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The Enzymatic Formation of Methanol from S-Adenosylmethionine by Various Tissues of the Rat

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SUMMARY

An enzyme which forms methanol from *S*-adenosylmethionine is demonstrated to be present in a variety of mammalian tissues. That one of the products is methanol was determined by two means: (a) crystallization as [*methyl*]dinitrobenzoate, and (b) synthesis of ^{35}S-labeled *S*-adenosylmethionine and demonstration that ^{35}S was not incorporated into the volatile product. Highest levels of enzyme activity are found in the pituitary and hypothalamus. Other areas which contain the enzyme include brain, kidney, liver, lung, and heart. The enzyme from brain has a K_m of 2.5 μM for *S*-adenosylmethionine. Enzyme activity is found predominantly in the supernatant fraction of brain homogenates.

INTRODUCTION

In recent years *S*-adenosylmethionine has been shown to have a widespread role in biological methylating reactions. *S*-Adenosylmethionine is known to be the methyl donor in the synthesis (1) and metabolism (2) of many biogenic amines, and in the synthesis of certain amino (3) and nucleic acids (4). A variety of enzymes, grouped under the general classification of methyltransferases, catalyze the donation of the methyl group from *S*-adenosylmethionine to the appropriate acceptor compound.

This report enlarges upon our earlier studies dealing with an enzyme in rat brain which catalyzes the transfer of the methyl group from *S*-adenosylmethionine to water, forming methanol (5). This enzyme appears to be similar to a methanol-forming enzyme present in bovine pituitary, as described by Axelrod and Daly (6).

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MATERIALS AND METHODS

Reagents and chemicals. Organic solvents used in these studies were fluorometric grade, obtained from Matheson, Coleman, and Bell. They were not further purified before use. α-Ketoglutaric, fumaric, malic, and succinic acids were obtained from Sigma Chemical Company, as was *p*-chloromercuribenzoate.

Preparation of enzyme from tissue samples. For routine analysis of enzyme activity in brain, whole rat brains weighing 1.5–2.0 g were homogenized in equal volumes of isotonic potassium chloride at 0–4°, using a glass homogenizer and a mated, closely fitting glass pestle. All homogenates were centrifuged at either 30,000 or 105,000 $\times$ *g* for 1 hr at 0°. Samples of tissue other than brain were treated in the same manner.

Assay for enzyme activity. All assays were performed in 15-ml screw-top conical glass centrifuge tubes containing 100 μmoles of phosphate buffer (pH 8.0), 2–5 nmoles of *S*-(*methyl*-^{14}C]adenosylmethionine (methyl-

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^{14}C, 55 mCi/mmole, New England Nuclear), and 50 μl of the centrifuged supernatant fluid in a final reaction volume of 200 μl. Unless otherwise indicated, the supernatant fraction was used as enzyme source throughout these studies.

Tubes were capped and incubated at 37° for 30 min. The reaction was terminated by the addition of 0.5 ml of borate buffer, 0.5 M, pH 10. The radioactive methanol was extracted into 5 ml of toluene. The samples were shaken vigorously for 15 sec and centrifuged at low speed for 5 min. Four milliliters of the organic phase were withdrawn and divided into two 2-ml aliquots. To one aliquot, 10 ml of Bray's solution (7) were added, and the sample was counted in a liquid scintillation counter. The second aliquot was evaporated to dryness in a stream of warm air. Following evaporation, counting solution was added and the remaining radioactivity was measured. The radioactivity lost during the course of evaporation was taken as a measure of the volatile product formed, and was expressed as units of methanol synthesized. One unit equals 1 nmole of methanol formed per hour. Appropriate control tubes, containing either boiled enzyme (3–5 min) or no enzyme, were run in parallel with the enzyme samples.

Identification of radioactive volatile product as methanol. The enzymatically formed material was tentatively identified as methanol by esterifying the product with 3,5-dinitrobenzoyl chloride. In this reaction, the volatile alcohol is converted to the nonvolatile ester, methyl 3,5-dinitrobenzoate.

