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Properties of α-Dehydrobiotin-Resistant Mutants of *Escherichia coli* K-12

MAX A. EISENBERG, BRIAN MEE, OM PRAKASH, AND MARCIA R. EISENBERG

Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York 10032

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We have isolated four classes of mutants resistant to α-dehydrobiotin, a biotin analogue. One mutant group, referred to as *bioR*, shows high excretion levels of biotin vitamers, derepressed levels of the biotin biosynthetic enzymes, and resistance to repression by biotin. This mutation has been mapped between *argC* and *bfe* at min 79. A second class of mutants, with lesions in the *bioA* operon at min 17.5, shows derepressed levels of the dethiobiotin synthetase enzyme and has been tentatively designated as *bioO* mutants. The other two mutant groups show alterations in permeability: biotin uptake is markedly reduced in one, whereas in the other proline uptake is also affected. The former mutation lies near *metE* at min 75 and has been designated as *bioP*. The permeability mutants in the second group also show poor growth on minimal media, suggesting a generalized permeability effect. This mutation, designated as *P*, has not been mapped.

We had previously reported the isolation of *Escherichia coli* mutants resistant to the biotin analogue α-dehydrobiotin (2). One group of mutants was characterized by the high excretion levels of biotin and the intermediates formed during biotin biosynthesis, as well as by the derepressed levels of two of the biotin-synthesizing enzymes. In addition, these two enzymes were not repressed by concentrations of biotin that normally cause essentially complete repression in the parental strain. This would suggest that in these mutants the regulation of the synthesis of these enzymes was altered. A mutant with similar properties was isolated by Pai (9) on the basis of its higher biotin excretion level and in subsequent studies was shown to be a regulatory mutant rather than a permeability mutant (10). The mutant from both laboratories was referred to as *bioR*. Another mutant with similar properties was isolated by Campbell et al. (1) but had the additional properties of requiring high concentrations of biotin for growth even though the synthesis of the biotin enzymes appeared to be derepressed. In addition, the mutant also showed a decreased ability to accumulate biotin from the medium. This mutant was referred to as *bir*, for biotin retention.

On examining the α-dehydrobiotin mutants from a number of strains of *E. coli*, we discovered among them two classes of permeability mutants, one of which appeared to affect only the uptake of biotin. This report describes in greater detail the properties of the various mutant groups resistant to the action of α-dehydrobiotin and their mapping by P1 transduction.

MATERIALS AND METHODS

Media. Cultures grown overnight in Vogel-Bonner minimal medium (14) with 0.25% vitamin-free casein hydrolysate (Nutritional Biochemicals Co.) were used to determine both the excretion levels of biotin and biotin vitamers and the enzymatic activity of two of the biotin-synthesizing enzymes. In the absence of casein hydrolysate, the required amino acid supplementation of 50 μg/ml was used. Luria-magnesium broth, Luria-calcium agar, and Luria top agar were used for the preparation of P1 lysates and for the transduction experiments (12). The transductants were isolated on minimal medium that contained the required nutrient supplementation or 7 μg of the inhibitor α-dehydrobiotin per ml. Nutrient agar containing 100 or 250 μg of rifampin per ml was used to isolate rifampin-resistant strains and to score subsequently for the *rif* marker.

Bacterial strains. The bacterial strains used in this study are listed in Table 1.

Reagents. α-Dehydrobiotin, obtained from L. J. Hanka, was chromatographed on Dowex-1-formate column to remove traces of biotin and biotin vitamers. 7-Keto-8-aminopelargonic acid and 7,8-diaminopelargonic acid were prepared as described previously (3). *S*-adenosyl-L-methionine was extracted from yeast and purified by column chromatography (5). Rifampin, adenosine triphosphate, pyridoxal phosphate, and all amino acids were purchased from Sigma

