Biochemical and Physiological Properties of Methionyl-sRNA Synthetase Mutants of *Salmonella typhimurium*

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SUMMARY

Two methionine auxotrophs (*metG*) of *Salmonella typhimurium* with the structural genes of the regulon intact have been studied. They possessed abnormal growth kinetics and the effect of the *metG* mutation on protein, ribonucleic acid and DNA synthesis suggested that these strains were impaired in their ability to synthesize protein; since they were able to synthesize methionine, but still required it for growth, they might have been defective in methionine activation for protein synthesis. To test this, the activity of methionyl-sRNA synthetase (L-methionine: sRNA ligase (AMP) EC 6.1.1.10) was determined in enzymic extracts of wild-type and *metG* strains. By using an acylation reaction the activation of methionine for protein synthesis was shown to be very decreased in *metG* extracts and this was reflected *in vivo* by a decreased level of charged methionyl-sRNA in mutant bacteria; in the pyrophosphate exchange assay mutants showed greatly increased K_m (methionine) values. The release of [³H]methionine from [³H]methionyl-sRNA was catalysed by wild-type extract, provided that pyrophosphate was present in the assay mixture, but not by the mutant extract. These results are discussed in relation to the two-part reaction catalysed by methionyl-sRNA synthetase. Mutant and wild-type enzyme behaviour differed at different pH values but not when subjected to chromatography on DEAE-cellulose or gel-filtration on Sephadex-G200. *MetG* mutants grown with limiting methionine had decreased values of all the biosynthetic enzymes except cystathionase, which was apparently de-repressed, suggesting that methionyl-sRNA was not the co-repressor for the methionine biosynthetic pathway.

INTRODUCTION

Recently, bacterial mutants which possess defective aminoacyl-sRNA synthetases for various amino acids have been isolated. Such mutants have been isolated on the basis of temperature sensitivity, e.g. valine (Eidlic & Neidhardt, 1965), alanine (Yanif, Jacob & Gros, 1965), phenylalanine (Eidlic & Neidhardt, 1965); analogue resistance, e.g. histidine (Roth & Ames, 1966), phenylalanine (Fangman & Neidhardt, 1964); as naturally occurring variants, e.g. glycine (Böck & Neidhardt, 1966; Neidhardt, 1966); and as amino acid-requiring auxotrophs, e.g. tryptophan (Hiraga, Ito, Hamada & Yura, 1967). *MetG* mutants of *Salmonella typhimurium* were originally isolated as methionine bradytrophs by Smith & Childs (1966). All the methionine biosynthetic enzymes were shown to be present but at apparently decreased values (Foster, cited by Smith & Childs, 1966) which originally led to the suggestion that

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metG mutants were super-repressed for the methionine biosynthetic enzymes. The present work shows that the requirement for methionine is due to an altered methionyl-sRNA synthetase.

In recent years, evidence that some amino acids must be attached to their specific sRNAs before they are able to bring about repression of their biosynthetic enzymes has been put forward. Perhaps the best-known example is that of histidine, where mutation in either of two genes, *hisS* or *hisR*, results in de-repression of the histidine biosynthetic enzymes (Roth & Ames, 1966; Silbert, Fink & Ames, 1966). In both cases there is a decrease in the amount of histidyl-sRNA formed, due to an increased K_m (histidine) of the histidyl-sRNA synthetase (*hisS*) or a decrease in the amount of active sRNA_{his} synthesized by the mutant bacteria (*hisR*). Since *metG* mutants have an altered methionyl-sRNA synthetase, the amounts of the methionine biosynthetic enzymes in these strains were studied. Some of these results have previously been reported briefly (Gross & Rowbury, 1969).

METHODS

Organisms. All the strains of *Salmonella typhimurium* used in this work were maintained on slopes of Oxoid nutrient agar, subcultured monthly and stored at 4° after incubation for 18 h. at 37°. The mutants *metG319* and *metG419* were isolated from the wild-type strain LT-2 and an Hfr derivative of LT-2 (HfrB2) was used as wild-type in these experiments. The mutant *metB23* is a methionine auxotroph and lacks the enzyme cystathionine synthetase. All strains were supplied by Dr D. A. Smith.

Growth of organisms. Organisms were grown on the glucose mineral salts medium (MM) of Davis & Mingioli (1950) supplemented with 0.04 mM-DL-methionine for the growth of methionine mutants. Conditions for growth, harvesting and preparation of enzymic extracts were as described by Rowbury & Woods (1961).

Phage propagation and assay. Phage P22.L4 is a virulent mutant of phage P22 (Smith & Levine, 1967) and was provided by Dr D. A. Smith. It was propagated on strain *metG319*. Phage (5×10^4 infective particles/ml.) were added to organisms growing exponentially in nutrient broth. Incubation was for 120 min. at 37°, after which cultures were shaken with a few drops of chloroform. Cell debris was removed by centrifugation (3000g for 10 min.) and the preparations stored over chloroform. For phage assay, samples were diluted in phosphate buffer (0.05 M, pH 7.4) and portions added to tubes of 4 ml. soft agar (0.2 % MgCl₂, 1 % Oxoid broth no. 2, 0.3 % NaCl, 0.75 % Difco Bacto agar). After adding 3×10^7 indicator bacteria (strain *metG319*) the soft agar was poured on to a basal agar (25 ml.) consisting of 1 % Trypticase agar base, 1.4 % Difco Bacto agar, 0.5 % NaCl. Plates were incubated at 37° overnight.

