[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY]

A Synthesis of Radioactive Methylhistidines[1]

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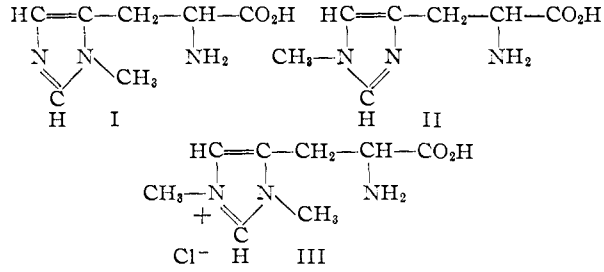
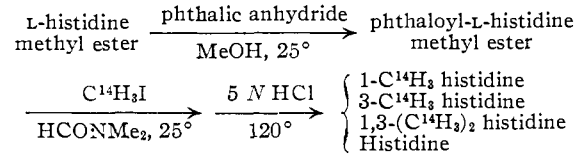
The methyl ester of phthaloyl-L-histidine was synthesized. This ester was methylated with $C^{14}H_3I$, and then acid hydrolysis was employed to remove the phthaloyl and ester groups. The products were separated by ion-exchange chromatography; they were 1-$C^{14}H_3$-L-histidine, 3-$C^{14}H_3$ histidine, 1,3-$(C^{14}H_3)_2$-L-histidine chloride and histidine.

Biochemical studies of the function and the metabolism of methyl-L-histidine compounds in animals would be facilitated by the availability of 1-methyl-L-histidine (I), 3-methyl-L-histidine (II) and 1,3-dimethyl-4-(β-carboxyl-β-aminoethyl)-imidazolium chloride or 1,3-dimethyl-L-histidine (Cl^-) (III) labeled with C^{14} in the methyl groups. The present methods were developed to provide an



efficient path for the synthesis of these labeled compounds; a path free of undesirable by-products with the C^{14}-methyl substituent on the α-amino nitrogen.

Early attempts[3] to synthesize the methylhistidines by methylation of phthaloyl histidine were thwarted by difficulties in the separation of products. Syntheses which did not involve L-histidine as an intermediate led to racemic products.[4] More recently, both 1-Me- and 3-Me-L-histidines have been prepared by methylation of L-histidine in liquid ammonia.[5] However, the existence of by-products methylated on the α-amino nitrogen renders this procedure unpromising for the specific incorporation of $C^{14}H_3$- groups on the imidazole ring. Protection of the α-amino group during methylation would be necessary in order to eliminate products methylated at the α-amino position. Tallan, Stein and Moore[5] methylated phthaloylhistidine but they reported that racemization occurred during the formation of the phthaloylhistidine. Attempts by this writer to form phthaloyl-L-histidine by heating an intimate mixture of L-histidine and phthalic anhydride[6] to 180° or by reaction of this mixture in boiling glacial acetic acid[7] led to racemization. However, it was found that the methyl ester of phthaloyl-L-histidine could be formed by reaction of the methyl ester of L-histidine and phthalic anhydride in anhydrous methanol by the general procedure of King and Kidd[8] for phthaloylamino acids.



Methylation of the methyl ester of phthaloyl-L-histidine occurred smoothly in dimethylformamide. The product, following hydrolysis of the phthaloyl and methyl ester groups, was a mixture of 1-methyl-L-histidine, 3-methylhistidine,[9] 1,3-dimethyl-L-histidine (Cl^-) and histidine. These amino acids were separated on a Dowex 50 (Na^+) ion-exchange column[5] and a representative separation is depicted in Fig. 1. The per cent. recovery of

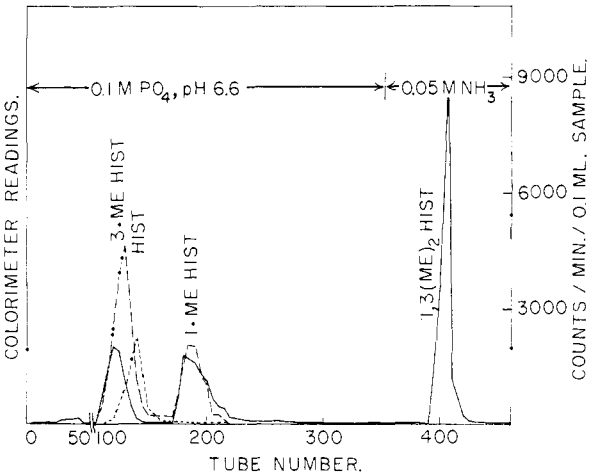


Fig. 1.—Separation of the methylhistidines on a column of Dowex 50 (Na^+); --- quantitative ninhydrin analysis[11]; ---- Pauly diazo analysis[12]; —— radioactivity.