TABLE 1. *Bacterial strains*

Designation	Genotype	Origin of source
PA309	F⁻*thr leu trp his arg thi*	M. C. Jones-Mortimer (7)
PA309-19	F⁻*thr leu trp his arg thi bioO*	α-DHB[a] mutant of PA309 (this work)
AB313	Hfr *leu thi str tsx*	K. B. Low
AB313-136	Hfr *leu thi str tsx bioR*	α-DHB[a] mutant of AB313 (this work)
Y10-1	F⁻*thr leu thi*	C. R. Fuerst
#2	F⁻*thr leu thi bioR*	α-DHB[a] mutant of Y10-1 (this work)
#2-1	F⁻*thr leu thi bioR bfe*	*bfe*-Resistant strain of #2 (this work)
18-3	F⁻*thr leu thi bioP*	α-DHB[a] mutant of Y10-1 (this work)
21-4	F⁻*thr leu thi P*	α-DHB[a] mutant of Y10-1 (this work)
T50-1	F⁻*thr leu thi (bio)* Δ	C. R. Fuerst
AB1157	F⁻*thr leu pro his arg thi*	A. Campbell (1)
AB1157-1	F⁻*thr leu pro his arg thi rif*	Rifampin-resistant strain of AB1157 (this work)
AB2070	F⁻*pro trp his ilv met thi*	E. A. Adelberg (15)
C959	F⁺*leu pro thr arg his thi*	A. Campbell (1)
C965	F⁻*leu pro thr his thi bir*	A. Campbell (1)
RM36	*thy tonB*	R. Moyer
RM60	*bfe tonB*	R. Moyer

[a] Resistance to α-dehydrobiotin.

Chemical Co. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was purchased from the Aldrich Chemical Co. Tryptone broth, nutrient broth, and agar were products of the Difco Co. All other chemicals were reagent grade.

Mutagenesis. The cultures were grown in tryptone broth at 37 C with shaking until a Klett reading of 100 was attained with a no. 66 filter. The cells were harvested by centrifugation, washed once in phosphate-buffered saline, and resuspended in 5 ml of phosphate-buffered saline. Nitrosoguanidine was added to a final concentration of 100 μg/ml, and the cell suspension was incubated with shaking for 30 min at 37 C. Samples of 0.1 ml were then distributed in a number of tubes containing 2.0 ml of tryptone broth, and the tubes were incubated for 2 h at 37 C for expression. Samples from each tube were then spread on Vogel-Bonner minimal medium plates, and a crystal of α-dehydrobiotin was added. The plates were incubated for 24 to 48 h at 37 C, and colonies from within the zone of inhibition were selected. The colonies were purified by restreaking on minimal medium and placing a filter paper disk with 200 ng of α-dehydrobiotin into the center of the streaked area.

Lysates and transduction. P1CMclr 100, a temperature-sensitive phage carrying a chloramphenicol resistance marker, was used for the preparation of the lysates. The method of Rosner (12) for confluent lysis was followed, except that the plates were incubated at 41 C overnight instead of 6 to 7 h. Titers of 5×10^9 to 10^{10} were obtained. The transductions were also carried out by the procedure of Rosner (12). The rifampin resistance marker (*rif*) was selected by the procedure of R. Moyer of this department, as follows. A culture was grown to early log phase (3×10^8 cells per ml), washed once with 0.85% saline, recentrifuged, and resuspended in 0.10 the volume of saline. Ethylenediaminetetraacetate was added to a final concentration of 2×10^{-4} M, and the cells were slowly shaken for 2 min. The suspension was diluted 10-fold with saline, and samples were spread on a nutrient agar plate containing 250 μg of rifampin per ml. Colonies were selected after 24 to 36 h of incubation at 37 C and purified on the same medium. The marker *bfe*, which confers resistance to phage BF23, was introduced by selecting colonies that were resistant to colicins Ib and E3. The *bfe* locus occurs at 79 min on the chromosome. Since phage BF23 and colicin E3 absorb to a common cell receptor, resistance to colicin E3 confers concomitant resistance to phage BF23.

