Lysine 2,3-Aminomutase: Role of *S*-Adenosyl-L-methionine in the Mechanism. Demonstration of Tritium Transfer from (2*RS*,3*RS*)-[3-^{3}H]Lysine to *S*-Adenosyl-L-methionine

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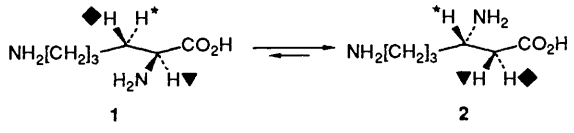
(2*RS*,3*RS*)-[3-^{3}H]Lysine has been synthesized, and incubated with lysine 2,3-aminomutase in the presence of *S*-adenosyl-L-methionine (SAM). After incubation, the SAM was converted, by heating, into methylthioadenosine (MTA). The latter contained *ca.* 1–6% of the incubated tritium, consistent with an exchange of SAM hydrogens (probably at C-5′) with the 3-*pro-R* hydrogen of lysine. The results support a mechanism for lysine 2,3-aminomutase in which SAM acts as hydrogen carrier.

Lysine 2,3-aminomutase catalyses the reversible interconversion of L-α-lysine **1** and (3*S*)-β-lysine **2**.[1] The enzyme requires pyridoxal phosphate (PLP), ferrous [iron(II)] ion and *S*-

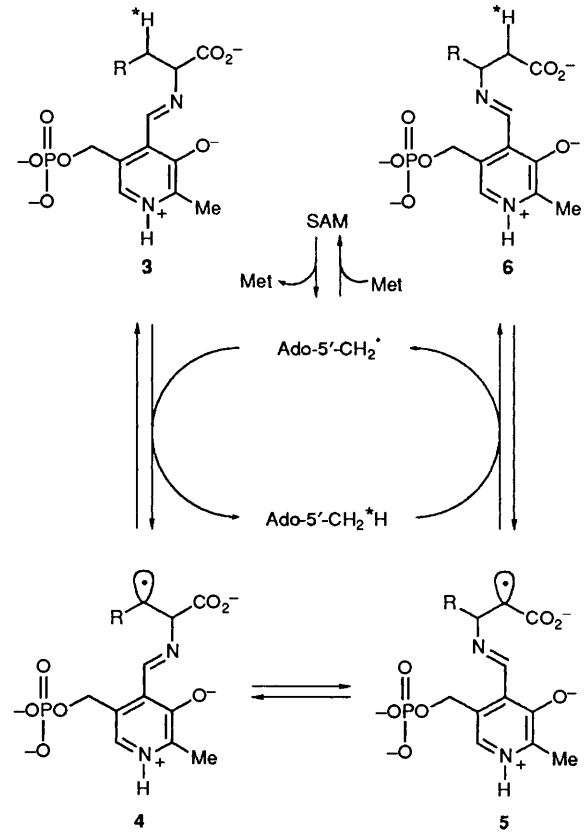


1 **2**

adenosyl-L-methionine (SAM) for catalytic activity. It has been suggested by Barker[2] that SAM plays a hydrogen-carrying role in the aminomutase reaction, analogous to the role played by adenosylcobalamin (AdoCbl) in vicinal 1,2-interchange reactions catalysed by AdoCbl-dependent enzymes. Such a mechanism (Scheme 1) would be consistent with the predominant intermolecularity established for the C-3-to-C-2 hydrogen transfer in the conversion of L-α-lysine **1** into (3*S*)-β-lysine **2**.[3–5] In contrast, the amino-group transfer proceeds intramolecularly.[3] Recently, Frey and co-workers demonstrated that this hydrogen transfer also takes place intramolecularly to a minor degree.[6]

Several years ago, one of us (D. J. A.) reported the results of experiments designed to test the proposed role of SAM as hydrogen carrier in the interconversion of α- and β-lysine.[4] It was rationalized that, according to the proposed mechanism, incubation of L-[3,3-$^2H_2$]lysine with lysine 2,3-aminomutase should result in incorporation of deuterium into the C-5′-methylene group of the coenzyme, SAM. After thermal degradation of the SAM to 5′-methylthioadenosine (MTA), the C-5′ deuterium might be detectable by ^{2}H NMR spectroscopy. The experiment was conducted by incubation of the enzyme with L-[3,3-$^2H_2$]lysine in the presence of SAM, the latter being used in large excess over the amount necessary for full activation of the aminomutase. This strategy was based on the expectation, based on published equilibrium dialysis experiments,[9] that unbound and enzyme-bound SAM would undergo rapid exchange. In that experiment, no deuterium was detectable in the isolated MTA, and it was therefore tentatively concluded that SAM does not serve as hydrogen carrier in the reaction.

In contrast to the above results, Frey and Moss[7,10] recently reported the formation of tritiated α- and β-lysine upon incubation of unlabelled α-lysine with the aminomutase in the presence of [5′-^{3}H]SAM. Their experiments were conducted with approximately stoicheiometric amounts, rather than large excesses, of SAM. Their tritium-transfer experiments were performed only in the SAM ⟶ α/β-lysine direction. In view of the conflicting results from our two groups, we thought it desirable to re-examine the question of hydrogen (tritium) transfer in the opposite direction over a range of SAM : enzyme ratios. We now report the synthesis of the [3-^{3}H]lysine required for this study, and its use in the above stated experiment.

