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S-Adenosylmethionine: The Relation of Configuration at the Sulfonium Center to Enzymatic Reactivity[1]

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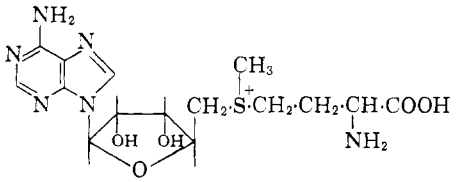
The sulfonium diastereoisomers of S-adenosyl-L-methionine have been resolved enzymatically and their optical rotations determined. It has also been shown that the methionine activating enzymes of yeast and liver yield products of the same steric configuration and that the steric requirements of guanidinoacetate methylpherase, the S-adenosylmethionine cleaving enzyme of yeast and catechol O-methyl transferase are identical with respect to the sulfonium center. (±)-S-Adenosyl-D-methionine has been synthesized by the chemical methylation of S-adenosyl-D-homocysteine, and its utilization by guanidinoacetate methylpherase has been investigated.

It is well known that the enzymatic activation of methionine for transmethylation involves the formation of a sulfonium compound, S-adenosylmethionine.[3,4] The structure of this nucleoside



has been established both by degradation[5] and by synthesis of the sulfonium compound itself[6] and of its product, S-adenosylhomocysteine.[7]

S-Adenosylmethionine synthesized by the condensation of 5′-methylthioadenosine and DL-2-amino-4-bromobutyric acid has previously been shown to be 50% utilized in the enzymatic methylation of guanidinoacetic acid.[6] The occurrence of stereoisomerism about triply substituted sulfonium atoms has been well documented[8] so that it may be presumed that the synthetic compound was a mixture of four diastereosiomers. However, it was not possible at the time of the first experiments to ascertain the relative stereospecificities of guanidinoacetate methylpherase toward the sulfonium center and the α-amino carbon atom.

More recently an enzymatic synthesis of S-adenosyl-L-homocysteine has been described which involves a condensation of adenosine and L-homocysteine. The enzyme is specific for the L-configuration of the amino acid and large amounts of the nucleoside may be readily obtained in pure form. Upon treatment with methyl iodide a product was obtained which, although chemically identical with S-adenosylmethionine, was only 50% utilized in the enzymatic synthesis of creatine.[9]

These results suggested that the sulfonium diastereoisomers might differ in their enzymatic reactivity and a more complete investigation of the relationship between enzymatic reactivity and configuration in certain diastereoisomers of S-adenosylmethionine was undertaken.

Results and Discussion

S-Adenosylmethionine synthesized by the methionine activating enzyme of rabbit liver was almost completely utilized as a methyl donor for the synthesis of creatine in the presence of guanidinoacetate methylpherase (curve 1, Fig. 1).

By contrast, S-adenosylmethionine formed by chemical methylation of S-adenosyl-L-homocysteine was utilized only to the extent of 50% under the same conditions (curve 2, Fig. 1). As shown in the figure, the enzyme remained active when creatine synthesis had ceased since addition of further amounts of enzymatically synthesized S-adenosylmethionine supported formation of more creatine. It should be emphasized that, in experiments not shown here, the *initial rates* of creatine formation observed with saturating amounts of the two preparations of S-adenosylmethionine were identical.

Interpretation of these results as due to preferential enzymatic utilization of one sulfonium diastereoisomer depends upon the demonstration that the two preparations differ in their relative content of sulfonium diastereoisomers and that they differ only in this respect. These stipulations were satisfied by the following experiments. First, it was shown that the optical rotations of samples of S-adenosylmethionine prepared by the methionine activating enzymes of liver[10] or yeast[11] were almost identical ($[\alpha]^{24}_{589}$ *ca.* (+)48°)[12] but significantly different from that of the compound prepared by the chemical methylation of S-adenosyl-L-homocysteine ($[\alpha]^{24}_{589}$ (+)52°) (Table I).

(1) Fourteenth paper in a series on enzymatic mechanisms in transmethylation.

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(3) G. L. Cantoni, THIS JOURNAL, **74**, 2942 (1952).

(4) G. L. Cantoni, *J. Biol. Chem.*, **204**, 403 (1953).

(5) J. Baddiley, G. L. Cantoni and G. A. Jamieson, *J. Chem. Soc.*, 2662 (1953).

(6) J. Baddiley and G. A. Jamieson, *ibid.*, 4280 (1954).

(7) J. Baddiley and G. A. Jamieson, *ibid.*, 1085 (1955).

(8) W. J. Pope and S. J. Peachey, *ibid.*, 1072 (1900); S. Smiles, *ibid.*, 1174 (1900); E. Wedekind, *Ber.*, **58**, 2510 (1925); M. P. Balfe, J. Kenyon and H. Phillips, *J. Chem. Soc.*, 2554 (1930).

(9) G. de la Haba and G. L. Cantoni, *J. Biol. Chem.*, in press.

(10) G. L. Cantoni, in "Biochemical Preparations," Vol. V, D. Shemin, Ed., John Wiley and Sons, Inc., New York, N. Y., 1957, pp. 58–61.

(11) S. H. Mudd and G. L. Cantoni, *J. Biol. Chem.*, **231**, 481 (1958).

