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Mode of Action of α-Dehydrobiotin, a Biotin Analogue

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α-Dehydrobiotin, like biotin, represses coordinately the 7,8-diaminopelargonic acid aminotransferase and the dethiobiotin synthetase enzymes that are encoded on the *l* and *r* strands, respectively, of the *bioA* operon. The rate of synthesis for both enzymes is inhibited about 80% in the presence of α-dehydrobiotin. Homobiotin and α-methylbiotin are less effective than α-dehydrobiotin in repressing the synthesis of the two enzymes. The selective repression of transcription from *l* strand by α-dehydrobiotin and homobiotin, previously reported in hybridization experiments, is not observed at the enzyme level. A combination of equal concentrations of biotin and α-dehydrobiotin which was reported to enhance selectively the level of messenger ribonucleic acid transcribed from the *l* strand does not increase the rate of synthesis of the aminotransferase enzyme. Instead, the enzymes encoded on both strands are essentially completely inhibited as with biotin alone. Strain differences have been ruled out to account for the different results obtained by the two methodologies. Our evidence would suggest that α-dehydrobiotin acts like biotin, presumably as the co-repressor, in the repression of the *bioA* operon. The low rates of enzyme synthesis observed in the presence of the biotin analogue is the result of incomplete repression due to a lower affinity of either the analogue for the repressor or of the co-repressor/repressor complex for the operator. While our evidence would support the concept of a two promoter/operator complex, both would have to respond equally to biotin and its analogues. The evidence, however, does not rule out other possible alternative models for the regulation of the biotin operon.

A model for the divergent transcription of the *bioA* operon in *Escherichia coli* K-12 (Fig. 1) was proposed by Guha et al. (5) on the basis of hybridization of pulse-labeled *bio*-specific messenger ribonucleic acid with deoxyribonucleic acid from λ*biot124* phages that had deletions into the *bioA* operon. In the proposed model, the message for 7,8-diaminopelargonic acid aminotransferase, coded for by cistron *A*, is transcribed leftward on the *l* strand while the message for the remaining cistrons *B*, *F*, *C* and *D* is transcribed to the right on the *r* strand. Support for this model also came from two independent lines of investigation. Krell et al. (10), in a study of biotin escape synthesis in lambda lysogens with deletions in the phage b_2 region, observed that constitutive synthesis of the product of the *bioA* cistron was controlled by phage replication while the enzyme product of the *bioD* cistron, dethiobiotin synthetase, was under transcriptional control. In an *E. coli* mutant in which the *gal* and *bio* operons are fused as the result of a deletion (from within the *galE* cistron, through the λ attachment site, and into the *bioA* cistron), Ketner and Campbell (9) showed that the remaining *gal* cistrons come under biotin control. The synthesis of the galactokinase enzyme, encoded on the *l* strand, and the dethiobiotin synthetase enzyme, encoded on

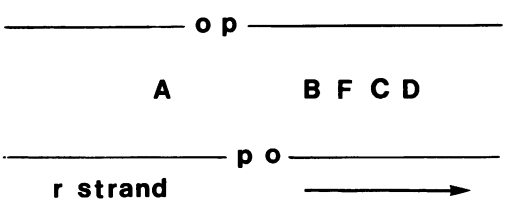

FIG. 1. *Proposed model for the divergent transcription of the bioA operon at 17 min on the E. coli chromosomal map. The capital letters represent cistrons coding for the following enzymes: 7,8-diaminopelargonic acid aminotransferase (A); biotin synthetase (B); 7-keto-8-aminopelargonic acid synthetase (F); step prior to 7-keto-8-aminopelargonic acid synthetase (C); and dethiobiotin synthetase (D).*

the *r* strand, was repressed by biotin.

A promoter/operator complex for each strand was favored in the original model for divergent transcription, and evidence to support this suggestion was provided by Vrancic and Guha (16) when they found that the two biotin analogues, α-dehydrobiotin and homobiotin, completely inhibited transcription from the *l* strand with little or partial inhibition from the *r* strand.

