A mixture of 33.5 g. (0.174 mole) of 4-amino-di-*n*-propylaniline[12] and 122 g. (0.183 mole) of aluminum sulfate·18H_2O in 440 cc. of water was treated with 62.5 g. (0.396 mole) of sodium thiosulfate in 330 cc. of water and 23.8 g. (0.175 mole) of zinc chloride in 75 cc. of water. To the stirred mixture was added 14.5 g. (0.049 mole) of potassium dichromate in 188 cc. of water dropwise over two hours while cooling in an ice-bath. The stirring was continued for one hour and the solid was collected, washed twice with water and once with 1:1 alcohol–ether. Nineteen and three-tenths grams (36%) of a light purple product was obtained, melting with decomposition from 193°. Five grams was purified by solution in 500 cc. of 95% alcohol, treatment with Darco, and concentration to about 200 cc. Three and fifty-five one-hundredths grams of colorless, finely-divided crystals that melted and decomposed from 208° was obtained.

Anal. Calcd. for $C_{12}H_{20}N_2O_3S_2$: N, 9.2. Found: N, 9.1.

Benzophenothiazines.—The three compounds were prepared and purified similarly. The details of one preparation follow.

5-(*p*-Tolylimino)-9-dipropylaminobenzo[a]phenothiazine.—A mixture of 14.9 g. (0.05 mole) of 2-amino-5-di-*n*-propylaminophenylthiosulfuric acid, 11.4 g. (0.05 mole) of 1-*p*-tolylaminonaphthalene,[13] 4.1 cc. of concentrated hydrochloric acid and 375 cc. of glacial acetic acid was stirred at room temperature while adding 184 cc. of 10% potassium dichromate in water over 30 minutes. The resulting dark blue-green mixture was stirred for one hour at room temperature and four hours on a steam-bath. After cooling, the reaction solution was poured into 600 cc. of concentrated aqueous ammonia and 750 cc. of water with stirring. The precipitate was collected, dried, and extracted with three 300-cc. and two 150-cc. portions of boiling benzene. The cooled benzene solutions were combined and treated with 750 cc. of 10% hydrochloric acid and shaken vigorously to precipitate the insoluble hydrochloride of the product. The dark blue precipitate was collected, washed with 10% hydrochloric acid and benzene, and dissolved in 1 liter of alcohol. The alcoholic solution was made basic by the addition of aqueous ammonia, and water was added to completely precipitate the dye base. Fifteen grams (66%) of a dark purple-red solid, m.p. 145–160°, was obtained. Crystallization of 9.7 g. of this from 970 cc. of heptane using Darco gave 5.27 g. of dark brown, finely-divided needles, m.p. 174–176°. Crystallization of the remainder with slower cooling gave 3.26 g. of green crystals with a metallic luster, m.p. 197–199°. The lower-melting form could be crystallized (by working rapidly) without raising the melting point, but seeding of such a solution with the higher-melting form gave the latter. The total yield was 39%.

Anal. Calcd. for $C_{29}H_{29}N_3S$: C, 77.1; H, 6.5; N, 9.3. Found: C, 77.2; H, 6.7; N, 9.2.

The compound showed maximum absorption at 667 mμ in alcoholic hydrochloric acid and 550 mμ in alcoholic ammonium hydroxide.

9-Dimethylamino-5-phenyliminobenzo[a]phenothiazine.—The compound was prepared in 15% yield in the same manner as the dipropyltolyl homolog. Only one melting point was observed, 244–249°.

Anal. Calcd. for $C_{24}H_{19}N_3S$: C, 75.4; H, 5.3; N, 11.0. Found: C, 75.2; H, 5.2; N, 10.8.

The compound showed absorption maxima at 667 mμ in alcoholic hydrochloric acid and at 530 mμ in alcoholic ammonium hydroxide.

9-Diethylamino-5-phenyliminobenzo[a]phenothiazine.—The compound was prepared in 30% yield by the method used above. Only one melting point was observed, 235–241°.

Anal. Calcd. for $C_{26}H_{23}N_3S$: C, 76.3; H, 5.7; N, 10.3. Found: C, 76.3; H, 5.8; N, 10.1.

The compound showed an absorption maximum at 680 mμ in alcoholic hydrochloric acid. The absorption in base was not measured.

