Studies on the Mechanism of Lysine 2,3-Aminomutase

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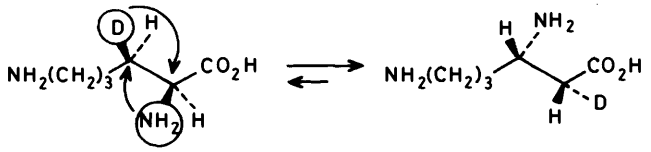
A modified purification procedure for lysine 2,3-aminomutase from *Clostridium subterminale* strain SB4 has been developed. The enzyme was shown to be free of α-lysine racemase activity by use of Marfey's reagent. The isotope effect for transfer of the 3-*pro-R* hydrogen of α-lysine to the 2-*pro-R* position of β-lysine was determined to be $k_H/k_D = 2.9 \pm 0.3$ by a competition experiment between L-[3,3-$^2H_2$]lysine and L-[2-$^2H_1$]lysine. No kinetic isotope effect was detected for α-deuterium substitution of α-lysine. Intermolecular transfer of deuterium was detected in two crossover experiments. Conversion of a mixture of L-[3,3-$^2H_2$]lysine and L-[4,4,5,5-$^2H_4$]lysine to β-lysine gave a product showing an enhanced $M + 5$ peak in the mass spectrum. Similarly a mixture of L-[3,3-$^2H_2$]lysine + L-[UL-^{13}C]lysine gave β-lysine having an enhanced $M + 7$ peak in the mass spectrum. These results suggest that the hydrogen migration might involve a hydrogen carrier in a manner similar to the mechanisms of adenosylcobalamin-dependent vicinal interchange reactions. A theory that *S*-adenosyl-L-methionine might be acting as this hydrogen carrier was tested by co-incubating a mixture of L-[3,3-$^2H_2$]lysine and an excess of *S*-adenosyl-L-methionine. After incubation, the *S*-adenosyl-L-methionine was degraded to 5'-methylthioadenosine, which was shown by ^2H n.m.r. to be devoid of deuterium. The results show that *S*-adenosyl-L-methionine does not serve as hydrogen carrier in the reaction.

Several years ago, we reported the results of our studies on the stereochemistry of lysine 2,3-aminomutase, an enzyme in *Clostridia* which catalyzes the reversible interconversion of L-α-lysine (**1**) and (3*S*)-β-lysine (**2**).[1] A similar or identical enzyme in



(**1**) (**2**)

Streptomyces produces the β-lysine found in certain antibiotics such as the streptothricins.[2] The reaction, with both enzymes, was shown to proceed with transfer of the amino group from C-2 of α-lysine to C-3 of β-lysine, with concurrent transfer of the 3-*pro-R* hydrogen of α-lysine to the 2-*pro-R* position of β-lysine, thus inverting the configuration at both migration termini, (**1**) ⟶ (**2**). Studies with [2-^{15}N,3-^{13}C]-α-lysine revealed that the amino group migration was completely intramolecular. However, incubations of [3,3-$^2H_2$]-α-lysine, admixed with unlabelled α-lysine, resulted in the formation, as the major product, of monodeuteriated β-lysine. This result suggested that the hydrogen migration might be intermolecular. In this regard, the reaction is reminiscent of a variety of other vicinal interchange reactions,[3] including several aminomutase reactions,[4] catalyzed by enzymes which require adenosylcobalamin (AdoCbl) for activity. In contrast to these enzymes, lysine 2,3-aminomutase does not use AdoCbl as coenzyme, but instead is dependent upon pyridoxal phosphate (PLP), *S*-adenosyl-L-methionine (SAM), and ferrous ion for maximal activity.[5] Despite the above results,[1,2] the mechanism of lysine 2,3-aminomutase remained unclear.

We now report the results of our continuing studies on this enzyme. In particular, we report the determination of the isotope effect for the C-3 to C-2 hydrogen migration. In addition, we report the results of crossover experiments which conclusively demonstrate the intermolecularity of this hydrogen migration. Finally, we report the results of an investigation of the possible involvement of *S*-adenosylmethionine as a hydrogen carrier in the hydrogen migration process.*

Results

Preliminary Studies.—Most of the studies described in our earlier report[1] were carried out with a crude cell-free extract of *Clostridium subterminale* strain SB4, ATCC29748 (C.SB4). For the present work, we have used lysine 2,3-aminomutase purified essentially according to Barker *et al.*,[5] but with the use of several adaptations which allowed the completion of the purification from wet cells in *ca.* 30 h elapsed time, including an overnight chromatographic step. The acetone precipitation step[5] was omitted, as it did not appear to improve the specific activity of the enzyme. The resultant enzyme cleanly converted α-lysine into β-lysine, and was completely free of α-lysine racemase activity or any other activity (other than lysine 2,3-aminomutase) that caused consumption of L-α-lysine.

α-Lysine racemase activity was examined in a qualitative manner by a sensitive technique using Marfey's reagent,[6,7] which allows the separation of L- and D-α-lysine as diastereoisomeric derivatives by h.p.l.c. Using this technique, less than 1% of D-lysine formation from L-lysine can easily be detected, a much higher sensitivity than can be achieved by the classical respirometric technique.[8] Thus, after *aerobic* (which deactivates lysine 2,3-aminomutase) incubation of the crude enzyme (cell-free extract) with L-lysine, considerable formation of D-lysine and reduction of total lysine content could be detected (Figure 1*a*). The enzyme purified through the ammonium sulphate and heat steps[5] was devoid of racemase activity, but still contained unidentified enzymatic activity which resulted in a reduction of L-lysine content after 24 h incubation (Figure 1*b*). However, the enzyme further purified by DEAE Sephadex A-50 column chromatography, when incubated aerobically with L-lysine, caused no change in the L-lysine concentration (Figure 1*c*).

* This work has been reported in preliminary communications: D. J. Aberhart, 15th IUPAC Symposium on the Chemistry of Natural Products, The Hague, August 17—22, 1986, Abstracts PB 35; Aberhart, D. J., 193rd American Chemical Society National Meeting, Denver, April 5—10, 1987, Abstracts ORG 300.