amino acids introduced into the column was 75% and the per cent. recovery of radioactive compounds was 78%. The average composition of the products of five preparations was 40% histidine and 20% each of 1-Me-histidine, 3-Me-histidine and 1,3-$(Me)_2$-histidine (Cl^-).

The methylhistidines were characterized by paper chromatography,[10] by the ninhydrin analy-

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(3) W. Keil, *Z. physiol. Chem.*, **208**, 67 (1932).

(4) (a) W. Sakami and D. W. Wilson, *J. Biol. Chem.*, **154**, 215 (1944); (b) R. G. Jones and K. C. McLaughlin, THIS JOURNAL, **71**, 2444 (1949).

(5) H. H. Tallan, W. H. Stein and S. Moore, *J. Biol. Chem.*, **206**, 825 (1954).

(6) J. C. Sheehan and V. S. Frank, THIS JOURNAL, **71**, 1856 (1949).

(7) G. Wanag and A. Veinbergs, *Ber.*, **75B**, 1558 (1942).

(8) F. E. King and D. A. A. Kidd, *J. Chem. Soc.*, 3315 (1949).

(9) The specific optical rotation of the 3-Me-histidine could not be determined, for the preparation was not completely free of histidine (10%). However, the route of synthesis would indicate that only the L-isomer was formed.

(10) (a) R. W. Cowgill, *Anal. Chem.*, **27**, 1519 (1956); (b) R. W. Cowgill and B. Freeburg, *Arch. Biochem. & Biophys.*, in press.

sis[11] for free α-amino nitrogen, and by analysis[12] for histidine unsubstituted on the ring nitrogen. Preparations that contained C^{14} were also analyzed by radioactivity techniques. Both the 1-Me- and 3-Me-histidines were found to move on paper chromatograms in five solvent systems[10b] at rates identical with those of authentic samples of the compounds.[13] The 1-Me-, 3-Me- and 1,3-$(Me)_2$-histidines all gave spots on paper chromatograms that were radioactive, gave a blue-green color with the ninhydrin spray,[14] but gave no color with the Pauly diazo spray[15] that is reactive only with those imidazole compounds that bear no substituent on the imidazole ring nitrogen. To ensure that methylation had not occurred on the α-amino group of the 1,3$(Me)_2$-histidine, this compound was analyzed for α-amino nitrogen by the Cocking and Yemm[11] modification of the quantitative ninhydrin method. On a molar basis, the color intensity was 80% of that of histidine. This discrepancy might be due to some decrease in color formation by the presence of the imidazolium structure of the 1,3-$(Me)_2$-histidine (Cl^-), but the difference in color intensity is not of the magnitude that would be anticipated if the α-amino group were methylated. The quaternary-base character of this compound was shown by the fact that it did not migrate toward the anode during electrophoresis at *p*H 9.5, and by titration data. pK values were determined in 0.1 *M* KCl. For 1-Me-histidine, $pK'_1 = \sim 2$, $pK'_2 = 6.58$ and $pK'_3 = 8.60$; for 1,3$(Me)_2$-histidine (Cl^-), $pK'_1 = \sim 2$, $pK'_2 = 7.95$, and $pK'_3 > 10.5$. pK'_1, pK'_2 and pK'_3 of 1-Me-histidine are assigned to the carboxyl, imidazole and α-amino groups, respectively, from comparison with values of histidine and 1-Me-histidine reported in the literature.[16] For 1,3-$(Me)_2$-histidine, the pK'_1, pK'_2 and pK'_3 values are assigned to the carboxyl, α-amino and imidazolium groups, respectively.

