Brain Research, 96 (1975) 407–412

The homologous methylation of tRNA in rat brain

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(Accepted June 11th, 1975)

Although several reports describing the properties of cerebral tRNA methyltransferases and of the regional variations in their activities with age have appeared[4,6,9,10,20,22], the activity of these enzymes has always been measured in terms of the number of [^{14}C-methyl] or [^{3}H-methyl] groups transferred from appropriately labeled S-adenosyl-L-methionine onto tRNAs purified from non-neural sources[4,6,9,10,20,22]. Studies on cerebral methylation of tRNA *in vivo* have been few[7,8,21] and thus the specificity spectrum of the tRNA methyltransferases of neural tissue as well as the role these enzymes play in the processes of maturation and modification[16] of neuronal[1] and glial tRNAs is wholly unknown.

While exploring the consequences of a prolonged depletion of cerebral S-adenosyl-L-methionine[17] on cerebral methylations[18], we noted a significant transfer of [^{14}C-methyl] groups from S-adenosyl-L-[^{14}C-methyl]methionine onto tRNAs of the same brain extracts from which we prepared the tRNA methyltransferases, *i.e.*, we observed homologous tRNA methylation, a reaction described only once before in a mammalian system, *viz.* in rat liver[5]. In the present communication we provide evidence that the homologous methylation of brain tRNA involves principally two positions of its guanine residues.

Male Sprague–Dawley rats were decapitated and the cerebral cortex excised and weighed. A 33% (w/v) homogenate prepared in medium A of Pegg[14] was centrifuged at 104,000 × *g* for 60 min and the supernatant used directly as a source of methyltransferases. S-adenosyl-L-[^{14}C-methyl]methionine (58 mCi/mmole) was from New England Nuclear, Boston, Mass., and *E. coli* B. from Schwarz-Mann, Orangeburg, N.Y. [^{14}C-methyl] transfer was determined both by the 'rate' and the 'total extent' assays, as described by Pegg[14]. The rate of methylation is expressed as nmoles of [^{14}C-methyl]/mg of protein/30 min, while the 'total extent' methylation is expressed as nmoles of [^{14}C-methyl]/mg of protein/60 min, each being measured under appropriately optimized conditions[15]. Incubations were at 37 °C. Endogenous methylation is defined as the methylation occurring in the absence of *E. coli* B. tRNA, while total methylation is defined as the methylation occurring in its presence. The

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TABLE I

THE METHYLATION ACTIVITY OF RAT BRAIN CORTEX

A: for the 'total extent' assay, each tube contained 7 nmoles of S-adenosyl-[^{14}C-methyl]methionine, 0.15 μmoles of Tris–HCl buffer, pH 8.6 and about 1.1 mg of brain cortex protein in addition to 150 μg of E. coli B. tRNA, as indicated. Incubations were at 37 °C for 60 min in a total volume of 0.67 ml. To determine the extent of [^{14}C-methyl] transfer, triplicate 0.25 ml aliquots were placed on discs of Whatman No. 1 filter paper and were processed according to Pegg[14]. Their radioactivity was determined in a Unilux II (Searle Analytic, Des Plaines, Ill.) spectrometer. The values are the means ± S.E.M. of 6 individual determinations in triplicate. B: for the 'rate' assay, the conditions were as in A except that only 0.15 mg of brain cortex protein was incubated. The time of incubation was 30 min. The samples were processed as in A. The values are the means ± S.E.M. of 6 individual determinations in triplicate. C: following incubation as in B, triplicate 0.1 ml aliquots were withdrawn and mixed successively with 0.1 ml of 0.8 *N* $HClO_4$ and 0.8 ml of 0.2 *N* $HClO_4$. The samples were then processed according to Fleck and Begg[2]. Following the alkaline treatment (0.4 ml of 0.3 *N* KOH at 37 °C for 1 h), the clear solution containing the RNA hydrolysis products was acidified and the resulting pellet was centrifuged off and washed with cold 0.2 *N* $HClO_4$. The washings and the original acidified supernatant were combined and their radioactivity determined. The residual pellet was suspended in 1 ml of water and mixed with 10 ml of PCS solubilizer (Amersham-Searle, Arlington Heights, Ill.). Its radioactivity represents that of the 'protein' fraction. See text for additional details.

