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ENZYMES CATALYZING THE *DE NOVO* SYNTHESIS OF METHYL GROUPS IN THE BRAIN AND OTHER TISSUES OF THE RAT

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Abstract—Rat brain contains all three of the enzymes required for *de novo* synthesis of the methyl group of methionine (serine transhydroxymethylase, methylene reductase, and [B_{12}]transmethylase) in activities comparable to those found in liver and kidney. The activities of methylene reductase in female kidney, and of [B_{12}]transmethylase in female brain and kidney, are higher than in the corresponding male tissues. Liver and kidney extracts contain an inhibitor of methylene reductase not present in brain extracts. This inhibitor differs from *S*-adenosylmethionine (SAM), which also inhibits methylene reductase in both liver and brain homogenates. The administration of L-DOPA to rats, which has been previously shown to deplete brain *S*-adenosylmethionine, also reduces the activity of brain [B_{12}]transmethylase if assayed without added SAM. Since SAM is required for activity of this enzyme, its decreased activity probably results from the decline in brain SAM concentration. *De novo* synthesis of methyl groups could be a mechanism by which the brain maintains its level of methionine in the face of increased methyl group utilization after administration of L-DOPA.

THE CATECHOL amino acid L-DOPA administered to rats in doses similar to those for the treatment of Parkinson's disease has been shown to depress the concentrations of *S*-adenosylmethionine (SAM) in brain and other tissues (WURTMAN *et al.*, 1970*a*; ORDÓÑEZ and WURTMAN, 1973). This depletion is caused by the utilization of large quantities of methyl groups for the 3-*O*-methylation of the L-DOPA, a major route of metabolism for the exogenous amino acid (WURTMAN *et al.*, 1970*b*). Repeated administration of L-DOPA causes the methionine concentration of the serum to decrease, but does not produce a similar effect in brain (ORDÓÑEZ and WURTMAN, 1973). The failure of brain methionine levels to fall even when unusually large amounts of methionine must have been utilized to form SAM suggested that brain might be capable of regenerating methionine from homocysteine (ELFORD *et al.*, 1965).

Early studies by LANGER (1964) were interpreted as showing that brain, unlike liver and kidney, was incapable of synthesizing the methyl group of methionine. However, more recent reports have identified individual enzymes within rodent or bovine brain that are neccessary for this process [N5-methyl tetrahydrofolate-homocysteine (cobalamin) methyltransferase; (B_{12}-transmethylase; FINKELSTEIN *et al.*, 1971); N5-methyl-tetrahydrofolate-NAD oxidoreductase (methylene reductase, EC 1.1.1.68; BRODERICK *et al.*, 1972); and L-serine-tetrahydrofolate-5, 10-hydroxymethyl transferase (serine transhydroxymethylase, EC 2.1.2.1.; BRODERICK *et al.*, 1972; BRIDGES, 1968].

The present report demonstrates that all of the enzymes required for *de novo* synthesis of the methyl group of methionine are present in rat brain, at a level of

Abbreviations used: SAM, *S*-adenosylmethionine; B_{12}-transmethylase, N5-methyl-tetrahydrofolate-homocysteine (cobalamin)methyltransferase; methylene reductase, N5-methyl-tetrahydrofolate-NAD-oxidoreductase; serine transhydroxymethylase, L-serine-tetrahydrofolate-5,10-hydroxymethyl transferase; THFA, tetrahydrofolic acid.

activity comparable to those of the liver and kidney. Since some properties of these enzymes differ from those of the corresponding enzymes in liver and kidney, the mechanisms which control methyl group synthesis in brain may differ from those operating in other tissues.

MATERIALS AND METHODS

Sprague–Dawley male rats (Charles River Laboratories, Wilmington, Mass.) were housed five per cage and exposed to light (Vita-Lite, Duro-Test Corp., North Bergen, NJ, U.S.A.) between 9 a.m. and 9 p.m. daily They had free access to Purina Laboratory Rat Chow and water. Animals were killed at 11 a.m.; the tissues to be assayed were frozen on dry ice and homogenized within 1–2 h of sacrifice. Individual brain regions were dissected according to the scheme of BAUMGARTEN *et al.* (1971).

