

REGULATION OF METHIONINE SYNTHESIS IN *SALMONELLA*  
*TYPHIMURIUM*: MUTANTS RESISTANT TO INHIBITION BY  
ANALOGUES OF METHIONINE

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Received September 26, 1967

**B**IOCHEMICAL and genetical experiments with methionine auxotrophs of *Salmonella typhimurium* (SMITH 1961; SMITH and CHILDS 1966; CHILDS and SMITH unpublished) have resulted in the recognition of six structural genes (*metA*, *B*, *C*, *E*, *F* and *H*) concerned with well defined sequential steps in the biosynthesis of methionine (Figure 1). Mutants of another gene (*metG*) do not have a specific enzyme deficiency: they are leaky due to a low level of activity of all the methionine enzymes. Methionine inhibits the activity of homoserine *O*-transsuccinylase (*metA*) and represses the synthesis of all the enzymes of the pathway (ROWBURY 1964). Two pairs of genes (*metA* and *H* and *metB* and *F*) are linked closely enough to be co-transducible whereas the *metC*, *E* and *G* genes are each located in well separated regions of the circular bacterial genome (Figure 3). On the basis of this partial clustering of genes concerned with related functions, it seemed likely that the mode of regulation of methionine synthesis in this organism would be sufficiently different from that of the control of lactose (BECKWITH 1967) and arabinose fermentation (ENGLESBERG *et al.* 1965) in *Escherichia coli*, histidine (AMES *et al.* 1967) and leucine (BURNS *et al.* 1966) synthesis in *S. typhimurium* and tryptophan synthesis in both organisms (BLUME and BALBINDER 1966; YANOFSKY and LENNOX 1959) to justify further investigation.

Mutants of bacteria resistant to inhibition by analogues of metabolic end products have often been shown to be abnormal in regulation of the biosynthesis of the normal end product (COHEN and JACOB 1959) and to be readily amenable to both biochemical and genetic analyses. The methionine analogues  $\alpha$ -DL-methyl methionine, DL-ethionine and DL-norleucine were found to be inhibitory and this paper is concerned with the isolation, phenotypic characterisation, enzymic abnormalities and mapping of mutants resistant to these analogues. Preliminary results (SMITH and LAWRENCE 1966) suggested that the regulation of methionine synthesis in *S. typhimurium* possesses some unique features.

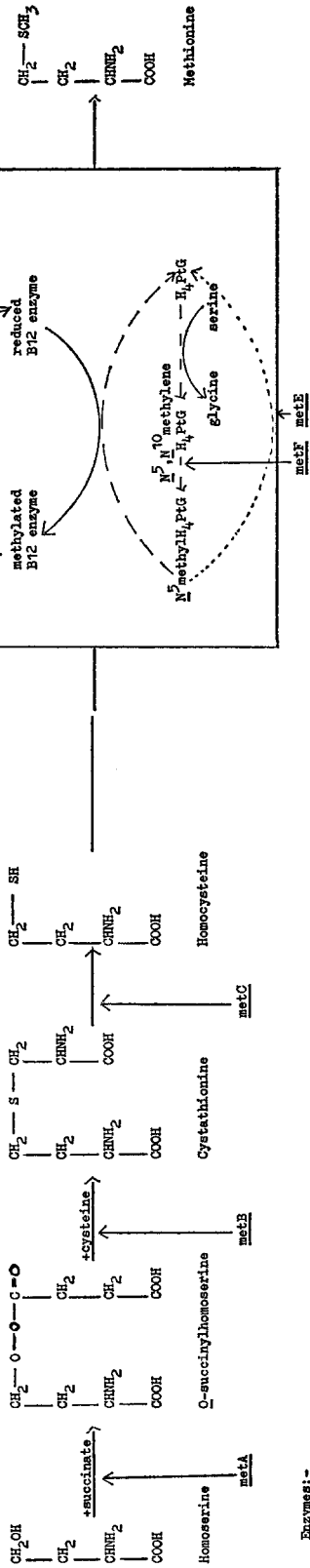


FIGURE 1.—The pathway of methionine biosynthesis in *Salmonella typhimurium*.  $\xrightarrow{\text{H}_4\text{PtG}_3}$  only;  $\xrightarrow{\text{H}_4\text{PtG}_1}$  or  $\text{H}_4\text{PtG}_2$ .

## MATERIALS AND METHODS

All the methionine (*met*) mutants of *Salmonella typhimurium* used were derived from strain LT-2. The *metA*, *B*, *C*, *E*, *F* and *G* mutants were some of those used by SMITH (1961) and SMITH and CHILDS (1966). *MetH463* is one of 8 recently isolated mutants with defects in the cobalamin (vitamin B12) dependent methylation of homocysteine (CHILDS and SMITH unpublished). The *S. typhimurium* Hfr strains A, B2 and H5 were obtained from Dr. K. E. SANDERSON of the Department of Biology, The University, Calgary, Alberta, Canada. They are described by SANDERSON and DEMEREC (1965). The maintenance of stock cultures, preparation and washing of liquid cultures of bacteria, propagation, assay and maintenance of phage P22 and the media used in experiments other than those concerned with enzyme studies, were also as previously described (SMITH 1961; SMITH and CHILDS 1966). Incubation was at 37°C unless otherwise stated. The optical density of liquid cultures was measured in a Unicam SP500 spectrophotometer at a wavelength of 650 m $\mu$ . Alterations to terminology and nomenclature previously used (SMITH and CHILDS 1966) and the labelling of new mutants are both in accordance with the proposals of DEMEREC, ADELBERG, CLARK and HARTMAN (1966).

*Measurement of growth rates of bacteria:* Stationary phase minimal medium (MM) cultures were diluted 1:4 in fresh MM and incubated on a reciprocating shaker (60 strokes per min) for one hour. The optical densities of these log phase cultures were then adjusted to 0.05 and 50 ml volumes incubated with shaking in 250 ml flasks and their optical densities measured at 30–60 min intervals and plotted on a log-linear scale until the stationary phase was reached. Samples were returned to the flask after measurement. Mean generation times were calculated from the slope of the straight line obtained during the logarithmic phase of growth.

*Isolation of analogue resistant mutants:* About  $3 \times 10^8$  analogue sensitive bacteria (either HfrA, B2 or H5) were spread onto the surface of minimal agar (MA) plates containing either  $\alpha$ -methyl methionine (6.1 mM), ethionine (6.1 mM) or norleucine (7.5 mM) i.e. all at 1 mg/ml. After incubation for 48 hr resistant colonies were picked and taken through two single colony isolations on either MA + analogue or nutrient agar (NA). Duplicate stock cultures were finally established from single colonies after re-checking analogue resistance.

