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The Isolation, Characterization and Amino Acid Sequence of a Melanocyte-stimulating Hormone from Bovine Pituitary Glands

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A melanocyte-stimulating hormone has been isolated from bovine posterior lobe powder by a procedure similar to that employed previously for the isolation of porcine β-MSH. The isoelectric point of the bovine hormone has been found to be more basic than that of the porcine polypeptide. By means of chemical and enzymic degradations, the structure of the bovine hormone has been shown to be

H.Asp.Ser.Gly.Pro.Tyr.Lys.Met.Glu.His.Phe.Arg.Try.Gly.Ser.Pro.Pro.Lys.Asp.OH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

The sequence of amino acids in the bovine hormone differs from that in the porcine in the substitution of a serine residue for a glutamic at position 2. A comparison is made between this "seryl-β-MSH" and the bovine MSH preparation obtained by Benfey and Purvis.[1]

The purification and isolation of one or the other of two porcine melanocyte-stimulating hormones (MSH) have been described recently by a number of investigators[1-4] and the structure of one of these hormones, β-MSH, has been elucidated[4a,5,6] as well. On the other hand, although considerable effort was expended prior to 1950 on the purification of a bovine MSH,[7] interest in this hormone has been superseded by the recent focus on the porcine hormones, so that only a few reports in the past few years have been concerned with attempts to purify the bovine hormone. In part, the lack of interest in the latter hormone derives from the fact that beef glands are not as rich a source of MSH activity as are pig glands; thus, various workers have reported that pig posterior lobes[8] possess 5 times[9] or 2 times[10] as much MSH activity as does an equal weight of beef posterior lobes. Nevertheless, concentration of bovine MSH activity has been achieved by Landgrebe and Mitchell,[9] and the purification of a bovine MSH by means of countercurrent distribution has been reported by Benfey and Purvis.[1b] The present communication describes the isolation and structure of a bovine MSH which differs markedly in amino acid composition and distribution behavior from that reported by Benfey and Purvis. A preliminary report of these data has appeared recently.[11]

Isolation Procedure

Crude MSH Concentrate.—This concentrate was prepared in exactly the same manner as the concentrate from pig glands.[4]

Purification by Zone Electrophoresis on Starch.—Preliminary experiments indicated that the MSH activity in the bovine crude concentrate had a greater cathodic mobility at *p*H 4.9 than did that in the porcine crude concentrate. Attempts were therefore made to perform the electrophoresis at a higher *p*H, and a *p*H 6.5 pyridine–acetic acid buffer was chosen. Although excellent resolution was obtained at this *p*H, the use of this particular buffer was discontinued because of certain technical difficulties. Among these were the increased trapping of bubbles in the starch during packing and the incomplete sedimentation of the individual starch segments during centrifugation in the presence of the buffer. Consequently, further purification by zone electrophoresis on starch was carried out with the same buffer and by the same procedure as that employed for the porcine hormone[4,12] except that only 100 mg. was used per trough; at higher loads the resolution was poor. A typical distribution pattern of material revealed by the reagent of Lowry, *et al.*,[13] is shown in the upper half of Fig. 1. The bulk of the MSH activity[14] was concentrated in segments 22–31; the extracts of these segments were pooled, filtered and lyophilized. The recovered powder was routinely rerun under identical conditions at a load of 100 mg. per trough. The pattern obtained from such a rerun is shown in the lower half of Fig. 1.

Purification by Countercurrent Distribution.—The purified material (200–400 mg.) obtained from zone electrophoresis was submitted to countercurrent distribution in the automatic all-glass apparatus[15] and in the same system that was used for porcine β-MSH,[4] namely, 2-butanol–aqueous 0.5% trichloroacetic acid; the other details of the procedure were similar to those used in connection with the porcine hormone. The volume of the lower phase was 5.0 ml., and that of the upper phase, 5.4 ml.

In a typical experiment, after a forerun of 16 transfers, 370 mg. of material containing approximately 90 mg. of starch from the electrophoretic run was added to the combined lower phases of the first two tubes. The mixture was stirred and centrifuged. The supernatant fluid was collected with a pipet, and the precipitate (insoluble starch) was washed once with the combined upper phases of the first two tubes. The upper and lower phases were put back into the machine which was then shaken manually to permit equilibration of the phases in the first two tubes. After the phases had separated, the remainder of the run was carried out automatically. After 240 transfers, the optical density of the contents of every fourth tube (lower phase from tubes 0 to 160; upper phase from tubes 161 to 239) was determined at 277 mμ. Over 95% of the material was present

(1) (a) B. G. Benfey and J. L. Purvis, THIS JOURNAL, **77**, 5167 (1955); (b) *Biochem. J.*, **62**, 588 (1956).

(2) A. B. Lerner and T. H. Lee, THIS JOURNAL, **77**, 1066 (1955).

(3) J. O. Porath, P. Roos, F. W. Landgrebe and G. M. Mitchell, *Biochim. Biophys. Acta*, **17**, 598 (1955).

(4) (a) I. I. Geschwind, C. H. Li and L. Barnafi, THIS JOURNAL, **78**, 4494 (1956); (b) I. I. Geschwind and C. H. Li, *ibid.*, **79**, 615 (1957).

(5) J. I. Harris and P. Roos, *Nature*, **178**, 90 (1956).

(6) I. I. Geschwind, C. H. Li and L. Barnafi, THIS JOURNAL, **79**, 620 (1957).

(7) H. Waring and F. W. Landgrebe, in "The Hormones," Vol. II, Ed. by G. Pincus and K. V. Thimann, Academic Press, Inc., New York, N. Y., 1950.

(8) As used in this paper, "posterior lobe" denotes posterior plus intermediate lobes of the pituitary.

(9) F. W. Landgrebe and G. M. Mitchell, *Quart. J. Exper. Physiol.*, **39**, 11 (1954).

(10) T. H. Lee and A. B. Lerner, *J. Biol. Chem.*, **221**, 943 (1956).

(11) I. I. Geschwind, C. H. Li and L. Barnafi, THIS JOURNAL, **79**, 1003 (1957).

(12) The run was carried out at 4°. In a previous publication,[4b] because of a typographical error, this temperature was erroneously reported as 40°.

(13) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

(14) The melanocyte-stimulating activity was determined by the method described by K. Shizume, A. B. Lerner and T. B. Fitzpatrick (*Endocrinology*, **54**, 553 (1954)); the unit is the same as that described by these investigators.

(15) L. C. Craig, W. Hausmann, E. H. Ahrens and E. J. Harfenist, *Anal. Chem.*, **23**, 1276 (1951).

