Diastereospecific, Enzymically Catalysed Transmethylation from *S*-Methyl-L-methionine to L-Homocysteine, a Naturally Occurring Process

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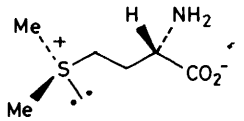
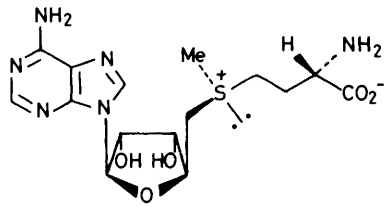
A known catabolic pathway of *S*-methyl-L-methionine in higher plants: donation of a methyl group to L-homocysteine resulting in the production of two molecules of L-methionine, is subjected to stereochemical studies. The two, diastereoisomeric (2-^{2}H, *methyl*-^{13}C)-*S*-methyl-L-methionines are synthesized and utilised in transmethylation reactions with L-homocysteine as the acceptor and an enzyme preparation from jack beans as a catalyst. The resulting, variously labelled methionine species are converted into butyl esters of the *N*-trifluoroacetylated derivatives and, as such, subjected to g.l.c. combined with mass spectrometry in two ionisation modes. Experimentally determined parameters such as mass peak intensities, isotopic enrichment factors, diastereoisomeric purities, and protein-derived methionine, are utilised for calculating the stereoselectivity in the enzyme transfer of the diastereoisotopic methyl groups from *S*-methyl-L-methionine to L-homocysteine. Together, the independent results from the two series of diastereoisomers reveal an enzymic preference of the *pro*-(*R*)-methyl group to the extent of 94% or more.

Thirty years ago, *S*-methyl-L-methionine (**1**) was discovered as a naturally occurring compound by two independent groups of investigators: Shive and co-workers isolated the long known compound from cabbage juice and provided evidence for its presence also in the foliage of several other vegetables,[1] whereas Challenger and Hayward identified an alkali-labile progenitor of dimethyl sulphide in asparagus tips as (**1**).[2] Since then, many additional findings of (**1**) have been reported, all in higher plants or products derived therefrom.[3a] Over the years, (**1**) has attracted considerable interest because of its alleged therapeutic activities.[3b]



(1)

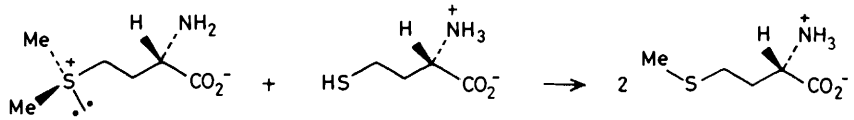


(2)

methionine.[3c] Two catabolic pathways of (**1**) are known: (i) elimination of dimethyl sulphide, with concurrent formation of L-homoserine, catalysed by enzymes from bacteria, yeast, and higher plants, and (ii) enzyme-catalysed demethylation to methionine.[3d] We shall here be concerned only with the latter.

Yeast, and other micro-organisms, contain homocysteine-dependent enzymes indiscriminately accept (**1**) or (**2**); methyltransferases of mammalian tissues seem to possess an ever more limited substrate specificity.[3d] From higher plants, the typical locus for (**1**), homocysteine-dependent transmethylases recognising both (**1**) and (**2**) as substrates have been partially purified. Thus, Abrahamson and Shapiro[4] described a stable enzyme preparation from jack bean (*Canavalia ensiformis*) meal, which we have used in the present study, whereas Dodd and Cossins[5] characterised a similar enzyme from young cotyledons of peas. To what extent specific enzymes, recognising only (**1**) or (**2**), are operating in higher plants remains to be defined.

Considering the reaction shown in Scheme 1 it is obvious that the diastereotopic methyl groups in (**1**) will, in principle, be transferable to L-homocysteine (**3**) at different rates, regardless of the involvement of chiral reagents, *e.g.* enzymes. However, model studies, conducted on the non-catalysed transfer of a methyl group from (*R*,*S*)-2-butylmethylsulphonium ion to *p*-thiocresolate ion and adamantane-1-thiolate, revealed a hardly measurable difference in the rate of transfer of the diastereotopic methyl groups when corrections were made for isotope effects.[6] Hence, it appeared interesting to study the biologically important, enzyme-catalysed reaction of Scheme 1 with a view to establishing: (i) whether any rate difference exists in the



(1) (3)

Scheme 1.

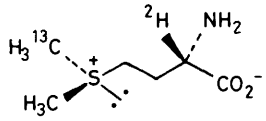
The biosynthesis of (**1**), studied in various plant tissues, or in cell-free extracts of these, consists in methyl group transfer from the ubiquitous *S*-adenosyl-L-methionine (**2**) to L-

transfer of the two methyl groups of (**1**), and (ii) if so, which methyl group is preferentially transferred. Choosing the stable jack bean methyltransferase[4] as the catalyst conveys biological

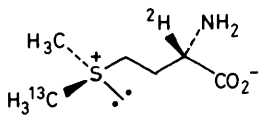
significance to the experiment since growing plants of jack beans are an established source of (**1**).[7] We report the results of our experiments.*

Results and Discussion

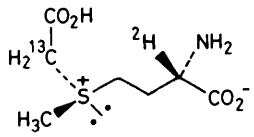
Our approach to the selectivity problem required synthetic access to the diastereoisomeric substrates (**4**) and (**5**). Commercially available (2-^{2}H)-D,L-methionine was resolved[8] to give enantiomerically homogeneous (2-^{2}H)-L-methionine (95 atom-% ^{2}H), which, upon reaction with (2-^{13}C)-bromoacetic acid, was transformed into an approximately 1:1 mixture of the diastereoisomeric sulphonium dicarboxylic acids, (**6**) and (**7**).



