# Diastereoisomers of Formaldehyde Derivatives of Tetrahydrofolic Acid and Tetrahydroaminopterin

S. B. HORWITZ, G. KWOK, L. WILSON, AND R. L. KISLIUK[1,2]

*Department of Pharmacology, Tufts University School of Medicine, Boston, Massachusetts 02111*

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Previous work on the diethylaminoethylcellulose chromatography of $CH_2O$ derivatives of tetrahydrofolic acid leading to the isolation of diastereoisomers, designated as 5,10-methylene derivatives, was repeated using formaldehyde-$^{14}C$. The isolated material contained 0.7–0.8 mole of formaldehyde/mole of tetrahydrofolate rather than 1 required for the 5,10-methylene structure. This finding was verified by chemical determination of $CH_2O$ on the isolated material. Comparable experiments with $CH_2O$ derivatives of tetrahydroaminopterin yielded 0.7 mole of $CH_2O$/mole of tetrahydropteridine. These results suggest that the compounds designated as diastereoisomers of 5,10-methylenetetrahydrofolate are actually complexes which contain 4 molecules of tetrahydropteridine and 3 molecules of formaldehyde. The diastereoisomer complex of $CH_2O$ and tetrahydroaminopterin corresponding chromatographically to the diastereoisomer complex of $CH_2O$ and tetrahydrofolate, which is biologically active, was more inhibitory to the growth of *Pediococcus cerevisiae* and *Streptococcus faecalis* than that corresponding to the biologically inactive diastereoisomer complex of formaldehyde and tetrahydrofolate. When the diastereoisomer complexes of tetrahydroaminopterin were combined a synergistic effect on growth inhibition was obtained. Each diastereoisomer complex inhibited mouse tumor dihydrofolate reductase to the same extent. Neither diastereoisomer complex showed significant inhibition of *Escherichia coli* thymidylate synthetase.

While searching for more effective antileukemic agents it was found that dihydroaminopterin and tetrahydroaminopterin are generally more effective inhibitors of biological systems than aminopterin.[3] Since an asymmetric center is present in tetrahydroaminopterin at C-6 in addition to the one in L-glutamic acid, it was of interest to investigate the biological activity of the individual diastereoisomers. After isolating diastereoisomers of tetrahydroaminopterin combined with $CH_2O$-$^{14}C$ in the manner described for the isolation of diastereoisomers of 5,10-methylenetetrahydrofolate,[4] we observed that the formaldehyde recovered in the isolated compounds was less than that predicted for the 5,10-methylene structure. The present report contains evidence which supports the view that diastereoisomers are separated by this method; however, they cannot be pure 5,10-methylene derivatives.

**Diastereoisomers of Formaldehyde Derivatives of Tetrahydrofolate.**—Figure 1 shows the pattern obtained on chromatography of a mixture of $CH_2O$-$^{14}C$ and *dl*-L-tetrahydrofolic acid. Four peaks (A′, B′, A, B) were obtained when either radioactivity or absorption at 295 mμ was measured. Peaks A and B are eluted at the same point as the diastereoisomers of 5,10-methylenetetrahydrofolate reported by Kaufman, *et al.*[4a] However, the ratio of $CH_2O$-$^{14}C$ to tetrahydrofolate is 0.7 rather than the expected value of 1. Technical errors in counting or in the determination of the specific activity of the $CH_2O$-$^{14}C$ were excluded. The ratio of $CH_2O$-$^{14}C$ to tetrahydrofolate for peaks A′ and B′ is about 1.5. All four peaks had the same spectrum[4a] with a peak at 295 mμ.

In order to verify the formaldehyde content of the isolated diastereoisomers 150 mg of the formaldehyde-tetrahydrofolate complex was chromatogrammed on a 35 × 40 cm diethylaminoethylcellulose column. Ten tubes from peak A and ten from peak B, taken from the region of maximum absorbance, were analyzed colorimetrically for formaldehyde[5] with a correction applied for the color obtained with tetrahydrofolate alone. The ratio of $CH_2O$ to tetrahydrofolate in peak A was 0.73 ± 0.05 (std dev) and peak B 0.74 ± 0.05 (std dev) in good agreement with the values obtained by radioactivity.

