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# Regulation of Homocysteine Biosynthesis in *Salmonella typhimurium*[1]

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The regulation of the homocysteine branch of the methionine biosynthetic pathway in *Salmonella typhimurium* has been reexamined with the aid of a new assay for the first enzyme. The activity of this enzyme is subject to synergistic feedback inhibition by methionine plus *S*-adenosylmethionine. The synthesis of all three enzymes of the pathway is regulated by noncoordinate repression. The enzymes are derepressed in *metJ* and *metK* regulatory mutants, suggesting the existence of regulatory elements common to all three. Experiments with a methionine/vitamin $B_{12}$ auxotroph (*metE*) grown in a chemostat on methionine or vitamin $B_{12}$ suggested that the first enzyme is more sensitive to repression by methionine derived from exogenous than from endogenous sources. *metB* and *metC* mutants grown on methionine in the chemostat did not show hypersensitivity to repression by exogenous methionine. Therefore, it appears that the *metE* chemostat findings are peculiar to the phenotype of this mutant; such evidence suggests a possible role for a functional methyltetrahydrofolate-homocysteine transmethylase in regulating the synthesis of the first enzyme. Thus there appear to be regulatory elements which are common to the repression of all three enzymes, as well as some that are unique to the first enzyme. The nature of the corepressor is not known, but it may be a derivative of *S*-adenosylmethionine. *metJ* and *metK* mutants of *Salmonella* have a normal capacity for *S*-adenosylmethionine synthesis but may be blocked in synthesis or utilization of a corepressor derived from it.

Methionine is synthesized in *Salmonella typhimurium* by a highly branched pathway, the two terminal branches converging in the methylation of homocysteine by $N^5$-methyltetrahydrofolate ($CH_3FH_4$) (Fig. 1). This paper deals with studies on the regulation of the subpathway leading to homocysteine. Three enzymes catalyze the sequential reactions unique to this pathway: homoserine *O*-transsuccinylase, forming *O*-succinylhomoserine from succinyl coenzyme A (CoA) and homoserine (25); cystathionine $\gamma$-synthase, forming cystathionine from *O*-succinylhomoserine and cysteine (4, 5); and $\beta$-cystathionase, forming homocysteine from cystathionine (4). The unlinked structural genes for the enzymes are *metA*, *metB*, and *metC*, respectively. Previous studies, reviewed by Smith (32), have shown that there is feedback control of the first enzyme (20) and noncoordinate repression by methionine of synthesis of the second and third enzymes (18), the actual corepressor possibly being *S*-adenosylmethionine (SAM) or something derived from it (8). Because of the lack of an assay suitable for use in crude extracts of *Salmonella*, little is known about the repressibility of the first enzyme. Lawrence (17) recently reported experiments showing that the first enzyme is derepressed in methionine regulatory mutants known to have derepressed second and third enzymes (18). However, he assayed the enzyme by measuring the appearance of free CoA in the reaction mixture, a procedure which is subject to interference in crude extracts (*see below*).

The discovery that $N^5$-methyltetrahydrofolate polyglutamates ($CH_3FH_4G_n$) are allosteric activators of cystathionine $\gamma$-synthase in *Neurospora crassa* (29) prompted us to investigate the possibility of a regulatory role for folate derivatives in *Salmonella*. No regulatory function for folates was found. However, in the course of these studies we developed

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assays for the transsuccinylase which, in conjunction with the use of a chemostat, made it possible to show that the repression of this enzyme is not only noncoordinate with that of the second and third enzymes, but probably makes use of a different mechanism.

## MATERIALS AND METHODS

**Materials.** The preparation has been described of succinyl CoA (31) and *O*-succinylhomoserine (6). L-Cystathionine, L-homoserine, $^3$H-L-homoserine, SAM, L-cystine, L-homocystine, and lactate dehydrogenase (L-lactate:nicotinamide adenine dinucleotide oxidoreductase, EC 1.1.1.27) were obtained from Calbiochem. Vitamin $B_{12}$, pyridoxal phosphate, and reduced nicotinamide adenine dinucleotide (NADH) were from Sigma Chemical Co., and L-methionine was from Nutritional Biochemicals Corp. Materials for the SAM synthetase assay included reduced glutathione from Nutritional Biochemicals Corp., adenosine triphosphate (ATP) from P-L Laboratories, and $^{14}$C-ATP from Calatomic. Methyltetrahydrofolate monoglutamate ($CH_3FH_4G_1$), diglutamate ($CH_3FH_4G_2$), triglutamate ($CH_3FH_4G_3$), and methylcobalamin were the generous gifts of W. Sakami (folates) and T. C. Stadtman.