*The excretion of methionine by analogue resistant mutants:* Loopfuls of saline suspensions of mutant and wild type colonies were streaked parallel to each other and about 2.5–3 cm apart on MA and incubated for 24 hr. Similar inocula of the methionine auxotroph *metF185* (a deletion mutant responding only to methionine—SMITH and CHILDS 1966) were then streaked at right angles to and between the two streaks and incubation continued for a further 48 hr. Excretion of methionine was indicated by relatively denser growth of the indicator inoculum nearest the analogue resistant inoculum. This was usually visible with the naked eye; occasionally the use of a stereoscopic binocular microscope (25 $\times$  magnification) was necessary to detect excretion at 37°.

For testing the excretion of methionine in liquid medium, organisms of the appropriate strain (1.0–1.5 mg dry wt./ml or approximately  $2-3 \times 10^9$  bacteria/ml) from overnight cultures in MM were inoculated in fresh medium and incubated on a reciprocating shaker (80 strokes per min) for 3–4 hr. Methionine in samples of the supernatant fluids (after removal of organisms by centrifugation) was estimated microbiologically as described by ROWBURY and WOODS (1964a) using *Escherichia coli* 3/62 (a strain which responds only to methionine). Methionine was also detected by paper chromatography; in such cases organisms were incubated as described above in MM but with the addition of S<sup>35</sup>-cysteine (specific activity 32 mc./mM, at a final concentration of 5  $\mu$ c./ml.) and after incubation samples of the supernatant fluids were spotted on paper chromatograms which were developed in *n*-butanol-propionic acid-water (47:22:31) for 16 hr. After spraying with ninhydrin (0.2% in *n*-butanol saturated with water) and drying at 80°, the chromatograms were scanned using a mica end-window Geiger-Müller tube (General Electric Co. type 2B2) in conjunction with a Scaler 1700 (Isotope Developments Ltd., Reading, Berks.).

*Uptake of C<sup>14</sup> from labelled methionine and ethionine:* C<sup>14</sup>-methyl L-methionine (specific activity 25 mc./mM) supplied by the Radiochemical Centre, Amersham, Bucks., U.K. and C<sup>14</sup>-ethyl 1-C L-ethionine (specific activity 2 mc./mM) supplied by Nuclear Research Chemicals,

Orlando, Florida, U.S.A. were used. Carrier L-methionine or DL-ethionine was added to 50 ml of mid log phase MM cultures (optical density about 0.2) in 250 ml flasks to give a final concentration of 0.034 and 0.031 M respectively ( $5 \mu\text{g/ml}$ ) followed by  $\text{C}^{14}$  methionine ( $3 \times 10^3$  d.p.m.) or  $\text{C}^{14}$  ethionine ( $1.3 \times 10^4$  d.p.m.). These cultures were incubated with shaking and 3 ml samples removed immediately and at 30 minute intervals over a period of 2 hr. The optical density of each sample was measured and duplicate 1 ml volumes transferred to a filter (Millipore  $0.45 \mu$ , 2.5 cm diameter) and washed with 10 ml MM. Each filter was then placed in 5 ml of scintillation fluid xylene Pop-op Ppo in a 15 ml scintillator ampoule and counting carried out either for 10 min or up to 10,000 c.p.m. with a Nuclear Chicago 720 Liquid Scintillator.

*Growth of organisms for enzyme assays:* Organisms were grown in MM with shaking for 16–18 hr at  $37^\circ$  (or  $25^\circ$ ), harvested (15 min at  $2000 \times g$ ), washed with one culture volume of water and resuspended in phosphate buffer (133 mM, pH 7.4). Such suspensions (20–30 mg dry wt./ml) were used both for the preparation of enzymic extracts as described previously for *E. coli* (Rowbury and Woods 1964a), and after dilution, directly and where appropriate for enzyme assays.

*Enzyme assays:* As previously described for *E. coli*, the activities of cystathionine synthetase (*metB*) and cystathionase (*metC*) were measured in enzymic extracts (Rowbury and Woods 1964a,b) and those of homoserine O-transsuccinylase (*metA*) and the homocysteine methylase complex (*metE*, *F* and *H*) in the presence or absence of cobalamin in intact organisms (Rowbury and Woods 1961, 1964b).

*Conjugation and transduction:* Plate mating was achieved by spreading onto selective media 0.2 ml quantities of  $10^{-2}$  broth dilutions of 1:20 mixtures of Hfr and  $\text{F}^-$  bacteria from saturated broth cultures. After incubation for 48 hr, plates were replicated directly for the scoring of unselected markers. The standard P22 phage transduction technique as previously described (Smith 1961) was used except that recipient bacteria were from saturated cultures and 0.2 ml instead of 0.1 ml quantities of the transduction mixtures spread on each plate of selective medium.

## RESULTS

*Inhibitory effects of analogues and their reversal:* The four analogues  $\alpha$ -methyl methionine, DL-ethionine, DL-methionine sulphone and DL-norleucine were tested for the inhibition of growth of the Hfr strains on MA + analogue at 6.1, 6.1, 6.7 and 7.5 mM respectively ( $1 \text{ mg/ml}$ ). All except methionine sulphone were inhibitory although none was completely restricting. The amount of residual growth was greatest in the presence of  $\alpha$ -methyl methionine. The relative effects of different concentrations of the analogues on the growth rate of HfrB2 were titrated in MM supplemented with analogues at concentrations ranging from  $1 \mu\text{g/ml}$  to  $1 \text{ mg/ml}$ . The minimum inhibitory concentration of  $\alpha$ -methyl methionine was  $1 \mu\text{g/ml}$  and of both ethionine and norleucine  $10 \mu\text{g/ml}$ . For  $\alpha$ -methyl methionine the lowest concentration giving maximum inhibition was  $5 \mu\text{g/ml}$ ; for ethionine and norleucine it was  $1 \text{ mg/ml}$ .

Differences in the nature of the inhibition of growth by each analogue were revealed when the effects on the growth rate of HfrB2 in MM of the addition of analogues at a final concentration of  $1 \text{ mg/ml}$  were measured. It can be seen that inhibition by  $\alpha$ -methyl methionine clearly differed from that by ethionine and norleucine (Figure 2). In cultures initially containing analogue the effect of  $\alpha$ -methyl methionine (Figure 2A) was more inhibitory than ethionine (Figure 2B) or norleucine (Figure 2C). When  $\alpha$ -methyl methionine was added to mid log phase cultures an immediate cessation of growth resulted (Figure 2A). Inhibi-