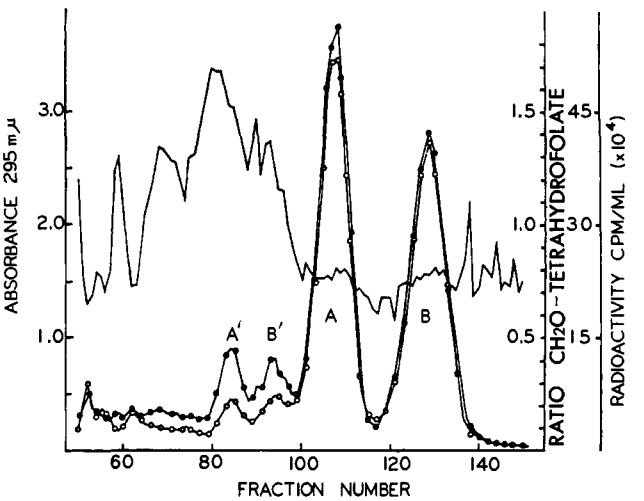


Figure 1.—Separation of diastereoisomers of formaldehyde derivatives of tetrahydrofolate on DEAE. ○—○, absorbancy at 295 mμ; ●—●, radioactivity in cpm/ml × $10^4$; ——, ratio of moles of formaldehyde to moles of tetrahydrofolate.

It has been demonstrated that peak B (and not peak A) is microbiologically and enzymatically active and therefore is the naturally occurring diastereoisomer.[4] In the present study each peak was assayed for its ability to act as a cofactor for thymidylate synthetase (Table I). Only peaks B and B′ were active.

**Diastereoisomers of Formaldehyde Derivatives of Tetrahydroaminopterin.**—Figure 2 shows the pattern

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(2) Leukemia Society Scholar.

(3) (a) R. L. Kisliuk, *Nature*, **188**, 584 (1960); (b) R. L. Kisliuk and M. D. Levine, *J. Biol. Chem.* **239**, 1900 (1964).

(4) (a) B. T. Kaufman, K. O. Donaldson, and J. C. Keresztesy, *ibid.*, **238**, 1498 (1963); (b) B. V. Ramasastri and R. L. Blakley, *Biochem. Biophys. Res. Commun.*, **12**, 478 (1963); (c) Y. C. Yeh and D. M. Greenberg, *Biochim. Biophys. Acta*, **105**, 279 (1965).

(5) T. Nash, *Biochem. J.*, **55**, 416 (1953).

TABLE I

FORMALDEHYDE DERIVATIVES OF DIASTEREOISOMERS OF TETRAHYDROFOLATE AS COFACTORS FOR THYMIDYLATE SYNTHETASE[a]

| Peak | Concn, $M$ | % act. |
|---|---|---|
| A′ | $1.9 \times 10^{-5}$ | 0 |
| B′ | $2.3 \times 10^{-5}$ | 25 |
| A | $6.1 \times 10^{-5}$ | 0 |
| B | $4.7 \times 10^{-5}$ | 97 |

[a] The tube containing the greatest concentration of compound from each of the four peaks was tested. The control reaction which contained $3 \times 10^{-4}$ $M$ *dl*-L-tetrahydrofolate was taken as 100% activity. The $K_m$ for *dl*-L-tetrahydrofolate was $4.5 \times 10^{-6}$ $M$ in this reaction: A. J. Wahba and M. Friedkin, *J. Biol. Chem.*, **237**, 3794 (1962).

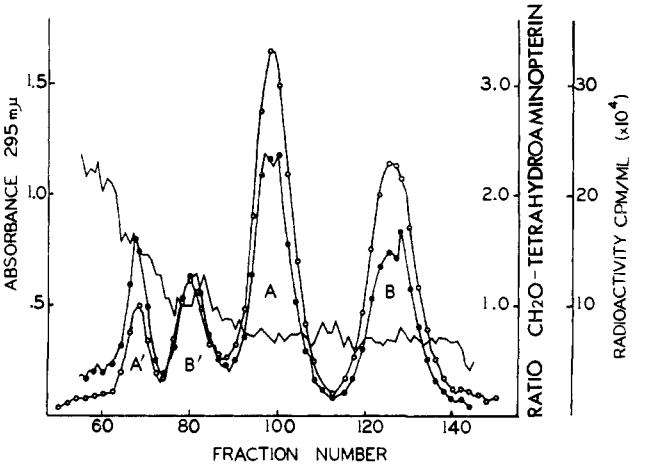


Figure 2.—Separation of diastereoisomers of formaldehyde derivatives of tetrahydroaminopterin on DEAE. O–O, absorbancy at 295 mμ; ●–●, radioactivity in epm/ml × $10^4$; ——, ratio of moles of formaldehyde to moles of tetrahydroaminopterin.