Experimental Part

Reaction of the methyl ester of L-histidine with phthalic anhydride.[8] Twelve g. (0.05 mole) of the methyl ester of L-histidine dihydrochloride[17] was suspended in 75 ml. of anhydrous methanol. The free base was formed by treating this solution with 60 ml. (damp volume) of Dowex 2 (OH^-) in anhydrous methanol. The resin was removed by filtration and then 14.2 g. (0.057 mole) of phthalic anhydride was added to the solution of the methyl ester of histidine. The reaction mixture was stored at room temperature overnight, before it was concentrated on a bath at 70° to about 30 ml. First acetone (about 170 ml.) and then ether were added slowly to bring about precipitation of 13 g. of the products as crystalline or oily material. (The product was usually an oil unless the solution was seeded.) A further 1.5 g. of oil was obtained on concentration of the mother liquor and addition of more ether. Total yield was 93.5%. The product was crystallized from water as clear, cubic crystals. A 24-tube countercurrent distribution of this product between 0.1 *M* aqueous phosphate buffer of *p*H 6.5 and benzene revealed that it was a mixture of approximately 85% as the methyl ester of phthaloyl-L-histidine and 15% as the methyl ester of *o*-carboxybenzoyl-N-histidine. The proportions of these two compounds in the crude product varied from batch to batch and in four preparations ranged from 50–95% as the methyl ester of phthaloylhistidine.

Conversion of the methyl ester of *o*-carboxybenzoyl-N-histidine to the methyl ester of phthaloylhistidine.[8] Ten ml. of freshly distilled thionyl chloride was dissolved in 40 ml. of benzene and 5 g. of the mixture of methyl esters of *o*-carboxybenzoyl-N-histidine and phthaloylhistidine was added slowly and with stirring. The suspension of fine, white, granular precipitate was allowed to stand at room temperature overnight. The solvent was evaporated and the residual product was crystallized from a mixture of anhydrous methanol and ether. The white crystals of methyl ester of phthaloyl-L-histidine hydrochloride amounted to 3.3 g. (63% yield). The product was recrystallized from 10% ethanol; m.p. 200–200.7°; Keil[3] reported 238–240° for the methyl ester of phthaloyl-DL-histidine·HCl.

Anal. Calcd. for $C_{15}H_{14}O_4N_3Cl$: C, 53.66; H, 4.20. Found: C, 53.63, 54.07; H, 4.52, 4.29, optical rotation, $[\alpha]^{20}D$ −101° (α = −1.96; 0.00972 g./ml. in H_2O; 2 dm. tube).

The hydrochloride in aqueous solution was readily converted to the free base by addition of slightly over one equivalent of NaOH (*p*H > 9). The methyl ester of phthaloyl-L-histidine was recrystallized from 10% ethanol; m.p. 182–182.5°; Keil[3] reported 188° for the methyl ester of phthaloyl-DL-histidine.

Anal. Calcd. for $C_{15}H_{13}O_4N_3$: C, 60.19; H, 4.38. Found: C, 60.31; H, 4.57; optical rotation, $[\alpha]^{20}D$ −111.7° (α = −1.65°; 0.0074 g./ml. in 0.1 *N* HCl; 2 dm. tube). Hydrolysis by 48% HBr at reflux temperature for 9 hours gave histidine of $[\alpha]^{20}D$ −40° (H_2O).

Methylation of the methyl ester of phthaloyl-L-histidine. One and one-half g. (0.005 mole) of the methyl ester of phthaloyl-L-histidine free base was dissolved in 7 ml. of dimethylformamide. Methyl iodide (0.005 mole that contained 0.5 millicurie of C^{14}) was added and the flask was securely stoppered. The mixture was stored at room temperature for three days, and then it was concentrated *in vacuo* at 50° to a sirup. The sirup was dissolved in 3 ml. of constant boiling hydrochloric acid and was heated in an autoclave at 15 p.s.i. (121°) for 7 hours. The solution then was concentrated *in vacuo* at 50° to yellow salts, and the salts were extracted with a total of 10 ml. of water. The residue of phthalic acid amounted to 0.916 g.; 110% of theory, as phthalic acid. The solution of methylated products contained a total of 3.96×10^7 c.p.m.,[18] which represents 71% of the initial activity of the methyl iodide.