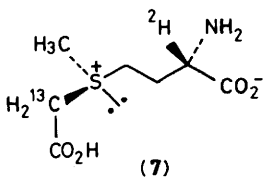
(**4**)



(**5**)



(**6**)



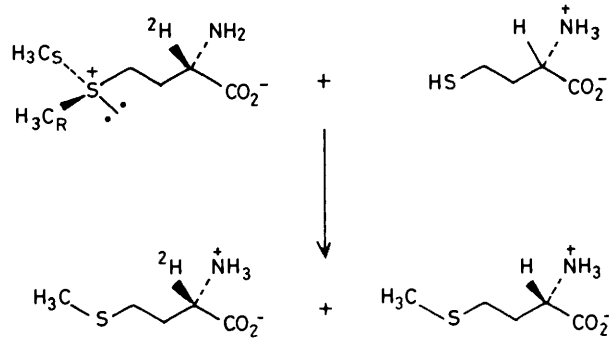
(**7**)

The non-labelled counterparts of (**6**) and (**7**) have recently been prepared, separated, and stereochemically identified by Cornforth *et al.*[9] Taking advantage of their data, (**6**) and (**7**) were separated and identified (see Experimental section).

Thermal decarboxylation of (**6**) and (**7**), under carefully controlled conditions, afforded (**4**) and (**5**), respectively, in yields of *ca.* 50%. N.m.r. spectroscopy (400 MHz) revealed a content of 13% of (**5**) in (**4**), and *ca.* 5% of (**4**) in (**5**).† ^{13}C Contents of 75 atom-% in both *S*-methylmethionines, (**4**) and (**5**), were established by mass spectrometric analysis of the dimethyl sulphone resulting from base-induced elimination of dimethyl sulphide from the two substrates, followed by oxidation.

With the stereoisomerically and isotopically well-defined *S*-methylmethionines, (**4**) and (**5**), in hand, the scene was set for the enzymic transfer reaction (Scheme 1), the stereochemical course of which can be deduced from the product composition in the following way. Suppose that, with (**4**) as the donor, the *pro*-(*S*)-methyl group is exclusively transferred to L-homocysteine (**3**) (Scheme 2); the product in this case will consist of an equimolar mixture of (2-^{2}H)-L-methionine and (^{13}C-*methyl*)-L-methionine; in case of exclusive transfer of the *pro*-(*R*) methyl group from (**4**), however, the outcome will be one molecule of (2-^{2}H)-(^{13}C-*methyl*)-L-methionine and one of L-methionine. Mass spectrometry can tell the two situations apart. Repetition of the experiment with (**5**) as the donor, permits an independent control of the stereochemical course of the enzymically catalysed reaction.

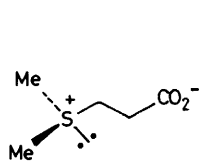
A solution of partially purified *S*-methylmethionine, homocysteine transferase, containing only one enzyme catalysing the transfer reaction, was obtained from jack bean meal according to literature directions.[4] When incubated with (**4**), or (**5**), and a 50—60% molar excess of L-homocysteine‡ in 0.4M-phosphate buffer (pH 6.5), this preparation brought about the *quantitative* conversion into L-methionine within 2 h at 39 °C, as ascertained by ^{1}H n.m.r. spectroscopy control in the non-labelled series serving as a model. Control experiments demonstrated that the corresponding uncatalysed reaction contributed less than 1% to the overall production of methionine and hence could be neglected. Another minor, though non-negligible, secondary source of methionine was traced to the enzyme solution; appropriate corrections were introduced (see Experimental section).



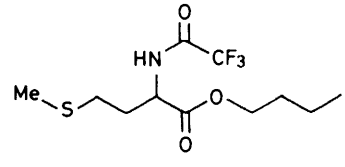
Scheme 2.

The enantiospecificity of the enzymic transfer reaction was studied under comparable conditions. Setting the extent of the above L:L-reaction to 100, the conversions observed for the *S*-methyl-L-methionine:D-homocysteine, *S*-methyl-D-methionine:L-homocysteine, and *S*-methyl-D-methionine:D-homocysteine combinations were roughly 10, 4, and 0%, respectively, based on ^{1}H n.m.r. spectroscopic analyses. The first two of these figures may well reflect a considerable overestimate in view of the total conversion of the L:L-isomer on which the comparison was based.

In our hands, dimethyl-β-propiothetin (**8**) was not at all recognised by the enzyme as a methyl group donor, a finding distinctly at variance with the literature statement that (**8**) serves equally well as *S*-methyl-L-methionine as a substrate for the enzyme;[4] we, as little as the investigators quoted,[10] have any immediate explanation for the discrepancy.



(**8**)



(**9**)

After total consumption of the substrates (**4**) and **5**), the amino acid fractions were isolated from the reaction mixtures by ion-exchange technique. In our preliminary report the trimethylsilyl esters of the *N*-trimethylsilylmethionines were utilised for g.l.c., combined with mass spectrometry (m.s.). Though applicable, the silylated derivatives suffer from limited

* For preliminary accounts of this work, see *Pure Appl. Chem.*, 1980, **52**, 157 (symposium lecture) and *J. Chem. Soc., Chem. Commun.*, 1980, 19.

† These values are considered more reliable than those reported in our preliminary reports, based on 270 MHz spectra.

