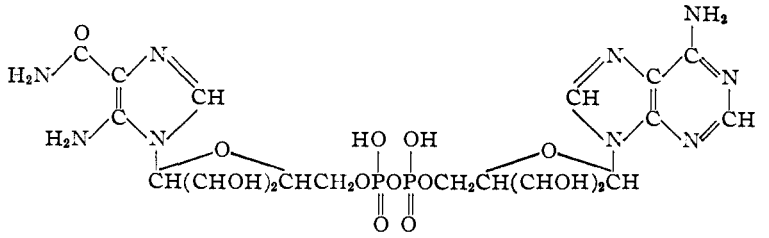
4-amino-5-carboxamido-imidazole, the known precursor of purines.[2] The enzyme was soluble, highly purified, beef-spleen DPNase. The reaction proceeded to completion according to the equation

The Imidazole + DPN ⟶ Nicotinamide + H^+ +



The dinucleotide was isolated in relatively pure form, and its structure established.

Although DPNases from animal sources have not previously been separated from cellular particles,[3,4] it was found possible to do so by treatment of washed beef-spleen cell fragments with isoamyl alcohol plus desoxyribonucleic acid. The solubilized enzyme was then purified by conventional methods (45 U./mg. protein).

The dinucleotide was formed by incubation of 33 units of this enzyme with 26.1 micromoles of DPN and 3000 micromoles of the imidazole at *p*H 7.5 in 10 ml. for 16 hr. at 37°. It was then isolated by paper electrophoresis (4°, 0.2 *M* ammonium acetate, *p*H 5.0). Its mobility toward the anode was 2.2 times that of DPN, whereas for the free imidazole it was 0, and for the nicotinamide-free hydrolytic product of DPN it was 2.5. Yield was 29 per cent. of the DPN, R_F 0.26 in the ethanol-acetic acid system[4] and 0.56 in the isoamyl alcohol K_2HPO_4 system,[5] both at 4°. It gave the test for diazotizable amines[6] and, when analyzed quantitatively, showed a ratio of imidazole to adenine to ribose to phosphate of 1.0:1.1:2.0:1.9.

The structure of the new dinucleotide was further established by cleavage with Kornberg's pyrophosphatase.[7] The products were isolated by electrophoresis and shown to be adenosine-5′-phosphate and a mononucleotide, the 5′-phosphate of the amino-carboxamidoimidazole riboside. This compound has been postulated by Greenberg[8] and by Buchanan[9] as the precursor of inosinic acid in pigeon liver and in bacteria. Mobility relative to DPN was 1.9, for adenosine-5′-phosphate was 1.6. It had R_F 0.69 in the isoamyl alcohol K_2HPO_4 system.[5]

These results strongly suggest that inosinic acid and its derivatives could arise in living things through the action of DPNase with this new dinucleotide as an intermediate. Such a pathway could be an alternative to the one viewed by Greenberg[8] or even the one he has been investigating.

The conversion of DPN to this new dinucleotide can be clearly distinguished from the exchange reactions of DPN with isoniazide and with β-acetyl-pyridine which have been described by Zatman, *et al.*[10] In these exchange reactions, (a) the energy rich quaternary linkage is not lost; (b) no H^+ is formed; and (c) the susceptibility to DPNase attack is not lost. On the contrary, in the new reaction, the quaternary linkage is lost, a H^+ is formed and the product is no longer attacked by DPNase. Similar features which distinguish between base exchange without loss of "onium" linkage and destruction of this linkage have been discussed fully in the case of thianiinase.[1]

FROM THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH NEW YORK, N. Y.
S. G. A. ALIVISATOS[11]
D. W. WOOLLEY

RECEIVED DECEMBER 27, 1954

(2) W. Shive, W. W. Ackermann, M. Gordon, M. E. Getzendaner and R. E. Eakin, THIS JOURNAL, **69**, 725 (1947).

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(11) Damon Runyon Memorial Fellow.