[^{14}C-methyl] transfer determined in	*Endogenous methylation (— E.coli B. tRNA)*	*Total methylation (+ E.coli B. tRNA)*	*E. coli B. methylation (total—endogenous)*
A. 'Total extent' assay§,*	1.00 ± 0.14	1.60 ± 0.18	0.60 ± 0.09
B. 'Rate' assay§,**	1.043 ± 0.10	1.348 ± 0.09	0.305 ± 0.05
C. 'Rate' assay followed by Fleck and Begg procedure§§			
(a) RNA fraction	0.854 ± 0.11	1.088 ± 0.09	0.234 ± 0.09
(b) Protein fraction	0.131 ± 0.02	0.173 ± 0.05	—
Recovery, % [a + b/B]	94	93.2	
Plus spermidine (5 m*M*)			
(c) RNA fraction	0.675 ± 0.14	1.118 ± 0.10	0.443 ± 0.12
(d) Protein fraction	0.128 ± 0.02	0.130 ± 0.02	—
Recovery, % [c + d/B]	76.8	92.3	

§ Ref. 14.
§§ Ref. 2.
* nmoles [^{14}C-methyl]/mg of protein/60 min.
** nmoles [^{14}C-methyl]/mg of protein/30 min.

latter process thus comprises the sum of the methylations of endogenous [^{14}C-methyl] acceptors (proteins and tRNAs) and of the added *E. coli* B. tRNA. The procedure of Fleck and Begg[2] was used to separate the [^{14}C-methyl] products formed under both conditions of incubation into [^{14}C-methyl]protein and [^{14}C-methyl]tRNA fractions. The latter fraction was further resolved into [^{14}C-methyl]purine bases and [^{14}C-methyl]pyrimidine nucleotides by means of chromatography on Dowex-50[3]. Purified [^{14}C-methyl] tRNA[13] was hydrolyzed to its constituent bases in trifluoroacetic acid[11] and the bases were separated by TLC[11]. Autoradiograms were prepared

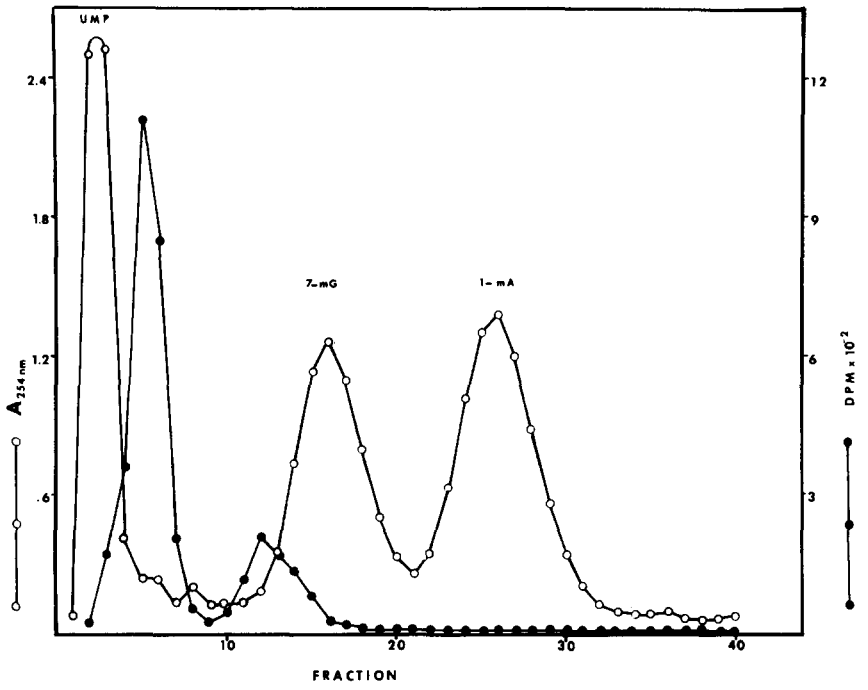

Fig. 1. Ion-exchange chromatography of the [^{14}C-methyl] products derived by alkaline hydrolysis of the RNA fraction[2]. The acidified supernatant (1 ml) resulting from the alkaline hydrolysis step[2] (see legend to Table I, C) was applied to a Dowex-50 column (see text for details), equilibrated with 3.63 *M* ammonium acetate buffer, pH 4.15 and elution with the same buffer was carried out at a rate of about 1 ml/min; 3.5 ml/tube was collected. A mixture of 5′-uridylic acid (tubes 2–5), 7-methyl guanine (tubes 13–19) and 1-methyl adenine (tubes 22–30) was run through the column for purposes of calibration. The radioactivity in each tube was determined after mixing 2 ml of effluent with 10 ml of 'Scintisol Complete' (Isolab, Inc., Akron, Ohio).