L-DOPA was a gift of the Hoffmann-LaRoche Co. (Nutley, NJ, U.S.A.). *dl*-L-N5-methyl[^{14}C]-methyltetrahydrofolate (N5-methyl THFA, barium salt, 54 mCi/mmol) was purchased from Amersham-Searle Co. (Arlington Heights, IL, U.S.A.) and DL-serine-3[^{14}C]from New England Nuclear (Boston, MA, U.S.A., 5·45 mCi/mmol). All other chemicals were of the highest purity commercially available.

Tissues were weighed and homogenized in 2 vol. (brain) or 10 vol. (liver and kidney) of 0·1 M-potassium phosphate buffer (pH 7·5) containing 10^{-3} M reduced glutathionine. After centrifugation at 34,000 *g* for 30 min, the supernatant fluids were dialysed for 1 h each against three changes of the phosphate buffer, except when indicated.

Enzyme assays. Serine transhydroxymethylase was assayed by a modification of the method of MACKENZIE (1950). The incubation mixture, kept in a Thunberg tube, contained 0·25 μCi of DL-serine-3[^{14}C], 2·0 mM-L-serine, 0·2 mM-pyridoxal phosphate, 6 mM tris buffer (pH 7·4), the dialysed crude protein extract (kept in the side arm of the tube until the reaction was allowed to start) and approx. 0·1 mM-tetrahydrofolate (THFA) in a final vol. of 1 ml. The THFA solution was prepared by saturating 15 ml of tris buffer containing 34 mM-ascorbic acid with 25 mg of *dl*-L-THFA, and using 0·1 ml of the final solution per assay tube. The mixture was kept at 37°C. The air was then evacuated from the Thunberg tube and replaced with N_2 to prevent decomposition of the THFA, and the reaction was initiated by mixing the enzyme with the rest of the components. After a 45-min incubation, the reaction was stopped by adding 1 ml of 20% TCA. The reaction mixture was then transferred to a centrifuge tube, and the Thunberg tube was rinsed with an additional 2 ml of 10% TCA, which was also transferred to the centrifuge tube. After centrifugation the supernatant fraction was brought up to pH 4·9; 0·1 ml of formaldehyde (1·28 M) was then added and the [^{14}C]formaldehyde formed during the reaction was precipitated with 20 ml of 0·4% 5,5′-dimethyl-1,3-cyclohexanedione (Dimedon). The precipitate was allowed to stand overnight before collection by filtration. After drying, the samples were counted in toluene phosphor. Blanks were obtained by adding the crude enzyme extracts at the end of the incubation period.

Methylene reductase was assayed in the reverse direction (i.e. by measuring the formation of 5,10 methylene THFA from N5-methyl THFA) using menadione as electron acceptor by a modification of the method of KUTZBACH and STOKSTAD (1971). The menadione and FAD in the incubation mixture quenched the toluene phosphor used to count the radioactive product. To reduce this quenching the following minor modifications were introduced: the reaction was initiated by adding 0·1 ml of a saturated solution of menadione prepared at 100°C and then allowed to cool to 30°C prior to filtration of the excess menadione. The final reaction mixture contained 0·8 mM-FAD and 26 μM-N5-methyl-[^{14}C]THFA; the reaction was stopped after 15 min instead of 60 min. Under these conditions the assay was linear with protein concentrations of up to 1·5 mg per incubation sample and was linear with time for up to 30 min. Unless otherwise specified, dialyzed liver and kidney extracts were further diluted 2× and 4× respectively with dialysis buffer; 0·1 ml aliquots were taken for assay.

B_{12}-transmethylase was assayed aerobically using vitamin B_{12} and 2-mercaptoethanol as the reducing system (TAYLOR and WEISSBACH, 1970). The final concentrations of SAM that yielded optimal enzyme activities were 0·1 mM for brain extracts and 0·5 mM for kidney and liver extracts. 0·1 ml of the dialysed extracts were used for assay.

The protein present in the soluble dialysed fraction was determined by the method of LOWRY *et al.* (1951).