α-Dehydrobiotin was isolated from culture filtrates of *Streptomyces lydicus* by Hanka et al. (6) and shown to function as a biotin antimetabolite. The antibiotic inhibited the growth of *E. coli* in a synthetic medium, and this inhibition could be overcome by biotin. The antibiotic, which appears to be a degradation product of biotin, was especially effective as a growth inhibitor of mycobacteria (7, 8). Eisenberg (1) had previously reported the isolation of mutants which are resistant to α-dehydrobiotin. These have been recently characterized as either regulatory or permeability mutants by Eisenberg et al. (3a). In addition, α-dehydrobiotin was observed to inhibit the formation of two of the biotin biosynthetic enzymes, suggesting that the analogue may function in a manner similar to biotin in repressing the *bioA* operon.

This study provides the details to support the above suggestion as to the mode of action of α-dehydrobiotin, and it will also be shown that the two enzymes encoded on different strands are coordinately repressed by biotin and α-dehydrobiotin. The extreme differential inhibition of enzyme synthesis that would be predicted from the results of Vrancic and Guha (16), where reportedly no transcription occurs from the *l* strand, is not borne out by this study as significant synthesis of both enzymes takes place under our condition of repression with α-dehydrobiotin.

MATERIALS AND METHODS

Bacterial strains. Strain *bioB105*, a single-site mutant of *E. coli* K-12, strain Y10-1, was the test organism for most of these studies. This mutation, which blocks the synthesis of biotin from dethiobiotin, lies on the *r* strand most proximal to the operator region (13, 14). Mutant *bioC315* (strain HfrH), whose lesion is also on the *r* strand but more distal to the operator region, was used for comparison as was the biotin prototroph, strain W3350, kindly furnished by A. Guha.

Culture media. For the biotin mutants, the medium of Vogel-Bonner (15) was used after supplementation with a final concentration of 0.5% glucose, 0.01% L-leucine, 0.005% thiamine hydrochloride, and 0.25% vitamin-free casein hydrolysate (Nutritional Biochemical Co.). For strain W3350 the minimal medium recommended by Guha et al. (5) was used.

Chemicals. The preparation of 7-keto-8-aminopelargonic acid and 7,8-diaminopelargonic acid have been previously described (3). *S*-adenosyl-L-methionine was prepared by perchloric acid extraction of yeast and purified by column chromatography (4). Adenosine triphosphate and pyridoxal phosphate were products of Sigma Chemical Co. The α-dehydrobiotin and α-methylbiotin were gifts from L. J. Hanka of Upjohn and Co., Kalamazoo, Mich. The α-dehydrobiotin was further purified on a Dowex-1-formate column with 0.012 M formic acid as the eluent. The small amounts of biotin, dethiobiotin, and 7,8-diaminopelargonic acid which were present in the crystalline preparation could readily be separated and removed by the above procedure. Homobiotin was a generous gift from W. E. Scott of Hoffman-LaRoche Lab., Nutley, N.J. All other chemicals were reagent grade.

Repression. An overnight culture grown either in tryptone broth or minimal medium with added biotin was used as a source of inoculum for fresh minimal medium containing 5 ng of biotin/ml which represses the biotin operon almost completely. The culture was grown at 37 C with rapid shaking (200 rpm) to an absorbance at 650 nm (A_{650}) of 0.5 (6×10^8 cells/ml). The cells were removed by centrifugation in the cold, washed twice with 100 ml of cold medium, and distributed among a number of flasks, each containing 40 ml of minimal medium and a different concentration of biotin or α-dehydrobiotin. The flasks were incubated for 2 h at 37 C, and the contents of each flask were poured into a centrifuge bottle (250 ml) containing 20 ml of frozen minimal medium with 100 μg of chloramphenicol/ml. The bottles were shaken until the medium thawed, and the cells were removed by centrifugation. After washing with cold 0.85 saline, the cells were suspended in 2 ml of 0.05 M phosphate buffer, pH 7.0, containing 0.003 M 2-mercaptoethanol and were disrupted by sonic oscillation (Bronson model W140D) by using 30-s bursts with 30-s cooling periods for a total sonic treatment time of 2.5 min. The suspension was centrifuged at 30,000 rpm (Beckman, type 40 rotor) for 20 min, and the supernatant fluid was dialyzed against the same buffer containing 0.005 M pyridoxal phosphate.

