[CONTRIBUTION FROM THE RESEARCH LABORATORIES, MERCK & CO., INC.]

Synthesis of Biocytin

BY DONALD E. WOLF, JOHN VALIANT, ROBERT L. PECK AND KARL FOLKERS

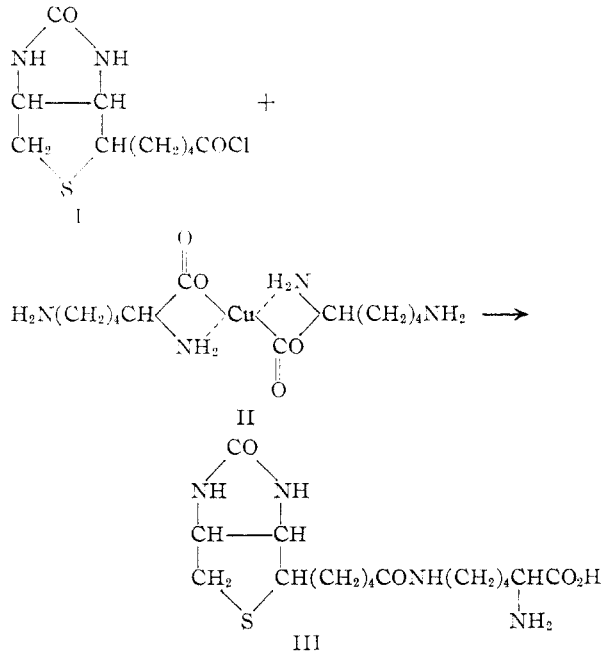
ε-N-Biotinyl-L-lysine has been synthesized by the reaction of biotin acid chloride with the copper–chelate complex of L-lysine, and by the reaction of biotin acid chloride with α-N-formyl-L-lysine followed by hydrolytic removal of the formyl group. Synthetic ε-N-biotinyl-L-lysine and biocytin from yeast are identical; thus, synthesis confirms the structure assigned to biocytin.

ε-N-Biotinyl-L-lysine has been synthesized and found to be identical with natural biocytin. The aspects of synthesis constitute final proof of structure, particularly in this case where paucity of natural biocytin severely limited its use.[1]

Biocytin is the conjugate of biotin which was discovered in controlled autolysates of yeast. Isolation of biocytin from yeast extract,[2] and its structure determination[3] have been described in accompanying papers. Degradation of biocytin by acid hydrolysis gave biotin and L-lysine. Having identified the two portions of the molecule and having shown that the α-amino group of lysine is apparently free in biocytin, it remained to synthesize ε-N-biotinyl-L-lysine for comparison with the natural material.

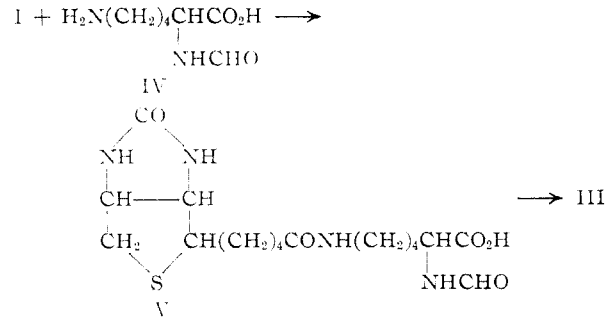
The synthesis of this compound from L-lysine involves the protection of the α-amino group of the diamino acid during attachment of the biotinyl group to the ε-amino group. Fischer and Zemplén[4] observed the formation of copper complexes by the α- and β-amino acids, but not by the γ-, δ- and ε-amino acids. Kurtz[5] utilized this observation for converting the copper complex of ornithine into citrulline; the α-amino group was unreactive toward urea since it was part of the copper chelate. Similarly, Neuberger and Sanger[6] converted the copper complex of lysine into the ε-N-acetyl- and ε-N-carbobenzoxylysine derivatives. Biotin acid chloride (I) has been prepared[7] from biotin by reaction with thionyl chloride. This acid chloride was allowed to react with the copper–chelate complex of lysine (II). To avoid excessive hydrolysis of biotin acid chloride, the reaction was carried out at −15°. Purification and separation from biotin yielded ε-N-biotinyl-L-lysine (III). This derivative can be separated from biotin because of its solubility in acidic solutions. Its solubility in water is much greater than that of biotin, and addition of acetone to an aqueous solution causes separation of ε-N-biotinyl-L-lysine in crystalline form.

An alternative method of synthesis of ε-N-biotinyl-L-lysine from L-lysine involves the protection of the α-amino group with a formyl group. α-N-Formyl-L-lysine (IV) was obtained by formylating ε-N-carbobenzoxy-L-lysine,[6] and subsequently removing the carbobenzoxy group by catalytic hydrogenation. The α-N-formyl-L-lysine and biotin acid chloride reacted to give ε-N-biotinyl-α-N-formyl-L-lysine (V). Selective hydrolysis removed the formyl group to yield ε-N-biotinyl-L-lysine (III).