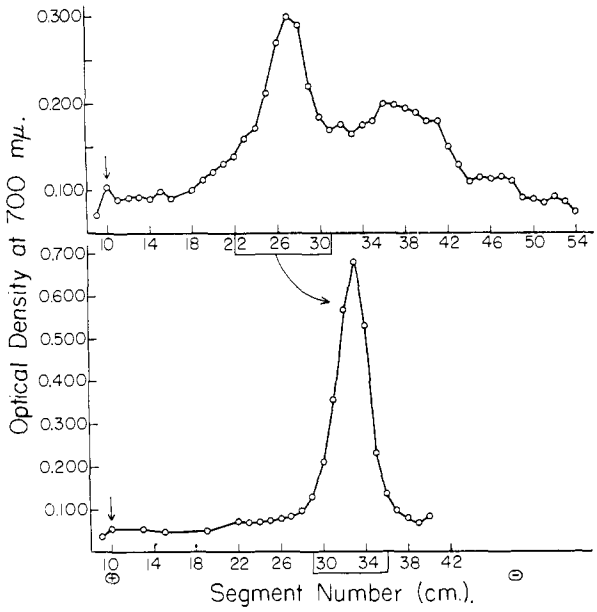

Fig. 1.—Upper, pattern obtained from zone electrophoresis of crude bovine MSH concentrate on starch; 60 cm. trough, 0.1 *M* pyridine-acetic acid buffer at *p*H 4.9, 230 volts, 48 hours. The activity is concentrated in segments 22–31. Lower, electrophoretic pattern obtained with material that had previously been purified by zone electrophoresis on starch. Conditions the same as above.

in a major peak with a K value of approximately 0.6 and a trailing shoulder with a K value of about 0.4. The remaining optical density was present as a high, irregular baseline extending from tubes 162 to 222. The contents of these latter tubes were then removed, and were replaced by fresh upper and lower phases. The machine was then set for cycling and was allowed to proceed automatically to a total of 702 transfers. The optical densities of the lower phases of every fourth tube were then determined, and, once the main peaks were located, the contents of every other tube making up the peaks also were analyzed.

The distribution obtained is shown in the upper part of Fig. 2. There are present one main peak (K = 0.53) and two smaller peaks (K = 0.37 and 0.30). More than 90% of the biological activity was located in the main peak.

The contents of tubes 226–250 (pool I) and of tubes 251–278 (pool II) were separately pooled, concentrated in a rotary evaporator, extracted with diethyl ether and subsequently lyophilized. The distribution apparatus was then filled with fresh upper and lower phases; pool I (87 mg.) was added to the lower phase of the first tube of the upper train and pool II (98 mg.) was added to the lower phase of the first tube (tube 120) of the lower train. The machine was set for recycling and allowed to proceed automatically for 251 transfers. Analysis of lower phases in alternate tubes revealed the distribution shown in the lower half of Fig. 2. Both peaks have practically identical partition coefficients (0.52, 0.53) and both show some trailing, which is more evident in the larger peak (pool II). A small but definite peak with a K value of approximately 0.3 is also seen. The material representing each of the two main peaks was individually pooled, concentrated and lyophilized; 69 mg. of pool I and 72 mg. of pool II were obtained (13.1% N; trichloroacetate). From 1 kg. of acetone-dried powder of bovine posterior pituitaries, approximately 150 mg. of MSH was isolated. The average activity of such preparations is 0.2×10^7 u./mg.[14]

Characterization

Amino Acid Analysis.—Quantitative amino acid analyses by the method of Levy[16] have been performed on 3 different samples of MSH obtained following countercurrent distribution. In all cases, 2 to 3-mg. samples were hydrolyzed

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

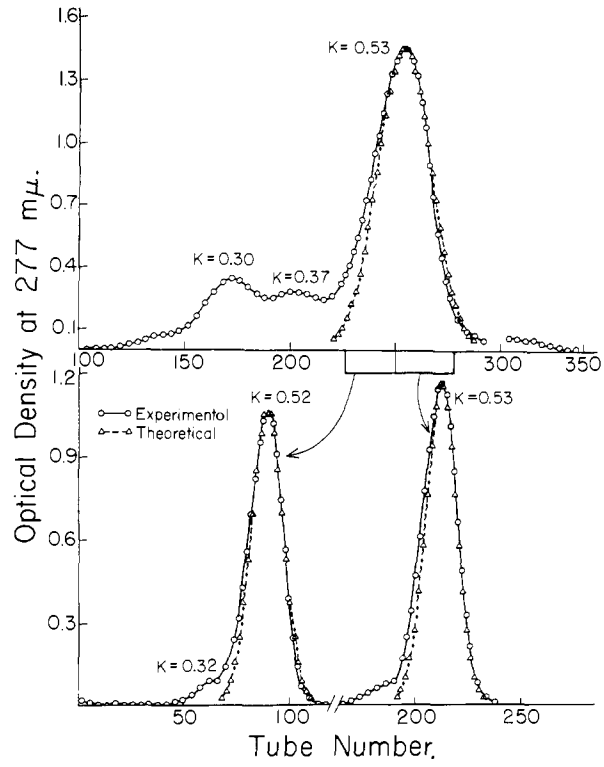

Fig. 2.—Upper, countercurrent distribution (702 transfers) of material obtained from zone electrophoresis on starch. System, 2-butanol-0.5% aqueous trichloroacetic acid. Lower, countercurrent distribution (251 transfers) of the two halves of the main peak obtained above (upper, Fig. 2). The material found in tubes 226–250 after the first distribution was placed in tube 0 and that found in tubes 251–278 was placed in tube 120. System same as above.

at 100° in 5.7 N HCl for 24 hours. The hydrolyzates were dinitrophenylated according to the method of Sanger[17] in 63% ethanol containing 2% $NaHCO_3$ and 4% fluorodinitrobenzene (FDNB). The reaction mixture was shaken for 3 hours at room temperature on a simple shaker designed by L. Barnafi.[18] The dinitrophenylated amino acids were then submitted to two-dimensional chromatography on paper, in the "toluene" and 1.6 M phosphate systems described by Levy[16]; each sample was run in triplicate or quadruplicate. The yellow spots were excised and eluted, and the optical densities were determined. In this manner the molar ratios for all the amino acids, with the exception of tryptophan, were obtained. Tryptophan[19] and amide ammonia[20] were estimated separately. In order to determine the optical configuration of the amino acids, a fourth sample was hydrolyzed and submitted to microbiological assay.[21] The results of the FDNB analyses on three different countercurrent preparations of bovine MSH and of the single microbiological assay (Table I) suggest the following empirical formula for the bovine MSH

$$Asp_2Glu_1Ser_2Gly_2Pro_3Met_1Phe_1Tyr_1Lys_2His_1Arg_1Try_1$$

All the optically active amino acids are of the L-configuration (see Discussion for configuration of tryptophan).