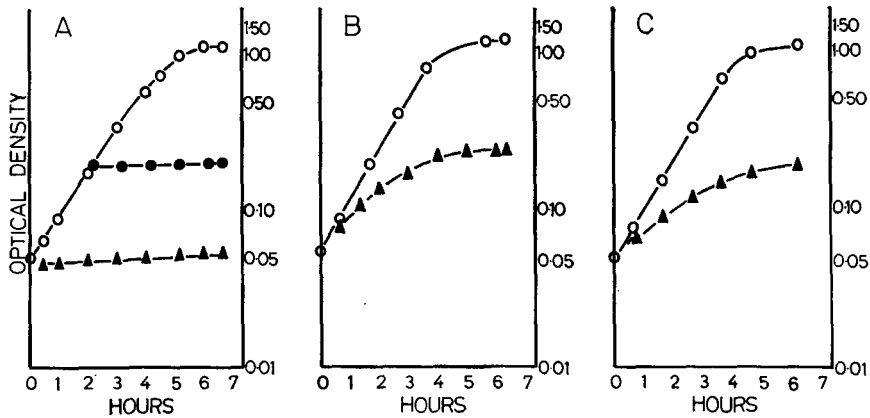


FIGURE 2.—The effect of the addition of methionine analogues at 1 mg/ml upon the growth of wild type organisms in minimal medium. ○—no analogue; ▲—analogue added at beginning of growth period; ●—analogue added in mid log phase of growth. A  $\alpha$ -methyl methionine; B ethionine; C norleucine.

tion of growth on MA by all three analogues could be overcome by the addition of DL-allo-cystathionine, DL-homocysteine or DL-methionine but not homoserine, vitamin B12, L-cysteine, sulphate, thiosulphate or sulphite to the medium. Using HfrB2 the relative efficiencies of either cystathionine, homocysteine or methionine in overcoming inhibition by each of the three analogues at 1 mg/ml in MM was titrated. Complete reversal of inhibition was only achieved by methionine and homocysteine at concentrations at least equivalent to that of the analogues.

*Isolation and characterisation of analogue resistant mutants:* Mutants resistant to  $\alpha$ -methyl methionine, ethionine and norleucine all arose at the relatively high frequency of  $c. 3 \times 10^{-7}$ . Initially only analogue resistant mutants were isolated which, in streak tests on MA at 25° or 37° or both temperatures excreted methionine. Thirteen  $\alpha$ -methyl methionine resistant and 17 ethionine resistant mutants fell into this category. In cross resistance tests on MA plates each supplemented with a different analogue at 1 mg/ml, 12 of the former and 7 of the latter were resistant only to either  $\alpha$ -methyl methionine or ethionine, respectively. The remaining 1  $\alpha$ -methyl methionine and 10 ethionine resistant mutants were resistant to all three analogues. Later, 4 mutants resistant to norleucine were isolated. They did not excrete methionine but were also resistant to inhibition by both  $\alpha$ -methyl methionine and ethionine. Thus, in summary, these presumptive methionine regulatory mutants fell into three main categories (Table 1); those resistant either to  $\alpha$ -methyl methionine (12-*metI*701–12 inclusive) or ethionine (7-*metJ*-713–719) and those resistant to both of these analogues and to norleucine (15-*metK*720–734). All *metI* and *J* mutants and 11 *metK* (720–730 inclusive) excreted methionine. The remaining 4 *metK* (731–734 inclusive) mutants were non-excretors.

The streak tests for methionine excretion on MA were carried out at 25° as well as 37°. Mutants appeared to differ in the relative amounts of methionine

TABLE 1

*The phenotypes of analogue resistant mutants and the strains from which they were derived*

Mutant	Parent strain	Medium of isolation	Analogue resistance on MA			Mean generation time in MM (min.)*			
			AM	ETH	NOL	No analogue	AM	ETH	NOL
	HfrA (SR305)	....	s	s	s	55.5(2)	..	..	..
	HfrB2	....	s	s	s	55.7(12)	..	..	..
	HfrH5	....	s	s	s	52(3)	..	..	..
<i>metI701</i>	HfrB2	AM	r	s	s	57	56	..	..
<i>I702</i>	HfrB2	AM	r	s	s	58	58	..	..
<i>I703</i>	HfrB2	AM	r	s	s	52.3(3)	52	55	59
<i>I704</i>	HfrB2	AM	r	s	s	55	55	55	55
<i>I705</i>	HfrB2	AM	r	s	s	55	60	64	..
<i>I706</i>	HfrB2	AM	r	s	s	59	57	..	..
<i>I707</i>	HfrB2	AM	r	s	s	56	55	..	..
<i>I708</i>	HfrA	AM	r	s	s	54	53	..	..
<i>I709</i>	HfrA	AM	r	s	s	56	58	60	..
<i>I710</i>	HfrA	AM	r	s	s	56	60	67	..
<i>I711</i>	HfrA	AM	r	s	s	58	65	68	..
<i>I712</i>	HfrA	AM	r	s	s	58	59	66	..
<i>metJ713</i>	HfrB2	ETH	s	r	s	61.5(7)	..	72	..
<i>J714</i>	HfrB2	ETH	s	r	s	63	..	72	..
<i>J715</i>	HfrB2	ETH	s	r	s	66	..	70	..
<i>J716</i>	HfrA	ETH	s	r	s	61	..	67	..
<i>J717</i>	HfrA	ETH	s	r	s	65.6(3)	..	72	..
<i>J718</i>	HfrA	ETH	s	r	s	63	..	75	..
<i>J719</i>	HfrA	ETH	s	r	s	63	..	71	..
<i>metK720</i>	HfrB2	ETH	r	r	r	72	78	78	85
<i>K721</i>	HfrB2	ETH	r	r	r	71.5(6)	69	69	75
<i>K722</i>	HfrB2	ETH	r	r	r	72	74	74	78
<i>K723</i>	HfrB2	ETH	r	r	r	76	78	78	82
<i>K724</i>	HfrB2	ETH	r	r	r	71	73	73	77
<i>K725</i>	HfrB2	ETH	r	r	r	71	73	73	77
<i>K726</i>	HfrB2	ETH	r	r	r	77	79	82	84
<i>K727</i>	HfrB2	ETH	r	r	r	74	74	76	79
<i>K728</i>	HfrA	ETH	r	r	r	73(3)	79	77	80
<i>K729</i>	HfrA	ETH	r	r	r	82.5(2)	87	87	87
<i>K730</i>	HfrB2	AM	r	r	r	67.3(3)	71	66	72
<i>K731†</i>	HfrH5	NOL	r	r	r	55	69	58	59
<i>K732†</i>	HfrH5	NOL	r	r	r	60(3)	65	61	65
<i>K733†</i>	HfrH5	NOL	r	r	r	58	64	59	65
<i>K734†</i>	HfrH5	NOL	r	r	r	59	64	59	67

Abbreviations used: *met* = methionine mutants; s = sensitivity; r = resistance; Hfr = high frequency male or donor; AM =  $\alpha$ -DL-methyl methionine; ETH = DL-ethionine; NOL = DL-norleucine; \* an average if > 1 measurement made; ( ) = number of times measurement made if > 1; † = mutants not excreting methionine.

they excreted at both temperatures but there was an overall tendency for greater excretion at 25° than at 37°. Twenty-seven of the 30 excreting mutants excreted more at 25°. The remaining three mutants (*metJ716*, *K718* and *K719*) excreted more at 37°. Methionine excretion in MM at 25° and 37° (assayed by the growth response of *E. coli* 3/62) was measured for 5 mutants which included representatives of all the three groups of analogue resistant mutants (Table 2). Variation between mutants in the amount of methionine excreted is illustrated by the excretion by two mutants (*metI701* and *706*) of two to four times as much methionine as the other three (*metJ714*, *K721* and *K725*) at 25°. Also, for three of the mutants (*metJ714*, *K721* and *K725*) methionine excretion was more marked at 25° than at 37°.