Synthetic ε-N-biotinyl-L-lysine and natural biocytin were found to be identical by chemical[3] and biological[8] comparisons.

Reaction of ε-N-carbobenzoxy-L-lysine with biotin acid chloride gave ε-N-carbobenzoxy-α-N-biotinyl-L-lysine.



Experimental

ε-N-Biotinyl-L-lysine.—To a boiling aqueous solution of 1.64 g. of L-lysine monohydrochloride in 10 ml. of water, was added an excess of solid copper carbonate. The blue solution was cooled to about −15° in an ice-salt-bath, and a

(1) L. D. Wright, E. L. Cresson, H. R. Skeggs, R. L. Peck, D. E. Wolf, T. R. Wood, J. Valiant and K. Folkers, *Science*, **114**, 635 (1951).

(2) L. D. Wright, E. L. Cresson, H. R. Skeggs, T. R. Wood, R. L. Peck, D. E. Wolf and K. Folkers, THIS JOURNAL, **72**, 1048 (1950); **74**, 1996 (1952).

(3) R. L. Peck, D. E. Wolf and K. Folkers, *ibid.*, **74**, 1999 (1952).

(4) E. Fischer and G. Zemplén, *Ber.*, **42**, 4878 (1909).

(5) A. C. Kurtz, *J. Biol. Chem.*, **122**, 477 (1938).

(6) A. Neuberger and F. Sanger, *Biochem. J.*, **37**, 515 (1943).

(7) D. E. Wolf, J. Valiant and K. Folkers, THIS JOURNAL, **73**, 4142 (1951).

(8) L. D. Wright, E. L. Cresson, K. V. Liebert and H. R. Skeggs, *ibid.*, **74**, 2004 (1952).

chloroform suspension of biotin acid chloride,[7] prepared from 1 g. of biotin, was added in portions over a 15-minute period. The *p*H of the mixture was kept above 8 by additions of 2.5 *N* sodium hydroxide solution, and the mixture was stirred vigorously throughout the addition of the biotin acid chloride. Stirring was continued for an additional one-half hour, while the mixture was allowed to warm to room temperature. The mixture was then centrifuged, and the blue aqueous layer was removed. The chloroform phase was washed with water, and dilute hydrochloric acid. All the washings were then combined with the original blue aqueous phase and the *p*H was adjusted to 2 with hydrochloric acid. The copper was removed by treatment with hydrogen sulfide and filtration through supercel. The filtrate was concentrated to dryness at reduced pressure and the residue was extracted with several small volumes of water. This extract was filtered and then subjected to a 10-plate countercurrent distribution, using an organic layer of equal parts of chloroform and *o*-cresol and an aqueous layer of equal volume which was adjusted to *p*H 3 with hydrochloric acid. Plates 4–9 contained most of the ninhydrin-reacting material. The contents of these plates were combined and ten volumes of petroleum ether was added. The aqueous layer was separated and the organic layer was washed three times with small volumes of water. The combined aqueous extract was washed with ether and lyophilized. The colorless residue was dissolved in water and acetone was added until a turbidity appeared. ε-N-Biotinyl-L-lysine was obtained as a colorless crystalline precipitate; yield 420 mg. (28% of the theoretical).

A quantity of 1.58 g. of crude ε-N-biotinyl-L-lysine which was obtained from several similar preparations was subjected to a 12-plate countercurrent distribution, using the same solvent system. Plates 4–8 yielded 715 mg. of colorless crystalline biocytin. Recrystallization of this material from a water–acetone mixture yielded 498 mg. of biocytin, m.p. 228–229°, $[\alpha]^{25}D$ +53°, *c* 1.05 g./100 ml., 0.1 *N* sodium hydroxide.

Anal. Calcd. for $C_{16}H_{28}N_4O_4S$: C, 51.59; N, 7.58; N, 15.04. Found: C, 51.44; H, 7.35; N, 14.83.

α-N-Formyl-L-lysine.—Twenty-nine and one-half grams of ε-N-carbenzoxy-L-lysine was dissolved in a mixture of 180 ml. of 98% formic acid and 20 ml. of acetic anhydride. This solution was allowed to stand at room temperature about 20 hours under anhydrous conditions. About 25% by volume of water was then added, and the solution was concentrated *in vacuo* to a residual clear oil. The ε-N-carbobenzoxy-α-N-formyl-L-lysine was dissolved in methanol and hydrogenated at 40-pounds pressure and at room temperature, using 35 g. of palladium catalyst on Darco. The catalyst was removed by centrifugation and filtration and was washed with slightly ammoniacal methanol. The combined filtrate and washings were neutralized with glacial acetic acid and concentrated *in vacuo* to an almost colorless oil, 13.8 g. (75% yield). α-N-Formyl-L-lysine was obtained in colorless crystalline form, m.p. 193–193.5°, by dissolving the oil in a mixture of water and methanol, and adding acetone until turbidity appeared.