Several whole rat brains were pooled, and the supernatant fraction was prepared as described earlier. Portions of this supernatant fraction were incubated with the appropriate reagents. To increase the amount of radioactivity formed, *S*-[*methyl*-^{3}H]adenosylmethionine (4.2 Ci/mmole, Nuclear-Chicago) was used as substrate. Following incubation, the [^{3}H]methanol was extracted into 5 ml of ethyl acetate. This solvent was used because it gave higher yields of [*methyl*]dinitrobenzoate. The ethyl acetate phase was withdrawn, and 50 μl of unlabeled methanol and 0.4 g of 3,5-dinitrobenzoyl chloride were added. The reaction was allowed to proceed at room temperature for several hours. The precipitate which formed on cooling the solution in an ice bath was filtered, washed with 2% sodium carbonate, and recrystallized to constant specific activity. The crystals obtained were taken up in a small volume of toluene and applied to a thin-layer chromatography plate (250 μ, silica gel).

Ascending chromatography was carried out in 1-butanol–acetic acid–water (4:1:1) and in 2-propanol–5% ammonia (8:2). Authentic radioactive methyl dinitrobenzoate, formed by carrying [^{14}C]methanol (5 mCi/mmole, New England Nuclear) through the esterification procedure, was chromatographed on the same plate as the enzymatically formed material. Strips 2 × 1 cm were chipped from the plate, eluted in 0.5 ml of water, and centrifuged. Following centrifugation, the solution was decanted and its radioactivity was determined.

Synthesis of ^{35}S-[methyl]adenosylmethionine. ^{35}S-[*methyl*]Adenosylmethionine was synthesized from [^{35}S]L-methionine (118 mCi/mmole, Amersham) and ATP by incubation with the partially purified rabbit liver enzyme described by Cantoni and Durell (8). After incubation for 2 hr at 37°, the reaction was stopped by the addition of 5 ml of 10% perchloric acid, and the protein was precipitated by centrifugation. The pH of the solution was adjusted to 7.0 with 5 N KOH; the $KClO_4$ precipitate was removed by centrifugation. The material was then purified by modifications of the method of Schlenk and De Palma (9). The supernatant solution was applied to a Dowex 50-X8 (H^+) column (3.0 × 0.7 cm) which had been washed with 6 N and 1 N HCl until the absorbance at 256 nm was less than 0.02. Following application of the material, the column was washed with 1 N HCl. Seventeen ml. fractions were collected, and the absorbance at 256 nm was monitored. Washing was continued until the absorbance at 256 nm was less than 0.02; approximately 1500 ml were required. The ^{35}S-[*methyl*]adenosylmethionine was eluted with 6 N HCl; elution began almost immediately and was complete after 85 ml of acid.

The first three fractions contained the bulk of the radioactive material absorbing at 256 nm. These were pooled, giving a

volume of approximately 60 ml. To this was added 0.5 μmole of highly purified unlabeled *S*-adenosylmethionine. Then 1 ml of a 20% solution of phosphotungstic acid was slowly added, with gentle mechanical stirring. The material was allowed to stand for 5 hr at 0°. After this time the precipitate was collected by centrifugation and washed with 2 volumes of acetone–water (1:1). The acetone–water phase was then extracted with 4 volumes of isoamyl alcohol–ether (1:1). After separation of the layers, the organic phase was removed by aspiration, and the extraction process was repeated four times with 2 volumes of the isoamyl alcohol–ether. Residual acetone and isoamyl alcohol were removed from the water phase by several extractions into 5 volumes of anhydrous ether. The water phase was concentrated by evaporation under vacuum. This was then applied to a Dowex 1-HCO_3 column. This step removes *S*-adenosylhomocysteine, while *S*-adenosylmethionine passes through the column. The effluent was collected and combined with 2 ml of the water with which the column had been washed. The purified material had a final concentration of 33.9 μM, and had a specific activity of 10.2 mCi/mmole. The material was acidified to pH 4 with H_2SO_4 and stored frozen until use.