obtained on chromatography of a mixture of $CH_2O$-$^{14}C$ and *dl*-L-tetrahydroaminopterin. Four peaks (A′, B′, A, B) were obtained as with tetrahydrofolate. The molar ratio of $CH_2O$-$^{14}C$ to tetrahydropteridine was 0.7 for peaks A and B, 1.5–2 for peak A′, and 1–1.5 for peak B′. All four peaks had spectra similar to those reported for the tetrahydrofolate derivatives.[4a] The maximum absorbancy was at 292 mμ.

One explanation for the occurrence of four peaks could be that the tetrahydroaminopterin contains D-glutamic acid and that peaks A′ and B′ represent the other possible pair of diastereoisomers. This is not the case, however, because analysis of glutamic acid[6] isolated after acid hydrolysis of tetrahydroaminopterin showed that the content of D-glutamic acid was less than 2%. Peaks A′ and B′ probably are diastereoisomers of *dl*-L-tetrahydroaminopterin bearing additional $CH_2O$.

Peak B which corresponds to the biologically active diastereoisomer strongly inhibits the growth of *Pediococcus cerevisiae* and *Streptococcus faecalis* (Figure 3). Peak A is somewhat less inhibitory (Table II) but when added to peak B at levels which alone produced no inhibition it potentiated the activity of peak B (Table II).

Peak B′ was more inhibitory than peak A′. The inhomogeneity as regards growth inhibition (Figure 3) of peak B′ may be caused by its overlapping with

(6) P. Polgar and A. Meister, *Anal. Biochem.*, **12**, 338 (1965).

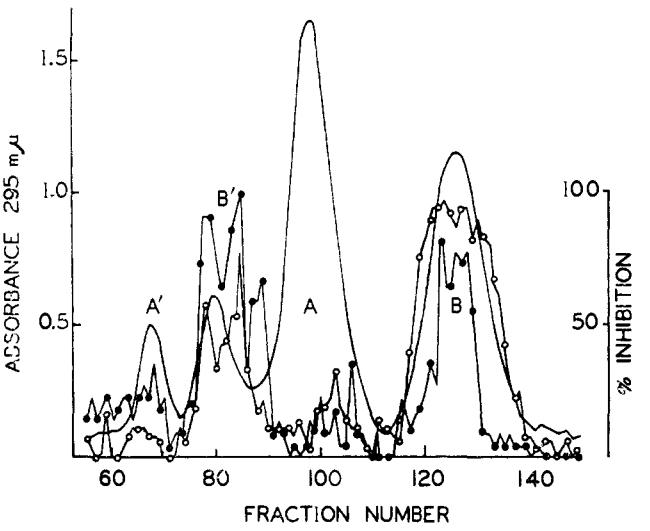


Figure 3.—Inhibition of the growth of *P. cerevisiae* and *S. faecalis* R by diastereoisomers of formaldehyde derivatives of tetrahydroaminopterin. Each tube was diluted 1:250,000 for *S. faecalis* and 1:5000 for *P. cerevisiae*. All dilutions were made in potassium ascorbate (6 mg/ml). ——, absorbancy at 295 mμ; O–O, % inhibition of the growth of *P. cerevisiae*; ●–●, % inhibition of the growth of *S. faecalis* R.

TABLE II

THE EFFECT OF FORMALDEHYDE DERIVATIVES OF DIASTEREOISOMERS OF TETRAHYDROAMINOPTERIN ON THE GROWTH OF *Pediococcus cerevisiae*[a]

| Peak A | | Peak B | | Peak A + peak B |
|---|---|---|---|---|
| Concn, mμg/ml | % inhib | Concn, mμg/ml | % inhib | % inhib |
| 3.0 | 0 | 2.8 | 9 | 32 |
| 3.6 | 0 | 3.2 | 11 | 45 |
| 3.9 | 0 | 3.3 | 18 | 50 |
| 5.4 | 0 | 4.8 | 45 | 80 |
| 10.9 | 2 | 6.8 | 57 | 91 |
| 16 | 50 | ... | ... | ... |

[a] Aliquots from peak A and peak B were diluted in potassium ascorbate, pH 6.0, 6 mg/ml, prior to assay.

peak A resulting in a synergistic increase in inhibition as seen in Table II.

