

Methionine Transport in Wild-type and Transport-defective Mutants of *Salmonella typhimurium*

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SUMMARY

Salmonella typhimurium possesses a permease specific for L-methionine (K_m of 0.1 to 0.2 μ M). Competition studies have shown that the permease has little or no affinity for the other L-amino acids commonly found in proteins. Methionine uptake was competitively inhibited by the growth inhibitory analogues DL-ethionine, α -methyl-DL-methionine and DL-methionine-DL-sulphoximine. Mutants resistant to α -methyl-methionine and methionine sulphoximine have been isolated which were severely defective in the methionine specific permease. Two of these mutants, *metP760* and *metP761*, mapped away from all previously located methionine structural and regulatory genes.

INTRODUCTION

Lawrence, Smith & Rowbury (1968) in a study of the regulation of methionine biosynthesis in *Salmonella typhimurium*, characterized mutants of three genes, *metA* (feedback-resistant, fbr), *metJ* and *metK*, all of which were resistant to growth inhibitory analogues of methionine (Fig. 1). *MetA*(fbr) mutants were resistant to feedback inhibition by methionine; *metJ* mutants were derepressed for the methionine biosynthetic enzymes and may possess an altered methionine aporepressor; some *metK* mutants had derepressed levels of the methionine enzymes while the remainder were normally regulated (Lawrence *et al.* 1968; Chater, 1970; Chater & Rowbury, 1970). Both types of *metK* mutants appear to have reduced S-adenosyl methionine synthetase activity (A. Hobson, personal communication). *MetA*(fbr), *metJ* and derepressed *metK* mutants excreted methionine. The excreting *metK* mutants showed reduced uptake of methionine and ethionine, and since it was proposed that these mutants might have permease defects, we have therefore undertaken a study of methionine transport in *S. typhimurium*.

Cohen & Monod (1957) first established the existence of specific transport systems for valine, phenylalanine and methionine in *Escherichia coli* which were independent from one another. Although these systems failed to transport the D-isomers of the amino acids, they were able to transport certain structural analogues of the L-isomers. Piperno & Oxender (1968) confirmed and extended these results by showing that the L-methionine

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permease did not transport other protein amino acids, nor did it transport D-methionine. This permease had a Michaelis constant (K_m) of $2.3 \mu\text{M}$ for methionine and L-ethionine competed with methionine for transport.

In the present work we describe a methionine-specific permease in *Salmonella typhimurium*. Several methionine analogues compete with methionine for transport, and certain analogue-resistant mutants are defective in methionine transport. An excreting *metK* mutant was tested for methionine uptake; when methionine excretion was prevented by introducing a genetic block in methionine synthesis, increased methionine transport activity was observed. Some of the results presented here have previously appeared as a preliminary publication (Ayling & Bridgeland, 1970).

METHODS

Media. Nutrient agar (NA) and nutrient broth (NB) were obtained from Oxoid Ltd. Minimal medium (MM) contained (g/l): K_2HPO_4 (laboratory grade), 10.5; KH_2PO_4 , 4.5; trisodium citrate $2\text{H}_2\text{O}$, 0.47; $(\text{NH}_4)_2\text{SO}_4$, 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; D-glucose, 2 (for growth experiments) or 4 (growing cells for transport assays). Minimal agar (MA) was prepared by solidifying minimal medium with 1.5% (w/v) Oxoid no. 1 agar.

Chemicals. MM and MA were supplemented where necessary to satisfy the requirements of auxotrophic strains with L-serine (200 $\mu\text{g/ml}$), other amino acids (20 $\mu\text{g/ml}$) and vitamin B_{12} (0.1 $\mu\text{g/ml}$). Methionine analogues were added to MA at the following concentrations to score analogue resistance: DL-ethionine (Koch-Light Laboratories, Colnbrook, Buckinghamshire, and Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.), 1 mg/ml (6.1 mM); DL-methionine-DL-sulphoximine (Koch-Light Laboratories and Sigma Chemical Co, London) 0.1 mg/ml (0.55 mM); α -methyl-DL-methionine (Sigma), 1 mg/ml (6.1 mM); DL-norleucine (Koch-Light Laboratories), 1 mg/ml (7.6 mM). L- and D-Methionine were obtained from Sigma, and L-[methyl- ^{14}C]methionine (53 or 60 $\mu\text{Ci}/\mu\text{mol}$) from the Radiochemical Centre, Amersham, Buckinghamshire.

Bacterial strains. All strains were ultimately derived from *Salmonella typhimurium* LT2; they are described in Table 1.

Mutants resistant to methionine sulphoximine were isolated by spreading 0.1 ml quantities of an overnight NB culture on MA + 0.1 mg/ml methionine sulphoximine. After 2 days of incubation at 37 °C, resistant colonies were picked off, purified by streaking out twice on NA, and their resistance to analogues was checked by streaking saline suspensions on the relevant media. Excretion of methionine was detected on plates by the cross-feeding of the methionine-specific auxotroph, *metF185* (Lawrence *et al.* 1968).

Transduction and conjugation. Stocks of the temperate phage P22 and a non-lysogenizing derivative L-4 (Smith & Levine, 1967), kindly supplied by Dr H. O. Smith, were propagated, assayed and maintained as described by Smith (1961). Transduction and conjugation analysis were as described by Ayling & Chater (1968).

Measurement of methionine accumulation. The method is based on that of Kessel & Lubin (1965).

