Minimal amounts of progesterone-like activity were found in the plasma of male snakes. The possible role of this substance in the reptile and its evolutionary significance are discussed.

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Bioautography of Biotin and Certain Related Compounds. (21140)

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The existence in culture filtrates of *Aspergillus niger* grown in the presence of added pimelic acid of a previously unrecognized component of natural material with biological activity equal to that of biotin in satisfying this growth factor requirement of *Neurospora crassa* has been described by Wright and Cresson(1). The factor was isolated from a large quantity of *Aspergillus niger* culture filtrate and identified as biotin L-sulfoxide(2,3). It was established that biotin L-sulfoxide originates from an *in vivo* oxidation of biotin rather than an *in vitro* (purely chemical) oxidation. Biotin L-sulfoxide has activity equal on a molar basis to biotin, biotin D-sulfoxide, biocytin, and desthiobiotin in satisfying the biotin requirement of *Neurospora crassa*. For a number of other biotin-requiring microorganisms thus far examined it is less active than biotin or the D-sulfoxide. Biotin L-sulfoxide has an ubiquitous occurrence and may be expected to have some function in metabolism.

During the investigations involved in the discovery, isolation, and characterization of biotin L-sulfoxide considerable bioautographic data with biotin, biotin L-sulfoxide, biotin D-sulfoxide, biocytin, oxybiotin, and desthiobiotin as well as mold filtrates were accumulated where butanol-water-acetic acid was used as the developing solvent and *Neurospora crassa* was the assay organism. These data are being published in detail for the interest they may have for those concerned with elucidating various aspects of the biochemistry of "naturally-occurring" forms of biotin.

Methods. Synthetic D-biotin, DL-desthiobiotin and DL-oxybiotin (O-heterobiotin) were obtained from Hoffmann-LaRoche, Inc., synthetic biotin D-sulfoxide and biotin L-sulfoxide(4) were kindly supplied by Dr. D. B. Melville, natural biotin L-sulfoxide was isolated from *Aspergillus niger* culture filtrate as described previously(2), biocytin was synthesized in the laboratories of the Chemical Division of Merck and Co., Inc.(5). The solvent mixture for the paper chromatograms was prepared by shaking together one part