Peaks A and B caused identical inhibition of mouse tumor dihydrofolate reductase (Table III). The bi-

TABLE III

THE INHIBITION OF MOUSE TUMOR DIHYDROFOLATE REDUCTASE BY FORMALDEHYDE DERIVATIVES OF DIASTEREOISOMERS OF TETRAHYDROAMINOPTERIN[a]

| Peak | Concn, $M$ | pH | % inhib |
|---|---|---|---|
| A | $2.8 \times 10^{-6}$ | 8.8 | 11 |
| B | $3.4 \times 10^{-6}$ | 9.0 | 12 |
| A | $2.8 \times 10^{-6}$ | 7.2 | 71 |
| B | $3.4 \times 10^{-6}$ | 7.2 | 74 |
| A | $1.8 \times 10^{-6}$ | 7.2 | 50 |
| B | $1.8 \times 10^{-6}$ | 7.2 | 50 |

[a] Fractions were neutralized with 1 $N$ HCl after adding 2-mercaptoethanol to 0.2 $M$. The control incubation contained the same amount of 0.4 $M$ $NaHCO_3$, pH 9.4, which was neutralized as above (see text).

carbonate buffer used to elute the diastereoisomers resulted in a final pH of 9 in the enzyme assay mixture. Under these conditions peaks A and B were both poor inhibitors. When the pH was lowered to 7.2, both diastereoisomers became quite inhibitory. A similar

pH effect was observed with tetrahydroaminopterin in the absence of formaldehyde. Thus removal or migration of formaldehyde does not account for the increased inhibition at lower pH.

Peaks A′, B′, A, and B showed no inhibition of *Escherichia coli* thymidylate synthetase when tested at $10^{-5}$ *M* either at pH 9.0 or 7.4. This result was unexpected because *dl*-L-tetrahydroaminopterin inhibits this enzyme 50% at the same concentration.[3b] Further investigation revealed that reaction of *dl*-L-tetrahydroaminopterin with 0.1 *M* formaldehyde abolished inhibition. Details of the interaction of formaldehyde with tetrahydroaminopterin will be reported separately.

**Recovery of Diastereoisomers.**—Data on the recovery of diastereoisomers based on absorption at 295 mμ is shown in Table IV. We have consistently observed with tetrahydroaminopterin derivatives that peak B is smaller than peak A. With tetrahydrofolate, the diastereoisomers were present in equal amounts as observed earlier.[4a]

TABLE IV
RECOVERY OF DIASTEREOISOMERS AFTER CHROMATOGRAPHY

| | % of total eluted absorbing material (295 mμ) in each fraction | |
|---|---|---|
| Peak | Tetrahydrofolate | Tetrahydroaminopterin |
| A′ | 5 | 9 |
| B′ | 5 | 12 |
| A | 45 | 43 |
| B | 45 | 36 |
| | Recovery of tetrahydropteridine added to column, % | |
| | 60 | 40 |

The ratio of formaldehyde to tetrahydropteridine of about 0.75 observed for peaks A and B shows that they cannot be pure 5,10-methylene compounds since there are four tetrahydropteridine molecules for every three molecules of $CH_2O$. These peaks may contain 50% tetrahydropteridines linked intermolecularly between the 5 positions (or between the 5 and 10 or 5 and 8 positions) giving a ratio of 0.5, plus 50% which are intramolecular 5,10-methylene compounds having a ratio of 1. The extent to which these complexes dissociate under various assay conditions is not known.

## Experimental Section

**Reduced Pteridines.**—Tetrahydrofolic acid and tetrahydroaminopterin were synthesized by reduction in AcOH.[7] The analysis of tetrahydroaminopterin prepared in this manner has been reported.[3b]

**5,10-Methylene Derivatives of Tetrahydropteridines.**—The method described by Kaufman, *et al.*,[4a] was followed except for minor modifications. Tetrahydropteridine (10 mg, 22 μmoles) was added to 1 ml of acetate buffer, pH 5.5, containing 50 μmoles of $CH_2O$-$^{14}C$ (33 μCi/μmole), and the solution was brought to pH 7.0 with 1 *N* KOH and immediately added to a DEAE column.