**Bacterial strains.** The following *Salmonella* strains were obtained from K. E. Sanderson of the University of Calgary, Canada: Lt-2 (wild type), *metA15*, *metB16*, *metB36*, *metC50*, *metE47*, *metF96*, and *metG419*. The phenotypes are shown in Fig. 1; *metE47* lacks the $B_{12}$-independent homocystine transmethylase (1), and *metG419* has a defect (increased $K_m$) in methionyl transfer ribonucleic acid (tRNA) synthetase (9). The following are either deletions or had zero reversion (33): *metA15*, *metB36*, *metC50*, and *metE47*. Other *Salmonella* strains were the generous gift of D. Smith of the University of Birmingham, England: *metJ713* and *metK721*, both methionine analogue-resistant mutants which overproduce methionine and have nonrepressible levels of cystathionine $\gamma$-synthase and $\beta$-cystathionase (18); and the double mutant *metE205*, *metH465*, which lacks both $B_{12}$-dependent and $B_{12}$-independent homocysteine transmethylases (2). R. C. Greene of Duke University generously provided the following *E. coli* strains: K-12, wild type; E31 (*metJ*), a regulatory mutant with nonrepressible levels of SAM synthetase, cystathionine $\gamma$-synthase, and $\beta$-cystathionase (12); and E40 (*metK*), a mutant deficient in SAM synthetase with nonrepressible levels of cystathionine $\gamma$-synthase and $\beta$-cystathionase (8).

**Bacterial cultures.** The minimal medium used in all experiments was Vogel and Bonner's medium E, which contains (in grams per liter): $K_2HPO_4$, 10; $NaNH_4HPO_4$, 3.5; citric acid·$1H_2O$, 2; $MgSO_4 \cdot 7H_2O$, 0.2; and glucose, 0.2. For cultivation of auxotrophs and studies of repression, various amino acids or vitamin $B_{12}$ were added in the concentrations indicated in the tables. For flask culture experiments, 50 ml of medium in a Klett side arm flask was first inoculated from a slant. After overnight growth on a rotary shaker at 37 C, the contents were used to inoculate 500 ml of the same medium in a Fernbach flask. The cells were incubated at 37 C on a rotary shaker, harvested by centrifugation at mid-log phase (approximately 170 Klett units), and then washed and extracted as described below. When enzyme levels were measured in stationary-phase cells (see Table 5), the cultures were incubated for 24 hr, i.e., about 20 hr after cessation of exponential growth.

The methods used for the chemostat cultures were modeled after those of Novick and Szilard (24), with the apparatus of Tabor and Tabor (35). The growth vessel was a 1-liter bottle with a side arm at the 700-ml level. The afternoon before beginning each experiment, a 700-ml Fernbach flask culture was grown to late log phase and then stored overnight at 2 C. The flask culture medium contained the limiting nutrient at the same concentration to be used in the chemostat. At the beginning of the experiment, the flask culture was used to inoculate the growth vessel. Fresh sterile medium was then pumped into the growth vessel at a constant flow rate from a 10-liter reservoir. Flow rates were adjusted to give the desired generation time for each experiment. Generation time, $\tau$, is a chemostat function defined by Novick and Szilard (24) as the reciprocal of growth rate, $\alpha$, where $\alpha = (1/n)(dn/dt)$, and $n$ = number of bacterial cells per ml. $\tau$ is also equal to $v/w$, where $v$ is the chemostat growth vessel volume and $w$ is the rate of infusion of medium into the growth vessel. Doubling time is equal to $\tau \times ln2$. The growth vessel was kept at 37 C in a constant temperature water bath while air was bubbled through the medium to maintain aeration and mixing. Growth medium and cells flowed out of the chemostat through the side arm into a cylinder immersed in an ice bath. The effluent was discarded until at least three chemostat volumes (2 liters) had passed through the side arm; the effluent cells were then harvested periodically by centrifugation and, at the end of the experiment, were combined with the cells remaining in the chemostat growth vessel. In a few experiments, the latter were harvested and extracted separately.

Cell density was routinely measured by absorbance at 550 nm in a cuvette of 1-cm light path, using a Zeiss spectrophotometer, after the absorbance had been calibrated against both chamber counts and viable colony counts. Cell densities were controlled at about $10^8$ cells per ml by limiting the concentrations of nutrients in the reservoir to $12 \times 10^{-6}$ to $14 \times 10^{-6}$ M in the case of L-methionine, or $3 \times 10^{-11}$ to $5 \times 10^{-11}$ M in the case of vitamin $B_{12}$. The rate of aeration was shown not to limit growth under these conditions.

Limitation of vitamin $B_{12}$ in flask cultures did not yield a sharp end point of growth, but a transition point, beyond which progressively slower growth continued until the maximum vitamin concentation per cell reached between 10 and 100 molecules per cell. However, in chemostat cultures it was possible to maintain a constant cell density throughout each experiment by vitamin limitation.

**Enzyme preparations and assays.** Cells were harvested by centrifugation and then washed in several volumes of the following buffer: potassium phos-

phate, *p*H 7.3, 50 mM; ethylenediaminetetraacetic acid, 1 mM; and dithiothreitol, 1 mM. Washed cells were suspended in two or more volumes of the same buffer and extracted with a Branson LS75 sonifier in four 30-sec bursts. Small volumes (1 ml) were extracted with a Branson W185 sonifier by using the microtip and four 15-sec bursts. The crude extracts were centrifuged at 15,000 × *g* for 20 min, and the supernatant fluid was applied to a column of Sephadex G25 (coarse beads, bed volume 10 times the supernatant volume), which had been equilibrated and was then eluted with the above buffer. The entire procedure was carried out at 2 C, and the gel filtered protein fractions were either assayed at once or stored at −20 C.