ISOLATION OF HOMOGENEOUS MELANOCYTE STIMULATING HORMONE FROM HOG PITUITARY GLAND

Sir:

The melanocyte stimulating hormone[1] (MSH), elaborated by the pituitary gland, darkens the skin of many chordates,[2] including man.[3] Although some investigators[4,5] suggest that MSH is not a distinct substance but an adrenocorticotropin, evidence is good that the major portion of MSH activity is distinct from other pituitary hormones.[2] We wish to report the isolation of a fraction homogeneous to electrophoresis and to countercurrent distribution possessing the main MSH activity of the pituitary gland.

One hundred g. of acetone dried hog posterior pituitary powder[6] (4–6 × 10^7 MSH u./g.)[7] mixed with 250 ml. acetone and 1 l. acetic acid, was heated to 50° for 10 min. and centrifuged. The residue was separated and re-extracted. The combined extracts (1900 ml.) were mixed with acetone (950 ml.) and 10 ml. saturated NaCl.[8] The supernate obtained by centrifugation was mixed with petroleum ether (5400 ml.) and allowed to stand overnight at −5°. The precipitate was acetone washed and then dried *in vacuo*. Sixteen to 18 g. of product

(1) Melanocyte stimulating hormone has been referred to also as melanophore hormone, melanophore dilating principle, intermedin, etc.

(2) H. Waring and F. W. Landgrebe in "The Hormones," edited by G. Pincus and K. V. Thimann, Academic Press, Inc., New York, N. Y., Vol. 2, 1950, p. 427.

(3) A. B. Lerner, K. Shizume and I. Bunding, *J. Clin. Endocrinol. & Metab.*, **14**, 1463 (1954).

(4) P. H. Bell, THIS JOURNAL, **76**, 5565 (1954).

(5) N. G. Brink, G. E. Boxer, V. C. Jelinek, F. A. Kuehl, Jr., J. W. Richter and K. Folkers, *ibid.*, **75**, 1960 (1953).

(6) We are grateful to the Armour Laboratories and Wilson & Company for supplying us with a total of 3 kg. of hog posterior pituitary powder.

(7) An MSH unit is defined by K. Shizume, A. B. Lerner and T. B. Fitzpatrick, *Endocrinology*, **54**, 553 (1954).

(8) F. W. Landgrebe and K. A. Munday, *Quart. J. Exp. Physiol.*, **39**, 11 (1954).

(1–2 × 10^8 MSH u./g.) was obtained. Eight grams was mixed with 150 ml. of 0.1 *N* acetic acid and centrifuged. Sixteen grams oxycellulose was added to the supernate and the mixture shaken for 75 min. Oxycellulose was removed by centrifugation, washed with 0.1 *N* acetic acid, then shaken in 100 ml. of 80% acetic acid for 60 min. The supernate was diluted with equal quantities of water. Lyophilization yielded 0.35–0.5 g. product (1–2 × 10^9 MSH u./g.). One and a half grams of this fraction was distributed through a 12-tube countercurrent system at 5° using *sec*-BuOH and 0.5% aqueous trichloroacetic acid. The contents of tubes 4–6 were combined and lyophilized. Approximately 0.5 g. of solids (3–4 × 10^9 MSH u./g.) was obtained. Forty mg. was subjected to paper electrophoresis at 5°, 18 volts/cm., 8–10 hours, *p*H 8.9 using barbiturate–acetate–hydrochloric acid buffer (u = 0.056). Four components were visualized with 1% brom phenol blue staining. That moving fastest toward the cathode was extracted with 20% acetic acid and lyophilized. The product was dissolved in 1 ml. of 0.2 *N* acetic acid and subjected to paper electrophoresis at *p*H 4.9 using pyridine–acetic acid buffer (u = 0.1), 5°, 18 volts/cm., 10–12 hours. Staining revealed a single component moving toward the cathode. The active area was extracted with 20% acetic acid and lyophilized. The white solid, 2.5 mg., (1.5–2.5 × 10^{10} MSH u./g.) represented about 30% of the total MSH activity placed on the first electrophoretic run at alkaline *p*H. Ninhydrin reaction of hydrolyzed extracts of different parts of the filter paper run at *p*H 4.9 showed MSH activity associated with the predominant color response.