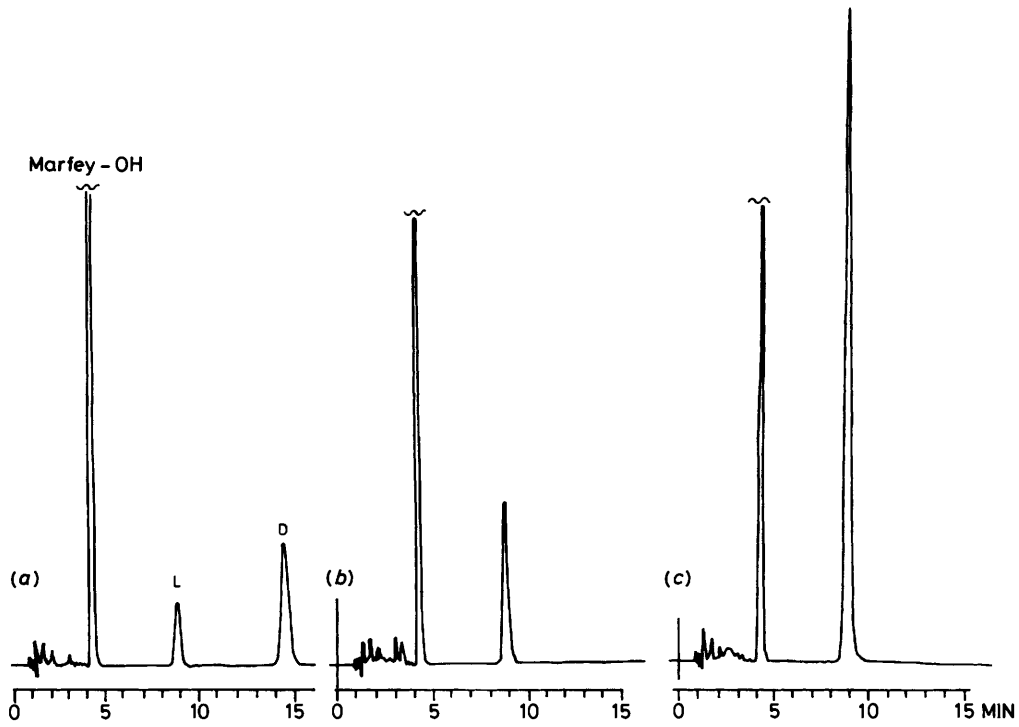
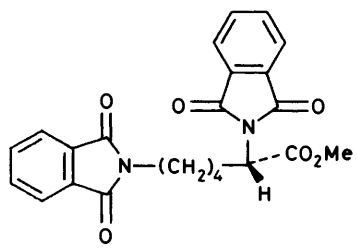
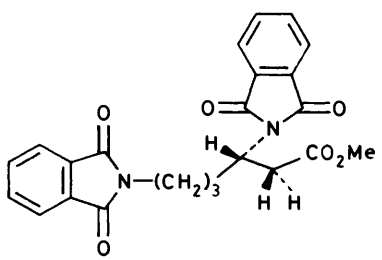


Figure 1. H.p.l.c. chromatograms (conditions, see Experimental section) of L-lysine treated aerobically with lysine 2,3-aminomutase at various stages of purification, followed by treatment with Marfey's reagent: (*a*) crude cell-free extract of C.SB4; (*b*) enzyme purified through ammonium sulphate and heat steps; (*c*) enzyme purified by DEAE Sephadex A-50 chromatography

Isotope Effect Studies.—In our previous conversions of mixtures of unlabelled DL-α-lysine and DL-[3,3-$^2H_2$]-α-lysine into β-lysine by crude or partially purified lysine 2,3-aminomutase, the deuterium content of the isolated β-lysine was always substantially lower than that of the starting material, suggesting the involvement of a substantial isotope effect in the hydrogen migration. The first objective of our present work was to evaluate the magnitude of k_H/k_D for the C-3 hydrogen migration. The isotope effect was evaluated by use of competition experiments* (which yield a $V_{max.}/K_m$ isotope effect[9]). In the first of these experiments, an equal mixture of L-[3,3-$^2H_2$]lysine and L-[2-$^2H_1$]lysine [D_0 2.0%, D_1 52.4%, D_2 45.6% by mass spectrometry on the di-*N*-phthaloyl methyl ester derivative, (**3**)], was incubated with the enzyme. After conversion of *ca.* 40% of the α-lysine into β-lysine, the latter was isolated as the *N*,*N*-diphthaloyl methyl ester derivative, (**4**), and its ^2H n.m.r. spectrum recorded (Figure 2). As shown in our previous work,[1] signals at δ 4.71 and δ 3.19 correspond to deuterium atoms at C-3 and C-2-*pro-R* of β-lysine, respectively, derived from L-[3,3-$^2H_2$]lysine. The signal at δ 2.79 corresponds



(**3**)



(**4**)

to a C-2-*pro-S* deuterium of β-lysine derived from L-[2-$^2H_1$]lysine. By comparing the area of the δ 2.79 peak with the areas of the δ 3.19 or δ 4.71 peaks (which, as before[1] are approximately equal), an estimate of the relative rates of conversion of the two labelled lysines could be derived. The relative area of *ca.* 3.3, after correction for the slight deviation from equality of the amounts of the two precursors seen in the mass spectrum of derivative (**3**) (52% D_1, 46% D_2) gives a value of $k_H/k_D \simeq 2.9$. The ^2H n.m.r. results were supported by mass spectrometric analysis of the β-lysine *N*,*N*-diphthaloyl methyl ester derivative (**4**), which showed D_0 4.4%, D_1 70.8%, D_2 24.8%.

* Attempts at direct measurement of the relative rates of conversion of L-lysine and L-[3,3-$^2H_2$]lysine by h.p.l.c. did not give very satisfactory results. Although a *ca.* 3-fold greater rate of conversion of the unlabelled substrate could be seen, the data were complicated by several factors: accurate estimation of α- and β-lysine concentrations in reaction mixtures containing, initially, 1 mg ml^{-1} of L-α-lysine is complicated by chromatogram noise. In particular, the determination of β-lysine concentration at low conversions (< 10%) is estimated to be subject to an error of at least ± 10%. In addition, it should be noted that lysine 2,3-aminomutase is extremely oxygen-sensitive and is isolated in an inactive form which requires reactivation by anaerobic incubation with sodium dithionite ($Na_2S_2O_4$),[5] a process which may take as much as one hour. In all of our studies, this activation step was carried out *in situ* in the presence of substrate. Thus accurate assignment of t = 0 for the conversions could not be made.