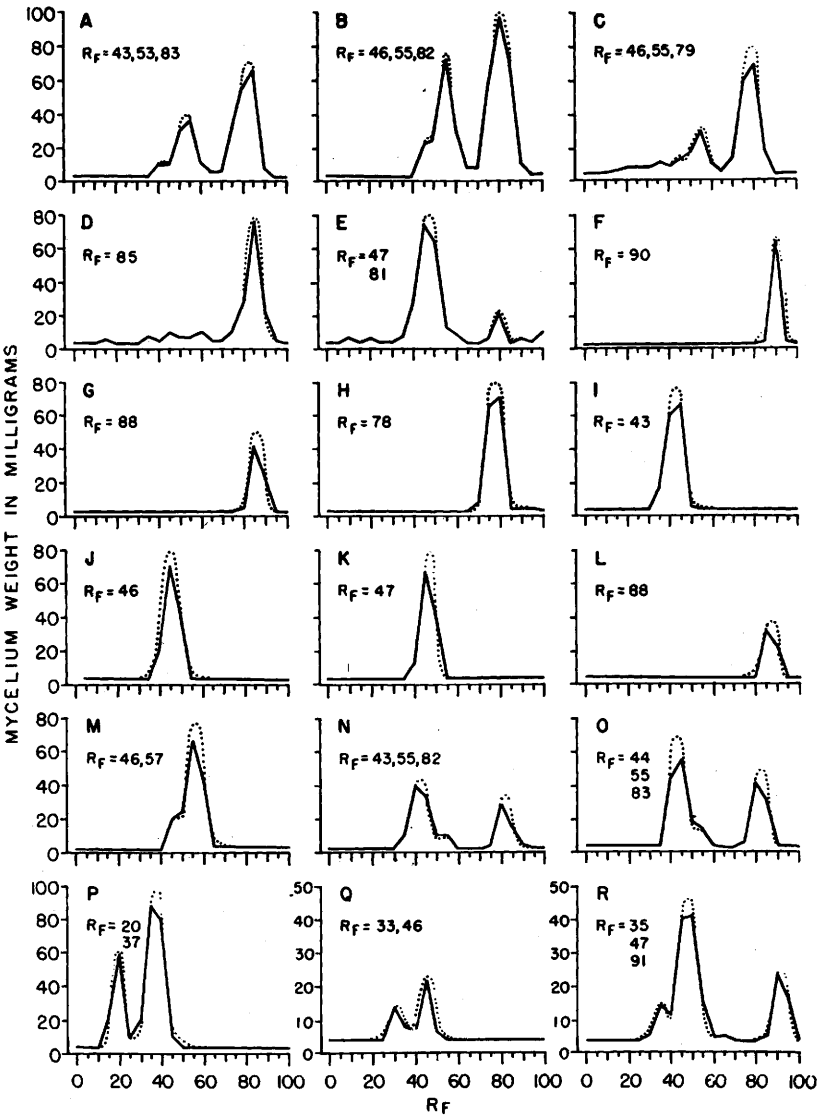

FIG. 1. Summary of bioautographic data. A, Synthetic biotin; B, synthetic biotin aerated; C, synthetic biotin shaken with *Aspergillus niger* synthetic medium; D, synthetic biotin shaken with autoclaved culture of *Aspergillus niger*; E, *Aspergillus niger* culture filtrate grown with added biotin; F, synthetic desthiobiotin; G, synthetic biotin treated with Raney nickel; H, synthetic oxybiotin; I, high potency biotin L-sulfoxide concentrate; J, synthetic biotin L-sulfoxide; K, crystalline biotin L-sulfoxide isolated from *Aspergillus niger* culture filtrate; L, high potency biotin L-sulfoxide concentrate treated with Raney nickel; M, synthetic biotin D-sulfoxide; N, high potency biotin L-sulfoxide concentrate treated with zinc and hydrochloric acid; O, high potency biotin L-sulfoxide concentrate treated with aluminum and sodium hydroxide; P, synthetic biocytin; Q, *Aspergillus niger* culture filtrate; R, *Aspergillus niger* culture filtrate grown with added pimelic acid.

glacial acetic acid, 4 parts n-butanol, and 5 parts water. The butanol phase (upper layer) was placed in the bottom of a large chromatographic jar and was the developing solvent. The aqueous phase (lower layer) was placed in a receptacle in the bottom of the jar. One-hundredth γ amounts of biotin (or its equivalent in the form of the derivative studied) in 0.01-0.02 ml of 50% ethanol were placed one inch from the edge of Whatman No. 1 filter paper sheets and allowed to dry. Development of the chromatograms was by the

ascending technic(6). Sixteen to 18 hours was the development time. Development was at room temperature which was not excessive during the period of this study. Under these conditions of paper chromatography the solvent travelled about 30 cm. After development the papers were air dried in a hood. Location of the areas of microbiological activity with *Neurospora crassa* was accomplished by cutting the paper into sections which were eluted in Erlenmeyer flasks with 25 ml of basal medium. The medium used had the following composition per liter: sucrose, 20 g; ammonium tartrate, 5 g; ammonium nitrate, 1 g; potassium dihydrogen phosphate, 1 g; magnesium sulfate (heptahydrate), 0.5 g; sodium chloride, 0.1 g; calcium chloride, 0.086 g; trace elements(7), 1 ml; p-aminobenzoic acid (PABA), 10 mg. After removal of the paper sections following a one-hour elution period the flasks were plugged, autoclaved, seeded with *Neurospora crassa*—PABA-less—and incubated at 30°C for 3-4 days. The extent of growth of the mold was determined by collecting the mycelium on a wire loop, squeezing to insipient dryness and then drying to constant weight at 80° (usually 18 hours). It was convenient to cut the paper chromatograms into 21 sections such that each section represented 0.05 of an R_F unit. When the mycelium weight was plotted as a function of the paper section number a smooth curve connecting the points permitted localization of the R_F by interpolation to within 0.02 unit.

Results and discussion. The bioautographic data for the various compounds and culture filtrates are summarized in Fig. 1 in which weight of mycelium per flask is plotted as a function of the paper section number which in turn is a function of the R_F value.

As pointed out first by Davis(8), even synthetic biotin (A) yields more than one area of microbiological activity. He observed that if biotin be paper chromatographed with butanol-acetic acid as the developing solvent and the paper then applied to a plate containing medium seeded with a biotin-requiring mutant of *Escherichia coli* (bioautography) 2 areas of activity are observed with R_F values of 0.89 and 0.58. Subsequent investigation showed that the material with R_F of 0.89 corresponds to biotin, while that with R_F 0.58 corresponds to "biotin sulfoxide". The latter apparently is produced from biotin by some component of the filter paper. With an organism such as *Neurospora crassa* which responds equally well to both sulfoxides of biotin(3) 3 areas of activity are observed corresponding to biotin, biotin D-sulfoxide, and biotin L-sulfoxide. In agreement with the results of Melville *et al.*(4) the *in vitro* oxidation of biotin, in this case presumably by a component of the filter paper rather than hydrogen peroxide, leads to the formation of the D-sulfoxide in much the larger amount. With an organism such as *Saccharomyces cerevisiae* or the *Escherichia coli* mutant of Davis which apparently responds to only the D-sulfoxide only one "sulfoxide" would appear to be formed. The solvent mixture described is the only one thus far examined that yields effective separation of the 2 sulfoxides. As indicated by the data, biotin is not oxidized by any one of a number of methods of aeration. In one instance (B) a 1 γ per ml aqueous solution was aerated by a vigorous stream of compressed air at room temperature for 18 hours. In a second experiment (C), biotin (0.05 γ/ml) was shaken (200 rpm, 30°C for 5 days) with a sterile synthetic medium used for the growth of *Aspergillus niger*(1). In this instance the pH of the medium was taken to that (pH 2.5) usually resulting from the growth of the mold on the medium. In a third experiment (D) a similar amount of biotin was shaken under similar conditions with a 5-day culture of *Aspergillus niger* which had been autoclaved to stop enzyme activity. When a similar amount of biotin was added to the medium prior to growth of *Aspergillus niger* the added biotin in the presence of the growing mold was converted to biotin L-sulfoxide (E).