Separation of the methylation products on an ion-exchange column. The mixture of methylhistidines and histidine was introduced into a 7 × 35 cm. column of Dowex 50 (Na^+) described by Tallan, Stein and Moore,[5] and elution was made with 0.1 *M* phosphate of *p*H 6.6. The progress of the elution is shown in Fig. 1. The 1,3-dimethylhistidine was held tightly on the column but could be removed by 0.05 *M* ammonia.

Each fraction in the eluate was desalted and recovered by the procedure of Tallan, Stein and Moore.[5] Of the 3.96×10^7 c.p.m. introduced onto the column, 16% was recovered as 3-methylhistidine, 18% as 1-methylhistidine and 44% as 1,3-dimethylhistidine. This represents about 0.62×10^5 ct./min./mg. for 1-Me- and 3-Me-histidines and 1.0×10^5 ct./min./mg. for 1,3-$(Me)_2$-histidine (Cl^-).

1-Methyl-L-histidine·2HCl.—The desalted fraction from the ion-exchange column which contained the 1-methylhistidine (300 mg.) as the dihydrochloride salt was dissolved in 1.0 ml. of water and 1-Me-histidine·2HCl crystallized upon addition of ten volumes of 1:1:: acetone: absolute ethanol. This salt was recrystallized from 99% ethanol.

Anal. Calcd. for $C_7H_{13}O_2N_3Cl_2$: C, 34.72; H, 5.41. Found: C, 34.95; H, 5.47; $[\alpha]^{20}D$ −17.6° ± 3.0° (α = −0.0058°; 0.0066 g. as free base/ml. in H_2O of *p*H 9; 0.5 dm. tube). Linnewek and Linnewek[19] reported $[\alpha]^{18}D$

(11) E. C. Cocking and E. W. Yemm, *Biochem. J.*, **58**, XII (1954).

(12) K. K. Koessler and M. T. Hanke, *J. Biol. Chem.*, **89**, 497 (1919).

(13) Samples of 1-Me-histidine for comparison were a sample of 1-Me-L-histidine obtained by hydrolysis of anserine and a sample of 1-Me-DL-histidine prepared by a different synthetic path (California Foundation for Biochemical Research). An authentic sample of 3-Me-L-histidine was kindly supplied by Dr. H. H. Tallan.

(14) 0.1% ninhydrin in 95% ethanol that contained 5% collidine. The sprayed paper was heated at 100–110° to bring out the colors.

(15) B. N. Arues and H. K. Mitchell, THIS JOURNAL, **74**, 252 (1952).

(16) A. Deutsch and P. Eggleton, *Biochem. J.*, **32**, 209 (1938).

(17) E. Fischer and L. H. Cone, *Ann.*, **363**, 107 (1908).

(18) Counter efficiency was 5% and all counts are expressed as measured at this efficiency.

(19) W. Linnewek and F. Linnewek, *Z. physiol. Chem.*, **189**, 80 (1930).

$-25.8°$ (H_2O) for 1-Me-L-histidine free base isolated from the hydrolysis of anserine.

1-Methyl-L-histidine Diflavianate.—The diflavianate salt was formed by the general method of Vickery,[20] and it was recrystallized from water; m.p. 234.7° dec.

Anal. Calcd. for $C_{27}H_{23}O_{16}N_7S_2$: C, 40.65; H, 2.92; N, 12.29. Found: C, 40.45; H, 3.08; N, 12.09.

3-Methylhistidine.—The 3-Me-histidine fraction from the ion-exchange column contained 10% histidine. In the radioactive tracer experiments for which this preparation was to be used,[10b] the contamination by histidine was not deleterious, and no further purification was attempted.

1,3-Dimethyl-L-histidine (Cl^-) or 1,3,-Dimethyl-4-(β-carboxyl-β-aminoethyl)-imidazolium Chloride.—The dry, desalted fraction from the ion-exchange column that contained 1,3-$(Me)_2$-histidine was taken up in about 1.0 ml. of H_2O, treated with Norite, filtered and adjusted to about *p*H 8 with ammonia. Acetone was added to the appearance of fluffy white crystals upon cooling the mixture. The crystals were washed on the filter funnel with acetone and dried *in vacuo* at 80°. The hygroscopic salt must be stored under desiccation.

Anal. Calcd. for $C_8H_{15}O_2N_3Cl$: C, 43.50; H, 6.86. Found: C, 43.4; H, 6.7. $[\alpha]^{20}D$ $+18.1° \pm 1.6°$ ($\alpha = 0.23°$; 0.0125 g./ml. in 1 *N* HCl; 1 dm. tube).