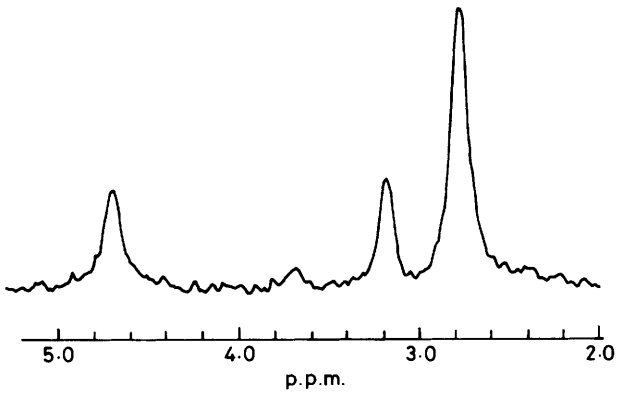


Figure 2. 76.77 MHz ^2H n.m.r. spectrum of β-lysine *N,N*-diphthaloyl methyl ester derivative (**4**) (labelling not shown), in $CHCl_3$ solution, isolated after incubation of approximately equal mixture of L-[3,3-$^2H_2$]lysine + L-[2-$^2H_1$]lysine with lysine 2,3-aminomutase (*ca.* 40% conversion of α ⟶ β lysine). Conditions: SW 2000 Hz, repetition rate 1.024 s, RD, zero, PW 20 μs, LB 1 Hz, 0.977 Hz/pt; 2324 scans

The rationale behind the competition experiment assumes that k_H/k_D for the C-2 hydrogen migration would be close to 1.0. Since the C-2–H bond is apparently not broken in the reaction, this seems a reasonable assumption and was confirmed by converting an equal mixture (D_0 49.5%, D_1 50.5% by MS) of unlabelled L-lysine and L-[2-$^2H_1$]lysine. After conversion of *ca.* 75% of the α-lysine into β-lysine, the product was again isolated as (**4**), which was shown (mass spectrum) to be D_0 51.2%, D_1 48.8%. The slight difference in the percentages between the starting material and product is not considered to be significant. Thus α-deuterium substitution of lysine had virtually no detectable effect on the reaction rate of lysine 2,3-aminomutase.

A similar competition experiment was also conducted using an equal mixture (D_0 50%, D_2 50%, mass spectrum) of unlabelled L-lysine and L-[3,3-$^2H_2$]lysine. After conversion by lysine 2,3-aminomutase of *ca.* 35% of the α-lysine into β-lysine, the latter was isolated as the derivative, (**4**), which showed D_0 59.9%, D_1 27.9%, and D_2 12.2%. This result can be interpreted in terms of the previously suspected crossover mechanism, in which deuterium from the D_2 substrate is transferred to the D_0 substrate, generating D_1-β-lysine. Thus, one-half of the 27.9% of the D_1-β-lysine would be formed from D_0-α-lysine and the remainder from the D_2-α-lysine (of course, the deuterium in the D_1-β-lysine from the two sources would not be in the same location). This being the case, it can be calculated that 73.9% of the β-lysine was formed from D_0-α-lysine and 26.1% from D_2-α-lysine, a ratio of 2.8. The ^2H n.m.r. of the product, (**4**), obtained from this last experiment could not, of course, yield relative rate data, but it confirmed the previous observations[1] of approximately (± 10% due to spectral noise) equal amounts of deuterium at C-3 and C-2-*pro-R* of the β-lysine derivative. When all of the above data are taken into consideration, it appears reasonable to conclude that for the migrating 3-*pro-R* hydrogen atom of L-lysine, $k_H/k_D \simeq 2.9 \pm 0.3$, thus indicating that this hydrogen migration is the rate-determining step in the rearrangement.

Crossover Studies.—We had already shown in several incubations of mixtures of unlabelled lysine and [3,3-$^2H_2$]lysine that a substantial amount of monodeuteriated α-lysine was always formed. These results suggested that the hydrogen migration process was partially intermolecular. However, the formation of D_1-α-lysine could also have been the result of exchange of deuterium with the medium, although this was unexpected considering Barker's report[5] that unlabelled α- or β-lysine incubated with purified lysine 2,3-aminomutase in tritiated water did not undergo exchange.

We therefore undertook experiments designed to rigorously confirm crossover between substrate molecules of hydrogen originating from C-3 of α-lysine in its transfer to the 2-*pro-R* position of β-lysine. Two such experiments have been carried out. In the first, a mixture of L-[3,3-$^2H_2$]lysine plus L-[4,4,5,5-$^2H_4$]lysine (D_0 0.4%, D_1 1.8%, D_2 47.9%, D_3 1.2%, D_4 48.1%, and D_5 0.6% by mass spectrum) was converted to β-lysine, which was isolated as (**4**). The product showed D_0 1.8%, D_1 12.8%, D_2 11.8%, D_3 2.4%, D_4 59.4%, and D_5 11.7%. It is satisfying to note that the percentage of D_5 product corresponds reasonably closely to that of the D_1 product. The combined percentage (24.6%) of the products, $D_1 + D_2$, comprising the carbon skeleton derived from L-[3,3-$^2H_2$]lysine compared with the combined percentage (71.1%) of the products, $D_4 + D_5$, comprising the carbon skeleton derived from L-[4,4,5,5-$^2H_4$]lysine (ignoring the minor D_0 and D_3 products, if real) indicates again a $k_H/k_D \simeq 2.9$ for the hydrogen transfer. This calculation assumes that deuterium substitution at C-4 or C-5 has no effect on the rate. Apparently *ca.* one-half of the migrating 3-*pro-R* deuterium atoms of α-lysine underwent crossover in their transfer to the 2-*pro-R* position.

Similarly, an equal mixture of L-[3,3-$^2H_2$]lysine and L-[UL-^{13}C]lysine (85% ^{13}C) was converted into β-lysine by the enzyme. The product, isolated as (**4**), showed in the mass spectrum, Figure 3*b*, an enhanced intensity for the *m/z* 427 and 421 peaks, and reduced intensity for the *m/z* 422 peak, relative to the corresponding peaks in the starting materials, Figure 3*a*. Thus, again it is obvious that crossover of migrating deuterium between labelled substrate molecules has occurred. It was hoped that the ^{13}C and/or ^2H n.m.r. spectra of the product obtained from this last experiment might reveal confirming evidence of ^{13}C-^2H coupling. However, the ^{13}C n.m.r. spectrum was too extensively ^{13}C-^{13}C coupled to allow confident assignment of