An overnight culture in MM (10 ml) was diluted with 50 ml of fresh MM and grown for 75 min. A solution of chloramphenicol (Chloromycetin, Parke-Davis, Hounslow, Middlesex) in MM (0.1%, w/v) was then added to the exponentially growing culture to give a final concentration of 200 $\mu\text{g/ml}$. After a further 30 min of incubation, by which time growth had almost ceased, the culture was harvested by centrifugation at room temperature (20 to 23 °C). The bacteria were washed with the culture volume of

Table 1. *List of Salmonella typhimurium strains used in these studies*

Strain	Source	Strain	Source
1. HfrA	K.E.S.	14. <i>metA43 metE205</i>	13 × 10 (transduction)
2. HfrA <i>metP760</i>	D.A.L. selected on MA + AMM from 1	15. <i>metB23 metP760</i>	K.F.C.
3. HfrA <i>metP761</i>	Selected on MA + METX from 1	16. <i>metE205 metP761</i>	14 × 3 (conjugation)
4. HfrA <i>metx-r31*</i>	Selected on MA + METX from 1	17. <i>leuA37</i>	K.E.S.
5. HfrH5	K.E.S.	18. <i>proB45</i>	K.E.S.
6. HfrH5 <i>metx-r15*</i>	Selected on MA + METX from 5	19. <i>purE8</i>	K.E.S.
7. HfrH5 <i>metx-r16*</i>	Selected on MA + METX from 5	20. <i>serA11</i>	K.E.S.
8. HfrH5 <i>metx-r17*</i>	Selected on MA + METX from 5	21. HfrB2	K.E.S.
9. <i>metA15</i>	D.A.S.	22. HfrB2 <i>metK721</i>	D.A.L.
10. <i>metA43</i>	D.A.S.	23. <i>argF111 serA13</i>	K.F.C.
11. <i>metB23</i>	D.A.S.	24. <i>argF111 metK721</i>	23 × 22 (transduction)
12. <i>metF185</i>	D.A.S.	25. HfrA <i>purD55</i>	K.E.S.
13. <i>metE205 metH463</i>	D.A.S.	26. HfrA <i>metA15</i>	25 × 9 (transduction)
		27. <i>metA15 metK721</i>	24 × 26 (conjugation)
		28. wild-type (wt/1)	11 × 2 (conjugation)

Abbreviations: AMM = α -methylmethionine; METX = methionine sulphoximine. K.E.S. = Dr K. E. Sanderson; D.A.L. = Dr D. A. Lawrence; D.A.S. = Dr D. A. Smith; K.F.C. = Dr K. F. Chater.

In transduction and conjugation crosses the recipient is given first, donor second. All HfrA strains carry the additional markers *hisD23 gal-50*; HfrH5 strains carry *leu-256*.

* Temporary phenotypic designations.

MM + 200 μ g/ml chloramphenicol (chloramphenicol medium, CM) at 0 °C except where otherwise indicated, and resuspended in 5 ml of CM. This medium did not contain the growth factors required by auxotrophic strains. The turbidity of bacterial suspensions was determined and adjusted to correspond to a dry weight content of approximately 0.8 mg/ml. The suspension was stored at 0 °C except where otherwise indicated.

Assays of methionine transport were performed in a 25 ml beaker immersed in a water bath at 25 °C. The bacterial suspension was added to CM already at 25 °C to give a final concentration of 50 μ g/ml dry weight bacteria, usually in a volume of 5 ml. This suspension was shaken for 2 min, shaking was then stopped and the assay initiated by the rapid addition of a solution of [14 C]methionine (specific activity 53 μ Ci/ μ mol, unless otherwise indicated) in CM from a syringe. Unlabelled amino acids and analogues being examined for their effect on [14 C]methionine uptake were added to the methionine solution. A spring-loaded syringe was used to mix the assay system and to transfer 1 ml samples at intervals of 10 or 15 s to membrane filters (25 mm diameter, 0.4 μ m pore size, Millipore, U.K. Ltd, London). Filtration was complete within 2 to 3 s at which instant the walls of the filter holder were rinsed twice with 5 ml of CM at room temperature. In each experiment, one sample was transferred with rapid mixing to a tube containing 1 ml of 10% trichloroacetic acid in CM and allowed to stand for at least 15 min at room temperature. The sample was then filtered, the tube rinsed twice with 2.5 ml of 5% trichloroacetic acid in CM and these washings transferred to the filter. The walls of the filter holder were then rinsed with 5 ml of the same fluid.

The 14 C content of material precipitated by trichloroacetic acid gives an estimate of the incorporation into cellular macromolecules. This fraction was determined routinely to confirm that treatment of bacteria with chloramphenicol had prevented the incorporation of intracellular 14 C into these materials.

The filters were dried under an infra-red lamp and covered with 5 ml of scintillant (6 g of 2,5-phenyloxazole and 0.4 g of 1,4-di[2-(5-phenyloxazolyl)]benzene in 1 l of xylene). The 14 C content was determined by liquid scintillation spectrometry. Counting efficiencies

were estimated by the channel ratio method using a quench calibration curve prepared by adding varying proportions of chloroform to a solution of [^{14}C]hexadecane in scintillation fluid. At least 94% of the ^{14}C accumulated by bacteria remained attached to the filter if it was removed from the scintillant. Nevertheless self-absorption was shown to be negligible by taking a filter whose ^{14}C content had been estimated as above and treating it with hyamine hydroxide (Hansen & Bush, 1967). This dissolved 90% or more of the ^{14}C , which was then estimated in a one-phase system using a 1:2 (v/v) mixture of triton X-100 and the scintillant described above.

Extraction and analysis of radioactivity from the intracellular pool. Bacteria were exposed to $1\ \mu\text{M}$ -[^{14}C]methionine for 30 s under the conditions of the transport assay. The bacteria were filtered off, washed with CM and the filter plunged rapidly into 2 ml of 70% ethanol at 0°C . The filter was rinsed with fresh ethanol at 0°C and the combined ethanol extracts concentrated in a rotary evaporator. The residue was dissolved in water and analysed by descending chromatography on paper (Whatman no. 3) in *n*-butanol:acetic acid:water (12:3:5, by vol.). Radioactive areas were located with a strip scanner, cut out and their ^{14}C content determined by liquid scintillation spectrometry.

RESULTS

General factors affecting transport rates and their estimation. Chloramphenicol-treated bacteria accumulated [^{14}C]methionine at a rate which was constant for about 80 s and then declined steadily, presumably as equilibrium between influx and efflux was established (Fig. 2). Chloramphenicol treatment apparently increased the methionine pool (i.e. the difference between ^{14}C accumulated by whole cells and that incorporated into trichloroacetic acid precipitable material), but had little effect on the initial rate of methionine accumulation.