‡ Generated *in situ* from 3 molar equivalents of virtually racemic homocysteine γ-thiolactone hydroiodide by alkali hydrolysis, or, alternatively, from 1.5—1.6 molar equivalents of pre-synthesized L-homocysteine. Identical results were obtained, irrespective of the source of L-homocysteine.

stability under g.l.c. conditions and low intensities of the molecular ion peaks in their mass spectra. In the later phases of our work we hence took recourse to the butyl esters of the *N*-trifluoroacetylmethionines (**9**),[11,12] possessing higher thermal stability and producing molecular ions of sizeable intensities in their mass spectra. When the *N*-trifluoroacetylated butyl esters of methionine derived from (**4**) and (**5**) were subjected to g.l.c.-m.s. analysis, signs of separation of isotopic species were noticeable in the g.l.c. pattern. In order to overcome this problem, 20—50 scans of narrow 'mass windows' were made throughout the g.l.c. profiles and a weighted average was calculated. When applied to derivatised, natural L-methionine this procedure gave the figures presented in Table 1 and used in all subsequent corrections for natural abundances.

The experimentally acquired knowledge of the relative peak intensities of the various isotopic species deriving from enzymic transmethylation reactions with (**4**) or (**5**) as donors were translated into stereospecificity terms as follows. If '*a*' denotes the number of μmol of *S*-methylmethionine required to produce a final yield of 1.00 μmol of methionine (including protein-derived methionine); *h* and *c* the mole fractions of ^{2}H- and ^{13}C-labelled species, respectively, in (**4**) and (**5**), *d* the fractional diastereoisomeric purity of (**5**) (taken to be 0.95 and 0.13 in the two series, respectively); x_5 the fraction of molecules of (**5**) transferring the *pro*-(*R*)-methyl group, *i.e.* ^{13}C-*methyl*; and x_4 the fraction of molecules of (**4**) transferring the *pro*-(*R*)-methyl group, *i.e.* ^{12}C-*methyl*, the following equations obtain, where I_1, I_2, and I_3 stand for the relative intensities ($I + I_2 + I_3 = 1$) corrected for natural abundance contributions, of three consecutive mass peaks starting with the lowest mass:

$$I_1 = -x_5 adhc + x_4(ahc - adhc) + adhc - ah - ac + 1$$
$$I_2 = 2x_5 adhc - 2x_4(ahc - adhc) - 2adhc + ah + ac$$
$$I_3 = -x_5 adhc + x_4(ahc - adhc) + adhc$$

Upon solving these equations it appears that x_4 and x_5 deviate only slightly from each other; the difference becomes marginal, as apparent from the equations, when the diastereoisomeric purity, as here, is high (*i.e.* *d* close to 1 or 0). Hence, a common value, *x*, can be calculated, with good approximation, from the three independent equations:

$$x = \frac{d + \frac{1 - I_1}{hca} - \frac{1}{h} - \frac{1}{c}}{2d - 1} = \frac{d + \frac{I_2}{2hca} - \frac{1}{2c} - \frac{1}{2h}}{2d - 1} = \frac{d - \frac{I_3}{hca}}{2d - 1}$$

For $d = 0.95$, we determine x_5 from the equations; for $d = 0.13$, x_4. The three *x*-values calculated from the three equations are averaged (weighted reciprocally to the standard deviation on the value) and the results are presented in Table 2 together with a numerical expression, *S*, of the stereoselectively defined as $S = 2x - 1$.

It appears from the results in Table 2 that the enzyme-catalysed methyl group transfer from *S*-methyl-L-methionine to L-homocysteine (Scheme 2) takes place with very high, albeit not necessarily complete specificity, the *pro*-(*R*)-methyl group being transferred to the extent of 94% or more.

Whether the less than 100% stereospecificity observed actually reflects a limited enzymic stereospecificity or rather should be atributed to experimental factors is difficult to decide unequivocally in view of the numerous parameters involved in the experimental work. There can be no doubt, however, that Nature here, once again, reveals its three-dimensional partiality. Combined with the recently adduced demonstration by Arigoni[13] of the transmethylation being accompanied by inversion of the migrating methyl group, we now possess a detailed insight into the stereochemical course of the jack bean enzyme-catalysed *S*-methylation of L-homocysteine with *S*-methyl-L-methionine as the donor molecule, a reaction operating in higher plants and possibly of considerable regulatory significance.

Table 1. Natural abundance contributions for various ions in the mass spectrum of the butyl ester of unlabelled *N*-trifluoroacetylmethionine (**9**)

Experiment [a]	I_1 [b]	I_2 [b]	I_3 [b]
EI	1.000(301) [c]	0.141(302) [c]	0.062(303) [c]
Calculated	1.000	0.136 [d]	0.059 [d]
CI [e,f]	1.000(319)	0.140(320)	0.059(321)
Calculated	1.000	0.140 [d]	0.059 [d]

[a] EI denotes electron impact; CI chemical ionisaton, with ammonia as the reactant gas. [b] Relative line intensities; $I_1 = 1.000$. [c] Figures in parentheses indicate the *m/z*-value of the ion considered. [d] Based on a value of 1.08 for natural ^{13}C-abundance. [e] The ion $[C_{11}H_{18}F_3NO_3S + NH_4]^+$ is considered. [f] Average of two determinations.