TABLE

Amino acid[9]	Per cent.[12]	Molecular ratio[13]
Aspartic	3.8	3
Glutamic	5.3	3
Serine	3.4	3
Glycine	2.3	3
Tyrosine	4.1	2
Lysine	5.3	3
Arginine	4.0	2
Valine	2.9	2
Phenylalanine	4.7	3
Alanine	1.4	1
Cystine[10]	5.3	2
Proline	3.7	3
Leucine	1.7	1
Threonine	1.3	1
Histidine	0.6	0
Tryptophan[11]	...	2
Total	49.8	34

(9) Semi-quantitative amino acid analyses were done by A. M. Gross and W. F. White of the Research Department, Armour Laboratories using filter paper chromatography and determining the intensity of ninhydrin stained areas with a densitometer; J. F. Rowland and A. M. Gross, *Anal. Chem.*, **26**, 502 (1954).

(10) Cystine and cysteine are not distinguished in the analysis. However, cysteine is probably absent because MSH is not oxidized and reduced readily as would be expected were this amino acid present. Methionine was not tested for.

(11) Tryptophan was determined by ultraviolet absorption after subtracting tyrosine from the total value; A. B. Lerner and C. P. Barnum, *Arch. Biochem.*, **10**, 417 (1946).

(12) Tryptophan, methionine, moisture and ash were not included in the total amino acid per cent. analysis.

(13) Molecular ratios are given in whole numbers and represent only approximate values.

The active fraction moved as a single component (staining with bromophenolblue) on paper electrophoresis at *p*H 1.4, 4.9, 8.9, 11.3 and 12.2. Since at *p*H 11.3 movement towards the anode was slight compared with dextran, the iso-electric *p*H was estimated to be in the region of 10.5–11. On the basis of amino acid composition minimum molecular weight was estimated at 4500. MSH activity of the final product was approximately 500 times that of the original hog posterior pituitary powder with little ACTH activity.[14] This fraction behaved as a single component when distributed in a 97 tube countercurrent apparatus employing the solvents described previously. Although the MSH preparation, assumed to be a polypeptide, was tested by electrophoresis and countercurrent distribution, other criteria for homogeneity remain to be satisfied.

(14) MSH has little if any vasopressin or ACTH activity. Assays set to detect one unit each of ACTH or vasopressin, using 378 and 449 mcg. of MSH, respectively showed no activity.

DIVISION OF DERMATOLOGY AARON B. LERNER
UNIVERSITY OF OREGON MEDICAL SCHOOL TEH H. LEE
PORTLAND 1, OREGON

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A NEW METHOD OF FORMING PEPTIDE BONDS

Sir:

We wish to describe a new and very useful method of forming peptide or other amide bonds. The two components, one containing a free carboxyl function and the other a free amino group, couple directly and rapidly in high yield on treatment with N,N′-dicyclohexylcarbodiimide at room temperature.

In contrast to other schemes for carboxyl activation involving mixed anhydride formation, the reaction is not sensitive to moisture; indeed, it may be carried out in aqueous solution. The remarkable selectivity of the reagent is attested by the successful use of carbobenzoxyserine as an acylating moiety without protection of the hydroxyl group. No racemization was detected employing as the acylating agent a dipeptide derivative in which an optically active amino acid furnished the free carboxyl function (carbobenzoxyglycyl-L-phenylalanine), an observation of considerable importance in the synthesis of larger peptides by joining units containing two, three or more amino acids. The co-product, N,N′-dicyclohexylurea, has a very low solubility in most organic or aqueous solvents, and, in all cases tried, is easily separated.

$$RCO_2H + NH_2R' + C_6H_{11}N{=}C{=}NC_6H_{11} \longrightarrow RCONHR' + C_6H_{11}NHCONHC_6H_{11}$$

The simplicity, convenience and efficiency of this technique may be illustrated by the synthesis of a tripeptide derivative. After a 4-hour period at room temperature, a solution in tetrahydrofuran of carbobenzoxyglycyl-L-phenylalanine containing a slight excess of crystalline N,N′-dicyclohexylcarbodiimide[1] and ethyl glycinate was treated with a small amount of acetic acid (to decompose the

(1) Readily prepared by the method of R. Herbeck and M. Pezzati, *Ber.*, **71**, 1933 (1938).