Assay procedures were as previously described for cystathionine γ-synthase (assay A, reference 13), β-cystathionase (10), and SAM synthetase (8); the elimination rates observed in the cystathionine synthase assay were multiplied by five to give the correct rates for cystathionine synthesis (13).

Two assays devised for the homoserine transacetylase of *Neurospora* (23) were adapted to the bacterial homoserine *O*-transsuccinylase, with somewhat unsatisfactory results. In the first assay, the enzyme was incubated with $^3$H-L-homoserine and succinyl CoA. The *O*-succinylhomoserine synthesized was converted to *N*-succinylhomoserine by alkali treatment and then applied to a Dowex-50 ($H^+$) column as previously described (23). In the second assay, which is based on the ability of the enzyme to catalyze the exchange between homoserine and *O*-succinylhomoserine, *O*-succinylhomoserine was substituted for succinyl CoA. In both assays, the separation of $^3$H-*O*-succinylhomoserine from $^3$H-homoserine is achieved because *N*-succinylhomoserine is not retained by Dowex-50 ($H^+$), whereas the protonated amino acid is. The reaction mixtures for both assays contained, in 0.5-ml volume: 50 μmoles of potassium phosphate, *p*H 7.5; 1.5 μmoles of succinyl CoA or 5 μmoles of *O*-succinyl-L-homoserine; 2 μmoles of $^3$H-L-homoserine; and sufficient extract to yield 0.1 to 0.3 μmole of labeled succinylhomoserine in 20 min at 37 C.

Much better results were obtained by coupling the transsuccinylase with a large excess of purified (11, 14) cystathionine γ-synthase, to give the overall reaction: L-homoserine + succinyl CoA → α-ketobutyrate + succinate + CoA + $NH_3$. The reaction mixtures contained, in a final volume of 1 ml: 1.5 μmoles of succinyl CoA; 2.0 μmoles of L-homoserine; 0.25 to 1.0 mg of extract protein; and 1.2 units of purified cystathionine γ-synthase. One unit of the purified cystathionine synthase is the amount catalyzing the formation of 1 μmole of α-ketobutyrate by gamma elimination from *O*-succinylhomoserine in 1 min. After 5 min at 37 C, the reactions were stopped by the addition of 0.1 ml of 1.5% trichloroacetic acid. After centrifugation, samples of the supernatant fluid were assayed for α-ketobutyrate with lactate dehydrogenase (14). Each reaction mixture was matched with a blank lacking succinyl CoA. The assay gave rates which were linear over a wide range of reaction time and over a range of transsuccinylase concentration catalyzing the synthesis of from 8 to 240 nmoles of *O*-succinylhomoserine per min. The rates were about 10 times higher than those reported by Schlesinger (28). The difference may be due to end-product inhibition of the enzyme by *O*-succinylhomoserine, since rates similar to Schlesinger's were found when the purified cystathionine γ-synthase was not added until after the completion of the reaction or when the radioactive synthesis assay was used. Most extracts were assayed by both the exchange assay and the coupled synthesis assay, and the rate ratio was 1:8.

We found that the enzyme could not be reliably assayed in crude extracts by measuring the liberation of free CoA as described by Lawrence (17). The method gave very high blanks due to enzymatic hydrolysis of succinyl CoA and to spontaneous hydrolysis and succinylation of the amino group of homoserine. The different extraction procedures used by Lawrence may have altered the portion of the blank which is due to enzymatic hydrolysis of succinyl CoA.

Proteins were determined by the method of Layne (19). All enzyme activities are expressed as nanomoles of product formed per minute per milligram of protein.

## RESULTS

**Studies of a regulatory role for folate derivatives in homocysteine biosynthesis.** In *N. crassa*, the second enzyme in the homocysteine pathway, cystathionine γ-synthase, is the target for allosteric regulation, being inhibited by SAM and activated by $^5$*N*-methyltetrahydrofolate polyglutamates ($CH_3FH_4G_n$) (29). In *E. coli*, it is the first enzyme, homoserine *O*-transsuccinylase, which is regulated, being subject to synergistic inhibition by methionine plus SAM (20). Table 1 shows that the same is true for *Salmonella* although the synergism appears much less marked. Lawrence has recently reported similar findings for this enzyme (17). The synergistic inhibition of the *Salmonella* transsuccinylase was observed only with the direct synthesis assays and not with the assay based on the exchange of homoserine into *O*-succinylhomoserine. This raises the question whether the exchange reaction is catalyzed by transsuccinylase or by another enzyme. We believe both reactions are catalyzed by the same enzyme since the rates changed in parallel in all of the flask culture experiments reported here.