on X-ray film, Type RB-254 (Eastman-Kodak, Rochester, N.Y.). Protein was determined according to Lowry *et al.*[12]. Spermidine phosphate (ICN Biochemicals, Cleveland, Ohio) was used at a concentration of 5 m*M*. The ^{12}C-methylated purines and pyrimidines were obtained commercially (Sigma Chem. Co., St. Louis, Mo., or Cyclo Chem. Corp., Los Angeles, Calif.), except for N_2^2-dimethyl guanine, which was a generous gift of Dr. M. Klagsbrun, Harvard Medical School, Cambridge, Mass.

The overall capacity of tRNA methyltransferases from rat cerebral cortex to transfer [^{14}C-methyl] groups to endogenous acceptors was determined by comparing this process in the presence and absence of added *E. coli* B. tRNA. The results (Table I) show that approximately 60% of the transfer measured in the 'total extent' mode[15] was onto endogenous acceptors and that this proportion was increased to about 77% when the 'rate' assay was applied[15]. A quantitative separation of the [^{14}C-methyl] products[2] (Table I, rows C,a and C,b) revealed more than 85% of the recovered radioactivity in the RNA fraction, irrespective of the presence of *E. coli* B. tRNA. Furthermore, the data also show that over 93% of the radioactivity was

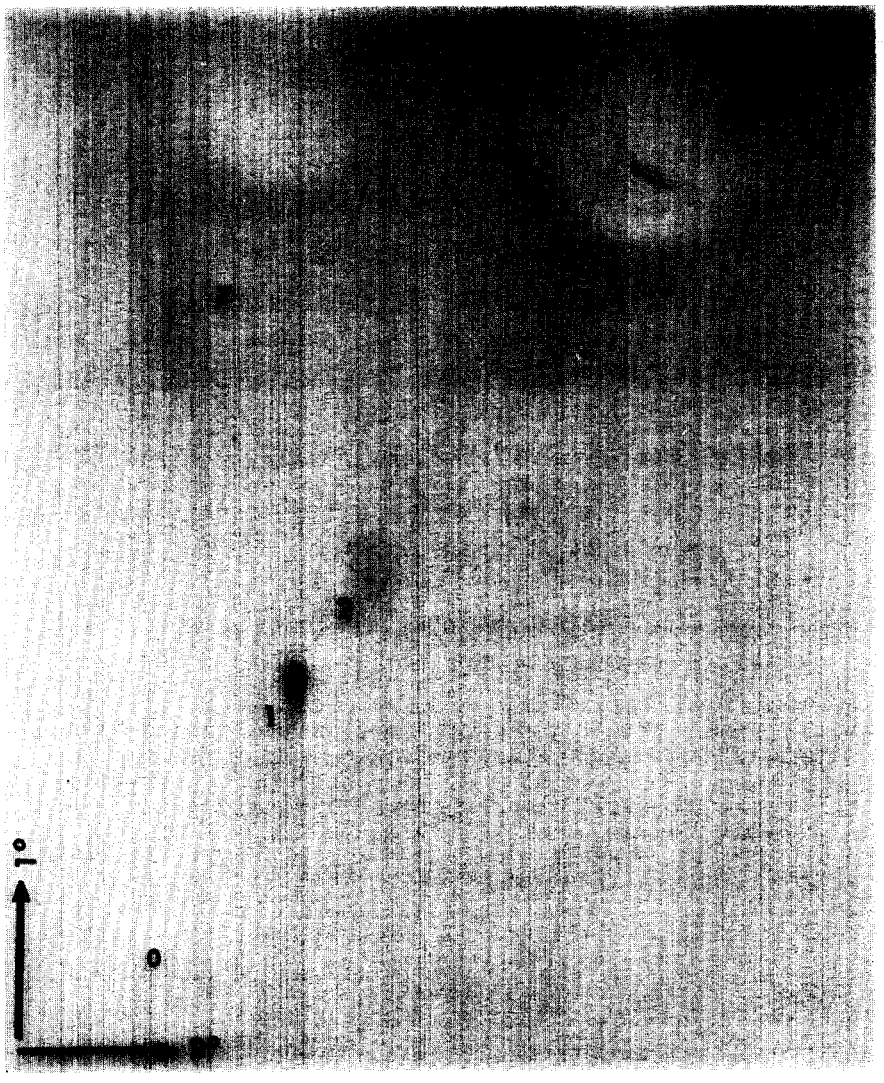

Fig. 2. Bi-dimensional thin-layer chromatography of [^{14}C-methyl]tRNA bases: autoradiogram of the CF_3COOH hydrolysate of [^{14}C-methyl]tRNA formed *in vitro* by 3-day-old brain tRNA methyltransferases. The hydrolysis of the purified [^{14}C-methyl]tRNA[13] was according to Klagsbrun[11]. The [^{14}C-methyl] bases were separated in methanol–water–conc. HCl (70:10:20) in the first direction and *n*-butanol–glacial acetic acid–water (68:17:17) in the second direction. The front of the solvents was allowed to rise to 14 cm. The numerals designate the following: 0, origin; 1, (1-methyl guanine + 7-methyl guanine); 2, N^2-methyl guanine; and 7, unidentified.

recovered by the Fleck and Begg[2] procedure. In separate experiments (C. E. Salas and O.Z. Sellinger, unpublished observations) we could show that the [^{14}C]protein fraction was insensitive to the action of RNAse-free DNAse (Worthington Biochemical Corp., Freehold, N.J.) and thus contained no ^{14}C-methyl-labeled DNA. Spermidine stimulated the methylation of *E. coli* B. tRNA by about 85%, as indicated by comparing values in rows C,c and C,a of the (total, endogenous) column (Table I) while,