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

(18) This shaker has as its basic component a vacuum-operated automobile windshield-wiper motor.

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

(20) A. L. Levy, I. Geschwind and C. H. Li, *J. Biol. Chem.*, **213**, 187 (1955).

(21) The microbiological assay was carried out by Shankman Laboratories, Los Angeles. Only organisms which respond solely to the L-form were used. No determination of glycine was made, and in the case of glutamic acid only a few determinations were made.

The minimum molecular weight calculated from this formula is 2135; the theoretical nitrogen content is 17.0%.

Determination of Isoelectric Point.—The electrophoretic mobilities of bovine MSH on starch were determined as previously described.[4] As shown in Fig. 3, the isoelectric point of the hormone was found to be *p*H 7.0.

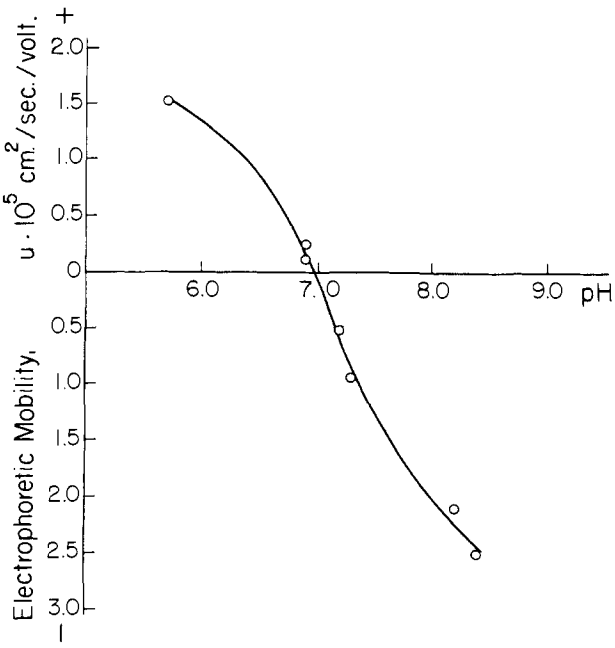

Fig. 3.—Electrophoretic mobility of bovine MSH on starch as a function of *p*H. The mobilities have been corrected for electroösmosis.

Degradation Procedures

N-Terminal Amino Acid Sequence.—The N-terminal residue of the MSH molecule was determined on 3 mg. of MSH by the FDNB[17] procedure; the sole ether-soluble DNP amino acid found following acid hydrolysis was identified by the chromatographic procedure of Levy[16] as DNP aspartic acid; a yield of 0.6 μM per 3 mg. of the dinitrophenylated MSH was obtained. This represents a recovery of 0.6 mole/mole, assuming that 5 DNP residues (reaction with one α-NH_2, two ϵ-NH_2, one O–Tyr and one Im–His) have been introduced into the dinitrophenylated hormone, thereby increasing the molecular weight of the hormone from approximately 2100 to 3000. Since there have been reports by other workers that N-terminal aspartic or asparagine residues may be lost occasionally in the course of dinitrophenylation,[22,23] this possibility was investigated. Examination of the ether extracts of the acidified dinitrophenylation reaction mixture revealed the presence of approximately 0.05 mole/mole of DNP aspartic acid. This value, though extremely small, was observed in two different experiments.

The aqueous solution remaining after ether extraction of the hydrolyzate of the DNP peptide was chromatographed on paper in 1 M Na_2HPO_4, a system which permits the separation of α- and ϵ-DNP lysines.[24] A recovery of 1.7 μM of ϵ-DNP lysine was obtained. No other water-soluble, colored, DNP amino acid was evident.

The sequence of amino acids at the N-terminus was determined by means of the paper-strip modification[25] of the phenyl isothiocyanate procedure[26]; 5 mg. of the hormone was employed for each experiment. The phenylthiohydantoins (PTH) obtained at each step were identified following chromatography of an aliquot on paper in the heptane–pyridine system of Sjöquist.[27] Another aliquot was hydrolyzed with constant boiling HCl at 150° for 16 hours[28] to regenerate the parent amino acids which were identified after chromatography in the system *n*-butanol–acetic acid–water.[29] The degradation procedure was employed successfully for seven steps, revealing in order the following amino acids: Asp, Ser, Gly, Pro, Tyr, Lys and Met. In the second step, along with the PTH of serine, a very definite spot of the PTH of glycine was also evident on the chromatograms. This latter spot reached its maximum at the third step, was still evident at the fourth, and was no longer visible by the fifth step.

TABLE I

MOLAR RATIOS OF AMINO ACIDS IN BOVINE β-MSH

Amino acid	MSH preparations CCI[a]	CCII[a]	CCIII[b]	CCIV[c]	Residues to the nearest integer
Glutamic acid	1.1	0.9	0.88	0.82	1
Aspartic acid	1.8	2.2	2.08	2.10	2
Serine[d]	2.0	1.9	2.05	1.98	2
Glycine	2.0	2.0	1.98		2
Proline	3.0	3.0	3.13	2.90	3
Methionine	1.2	1.0	1.00	0.97	1
Phenylalanine	1.0	1.1	0.90	1.11	1
Tyrosine	0.9	1.1	1.00	0.93	1
Lysine	1.8	1.9	1.95	2.10	2
Histidine	1.0	1.1	1.00	1.02	1
Arginine	0.9	1.0	1.13	0.89	1
Tryptophan	1.2	1.0			1
Amide NH_3		0.0	0.0		..
				Total	18

[a] Analysis by FDNB method[16]; average of three chromatograms. [b] Analysis by FDNB method; average of four chromatograms. [c] Analysis by microbiological assay.[21] [d] Corrected for 10% destruction.