STAMFORD, CONNECTICUT RECEIVED SEPTEMBER 26, 1951

(12) W. A. Jacobs and M. Heidelberger, *J. Biol. Chem.*, **21**, 114 (1915).

(13) Prepared in 60% yield by the method of H. H. Hodgson and E. Marsden (*J. Soc. Chem. Ind. Trans.*, **58**, 156 (1939)) from *p*-toluidine and 1-naphthylamine using 1 mole % of HI as catalyst.

[CONTRIBUTION FROM THE RESEARCH DIVISION, SHARP AND DOHME, INC., AND THE RESEARCH LABORATORIES, MERCK AND CO., INC.]

Isolation of Crystalline Biocytin from Yeast Extract

BY LEMUEL D. WRIGHT, EMLEN L. CRESSON, HELEN R. SKEGGS, THOMAS R. WOOD,[1] ROBERT L. PECK, DONALD E. WOLF AND KARL FOLKERS

Biocytin has been isolated in crystalline form from yeast extract. The isolation involved adsorption on norit A, elution with aqueous ammonia, adsorption on superfiltrol–celite, elution with aqueous ethanolic ammonia, chromatography on superfiltrol–celite, chromatography on alumina, partition with butanol and cresol, countercurrent distribution and crystallization from water.

The isolation of crystalline biocytin from yeast extract was reported.[2] The present paper describes details of the procedures employed for the isolation of biocytin in crystalline form.

The term biocytin (Gr. Kútos, cell) has been used to designate the predominant form of biotin occurring in many solubilized natural products, especially those originating from the controlled autolysis of actively metabolizing material such as yeast extract.[2,3] Biocytin is characterized microbiologically by its availability as a source of biotin to *Lactobacillus casei, Lactobacillus delbrückii* LD5, *Lactobacillus acidophilus, Streptococcus fecalis* R, *Neurospora crassa* and *Saccharomyces carlsbergensis* and by its unavailability as a source of biotin to *Lactobacillus arabinosus, Lactobacillus pentosus* and *Leuconostoc mesenteroides* P-60. When subjected to strong acid hydrolysis (at least 3 *N* at 120° for 1 hour) biocytin yields biotin, or its microbiological equivalent, as a moiety.

When any natural product is isolated in pure form for the first time, there is a question as to whether or not the pure substance obtained is identical with the factor as it occurs in natural materials. Consideration of the data obtained during the course of the isolation work furnishes considerable presumptive evidence that the crystalline substance isolated is identical with biocytin as it occurs in yeast extract. Thus, although a number of steps were essential in the isolation procedure so that the

(1) E. I. du Pont de Nemours & Company Inc., Newark, Delaware

(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); 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).

(3) L. D. Wright and H. R. Skeggs, *Proc. Soc. Exptl. Biol. Med.*, **56**, 95 (1944).

over-all yield was low, no single step was associated with marked unaccountable loss of biocytin, nor was a "split" in microbiological activity other than that associated with the separation of biotin from biocytin ever encountered. The isolation steps did not involve drastic conditions such as extremes of *p*H, heat or oxidizing conditions. Moreover, the isolation procedures did not involve the formation and decomposition of a derivative. Countercurrent distribution in different systems showed only one biotin-containing peak. Finally, crystalline biocytin has a microbiological spectrum of activity identical with that of the biotin complex of yeast extract, and is identical with the biotin complex of yeast extract as shown by bioautographic paper-strip chromatography. It thus appears evident that crystalline biocytin is identical with the naturally occurring form.

The following steps were employed to obtain crystalline biocytin from yeast extract: adsorption on norit A; elution with aqueous ammonia; adsorption on superfiltrol–celite; elution with aqueous ethanolic ammonia; chromatography on superfiltrol–celite; chromatography on alumina; partition with butanol and cresol; countercurrent distribution; and crystallization from water. Some 5,000 pounds of yeast extract, representing about 25 tons of original yeast, were processed in order to obtain sufficient biocytin for completing the isolation and structural studies.

The structure determination[4] and synthesis[5] of biocytin are described in accompanying papers.

