sintering at 215°. A second crop of crystals weighing 1.2 mg. (m.p. about 225–235° (dec.)) was obtained by repetition of evaporation and washing. The biotin content of this crystalline sample was found to be about 40.3% by microbiological assay. Recrystallization of combined crops from water by slow evaporation gave crystals which melted on the microblock at 228–232° (dec.) with preliminary sintering at 222°. The crystals gave a positive ninhydrin test.

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RECEIVED OCTOBER 29, 1951

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

Structure Determination of Biocytin as ε-N-Biotinyl-L-lysine

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Biocytin has been shown to be ε-N-biotinyl-L-lysine by degradative studies. Hydrolysis of biocytin yields biotin and L-lysine. Evidence was found which shows that the α-amino group of lysine is free in biocytin, and that biotin is attached at the ε-amino group. Comparison of biocytin from yeast and synthetic ε-N-biotinyl-L-lysine showed that they are identical.

The isolation of biocytin has been described,[1] and a preliminary report on structure has appeared.[2] Biocytin is ε-N-biotinyl-L-lysine. This conclusion has been confirmed by synthesis[3] and by the biological activity of the synthetic compound.[4]

Only milligram amounts of pure biocytin were available for degradative study, partly because of the low yields in the final crystallizations. Biocytin crystallized from very concentrated aqueous or aqueous methanol solutions of highly purified concentrates. Removal of mother liquors and washing the crystals with fresh solvent markedly diminished the yield of crystalline biocytin. It was later found that recrystallization of synthetic biocytin,[3] which was also available in larger quantities, presented less difficulty. Recrystallization of biocytin from solutions containing chloride ions, but not in much excess, gave a crystalline mixture of biocytin and its hydrochloride, as judged by analytical data. Paucity of pure crystalline material prevented adequate elementary microanalyses therefore, structural methods requiring less material were used.

A 700-μg. sample of biocytin was hydrolyzed in acid solution to give biotin and L-lysine. The biotin was isolated as the crystalline free acid, and characterized by solubility, melting point determinations, and microbiological assay. The presence of L-lysine in the hydrolysate was demonstrated by paper-strip chromatography,[5] microbiological assay, and by chromatography on starch.[6] Paper-strip chromatography showed only the lysine spot, and no other cleavage products could be detected.

The amounts of biotin and lysine found in acid hydrolysates of biocytin were 60% and 34%, respectively. These amounts corresponded to a 1:1 molar ratio. The yield of each component was a little less than the theoretical, evidently due to incomplete hydrolysis of biocytin. There was no evidence of carbon dioxide or ammonia formation during acid hydrolysis. Thus, biocytin appeared to be either ε-N-biotinyl-L-lysine or α-N-biotinyl-L-lysine.

It is known that alkaline hydrolysis of biotin at 140° results in cleavage of the ureido ring to give the diamine degradation product of biotin.[7] Treatment of the diamine with phosgene affects resynthesis of biotin on an analytical basis.[8] The corresponding alkaline hydrolysis of biocytin followed by treatment with phosgene and biological assay showed the presence of 55% of biotin. Thus, alkaline hydrolysis of biocytin gave substantially the same amount of biotin as was produced by acid hydrolysis.

Evidence was found for a free amino group in biocytin. The ninhydrin reaction was positive and indicated an α-amino group. Reaction of biocytin with 2,4-dinitrofluorobenzene[9] readily gave a yellow 2,4-dinitrophenyl derivative which did not react with ninhydrin. Hydrolysis of the 2,4-dinitrophenyl derivative of biocytin yielded about one equivalent of biotin. Microbiological assay of the hydrolysate showed that lysine was absent.

Reaction of both ε-N-biotinyl-L-lysine and α-N-biotinyl-L-lysine with nitrous acid would be expected to give products which would not react with ninhydrin under the test conditions. The nitrous acid product from ε-N-biotinyl-L-lysine would also be expected to undergo hydrolysis to a lysine derivative which would not react with ninhydrin, because of the removal of the α-amino group. The ε-amino group of lysine does not react with ninhydrin under the conditions used. The nitrous acid product from the α-N-biotinyl-L-lysine would be expected to hydrolyze to a lysine derivative which would react with ninhydrin, because the α-amino group was retained.

Reaction of biocytin with nitrous acid gave a product which did not react with ninhydrin. Hydrolysis of this product yielded a lysine derivative which did not react with ninhydrin. Thus, it was concluded, biotin is attached to the ε-amino group of lysine, and biocytin is ε-N-biotinyl-L-lysine, I.