(12) In preliminary experiments designed to determine the most suitable conditions for polarimetric measurements on the various diastereoisomers of S-adenosylmethionine, it was found that although the specific rotation of the nucleoside was virtually unchanged in salt concentrations (equimolar acetic acid/potassium acetate) ranging from 0.5 to 7 molar, it become increasingly less dextrorotatory at *p*Hs above 4.5. This observation may account for the discrepancy between the present values and the value $[\alpha]^{24}_{589}$ (+)16.8° (*c* 1% in water) recently recorded for crystalline S-adenosylmethionine bromide (J. A. Stekol, E. I. Anderson and S. Weiss, *J. Biol. Chem.*, **233**, 425 (1958)) since an aqueous solution of the sulfonium halide would be expected to be substantially neutral.

TABLE I

OPTICAL ROTATIONS OF DIASTEREOISOMERS[a]

Compound	Source	Concn.,[b] %	Specific rotation[c] 589 mμ	436 mμ
(−)-S-Adenosyl-L-methionine[f]	Methionine activating enzyme of rabbit liver	1.78	(+)48.5°	(+)100.5°
	Methionine activating enzyme of baker's yeast	0.92	(+)47.2°	(+)101.2°
(±)-S-Adenosyl-L-methionine	Chemical methylation of S-adenosyl-L-homocysteine	1.81	(+)52.2°	(+)106°
(+)-S-Adenosyl-L-methionine	Enzymatic resolution by guanidinoacetate methylpherase	1.83	(+)57°	(+)115°
(±)-S-Adenosyl-D-methionine	Chemical methylation of S-adenosyl-D-homocysteine	1.29	(+)16°	(+) 33°
	CHEMICAL DEGRADATION PRODUCTS			
5'-Methylthioadenosine[e]	Authentic sample	1.01	(+)15°	
	From (−)-S-adenosyl-L-methionine	0.37	(+)11°	
	From (±)-S-adenosyl-L-methionine	.30	(+)13°	
L-α-Amino-γ-butyrolactone[f]	From authentic L-homoserine	.16	(−)35°	
	From (−)-S-adenosyl-L-methionine	.24	(−)25°	
	From (±)-S-adenosyl-L-methionine	.18	(−)32°	

[a] All rotations were determined in Rudolph Photoelectric Polarimeter (Model 80) in a 1 dcm. tube with detachable end-plates, at room temperature (25°). [b] Concentrations were determined from the 259 mμ absorption in the case of the nucleosides (E 15,400) and from ninhydrin determinations in the case of the aminolactone. [c] The rotations are considered to be accurate to within (±)1° in the case of the sulfonium compounds, (±)2° for the samples of 5'-methylthioadenosine and (±)3° for the lactone. [d] The solvent for the sulfonium nucleosides was an acetic acid solution containing potassium acetate from the buffered Amberlite IRC-50 (XE-64) column. The solutions were adjusted to pH 3.5 before reading and a 5 molar mixture of equal amounts of acetic acid and potassium acetate was used as a blank. [e] In 0.3 N acetic acid. [f] In 5 N hydrochloric acid. This solution would therefore be an equilibrium mixture of the lactone and hydroxy acid.

Second, to prove that the compounds differed only in the configuration of the sulfonium group and not in chemical and configurational changes in the rest of the molecule the compounds were degraded to 5'-methylthioadenosine and homoserine. The optical activities and chemical properties of these compounds derived from both types of S-adenosylmethionine were the same (Table I, and Experimental). As a further check that the L-configuration at the α-carbon atom had been retained, the derived samples of homoserine were subjected to treatment with D-amino acid oxidase and were not oxidized, while authentic DL-homoserine carried through the same isolation procedure was attacked by this enzyme. These experiments demonstrated that the rotatory differences between the two preparations were not due to chemical changes within the molecule but solely to the isomerism about the sulfonium atom and that enzymatically synthesized S-adenosylmethionine is correctly described as (−)-S-adenosyl-L-methionine.[13]