Experimental

General.—M.p.s were determined in capillary tubes in a heated bath and are uncorrected. ^{1}H N.m.r. spectra were recorded on Bruker HXE-90 or HX-270 instruments (FT mode), ^{13}C n.m.r. spectra (at 22.63 MHz) on a Bruker WH-90 spectrometer. G.l.c. was performed on a Perkin-Elmer Sigma 1 instrument. Mass spectra were obtained on a VG Micromass 7070 instrument operated at 70 eV (in the EI-mode) with open collector slit in order to produce flat-topped peaks. The signals were monitored by means of a u.v. recorder. A Pye Unicam Series 204 gas chromatograph was attached through all-glass transfer lines (kept at 200 °C), to the ion source (at 220 °C) of the mass spectrometer. The glass column used (1.5 m × 2.2 mm i.d.) was packed, unless otherwise specified, with 3% OE-17 on Chromosorb W, HP, isothermally operated at 145 °C. Ion-exchange chromatography was performed on a Kontron Liquimat III Amino Acid Analyzer, column: 25 cm × 4 mm i.d.; resin: Durum DC-4; buffer: Durum pico buffer system I; further conditions as specified in the text. Optical rotations were determined on a Perkin-Elmer 141 polarimeter. D-Methionine (>99%) was from 'EGA-Chemie,' (2-^{2}H)-D,L-methionine from Merck, Sharp and Dohme, Canada (cat. no. MD-1111, 98 atom-% ^{2}H), and (*^{13}C-methyl*)-L-methionine (cat. no. C232, 90 atom-% ^{13}C), (2-^{13}C)-bromoacetic acid (cat. no. C121, 90 atom-% ^{13}C), and ^{13}C-methyl iodide (cat. no. C27, 90 atom-% ^{13}C) all from Stohler Isotope Chemicals.

Non-labelled Compounds

L- *and* D-*Homocysteine* [(**3**), *and Mirror Image*].—L- or D-Methionine (2.01 g) was dissolved in liquid ammonia (40 ml). Sodium (1.23 g) was added to the solution in 10—20 portions. When the blue colour persisted, anhydrous ammonium iodide was added portionwise to discharge the colour (4.4 g). After evaporation under reduced pressure, the residue was taken up in oxygen-free water (5 ml), and the pH adjusted to 6—6.5 by addition of concentrated hydrogen iodide (*ca.* 4 ml). Oxygen-free ethanol (100 ml) was added to the filtered solution, and the almost colourless homocysteine (1.4 g, 79%) was collected; δ_H (90 MHz; NaO^2H in ^2H_2O, containing sodium 4,4-dimethyl-4-silapentanesulphonate (DSS) as internal reference) 3.28 (1 H, m, 2-H), 2.3—2.6 (2 H, m, 4-H), and 1.6—1.9 (2 H, m, 3-H). The contents of homocystines in the synthetic products were lower than 3% as estimated by ^{1}H n.m.r. spectroscopic comparison with a mixture of homocystine and *meso*-homocystine prepared according to the literature:[14]

Table 2. Mass spectrometric results of the analysis of *N*-trifluoroacetylated butyl esters of methionine derived from *S*-methylmethionine and homocysteine by enzymically catalysed transmethylation

Experiment [a]	Diastereoisomer	I_1 [b]	I_2 [b]	I_3 [b]	x [c,d]	S% [b]
CI	(5)	0.240(319)	0.731(320)	0.029(321)	0.95 ± 0.02	90 ± 4
	(4)	0.491	0.221	0.289	1.00 ± 0.02	100 ± 4
EI	(5)	0.240(301)	0.727(302)	0.033(303)	0.94 ± 0.02	88 ± 4
	(4)	0.486	0.221	0.294	1.00 ± 0.02	100 ± 4

[a] CI denotes chemical ionisation, with ammonia as the reactant gas; EI electron impact. [b] See text for definition. [c] See text for calculation method. [d] The limits given are derived by differentiating the three equations (see text) with regard to the various parameters, inserting the values: a = 0.47 ± 0.01 [for diastereoisomer (5)]; 0.48 ± 0.01 [for diastereoisomer (4)]; h = 0.95 ± 0.01, c = 0.75 ± 0.01; d = 0.95 ± 0.02, and 0.13 ± 0.02 (for the two series); $I = I_i \pm 0.005$, and, finally, forming the weighted average of the three results.

δ_H (90 MHz, NaO^2H, ^2H_2O, DSS) 3.34 (2 H, dd, 2-H), 2.77 (4 H, t, 4-H), and 1.8—2.3 (4 H, m, 3-H).*

Homocysteine γ-Thiolactone Hydroiodide.—The lactone salt was prepared from L-methionine as described by Baernstein;[15] δ_H (90 MHz, ^2H_2O, DSS) 4.37 (1 H, dd, 2-H), 3.4—3.6 (2 H, m, 4-H), and 2.0—3.0 (2 H, m, 3-H); $[\alpha]_D^{25}$ +0.2° (*c* 2.7, H_2O). In view of the reported value: $[\alpha]_D^{26}$ +21.5° (*c* 1 in H_2O) for the lactone hydrochloride, [16] the synthesis, in our hands, was accompanied by virtually complete racemisation.

S-*Methyl*-L-*methionine Hydrobromide*, (1)·HBr.—The salt was produced following the directions of Toennies and Kolb,[17] m.p. 144—146 °C (decomp.) [lit.,[18] 138—139 °C (decomp.)]; $[\alpha]_D^{24}$ +20.9° (*c* 1.8 in H_2O) (lit.,[18] +19.1°); δ_H (90 MHz, ^2H_2O, DSS) 3.89 (1 H, t, 2-H), 3.4—3.6 (2 H, m, 4-H), 2.96 (6 H, s, MeS^+), and 2.2—2.5 (2 H, m, 3-H);† δ_C [22.63 MHz, ^2H_2O and H_2O (1:1), external dioxane at δ_C 67.4] 173.4 (C=O), 53.9 (2-C), 40.5 (4-C), 26.1 (3-C), and 25.9 (MeS^+) (coinciding signals were observed for the diastereotopic *S*-methyl groups throughout the pH-range (1—12).†‡