When plots of methionine accumulated with time are extrapolated through zero on the time axis, quite substantial values are obtained (Fig. 2). There are probably two reasons for this. First, bacteria would presumably continue to accumulate methionine for 2 to 3 s after they were squirted on to the filter, which is the time recorded. Secondly, the filters themselves bind a small and variable amount of ^{14}C . The ^{14}C apparently accumulated at zero time was not due to exchange between the methyl group of the [methyl- ^{14}C]methionine and a component of the bacterial membrane or wall, because a similar effect was obtained with [carboxyl- ^{14}C]methionine.

Plots of methionine, accumulated with time obtained with bacteria which had been stored at 0°C , were generally linear over the 1 min assay period (Fig. 2) and rates of transport were calculated from the slopes of these plots. However, with bacteria which had been stored at 5°C , plots of methionine accumulated with time were often curvilinear (Fig. 3), suggesting that equilibrium between influx and efflux of [^{14}C]methionine had been approached during the assay period. In this case, rates of transport were estimated from the slope of a line drawn through the points obtained from the first two, three or four samples, depending on the degree of curvature of the plot. Rates of methionine transport at 25°C declined by approximately 20%/h when bacteria were stored at 0°C and by only 5%/h when stored at 5°C .

Rates of methionine transport were proportional to the concentration of bacteria in the assay system over the range 0 to $125\ \mu\text{g}$ dry weight of bacteria per ml.

Fate of intracellular methionine. When bacteria were incubated with [$1\text{-}^{14}\text{C}$]methionine in CM under normal assay conditions, 67% of the ^{14}C accumulated in 30 s was shown to be present as methionine or methionine sulfoxide. The latter compound was probably

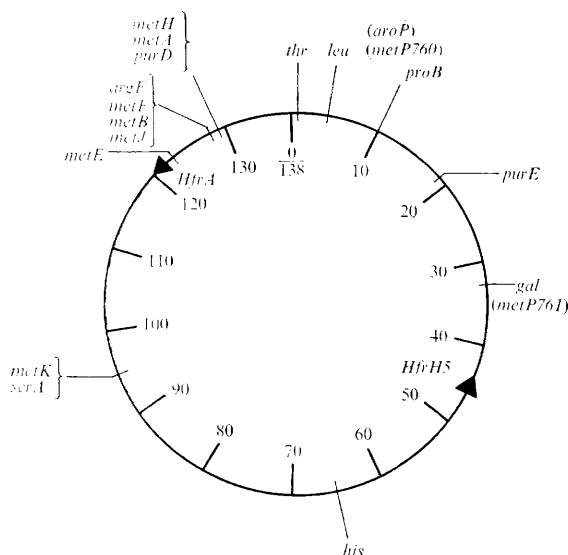


Fig. 1

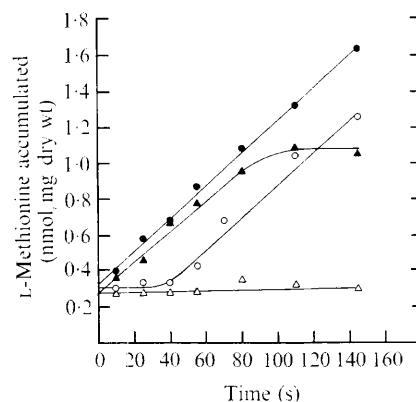


Fig. 2

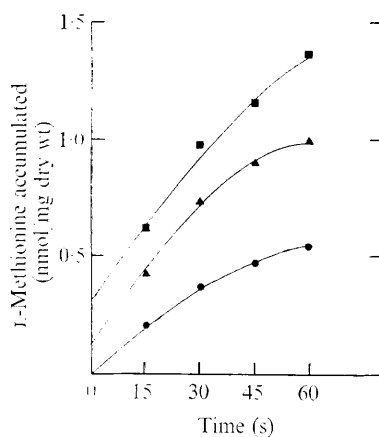


Fig. 3

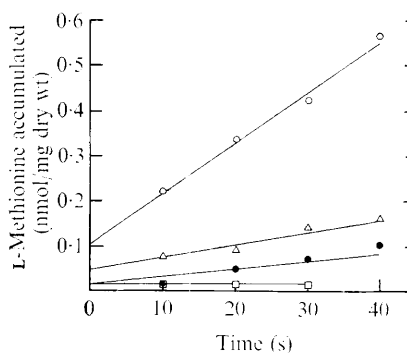


Fig. 4

Fig. 1. Abbreviated linkage map of *Salmonella typhimurium* (after Sanderson, 1970). Resistance to α -methylmethionine (*metA*, *fbr*), ethionine (*metJ*), α -methylmethionine or ethionine or norleucine (*metK*). Aromatic amino acid permease (*aroP*), methionine-specific permease (*metP*). Inability to utilize galactose (*gal*). Bracketed markers, }, are co-transduced, with gene order and orientation as shown. (), markers whose position known approximately. The arrows indicate points of entry of Hfr donors. The map is marked in 10 min intervals.

Fig. 2. Effect of chloramphenicol treatment on the uptake of methionine and its incorporation into trichloroacetic acid insoluble material by wild-type. Bacteria (strain HfrA) were grown and harvested as described in the Methods except that one half of the culture was not treated with chloramphenicol. After harvesting, bacteria were assayed at 25 °C for uptake of 5 μ M-L-[¹⁴C]methionine (10.7 μ Ci/ μ mol). \blacktriangle , \triangle , Chloramphenicol-treated bacteria; \bullet , \circ , untreated bacteria (closed figures represent accumulation by whole cells, open figures represent incorporation into trichloroacetic acid insoluble material).

Fig. 3. Uptake of methionine by wild-type bacteria. Bacteria (strain HfrA) were harvested and then washed and stored at 5 °C. Uptake of L-[¹⁴C]methionine (60 μ Ci/ μ mol) at 25 °C was assayed at 0.2 (\bullet), 0.5 (\blacktriangle) and 1.0 (\blacksquare) μ M concentrations.