Assays. An assay for α-dehydrobiotin sensitivity was standardized to give reproducible and quantitative results. Cells were grown overnight in minimal medium, washed once in 0.85% saline, and resuspended in saline. Sufficient cells were added to 2.5 ml of 0.7% minimal medium top agar to give a final concentration of 10^8 cells per ml. The overlay was poured and permitted to solidify. Concentrations of α-dehydrobiotin varying from 0.01 to 7.0 μg were added to 6-mm filter paper disks which were then placed onto the agar surface. After overnight incubation at 37 C, the zones of inhibition could be measured. True biotin and "total" biotin were determined on the filtrates from the cultures grown overnight. True biotin was assayed with strain *bioB*-105, which cannot synthesize biotin from dethiobiotin and hence will only grow in the presence of added biotin. Total biotin includes, in addition to biotin, all of the intermediates in the pathway of biotin biosynthesis (7-keto-8-aminopelangonic acid, 7,8-diaminopelangonic acid, and dethiobiotin) as well as biotin-*d*-sulfoxide and biocytin. Strain *bioC*-315, which is blocked early in the biosynthetic pathway and responds to the above biotin vitamers, was used as the assay organism. (The term biotin vitamer refers to those compounds capable of replacing the biotin requirement of an organism.) The 7,8-diaminopelangonic acid aminotransferase and the dethiobiotin synthetase enzyme activities were determined, by procedures described

previously (4,5), on the dialyzed crude extracts prepared from stationary-phase cultures or cultures grown to mid-log phase. For the repression studies, the extracts were prepared from cells grown to mid-log phase in various concentrations of biotin.

Biotin uptake studies. Cells were grown from a 1% inoculum to mid-log phase (120 Klett units at 66 nm). The cells were washed once with distilled water warmed to 37 C and then suspended in 0.05 M phosphate buffer (pH 6.6). The uptake medium contained (per milliliter): potassium phosphate (pH 6.6); 50 μmol; glucose, 20 μmol; magnesium sulfate, 1 μmol; chloramphenicol, 100 μg; and cells (dry weight), 1.0 to 1.2 mg. The reaction mixture was equilibrated for 10 min at 37 C, and 75 ng of [^{14}C]biotin (20 mCi/mmol) was added to start the reaction. Portions of 2 ml each were withdrawn at various time intervals and rapidly added to 4 ml of cold 0.9% saline previously placed on a membrane filter (0.65-μm pore size, 47-mm diameter; Millipore Corp.). Filtration was completed within 10 to 12 s, and the filters were washed once with 4 ml of cold saline. The filters were placed in counting vials and dried at 80 C for 1 h, and 15 ml of toluene containing 6 g of 2,5-di-phenyloxazole and 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per liter was added. The radioactivity on the filters was counted at 4C in an Ansitron liquid scintillation counter.

RESULTS

A total of 35 α-dehydrobiotin-resistant mutants was isolated from three different strains of *E. coli*. When the filtrates of overnight cultures were tested for their excretion of "total" and true biotin, two main groups could be readily distinguished: the very high excretors and those with the parental level. A small third group was also detected with excretion levels somewhat higher than the parental strains. Assays for the aminotransferase and dethiobiotin synthetase enzymes indicated very large increases in activities in the group showing the highest vitamer excretion level. The increase in enzymatic activity varied two to fivefold for the aminotransferase enzyme and five- to tenfold for the dethiobiotin synthetase enzyme. Additional parameters such as α-dehydrobiotin sensitivity, biotin repression, permeability, as well as mapping, were required to distinguish the low excretors. At present there are four α-dehydrobiotin-resistant mutant groups whose properties are described below and summarized in Table 2 along with data for the parental strains.