Digestion with Leucine Aminopeptidase (LAP).[30]—Digestion with LAP was employed as an enzymic approach for determining the sequence of amino acids at the N-terminus. One ml. of the enzyme solution (0.6 mg. enzyme) treated with diisopropyl fluorophosphate[31] was added to 0.6–0.7 μM[32] of the hormone in 1 ml. of solution (0.02 M tris buffer, *p*H 8.1; 0.002 M Mg^{++}) to give an enzyme:substrate molar ratio of 1:300–1:350. The resulting solution was incubated at 40°. At designated intervals up to 24 hours, aliquots of the reaction mixture were removed and allowed to react with FDNB. The free DNP amino acids were then extracted from the acidified reaction mixture into ether and the amounts of the DNP amino acids were quantitatively determined by the method of Levy. The rate of release of amino acids by LAP is plotted in Fig. 4a and, for comparison, the rate of release of amino acids from porcine β-MSH is presented in Fig. 4b. Even after 24 hours, only 0.43 mole per mole of the N-terminal aspartic acid of bovine MSH had been released (these results are typical of three different digestions carried out with different batches of bovine MSH). The only other amino acid found was serine, the amount of which also increased steadily during the 24 hours of digestion. A comparison of the results of these experiments on bovine MSH with those obtained with β-MSH will be reserved for the Discussion.

C-Terminal Amino Acid Sequence.—The sole approach applied to the investigation of the sequence of amino acids at the C-terminus was enzymic. For these investigations carboxypeptidase (Worthington) was treated with a 50-fold molar excess of DFP, and 0.6 mg. of the treated enzyme was incubated at 40° with 1 μM of the hormone (enzyme:sub-

(22) E. O. P. Thompson, *Biochim. Biophys. Acta*, **10**, 633 (1953).
(23) F. Weygand and R. Junk, *Z. physiol. Chem.*, **300**, 27 (1955).
(24) J. Cummins, personal communication.
(25) H. Fraenkel-Conrat, THIS JOURNAL, **76**, 3606 (1954).
(26) P. Edman, *Acta Chem. Scand.*, **4**, 283 (1950).
(27) J. Sjöquist, *ibid.*, **7**, 447 (1953).
(28) A. L. Levy, *Biochim. Biophys. Acta*, **15**, 589 (1954).
(29) S. M. Partridge, *Biochem. J.*, **42**, 238 (1948).
(30) D. H. Spackman, E. L. Smith and D. M. Brown, *J. Biol. Chem.*, **212**, 255 (1955). Some of the enzyme used was a gift from Dr. L. K. Ramachandran.
(31) R. L. Hill and E. L. Smith, unpublished manuscript (personal communication).
(32) The molecular weight of MSH assumed in this paper is the minimal value obtained from the amino acid analyses—2135. At all times weights of materials were corrected to the true peptide weight (based on N Content).

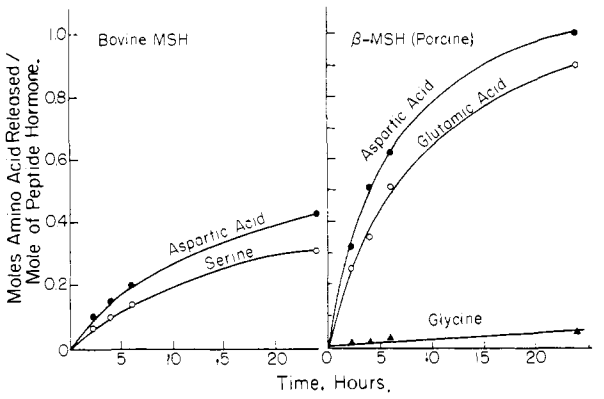

Fig. 4.—Liberation of amino acids from bovine MSH (left) and porcine β-MSH (right) by leucine aminopeptidase.

strate molar ratio 1:55) in 1 ml. of 1% $NaHCO_3$. At designated intervals aliquots were removed, were subjected to dinitrophenylation, and were otherwise treated in the same manner as the aliquots from the leucine aminopeptidase experiment reported above. After 24 hours only one free amino acid, aspartic acid, was found to be present, in a yield of 0.2–0.25 mole per mole.

Chymotryptic Digestion.—Approximately 6 μM of the hormone in aqueous solution (2 ml.) was incubated with 0.75 mg. of chymotrypsin (Armour). The solution was adjusted with NH_4OH to *p*H 8.3, at which *p*H the solution was still slightly turbid. Digestion was carried out at 40° for 18 hours, at the end of which time the solution was clear. The products of digestion were separated by zone electrophoresis on paper in a γ-collidine–acetic acid–water buffer[33] of *p*H 7.0. The details of this procedure were exactly the same as those reported for the enzymic digests of β-MSH.[6] At the termination of the electrophoretic run, guide-strips were cut from the dried paper and sprayed with ninhydrin. The pattern obtained was similar to that seen with β-MSH,[6] with the exception that in the digestion products from the bovine hormone the only band that migrated toward the anode (Ch-5B) had a lesser mobility than did the analogous band derived from the porcine hormone (Fig. 5). The mobilities of the three major bands moving toward the cathode were apparently the same in the digests of the bovine and porcine hormones. Spray reagents specific for the various amino acids revealed in which bands arginine,[34] tyrosine,[35] histidine[36] and tryptophan[37] were located; in each case the results were the same as those observed previously with the digest of β-MSH.

Each band was demarcated on the paper, with the guide-strips serving as reference, and was subsequently eluted with 1 *N* NH_4OH as previously described.[6]

Amino Acid Analyses of the Peptides Obtained from the Chymotryptic Digest.—From each eluate one-tenth of the solution was evaporated to dryness and hydrolyzed in 5.7 *N* HCl for 24 hours at 110°. The hydrolyzate was then taken for duplicate quantitative amino acid analysis by the method of Levy.[16] The results are presented in Table II.

The empirical formulas for each of the peptides, as derived from the amino acid analyses, are as follows: Ch-2B (Arg,Try); Ch-3B (Lys,Met,Glu,His,Phe); Ch-4B (Gly, Ser,Pro_2,Lys,Asp); and Ch-5B (Asp,Ser,Gly,Pro,Tyr).

N-Terminal Amino Acids and Amino Acid Sequences of the Peptides Obtained from the Chymotryptic Digest.—From each eluate two-tenths of the solution was allowed to react with FDNB (2%) for 2 hours. The DNP-peptides were extracted from the acidified reaction mixture into ether or ethyl acetate, taken to dryness, and hydrolyzed in 5.7 *N* HCl for 16 hours at 110°. Each peptide possessed only one N-terminal amino acid. From Ch-2B, DNP-arginine was found; from Ch-3B, diDNP-lysine; Ch-4B, DNP-glycine and ε-DNP lysine; and from Ch-5B, DNP-aspartic acid. The yield of DNP-glycine from Ch-4B was approximately 25% of the theoretical value.[38]

The N-terminal amino acid sequences of peptides Ch-3B and Ch-4B were investigated by allowing one-quarter of the eluate to react with DFP-treated leucine aminopeptidase,[30] with the tris buffer employed above. With peptide Ch-3B and 37.5 μg. of the enzyme (E:S = 1:1 × 10^4), the peptide was completely hydrolyzed within 30 minutes at room temperature. By taking aliquots at very short intervals after the addition of the enzyme, it was possible to determine (Table III) that lysine was the N-terminal amino acid, and that, following its release, methionine was released by the enzymic action. Subsequently glutamic acid, histidine and phenylalanine all appeared almost simultaneously. In contrast to its effect on Ch-3B, the enzyme released only one amino acid, glycine, from Ch-4B even after reaction for 24 hours at 40° at an enzyme to substrate molar ratio of 1:625. After 2 hours of digestion all of the glycine had appeared, and no other amino acid appeared subsequently.

