Methionine synthesis in *Proteus mirabilis*

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SUMMARY

Sixty different isolates of methionineless auxotrophs of *Proteus mirabilis* were arranged in nine biochemical groups according to their growth responses to methionine or its precursors. These requirements suggested that *P. mirabilis* possesses a route for methionine biosynthesis which is similar to the pathway operating in *Escherichia coli* and *Salmonella typhimurium*. In contrast to findings with the latter organisms syntrophism was not observed between these mutants of *P. mirabilis* even with sonically disrupted potential feeder strains. Two methionineless auxotrophs of *E. coli* fed auxotrophs of *P. mirabilis* which had metabolic blocks earlier in this pathway. These results, which suggested an inability of methionineless auxotrophs of *P. mirabilis* to accumulate precursors of metabolic blocks, were confirmed by a quantitative comparison of methionine precursors in wild-type and mutant strains of *P. mirabilis* and *E. coli*. The presence of *S*-methylcysteine (SMC) was demonstrated in wild-type and methionineless auxotrophs of *P. mirabilis* and *E. coli*. The growth responses of methionineless auxotrophs of *P. mirabilis* to SMC supported a hypothesis for the participation of this amino acid in the synthesis of methionine via an alternative route.

INTRODUCTION

Syntrophism amongst methionineless auxotrophs of bacteria has been used to study the sequence of intermediates in the synthesis of methionine. The reports of Lampen, Roepke & Jones (1947) and of Davis & Mingioli (1950) dealt with such mutants of *Escherichia coli*; syntrophism occurred between auxotrophs with different nutrient requirements. Demerec *et al.* (1955) and Smith (1961) demonstrated syntrophism amongst different phenotypic groups of methionineless auxotrophs of *Salmonella typhimurium*. Clowes (1958) reported syntrophism among cysteineless mutants of *S. typhimurium*. Figure 1*a* represents the pathway of methionine formation by micro-organisms as revealed by the syntrophism tests of the above workers. It proceeds via cysteine and *O*-succinylhomoserine to form cystathionine, which is cleaved to homocysteine and then methylated to methionine. A second pathway was demonstrated by Wiebers & Garner (1963) who isolated from *Neurospora crassa* an enzyme which was capable of forming homocysteine from homoserine and H₂S. Ragland & Liverman (1956) suggested a third route, namely a transthiomethylation of the thiomethyl group of *S*-methylcysteine (SMC) to homoserine to yield methionine (Fig. 1*b*). These authors isolated SMC from extracts of *N. crassa* and reported that SMC could serve as sole sulphur source for certain strains. The results of Wiebers & Garner (1964) indicated the participation of SMC in the biosynthesis of methionine in

N. crassa by a pathway which excludes cysteine. The same may be true for yeasts (Maw, 1961). Wolff, Black & Downey (1956) showed the synthesis of SMC in bakers yeast. Roberts *et al.* (1955) reported that SMC could be utilized by *E. coli* in isotope competition experiments and also by a cysteine-requiring mutant of this organism.

The present paper deals with the pathway for methionine synthesis in *Proteus mirabilis*, the absence of syntrophism among methionineless mutants of *P. mirabilis* and the presence, in *P. mirabilis* and in *Escherichia coli*, of *S*-methylcysteine which had not previously been shown to occur in bacteria.

Fig. 1. Two possible pathways of methionine synthesis. *a*, Classical pathway; *b*, alternate pathway. * Adenosine-5'-sulphatophosphate; † 3'-phospho-adenosine-5'-sulphatophosphate; ‡ *S*-methylcysteine. *metF*, *cysE*, *hser/met*, etc., indicate metabolic blocks.

METHODS

Media. The minimal medium was a modification of that of Davis & Mingioli (1950) (g./l.): K_2HPO_4 , 10.5; KH_2PO_4 , 4.5; $\text{Na}_3\text{-citrate} \cdot 2\text{H}_2\text{O}$, 0.47; $(\text{NH}_4)_2\text{SO}_4$, 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.102; glucose (autoclaved separately), 2.5; agar 17.5; distilled water 1 l. The pH of this medium was 7.2. For the growth of *Proteus mirabilis* strains nicotinic acid (7.5 $\mu\text{g./ml.}$, membrane filtered) was added. Top-layer agar contained 0.7% (w/v) agar. The sulphur-free medium had $(\text{NH}_4)_2\text{SO}_4$ and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ replaced by equivalent amounts of NH_4Cl and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, respectively.