Incorporation of [¹⁴C]phenylalanine into protein. This was followed by a method based on that of Neale & Tristram (1963). Exponentially growing cultures in MM medium, supplemented where necessary, were harvested and washed with two culture volumes of MM medium. The washed bacteria were resuspended in 10 ml. fresh MM medium at equivalents of 125 µg. dry weight bacteria/ml. and incubated with aeration at 37°. Incorporation was initiated by addition of [¹⁴C]phenylalanine (1.25 µCi, 7 mCi/mM) and 1 ml. samples were transferred at intervals into an equal volume of 10 % trichloroacetic acid and allowed to stand for 30 min. at room temperature. Samples were

heated at 100° for 30 min. to remove nucleic acids, cooled and filtered through a Millipore filter (type HA). The precipitate was washed with two 2 ml. quantities of 5 % trichloroacetic acid (TCA), followed by a few drops of ether, mounted on an aluminium planchet and counted with a thin end-window Geiger-Müller tube (I.D.L. Ltd, Reading, Berkshire).

Incorporation of [¹⁴C]uracil into RNA. The method was essentially similar to that used to follow incorporation of [¹⁴C]phenylalanine into protein except that samples were not heated. The incubation mixture contained 0.6 µCi, [¹⁴C]uracil (50 mCi/mM). After standing for 30 min. at room temperature, samples were filtered and counted as before.

Incorporation of [³H]thymidine into DNA. Organisms growing logarithmically in MM medium + L-methionine (mM) + uridine (1.5 mM) + [³H]thymidine (2 µg./ml; 0.1 µCi/µg.) were rapidly harvested and washed with cold MM medium. They were then resuspended at equiv. 50 µg. dry wt./ml. in an incubation mixture which contained in 5 ml. of MM medium, 7.5 µmoles uridine and 10 µg. [³H]thymidine (0.1 µCi/µg.) with L-methionine and/or chloramphenicol (100 µg./ml.) added as required. Incubation was with shaking at 37° and samples were removed at 15 min. intervals into equal volumes of cold 10 % TCA. After standing overnight samples were filtered on Millipore filters (type HA), and after washing the filters with 5 % TCA containing 100 µg./ml. thymidine and drying they were placed in 10 ml. of scintillation fluid (0.6 % butyl PBD in toluene + methanol, 3 + 1, v/v) and counted.

Induction of D-serine deaminase (D-serine hydrolase (deaminating), EC 4.2.1.14). Overnight cultures of organisms were used to inoculate 300 ml. MM medium with or without 0.1 mM-DL-methionine and incubated at 37°. Once bacteria had attained the logarithmic phase of growth a sample was removed and a cell-free extract made, D-serine was then added to the culture flask to 3 mM. Extracts were made after 1, 2 and 3 h. and assayed for D-serine deaminase activity. The reaction mixture contained, in a total volume of 2 ml., 133 µmoles phosphate buffer (pH 7.5), D-serine (12 µmoles) and enzyme extract. Incubation was for 20 min. at 37° and the reaction then stopped by adding 0.5 ml. 25 % trichloroacetic acid. Precipitated protein was removed by centrifugation and pyruvate present in the supernatant fluid estimated by the method of Wijesundera & Woods (1962).

Assay for amino acid-dependent ATP-pyrophosphate exchange. The activation of methionine was measured by this method and the reaction mixture contained in a volume of 1 ml., 50 µmoles tris-HCl buffer (pH 7.5), 80 µmoles MgCl₂, 2 µmoles ATP, 3 µmoles. β-mercaptoethanol, 2 µmoles [³²P]pyrophosphate (100 to 200 counts/min./nmole), limiting enzyme and various amounts of methionine. Reaction was started by adding extract and after incubation for 15 min. at 37° protein was precipitated by adding 2 ml. 7.5 % TCA. After removal of the precipitate, 0.5 ml. charcoal suspension (10 % Darco G20 in 0.10 M-Na₄P₂O₇) and 4 ml. 1 % H₃PO₄ in 0.10 M-Na₄P₂O₇ was added to each tube. The charcoal was collected by filtration, washed six times with 4 ml. quantities of 0.05 M-sodium acetate buffer (pH 4.5), and radioactivity assayed by using a mica end-window Geiger-Müller tube.

Assay for aminoacyl-sRNA formation. The method used to follow methionyl-sRNA formation was based on that of Fangman & Neidhardt (1964). The reaction mixture contained, in a final volume of 0.5 ml., 25 µmoles tris-HCl buffer (pH 7.5), 1.5 µmole ATP, 1.5 µmole β-mercaptoethanol, 4 µmoles MgCl₂, 0.5 µCi [³H]methionine (30 mCi/

mm), 1.7 mg. sRNA and a limiting amount of enzyme. Reaction was started by adding enzymic extract, and after incubation for 5 to 20 min. at 37° a 0.3 ml. sample was withdrawn on to a rectangle of Whatman's 120-drop reaction paper impregnated with casein, and dropped into ice-cold 10 % TCA. Papers were then treated by the method of Mans & Novelli (1961) to remove lipid, dried and counted by scintillation in a mixture of 0.6 %, 2,5-diphenyloxazole, and 0.01 % 1,4-bis-(2,5-phenyloxazolyl) benzene in sulphur-free toluene.

AMP-dependent release of methionine from methionyl-sRNA. The release of [³H] methionine from [³H]methionyl-sRNA was followed by the technique of Hervé & Chapeville (1963). The reaction mixture contained, in a total volume of 0.5 ml., 50 μ moles tris-HCl buffer (pH 7.5), 8 μ moles MgCl₂, 2.5 μ moles AMP, 2.5 μ moles Na₄P₂O₇, [³H]methionyl-sRNA and limiting enzyme. Incubation was at 37° and 0.1 ml. samples were withdrawn at intervals on to Whatman 120-drop reaction paper and treated as above.

Assay of the methionine biosynthetic enzymes. The methionine biosynthetic enzymes were assayed as described previously (Rowbury & Woods, 1966; Rowbury, Lawrence & Smith, 1968).

Estimation of in vivo charged sRNA. The fraction of methionyl-sRNA existing in a charged form was determined by the method of Böck, Faiman & Neidhardt (1966), which uses the periodate method to destroy the amino acid-accepting capacity of uncharged sRNA.