Group I (bioR). Group I (*bioR*) mutants were the highest vitamer excretors and also showed derepressed levels of the aminotransferase and dethiobiotin synthetase enzymes. In addition, the synthesis of these enzymes was highly resistant to repression by biotin (Table 3). No apparent repression was observed at 25,000 times the concentration of biotin normally required to repress completely the biotin operon in the parental strain. Similar results have been reported for the *bioR* mutant isolated by Pai (9). For comparison, we have also included one of Campbell's *bir* mutants with a *bir*$^+$ control. At 5 μM biotin, the *bir* mutant was 70% repressed whereas the *bioR* mutant was completely resistant. The *bir* mutant was also less

TABLE 2. *Summary of the constitutive properties of mutant groups resistant to α-dehydrobiotin*

Strain	Biotin excretion (ng/ml)		Enzyme activity (nmol/mg of protein per 10 min)		Mapping position (min)	Relative biotin uptake (%)
	Total	True	Amino-transferase	Dethiobiotin synthetase		
Parental						
Y10-1	4	0	0.32	0.10		100
AB313	4	0	0.31	0.11		
PA309	4	0	0.34	0.22		100
Group I (*bioR*)						
Y10-1, #2	79	40	1.19	0.52	79	
Y10-1, 6-1	110	88	0.87	0.55	79	
AB313-136	73	74	0.92	0.86		
Group II (*bioO*)						
PA309-19	4	0	0.21	0.37	17	100
Group III (*bioP*)						
Y10-1, 18-3	13	5	0.32	0.14	75	10
Group IV (*P*)						
Y10-1, 21-4	6	0	0.33	0.21	?	22

TABLE 3. *Biotin repression of the aminotransferase and dethiobiotin synthetase enzymes in bioR and bir mutants of E coli*

Biotin (μM)	Aminotransferase sp act[a]				Dethiobiotin synthetase sp act[b]			
	AB313 (*bioR*⁺)	AB313-136 (*bioR*⁻)	C959 (*bir*⁺)	C965 (*bir*⁻)	AB313 (*bioR*⁺)	AB313-136 (*bioR*⁻)	C959 (*bir*⁺)	C965 (*bir*⁻)
0	0.32	1.17	0.35	0.61	0.34	1.59	0.71	0.93
0.02	0.05	1.43	0.05	0.45	0.14	2.11	0.11	0.99
5	0	1.44	0	0.22	0	1.74	0	0.27
500	0	1.29	0	0.14	0	1.89	0	0.24

[a] Specific activity: nanomoles of 7,8-diaminopelargonic acid/milligram of protein per 10 min.
[b] Specific activity: nanomoles of dethiobiotin/milligram of protein per 10 min.

resistant to α-dehydrobiotin inhibition than the *bioR* mutant; inhibition was observed at 1 μg/disk, whereas the *bioR* mutant was resistant up to 7 μg/disk. The *bioR* mutant grew well from a small inoculum, 5×10^5 cells per ml and did not require added biotin for optimum growth. The rate of [^{14}C]biotin uptake in the *bioR* mutant as compared to the parent strain Y10-1 was decreased by approximately 50% (Fig. 1), but the total biotin accumulation was reduced only 15 to 20%. Pai (10), however, found about 90% inhibition of both the uptake rate and the total biotin accumulation with his *bioR* mutant.