Chemicals. Carrier-free ^{35}S -sulphate in aqueous solution (pH 6.5–7.5) was obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England, and amino acids from the Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A. Analytical grade sulphate, sulphite, thiosulphate and sulphide were used to test growth responses to inorganic precursors of cysteine (Postgate, 1963).

Organisms. Mutants of *Proteus mirabilis* strain 13 (Coetzee & Sacks, 1960) were obtained by treatment with ultraviolet radiation, manganese chloride or hydrogen peroxide (Demerec, Bertani & Flint, 1951). The penicillin method of Lederberg & Zinder (1948) and Davis (1948) was used to select auxotrophic mutants which were isolated by the replica-plating technique of Lederberg & Lederberg (1952). For the isolation of methionineless mutants of *Escherichia coli* strain CA-7 (obtained from Professor Fredericq, Liège) the more selective method of Gorini & Kaufman (1960) was used. The nutritional requirements of mutants were determined auxanographically (Lederberg, 1946); sulphur sources were applied in drops containing 3 mg. S/ml. The abbreviations *metF*, *cysE*, *hser/met*, etc., are used to indicate specific metabolic blocks in the methionine pathway (Sanderson & Demerec, 1965) as shown in Fig. 1 and correspond to the growth requirements listed in Table 1. Organisms were maintained on nutrient agar slopes at 4°.

Disruption of organisms. Extracts of organisms were prepared from suspensions (30 ml.; equiv. 500 mg. dry wt. bacteria) by ultrasonic treatment in a Raytheon model S102A oscillator (9 kcyc./sec. for 2 hr).

Syntrophism. This was determined by the following techniques. (a) Parallel streaking (Demerec *et al.* 1955; Smith, 1961; Clowes, 1958). Parallel streaks of mutants were made about 2–3 mm. apart on minimal medium enriched with either methionine (2 µg./ml.) or 0.2% (w/v) Difco nutrient broth powder. (b) Auxanographic technique (Clowes, 1958). Liquid cultures of organisms grown with limiting amounts of methionine (2 µg./ml.) were centrifuged down and the supernatant fluids and the deposits (after ultrasonic treatment) were tested auxanographically for their ability to support growth of other mutants. Testing of disrupted cell material for feeding ability eliminated the possible impermeability of feeding strains for accumulated metabolites. (c) Replica plating (Smith, 1961). Inocula of 6 methionineless mutants were spread over well-separated areas of about 1 cm. diameter on each of a number of Difco SS agar plates (Difco MacConkey agar for *Escherichia coli*), over a template. After overnight incubation these plates were replicated (Lederberg & Lederberg, 1952) to single enriched minimal medium plates on which different methionineless auxotrophs were spread. Syntrophism was evident as haloes of growth round replicated areas. (d) Turbidimetric technique (Lampen *et al.* 1947; Davis & Mingioli, 1950). Overnight broth cultures of test strains were washed twice with saline and resuspended in the original volume of saline. Tubes containing 40 ml. enriched minimal medium were inoculated separately with 0.5 ml. of these washed suspensions. Similar tubes were inoculated with 0.25 ml. of washed suspensions of each of two different strains to yield a total inoculum volume of 0.5 ml., analogous to that of tubes with single inoculum. After 24 hr incubation the turbidity of cultures was compared. Syntrophism was indicated by mixed inocula yielding heavier growth than single inocula. (e) Colonial syntrophism (Demerec *et al.* 1955). Pairs of mutants were plated in proportions so that one would form a background growth on the surface of slightly enriched minimal medium and might feed the small number of organisms of the other mutant present in the mixture. Apart from the turbidimetric technique, syntrophism experiments were observed for at least 7 days. Incubation temperature was 37°.