Fig. 4. Effect of (i) temperature and (ii) omission of glucose on the uptake of methionine by wild-type bacteria. The bacteria (HfrA) were washed and resuspended in CM or glucose-free CM and stored at 5 °C until assayed. Uptake of 0.5 μ M-L-[¹⁴C]methionine (60 μ Ci/ μ mol) was determined at 25 °C (\circ), 15 °C (\triangle) and 0 °C (\square), all in the presence of 0.4 % glucose; uptake at 25 °C in the absence of glucose (\bullet).

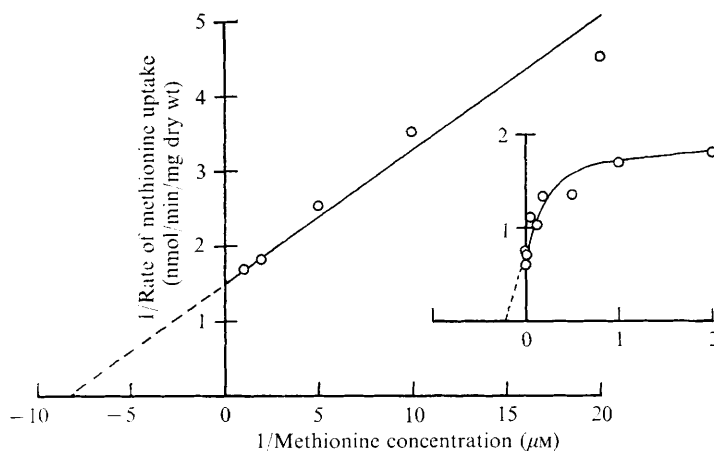


Fig. 5. Double reciprocal plot for methionine uptake by HfrA. Accumulation of methionine after 10, 25, 40 and 55 s was determined over a range of methionine concentrations from 0.05 to 1 μM . Initial rates of uptake were calculated from the slopes of plots of methionine accumulation. Inset, similar data obtained over the concentration range 0.5 to 100 μM . The specific activity of the L-[^{14}C]methionine employed was 54 $\mu\text{Ci}/\mu\text{mol}$ for concentrations 0.05 to 1 μM , 10.7 for 2 to 10 μM and 5.4 for 20 to 60 μM and 1.5 for 100 μM .

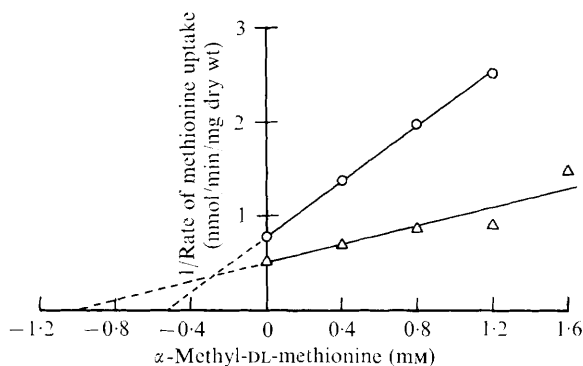


Fig. 6. Competitive inhibition of methionine uptake by α -methylmethionine in strain HfrA. Bacteria were washed and resuspended in CM at 20 to 23 $^{\circ}\text{C}$. Rates of methionine uptake were determined from the difference between two samples taken at 15 and 30 s. Uptake of 0.1 (○) and 0.4 (△) μM -L-[^{14}C]methionine (6 $\mu\text{Ci}/\mu\text{mol}$) was determined in the presence of increasing concentrations of α -methyl-DL-methionine.

formed from methionine by atmospheric oxidation during extraction and chromatography (Greenstein & Winitz, 1961). Piperno & Oxender (1968) have similarly shown in *Escherichia coli* that over 50% of methionine was metabolized after 2 min. The relatively low proportion of ^{14}C remaining in cellular methionine is perhaps not surprising in view of the fact that methionine may be converted to S-adenosylmethionine, in which form its methyl group may be transferred to a variety of compounds.

Evidence that methionine transport is an active process. A first approximation of the overall concentration of methionine within the bacterial cell may be estimated from the data obtained in transport assays. It will be assumed that the accumulated ^{14}C is present entirely in methionine at a uniform concentration, and that 1 g dry wt of bacteria is equiva-

Table 2. *The effect of amino acids and methionine analogues on the uptake of methionine by HfrA*

The bacteria were stored at 5 °C until assayed. The final methionine concentration was 0.2 μM (53 $\mu\text{Ci}/\mu\text{mol}$). A single sample at 30 s was used to determine the amount of methionine accumulated. The results are expressed as the percentage of methionine accumulated without the amino acids or analogues.

Amino acid or analogue	Methionine accumulated in 30 s	Amino acid or analogue	Methionine accumulated in 30 s
None	100	Cysteine	105
Glutamate	95.5	Lysine	100.5
Glutamine	103.5, 97.7, 96	Tyrosine	100
Leucine	107	Tryptophan	108.3
Isoleucine	107.5	Phenylalanine	104.5, 79.2, 108
Threonine	101.5	Serine	109
Valine	99.5	Histidine	106
Homocysteine thiolactone	109	D-Methionine	77.2
Alanine	98.3	L-Methionine	4.3, 1.8
Arginine	89.6	L-Methionine-DL-sulphoxide	98
Aspartic acid	89	α -Methyl-DL-methionine*	45.7
Proline	82	DL-Ethionine*	14
Glycine	91.3	DL-Methionine-DL-sulphoximine*	63.5
Asparagine	99.4	DL-Norleucine*	59

Final concentration of amino acid or analogue = 20 μM except where indicated.

* Final concentration = 800 μM .

lent to an intracellular water volume of 4 ml (this value was obtained for *Escherichia coli* by Roberts *et al.* 1955). In Fig. 3 the methionine accumulated after 55 s by 1 mg dry weight of bacteria at an external methionine concentration of 0.2 μM is 0.54 nmol, so that the intracellular methionine concentration is 135 μM . Thus the ratio of intracellular to extracellular concentration is 680. It can be seen that methionine has been transported against a concentration gradient, indicating that an active, energy-consuming process is involved. This conclusion is supported by the finding that the rate of methionine accumulation was reduced by 80% in the absence of glucose (Fig. 4). The rate of methionine accumulation was also reduced, by 60% and 25%, when bacteria were incubated with potassium cyanide (5 mM) and 2,4-dinitrophenol (1 mM) respectively, both of which inhibit the formation of ATP by respiratory-chain phosphorylation. Finally, the rate of transport was reduced by 74% at 15 °C, and was almost zero at 0 °C (Fig. 4).