The kinetics of enzyme synthesis in the presence of the biotin analogues, starting with a fully repressed biotin operon, was determined on cells grown in the manner described above. The washed cells were distributed between two flasks each containing 300 ml of minimal medium. Either α-dehydrobiotin, homobiotin, or α-methylbiotin was added to one flask, and no additions were made to the second flask, which served as a control. Previous studies in this laboratory showed that in the absence of biotin, maximum derepression of the biotin-synthesizing enzymes occurred in about 2 h in a biotin auxotroph. Samples of 40 ml were removed at 20-min intervals from each flask over the 2-h incubation period at 37 C and added to the frozen chloramphenicol minimal medium. The

crude extracts were prepared in the manner described above.

Assays. The crude extracts were assayed for the 7,8-diaminopelargonic acid aminotransferase and dethiobiotin synthetase activities by the procedures described by Eisenberg and Stoner (4) and Eisenberg and Krell (2), respectively.

RESULTS

Preliminary studies with α-dehydrobiotin indicated that the biotin analogue could repress the synthesis of the dethiobiotin synthetase enzyme. When the assay for the aminotransferase enzyme became available, it was possible to test the proposed model for divergent transcription by determining if the enzymes encoded on each strand are coordinately repressed or not. The effectiveness of the analogue was therefore studied as a function of concentration starting with a fully repressed operon to decrease the background levels of the enzymes. In Fig. 2 are shown the results of this study, and in the insert are the results obtained from a similar study with biotin for comparison. The synthesis of both the aminotransferase enzyme (encoded on the *l* strand) and the dethiobiotin synthetase enzyme (encoded on the *r* strand) was repressed with increasing concentrations of biotin. Repression of the synthesis of both enzymes was also observed with increasing concentrations of α-dehydrobiotin, reaching a maximum between 60 to 80 ng/ml. While repression of enzyme synthesis was essentially complete with 5 ng of biotin/ml, only 73 and 81% repression was attained with the biotin analogue for the aminotransferase and dethiobiotin synthetase enzymes, respectively.

Some indication of the kinetics of this limited repression was obtained by comparing the activity of each enzyme in the presence and absence of α-dehydrobiotin as a function of time over the 2-h period (Fig. 3). After the removal of biotin and in the absence of α-dehydrobiotin, there was an initial lag period of about 20 min in the synthesis of both enzymes, and then the specific activity of both enzymes increased linearly for about 40 to 60 min before falling off. When α-dehydrobiotin was present in the medium, a similar initial lag period was observed, and this was also followed by a linear increase in the rate of enzyme synthesis but at a much reduced level. After 60 to 80 min, the rate fell nearly to zero and remained as such. The final specific activities at the end of 2 h in the presence of α-dehydrobiotin were similar to those obtained in the previous experiment.