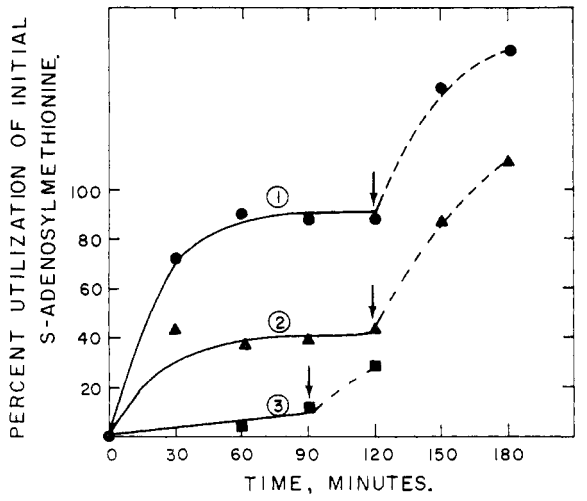

Fig. 1.—Utilization of sulfonium diastereoisomers of S-adenosylmethionine by guanidinoacetate methylpherase. All reaction mixtures contained potassium phosphate (pH 7.4) 50 μmoles/ml., guanidinoacetic acid, 3.8 μmoles/ml., freshly neutralized reduced glutathione, 8 μmoles/ml. The reaction was started by the addition of enzyme. Curve 1, S-adenosyl-L-methionine (formed by rabbit liver enzyme), 0.24 μmole/ml.; guanidinoacetate methylpherase, 5.3 units/μmole substrate; Curve 2, S-adenosyl-L-methionine (formed by methylation of S-adenosyl-L-homocysteine), 0.30 μmole/ml.; guanidinoacetate methylpherase, 5.5 units/μmole substrate; Curve 3, S-Adenosyl-L-methionine (resolved by treatment with S-adenosyl-L-methionine cleaving enzyme), 0.30 μmole/ml., guanidinoacetate methylpherase, 5.8 units/μmole substrate. The mixtures were incubated at 37° and aliquots removed at the times shown for assay of creatine formation. At the arrow, enzymatically formed S-adenosyl-L-methionine (0.23 μmole per ml.) was added to each vessel. Utilization was calculated on the basis of creatine formation.

To obtain the epimeric sulfonium isomer, a large scale preparation was carried out in which (±)-S-adenosyl-L-methionine was allowed to react as fully as possible in the presence of guanidinoacetate methylpherase. Approximately 50% of the initial material was unreactive. The inactive sulfonium compound was reisolated from the incubation mixture and shown to be chemically identical with enzymatically synthesized S-adeno-

(13) In considering the four diastereoisomers possible in S-adenosylmethionine, it will be convenient to use the conventional designations D- and L- to describe configuration about the α-carbon atom while using the terms (+) and (−) to signify the contribution of the sulfonium group to the rotation of the whole molecule; thus, the completely racemic product will be described as (±)-S-adenosyl-DL-methionine. One of us (S. H. M.) recently used the term *allo*-S-adenosyl-L-methionine to describe the enzymatically inactive diastereoisomer before the optical relationships between the various forms had been determined.[14] In the light of the results presented here this nomenclature is now superseded by the term (+)-S-adenosyl-L-methionine.

There has recently been a tendency to give the prefix D- or L- a nutritional as distinct from a configurational significance. The term S-adenosyl-D-methionine has been used to describe a product isolated from yeast fed D-methionine and known to contain significant amounts of the nucleoside synthesized from endogenous L-methionine. (See footnote 10 and S. K. Shapiro, A. N. Mather, *J. Biol. Chem.*, **233**, 631 (1958)). We feel that the nomenclature suggested on the basis of the present experiments may be preferable,

syl-L-methionine. However, after intensive purification, its specific rotation ($[\alpha]_{5890}^{24}$ (+)57°) was of the value expected for (+)-S-adenosyl-L-methionine on the basis of the results obtained with the enzymatically and chemically prepared analogs.

Studies similar to those reported above have been carried out with the yeast enzyme which catalyzes the decomposition of S-adenosylmethionine to 5'-methylthioadenosine and α-amino-γ-butylrolactone.[14] When (±)-S-adenosyl-L-methionine was incubated with relatively large amounts of this enzyme the reaction ceased after the utilization of 50% of the initial material (Fig. 2).

To show that this was indeed due to a preferential decomposition of one diastereoisomer, the unreacted material from a similar, larger-scale experiment was isolated. This material, which was identical with S-adenosylmethionine on the basis of chromatographic and electrophoretic data, and in the nature of its decomposition products, was inactive as a substrate for guanidinoacetate methylpherase (curve 3, Fig. 1).

Because the cleaving enzyme has a relatively low substrate affinity and is inhibited by the product 5'-methylthioadenosine, it is best studied using initial rates of reaction. Such studies (Table II)

TABLE II

COMPARISON OF SULFONIUM DIASTEREOISOMERS OF S-ADENOSYLMETHIONINE AS SUBSTRATE FOR S-ADENOSYLMETHIONINE CLEAVING ENZYME[a]

Expt.	Method of preparation of S-adenosylmethionine	Initial S-adenosylmethionine, μmoles/ml.	5'-Methylthioadenosine formed, μmoles	Rate, as % of control
1	Rabbit liver enzyme	2.6	0.26	..
	(+)-S-adenosyl-L-methionine from guanidinoacetate methylpherase resolution	2.6	0	0
2	Rabbit liver enzyme	4.2	0.41	..
	(+)-S-adenosyl-L-methionine from cleaving enzyme resolution	4.0	.01	3
3	Rabbit liver enzyme	2.06	.33	..
	Rabbit liver enzyme	4.12	.38	..
	Methylation of S-adenosyl-L-homocysteine	4.12	.18	47

[a] The substrates were incubated with the partially purified enzyme for 2 hr. at 37° at *p*H 5.6 in a final volume of 0.5 ml. The extent of reaction was measured by the formation of 5'-methylthioadenosine. A control, incubated in the absence of enzyme, was subtracted from each experimental value.

showed that the S-adenosylmethionine obtained by resolution of (±)-S-adenosyl-L-methionine with guanidinoacetate methylpherase or with the cleaving enzyme itself is not attacked at a significant rate by the latter enzyme. Taken together these data prove that the two enzymes exhibit identical stereospecificity with respect to the sulfonium center.