C-Terminal Amino Acid Sequences of the Peptides Obtained from the Chymotryptic Digest.—From each eluate two-tenths of the solution was allowed to react for 24 hours at room temperature with 0.6 mg. of DFP-treated carboxypeptidase. The solution was then allowed to react with FDNB (3%) for 3 hours, and the DNP amino acids formed were identified by the Levy procedure.[16] The results are presented in Table IV. It will be seen that, aside from what is observed with the dipeptide Ch-2B, only from peptide Ch-3B was more than one amino acid obtained; the results indicate that in this particular peptide the C-terminal sequence is . . . .His.Phe.

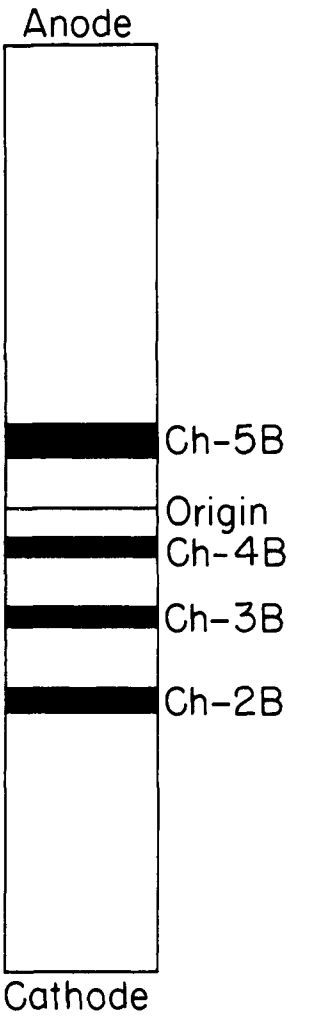

Fig. 5.—Resolution of a chymotryptic digest of bovine MSH by zone electrophoresis on paper. System, γ-collidine–acetic acid–water at *p*H 7.0; 11 volts/cm. for 6 hours.

Stepwise Degradation of Peptide Ch-4B.—In order to determine the sequence of amino acids in Ch-4B, the final quarter of the eluate containing Ch-4B was taken for stepwise degradation by a modification[39] of the phenyl isothiocyanate reaction. The reaction cycle was repeated for four steps. The phenylthiohydantoins formed were identified by chromatography of an aliquot on paper in the heptane–pyridine system, and further identified by regeneration of the parent amino acid from another aliquot (*vide supra*). In this manner the sequence gly.ser.pro.pro was formulated. Finally, the aqueous solution remaining after extraction of the fourth phenylthiohydantoin was taken to dryness and then allowed to react with FDNB. After hydrolysis of the

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

(34) J. P. Jepson and I. Smith, *Nature*, **172**, 1100 (1953).

(35) R. Acher and C. Crocker, *Biochim. Biophys. Acta*, **9**, 704 (1952).

(36) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463 (1951).

(37) I. Smith, *Nature*, **171**, 43 (1953).

(38) No values for the recoveries of the other DNP N-terminal amino acids are given because the amounts of DNP peptides available were too small to weigh. The recovery of DNP-glycine was obtained by comparing it with the recovery of ε-DNP-lysine, also obtained from the same peptide.

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

TABLE II

AMINO ACID ANALYSES[a] OF THE PEPTIDES OBTAINED FROM CHYMOTRYPTIC DIGEST OF BOVINE β-MSH

Amino acid	Peptide, μM Ch-2B	Ch-3B	Ch-4B	Ch-5B
Glutamic acid		0.241 (1.04)[b]		
Aspartic acid			0.266 (0.97)	0.273 (1.02)
Serine			.274 (1.00)	.234 (0.90)
Glycine			.292 (1.07)	.292 (1.09)
Proline			.566 (2.06)	.248 (0.93)
Methionine		0.265 (1.15)		
Phenylalanine		.236 (1.02)		
Tyrosine				.251 (0.94)
Lysine		.202 (0.88)	.256 (0.94)	
Histidine		.210 (0.91)		
Arginine	0.249			
Tryptophan	0.059			

[a] Analysis on an aliquot equal to 1/20 the total amount of peptide; average of 2 such analyses. [b] Values in parentheses are the molar ratios for each amino acid in the peptide.

TABLE III

THE RELEASE OF AMINO ACIDS FROM PEPTIDE CH-3B BY THE ACTION OF LEUCINE AMINOPEPTIDASE[a]

Amino acid	Time[b] 0 μM	5 μM	10 μM	15 μM	20 μM	60 μM
Lysine	0.005	0.038	0.055	0.060	0.062	0.064
Methionine	...	.023	.042	.050	.054	.064
Glutamic acid	...	.007	.026	.041	.047	.064
Histidine	...	.010	.029	.040	.043	.064
Phenylalanine	...	.008	.027	.042	.046	.064

[a] Reaction carried out at room temperature; Enzyme: substrate molar ratio = 1:1 × 10⁴. [b] Minutes after addition of enzyme.

TABLE IV

C-TERMINAL AMINO ACIDS RELEASED BY CARBOXYPEPTIDASE FROM THE PEPTIDES OBTAINED FROM CHYMOTRYPTIC DIGEST OF BOVINE β-MSH

Peptide	Amino acids
Ch-2B	Tryptophan (0.875 μM)[a]
Ch-3B	Phenylalanine (0.871 μM); histidine (0.126 μM)
Ch-4B	Aspartic acid (0.432 μM)
Ch-5B	Tyrosine (0.884 μM)

[a] DNP-arginine was obtained from the water-soluble fraction.

DNP product and extraction of any ether-soluble components, paper chromatography in 1 *M* Na_2HPO_4[24] revealed the presence of a yellow spot with the same R_f as α-DNP-lysine (both α-DNP- and ε-DNP-lysine were run as controls on the same sheet). From the ether-soluble fraction only dinitrophenol was obtained in any yield.