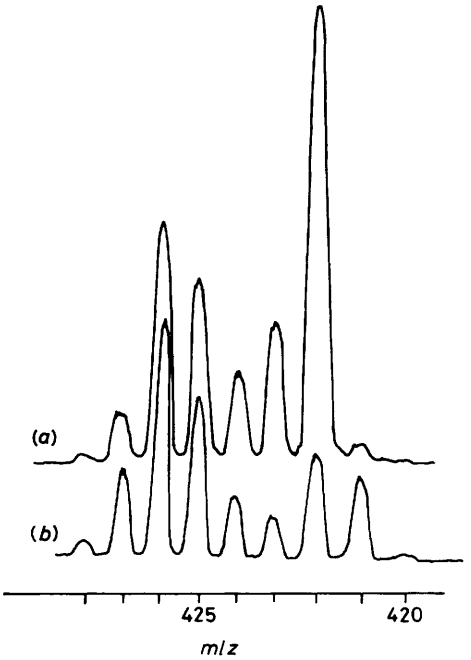
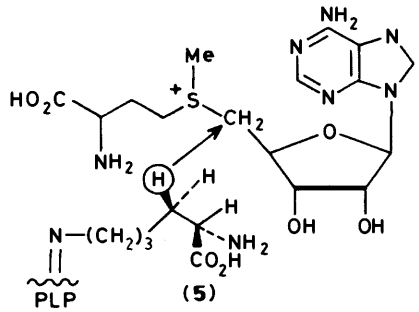


Figure 3. Molecular ion regions of electron impact mass spectra (70 eV): (*a*) Mixture of L-[3,3-$^2H_2$]-α-lysine + L-[UL-^{13}C]-α-lysine *N,N*-diphthaloyl methyl ester derivative. (*b*) β-Lysine *N,N*-diphthaloyl methyl ester derivative obtained after incubation of L-[3,3-$^2H_2$]-α-lysine + L-[UL-^{13}Cl-α-lysine with lysine 2,3-aminomutase

peaks in the δ 36 region (C-2) to ^{13}C-^{2}H coupled resonances. Also the ^{2}H n.m.r. spectrum was too noisy to allow detection of ^{13}C-coupled ^{2}H resonances. Nevertheless, all of the above evidence strongly indicates that the hydrogen migration process is a partially intermolecular one, and that intermolecular hydrogen transfer occurs in approximately half of the turnovers under the conditions used in our experiments.

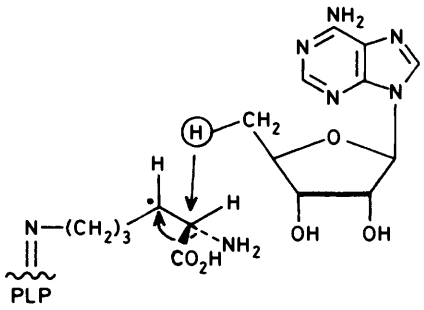
A Test of S-*Adenosylmethionine as Hydrogen Carrier.*—The above established intermolecularity of the hydrogen transfer suggested that there might be some hydrogen carrier involved in the catalytic mechanism in a manner analogous to the mechanisms of AdoCbl-dependent vicinal interchange reactions.[3] Since lysine 2,3-aminomutase does not require AdoCbl for activity, the question arises as to what might be the nature of this putative hydrogen carrier, if indeed any such entity actually exists. In this regard, we were intrigued by a suggestion by Barker[10] that *S*-adenosylmethionine (SAM) might be serving in this role. Reversible homolytic cleavage of SAM between the 5′-carbon and the sulphur atom would generate a 5′-deoxyadenosyl radical, capable, in principle, of abstracting the 3-*pro-R* hydrogen of L-lysine and then returning it to the 2-*pro-R* position after intramolecular shift of the amino group from C-2 to C-3, (**5**) ⟶ (**6**) (Scheme 1). The suggested cleavage of SAM in the lysine 2,3-aminomutase reaction may be regarded as analogous to the reported cleavage of SAM to form 5′-deoxyribose in the pyruvate-formate lyase reaction.[11]

We now report the results of a study which suggests that, in fact, SAM does not perform such a hydrogen-carrying function in this reaction. The rationale behind these experiments was as follows, Scheme 2. Transfer of the 3-*pro-R* hydrogen of α-lysine



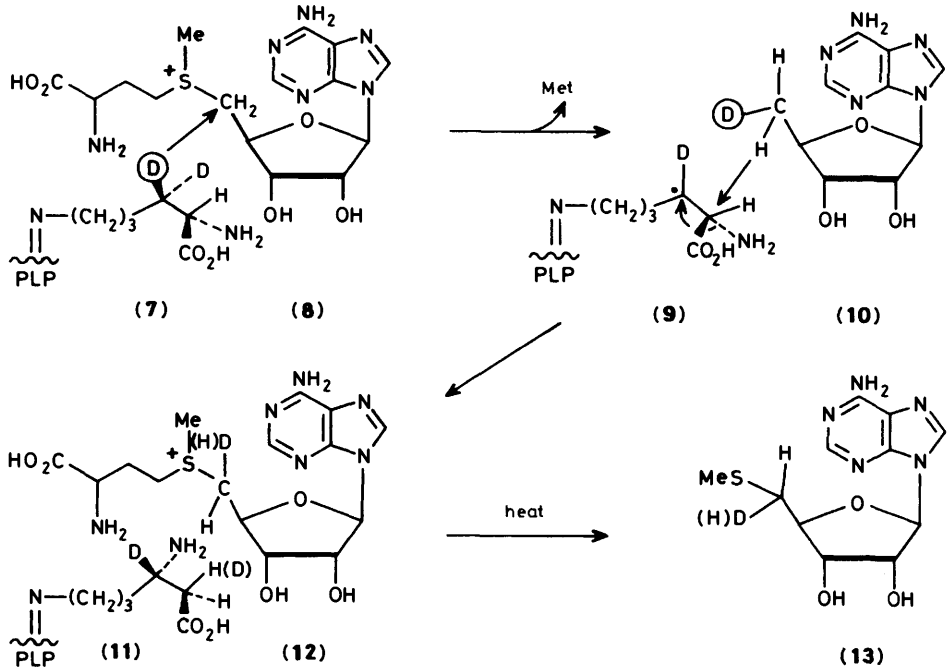
(**5**)

Met

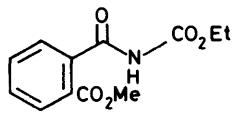


(**6**)

Scheme 1.