DEAE-cellulose chromatography. Crude enzymic extract (5 ml., 10 mg. protein/ml.) was added to a column (25 $\times$ 1 cm.) of DEAE-cellulose (Whatman DE 11) and eluted with 400 ml. of a linear gradient of NaCl (0.05 to 0.5 M) at a flow rate of approximately 30 ml./h. Eighty 5 ml. fractions were collected and assayed for protein and synthetase activity.

Gel filtration on Sephadex G-200. One ml. crude extract (10 mg. protein) was added to the top of a column (80 $\times$ 1 cm.) of Sephadex-G 200 and eluted with 0.01 M-tris-HCl buffer (pH 7.5). Eighty 1 ml. fractions were collected and assayed for protein and synthetase activity. The void volume of the column was determined by measuring the exclusion of blue dextran; bovine serum albumin was used as a marker protein.

Chemicals. These were usually obtained from British Drug Houses Ltd, Poole, Dorset, and were of 'Analar' grade. Radioactive chemicals were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, and the sRNA used in the assay of methionyl-sRNA synthetase was a gift from the Microbiological Research Establishment, Porton.

Protein determinations. The spectrophotometric method of Layne (1957) was used.

RESULTS

Growth kinetics of metG mutants. The growth of the *metG* mutants *metG319* and *metG419* on different concentrations of DL-methionine was followed in MM medium; the results obtained are presented in Fig. 1(b) and 1(c) respectively. For comparison, the growth of a methionine auxotroph, *metB23*, on different methionine concentrations is shown in Fig. 1(a). As expected, the growth rate of *metB23* did not vary significantly at different methionine concentrations and continued until the exogenous methionine supply had been exhausted. Growth then ceased and the final bacterial

concentration was approximately proportional to the original concentration of DL-methionine. In contrast, the growth rate of *metG* mutants varied with methionine concentration.

These results are very like those obtained by Doolittle & Yanofsky (1968) with a tryptophanyl-sRNA synthetase mutant of *Escherichia coli* and suggested that *metG* mutants might be defective in their ability to activate methionine for protein synthesis. It was also possible that *metG* mutants were altered in the synthesis of S-adenosyl-methionine (SAM) so that SAM synthesis was decreased in the absence of methionine.

Fig. 1. Growth of *metB23*, *metG319* and *metG419* mutants of *Salmonella typhimurium* at different concentrations of DL-methionine. Overnight cultures were used to inoculate fresh MM medium containing DL-methionine at the required concentration. Cultures were shaken at 37° and extinction readings taken at intervals. Results are plotted for *metB23*, *metG319* and *metG419* in (a), (b) and (c) respectively. The concentrations of DL-methionine were: ○, 80 μM; ○●, 40 μM; ○, 30 μM; ◐, 20 μM; ●, 10 μM.

The behaviour of the *metG* mutants in the presence of the methionine analogues ethionine and norleucine did not support this view. Most methionine-requiring auxotrophs can grow and make protein for some time when ethionine or norleucine replace the methionine supplement, since the analogues can replace methionine in proteins. *MetG* mutants, however, showed no growth at all in the presence of the analogues, indicating that a process which can utilize the analogues was altered. Since these analogues are not utilized in SAM synthesis (Gross, 1969) it seemed likely that the process altered was protein synthesis.

Protein synthesis in metG319 and metB23 mutants: incorporation of [¹⁴C]phenylalanine into the hot TCA-insoluble fraction. Since *metG* mutants appeared to be abnormal in their growth kinetics it was decided to examine the effect of methionine starvation on protein synthesis in *metG* mutants. Protein synthesis was observed by following

the incorporation of [14 C]phenylalanine into a hot TCA-insoluble fraction and by following the synthesis of the inducible enzyme D-serine deaminase. Results presented in Fig. 2 show that in the absence of methionine, incorporation of [14 C]phenylalanine by *metG319* and *metB23* was very much decreased when compared with incorporation by wild-type organisms. In the presence of 5 μ M-DL-methionine the rate of incorporation by *metG319* was stimulated for about 30 min. but reached a maximum considerably lower than that for *metB23* and wild type.

Protein synthesis in metG319 and metB23 mutants: Induction of D-serine deaminase. Protein synthesis was also estimated by following the induction of the enzyme D-serine

Fig. 2

Fig. 3

Fig. 2. Incorporation of [14 C]phenylalanine into protein by *metB23* and *metG319* mutants. Exponentially growing bacteria were harvested, washed and used to inoculate MM medium containing the indicated supplements. Cultures were shaken at 37° and 1 ml. samples taken at intervals into an equal volume of 10 % trichloroacetic acid. Samples were treated as described in Methods and assayed for radioactivity. Plotting [14 C]phenylalanine incorporation against time gave the following curves: Δ , wild-type (MM); $\blacktriangle$, wild-type (5 μ M-L-methionine); ∇ , *metB23* (MM); $\blacktriangledown$, *metB23* (5 μ M-L-methionine); $\square$, *metG319* (MM); $\blacksquare$, *metG319* (5 μ M-L-methionine).

Fig. 3. Induction of D-serine deaminase synthesis in wild-type and *metG319* strains. D-serine (3 mM) was added to exponentially growing cultures, samples taken at intervals and assayed for D-serine deaminase activity. Assay procedure and growth conditions are given in Methods. Plotting D-serine deaminase activity (μ moles pyruvate formed/min./mg. protein) against time gave the following curves: $\square$, *metG319* (MM); Δ , wild-type (MM); $\blacksquare$, *metG319* (0.1 mM-DL-methionine); $\blacktriangle$, wild-type (0.1 mM-DL-methionine); Δ , wild-type (MM, in absence of D-serine).

deaminase, following addition of the inducer D-serine. Results presented in Fig. 3 show that in the absence of added methionine, *metG319* did not synthesize the enzyme even after addition of the inducer, whereas wild-type organisms began rapid synthesis of the enzyme directly after addition of the inducer. In the presence of 0.1 mM-DL-methionine, *metG319* synthesized D-serine deaminase at a rate somewhat less than that of the wild-type. These results confirmed that the *metG* mutation very much decreased the overall rate of protein synthesis unless exogenous methionine were present.