RESULTS

Verification of enzymatically formed product as methanol. The authentic [*methyl*-^{14}C]dinitrobenzoate was recrystallized to constant specific activity and melting point. The melting point range of the purified crystals was 106–108°. This agreed with published values (10) of 107° and justified the use of this material as a standard of comparison. These crystals were compared chromatographically with the ester synthesized from the enzymatically formed [^{3}H]methanol. The results of the chromatography in 1-butanol–acetic acid–water (Fig. 1) showed the radioactive peaks to be superimposable. Similar results were obtained in 2-propanol–5% ammonia. These data strongly suggested that the two compounds were identical and that methanol was indeed a product of the enzymatic reaction. The over-all recovery

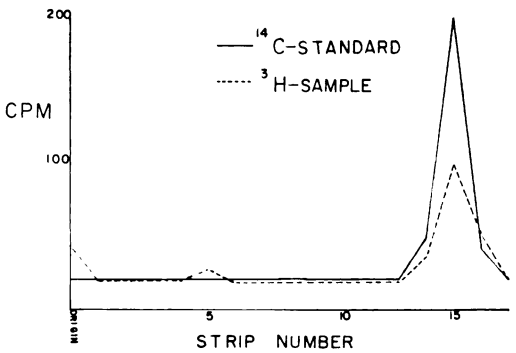

FIG. 1. *Chromatography of authentic [methyl-^{14}C] dinitrobenzoate (standard) and the ester formed by reaction of enzymatically formed [^{3}H]methanol with 3,5-dinitrobenzoyl chloride (sample)*

Chromatographic methods are described under MATERIALS AND METHODS.

of the authentic [^{14}C]methanol used to synthesize the [*methyl*-^{14}C]dinitrobenzoate was about 10%. This was low, but somewhat milder conditions were used to form the ester than would usually be employed. The recovery of the enzymatically formed [^{3}H] methanol through all the procedures of esterification was about 8%. These results suggest that most and probably all of the enzymatically formed volatile product is methanol.

Contribution of sulfur atom to volatile product. When equimolar concentrations of either ^{35}*S*-[*methly*]adenosylmethionine or *S*-[*methyl*-^{14}C]adenosylmethionine were incubated with methanol-forming enzyme from various rat organs, no significant ^{35}S-containing volatile product was seen (Table 1). Substantial amounts of ^{14}C-containing volatile product were isolated from braini, kdney, and lung. These data were taken as evidence that the ^{35}S atom was not a constituent of the volatile product formed. The chromatographic similarity between the enzymatically formed product and authentic methanol, the similarity in recoveries throughout the esterification procedure, and the lack of any sulfur-containing volatile product led to the conclusion that a single volatile product had been formed, and that it was methanol.

Assay for methanol-forming activity. The assay for enzyme activity was linear for at least 2 hr of incubation at 37° and also was

TABLE 1

Volatile product formation from S-[methyl-^{14}C]- and ^{35}S-[methyl]adenosylmethionine in various rat organs

Each value is the mean and standard error of five determinations. Organs from the rat were weighed and homogenized in 4 volumes of potassium phosphate buffer, 0.05 M, pH 7.9. An aliquot of the 30,000 × *g* supernatant fraction, containing the equivalent of 33 mg of tissue, was incubated with *S*-adenosylmethionine isotopically labeled with either ^{35}*S*-[*methyl*]adenosylmethionine (3.39 nmoles/incubation tube) or *S*-[*methyl*-^{14}C]adenosylmethionine (3.61 nmoles/tube) in a final volume of 250 μl. The starting radioactivity was: ^{35}*S*-[*methyl*]adenosylmethionine, 47,172 dpm; *S*-[*methyl*-^{14}C]adenosylmethionine, 342,526 dpm. In none of the tissues studied was any ^{35}S-containing volatile product seen. In all cases, when ^{35}S "dried" values were subtracted from "wet" values, the results ranged from +15 to −27 dpm, and were within the range of variability of this assay. On the basis of the amount of [^{14}C]methanol formed, kidney and brain did not differ significantly, although both differed from lung, heart, and liver ($p < 0.001$). Lung, but not heart, differed ($p < 0.05$) from liver.