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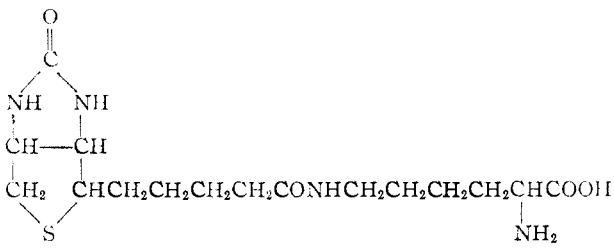

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Comparison of natural biocytin with synthetic biocytin[3] showed no difference in properties. There was no difference in melting point behavior, or paper-strip R_F values. The infrared spectra of natural and synthetic biocytin were identical. The rate of acid hydrolysis was the same for both preparations, as were biotin content, paper-strip chromatography with bioautographic visualization, stability, avidin-combinability, and growth-stimulating properties for all microörganisms tested.[4]

Experimental

Additional Properties of Crystalline Biocytin.—When biocytin was isolated by the procedures described,[1,2] it was recrystallized best from water although aqueous methanol and aqueous acetone were used successfully. When the biocytin was allowed to crystallize rapidly from aqueous methanol or acetone, the product melted at 228–230° (dec.). When the crystalline biocytin was allowed to separate slowly, it was found to sinter slightly at 227° and melt at 245–252° (dec., microblock).

A sample of biocytin was recrystallized from aqueous acetone in the presence of about one equivalent of hydrochloric acid. The recovered crystalline material melted at about 227° (dec., microblock), and showed the presence of halogen.

Anal. Calcd. for $C_{16}H_{29}N_4O_4SCl$: C, 46.99; H, 7.15; N, 13.70. Found: C, 46.55; H, 6.74; N, 14.20.

A sample of biocytin which was recrystallized from neutral aqueous acetone was analyzed.

Anal. Calcd. for $C_{16}H_{28}N_4O_4S$: C, 51.59; H, 7.58; N, 15.04. Found: C, 52.80; H, 7.60; N, 14.71.

Only end absorption in the ultraviolet was observed for aqueous or aqueous methanolic solutions of biocytin.

Acid Hydrolysis of Biocytin to Biotin.—A sample of pure crystalline biocytin weighing 700 μg. was dissolved in 1.5 ml. of 3 *N* hydrochloric acid, and the solution was heated at 120° for two hours in a sealed tube. After cooling, the solution was transferred to a 15-ml. centrifuge tube, and evaporated to dryness in a stream of dry air. The glassy residue was dissolved in a total of 0.15 ml. of warm water, including wash water, and the solution was transferred to a 5-ml. centrifuge tube. The solution was then evaporated while warm to a volume of 0.08 ml. and allowed to cool slowly. Crystals separated on the walls of the tube. The supernatant solution was removed, and the residue was recrystallized from 0.08 ml. of water. The product, m.p. 212–215° (microblock), was dissolved in 0.2 ml. of hot water, and the solution was filtered through Darco G-60. The filtrate was evaporated to a volume of about 0.1 ml., and colorless crystals separated. After one more crystallization, the crystals melted at 230–231° with no change in melting point on further recrystallization. When mixed with authentic biotin (m.p. 230–231°), the crystalline mixture melted at 230–231°.

A solution of 62 μg. of the biotin from biocytin in 6.2 ml. of 20% ethanol, and a corresponding solution of authentic biotin were used for comparative bioassays. This assay of the solution of crystals from biocytin showed 11.1 μg./ml. of biotin, and the assay of the authentic sample showed 10.1 μg./ml. Thus, the identity of the crystals from biocytin is shown again to be biotin.

A 93-μg. sample of biocytin was hydrolyzed in 0.5 ml. of 2.5 *N* hydrochloric acid at 130° for two hours. Microbiological assays on the neutralized hydrolysate showed the presence of about 60% of biotin (calcd., 65.6%; found, 58.7 61.3%).

Alkaline Hydrolysis of Biocytin and Resynthesis of Biotin.—A sample of biocytin weighing 54 μg. was hydrolyzed in a solution of 20.5 mg. of barium hydroxide octahydrate in 0.1 ml. of water at 140° for 22 hours. The hydrolysate was worked up, treated with phosgene and submitted for biotin assay as previously described.[8] The assay showed the presence of biotin corresponding to 54.5% of the biocytin sample used; calcd., 65.6%.