Discussion

The procedure employed for the isolation of bovine β-MSH is patterned after the procedure developed for β-MSH of porcine origin,[4] from which it differs only in minor details. Once again, the most important single step in the isolation procedure appears to be the selective adsorption of the hormone on oxycellulose. As already has been shown,[9] this one procedure permits approximately a 40-fold purification of bovine melanocyte-stimulating activity.

The material eluted from oxycellulose has been submitted to zone electrophoresis on starch. In the course of this procedure, certain reproducible effects have been observed with the bovine hormone which have not been previously seen with a porcine crude concentrate. Thus, it may be noticed (Fig. 1) that the material purified by a single electrophoretic run possesses a greater cathodic mobility than does the crude material (migration of 17 and 23 cm. for the crude and purified materials, respectively). Futhermore, the pattern for the purified material appears considerably sharpened (note in the original electrophoretic pattern the broad cut from which the purified material was obtained). Both effects can be explained on the basis of an interaction between the hormone and some other component(s) present in the crude material, although it is possible that some complete and irreversible alteration in the hormone occurs in the course of the first isolation from starch.

The amino acid and N-terminal group analyses of material which has been twice submitted to zone electrophoresis, and of the product obtained from the countercurrent procedure, are identical. In this respect the bovine material purified by zone electrophoresis differs from the porcine hormone at the comparable stage of the isolation procedure. In the latter case, a number of contaminating amino acids not present in the final product are present in the electrophoretic product.[4] Despite the apparent purity of the bovine hormone at this point in the isolation scheme, countercurrent distribution has resulted in the resolution of three components of differing partition coefficients. However, the quantitative amino acid analysis of each of these components is identical. The entire pattern is highly reminiscent of that observed with the porcine hormone, with the exception that the *K* values for the bovine components are generally, and reproducibly, 15% lower than those obtained for the components of porcine origin. Because of the lack of sufficient quantities of purified porcine and bovine hormones and of the large number of transfers that would be required to separate components with *K* values of 0.64 (porcine) and 0.53 (bovine) in this system, no attempt has been made to see whether the *K* values obtained for the bulk of the hormone from the two species are indeed really different and whether the analogous components from the different species may be resolved.

It is of some interest at this point to compare the isolation procedure described above with that used by Benfey and Purvis[1b] for their bovine MSH preparation. The starting material used by these investigators was obtained by the methods developed by Stehle,[40] in which dilute acetic acid extracts are treated, in order, with ethanol, methanol and ethyl acetate, and are then further fractionated by precipitation with picric acid. When such material was submitted to countercurrent distribution in the same system that we have employed, namely, 2-butanol–aqueous 0.5% trichloroacetic acid, a *K* value of 7 was obtained, in contrast with the value found in this paper of 0.53 for the bovine hormone. The system actually used by Benfey and Purvis for the purification of their bovine preparation was 2-butanol–aqueous 0.1% trichloroacetic acid, in which the melanocyte-stimulating

(40) (a) R. L. Stehle, *Rec. Canad. Biol.*, **3**, 408 (1944); (b) *Brit. J. Pharmacol.*, **8**, 435 (1953).

activity had a K value of 0.74–0.85.[1b] The preparation of Benfey and Purvis also varies considerably from the bovine preparation reported herein with respect to its amino acid composition. For example, it contains large quantities of alanine, leucine and valine (3, 3 and 5 residues, respectively, per mole consisting of a total of 47 residues). In addition, cystine, isoleucine and threonine are present. None of these six amino acids is present in the bovine β-MSH, whose analysis is given in Table I. There are, as well, great differences in the quantities of those amino acids that are common to both preparations. In the light of the marked differences in K values and amino acid composition between the two, these preparations would appear to be unrelated. Although there is apparently no connection between the bovine preparation of Benfey and Purvis and the porcine α-MSH of Lerner and Lee[2,10] the isolation of two unrelated melanocyte-stimulating hormones from bovine posterior lobes[8] is reminiscent of the discovery of α- and β-MSH in porcine posterior lobes.

The amino acid analyses recorded in Table I indicate that the composition of the bovine hormone differs from that of β-MSH in that the former contains one more residue of serine and one less residue of glutamic acid than does the porcine hormone. The substitution of a neutral residue for an acidic one is sufficient to explain the more basic isoelectric point of the bovine preparation. There is illustrated in Fig. 6 a comparison of the mobilities of the bovine hormone and porcine β-MSH in a pyridine-acetic acid buffer, pH 4.9. To one trough, 1 mg. each of the bovine and porcine hormones was added at the origin; to a parallel trough 1 mg. of the bovine preparation alone was added. The separation achieved, effected during a 24 hour run at 5 v./cm., unequivocally illustrates the greater cathodic mobility of the bovine preparation at this pH. Knowledge of the amino acid composition also permits the calculation of the isoionic point of the hormone, pH 7.2, which compares favorably with the experimentally determined isoelectric point, 7.0. The isoionic point calculated from the data of Benfey and Purvis is approximately 6.8, if a complete absence of amide groups is assumed. Waring and Landgrebe[7] reported that in crude bovine posterior lobe extracts the melanocyte-stimulating activity had an isoelectric point of approximately 4.1. Ketterer and Kirk[41] have stated that the "melanophore-expanding activity of ox posterior lobe powder is complex," but that the "center of gravity for all. . .activity" was in the region of 4.8–4.9. In the light of the findings reported above, which demonstrated decreased cathodic mobilities in crude MSH preparations, one may suspect that interactions of the type suggested above may satisfactorily explain the observations of Waring and Landgrebe and Ketterer and Kirk.

At each pH utilized for the determination of the isoelectric point, as well as in pyridine–acetic acid buffers of pH 4.9 and 6.4, the material purified by the countercurrent procedure appeared to be homogeneous. The determination of the mobility at pH 8.4 was performed in a Veronal buffer of 0.1 ionic strength, and in this buffer the material also appeared to be homogeneous. However, in a borate buffer of similar pH (in the trough) and ionic strength, the spread of the material is considerable. This specific effect of borate buffer has been previously observed at times in this Laboratory by I. D. Raacke, and apparently is due to some interaction between material, buffer and starch.