Desthiobiotin, as expected, yields only one area of microbiological activity (F). Raney nickel treatment of biotin gives a product (G) with only one area of activity corresponding to desthiobiotin. Desthiobiotin and biotin are not well separated by any solvent system thus far examined.

Oxybiotin, like desthiobiotin, yields only one area of microbiological activity(H). Its

R_F value in the butanol-water-acetic acid system is only slightly less than that of biotin (0.78 vs. 0.83) so that differentiation in this system is not feasible. On the other hand, the R_F value of oxybiotin is significantly lower than that of desthiobiotin (0.78 vs. 0.89) and differentiation of these 2 compounds in this system may be accomplished.

Biotin L-sulfoxide in the form of a concentrate (I) or as the synthetic (J) or isolated crystalline material (K) migrates as an entity. There appears to be no equilibrium between it and the D-sulfoxide originating from the oxidative tendency of the filter paper. Reduction of biotin L-sulfoxide in the form of a concentrate with Raney nickel leads to material (L) that yields only one zone of activity attributable to desthiobiotin. The sample of biotin D-sulfoxide used gave 2 areas of microbiological activity (M), one corresponding to biotin L-sulfoxide. Since the D-sulfoxide is the predominant form obtained on *in vitro* oxidation and the L-sulfoxide appeared to be unaffected by the filter paper, it is suggested that the sample of the D-sulfoxide examined contained a small amount of L-sulfoxide as a contaminant. When biotin L-sulfoxide in the form of a concentrate was reduced with zinc and hydrochloric acid (N) or aluminum and sodium hydroxide (O), the product on bioautography gave 3 zones of microbiological activity. This is the expected finding if it be assumed that reduction was incomplete and the biotin formed by reduction was oxidized by a component of the filter paper to give the 2 sulfoxides in the proportions previously described.

Crystalline synthetic biocytin (ϵ-N-biotinyl-L-lysine) (P) yields more than one area of microbiological activity when bioautographed with *Neurospora crassa*. It is suggested that a component of the filter paper oxidizes biocytin in a manner analogous to that observed with biotin.

Aspergillus niger culture filtrate obtained after growth on a synthetic medium without added pimelic acid (Q) contains 2 biotin derivatives detectable by bioautography with *Neurospora crassa*. The compounds from their R_F values and microbiological activity (1) are believed to be biocytin and biotin L-sulfoxide. Note in particular the complete absence of biotin. Culture filtrate obtained after growth, with added pimelic acid (R) contains biocytin, much more biotin L-sulfoxide than present without added pimelic acid, and, in addition, biotin and/or desthiobiotin or related derivative.

It is appreciated that correspondence in R_F values in a single solvent system does not serve to establish the identity of 2 compounds. The solvent system butanol-water-acetic acid described here has been found useful in obtaining *presumptive* evidence for the existence of the biotin derivatives described because, (a) the R_F values for the various derivatives are nicely spread out permitting, for example, separation of the 2 diameric sulfoxides, and (b) the R_F values in this solvent system are essentially uninfluenced by unrelated contaminants. To obtain *conclusive* evidence for the existence of a specific biotin derivative a large number of solvent systems must be employed. R_F values of biotin derivatives in most other solvent systems studied are markedly influenced by salts and other extraneous material so that some sort of preliminary concentration as, for example, 2-way paper chromatography usually is essential.

Summary. R_F values determined bioautographically where butanol-water-acetic acid was the solvent system and *Neurospora crassa* was the indicating organism are summarized for biotin, desthiobiotin, oxybiotin, biocytin, biotin L-sulfoxide, biotin D-sulfoxide, and the microbiologically active components of *Aspergillus niger* culture filtrate.

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