We next tested the effect on transsuccinylase activity of adding $CH_3FH_4G_3$ to a gel-filtered ammonium sulfate fraction of this enzyme from the wild type; there was no activation (Table 1). The small inhibition observed is encountered with many enzymes when treated with polyglutamate derivatives of

TABLE 1. *Feedback inhibition of homoserine O-transsuccinylase*[a]

| Inhibitor concn (mM) | | | Specific activity[c] | Percent inhibition |
|---|---|---|---|---|
| L-Methionine | SAM[b] | $CH_3FH_4G_3$[b] | | |
| 0 | 0 | 0 | 1,033 | |
| 0.10 | 0 | 0 | 619 | 40 |
| 0 | 0.06 | 0 | 898 | 13 |
| 0.10 | 0.06 | 0 | 328 | 68 |
| 0 | 0 | 0.05 | 750 | 27 |
| 0.10 | 0.06 | 0.05 | 282 | 73 |

[a] Enzyme preparation used was slightly purified (approximately threefold) by ammonium sulfate precipitation. Homoserine *O*-transsuccinylase activity was measured by coupling it to cystathionine γ-synthase as described.

[b] SAM, *S*-Adenosylmethionine; $CH_3FH_4G_3$, methyltetrahydrofolate triglutamate.

[c] Expressed as nanomoles of product formed per minute per milligram of protein.

folate (B. T. Kaufman, *unpublished data*). Since gel filtration does not always separate polyglutamates from protein (29), we also examined a *metF* mutant, blocked in the synthesis of $CH_3FH_4$ (Fig. 1). We were encouraged by finding no transsuccinylase activity (Table 2) in extracts from flask cultures grown on limiting methionine (0.1 mM limits growth; 0.5 mM does not). However, activity was not restored by any of the following: $CH_3FH_4G_1$, $CH_3FH_4G_2$, $CH_3FH_4G_3$, $N^5$-formyltetrahydrofolate monoglutamate, methylcobalamin ($10^{-4}$ M), or boiled extract of wild type. Moreover, none of these additions affected the activities of cystathionine γ-synthase or β-cystathionase in the *metF* extract, the levels of the latter two enzymes being similar to wild type (Table 2).

Thinking that the activation of transsuccinylase by folates might be able to be manifested only in vivo, we next examined a *metE* mutant. This mutant should accumulate large amounts of methylfolates since in the absence of $B_{12}$ it is blocked in the methylation of homocysteine (Fig. 1). However, extracts of this strain grown on limiting methionine were also found to have no transsuccinylase, and levels of the other two enzymes were comparable to wild type (Table 2). In fact, transsuccinylase could not be detected in any methionine auxotroph (Table 2) grown on low levels of methionine which only partially reduced the enzyme level in wild type. The levels of the other two enzymes were little affected by methionine. Table 2 also shows that extracts of *metA15* mutants grown on low methionine have high levels of cystathionine γ-synthase and β-cystathionase, an unexplained phenomenon that we have made use of for many years in purifying these enzymes (10, 14).

*metE* mutants will respond alternately to methionine or vitamin $B_{12}$. Extracts of cells grown on non-growth-limiting levels ($7.4 \times 10^{-9}$ M) of the vitamin had high transsuccinylase activity (Table 2). This observation, somewhat reminiscent of an earlier one in the arginine pathway (30), suggested that exogenous and endogenous methionine might affect the repression of the transsuccinylase differently. The conclusion was not supported by the finding that transsuccinylase was still absent from extracts of *metB* and *metC* grown on homocystine instead of methionine (Table 2). Since the intracellular concentration of nutrients cannot be controlled by limiting them in flask cultures, we examined this question further with the use of a chemostat.

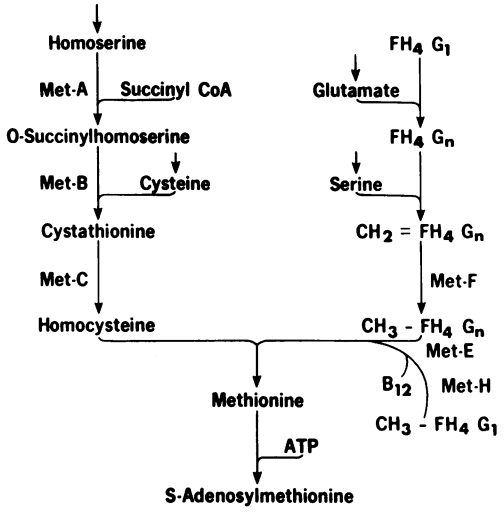


FIG. 1. *Pathway of methionine biosynthesis in Salmonella. The structural gene designations and their respective enzymes are as follows: metA, homoserine O-transsuccinylase; metB, cystathionine γ-synthase- metC, β-cystathionase; metE, non-$B_{12}$ methyltetrahydrofolate-homocysteine transmethylase; metF, methylenetetrahydrofolate reductase; and metH, $B_{12}$-dependent methyltetrahydrofolate-homocysteine transmethylase. Not shown in the figure are metG, methionyl-tRNA synthetase; and metJ and metK, methionine regulatory genes. The following abbreviations are used in the figure: $FH_4G_1$, tetrahydrofolate monoglutamate; $FH_4G_n$, tetrahydrofolate polyglutamate; $CH_2{=}FH_4G_n$, methylenetetrahydrofolate polyglutamate: $CH_3{-}FH_4G_1$, methyltetrahydrofolate monoglutamate; and $CH_3FH_4G_n$, methyltetrahydrofolate polyglutamate.*