Organ	Volatile product formed	
	^{35}S	^{14}C
	dpm/hr/33 mg	
Kidney		708 ± 55
Brain		587 ± 3
Lung		275 ± 12
Heart		259 ± 15
Liver		171 ± 42

linear with respect to protein concentration over the range tested. The recovery of added radiolabeled methanol was 85–90%. Enzyme activity was virtually abolished by boiling the supernatant fraction for 5 min, although traces of volatile product-forming activity could be detected in the boiled preparation. The enzyme showed a pH optimum of 8.0 and a temperature optimum of 43°. However, at this temperature, a small degree of breakdown of *S*-[*methyl*] adenosylmethionine occurred, and the blanks fluctuated accordingly. For this reason, all incubations were performed at 37°.

Properties of the enzyme. Figure 2 shows a Lineweaver-Burk plot for the methanol-forming enzyme. The crude supernatant fraction was used as enzyme source. From this curve, a K_m value of 2.5 μM for *S*-adenosylmethionine was obtained. The effects of a variety of compounds on enzyme activity were also tested. *p*-Chloromercuribenzoate had no effect on enzyme activity at concentrations up to 1 mM; similarly, mercaptoethanol at 1 mM did not enhance enzyme activity. Succinic, fumaric, malic, and α-ketoglutaric acids at 1 mM were without effect on enzyme activity. EDTA at a concentration of 1 mM had no effect on enzyme activity.

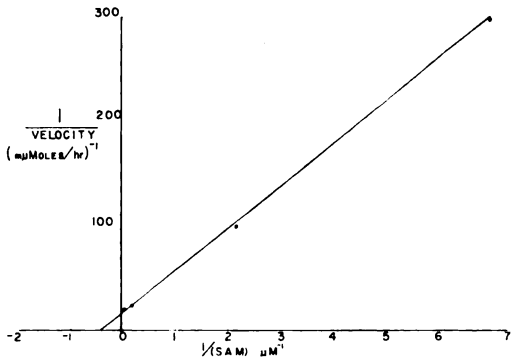

FIG. 2. *Double-reciprocal plot after the method of Lineweaver and Burk*

The estimated K_m of the methanol-forming enzyme is 2.5 μM. Determinations were performed in triplicate; each point is the mean of those values. Individual determinations agreed within 10% of each other. The K_m was estimated by regression analysis of the data points. SAM, *S*-adenosylmethionine.

Subcellular distribution of methanol-forming enzyme. The methanol-forming enzyme found in rat brain is localized almost exclusively in the supernatant fraction of centrifuged homogenates. When the 105,000 × *g* pellet and supernatant fraction were compared, over 95% of the enzyme activity was found in the supernatant. Differential ultracentrifugation following very gentle homogenization (three to five slow, hand-driven turns in a Duall tissue grinder with a fitted glass pestle) in 0.32 M sucrose failed to reveal any significant particulate distribution of the enzyme.

Distribution of enzyme within areas of rat brain. Table 2 shows the distribution of the enzyme in various areas of rat brain. The

TABLE 2

Distribution of methanol-forming enzyme in areas of rat brain

The activity of the methanol-forming enzyme was determined as described under MATERIALS AND METHODS. The enzyme values for hypothalamus and mesencephalon were significantly greater than the medullary level ($p < 0.01$). The sensitivity of this method allows detection of as little enzyme activity as 0.02 unit/g. Values below this are listed as "trace." Values are the means and standard errors of five to seven determinations.

Brain area	Methanol-forming enzyme
	units/g (wet wt)
Pituitary (whole)	53.64 ± 2.52
Hypothalamus	3.26 ± 0.19
Mesencephalon	1.87 ± 0.09
Medulla	1.46 ± 0.10
Pons	1.41 ± 0.06
Diencephalon	1.39 ± 0.07
Cortex	Trace

TABLE 3

Distribution of methanol-forming enzyme in various organs of the rat

Methanol-forming activity was assayed on the organs listed. Values for brain and kidney are significantly greater than those in the other organs ($p < 0.01$). Values for heart are greater ($p < 0.05$) than liver. The sensitivity of the assay allows detection of as little activity as 0.02 unit/g. All values below this are recorded as "trace." Values are the means and standard errors of five determinations.