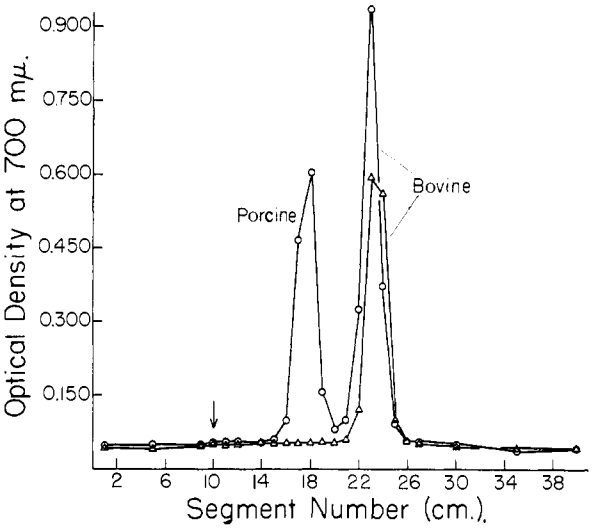

Fig. 6.—Patterns obtained from zone electrophoresis on starch of a purified bovine MSH (Δ) and of a mixture of bovine MSH and porcine β-MSH (O). Origin for both runs is designated by the arrow; 40 cm. trough, 0.1 M pyridine-acetic acid buffer at pH 4.9, 200 volts, 24 hours.

The material obtained by the recorded isolation procedure was considered sufficiently pure to permit an investigation of the structure of the hormone. In the light of the known structure of porcine β-MSH and the concept that the heptapeptide core[5,6] common to β-MSH and the corticotropins would probably remain inviolate, it was assumed that a simple substitution of serine for glutamic acid occurred at the second residue in the β-MSH structure to produce the bovine hormone. Preliminary support for this idea was forthcoming from the N- and C-terminal group analyses. Further support was obtained from the degradation of the N-terminal sequence with phenyl isothiocyanate which revealed a serine residue next to the N-terminal aspartic acid. However, at this point a fair amount of glycine was usually evident also. Apparently under the acidic conditions needed for the cyclization of the terminal aspartic phenylthiocarbamate, the particularly labile seryl–glycine bond is partially broken. No such effect was observed with the comparable glutamyl–glycine bond of the porcine β-MSH. In all, the phenyl isothiocyanate method was employed for the elucidation of a sequence of seven consecutive amino acids. At each step the quantities of phenylthiohydantoins formed were determined spectrophotometrically,[25] but, as reported previously,[6] the amount of identifiable phenylthiohydantoins evident on the chromatograms was much less than that indicated by spectrophotometry. Recently, Elliott and Peart[42] reported similar findings in the course of

(41) B. Ketterer and R. L. Kirk, *J. Endocrinol.*, **11**, 19 (1954).

(42) D. F. Elliott and W. S. Peart, *Biochem. J.*, **65**, 246 (1957).

the degradation of hypertensin. These findings may be explained in terms of Edman's[43] recent formulation of the mechanism of the phenyl isothiocyanate degradation.

For confirmation of the N-terminal sequence, leucine aminopeptidase[30] was employed. Although the enzyme promoted the release of aspartic acid and serine, only 0.43 mole of the N-terminal aspartic acid was released from the bovine hormone in 24 hours under the conditions chosen (Fig. 4a). Under the same conditions, 1.0 mole of aspartic acid was released from porcine β-MSH (Fig. 4b). Thus the rate of release of identical N-terminal amino acids from two different substrates was markedly modified by the nature, in each instance, of the adjacent amino acid. It may be further noted that in both cases little or none of the third amino acid, glycine, appeared. Although glycine is the amino acid most slowly released by LAP,[44] the failure of the enzyme to release glycine from both hormones is believed to be due to the glycyl–proline linkage which is involved. It has been our general experience that amino acids in peptide linkage with the imino group of proline are not released. Thus, it will also be recalled that LAP hydrolyzed the release of only the terminal glycine of peptide Ch-4B. The initial sequence found for this peptide was Gly·Ser·Pro. . . . Here, too, a bond involving the imino group of proline was not split.[45] On the other hand, Elliott and Peart[42] have found that LAP (enzyme to substrate ratio unrecorded) promoted the release of proline, and the amino acids which precede it, from the hypertensin molecule. Consequently, we have reported our experiences with this enzyme without attempting to generalize our findings.

Since submission of the intact hormone to stepwise chemical and enzymic degradative procedures permitted the identification of only seven amino acids at the N-terminus along with the C-terminal aspartic acid, proteolysis with chymotrypsin was employed to cleave the hormone into the four component peptides which, on the basis of the aromatic amino acid content of the hormone, are theoretically possible. Of the four peptides resolved by paper electrophoresis, three were identical in terms of mobility, N- and C-terminal groups, and amino acid analysis, with peptides obtained from the chymotryptic digestion of porcine β-MSH.[6] On the other hand, the only peptide which migrated toward the anode during the electrophoretic run (Ch-5B) did so at a lesser mobility than that observed with peptide Ch-5 from the porcine hormone hydrolysate. Furthermore, though the N- and C-terminal groups of Ch-5B were identical with those found for the comparable porcine peptide, its amino acid composition differed in one more serine and one less glutamic acid, thus explaining the decreased mobility of the bovine peptide.

With respect to the analyses of the peptides obtained by chymotryptic digestion, reference should be made to a number of findings. First, from the amino acid analyses it may be calculated that approximately 5 μmoles of each peptide was obtained. This value represents a recovery of greater than 80% of theory, without the application of any correction for losses encountered through guide strips, incomplete elution, etc. Second, the amount of C-terminal aspartic acid released by carboxypeptidase from Ch-4B is greater than that obtained from the intact hormone (Table IV). A similar finding was made for the comparable porcine peptide and hormone. Third, in the course of the action of leucine aminopeptidase on Ch-3B, after lysine and methionine are released, the following three amino acids, *i.e.*, glutamic acid, histidine and phenylalanine, are released almost simultaneously (see Table III). This may be explained as due to the rate-limiting release of glutamic acid, followed by rapid hydrolysis of the bond between histidine and phenylalanine. This effect is the same as that observed by White[48] during the hydrolysis by LAP of Corticotropin A, in which the same sequence . . .Glu.His.Phe. . . occurs. Finally, the release of tryptophan from Ch-2B by carboxypeptidase supplements the data from microbiological assay presented in Table I by establishing the L-configuration for this amino acid; thus, it has been found that all the amino acids in this hormone are of this configuration. The large number of amino acids released by LAP and by carboxypeptidase from the intact molecule and from the peptides obtained from the chymotryptic digest is consistent with this finding.

The only peptide whose structure was not readily determined either from the analytical data or from the results of the action of LAP was Ch-4B. As mentioned above, LAP promoted the release of only the N-terminal glycine, and so, in order to determine the sequence of amino acids in this peptide, the phenyl isothiocyanate procedure was employed. After the first four amino acids had been removed, the remaining dipeptide (ϵ-phenylthiocarbamyl-lysyl-aspartate) was dinitrophenylated to give α-DNP-ϵ-phenylthiocarbamyl-lysyl-aspartate, which upon hydrolysis gave rise to α-DNP-lysine and aspartic acid.