Kinetic studies of methionine transport in wild-type. Rates of methionine transport were determined on wild-type bacteria (strain HfrA *hisD23*) over the concentration range 0.05 to 100 μM . A Lineweaver–Burk (1934) double reciprocal plot of the data is shown in Fig. 5. The plot is biphasic, with the break occurring at 2 to 5 μM (reciprocal methionine concentration of 0.5 to 0.2 μM^{-1}). The possible significance of this break and of the steep limb of the plot will be considered in the final discussion. The permease system represented by the less steep limb of the plot over the concentration range 0.05 to 2 μM has a K_m of approximately 0.13 μM and a maximum velocity (V) of approximately 0.7 nmol/min/mg dry wt bacteria. Kinetic data obtained with bacteria which had been stored at 5 °C produced similar values for K_m (0.11 μM) but larger values for V (1.5 nmol/min/mg dry wt). The difference in estimates obtained for V probably reflects the variability in uptake rates (v) for bacterial suspensions prepared at different times; at a methionine concentration of 0.1 μM , the rate of uptake for bacteria which had been stored at 0 °C was 1.03 ± 0.35 nmol/

min/mg dry wt (mean of 5 determinations \pm standard deviation); for bacteria stored at 5 °C it was 1.01 ± 0.25 nmol/min/mg dry wt (3 determinations).

Specificity of the methionine permease. The ability of other protein amino acids to compete with methionine for a component of the methionine permease was tested by adding them one at a time to the assay system at concentrations 100-fold that of methionine ($0.2 \mu\text{M}$). Whereas unlabelled L-methionine reduced ^{14}C accumulation by 96% or more, none of the other L-amino acids caused more than 20% reduction (Table 2). D-Methionine caused 22% reduction, which may represent genuine competition by the D-stereoisomer or the presence of L-methionine impurity in the D-methionine. DL-Methionine-DL-sulphoxide was also tested at a concentration of $20 \mu\text{M}$, and had no effect on methionine uptake.

Substantial reduction of [^{14}C]methionine accumulation was caused by the methionine analogues α -methyl-DL-methionine, DL-ethionine, DL-methionine-DL-sulphoximine and DL-norleucine, but only when their concentration was 4000-fold that of methionine (Table 2). The affinities of the analogues for the methionine permease will be inversely proportional to their inhibitor constants (K_i 's), providing that they inhibit methionine transport in a truly competitive manner. This was the case for the first three analogues (norleucine was not tested) and their K_i 's were determined by the graphical method of Dixon (1953); Fig. 6 shows the results for α -methylmethionine. The estimated K_i 's were DL-ethionine, 16 to $30 \mu\text{M}$; α -methyl DL-methionine, 0.17 to 0.26 mM ; and DL-methionine-DL-sulphoximine, 0.3 to 0.38 mM . Because the K_i 's of the analogues, especially α -methylmethionine and methionine sulphoximine, are so high compared with the K_m for methionine, the possibility arises that the inhibition observed is due to traces of L-methionine in the analogue. However, this seems unlikely, since as will be shown later, mutants *metP760* and *761* are simultaneously defective in methionine transport and resistant to α -methylmethionine and methionine sulphoximine.

Isolation and characterization of transport-defective mutants. The first mutant shown to possess a defect in methionine transport was *metP760*, which was originally isolated by Lawrence (1967) as one of 13 α -methylmethionine-resistant mutants. The other 12 mutants were feedback inhibitor-resistant mutants mapping in the *metA* gene (Lawrence *et al.* 1968; Chater & Rowbury, 1970).

Of 45 new mutants in HfrA *hisD23* and HfrH5 *leu-256* selected as resistant to methionine sulphoximine, 13 were cross-resistant to α -methylmethionine but none were resistant to ethionine or norleucine. Five of the 45 new mutants were selected for further study, *metP761* and *metx-r31* from HfrA, and *metx-r15*, *-r16* and *-r17* from HfrH5, together with *metP760*. Of these six mutants only the four which were also α -methylmethionine-resistant showed reduced methionine transport (*metP760*, *metP761*, *metx-r16* and *-r17*). *MetP760* and *metP761* were selected for detailed study because they showed the lowest rates of methionine transport.

Unlike some of the previously isolated α -methylmethionine-resistant mutants in *metA* and *metK* (Lawrence *et al.* 1968), none of the four transport mutants excreted methionine at 25 °C or 37 °C in amounts which could be detected by cross-feeding on MA.

The effect of α -methylmethionine and methionine sulphoximine on the growth of *metP760* and *metP761*, and the analogue-sensitive parent strain HfrA *hisD23* was studied in minimal medium. Whereas growth of the sensitive strain was completely inhibited by $61 \mu\text{M}$ - α -methylmethionine (Fig. 7*a*), growth of the mutants *metP760* (Fig. 7*c*) and *761* (Fig. 7*d*) was unaffected. Both mutants showed similar degrees of resistance to 6.1 mM - α -methylmethionine. Growth of the parent strain was completely inhibited by $55 \mu\text{M}$ -methionine sulphoximine (Fig. 7*b*), whereas growth of *metP760* (Fig. 7*c*) was only par-