Fig. 4

Fig. 5

Fig. 4. Incorporation of [^{14}C]uracil into RNA by *met B23* and *metG319*. Overnight cultures were used to inoculate fresh MM medium containing [^{14}C]uracil and supplemented with methionine where indicated. Cultures were shaken at 37° and 1 ml. samples taken at intervals into an equal volume of cold 10% TCA and treated as described in Methods. Plotting [^{14}C]uracil incorporation against time gave the following results: Δ , wild-type (MM); $\blacktriangle$, wild-type ($5 \mu\text{M-L-methionine}$); ∇ , *metB23* (MM); $\blacktriangledown$, *metB23* ($5 \mu\text{M-L-methionine}$); $\square$, *metG319* (MM); $\blacksquare$, *metG319* ($5 \mu\text{M-L-methionine}$).

Fig. 5. Effect of chloramphenicol on [^{14}C]uracil incorporation in *metG319*. Experimental details were as described above, and chloramphenicol ($150 \mu\text{g./ml.}$) was added where indicated. Plotting [^{14}C]uracil incorporation against time gave the following results; $\blacktriangle$, wild-type (+chloramphenicol); $\blacksquare$, *metG319*(+chloramphenicol); $\square$, *metG319* (no chloramphenicol).

RNA synthesis in *metG319*. Strains of bacteria which are 'stringent' in their synthesis of RNA are unable to synthesize RNA if starved of an amino acid. Chloramphenicol allows RNA synthesis under these conditions (Aronson & Spiegelman, 1961). Results presented in Fig. 4 indicate that in the absence of methionine, incorporation of [^{14}C]uracil into a cold TCA-insoluble fraction was much decreased in *metG319* as compared with wild-type. Some incorporation occurred in *metB23* in the absence of methionine, possibly due to utilization of an existing methionine pool which, because of its defec-

tive methionyl-sRNA synthetase, could not be used by *metG319*. In the presence of a low concentration of DL-methionine (5 μ M), incorporation of [14 C]uracil was greatly increased in *metG319* and *metB23*. In *metB23* the rate and final degree of incorporation of [14 C]uracil was indistinguishable from wild-type, whereas that of *metG319* was still slightly less, probably due to the altered synthetase which prevented full utilization of the proffered methionine. These results indicate that RNA synthesis in *metG319* was stringently controlled. In the presence of 100 μ g./ml. chloramphenicol, incorporation of [14 C]uracil was allowed in *metG319* even in the absence of added methionine and to an extent comparable to wild-type (Fig. 5).

Table 1. *The effect of methionine-limitation on multiplication of phage P22.L4 in Salmonella typhimurium metG319*

Organisms growing logarithmically in MM medium + L-methionine (mm) were rapidly harvested, washed with cold MM medium and after suspension in MM medium ($\pm$ L-methionine mm) at equiv. 0.05 mg. dry wt organisms/ml. with the addition of 2 to 3×10^4 phage/ml. (prepared as in Methods) were incubated with shaking at 37°. At intervals, samples were removed, treated with chloroform, and after bubbling-off excess chloroform, appropriate dilutions were plated with strain *metG319* as indicator in a soft agar overlay (Rowbury, 1968).

Time	Infective phage particles/ml.	
	L-Methionine absent	L-Methionine present
Zero	2.5×10^4	2.2×10^4
1½ h.	4.6×10^8	3.1×10^7
4 h.	1.2×10^8	2.5×10^{11}

Synthesis of DNA in metG mutants. The results so far described suggest that in the absence of methionine, protein synthesis rather than SAM synthesis was affected primarily in *metG319*. Measurements of DNA synthesis in the presence and absence of methionine supported this view. When protein synthesis is inhibited in bacteria, e.g. by adding chloramphenicol or limiting a required amino acid, any rounds of DNA synthesis at present in progress are completed but new rounds do not begin (Maaløe & Hanawalt, 1961). For organisms growing in minimal medium with no gap between rounds of replication, there is generally about a 40 % increase in DNA on amino acid starvation (Donachie, 1969). Limitation of SAM, on the other hand, allows a 100 % increase in DNA (Lark 1968).

When *Salmonella typhimurium metG319* was shifted from minimal medium containing methionine (mm) to methionine-free medium there was an increase in DNA of 47 % as compared with 41 % in presence of chloramphenicol. Clearly, the lesion in *metG319* allowed only very limited re-initiation of DNA synthesis and therefore affected protein synthesis rather than SAM synthesis. In addition, the formation of phage was completely inhibited in methionine-starved *metG319*; the multiplication of P22.L4 was prevented unless methionine is added (Table 1).

Methionyl-sRNA formation in wild-type and metG319. To test the theory that the *metG* phenotype resulted from decreased ability to catalyse the formation of methionyl-sRNA, the formation of methionyl-sRNA by extracts of wild type and *metG319* was followed over a period of 20 min.; the results obtained are presented in Fig. 6. It can be seen that although the wild-type extract catalysed the rapid formation of methionyl-sRNA, the *metG319* extract was practically devoid of activity.

Methionyl-sRNA formation by a mixture of wild-type and metG319 extracts. The inability of *metG319* extract to catalyse methionyl-sRNA formation may mean that the *metG* gene specifies methionyl-sRNA synthetase and that mutation leads to a decrease in enzymic activity, but there are two other possible explanations. First, the *metG* gene may be involved in the synthesis of a cofactor required for methionyl-sRNA synthetase activity; secondly, mutation in *metG* may result in accumulation of an inhibitor of methionyl-sRNA synthetase activity. To investigate these latter two

Fig. 6

Fig. 7

Fig. 8

Fig. 6. Methionyl-sRNA formation by a mixture of wild-type and *metG319* extracts. The assay mixture is described in the Methods. Plotting methionyl-sRNA formation against time gave the following curves: Δ , wild-type; $\square$, *metG319*; $\blacklozenge$, a mixture of equal quantities of wild-type and *metG319* extracts.