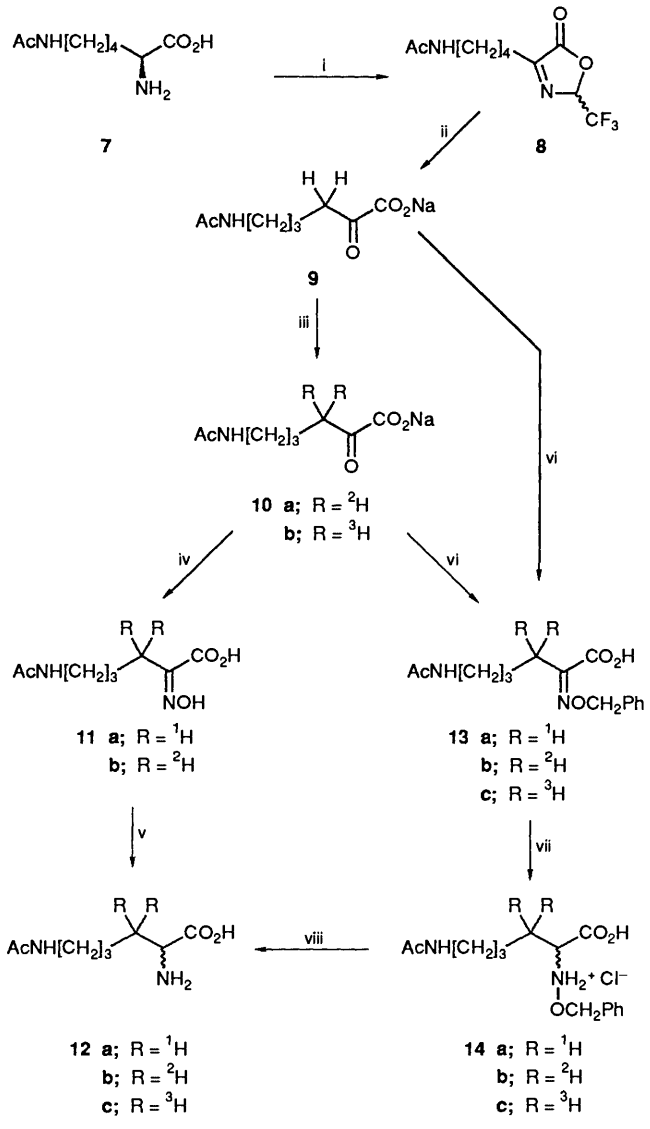


3 **6** **4** **5**

Scheme 1 Hypothetical mechanism for interconversion of α- and β-lysine by lysine 2,3-aminomutase involving 5′-deoxyadenosyl radicals. Interconversion of intermediates **4** and **5** has been proposed to proceed *via* an aziridine intermediate (not shown).[3,7,8]

Results

Synthesis of (2RS,3RS)-[3-^{3}H]*Lysine*.—This compound was prepared by the route outlined in Scheme 2. The pathway is an adaptation of a method previously used for the synthesis of [3-^{3}H]lysine by Stein and Englard.[11] Their synthesis produced, from tritiated water having a specific activity of *ca.* 18 mCi mmol^{-1}, (±)-[3-^{3}H]lysine having a specific activity of only *ca.* 0.3 mCi mmol^{-1}, which was insufficient for our purposes. In



Scheme 2 Synthesis of labelled acetyllysines **12b** and **12c**. *Reagents and conditions:* i, TFAA then heat; ii, aq. NaOH; iii, Na_2CO_3 and ^2H_2O or [^{3}H]water; iv, NH_2OH·HCl; v, H_2–various catalysts; vi, H_2NOCH_2-Ph·HCl; vii, $NaCNBH_3$–TFA; viii, H_2–Pt(C), aq. HCl.

addition, certain steps (evaporation of tritiated water solutions, elevated pressure hydrogenation) were deemed undesirable from a radiation safety point of view. Therefore, the following procedure was developed, first using deuterium labelling in attempts to optimize the incorporation of label from water into intermediate **10** and to minimize losses of label in subsequent steps.

Keto acid salt **9** was prepared from N^6-acetyl-L-lysine **7** *via* the oxazolone **8** by extensive modification of the procedure of Stein and Englard.[11] The salt **9** was then smoothly converted under mild conditions into the α-deuteriated analogue **10a**. The corresponding oxime **11b** was prepared in the exchange solution and it was then precipitated with MeCN. Catalytic hydrogenation of oxime **11b** at atmospheric pressure using a wide variety of catalysts gave either low yields of N^6-acetyl-[3-$^2H_2$]lysine **12b** (using Raney nickel, Pd–$BaSO_4$, Rh–Al or Pt–S–C as catalyst) or else proceeded with extensive exchange of the β-hydrogens with the solvent (runs with 10% Pt–C or Pd–C–aq. NH_3 as catalyst). Similarly, when unlabelled oxime **11a** was hydrogenated in D_2O in the presence of platinum or 10% Pt/C as catalyst, significant incorporations of deuterium at both C-2 and C-3 were observed. These results could be rationalized by postulating that the reductions proceeded with initial N–O bond hydrogenolysis[12] to yield C=N–metal or C=N–H intermediates which could then either undergo tautomerization, resulting in hydrogen exchange, or hydrolysis to the α-keto acid, resulting in low yields of amino acids **12**. We reasoned that hydride reduction of the C=N bond prior to N–O bond hydrogenolysis might circumvent these problems. While reduction of oxime **11a** with $NaCNBH_3$[13–15] gave mixtures of products, the *O*-benzyl oxime **13a** underwent smooth reduction to the salt **14a** with $NaCNBH_3$ in the presence of trifluoroacetic acid (TFA). Subsequent reduction (10% Pt/C, H_2) of compound **14a** gave a good yield of the free amino acid **12a**. Similarly, deuteriated *O*-benzyl oxime **13b** (not isolated; prepared *in situ* from **10a**) was converted *via* **14b** into **12b**, essentially without loss of deuterium. ^{2}H NMR spectroscopy showed that 8% of the deuterium was located at C-2[13] with the remainder located at C-3. Acidic hydrolysis of compound **12b** then afforded (±)-[3-$^2H_2$]lysine.