Experimental

Determination of Biocytin.—Biocytin was determined by a differential assay in which the difference between "total biotin" and "free biotin," as determined by microbiological assay with *Lactobacillus arabinosus*[2,6] represents biocytin. In the determination of "total biotin," a sample expected to contain 0.05–5 γ of "total biotin" in 1 ml. was first autoclaved for one hour at 120° with 1 ml. of 6 N H_2SO_4. Following autoclaving, the hydrolyzed material was neutralized and diluted to a suitable volume for assay. "Free biotin" was determined by assay of a suitable aliquot of the same sample without prior acid hydrolysis. Biocytin was expressed in terms of biotin used as a standard in the microbiological assays. As shown below, biotin is not adsorbed on superfiltrol–celite. Biocytin concentrates which had undergone a superfiltrol–celite adsorption and the subsequent elution steps were essentially free of biotin. Thus, except for occasional checks, it was not necessary to make a correction for free biotin in following the biocytin fractionation after the superfiltrol–celite adsorption and elution stage.

Adsorption and Elution with Norit.—One hundred pounds of Zymezate brand yeast extract (Standard Brands, Inc.) was dissolved in 250 gallons of water. Sufficient powdered lime was added to bring the *p*H to about 9. Fifty pounds of supercel was added and the mixture was filtered through a filter press. The filtrate plus washings (total volume about 300 gallons) was neutralized to *p*H 7 with hydrochloric acid and then stirred for one hour with 80 pounds of norit A. The norit A was removed by filtration through a filter press and washed with 100 gallons of water containing 5% concentrated aqueous ammonia. The norit A was eluted by stirring with 120 gallons of 50% ethanol containing 5% concentrated ammonium hydroxide. The elution step was repeated and the combined eluate was concentrated *in vacuo* to a volume of about 20 liters. From 1,140 liters of yeast extract containing 38 kg. of solids (biotin content, 48 mg.; biocytin content 130 mg.); there were obtained the following sequential fractions: lime filtrate, 1,140 liters, 36 kg. of solids (biotin, 50 mg.; biocytin, 134 mg.); first norit A eluate, 380 liters, 2.88 kg. of solids (biotin, 23 mg.; biocytin, 68 mg.); second norit A eluate, 380 liters, 1.72 kg. of solids (biotin, 5 mg.; biocytin, 22 mg.). The norit A filtrate from the adsorption step contained 24 mg. of free biotin, but only 6 mg. of biocytin. The aqueous ammonia wash of the norit A removed a further 4 mg. of free biotin but no biocytin. The concentration of biocytin in this step attains about 5–10-fold.

Adsorption and Elution with Superfiltrol–Celite.—An amount of norit eluate containing 3.83 kg. of dry matter (biotin, 24.8 mg.; biocytin, 67.2 mg.) was diluted to 40 liters, neutralized to *p*H 7.0 and clarified by filtration through paper. The clarified filtrate was poured onto a large chromatographic column, two feet in diameter, six feet in height, charged with 100 pounds of a superfiltrol–celite (1:2) mixture. When the adsorbent had taken up the norit eluate, 128 liters of water was added to the column. This aqueous wash removed 22.9 mg. of biotin and a total of 2.51 kg. of dry matter and was discarded. The superfiltrol–celite column was eluted then with 160 liters of 50% ethanol containing 5% concentrated ammonium hydroxide. The eluate was evaporated *in vacuo* to about 8 liters. There was present in the eluate about 0.76 kg. of solids containing 46.7 mg. of biocytin. This step is effective in concentrating biocytin about 5-fold to yield concentrates containing 50–100 γ of biocytin as biotin/g.

Chromatography on Superfiltrol–Celite.—The eluate obtained from the processing of about 4 kg. of solids with superfiltrol–celite was neutralized to *p*H 7.0 and diluted to a concentration of 10%. Usually, about 1 kg. of dry matter was obtained, so that the total volume was about 10 liters. This solution was mixed with a little supercel and clarified by centrifugation and filtration. The clarified filtrate was poured onto a large chromatographic column (1.5 feet in diameter; 4 feet in height) charged with an amount of superfiltrol–celite mixture equal to 15–20 times the weight of the dry matter (usually about 30–40 pounds of adsorption mixture). It is essential that a ratio of 15–20 times as much superfiltrol–celite mixture as dry matter to be chromatographed be employed in this step. With less adsorbing agent no separation of biocytin from inert dry matter is obtained. With larger amounts of adsorbing agent the biocytin is eluted poorly by water and considerable irreversible adsorption occurs.