S-*Methyl*-L- *and* S-*methyl*-D-*methionine Toluene*-p-*sulphonate* (1, *Salt with Toluene*-p-*sulphonic Acid, and Enantiomer*).—A solution of L- or D-methionine (513 mg) and methyl toluene-*p*-sulphonate (1 320 mg) in formic acid (2 ml) was kept at 39 °C for 6.5 h, and then concentrated under reduced pressure. The oil was triturated with ether and it crystallised on addition of methanol (15 ml); after addition of ether (15 ml) the crystalline salt was isolated (1 057 mg, 92%), m.p. 148—149 °C (decomp.) (from methanol–water, 95:5); $[\alpha]_D^{24}$ +15.1° (*c* 2.0 in water) (Found: C, 46.65; H, 6.35; N, 4.1; S, 19.0. $C_{13}H_{21}NO_5S_2$ requires C, 46.55; H, 6.31; N, 4.18; S, 19.12%). D-Enantiomer, m.p. 148—149 °C (decomp.); $[\alpha]_D^{24}$ −15.3° (*c* 2.1 in water).

S-*Methyl*-L-*methionine Bis*-2,4,6-*trinitrobenzenesulphonate*, (1).2$(NO_2)_3C_6H_2SO_3H$.—The salt crystallised in almost quantitative yield on mixing the above hydrobromide of (1) (123 mg) and 2,4,6-trinitrobenzenesulphate acid tetrahydrate (412 mg) in water (3 ml). The air-dried salt contained 9.0% water (Karl Fischer) and was partially dehydrated over P_2O_5 *in vacuo*, δ_H [90 MHz, 6 mg in $(C^2H_3)_2SO$ (400 μl) containing ^2HCl in ^2H_2O (25 μl, 7M), and DSS] 4.14 (1 H, br t, 2-H), 3.61 (2 H, br t, 4-H), 3.02 (3 H, s, MeS^+), 3.00 (3 H, s, MeS^+), and 2.39 (2 H, br q, 3-H).

Bis-2,4,6-*Trinitrobenzenesulphonates of* (C_SS_S)- *and* (C_SS_R)-S-*Carboxymethylmethionine* [*Unlabelled* (7) *and* (6)].—An approximately 1:1 mixture of the hydrobromides of unlabelled (7) and (6), prepared upon heating of L-methionine (984 mg) and bromoacetic acid (947 mg) in water (7 ml) to 40 °C for 10 h, was converted into diastereoisomeric salts essentially as described,[9] yet with the following minor modifications: the reaction mixture was diluted with water (to 40 ml), 1.0M-iodine (3.3 ml), and potassium iodide (1.20 g) were added, and the mixture was left at 3 °C overnight to give 'black, shiny, pyramid-shaped crystals.'[9] Next day, three portions (0.7 ml each) of 1.0M-iodine were added at 3-h intervals without intermittent filtrations. On the third day, two more portions (0.7 ml each) were added, spaced by 4 h, and, finally, on the fourth day, the crystals consisting of the pentaiodide of (6) were collected, washed with water, and air-dried (2.36 g, or 84%, based on a molecular weight of 843,[9] and a 1:1 composition of diastereoisomers in the original reaction mixture).

To the filtrate, 1.0M-iodine (1 ml) was added and the solution was left for a further 3 days at 3 °C, at the end of which the needles, apparently consisting of the pentaiodide of homogeneous (7), had formed. Three additional portions (1.0 ml each) of 1M-iodine were added with 3-h intervals; next day, the pentaiodide of (7) was collected (1.16 g, 41%).

The two pentaiodides were separately suspended in water (5 ml) and extracted with ether until no more iodine could be removed. After removal of ether from the filtered solutions, the diastereoisomeric purity of the two hydroiodides of (6) and (7) was controlled by ion-exchange amino acid chromatography (column temperature 40 °C and buffer pH 2.70) and n.m.r. spectroscopic analyses. Chromatography revealed ratios of 96:4 and 4:96 between (6) and (7) in the two pentaiodide fractions. Homoserine was identified as an impurity (<2%) in both salts. The hydroiodide solutions were each diluted with water (to 15 ml); on adding 2,4,6-trinitrobenzenesulphonic acid (10% molar excess) to the two solutions, the corresponding hydrated salts precipitated in yields of 88% for the salt of (6), 80% for that of (7), both based on the amount of pentaiodide; δ_H for the hydroiodide of (7) (90 MHz, ^2H_2O, DDS) 4.27 (2 H, centre of AB system, $HO_2CCH_2S^+$), 4.04 (1 H, t, 2-H), 3.3—3.9 (2 H, m, 4-H), 2.97 (3 H, s, MeS^+), and 2.3—2.6 (2 H, m, 3-H); δ_H values for the hydroiodide of (6) were similar. In mixtures of the two, however, a 2.3 Hz displacement of the 2-H signal for (6)

* The synthetic enantiomers contain more than 95% L- and 95% D-homocysteine according to h.p.l.c.-analyses with a chiral mobile phase, kindly performed by Professor E. Gil-Av, Dr. N. Grünberg, and Dr. S. Weinstein at the Weizmann Institute of Science, Rehovot, Israel.