Fig. 7. AMP-dependent release of methionine from methionyl-sRNA, catalysed by extracts of wild-type and *metG319* strains. Assay procedure as described in Methods. Plotting methionyl-sRNA remaining against time gave the following curves: Δ , wild-type; $\square$, *metG319*; $\blacklozenge$, wild-type in the absence of pyrophosphate.

Fig. 8. The effect of pH on the activity of methionyl-sRNA synthetase from wild-type and *metG319* extracts. Enzyme activity was determined by the [32 P]PP_i exchange assay. The following buffers were used to obtain the required pH values: sodium acetate (pH 4.8 to 5.6), tris-maleate (pH 5.6 to 7.0), tris-HCl (pH 7.0 to 8.9) and glycine-NaOH (pH 8.9 to 10.6). Results were plotted, and the following curves were obtained: Δ , wild-type; $\square$, *metG319*.

possibilities, the formation of methionyl-sRNA by a mixture of the two extracts was followed. The rationale behind this experiment was that if *metG* specified methionyl-sRNA synthetase, then the combined activity of the two extracts would be equal to the sum of their individual activities when each was assayed alone. If the difference in activities were due to the presence of a cofactor in the wild type or an inhibitor in *metG*, then their combined activities would be greater or less than their individual activities unless the cofactor were limiting. The results presented in Fig. 7 show that the amount of methionyl-sRNA formed by a mixture of equal quantities of the two extracts was equal to the sum of the activities of the wild-type and *metG319* when assayed separately. It must therefore be concluded that the *metG* gene of *Salmonella typhimurium* is the structural gene for methionyl-sRNA synthetase.

Amounts of in vivo charged sRNA_{m+f} in wild-type and metG319. In vitro studies on the formation of [3 H]methionyl-sRNA provide good evidence that *metG* mutants

possess a methionyl-sRNA synthetase with decreased activity. To support this evidence, sRNA was extracted from mutant and wild-type bacteria and the percentage of sRNA_{m+f} 'charged' with methionine determined. Samples of extracted sRNA were subjected to periodate oxidation to destroy the amino acid-accepting capacity of 'uncharged' sRNA, and after removal of attached amino acids from oxidized and unoxidized samples they were recharged *in vitro* with [³H]methionine. By comparing the amounts of [³H]methionine attached to treated and untreated samples, the percentage of charged sRNA_{m+f} was calculated (Table 2). sRNA_{m+f} from wild-type organisms was approximately 100 % charged, whereas that from *metG319* was

Table 2. *In vivo* amounts of charged sRNA_{m+f}

Organisms were grown on MM medium containing a low (0.04 mM) or a high (1 mM) concentration of DL-methionine. sRNA was extracted from the bacteria, half of it was oxidized with periodate, and then both untreated and treated samples were charged to completion with [³H]methionine.

Strain	sRNA _{m+f} charged (%)	
	Low methionine	High methionine
Wild-type (HfrB2)	99.9	99.8
<i>metG319</i>	60.1	98.1
<i>metG419</i>	59.5	97.2

Table 3. *K_m* (methionine) values of methionyl-sRNA synthetases from wild-type and mutant strains of *Salmonella typhimurium* as measured by pyrophosphate exchange

The assay procedure was that described in the Methods, and the reaction mixture contained the following ranges of concentrations of L-methionine: 5 to 250 μM for HfrB2, 1.0 to 2.5 mM for *metG319*, and 1.0 to 5.0 mM for *metG419*.

Strain	<i>K_m</i>
Wild-type (HfrB2)	1.8×10^{-5} M
<i>metG319</i>	1.0×10^{-2} M
<i>metG419</i>	1.0×10^{-2} M

approximately 60 % charged. Taking into account the very much decreased activity of the mutant enzyme, it is surprising that this figure was not lower. However, Martin, Yegian & Stent (1963) found that under conditions of methionine starvation most of the methionine-accepting capacity of sRNA_{m+f} was protected against periodate oxidation. These authors later proposed (Yegian, Stent & Martin, 1966) that this protection might stem from *N*-formylmethionine which became trapped on its sRNA_f due to cessation of growth of polypeptide chains.

AMP-dependent releases of methionine from methionyl-sRNA. The above results show that extracts of *metG319* were very low in their ability to form methionyl-sRNA. However, the formation of this compound occurred in two stages:

- (1) methionine + ATP + enzyme $\rightleftharpoons$ methionine-AMP-enzyme + PP_i;
- (2) methionine-AMP-enzyme + sRNA_m $\rightleftharpoons$ methionyl-sRNA_m + AMP + enzyme.

To determine whether reaction (2), the 'transfer' step, was affected, the release of methionine from methionyl-sRNA, catalysed by extracts of wild-type and *metG319* organisms, was followed. The results (Fig. 7) showed that only the wild-type extract catalysed the release of methionine to any extent. This would suggest that *metG319*

lacks transfer reaction activity; but it can be seen that wild-type extract was unable to catalyse this reaction when pyrophosphate was not present in the reaction mixture. This suggests that reversal of the transfer step also required reversal of reaction (1) so that lack of activity with *metG319* extract may reflect an alteration in either partial reaction.

K_m (methionine) determinations for methionyl-sRNA synthetases from wild-type and *metG319*. Preliminary attempts to determine the affinity of the *metG319* enzyme for methionine by using the acylation reaction were largely unsuccessful for technical reasons. It was therefore decided to use the methionine-dependent [³²P]pyrophosphate exchange assay which allowed much higher concentrations of methionine to be used. This assay measures reaction (1) above, the 'activation' step, so that it was also possible to see whether this stage of the reaction was affected by the *metG* mutation. The results (Table 3) show that both *metG319* and *metG419* had greatly increased *K_m* (methionine) values as compared with wild-type.