When the adsorbent had taken up the above-mentioned clarified filtrate, the column was developed with water and 2-liter eluate fractions were collected. About 40 such eluate fractions were usually taken in order to obtain a satisfactory recovery of the biocytin which was achieved in about 72 hours of continuous operation. The fractions were assayed for biocytin and total solids (dry matter); those fractions containing most of the biocytin were combined and concentrated *in vacuo* to dryness. This chromatographic step is effective in concentrating biocytin 5–10-fold to yield con-

TABLE I

DATA ON TYPICAL ELUATE FRACTIONS FROM A SUPERFILTROL-CELITE CHROMATOGRAM OF BIOCYTIN CONCENTRATE

Fraction	Solids, g.	Biocytin content, mg.	(As biotin), γ/g.
1	38.02	0	0
5	53.06	0.24	5
7	69.70	.44	6
11	20.72	.40	19
17	6.78	.64	94
23	3.86	1.04	270
33	2.36	1.40	594
39	2.02	1.24	614
45	1.68	0.84	500
51	1.46	.44	301
55	1.70	.26	153
Starting sample (9.9 liters)	974.06	46.95	48

(4) R. L. Peck, D. E. Wolf and K. Folkers, THIS JOURNAL, **74**, 1999 (1952).

(5) D. E. Wolf, J. Valiant, R. L. Peck and K. Folkers, *ibid.*, **74**, 2002 (1952).

(6) L. D. Wright, *Biological Symposium*, **13**, 290 (1947).

centrates containing 200–500 γ of biocytin as biotin/g. Data for a typical column are summarized in Table I.

Chromatography on Alumina.—The dry, combined active material obtained as described in the preceding section was suspended in 180 ml. of water. To this was added 1020 ml. of ethanol, and the mixture was shaken for about ten minutes to achieve the separation of a precipitate. The mixture was centrifuged and the supernatant was decanted. The precipitated solids were resuspended in 180 ml. of water and again shaken with 1020 ml. of ethanol. Following centrifugation of this suspension, the supernatant solution was added to that obtained from the first precipitation. The insoluble residue contained no biocytin and was discarded. The combined 85% ethanol solution containing about 30 g. of dry matter was poured onto a chromatographic column containing 5 kg. of acid- and water-washed alumina.

Care must be taken in the selection of the alumina used in the chromatography. Unactivated alumina fails to bring about any separation of the biocytin from inert dry matter while highly activated alumina may adsorb biocytin so that it cannot be eluted without lowering the ethanol content of the developing solution to such an extent that biocytin is then eluted together with inert dry matter.

When the alumina had taken up the 85% ethanol solution, the column was developed with 50% ethanol; two-liter eluate fractions were taken. Satisfactory recovery of the biocytin required collection of about 20 such fractions which was achieved in about 48 hours of continuous operation. The fractions were assayed for biocytin and dry matter; those fractions containing most of the biocytin were combined and concentrated *in vacuo* to a volume of about 200 ml. Table II shows data from a typical column. Ethanol precipitation of inert matter, followed by chromatography of the ethanol solution on alumina is effective in concentrating biocytin about 10-fold to yield concentrates containing 4,000–10,000 γ of biocytin as biotin/g.

TABLE II

DATA ON THE FRACTIONS[a] FROM CHROMATOGRAPHY OF BIOCYTIN CONCENTRATE ON ALUMINA

Fraction	Solids, g.	Biocytin content, mg.	(As biotin), γ/g.
1	0.30	0.032	107
2	3.94	.080	22
3	0.78	.080	10
5	1.04	.06	575
6	1.44	2.86	2,100
7	0.78	3.60	4,600
9	.14	2.62	18,700
11	.02	1.32	66,000
13	.10	0.74	7,400
16	.04	.24	6,000
Starting sample	26.89	19.842	738

[a] Fractions 6–16 combined and evaporated *in vacuo* gave a solution in which there was 2.88 g. of solids containing 18.14 mg. of biocytin as biotin (6,290 γ/g.).

The alumina chromatography step and the steps preceding it have been carried out about 75 times in the processing of sufficient yeast extract to obtain the small amounts of crystalline biocytin which have been used for characterization studies.

Partition with Butanol and Cresol.—Partition with butanol and with cresol effected some concentration of biocytin and was of value in removing considerable amounts of surface-active material and inorganic salts which would be troublesome in subsequent operations. This procedure was carried out with nearly every lot of processed yeast extract. It was convenient to stockpile concentrates at this stage of the purification. In the lyophilized state, the product is stable.