RESULTS

Activities of the enzymes catalyzing the de novo *synthesis of methyl groups.* Under optimal conditions, significant activities of all three enzymes could be demonstrated in rat brain (Table 1). The activities of serine transhydroxymethylase and methylene

TABLE 1.—SPECIFIC ACTIVITIES OF THE ENZYMES CATALYSING THE *de novo* SYNTHESIS OF METHYL GROUPS IN RAT TISSUES

Enzyme	Activity (nmol mg^{-1} min^{-1}) Brain	Liver	Kidney
Serine transhydroxymethylase	0·272	5·591	2·168
Methylene reductase	0·028	0·184	0·322
B_{12}-transmethylase	0·029	0·034	0·146

Data represents means of 20 individual determinations from groups of male rats weighing 100 g.

reductase were lower in brain than in liver or kidney. In confirmation of the findings of FINKELSTEIN *et al.* (1971), the activities of the B_{12}-transmethylase were similar in brain and liver, and both were lower than in kidney. Within the brain [B_{12}]transmethylase activities did not differ markedly from region to region (Table 2).

TABLE 2.—REGIONAL DISTRIBUTION OF B_{12}-TRANSMETHYLASE IN RAT BRAIN

Area	Activity (nmol mg^{-1} min^{-1})
Spinal cord	0·016
Cerebellum	0·011
Pons, Medulla Oblongata	0·017
Mesencephalon	0·018
Striatum	0·015
Thalamus	0·020
Rest of forebrain	0·015

Seven brain regions were dissected from each of five brains from male rats; the tissues representing each region were then pooled and assayed. Data represents means of two duplicate determinations.

The activities of [B_{12}]transmethylase in kidney and brain extracts from female rats were significantly higher than in extracts prepared from male tissues; no sex-related differences were observed in liver extracts (Table 3). The activity of methylene reductase was higher in kidneys of female rats than in males (Table 3); no sex-related differences in the activity of this enzyme were found in extracts from brain or liver, nor in tissue serine transhydroxymethylase activities.

Inhibition of methylene reductase by tissue extracts. The activities of methylene reductase and B_{12}-transmethylase in brain were significantly lower than that of serine transhydroxymethylase, suggesting that one of the two former enzymes might control the rate at which the brain can regenerate methionine from homocysteine (Table 1). For this reason, studies were performed to examine mechanisms that might control the activities of these enzymes in rat brain *in vivo*.

TABLE 3.—SEX-DEPENDENCE OF THE ENZYMES INVOLVED IN *de novo* SYNTHESIS OF METHYL GROUPS

Tissue		Specific activities (nmol mg^{-1} min^{-1}) Methylene reductase	$[B_{12}]$transmethylase
Brain	M	0·034 ± 0·001	0·031 ± 0·001
	F	0·033 ± 0·001	0·036 ± 0·001*
Kidney	M	0·258 ± 0·010	0·112 ± 0·007
	F	0·314 ± 0·012*	0·161 ± 0·010*
Liver	M	0·161 ± 0·005	0·024 ± 0·003
	F	0·174 ± 0·008	0·030 ± 0·003

Data represents means ± S.E. from groups of six animals each, weighing 250 g.
* $P < 0·01$ differs from male controls.

When tissue extracts were assayed for methylene reductase activity with or without previous dialysis, it was found that undialysed liver extracts were inhibited up to 70%, depending upon the amount of dilution of the extract prior to assay (Fig. 1); brain extracts lacked evidence of a dialysable inhibitor. To discard the possibility of poor product recovery being responsible for the observed inhibition, samples at 2 × dilution (Fig. 1) were incubated under identical conditions as experimental samples, with the exception that N5-methyl THFA was substituted with [^{14}C]formaldehyde. Recoveries ranged from 95 to 97 per cent of those obtained from control tubes lacking the crude protein extracts. No differences were found between dialysed or undialysed extracts.