Effect of pH on the activity of methionyl-sRNA synthetase from wild-type and metG319. It was of interest to investigate whether the decreased activity of methionyl-sRNA synthetase from *metG* mutants was associated with an alteration in its pH optimum. The rate of pyrophosphate exchange was determined with a range of buffers of different pH values. The results are presented in Fig. 8. The activities of wild-type and *metG319* enzymes showed little activity below pH 5 but increased with pH until pH 7.0 was reached. At pH values greater than 7.0, activity decreased until pH 7.2 was reached. From this point on the behaviour of wild-type and *metG319* differed considerably. At pH values greater than 7.2 the activity of *metG319* continued to decrease whereas that of the wild-type increased to a new maximum at pH 7.5 and remained fairly constant until pH 8.5. With *metG319* only a shoulder of activity remained between pH 7.9 and 8.8, suggesting that in the wild-type organisms the bulk of methionyl-sRNA synthetase activity was associated with a broad pH optimum and that *metG* organisms largely lacked this component.

Chromatography of wild-type and metG319 extracts on DEAE-cellulose. The different behaviour of *metG319* and wild-type methionyl-sRNA synthetase when measured over a range of pH values reflected the difference in enzymic activities of the two strains. To investigate the possibility that this difference was reflected in other physico-chemical properties, enzymic extracts were subjected to chromatography on DEAE-cellulose. The results in Fig. 9(a) for the wild-type and Fig. 9(b) for *metG319* show that both enzymes had very similar elution profiles, the slight displacement of the *metG319* peak in Fig. 9(b) being within the limits of variation imposed by the experimental technique.

However, enzyme from both strains was eluted in two more or less equal peaks of activity. The reason for this may be that, as suggested by Cerhová & Rychlík (1967) for *Escherichia coli*, *Salmonella typhimurium* contains two species of methionyl-sRNA synthetase, one catalysing the attachment of methionine to sRNA_m and the other to sRNA_r. If this be so, then this must reflect a species difference between *S. typhimurium* and *E. coli*, because Hartley & Henrikson (1967), Cassio & Waller (1968) and Bruton & Hartley (1968) have all shown that in *E. coli* a single enzyme catalyses the attachment of methionine to both types of sRNA. In view of these results it seems unlikely that there are two species of methionyl-sRNA synthetase in *S. typhimurium*. Alternatively, it may be that the smaller pH peak and one of the elution peaks represents a

breakdown product or a precursor of the mature enzyme, or it may represent active subunits of an oligomeric protein. The fact that native methionyl-sRNA synthetase can exist as active subunits is suggested by the results of Hartley & Heinrikson (1967) and Bruton & Hartley (1968).

Fig. 9. DEAE-cellulose chromatography of wild-type and *metG319* methionyl-sRNA synthetase. Fractions were assayed for enzyme activity by the pyrophosphate exchange assay. Results obtained for wild type and *metG319* are presented in (a) and (b) respectively. Enzyme activity expressed as μ moles pyrophosphate exchanged/15 min./mg. protein for wild-type and μ moles pyrophosphate exchanged/15 min./mg. protein for *metG319*; $\square$, enzyme activity; $\bullet$, protein concentration.

Gel-filtration on Sephadex G-200. The behaviour of methionyl-sRNA synthetase on DEAE-cellulose at a range of pH values might be expected to reflect the ionic properties of the enzyme molecule. To investigate the possibility of a difference in the molecular sizes of wild-type and mutant enzymes, they were subjected to gel-filtration

Fig. 10. Gel-filtration of wild type and *metG319* methionyl-sRNA synthetases on Sephadex G-200. Fractions were assayed for enzyme activity by the pyrophosphate exchange assay. Results obtained for wild type and *metG319* are presented in (a) and (b) respectively. Enzymic activity, □, as μ moles pyrophosphate exchanged/15 min./mg. protein, and protein concentration, ●, are plotted for both strains, and the elution profile of serum albumin, ○, is included in (a).

on Sephadex G-200. The results (Fig. 10(a) for wild-type, Fig. 10 (b) for *metG319*) showed that there was no significant difference between the elution patterns of wild-type and *metG* mutants, the slight displacement of the *metG319* peak in Fig. 10(b) being within the limits of variation imposed by the experimental technique.

Amounts of the methionine biosynthetic enzymes in Salmonella typhimurium metG319 and metG419. Since protein synthesis in strain *metG319* is limited by the supply of methionyl-sRNA, it was interesting to measure the amounts of the methionine biosynthetic enzymes under conditions where growth was limited by methionine supply, since it is the aminoacyl-sRNA rather than the free amino acid which acts as co-repressor in some biosynthetic pathways (Neidhardt, 1966). Table 4 shows representative

Table 4. *Amounts of methionine-biosynthetic enzymes in Salmonella typhimurium metG319 and metG419*

Organisms were grown at 30° in MM medium plus 0.02 mM-L-methionine (low methionine) or mM-L-methionine (high methionine). Enzyme assays as described in Methods.

Strain	Methionine	Cystathionine synthetase (μ mole/mg. dry wt/h.)	Cystathionase (μ mole pyruvate/mg. dry wt/h.)	Homocysteine methylase (μ mole methionine formed/mg. dry wt/h.)
HfrB2	Low	0.200	0.53	0.33
	High	0.061	0.40	0.08
<i>metG319</i>	Low	0.030	0.93	0.17
	High	0.015	0.45	0.09
<i>metG419</i>	Low	0.065	0.93	0.28
	High	0.018	0.78	0.09

results for organisms grown at 30° with 0.02 mM-L-methionine (low methionine) or mM-L-methionine (high methionine). Values for cystathionine synthetase and the homocysteine methylase complex were lower than in wild-type (preliminary results suggest this also applies to the amounts of the methionine-specific aspartokinase and homoserine dehydrogenase). The values of cystathionase were somewhat higher than in wild-type; this observation has been repeated several times. It therefore appears that methionyl-sRNA is not the co-repressor, at least not for the whole biosynthetic pathway, since conditions which restrict methionyl-sRNA synthetase in *metG* mutants lead to a slight repression of cystathionine synthetase, the homocysteine methylase complex and the methionine-specific aspartokinase and homoserine dehydrogenase. This repression may be due to a build-up of free methionine because it cannot be readily activated. The de-repression of cystathionase does not fit this pattern and is not at present explicable.