† Dissolved in ^2HCl-containing ^2H_2O, we observed the following δ_H values: 4.28 (1 H, t, 2-H), 3.4—3.7 (2 H, m, 4-H), 2.99 (6 H, s, MeS^+), and 2.3—2.7 (2 H, m, 3-H). These shifts are clearly at variance with the δ_H values (3.89, 3.48, 2.97, 2.80, and 2.40) reported for the dimethanesulphonate of (1).[19] Also, our δ_C-shift measured in ^2H_2O, containing an excess of methanesulphonic acid: 171.1 (C=O), 52.1 (2-C), 40.0 (4-C), 25.8 (MeS^+), and 25.4 (3-C), deviate from those reported: 170.9, 51.9, 39.9, 39.4, 25.5 (MeS^+), and 25.2 (MeS^+).[19]

‡ In contrast with our findings of isochronic *S*-methyl groups throughout a wide pH-range, Dyckes and his co-workers[20] reported a difference of 0.04 in the δ_C values of the *S*-methyl groups in *S*-methionine hydroiodide at pH 7.

towards higher field was noticed, a feature permitting the disclosure of 2—3% of (**6**) as an admixture in (**7**) and *vice versa*; δ_H for the 2,4,6-trinitrobenzenesulphonates of (**7**) and (**6**) [90 MHz, $(C^2H_3)_2SO$, ^2HCl, ^2H_2O, DSS as above] 4.73 (2 H, br, $HO_2CCH_2S^+$), 4.15 (1 H, br t, 2-H), 3.72 (2 H, br t, 4-H), 3.08 (3 H, s, two peaks separated by 1.9 Hz, MeS^+ groups in the diastereoisomers), and 2.3—2.6 (2 H, br q, 3-H); δ_C [aqueous $KHCO_3$, C^2H_3CN, 5:1 v/v)] for the mixture of hydrobromides of (**7**) and (**6**) 174.1 (1-C), 168.7 (CH_2CO_2H), 54.0 (2-C), 48.1 (4-C), 38.9 and 38.7 [$C(O)CH_2S$, separation 4.9 Hz between the diastereoisomers], 26.6 (3-H), and 24.0 (MeS^+) (dioxane at δ 67.4 as internal reference). Spectra recorded in very alkaline or very acidic solutions exhibited even smaller differences between the diastereoisomers. Assignments are based on chemical shifts at varying pH values and on undecoupled spectra.

Dimethyl-β-propiothetin (**8**) *Toluene*-p-*sulphonate*.—3-Methylthiopropionitrile[21] (10.0 g) was refluxed in concentrated hydrochloric acid (40 ml) for 1.5 h. The mixture was cooled and diluted with water (20 ml) and then extracted with dichloromethane to give the oily 3-methylthiopropanoic acid. Heating of the acid and methyl toluene-*p*-sulphonate to 100 °C for 2 h gave a viscous oil which was extracted with ether and ethyl acetate. The residue crystallised after 3 days at −20 °C and, on trituration with acetone, gave the thetin toluene-*p*-sulphonate (64% from the crude acid), m.p. 91.5—92.5 °C [from acetonitrile–acetone, 1:1 (v/v)] (Found: C, 47.0; H, 5.9; S, 20.8. $C_{12}H_{18}O_5S_2$ requires C, 47.04; H, 5.92; S, 20.93%); δ_H (90 MHz, ^2H_2O, DSS) 7.3—7.8 (4 H, m, ArH), 3.52 (2 H, t, 3-H), 2.97 (2 H, t, 2-H), 2.92 (6 H, s, MeS^+), and 2.39 (3 H, s, arom. CH_3).

Labelled Compounds

(2-^{2}H, ^{13}C-methyl)-D,L-*Methionine*.—(2-^{2}H)-D,L-Methionine (219 mg) was converted into the dianion of the corresponding homocysteine by dissolution in liquid ammonia (10—15 ml) and addition of sodium (167 mg) and ammonium iodide (259 mg) as described above. To the cooled solution (−78 °C), (^{13}C)-methyl iodide (223 mg, 93% ^{13}C according to ^{1}H 90 MHz n.m.r. spectroscopy) was added; after 1 h at −78 °C the solution was allowed to come to room temperature, leaving a solid that was dissolved in water (9 ml); the pH value of the solution was adjusted to 6.5 with aqueous hydroiodic acid. After filtration (Hyflo-Supercel) and evaporation to dryness, the residue was redissolved in water (1.5 ml). Addition of ethanol (30 ml) caused the labelled methionine to separate (175 mg, 79%); δ_H (90 MHz, NaO^2H, ^2H_2O, DSS) 3.32 (dd, trace of 2-H), 2.88 and 1.33 (centre at 2.11) (3 H, *S*-^{13} C-*methyl*, $J(^{13}C\text{-}H)$ 139.3 Hz), 2.56 (2 H, dt, 4-H), 2.11 (9% of total *S*-Me, s, ^{12}C-*methyl*), and 1.7—2.0 (2 H, dt, 3-H). The n.m.r. spectrum precluded the presence of (2-^{2}H)-homocysteine or (2-^{2}H)-homocystine in the preparation.

(C_S,S_S)-*and* (C_S,S_R)-(2-^{2}H, carboxymethyl-1-^{13}C)-S-*carboxymethylmethionine*, *Bis*-2,4,6-*trinitrobenzenesulphonate* (**6**)·2X *and* (**7**)·2X [X = $(NO_2)_3C_6H_2SO_3H$].—(2-^{2}H)-D,L-Methionine was resolved with (+)-α-bromocamphor-π-sulphonic acid according to the literature procedure,[8] to give (2-^{2}H)-L-methionine, $[\alpha]_D^{24}$ +23.7° (*c* 3.1, 1M-hydrochloric acid) (lit.,[8] +23.4° (*c* 3.1 1M-hydrochloric acid) for the 2-^{1}H-compound). ^{1}H-N.m.r. spectroscopy revealed a content of 96% of 2-^{2}H- and 4% of 2-^{1}H-substitution whereas the ratio 94:6 was established by g.l.c. combined with m.s.