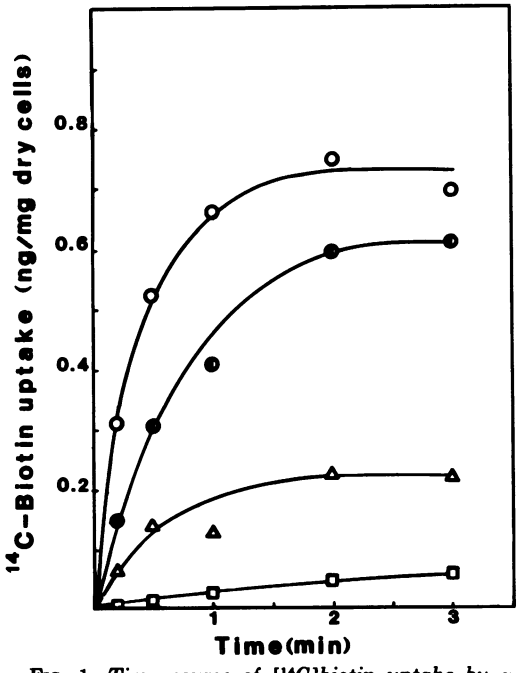

FIG. 1. *Time course of [^{14}C]biotin uptake by α-dehydrobiotin-resistant mutants of E. coli: Y10-1 (wild type), ○; #2 (bioR), ●; 18-3 (bioP), △; 21-4 (P), □. Cells were suspended in 0.05 M phosphate buffer (pH 6.6) with 75 ng of [^{14}C]biotin per ml of uptake medium.*

Our initial attempts to map the *bioR* mutation in strain AB313-136 by P1 transduction were unsuccessful since this strain proved to be a λ lysogen. Preliminary mapping by mating experiments place this mutation tentatively at min 75 on the *E. coli* chromosome map (2). With strain 2, we were able to show about 50% co-transduction with *argC* at min 79. Pai (9) had previously shown a similar co-transduction with *argC* with his *bioR* mutant. The *bir* mutation was mapped by Campbell et al. (1) between the rifampin and the thiamine markers at min 79.5. P1 transduction with *arg*, *rif*, and *bfe* markers placed the *bioR* mutation closer to the *bfe* locus. From the data in Table 4, it was

TABLE 4. *Mapping of the bioR locus*[a]

Selected phenotype	Unselected phenotype	No. of transductants
α-DHB[b]	Arg⁺ Bfe^R Rif^R	14
	Arg⁻ Bfe^R Rif^R	19
	Arg⁺ Bfe^S Rif^R	2
	Arg⁻ Bfe^S Rif^R	3
	Arg⁺ Bfe^R Rif^S	38
	Arg⁻ Bfe^S Rif^S	2
	Arg⁺ Bfe^S Rif^S	6
	Arg⁻ Bfe^R Rif^S	34

[a] The P1 lysate was prepared on Y10-1, #2-1 (Arg⁺ BioR⁻ Bfe^R Rif^S) and used to transduce AB-1157 (Arg⁻ BioR⁺ Bfe^S Rif^R) to α-dehydrobiotin resistance. The α-dehydrobiotin-resistant transductants selected in this way were seeded onto Vogel-Bonner casein medium that contained 7 μg of α-dehydrobiotin per ml and incubated at 41 C. The colonies were scored for their arginine phenotype by replica plating on minimal medium with 50 μg of arginine per ml and for the Rif^R phenotype on nutrient agar with 250 μg of rifampin per ml. The Bfe phenotype was scored by cross-streaking each transductant through a BF23 streak made from a lysate containing 10^{10} phage per ml using a 4-mm loop.
[b] α-DHB, Resistance to α-dehydrobiotin.

determined that *bioR* co-transduced with *bfe* 89% and with *argC* and *rif*$^+$ 51 and 68%, respectively. Since the dominant *rif* marker was shown by Kirschbaum and Konrad (8) to be co-transduced 88% with the *bfe* locus, the gene order for the various markers must be as shown in Fig. 2.

Group II (bioO). At present group II (*bioO*) contains only one member that was selected for further study because of the unusual differential pattern of expression of the two biotin enzymes. The constitutive activity of the dethiobiotin synthetase enzyme was found to be consistently higher than the parental strain (65%), whereas the animotransferase activity (Table 2) was consistently lower (40%). In the *bioA* operon, which shows divergent transcription, the cistron coding for the aminotransferase enzyme is transcribed leftward on the *l* strand, whereas the cistron for the dethiobiotin synthetase activity is transcribed to the right on the *r* strand. This mutation appears to have altered the regulation of the *bioA* operon, so that transcription from the *l* strand is repressed whereas that from the *r* strand is derepressed.