(**7**) (**8**) (**9**) (**10**) (**11**) (**12**) (**13**)



(**14**)

Scheme 2. Hypothetical mechanism for lysine 2,3-aminomutase illustrating the possible role of *S*-adenosylmethionine as a hydrogen carrier

(7), to a 5′-deoxyadenosyl radical formed from SAM (8), would produce 5′-deoxyadenosine (10). By analogy with the chemistry of AdoCbl-dependent enzymes,[3] the three hydrogens of the newly formed methyl group would be expected to be enzymically indistinguishable. If deuterium from L-[3,3-$^2H_2$]lysine, (7), were transferred to the acceptor, then a monodeuteriated methyl group would be formed, as in (10). Assuming free rotation of the methyl group in the active site of the enzyme,* this deuterium would be returned to the substrate in, at most, one turnover out of three. In fact, return of deuterium might be less than one in three if an isotope effect were to prevail in this second step. The overall result of these events would be the accumulation of 5′-deuterium-labelled SAM. Through equilibrium dialysis experiments with [^{14}C]SAM, Zappia *et al.*[12] have shown that SAM does not bind tightly to lysine 2,3-aminomutase. Their results indicate rapid exchange of bound and unbound SAM with the enzyme. In this regard, lysine 2,3-aminomutase differs from AdoCbl-dependent enzymes, where exchange of bound and unbound AdoCbl is apparently very slow.[13]

We therefore decided to test the involvement of SAM as a hydrogen carrier by incubating L-[3,3-$^2H_2$]lysine with lysine 2,3-aminomutase in the presence of a large pool of unlabelled SAM, followed by examination of the recovered SAM for deuterium content. A mixture of L-[3,3-$^2H_2$]lysine (169 μmol, 25 mg) and SAM (57 μmole, 25 mg) was incubated with the enzyme, following the conversion of α-lysine to β-lysine by h.p.l.c. Two runs were carried out, the first being conducted for 22 h (*ca.* 108 μmol of α-lysine converted into β-lysine), the second for 46 h (*ca.* 122 μmole converted). At the end of the incubation period, the reactions were terminated by heating the mixtures in boiling water for 30 min. During this procedure, the remaining SAM was converted in high yield into 5′-methylthioadenosine (MTA) (13).[14] Since this compound still retains the 5′-methylene group of interest, this degradative reaction was not expected to be detrimental to our purposes. In fact for several reasons, the reaction facilitated our studies. MTA is much more stable than SAM and is easily separable from α- and β-lysine by cation exchange followed by preparative h.p.l.c.,[15] whereas the separation of SAM from α- and β-lysine proved to be quite troublesome. In the ^{1}H n.m.r. spectrum of MTA, the signals for the 5′-methylene group (δ 3.20 and 3.25) are well separated from all other signals, whereas in the ^{1}H n.m.r. spectrum of SAM,[16] the methionine group hydrogens appear in approximately the same region.

After purification (in *ca.* 50—60% overall yield) of the MTA recovered from the two runs, its ^{2}H n.m.r. spectrum was run in ^{2}H-depleted water, containing a carefully measured amount of either [$^2H_9$]-t-butyl alcohol (δ 1.40, 0.18 μmole, run 1) or potassium [^{2}H]formate (δ 8.30, 2.35 μmole, run 2). The spectra showed only the resonances due to the added reference compounds (S/N ≃ 20) and HOD, and no signal was detectable at *ca.* δ 3.2 where any 5′-^{2}H would have appeared. It is estimated that >1.0 μDaltons of deuterium at C-5′ could have been detected, far less than the amount of deuterium transfer which took place in the aminomutase reactions.

The α- and β-lysine were recovered from the 22 h incubation mixture as the derivatives (3) and (4). Mass spectrometric examination of these revealed that a small amount of exchange had occurred in the α-lysine derivative (D_0 7.3%, D_1 5.0%, and D_2 86.9%), but a larger amount of exchange (D_0 3.8%, D_1 21.2%, D_2 70.8%, and D_3 4.2%) was seen in the β-lysine product. Since the reaction was allowed to proceed nearly to equilibrium, reversal of the reaction on partially exchanged β-lysine is undoubtedly responsible for, at least, the D_1 α-lysine product formed. The exchange of the β-lysine was somewhat surprising, since no unlabelled carrier was added in these experiments, in contrast to the experiments discussed earlier.

In a control experiment, unlabelled L-lysine and SAM were co-incubated with lysine 2,3-aminomutase in a medium in which most of the water was replaced by D_2O. After conversion of *ca.* 70% of the α-lysine into β-lysine, the mixture was heated as before followed by isolation of the MTA, and of the α- and β-lysine as compounds (3) and (4). Mass spectrometric examination of the MTA revealed the presence of a substantial amount of mono- and dideuteriated molecules (D_0 50.3%, D_1 33.3%, and D_2 16.4%). The formation of monodeuteriated MTA was not unexpected since the exchange of C-8 hydrogens of adenine and its derivatives upon heating in D_2O is well precedented.[17] However it was rather disconcerting to note the exchange of a second hydrogen atom. The identity of this second exchanged hydrogen atom was revealed by a ^{2}H n.m.r. spectrum which showed two peaks, of roughly equal intensity, at δ 3.15 and δ 8.65, the former corresponding to the chemical shift of the 5′-methylene group of MTA. However, the observed extent of exchange (*ca.* 25% per position) corresponds to only *ca.* 12% of the total amount of hydrogen located at C-5′. Furthermore, virtually identical results (D_0 55.7%, D_1 31.5%, and D_2 12.9%) were obtained for MTA recovered after a blank run from which the lysine 2,3-aminomutase was omitted. Thus the C-5′ hydrogen-deuterium exchange is exclusively a result of heating SAM in D_2O and not the result of equilibration of the 5′-hydrogens by lysine 2,3-aminomutase (or by a contaminating enzyme). That the C-5′ hydrogen exchange occurs at the SAM stage rather than on MTA itself was shown by heating MTA alone in D_2O. Only exchange of the C-8 hydrogen was observed by ^{2}H n.m.r. of the re-isolated MTA.

The α- and β-lysine derivatives isolated from the D_2O run showed only a small amount of deuterium incorporation (α-lysine = D_0 94.6%, D_1 5.4%; β-lysine = D_0 92.5%, and D_1 7.5%), roughly consistent with previous observations.[5]

Discussion.—The crossover results presented in an earlier part of this paper conclusively establish that the C-3 to C-2 hydrogen migration of lysine 2,3-aminomutase is an intermolecular process. Such a process can most readily be explained by proposing the action of a hydrogen-carrying entity in the active site of the enzyme. Despite the attractiveness of the proposed theory that SAM might serve such a role,[10] the overall conclusion which must be drawn from the above experiments is that SAM does *not* act in this manner. The absence of detectable hydrogen at the 5′-position of MTA isolated after heating the reaction mixture is inconsistent with such a mechanism, and the absence of 5′-deuterium cannot be explained by invoking enzymic or complete chemical exchange of these deuterons after the aminomutase reaction is complete. Also the failure of the isolated β-lysine derived from L-[3,3-$^2H_2$]lysine to have suffered extensive loss of C-2 deuterium is inconsistent with such a process. If deuterium transferred to C-5′ of the carrier had completely exchanged with the medium, then only C-3-monodeuteriated β-lysine could have been formed, which was not the case. It has been assumed in this investigation that if SAM were acting as a hydrogen acceptor, that hydrogen acceptance would be at the 5′-position and not at some position of the methionine residue which is eliminated in the degradation to MTA. This seems to be a reasonable assumption to make as a working hypothesis.