A further point indicated by the data of Table II, experiment 3, is that (+)-S-adenosyl-L-methionine sigifincantly inhibits the utilization of (−)-S-adenosyl-L-methionine by the cleaving enzyme, as do some other 5'-alkylthioadenosine derivatives.[14] This inhibition could be overcome by the addition of further amounts of (−)-S-adenosyl-L-methionine. Thus, in another experiment two micromoles of (±)-S-adenosyl-L-methionine (*i.e.*, one micromole of the (+) and one micromole of the (−)compound) were cleaved at 59% of the rate of one micromole of (−)-S-adenosyl-L-methionine. However, in the presence of an additional five micromoles of (−)-S-adenosyl-L-methionine, the degree of inhibition was so slight as to allow cleavage at 97% of the rate of the control, in which six micromoles of the (−)-compound were used.

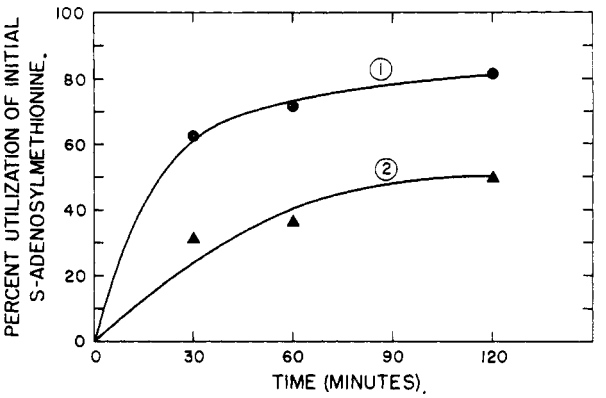

Fig. 2.—Utilization of sulfonium diastereoisomers of S-adenosylmethionine by cleaving enzyme of yeast. Each vessel contained in 0.5 ml.; potassium phosphate, *p*H 5.6, 100 μmoles; cleaving enzyme, specific activity 0.41, 1.4 mg. Curve 1, S-adenosyl-L-methionine (formed by the rabbit liver enzyme), 0.29 μmole; Curve 2, S-adenosyl-L-methionine (formed by methylation of S-adenosylhomocysteine), 0.30 μmole. The reaction was started by the addition of enzyme and the vessels were incubated at 37°. The reaction was stopped at the times shown for assay of the formation of 5'-methylthioadenosine.[14]

The inhibitory power of (+)-S-adenosyl-L-methionine raised the possibility that the non-utilization of this substance by the cleaving enzyme might be due to substrate inhibition. However, when a series of concentrations of (+)-S-adenosyl-L-methionine from 4×10^{-3} *M* to 5×10^{-4} *M* was assayed as substrate, a significant rate of cleavage was not detected.

To extend these studies, a third enzyme, catechol O-methyl transferase,[15] which transfers the methyl group of S-adenosyl-L-methionine to epinephrine, was examined.

In this case also the enzyme used at most only 50% of (±)-S-adenosyl-L-methionine. Furthermore, as shown in Table III, it was unable to utilize the inactive diastereoisomer obtained by resolution with either of the two previous enzymes.

In order to clarify the matter of the specificity of guanidinoacetate methylpherase for diastereoisomers differing in configuration at the α-carbon atom (±)-S-adenosyl-D-methionine was prepared by methylation of S-adenosyl-D-homocysteine. The enzyme attacked this substrate, but at a rate

(14) S. H. Mudd, *J. Biol. Chem.*, in press.

(15) J. Axelrod and R. Tomchick, *ibid.*, **233**, 702 (1958).

TABLE III

COMPARISON OF SULFONIUM DIASTEREOISOMERS OF S-ADENOSYLMETHIONINE AS SUBSTRATE FOR CATECHOL O-METHYL TRANSFERASE[a]

Method of prepn. of S-adenosylmethionine	Initial S-adenosyl-methionine, milli-micromoles	4'-Methoxy-epinephrine formed, milli-micromoles	Rate, as % of control
Rabbit liver enzyme	52	12.1	..
(+)-S-Adenosyl-L-methionine from:			
(i) Guanidinoacetate methylpherase resolution	53	0.5	4
(ii) Cleaving enzyme resolution	52	0	0

[a] All vessels contained in 0.6 ml.: potassium phosphate, *p*H 7.4, 25 μmoles; L-epinephrine bitartrate, 600 milli-micromoles; $CoCl_2$, 5 μmoles; catechol O-methyl transferase (first calcium phosphate eluate,[15] 80 μgrams); and S-adenosylmethionine as indicated. Incubation was for 90 minutes at 37°.

slow in comparison to that found with (−)-S-adenosyl-L-methionine (Table IV). To prove that this result was not due to a slow racemization at the α-carbon atom, the S-adenosylhomocysteine arising from the two reactions was isolated and examined by an enzymatic assay which is specific for S-adenosyl-L-homocysteine[9] (Table V). This assay showed conclusively that racemization had not occurred and that guanidinoacetate methylpherase will attack both α-carbon diastereoisomers.