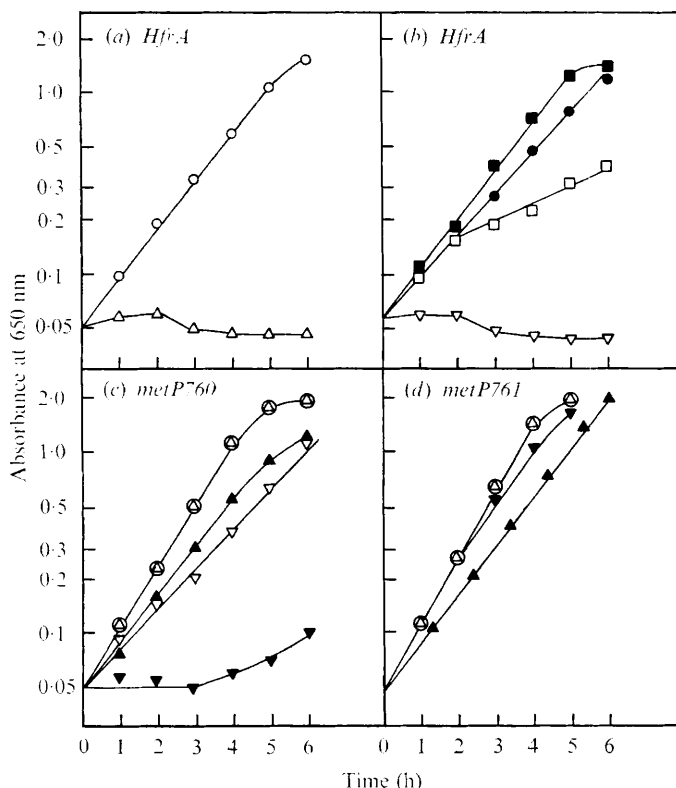


Fig. 7. Growth of HfrA and its analogue-resistant mutants, *metP760* and *metP761*, in the presence of α -methylmethionine and methionine sulfoximine. The experiment was performed as described by Lawrence *et al.* (1968). Media were minimal medium (○); MM + α -methylmethionine, 61 μ M (△) and 6.1 mM (▲); MM + methionine sulfoximine, 55 μ M (▽) and 0.55 mM (▼); MM + 55 μ M-methionine sulfoximine + 67 μ M-L-methionine (●); MM + 55 μ M-methionine sulfoximine + 68 μ M-L-glutamine (□) or 0.68 mM-L-glutamine (■).

tially inhibited. *MetP760* was completely inhibited by 0.55 mM-sulfoximine over a period of 3 h (Fig. 7c), whereas *metP761* was still fully resistant (Fig. 7d).

The inhibition of growth of the analogue-sensitive parent strain by 55 μ M-sulfoximine was almost completely abolished by 67 μ M-L-methionine (Fig. 7b). Lawrence *et al.* (1968) have similarly shown that α -methylmethionine inhibition is prevented by methionine. If it is assumed that these two analogues can only enter the bacterium by the methionine permease, then methionine could block their entry because it possesses a much higher affinity for the permease. The methionine sulfoximine inhibition of the parent strain is also prevented by L-glutamine at 0.68 mM (Fig. 7b) although glutamine is not transported by the methionine permease (Table 2). However, methionine sulfoximine inhibits glutamine synthetase in *Escherichia coli* (Weisbrod & Meister, 1967), and if it also inhibits this enzyme in *Salmonella typhimurium*, glutamine would bypass the metabolic block caused by this inhibition.

Methionine transport in analogue-resistant mutants. The six analogue-resistant strains *metP760*, *metP761*, *metx-r31* (in HfrA *hisD23*) and *metx-r15*, *metx-r16* and *metx-r17* (in HfrH5 *leu-256*) were tested for transport activity at a methionine concentration of 1 μ M (Fig. 8). Of these mutants, the four which are resistant to sulfoximine and α -methyl-

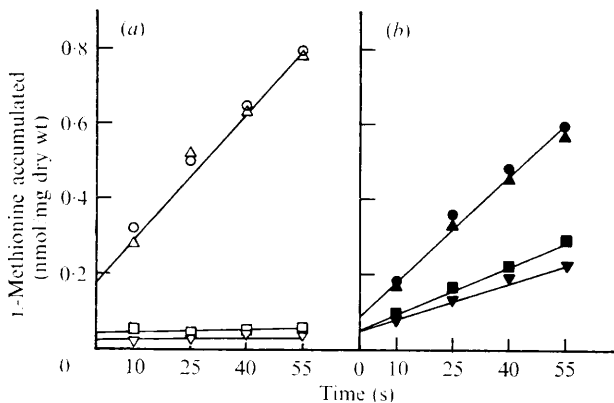


Fig. 8. Uptake of methionine by HfrA and HfrH5 and their analogue-resistant mutants. Uptake of $1 \mu\text{M}$ -L-[^{14}C]methionine ($53 \mu\text{Ci}/\mu\text{mol}$) was determined at 25° . Strains were HfrA (\circ), HfrA metx-r31 (\triangle), HfrA *metP760* (∇), HfrA *metP761* (\square), HfrH5 (\bullet), HfrH5 metx-r15 (\blacktriangle), HfrH5 metx-r16 (\blacktriangledown), HfrH5 metx-r17 (\blacksquare).

methionine in MM show either negligible uptake (*metP760* and *metP761*, Fig. 8a) or reduced uptake (metx-r16 and metx-r17, Fig. 8b). The two mutants which are resistant only to sulphoximine (metx-r31, Fig. 8a and metx-r15, Fig. 8b) do not show any reduction in uptake compared with their respective analogue-sensitive parents.

Kinetic studies of transport in metP760 and metP761. Rates of methionine transport were determined for *metP760* over the concentration range 0.2 to $60 \mu\text{M}$. The double reciprocal plot of these data (Fig. 9) is linear over the concentration range 0.2 to $10 \mu\text{M}$ (i.e. $1/S$ values of 5 to 0.1 respectively). The K_m was estimated to be 10 to $20 \mu\text{M}$, which is two orders of magnitude greater than that for wild-type ($0.13 \mu\text{M}$). A value of $0.5 \text{ nmol/min/mg dry wt}$ was obtained for V which is of the same order as that obtained for wild-type (0.7). At methionine concentrations greater than $10 \mu\text{M}$ the double reciprocal plot appears to become biphasic (inset to Fig. 9).

A double reciprocal plot of similar data for *metP761* (Fig. 10) is linear over the concentration range 0.7 to $5 \mu\text{M}$ ($1/S$ values of 1.4 to 0.2 respectively); estimated values for the K_m were $7 \mu\text{M}$, and for V , $0.25 \text{ nmol/min/mg dry wt}$. Thus both mutants possess permeases with K_m 's which are considerably greater than that of their wild-type parent, that is to say with affinities for methionine which are considerably less than that of the wild-type permease.