TABLE 2. *Enzyme levels in Salmonella wild-type and methionine auxotrophs grown in flask culture*

| Gene | Allele | Nutrient | Concn (mM) | Specific activity[a] | | |
|---|---|---|---|---|---|---|
| | | | | Transsuccinylase | Cystathionine γ-synthase | β-Cystathionase |
| Wild type | (Lt-2) | None | | 10 | 30 | 30 |
| | | L-Methionine | 0.05 | 3 | 30 | 20 |
| | | | 0.50 | 0 | 5 | 15 |
| *metA* | 15 | L-Methionine | 0.05 | | 185 | 72 |
| | | L-Cystathionine | 0.05 | | 310 | 63 |
| | | L-Homocystine | 0.03 | | 350 | 70 |
| | | L-Homocystine | 0.50 | | 35 | 41 |
| | | *O*-Acetyl-L-homoserine | 0.50 | | 20 | 30 |
| *metB* | 16 | L-Methionine | 0.05 | 0 | | 11 |
| | | L-Homocystine | 0.05 | 0 | | 7 |
| *metC* | 50 | L-Methionine | 0.05 | 0 | 55 | |
| | | L-Homocystine | 0.03 | 0 | 56 | |
| *metE* | 47 | L-Methionine | 0.05 | 0 | 75 | 18 |
| | | Vitamin $B_{12}$ | $7.4 \times 10^{-6}$ | 21 | 25 | 16 |
| *metF* | 96 | L-Methionine | 0.05 | 0 | 50 | 24 |
| *metE*, *metH* | 205 465 | L-Methionine | 0.05 | 0 | 165 | 34 |

[a] Expressed as nanomoles of product formed per minute per milligram of protein.

**Chemostat experiments.** Figure 2 shows the levels of the three enzymes of homocysteine biosynthesis in *metE47* grown in a methionine chemostat, plotted as a function of generation time (defined above). The longer the generation time, the lower the intracellular concentration of methionine (24). The shortest generation time used, 75 min, corresponds to a doubling time of 52 min (doubling time = $\tau \times ln2$) which exceeds the maximum doubling times of this strain in flask culture of 35 min on methionine and 40 min on vitamin $B_{12}$. As expected, the synthesis of the second and third enzymes was progressively repressed in parallel as the generation time was increased up to 4 hr. However, it was virtually impossible to elicit any synthesis of the transsuccinylase by lowering the intracellular concentration of methionine, a very low level of this enzyme finally appearing at a generation time greater than 5 hr. Figure 3 shows the results obtained with the same mutant in a vitamin $B_{12}$ chemostat. The pattern seems to recapitulate that of Fig. 2 with everything displaced towards shorter generation times. But the most striking results were that all three enzymes were fully derepressed at generation times between 2 and 3 hr, and that there was no measurable transsuccinylase at short generation times, although the mutant could not grow on vitamin $B_{12}$ without the functioning of this enzyme. The results so far supported the conclusion that exogenous methionine was more effective than endogenous in repression of the transsuccinylase.

At very long generation times the levels of all three enzymes declined in both the methionine and vitamin $B_{12}$ chemostats. Possible explanations for this decline are: (i) harvesting of cells before they reached a steady state; (ii) decreased protein content in general; (iii) loss of viability; and (iv) genetic change to countervene the possibly noxious accumulation of high concentrations of methionine precursors. None of these possibilities has been specifically ruled out. In several experiments, the proportion of viable cells was checked by comparing plate and chamber counts; the proportion was the same in these experiments. Reversion to prototrophy was never observed except at the longest generation time (5.8 hr) in the methionine chemostat when 0.25% prototrophs were found.

Similar experiments were next undertaken with nonreverting (33) *metB* and *metC* mu-

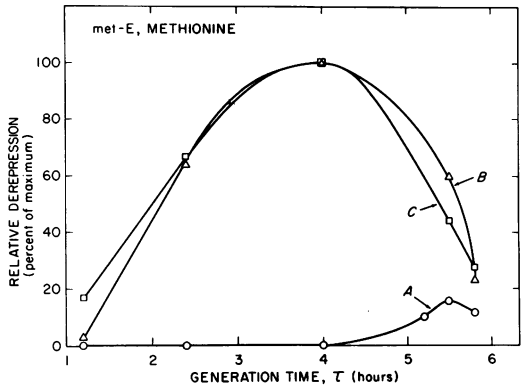


FIG. 2. *Enzyme levels in metE47 grown in the methionine chemostat. Methods used for growth of chemostat cultures, enzyme preparations, and assays are described in the text. Derepression, plotted as relative activity, is shown as a function of generation time, a chemostat function inversely proportional to growth rate. A = homoserine O-transsuccinylase (first enzyme); B = cystathionine γ-synthase (second enzyme); and C = β-cystathionase (third enzyme).*

