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EVIDENCE FOR THE BIOSYNTHESIS OF SELENOBIOTIN

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SUMMARY

Chemically synthesized selenobiotin is, like sulfur biotin, able to bind to avidin. This observation was used to help identify biologically synthesized selenobiotin as an excretion product of Phycomyces blakesleeanus. The identification of [^{75}Se]selenobiotin was based on the highly specific binding of biotin to avidin used as an affinity ligand to Sepharose, on its release from the complex by proteolytic treatment, and its chromatographic behavior relative to [^{14}C]biotin standards. These results represent the first evidence of a biological synthesis of a heterocyclic ring that contains selenium in place of sulfur.

The selenium atom within most of the biologically synthesized organic selenium compounds thus far identified exists in acyclic form such as in dimethylselenide (1), in the trimethylselenonium ion (2), and in selenocysteine (3, 4); or it is external to the ring as in 4-selenouridine (5, 6). The existence of a biologically synthesized cyclic selenium compound such as selenobiotin has never been demonstrated, though chemical synthesis has been described (7, 8). The chemically synthesized selenobiotin mimics biotin in a number of its biological properties; for example, it replaces biotin as a growth factor for several microorganisms, is incorporated into carboxymethylases without loss of enzyme activity, and with Escherichia coli, the selenoacetyl-CoA carboxylase is as active as the normal enzyme.

Since a chemical synthesis does not necessarily prove the existence of a biological route of synthesis, experiments were undertaken to see if selenobiotin could be biologically synthesized. This paper will present evidence

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**Initial experiments were carried out with [^{75}Se]selenite, but approximately 60 per cent of the radioactivity was volatilized during the growth period.

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that Phycomyces blakesleeanus, a fungus, synthesizes a seleno-compound similar in many of its properties to biotin.

MATERIALS AND METHODS

Chemicals

D-[carbonyl-^{14}C]biotin ([^{14}C]biotin) and [^{75}Se]selenate ($^{75}SeO_4^=$) were obtained from Amersham Corporation, Arlington Heights, Illinois. Highly purified avidin was purchased from Sigma Chemical Company, St. Louis, Missouri. Chemically synthesized selenobiotin was kindly supplied by Dr. A. Marquet, Universite Pierre et Marie Curie, Paris, France. Stock cultures of P. blakesleeanus were maintained on Sabouraud dextrose agar slants at 2°C. All other chemicals were of the highest purity available.

Culture Conditions

Cultures of P. blakesleeanus were maintained on Eisenberg's medium (9) modified by the addition of pimelic acid and the substitution of urea for L-asparagine (10). Ferric citrate and trace element solutions, described by Hammer et al. (11), were used to supplement the medium. P. blakesleeanus was grown in 500 ml of autoclaved culture medium (121°C, 15 min) containing 50 µCi [^{75}Se] selenate** in Fernbach flasks at room temperature for 8-14 days.

Radioactivity Measurements

^{75}Se was counted in a Packard auto-gamma scintillation spectrometer; ^{14}C was counted in a Packard tri-carb liquid scintillation spectrometer in vials with 10 ml Bray's solution (12). Since ^{75}Se decays with both beta and gamma radiation, it was necessary to correct for ^{75}Se contribution to the values obtained in the beta counter. A concentration range of ^{75}Se was counted in both the gamma and beta spectrometers; a log-log plot gave a straight line. A sample containing both ^{75}Se and ^{14}C is first counted in the gamma spectrometer. The value in the beta counter is then corrected for ^{75}Se contribution to obtain the actual ^{14}C count.

Identification of [^{75}Se]Selenobiotin

Descending paper chromatography. Whatman #1 filter paper was used with the solvent system: ethanol-t-butanol-formic acid-water (24:8:2:6). Before chromatography, the chamber was saturated with the solvent; chromatograms were run 12-14 h (25-30 cm), cut into strips (1 cm) and counted for ^{75}Se and ^{14}C.

Biotin-Avidin Binding

Method 1 - Spectrophotometric titrations of avidin with biotin and synthetic selenobiotin were carried out according to the method of Wei (13) using a Gilford spectrophotometer.

Method 2 - A Sepharose-avidin affinity column was prepared according to the method of Green and Toms (14) modified by replacing 1,3-diaminopropane with ethanolamine. A column (1.6 x 10 cm) was prepared from 20 ml of the suspended conjugate. After the 8-14 day growth period the P. blakesleeanus medium, containing biotin, its vitamers, and presumably [^{75}Se]selenobiotin, was filtered through a Buchner funnel and then through a milipore filter (0.3 µ). The clear, yellow filtrate was added slowly (1-2 ml/min) to the column and washed with 0.01 M sodium phosphate buffer, pH 7.0, until radioactivity could no longer be detected in the wash. The suspension was removed from the column and placed in a

scintillation vial for counting of bound ^{75}Se. The conjugate was then filtered, washed with distilled water and suspended in 15 ml of 0.01 M NH_4HCO_3 buffer, pH 7.6; 10 mg pronase was then added to the washed conjugate. The mixture was incubated with stirring at 37°C for 24 h. After filtration, the pronase incubation of the conjugate was repeated, the combined filtrates were counted for released ^{75}Se, and then evaporated to dryness on a rotary evaporator. The residue was dissolved in 0.1 ml water, [^{14}C]biotin (0.015 µCi) added as a marker, and the sample was chromatographed.