In a similar manner, tritiated keto acid **10b**, prepared by equilibration of compound **9** with tritiated water (specific activity 13.7 mCi mmol^{-1}) was converted into (±)-[3-^{3}H]lysine having a specific activity of 11.6 mCi mmol^{-1}. Assuming *ca.* 8% of the tritium to be located at C-2 by analogy with the deuterium-labelling results, the C-3 specific activity would be *ca.* 10.6 mCi mmol^{-1}, which is *ca.* 79% of the maximum possible value.

Lysine 2,3-Aminomutase-catalysed Transfer of Lysine C-3 *Hydrogens to* S-*Adenosyl*-L-*methoinine.*—Prior to carrying out the hydrogen (tritium) crossover experiments, we attempted to increase the specific activity of our enzyme over that used in our previous work.[4,5] That enzyme preparation had a specific activity of *ca.* 0.3 units mg^{-1} of protein, even though SDS-PAGE gels of this protein showed one major band with only minor traces of impurities. In contrast, Chirpich *et al.*[1] and Frey and co-workers[6,7,10] have reported for lysine 2,3-aminomutase specific activities as high as 12–14 units mg^{-1} protein. Frey's initial experiments[7] with [5′-^{3}H]SAM were done with an enzyme having a specific activity of *ca.* 3 units mg^{-1} protein and, in later studies,[6] enzyme of the highest reported specific activity was used. By adoption of their use of 0.1 mmol dm^{-3} L-lysine in all isolation buffers,[10] and other modifications (see Experimental) for activating the enzyme, the specific activity of our lysine 2,3-aminomutase preparation was raised to *ca.* 3.5 units mg^{-1} protein. Aside from the inclusion of 0.1 mmol dm^{-3} L-lysine in all solutions used in the purification, the enzyme was isolated as previously described[4] and gave similar SDS gels.

The activated enzyme (*ca.* 10 nmol) was then incubated in four simultaneous runs with an excess of (2*RS*,3*RS*)-[3-^{3}H]lysine and SAM, the latter added in amounts ranging from 5–1000 nmol (experiments 1–4, Table 1). After extensive incubation, additional SAM (*ca.* 8 μmol) was added as carrier. The mixture was then heated at 100 °C to stop the reaction and to convert the SAM into MTA. The latter was then separated from the substrate as in the previous work.[4] The crude MTA was then subjected to a rigorous chromatographic protocol involving cation-exchange HPLC, followed by reversed-phase preparative HPLC.[16,17] At all steps of purification, the major radioactive peak corresponded to MTA. However, we noticed in preliminary experiments that substantial losses of tritium occurred at various stages of the purification. This problem was eventually traced to the formation of MTA sulphoxides which, if formed prior to isotopic dilution, resulted in lower MTA specific activity after HPLC and crystallization. After inclusion of several reductive treatments with dithiothreitol in the purification protocol,[18] MTA sulphoxides were reconverted into MTA, and no significant tritium losses were observed during the purification.

Table 1 Radioactivity data for MTA recovered from Lysine 2,3-aminomutase incubations with (2*RS*,3*RS*)-[3-^{3}H]lysine

Incubation experiment no.	SAM added (nmol)	Purification[a] stage	MTA specific activity (μCi mmol^{-1})		^{3}H SAM[d] formed in incubation (μCi)	% ^{3}H recovered as MTA[e]	Specific activity of SAM prior to adding carrier (mCi mmol^{-1})[f]
			Measured[b]	Corrected[c]			
1	5	i	50.9		0.35	1.2	84
		ii	1.3	53.7			
		iii	1.3	51.7			
2	20	i	49.4		0.34	1.2	20.5
		ii	1.11	44.6			
		iii	0.98	39.2			
3	100	i	55.6		0.39	1.4	4.5
		ii	1.07	53.7			
		iii	1.03	51.5			
4	1000	i	228		1.75	6.1	1.8
		ii	6.8	274			
		iii	6.8	270			

[a] Purification stages: i, MTA purified through all chromatographic steps, prior to addition of additional carrier for crystallization; ii, crystallized twice; iii, crystallized three times. [b] MTA quantity in counted sample at stages i and iii measured by cation-exchange HPLC, and at stages ii and iii by weighing vacuum-dried crystalline material. [c] Measured values multiplied by dilution factor for comparison with specific activities at stage i prior to recrystallization. [d] Calculated for purification stage i. Calculated as measured specific activity of MTA × (μmol SAM dilution with carrier, before heating, *i.e.* 7.7–8.3 μmol). [e] Percentage recovery of tritium in (2*RS*,3*RS*)-[3-^{3}H]lysine. [f] Calculated assuming that the SAM content of isolated lysine 2,3-aminomutase is negligible by comparison with the amount added prior to incubation (*i.e.*, 5–1000 nmol).