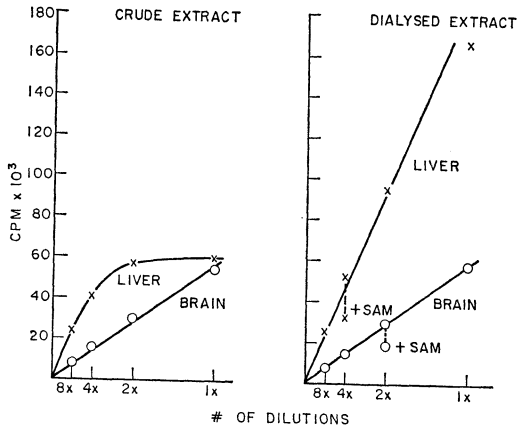

FIG. 1.—The effect of dialysis and SAM on methylene reductase activity present in tissue extracts. Crude extracts containing methylene reductase activity were prepared by homogenizing brain and liver in 2 and 10 vol. respectively of phosphate buffer as described in Materials and Methods. The protein concentration in both preparations was approximately 10 mg/ml. The crude extracts were further diluted before assay as specified. The effect of SAM was measured at a final concentration of 0·8 mM in the assay system. Each point represents the mean of two duplicate determinations.

KUTZBACH and STOKSTAD (1967) reported that SAM inhibited methylene reductase activity in liver extracts *in vitro*. When we added SAM to the final assay mixture in concentrations similar to those present *in vivo* (0·8 mM), the methylene reductase activity was inhibited in dialysed extracts of brain and liver to a similar extent (36%, Fig. 1). This inhibition is too small to account for the extent of inhibition in liver undialysed extracts, suggesting an inhibitor other than SAM to be present in the tissue.

The dialysable inhibitor is also present in kidney (Table 4). When brain extracts are added to undialysed preparations of liver or kidney, the methylene reductase activity present in brain becomes inhibited (Table 4), proving that the enzyme of brain origin is similarly sensitive to the inhibitor but the inhibitor is not present in brain tissue.

TABLE 4.—INHIBITION OF METHYLENE REDUCTASE ACTIVITY BY LIVER AND KIDNEY EXTRACTS

	Methylene reductase activity (counts/min) Liver	Kidney
Dialysed extract (A)	136,905	97,235
Undialysed extract (B)	112,275	77,859
Brain activity added to undialysed extract (C)	30,837	29,112
Undialysed extract + Brain (B + C)	121,641	94,503
$\frac{(B + C) - B}{C} \times 100$*	30%	57%

Data are means of duplicate determinations.
* Per cent activity remaining in brain extract after inhibition.

Regulation of brain [B_{12}]*transmethylase*. When the activity of the B_{12}-transmethylase was assayed without added SAM, the dialysed crude preparations of brain and kidney extracts showed an apparent 'SAM-independent' activity (Table 5). Administration of L-DOPA to rats to cause depletion of SAM in kidney and brain caused a decline in this residual activity in brain extracts, but not in kidney extracts (Table 5). Animals receiving a particular dose of L-DOPA chronically did not exhibit a greater change than those given only a single injection; among rats treated chronically, but not acutely the larger dose of L-DOPA (250 mg) had a greater effect than the smaller dose ($P < 0{\cdot}05$; Table 5).

We further attempted to correlate this effect of L-DOPA with the concentrations of SAM that could be expected to be present in our tissue extracts after dialysis. When SAM was incubated with tissue extracts prior to dialysis in concentrations similar to those present in the complete system during assay, an enhanced activity was still evident after prolonged dialysis (Table 6); however, treatment with albumin-coated charcoal (GOTTLIEB *et al.*, 1972) rendered the preparations from all tissues totally inactive unless they were supplied with additional SAM.

TABLE 5.—THE SAM-INDEPENDENT B_{12}-TRANSMETHYLASE ACTIVITY OF RAT TISSUE EXTRACTS: EFFECT OF L-DOPA TREATMENT

	Per cent of full activity		
	Brain	Liver	Kidney
Control	20·3	4·7	16·7
Acute DOPA (100 mg/kg)	10·2*	4·5	—
Chronic DOPA (100 mg/kg)	12·9†	3·8	15·5
Acute DOPA (250 mg/kg)	7·7†	4·7	15·5
Chronic DOPA (250 mg/kg)	8·2†‡	6·2	16·5

Groups of six animals weighing 100 g were killed one h after receiving one or the last of ten daily injections of L-DOPA i.p. or its diluent (0·05 N-HCl). Dialysed tissue extracts from each animal were assayed by duplicate determinations in the complete system (+ SAM) or without added SAM (SAM-independent activity). Data represents the per cent of full activity remaining in the −SAM system.

