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In Vitro Studies of 5,10-Methylenetetrahydrofolate Reductase: Inhibition by Folate Derivatives, Folate Antagonists, and Monoamine Derivatives

*J. L. Hollinger, O. R. Hommes, Th. J. J. M. van de Wiel, J. C. N. Kok, and M. J. T. Jansen

**Diaconessenhuis, Department of Neurology, Arnhem, The Netherlands; and Radboud University Hospital, Department of Neurology, Nijmegen, The Netherlands*

Abstract: Folate monoglutamates and folate antagonists have an inhibitory action on the activity of 5,10-methylenetetrahydrofolate reductase in rat brain. The type of inhibition was studied for dihydrofolic acid using the Lineweaver–Burk transformation. Some of the monoamine alkaloids, the *in vitro* products of 5,10-methylenetetrahydrofolate reductase, have either a stimulatory or inhibitory effect on the enzyme activity. **Key Words:** 5,10-Methylenetetrahydrofolate reductase—Folate derivatives—Folate antagonists—Monoamine derivatives. **Hollinger J. L. et al.** *In vitro* studies of 5,10-methylenetetrahydrofolate reductase: Inhibition by folate derivatives, folate antagonists, and monoamine derivatives. *J. Neurochem.* **38**, 638–642 (1982).

The enzyme 5,10-methylenetetrahydrofolate reductase (EC.1.1.68) catalyzes the reaction:

$$\text{5,10-methylene-THF} + \text{NADPH} + \text{H}^{+} \longrightarrow \text{5-methyl-THF} + \text{NADP}^{+}$$

This reaction is reversible in the presence of nonspecific electron acceptors (Kutzbach and Stokstad, 1971) (THF = tetrahydrofolic acid). The 5,10-methylenetetrahydrofolate reductase is capable of forming 5,10-methylenetetrahydrofolate, which because of an equilibrium reaction yields formaldehyde and tetrahydrofolic acid (Donaldson and Keresztesy, 1961). In the presence of high concentrations of monoamines, a cyclization of the monoamine with formaldehyde occurs spontaneously, with resultant formation of an alicyclic alkaloid (Lauwers et al., 1975; Meller et al., 1975; Rosengarten et al., 1975). The reaction of monoamines with formaldehyde (the Pictet–Spengler condensation) is a well-established process.

The reaction products with catecholamines are derivatives of 1,2,3,4-tetrahydroisoquinolines, and with indolamines of 1,2,3,4-tetrahydro-β-carbolines.

The physiological and pathological significance of the formaldehyde-derived monoamine alkaloids is, at present, being studied.

Rommelspacher et al. (1979) demonstrated the existence of 6-hydroxytetrahydro-β-carboline, the alkaloid from serotonin and formaldehyde, both in man and rat. The alkaloid from tryptamine and formaldehyde (1,2,3,4-tetrahydro-β-carboline) has been identified *in vivo* in rat brain by Barker et al. (1979). Folate compounds administered exogenously are known to cause convulsive activity (Obbens and Hommes, 1973; Hommes et al., 1979). The monoamine derivatives 4-methoxydopamine and *N*-methyldopamine, and 1,2,3,4-tetrahydrodimethyl-β-carboline have also been shown to cause convulsions in the rat, whereas the alkaloid of dopamine exhibits anticonvulsant activity (Hollinger and Hommes, unpublished). Experiments have been carried out, therefore, to determine the effects of these substances on reductase activity, on the

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Address correspondence and reprint requests to M. J. T. Jansen, Radboud University Hospital, Laboratory of Neurology, Nijmegen, The Netherlands.

Abbreviation used: THF, Tetrahydrofolic acid.

assumption that the convulsive behaviour induced by folate is dependent upon the formation of alkaloids as a result of reductase activity.

MATERIALS AND METHODS

5-[^{14}C]methyltetrahydrofolic acid barium salt, specific activity 57 mCi/mmol, was purchased from Radiochemical Centre, Amersham, England. Folic acid, dihydrofolic acid, tetrahydrofolic acid, methotrexate, pyrimethamine, sodium glutamate, and *N*-para-aminobenzoyl-L-glutamic acid were purchased from Sigma Chemical Co. 4-Methoxydopamine HCl, 4-methoxydopamine derivative, epinine, epinine derivative, and 1,2,3,4-tetrahydro-2,9-dimethyl-β-carboline were gifts from P. Laduron, Janssen Pharmaceutica, Beerse, Belgium.