Isolation of free amino acids. Strains were grown for 20 hr on a sulphur-free medium supplemented with ³⁵S-sulphate (0.5 µc./ml.) as the sole sulphur source. For methionineless mutants the medium was enriched with DL-methionine (7 µg./ml.). After

20 hr incubation, by which time all free sulphur-containing amino acids would be metabolized and replaced by intermediates containing the isotope (Cowie, Bolton & Sands, 1950; Roberts *et al.* 1955) the organisms were harvested and a 30 ml. suspension (equiv. 500 mg. dry wt. bacteria) prepared in distilled water. Bacterial concentrations were also estimated by measuring the extinction at $630\text{ m}\mu$ (E_{630}) and by determination of the protein content of disrupted extracts by the biuret method of Gornall, Bardawill & David (1949) with bovine albumin as standard. Disrupted cell material was centrifuged at $151,000g$ in a Spinco Model L ultracentrifuge for 30 min. Proteins and peptides in the supernatant fluid were precipitated with a final concentration of 10% perchloric acid and centrifuged. The supernatant fluid was neutralized with 5 N-KOH followed by centrifugation and filtration. The filtrate was passed through a column ($25 \times 1\text{ cm.}$) of cation exchange resin (Amberlite IR 120, H^+ form) followed by 100 ml. water to remove carbohydrates, salts, inorganic sulphur precursors of cysteine and excess ^{35}S -sulphate. The amino acids were then eluted with N-NH₄OH (50 ml.), concentrated to dryness under vacuum and one half of the material oxidized with performic acid (Leggett Bailey, 1962; Moore, 1963). Unoxidized sulphur-containing amino acids possess different R_f values than the oxidized derivatives, and experiments were done with both types of preparation (Wiebers & Garner, 1964; Roberts *et al.* 1955).

Chromatography of amino acid mixtures. Two-dimensional chromatograms on Whatman no. 3 MM paper were developed for 16 hr by the descending technique. The solvents used were: first dimension, *n*-butanol + formic acid + water (77 + 10 + 13, by vol.); second dimension, *n*-butanol + pyridine + water (1 + 1 + 1, by vol.). Amino acids were located by dipping into 0.2% (w/v) ninhydrin in acetone and heating at 100° for 5 min. The identity of amino acids was confirmed by paper electrophoresis, thin-layer chromatography and auxanography, using samples eluted from uncoloured chromatograms. Paper electrophoresis was done on Whatman no. 3 MM paper with a pH 1.85 buffer consisting of 2.5% (v/v) formic acid + 7.8% (v/v) acetic acid (Leggett Bailey, 1962). Thin-layer chromatography was done on activated silica-gel G layers according to Randerath (1964). For auxanographic identification drops of eluates from paper chromatograms were spotted on minimal medium plates seeded with auxotrophs with known nutrient requirements in top layers. Positive results were obtained after 24–48 hr incubation.

Quantitative comparison of sulphur-containing amino acids. The radio-activity of the sulphur-containing amino acids was determined by counting rectangular pieces ($0.5 \times 2.0\text{ cm.}$) of the two-dimensional chromatograms in a Packard Model 3003 tricarb liquid scintillation spectrometer. The efficiency of the counter for ^{35}S was 62% and sufficient counts were recorded to give a statistical error of less than 2%. Counts were corrected for isotopic decay. The scintillation fluid was 0.4% (w/v) *p*-terphenyl + 0.04% (w/v) 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene in toluene, + 1,4-dioxane (7 + 3, by vol.). Similar determinations were carried out after separation and identification by paper electrophoresis. The radioactivity of the sulphur-containing amino acids/mg. dry wt. organisms grown on ^{35}S -sulphate was used as standard for comparison of the amounts of these amino acids in mutant and wild-type strains of *Proteus mirabilis* and *Escherichia coli*. These estimates are minimal since no attempt was made to compensate for excretion of accumulated metabolites during growth or losses during isolation and chromatography.