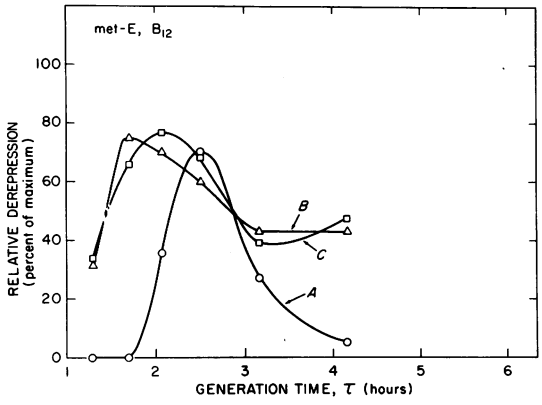


FIG. 3. *Enzyme levels in metE47 grown in the vitamin $B_{12}$ chemostat. Procedures are as described in the legend to Fig. 2 and the text. A = homoserine O-transsuccinylase (first enzyme); B = cystathionine γ-synthase (second enzyme); and C = β-cystathionase (third enzyme).*

tants (Table 3); *metC* mutants are leaky (7), but the doubling time of *metC50* in minimal medium is 5 hr. It was not possible to compare endogenous and exogenous methionine with these mutants because the methionine precursors which they can utilize would not sustain growth in the chemostat at reasonably short generation times. In the case of homocystine, this was apparently due to low affinity for some rate-limiting step and, in the case of cystathionine, to low velocity of growth. The results with methionine (Table 3) show that in both mutants transsuccinylase was fully derepressed at a generation time of 2.5 hr, at which the enzyme was fully repressed in *metE47*. This result suggested that the apparent extreme repressibility of transsuccinylase by exogenous methionine might be peculiar to, or in some way related to, the specific phenotype of the *metE* mutant.

**Corepressor for the enzymes of homocysteine biosynthesis.** The chemostat experiments with *metE47* suggested that the corepressor for transsuccinylase might be different from the corepressor for the other two enzymes and more immediately derived from exogenous methionine. *metG419*, a $K_m$ mutant for methionyl-transfer ribonucleic acid (tRNA) synthetase, has been reported to have normally repressible cystathionine γ-synthase and β-cystathionase (9). The results in Table 4 indicate that methionyl-tRNA is also not part of the repressor for transsuccinylase, which was normally repressed by methionine in this mutant.

*metJ713* and *metK721* were isolated as methionine analogue-resistant mutants and were found to have nonrepressible levels of cystathionine γ-synthease and β-cystathionase (18), as well as of the methionine-specific β-aspartokinase (L-aspartate 4-phosphotransferase, EC 1.2.1.11) and homoserine dehydrogenase (L-homoserine:NAD oxidoreductase, EC 1.1.1.3) (27). The results of Table 4 show that transsuccinylase levels in these mutants parallel those of the other enzymes of homocysteine biosynthesis. Lawrence has also recently reported derepression of transsuccinylase in these mutants (17).

Greene et al. (8) have isolated *E. coli* mutants with nonrepressible levels of cystathionine γ-synthase and β-cystathionase. One of them, E40, mapped in the region of the *metK* gene of *Salmonella* although its exact genetic identity has not been established (34; R. C. Green, *personal communication*). Transsuccinylase was also derepressed in this mutant (Table 4). Greene et al. (8) found that the mutant had an extremely low level of SAM synthetase, suggesting that SAM, or something derived from it, might be a corepressor. Table 5 shows that, in contrast to this *E. coli* mutant, *Salmonella metK721* had wild-type levels of SAM synthetase. *Salmonella metJ713* had derepressed levels of SAM synthetase (Table 5) like *E. coli* E31 (*metJ*) (12).

*Salmonella* transsuccinylase was found to diverge from the other enzymes of homocysteine biosynthesis in one other respect, in that

TABLE 3. *Enzyme levels in metB, metC, and metE mutants grown in the chemostat*[a]

| Gene | Allele | Nutrient | Generation time (hr)[c] | Specific activity[b] | | |
|---|---|---|---|---|---|---|
| | | | | Transsuccinylase | Cystathionine γ-synthase | β-Cystathionase |
| *metB* | 36 | L-Methionine | 2.6 | 600 | | 200 |
| *metC* | 50 | L-Methionine | 2.6 | 260 | 140 | |
| *metE* | 47 | L-Methionine | 2.4 | 0 | 225 | 63 |
| | | Vitamin $B_{12}$ | 2.5 | 230 | 210 | 65 |

[a] Conditions for growth of organisms in the chemostat, extraction of cells, and enzyme assays are described in the experimental procedures.
[b] Expressed as nanomoles of product formed per minute per milligram of protein.
[c] Generation time is a chemostat function which is the reciprocal of growth rate (see text).