Preparation of rat brain homogenates

Wistar rats of 160–200 g body weight were killed by decapitation. Their brains were rapidly removed and washed in 0.25 M-cold sucrose. The complete brain was homogenized for 1 min in 10 vol. of 0.25 M-cold sucrose with a Potter–Elvejhem homogenizer. The suspension was centrifuged for 20 min at 600 *g* to remove cell debris. The supernatant was stored at −20°C until required for enzyme assays.

Enzyme assays in rat brain homogenates

The 5,10-methylenetetrahydrofolate reductase was estimated in the reverse direction. We did not use an electron acceptor in the incubation mixture because we used crude enzyme preparations. Crude homogenates contained oxidizing substances, such as FAD, sufficient that addition of such electron acceptors did not further stimulate the enzyme activity. Only purified enzyme demonstrated a requirement for oxidizing substances in the enzyme assay (Stebbins et al., 1976).

The method we used was based on that of Laduron et al. (1975) with 4-methoxydopamine as the formaldehyde-binding substance. The incubation mixture contained 100 μl 0.5 M-sodium phosphate buffer, pH 6.4, 150 μl glass-distilled water or 100 μl of various concentrations of folate derivatives or monoamine derivatives, and 50 μl glass-distilled water, 100 μl 4-methoxydopamine 0.1 M, 50 μl 5-[^{14}C]methyltetrahydrofolic acid (10.0 μCi), and 100 μl enzyme preparation. The blank was prepared without enzyme or with boiled enzyme. After incubation for 2 h in a water bath at 37°C, the reaction was stopped by adding 1.0 ml of a 0.5 M-sodium borate buffer pH 10.0. The reaction products were extracted in 10.0 ml of a toluene–isoamyl alcohol 2:3 mixture from the aqueous phase, previously saturated with 1 g sodium chloride. After mixing on a Vortex mixer for 1 min, the tubes were centrifuged for 15 min at 1545 g. Four milliliters of the organic phase were added to a vial with 10.0 ml Instagel

TABLE 1. *K_i values of folate derivatives and folate antagonists for the 5,10-methylenetetrahydrofolate reductase*

Substance	50% inhibition concentration (moles/liter)
Dihydrofolic acid	7.0×10^{-5}
Folic acid	3.5×10^{-4}
Tetrahydrofolic acid	1.0×10^{-3}
Methotrexate	1.7×10^{-5}
Pyrimethamine	3.7×10^{-4}
S-adenosyl-L-methionine	1.8×10^{-4}
N-*p*-aminobenzoyl-L-glutamic acid	No inhibition
Sodium glutamate	No inhibition

Each value represents the incubation concentration (moles/liter) of inhibitor necessary to produce a 50% inhibition of enzyme activity with 5-[^{14}C]methyltetrahydrofolic acid (1.74 nmol/liter) as methyl donor and 4-methoxydopamine as the formaldehyde-binding substance in rat brain homogenates.

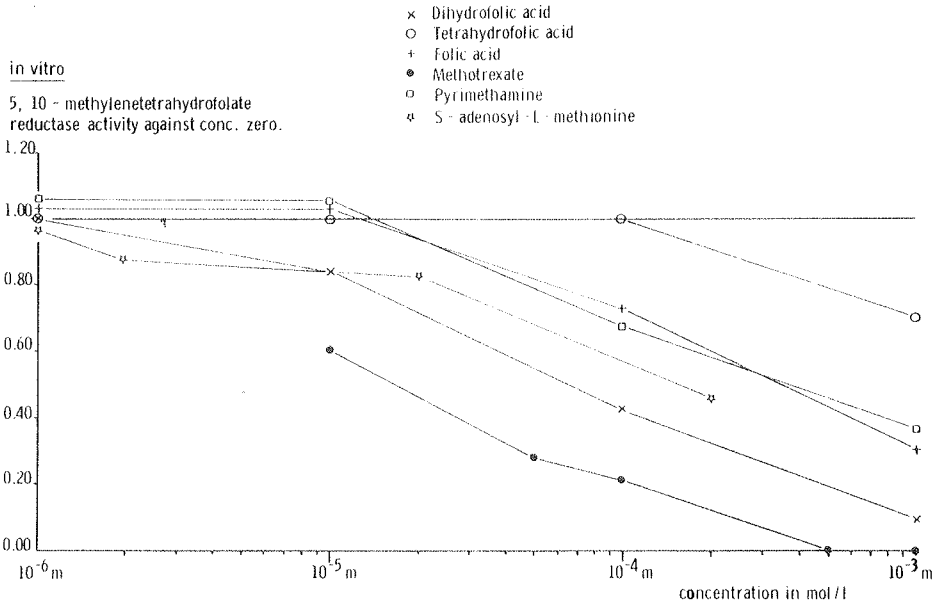

FIG. 1. Inhibition of the 5,10-methylenetetrahydrofolate reductase activity by various concentrations of folate derivatives and folate antagonists in rat brain homogenates *in vitro*. Reductase activity was measured with 4-methoxydopamine as the formaldehyde-binding substance.

and the radioactivity was measured with a liquid scintillation counter. The enzyme activity was expressed in nanomoles ^{14}C products per hour per gram tissue.