Organs	Enzyme activity
	unit/g (wet wt)
Brain (whole)	0.47 ± 0.01
Kidney	0.56 ± 0.01
Liver	0.14 ± 0.03
Lung	0.22 ± 0.01
Heart	0.21 ± 0.01
Spleen	Trace
Pancreas	Trace
Adrenal	Trace

enzyme is most highly localized in the pituitary. Appreciable quantities of activity are present in the hypothalamus and diencephalon. Only trace amounts of activity were found in the cortex. Enzyme activity has been noted in the brain tissue of calf, frog, rabbit, and man.

Organ distribution of methanol-forming enzyme in the rat. Table 3 shows the distribution of enzyme activity in various tissues of the rat. The enzyme is found in several organs. The pancreas and adrenal glands show only traces of activity. Mouse adrenal, however, seems to possess substantial amounts of methanol-forming activity.

DISCUSSION

The finding of a methanol-forming enzyme provokes inquiry as to the biological role of such an enzyme. The action of the methanol-forming enzyme results in the synthesis of methanol and *S*-adenosylhomocysteine (6). Eithes of these could be the principal product of the reaction.

Methanol. Methanol has been studied primarily in the context of methanol intoxication. The toxicity of methanol has been the subject of a number of studies and reviews (11). When ingested in sufficient quantity, methanol leads to metabolic acidosis, blindness, and ultimately death. This is not due directly to the alcohol itself, but to its conversion to formaldehyde and formic acid. The enzymes which carry out these conversions, alcohol and aldehyde dehydrogenases, have been reported in a number of tissues, particularly brain and liver (12, 13). Thus the same organs which carry out methanol synthesis also carry out its degradation to potentially toxic compounds. However, the amounts of methanol formed by the enzyme we are describing are such that, even if completely metabolized to formic acid, they would not be expected to alter the acid-base balance of the animal.

The possibility of a direct biological role of methanol is, at this time, speculative. Eriksen and Kulkarni (14) have shown that methanol is present as an endogenous compound in the breath of normal human subjects. Majchrowicz and Mendelson (15) have demonstrated recently that blood methanol levels are increased during ethanol intake in humans, and have postulated that methanol or its metabolites may contribute to toxic disorders during high ethanol intake or withdrawal. Mazur *et al.* (16) have demonstrated an effect of methanol and its metabolites on the contractile amplitude and coro-

nary flow of the isolated rabbit heart. It is generally accepted that methanol has inebriant effects in high doses, and it is possible that methanol production within discrete areas of the brain might in some way, either directly or as a consequence of its metabolism, influence neuronal function. Baldessarini and Kopin (17) have reported the *S*-[*methyl*]adenosylmethionine levels in rat brain to be of the order of 20 μM, well above the K_m for the methanol-forming enzyme. Thus, in the brain at least, the enzyme may be saturated by its substrate.

S-Adenosylhomocysteine. The possibility exists that the methanol-forming enzyme is, in reality, an "*S*-adenosylhomocysteine-forming enzyme." Salvatore *et al.* (18) have reported that the tissue levels of *S*-adenosylhomocysteine are of the same order of magnitude as the *S*-adenosylmethionine levels. A biological role for *S*-adenosylhomocysteine has recently been suggested by Zappia *et al.* (19) and by Deguchi and Barchas (20). These studies suggest that *S*-adenosylhomocysteine acts as a potent inhibitor in a number of transmethylation reactions. The mechanism whereby *S*-adenosylhomocysteine exerts its inhibitory role appears to involve competition with *S*-adenosylmethionine for enzyme binding sites. The affinity of *S*-adenosylhomocysteine for methyltransferases is as much as 7 times greater than the affinity of *S*-adenosylhomocysteine for the enzyme.

A possible role of the methanol-forming enzyme might therefore be in the formation of *S*-adenosylhomocysteine, which would then act as a local modulator of transmethylation reactions. However, in most of the tissues studied in this report, methanol-forming enzyme activity is too low to affect methyltransferase activity appreciably *in vivo*. In brain, however, the methyltransferase involved in epinephrine synthesis has been shown (21) to be present in very low amounts, and both its activity and regional distribution parallel those of the methanol-forming enzyme. Furthermore, although the activity of the methanol-forming enzyme is low on a per gram of brain basis, its functional importance in the formation of *S*-adenosylhomocysteine would depend upon its cellular localization and that of its substrate.