TABLE IV

UTILIZATION OF (±)-S-ADENOSYL-D-METHIONINE BY GUANIDINOACETATE METHYLPHERASE[a]

Time, min.	Creatine formation, μmoles/ml. (−)-S-Adenosyl-L-methionine	(±)-S-Adenosyl-D-methionine
0	0	0
30	0.25	0.02
90	.27	.05
120	.25	.08

[a] Each tube contained in μmoles/ml., in a total volume of 5 ml.: potassium phosphate, 50; guanidinoacetic acid, 4; freshly neutralized reduced glutathione, 8; S-adenosylmethionine, 0.3. Guanidinoacetate methylpherase (5 units/ml., specific activity 0.66) was added to initiate the reaction. Aliquots were withdrawn at the times indicated for assay of creatine formation.

TABLE V

CONFIGURATION S-ADENOSYLHOMOCYSTEINE[a]

	S-Adenosylhomocysteine from (±)-S-Adenosyl-D-methionine	(±)-S-Adenosyl-L-methionine
	Optical density at 265 mμ	
Initial value	0.400	0.504
Final value	0.395	0.331
% reaction	2	95

[a] All vessels contained potassium phosphate, *p*H 7.4, 100 μmoles; dimethylacetothetin, 100 μmoles; thetin methylpherase,[21] 37 units (specific activity 179) and adenosine deaminase (100 μ grams of a tryptic digest of bovine intestinal mucosa obtained from Armour and Co.). At zero time S-adenosyl-L-homocysteine enzyme,[9] 15 units (specific activity 27) was added and the reaction followed until there was no further drop in the absorption at 265 mμ.

The present results differ quantitatively from those originally reported for S-adenosylmethionine prepared by the condensation of 5′-methylthioadenosine and DL-2-amino-4-bromobutyric acid.[6] This difference may be due to variations in the amounts and purity of the enzyme used in the two experiments or to differing diastereoisomeric composition of the two products prepared by different synthetic routes or to variations in the assay technique. The record of the original experiments is not available to us.

The ability of enzymes to discriminate between pairs of compounds differing in configuration about carbon atoms, double bonds and ring structures is well known. While extension of such enzymatic studies to other types of asymmetric centers has been limited, stereospecific effects have been shown with compounds asymmetric about a tetracovalent phosphorus atom in their rate of enzymatic hydrolysis[16] and in their ability to inactivate cholinesterase.[17]

Although the natural occurrence of optically active derivatives of sulfoxides has long been established[18] and the stereospecific nature of the enzymatic cleavage of alliin[19] and the enzymatic reduction of the methionine sulfoxides[20] has been clearly defined, only one previous enzymatic study of an asymmetric sulfonium compound (sterically analogous to the sulfoxides) has been reported.[21] The enzyme catalyzing the transfer of the methyl group of methylethylacetothetin to L-homocysteine was found to utilize 90% of a racemic substrate, and it was considered that the enzyme "does not exhibit absolute optical specificity toward the sulfonium group, or that methylethylacetothetin is easily racemized."

The present series of experiments, therefore, comprises the major part of recorded experience bearing upon the problem of stereospecific interactions between sulfonium compounds and enzymes. To date, five enzymes involved in the metabolism of S-adenosylmethionine have been investigated in this regard. Two synthesize the nucleoside, while three carry out transalkylation reactions requiring it as a substrate. It is of some interest that without exception these enzymes show a marked degree of specificity for one sulfonium diastereoisomer. Extrapolation of these findings would lead to the supposition that stereospecific enzyme action will be the rule among sulfonium compounds.

In the light of these results it is plausible to suggest that the Se-adenosylselenomethionine, formed by the methionine activating enzymes of yeast and rabbit liver and utilized by guanidinoacetate methylpherase as a methyl donor,[22] has the same configuration about its asymmetric selenium atom as that found in (−)-S-adenosyl-L-methionine.

So far as we are aware the present work consti-

(16) F. C. G. Hoskin and G. S. Trick, *Can. J. Biochem. Physiol.*, **33**, 963 (1955).

(17) H. S. Aaron, H. O. Michel, B. Witten and J. I. Miller, THIS JOURNAL, **80**, 456 (1958).

(18) For refs. see R. L. M. Synge and J. C. Wood, *Biochem. J.*, **64**, 252 (1956). Recent crystallographic studies on (+)-S-methylcysteine sulfoxide permit the assignment of absolute configurations in this series (R. Hine and D. Rogers, *Chemistry & Industry*, 1428 (1956)).

(19) A. Stoll and C. Seebeck, *Adv. Enzymology*, **11**, 377 (1951).

(20) S. Black and E. M. Harte, *Federation Proc.*, **17**, 191 (1958).

(21) J. Durell, D. G. Anderson and G. L. Cantoni, *Biochim. Biophys. Acta*, **26**, 270 (1957).

(22) S. H. Mudd and G. L. Cantoni, *Nature*, **180**, 1052 (1957).

tutes the first biological resolution of a sulfonium compound. It is hoped that the availability of enzymatically reactive and inactive forms of S-adenosyl-L-methionine will facilitate studies on the mechanism of enzymatic transmethylation.