In the preparative reversed-phase HPLC purifications of MTA, a minor amount of an additional radioactive substance was collected which eluted prior to the main bulk of the MTA. After modification of the reversed-phase solvent protocol so that a variety of related adenine nucleosides were cleanly separated, this minor radioactive peak was found to co-elute with 5′-deoxyadenosine (5′-dA). On the other hand, 5′,8-anhydroadenosine[19,20] was not detected. These compounds are possible products of radical-based cleavage of SAM.[17,20]

After chromatography and addition of more non-radioactive MTA as carrier, the MTA was recrystallized, and its specific activity determined. In all experiments, a substantial amount of tritium was detected in the MTA, and the specific activities did not vary significantly during the later purification steps (Table 1). The amount of tritium transferred to the coenzyme was essentially independent of the amount of added SAM, except in run 4 where 1000 nmol of SAM were added. In this run, for unknown reasons, a *ca.* five-fold higher specific activity was observed in the isolated MTA.

The percentage of incubated tritium in lysine which was transferred to SAM in each exchange mixture is shown in Table 1, as calculated from the specific activities of the isolated MTA and the amount of carrier (*ca.* 6.7–8.3 μmol) added in the purification to 'stage 1'. It should be noted that the tritiated lysine is racemic at both C-2 and C-3. Only L-lysine is a substrate of lysine 2,3-aminomutase,[1] and only its 3-*pro-R* hydrogen is transferrable.[3,5]

An attempt was made to determine the amount of SAM present in our lysine 2,3-aminomutase. The enzyme was denatured under mild conditions to minimize decomposition of SAM. After chromatography on cation-exchange cellulose (CM-52), the SAM content was estimated by cation-exchange HPLC. Very little SAM was detectable, and therefore the precision of the measurement is poor, but it is estimated that 10 nmol of our enzyme contained only *ca.* 1.5 ± 1.0 nmol of SAM, not enough to account for the apparent high specific activities reached by SAM after incubation with enzyme in the presence of tritiated lysine. A control experiment in which bovine serum albumin was spiked with known amounts of SAM indicated *ca.* 87% recovery of SAM after ion-exchange chromatography.

Discussion

The results described in this paper show that, in contrast to our earlier conclusion,[4] lysine 2,3-aminomutase catalyses extensive hydrogen transfer from C-3 of lysine to SAM (presumably to the C-5′ position). Although the location of tritium in the labelled SAM was not determined, it is tentatively assumed to be located at C-5′ by analogy with the results of Frey and Moss.[7,10] The results support Barker's proposed mechanism for this reaction (Scheme 1).[2] The reasons for the formation of SAM having apparently higher specific activity than the incubated lysine in runs with low SAM:enzyme ratios (runs 1 and 2) are unclear at present. This phenomenon could, at least in part, be rationalized if the isolated enzyme contained a substantial amount of SAM, so that during incubations the enzyme contained significantly more than the 5 or 20 nmol of SAM added prior to incubations in runs 1 and 2. However, an attempt to measure the SAM content of the isolated enzyme revealed that, after denaturation, only *ca.* 0.15 ± 0.10 moles of SAM per mole of enzyme could be detected by HPLC. This does not exclude the possibility that SAM could also be covalently bound to the enzyme and thus not be removed by SDS denaturation. If this were the case, the enzyme–cofactor linkage would still have to permit thermal extrusion of MTA.

The earlier failure to detect transfer of deuterium to SAM from [3,3-$^2H_2$]lysine can be explained by proposing that the exchange of enzyme-bound SAM with unbound cofactor is quite slow, so that the extent of deuterium transfer to the cofactor pool was only a small fraction of that required for ^{2}H NMR detection. This conclusion conflicts with that of Zappia and Ayala.[9] Our results support and complement those of Frey and co-workers,[6,7,10] who demonstrated tritium transfer from [5′-^{3}H]SAM to α- and β-lysine by activated lysine 2,3-aminomutase.

The reaction has been proposed to involve the intermediacy of adenosin-5′-yl free radicals, which then abstract 3-*pro-R* hydrogens from L-lysine to form reversibly 5′-deoxyadenosine as intermediate hydrogen carrier.[2,10] In fact, chromatographic evidence for the formation of a small amount of radioactive 5′-deoxyadenosine was found during the purification of the MTA derived from enzyme-labelled SAM. The identity of the tritium-labelled peak corresponding to 5′-deoxyadenosine has not yet been confirmed spectroscopically due to a lack of sufficient material. An alternative product, 5′,8-anhydroadenosine, which could conceivably have been formed from adenosyl radicals,[19,20] was not chromatographically detectable.

Experimental

General.—^{1}H NMR spectra were recorded on a Bruker WM-250 or AM-400WB instrument, with internal tetramethylsilane (TMS) or $Me_3SiCD_2CD_2CO_2Na$ (TMSP) as standard. For ^{2}H NMR spectra taken in ^{2}H-depleted water, $(CD_3)_3COD$, δ_{CD} 1.27, was used as internal standard, and for spectra taken in $CHCl_3$, the natural-abundance $CDCl_3$ peak, δ 7.27, was used. Mass spectra were performed on AEI MS-30 (EI spectra), Finnigan 4000 (CI spectra) and VG 770E-HF (FAB spectra) instruments.