As a control, biotin methyl ester was subjected to identical conditions of alkaline hydrolysis and phosgene treatment, and also assayed. Duplicate assays showed the presence of 86 and 97% of biotin, respectively, indicating nearly quantitative resynthesis.

L-Lysine from Biocytin.—An acid hydrolysis of biocytin was carried out as described above. A portion of the hydrolysate was chromatographed on a paper-strip using water-saturated phenol as solvent.[5] A single spot, R_F 0.50, was observed when the strip was sprayed with ninhydrin solution. Comparative chromatography of authentic L-lysine showed the same R_F value. Using a collidine–lutidine mixture, lysine and the biocytin hydrolysate showed ninhydrin spots having the same R_F value, 0.12.

Another portion of the hydrolysate was examined by means of microbiological amino acid assays. The only amino acid which was present appeared to be L-lysine.

A 288-μg. sample of biocytin was hydrolyzed in 0.5 ml. of 2.5 *N* hydrochloric acid for one and one-fourth hours at 130°, and the solution was evaporated to dryness. The residue was dissolved in 6 ml. of water and the solution was continuously extracted for 24 hours (with water replacement) with *n*-butanol to remove free biotin. The butanol extracts were combined, evaporated to dryness, and bioassayed for biotin. The biotin found by assay amounted to about 130 μg. or 45%; calcd., 65.6%. Microbiological assay of the butanol-extracted aqueous solution showed the presence of about 72 μg. of lysine or 25%; calcd., 39.3%. The molar ratio of lysine to biotin in this experiment is about 1:1.

The specificity of the microbiological assay for L-lysine was checked. No response was given with D-lysine, DL-ornithine or any amino acid tested other than L-lysine.

Partition chromatography of a similar biocytin hydrolysate on starch[6] showed only one significant ninhydrin-reacting peak and a minor peak. The major peak was identified as lysine by its position in the emerging effluent, and by the fact that the same peak was obtained when lysine was added to another portion of the biocytin hydrolysate. The minor peak apparently represents a small amount of unhydrolyzed biocytin. The lysine recovered chromatographically from the biocytin hydrolysate corresponded to 33.8%; calcd., 39.3%.

Evidence for a Free Amino Group in Biocytin.—To a solution of 951 μg. of biocytin in 0.1 ml. of 5% sodium bicarbonate was added 0.5 ml. of 10% 2,4-dinitrofluorobenzene.[9] The mixture was stirred for two hours. The solution was diluted to a volume of 1 ml., extracted three times with 2-ml. portions of ether, made slightly acid with dilute hydrochloric acid, and again extracted with ether. The residual aqueous solution was yellow and contained an oily yellow precipitate of N-(2,4-dinitrophenyl)-biocytin. After hydrolysis was carried out, a determination for lysine was made. No lysine was found in the hydrolysate.

The same procedure was also carried out with lysine, with ε-N-carbobenzoxy-α-N-biotinyl-L-lysine,[3] and with no added test substance. The final ether-extracted aqueous solutions in the latter two cases were colorless. In the experiment with lysine, the final aqueous solution was yellow. Hydrolysis and lysine assay showed no lysine. The experiment with ε-N-carbobenzoxy-α-N-biotinyl-L-lysine yielded about 16% lysine; calcd., 28%. Lysine assay after hydrolysis of ε-N-carbobenzoxy-α-N-biotinyl-L-lysine gave a lysine content of 29%; calcd., 28%.

Linkage between Biotin and L-Lysine in Biocytin.—A solution of 500 μg. of biocytin in 0.05 ml. of 6 *N* hydrochloric acid was exposed to nitrous oxide fumes for ten minutes,[5] then evaporated to dryness, and diluted with water and evaporated several times to remove traces of nitrous oxide. Hydrolysis and subsequent paper-strip chromatography showed no ninhydrin-positive spot. Also, a bioassay showed no lysine to be present. A biotin assay showed biotin to be present in the hydrolysate, but at low concentration, probably as a result of partial destruction by the nitrous oxide.

