Technical Papers

The Elucidation of Biocytin

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In 1944, Wright and Skeggs (*1*) had been studying the biotin content of natural products by microbiological assays using *Lactobacillus casei* and *Lactobacillus arabinosus*. When certain extracts of natural products, particularly those from the controlled autolysis of actively metabolizing yeast, were assayed, it was found that the results were decidedly higher when the assays were conducted with *L. casei* rather than with *L. arabinosus*. It was evident that the assays with *L. arabinosus* were measuring the biotin content of the extracts, and that the assays with *L. casei* were measuring not only the biotin content of the extracts, but also a biotin complex. Hydrolysis degraded the biotin complex to biotin. The biotin complex was designated biocytin (from Gr. *Kútos,* 'cell').

Biocytin can also be utilized for growth by *L. delbrückii* LD_5, *L. acidophilus, Streptococcus fecalis* R, *Neurospora crassa,* and *Saccharomyces carlsbergenis*. Biocytin is not available per se for growth to *L. pentosus* and *Leuconostoc mesenteroides* P-60 in addition to *L. arabinosus*. Enzymatic treatment of biocytin-containing extracts with pepsin, papain, takadiastase, mylase, or polidase did not cleave the complex and release biotin.

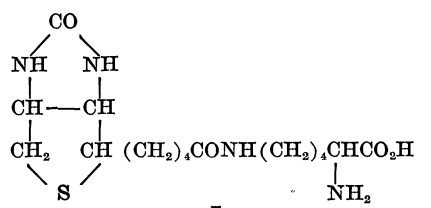
In 1946, a cooperative research program was begun by the Research Division of Sharp & Dohme, Inc., and the Research Laboratories of Merck & Co., Inc., to attempt to isolate biocytin in pure form. Research on its structure and synthesis might be expected to follow.

In December 1949, a joint announcement was made on the isolation of crystals of biocytin from yeast extract (*2*). The amount obtained was very small, however. Only 1.5 mg of recrystallized material was available. Recrystallization was difficult; the contaminating gummy impurities were hard to remove and resulted in low recovery of recrystallized material. This product showed $40 \pm 4\%$ of biotin by hydrolysis and microbiological assay, and melted at 230°–240° (dec). It was definite that only one biologically active complex, biocytin, was present during the purification.

The effort on isolation and crystallization was continued, and we are now able to report further properties of crystalline biocytin, as well as its structure determination and synthesis. Full details of the isolation scheme (*3*), structure determination (*4*), synthesis (*5*), and biological studies (*6*) will appear elsewhere in due time.

To complete this investigation, about 5,000 lbs of yeast extract, representing about 25 tons of yeast, were processed. The steps utilized in the isolation of biocytin consisted of adsorption on norit A, elution with aqueous-ethanolic ammonia, chromatography on superfiltrol-celite, chromatography on alumina, partition with butanol and cresol, countercurrent distribution between cresol-chloroform and water, and crystallization from water. The biocytin, after slow crystallization from aqueous acetone, melted at 245°–252° (dec, microblock). The product from rapid crystallization in aqueous methanol or acetone melted at 228°–230°. Recrystallized samples yielded about 60% of biotin by acid hydrolysis and microbiological assay. The same result was obtained when alkaline hydrolysis was used, followed by reaction with phosgene to convert the biotin diamine to biotin.

Paucity of adequately recrystallized material permitted only two C—H and N analyses. One set of analyses revealed a hydrochloride in reasonable agreement with $C_{16}H_{29}N_4O_4SCl$, and the other analyses revealed that the next sample was essentially the free base of composition $C_{16}H_{28}N_4O_4S$. Hydrolysis of a 700-μg sample of biocytin yielded crystalline biotin. Paper chromatography of the hydrolysate revealed one ninhydrin-reacting spot, which was identified as lysine by its R_F value. Chromatography on starch and microbiological assay confirmed the identification of lysine. Biocytin possesses a free amino group, as evidenced by reaction with ninhydrin and 2,4-dinitrofluorobenzene. Reaction of biocytin with nitrous acid, followed by hydrolysis, yielded a lysine derivative which did not react with ninhydrin under the test conditions. Thus, the evidence from a few milligrams of natural biocytin indicates that biocytin has the structure of ε-*N*-biotinyl-L-lysine (I).



