# ENZYMATIC REDUCTION OF D-BIOTIN-d-SULFOXIDE WITH CELL-FREE EXTRACTS OF ESCHERICHIA COLI

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Summary. D-biotin-d-sulfoxide can fulfill the growth requirement for E. coli mutants deficient in biotin biosynthesis. In vitro reduction of D-biotin-d-sulfoxide to biotin requires a protein cell-free extract and NADPH. Preliminary experiments suggest that more than one protein participates in the reaction.

The presence of biotin sulfoxide in culture filtrates of various microorganisms as well as the occurrence of the d-isomer in cows milk has been well documented (1,2,3,4). The d-isomer was also found to be present in all preparations of authentic biotin (5). Melville et al. showed that the l and d-isomers have different growth promoting properties for yeast and lactobacilli species and that biotin appeared in cultures of yeast supplied with biotin-d-sulfoxide. This prompted these authors to suggest the existence of a highly specific d-sulfoxide reductase (6).

While searching for an intermediate in the conversion of dethiobiotin to biotin we discovered that biotin dependent mutants of E. coli were able to utilize D-biotin-d-sulfoxide in place of biotin. Four genes required for sulfoxide utilization have been found and mapped (7). In this paper we reported the conversion of biotin-d-sulfoxide to biotin with protein cell-free extracts and preliminary characterization of the reaction.

Materials and Methods. Synthesis of D-biotin-d-sulfoxide (BDS). The d-sulfoxide of biotin was synthesized and purified according to the procedures of B.D. Melville (8). Paper chromatographic analysis (9,10) with a

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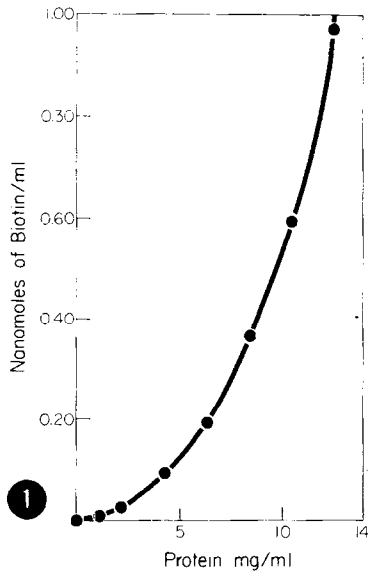
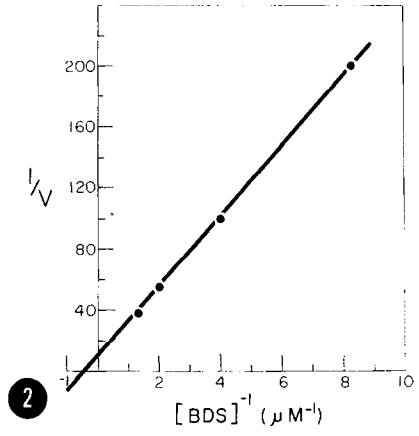
n-butanol:formic acid:water (4:1:1) solvent system revealed a major spot with an $R_F$ of 0.55 and a minor spot with an $R_F$ of 0.76, corresponding to BDS and biotin respectively. BDS solutions contained less than 0.01% biotin by biological tests. The biotin did not interfere with our experiments as diluted solutions of BDS were routinely used. As predicted crystals of BDS melted at 208-212°C (8). BDS crystals were also analyzed by mass spectrometry (MS-9, direct and 70 eV, Stanford University). The molecular ion ($M^+$, m/e 260) was clearly visible and the fragmentation products supported the conclusion that the major component in the crystals was BDS.

In vitro assay for D-biotin-d-sulfoxide reductase. To prepare the crude extract, cells were grown to stationary phase in synthetic medium containing 0.1% glucose, 0.1% caseamino acids and $10^{-2}$ µg/ml of biotin (10). The cells were washed in 0.9% NaCl, resuspended at 200 times the original concentration in 0.05 M potassium buffer (pH 7.0) containing 2.0 mM 2-mercaptoethanol. Following sonication the suspension was clarified by centrifugation (10,000 rpm, 20 minutes) and the supernatant dialyzed 20 hours at 4°C against two, 1 liter changes of buffer. The protein concentration was determined by the method of Warburg and Christian (11).

The reaction mixture (1 ml) contained 0.4 mg NADPH, 9.6 nmoles BDS and 1-15 mg protein. The reaction was started by adding BDS following preincubation of the other ingredients at 37°C for 15 minutes. After 30 minutes the reaction was stopped by addition of trichloroacetic acid (0.2 mmoles). The reaction mixture after clarification by centrifugation was assayed for biotin by the disk microbiological method (12). The assay organism (strain SA291) carried a deletion which removed the entire biotin gene cluster and the *bisA* gene (genes *bisA*, *bisB*, *bisC*, *bisD* are required for BDS utilization) (7,10). Therefore it could only respond to biotin.

The reaction mixture for Figure 1 contained 1.7 mg of protein extract and after 30 minutes incubation 100 µl of the mixture was assayed. The reaction mixture for Figure 2 contained 3.7 nmoles of BDS.