Liquid scintillation counting was performed with a Packard TriCarb model 2250 instrument. Samples were dissolved in Ecolume (ICN Radiochemicals) (10 cm^3). HPLC radiochromatograms were produced by manually collecting 15–20 second fractions of eluant (*ca.* 0.25–0.33 cm^3) in liquid scintillation counting vials, followed by addition of Ecolume and counting. Radioactive zones on TLC plates were detected with a Bioscan System 200 imaging scanner.

Materials.—Sodium dihydrolipoate solution (0.15 mol dm^{-3}) was prepared by addition of Na_2CO_3 (29.4 mg) to a suspension of dihydrolipoic acid[21] (58 mg) in degassed water (1.85 cm^3). *S*-Adenosyl-L-methionine (SAM) hydrochloride salt was purified as described by Chirpich *et al.*[1] The resultant solution in dil. HCl was lyophilized twice (additional water being added after the first time), after which the residue was redissolved in water. The SAM concentration was estimated from the absorbance at 260 nm (ε 15 400). Analysis by HPLC typically indicated the presence of 1–2% MTA and no *S*-adenosyl-L-homocysteine. HPLC was performed on an Alltech Associates Partisil SCX column (0.46 cm × 25 cm; 10 μm particle size) with ammonium formate buffer (10 mmol dm^{-3}; pH 4.0) as eluant at 1.2 cm^3 min^{-1}. SAM solutions of appropriate concentrations were freeze–thaw degassed and then frozen and sealed in ampules under vacuum and stored at −20 °C. Sodium cyanoborohydride was purified by a published method.[13] MTA sulphoxide (mixed stereoisomers) was prepared as described,[22] and purified by chromatography on Amberlite XAD-4 resin as described by Mills *et al.*,[23] followed by recrystallization from absolute EtOH–Pr^{i}OH, m.p. 111–128 °C. 5′,8-Anhydroadenosine was obtained as described by Hay and Finke.[20] This product was further purified by preparative TLC on silica gel (solvent 77% EtOAc–20% Pr^{i}OH–3% HOAc), followed by recrystallization from water,[19] m.p. 295 °C (decomp.).

Synthesis of (2RS,3RS)-[3-^{3}H]*Lysine.*—N^6-Acetyl-L-lysine **7**, (0.94 g, 5 mmol) was added in small portions during 10 min to trifluoroacetic anhydride (TFAA) (2.1 cm^3, 14.9 mmol). The mixture was stirred at 60 °C for 90 min, after which the residual TFAA (and TFA) was evaporated off under reduced pressure. The residual liquid was heated at 115 °C under N_2 for 15 min. Additional TFA was removed by rotary evaporation of the cooled mixture, and then the residual oil was distilled in a kugelrohr apparatus to give 4-(4′-*acetamidobutyl*)-2-*trifluoromethyloxazol*-5(2H)-*one* **8**, as a viscous, yellow oil (0.965 g, 72.5%), b.p. 135–150 °C (0.1 mmHg); δ($CDCl_3$) 1.62 (2 H, apparent quintet, *J* 6 Hz, 2′-H_2), 1.82 (2 H, apparent quintet, *J* 7–8 Hz, 3′-H_2), 1.98 (3 H, s, NAc), 2.74 (2 H, dt, *J* 2.2 and 7.5 Hz, 1′-H_2), 3.29 (2 H, apparent quintet, *J* 6.7 Hz, 4′-H_2), 5.76 (1 H, br s, NH) and 6.12 (1 H, dq, $J_{H,H}$ 2.1 Hz, $J_{H,F}$ 4.2 Hz, 2-H). High-resolution MS (EI, 30 eV) M^+ 266.0876 ($C_{10}H_{13}F_3N_2O_3$ requires M, 266.0876).

The product was dissolved in diethyl ether (20 cm^3) and treated with 1 mol dm^{-3} aq. NaOH (3.8 cm^3) and the two-phase mixture was stirred overnight in an open flask. Upon treatment of the resultant aq. solution with acetone (50 cm^3) a precipitate was formed which, after several hours storage at 4 °C, was collected by suction filtration to yield 6-acetamido-2-oxohexanoic acid sodium salt **9**, as light yellow crystals (0.323 g, 43% from **8**). A portion recrystallized from absolute EtOH had m.p. 197–198 °C, δ(D_2O) 1.58 (4 H, m, γ- and δ-H_2), 1.99 (3 H, s, NAc), 2.77 (2 H, t, *J* 6.9 Hz, β-H_2) and 3.18 (2 H, t, *J* 6.4 Hz, ε-H_2).

The product **9** (42 mg, 0.2 mmol), Na_2CO_3 (5 mg, 0.05 mmol) and [^{3}H]water (0.2 cm^3, 0.15 Ci) were magnetically stirred in a septum-sealed conical reaction vial while being heated at 65 °C for 3 h. After cooling to 25 °C, the mixture was chilled (−78 °C) and treated with a solution of *O*-benzylhydroxylamine hydrochloride (64 mg cm^{-3} MeOH; 0.5 cm^3, 0.2 mmol). The mixture was allowed to warm to 25 °C and, after being stirred for several minutes, was treated with TFA (50 mm^3, 0.65 mmol) and $NaCNBH_3$ (2 mol dm^{-3}; 0.2 cm^3 MeOH, 0.8 mmol) added by syringe. The mixture was stirred for 18 h at room temperature with further additions of TFA (total 75 mm^3, 0.965 additional mmol) and $NaCNBH_3$ solution (total 300 mm^3, 1.2 additional mmol) in portions during the first 50 min. In the development of this procedure with deuteriated materials, this reduction was followed by ion-pair HPLC using a Waters Associates Resolve C_{18} column (0.39 × 15 cm) with 18% MeOH–82% 0.1 mol dm^{-3} sodium acetate buffer, pH 5.0, containing sodium heptane-1-sulphonate (5 mmol dm^{-3}) as eluent at 1.0 cm^3 min^{-1} and detection by UV at 254 nm.