There was, however, a small amount of deuterium loss in the

* Free rotation of methyl groups in the active sites of enzymes is strongly supported by a plethora of studies with chirally labelled methyl groups in which preferential abstraction of a hydrogen atom is governed only by kinetic isotope effects and not steric effects. For a review, see H. G. Floss, M.-D. Tsai, and R. W. Woodard, *Topics in Stereochemistry*; ed. E. L. Eliel, S. H. White, and N. L. Allinger, Wiley, New York, 1984, vol. 15, pp 253–321.

isolated β-lysine, and it may be that the actual, as yet unidentified, hydrogen carrier (if any) has the ability to undergo a limited amount of exchange with the medium. Although relatively little exchange of deuterium into the lysines was observed in incubations in D_2O, this may be the result of some isotope effect which discriminates against solvent deuterium. Thus, the role of SAM in the reaction is probably that of an allosteric effector, as previously suggested.* Obviously further work on this enzyme will be required to define the nature of the hydrogen carrying function, as well as to elucidate the role of pyridoxal phosphate in the reaction, which has not been addressed in the present work.†

Experimental

General.—^{1}H n.m.r. spectra were recorded on a Varian EM-360 or Bruker WM-500 instrument. ^{2}H- and ^{13}C-n.m.r. spectra were recorded on a Bruker WM-500 instrument. Mass spectra were recorded by Shrader Analytical and Consulting Laboratories, Detroit, Michigan on an AEI·MS-30 mass spectrometer at 70 eV, or by Morgan Schaffer Corporation, Montreal, Quebec, Canada, on a Perkin Elmer RMU-6D instrument at 70 eV. Liquid chromatography was performed with a Waters instrument equipped with an M-6000A pump, a U6K injector, and a Model 450 variable wavelength detector. L-[UL-^{13}C]lysine hydrochloride (85% ^{13}C) was obtained from Cambridge Isotope Laboratories.

L-[4,4,5,5-$^2H_4$]Lysine hydrochloride (98 atom % ^{2}H) was obtained from Merck. A ^{13}C n.m.r. (D_2O + 0.10M KPO_4, pH 7.0 with internal dioxane, δ 67.8 as reference) confirmed that virtually all of the deuterium was located at C-4 and C-5: δ 22.69 and 27.59 (very weak multiplets), other peaks singlets of apparently normal intensity at δ 31.10, 40.32, 55.67, and 175.78.

(2*RS*)-[2-$^2H_1$]Lysine and (2*RS*)-[3,3-$^2H_2$]lysine, available from our previous work,[1] were resolved by a published procedure[1,8] to yield optically pure (2S)-[2-$^2H_1$]lysine and (2S)-[3,3-$^2H_2$]lysine. The optical purity was confirmed by using Marfey's reagent[7,8] (see below, racemase tests) which showed no trace of D-lysine.

Purification of Lysine 2,3-*Aminomutase.*—The procedure (once developed) was normally carried out without assays for protein or enzyme activity at intermediate stages, due to the extensive amount of time required for the latter assay. The procedure was not conducted in a cold room, but enzyme solutions and buffers were kept in ice-water for as long as was convenient.

Clostridium subterminale strain SB4 (ATCC 29748) was grown on the medium previously described,[1,5] minus methylene blue (which stained the tubing and filters used for collecting cells). The medium (30 l in two 5 gal carboys) was equilibrated at 37 °C for 24 h prior to inoculation with 100 ml of fresh inoculum. Growth of cells was allowed to proceed in the stationary medium for 12 h, and then the cells were collected by tangential filtration, using a Millipore Pellicon cassette system equipped with an 0.22 μm Durapore filter, to a volume of *ca.* 1—2 l, followed by centrifugation. The cells (75 g) (wet), were suspended in cold (4 °C) standard[1,5] buffer (200 ml) (not deaerated), and the suspension was passed, in portions, through a cold 40 ml French pressure cell at 20 000 p.s.i. The exudate was centrifuged at 0 °C for 30 min at 40 kg, and the supernatant (226 ml) was decanted. This was treated with an equal volume of 5% streptomycin sulphate added continuously over 30 min while the mixture was being stirred in an ice-water bath. The mixture was centrifuged (4 °C, 30 min, 40 kg) and the supernatant (432 ml) was decanted.

This was treated with powdered enzyme-grade ammonium sulphate (104 g) added in 1.73 g portions every 30 s, while being stirred in an ice-water bath. Stirring was continued for an additional 30 min, and then the mixture was centrifuged (4 °C, 30 min, 40 kg). Very little precipitate was obtained in this step. The supernatant (460.2 ml) was further treated with ammonium sulphate (28.9 g) added in 0.48 g portions every 30 s, stirring at 4 °C for an additional 30 min. After centrifugation (4 °C, 30 min, 40 kg), the precipitate was redissolved in 10 ml of 30 mM Tris chloride, 10 mM dithiothreitol, 0.1 mM pyridoxal phosphate buffer, pH 7.8. The solution, in a 25 ml Erlenmeyer flask sealed with a serum cap through which was inserted a thermometer, was continuously flushed with argon *via* syringe needles. The flask was shaken in a 70 °C water bath, allowing the internal temperature to be raised to 59—60 °C over 2 min and then kept at 60—61 °C for a further 4.5 min. The mixture was then rapidly cooled in ice-water and centrifuged (4 °C, 30 min, 40 kg). The supernatant (*ca.* 8 ml) was diluted with standard buffer (40 ml) containing NaCl (0.1M). The solution was concentrated to *ca.* 10 ml in an Amicon 65 ml stirred filtration cell using a PM 30 membrane filter at 40 p.s.i.