Further liquid culture experiments at 25° with representatives of all three groups (*metI706*, *J714* and *K721*) involving the use of S<sup>35</sup>-cysteine confirmed that the compound excreted was methionine in that isotope from cysteine was incorporated into a compound which behaved like methionine on paper chromatograms. *MetI706* incorporated about four times as much isotope as *metJ714* and *K721* which is in accordance with the measurements recorded in Table 2.

The growth rates in MM of the three parental strains and all analogue resistant mutants were measured (Table 1). Mean generation times for HfrA, B2 and H5 were 55.5, 55.7 and 52 min respectively. The average mean generation time for *metI* mutants and *metK* mutants which did not excrete methionine (56 min, var. 51–59 and 58 min, var. 55–60 respectively) were very similar, whereas those for *metJ* (63.3 min, var. 62–67) and *metK* mutants which did excrete methionine (73.4 min, var. 70–85) differed both from each other and from the *metI* and non-excreting *metK* mutants. Additions of 0.134 mM (20 µg/ml) methionine to cultures of the more slowly growing *metJ* and *metK* mutants resulted in no increase in growth rate.

To facilitate comparison with the analogue cross-resistance of the mutants on solid medium (MA) the growth of each mutant was also tested at least once in liquid medium (MM) containing either  $\alpha$ -methyl methionine, ethionine or nor-

TABLE 2

*Excretion of methionine by analogue-resistant strains*

Strain	Methionine excreted (mµmole/mg. dry wt. organisms/hr.)	
	At 25°	At 37°
HfrB2	0	0.5
<i>metI701</i>	10.7	**
<i>metI706</i>	17.5	21.4
<i>metJ714</i>	3.7	0.5
<i>metK721</i>	7.6	2.5
<i>metK725</i>	4.6	0

\*\* not tested

Abbreviations as in TABLE 1.

leucine at 1 mg/ml. Where growth was obtained the mean generation time was calculated (Table 1). It is seen that the resistance of *metJ* mutants to ethionine alone is confirmed but that of *metI* mutants to  $\alpha$ -methyl methionine alone is not. Seven of the 12 *metI* mutants (*metI703, 704, 705, 709, 710, 711* and *712*) showed some cross-resistance to ethionine and two some cross-resistance to norleucine (*metI703* and *704*). Also, although the *metK* mutants were confirmed in their cross-resistance to all three analogues, there was a marked tendency for slower growth in analogue supplemented MM than in MM especially in the presence of norleucine.

*Uptake of C<sup>14</sup> ethionine and C<sup>14</sup> methionine by analogue resistant mutants:* These experiments were designed to find out if any ethionine resistant mutants (*metJ* and *K*) possessed defects in the uptake of C<sup>14</sup> ethionine. All *metJ* and *K* mutants were tested and their responses were compared with the uptake of C<sup>14</sup> methionine by two *metJ* (*713* and *717*) and 4 *metK* (*721, 728, 730* and *732*)

TABLE 3

*Uptake of C<sup>14</sup> from labelled ethionine and methionine*

Strain	Uptake ( $\mu\text{g/hr/ml}$ culture)	
	ethionine	methionine
HfrA (SR305)	0.140	1.559
HfrB2	0.180	3.236
HfrH5	0.141	2.446
<i>metI703</i>	0.099	2.062
705	0.093	....
<i>metJ713</i>	0.110	1.889
J714	0.131	....
J715	0.136	....
J716	0.105	....
J717	0.074	2.978
J718	0.098	....
J719	0.090	....
<i>metK720</i>	0	....
K721	0	1.705
K722	0	....
K723	0	....
K724	0	....
K725	0	....
K726	0	....
K727	0	....
K728	0	1.706
K729	0	....
K730	0	2.245
K731†	0.066	....
K732†	0.016	4.080
K733†	0.027	....
K734†	0.023	....

Abbreviations as in TABLE 1.



mutants and the uptake of both labelled compounds by all three parental strains. Results are expressed as uptake in  $\mu\text{g/hr/ml}$ . (Table 3).

Both ethionine and methionine were taken up by *metJ* mutants although three (*metJ717*, *718* and *719*) took up less ethionine than other *metJ* mutants and the parental strains. In contrast the methionine excreting *metK* mutants (720–730 inclusive) took up no ethionine and the non-excreting mutants (731–734 inclusive) very little although methionine uptake was, on the whole, unaffected. Uptake of ethionine and methionine was linear in all cases except that for *metI703* it appeared to be exponential.

*Regulation of methionine-forming enzymes in the wild-type and analogue resistant mutants:* Most of the analogue resistant mutants excreted methionine (Tables 1 and 2) and so their methionine enzymes might have been less sensitive to regulation by methionine. The effect of growth in the presence of methionine on the cystathionine synthetase (*metB*), cystathionase (*metC*) and homocysteine methylase complex (*metE*, *F* and *H*) (Figure 1) in wild type (HfrB2) and several analogue-resistant mutants was therefore tested either at 25° or 37° or at both temperatures. Two *metI*, two *metJ* and four *metK* mutants were each used in some or all of the experiments. The data appear in Tables 4, 5 and 6.

All the methionine-forming enzymes of wild-type *S. typhimurium* HfrB2 tested were subject to repression at both 25° and 37° but the repression was apparently non-coordinate. Thus, when the organisms were grown with 10 mM (1.49 mg/ml) methionine, cystathionine synthetase (Table 4) and the enzymes of the homocysteine methylase complex (Table 6) were repressed by 80–95% but cystathionase by only 40–45% (Table 5). Enzyme levels were about twice as high in organisms cultured at 25° than in those grown at 37° (e.g. the cystathionase, Table 5).

Enzyme levels in the *metI* strains tested were similar to those in HfrB2 and growth in the presence of methionine resulted in a similar degree of repression i.e., a marked repression of the cystathionine synthetase (Table 4) and homocysteine methylase complex (Table 6) but only about 40% repression of the cystathionase (Table 5).

TABLE 4

*The effect of methionine on the formation of cystathionine synthetase at 25°*

Strain	Cystathionine synthetase activity ( $\mu\text{mole cystathionine/mg protein/hr}$ )	
	Organisms cultured without methionine	Organisms cultured with methionine
HfrB2	0.75	0.14
<i>metI706</i>	0.34	0.10
<i>metJ714</i>	16.0	14.7
<i>metK721</i>	8.9	10.7
<i>metK731</i> †	0.90	0.21

Methionine concentration 10mM (1.49 mg/ml).  
Abbreviations as in TABLE 1.