1,3-Dimethyl-L-histidine Diflavianate.—The diflavianate was prepared by the general method of Vickery,[20] and it was recrystallized from water; m.p. 237.5° dec.

Anal. Calcd. for $C_{28}H_{25}O_{18}N_7S_2 \cdot H_2O$: C, 40.53; H, 3.28. Found: C, 40.58, H, 3.17.

(20) H. B. Vickery, *J. Biol. Chem.*, **71**, 303 (1927).

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Purines. VII. The Preparation of Certain 6-Alkylamino- and 6-Dialkylaminopurines[1]

BY MELVIN SUTHERLAND AND BERT E. CHRISTENSEN

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The procedures for the preparation and isolation of high molecular weight alkylamino- and dialkylaminopurines are described. Twenty such compounds have been prepared by the methods described herein.

A great deal of interest has centered in the 6-substituted purines following the recent discovery of the biological importance of kinetin[2] and 6-mercaptopurine.[3] This activity has been divided between the development of new synthetic routes to kinetin itself and to the synthesis[4] and testing of many of its analogs.

Miller and co-workers originally prepared 6-furfurylaminopurine by the aminolysis (in a sealed tube) of 6-methylmercaptopurine[2] following the procedure of Elion and Hitchings. Since then a number of other approaches to the synthesis of kinetin have been reported. One of the most recent methods for the preparation of the 6-substituted aminopurines involves the synthesis and reduction of the corresponding 6-amidopurine with lithium aluminum hydride.

In this Laboratory an aminolytic procedure based on the use of 6-chloropurine was successfully applied to the preparation of a number of 6-substituted purines.[4] Since this procedure appeared to have a wide degree of applicability and in view of the possibility of turning up other biologically active analogs, this work has been expanded to include a number of other potentially active purine derivatives. Recently several new 6-substituted aminopurines have been reported to possess activity.[5] Since this activity may be dependent in part on fat solubilities of the compound, this Laboratory has prepared a number of high molecular weight alkylaminopurines with the side chain in the 6-position.

This was done by refluxing the chloropurine with different aliphatic amines in *n*-butyl alcohol according to the procedure described earlier.[4] However, due to solubility characteristics of the aliphatic amines, it was not possible to isolate the reaction product by the usual crystallization techniques. The lower molecular weight homologs were isolated by steam distilling the unreacted aliphatic amine from the aqueous basic solution of the reaction mixture. The course of the steam distillation could be followed by the slow progression of the insoluble aliphatic amine through the condenser. The product was then obtained by the ethereal extraction of the basic media left in the distilling flask.

As the aliphatic side chain increased in length the products became contaminated with traces of the starting amine which could not be removed by continuous distillation with steam.

Attempts to remove the unreacted amine by co-distillation at reduced pressures with diphenyl ether were quite successful but no practical way was found for isolating the product in turn from the diphenyl ether. Co-distillation under similar conditions with ethylene glycol was successfully applied to the isolation of 6-isohexylaminopurine, but the process proved to be impractical for the distillation of the higher homologs.

Finally it was observed that it was possible to remove amines as large as octadecylamine from sirupy mixtures of 6-substituted purines by the use of super-heated steam. This was the procedure which was adopted for the isolation of the higher molecular weight 6-substituted purines.

One rather interesting steric effect was noted in

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(2) C. O. Miller, F. Skoog, F. S. O. Kumura, M. H. Von Saltza and F. M. Strong, THIS JOURNAL, **77**, 2662 (1955).

(3) C. T. Bahner, B. Stump and M. E. Brown, *ibid.*, **75**, 6301 (1953).

(4) J. W. Daly and B. E. Christensen, *J. Org. Chem.*, **21**, 177 (1956); M. W. Bullock, J. W. Hand and E. L. R. Stokstad, THIS JOURNAL, **78**, 3693 (1956).

(5) C. G. Skinner, W. Shive, R. G. Ham, D. C. Fitzgerald, Jr., and R. E. Eakin, *ibid.*, **78**, 5097 (1956); R. G. Ham, R. E. Eakin, C. G. Skinner and W. Shive, *ibid.*, **78**, 2648 (1956).