DISCUSSION

The present study investigated the effect of the *metG* mutation on the growth characteristics and protein, RNA and DNA synthesis in *Salmonella typhimurium*. Auxotrophic mutants of *Escherichia coli* analogous to *metG* have also been isolated with requirements for tryptophan (Doolittle & Yanofsky, 1968; Kano, Matsushiro & Shimura, 1968), histidine (Nass & Neidhardt, 1966) and glycine (Folk & Berg, 1968).

Of these, the tryptophanyl-sRNA synthetase mutants have been shown to possess growth characteristics similar to those of *metG* mutants (Doolittle & Yanofsky, 1968; Ito, Hiraga & Yura, 1969). It was possible that the *metG* mutants were altered in SAM synthetase rather than in methionyl-sRNA synthetase. However, the very low rate of incorporation of [¹⁴C]phenylalanine into protein in MM medium (Fig. 2), lack of synthesis of D-serine deaminase in the presence of inducer (Fig. 3), and decrease of DNA (Table 1) and RNA (Fig. 4) synthesis in *metG* mutants all establish that protein synthesis is affected in *metG* mutants. Further experiments on the synthesis of methionyl-sRNA *in vitro* showed that *metG* mutants possessed a methionyl-sRNA synthetase with much decreased affinity for methionine. Results obtained using the pyrophosphate exchange assay support this conclusion and show that the K_m (methionine) values of methionyl-sRNA synthetases from *metG* mutants are greatly increased.

The release of [³H]methionine from [³H]methionyl-sRNA was catalysed by a wild-type extract but not by *metG319*, suggesting that the latter is very restricted in its ability to catalyse this reaction. However, since removal of methionine from sRNA appears to require reversal of both partial reactions, this may reflect only the defective first step. Clarification of this problem might result from a study of the transfer of radioactive methionine from chemically synthesized methionyl-adenylate to sRNA.

Evidence that methionyl-sRNA is not the co-repressor of methionine biosynthesis is presented in Table 4; further support comes from studies of methionine-analogue resistant strains in which the biosynthetic enzymes are de-repressed in two groups of resistant strains but in neither are there obvious alterations in amounts of methionyl-sRNA synthetase or in sRNA (T. S. Gross & R. J. Rowbury, unpublished). Since limitation of methionine will inevitably limit *N*-formyl-methionyl-sRNA also, it seems unlikely that this compound is involved in repression. Methionine may either act directly as co-repressor or, as in *Escherichia coli* (Greene, Su & Holloway, 1970), SAM may have a role. So far no evidence for altered SAM metabolism has been obtained in de-repressed strains of *Salmonella typhimurium*.

The experiments on the multiplication of P22.L4 phage in *metG319* demonstrate that the phage is dependent on the host methionyl-sRNA synthetase and does not produce its own specific enzyme or an active altered host enzyme. In at least one case, an inactive synthetase is activated by the presence of phage in *Escherichia coli* (Neidhardt, 1966).

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REFERENCES

- ARONSON, A. I. & SPIEGELMAN, S. (1961). Protein and ribonucleic acid synthesis in a chloramphenicol inhibited system. *Biochimica et biophysica acta* **53**, 70-84.
- BÖCK, A., FAIMAN, L. E. & NEIDHARDT, F. C. (1966). Biochemical and genetic characterization of a mutant of *Escherichia coli* with a temperature-sensitive valyl ribonucleic acid synthetase. *Journal of Bacteriology* **92**, 1076-1082.
- BÖCK, A. & NEIDHARDT, F. C. (1966). Location of the structural gene for glycyl ribonucleic acid synthetase by means of a strain of *Escherichia coli* possessing an unusual enzyme. *Zeitschrift für Vererbungslehre* **98**, 187-192.
- BRUTON, C. J. & HARTLEY, B. S. (1968). Subunit structure and specificity of methionyl-transfer-ribonucleic acid synthetase from *Escherichia coli*. *Biochemical Journal* **108**, 281-288.