TABLE 5

*The effect of methionine on cystathionase formation*

Strain	Cystathionase activity ( $\mu\text{g}$ pyruvate/mg protein/hr)			
	Organisms cultured at 25°		Organisms cultured at 37°	
	Methionine absent	Methionine present	Methionine absent	Methionine present
HfrB2	63	35	39	24
<i>metI701</i>	**	**	34	15
<i>metI706</i>	64	37	36	24
<i>metJ714</i>	118	122	73	70
<i>metJ715</i>	**	**	84	76
<i>metK720</i>	108	94	64	43
<i>metK721</i>	114	113	60	61
<i>metK725</i>	87	95	43	26
<i>metK731+</i>	55	36	**	**

\*\* not tested. Methionine concentration 10mM (1.49 mg/ml).

Other abbreviations as in TABLE 1.

In contrast to the apparently normal enzyme levels and repressibility of *metI* strains, the enzymes of *metJ* and *metK* strains which excreted methionine were almost insensitive to repression by methionine. This was apparent in two ways. Firstly, after culture in MM (i.e. with methionine) enzyme levels were generally higher in *metJ* and *metK* mutants than in wild type; this derepression was most marked for cystathionine synthetase (Table 4). Secondly, these *metJ* and *metK* mutants were either insensitive, or only slightly sensitive, to repression by exogenous methionine. Thus the cystathionase of the *metJ* mutants was clearly non-repressible by 10 mM methionine at either 25° or 37° as was that of the *metK* mutants at 25° (Table 5), but this enzyme and those of the methylase complex (Table 6) in *metJ* and methionine excreting *metK* strains were, with the excep-

TABLE 6

*The effect of methionine on the formation of homocysteine methylase at 37°*

Strain	Methionine formed ( $\mu\text{mole}/\text{mg}$ dry wt./hr)			
	Cobalamin present		Cobalamin absent	
	Organisms cultured without methionine	Organisms cultured with methionine	Organisms cultured without methionine	Organisms cultured with methionine
HfrB2	300	45	60	4
<i>metI701</i>	220	70	73	0
<i>metI706</i>	340	60	80	15
<i>metJ714</i>	580	410	400	305
<i>metJ715</i>	485	280	295	130
<i>metK720</i>	765	655	480	510
<i>metK721</i>	690	590	600	530
<i>metK725</i>	885	550	450	380
<i>metK731+</i>	283	76	136	4

Methionine concentration 10mM (1.49 mg/ml).

Abbreviations as in TABLE 1.

tion of the cystathionase of *metK721* and the methylase of *K720*, somewhat repressible at 37°. In fact, the repressibility of the cystathionase of these *metK* mutants, except *metK721*, at this temperature, was similar to that of the wild-type enzyme (30–40%—Table 4) although that of the methylase of *metJ* and *metK* mutants was 30–55% and 10–40% respectively, clearly lower than the 85–95% repressibility of this enzyme in wild type (Table 6).

In *metK731*, a representative of the *metK* mutants which did not excrete methionine, the cystathionine synthetase and cystathionase levels at 25° (Tables 4 and 5) and homocysteine methylase levels at 37° (Table 6) were very similar to wild type. These enzymes in this mutant were also as repressible by methionine as those of the wild type.

Thus the data of Tables 4, 5 and 6 indicate that *metI* mutants and non-excreting *metK* mutants have enzyme levels similar to the wild-type HfrB2 and are repressible by methionine whereas *metJ* mutants and excreting *metK* mutants have higher enzyme levels than HfrB2 and are essentially non-repressible, i.e., they are derepressed.

The immediate inhibitory effect of  $\alpha$ -methyl methionine on growth of wild-type *S. typhimurium* (Figure 2) suggested that the analogue might mimic methionine as a feedback inhibitor of homoserine *O*-transsuccinylase (*metA*). The marked methionine excretion by *metI* mutants associated with unaltered repressibility might then be due to an altered feedback inhibitory response to methionine and  $\alpha$ -methyl methionine. It has not yet been possible to test the effect of the analogue on resistant strains as it is difficult to assay homoserine-*O*-transsuccinylase in the presence of cystathionine synthetase and no analogue resistant mutants possessed a deficiency in the cystathionine synthetase structural gene (*metB*). However, the activity of homoserine-*O*-transsuccinylase of *metB23* was reduced by 91% in the presence of 0.05 mM (8.2  $\mu$ g/ml)  $\alpha$ -methyl methionine although concentrations up to 1 mM (163  $\mu$ g/ml) did not affect the other methionine-forming enzymes.

*Mapping the sites of mutation of analogue resistant mutants: A. Conjugation.* All analogue resistant mutants were derived from Hfr strains. In the case of derivatives of HfrA and H5 this facilitated determination of the approximate location of their sites of mutation by measuring the relative frequency of analogue resistance amongst prototrophic recombinants in conjugation between each of them and representative mutants of the *metE*, *metB*, *metA*, *pyrC*, *tyrC*, *cysC*, *serA* and *argE* genes (Figure 3). (The fertility of HfrB2 derivatives was too low to permit this.) Plate mating was carried out on MA and followed by replication to MA and MA supplemented with the appropriate analogue. The number of recombinants screened in each cross was seldom less than 50 and usually greater than 100.

In crosses involving the 11 derivatives of HfrA, all 5 *metI* mutants gave the highest frequency of analogue resistant recombinants with the *metA* recipient (98–100%), all 4 *metJ* mutants with the *metB* recipient (92–100%) and the two *metK* mutants with the *serA* recipient (40–70%). All 4 *metK* derivatives of HfrH5 also gave the most analogue resistant recombinants with the *serA* recipient (65–77%).

