydrin and a positive Ehrlich test for tryptophan. N-Terminal residue analysis by the phenyl isothiocyanate method[37,38] revealed tryptophan at the N-terminus. Amino acid analyses were performed on the recovered peptides to give the molar ratios shown in Table III.

Proposed Structure of Equine α-MSH.—On the basis of the above data obtained from enzymic digestion, together with information derived from analysis of C- and N-terminal residues and from amino acid composition, the following tentative structure may be formulated for α_e-MSH:



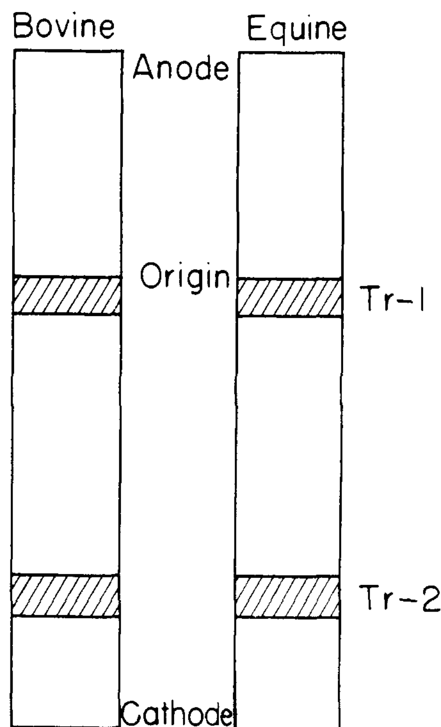


Fig. 3.—Resolution of tryptic digests of α_e-MSH and α_b-MSH by zone electrophoresis on paper; conditions same as in Fig. 2.

It is evident that the amino acid sequence of the equine α-MSH has been almost completely determined. It would appear from the identical behavior manifested by it and the bovine α-MSH

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(38) H. Fraenkel-Conrat and J. I. Harris, THIS JOURNAL, **76**, 6058 (1954).

that these two peptide hormones are also structurally identical, at least with respect to amino acid sequence. In addition, it can be said that the carboxyl group of the C-terminal amino acid residue, valine, is blocked, probably by an amide structure, since very mild acid hydrolysis leaves the valine susceptible to removal by carboxypeptidase digestion. Both α_p-MSH[15,16] and α_b-MSH[11,12] have been shown to be blocked at the N-terminus by an acetyl residue. The failure to react with ninhydrin on the part of the N-terminal amino group of α_e-MSH indicates similar blocking at this terminus of the equine hormone as well, possibly also by an acetyl group.

Acknowledgment.—We wish to thank the National Institutes of Health of the United States Public Health Service for a grant (G2907) which made this work possible.

[CONTRIBUTION FROM THE BIOCHEMISTRY SECTION, ARMOUR AND COMPANY, CENTRAL RESEARCH LABORATORIES, CHICAGO 9, ILLINOIS]

The Fractionation of Insulin on Diethylaminoethylcellulose

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RECEIVED JANUARY 29, 1960

Insulin was separated into two components on the anion exchanger diethylaminoethylcellulose, using stepwise elution with carbon dioxide–water solution and 0.1 M ammonium phosphate. The fractions were compared as to biological properties, crystallizability, solubility, amino-acid content, optical rotation and electrophoretic mobility. They were identical in qualitative amino-acid content but displayed significant differences in physical configuration as well as in net charge. Their apparent interrelationship is that of native and partially denatured protein forms. It is postulated that the two insulins are composed of polymeric forms each containing a different monomeric species.

Introduction

Heterogeneity in purified insulin preparations has been demonstrated by means of several criteria.[1-7] In his sedimentation studies, Fredericq[1] has obtained evidence that the component differences lie in the elementary sub-units of the insulin polymer. The classical work of Sanger[8] shows that these differences are not concerned with the nature or sequence of the constituent amino acids; the fractionation studies of Fredericq,[1] Harfenist and Craig[2], and Timasheff, Brown and Kirkwood[3] collectively suggest that the differences can be attributed to variations in a few chemical groups, resulting in slight differences in net molecular charge. Indeed, Harfenist and Craig[2] have actually showed that their A and B components, isolated by countercurrent distribution, differ by only a single amide group per 6000 molecular-weight unit.