TABLE 4. *Enzyme levels in methionine regulatory mutants of Salmonella and Escherichia coli grown in flask culture*

| Organism | Gene | Allele | L-Methionine concn (mM) | Specific activity[a] | | |
|---|---|---|---|---|---|---|
| | | | | Transsuccinylase | Cystathionine γ-synthase | β-Cystathionase |
| *Salmonella* | Wild type | (Lt-2) | 0 | 10 | 30 | 30 |
| | | | 0.05 | 3 | 30 | 20 |
| | | | 0.50 | 0 | 5 | 15 |
| | *metG*[b] | 419 | 0.05 | 25 | 55 | 39 |
| | | | 0.50 | 0 | 5 | 14 |
| | *metJ* | 713 | 0.50 | 340 | 200 | 24 |
| | *metK* | 721 | 0.50 | 360 | 145 | 22 |
| *E. coli* | Wild type | (K-12) | 0 | 25 | 10 | 55 |
| | *metK*[c] | E40 | 0.05 | 320 | 300 | 210 |

[a] Expressed as nanomoles of product formed per minute per milligram of protein.
[b] *metG419* is a mutant with a defective methionyl-tRNA synthetase ($K_m$ for methionine 100 times higher than wild type). The remaining *Salmonella* mutants have methionine regulatory defects.
[c] Although *E. coli* strain E40 maps in the same region as the *metK* gene of *Salmonella*, the exact genetic identity of this mutant is uncertain (26).

it fell to very low levels after the wild type had entered resting phase (Table 6). However, in resting phase cultures of both *metJ713* and *metK721* transsuccinylase was derepressed in parallel with the other enzymes (Table 6). Our observation that the first enzyme is repressed in resting phase cells of the wild type differs from the results reported by Lawrence (17). However, his results are based on an assay subject to high nonenzymatic blanks (*see above*) and may not have revealed the low activities.

## DISCUSSION

Many amino acids have additional functions besides serving as building blocks for protein, but methionine is conspicuous in this respect. It contributes to membrane phospholipid (the methyl groups of choline), is a source of polyamines, a general methylating agent (via SAM), and the initiator of protein synthesis (via a particular species of methionyl-tRNA). It would, then, not be surprising if the regulation of its synthesis were to reveal some unusual features. Methionine synthesis is also of interest as an example of the general problem of regulation of the synthesis of a group of sequential enzymes coded by unlinked genes.

**Feedback control and the possible role of folate derivatives in regulating homocysteine biosynthesis.** In *N. crassa* the converging pathways leading to the synthesis of homocysteine and of the methyl group are coordinated by the balance between two allosteric controls: one is activation of cystathionine γ-synthase, the second enzyme of the

TABLE 5. *S-adenosylmethionine synthetase in regulatory mutants of Salmonella and Escherichia coli*[a]

| Organism | Gene | Allele | L-Methionine concn (mM)[b] | Specific activity[c] |
|---|---|---|---|---|
| *E. coli* | Wild type | (K-12) | 0 | 1.95 |
| | *metK* | (E40) | 0.05 | 0 |
| *Salmonella* | Wild type | (Lt-2) | 0 | 1.82 |
| | *metJ* | 713 | 0.10 | 3.67 |
| | | | 0.50 | 6.87 |
| | *metK* | 721 | 0 | 1.20 |
| | | | 0.50 | 1.43 |

[a] Cells were grown in flask culture and harvested during mid-log-phase growth as described in the experimental procedures. *S*-adenosylmethionine synthetase was assayed as described by Greene et al. (15), except that desalted extracts rather than toluenized cells were used.

[b] Concentration of L-methionine in the growth medium.

[c] Expressed as nanomoles of product formed per minute per milligram of protein.

TABLE 6. *Enzyme levels in Salmonella methionine regulatory mutants after cessation of growth in flask culture*[a]

| Gene | Allele | L-Methionine concn (mM) | Specific activity[b] | | |
|---|---|---|---|---|---|
| | | | Transsuccinylase | Cystathionine γ-synthase | β-Cystathionase |
| Wild type | (Lt-2) | 0 | 2 | 30 | 20 |
| *metJ* | 713 | 0.50 | 520 | 370 | 67 |
| *metK* | 721 | 0.50 | 240 | 140 | 57 |

[a] Cultures were incubated at 37 C for 24 hr (approximately 16 hr after cessation of exponential growth). Harvesting and extraction of cells and assays used are described in the text.

[b] Expressed as nanomoles of product formed per minute per milligram of protein.

homocysteine pathway, by $CH_3FH_4G_n$, the end product of the methyl pathway; and the other is the feedback inhibition of cystathionine γ-synthase (as well as methylenetetrahydrofolate reductase in the methyl pathway) by SAM (29). In *E. coli* it is the first enzyme of the homocysteine pathway, homoserine *O*-transsuccinylase, whose activity is regulated through synergistic feedback inhibition by SAM plus methionine (20). The same is true in *Salmonella* although the synergism seems to be weaker (Table 1). There is also marked inhibition of the *Salmonella* transsuccinylase by its product, *O*-succinylhomoserine. We have found no evidence that the activity of this enzyme, or of the other two, is affected by folate derivatives (Table 1). It also appears that $CH_3FH_4$ plays no role in regulating the synthesis of the three enzymes since repression by methionine was the same in both *metE* and *metF* mutants (Table 2). Thus, the coordination of the converging pathways must be accomplished in some other way.