Experimental

Guanidinoacetate Methylpherase.—This enzyme was purified from pig liver and its activity determined as previously described.[23]

S-Adenosylmethionine Cleaving Enzyme.—This enzyme was prepared from baker's yeast, partially purified and assayed as described.[14]

Catechol O-methyl Transferase.—This enzyme was kindly supplied by Dr. J. Axelrod.[15]

S-Adenosyl-L-homocysteine.—This compound was synthesized by the condensation of adenosine and L-homocysteine by a partially purified enzyme from rat liver. The product was purified and crystallized before use, as described.[9] It had $[\alpha]^{24}_{5890}$ (+)40° (0.68% in 0.1 *N* HCl).

S-Adenosyl-D-homocysteine.—This compound was prepared from 2′,3′-isopropylidene adenosine (5.6 g.) essentially by the method of Baddiley and Jamieson[7] and isolated by the procedure of de la Haba and Cantoni[9] with the following modification. The acid solution resulting from the decomposition of the phosphotungstate was neutralized by passing it through a column of Dowex 3 in the hydroxide form and washing the column with water until the effluent was substantially free of ultraviolet (260 mμ) absorbing material. The eluate was concentrated to about 10 ml. and adjusted to *p*H 7 with a little hydriodic acid. The fraction precipitating between 50–95% ethanol concentration was repeatedly recrystallized from water to give fine white crystals (65 mg.) of S-adenosyl-D-homocysteine, m.p. 209–212° dec., $[\alpha]^{24}_{5890}$ (+) 7.5° (0.63% in 0.1 *N* HCl).

Anal.[24] Calcd. for $C_{14}H_{20}O_5N_6S$: C, 43.75; H, 5.21; S, 8.32. Found: C, 43.33; H, 5.55; S, 8.11.

S-Adenosyl-L-methionine.—This compound was synthesized enzymatically from adenosine triphosphate and L-methionine with the methionine activating enzyme of rabbit liver[10] or the yeast activating enzyme purified through the ammonium sulfate A step.[11,25]

Methylation of S-Adenosylhomocysteine.—S-Adenosylhomocysteine (50 mg.) was dissolved in formic acid (0.8 ml.). Methyl iodide (0.5 ml.) was dissolved in the solution by vigorous shaking. The tube was stoppered and kept in the dark for 5 days at room temperature. At the end of this time the contents were lyophilized, dissolved in a few milliliters of water, extracted three times with ether and relyophilized. The powder was dissolved in water, and S-adenosylmethionine separated from unreacted S-adenosylhomocysteine as described below.

Separation of S-Adenosylhomocysteine and S-Adenosylmethionine.—A 6 × 1 cm. column of Amberlite IRC 50 (XE-64) previously buffered with 0.01 *M* potassium phosphate, *p*H 7.0, was employed. The mixture was applied to the column and elution of S-adenosylhomocysteine effected with the same buffer (20 ml.). The column was washed with 0.25 *N* acetic acid (20 ml.) to remove the last traces of S-adenosylhomocysteine, and S-adenosylmethionine was then eluted with 20 ml. of 4 *N* acetic acid. This eluate was lyophilized, dissolved in water and adjusted with dilute sodium hydroxide to *p*H 6.0.

Prior to use with guanidinoacetate methylpherase, S-adenosylmethionine was applied to a column of Amberlite XE-64 (hydrogen form, size 6 cm. × 1 cm.), the column washed with water and with 0.1 *N* hydrochloric acid (20 ml.). The sulfonium compound was then eluted with 0.3 N hydrochloric acid and the eluate lyophilized and dissolved in water as described above.

Resolution of (±)-S-Adenosyl-L-methionine (I).—A reaction mixture containing potassium phosphate buffer (*p*H 7.4, 1000 μmoles), S-adenosyl-L-methionine (chloride salt, 98 μmoles, prepared by methylation of S-adenosyl-L-homocysteine), guanidinoacetic acid (64 μmoles, *p*H 7), freshly neutralized reduced glutathione (163 μmoles) and 113 units of guanidinoacetate methylpherase (specific activity 0.7) in a final volume of 22 ml. was incubated for 120 minutes at 37°. The reaction was stopped with 1.16 ml. of 100% trichloroacetic acid. The mixture was cooled in an ice-bath and centrifuged. To the supernatant fluid was added an equal volume of 1.5% ammonium reineckate in 5% trichloroacetic acid. The reineckate was decomposed in the usual way[10] and the product chromatographed on a buffered column of Amberlite XE-64 as in the above separation. The recovery of S-adenosylmethionine indicated that less than 50% of the initial material had reacted. The recovered nucleoside was again treated with 63 units of guanidinoacetate methylpherase (specific activity 0.5) in a reaction mixture containing potassium phosphate buffer (*p*H 7.4, 2,000 μmoles), guanidinoacetic acid (154 μmoles, *p*H 7), freshly neutralized reduced glutathione (326 μmoles), in a final volume of 40 ml. The reaction mixture was incubated 120 minutes at 37° and the reaction stopped with 2.0 ml. of 100% trichloroacetic acid. The S-adenosylmethionine was precipitated as above and chromatographed on Amberlite XE-64. The material eluted with 4 *N* acetic acid was then subjected to paper chromatography (acid washed Whatman 3 MM) in butanol–acetic acid–water (4:1:5) and the strip corresponding to S-adenosylmethionine eluted with water and again treated on a buffered column of Amberlite XE-64. The eluate was lyophilized, taken up in 5 *N* acetic acid, adjusted to *p*H 3.6 and its optical rotation determined.