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Synthetic biotin acid chloride (*7*) had been previously prepared. When it was allowed to react with the copper-chelate complex of L-lysine, ε-*N*-biotinyl-L-lysine was obtained. Reaction of biotin acid chloride with α-*N*-formyl-L-lysine to give ε-*N*-biotinyl-α-N-formyl-L-lysine, followed by hydrolytic removal of the formyl group, also yielded ε-N-biotinyl-L-lysine.

Synthetic ε-*N*-biotinyl-L-lysine and biocytin isolated from yeast extract were compared by a number of chemical and biological tests (*4, 6*). The data revealed that the two products are identical. This comparison has included infrared absorption spectra, microbiolog-

ical activities, combination with avidin, activity in stimulating the aspartic acid deaminase system, behavior on paper chromatograms as determined by bioautographic procedures, behavior toward commercial enzymes, and rates of hydrolysis.

References

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The Function of the Cups of *Polyporus conchifer*

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No special significance has been ascribed previously to the curious cup-shaped structure developed at the base of the fruit-body of *Polyporus conchifer* (Schw.) Fr. Growing upon dead branches of elm, the fungus is widely distributed in North America. As generally collected in the autumn, the fruit-body is small, white, grey, or pale-brown, thin-textured, and shelving. The fertile pileus is usually semicircular or kidney-shaped in outline and about 1×4 cm in size. The underside of the flat pileus bears a layer of pores within which basidia are developed.

At the base of the fruit body on the upper side is a small vaselike structure, 4–6 mm in diameter and 5 mm deep. This has generally been described as "sterile," and, as far as the present writer has been able to discover, only Lloyd (*1*) has ever commented upon the unusual nature of a polypore which produces cups in addition to the regular shelving pileus.

It appeared probable that the cups of *Polyporus conchifer* might serve to disperse some kind of reproductive structures and be similar to the splash cups of the Nidulariaceae, liverworts, and mosses to which attention has been drawn recently (*2*). Observations just completed have revealed that special spores are, in fact, disseminated by raindrops falling into the cups of *P. conchifer*.

Fruit-body formation begins during the late summer with the development of the cupulate portion. The cups always grow only on the upper sides of elm branches. In the autumn, the flat pore-bearing portion develops as an outgrowth from one side of the cup. The fungus discharges basidiospores throughout the autumn, but by spring the flat pileus has broken away from the cup entirely. The cups without their spore-bearing pilei are remarkably like those of *Crucibulum vulgare* Tul.

Cups examined from early February 1951 at intervals of 2 weeks until July were always empty, whatever material they had contained evidently having been dispersed. On August 10, large numbers of new cups were found in several stages of development. Some new cups grew from within the old, but most of them developed independently. Every new cup collected at this time contained small dark-brown granules of various sizes, mostly split off from the inside of the cup at the base of the youngest cups, but formed from the inner rim of older cups.

When a drop of water was placed in a fungus cup under the binocular microscope, the dark masses were seen to swell rapidly and almost instantly. One would judge that some hydrophilic colloid is present, because of the rapidity with which the dark masses absorb water. The contents of the cup became cloudy as absorption of water progressed, and when the cloudy drop was transferred to a slide and examined it was found to consist of a suspension of countless minute rod-shaped spores, 3 μ in diameter and 3–8 μ in length.

Transferred to a hanging drop of nutrient agar, the spores germinated in 24–36 hr at room temperature, and the germination percentage was very high. Very young germ tubes bore clamp connections, from which it seems likely that the spores are binucleate. Although the actual process of their formation has not yet been studied, their occurrence mostly in chains and their rod shape suggest that they are oidia.

By allowing small drops of water to fall 8 ft into fresh cups in the laboratory, oidia were observed to be splashed as much as 4 ft from the cups.

It is clear that the cups of *Polyporus conchifer* are special organs for the dissemination of oidia by rain. This occurs mostly before the shelf portion of the fruit body has formed and therefore before basidiospore discharge has begun. The reproductive period of the fungus is thus greatly extended: oidia are splashed from the cups in summer, and basidiospores are shed from the pilei in the autumn.

References

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Volatile Silica Affecting Plant Ash Analyses

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In a study on the mineral metabolism of plants, the following experiment was performed. Three g of the seed of a kind of turnip, *Brassica ceruna*, were spread on moist filter paper, 11 cm in diameter. The paper was supported in a moist chamber by 4 horizontal

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