A 390-ml. solution of 1.38 g. of active material containing 14.04 mg. of biocytin as biotin (10,170 γ/g.) obtained as a product of the previous step, was brought to *p*H 6 with a drop or two of 1 *N* hydrochloric acid, and repeatedly extracted in a separatory funnel with butanol. The butanol extraction removed considerable colored inert material and any trace of free biotin. The 375 ml. of aqueous phase (12.38 mg. of biocytin as biotin) was shaken with an equal volume of freshly redistilled mixed cresols. The mixture was centrifuged and the two layers separated by aspiration. The residual aqueous phase contained only 1.98 mg. of biocytin as biotin. The cresol extract was placed in a large separatory funnel, 5 volumes of diethyl ether was added, and the cresol–ether mixture was extracted with water until colorless extracts were obtained. The combined aqueous extracts were extracted several times with ether to remove cresol. The aqueous phase was then concentrated *in vacuo* to a volume of about 100 ml. This solution was dried by lyophilization to give 262 mg. of solids containing 10.0 mg. of biocytin as biotin (38,170 γ/g.). The butanol and cresol partition steps are effective in concentrating biocytin 3–5-fold to yield concentrates containing 20,000–50,000 γ of biocytin as biotin/g.

Rechromatography on Alumina.—The 262 mg. of lyophilized material from the previous step was dissolved in 100 ml. of 85% ethanol and poured onto a chromatographic column containing 500 g. of acid-washed alumina. The column was developed with 50% ethanol, and 100-ml. fractions were collected. The separate fractions were assayed for biocytin. Fractions 11–23, containing most of the biocytin, were combined and concentrated *in vacuo* to a volume of about 200 ml. This concentrate was subjected to butanol and cresol partition as previously described. Lyophilization of the final aqueous solution gave 22.5 mg. of dry residue containing 6.3 mg. of biocytin as biotin (280,000 γ/g.). Rechromatography on alumina was effective in concentrating biocytin about 5–10-fold to yield concentrates containing 250,000–300,000 γ of biocytin as biotin/g.

Countercurrent Distribution and Crystallization of Biocytin.—The system found most useful for the countercurrent distributions for purification work comprised mutually saturated phases of water at *p*H 3 (dilute hydrochloric acid) and a 1:1 mixture of chloroform and *o*-cresol. Biocytin showed a partition coefficient of about unity in this system. Since biocytin is very soluble in *o*-cresol and practically insoluble in chloroform, varying the chloroform content of the solvent phase permitted shifting the location of the biocytin in the system. About three 9-tube distributions were necessary to increase the biocytin (as biotin) content of concentrates from 1.25% to 15–16%. The distributions were carried out in centrifuge tubes, since the emulsions encountered could be broken most effectively by centrifugation. On completion of a distribution, the content of each tube was quantitatively transferred to a separatory funnel, mixed with 3–5 volumes of ether, shaken and separated into two layers. The aqueous layer was removed, and the solvent layer was extracted several times with small portions of water. The combined aqueous solutions were extracted twice with one volume of ether to remove cresol, and dried from the frozen state. The most active fractions were combined and redistributed. A typical latter-stage distribution is illustrated in Table III.

TABLE III

9-TUBE COUNTERCURRENT DISTRIBUTION OF 53 MG. OF BIOCYTIN CONCENTRATE (10.9% BIOTIN). AQUEOUS PHASE MIGRATED

System: water at *p*H 3 *vs.* 1:1 mixture of chloroform and *o*-cresol, mutually saturated

Tube no.	Wt. of material in tube, mg.	Biocytin content (as biotin), %
1	6.9	. .
2	7.4	4.9
3	7.6	11.1
4	9.2	14.9
5	6.4	14.4
6	4.1	16.2
7	4.0	12.0
8	3.7	6.5
9	4.2	2.4

Fractions 3, 4 and 5, summarized in Table III, deposited crystals when slowly evaporated from concentrated aqueous solutions. The sirupy mother liquors surrounding the crystals in fraction 3 were carefully removed and the crystals were washed with aqueous methanol, methanol and dried; yield 1.5 mg., m.p. about 230–240° (dec.) with preliminary

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

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

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.

(1) 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).

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