(II) A 10-ml. reaction mixture containing (±)-S-adenosyl-L-methionine (25 μmoles), potassium phosphate buffer (*p*H 5.6, 500 μmoles) and 25 units of S-adenosylmethionine cleaving enzyme (specific activity 0.14) was incubated for 3 hr. at 37°. At the end of this period the reaction was stopped with 1 ml. of 30% perchloric acid. The mixture was cooled in an ice-bath and centrifuged. The supernatant fluid was brought to *p*H 6.7 with dilute potassium hydroxide and the solution clarified by centrifugation.

S-Adenosyl-L-methionine was isolated from the mixture on a buffered column of Amberlite XE-64 as described above.

Degradation of S-Adenosyl-L-methionine.—S-Adenosyl-L-methionine (prepared with the rabbit liver enzyme) in acetate buffer (0.01 *M*, *p*H 4.8) was heated at 100° for 15 minutes, to degrade the sulfonium compound to 5′-methylthioadenosine, homoserine and homoserine lactone and the mixture applied to a column of Amberlite XE-64 (size, 6 × 1 cm.) previously buffered at *p*H 5.5 with 0.01 *M* acetate. Homoserine and homoserine lactone passed directly through the column upon washing with a little water. This eluate was lyophilized and the residue dissolved in 5 *N* hydrochloric acid to convert to an optically stable equilibrium mixture of homoserine and its lactone. 5′-Methylthioadenosine was eluted from the column with 0.25 *N* acetic acid. In order to free the solution from large amounts of contaminating potassium acetate, this solution was lyophilized, taken up in water and applied to a small column of Dowex 50 (hydrogen form). The nucleoside was eluted with 20% aqueous ammonia (v./v.), the eluate lyophilized and the residue dissolved in 0.3 *N* hydrochloric acid. S-Adenosyl-L-methionine prepared by the methylation of S-adenosyl-L-homocysteine and an authentic sample of L-homoserine were carried through a similar series of reactions and the optical rotations of the products determined (Table I).

Chemical Identification of Diastereoisomers.—1. When examined by paper electrophoresis at *p*H 5.2 in 0.2 *M* acetate buffer in a potential gradient of 16 v./cm., the rates of migration of the enzymatic, synthetic and resolved compounds toward the cathode were identical (4.4 cm./hr.).

2. When treated with 0.1 *N* sodium hydroxide at room temperature for 15 minutes a solution of the resolved compound showed the characteristic decomposition of the sulfonium compound as shown by the disappearance of the adenosine spectrum and the appearance of the adenine spectrum (λ_{max} 268 mμ in 0.1 *N* sodium hydroxide).[26] No

(23) G. L. Cantoni and P. J. Vignos, Jr., in "Methods in Enzymology," Vol. II, S. P. Colowick and N. O. Kaplan, Eds., Academic Press, Inc., New York, N. Y., 1955, pp. 260–263.

(24) Analysis by Dr. W. Alford and his staff.

(25) It has been observed that certain preparations of S-adenosylmethionine synthesized with the rabbit liver enzyme are utilized only to the extent of 80–90% by guanidinoacetate methylpherase while the nucleoside synthesized with the more highly purified yeast enzyme is utilized to 100%. From the reaction mixture, enzymatically inactive material chemically identical with S-adenosylmethionine, can be isolated. The reason for this discrepancy is under investigation but the possibilities of a non-stereospecific synthesis by the rabbit liver enzyme or the presence of sulfonium or amino acid racemases must be considered.

(26) L. W. Parks and F. Schlenk, *J. Biol. Chem.*, **230**, 295 (1958).

difference could be detected in the relative rates of decomposition of the enzymatic and synthetic nucleosides in 0.1 *N* potassium hydroxide as determined by the change in absorption at 280 mμ.

3. The various diastereoisomers, and the 5'-methylthioadenosine and L-homoserine arising from their decomposition products, had R_fs identical with those of authentic samples in the following solvent systems.

A. Ethanol (300), water (100), concd. hydrochloric acid (1). R_fs: S-adenosylmethionine, 0.12; 5'-methylthioadenosine, 0.62; L-homoserine, 0.80.

B. Methyl Cellosolve (112), water (12), acetic acid (1). R_fs: S-adenosylmethionine, 0.05; 5'-methylthioadenosine, 0.65; L-homoserine, 0.42.

C. 1-Propanol (6), ammonium hydroxide (3, d., 0.880), water (1). R_fs: 5'-methylthioadenosine, 0.88; L-homoserine, 0.76.

Configuration of S-Adenosylhomocysteine Isolated from Guanidinoacetate Methylpherase Reaction.—A reaction mixture containing potassium phosphate (30 μmoles, *p*H 7.4), guanidinoacetic acid (3.8 μmoles), freshly neutralized reduced glutathione (8.2 μmoles), (±)-S-adenosyl-D-methionine (4 μmoles, prepared by the methylation of S-adenosyl-D-homocysteine) and guanidinoacetate methylpherase (9 units) in a final volume of 1 ml. was incubated for 2 hr. at 37°. The reaction was terminated by the addition of 0.05 ml. of 30% perchloric acid. The precipitate was removed by centrifugation, the supernatant fluid brought to *p*H 6.7 and placed on a buffered Amberlite XE-64 column as for the separation of S-adenosylhomocysteine and S-adenosylmethionine. The control mixture contained all the components, but the enzyme was added after addition of perchloric acid. A similar pair of vessels was incubated with the corresponding chemically synthesized (±)-S-adenosyl-L-methionine (3.8 μmoles).