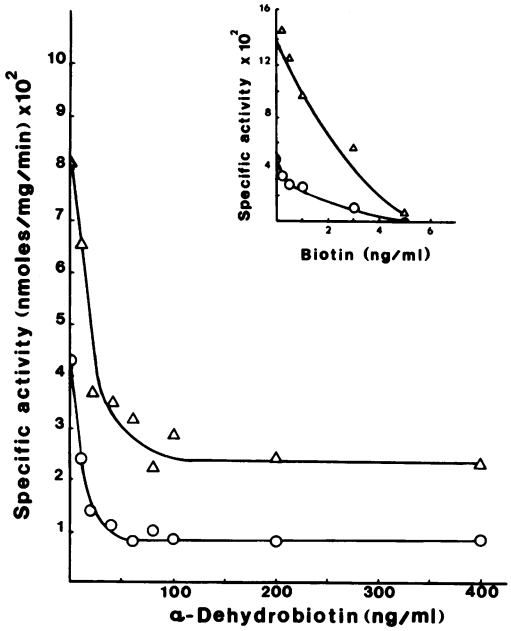

FIG. 2. *The effect of various concentrations of α-dehydrobiotin on two biotin-synthesizing enzymes. Cells grown to mid-log phase under conditions of biotin repression were washed, distributed in minimal medium with the indicated concentrations of α-dehydrobiotin and biotin, and incubated at 37 C for 2 h. The crude extract preparations obtained from each sample was assayed for aminotransferase activity (Δ) and dethiobiotin synthetase activity (O). Details are given in Materials and Methods.*

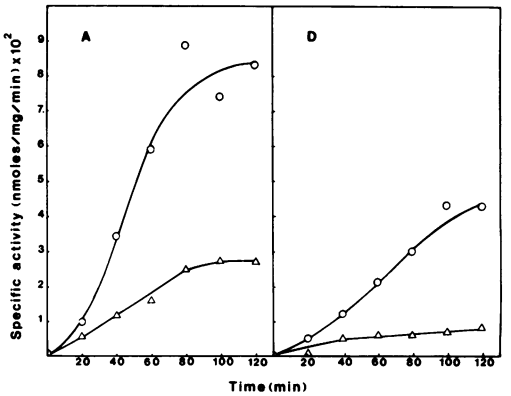

FIG. 3. *The kinetics of enzyme synthesis in the presence of α-dehydrobiotin in strain bioB105. Cells grown to mid-log phase under conditions of biotin repression were distributed in minimal medium with no additions (O) and with α-dehydrobiotin (0.2 μg/ml) (Δ), and incubated at 37 C. The crude extracts, prepared from samples removed at specified time intervals, were assayed for aminotranferase activity (A) and dethiobiotin synthetase activity (D). Details are given in Materials and Methods.*

A replot of the above data to give the differential rate of enzyme synthesis, i.e., the proportion of enzyme synthesized to the total protein synthesized, is shown in Fig. 4. Relative to the control, the rate of synthesis of both enzymes was reduced about 80% in the presence of α-dehydrobiotin over the 2-h incubation period during which time the cells had undergone 1.5 divisions. The clustering of points in the differential plot at the later time intervals would suggest that the synthesis of the enzymes had essentially ceased in the biotin auxotroph. The absence of biotin in the medium would have limited the growth of the auxotroph. This general pattern of repression has been observed repeatedly, with the differential rate of enzyme synthesis for both enzymes varying from 20 to 50% of the control value and completely independent of the α-dehydrobiotin concentration over the range of 0.1 to 2.0 μg/ml. In no instance has the addition of the biotin analogue resulted in the complete repression of the aminotransferase enzyme and the partial repression of the dethiobiotin synthetase enzyme as would be predicted from the hybridization studies of Vrancic and Guha (16).

The possibility that these results were peculiar to this mutation, which lies most proximal to the operator region, was ruled out when mutant *bioC315* was used as the test organism. The results shown in Fig. 5, with 1 μg of α-dehydrobiotin/ml, indicate a pattern similar to that observed with the *bioB* mutant except that the final levels of repression were not as high. The differential rate of enzyme synthesis for both enzymes was about 40% of the control values. It is also evident (Fig. 5) that a mixture of equal concentrations of biotin and α-dehydrobiotin (1 μg/ml) almost completely repressed the synthesis of both enzymes. Although not shown in the figure, biotin alone at the same concentration repressed the two enzymes to the same extent as the mixture. Similar results were also obtained with the *bioB* mutant. In the hybridization experiment of Vrancic and Guha (16), about a fourfold increase was observed in the *bio* mRNA transcribed from *l* strand.