The mixture was chilled to −78 °C, and dry cation-exchange resin (Dowex 50 W-X8, H^+-form; 50–100 mesh; *ca.* 1.5 mequiv. H^+) was added. The vial was resealed, vented with a syringe needle and allowed to warm to room temperature. The mixture was then magnetically stirred for 1 h. This step was repeated with an additional portion of cation-exchange resin (300 mg). The contents of the vial were then transferred to a column (1 × 6 cm) containing the same resin, and the column was eluted with water (80 cm^3). Then elution with 2 mol dm^{-3} NH_4OH (20 cm^3) and evaporation of the eluate gave N^6-acetyl-N^2-benzyloxy-[3-^{3}H]lysine **14c**. A radiochromatogram (silica gel TLC; solvent 4% 2 mol dm^{-3} NH_4OH in propan-2-ol, eluted twice) showed *ca.* 91% of the radioactivity at R_f 0.25, coeluting with the unlabelled analogue **14a**.

The product **14c**, in water (4 cm^3) plus 6 mol dm^{-3} HCl (0.025 cm^3), was added to a slurry of 10% Pt–C (which had been prehydrogenated for 20 min) in a 10 cm^3 round-bottom flask. The mixture was stirred under H_2 at atmospheric pressure at 25 °C for 9 h, and then filtered to yield a solution of (2*RS*,3*RS*)-N^6-acetyl-[3-^{3}H]lysine **12c**. Radiochromatography (silica gel TLC; solvent 10% 2 mol dm^{-3} NH_4OH in 95% EtOH) showed a single radioactive zone (>98%), R_f 0.30, comigrating with authentic unlabelled compound **12a**.

The above filtrate (*ca.* 10 cm^3) was treated with Dowex 50W-X8 cation-exchange resin (H^+-form; 50–100 mesh; 1.0 g) at reflux for 44 h.[24] After cooling, the mixture was added to a column (1 × 6 cm) of the same resin, and the column was washed successively with 0.5 mol dm^{-3} HCl (10 cm^3), water (20 cm^3) and 2 mol dm^{-3} NH_4OH (16 cm^3), discarding the first 6 cm^3 of the last eluate (neutral part), after which the lysine-containing eluate was collected and lyophilized. After redissolution in water (5 cm^3), lyophilization again gave (2*RS*,3*RS*)-[3-^{3}H]lysine (0.62 mCi), which was redissolved in water (1.3 cm^3) plus 6 mol dm^{-3} HCl (25 mm^3). Radiochromatography (silica gel TLC; 50% 2 mol dm^{-3} NH_4OH in 95% EtOH) showed a single peak, R_f 0.18, corresponding to lysine. HPLC analysis[25] and liquid scintillation counting of suitably diluted samples indicated a total yield of 9.7 mg of (±)-lysine hydrochloride (26.8% from **9**). The specific activity is thus 11.6 ± 0.4 mCi $mmol^{-1}$ which is 79% of the theoretical maximum, assuming 8% of the tritium to be located at C-2.

The deuteriated analogue (2*RS*,3*RS*)-N^6-acetyl-[3-$^2H_2$]lysine **12b** was obtained by the same procedure as described for

12c, except that D_2O was used in place of [^{3}H]water. The residue obtained after reduction of intermediates **13b** and **14b** (which were not isolated) and lyophilization was recrystallized from aq. EtOH to give compound **12b** as needles, m.p. 238–240 °C (decomp.); δ(D_2O) 1.41 (2 H, m, γ-H_2), 1.56 (2 H, m, δ-H_2), ~1.88 (<0.1 H, m, residual β-CH^2H), 1.99 (3 H, s, NAc), 3.19 (2 H, t, *J* 6.8 Hz) and 3.70 (0.85 H, s, α-H); δ_D(H_2O) 1.91 (rel int 92%, br s, β-$^2H_2$) and 3.79 (8%, br s, α-^{2}H). A portion of compound **12b** was converted as previously described[4] into the di-*N*-phthaloyl methyl ester derivative, δ_D($CHCl_3$) 2.28 (94%, br s, β-$^2H_2$) and 4.82 (6%, br s, α-^{2}H); CI MS (NH_3) (+) 442 (2%), 441 (7) and 440 (13, M + NH_4 isotopomers); 425 (11), 424 (54), 423 (100) and 421 (4, M + 1 isotopomers), 365 (7), 364 (31), 363 (71), 362 (35), and 361 (9, *M* − CO_2CH_3 isotopomers); (−) 424 (13%), 423 (56) and 422 (100, M + e^- isotopomers).