When the mutant and the parental strain were grown in the presence of 5 ng of biotin per ml, the two enzymes were essentially completely repressed in parental strain AB309, but only 60% repressed in the mutant (Table 5). A twofold increase in the concentration of biotin in the medium produced little change in the degree of repression of the mutant. Preliminary mating experiments placed this mutation within the *bioA* operon at min 17.5 on the chromosome map of *E. coli* (13). P1 transduction of a biotin deletion mutant, T50-1 (deleted from the λ attachment site through *uvrB* locus), with lysates prepared from the group II mutant yielded 14 transductants that were prototrophic for biotin. Thirteen of these transductants were resistant to 500 ng of α-dehydrobiotin per disk, whereas the parental strain was sensitive even to 10 ng/disk. Permeability studies did not reveal any impairment in the uptake of radioactive biotin from the medium.

Group III (bioP). The members of group III (*bioP*) showed somewhat higher levels of excretion of both total and true biotin than the parental strain Y10-1. The group III mutants are also resistant to 5 μg of α-dehydrobiotin per disk. Since the resistance to the biotin analogue did not manifest itself in any alteration of the control of the biotin biosynthetic enzymes (Table 2), an alteration in the permeability of the cell membrane to α-dehydrobiotin had to be considered. This biotin analogue had previously been shown to inhibit biotin uptake by strain Y10-1, presumably by competing with biotin for the transport protein (11). Therefore, the resistance to the biotin analogue could also manifest itself in the inhibition of biotin uptake by the mutant if α-dehydrobiotin were transported by the same mechanism. That this is indeed the case is shown in Fig. 1. The rate of biotin uptake and the final steady-state level were reduced over 90% compared to the parent strain. The residual biotin taken up appeared to be mostly in the bound form. Transduction with P1 phage indicated that this mutation did not co-transduce with *argC* or *rif* markers and, hence, occupied a locus different from *bioR*. However, it was co-transduced 96% with *metE* (Table 6) and 10% with *ilv*, suggesting the following order for the locus at min 75: *ilv–bioP–metE*.

Group IV (P). The members of group IV (*P*) have all the characteristics of the parental strain, except that they are resistant to 2 to 3 μg of α-dehydrobiotin per disk and show a slower growth rate in the casein minimal growth medium. Biotin uptake studies showed a decreased uptake that was less marked than in the group III mutants (Fig. 1). However, unlike the group III mutants, the members of this group showed a decrease in the rate of proline uptake as well as a marked decrease in the maximal internal concentrations reached in 2 min (Fig. 3). The α-dehydrobiotin resistance marker in this mutant was not co-transduced with any of the markers at min 79, 75, and 17.5. No further attempt was made to map this mutation, and

TABLE 5. *Biotin repression of the aminotransferase and dethiobiotin synthetase enzymes in strain AB309-19 (bioO)*[a]

Biotin (ng/ml)	7,8-Diaminopelargonic acid aminotransferase sp act		Dethiobiotin synthetase sp act	
	309 (bioO$^+$)	309-19 (*bioO*)	309 (*bioO*$^+$)	309-19 (*bioO*)
0	1.03	0.62	0.22	0.37
5	0.08	0.25	0	0.14
10	0.06	0.20	0	0.15

[a] See footnotes *a* and *b*, Table 3.

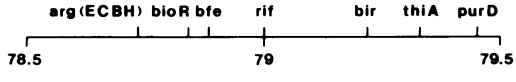

FIG. 2. *Genetic position of the bioR region on the E. coli chromosome. The order and relative distances of arg(ECBH), bfe, rif, bir, thiA, and purD are from Taylor and Trotter (10). The position of bioR is from the data presented in Table 4. bioR is 89% co-transducible with bfe.*

TABLE 6. *Mapping of the bioP locus*[a]

Selected phenotype	Unselected phenotype	No. of transductants
Ilv+	α-Dehydrobiotin resistant	2
	α-Dehydrobiotin sensitive	21
Met+	α-Dehydrobiotin resistant	22
	α-Dehydrobiotin sensitive	1