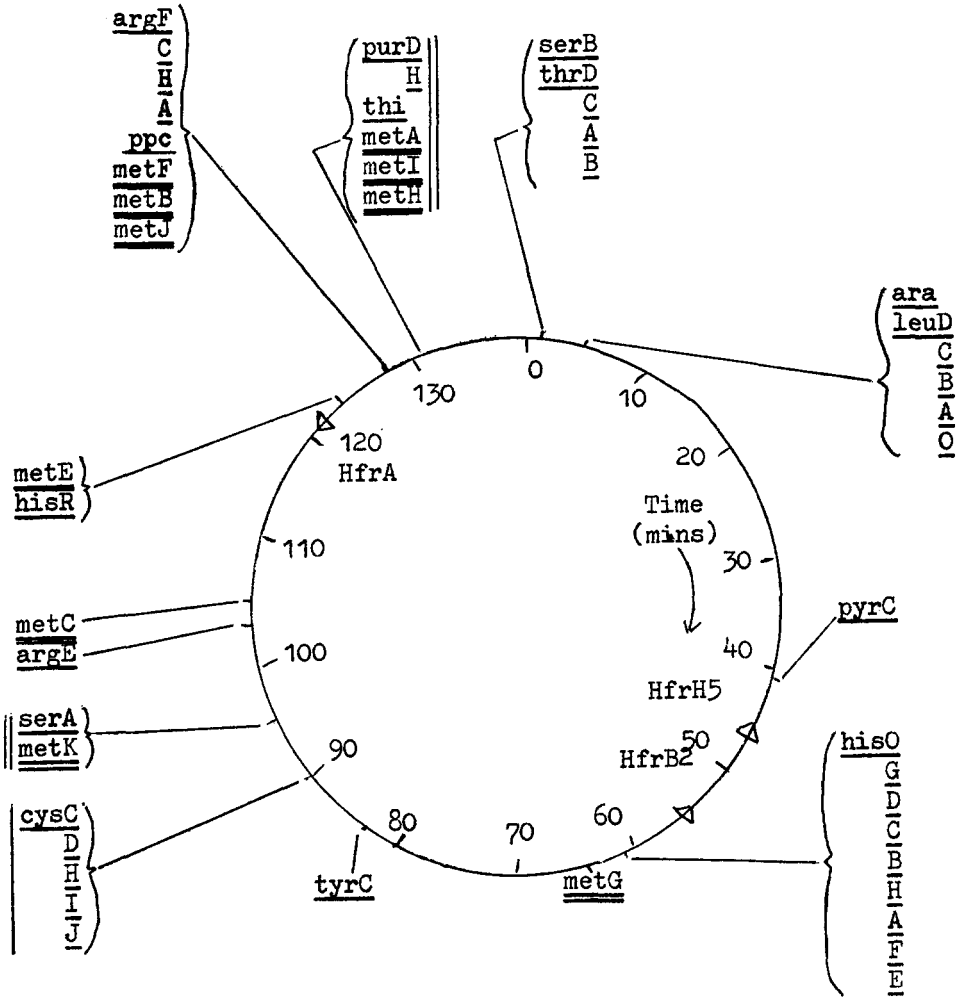


FIGURE 3.—Part of the circular linkage map of *Salmonella typhimurium*. (After SANDERSON and DEMEREC 1965; SANDERSON, unpublished; AYLING, unpublished).

The time scale on the inside of the circle is based on the time of entry of the different genes during Hfr conjugation.

- } = transducing fragment; gene order and orientation known.
- |} = transducing fragment; gene order known, orientation unknown.
- ||} = transducing fragment; gene order and orientation unknown.

Symbols refer to genes determining:

1. Requirements for arginine (*arg*), cysteine (*cys*), histidine (*his*), leucine (*leu*), methionine (*met*), purine (*pur*), pyrimidine (*pyr*), serine (*ser*), thiamin (*thi*), threonine (*thr*), tyrosine (*tyr*).
2. Utilization of arabinose (*ara*).
3. Deficiency of phosphoenolpyruvate carboxylase (*ppc*).

**B. Transduction.** It seemed likely that the location of the sites of mutation of the analogue resistant derivatives of HfrB2 would be similar to those suggested by the results of the conjugation experiments using the derivatives of HfrA and

HfrH5. This was confirmed in exploratory co-transduction experiments in which relative frequency of donor phenotype recombinants (CLOWES 1958) was determined in crosses between the relevant 7 *metI*, 3 *metJ* and one *metK* mutant and *metA*, *metB* and *serA* recipients. The results obtained prompted more precise mapping of the site of mutation to analogue resistance in each mutant in further co-transduction experiments both by repeating and extending the exploratory experiments and including as recipients, where appropriate, mutants of the *metH* and *metF* genes which are themselves co-transducible with the *metA* and *B* genes respectively (CHILDS and SMITH unpublished; SMITH 1961).

Before this could be done two preliminary steps had to be carried out. The derivatives of HfrA were found to be "rough" and were not lysed by either the H1 or the H4 strain of phage P22. It was, therefore, necessary to transfer the analogue resistance marker from the HfrA derivatives to appropriate "smooth" phage sensitive bacteria by conjugation. Analogue resistant prototrophic recombinants were obtained in plate mating experiments as previously described. They were then taken through two single colony isolations and their methionine excreting properties and analogue cross-resistance determined to confirm identity with the phenotype of the original mutant before normal propagation of transducing phage. Secondly, all *metH* mutants were initially derived from *metE205* because, as can be seen from Figure 1, a deficiency for cobalamin dependent  $N^5$ -methyltetrahydrofolate-homocysteine transmethylase in the indirect methylation of homocysteine (*metH*) can only be detected if direct methylation does not occur, i.e., that there is also a deficiency for  $N^5$ -methyltetrahydropteroyltriglutamate-homocysteine transmethylase (*metE*) (CHILDS and SMITH unpublished). Thus, each *metH* mutant carries mutations in each of two genes located in different transducing fragments (Figure 3). To permit the growth of *metH*<sup>+</sup>*metE205* recombinants it was, therefore, necessary always to supplement the selective MA with vitamin B12 to which *metE* mutants respond as an alternative to methionine. *metHmetE205*<sup>+</sup> recombinants also grow on this medium because with the *metE* enzyme functional the effect of a *metH* deficiency is masked. In transduction between *metHmetE205* × wild type a total of 360 recombinant colonies selected on four MA + B12 plates were replicated to MA. The application of a heterogeneity  $\chi^2$  test to the relative numbers of *metH*<sup>+</sup>*metE205* and *metHmetE205*<sup>+</sup> recombinants obtained indicated that their ratio did not differ significantly from 1:1 ( $P = 0.5$ ). Thus, when the frequency of co-transduction of analogue resistance was being scored in crosses involving *metH* recipients the number of wild type recombinants obtained was always halved.