Some observations in our laboratory concerning the substrate specificity of the methanol-forming enzyme suggest that it may catalyze methanol formation from *S*-adenosylmethionine derivatives as well as the parent compound. The most active of these derivatives appears to be thioribosylmethionine. It will be necessary to prepare enough of this material for kinetic comparison with *S*-adenosylmethionine to determine the substrate for which the enzyme has the highest affinity. These early results suggest that the enzyme may be involved in the demethylation of *S*-adenosylmethionine and its derivatives.

Thus, although a biological role cannot yet be definitively ascribed to the enzyme, the evidence at hand seems sufficient to merit further investigation. We have found the enzyme to be widely distributed in rat organs. Other workers also have found the enzyme in a variety of tissues. Apart from the initial finding by Axelrod and Daly of enzyme activity in rat pituitary, Molinoff and Axelrod[2] have noted activity in the bovine adrenal, and Creveling[3] has found enzyme activity in rat heart. A more recent report has described the presence of the methanol-forming enzyme in the red blood cells of humans and a number of mammalian species (22). The facts that in the rat the enzyme is widely distributed, that the substrate levels in brain approximate the K_m, and that methanol has been found in human breath and in the blood of persons with an intake of ethanol all suggest that the reaction forming methanol from *S*-adenosylmethionine may play a biological role.

REFERENCES

1. J. Axelrod, *J. Biol. Chem.* **237**, 1657 (1962).
2. J. Axelrod, *Science* **126**, 400 (1957).
3. S. K. Shapiro, *Biochim. Biophys. Acta* **29**, 405 (1958).
4. E. Fleissner and E. Borek, *Proc. Nat. Acad. Sci. U.S.A.* **48**, 1199 (1962).

[2] P. Molinoff and J. Axelrod, personal communication.

[3] C. Creveling, personal communication.

5. R. D. Ciaranello and J. D. Barchas, *Pharmacologist* **9**, 248 (1967).
6. J. Axelrod and J. Daly, *Science* **150**, 892 (1965).
7. G. A. Bray, *Anal. Biochem.* **1**, 279 (1960).
8. G. L. Cantoni and J. Durell, *J. Biol. Chem.* **225**, 1033 (1957).
9. F. Schlenk and R. E. De Palma, *J. Biol. Chem.* **229**, 1051 (1957).
10. "Handbook of Chemistry and Physics," Ed. 45. Chemical Rubber Co., Cleveland, 1965.
11. J. R. Cooper and M. N. Kini, *Biochem. Pharmacol.* **11**, 405 (1962).
12. V. G. Erwin and R. A. Deitrich, *J. Biol. Chem.* **241**, 3533 (1966).
13. N. H. Raskin and L. Sokoloff, *Science* **162**, 131 (1968).
14. S. P. Eriksen and A. B. Kulkarni, *Science* **141**, 639 (1963).
15. E. Majchrowicz and J. Mendelson, *Fed. Proc.* **29**, 649 (1970).
16. M. Mazur, A. Dzialek, J. Lembke and W. Dologicz, *Agressologie* **10**, 317 (1969).
17. R. Baldessarini and I. J. Kopin, *J. Neurochem.* **13**, 769 (1966).
18. F. Salvatore, V. Zappia and S. K. Shapiro, *Biochim. Biophys. Acta* **158**, 461 (1968).
19. V. Zappia, C. R. Zydek-Cwick and F. Schlenk, *J. Biol. Chem.* **244**, 4499 (1969).
20. T. Deguchi and J. Barchas, *J. Biol. Chem.* **246**, 3175 (1971).
21. R. D. Ciaranello, R. E. Barchas, G. S. Byers, D. W. Stemmle and J. D. Barchas, *Nature* **221**, 368 (1969).
22. J. Axelrod and C. K. Cohn, *J. Pharmacol. Exp. Ther.* **176**, 650 (1971).