The 2.2 × 25 cm column was prepared by washing with 0.5 *N* KOH until the washings were colorless, with $H_2O$ until the effluent was neutral, with 1 l. of 0.4 *M* $NaHCO_3$ buffer, pH 9.5, and finally with 1 l. of $4 \times 10^{-3}$ *M* $HCO_3^-$, pH 9.5. The water-jacketed column was kept at 0°. The compounds were eluted with a $HCO_3^-$ gradient. Five-milliliter portions were collected and the concentrations of folate derivatives were estimated by the absorbancy at 295 mμ using a value of 28,000 for the extinction coefficient at this wavelength.

**Radioactivity Measurement.**—Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrophotometer Series 314 E with dioxane–anisole–dimethoxyethane (6:1:1) containing 1.2% of 2,5-diphenyloxazole and 0.05% of 1,4-bis-2-(5-phenyloxazolyl)benzene[8] as counting fluid. Aliquots (20 μl) were added to 15 ml of scintillation fluid. The absence of quenching was determined with internal $^{14}C$-toluene standards.

**Assays.**—The enzymatic and microbiological assays were carried out as described.[3b,9]

**Determination of D-Glutamic Acid.**—Tetrahydroaminopterin was hydrolyzed by autoclaving for 3 hr in 3 *N* HCl in a sealed tube. Glutamate was isolated by Dowex 50 chromatography and assayed for D-glutamate.[6]

(7) B. L. O'Dell, J. M. Vandenbelt, E. S. Bloom, and J. J. Pfiffner, *J. Am. Chem. Soc.*, **69**, 250 (1947).

(8) J. D. Davidson and P. Feigelson, *Intern. J. Appl. Radiation Isotopes*, **2**, 1 (1957).

(9) S. B. Horwitz and R. L. Kisliuk, *J. Med. Chem.*, **11**, 907 (1968).

# Synthesis and Antiinflammatory Activity of 2-Aryl-2-α-piperidyl-1,3-dioxanes

PAUL AEBERLI, WILLIAM J. HOULIHAN,[1] AND E. I. TAKESUE

*Sandoz Pharmaceuticals Research Laboratories, Hanover, New Jersey 07936*

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A series of 2-aryl-2-α-pyridyl- and 2-aryl-2-α-piperidyl-1,3-dioxanes have been prepared and evaluated for antiinflammatory activity. The most active members, 2-aryl-2-α-piperidyl-5,5-diphenyl derivatives, were twice as potent as phenylbutazone.

The interest in obtaining a nonsteroidal antiinflammatory agent is indicated by the amount of research that has been carried out in this area during the last few years.[2] In our laboratories we have found that certain 2-aryl-2-α-pyridyl- and 2-aryl-2-α-piperidyl-1,3-dioxanes[3] (II–IV) possess activity in the antiinflammatory[4] area. The present paper reports on the synthesis and antiinflammatory activity of some analogs of II–IV.

**Chemistry.**—The preparation of the 1,3-dioxanes reported in this work was accomplished by the proce-

(1) To whom inquiries should be addressed.

(2) T. Y. Shen in "Annual Reports in Medicinal Chemistry, 1966," C. K. Cain, Ed., Academic Press, New York, N. Y., 1966, pp 217–226; R. A. Scherrer in "Annual Reports in Medicinal Chemistry, 1965," C. K. Cain, Ed., Academic Press, New York, N. Y., 1965, pp 224–232; M. W. Whitehouse, *Progr. Drug Res.*, **8**, 321 (1965); International Symposium on Non-Steroidal Anti-Inflammatory Drugs, Milan, Sept 1964, S. Garattini and M. N. Dukes, Ed., Excerpta Medica Foundation, New York, N. Y., 1965; Abstracts of the 9th National Medicinal Chemistry Symposium of the American Chemical Society, Minneapolis, Minn., June 1964, p 11.

(3) Arabic numerals refer to compounds described also in the tables, while Roman numerals refer to compounds mentioned only in the text.

(4) The closely related 2-$R_1$-2-$R_2$-4- and -5-α-piperidyl-1,3-dioxanes have recently been reported to possess antiinflammatory, antispasmodic, local anesthetic, and preferential ganglionic blocking activity: W. R. Hardie, U. S. Patent 3,256,289 (1966); *Chem. Abstr.*, **65**, 7190 (1966).