The percentage of analogue resistant wild-type recombinants was determined in transduction between *metA94* and *metH463* and all *metI* mutants, *metB23* and *metF96* and all *metJ* mutants, and *serA11* and all *metK* mutants. The results appear in Table 7, and they are incorporated in Figure 3. First, all *metI*, *J* and *K* mutants, respectively, yielded similar numbers of analogue resistant recombinants in each relevant cross, thus indicating that each group comprised mutants whose sites of mutation were closely linked probably within the same gene. Secondly, in addition to confirming the co-transduction of *metI* with *metA* (96.7%, var. 95–100) and *metJ* with *metB* (93.6%, var. 90–97), co-transduction

TABLE 7

*Frequency of co-transduction of metI, J and K with linked genes*

(a) <i>metI</i> mutants						
Donor	<i>metA94</i>		Recipient		<i>metH463</i>	
	Nos.	%	Nos.	%	Nos.	%
<i>metI701</i>	80	100	155	40		
702	264	97	117	43		
703	244	98	76	53		
704	185	97	422	43		
705	215	96	408	31		
706	406	99	1158	46		
707	164	95	462	45		
708	578	98	187	34		
709	364	96	252	43		
710	199	99	263	48		
711	513	98	311	43		
712	1014	99	620	51		

(b) <i>metJ</i> mutants						
Donor	<i>metB23</i>		Recipient		<i>metF96</i>	
	Nos.	%	Nos.	%	Nos.	%
<i>metJ713</i>	617	96	777	52		
714	1342	96	621	49		
715	993	95	384	43		
716	283	90	258	43		
717	257	97	328	43		
718	558	94	455	41		
719	912	88	302	31		

(c) <i>metK</i> mutants			
Donor	Recipient		%
	Nos.	<i>serA11</i>	
<i>metK720</i>	1604	1.5	
721	881	1.1	
722	137	3.0	
723	214	1.6	
724	1282	1.2	
725	830	1.3	
726	1939	1.3	
727	435	2.3	
728	375	1.3	
729	390	1.0	
730	1231	1.7	
731†	819	2.54	
732†	214	0.47	
733†	395	1.52	
734†	313	1.0	

Abbreviations as in TABLE 1. In all these experiments prototrophic recombinants were selected and their analogue resistance scored as the unselected marker; the percentage given for each cross is that of analogue resistant prototrophs.

of *metI* with *metH* (43.25%, var. 31–53) and *metJ* with *metF* (43.4%, var. 31–52) was also established, clearly indicating that *metI*, *A* and *H* were linked and located on one transducing fragment and *metJ*, *B* and *F* on another. Thirdly, the co-transduction of *metK* with *serA* was also confirmed. However, it occurred at a very low frequency (1.52%, var. 0.5–3.0). The reliability of these data was tested in reconstruction experiments designed to eliminate spontaneous mutation of *serA11* or donor bacteria in *metJ* and *K* phage preparations as a source of analogue resistant colonies in these experiments. Recombinants obtained in transduction between *serA11* and wild type (HfrB2) and between an unlinked *thrA* mutant and *metJ714* and each of five different *metK* mutants (720, 721, 723, 724 and 726) were screened for analogue resistance. None of the 505 recombinants from the first cross, or 664, 1037, 960, 270 and 796 recombinants, respectively, from each of the other 6 crosses, was analogue resistant.

## DISCUSSION

Clearly the overproduction of methionine by *metI*, *J* and some *K* mutants (Tables 1 and 2) strongly suggests that they are deficient in the regulation of methionine synthesis and this conclusion is supported by the evidence for derepression of cystathionine synthetase, cystathionase and the homocysteine methylase complex in the *metJ* and methionine excreting *metK* mutants (Tables 4, 5 and 6). Recent work shows that a methionine-specific aspartokinase and a methionine-specific homoserine dehydrogenase are also derepressed in these mutants (ROWBURY, unpublished). To facilitate speculation on the likely nature of the *metI*, *J* and *K* mutants, their main properties, given in detail in Tables 1–7 inclusive, are summarized in Table 8. Each group will be considered separately.

1). *MetI*. These mutants are resistant only to inhibition by  $\alpha$ -methylmethionine and this analogue is quite different from ethionine and norleucine in its influence

TABLE 8

*Summary of properties of methionine regulatory mutants*

Strains	Analogue resistance on MA			Excretion of methionine	Average generation time in MM (min)	Uptake of $C^{14}$ from ethionine	Repression of methionine-forming enzymes by 10mM methionine	Linkage (average % co-transduction)
	AM	ETH	NOL					
<i>metI</i>	r	s***	s***	+	56	+	+	{ <i>metA</i> 96.7 <i>metH</i> 43.25
<i>metJ</i>	s	r	s	+	63.5	+	—	{ <i>metB</i> 93.6 <i>metF</i> 43.4
<i>metK</i>	{ r	{ r	{ r	+	73.4	—	—	} <i>serA</i> 1.52
	{ r	{ r	{ r	—	58	+ / —	+	
wild type	s	s	s	—	54	+	+	

Abbreviations as in previous tables.

\*\*\* Some mutants show resistance to ethionine and norleucine in liquid medium.

upon the growth of wild type organisms (Figure 2) in that its effect is instantaneous rather than gradual. This could indicate the response of a particularly sensitive control mechanism. Although *metI* mutants excrete methionine, their growth rates and the repressibility of their methionine-forming enzymes by methionine are very similar to those of wild-type organisms. Also their sites of mutation are very close to *metA*, the structural gene for homoserine-*O*-transsuccinylase, the first enzyme of the methionine pathway, and in strain *metB23* the activity of this enzyme is reduced in the presence of low concentrations of methionine or  $\alpha$ -methylmethionine which do not affect the other enzymes of the pathway. All this evidence would indicate that *metI* mutants are feedback inhibitor resistant (MOYED 1961; UMBARGER 1961).

However, there are two reasons for reservations about this conclusion. Firstly, if homoserine-*O*-transsuccinylase is subject to feed-back inhibition then it is likely to be an allosteric protein (MONOD, CHANGEUX and JACOB 1963) and, therefore, probably a multimer (CRICK and ORGEL 1964). If this were the case, evidence of intragenic complementation between *metA* mutants would be anticipated, but in appropriate transduction analysis involving crosses between 42 of 44 *metA* mutants, no abortive transduction was detected (SMITH and CHILDS 1966). On the other hand it should be noted that complementation does not occur between *hisG* mutants of *S. typhimurium* (LOPER *et al.* 1964) although there is evidence for sub-unit structure of the relevant enzyme (VOLL, APELLA and MARTIN 1967). Secondly, in transduction between *metA43*, a deletion mutant failing to recombine with all known *metA* mutants (SMITH and CHILDS 1966), and *metI* mutants the number of analogue resistant wild type recombinants was 99%, i.e., similar to that using *metA* point mutants as recipients. This would indicate that mutation resulting in a feedback inhibitor resistant homoserine-*O*-transsuccinylase does not occur anywhere within the *metA* gene, a situation different from that for the *hisG* gene which specifies the feedback inhibitor sensitive first enzyme of histidine synthesis in *S. typhimurium* (SHEPPARD 1964). In this gene 7, sites of mutation to feedback inhibitor resistance are located near the middle of this gene and one towards the end of it. It could be that homoserine-*O*-transsuccinylase comprises two different polypeptides; one (*metA*) concerned with enzymic activity and the other (*metI*) concerned with interaction with the inhibitor methionine.