The commercial (2-^{13}C)-bromoacetic acid, purchased as containing ≥90 atom-% ^{13}C, in fact contained 75—80% of (2-^{13}C)-, 10—12% of (1-^{13}C)-, and 10—13% of non-labelled bromoacetic acid acording to ^{1}H n.m.r. spectroscopic analysis (60 MHz).

The labelled reactants were utilised in the synthesis of the dicarboxylic acids as outlined above, with the sole modification that non-labelled, additional bromoacetic acid was introduced to ensure complete alkylation of methionine, even small amounts of which may seriously impede separation of the diastereoisomeric dicarboxylic acid salts. From (2-^{2}H)-L-methionine (985 mg) and (2-^{13}C)-bromoacetic acid [930 mg total, close to 75 atom-% (2-^{13}C) labelled material (*vide supra*)] a 1:1 mixture of the bromides was obtained, and, in turn, converted as described above into the doubly labelled pentaiodides of (**7**) [1.15 g, 41% (based on methionine, M^+, 854)] and (**6**) (2.34 g, 84%). After conversion into the hydroiodides, amino acid chromatography (column temperature 40 °C, buffer pH 2.70), revealed isomer ratios of 96:4 and 3:97 between (**7**) and (**6**) for the two isomers. Conversion of the two diastereoisomers into their bis-2,4,6-trinitrobenzenesulphonates, as described above for the non-labelled counterparts, was achieved in high yields.

(C_S,S_S)-*and* (C_S,S_R)- (2-^{2}H, methyl-^{13}C)-S-*Methylmethionine Bis*-2,4,6-*trinitrobenzenesulphonate*, (**4**)·2X *and* (**5**)·2X [X = $(NO_2)_3C_6H_2SO_3H$].—The dried (P_2O_5; 0.5 mmHg; 12 h) 2,4,6-trinitrobenzenesulphonate of (**7**) (401 mg) was dissolved in hexamethylphosphoramide (3 ml); pyridine (1.3 ml) was added and the efficiently stirred mixture was heated to 58—60 °C for 15 min. The mixture was then poored into cold ether (50 ml) and the precipitate collected by centrifugation. After dissolving the precipitate in hydrochloric acid and evaporation, the residue was suspended in water (3 ml) and sodium acetate added to adjust the pH value to 4—5. The filtered solution and aqueous washing (total of 5 ml) was transferred to an IR-120 ion exchange column (*ca.* 10 ml, Na^+-form). The column was rinsed, first with water (20 ml), then quickly with dimethyl sulphoxide (20 ml) to remove the slightly soluble pyridinium trinitrobenzenesulphonate, and finally with water. The doubly labelled *S*-methylmethionine (**5**) was eluted with 2M-aqueous ammonia (40 ml); ammonia was quantitatively removed by partial evaporation, the concentrated solution made acidic with hydrochloric acid, and the mixture finally taken to dryness. The residue was redissolved in water (5 ml), and the solution filtered through charcoal. From the concentrated solution (3 ml), the trinitrobenzenesulphonate of (**5**) precipitated upon addition of trinitrobenzenesulphonic acid (379 mg, tetrahydrate). The air-dried salt (222 mg, 53%, based on M^+ 824), according to ^{1}H n.m.r. spectroscopy [400 MHz, $(C^3H)_2SO$, ^2HCl as above], contained 5% of the diastereoisomeric salt of (**4**) as a contaminant.

In the same fashion, the salt of (**4**) (202 mg, 48%) was obtained from the salt of (**6**) (402 mg). ^{1}H N.m.r. spectroscopic analysis revealed a content of 13% of the diastereoisomeric salt of (**5**) as a contaminant.

Transmethylation Reactions

The enzyme for the present studies was prepared according to literature directions (buffer extraction, precipitation with ammonium sulphate, and dialysis),[4] with the application of 0.02M rather than 0.01M phosphate buffer (pH 6.9) as the sole modification. Whole jack beans ('ICN Nutritional Biochemicals,' Catal. no. 102 109), after grinding and defatting, or jack bean meal ('Sigma Chemical Co.,' Catal. no. J-0125), as such, were used for the enzyme preparations with comparable results. The enzyme was remarkably stable, enduring year-long storage at −20 °C and repeated thawing and freezing.

The bis-2,4,6-trinitrobenzenesulphonate of (**5**) (30 ml) was converted into the corresponding hydrochloride by passing its solution in 50% aqueous acetonitrile (v/v) (0.6 ml) through an IRA-400 ion exchange column in the chloride form (*ca.* 1.1 ml). The column was eluted, first with the same solvent (0.4 ml) and then with water (4 ml). After charcoal treatment, filtering and

freeze-drying, the residue was dissolved in water (0.75 ml) to give a solution which, according to amino acid chromatography (standard programme for protein amino acids), contained 0.93 μmol of the hydrochloride of (**5**) per 25 μl. Analogously, a solution of the hydrochloride of (**4**) was prepared, containing 1.18 μmol per 25 μl. These solutions were utilised in the enzymic reacions, carried out under two sets of conditions:

(a) Virtually racemic homocysteine γ-thiolactone hydroiodide (*vide supra*) (30 μmol, 100 μl) was mixed with 7.5M-sodium hydroxide (1 500 μmol, 200 μl) under nitrogen and kept for 5 min, when primary potassium phosphate (2 000 μmol, 2 000 μl) was added to bring the pH value to 6.4—6.6. Portions of this solution (300 μl) were mixed with the enzyme solutions (300 μl) and the labelled *S*-methylmethionines (25 μl). The reaction mixtures were kept at 39 °C for 2 h, when the protein was precipitated by heating to 100 °C for 1 min. Water (1 ml) was added, and the supernatant liquid, after centrifugation, combined with three aqueous washings (1 ml each) and transferred to an IR-120 ion exchange resin (5.7 ml), in the protonated form. After being washed neutral with water (*ca.* 15 ml), the column was eluted with 2.3M-pyridine (60 ml, 0.5 ml per min), and the amino acid isolated by concentration to dryness. Finally, it was converted into the *N*-trifluoroacetylated butyl ester (*vide infra*). Under these conditions (*i.e.* 0.4M-phosphate buffer) the transmethylation was complete after less than 2 h.