* $P < 0·01$ differs from control.

† $P < 0·001$ differs from control.

‡ $P < 0·05$ differs from chronic DOPA (100 mg/kg).

TABLE 6.—EFFECT OF DIALYSIS AND CHARCOAL TREATMENT ON 'SAM-INDEPENDENT' B_{12}-TRANSMETHYLASE ACTIVITY

	Per cent of full activity remaining after incubation	
	0 h (non-dialysed)	3 h (dialysed)
Brain	85	60
Liver	85	60
Kidney	90	70
Charcoal added (all tissues)	0	0

Crude extracts containing B_{12}-transmethylase activity were prepared by homogenizing the tissues in phosphate buffer as described in Materials and Methods. After addition of SAM to a final concentration of 0·1 mM for brain and 0·5 mM for liver and kidney the crude extracts were incubated for 15 min at 37°C. At the end of the incubation period, samples were either assayed immediately or dialysed for 3 h previous to assay. Charcoal was added in all cases immediately after incubation. The B_{12}-transmethylase activity measured in the absence of added SAM during assay was compared to duplicates containing the complete incubation system (+ SAM). Data are expressed as per cent of the full activities. Each point represents the average of two determinations.

To show that the observed effects of SAM were caused by SAM binding to proteins present in the extracts and not to some long-lasting activating mechanisms, [^{14}C]SAM was added to tissue preparations at the same concentrations as in the previous experiments; the distribution of radioactivity between the extract and the dialysis medium was estimated after 3 or 24 h of dialysis. After 24 h, the ratios of protein-bound to free radioactivity were: brain 7:1, liver 3:1, and kidney 2:1, indicating

that SAM binds to proteins in the crude extracts. Ratios at 3 h of dialysis were about 80 per cent of those observed at 24 h.

DISCUSSION

When rats receive repeated injections of L-DOPA, the methionine levels in serum are depressed while brain methionine concentrations remain unchanged. Furthermore, prolonged administration of L-DOPA (for up to 10 days) under conditions in which the amount to be methylated is in excess of the amount of methyl groups provided in the diet does not have any effect on the brain methionine levels (ORDÓÑEZ and WURTMAN, 1973).

At least three possible mechanisms could explain these observations. Increased transmethylations in brain tissue could produce an augmented synthesis of methyl groups, or could somehow accelerate the brain uptake of methyl groups (as methionine or as N5-methyl THFA) from the circulation. Another possibility is that transmethylations cause the accumulation of metabolites (e.g. *S*-adenosylhomocysteine; DEGUCHI and BARKAS, 1971) that inhibit the 3-*O*-methylation of L-DOPA or of other brain substrates.

The present observations indicate that the brain is capable of regulating methionine by methylating the homocysteine formed during transmethylation. This possibility was not confirmed in an earlier study (LANGER, 1964); however, in that study, SAM was not added to the experimental system. Brain contains relatively low amounts of both methionine and methionine-activating enzyme (MUDD *et al.*, 1965), hence the amount of endogenous SAM present may have been insufficient to fully activate the B_{12}-transmethylase. Our observations support this explanation (Table 5). All the enzymes needed for the *de novo* synthesis of methyl groups are present in brain (Table 1). The first two enzymes in the pathway, serine transhydroxymethylase and methylene reductase, are present in brain at lower activity levels than in liver or kidney while the activity of the B_{12}-transmethylase in brain is comparable to that of the enzyme in liver, as first shown by FINKELSTEIN *et al.* (1971).

One possible explanation for the relatively smaller amounts of activity of brain serine transhydroxymethylase and methylene reductase when compared to other tissues is that the brain actively concentrates N5-methyl THFA from the circulation (LEVITT *et al.*, 1971), thus lowering the requirements for synthesis of this compound *in situ*.