Effect of transport mutations on growth of methionine auxotrophs. The methionine auxotrophs *metB23* and *metE205* (Smith & Childs, 1966) grow at wild-type rate at 37°C in MM supplemented with as little as $7 \mu\text{M}$ -L-methionine. Since the kinetic data in Fig. 9 and 10 show that at this concentration *metP760* transports methionine at about one-third and *metP761* at about one-fifth of the wild-type rate, we expected that the double mutants *metB23 metP760* and *metE205 metP761* would grow comparatively slowly or not at all under these conditions. In fact both double mutants grew at the same rates as the auxotrophs from which they were respectively derived, suggesting that the methionine requirements of the auxotrophs may be satisfied by the permease working at less than its maximum rate. Alternatively it may be that the kinetic parameters for the permease operating in chloramphenicol-treated bacteria differ from those in growing bacteria. Differences in the K_m of the histidine-specific permease have been found for these different conditions (Rosen & Vasington, 1971).

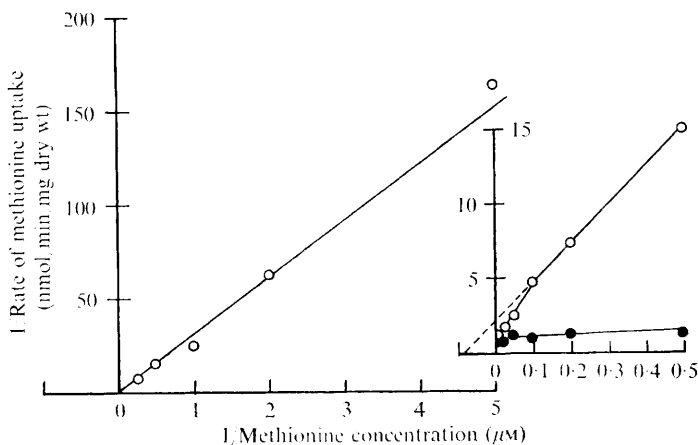


Fig. 9. Double reciprocal plot for methionine uptake by HfrA *metP760*. Rates of uptake were determined as described in Fig. 5. The main figure shows data obtained over the concentration range 0.2 to 5 μ M-L-methionine. Inset – data obtained over the concentration range 2 to 60 μ M. \circ , Strain HfrA *metP760*; \bullet , HfrA (data from Fig. 5 for comparison).

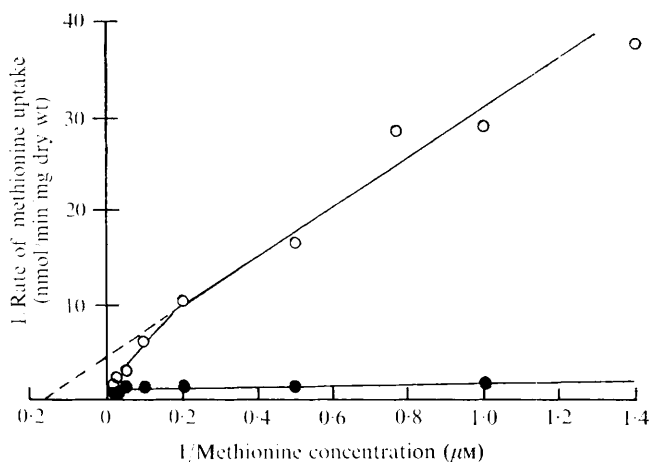


Fig. 10. Double reciprocal plot for methionine uptake by HfrA *metP761*. Rates of methionine uptake were determined as described in Fig. 5 over the concentration range 0.7 to 60 μ M-L-[14 C]-methionine. \circ , Strain HfrA *metP761*; \bullet , HfrA for comparison (data from Fig. 5).

Genetic studies on analogue-resistant mutants. Chater (1969) concluded from HfrA mediated conjugation analysis that *metP760* was located in the *pro-leu* region of the linkage map, since this mutant showed 58% linkage with *leuA37* and 69% with *proB45*, and less linkage with more proximal and distal auxotrophic markers. No co-transduction has been detected between *metP760* and *leuA37* or *proB45*. Preliminary genetic analysis indicates that *metP761* is located near *gal-50*. If this were so, then *metP760* and *metP761* are mutations in widely separated genes. However, until this is confirmed, both mutants are given the same tentative gene designation.

Mutations in an aromatic amino acid transport system (*aroP*) which confer resistance to the glutamine analogue azaserine are located between *proA* and *leu* (Ames, 1964; Ames & Roth, 1968), i.e. they may be in the vicinity of *metP760*. However, *aroP504* was sensitive

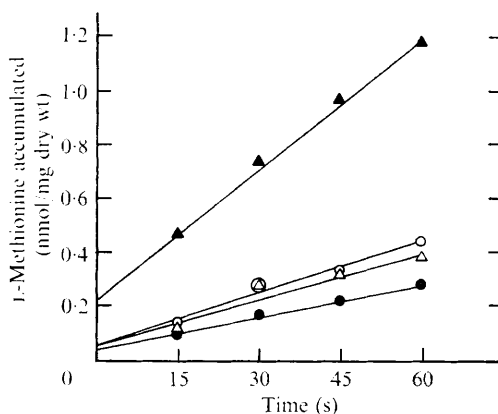


Fig. 11. The effect of introducing a genetic block in methionine biosynthesis on the rate of methionine uptake by a *metK* strain. Bacteria were grown in MM + 60 μ M-L-methionine. After incubation with chloramphenicol the cultures were washed twice with CM and stored at 0 °C. Methionine uptake was determined at a concentration of 0.2 μ M (53 μ Ci/ μ mol). Strains: HfrB2 (○); HfrB2 *metK721* (●); *metA15* (△); *metA15 metK721* (▲).

to the two methionine analogues methionine sulfoximine and α -methylmethionine, and *metP760* and *metP761* were sensitive to azaserine. Consequently the methionine and aromatic amino acid transport systems are quite distinct, although genes determining these systems may be fairly close on the linkage map (Fig. 1).