Anal. Calcd. for $C_7H_{14}O_3N_2$: C, 48.26; H, 8.10; N, 16.09. Found: C, 48.60; H, 8.00; N, 16.03.

ε-N-Biotinyl-α-N-formyl-L-lysine.—Thirteen and one-half grams of biotin was converted to biotin acid chloride as previously described.[7] To the dry biotin acid chloride, was added 9.6 g. of α-N-formyl-L-lysine. The mixture was cooled in an ice-bath and 25 ml. of ice-cold pyridine was added with shaking. The temperature was maintained at 0–5° for 20 minutes, then allowed to rise to room temperature over an additional 40-minute period. The clear orange solution was evaporated at reduced pressure. The residue was dissolved in water and this solution was evaporated again to remove most of the remaining pyridine. The residue was washed with dilute hydrochloric acid, and water. It was then dissolved in 2% sodium bicarbonate solution. After filtration the filtrate was adjusted to *p*H 3 with hydrochloric acid. The light tan, gelatinous precipitate was washed with water and dried *in vacuo* over phosphorus pentoxide; weight 15.1 g. (68.5% yield).

The benzylamine salt of ε-N-biotinyl-α-N-formyl-L-lysine was made by suspending the free acid in methanol and adding benzylamine until all the solid material was in solution. Addition of ethyl ether precipitated an almost colorless salt, and several reprecipitations yielded colorless crystals, m.p. 112–113°.

Anal. Calcd. for $C_{24}H_{37}O_5N_5S$: C, 56.78; H, 7.35; N, 13.80. Found: C, 55.69; H, 7.09; N, 13.50.

ε-N-Biotinyl-L-lysine.—Approximately 500 mg. of ε-N-biotinyl-α-N-formyl-L-lysine was dissolved in 20 ml. of 6 *N* hydrochloric acid, and the solution was heated at 65° for 30 minutes. The clear solution was then evaporated *in vacuo*, and the excess hydrochloric acid was removed by repeated addition of water and evaporation. The residue was triturated with water, and the aqueous extracts were filtered, combined, and concentrated to dryness at reduced pressure. This residue was subjected to a 12-plate countercurrent distribution using an organic layer of equal parts of chloroform and *o*-cresol and an aqueous layer of equal volume which was adjusted to *p*H 3 with hydrochloric acid. Plates 8 and 9 were combined, ten volumes of petroleum ether was added and the aqueous layer was separated. The organic layer was washed several times with water and the washings combined with the aqueous layer. The combined water extract was washed with ether and lyophilized. The residue of ε-N-biotinyl-L-lysine was dissolved in a minimum of water and precipitated as a white solid product by addition of acetone.

Anal. Calcd. for $C_{16}H_{28}N_4O_4S$: C, 51.59; H, 7.58; N, 15.04. Found: C, 51.63; H, 7.18; N, 15.23.

α-N-Biotinyl-ε-N-carbobenzoxy-L-lysine.—The biotin acid chloride from 400 mg. of biotin was cooled in an ice-bath protected from moisture. The methyl ester hydrochloride from 700 mg. of ε-N-carbobenzoxy-L-lysine was dissolved in 8 ml. of pyridine, cooled in ice and added in portions to the biotin acid chloride. The reaction mixture was shaken to provide mixing and allowed to stand at room temperature for two hours. It was then concentrated at reduced pressure and dried over phosphoric anhydride in a vacuum. The oily residue was dissolved in chloroform, washed with 1 *N* hydrochloric acid, 2% sodium bicarbonate solution and finally with water. The chloroform solution was dried over magnesium sulfate and evaporated at reduced pressure giving a yellow gum weighing 259 mg.

Efforts to crystallize the α-N-biotinyl-ε-N-carbobenzoxy-L-lysine methyl ester failed so the ester was saponified by treatment with 0.5 *N* aqueous sodium hydroxide. After trituration at room temperature about 30 minutes the ester had all dissolved. The solution was filtered and acidified with 6 *N* hydrochloric acid in an ice-bath. The α-N-biotinyl-ε-N-carbobenzoxy-L-lysine precipitated as a brown gum. It was washed with water and dried in a vacuum. It weighed 180 mg.

The crude acid was crystallized by treating a solution in aqueous methanol with a little charcoal, then diluting further with water until cloudy. The first crop weighed 127 mg. and when pure, melted at 135–136°; $[\alpha]^{28}D$ +34°, *c* 1.04 g./100 ml. 0.1 *N* sodium hydroxide.

Anal. Calcd. for $C_{24}H_{34}N_4O_6S$: C, 56.90; H, 6.77; N, 11.06. Found: C, 56.60; H, 6.59; N, 10.97.

RAHWAY, NEW JERSEY RECEIVED OCTOBER 29, 1951