The specific activities of methylene reductase and [B_{12}]transmethylase were found to be similar in brain, while the specific activity of serine transhydroxymethylase was approximately ten times higher (Table 1); this suggests that one of the two former enzymes could control the net rate of methionine synthesis. This rate could be regulated either by the total amount of enzyme present in the tissue, or by activatory or inhibitory metabolites. An example of the first possibility is evident from the results obtained in the sex-dependence studies (Table 3). The methylene reductase activity was found to be significantly higher in the kidneys of female rats with no significant differences between the sexes in the other organs studied. The [B_{12}]transmethylase activity in different organs was also affected by sex, but in a way opposite to that previously reported (FINKELSTEIN *et al.*, 1971); the kidney and brain enzyme levels are higher in female rats, with no significant sex differences in the activities present in liver (Table 3). Nevertheless, the levels of both enzymes in female liver are

higher than in the male control. These results probably reflect higher methionine utilization in female animals, as recently suggested by YAMASAKI and NATORI (1972), who found a lower methionine concentration in female liver than in male.

Areas rich in catecholamines (e.g. striatum, mesencephalon) could have been expected to have high levels of the enzyme [B_{12}]transmethylase because of the methylations involved in the inactivation of these neurotransmitters (AXELROD, 1965); however, we did not find this to be true. The distribution of [B_{12}]transmethylase in brain was found to be relatively uniform. The cerebellum showed the lowest levels of activity and the thalamus, the highest (Table 2).

When searching for possible metabolites that could regulate the pathway being studied in the brain, we turned our attention to the studies of KUTZBACH and STOKSTAD (1967, 1971), who demonstrated an effect of SAM on the activity of liver methylene reductase. The enzyme present in brain tissue shows a similar inhibition of activity by SAM (Fig. 1). Furthermore, it is apparent that additional factors regulate this enzyme in liver and kidney, but are not operative in brain (Fig. 1 and Table 4). Our data show that a dialysable molecule, not present in brain extracts, has a large inhibitory effect on the enzyme activity in liver and kidney extracts. This inhibitor is not SAM; however, its further characterization awaits future study.

Another possible point of regulation of the pathway of *de novo* synthesis of methyl groups by SAM is at the level of the [B_{12}]transmethylase (TAYLOR and WEISSBACH, 1969). It is not known if the enzyme is fully activated under physiological conditions. If this were not the case, fluctuations in the levels of SAM could have negative effects on the activity of the enzyme. Our results indicate that brain [B_{12}]transmethylase, when assayed *in vitro* and in the absence of externally supplied SAM, is dependent upon the levels of SAM originally present in the tissue (i.e. it decreases following the methylation of exogenous L-DOPA) (Table 5). That this effect of L-DOPA on brain [B_{12}]transmethylase is related to SAM is indicated by the experiments in which charcoal completely abolished the residual activity (Table 6), and by the finding that SAM binds to proteins in the crude extract. TAYLOR and WEISSBACH (1969) have previously shown binding of SAM to purified [B_{12}]transmethylase of bacterial origin; MANGUM and NORTH (1971) have reported that SAM-independent activity remains in [B_{12}]transmethylase from pig kidney after extensive purification of the enzyme. These two findings appear to be related. Our kidney preparations had 20 per cent of the full activity of [B_{12}]transmethylase remaining after dialysis (Table 5); however, this residual activity was not affected by L-DOPA, which causes a fall in the SAM concentrations in this tissue (ORDÓÑEZ and WURTMAN, 1973), but was completely abolished by charcoal treatment (Table 6). The differential effects of L-DOPA in the different tissue preparations could be explained by assuming different strengths of binding of SAM to the [B_{12}]transmethylase molecules present in the preparations. Our experiment showing binding of SAM to crude tissue extracts cannot give information related to this hypothesis because of the mixed nature of the proteins present in the preparation.

In conclusion, the possibility that brain tissue maintains its methionine concentrations during increased transmethylation by increasing the synthesis of methyl groups *de novo* has been shown to be feasible. The net rate of this pathway *in vivo* could be regulated by hormonal interactions (as shown by the sex-dependent variations in enzyme levels), while the 'fine tuning' control could involve SAM.

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