Michaelis–Menten kinetics

Whenever kinetic properties similar to Michaelis Menten kinetics were obtained, the parameters K_m and V_{max} were determined by means of the Lineweaver–Burk transformation (Lineweaver and Burk, 1934).

RESULTS

Kinetic properties of 5,10-methylenetetrahydrofolate reductase of rat tissue

The values for K_m (1.45 ± 0.25 μM) and V_{max} (7.43 ± 0.33 nmol/h/g tissue, mean ± S.D.) were determined by measuring the reaction velocities at different concentrations of 5-[^{14}C]methyltetrahydrofolic acid with crude enzyme preparations from rat brain. We have been unable to obtain from the literature K_m and V_{max} values for the 5,10-methylenetetrahydrofolate reductase activity of crude rat brain homogenates.

Inhibition of the 5,10-methylenetetrahydrofolate reductase by folate derivatives and folate antagonists *in vitro*

The following substances were tested: folic acid, dihydrofolic acid, tetrahydrofolic acid, methotrexate, pyrimethamine, *N*-para-aminobenzoyl-L-glutamic acid, sodium-glutamate, and *S*-adenosyl-L-methionine. The results are presented in Table 1 and Fig. 1. For the folate monoglutamates and folate antagonists an inhibition of the 5,10-methylenetetrahydrofolate reductase could be demonstrated on brain, which was most pronounced in the case of dihydrofolic acid and methotrexate. *S*-adenosyl-L-methionine was also shown to inhibit the enzyme activity. For sodium-glutamate, which is epileptogenic only in high doses, and *N*-para-aminobenzoyl-L-glutamic acid, a constituent part of the folate molecule, no inhibition was shown. Using the Lineweaver–Burk transformation, dihydrofolic acid was found to show competitive inhibitory activity on the enzyme (Fig. 2).

Influence of monoamine derivatives on brain 5,10-methylenetetrahydrofolate reductase activity *in vitro*

Different effects on the 5,10-methylenetetrahydrofolate reductase activity were observed with the cyclized monoamine derivatives (Table 2). The dopamine derivative was shown to increase the enzyme activity whereas the epinine derivative and the β-carboline derivative had inhibitory effects. Epinine had no effect.

DISCUSSION

Cellular folates are present mostly as their polyglutamate derivatives. In this form they have been shown to be better substrates (lower K_m) than monoglutamates for folate-dependent enzymes, and may function as natural substrates and as regulators of folate metabolising enzymes (Coward et al., 1974; Cheng et al., 1975). We have performed our studies on the inhibition of 5,10-methylenetetrahydrofolate reductase, however, with folate monoglutamates, because polyglutamates were not available. The inhibition by dihydrofolic acid was found to be competitive with respect to 5-methyltetrahydrofolic acid, in agreement with the observations of Matthews and Haywood (1979), who used purified reductase from pig liver. The more pronounced inhibition by dihydrofolic acid than that caused by folic acid or tetrahydrofolic acid is probably due to the formation of an intermediate product, an N^5-methyldihydrofolate derivative, in the enzyme reaction (Matthews and Haywood 1979).