RESULTS

Methionineless auxotrophs. Fifty-two isolation experiments yielded 2066 auxotrophic strains of *Proteus mirabilis* of which the largest group, 380, required methionine for growth; of these, auxotrophs responding to cysteine were especially abundant. These findings are analogous to those of Horowitz (1955) with mutants of *Neurospora crassa*. About 50% of our methionineless proteus auxotrophs were leaky. A final group of 60 auxotrophs was chosen on the basis of one phenotypically distinguishable type per isolation experiment. Mutant *ser/met*-544 was leaky but is included as the only representative of its type. The growth responses shown in Table 1 indicate that these

Table 1. *Grouping and growth requirements of methionineless auxotrophs of Proteus mirabilis*

Growth requirements were determined by applying possible precursors in drops of appropriate concentration on minimal medium containing auxotrophs in a top layer.

Auxotroph group	Growth requirements									
	SO ₃ ²⁻	S ₂ O ₃ ²⁻	S ²⁻	Ser	Cys	Hser	Cyst	Hcys	B12	Met
<i>metF</i>	—	—	—	—	—	—	—	—	—	+
<i>metE</i>	—	—	—	—	—	—	—	—	+	+
<i>metA/B</i>	—	—	—	—	—	—	+	+	—	+
<i>hser/met</i>	—	—	—	—	—	+	+	+	—	+
<i>cysE</i>	—	—	—	—	+	—	+	+	—	+
<i>ser/met</i>	—	—	—	+	+	—	+	+	—	+
<i>cysG</i>	—	+	+	—	+	—	+	+	—	+
<i>cysC</i>	+	+	+	—	+	—	+	+	—	+
<i>cysA</i>	+	—	+	—	+	—	+	+	—	+

Cys, Cysteine; Cyst, cystathionine; Hcys, homocysteine; Hser, homoserine; Met, methionine; Ser, serine; B12, vitamin B12; +, growth equivalent to that with methionine as supplement; —, no growth.

Table 2. *Methionineless strains of Proteus mirabilis*

Auxotrophic mutants were obtained by treatment of the wild-type strain with mutagenic agents, selected with penicillin and isolated by replica-plating. Sixty auxotrophs were chosen on the basis of one phenotypically distinguishable type per isolation experiment.

The growth requirements for each group are listed in Table 1.

Auxotroph group	Auxotrophic strains
<i>metF</i>	286, 291, 974, 1445, 1710.
<i>metE</i>	244, 380, 452, 505, 572, 687, 855, 933, 1001, 1261.
<i>metA/B</i>	253, 1303, 1599.
<i>hser/met</i>	1430.
<i>cysE</i>	329, 448, 554, 663, 699, 811, 846, 1002, 1602, 1638.
<i>ser/met</i>	544 (leaky).
<i>cysG</i>	243, 306, 373, 435, 470, 523, 545, 576, 756, 845, 921, 1080, 1114, 1174, 1262, 1648, 1795, 1803.
<i>cysC</i>	484, 666, 697, 766, 865, 1148, 1796.
<i>cysA</i>	354, 832, 932, 1211.

mutants could be separated into nine biochemical groups. The nomenclature of each group corresponds to the metabolic block indicated in Fig. 1. Auxotrophs responding to methionine, homocysteine, cystathionine or homoserine are referred to as *hser/met*

(Table 2). It is not known where the metabolic block in the homoserine pathway is since the growth response of these mutants to possible precursors of homoserine was not tested. The same holds for the *ser/met* mutant which responds to methionine, homocysteine, cystathionine, cysteine or serine (Table 2). The strains of *P. mirabilis* used in these experiments will be referred to as methionineless strains and they are listed in Table 2. All the auxotrophs in Table 2 except *hser/met*-1430 and *ser/met*-544, when supplemented with *S*-methylcysteine (SMC) in minimal medium, show growth equivalent to that with methionine. The two exceptions grew very poorly with SMC. Prototrophic strains of *P. mirabilis* and *Escherichia coli* utilized SMC as the only source of sulphur in accordance with the findings of Roberts *et al.* (1955) with *E. coli*. *Proteus mirabilis metE*-1001 was classified as *metE* because it responded either to methionine or to vitamin B 12 but not to homocysteine. However, this mutant was also found to grow when supplemented with threonine. The growth response of the auxotrophs to the possible intermediate *O*-succinyl-homoserine (Rowbury, 1964) was not tested and consequently *metA* and *metB* mutants were classed together in group *met A/B* which responded, to cystathionine, homocysteine or methionine. All *metE* auxotrophs, except *metE*-505, attained complete growth after 24 hr when supplemented with vitamin B 12. *MetE*-505 was fully grown, with vitamin B 12, only after 48 hr. When supplemented with methionine the *metE* mutants like all methionine auxotrophs showed full growth after 24 hr. This difference in response to vitamin B 12 was also noted by Davis & Mingioli (1950) with similar mutants of *E. coli*.