[a] The P1 lysate was prepared from strain 18-3 (Ilv+ Met+ BioP−) and used to transduce AB2070 (Ilv− Met− BioP+) to Ilv+ or Met+. The transductants were isolated on minimal medium containing all the required nutrilites except isoleucine and valine or methionine. The colonies were scored for α-dehydrobiotin resistance by inoculating each colony into a drop of the appropriate minimal medium and incubating it for 1.5 h at 37 C. A loopful was streaked on the same medium used to isolate the transductants and a filter paper disk containing 2 μg of α-dehydrobiotin was placed onto the center of the streak. After 24 h of incubation at 37 C, the streaks were examined for zones of inhibition.

for convenience we designated this gene as *P* for general permeability.

DISCUSSION

We have shown that selection of cells resistant to α-dehydrobiotin, a biotin analogue, yields four distinct classes of mutants with different physiological properties. Our group I mutants have all the characteristics expected of a mutation in a repressor gene. Their properties are identical to those described for a mutant isolated by Pai (9, 10), except that our *bioR* mutants do not show the marked inhibition of biotin uptake as observed in Pai's mutant. Pai (Abstr. Annu. Meet. Am. Soc. Microbiol., 1974, P276, p. 190) also recently reported the isolation of a *bioR* mutant resistant to α-dehydrobiotin.

We have tentatively classified the lesion in the group II mutant as an O^c because of the derepressed levels of the dethiobiotin synthetase enzyme and its location within the *bioA* operon itself. The partial repression of the aminotransferase enzyme is difficult to understand unless this mutant contains either a double mutation or a deletion. More extensive mapping may help clarify this point.

The group III and IV mutants that we have tentatively classified as representing transport mutants differ in their properties. In strain 21-4 (group IV), both biotin uptake and proline uptake are reduced, suggesting a generalized permeability defect. This postulation is supported by the fact that these mutants grow more slowly than the parental strain, indicating a possible impairment in the uptake of other essential metabolites as well. The exact nature of this mutation is still to be determined, but generalized permeability mutants have been reported in which the energy-coupling mechanism to transport has been disrupted (6). The biotin-specific mutation for which we suggest the gene marker *bioP* (group III) and the general permeability mutant (group IV) should prove useful in the study of the energy-coupling mechansim and biotin transport in vesicle preparations.

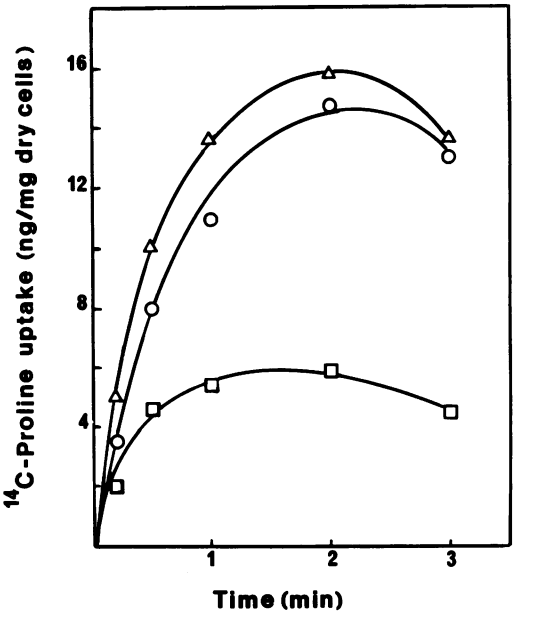

FIG. 3. *Time course of [^{14}C]proline uptake by α-dehydrobiotin-resistant mutants of E. coli: Y10-1 (wild type), O; 18-3 (bioP), Δ; and 21-4 (P), □. Procedure was as described in Fig. 1, except that 87 ng of [^{14}C]proline (100 mCi/mmol) was used in place of biotin.*

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