The material eluted from the column by 15 ml. of phosphate buffer 0.01 *M*, *p*H 6.7, was collected. The eluates from the incubated mixtures contained large amounts of ultraviolet absorbing material, while negligible amounts were found in the unincubated samples. It was shown by paper chromatography in three solvent systems that S-adenosylhomocysteine was the major component of this ultraviolet absorbing material. Aliquots of the eluates were assayed enzymatically for the presence of S-adenosyl-L-homocysteine using an enzyme which reversibly and specifically cleaves the L-form of this nucleoside.[9] Adenosine liberation was measured by means of adenosine deaminase (Table V).

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BETHESDA, MARYLAND

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, POLYTECHNIC INSTITUTE OF BROOKLYN]

Peptide Syntheses *Via* Amino Acid Active Esters[1]

BY MURRAY GOODMAN AND KENNETH C. STUEBEN

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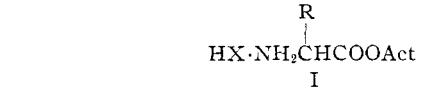
Amino acid active ester hydrobromides have been prepared. By use of these compounds, tripeptide derivatives have been synthesized in high over-all yield without isolation of a dipeptide intermediate. This has been accomplished by taking advantage of the difunctionality of the amino acid active ester as well as the difference in rate of reaction at the amino and active ester ends of the molecule.

Since 1950, several promising methods for peptide synthesis have been published. Among these are the mixed anhydride,[2-6] carbodiimide[7] and active ester[8] approaches. These new methods form amides at different rates. Thus, typical reaction times for peptide formation are 2-4 hours for mixed anhydrides, 5 hours for carbodiimides and 12–24 hours for the active esters. In this paper we wish to report the synthesis of difunctional amino acid derivatives of the type (where Act =



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CH_2CN or $-p-C_6H_4NO_2$) and the general use of these compounds in peptide synthesis.

Intermediates of a similar nature, *i.e.*, peptide active ester hydrohalides, have been used to form cyclic peptides.[9-11] Schwyzer[9] employed the *p*-nitrophenyl ester of a decapeptide in his synthesis of gramicidin-S. In addition, Kenner[10] synthesized a cyclic pentapeptide utilizing a *p*-nitrophenyl thiol ester of the linear pentapeptide hydrobromide. While the methods just described have been designed expressly for cyclization, amino acid active esters afford a general method for the preparation of peptides.

By taking advantage of the difunctionality of the amino acid active esters as well as the difference in rate among certain acylation reactions, we have been able to synthesize tripeptides directly *via* two consecutive reactions. The first (more rapid) acylation reaction is brought about at the amino end of compound I by means of N,N'-dicyclohexylcarbodiimide or mixed anhydrides. The

(1) This research was supported by a grant from the National Science Foundation, NSF G-4571. Presented before the 134th Meeting of the American Chemical Society, Chicago, Ill., September 8–12, 1958.

(2) (a) T. Wieland, W. Kern and R. Sehring, *Ann.*, **569**, 117 (1950); (b) T. Wieland and R. Sehring, *ibid.*, **569**, 122 (1950).

(3) R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951).

(4) (a) J. R. Vaughan, THIS JOURNAL, **73**, 3547 (1951); (b) J. R. Vaughan and R. L. Osato, *ibid.*, **74**, 676 (1952).

(5) T. Wieland and H. Bernhard, *Ann.*, **572**, 190 (1951).

(6) J. M. Kenner, *Chemistry & Industry*, 15 (1951).

(7) (a) J. C. Sheehan and G. P. Hess, THIS JOURNAL, **77**, 1067 (1955); (b) J. C. Sheehan, M. Goodman and G. P. Hess, *ibid.*, **78**, 1367 (1956); (c) J. C. Sheehan and J. J. Hlavka, *J. Org. Chem.*, **21**, 439 (1956).

(8) (a) R. Schwyzer, B. Iselin and M. Feurer, *Helv. Chim. Acta*, **38**, 69 (1955); (b) R. Schwyzer, M. Feurer, B. Iselin and H. Kägi, *ibid.*, **38**, 80 (1955); (c) R. Schwyzer, M. Feurer and B. Iselin, *ibid.*, **38**, 83 (1955); (d) B. Iselin, W. Rittel, P. Sieber and R. Schwyzer, *ibid.*, **40**, 373 (1957).

(9) R. Schwyzer and P. Sieber, *Angew. Chem.*, **68**, 518 (1956); *Helv. Chim. Acta*, **40**, 624 (1957).

(10) G. W. Kenner and J. M. Turner, *Chemistry & Industry*, 602 (1955).

(11) R. Schwyzer, B. Iselin, W. Rittel and P. Sieber, *Helv. Chim. Acta*, **39**, 872 (1956).