**Repression of enzyme synthesis.** Repression of the synthesis of the three enzymes is noncoordinate, with much more facile repression of transsuccinylase than of cystathionine γ-synthase and somewhat less marked repressibility of β-cystathionase than of the latter. Stringent repression of the transsuccinylase is illustrated by the fact that no activity of this enzyme could be shown in extracts of any methionine auxotroph grown in flask culture on methionine, even at growth-limiting concentrations (Table 2). The mere absence of this enzyme from *metA* extracts is accordingly no proof that this gene codes its structure. There seems little doubt from other types of evidence (25, 26) that it does.

**Evidence for common elements in repression of all three enzymes.** The strongest indication of elements common to the repression mechanism of all three enzymes is that all the enzymes are fully derepressed in the analogue-resistant mutants, *metJ713* and *metK721* (Table 4), and none is derepressed in the methionyl-tRNA synthetase mutant, *metG419*. An *E. coli* mutant tentatively designated *metK* has been shown to have a defective SAM synthetase, suggesting that SAM or something derived from it is a corepressor for all three enzymes. There is some uncertainty about this conclusion because the *Salmonella metK* mutant that we studied appears to have a normal SAM synthetase (Table 5). This problem may be clarified if it can be determined whether the latter mutant is blocked in the conversion of SAM to another product which is the actual corepressor.

The selective disappearance of transsuccinylase activity from wild-type *Salmonella* in resting phase may also reflect hypersensitivity to a common mechanism, particularly since it does not occur in either *metJ* or *metK* mutants (Table 6). Differing sensitivities to a common repression mechanism could also explain the noncoordinate pattern of repression of the three enzymes. The high levels of cystathionine γ-synthase and β-cystathionase in *metA* mutants (Table 2) may be another mani-

festation of a common control mechanism. This finding is reminiscent of proposals made for the histidine (15, 16) and arginine (21) pathways, that the first enzyme of the pathway might participate directly in the repressor complex.

**Evidence for elements in repression not shared by all three enzymes.** The possibility that regulation of transsuccinylase synthesis might involve elements not shared with the other two enzymes was raised by chemostat experiments with *metE47* (Fig. 2 and 3). These experiments at first suggested that exogenous methionine might selectively promote the repression of the transsuccinylase since it was virtually impossible to derepress this enzyme by limiting intracellular methionine derived from the medium (Fig. 2), whereas it could be derepressed by limiting vitamin $B_{12}$ (Fig. 3). However, when *metB* and *metC* mutants were grown in a methionine chemostat, transsuccinylase was easily derepressed (Table 3). These results are limited since it was not possible to cultivate *metB* and *metC* mutants in a chemostat limiting endogenous methionine synthesis with methionine precursors as nutrients. But they do suggest that the superrepression of transsuccinylase in the experiments of Fig. 2 is a function of the *metE* phenotype rather than of a difference in effectiveness of methionine derived from exogenous and endogenous sources.

At the two shortest generation times used in the vitamin $B_{12}$ chemostat (Fig. 3), no transsuccinylase activity could be detected at all, which seems curious since all of the enzymes of methionine biosynthesis are required for growth to occur under these conditions. In addition, methylenetetrahydrofolate reductase is almost completely repressed by vitamin $B_{12}$ (22); some investigators believe this finding is not unreasonable (3).

In the vitamin $B_{12}$ chemostat (Fig. 3), the direct effect of shortening the generation time is to reduce the intracellular concentration of a holoenzyme (the $B_{12}$-transmethylase) rather than of methionine or a methionine precursor. We assumed that any effects on the synthesis of the homocysteine biosynthetic enzymes would result from the consequent decrease in the intracellular concentration of "endogenously formed" methionine, but this may not be so. It has recently been proposed that the functional holoenzyme of the $B_{12}$-transmethylase is itself a component of the repression apparatus for methylenetetrahydrofolate reductase and the non-$B_{12}$-transmethylase (*see* Fig. 1; reference 3, 22; H. Kung, C. Spears, R. C. Greene, and H. Weissbach, *personal communication*). One unique characteristic of *metE* mutants grown on methionine is that they contain no functional transmethylase at all. The $B_{12}$-transmethylase is presumably present (22; Kung et al., *personal communication*), but only in apoenzyme form.

One could speculate that the presence of a functional transmethylase is somehow necessary for induction of the synthesis of transsuccinylase, thereby preventing the overproduction of homocysteine when it cannot be methylated. Transmethylase itself might be a structural component of an inductive regulatory complex (3, 22; Kung et al., *personal communication*). A more effective mechanism for coordinating the synthesis of homocysteine with that of the methyl groups would be afforded if the transmethylases could methylate a compound other than homocysteine, producing a product necessary for the induction of transsuccinylase synthesis.

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