Two methionine-requiring auxotrophs of *Escherichia coli* strain CA-7 were isolated and designated *metF*-1, -2. In minimal medium these mutants responded only to methionine and, in contrast to the *metF* mutants of *P. mirabilis*, did not grow with SMC.

Syntrophism among methionineless mutants of Proteus mirabilis and Escherichia coli. All syntrophism techniques gave positive results with several biochemically different arginine-requiring mutants of *P. mirabilis* (Coetzee, 1965). These experiments served as controls for the syntrophism techniques used. No syntrophism was detected between pairs of methionineless mutants of *P. mirabilis*. The only exception was *metE*-1001 which was fed by all the methionineless mutants of *P. mirabilis* and by the two *metF* auxotrophs of *E. coli*. These methionineless strains of *P. mirabilis* and the two *metF* mutants of *E. coli* also fed both threonine-requiring and glycine-requiring mutants of *P. mirabilis*, and it is possible that the ability to feed *metE*-1001 may be due to this mutant's growth response to threonine. This behaviour of *metE*-1001 was not further investigated.

The absence of syntrophism among methionineless auxotrophs of *Proteus mirabilis* pointed to an inability of these mutants to accumulate the precursors of metabolic blocks in a methionine pathway. Since syntrophism is known to occur among methionine-requiring auxotrophs of *Escherichia coli* (Lampen *et al.* 1947; Davis & Mingioli, 1950) the two *metF* mutants of this organism were tested for their ability to feed methionineless mutants of *P. mirabilis*. *MetF* mutants of *E. coli* fed all the methionineless mutants of *P. mirabilis* except those of groups *metF* and *metE*.

Quantitative comparison of sulphur-containing amino acids in wild-type and methionineless auxotrophs of Proteus mirabilis and Escherichia coli. The estimates of sulphur-containing precursors of methionine in wild-type and some methionineless auxotrophs of *P. mirabilis* and *E. coli* are listed in Table 3. These quantitative estimates confirmed

the results of syntrophism tests. Although *metF*-1710 and *metE*-855 contain about three times as much homocysteine and *metA/B*-1599 contains almost twice as much cysteine as the wild-type organism, this small accumulation of precursors of metabolic blocks is possibly not enough to produce syntrophism. In contrast, *E. coli metF*-1 contained about forty times as much homocysteine and more than thirty times the cystathionine content of the wild-type strain. Similar results were obtained with all representatives of each group of *P. mirabilis* mutants. As would be expected from its early block, *P. mirabilis cysC*-697 only contained traces of sulphur-containing precursors of methionine.

Table 3. *Sulphur-content of amino-acid precursors of methionine in wild-type and methionineless auxotrophs of Proteus mirabilis and Escherichia coli*

Organisms were grown on sulphur-free medium containing limiting amounts of ^{35}S -sulphate as sole sulphur source, harvested after 20 hr and sonically disrupted. Free amino acids from equivalent amounts of organisms (equiv. 500 mg. dry wt.) were isolated and separated by paper chromatography. The radioactivity of each amino acid/mg. dry wt. bacteria was taken as quantitative standard of comparison. Counts were corrected for isotopic decay which ranged between 9 and 22% when the measurements were made.