2). *MetI*. Methionine overproduction in these mutants is associated with resistance only to inhibition by ethionine, slightly slower growth rate in MM as compared with wild type (uninfluenced by the addition of methionine to the medium), almost unimpaired uptake of C<sup>14</sup>-ethionine and marked derepression of the methionine enzymes assayed. The location of the *metI* gene is interesting. It is closely linked to two methionine structural genes, *metB* and *F*, which specify cystathionine synthetase and N<sup>5</sup>,N<sup>10</sup>-methylene tetrahydrofolate reductase (one of the enzymes of the homocysteine methylase complex—Figure 1) respectively. In wild-type organisms this synthetase (Table 4) and the enzymes of the complex (Table 6) were repressed to a similar extent by methionine suggesting coordinate control. This is in contrast to the cystathionase (Table 5) specified by the *metC* gene which was much less sensitive to such repression. The results of recent preliminary experiments involving transduction between *metB* and *metF* mutants



and *argFmetJ* double mutants, and *metBmetJ* double mutants and *metF* mutants are compatible with the sequence *metJ-metB-metF-argF*, (DR. P. AYLING, unpublished observations). Thus the *metJ* mutants could be of the operator constitutive type (JACOB and MONOD 1961) *metJBF* comprising a methionine operon. Against this it must be remembered that the *metE* and *H* enzymes (*N*<sup>5</sup>-methyltetrahydropteroyltriglutamate and cobalamin dependent *N*<sup>5</sup>-methyltetrahydrofolate-homocysteine transmethylases respectively) are also part of the methylase complex and they are presumably coordinately repressed with cystathionine synthetase but the *metE* and *H* genes are not part of the *metJBF* cluster (Figure 3). Also, in *metJ* mutants the *metC* enzyme (cystathionase) is as derepressed as cystathionine synthetase and the methylase complex. This would not be expected if *metC* were in a separate unit of coordinate control.

The *metJ* gene could be a repressor gene but all in all the properties of *metJ* mutants may be most compatible with a more general metabolic defect resulting from abnormal regulation of methionine biosynthesis. HistidinyI-tRNA is involved in the regulation of histidine synthesis in *S. typhimurium* (ROTH *et al.* 1966); possibly methionyl-tRNA plays an analogous role in methionine synthesis and *metJ* mutants are in some way deficient in the synthesis or normal activity of methionyl-tRNA.

3). *MetK*. The methionine excreting mutants of this group are similar to *metJ* mutants in that their methionine-forming enzymes are derepressed but differ from them in their resistance to all three analogues, the possession of a much more reduced growth rate, inability to take up ethionine and the location of their sites of mutation some distance from any known *met* gene. This genetic evidence would eliminate consideration of these mutants as operator constitutives and their slow growth rate and inability to take up ethionine would not support a deficiency in the synthesis of a repressor unless the gene also specified, for example, a permease. The non-excreting *metK* mutants also result from mutation at sites near the *serA* gene but repression of their methionine-forming enzymes by methionine is virtually the same as that in wild type so they are, therefore, unlikely to be mutants of either operator or repressor genes. They could, however, possess a selective permeability defect resulting in less than 50% of the ability of wild-type organisms to take up ethionine but unimpaired uptake of methionine (Table 3).

As the frequency of co-transduction of *metK* and *serA* is approximately the same in crosses involving all *metK* mutants it seems likely that their sites of mutation are closely linked possibly within the same gene, mutation of which results in resistance to inhibition by all three methionine analogues but differing in other associated properties. (It is, of course, impossible to test complementation between excreting and non-excreting *metK* mutants.) On balance, two properties shared by the excreting *metK* and *metJ* mutants, namely, a slow growth rate unaffected by the presence of excess methionine and derepression of the methionine-forming enzymes, could indicate that these two types of mutants possess different methionyl-tRNA deficiencies. In this case it must also be postulated that in the wild type the *metK* gene is concerned with the induction of a system involved in the uptake of ethionine deficient in excreting mutants, and that in non-excreting

mutants repression functions are normal but uptake functions much reduced in efficiency.

It is felt that studies of the *metJ* and *K* mutants may indicate that charged methionyl-tRNA could be concerned in the regulation of methionine synthesis in *S. typhimurium* possibly as a co-repressor and that either the *metJ* or the *metK* genes specify either methionyl-tRNA synthetase or are concerned with the synthesis of methionine-specific tRNA itself. The role of the *metG* gene is at present obscure but although it appears not to be concerned with the synthesis of a methionine enzyme intra-genic complementation occurs between *metG* mutants (SMITH and CHILDS 1966) indicating its association with the synthesis of a protein.

Thus, the scattered gene system concerned with methionine synthesis possesses both unique features of its own and marked similarities to the more classical operons of, for example, the histidine (AMES *et al.* 1967) and tryptophan systems (BAUERLE and MARGOLIN 1966) in this organism. Much clarification is desirable and work has been started on possible defects in the synthesis of methionyl-tRNA in *metJ* and *K* mutants (ROWBURY and GROSS unpublished) and the ordering and orientation of the two clusters of methionine genes (AYLING, personal communication). Attempts are also being made to establish appropriate partial diploids to test the dominance or recessivity of the alleles of the regulatory genes (CHATER, personal communication). Additional support for the hypothesis proposed in this discussion is emerging from studies of suppressors of *metG* mutants (LAWRENCE, unpublished), and the recent identification of at least three genes concerned with the inter-relationship of cysteine and methionine synthesis (QURESHI, personal communication) may permit investigation of the control and integration of these two pathways.

The excellent technical assistance of Miss ELAINE WARDEN, Mr. M. OAKES, and Mr. M. QURESHI is gratefully acknowledged. One author (D.A.L.) was in receipt of a Science Research Council Studentship during the course of this work.

#### SUMMARY

The methionine analogues  $\alpha$ -methyl methionine, ethionine and norleucine were found to inhibit the growth of wild type *Salmonella typhimurium* in minimal medium. Inhibition by  $\alpha$ -methyl methionine differed from that by ethionine and norleucine in that it was instantaneous and more drastic. Twelve mutants resistant to inhibition by  $\alpha$ -methyl methionine (*metI*), 7 to ethionine (*metJ*) and 15 to  $\alpha$ -methyl methionine, ethionine and norleucine (*metK*) were isolated. All except 4 *metK* mutants overproduced and excreted methionine. The growth rates of *metI* and non-excreting *metK* mutants were unaltered in minimal medium but those of both *metJ* and excreting *metK* mutants were reduced. *MetI* and *J* mutants took up ethionine from their culture medium but excreting *metK* mutants did not and non-excreting mutants only at a reduced rate. The methionine-forming enzymes of *metI* and non-excreting *metK* mutants assayed were repressed by methionine as in wild type; those of *metJ* and excreting *metK*

mutants were not. In transduction experiments the sites of mutation of all *metI* and *metJ* mutants, respectively, were shown to be linked to each of the two separate pairs of co-transducible methionine structural genes, whereas those of the *metK* mutants were located together but in a different region of the bacterial genome. It is thought likely that the *metI* mutants are feedback inhibitor resistant and that *metJ* and *metK* mutants could be deficient in different stages in the synthesis of a co-repressor which may be methionyl-tRNA.

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