The concentrated solution was added to a 2.5 × 30 cm column of DEAE Sephadex A-50 set up in standard buffer/0.1M NaCl at 4 °C. The solution was washed into the column with additional standard buffer/0.1M NaCl (20 ml) and then the column was eluted with a linear gradient from 150 ml each of standard buffer/0.1M NaCl and standard buffer/0.4M NaCl, at 0.4 ml/min^{-1}, collecting 20 min fractions. Two yellow bands (at *ca.* 0.2M NaCl and 0.4M NaCl) were eluted. The first band (spanning *ca.* eight fractions) contained all of the lysine 2,3-aminomutase activity. The fractions were combined and concentrated at 4 °C in an Amicon filtration cell (PM30 membrane) to *ca.* 10 ml volume. Standard buffer (without NaCl) (40 ml) was added, and the solution was again concentrated to 10 ml. The product typically contained *ca.* 5 mg ml^{-1} of protein having *ca.* 0.2 units mg^{-1} of lysine 2,3-aminomutase activity, as measured by the following technique. A unit of lysine 2,3-aminomutase activity is defined[5] as the amount of enzyme forming 1 μmole of β-lysine per min under the assay conditions. The enzyme was used immediately for all incubations described in this paper and was not stored.

Assay of Lysine 2,3-*Aminomutase.*—Protein assays were performed by using the Bio-Rad protein assay method, with bovine serum albumin as standard.

Enzyme assay: L-lysine monohydrochloride (25 mg) in water‡ (5 ml, adjusted to pH 7.8 with NaOH) was placed in a 25 ml Wheaton bottle. The following (prepared in deaerated H_2O) were added: $FeSO_4 \cdot 7H_2O$ (5.9 mg ml^{-1}, 200 μl), dithiothreitol (4.1 mg ml^{-1}, 175 μl), pyridoxal phosphate (0.46 mg ml^{-1}, 175 μl), and *S*-adenosyl-L-methionine (2.0 mg ml^{-1}, 500 μl). The enzyme solution (*ca.* 1 ml, 5—10 mg protein) was added, and the bottle was filled nearly to capacity with cold (*ca.*

* Barker[5] reported that near maximal activity of lysine 2,3-aminomutase was obtained when 3.7 moles of SAM were added per mole of enzyme, which is a hexamer. Thus it is conceivable that there might be two binding sites for SAM: one serving in the role of an allosteric binding site with rapid exchange with unbound SAM, the other having tightly bound SAM which could be involved in the catalytic mechanism. If this were the case, our conclusions based on the failure to detect deuterium incorporation into non-bound SAM would be invalid.

† In Fig. 5, the pyridoxal phosphate is shown as having formed a Schiff base intermediate with the ε-amino group of lysine. However, this is not based on any experimental evidence and is presented only as a hypothesis.

‡ Glass distilled water was boiled and then cooled under nitrogen and kept under nitrogen until used.

10 °C) standard[1,5] buffer (degassed by vacuum pumping). Finally sodium dithionite (25 mg/ml^{-1}, 250 µl) was added, giving a straw coloured solution. The bottle was closed by a rubber stopper secured with an aluminium cap, and the air space was flushed with argon. A sample (*ca.* 0.1—0.2 ml) was immediately removed by syringe (replacing the volume by argon) for analysis at time = 0 (t = 0). This was placed in an Amicon Centrifree device and centrifuged at *ca.* 2 000 r.p.m. for 10 min. The bottle was then placed in a 37 °C water bath and incubated stationary for the duration of the run. Aliquots were removed at intervals and processed as for the t = 0 sample.

After centrifugation, the solution passing through the Centrifree filter was directly analyzed by h.p.l.c. for its content of α- and β-lysine, as previously described.[1] Solutions of L-α-lysine and (3*RS*)-β-lysine of known concentration (1.0 mg ml^{-1}) were used as standards.

Detection of α-Lysine Racemase Activity in Lysine 2,3-*Aminomutase Preparations using Marfey's Reagent.*—Samples (0.5 ml) containing *ca.* 2—5 mg of protein were placed in 25 ml vials. Blank runs were performed by using 0.5 ml of standard[5] buffer in place of enzyme solution. In 'boiled enzyme' runs, the 0.5 ml enzyme sample was heated (in the 25 ml vial) in a boiling water bath for 10 min and then cooled to room temperature.

To each sample was added L-lysine (0.80 ml, 0.10M, pH 7.8), tetrasodium pyrophosphate buffer (0.70 ml, 0.10M, pH 8.3), and pyridoxal phosphate (0.40 ml, 0.01M). The vials were incubated (aerobically) on a gyrotory shaker at 150 r.p.m. at 37 °C for 24 h.

A portion (0.2—0.3 ml) of each solution was placed in an Amicon Centrifree filter and centrifuged at *ca.* 2 000 r.p.m. for 10 min. A portion (0.10 ml) of each filtrate (corresponding to *ca.* 0.9—1.0 mg of lysine originally present in the incubation mixture) was freeze-dried in a 1 dram vial.

The residues were treated with Marfey's reagent[6] [N^2-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide][7] (0.40 ml, 1% in acetone) plus $NaHCO_3$ (1M, 80 µl) and heated with gentle oscillation in a water bath at 37 °C for 2 h. Each reaction mixture was treated with 2M HCl (80 µl) and then filtered through a Gelman Acro LC13 filter. The filtrates were kept at −78 °C prior to analysis.

The filtrates were then analysed by h.p.l.c. using the following conditions: column, Waters Nova-PAK C_{18}, 3.9 mm by 15 cm; solvent, 1.0 ml min^{-1} 30% MeCN/70% 0.05M triethylamine phosphate (7.0 ml freshly distilled triethylamine/1 l H_2O adjusted to pH 3.0 with concentrated H_3PO_4); detector, 340 nm, 2.0 AUFS; all injections were 2 µl. Under these conditions, the L-lysine derivative had a retention time of 8.8 min, whereas the D-lysine derivative (when present) had a retention time of 14.6 min. Typical chromatograms produced from samples of lysine 2,3-aminomutase at various stages of purification are shown in Figure 1.

α-Lysine Mixtures: Preparation of N,N-*Diphthaloyl Methyl Ester Derivative* (**3**).—The unlabelled and/or labelled lysines (*ca.* 15—17 mg of each of the pair) were dissolved in a little H_2O (2—3 ml), and, after thorough mixing, a portion of the solution was removed for preparation of the derivative (the remainder being used for incubations).

The removed portion (*ca.* 5 mg total lysine) was treated with *N*-ethoxycarbonylphthalimide[2] (25 mg), sodium carbonate (10 mg), and H_2O (1 ml), and the mixture stirred at 25—30 °C for 1 h. The mixture was acidified (1M HCl) and evaporated to dryness *in vacuo* at 25 °C. The residue was triturated with $CHCl_3$ and MeOH, and treated with an excess of diazomethane in ether for a few min. The solvent was evaporated under reduced pressure, and the residue chromatographed by preparative t.l.c. (solvent: 30% ether–benzene) on silica gel HF 254 + 366. The desired band was extracted with EtOAc, and, after filtration and evaporation of the solvent, the residue was crystallized and recrystallized from MeOH to yield N,N-*Diphthaloyl* L-*lysine methyl ester* (**3**), which was submitted for low resolution mass spectroscopy (see text for M^+ data).