When the kinetic studies were carried out with strain W3350, which was used in the hybridization experiments, the results shown in Fig. 6 were obtained. In the absence of any additions, the specific activity of both enzymes increased rapidly for about 60 min and then decreased. This would be expected in a biotin prototroph which is capable of synthesizing sufficient biotin to repress the biotin operon. In the presence of α-dehydrobiotin, the specific activity also increased initially and then fell to a constant value that was higher than the control

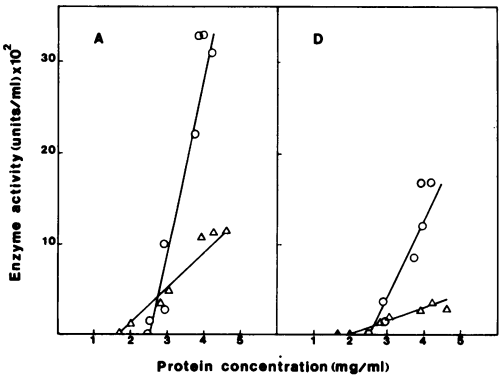

FIG. 4. *Differential rate of enzyme synthesis. A replot of data in Fig. 3.*

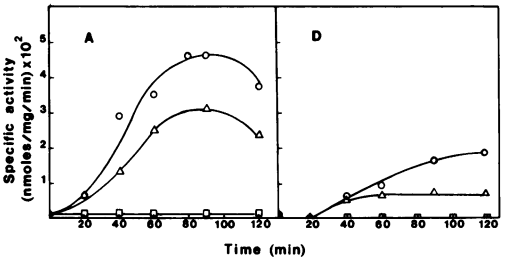

FIG. 5. *The kinetics of enzyme synthesis in the presence of α-dehydrobiotin in strain bioC315. Details as in Fig. 3. Symbols:* (○) *no addition;* (△) *α-dehydrobiotin (1 μg/ml); and* (□) *α-dehydrobiotin and biotin (1 μg/ml of each). Panels:* (*A*) *aminotransferase activity, and* (*D*) *dethiobiotin synthetase activity.*

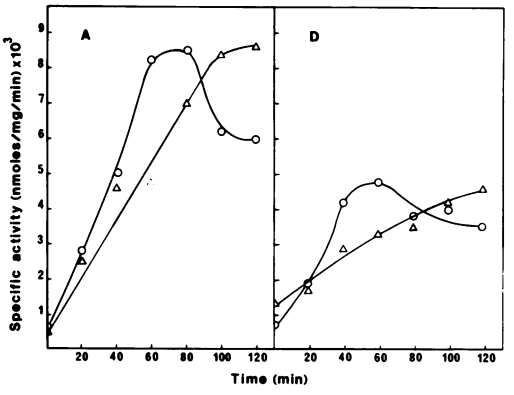

FIG. 6. *The kinetics of enzyme synthesis in the presence of α-dehydrobiotin in strain W 3350. Details as in Fig. 3. Symbols:* (○) *no additions, and* (△) *α-dehydrobiotin (1 μg/ml). Panels:* (*A*) *aminotransferase activity and* (*D*) *dethiobiotin synthetase activity.*

values at 2 h. The specific activity of both enzymes in this experiment was much lower than attained with the point mutants. The differential rate of synthesis, within the limits of error of the bioassay for these low enzyme activities, showed 60 and 80% repression of the aminotransferase and dethiobiotin enzymes, respectively. The combination of biotin and α-dehydrobiotin also resulted in almost complete repression of both enzymes as shown for the point mutants. These experiments with strain W3350 have also been repeated numerous times with considerable variability in the level of repression. However, the same overall pattern was always observed.

In the hybridization studies, 1 μg of homobiotin/ml was found to repress completely transcription from the *l* strand with little effect on the *r* strand. Homobiotin in the same concentration in our system was not an effective repressor of either enzyme (Fig. 7). The maximum level of repression after 2 h for both enzymes was markedly less than observed with α-dehydrobiotin. The differential rate of synthesis was decreased only 30% for the aminotransferase enzyme and about 40% for the dethiobiotin synthetase enzyme. α-Methylbiotin, a natural biotin analogue, was an even poorer repressor, as 100 μg/ml gave only about 38% repression of both enzymes after 2 h.