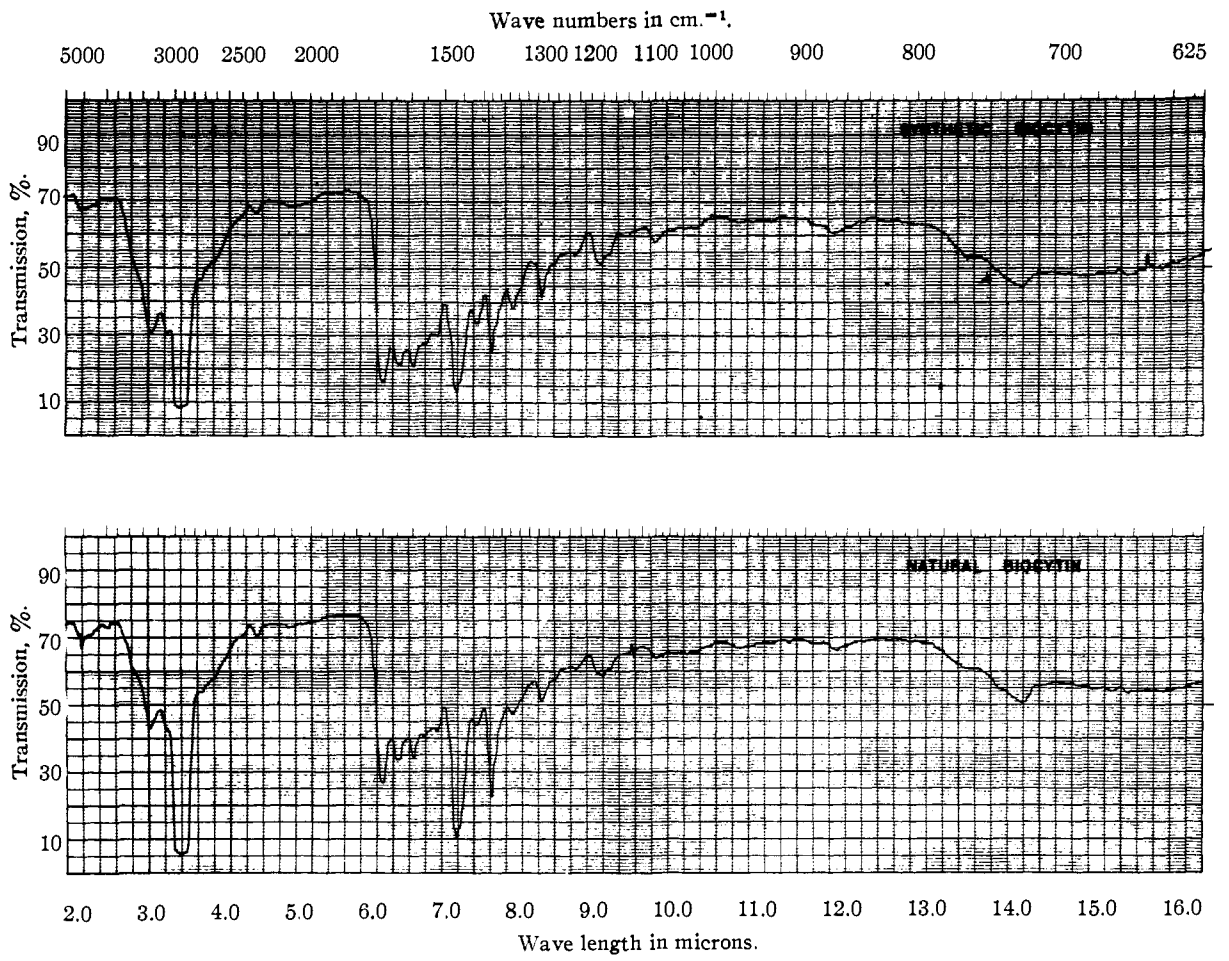

Fig. 1.

A comparative sample, which was not exposed to nitrous fumes, showed a typical lysine spot on paper chromatography. Both lysine and biotin were present in about equimolar amounts as shown by microbiological assays.

Comparison of Natural and Synthetic Biocytin.—When samples of both natural and synthetic[3] biocytin were obtained by slow crystallization from aqueous acetone, they were found to sinter at 225° and melt at 245–250°. There was no depression in the melting point behavior of a mixture. When both samples were allowed to crystallize rapidly from aqueous acetone, the natural and synthetic products melted at 228–230°, and there was no depression of the melting point of a mixture.

The infrared absorption spectra of natural and synthetic biocytin are identical as shown by the curves in Fig. 1.

Paper-strip chromatography of biocytin, visualized with ninhydrin, showed the same R_F value for natural and synthetic samples. In the system butanol:water:acetic acid (4:2:1), biocytin showed an R_F value of about 0.4.

Acknowledgment.—We wish to express our appreciation to Dr. J. P. Messerly for microbiological lysine assays, to Dr. N. R. Trenner and his associates for infrared spectra, and to Dr. G. E. Boxer and Miss V. C. Jelinek for the starch chromatography.

RAHWAY, NEW JERSEY RECEIVED OCTOBER 29, 1951