Methionine transport in an excreting metK strain. Lawrence *et al.* (1968) found that growing cultures of excreting *metK* mutants accumulated slightly less [14 C]methionine and much less [14 C]ethionine than wild-type over periods of 120 min. One explanation for these results, assuming that methionine and ethionine are transported into the cell by the same permease, is that excreted methionine competes with the [14 C]amino acids for uptake. An alternative explanation, which would account for the analogue resistance of both excreting and non-excreting *metK* mutants, is that all *metK* mutants possess a defect in the permease which transports methionine and its analogues. Chater (1970) found no difference in uptake of methionine by chloramphenicol-treated cells of wild-type and an excreting *metK* mutant under the assay conditions employed in the present studies. However, it is just possible that the *metK* mutant possessed a permease with a decreased affinity for methionine which was not revealed by the relatively high concentration (5 μ M) of methionine used by Chater. For this reason we have re-examined uptake of L-methionine in a *metK* strain at a concentration of 0.2 μ M.

The strain *metK721* transported methionine at a slightly reduced rate compared with its parent HfrB2 (Fig. 11). If this reduction is due to dilution of the [14 C]methionine by excreted methionine it should be possible to prevent this effect by introducing a genetic block in methionine biosynthesis. Consequently we examined uptake in the double mutant *metA15 metK721*, and in *metA15* (Smith & Childs, 1966) as a control (Fig. 11). Unexpectedly, *metA15 metK721* transported methionine several times more rapidly than *metA15*. The results of this experiment were confirmed using a modified assay procedure rather than a genetic block to eliminate the effects of methionine excretion on uptake. Chloramphenicol-treated bacteria (50 μ g) were collected on a membrane filter at room temperature (23 °C) and continuously washed for approximately 30 s with a 0.2 μ M solution of [14 C]methionine. Under these conditions *metK721* accumulated 0.49 nmol

methionine/min/mg dry wt compared with 0.17 nmol under standard assay conditions, whereas HfrB2 accumulated 0.23 nmol methionine in the modified assay compared with 0.28 nmol under standard conditions. Thus when the effects of methionine excretion on uptake are eliminated by genetic or technical means, the presence of the *metK72I* mutation is seen to increase substantially the rate of methionine uptake.

DISCUSSION

The present work shows that *Salmonella typhimurium* possesses a permease specific for L-methionine with a K_m of approximately 0.13 μM (represented by the less steep limb of the double reciprocal plot in Fig. 5). At low concentrations of methionine, the methionine analogues, ethionine, α -methylmethionine and methionine sulfoximine all competitively inhibit methionine transport, suggesting that they may be taken into the bacterial cell by the methionine-specific permease. This possibility is supported by the fact that the mutants *metP760* and *metP761*, both of which are severely defective in the methionine-specific permease, are resistant to α -methylmethionine and methionine sulfoximine. It is interesting that these two mutants are not resistant to ethionine because, of the analogues tested, this one appears to have the greatest affinity (i.e. smallest K_i) for the permease.

In *metP760* the methionine-specific permease is retained with a reduced affinity (increased K_m) for methionine, whereas in *metP761* it shows a reduced affinity for methionine and possibly a lowered maximum velocity as well.

There are several possible explanations for the biphasic form of the double reciprocal plot of transport data for wild-type (Fig. 5). Firstly there may be in addition to the methionine-specific permease a second permease with a lower affinity for methionine (K_m of approximately 10 μM , Fig. 5). The kinetics for histidine transport in *Salmonella typhimurium* show a biphasic double reciprocal plot; the less-steep limb of the plot results from the activity of a high-affinity histidine-specific permease ($K_m = 0.1 \mu\text{M}$) while the steep limb is due to a general aromatic permease with a low affinity for histidine ($K_m = 0.1 \text{ mM}$). This conclusion is firmly based on the existence of two classes of mutants, each lacking one of the permeases (Ames, 1964; Shifrin, Ames & Ames, 1966; Ames & Lever, 1970).

A second possibility is that at relatively high methionine concentrations passive diffusion makes a significant contribution to methionine uptake. However, the steep limb of the double reciprocal plot (Fig. 5) does not extrapolate through the origin, and therefore represents a saturable uptake process rather than simple diffusion so that this possibility can be excluded.

Thirdly, the biphasic plot might result from the presence of radioactive impurity in the [^{14}C]methionine. Up to 5% of [^{14}C]methionine used in these studies was present as methionine sulfoxide (although this may have been produced during chromatographic analysis). Freshly prepared solutions of unlabelled L-methionine also contained small amounts of methionine sulfoxide, although the actual amount has not been determined. To produce a biphasic double reciprocal plot, methionine sulfoxide would have to be transported by a permease distinct from the methionine-specific one: if methionine and the sulfoxide were both transported by the same permease, a linear plot would result. It is clear that the methionine-specific permease does not transport the sulfoxide because the analogue does not compete for uptake with [^{14}C]methionine (Table 2).

It is difficult to determine whether the steep limb of the plot results from the transport of methionine alone or methionine together with its sulfoxide because the methionine-specific permease will be functioning at full capacity at the high concentrations of methionine

needed to study the second permease. We hope to study the second permease in mutants defective in the methionine-specific permease.

The conclusion of Chater (1970) that *metK* mutants do not have a defective permease is supported by the present studies. Firstly, *metK72I*, far from having a defective permease, actually accumulates methionine several times more rapidly than wild-type when precautions are taken to prevent methionine excretion from interfering with the permease assay. Secondly, the mutations *metP760* and *76I*, which result in a defective permease, are located away from *metK*. The increased rate of transport in *metK72I* raises the interesting possibility that the permease is regulated by the same system as the methionine biosynthetic enzymes. Growth of bacteria in the presence of 10 mM-L-methionine does not reduce the rate of methionine uptake although the methionine enzymes are repressed under these conditions (Lawrence *et al.* 1968). It has been reported that mutations in either of two genes result in increased levels of the histidine specific permease (Ames & Lever, 1970; Krajewska-Grynkiewicz, Walczak & Klopotoski, 1971).

Current work is aimed at the accurate location of the *metP760* and *metP76I* mutations, and it is hoped to clarify the roles of the *metJ* and *metK* genes in the regulation of the methionine permease.

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