The derepression observed with α-dehydrobiotin in the first 60 min was initially thought to be the result of either a low permeability of the cells to the analogue or of a low rate of exchange of the analogue for biotin in a repressor/corepressor complex. Those two possibilities were explored by preincubating the cells in the presence of α-dehydrobiotin and an inhibitor of protein synthesis. The washed, biotin-repressed cells were suspended in fresh medium containing 0.2 μg of α-dehydrobiotin and 100 μg of chloramphenicol/ml and incubated for 30 min at 37 C. The cells were harvested by centrifugation, washed twice with fresh medium, resuspended in fresh medium containing 0.2 μg of α-dehydrobiotin/ml, and samples were taken at the usual time intervals. The control was preincubated in chloramphenicol alone. The overall pattern of residual repression (Fig. 8) is essentially the same as that observed in Fig. 3 without the preincubation period. In addition, the enzyme levels at zero time have not changed significantly from the untreated control.

DISCUSSION

We had previously reported the coordinate repression of the 7-keto-8-aminopelargonic acid synthetase and the dethiobiotin synthetase enzymes by biotin (12). Both of these enzymes are encoded for by cistrons on the *r* strand of the biotin operon. This study indicates a marked reduction in the specific activities of the two enzymes, the aminotransferase and dethiobiotin synthetase, with increasing concentrations of both biotin and α-dehydrobiotin. The former enzyme is encoded on the *l* strand and the latter enzyme is encoded on the *r* strand. Whereas essentially complete repression of the two enzymes is obtained with biotin, a maximum value of only 73 to 81% repression is attained with α-dehydrobiotin under the same conditions. Within the limits of error of the bioassay with crude extracts, the ratio of the two enzymes is essentially constant over the concen-

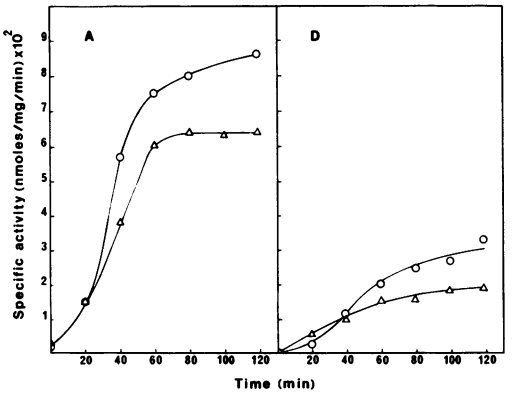

FIG. 7. *Kinetics of enzyme synthesis in the presence of homobiotin in strain bioB105. Details as in Fig. 3. Symbols:* (○) *no additions, and* (△) *homobiotin (1 μg/ml). Panels: (A) aminotransferase activity, and (D) dethiobiotin synthetase activity.*

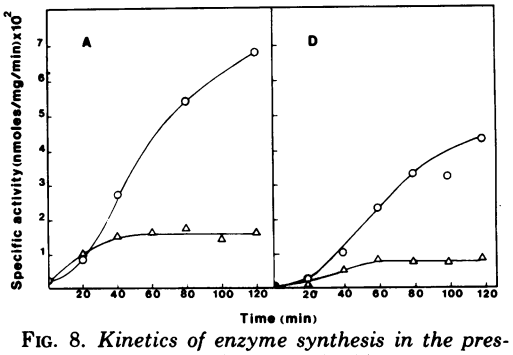

FIG. 8. *Kinetics of enzyme synthesis in the presence of α-dehydrobiotin in strain bioB105 after a 30-min preincubation period in α-dehydrobiotin and chloramphenicol. Details as given in the text and Fig. 3. Symbols:* (○) *no additions, and* (△) *α-dehydrobiotin (1 μg/ml). Panels: (A) aminotransferase activity, and (D) dethiobiotin synthetase activity.*

tration range used for both biotin and α-dehydrobiotin. The two enzymes would appear to be coordinately repressed by both biotin and α-dehydrobiotin. The other biotin analogues, homobiotin and α-methylbiotin, are not as effective as α-dehydrobiotin in repressing the biotin operon in the concentrations used.