(b) Mixing L-homocysteine (1.5 μmol, 25 μl), *S*-methyl-L-methionine (**1**) (as the toluene-*p*-sulphonate) (1.25 μmol, 25 μl), and the enzyme (300 μl) in a phosphate buffer (0.1M, 250 μl, pH 7.1), and keeping the mixture under nitrogen at 39 °C, resulted in a slower reaction. Under these conditions (*i.e.* 0.05M-phosphate buffer) the reaction had proceeded to the extent of 60, 90, and 100% in 2, 4.5, and *ca.* 20 h, respectively. In this case, heating did not cause precipitation of protein but addition of 0.9M-phosphate buffer (0.6 ml) served well for this purpose.

Analytical Methods

Extent of Reaction.—^{1}H N.m.r. spectroscopy (90 MHz) allowed the detection of ^{12}C-methionine and *S*-methylmethionine in mixtures down to *ca.* 0.14 and 0.07 μmol, respectively. In the ^{13}C labelled species, ^{13}C–H couplings interfered with the determinations; in these cases the reactions were performed under conditions identical with those prevailing in model experiments with the ^{12}C counterparts.

Quantitative G.l.c.—The amino acid residue from the enzymic reaction [procedure (a) above] was treated with hydrogen chloride in butanol [3.0 ml, freshly prepared from acetyl chloride (10 ml) in butanol (100 ml)] in a tared flask and heated to 100 °C for 30 min. After weighing, an aliquot (*ca.* 20%) was transferred to a Reacti Vial^R and volatiles were removed in a stream of nitrogen. Trifluoroacetic anhydride (100 μl of a 10% solution in dichloromethane) was added and the mixture was heated to 100 °C for 20 min. After evaporation, a solution of dimethyl suphide in ethyl acetate (20% v/v) was added, and the volatiles were again removed. The vial was closed, dimethyl sulphide (20 μl) and a standard solution of hexadecane in ethyl acetate (100 μl) were added, and the solution was ready for g.l.c. (glass column (2.4 m × 3 mm i.d.); 6.4% OV 17 on Chromosorb W, HP, 80—100 mesh; He gas (45 ml per min); 128—200 °C at 4 °C per min). Methionine solutions, in the absence of dimethyl sulphide, were found to undergo surprisingly fast changes. The g.l.c. response factor, relative to hexadecane, was found reproducible within ±2%.

Known quantities of methionine, carried through the entire procedure (a), were recovered to the extent of 91 and 97% in two experiments (corrected for protein-derived methionine; see below).

Two experiments, conducted in the absence of added *S*-methylmethionine, both revealed the presence of 0.65 μmol of methionine per ml of used enzyme solution. This correction was employed in calculating the chemical yields of methionine.

Two experiments, with unlabelled *S*-methylmethionine toluene-*p*-sulphonate, deomonstrated conversion of this substrate into methionine to an extent of 98 and 100%.

Determination of the ^{13}C-*Content in the Diastereoisomers of* H_2 (2-^{2}*H*, methyl-^{13}*C*)-S-*Methylmethionine* (**5**) *and* (**4**).—The bis-2,4,6-trinitrobenzenesulphonate of the *S*-methylmethionine species (9—11 mg) and triethylamine (*ca.* 1.1 μmol) in dimethyl formamide (110 μl) were heated to 125 °C for 15 min. To the cooled solution, 40% hydrogen peroxide (20 μml) was added, and the mixture was kept at 60 °C for 30 min to give a mixture of dimethyl sulphoxide and dimethyl sulphone. After centrifugation, the solution was subjected to g.l.c., combined with m.s. in the CI mode (ammonia as the reactant gas). Two dominating peaks at *m/z* 96 and 112, represented the molecular ions of dimethyl sulphoxide and sulphone, respectively. Since the sulphone g.l.c. profile and m.s. background proved superior to those of the sulphoxide, the latter were neglected. Scans of the mass region *m/z* 112—114 served to establish the ^{13}C-content of (**5**) as 74.6%, that of (**4**) as 74.8% applying corrections for natural abundance contributions obtained from the spectrum of the non-labelled species. In the calculations the value *c* 0.75 was consequently employed.

Enzyme-derived Methionine.—The quantity of methionine released from the enzyme during the transmethylation reaction was determined by g.l.c.-m.s. of the derivatives (**9**). Using the mass spectrometer as a detector (EI, 70 eV) at *m/z* 301, and the synthetic derivative (**9**) of L-methionine as an external standard, it was established that in experiments conducted at high phosphate concentration (0.41M) for 2 h at 39 °C, 0.39 μmol of methionine per ml of enzyme was produced. This correction was employed, in the guise of the parameter *a*, throughout the calculations. At low concentrations (0.052M), for 20 h at 39 °C, 0.95 μmol was produced per ml of enzyme.

Substrate Specificity.—Experiments with all four combinations of *S*-methyl-L-and-D-methionine and L- and D-homocysteine, were conducted as described under procedure (b) above. After 20 h, the L-L-combination had reacted completely, whereas the L/D-, D/L-, and D/D-combinations had reacted to the extent of less than 10, less than 4, and 0%, respectively. The reactions were followed by ^{1}H n.m.r. and the figures hence are only approximate.

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