conversely, this polyamine appeared to inhibit the methylation of endogenous tRNA by about 20%. A more comprehensive analysis of the effects of age on the pattern of homologous and heterologous methylation of tRNA in rat brain cortex is presented elsewhere[15]. When the alkaline hydrolysate[2] containing [^{14}C-methyl]tRNA breakdown products formed by endogenous methylation of 3-day-old cerebral cortex tRNA was acidified and eluted off a Dowex-50W-X8 (200–400 mesh) column (1 cm × 10 cm)[3], the radioactivity profile (Fig. 1) indicated the definite presence of [^{14}C-methyl]guanines[3] (fractions 11–15), in addition to large amounts of radioactive material eluting between the uracil and cytidine nucleotides (tubes 2–8, Fig. 1; *cf.* also Fig. 1 of ref. 3). Additional proof of the formation of [^{14}C-methyl]guanines during endogenous methylation is provided by the results of the TLC of a trifluoroacetic acid hydrolysate (Fig. 2) which shows two radioactive spots, identified as N^2-methyl guanine and (1 + 7)-methyl guanine.

Simon *et al.*[20] observed that rabbit liver tRNA is 10 times more effective as a substrate for rat brain tRNA methyltransferases than is homologous brain tRNA and 3–4 and 4–5 times less effective than either *E. coli* B. or *E. coli* K12 tRNA. These workers also showed that rat brain contains 'more than one methylating activity' and that cerebral methylases are about 7 times more active in fetal than in adult rat brain. More recently, Johnson *et al.*[6] compared tRNA methyltransferases in neonatal and adult mouse brain and noted significant methylation of *E. coli* tRNA but little, if any, homologous tRNA methylation. These workers stated that all of the methylation observed in the absence of *E. coli* tRNA was 'presumably the result of protein methylation', even though they failed to examine the nature of the [^{14}C-methyl] products so produced. Our results do not support the conclusion of Johnson *et al.*[6], inasmuch as they demonstrate that, on the contrary, a sizable portion of the 'endogenous methylation' in rat brain cortex involves homologous tRNA. These findings supplement the recent report by Jank and Gross[5], who found that homologous tRNA methylation in adult rat liver involves principally the transfer of [^{14}C-methyl] groups to cytidine and adenine residues to form 5-methylcytosine and 1-methyladenine. As shown in Fig. 2, tRNA methyltransferases from immature rat brain cortex introduce [^{14}C-methyl] groups principally on brain tRNA guanine residues, thus forming tRNAs containing N^2- and (1 + 7)-[^{14}C-methyl]guanines. Further work is in progress to delineate more fully the patterns of homologous tRNA methylation in brain as a function of age and cell type and of the role of spermidine in this process. The possibility that the observed results are not entirely the reflection of homologous tRNA methylation, but rather of a heterocellular process, whereby neuronal[19] tRNAs are methylated by glial tRNA methyltransferases and glial tRNAs by neuronal tRNA methyltransferases, will also be explored.

This research was supported by the United States Public Health Service, Grant NS-06294. CJC is a pre-doctoral trainee, NIMH 013034.

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