The assumption that folate compounds exert their

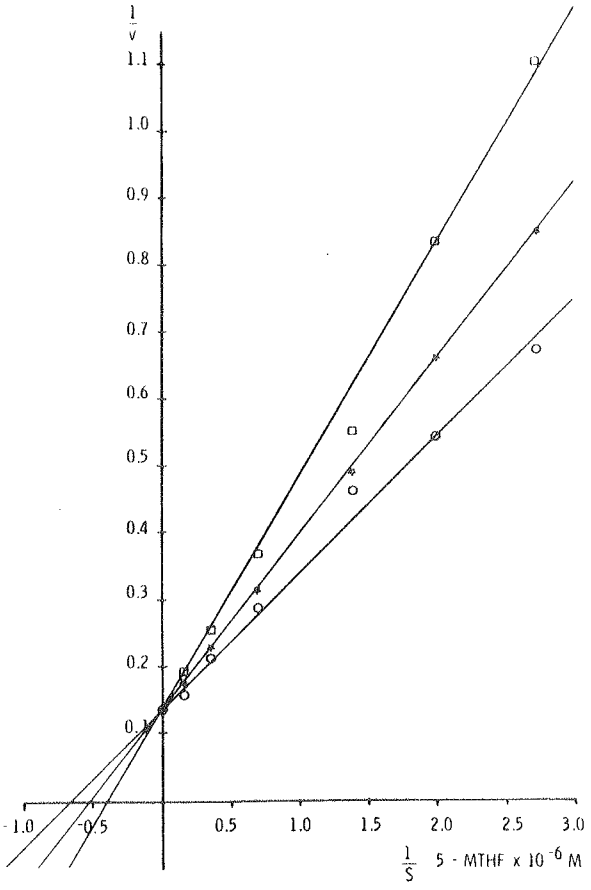

FIG. 2. Lineweaver–Burk plots for 5,10-methylenetetrahydrofolate reductase activity assayed at pH 6.4 with different concentrations of dihydrofolic acid. Each point represents the mean value obtained from duplicate determinations upon a single enzyme source prepared from pooled brains of eight rats. —○—, without dihydrofolic acid: K_m 1.38 μM; V_{max} 7.30 nmol/h·g tissue. —*—, dihydrofolic acid 1×10^{-5} M: K_m 1.87 μM; V_{max} 7.30 nmol/h·g tissue. —□—, dihydrofolic acid 2×10^{-5} M: K_m 3.11 μM; V_{max} 7.30 nmol/h·g tissue.

TABLE 2. *Effects of monoamine derivatives on the 5,10-methylenetetrahydrofolate reductase activity*

Substance	Concentration	Enzyme activity as percentage of the control
4-Methoxydopamine derivative[a]	1×10^{-2} M	100
	1×10^{-3} M	186
	1×10^{-4} M	116
Dopamine derivative[b]	1.72×10^{-3} M	204
	1.72×10^{-4} M	120
	1.72×10^{-5} M	105
Epinine[c]	1.61×10^{-3} M	100
Epinine derivative[d]	1.53×10^{-3} M	11
	1.53×10^{-4} M	47
	1.53×10^{-5} M	92
β-Carboline derivative[e]	1.94×10^{-3} M	15
	1.94×10^{-4} M	27
	1.94×10^{-5} M	76

Inhibitory and stimulatory effects of monoamine derivatives on the 5,10-methylenetetrahydrofolate reductase activity from a single enzyme source prepared from pooled rat brains. Reductase activity was measured with 4-methoxydopamine as the formaldehyde-binding substance.

[a] 1,2,3,4-Tetrahydro-7-methoxy-6-isoquinolinol.
[b] 1,2,3,4-Tetrahydro-6,7-isoquinolinediol.
[c] 4-[-2-(Methylamino)ethyl]pyrocatechol.
[d] 1,2,3,4-Tetrahydro-2-methyl-6,7-isoquinolinediol.
[e] 1,2,3,4-Tetrahydro-2,9-dimethyl-β-carboline.

convulsant effect by means of stimulation of monoamine alkaloid formation is not confirmed by our results.

The various monoamine derivatives have different effects on the 5,10-methylenetetrahydrofolate reductase activity. The dopamine derivative, which is anticonvulsive, stimulates the reductase activity. The epinine derivative and the β-carboline derivative, which were convulsive, inhibit the enzyme activity.

These results are the contrary of what we had expected. We had expected that the dopamine derivative, which is anticonvulsive, should inhibit the reductase activity. The epinine derivative and the β-carboline derivative, being convulsant, should stimulate the reductase activity.

It remains uncertain if the monoamine alkaloids demonstrated in brain tissue (Barker et al., 1979; Rommelspacher et al., 1979), and in human plasma and platelets (Kari et al., 1980) are produced by means of the reductase, which would require the reductase to act in the reverse of its normal physiological direction.

Kutzbach and Stokstad (1971) calculated that the enzymatic reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate has a $K_{eq} = 10$ at pH 7—thus, under normal physiological conditions, the rate of the reverse reaction is less than 1% of that of the forward one.

However, the possibility remains that *in vivo* monoamine alkaloid formation can take place during the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate in which an intermediate product, the N^5-methyldihydrofolate, is formed (Matthews and Haywood, 1979). We suggest that from this product the enzyme could transfer a one-carbon unit (in the form of formaldehyde) to monoamines.

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