RESULTS AND DISCUSSION

Chemically synthesized selenobiotin (7, 8, 15) has not been reported to bind to avidin. That selenobiotin can be bound by avidin was established by the spectrophotometric titration of synthetic biotin with avidin following the method of Wei (13). The spectral shift observed shows that complex formation did indeed occur (Fig. 1).

Direct application of the filtered culture medium, taken from an 8-14 day culture, to a Sepharose-avidin affinity column revealed significant binding of [^{75}Se] (3600 cpm). Subsequent pronase digestion of the complex released the [^{75}Se]-labeled material from the conjugate. Co-chromatography with [^{14}C]biotin yielded coincident ^{14}C and ^{75}Se radioactivity (Fig. 2). These results indicate both synthesis and excretion of [^{75}Se]selenobiotin by *P. blakesleeanus*.

Control experiments designed to eliminate the possibility of spurious binding of $^{75}SeO_4^=$ to the Sepharose-avidin conjugate were also carried out. These controls included one in which the solution contained $^{75}SeO_4^=$ (2×10^3 cpm) in

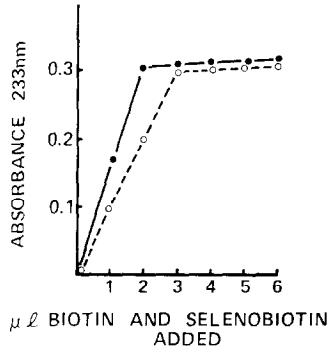

Figure 1: Spectrophotometric titration of synthetic selenobiotin (●——●) and biotin (o----o) with avidin in 0.2 M ammonium carbonate, pH 8.9. Selenobiotin or biotin (2-4 mg/ml) was added in 1 µl aliquots to an avidin solution (0.6 mg/3 ml). Increase in absorption at 233 nm due to the biotin-avidin complex formation was measured.

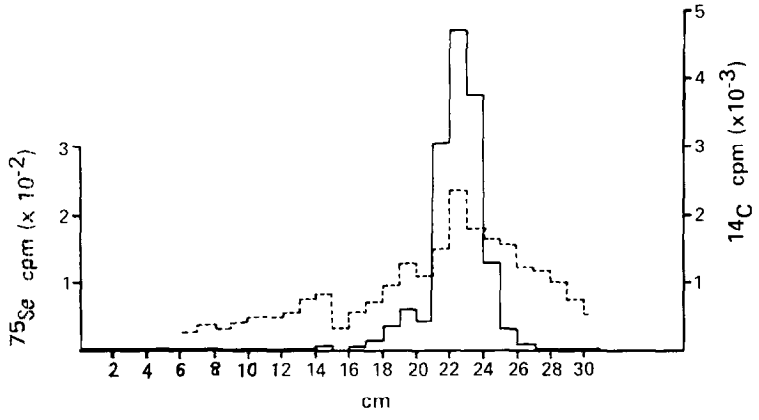

Figure 2: Chromatographic pattern of [^{75}Se]selenobiotin (------) and [^{14}C] biotin (———) run together on Whatman #1 filter paper. Each 1 cm strip was counted for ^{75}Se and ^{14}C. Solvent system: ethanol-t-butanol-formic acid-water (24:8:2:6).

water alone, and a second in which $^{75}SeO_4^{=}$ (2 x 10^6 cpm) was added to a filtered medium from a culture of P. blakesleeanus grown without $SeO_4^{=}$. In neither case was any binding to the Sepharose-avidin conjugate seen.

The assumption has been made in the past that sulfur and selenium, because of their chemical similarities, follow the same metabolic pathways. Distinct differences, however, both in animals and microorganisms, have been observed in the metabolic routes followed by sulfur and selenium. In each of the following enzymes the selenium occupies a specific position, distinct from that of the sulfur amino acids, within the polypeptide chain. Animal cells, for example, are able to incorporate the selenium from selenite into the enzyme glutathione peroxidase (16, 17, 18); and microorganisms can synthesize the selenoenzymes formic dehydrogenase (19) and glycine reductase (20). There are also scattered references which indicate an inability of some plants and microorganisms to assimilate selenium through the sulfur pathway (21).

Whether selenium can be incorporated biologically into cyclic compounds has remained in question. The synthesis of selenobiotin by P. blakesleeanus, strongly suggested by these experiments, demonstrates that the selenium atom can mimic the sulfur atom during its incorporation into a cyclic form.

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