- CASSIO, D. & WALLER, J.-P. (1968). Études de la méthionyl-tRNA synthétase d'*Escherichia coli*. III. Dissociation en sous-unités actives par action d'un facteur extrinsèque. *European Journal of Biochemistry* **5**, 33-41.
- CERHOVÁ, M. & RYCHLÍK, I. (1967). Isolation of two methionyl-tRNA synthetases from *Escherichia coli*. *Collection of Czechoslovakian Chemical Communications* **32**, 3808-3811.
- DAVIS, B. D. & MINGIOLI, F. S. (1950). Mutants of *Escherichia coli* requiring methionine or vitamin B₁₂. *Journal of Bacteriology* **60**, 17-28.
- DONACHIE, W. D. (1969). Control of cell division in *Escherichia coli*: Experiments with thymine starvation. *Journal of Bacteriology* **100**, 260-268.
- DOOLITTLE, W. F. & YANOFSKY, C. (1968). Mutants of *Escherichia coli* with an altered tryptophanyl-transfer ribonucleic acid synthetase. *Journal of Bacteriology* **95**, 1283-1294.
- EIDLIC, L. & NEIDHARDT, F. C. (1965). Role of valyl sRNA synthetase in enzyme repression. *Proceedings of the National Academy of Sciences of the United States of America* **53**, 539-543.
- FANGMAN, W. L. & NEIDHARDT, F. C. (1964). Demonstration of an altered aminoacyl ribonucleic acid synthetase in a mutant of *Escherichia coli*. *Journal of Biological Chemistry* **239**, 1839-1843.
- FOLK, W. R. & BERG, P. (1968). Mutant glycyl-tRNA synthetase and the resulting glycine auxotrophy in *E. coli*. *Federation Proceedings. Federation of American Societies for Experimental Biology* **27**, 796.
- GREENE, R. C., SU, C. H. & HOLLOWAY, R. (1970). S-adenosyl-methionine synthetase mutants of *Escherichia coli* K12 with impaired control of methionine biosynthesis. *Biochemical and Biophysical Research Communications* **38**, 1120-1126.
- GROSS, T. S. (1969). Ph.D. Thesis, University of London.
- GROSS, T. S. & ROWBURY, R. J. (1969). Methionyl-tRNA synthetase mutants of *Salmonella typhimurium* which have normal control of their methionine biosynthetic enzymes. *Biochimica et biophysica acta* **184**, 233-236.
- HARTLEY, R. L. & HEINRIKSON, B. S. (1967). Purification and properties of methionyl-transfer-ribonucleic acid synthetase from *Escherichia coli*. *Biochemical Journal* **105**, 17-24.
- HERVÉ, G. & CHAPEVILLE, F. (1963). Spécificité des enzymes d'activation à l'égard des acides ribonucléiques estérifiés par les acides aminés. *Biochimica et biophysica acta* **76**, 493-500.
- HIRAGA, S., ITO, K., HAMADA, K. & YURA, T. (1967). A new regulatory gene for the tryptophan operon of *Escherichia coli*. *Biochemical and Biophysical Research Communications* **26**, 522-527.
- ITO, K., HIRAGA, S. & YURA, T. (1969). Tryptophanyl transfer RNA synthetase and repression of the tryptophan operon in the *trpS* mutant of *Escherichia coli*. *Genetics* **61**, 521-538.
- KANO, Y., MATSUSHIRO, A. & SHIMURA, Y. (1968). Isolation of the novel regulatory mutants of the tryptophan biosynthetic system in *Escherichia coli*. *Molecular and General Genetics* **102**, 15-26.
- LARK, C. (1968). Effect of the methionine analogues, ethionine and norleucine, on DNA synthesis in *Escherichia coli* 15T⁻. *Journal of Molecular Biology* **31**, 401-414.
- LAYNE, E. (1957). In *Methods in Enzymology*, vol. 3, p. 451. Edited by S. P. Colowick & N. O. Kaplan. New York: Academic Press.
- MAALØE, O. & HANAWALT, P. C. (1961). Thymine deficiency and the normal DNA replication cycle. I. *Journal of Molecular Biology* **3**, 144-155.
- MANS, R. J. & NOVELLI, G. D. (1961). Measurement of the incorporation of radioactive amino acids into protein by a filter paper disc method. *Archives of Biochemistry and Biophysics* **94**, 48-53.
- MARTIN, E. M., YEGIAN, C. & STENT, G. S. (1963). The intracellular condition of soluble ribonucleic acid in *Escherichia coli* subjected to amino acid starvation. *Biochemical Journal* **88**, 46 p.
- NASS, G. & NEIDHARDT, F. C. (1966). Histidyl-sRNA synthetase and enzyme repression in *Escherichia coli*. *Bacteriological Proceedings*, p. 87.
- NEALE, S. & TRISTRAM, H. (1963). Effects of O-methyl-DL-serine on growth and protein synthesis in *Escherichia coli*. *Journal of Bacteriology* **86**, 1241-1250.
- NEIDHARDT, F. C. (1966). Role of amino acid activating enzymes in cellular physiology. *Bacteriological Reviews* **30**, 701-719.
- ROTH, J. R. & AMES, B. N. (1966). Histidine regulatory mutants in *Salmonella typhimurium*. II. Histidine regulatory mutants having altered histidyl-tRNA synthetase. *Journal of Molecular Biology* **22**, 325-334.
- ROWBURY, R. J. (1968). The inhibitory action of α -methylmethionine on *Escherichia coli*. *Journal of General Microbiology* **52**, 223-230.

- ROWBURY, R. J., LAWRENCE, D. A. & SMITH, D. A. (1968). Regulation of the methionine specific β -aspartokinase and homoserine dehydrogenase of *Salmonella typhimurium*. *Journal of General Microbiology* **54**, 337-342.
- ROWBURY, R. J. & WOODS, D. D. (1961). Further studies on the repression of methionine synthesis in *Escherichia coli*. *Journal of General Microbiology* **24**, 129-144.
- ROWBURY, R. J. & WOODS, D. D. (1966). The regulation of cystathionine formation in *Escherichia coli*. *Journal of General Microbiology* **42**, 155-163.
- SILBERT, D. F., FINK, G. R. & AMES, B. N. (1966). Histidine regulatory mutants in *Salmonella typhimurium*. III. A class of regulatory mutants deficient in tRNA for histidine. *Journal of Molecular Biology* **22**, 335-347.
- SMITH, D. A. & CHILDS, J. D. (1966). Methionine genes and enzymes of *Salmonella typhimurium*. *Heredity* **21**, 265-286.
- SMITH, H. O. & LEVINE, M. (1967). A phage P22 gene controlling integration on prophage. *Virology* **31**, 207-216.
- WIJESUNDERA, S. & WOODS, D. D. (1962). The catabolism of cystathionine by *Escherichia coli*. *Journal of General Microbiology* **29**, 353-366.
- YANIF, M., JACOB, F. & GROS, F. (1965). Mutations thermosensibles des systems activant la valine chez *E. coli*. *Bulletin de la Société de Chimie Biologique* **47**, 1609-1656.
- YEGIAN, C. D., STENT, G. S. & MARTIN, E. M. (1966). Intracellular conditions of *Escherichia coli* transfer RNA. *Proceedings of the National Academy of Sciences of the United States of America* **55**, 839-846.