Although no evidence is presently available concerning the role of biotin as the co-repressor of the biotin operon, the simplest explanation for the above results would be that α-dehydrobiotin must simulate the action of biotin in the repression of the biotin operon. The incomplete repression of the two enzymes would indicate that the co-repressor/repressor/operator complex formed in the presence of α-dehydrobiotin is not as efficient as that formed in the presence of biotin. The lower efficiency may be a reflection of the poorer binding of the α-dehydrobiotin to the repressor or of the co-repressor/repressor complex to the operator region.

In support of this postulation is the evidence obtained from the kinetic experiments. Starting with an essentially fully repressed operon, one observes a linear rate of derepression of the two enzymes encoded on different strands of the biotin operon after an initial lag period. The rate for both enzymes, in the presence of the analogue, is only 20% of the control, again pointing to the coordinate relationship between the enzymes encoded on the two strands. This residual derepression cannot be accounted for on the basis of the low permeability of the cells to the biotin analogue or to the slow dissociation of the biotin repressor/operator complex as the same pattern of derepression is observed after preincubation of the cells for 30 min with both α-dehydrobiotin and chloramphenicol. Unfortunately studies are not presently available concerning the rate of α-dehydrobiotin uptake by *E. coli* cells. However, it has been previously shown that the biotin analogue inhibits biotin uptake and presumably is transported by the same carrier protein (11). The biotin uptake in strain Y10-1 of *E. coli* reaches a steady-state level within 2 min. A 30-min preincubation with both the biotin analogue and chloramphenicol would have been ample time for sufficient α-dehydrobiotin to accumulate within the cells to repress the operon and alter the rate of the residual derepression. However, this was not the case. Similarly, this time interval is longer than the half time (15 to 20 min) required for the dissociation of the *lac* repressor from its operator (12). The preincubation data, instead, would suggest that the formation of the repression complex is rapid and what is being observed is a low rate of enzyme formation due to the decreased transcription from both strands as the result of the incomplete repression of the biotin operon. The requirement for de novo protein synthesis to maintain the observed low rate of enzyme synthesis is also verified by the chloramphenicol experiment as no enzyme synthesis occurred in the 30-min preincubation period over that of the control at zero time.

In none of the above studies has it ever been observed that transcription from the *l* strand is completely repressed by either α-dehydrobiotin or homobiotin as in the hybridization studies of Vrancic and Guha (16). The extreme differential repression of transcription by the biotin analogues would have been readily observed at the enzyme level, starting with a fully repressed operon to minimize the enzyme background. Similarly, the marked stimulation of transcription from the *l* strand by the addition of both biotin and α-dehydrobiotin as observed in the hybridization experiments would not easily have gone undetected by the sensitive bioassay procedure utilized. Instead, the same extensive enzyme repression was observed for the mixture of the two compounds as with biotin alone.

It is difficult to account for the marked discrepancy between our data obtained by assaying the translational product of *bio* messenger ribonucleic acid and the hybridization studies which presumably measured *bio* messenger ribonucleic acid. It cannot be attributed to strain differences as the pattern is the same in two biotin auxotrophs and the biotin prototroph. One obvious difference between the two investigations is the degree of repression of the biotin operon prior to the addition of the biotin analogues. In this system the biotin operon is essentially fully repressed whereas in the hybridization studies it is partially repressed. Whether the level of the biotin enzymes in the biosynthetic pathway has any influence on the nature of the repression would require a more extensive study. While our evidence would not rule out the possibility of two promoter/operator complexes, it would certainly require that the two complexes respond to biotin and the biotin analogues to the same degree in the repression of the biotin operon. The data, however, do not exclude other possible models for the regulation of the biotin operon such as one operator and two promoters.

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