Organism	Sulphur source					Total activity
	Methionine	Homo-cysteine	Cystathionine	Cysteine	SMC*	
	Radioactivity (counts/min./mg. dry wt. bacteria)					
<i>Escherichia coli</i>						
Wild type	136.0	124.0	96.0	128.0	118.0	602.0
<i>metF</i> -1	5.8	5800.0	3600.0	246.0	4400.0	14051.8
<i>Proteus mirabilis</i>						
Wild type	124.0	106.0	90.0	122.0	102.0	544.0
<i>metF</i> -1710	4.2	302.0	196.0	166.0	3.8	672.0
<i>metE</i> -855	3.6	294.0	192.0	158.0	3.8	651.4
<i>metA/B</i> -1599	3.8	3.4	2.8	214.0	4.2	228.2
<i>cysC</i> -697	4.0	3.6	3.0	3.8	3.2	17.6

* SMC, *S*-methylcysteine.

The chromatographic studies of methionine precursors led to the discovery of *S*-methylcysteine (SMC) in wild-type and mutant strains of *Proteus mirabilis* and *Escherichia coli* (Table 3). The identity of SMC was confirmed by two-dimensional paper chromatography, paper electrophoresis and thin-layer chromatography of oxidized and unoxidized samples of amino acids isolated from these organisms. SMC isolated from strains grown on ^{35}S -sulphate as sole sulphur source contained the ^{35}S isotope. Mutant strains of *P. mirabilis* which attained growth equivalent to that with methionine when supplemented with SMC contained negligible amounts of SMC themselves. The two *metF* mutants of *E. coli* which did not respond to SMC accumulated SMC in large quantities (Table 3); this suggested that they should feed *metF* and *metE* mutants of *P. mirabilis* which do respond to SMC. However, no such syntrophism was observed.

DISCUSSION

The growth requirements of the methionineless auxotrophs of *Proteus mirabilis* (Table 1) indicate that the route of methionine synthesis (Fig. 1*a*) is similar to that found in *Escherichia coli*, *Salmonella typhimurium* and *Neurospora crassa* (Meister, 1965; Cauthen, Foster & Woods, 1966). The absence of syntrophism between methionineless auxotrophs of *P. mirabilis* contrasts sharply with similar mutants of *E. coli* and *S. typhimurium*. According to growth requirements the *metF*, *metE* and *metA/B* mutants of *P. mirabilis* appear to be similar to auxotrophs of *S. typhimurium* with which Smith (1961) and Demerec *et al.* (1955) obtained syntrophism. Clowes (1958) showed cross-feeding with cysteineless mutants *cysD*, *cysC*, *cysE* and *cysA* of *S. typhimurium* whose growth requirements are much the same as the *cysG*, *cysC*, *cysE* and *cysA* mutants of *P. mirabilis*. Lampen *et al.* (1947) and Davis & Mingioli (1950) reported syntrophism between several methionineless strains of *E. coli* with similar blocks to the *P. mirabilis* strains studied here. The absence of syntrophism caused by impermeability of the cell membranes to intermediates in methionine synthesis was ruled out, furthermore the syntrophism techniques appeared to be reliable and all previous studies on methionine metabolism have indicated that these intermediates are stable. The explanation for this behaviour appears to lie in the inability of *P. mirabilis* mutants to accumulate sufficient quantities of methionine precursors to produce syntrophism. Evidence for this possibility was contributed by the finding that methionineless auxotrophs and wild-type strains of *P. mirabilis* contained equivalent amounts of methionine precursors. In contrast, a *metF* mutant of *E. coli* contained more than twenty times the methionine precursors than the wild-type strain.

The observation that all our mutants which require cysteine, homoserine and serine respond to methionine is noteworthy. This may mean that the methionine-cysteine pathway is reversible in *Proteus mirabilis* and that methionine can serve as a precursor for these amino acids. However, cysteineless mutants of *Escherichia coli* (Lampen *et al.* 1947) and *Salmonella typhimurium* (Demerec *et al.* 1955) were also found to respond to methionine although the pathway is unidirectional in these organisms (Delavier-Klutchko & Flavin, 1965). In contrast to reports on the latter two bacterial organisms the methionine-cysteine pathway is reversible in various fungi (Delavier-Klutchko & Flavin, 1965). The possible reversibility of this pathway in *P. mirabilis* has not been investigated but in the light of the above-mentioned reports on *E. coli* and *S. typhimurium* the growth response of cysteine, homoserine and serine requiring mutants to methionine does not provide any proof in this respect.