A slower running compound observed in the preparative t.l.c. separations was identified as the imide (**14**) from its ^{1}H n.m.r. spectrum: δ ($CDCl_3$) 1.20 (3 H, t, *J* 6 Hz), 3.90 (3 H, s), 4.11 (2 H, q, *J* 6 Hz), and 7.2—8.2 (5 H, m).

Conversions of Labelled Lysines to β-Lysine. Isolation of α- and β-Lysine N,N-*Diphthaloyl Methyl Ester Derivatives.*—A procedure virtually identical to that described above for enzyme assays was used for conversions of labelled lysines to β-lysines. The precursor was always added at a final concentration of 1.0—1.2 mg/ml^{-1}, and most conversions were carried out on 25 mg of substrate. In runs with high *S*-adenosyl-L-methionine (SAM) concentration designed to detect 5′-deuterium incorporation, SAM was added at 1.0 mg ml^{-1}. In D_2O runs, the amino acid and standard buffers were dissolved in D_2O instead of H_2O and adjusted to pH (meter reading) 7.8. Approximately 80—90% of the water in the final incubation mixture was replaced by D_2O.

After conversion of α- to β-lysine to the desired extent (monitored by h.p.l.c.), the reaction mixture was heated in a boiling H_2O bath for 30 min. The cooled mixture was then centrifuged briefly, and the supernatant added to a 2.0 × 15 cm column of Dowex 50W-X8, 50—100 mesh, H^+ form. After elution of acidic or neutral materials and washing of the column with H_2O, the column was eluted with 2M NH_4OH (200 ml) and the effluent evaporated under reduced pressure to yield the crude amino acid product mixture. This was dissolved in H_2O (5 ml) and treated with *N*-ethoxycarbonylphthalimide (125 mg) and Na_2CO_3 (50 mg) with vigorous stirring at room temperature for 1.5 h. The mixture was then acidified (1M HCl) and evaporated to dryness *in vacuo* at 25 °C. The residue was triturated with $CHCl_3$ and MeOH, and treated with an excess of diazomethane in ether. After evaporation of the solvents under reduced pressure, the residue was chromatographed by preparative t.l.c. using silica gel HF 254 + 366 (solvent: 30% ether–benzene). The band corresponding to the mixture of α- and β-lysine *N*,*N*-diphthaloyl methyl ester derivatives (which did not separate) was extracted with EtOAc. After evaporation of the solvent under reduced pressure, the residue was redissolved in a little $CHCl_3$ (0.2—0.3 ml) and the solution was diluted to *ca.* 2 ml with iso-octane. This solution was then chromatographed by preparative h.p.l.c., and the peaks corresponding to α- and β-lysine were collected. Chromatographic conditions: column, Waters µPorasil 7.8 mm × 30 cm stainless steel; solvent, 1% isopropyl alcohol–iso-octane at 8.0 ml min^{-1} detector, 254 nm. The retention times for the α- and β-lysine derivatives were *ca.* 15 and 20 min, respectively (depending on column load). The collected solutions were then evaporated under reduced pressure to yield the pure derivatives (**3**) and (**4**), *ca.* 5—10 mg, depending on the amount of conversion by the enzyme. The β-lysine derivative (**4**), had ^{13}C n.m.r. ($CDCl_3$) δ 25.54, 26.72, 36.67, 37.32, 47.62, 51.72, 123.20, 123.31, 131.80, 132.14, 133.72, 133.87, 133.95, 168.21, 168.24, and 171.11 (conditions, 125.80 MHz, SW 29 412 Hz, PW 4 µs, RD zero, LB 2 Hz, repetition rate 0.56 s).

Isolation of 5′-Methylthioadenosine (*MTA*) *from Incubation Mixtures.*—After the incubations were complete, the mixture was heated as before in a boiling H_2O bath for 30 min. The cooled mixture was then filtered through a PM-30 membrane in an Amicon filtration cell at *ca.* 40 p.s.i. The filtrate was added to a 1.5 × 20 cm column of Whatman CM52 anion exchange cellulose, and the acidic and neutral products collected including a column wash with H_2O. The effluent was lyophilized to yield the crude MTA. The column was then eluted

with 2M NH_4OH to yield, after evaporation under reduced pressure, the crude amino acid product mixture which was derivatized as described above.

The crude MTA was redissolved in *ca.* 3—4 ml H_2O, filtered through an 0.45 μm nylon filter, and separated by preparative h.p.l.c.[19] Chromatographic conditions: column, Nucleosil SA (Alltech Associates) 4.6 mm × 25 cm, 5 μm particle size; solvent, ammonium formate, 0.2M, adjusted to pH 4.0, at 1.0 ml min^{-1}; detector, 254 nm. MTA had a retention time of *ca.* 20 min, depending on column load. The collected peak was then lyophilized to yield MTA as a white powder (*ca.* 10 mg from 25 mg of SAM used in incubation mixture) which was (in some cases) submitted for low resolution mass spectroscopy.[20] For ^2H n.m.r. spectra, the powder was redissolved in a few ml of ^2H-depleted H_2O (Aldrich) and re-lyophilized. Samples were then dissolved in *ca.* 0.4 ml ^2H-depleted H_2O to which was added 10 μl concentrated HCl for solubility (samples of unlabelled MTA in D_2O treated with 10 μl HCl showed no decomposition over several days at 25 °C, by ^1H n.m.r.). To the ^2H n.m.r. samples was added either [$^2H_9$]-t-butyl alcohol (0.015 mg) or potassium [^2H]formate[21] (0.20 mg), and the spectra were then recorded: conditions (typical): SF 76.77 MHz, SW 2 000 Hz, PW 20 μs, RD zero, LB 2 Hz, repetition rate 1.024 s, *ca.* 5 000 scans. The 60 MHz ^1H n.m.r. of unlabelled MTA (D_2O + trace of HCl) had (relative to internal t-butyl alcohol, δ 1.40) δ 2.27 (3 H, s, MeS–), 3.12 (2 H, m, 5′-CH_2), 4.50 (1 H, m, 2′ or 3′-CH) 4.58 (1 H, m, 2′ or 3′-CH), 5.02 (1 H, m, 4′-CH), 8.61 (1 H, s, 2 or 8-CH), and 8.70 (1 H, s, 2 or 8-CH).

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