With the elucidation of the structure of peptide Ch-4B, the sequence of amino acids in the hormone could be formulated (Table V). It may be seen

(43) P. Edman, *Acta Chem. Scand.*, **10**, 761 (1956).

(44) E. L. Smith and D. H. Spackman, *J. Biol. Chem.*, **212**, 271 (1955).

(45) Another example that may be cited is the action of LAP (unpublished observations) on Tr-2,3, the C-terminal peptide derived from a tryptic hydrolysate of α-corticotropin.[46] The initial sequence of this peptide, as derived by the phenyl isothiocyanate procedure, is Val.Tyr.Pro. . . .[46] The enzyme releases valine from this peptide very rapidly, and even at enzyme to subtrate ratios of 1:30 for 24 hours no tyrosine is released, despite the fact that this amino acid is a very favorable substrate for the enzyme.[44] Once valine and tyrosine are removed from this peptide by the phenyl isothiocyanate procedure, LAP releases proline readily since LAP may split off proline with a *free* imino group. A final example derived from work in this Laboratory has been obtained with lactogenic hormone. The initial sequence of this hormone is Thr.Pro.Val. . . .[47] Employing the performic acid-oxidized protein as a substrate, J. Cummins (personal communication) has found that LAP does not promote the release of any amino acid. One may also refer to the findings of Smith and Spackman[44] that the dipeptide glycyl-proline is not attacked by LAP, although prolyl-glycine is split at a rate 2.7% of leucyl-leucine.

(46) C. H. Li, I. I. Geschwind, R. D. Cole, I. D. Raacke, J. I. Harris and J. S. Dixon, *Nature*, **176**, 687 (1955).

(47) R. D. Cole, I. I. Geschwind and C. H. Li, *J. Biol. Chem.*, **224**, 399 (1957).

(48) W. F. White, THIS JOURNAL, **77**, 4691 (1955).

TABLE V

THE SEQUENCE OF AMINO ACIDS IN BOVINE β-MSH ("SERYL-β-MSH")

Method	Substrate	Product	Sequence
FDNB	H[a]		Asp.
LAP	H		Asp.Ser.
PITC	H		Asp.Ser.Gly.Pro.Tyr.Lys.Met
Chymotrypsin	H	Ch-5B[b]	Asp(Ser,Gly,Pro)Tyr
Chymotrypsin	H	Ch-3B[b]	Lys(Met,Glu)His.Phe
LAP	Ch-3B		Lys.Met(Glu,His,Phe)
Chymotrypsin	H	Ch-2B[b]	Arg.Try
Chymotrypsin	H	Ch-4B[b]	Gly(Ser,Pro,Pro,Lys)Asp
LAP	Ch-4B		Gly.
PITC	Ch-4B		Gly.Ser.Pro.Pro(Lys,Asp)
Carboxypeptidase	H		Asp
Complete sequence:			Asp.Ser.Gly.Pro.Tyr.Lys.Met.Glu.His.Phe.Arg.Try.Gly.Ser.Pro.Pro.Lys.Asp
			1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

[a] H = hormone. [b] The N-terminal amino acid was obtained by the FDNB procedure, and the C-terminal amino acid or sequence by the carboxypeptidase method.

that of all the peptides found in the chymotryptic digest, only the dipeptide Arg.Try (Ch-2B) must have its position assigned by elimination, for the contiguity of Ch-5B and Ch-3B is assured by the results of the phenyl isothiocyanate procedure when applied to the intact hormone, and the position of Ch-4B must be C-terminal since it alone possesses a C-terminal aspartic acid. As suspected, the sequence of amino acids in the bovine hormone differs from that found for porcine β-MSH[5,6] only at the second residue. It is therefore suggested that porcine and bovine β-MSH be henceforth called "glutamyl-β-MSH" and "seryl-β-MSH," respectively. The interchange of a neutral amino acid for an acidic one has not been previously encountered among biologically active peptides derived from different species, where experience to date has demonstrated interchanges of the neutral-neutral or basic-basic types.

Finally, in a series of 8 separate comparative assays[14] the porcine hormone has been found to be approximately 2.5 times as active as the bovine hormone. Thus, the interchange of amino acids at the position of the second residue has resulted in a modification of the hormonal activity. Seryl-β-MSH is comparable to the porcine hormone, however, in that upon being heated in 0.1 *N* NaOH for 15 minutes, "protection"[7] occurs with little or no "potentiation,"[7] in confirmation of the findings of Landgrebe and Mitchell[9] with an oxycellulose-eluate of bovine posterior lobe powder.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, NEW MEXICO HIGHLANDS UNIVERSITY]

Potential Purine Antagonists. VIII. The Preparation of Some 7-Methylpurines[1]

BY RAJ NANDAN PRASAD[2] AND ROLAND K. ROBINS[3]

RECEIVED MAY 13, 1957

A new method of synthesis of 7-methyladenine (III) and 7-methylhypoxanthine (V) has been accomplished from 4-amino-1-methyl-5-imidazolecarbonitrile (II) and 4-amino-1-methyl-5-imidazolecarboxamide (IV), respectively. The chlorination of V yielded 6-chloro-7-methylpurine (VI). Various new 7-methyl-6-substituted purines have been prepared from VI. 2,6-Dichloro-7-methylpurine (XIV) has been prepared from 7-methylxanthine (XIII). Several new 2,6-disubstituted-7-methylpurine derivatives have been prepared from XIV.

In the course of a general program for the synthesis of potential purine antagonists as antitumor agents, it was discovered that 6-chloro-9-methylpurine[4] showed definite activity. It therefore seemed desirable to prepare 6-chloro-7-methylpurine (VI) as a candidate anti-tumor agent. Since preliminary methylation studies of 6-chloropurine gave a difficultly separable mixture of the 7-methyl and 9-methyl isomers, the possible preparation of 6-chloro-7-methylpurine (VI) from 7-methylhypoxanthine (V) was investigated. Earlier methods of preparation of 7-methylhypoxanthine[5] proved unsatisfactory for large scale preparation. This prompted investigation of a new route to the preparation of 7-methylhypoxanthine (V).

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(4) R. K. Robins and H. H. Lin, THIS JOURNAL, **79**, 490 (1957).

(5) (a) E. Fischer, *Ber.*, **30**, 2400 (1897); (b) **31**, 104 (1898).