The purpose of the present paper is to report the separation of purified insulin into two components apparently unlike those obtained in prior investigations. Fractionation was accomplished on the anion exchanger diethylaminoethylcellulose (DEAE-cellulose)[9] by using stepwise elution with carbon dioxide–water solution[10] and 0.1 M ammonium phosphate. The relation of the two fractions to each other as well as to the end products of other fractionation procedures is discussed.

(1) E. Fredericq, "Ciba Foundation Colloquia on Endocrinology: Internal Secretions of the Pancreas," **9**, J. and A. Churchill Ltd., London, 1956, p. 89–109.

(2) E. J. Harfenist and L. C. Craig, THIS JOURNAL, **74**, 3083 (1952); *ibid.*, **75**, 5528 (1953).

(3) S. N. Timasheff, R. A. Brown and J. G. Kirkwood, *ibid.*, **75**, 3121, 3124 (1953).

(4) N. K. Boardman, *Biochim. Biophys. Acta*, **18**, 290 (1955).

(5) J. Lens, *ibid.*, **2**, 76 (1948).

(6) R. R. Porter, Information supplied with IUPAC crystalline insulin, batch 2189.

(7) D. F. Waugh, D. F. Wilhelmson, S. L. Commerford and M. L. Sackler, THIS JOURNAL, **75**, 2592 (1953).

(8) F. Sanger, *Advances in Protein Chem.*, **7**, 1 (1952).

(9) E. A. Peterson and H. A. Sober, THIS JOURNAL, **78**, 751 (1956).

(10) M. A. Mitz and S. S. Yanari, *ibid.*, **78**, 2649 (1956).

Materials

Adsorbent.—The DEAE-cellulose preparations were prepared by the method of Peterson and Sober,[9] using Solka-Floc SW-40B. The preparations ranged in capacity from 0.4 to 0.6 meq. per g. After use, they were regenerated with 0.1 M sodium hydroxide.

Insulin.—The insulin preparations studied in detail were: (1) Armour crystalline zinc insulin, Lot 1, 24.2 units per mg.; (2) Armour crystalline zinc insulin, Lot 2, 24.5 units per mg.; (3) Armour crystalline zinc insulin, Lot 3, 22 units per mg.; (4) U.S.P. Reference Standard, crystalline zinc insulin, 24.2 units per mg.; and (5) IUPAC crystalline zinc insulin, Batch 2189, 23 units per mg. The preparations were of beef origin with the exception of the U.S.P. Reference Standard which was a mixture of beef and pork insulins.

Methods

Chromatographic Procedures.—The adsorbent column was prepared from an aqueous slurry of DEAE-cellulose in the free base form. Approximately 3 g. of exchanger were used for a column 33 × 0.9 cm. To insure even distribution of the exchanger, gentle air pressure was used during packing. The column was washed with one to two hold-up volumes of distilled water and a freshly prepared solution containing 90–100 mg. of insulin (adjusted to pH 3.0 with hydrochloric acid) was added. Distilled-water wash was continued until the effluent, which showed a small pH shift, returned to pH 7. The water reservoir was then saturated with carbon dioxide gas at atmospheric pressure. At the breakthrough volume of the carbon dioxide solution, conveniently identified by a sharp change in effluent pH (minimum, pH 4.1 at 25°), the initial protein peak (Insulin I) appeared in the effluent. After elution of Insulin I was complete, as determined by optical density measurement, the eluent was changed to 0.1 M ammonium phosphate, pH 4.3.[11] After the carbon dioxide had been displaced, the 0.1 M ammonium phosphate solution was adjusted to pH 3.3 with phosphoric acid and the second protein fraction (Insulin II) was then eluted.

(11) This solution satisfied two important requirements as an intermediate eluent. (a) It removed carbon dioxide as the bicarbonate ion and thus prevented the column collapse which results when carbon dioxide gas is liberated. (b) It prevented elution of the remaining protein at undesirable alkaline pH since it did not permit the effluent pH to rise above 7.1.