An unlabelled sample of compound **14a**, prepared as described above for **14c**, was converted into the hydrochloride, which gave needles, m.p. 133–134 °C (from water); δ(D_2O) 1.30 (2 H, m, γ-H_2), 1.47 (2 H, m, δ-H_2), 1.96 (3 H, s, NAc), 3.12 (2 H, t, *J* 6.6 Hz, ε-H_2), 3.51 (1 H, br s, α-H), *ca.* 4.8 (2 H, benzylic CH_2, obscured by HOD peak)* and 7.43 (5 H, s, Ph). High-resolution FAB MS (8 keV Xe beam, thioglycerol matrix) M + 1 *m/z*, 295.1631. $C_{15}H_{23}N_2O_4$ requires *m/z*, 295.1658. This product decomposed on storage in aq. MeOH during a period of 3–7 days.

Lysine 2,3-Aminomutase-catalysed Hydrogen-exchange Procedure.—Lysine 2,3-aminomutase was isolated essentially as previously described[4] except that all isolation buffers were supplemented with L-lysine (0.1 mmol dm^{-3}).[10] The enzyme solution (5.2 mg protein cm^{-3}) was subdivided into 1–2 cm^3 aliquots and, after rapid freezing of the solution in evacuated, sealed ampules, in solid CO_2–acetone, was stored at −70 °C until use.

A revised activation protocol was developed which yielded enzyme of higher specific activity than that obtained in our previous work.[4] Activation buffer was prepared from freeze–thaw–degassed Tris hydrochloride buffer (9 cm^3; 50 mmol dm^{-3}; pH 8.2) containing pyridoxal phosphate (0.1 mmol dm^{-3}), and was kept frozen in a septum-sealed 2-neck flask under Ar until it was used. Solid $Na_2S_2O_4$ (1.7 mg) and Fe $(NH_4)_2(SO_4)_2$·$6H_2O$ (3.9 mg) were added, then the head-space was evacuated and then purged with Ar, after which the buffer was allowed to thaw. Aq. sodium dihydrolipoate (0.15 mol dm^{-3}; 1.0 cm^3) was added by syringe, and the resultant solution was subjected to three more freeze–thaw–degas cycles.

Portions (0.6 cm^3) of the activation buffer were injected into Ar-purged septum-sealed vials (2 cm^3 capacity). Freshly thawed enzyme solution (0.55 cm^3; 2.85 mg protein, 10 nmol) was added by syringe, and the resultant solution was stored at 37 °C for 19 h.†

Septum-sealed ampoules containing freeze–thaw–degassed solutions of Na_2 EDTA (15 mg cm^{-3}) and unlabelled L-lysine hydrochloride (0.5 mol dm^{-3}; adjusted to pH 8.0 with Na_2CO_3) were Ar-purged by piercing their septa with 25 gauge needles and placing them in a vacuum desiccator which was then evacuated briefly and repressurized with Ar several times. Frozen SAM solution (see *Materials*) (0.5 cm^3; either 6.1 or 0.7 mmol dm^{-3}) was thawed, and the pH was adjusted to *ca.* 8.0 by addition of Tris-hydrochloride buffer, pH 8.8 (5 mm^3; either 1.5 or 0.1 mol dm^{-3}, respectively). These ampoules were then Ar-purged as above. Aq. (2*RS*,3*RS*)-[3-^{3}H]lysine·HCl (0.35 cm^3; 6.45 mg cm^{-3} water) was mixed with Na_2CO_3 (3 mg), and freeze–thaw–degassed, followed by Ar-purging.

At the end of the enzyme-activation period, additions were made by syringe to the enzyme solution of (a) EDTA solution (40 mm^3; prewarmed to 37 °C); (b) SAM (as required, see Table 1); (c) lysine (75 mm^3 of either unlabelled or tritiated; 37.5 μmol or 2.67 μmol, respectively). At intervals, aliquots were removed by syringe from the non-radioactive lysine control run for HPLC analysis of the conversion of α-lysine into β-lysine, as previously described.[25] Such analyses indicated a specific activity for the enzyme of *ca.* 3.5 units mg^{-1} protein.

After incubation for 36 h, unlabelled SAM solution (16.7 mmol dm^{-3}; pH 7.3) was injected into each incubation mixture to give a total of 7.7–8.3 μmol. Then the vials were immersed in boiling water for 30 min to convert the SAM into MTA.

Purification of MTA.—After being heated, the incubation mixture was centrifuged in an Amicon Centricon 30 filtration device at 3000 g. The filtrate was applied to a column of Whatman CM52 (0.5 × 11 cm), and the column was washed with water (10 cm^3). The eluate was lyophilized, and the residue was triturated with a mixture of propan-2-ol–EtOAc–acetic acid (77:20:3). The resultant suspension was applied to a column (0.5 × 4 cm) of silica gel (E. Merck; 0.040–0.063 mm), and the column was eluted with trituration solvent (7.5 cm^3). The eluate was evaporated under reduced pressure, and the residue was triturated with toluene (5 cm^3). After evaporation of the solvent (to remove acetic acid), the residue was dissolved in 10% MeOH–water (1 cm^3) and treated under N_2 with dithiothreitol (38 mg)[18] at 65–70 °C for 4–10 h, until all MTA sulphoxide was converted into MTA. The reduction was monitored by HPLC on an Alltech Partisil SCX column (10 μm particle size; 0.46 × 25 cm) controlled at 35 °C, with 2% propan-2-ol in ammonium formate buffer (0.05 mol dm^{-3}; pH 4.0) as eluant at 1.2 cm^3 min^{-1}.