The quantitative estimates of *S*-methylcysteine (SMC) in methionineless auxotrophs of *Proteus mirabilis* and *Escherichia coli* are in agreement with the growth responses of these strains to SMC. The *metF* mutants of *E. coli* which do not grow with SMC accumulate it in large quantities. The *metF* mutants of *P. mirabilis* which do grow with SMC contain negligible amounts of this amino acid. This difference in the *metF* metabolic blocks of *P. mirabilis* and *E. coli* cannot be explained. Previous reports of the occurrence of SMC in micro-organisms have been confined to *N. crassa* and yeasts. SMC and its sulfoxide occur in the non-protein nitrogen fraction of various plants (Morris & Thompson, 1955; Synge & Wood, 1956; Thompson, Morris & Zacharius, 1956). Bolton, Cowie & Sands (1952) did not detect it in *E. coli* strain B,

nor did they find homocysteine and cystathionine, although this may be due to the severity of the hydrolytic techniques used. In the present investigation the organisms were not hydrolysed and only free amino acids were examined.

The inability of auxotrophs to accumulate precursors of metabolic blocks has not been reported for other amino acid synthetic pathways in micro-organisms. The existence of more than one pathway leading to the same end-product may possibly influence the accumulation of intermediates in metabolic routes, although this has not been reported. The present findings support the hypothesis that methionine may be synthesized by more than one pathway. Ragland & Liverman (1956) suggested that methionine was synthesized alternatively by the transthiomethylation of the thiomethyl group of SMC to homoserine. According to Wolff *et al.* (1956) SMC is synthesized from serine and methanethiol in yeasts. The restricted growth of the homoserine-requiring methionineless mutant of *Proteus mirabilis* (*hser/met-1430*) when supplemented with SMC in minimal medium supports these findings since this amino acid is a precursor of methionine in the pathway *via* SMC. The ability of *metF*, *metE* and *metA/B* mutants of *P. mirabilis* to grow with SMC also supports the participation of SMC in the formation of methionine *via* a route which excludes cysteine (see Fig. 1). These results agree with the findings of Wiebers & Garner (1964) who encountered a cystathionineless mutant of *Neurospora crassa* which responded to SMC.

Maw (1961) reported the ability of *S*-methylcysteine (SMC) to annul the inhibition of yeast growth by *L*-ethionine. This methionine analogue is a competitive antagonist of methionine in reactions subsequent to the synthesis of the latter, e.g. incorporation into protein (Gross & Tarver, 1955) and conversion to *S*-adenosylmethionine (Parks, 1958). According to Maw (1961) the apparent absence of SMC from proteins argues against the possibility that it may annul the action of ethionine by substituting for methionine in one or more reactions essential for growth which have been blocked by ethionine. It is also doubtful whether SMC is an intermediate in the major metabolic reactions of methionine, such as protein synthesis and *S*-adenosylmethionine formation. Maw (1961) concluded that the most tenable explanation is that SMC gives rise to methionine by a separate pathway unaffected by ethionine. Even if SMC participates in the synthesis of methionine by an alternative route, the formation of methionine remains obscure since Wiebers & Garner (1964) presented data which indicate that neither SMC nor cystathionine are obligate precursors for methionine in *Neurospora crassa*. They proposed that SMC and cystathionine contribute to sulphur metabolism in general as alternative sources of reduced sulphur, or function as regulatory compounds. The fact that conventional methionineless auxotrophs can be isolated suggests that methionine synthesis by an alternative route does not satisfy the requirements of the organism except when exogenous SMC is present. This may mean that methionine synthesis *via* SMC is in turn dependent on the synthesis of methionine by the classical pathway or on the presence of methionine or a source of methyl groups. The role of SMC in the synthesis of methionine by *P. mirabilis* and *E. coli* is at present being investigated. This may yield information to explain the failure of methionine auxotrophs of *P. mirabilis* to accumulate precursors of metabolic blocks.

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