Results and Discussion. Strains of E. coli dependent on biotin for growth due to point mutations in each of the five biotin biosynthetic genes were able to metabolize D-biotin-d-sulfoxide (BDS) in place of biotin (Table I). Deletion mutations removing genes located from the gal operon through chlA (strain SA291) and those deleting the region from bioD through chlA rendered E. coli K12 incapable of utilizing BDS. It was also possible to select strains harboring point mutation which interfere with BDS metabolism. The bisA mutations were genetically located just to the right of chlA. The bisB, bisC and bisD mutations reside in genes located at various sites around the E. coli chromosome (7). The possibility that BDS is an intermediate in the biosynthesis of biotin from dethiobiotin was considered and excluded by the fact that strain C173 which is able to transform dethiobiotin into biotin failed to utilize BDS. Bioautographic analysis of the BDS solution using biotin dependent mutants to assay the chromatograms revealed a growth stimulating spot with an $R_F$ identical to BDS. This demonstrates that BDS rather than biotin or other impurities in the BDS solution was responsible for growth stimulation.

Table I. Growth Response of Bio-Mutants to D-Biotin-d-Sulfoxide

| Strain | Bio-mutation | Growth Response to D-biotin | Growth Response to D-biotin-d-sulfoxide |
|---|---|---|---|
| R879 | bioA24 | + | + |
| C562 | bioB562 | + | + |
| R872 | bioF3 | + | + |
| C541 | bioC541 | + | + |
| C519 | bioD519 | + | + |
| C249 | Δgal-bioB | + | + |
| C173 | ΔbioD-chlA | + | - |
| R876 | bioC, bisA | + | - |
| DD33 | bisB, bioA24 | + | - |
| DD34 | bisC, bioA24 | + | - |
| DD38 | bisD | + | - |

The conversion of BDS to biotin was achieved with cell-free extracts. The reaction required BDS, NADPH and a cell-free protein extract from a bis$^+$ culture (Table II). NADPH becomes limiting at concentrations less than 0.2 mg/ml. NADH could partially substitute for NADPH, but this is probably due to transhydrogenase activity in the crude extract. EDTA partially inhibited the reaction suggesting a requirement for metal ion. Cell-free extracts of strains carrying bio mutations were tested for BDS reductase activity and all were able to reduce BDS to biotin. Extracts from strains harboring point mutations or deletion mutations in bisA, bisB, bisC, bisD lacked BDS reductase activity. These have been shown to be separate genes, suggesting that at least 4 peptides participated in the reaction (10).

The rate of the reaction depends on the protein concentration (Fig. 1) and the BDS concentration (Fig. 2). The rate of the reaction did not follow first order kinetics when protein concentration was varied. Again this suggests that the reduction of BDS to biotin requires more than one protein. At 2.0 μM BDS the substrate is saturating the reaction and at concentrations of BDS greater than 19.0 μM substrate inhibition occurred. The possibility that some impurity in the BDS solution was poisoning the reaction has not

been excluded. The apparent Km for BDS was 2.5 μM which is similar to that found of dethiobiotin synthetase for its substrate, diaminopelargonic acid (12)

In three experiments cells were grown in synthetic media containing $1 \times 10^{-2}$ μg/ml biotin, washed with saline and resuspended in prewarmed synthetic medium. This was then divided into three parts. One portion received no biotin vitmer, the second received biotin and the third received BDS. After two hours incubation the BDS reductase specific activity was assayed and found to be the same in all three cultures. This suggests that BDS reductase is constitutively synthesized. However, the alternative that the small amounts of BDS present in commercial biotin (5) have induced enzyme synthesis has not been ruled out.

The biological importance of the diastereo-isomers of biotin sulfoxide were first studied by Melville et al. (6). The d-isomer was reported to be much more active in supporting growth of yeast and Lactobacillus arabinosus than the l-isomer which was only metabolized by Lactobacillus casei. In an aerobic environment biotin-sulfoxide may be one of the major forms of biotin

Table II. D-Biotin-d-Sulfoxide Reductase Activity

| Reaction mixture | Strain* | Biotin equivalent nmoles x $10^{-2}$ per mg protein |
|---|---|---|
| Complete | R879 | 5.3 |
| Complete | C173 | <0.3 |
| Complete | R879 | <0.3 |
| Complete | DD33 | <0.3 |
| Complete | DD34 | <0.4 |
| Complete | DD38 | <0.1 |
| Boiled extract | R879 | <0.4 |
| Boiled NADPH | R879 | <0.4 |
| Boiled D-biotin-d-sulfoxide | R879 | <0.4 |
| NADH replacing NADPH | R879 | 1.8 |
| 0.01 MEDTA | R879 | 0.9 |

*See Table I for a description of the bio mutations.

and offer an advantage to organisms able to scavenge this oxidized form of biotin. The reduction of BDS to biotin may be similar mechanistically to the

reduction of methionine sulfoxide which is a complex reaction requiring three protein fractions and NADPH (13).

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