The reduced mixture was then separated by preparative HPLC (Partisil SCX, same conditions as above). Pooled MTA-containing fractions were lyophilized, and the residue was redissolved in water and lyophilized again to remove ammonium formate. The residue was then chromatographed by preparative reversed-phase HPLC on a Nova-PAK Radial-PAK cartridge (0.8 × 10 cm, Waters Associates) with 20% MeOH in 50 mmol dm^{-3} aq. $NH_4H_2PO_4$ as eluent at 1.0 cm^3 min^{-1}. In addition to the main MTA-containing peak, a fore-run zone, containing some MTA sulphoxide and including the region where 5′-deoxyadenosine and 5′,8-anhydroadenosine elute, was also collected (see below, *Detection of 5′-Deoxyadenosine*). The MTA-containing fractions were pooled, then evaporated under reduced pressure to remove MeOH, and the remaining solution was lyophilized. The residue was dissolved in degassed water (10 cm^3) and the solution was applied to a column of Amberlite XAD-4 resin (0.6 × 18 cm). The column was washed with water (20 cm^3), followed by 5% EtOH–water (15 cm^3), followed by 25% EtOH–water (*ca.* 50 cm^3). MTA-containing fractions, identified by HPLC (SCX column), were pooled and evaporated. The yield of tritiated MTA plus MTA sulphoxide to this point was determined by HPLC, and then a 40–50-fold excess of unlabelled MTA was added as carrier for the purposes of crystallization. The product, dissolved in water (1 cm^3), was again treated with dithiothreitol (20 mg) at 65 °C as above. On cooling to 4 °C, crystalline MTA was formed. This was recrystallized twice from water (0.5–1.0 cm^3) for counting. The specific activities found are shown in Table 1.

Detection of 5′-Deoxyadenosine.—Fore-run fractions collected in the reversed-phase preparative HPLC purification of MTA (above) were pooled and lyophilized. The residue was

* δ 4.85 in [$^2H_5$]pyridine solution.

† Nearly identical results were obtained for activation periods of 5–28 h.

redissolved in water (3 cm^3) and partially desalted by slow elution (*ca.* 0.3 cm^3 min^{-1}) through a Waters Associates Sep-PAK C_{18} cartridge, followed by washing of the column successively with water (0.5 cm^3) and 50% MeOH–water (10 cm^3). The aq. MeOH eluate was evaporated in a stream of N_2 at 40 °C. After being redissolved in a small volume of water, small samples of the product were rechromatographed on the C_{18} Radial-PAK HPLC cartridge, with the following solvent program at 1.0 cm^3 min^{-1}: 0–4 min, 7% MeOH–50 mmol dm^{-3} aq. $NH_4H_2PO_4$; 4–11 min, linear gradient to 30% MeOH–50 mmol dm^{-3} aq. $NH_4H_2PO_4$; then isocratic with this solvent. The retention times for tested adenine nucleosides were: *S*-adenosyl-L-homocysteine 10.4 min; MTA sulphoxide stereoisomers 12.6 and 12.8 min; 5′,8-anhydroadenosine 14.3 min; 5′-deoxyadenosine 16.2 min; MTA 20.2 min. Samples of the eluate from one injection were collected for 20 s each for liquid scintillation counting.

Detection of S-*Adenosyl*-L-*methione* (*SAM*) *in Lysine* 2,3-*Aminomutase*.—Sodium lauryl sulphate (SDS) was added to a solution of lysine 2,3-aminomutase (0.5 cm^3; 2.6 mg protein, 10 nmol; pH adjusted to 6.9 with dil. HOAc) to give an SDS concentration of 60 mmol dm^{-3}. After 5 h at 37 °C, the mixture was centrifuged at 3000 g for 16 h in an Amicon Centricon filtration device (30 k dalton molecular weight cut-off) at room temperature. 10% aq. KCl[26] was added to the filtrate to give a concentration of 50 mmol dm^{-3}, followed by brief centrifugation (10 min; 12 000 g). The supernatant solution was chromatographed on a column of Whatman CM-52 (0.25 × 8 cm).[1] Elution first with five column-volumes of aq. NaOAc (5 mmol dm^{-3}; pH 5.0) gave MTA (*ca.* 0.4–0.7 nmol), estimated by HPLC as described above. Finally, elution with 0.04 mol dm^{-3} HCl (5 cm^3) gave SAM (*ca.* 0.5–2.0 nmol), also estimated by HPLC as described under *Materials*.

A control experiment was carried out in which bovine serum albumin (5.2 mg) in 10 mmol dm^{-3} Tris·HCl buffer (pH 6.9), containing 0.5 mmol dm^{-3} dithiothreitol and 60 mmol dm^{-3} SDS (0.5 cm^3), was mixed with SAM (0.7 mmol dm^{-3}; 14 mm^3, 10 nmol, also containing *ca.* 0.7 nmol of MTA) and treated as described for lysine 2,3-aminomutase. In this case, the recovery of MTA was *ca.* 1.7 nmol, and of SAM *ca.* 9.0 nmol.

Note added in proof. Moss and Frey have recently reported detection of [^{14}C]-5′-deoxyadenosine after incubation of [8-^{14}C]SAM with lysine 2,3-aminomutase. M. L. Moss and P. A. Frey, *J. Biol. Chem.*, 1990, **262**, 18112.

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